Effect of cereal grains on the formation of heterocyclic aromatic amines in fried beef patties

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Dedication

SOLI DEO GLORIA

Abstract

The effect of incorporating flours representing different anatomical parts of the grain of corn, wild rice, and oat as well as corn bran extract into beef patties on the formation of heterocyclic aromatic amines (HAAs) during grilling was investigated. Beef burgers containing 5 or 10% dry cereal solids or cereal extract adsorbed to a cellulose carrier were grilled for 7 minutes per side on an electric grill. The HAA content of the cooked material was assessed using an optimized solid-phase extraction method, reversed-phase HPLC separation, and UV and fluorescence detection. 9*H*-Pyrido[3,4-*b*]indole (norharman), 1-methyl-9*H*-pyrido[3,4-*b*]indole (harman), 9*H*-pyrido[2,3-*b*]indol-2-amine (AαC), 1-methyl-6-phenyl-1*H*-imidazo[4,5-*b*]pyridin-2-amine (PhIP), and 3,8-dimethyl-3*H*-imidazo[4,5-*f*]quinoxalin-2-amine (MeIQx) were detected in all burgers. Norharman and harman formation were significantly increased in fried beef patties grilled with wild rice hulls, wild rice flour, and oat hulls. Other treatments also tended to increase β-carboline (harman and norharman) and PhIP levels relative to plain beef patties. Due to the analytical set-up it was not possible to obtain a full set of reliable data about the effect of cereal materials on the formation of MeIQx, but some materials may be able to reduce the formation of this HAA. It is concluded that the addition of whole cereal materials in beef patties is not an effective way to reduce the formation of β-carbolines and PhIP during grilling. Definite conclusions about the effect of cereal materials on the formation of MeIQx and structurally related HAAs cannot be drawn from this study.

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Chapter 1. Introduction

Heterocyclic aromatic amines (HAAs) are formed in trace amounts in protein-containing foods heated at high temperatures. Creatine is a necessary precursor for formation of one class of HAAs (amino-imidazo-azaarenes); therefore they are formed only in meat-based products. Free radicals formed as a result of the Maillard reaction are assumed to take part in amino-imidazo-azaarene formation. The other class of HAAs (amino carbolines) are pyrolysis products of amino acids, and the involvement of free radicals in their formation has been suggested as well (*1*).

After ingestion, HAAs are metabolized by human phase I and phase II enzymes, which can lead to bioactivation of the HAAs. Bioactivated HAAs readily form adducts with DNA, increasing DNA mutations and the risk of tumor formation (*2*). The carcinogenic potential of HAAs behooves us to limit their ingestion, and since cooked meats are a major source of HAAs in human diets, reduction of HAA formation in commonly consumed meat products like hamburgers would be a practical way to lower HAA exposure.

Because free radicals are assumed to be part of the HAA formation pathway, antioxidants could reduce the formation of these potent mutagens during cooking by scavenging free radicals. Some, but not all, studies have reported a reduction in HAA formation when synthetic antioxidants were applied to meat prior to cooking. However, using synthetic antioxidants raises consumer acceptability concerns and prevents clean labeling. Natural antioxidant sources (for example, herbs and spices) have also been shown to effectively limit HAA formation, but some of these natural antioxidant treatments are strongly flavored (for example, 20% garlic marinades), reducing sensory acceptability of the cooked product (*3*).

Cereal grains are a promising option for effective HAA mitigation because of 1) their naturally occurring antioxidants, 2) their relatively mild flavor, and 3) their positive consumer image.

Therefore, the aim of this thesis was to investigate whether cereals (corn, wild rice, and oats), either as whole grains and/or milling fractions (bran, hulls) can reduce HAA formation in grilled beef patties. Because cereal materials may contribute amino acids and reducing sugars to the cooking matrix, the HAA-suppressing capacity of corn bran extract prepared using a method designed to minimize the recovery of these HAA precursors was also assessed.

Chapter 2. Literature review, hypothesis, and study objectives

2.1. Discovery of heterocyclic aromatic amines

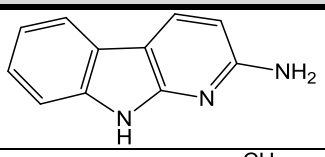
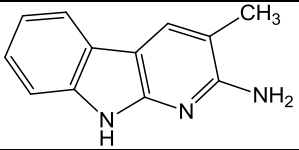
Mutagenicity of meat cooked at high temperatures was first observed in 1939 by Widmark, who produced malignant adenocarcinomas in mice by painting them with either an alcohol or petroleum extract of horse meat roasted at 275 °C (*5*). Identification of carcinogenic compounds present in cooked meat began in the mid-1960s, when several research groups isolated polycyclic aromatic hydrocarbons from grilled beef (*6, 7*). In 1977, Nagao et al. (*8*) used the Ames assay (*9*) to quantify the mutagenicity of both smoke condensate collected during broiling of beef steak and DMSO extract of the charred meat surface towards *Salmonella typhimurium* TA98. Mutagenicity of the charred meat extract was substantially higher than could be explained by polycyclic aromatic hydrocarbon content alone. Matsumoto et al. (*10*) tested pyrolysates of 18 individual amino acids for mutagenicity with the Ames assay; mutagenicity appeared at pyrolysis temperatures above 300 °C, with tryptophan pyrolysates showing much greater mutagenicity than the other amino acids. In a follow-up paper, the researchers described the mutagenicity of various pyrolyzed proteins and peptides (*11*). Around the same time, Commoner et al. (*12*) described a basic, solvent-extractable, mutagenic compound chromatographically distinguishable from benzo[*a*]pyrene in cooked hamburgers, and suggested that the formation conditions of this compound were similar to those of Maillard browning compounds. The researchers observed increased doneness levels of the hamburgers were associated with increased mutagenicity in the Ames assay but noted the cooking temperature (200 °C) was far below pyrolysis temperatures, indicating the possibility of two heat-induced mutagen types differing in formation temperature.

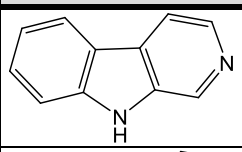
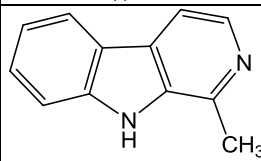
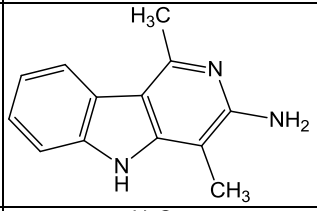
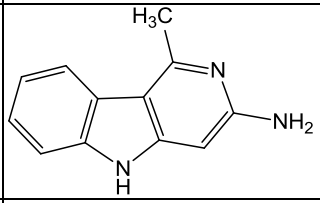
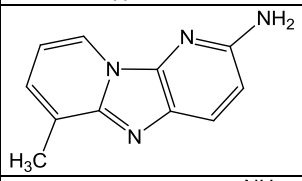
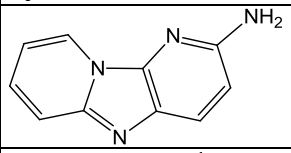
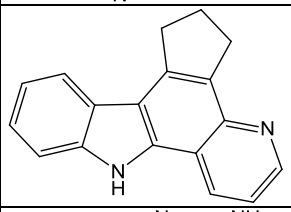
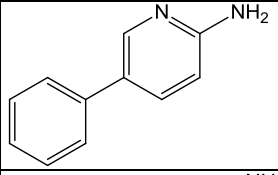
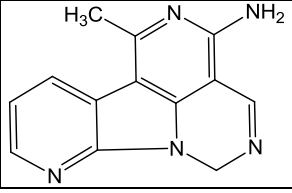
2.2. Isolation and characterization of heterocyclic aromatic amines

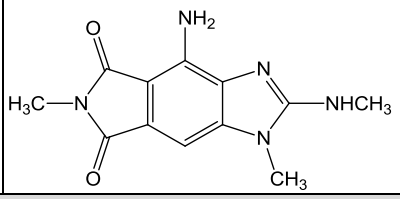
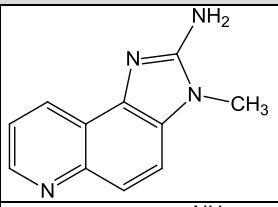
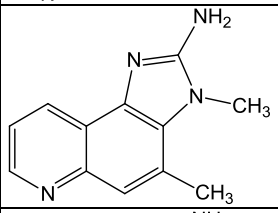
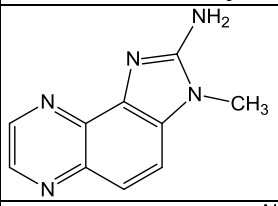
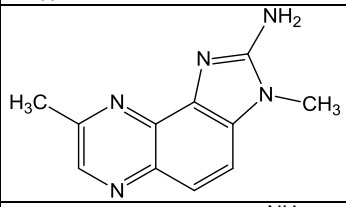
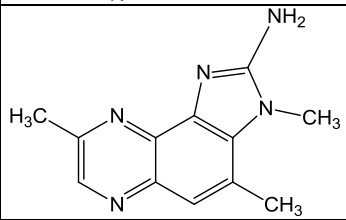
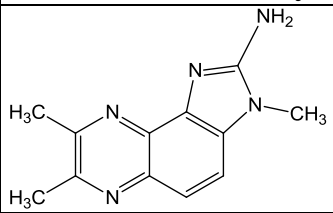
Many research groups worked to isolate and chemically characterize these newly discovered mutagenic compounds formed in cooked meat, fish, and other heated protein-rich materials. The compounds were classified as HAAs; over 20 HAAs have been identified to date (*13*). Some HAAs have only been isolated from individual pyrolyzed amino acids or pyrolyzed creatine and have not been recovered in foods; this discussion will focus on the HAAs recovered from foods, particularly beef.

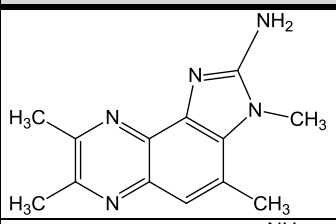
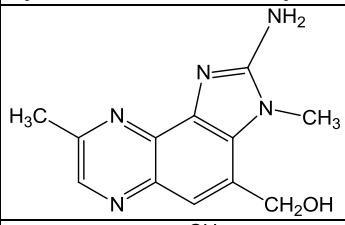
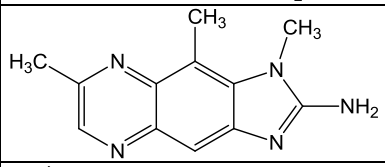
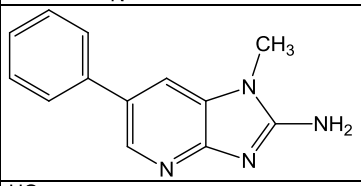
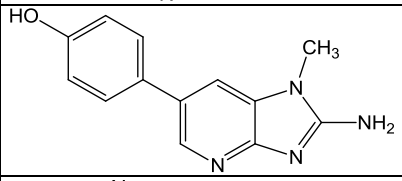
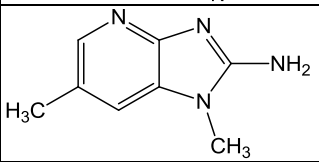
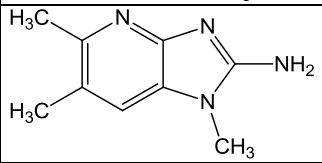
Historically, HAAs were grouped into two classes based on their mutagenicity after acidic nitrite treatment: 1) amino-carbolines (amino acid pyrolysis products), whose mutagenicity disappears after nitrite treatment, and 2) amino-imidazo-azaarenes, which retain their mutagenicity after nitrite treatment (*14, 15*). Some pyrolysis products which do not contain a carboline moiety have since been identified, but they are usually grouped together with the amino-carbolines in the literature. The amino-imidazo-azaarenes and the pyrolysis products differ in their formation conditions and mechanisms and will be discussed separately. For full chemical names, structures, and CAS numbers of the individual HAAs, see Table 1.

Table 1: Heterocyclic aromatic amines reported in cooked foods and model systems

Common name	Chemical name	Molecular formula	CAS[a] number	MW[b]	Structure
Amino carbolines and other pyrolysis products					
AαC	9*H*-Pyrido[2,3-*b*]indol-2-amine	$C_{11}H_9N_3$	26148-68-5	183	
MeAαC	9*H*-Pyrido[2,3-*b*]indol-2-amine, 3-methyl-	$C_{12}H_{11}N_3$	68006-83-7	197	

Common name	Chemical name	Molecular formula	CAS[a] number	MW[b]	Structure
norharman	9*H*-Pyrido[3,4-*b*]indole	$C_{11}H_8N_2$	244-63-3	168	
harman	9*H*-Pyrido[3,4-*b*]indole, 1-methyl-	$C_{12}H_{10}N_2$	486-84-0	182	
Trp-P-1	5*H*-Pyrido[4,3-*b*]indol-3-amine, 1,4-dimethyl-	$C_{13}H_{13}N_3$	62450-06-0	211	
Trp-P-2	5*H*-Pyrido[4,3-*b*]indol-3-amine, 1-methyl-	$C_{12}H_{11}N_3$	62450-07-1	197	
Glu-P-1	Pyrido[3',2':4,5]imidazo[1,2-*a*]pyridin-2-amine, 6-methyl-	$C_{11}H_{10}N_4$	67730-11-4	198	
Glu-P-2	Pyrido[3',2':4,5]imidazo[1,2-*a*]pyridin-2-amine	$C_{10}H_8N_4$	67730-10-3	184	
Lys-P-1	Cyclopenta[*c*]pyrido[3,2-*a*]carbazole, 1,2,3,8-tetrahydro-	$C_{18}H_{14}N_2$	69477-66-3	258	
Phe-P-1	2-Pyridinamine, 5-phenyl-	$C_{11}H_{10}N_2$	33421-40-8	170	
Orn-P-1	6*H*-2,5,6a,7-Tetraazafluoranthen-3-amine, 1-methyl-	$C_{13}H_{11}N_5$	78859-36-6	237	

Common name	Chemical name	Molecular formula	CAS[a] number	MW[b]	Structure
Cre-P-1	Pyrrolo[3,4-*f*]benzimidazole-5,7(1*H*,6*H*)-dione, 4-amino-1,6-dimethyl-2-(methylamino)-	$C_{12}H_{13}N_5O_2$	133883-91-7	259	
Amino-imidazo-azaarenes					
IQ	3*H*-Imidazo[4,5-*f*]quinolin-2-amine, 3-methyl-	$C_{11}H_{10}N_4$	76180-96-6	198	
MeIQ	3*H*-Imidazo[4,5-*f*]quinolin-2-amine, 3,4-dimethyl-	$C_{12}H_{12}N_4$	77094-11-2	212	
IQx	3*H*-Imidazo[4,5-*f*]quinoxalin-2-amine, 3-methyl-	$C_{10}H_9N_5$	108354-47-8	199	
MeIQx	3*H*-Imidazo[4,5-*f*]quinoxalin-2-amine, 3,8-dimethyl-	$C_{11}H_{11}N_5$	77500-04-0	213	
4,8-DiMeIQx	3*H*-Imidazo[4,5-*f*]quinoxalin-2-amine, 3,4,8-trimethyl-	$C_{12}H_{13}N_5$	95896-78-9	227	
7,8-DiMeIQx	3*H*-Imidazo[4,5-*f*]quinoxalin-2-amine, 3,7,8-trimethyl-	$C_{12}H_{13}N_5$	92180-79-5	227	

Common name	Chemical name	Molecular formula	CAS[a] number	MW[b]	Structure
TriMeIQx	3*H*-Imidazo[4,5-*f*]quinoxalin-2-amine, 3,4,7,8-tetramethyl-	$C_{13}H_{15}N_5$	132898-07-8	241	
4-CH_2OH-8-MeIQx	3*H*-Imidazo[4,5-*f*]quinoxaline-4-methanol, 2-amino-3,8-dimethyl-	$C_{12}H_{13}N_5O$	153954-29-1	243	
7,9-DiMeI*g*Qx	1*H*-Imidazo[4,5-*g*]quinoxalin-2-amine, 1,7,9-trimethyl-	$C_{12}H_{13}N_5$	156243-39-9	227	
PhIP	1*H*-Imidazo[4,5-*b*]pyridin-2-amine, 1-methyl-6-phenyl-	$C_{13}H_{12}N_4$	105650-23-5	224	
4'-OH-PhIP	Phenol, 4-(2-amino-1-methyl-1*H*-imidazo[4,5-*b*]pyridin-6-yl)-	$C_{13}H_{12}N_4O$	126861-72-1	240	
DMIP	1*H*-Imidazo[4,5-*b*]pyridin-2-amine, 1,6-dimethyl-	$C_8H_{10}N_4$	132898-04-5	162	
TMIP	1*H*-Imidazo[4,5-*b*]pyridin-2-amine, 1,5,6-trimethyl-	$C_9H_{12}N_4$	161091-55-0	176	

[a]CAS: Chemical Abstract Service
[b]MW: Molecular Weight

2.2.1. Amino-carbolines and other pyrolysis products

Amino acids are the sole precursor for this class of HAAs (except Cre-P-1). Therefore they may be found in heated, amino-acid-containing foods of either animal or vegetable origin (*1*).

Because high temperatures (>300 °C) are required for substantial formation of the amino-carbolines (*10*), many of these HAAs were first isolated from amino acid pyrolysis systems. However, low levels of some of these HAAs are found in model systems at normal cooking temperatures (*16*) and cooked foods.

In 1978, AαC and MeAαC (α-carbolines) were first isolated from pyrolyzed soybean globulin (*17*). The first positive identification of α-carbolines in normally cooked food was three years later in grilled beef (*18*). These compounds are formed by pyrolysis of tryptophan (*19*), and thus their formation in pyrolyzed proteins was shown to be positively correlated with tryptophan content (*20*).

The β-carbolines, norharman and harman, are abundantly formed in tryptophan pyrolysates (*21, 22*). However, compared to the other pyrolysis products, norharman and harman form easily at lower temperatures and are relatively abundant in foods. Arvidsson et al. (*23*) heated meat juice model systems at temperatures ranging from 100 to 225 °C. At 100 °C norharman and harman were the only HAAs formed; furthermore, they were the most prevalent HAAs at all studied temperatures. These low formation temperatures indicate norharman and harman have multiple formation pathways, including some that do not require pyrolysis. Norharman and harman are also biosynthesized by some plants and are endogenously formed in human tissues, as reviewed by Pfau and Skog (*24*).

The γ-carbolines, Trp-P-1 and Trp-P-2, were first characterized in tryptophan pyrolysates (*21*) and later in broiled beef (Trp-P-1) (*25*) and fish (both Trp-P-1 and Trp-P-2) (*26*). The concentrations of Trp-P-1 and Trp-P-2 formed in tryptophan pyrolysates are usually lower than those of the other mutagens formed (the α- and β-carbolines), but they contribute significantly to the mutagenicity of these pyrolysates as measured by the Ames assay (*19*).

The δ-carbolines, Glu-P-1 and Glu-P-2, were isolated from glutamic acid pyrolysates (*27*). Traces of Glu-P-1 were reported in fried beef (*28*), but other research groups did not detect δ-carboline formation in cooked meat or fish samples (*4, 29*).

Other pyrolysis products reported in the literature include Phe-P-1, Lys-P-1, Orn-P-1, and Cre-P-1. Phe-P-1, a phenylalanine pyrolysis product (*21*), has been detected in broiled sardines (*30*). Lys-P-1, Orn-P-1, and Cre-P-1 are pyrolysis products of lysine (*31*), ornithine (*32*), and creatine (*33*), respectively, but they have not been reported in foods.

2.2.2. Amino-imidazo-azaarenes

In contrast to the amino-carbolines and pyrolysis products, amino-imidazo-azaarenes (AIAs) require creatinine (the lactam formed from creatine) for their formation (*34*). Creatine is a biological molecule synthesized by vertebrates and stored primarily in skeletal muscle as an energy reservoir (*35*). As a result, AIAs are only formed in creatine-containing, animal-based products. The formation mechanisms for the IQ- and IQx-type AIAs are generally assumed to include reactions between creatine and Maillard browning products, which form rapidly during heating of amino acids and reducing sugars. However, some studies have reported AIA formation in model systems lacking reducing sugars. HAA formation mechanisms will be discussed in more depth in a later section.

The IQ- and IQx-type mutagens were reported to be responsible for 75% of the mutagenic activity in beef (*14*). The imidazoquinolines IQ and MeIQ were the first AIAs to be discovered and were initially isolated from broiled fish (*36*). Formation in cooked beef dishes or beef flavoring was reported afterwards (*15, 37*); however, many studies investigating HAAs in beef did not detect these two compounds (*4, 38*). The imidazoquinoxalines MeIQx and 4,8-DiMeIQx were first described in cooked beef (*15, 39-41*) and are the most frequently described IQ- or IQx-type HAAs in beef (*4, 42, 43*). Less common mutagenic IQx-type HAA compounds

reported in cooked meats or meat extracts include IQx (*44*); 7,8-DiMeIQx (*45*); TriMeIQx (this HAA is formed in model systems and used as an internal standard in extractions from cooked foods) (*46-48*); 4-CH_2OH-8-MeIQx (*49*); and 7,9-DiMeI*g*Qx (*50*). Recently, several new HAAs containing the I*g*Qx skeleton in their structure have been identified in cooked meats using LC-MS/MS (*51*). These compounds are similar to IQx-type compounds, but the three aromatic rings in an I*g*Qx structure are fused linearly, instead of the bent conformation displayed by the IQx-type. Toxicological studies of these compounds indicate mutagenic potential (*52*).

PhIP, first identified in cooked beef (*53*), is the most abundant imidazopyridine in cooked meats. Other imidazopyridines reported in beef include DMIP, TMIP, and (only in beef extract) 4-OH-PhIP (*42, 54, 55*).

2.3. Carcinogenic potential of heterocyclic aromatic amines

HAAs are only formed in very low concentrations in foods, but the presence of these compounds in the human diet is consequential because of their marked mutagenic and carcinogenic potential. The International Agency for Research on Cancer, which has reviewed and evaluated the human cancer risk of over 900 chemicals, classifies IQ as “2A-Probably carcinogenic to humans” and AαC, PhIP, and MeIQx as “2B-Possibly carcinogenic to humans” (*56*).

HAA mutagenicity was first demonstrated in bacteria using the Ames assay, which utilizes histidine-dependent *Salmonella typhimurium* strains (*9*). In this assay, the bacteria are plated onto histidine-free media and inoculated with rat liver preparation and the potential mutagen. The bacteria only grow if they develop a mutation to produce histidine. The number of bacterial colonies is related to the mutagenic strength of the compound. Researchers noted that liver preparation was necessary for the development of mutagenicity, indicating xenobiotic

metabolizing enzymes produced by the liver transformed the HAAs into DNA-reactive metabolites (*8*).

HAA mutagenicity has been suggested in human cell cultures via the formation of HAA-DNA adducts. Adducts elevate the risk of cancer development because if unrepaired, they may lead to inaccurate DNA transcription. If these mutations occur in key genes coding for cancer-preventative proteins within the human genome, the individual's cancer-preventative system will be compromised. HAA-DNA adducts were observed in human mammary epithelial cells treated with HAAs (*57*). Nauwelaers et al. (*58*) described various DNA adducts formed by hepatocyte cells inoculated with AαC, PhIP, or MeIQx. The formation mechanism of HAA-DNA adducts will be discussed in Section 2.4.

HAAs readily induce cancer in animal models. HAA-spiked diets (IQ, MeIQ, MeIQx, PhIP, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2 , AαC, and MeAαC) produced tumors in the liver, bladder, small and large intestine, blood vessels, prostrate, lymphoid tissue, and mammary glands of rats and mice (*59*). Adamson et al. (*60*) observed hepatic carcinomas in macaques given a daily dose of IQ.

In humans, epidemiological studies indicate that consumption of well-done meat (the principal dietary source of HAAs) is correlated with increased cancer risk. Nowell et al. (*61*) performed a case-control study of colorectal cancer patients and found intake of steak, pork chops, bacon, and sausage, and preference for a greater degree of meat doneness were all significantly associated with cancer. These results were seconded by Le Marchand et al., who showed that colorectal cancer risk is associated with a preference for well-done red meat in subjects with "fast" NAT2 and CYP1A2 (xenobiotic metabolizing enzymes) genotypes (*62*). Anderson et al. (*63*) found a preference for the barbecue cooking method in meat preparation was a risk predictor for pancreatic cancer. In addition to meat intake and cooking taste, other

lifestyle traits such as smoking and caffeine intake, which increase the expression of xenobiotic metabolizing enzymes (CYP450s), can modulate HAA-related cancer risk (*61*).

2.4. Absorption, metabolism, and excretion of heterocyclic aromatic amines

Upon ingestion in foods, HAAs are absorbed from the human GI tract into the portal vein and carried to the liver. HAA metabolism, which produces genotoxic HAA metabolites, occurs primarily in the liver. Therefore, limiting gut absorption of HAAs, for example by interactions with other non-absorbed constituents of the diet, reduces the number of HAAs that reach the liver, are metabolized and bioactivated. Instead, a portion of the meal's HAA load is excreted as unmetabolized compounds in the feces, thus reducing an individual's exposure risk to the ultimate mutagens/carcinogens. Lactic acid bacteria show HAA-binding ability (*64, 65*), which is hypothesized to occur via non-specific binding of the HAAs to the outer surface of the bacteria. Cereal fibers may also reduce gut absorption of HAAs, with increased lignification being associated with increased binding capacity (*66*).

After absorption and transportation to the liver, HAAs are metabolized by the phase I and phase II xenobiotic metabolizing enzymes (*2*). Phase I enzymes (primarily CYP1A2) attach a hydroxyl group to the HAA structure in various positions in preparation for conjugation by the phase II enzymes. HAA-metabolizing phase II enzymes include the N-acetyltransferases (NATs) and sulfotransferases (SULTs), which make the HAA structure more water-soluble and thus more readily excretable in the urine. UDP-glucuronsyl transferases (UGTs) also metabolize hydroxylated HAAs; glucuronidated metabolites are excreted primarily in the bile.

If phase I hydroxylation occurs at any part of the HAA structure except the exocyclic amino group, stable phase II conjugates are formed and excretion of the detoxicated HAAs can successfully occur. However, if phase I hydroxylation occurs at the exocyclic amino group, the

resulting phase II conjugates are bioactivated and unstable. These unstable esters undergo heterolytic cleavage to form a nitrenium ion, which is highly reactive. The electrophilic nitrenium ion readily attacks electron-rich double bonds in the DNA base guanine, forming a covalent DNA adduct. HAA nitrenium ions are stabilized by charge delocalization over the aromatic structure; nitrenium ion stability has been positively correlated with mutagenic potential (*67*). Figure 1 provides a sample mechanism of HAA-DNA adduct formation. The described mechanism is not valid for the β-carbolines norharman and harman which do not contain an exocyclic amino group, and are therefore not directly mutagenic. However, they do become mutagenic in the presence of some other chemicals, such as toluene, and are therefore described as "co-mutagenic" (*24*).

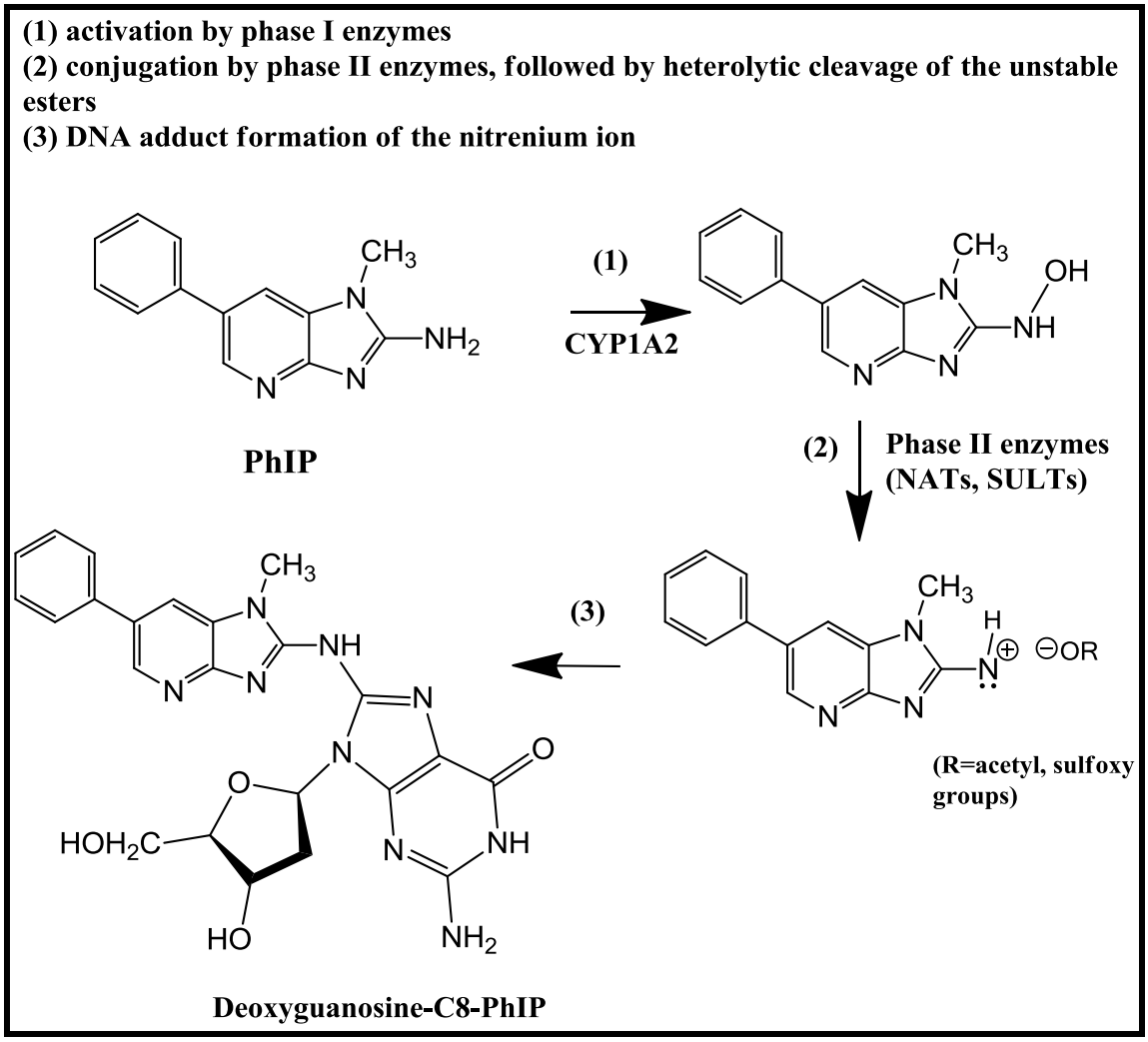


Figure 1: Mutagenic metabolism of PhIP. Figure adapted from Turesky (65). (NATs: N-acetyltransferases; SULTs: sulfotransferases)

2.5. Risk reduction strategies for heterocyclic aromatic amines

Potential strategies to minimize cancer risk from ingested HAAs include 1) reduction of HAA gut absorption, as described earlier, 2) minimization of HAA bioactivation by liver enzymes by intake of specific phytochemicals that alter expression patterns of phase I/phase II genes, 3) consumer education with respect to preparation of meat/fish and consumption of overcooked meat products, and 4) reduction of HAA formation during cooking. While all strategies are of academic interest, they have different shortcomings. The interaction of dietary constituents with phase I/phase II gene expression (for example, cruciferous vegetables) is complex and may

actually increase bioactivation of HAAs (*68*). Therefore, this strategy is far from being understood and cannot be considered prescriptive for HAA risk reduction at this time. A weakness of the consumer education strategy (reduce overall meat intake, use low-heat cooking methods, and avoid overcooked meat) is its dependency on consumer acceptance and implementation. A shared weakness of the first three approaches (reduction of absorption, limiting bioactivation, and consumer education with respect to the avoidance of overcooked meat) is that they are reactive and attempt to deal with an already-existent problem, i.e. HAAs in the diet. In contrast, consumer education with regard to proper preparation of meat/fish and also the fourth approach (reduction of HAA formation during cooking) are proactive and attack the problem at root level.

2.6. Formation mechanisms of heterocyclic aromatic amines

Knowledge of HAA formation mechanisms is vital to identify viable approaches for reducing HAAs during the cooking process. Uncoding these mechanisms is complex, however, because of the many factors simultaneously affecting HAA formation in meats, including cooking time and temperature, moisture content, level of Maillard reaction precursors, presence of antioxidants, and other characteristics. The differing formation mechanisms for the AIAs and the amino carbolines/pyrolysis products provide an added challenge. The following sections will summarize the research elucidating HAA formation, focusing on the major HAAs reported in beef (IQ-type, IQx-type, and PhIP for the AIAs and the α, β, and γ-carbolines for the pyrolysis products). General effects of time, temperature, moisture content, and precursors on HAA formation will be discussed first in Section 2.6.1; specific mechanistic research on HAA formation will be discussed in Sections 2.6.2-4.

2.6.1. Effect of time, temperature, moisture content, and precursors

Laser Reuterswärd et al. (*69*) observed a linear relationship between heating temperature and Ames assay mutagenicity of fried beef patties, indicating that higher temperatures promoted greater overall HAA formation. Individual HAAs differ in their specific heat requirements. The β-carbolines are formed most readily, being already observed at a 100 °C heating temperature. After extended periods (45 - 150 min) of low-temperature heating (125 °C), the IQx derivatives appear. If higher temperatures (200 - 225 °C) are used, the IQx derivatives begin forming in less than five minutes, with MeIQx forming more readily than 4,8-DiMeIQx (*70*). Compared to the IQx-type AIAs, PhIP requires harsher time/ temperature conditions, with a minimum formation temperature between 150 - 175 °C; temperatures of 200 °C or higher are required for rapid formation (*71*). The α- and γ-carbolines require even more severe environments for formation.

Once HAAs are formed they are susceptible to being degraded, with the AIAs being more unstable than the amino carbolines in some studies (*23, 72*). Another paper showed that AαC and PhIP were more heat-sensitive than the IQ- or IQx-type HAAs. However, this study used heated standard solutions of HAAs, which is a different matrix environment than meat (*73*).

Aqueous conditions promote IQx-type AIA formation; dry heating promotes PhIP (*42, 74*). Borgen et al. (*42*) reported that dry heating also promotes β-carboline production. In contrast, Bordas et al. (*48*) saw increased β-carboline formation with aqueous heating compared to dry heating.

HAA formation profiles are also affected by concentrations of precursors and amino acid profile. With respect to the AIAs (thought to form as a reaction between Maillard reaction products and creatinine) amino acids, reducing sugars, and creatine in the cooking system can

all affect the AIA levels. The concentration of individual amino acids present during cooking can affect formation levels of amino carbolines.

Creatine/creatinine concentrations were demonstrated to have the greatest impact on mutagenicity in various cooked beef products (*69, 75*). A study using heated beef flavor model systems observed that a five-fold increase in creatinine concentration produced substantially higher IQ, MeIQx, and PhIP levels compared to the native creatinine concentration (*48*). However, a study investigating the effect of creatine supplementation in swine diets on HAA formation from the cooked pork found that increased creatine content in the pork meat did not result in increased HAA levels (*76*).

With respect to amino acids, increasing the phenylalanine, alanine, and glycine levels in a beef flavor model system to 50 times the native concentration led to significant increases in PhIP and IQ compared to the control system (*48*). Another study comparing HAA formation in meat juice model systems from chicken, pork, or beef observed substantially higher PhIP formation in the dry-heated chicken system than the pork or beef juice models. These results could be partially explained by the higher phenylalanine content of the chicken compared to the other meats (*42*), since phenylalanine has been demonstrated to be a precursor of PhIP (*77*). Another study demonstrated that addition of tryptophan caused an explosion of β- and γ-carboline formation in a meat juice model system (*74*).

Reducing sugars, as precursors to the Maillard reaction, have been assumed to be necessary for production of the AIAs. The Maillard reaction's involvement is based on the findings of various model systems. In 1983, Jägerstad et al. (*78*) showed that heating glucose and creatine or creatinine with various amino acids produced mutagenicity; mutagenicity was highest in the threonine model system, followed by glycine. One year later, Jägerstad et al. (*79*) formed MeIQx by heating glucose, glycine, and creatinine. In 1993, Jägerstad's group

demonstrated that radio-labeled carbon atoms from glucose were incorporated into MeIQx and 4,8-DiMeIQx in a radio-labeled glucose-threonine-creatinine model system, proving that molecules originating from glucose may be incorporated into IQx-type HAAs (*80*).

The absolute necessity of glucose or other reducing sugars for the formation of IQx-type AIAs has not been shown by all studies. Dry-heating individual amino acids with creatinine produced MeIQx (*81*). The IQ- and IQx-type AIAs are believed to originate from reactions between creatinine and substituted pyridine and pyrazine compounds formed in the Maillard reaction, so the observation that dry-heated (200 °C) threonine or serine can produce small amounts of pyrazine products could explain these results (*82*). Pyrazine formation is favored by the presence of sugars, but not completely inhibited in their absence, indicating that IQ- and IQx-type AIA formation is theoretically possible without reducing sugar. IQ- and IQx-type formation will be discussed in more detail in Section 2.6.4.

A heated aqueous mixture of phenylalanine and creatine produced PhIP. Adding glucose to the system increased formation levels, but was evidently not essential (*83*). Murkovic's group has since completed several mechanistic studies on PhIP formation confirming that sugars were not a necessary precursor. This work will be discussed in Section 2.6.3.

It has been clearly demonstrated, however, that sugars substantially affect AIA levels in both model systems and cooked foods. Skog and Jägerstad (*84*) used an aqueous sugar-glycine-creatine or creatinine model system to demonstrate that adding molar amounts of sugars (glucose, fructose, lactose, or sucrose) up to half the molar amount of creatine/creatinine sharply increased the system's mutagenicity. Mutagenicity was sharply decreased when molar amounts above the molar amount of creatine/creatinine were used. The researchers suggested that this may be due to Maillard reaction products such as 5-hydroxy-2-methyl-furfural blocking creatine. This hypothesis would not explain the results from the sucrose model systems,

however, since sucrose is not a reducing sugar and cannot participate in the Maillard reaction unless heating conditions favor sucrose hydrolysis or breakdown into reactive fragments. A follow-up paper by the same group observed a dose-dependent inhibition of mutagenicity in fried beef patties containing either glucose or lactose (*85*). Kato et al. (*86*) found that adding 0.08% (w/w) of a reducing sugar (glucose, fructose, or lactose) to ground beef patties sharply increased their mutagenicity, but that adding higher amounts of these reducing sugars decreased mutagenicity.

2.6.2. Mechanisms of amino carboline formation

Specific mechanistic studies on amino carboline formation are scarce. Pyrolysis of amino acids produces reactive radical fragments which can condense to form heterocyclic structures (*1, 87*), although not all pyrolysis fragments are radicals (*87*). Milic et al. used electron spin resonance (ESR) to observe radical formation during lysine, aspartic acid, and glutamic acid pyrolysis (*88*); a later study also used ESR to demonstrate free radical formation during serine, threonine, or tyrosine pyrolysis (*89*). However, no ESR studies have been performed with tryptophan pyrolysates: the role of free radicals in amino carboline formation is unknown. Shulman and Simmonds (*90*) and Chiavari (*91*) used GC-MS to characterize amino acid pyrolysates and found that the major tryptophan products were all indoles. Sharma et al. (*22*) also investigated various tryptophan pyrolysis products (including norharman and harman) and noted the predominance of indole derivatives, indicating that the indole moiety of tryptophan is heat stable. The researchers suggested the pyrolysis formation of norharman and harman as a Diels-Alder product from 3-ethenylindole (formed from tryptophan after deamination and decarboxylation). See Figure 2 for a depiction of this reaction.

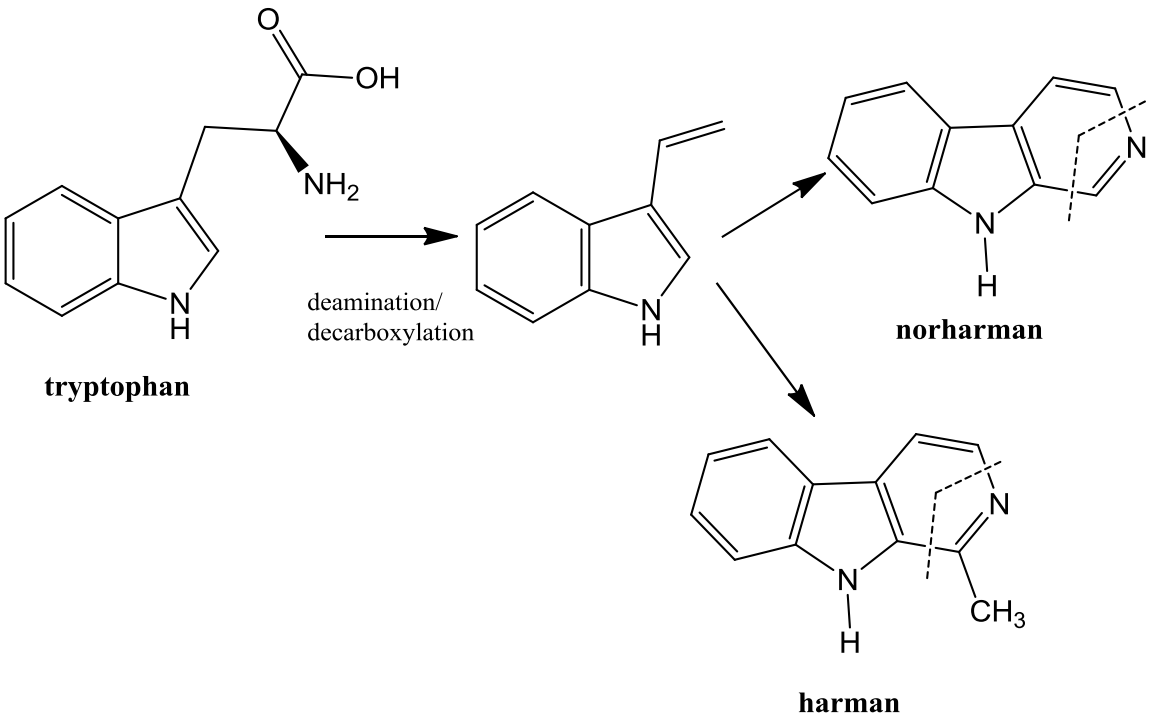


Figure 2: Norharman and harman as Diels Alder products of tryptophan (figure adapted from Sharma et al. (*22*))

Alternatively, the β-carbolines could be formed via a Pictet-Spengler condensation between tryptophan and Maillard reaction aldehydes. This reaction can occur at low temperatures: Herraiz et al. (*92*) successfully created β-carboline products from tryptophan and phenolic aldehydes at temperatures below 100 °C. Theoretically, a Pictet-Spengler reaction between tryptophan and formaldehyde (the Strecker degradation product of glycine) could produce norharman; a reaction between tryptophan and acetaldehyde could produce harman (Figure 3) (*93*). This reaction provides a possible explanation for the observed formation of norharman and harman far below pyrolysis temperatures.

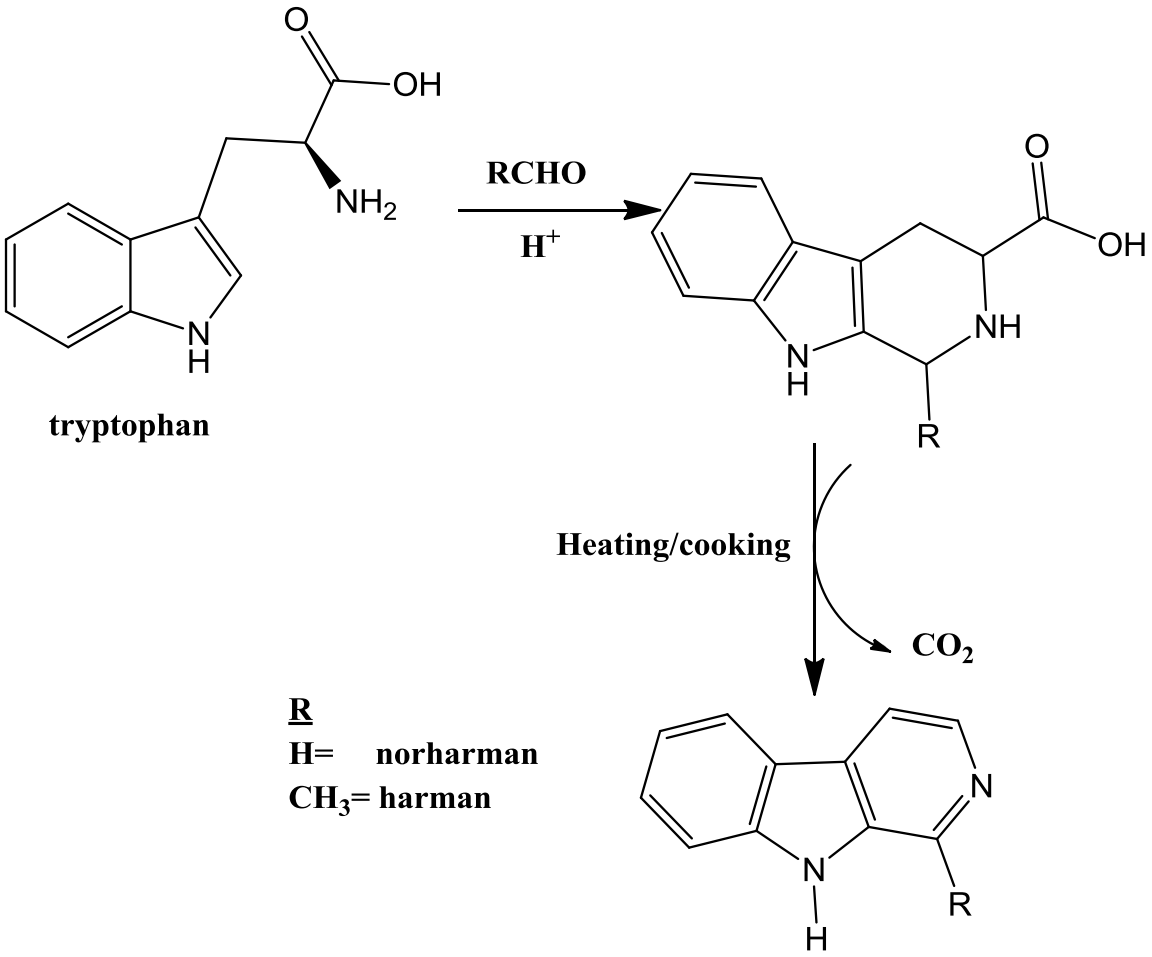


Figure 3: Norharman and harman as Pictet-Spengler condensation products of tryptophan and simple aldehydes (figure adapted from Herraiz (*93*))

2.6.3. Mechanisms of PhIP formation

Murkovic et al. (*94*) heated creatinine-phenylalanine model systems, using phenylalanine labeled at various positions with carbon-13 to elucidate PhIP formation. Results indicated that phenylacetaldehyde, a thermal degradation product of phenylalanine, is a key intermediate in PhIP formation. This was confirmed by greater PhIP formation in a phenylacetaldehyde-creatinine model system than in a phenylalanine-creatinine system. Later mechanistic work detected an aldol condensation product of phenylacetaldehyde and creatinine in both a model system and cooked meat. The researchers suggested that the final steps of the mechanism could involve free radical reactions between creatinine and the aldol condensation

product (Figure 4) (*77*). Evidence for the pyridine nitrogen coming at least partially from creatinine was provided by a model system in the same study using ^{15}N-labeled phenylalanine and unlabeled creatine: 10% of the PhIP formed incorporated no ^{15}N, indicating that all of the nitrogen atoms in the structure could come from creatinine. However, 77% of the PhIP formed had a molecular weight one Da higher than native PhIP and 13% of the PhIP formed had a mass two units heavier than native PhIP, indicating multiple mechanistic pathways for the formation of this compound.

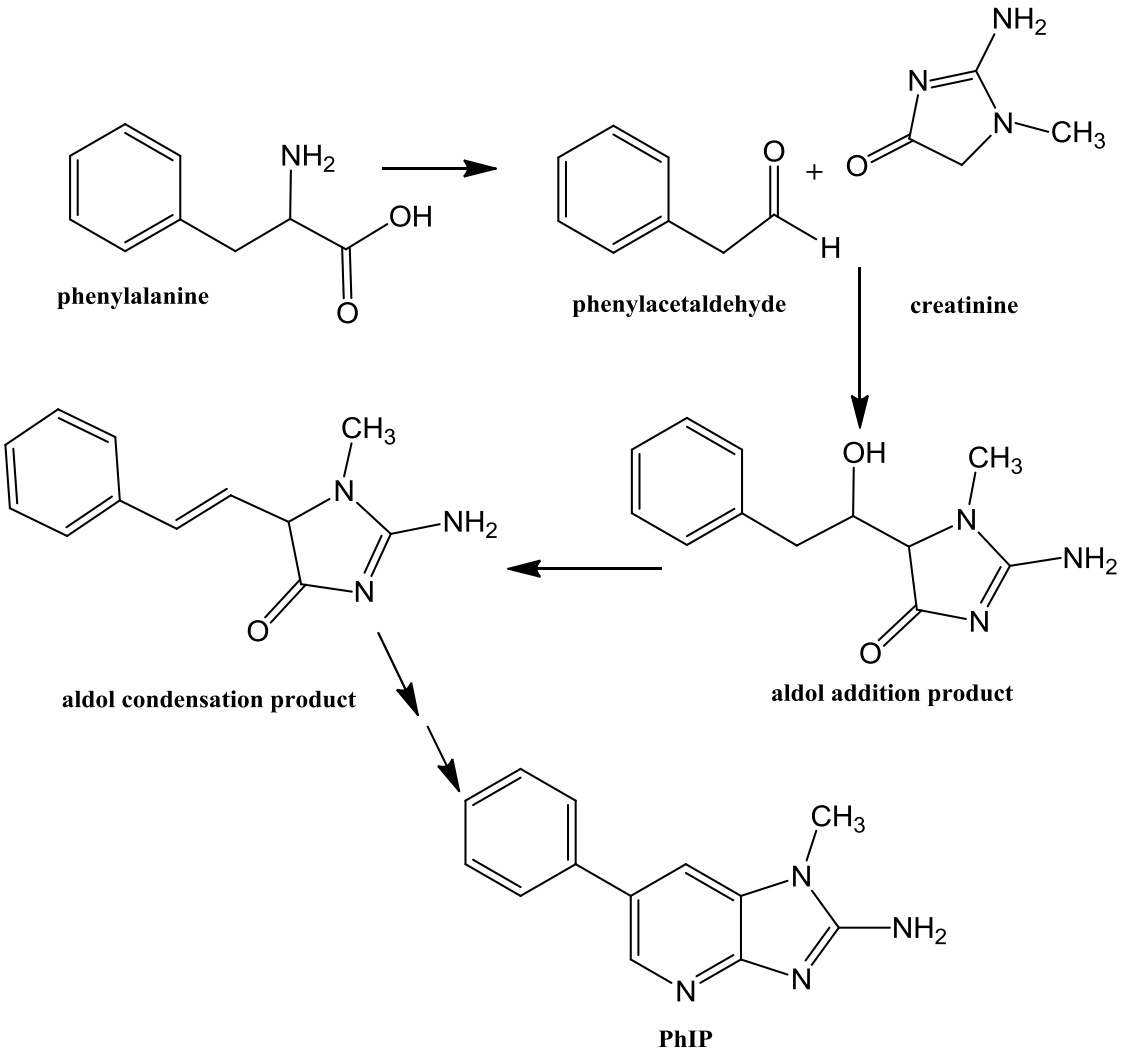


Figure 4: Suggested PhIP formation mechanism (adapted from Zöchling and Murkovic (*77*))

2.6.4. Maillard reaction: Pyridine, pyrazine, radicals, and IQ- and IQx-type heterocyclic aromatic amine formation

As stated previously, IQ- and IQx-type HAAs are assumed to arise from reactions between creatinine and pyridine- or pyrazine-based molecules produced in the Maillard reaction. In 1992 and 1993, two research groups suggested that IQx-type HAA formation may involve reactions between a pyrazine radical, aldehydes (such as Strecker aldehydes), and creatinine (Figure 5) (*95, 96*). A potential route for pyrazine formation via the Maillard reaction has been suggested to involve: 1) condensation between a reducing sugar and a primary amino group (usually provided by an amino acid) to form an imine (Schiff base) and 2), a reverse aldol reaction producing 2-carbon enaminol fragments which exist in tautomeric equilibrium with their keto form. The third, final step is a condensation of two of these fragments, producing a 1,4-disubstituted pyrazine molecule (Figure 5) (*95*). Evidence for the production of pyrazine radicals produced via the Maillard reaction first came from Namiki's group, who did ESR spectral work of various mixtures of sugars and amino acids (*97, 98*) and found spectra consistent with a 1,4-disubstituted pyrazine radical. Later work from this group indicated that this pyrazine radical had arisen from 2-carbon fragments derived from Schiff base products (*99*). The spectra changed based on which amino acid was used, but not on which sugar was used, which would match the pyrazine formation mechanism described previously, where the 1- and 4-position substituent on the pyrazine molecule originate from the amino acid. Milic and Piletic (*100*) produced ESR spectra characteristic of a substituted pyrazine radical by heating D-glucose and 4-aminobutyric acid. Roberts and Lloyd (*101*) and Hofmann (*102*) have also published ESR spectra of pyrazine radicals formed in the Maillard reaction. Note that this is not the sole pathway of pyrazine formation; pyrazines have also been detected in small quantities after dry-heating of serine, threonine, or tyrosine (*82*).

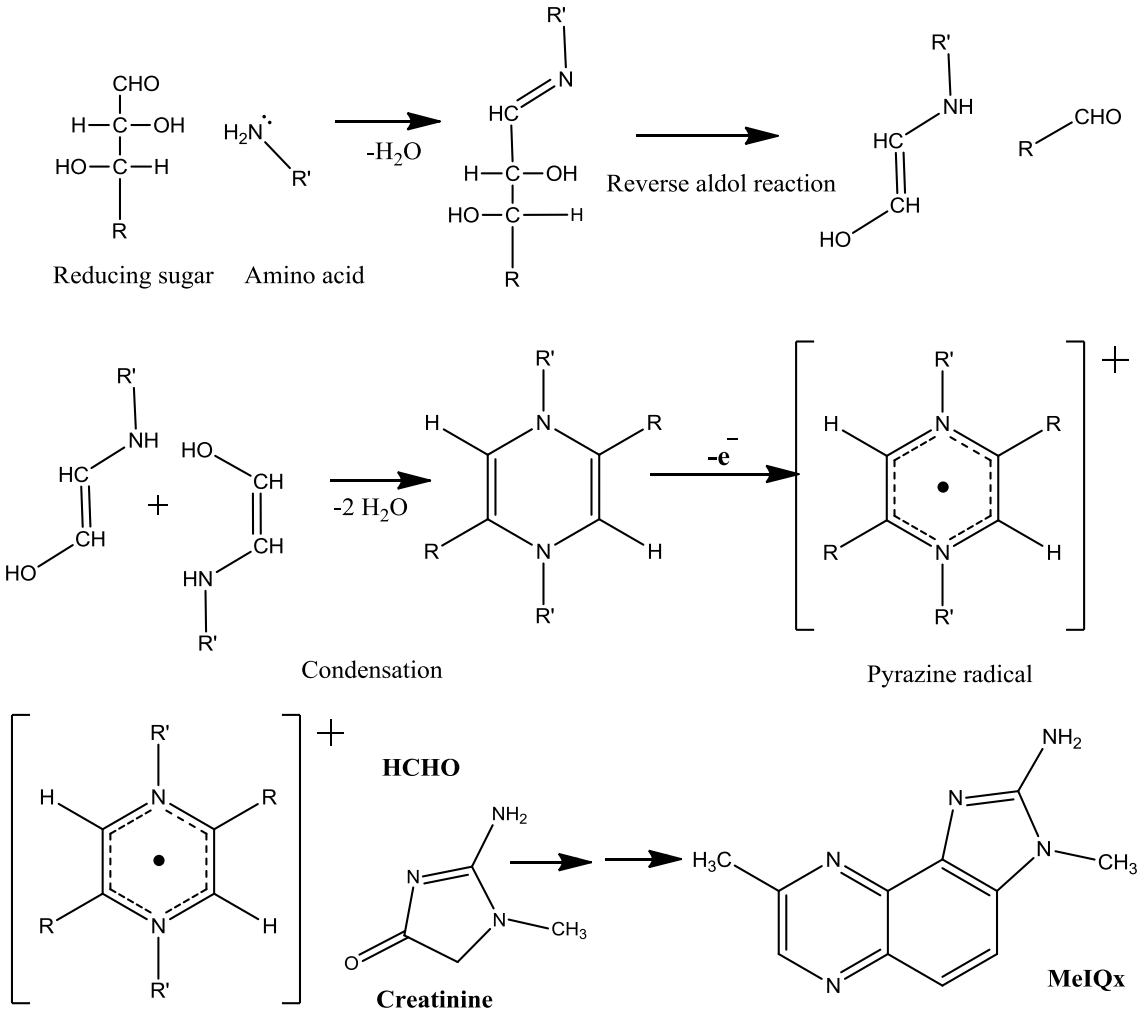


Figure 5: Formation of IQx-type heterocyclic aromatic amines

Evidence for the involvement of pyrazine radicals in IQx-type HAA formation was provided by Kato et al. (*103*), who produced MeIQx by heating glucose, glycine, and creatinine in diethylene glycol. ESR analysis of this reaction indicated the presence of a dicarboxymethyl pyrazine cation radical in the reaction mixture. Adding antioxidants such as butylated hydroxyanisole (BHA), epigallocatechin gallate (EGCG), sesamol, or esculetin to this model system reduced the intensity of the ESR spectra, reduced the amount of MeIQx formed (only EGCG was tested for this property), and reduced the mutagenicity (Ames assay) of the extract reaction mixture. The researchers suggested that the simultaneous reduction of formed MeIQx

and ESR spectral intensity observed in the systems with added antioxidants were due to the phenolic antioxidants' scavenging of the radical intermediate in MeIQx formation. Kikugawa (*104*) added EGCG, BHA, esculetin, and propyl gallate to heated glucose/glycine/creatinine model systems and observed both a decrease in mutagenicity (as measured by the Ames assay) and radical formation (according to ESR spectral intensity), noting that EGCG was especially effective.

The theoretical formation of pyridine radicals as precursors to the IQ-type HAAs has been hypothesized (*95*). Lee et al. (*105*) described the formation of IQ from a creatinine/acetylformaldehyde/ 2-methylpyridine system. However, no experimental work has been done to specifically assess whether or not radicals are involved in IQ formation.

2.6.5. Antioxidant-preventative effect theory

The proposed theory that HAA formation mechanisms involved free radicals and antioxidants therefore could suppress HAA formation during cooking by scavenging these free radicals received significant attention in the scientific community. A flood of papers describing the effect of scores of synthetic and natural antioxidants on HAA formation have been published in the past twenty years (*106*), even though the research foundation for this theory is somewhat slim. No actual mechanism for amino carboline formation has been published, and although pyrolysis of amino acids in general has been shown to generate free radicals, this has not been specifically demonstrated for tryptophan, the precursor of the α-, β-, and γ-carbolines. For PhIP, much of the formation mechanism is known. The first steps of this mechanism do not contain radical steps, and although Murkovic suggested that the final, unknown step of PhIP formation might involve radicals (*77*), the addition of thyme, marjoram, rosemary, or monascus red, which are assumed to contain antioxidants, to a phenylalanine-creatine model system increased the levels of PhIP formed (*107*).

The involvement of free radicals in IQx-type HAA formation has been most studied: antioxidants that effectively scavenge the pyrazine radical should reduce IQx-type HAA formation. However, any compound that prevents pyrazine formation would also reduce the formation of pyrazine radicals, regardless of whether the compound acts as an antioxidant. Emerging evidence suggests that HAA suppression by some of the antioxidants, originally touted as pyrazine radical scavengers, may not only be due to pyrazine radical scavenging, but also to suppression of pyrazine formation. This topic will be discussed further in Section 2.6.6. The following section (2.6.6.) will provide a sample of the work investigating the effects of synthetic and natural antioxidants on HAA formation, with an emphasis on studies using beef.

2.6.6. Effect of antioxidants on heterocyclic aromatic amine formation in cooked beef

Application of antioxidant spices (rosemary, thyme, sage, and garlic) applied to the surface of beef steak produced a reduction in IQ, MeIQ, MeIQx, 4,8-DiMeIQx, and PhIP compared to plain cooked beef (*108*). Balogh et al. (*109*) prepared beef patties with 1 and 10% Vitamin E or rosemary oleoresin (percentage based on fat content), and observed a significant reduction in PhIP with all treatments. MeIQx was significantly reduced in the 1% Vitamin E sample, but not with the other treatments. In another study, rosemary or grape seed extracts effectively reduced PhIP and MeIQx concentrations in cooked beef patties; reductions were correlated with two measures of antioxidant status (Trolox equivalents and “total phenolics” as determined by the Folin-Ciocalteu assay). However, norharman and harman were significantly increased with increasing amounts of these plant extracts (*110*). Cheng et al. (*111*) also observed a reduction in PhIP, MeIQx, and MeIQ levels in beef patties cooked with grape seed extracts; apple extract was also effective, with proanthocyanidins being the active HAA-suppressing compounds. Puangsombat et al. (*112*) observed a reduction in MeIQx and PhIP formation when

beef patties were cooked with spices (rosemary, turmeric, fingerroot, or galangal); HAA reduction was correlated both with antioxidant activity (as determined by the Trolox equivalence antioxidant capacity, or TEAC, assay) and "total phenolic" count (the total phenolics method measures all compounds with reducing properties at high pH). Quelhas et al. (*113*) observed a reduction in PhIP and AαC, but not MeIQx or 4,8-DiMeIQx, levels in fried beef that had been marinated with a green tea extract. Shin and Ustonol (*114*) observed a very slight reduction in MeIQx and 4,8-DiMeIQx in beef steaks marinated in an 11% garlic marinade relative to control steaks, but no reduction in PhIP. Gibis (*3*) also observed MeIQx reduction with garlic-based marinade. In a later paper, Gibis and Weiss (*115*) observed approximately 50 and 40% reduction of MeIQx and PhIP, respectively, in beef patties prepared with hibiscus extract marinade, but the norharman and harman content of the treated sample was significantly increased relative to the untreated patties. One percent carvacrol (a primary component of oregano extract) reduced MeIQ, MeIQx, and PhIP (*116*). Tart cherry added to beef patties produced between 33 and 81% reduction of the IQ and IQx-type HAAs; PhIP was reduced by around 90% (*117*).

In contrast to previous studies which showed a HAA-suppressing activity of many herbs, Damasius et al. (*118*) found that while thyme, savory, or oregano produced a slight reduction in PhIP in heated beef systems, other herbs had either no effect (rosemary, sweetgrass, and coriander) or resulted in greater PhIP levels. Neither radical-scavenging activity nor "total phenolic" count of the herb extracts were correlated with PhIP levels. Awney and Sindi (*119*) observed increased MeIQx and decreased PhIP levels (higher Ames assay mutagenicity overall) in beef Shawerma prepared with rosemary extracts. Persson et al. (*120*) observed an increase in PhIP, MeIQx, norharman, and harman levels in beef patties fried in fresh virgin olive oil with added rosemary compared to patties fried in oil only; after one year of storage, the oil plus

rosemary produced lower HAA levels compared to plain oil (also stored for one year). Melo et al. (*28*) reported no significant difference in HAA content between beef dishes cooked with antioxidant-rich ingredients, such as garlic, wine, olive oil, and onion, and beef cooked without these ingredients. Awney (*121*) observed dramatically increased IQ levels in beef Shawerma prepared with increasing amounts of green tea extract marinade or green tea + olive oil marinade. While the researchers detected and quantified IQ using ultraviolet (UV) light absorption, which increases the uncertainty of their results because of matrix interference during detection, they also assessed mutagenicity of the samples with the Ames assay and observed a corresponding increase in mutagenicity in the samples with the higher reported IQ levels. A pro-oxidant effect was suggested as the cause for the increase in IQ. Viegas et al. (*122*) quantified IQ, MeIQx, PhIP, and 4,8-DiMeIQx in fried beef dishes prepared with various marinades containing either beer or red wine alone or beer or red wine plus herbs reported to be high in antioxidants (garlic, ginger, thyme, rosemary, and red chili pepper). The radical-scavenging activity of each marinade was evaluated by the DPPH assay. Although the HAA content of the beef prepared with the herb-containing marinades was significantly lower than plain beef, there was no correlation between HAA reduction and radical-scavenging activity.

A potential drawback of using antioxidants from natural sources is their often-potent flavors, which may lead to decreased consumer acceptability (*3*). Messner and Murcovic (*123*) investigated the HAA-suppressing ability of a synthetic antioxidant: lyophilized samples of chicken, beef, pork, or turkey were heated with 0.01, 0.05, or 0.1% *t*-butylhydroquinone, but no difference in PhIP levels compared to untreated meat samples was observed.

2.6.7. Alternative mechanisms for heterocyclic aromatic amine suppression by known antioxidant compounds

Although many of the previously discussed studies reported a reduction in HAA levels when compounds with known antioxidant properties were added to beef before cooking, others found no effect, a mixed effect (reduction of some HAAs, but not others), or even an increase in HAA levels when antioxidant compounds were added. Most puzzling are those studies where radical-scavenging capacities of various treatments fail to correlate with HAA reduction patterns. However, it has to be pointed out that some studies used antioxidant tests, which do not measure radical scavenging activities, but only reducing properties of compounds or extracts.

Wang's group was the first to investigate the possibility of non-antioxidant mechanisms for HAA reduction by known antioxidant compounds, after being inspired by the results from their study where twelve different plant-based phenolic antioxidant compounds were tested for PhIP- suppressing ability in a model system (*124*). The TEAC assay was performed on each antioxidant, and the researchers noted that the compound with the lowest TEAC score, naringenin, was most effective at suppressing PhIP formation. However, it has to be kept in mind that the TEAC assay measures electron transfer reactions (reducing properties), whereas other tests actually measure radical scavenging by hydrogen atom transfer (*125*). In a groundbreaking paper published in 2008, they presented evidence that naringenin's inhibitory activity was due to its blocking of phenylacetaldehyde, a PhIP precursor (*126*). A subsequent study demonstrated that the powerful PhIP-inhibiting ability of EGCG was due to its scavenging of phenylacetaldehyde, and not its antioxidant capacity (*127*).

Recent research also indicated that EGCG may inhibit pyrazine radical formation by reacting with the imine precursor to pyrazine. Bin et al. (*128*) used electron paramagnetic

spectroscopy to monitor radical formation and observed that at up to a 20 mM concentration in a glyoxal-alanine model system, EGCG favored radical formation, but that at higher concentrations, radical formation was hindered. LC-MS data provided evidence for an EGCG adduct with glyoxal imine, indicating that EGCG could block this precursor.

Since research suggests that some antioxidant compounds may inhibit HAA formation without acting as antioxidants, this opens up a second line of research to find out new means of lowering HAA formation during cooking.

2.6.8. Cereal grains as an antioxidant source

Many natural antioxidant sources have been tested for their ability to reduce HAA formation in cooked beef, but no one has tested cereal grains to date. Hydroxycinnamic acids are the principal phenolic compounds in cereal grains; they are found in the plant cell walls of cereals, where they provide structural strength to the cell wall by forming diferulate or higher ferulate linkages between arabinoxylan molecules or arabinoxylans and lignin (*129, 130*). Because cereal cell walls are thickest in the outer layers of the kernel, hydroxycinnamic acids are concentrated in the bran fraction including the ferulic acid rich aleurone layer, the testa, and the pericarp. Corn (maize) has the highest ferulic acid content among the cereals (*131*). In a model system study, ferulic acid inhibited MeIQx formation by 43%, but had no effect on PhIP formation (*132*).

2.7. Laboratory methods for quantitative analysis of heterocyclic aromatic amines

2.7.1. Extraction techniques for heterocyclic aromatic amines

The foundation of accurate HAA quantification is an extraction procedure that both removes the meat matrix and recovers a high percentage of the HAAs. Challenges in HAA extraction include 1) the diversity of chemical structures included in the HAA class and 2) the

trace concentrations of individual HAAs in cooked meat (ng/g or less). An effective HAA extraction method must be inclusive enough to retain the range of HAA structures while eliminating enough of the matrix to permit chromatography. Matrix removal is critical for less selective detection methods hinging upon chromatographic separation, such as fluorescence emission and especially UV absorption.

HAA extraction methods exploit two characteristics inherent to HAA molecules: basicity and aromaticity. The basic fraction of cooked meat is isolated by mixing meat with a base (converting the amines to their basic, unprotonated, lipophilic form), and extracting the HAAs from the mixture into an organic solvent. Alternatively, the meat may be homogenized with an acid (converting the amines to their protonated, hydrophilic form) and extracted with an organic solvent, which segregates the HAAs into the aqueous fraction. Extensive clean-up is required, and may include selective isolation of the aromatic fraction with Amberlite, or blue cotton/chitin/rayon. An array of extraction methods have been developed, with liquid-liquid extraction (*133*), blue cotton or blue rayon adsorption extraction (*134*) and solid phase extraction (both two-fraction and single fraction)(*4, 29, 133, 135*) being prevalent techniques.

2.7.2. Methods to separate heterocyclic aromatic amines

High performance liquid chromatography (HPLC) is the most commonly used HAA separation technique (*4, 135*). Some novel separation methods have been developed: Jautz and Morlock (*136*) published a validation for an HPTLC (high performance thin layer chromatography) separation method. A follow-up paper compared the HPTLC method to a standard HPLC method; precision was slightly less with the HPTLC method, but overall results correlated with those achieved by the HPLC method (*137*). HAAs may also be separated by gas chromatography (GC), but must be derivatized to prevent broad, tailing peaks caused by the

adsorption of the polar amines to the column. An example of a GC method applicable to most of the HAAs commonly found in foods was published by Casal et al. (*138*).

2.7.3. Detection modes used for the HPLC analysis of heterocyclic aromatic amines

UV absorption and fluorescence emission were the most common detection methods used historically in HAA research (*29*). Importantly, only PhIP and the amino carbolines can be detected by fluorescence; the IQ- and IQx-type HAAs do not fluoresce and need to be detected by using the less selective UV absorption. Fluorescence detection is more sensitive than UV detection and detection limits comparable to mass spectrometry (MS) are theoretically possible (*4*). However, both UV absorption and fluorescence are dependent on chromatographic resolution for accurate quantification, making mass spectrometry the gold standard for HAA detection (*135*).

Because both the diverse chemical characters of the HAAs and the complex meat matrix lead to different recovery rates for the individual HAAs, they are usually quantified by standard addition. Although this method is time-consuming, it provides a more accurate estimate of individual recovery rate and concentrations for each of the HAAs compared to external or internal calibration methods.

Despite correction for individual recovery rates, reported HAA contents vary somewhat between studies. Some HAA contents of fried beef patties as reported in the literature are presented in Table 2.

Table 2: Heterocyclic amine content (ng/g ± standard deviation) of fried beef patties from literature

Reference	IQ	MeIQx	4,8-DiMeIQx	PhIP	norharman	harman	AαC
Balogh et al. (109) 6 min/side, 225 °C	2.8 ± 1.8	3.5 ± 1.0	3.0 ± 1.5	13.3 ± 6.0	not studied	not studied	not studied
Balogh et al. (109) 10 min/side, 225 °C	5.3 ± 3.5	5.8 ± 1.8	4.8 ± 4.0	31.4 ± 13.5	not studied	not studied	not studied
Britt et al. (117) 10 min/side, 225 °C	1.1 ± 0.7	9.2 ± 4.4	2.7 ± 1.2	12.0 ±2.1	not studied	not studied	not studied
Cheng et al. (111) 6 min/side, 210 °C	not studied	2.96 ± 0.49	0.95 ± 0.11	10.10 ± 0.85	not studied	not studied	not studied
Gibis (4) 4.5 min (double-sided grill), 230 °C	not detected	3.7 ± 0.68	1.1 ± 0.27	1.6 ± 0.47	14.3 ± 1.37	13.3 ± 1.37	not detected
Knize et al. (71) 10 min total, 230 °C	0.7 ± 0.3	7.3 ± 2.7	1.0 ± 0.6	32 ± 10	not studied	not studied	not studied
Persson et al. (43) 5 min (double-sided grill), 200 °C	not studied	0.6 ± 0.3	not studied	44.2 ± 36.6	15.3 ± 1.1	not studied	not studied

2.8. Study objective and hypothesis

Although the hypothesis that antioxidants prevent the formation of HAAs has not been mechanistically studied in sufficient detail, a preventive effect of antioxidants for the formation of HAAs has often been shown in the past. However, a HAA-reducing effect was neither observed for all antioxidant containing plant extracts nor for all HAAs. Many of the effective HAA-reducing plant extracts add strong flavors to the final meat products. The addition of cereals such as wild rice is well accepted by Minnesotan consumers as demonstrated by products such as wild rice sausages and meat loaf. As described above, cereal grains are a rich source of various types of antioxidants, including hydroxycinnamic acids. The objective of this study was therefore to investigate the effect on HAA formation levels of adding cereals (corn, oats, and wild rice) to beef patties before high-temperature grilling. The effect of ground whole grains and/or milling fractions (bran, hulls) or extracts of these cereal grains/milling fractions will be analyzed. Our hypothesis is that the antioxidants in the cereal grains such as

hydroxycinnamic acids and their derivatives will inhibit the formation of HAAs in the beef patties, particularly the IQx-type mutagens. Hydroxycinnamic acids and their derivatives are also interesting candidates for the second line of research on the prevention of HAA formation involving the interaction with Maillard reaction based intermediates. Based on its high levels of hydroxycinnamic acids, the corn bran material should be the most effective HAA inhibitor.

Chapter 3. Materials and methods

3.1. Materials, chemicals, and equipment

3.1.1. Beef

Ground beef was purchased from the University of Minnesota Meat Science Laboratory (Saint Paul, MN, USA). Beef was ground round from a 19-month old Angus heifer slaughtered and butchered at the University of Minnesota Meat Science Laboratory. Meat was sourced from a single muscle group from one animal to minimize variations in HAA precursors (*72, 139*). The beef was ground twice with a 3/16th inch plate, mixed once in a Leland Double Action mixer, bagged in one-pound lots, frozen immediately, and stored at -55 °C.

3.1.2. Cereals

Wild rice (*Zizania aquatica* L.) grain (2010 crop) and wild rice hulls (2011 crop) were obtained from Deerwood Rice Company in Deerwood, MN, USA. Oat (*Avena sativa* L.) hulls were donated by the Whole Grain Milling Company (Welcome, MN, USA). Whole corn (*Zea mays* L.) and corn bran were purchased from SEMO Milling Company (Cape Girardeau, MO, USA). All cereal materials were stored at -20 °C.

3.1.3. Materials and laboratory supplies

(a) Corn bran extracts preparation supplies. Cellulose fiber was purchased from Sigma Aldrich (St. Louis, MO, USA).

(b) Heterocyclic aromatic amine extraction supplies. Polypropylene centrifuge tubes (50 mL) with screw caps were from Sarstedt (Newton, NC, USA). Empty cartridges (25 mL), 20 µm frits, and Isolute HM-N (diatomaceous earth) were from Biotage (Charlotte, NC, USA). Solid phase extraction cartridges (Bond Elut LRC-PRS, 500 mg, 10 mL reservoir and BondElut LRC-C18, 500 mg, 10 mL reservoir) and cartridge adapters/couplers were from Agilent (Santa Clara, CA, USA). PTFE needles and plastic valves for vacuum manifold were from Chrom Tech (Apple Valley, MN,

USA). Reaction vials (Reacti-Vials, 5 mL) were from ThermoScientific (Waltham, MA, USA). Microcentrifuge tubes (1.7 mL) were from Corning Incorporated (Corning, NY, USA).

(c) High performance liquid chromatography supplies. HPLC vials (2 mL, 9 mm screw cap with PTFE septum) and glass vial inserts (50 µL) were from Chrom Tech (Apple Valley, MN, USA). The TSK-gel Super-ODS HPLC column (reversed-phase C18, 4.6 i.d. x 100 mm, 2.3 µm particle size, 140 Å pore diameter) and guard cartridge (4 i.d. x 4 mm, 2.3 µm particle size) were from Tosoh Bioscience (King of Prussia, PA, USA). The Kinetex PFP HPLC column (pentafluorophenyl phase, 4.6 i.d. x 100 mm, 2.6 µm particle size, 100 Å pore diameter), Kinetex XB-C18 HPLC column (reversed-phase C18 with isobutyl side chains, 4.6 i.d. x 100 mm, 2.6 µm particle size, 100 Å pore diameter), and SecurityGuard ULTRA guard cartridges for C18 columns were from Phenomenex (Torrance, CA, USA). Filters (mixed cellulose esters, 0.45 µm) for filtering aqueous mobile phase at preparation were from Millipore (Bedford, MA, USA).

3.1.4. Chemicals

HAA standard compounds (norharman, harman, AαC, IQ, MeIQx; 4,8-DiMeIQx, and PhIP) were purchased from Toronto Research Chemicals (North York, ON, Canada). Ammonium acetate, ammonium hydroxide, 1-butyl-3-methylimidazolium tetrafluoroborate, ethyl acetate (HPLC grade), hydrochloric acid, methanol (ACS reagent grade), orthophosphoric acid, sodium chloride, sodium sulfate, and triethylamine (≥99.5%) were from Sigma Aldrich (St. Louis, MO, USA). Acetone (HPLC grade), acetonitrile (HPLC grade) and sodium hydroxide were from Fisher Scientific (Waltham, MA, USA). Ethanol (200 proof) was from Decon Labs (King of Prussia, PA, USA). De-ionized distilled water was produced on site. For comprehensive information about these chemicals, including hazard ratings, see Appendix A.

3.1.5. Major instruments

Key instruments used in this study are listed below. For a comprehensive listing of instruments, see Appendix B.

(a) High performance liquid chromatography. The HPLC system consisted of the following Hitachi (Santa Clara, CA, USA) components: L-7100 pump, L-7250 autosampler, L-7300 column oven, L-7455 diode array detector, L-7485 fluorescence detector, and D-7000 data interface. Data were analyzed with the Hitachi D-7000 HSM software package.

(b) Electric grill. A Hamilton Beach 25331 Super Sear Nonstick Indoor Searing Grill (Southern Pines, NC, USA) and a Weber Q140 electric grill (Palatine, IL, USA) were compared; the Weber model was selected to cook all the hamburger patties used in the final data sets.

(c) Blender. Oster Osterizer Galaxie (Fort Lauderdale, FL, USA).

(d) Vacuum manifold. Model and brand are unknown. 8 positions were used concurrently.

(e) Centrifuge. Dynac Centrifuge (Parsippany, NJ, USA).

(f) Microcentrifuge. Biofuge 15, Heraeus Instruments (Buckinghamshire, England).

(g) Mill. FitzMill Homiloid hammer mill, The Fitzpatrick Company (Elmhurst, IL, USA).

(h) Karl Fischer coulometric titrater. Aquatest CMA, Photovolt (Indianapolis, IN, USA).

(i) Rapid Visco Analyzer. Model RVA-4, Newport Scientific (Warriewood, NSW, Australia).

3.2. Preparation of cereal materials

Cereal materials were milled to 100 mesh (149 micron). The fine particle size was chosen to maximize the contact surface area between beef and cereal during cooking. After milling, the cereals were frozen and lyophilized; the dried material was stored at -20 °C.

Moisture content of each milled, dried cereal material was determined in triplicate by the Karl Fischer coulometric titration method. Approximately 0.5 g of milled cereal sample and 12.5 mL of HPLC-grade methanol were weighed, placed in a 16.5 mL glass Pyrex tube, capped

tightly, and shook at 118 rpm for 18 h. Extracts were centrifuged at 600 *g* to settle particulates; approximately 1.5 mL of the clear supernatant was injected in the Aquatest CMA instrument. Triplicate methanol blanks were also prepared and analyzed in parallel with the cereal extracts.

Cooked corn and wild rice flours were prepared by stirring a flour-water suspension (4.0 g milled flour and 25 mL water) in a Rapid ViscoAnalyzer with a 35-min time-temperature program (0 to 0.5 min, heat to 50 °C; 0.5 to 3.0 min, 50 to 95 °C; 3.0 to 23.0 min, hold at 95 °C; 23.0 to 26.0 min, cool to 50 °C; 26.0 to 35.0 min, hold at 50 °C). Cooked paste was freeze-dried, and the moisture content of the dried material was determined by the Karl Fisher method as described previously.

3.3. Preparation of corn bran extract

An extract of milled, lyophilized corn bran was prepared, using a method designed to minimize the amino acids and reducing sugars (HAA precursors) retained in the extract. Aliquots of bran (6.09 g, the amount of bran needed to provide 6.00 g of dry solids) were stirred for 1 h with 50 mL acetone water (2:1, v/v) in a 250 mL Erlenmeyer flask. The suspension was filtered through Whatman No. 4 filter paper, and the pH of the filtrate was adjusted to pH 2 with concentrated hydrochloric acid. Sodium chloride (5 g) was added, and the filtrate was extracted twice with ethyl acetate (2 x 30 mL). The organic phases were combined, washed with 30 mL pH 2 water, dried over sodium sulfate, and evaporated under reduced pressure at temperatures below 60 °C.

The residual cereal material remaining on the filter paper was washed with acetone (60 mL, 2 x 30 mL) and allowed to dry before being removed from the filter paper. To release ester-linked hydroxycinnamic acids and their derivatives, the dry material was suspended in an amber glass bottle with 70 mL of 2M sodium hydroxide and stirred for 16 h. The hydrolyzed mixture was acidified with concentrated hydrochloric acid to <pH 2 to protonate the phenolic acids and

prepare them for solvent extraction. The slurry was extracted four times with ethyl acetate (180 mL total; 60 mL first extraction, then 3 x 40 mL). Centrifugation was necessary after each extraction to achieve phase separation. The organic phase was dried over sodium sulfate, and then evaporated under reduced pressure at <60 °C.

To incorporate the extract into the burger meat, a 6 g bed of cellulose was prepared in a plastic Petri dish. Acetone (500 µL) and ethyl acetate (500 µL) were added to the flask containing the dried residue from the acetone-water extraction, swirling after each addition. The suspension was adsorbed dropwise onto the cellulose bed. The dried residue from the extraction of the hydrolyzed cereal material was dissolved in 1 mL each of acetone and ethyl acetate and then adsorbed dropwise onto the cellulose bed. After evaporation of the solvents, the adsorbed extract was evenly blended into the cellulose bed with a spatula. The mixture was combined with 54 g of raw ground beef and cooked as described in Section 3.4. The extraction procedure was performed in duplicate and the resulting beef patties were cooked in parallel with plain beef patties.

3.4. Preparation of beef patties

Frozen beef was thawed by placing the 1-lb packages in a bowl under a steady drip of cold water for at least 2.5 h. Each package was double-bagged inside two plastic zippered bags during thawing. After thawing, meat and meat juices were mixed by hand to evenly distribute moisture.

Patties without cereal material were prepared by weighing 60.0 g aliquots of beef and shaping into a 9-cm disk using a jar lid. Patties with cereal material were prepared by first weighing the amount of milled, lyophilized cereal required to provide either 3 or 6 g of dry cereal solids (5 or 10% of a 60 g patty). See Table 3 for a list of cereal treatments cooked with beef. Patty ingredients were made up to 60 g with beef. The beef and cereal were blended by hand and shaped into a 9-cm disk with a jar lid.

Table 3: Summary of cereal treatments

Cereal treatment	***Moisture content, g/100 g****	***Amount of cereal material for 5 % treatment*****	***Amount of cereal material for 10 % treatment*****
Raw wild rice flour	2.18	3.065	6.13
Cooked wild rice flour	2.61	3.08	6.15
Wild rice hulls	0.608	3.02	6.04
Oat hulls	0.298	3.01	6.02
Raw corn flour	4.02	3.12	6.24
Cooked corn flour	4.94	3.15	6.30
Corn bran	1.59	3.05	6.10

**Dry basis moisture content of the milled, lyophilized material, as determined by Karl Fischer*
***Amount of cereal required to contribute 5 or 10% cereal solids to a 60 g patty*

Prior to cooking, the Weber grill was preheated on the highest setting for at least 30 min, according to the manufacturer's instructions. Four fixed cooking positions were assigned on the grill grate and four patties were cooked in each batch (three different cereal treatments and one "blank" patty without cereal material). Patties were grilled for 7 min per side on the highest grill setting. Each cereal treatment was cooked in triplicate; each replicate was cooked in a different cooking position. The grill grate and reflective lower pan were washed and the grill was preheated for 30 min between every cooking batch. After three replicates for an experimental treatment had been cooked, they were cooled to room temperature, weighed to determine cooking loss, finely ground in a blender, and combined. See Figure 6 for a sample cooking scheme.

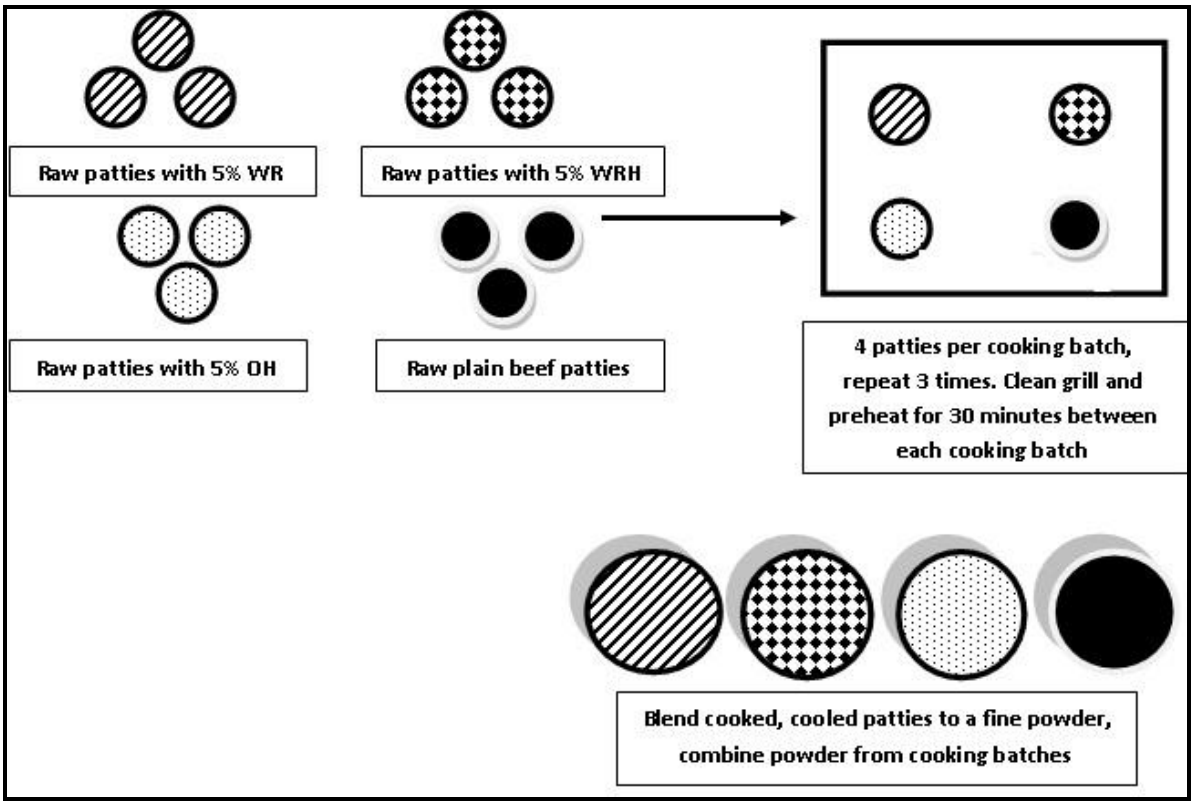


Figure 6: Sample cooking scheme (WR = wild rice, WRH = wild rice hulls, OH = oat hulls).

The purpose of this cooking method was to capture potential variability between cooking events and provide a valid comparison point for each experimental treatment. In contrast, if all three patties for a treatment were cooked in the same batch and then compared to a set of blank patties cooked in a separate batch, discerning whether observed differences were really caused by the treatment or only produced by variability between the cooking events (for example, pan temperature) would be impossible.

3.5. Extraction of heterocyclic aromatic amines from cooked beef

HAAs were extracted from cooked beef using a one-fraction, solid phase extraction method modified from Gibis (*4*). To soften the beef matrix and transform the HAAs to their basic, unprotonated form, 50 g of 1M NaOH and 20 g of cooked meat material were blended on high speed for 3-5 min until a homogenous, dough-like mixture was formed. This mixture was

rested for 1 h in a sealed jar and then two 10 g portions were removed into two 100 mL glass beakers. One portion was spiked with 135 µL of HAA standard spiking solution (22.5 ng/mL IQ, 92.25 ng/mL MeIQx, 22.2 ng/mL 4,8-DiMeIQx, 157.4 ng/mL norharman, 76.8 ng/mL harman, 109.8 ng/mL PhIP, and 21.8 ng/mL AαC in methanol). These HAA standards were chosen to provide a representative sample of the different classes of HAAs (IQ- and IQx-type, amino-imidazo-pyradines, and amino carbolines), focusing on the members of these classes most commonly reported in beef. The standard solution was stirred into the basic beef mixture with a spatula.

All following steps were performed in parallel on both the spiked and unspiked portion. Each treatment was extracted in duplicate, providing four data points for standard addition quantification (two unspiked and two spiked).

Diatomaceous earth (2.8 g) was stirred into the basic beef mixture, which was then transferred to a 50 mL polypropylene centrifuge tube. Ethyl acetate (25 mL) was added and evenly distributed with a few seconds of vigorous shaking. The tubes were laid flat on a shaker, shaken (118 rpm) for 20 min, vortexed on high speed for 4 min, and then centrifuged (900 *g*) for 15 min.

The supernatant was passed through a solid phase extraction cartridge tandem (blank cartridge filled with 2.8 g of diatomaceous earth coupled over a pre-conditioned (2 mL ethyl acetate) PRS cartridge). The beef-diatomaceous earth mixture was extracted a second time with 25 mL ethyl acetate (except the second extraction was not vortexed) and this supernatant was also passed through the cartridge tandem. The tandem was washed with 6 mL of ethyl acetate, uncoupled, and the PRS cartridge washed with another 6 mL ethyl acetate and dried under a gentle nitrogen flow for 1 h.

The dried PRS cartridges were washed with 6 mL HCl (0.01M) and then 6 mL HCl (0.1M)/methanol (90:10, v/v) to activate their cation exchange mechanism. The HAAs were eluted from the PRS cartridge onto a preconditioned C18 cartridge with 30 mL ammonium acetate solution (0.5M, pH 9), followed by 10 mL ammonium acetate (0.5M, pH 9)/methanol (90:10, v/v). C18 cartridges were preconditioned with 2 mL ammonium acetate (0.5M, pH 9), then 2 mL methanol. After elution of the HAAs from the PRS cartridge, the PRS-C18 cartridge tandem was uncoupled. The C18 cartridge was washed twice with 5 mL water and then dried under a nitrogen flow for a minimum of 1.5 h.

HAAs were eluted from the C18 cartridges into reaction vials with 1.2 mL of a 90:10 (v/v) mixture of methanol/ammonia (25%). The eluate was dried under nitrogen and then redissolved in 135 µL of triethylamine (0.01M, pH adjusted to 3.40)/methanol (75:25, v/v). The suspension was transferred to a 1.7 mL microcentrifuge vial and centrifuged (10,000 *g*) for 10 min; the supernatant was then transferred into HPLC vials. A portion of the supernatant was diluted by removing exactly 10 µL with a glass microliter syringe, adding 120 µL triethylamine (0.01M, pH 3.40)/methanol (75:25, v/v), and mixing. Both original and diluted concentration samples were injected into the HPLC.

Modifications to the original method included adding a rest time for the beef-sodium hydroxide mixture, changing the polarity of the solution used to dissolve the final extraction residue, and centrifuging the final solution. These modifications will be discussed in Chapter 4.

3.6. HPLC separation and analyte detection

3.6.1. Separation

Heterocyclic aromatic amines for all beef + cereal data sets were separated at 35 °C on a Kinetex XB-C18 HPLC column with a SecurityGuard ULTRA guard cartridge for C18 columns (Section 3.1.3). Injection volume was 50 µL, using the full-loop injection mode (2 x loop volume)

of the autosampler. Mobile phase flow rate was 1.1 mL/min, using three eluents to form a gradient: A) 0.01M triethylamine, pH adjusted to 3.40 with orthophosphoric acid, B) acetonitrile, and C) methanol/water (70:30, v/v). Eluents were degassed by sparging with helium gas prior to every set of analyses. The mobile phase gradient program is summarized in Table 4.

Table 4: Mobile phase gradient for heterocyclic aromatic amine separation

Gradient time, min	A: % 0.01M TEA, pH 3.40	B: % acetonitrile	C: methanol/water (70:30, v/v)
0.0	95	5	0
8.0	89	5	6
13.0	84	10	6
22.0	72	22	6
27.0	8	86	6
29.0	8	86	6
32.0	95	5	0
34.0	95	5	0

3.6.2. Detection

IQ, MeIQx, and 4,8-DiMeIQx were detected by UV absorption. PhIP, norharman, harman, and AαC were detected by fluorescence emission. UV absorption and fluorescence excitation and emission wavelengths were selected for maximum sensitivity, except for norharman, where less sensitive fluorescence excitation/emission wavelength parameters were used to increase the upper range of detectable norharman concentrations. Wavelengths were chosen based on literature and fluorescence spectra as determined by a Jasco spectrofluorimeter, followed by final optimization on the Hitachi HPLC system. Final wavelength parameters are provided in Table 5. Linearity of the detector response-HAA concentration relationship was confirmed by creating HAA standard curves, which are provided in Appendix C.

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Table 5: Detection wavelengths for heterocyclic aromatic amines

	λ, UV absorption	λ, fluorescence excitation	λ, fluorescence emission
IQ	270		
MeIQx	268		
4,8-DiMeIQx	270		
PhIP		337	395
norharman		354	450
harman		354	450
AαC		353	404

3.7. Standard addition quantification

HAA peaks were integrated using Hitachi's D-7000 HSM software. HAA concentrations in the injected extracts were quantified by standard addition. A sample calculation is found in Appendix D.

3.8. Statistical analysis

Confidence intervals (p=0.05) were calculated according to a standard method for samples quantified by standard addition (*140*). A sample calculation is provided in Appendix D. If the confidence intervals for two determinations do not overlap, the difference between their means is statistically significant. Means that are not statistically different from each other will always have overlapping confidence intervals. However, an overlapping confidence interval does not necessarily guarantee statistical insignificance; in some cases means that are statistically different from each other will also have overlapping confidence intervals (*141*).

Chapter 4. Results/discussion

4.1. Optimization of analysis methods for heterocyclic aromatic amines

All HAA analysis methods used in this study were based on a procedure published by Gibis (*4*). However, some of Gibis' method parameters were changed or additional method features were added to optimize HAA recovery, reproducibility, and chromatographic separation.

4.1.1. Selection of grill

Prior to optimization of analysis methods, establishment of a reproducible cooking was necessary. Our goal was to mimic the high-heat conditions experienced by a burger during typical gas or charcoal grilling and produce a similar HAA profile. To maximize temperature control and minimize variation between cooking events throughout the study, an electric grill was chosen over a gas or charcoal grill. Since electric grills often have low heat output, two grills were tested. We compared grilling results and HAA formation in beef patties cooked on a Hamilton Beach 25331 Super Sear Nonstick Indoor Searing Grill and a Weber Q140 electric grill on their highest temperature settings. However, the Hamilton Beach grill was already unable to produce burgers with the blackened exterior spots characteristic of a grilled burger. In contrast, the Weber Q140 grill produced visibly darker patties and was therefore used to grill all sample patties. The HAA formation based on this cooking method will be discussed later.

4.1.2. Development of an HPLC method

Broad, tailing peaks are a common problem in the chromatographic separation of amines on modified silica columns, because their amine moiety interacts with residual silanol groups on the column material. During the production of reversed phase silica materials silanol groups are partially derivatized with hydrophobic groups such as octadecyl (C18)-groups. However, since these groups are sterically cumbersome, some silanol residues remain

underivatized. Therefore, most modified silica phases are end-capped with compounds such as trimethylchlorosilane. Even end-capped phases contain, however, some silanol groups, which are prone to interactions with the amines. Thus, mobile phase additives, such as triethylamine and ionic liquids, are used to combat this problem by blocking residual silanol groups, i.e. interacting with the silanol groups instead of the analytes (*142, 143*). We compared the addition of 0.01M triethylamine or 1-butyl-3-methylimidazolium tetrafluoroborate (BMIm-BF_4) to the aqueous portion of the gradient, and found that although BMIm-BF_4 produced a much sharper IQ peak compared to triethylamine, the fluorescence baseline of the chromatograms using BMIm-BF_4 was more uneven than the baseline of the chromatograms using triethylamine (Figures 7 and 8). While using BMIm-BF_4 in the aqueous portion of the gradient would certainly have increased the detection limit of IQ, it would have prevented us from reliably identifying and quantifying small amounts of the other fluorescent HAAs. We therefore chose triethylamine for future method work.

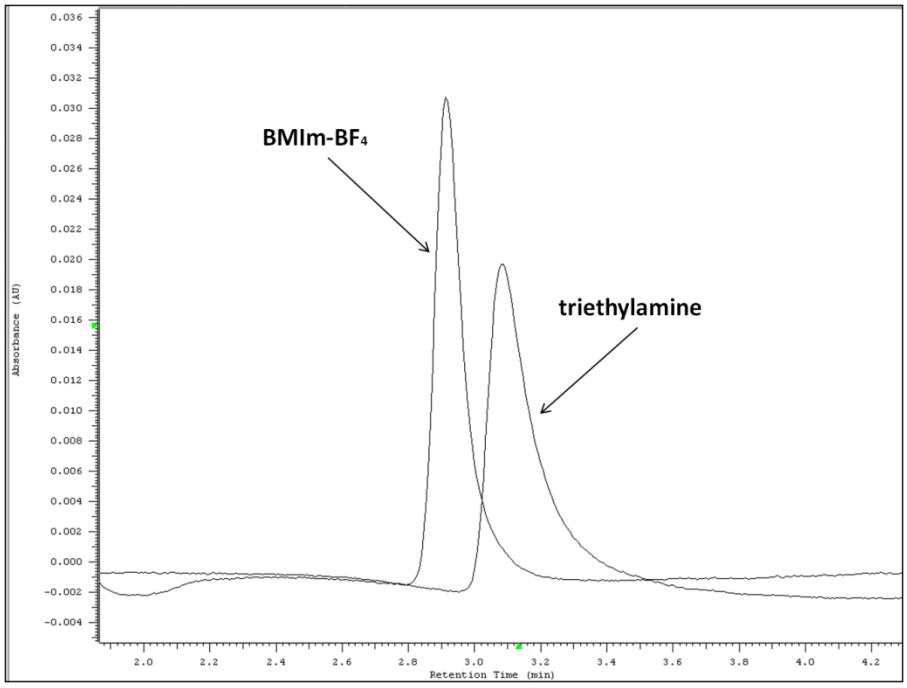

Figure 7: IQ peak shape with different mobile phase additives (UV absorption at 267 nm; BMIM-BF_4: 1-butyl-3-methylimidazolium tetrafluoroborate).

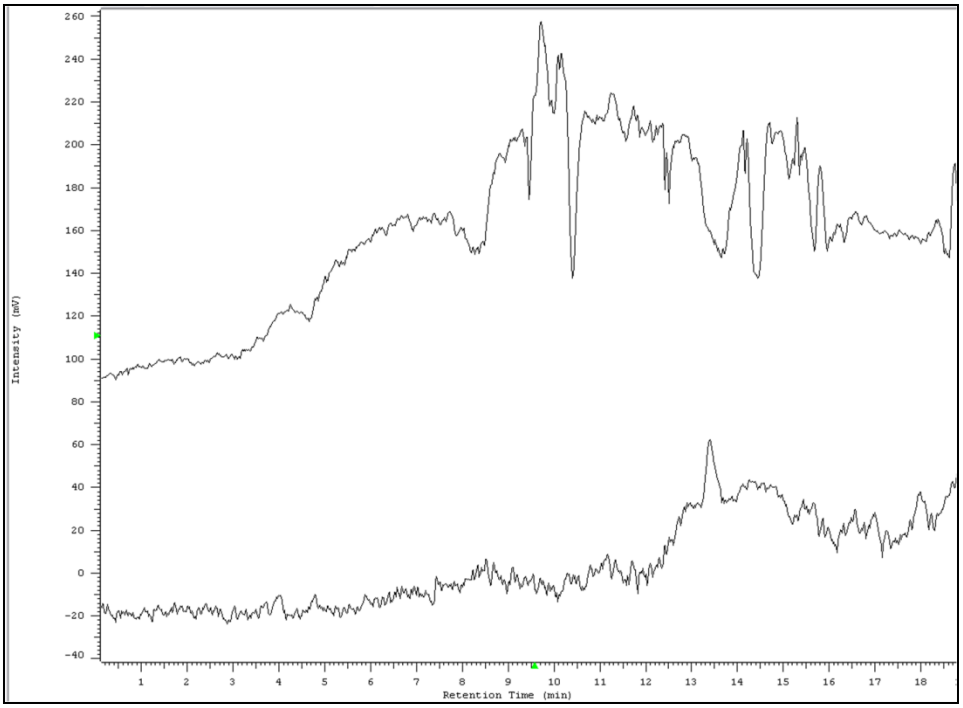

Figure 8: Fluorescence baselines of chromatograms recorded with 1-butyl-3-methylimidazolium tetrafluoroborate (BMIm-BF_4, upper trace) and triethylamine (lower trace) as mobile phase additives.

Three separate columns were compared for their HAA separation effectiveness and practical aspects of use: TSK-gel Super-ODS, Kinetex PFP, and Kinetex XB-C18. Super-ODS and Kinetex XB-C18 are C18-modified silica phases, whereas the Kinetex PFP column is a pentafluorophenyl-modified silica phase, which can provide superior separations for some aromatic compounds compared to other reversed-phase materials. The Kinetex XB-C18 column contains isobutyl-groups in addition to the C18-groups to shield residual silanol groups from interacting with the analytes.

A major drawback of the TSK-gel Super-ODS columns was their limited durability. Split peaks, indicative of dead-space development, began appearing after ~50 runs in three separate TSK-gel Super-ODS columns. Additionally, compared to both of the Kinetex column models, the TSK-gel column was less effective at HAA separation (Figures 9 and 10). Although the XB-C18

column produced an irregular IQ peak shape, it achieved near-baseline separation of 4,8-DiMeIQx and norharman, and was therefore selected for future method development.

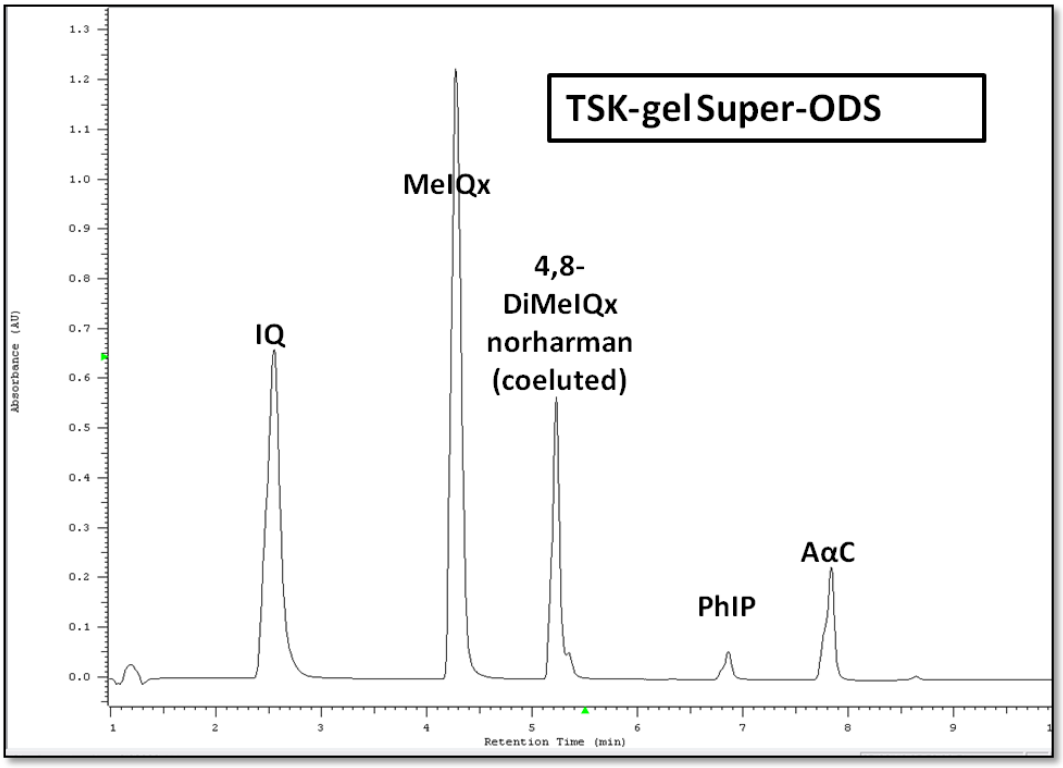

Figure 9: Separation of heterocyclic amine standards on TSK-gel Super-ODS column (UV absorption at 274 nm).

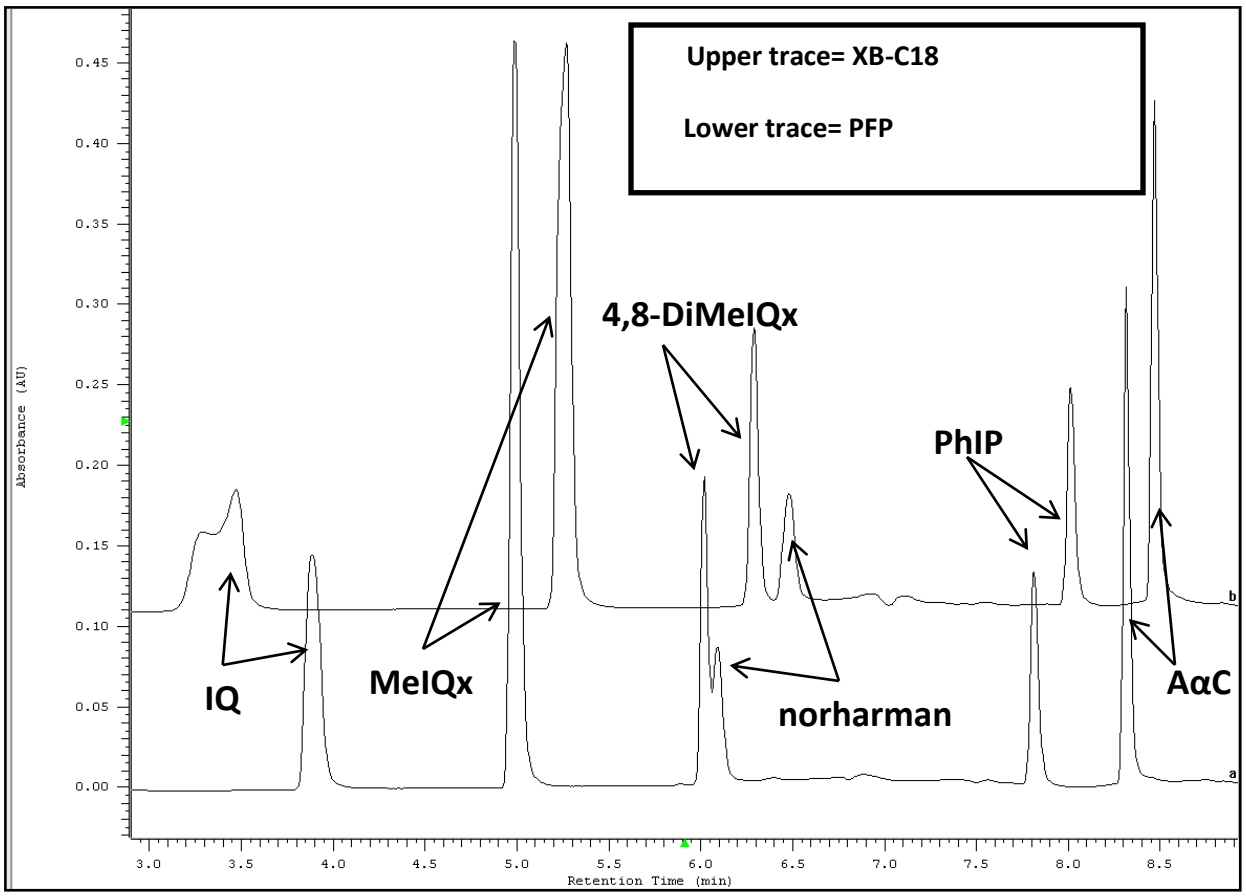

Figure 10: Separation of heterocyclic aromatic amine standards on Kinetex columns (UV absorption at 274 nm).

Our next method improvement step was to change the needle wash solution from pure methanol to 75:25 (v/v) water/methanol. This substantially improved IQ peak symmetry on the Kinetex XB-C18 column and also improved separation of norharman and 4,8-DiMeIQx, which were still prone to co-elution (Figure 11), depending on minor changes in the method.

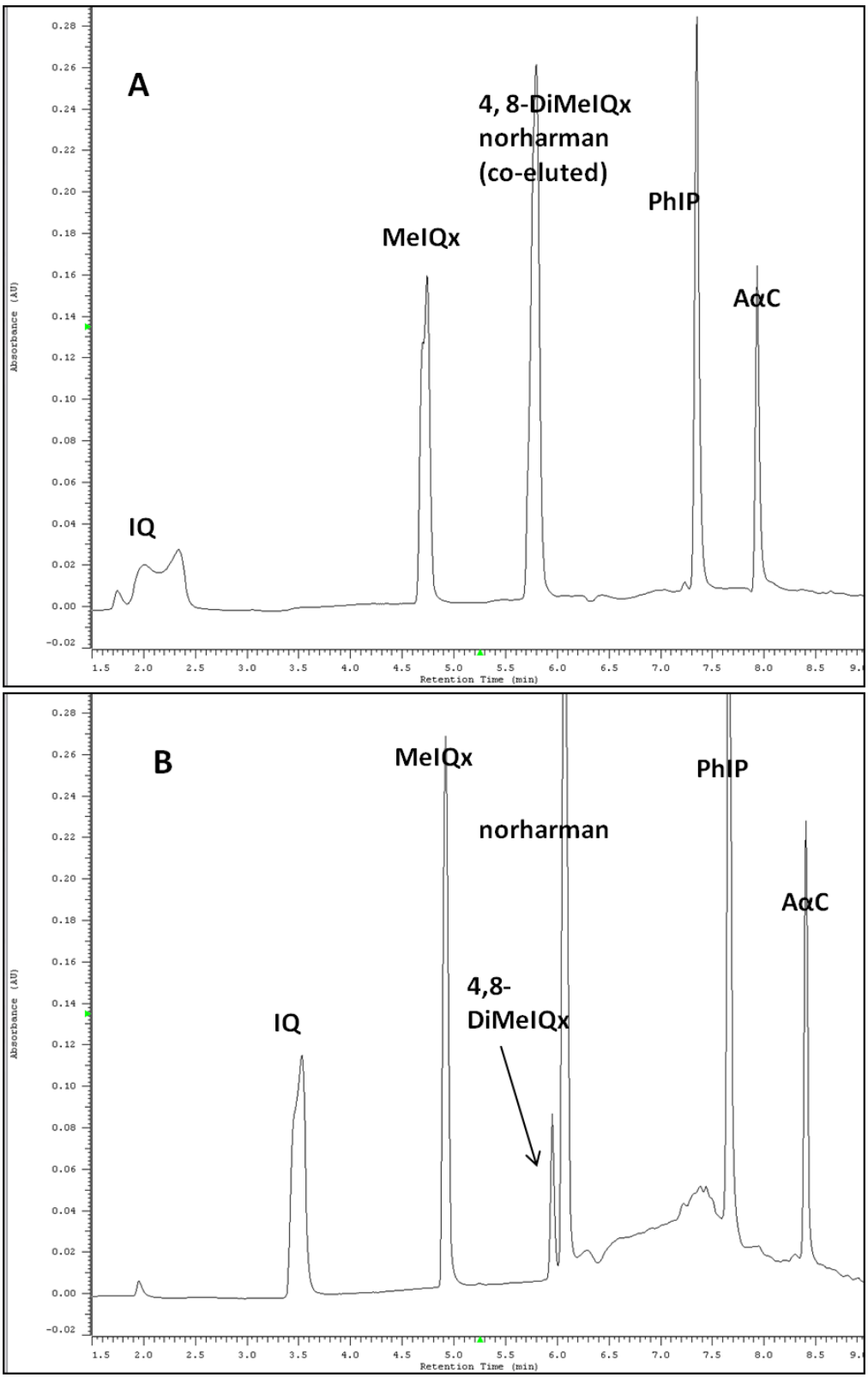

Figure 11: Effect of needle wash polarity. A = pure methanol wash. B = 75/25 water-methanol (UV absorption at 255 nm).

The final method optimization step was to improve HAA separation. Baseline separation of 4,8-DiMeIQx and norharman was achieved by lengthening the gradient (delayed incorporation of acetonitrile in the mobile phase) specified by Gibis (*4*) and adding a hold step around the elution times of these two compounds.

Although this improved gradient worked well for standard solutions, compounds from the beef matrix partially co-eluted with some analytes, which made additional method changes necessary, leading to a total method time (including column flush and equilibration) of 34 min, compared to the 23 min method reported by Gibis. Use of a second organic modifier (methanol/water, 70:30, v/v) enhanced separation. Additionally, the use of two aqueous phases (0.01M triethylamine adjusted to pH 3.00 and 4.00) was abandoned in favor of a single aqueous phase (0.01M triethylamine adjusted to pH 3.40). Finally, the column temperature was increased from 25 to 35 °C.

Figure 12 shows the separation of the analyzed fluorescent HAAs in a beef matrix achieved by the optimized method. However, although this method was optimized for beef extract, incorporation of cereals into beef created another source of matrix interference (compounds already present in the cereals or else formed during cooking from cereal precursors). Peak resolution and separation were susceptible to slight differences between sample runs, such as additional cereal-derived peaks or small variations in elution times. This challenge was especially evident with the less-selective UV detection method.

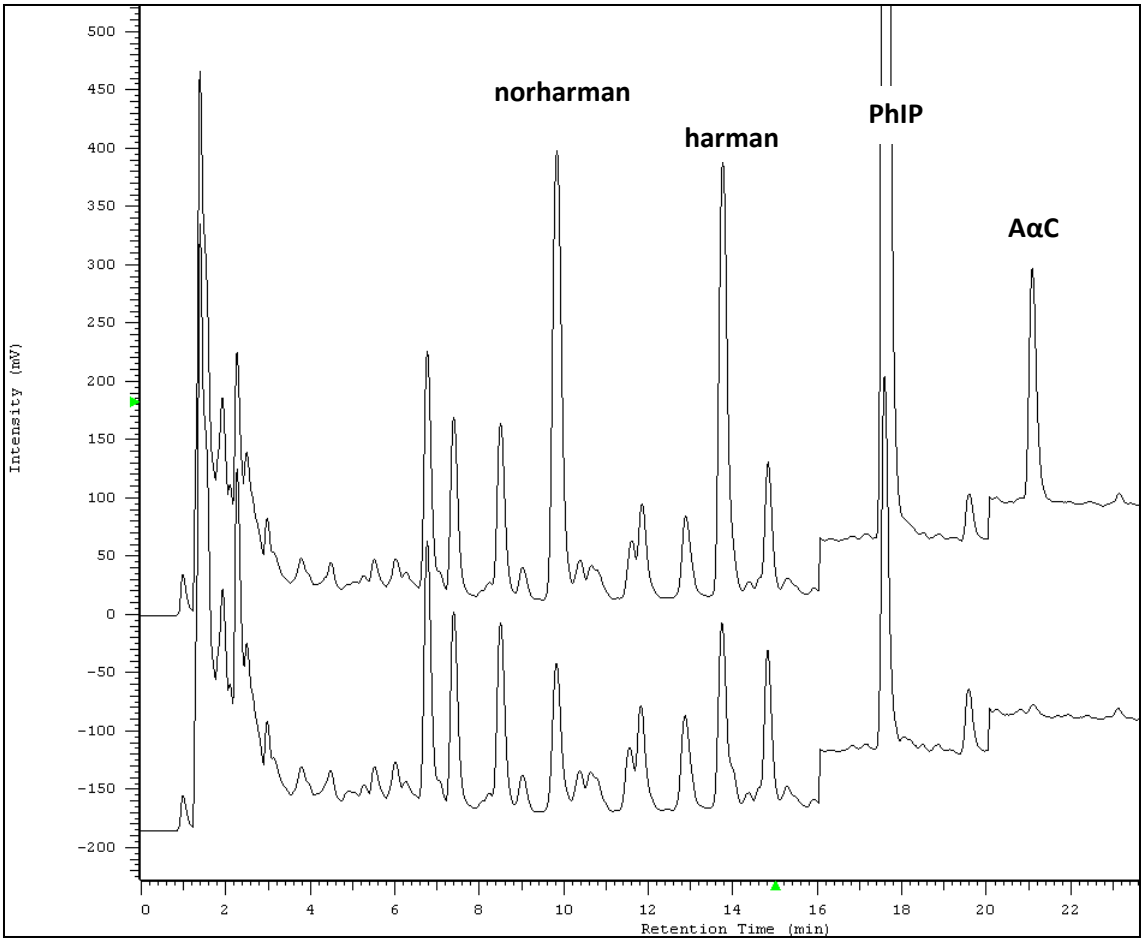

Figure 12: Separation of a beef extract with the optimized HPLC method. Upper trace is the spiked extract; lower trace is the unspiked extract (fluorescence detection).

4.1.3. Optimization of extraction method

In contrast to some other solid phase HAA extraction methods (*113, 118, 135, 144*), the Gibis method did not specify a rest period after blending the cooked beef material with sodium hydroxide solution. We observed that immediately after the blending step, the beef-sodium hydroxide mixture had a glutinous structure that was difficult to mix with diatomaceous earth and formed large lumps during the ethyl acetate extraction step. However, if the basic beef mixture was allowed to rest before incorporation of diatomaceous earth, the stiff, dough-like structure softened into a smooth slurry with few or no lumps during extraction. Therefore, the

first modification to Gibis' extraction protocol was to include a 1 h rest period after blending the cooked beef material with 1M sodium hydroxide solution.

Our second method optimization step was to compare recovery rates for select HAAs when one or two ethyl acetate extraction steps were used. Better recovery rates for norharman, PhIP, and MeIQx (tentatively) were obtained with two ethyl acetate extractions (Table 6), so a second ethyl acetate extraction step was incorporated into the standard HAA extraction procedure.

Table 6: Percent recovery with one vs. two ethyl acetate extractions

HAA	Percent recovery, one ethyl acetate extraction	Percent recovery, two ethyl acetate extractions
MeIQx	26.8*	49.6*
norharman	57.6	66.5
PhIP	50.5	60.6
AαC	33.8	28.8
**Potential coelution observed in UV absorption spectrum of MeIQx peak; values are therefore tentative.*		

Our final extraction method optimization step was to investigate the effect of increasing the polarity of the solution used to dissolve the final beef extract residue. As expected, sharper, narrower peaks were observed with increased solution polarity. The effect was especially pronounced for IQ, which elutes first in the gradient method (Figure 13). Comparing Figures 13A-C, it is clear that decreasing the methanol percentage in the injection solution from 100 to 25% dramatically improved IQ peak symmetry by decreasing both tailing and fronting. Too much organic phase in the injection solution can alter the initial separation environment by forming a small pocket of organic-rich solution within the mostly aqueous environment found at the beginning of a reversed-phase gradient, producing broad, asymmetric peaks (Figure 13A).

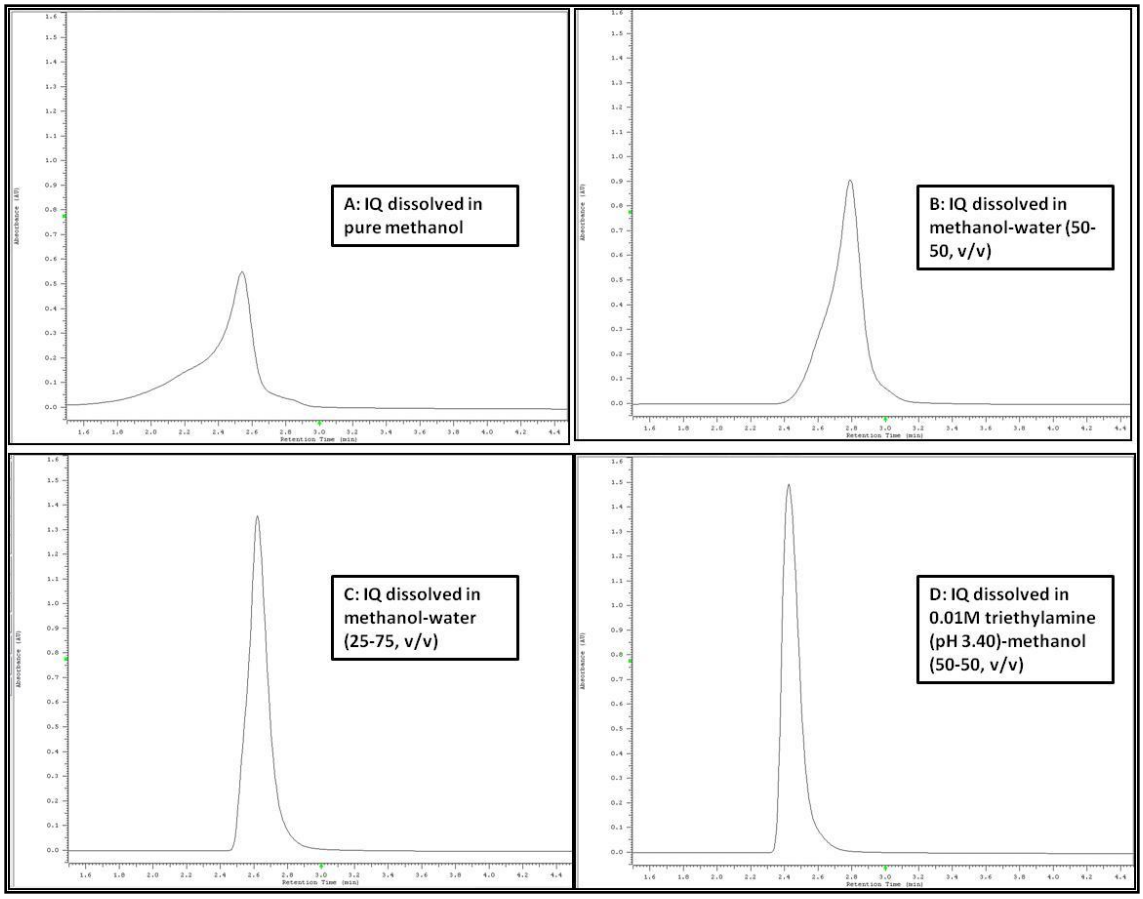

Figure 13: Effect of solution polarity on heterocyclic aromatic amine peak shape. 20 µL injection of IQ standard, UV absorption at 274 nm, all chromatograms similarly scaled.

Using water vs. the HPLC mobile phase A (0.01M triethylamine (pH 3.40)) as the aqueous part of the injection solvent was also compared (Figures 13B and 13D). The acidic mobile phase solution produced a sharper, more symmetric IQ peak than water alone. One possible explanation would be that the pKa of IQ is 5.86 ± 0.40, which is close to the typical pH of distilled water. In the more acidic mobile phase-methanol solution, IQ would exist almost entirely in its protonated form, while in the water-methanol system, the protonated and unprotonated forms would be equally abundant in the injection solution. Once again, when the injection solution produces varying environments in the adjacent mobile phase, IQ will be separated in its protonated and unprotonated forms at the beginning of the HPLC run. Because IQ interacts very little with the stationary phase and elutes rapidly, the resulting IQ peak is broad

and asymmetric (Figure 13B). This effect is less pronounced for compounds interacting more strongly with the stationary phase since the mobile phase environment changes quickly while the compounds are interacting with the stationary phase. Based on these results, we chose to use a 75:25 (v/v) 0.01M triethylamine (pH 3.40)/methanol solution as the injection solution.

Finally, it was not possible to completely redissolve the dried extract residue. After it was double-checked that the HAAs are soluble in the injection solution a centrifugation step was added to remove particulates. This protected the guard cartridge and column from clogging without filtration of the extract, which would have a) led to losses of a portion of the scarce volume, b) added an additional source of error by potential adsorption of the HAAs to the filter membrane, and c) added additional costs for membrane filters.

4.2. Analysis of heterocyclic aromatic amines from cooked beef

Of the seven HAAs analyzed in this study (IQ, MeIQx, 4,8-DiMeIQx, PhIP, norharman, harman, and AαC), all except IQ and 4,8-DiMeIQx (both are detected by UV absorption at 270 nm) were consistently recovered and identified in cooked beef patties. 4,8-DiMeIQx formation could not be assessed because its retention time coincided, depending on the sample, with a cluster of large matrix peaks. IQ formation was only confirmed in one set of cooked patties during the study, but its retention time also fell in a crowded section of the UV chromatogram, which seriously hampered its detection. The fact that both 4,8-DiMeIQx and IQ are not fluorescent prevented them from being detected by using more specific and sensitive fluorescence detection.

Analysis of MeIQx, the third non-fluorescing HAA, was also problematic throughout the study. Although MeIQx was detected in almost all samples, it would have not been scientifically sound to quantitatively analyze this compound in many chromatograms because of partial co-elution with matrix compounds (Figure 14). If the MeIQx peak was at least partially separated,

two-peak deconvolution was performed with the Hitachi HSM software. However, this option was only possible if a partial separation was present. Pure MeIQx has a major absorption peak at 268 nm and does not absorb in the range of 280-300 nm (Figure 14C). In the example from Figure 14, although the MeIQx peak from the spiked extract appears clean at first glance according to its spectrum from the diode array detector (DAD) (Figure 14D), the peak purity was only 95% and the peak clearly displays both left and right shoulders (upper trace, Figure 14B). The DAD spectrum for the MeIQx peak from the unspiked extract is clearly contaminated, with absorption in the 280-300 nm range (Figure 14E) that cannot be completely explained by the coeluting peak on the right. This indicates that there is a hidden peak underneath the MeIQx peak (seen as a left shoulder in the spiked extract).

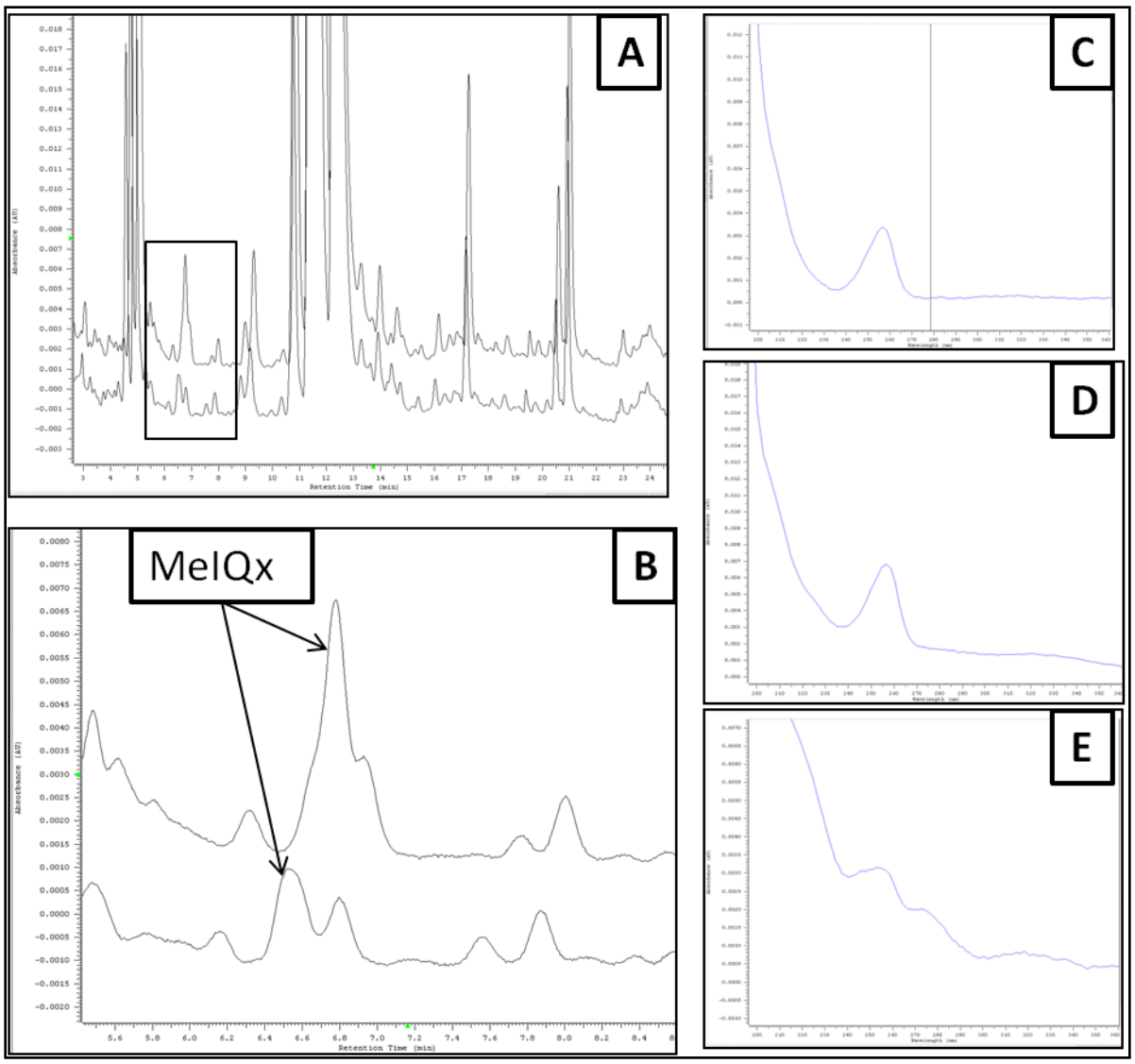

Figure 14: MeIQx co-elution example. A and B: UV absorption chromatograms at 268 nm. Upper trace = spiked extract, lower trace = unspiked extract. C: UV absorption spectrum of MeIQx standard. D: UV absorption spectrum of MeIQx peak from spiked extract. E: UV absorption spectrum of MeIQx peak from unspiked extract.

Norharman, harman, and PhIP were easily quantified in all samples due to the sensitivity and increased selectivity of fluorescence detection. Quantifiable levels (signal to noise ratio of > 9:1) of AαC were also observed in almost all samples, even though the calculated AαC concentrations in the burgers were very low (see Table 7 in Section 4.4). These results underscore the advantage of using a sensitive and more selective detection method such as

fluorescence. However, fluorescence detection cannot be used for the IQ- and IQx-type HAAs, which explains the growing use of LC-MS/MS instrumentation in HAA analysis (*135*).

4.3. Recovery rates of heterocyclic aromatic amines from beef matrices

Recovery rates (see Appendix D for a sample calculation) varied between individual HAAs. Recovery rates up to 106, 84, and 78% were obtained for norharman, harman, and PhIP, respectively. AαC recovery was lower, with up to 55% recovery being observed. These recovery rates are similar to those reported in the literature for the analysis of these HAAs from meat matrices (*4, 71*). In the few samples where MeIQx was quantified, recovery rates up to 81% were observed. The range of recovery rates among the HAAs is due to their diversity of chemical structures, resulting in differences in hydrophobicity, acid-base behavior, and solubility.

Recovery rates gradually increased over the course of the study, reflecting increased proficiency with the complex extraction method. Recovery rates also differed between treatments, with recoveries tending to be higher in the patties cooked with cereal than the plain beef. This is likely a result of the cereal material making the initial beef-sodium hydroxide-diatomaceous earth extraction matrix less glutinous, thus reducing the formation of lumps and increasing the beef-extraction solvent contact surface area.

Recovery rate variability between different HAAs within the same matrix and between different matrices underlines the value of using the standard addition quantification method, since use of an internal or an external standard cannot adequately correct for these individual variations. Besides standard addition, which is an extremely laborious way of quantification, stable isotope dilution would be another suitable HAA analysis method. However, stable isotope dilution analysis is costly, because the method necessitates stable isotopes for all analyzed HAAs and also a mass spectrometric detector.

4.4. Effects of cereal additives on heterocyclic aromatic amine levels in cooked burgers

Norharman, harman, PhIP, and AαC contents of the patties cooked with cereals are presented in Table 7. Please note that the values in Tables 7 and 8 represent the amounts of HAAs that are formed per g raw beef during cooking. For a sample calculation demonstrating how HAA levels from the cooked beef containing cereal additives were calculated into levels per g raw beef see Appendix D. In addition, chromatograms from the cereal treatments are found in Appendix E. Treatments are grouped according to cooking events, with the values for the plain sample cooked on the same pan presented for each cooking event. As mentioned previously, IQ and 4,8-DiMeIQx were not detected in these samples. MeIQx was detected in every sample, but not usually quantified because of matrix interferences. MeIQx was successfully quantified in duplicate in three samples from the same cooking event. These values are shown in Table 8 (see also Appendix E), and their UV chromatograms are provided in Appendix F.

Table 7: Norharman, harman, PhIP, and AaC contents of grilled beef patties* with and without cereal treatments

Sample		Norharman			Harman			PhIP			AαC		
		*ng HAA/g raw beef ± SD***	*upper 95% CI ****	*lower 95% CI ****	*ng HAA/g raw beef ± SD*	*upper 95% CI*	*lower 95% CI*	*ng HAA/g raw beef ± SD*	*upper 95% CI*	*lower 95% CI*	*ng HAA/g raw beef ± SD*	*upper 95% CI*	*lower 95% CI*
Set A	5% raw corn flour	4.42 ± 0.11	*5.21*	*3.62*	3.58 ± 0.19	*5.42*	*1.75*	6.21 ± 0.34	*10.08*	*2.34*	0.14 ± 0.13	*6.60*	*0 (-6.32)*
	5% corn bran	4.90 ± 0.41	*8.19*	*1.61*	2.92 ± 0.52	*7.70*	*0 (-1.86)*	3.95 ± 0.12	*4.96*	*2.94*	0.13 ± 0.02	*1.01*	*0 (-0.74)*
	5% cooked wild rice flour	5.34 ± 0.25	*7.33*	*3.35*	4.10 ± 0.44	*8.91*	*0 (-0.71)*	4.27 ± 0.06	*4.80*	*3.73*	0.14 ± 0.14	*7.00*	*0 (-6.73)*
	plain	2.08 ± 0.76	*6.25*	*0 (-2.08)*	1.30 ± 0.39	*3.73*	*0 (-1.13)*	2.99 ± 0.28	*5.28*	*0.70*	0.14 ± 0.06	*3.18*	*0 (-2.90)*
Set B	10% raw corn flour	6.31 ± 0.80	*12.73*	*0 (-0.10)*	4.93 ± 0.65	*12.29*	*0 (-2.42)*	4.96 ± 1.21	*15.48*	*0 (-5.56)*	0.36 ± 0.25	*1.59*	*0 (-0.87)*
	10% corn bran	7.07 ± 2.55	*29.86*	*0 (-15.72)*	3.86 ± 1.10	*14.56*	*0 (-6.85)*	2.11 ± 0.66	*5.69*	*0 (-1.47)*	0.17 ± 0.02	*0.25*	*0.10*
	10% cooked wild rice flour	12.24 ± 2.70	*48.34*	*0 (-23.85)*	11.30 ± 0.98	*33.94*	*0 (-11.33)*	7.28 ± 1.38	*23.59*	*0 (-9.02)*	0.33 ± 0.35	*1.98*	*0 (-1.33)*
	plain	4.63 ± 1.20	*14.95*	*0 (-5.68)*	2.27 ± 0.27	*4.58*	*0 (-0.05)*	2.09 ± 0.55	*5.70*	*0 (-1.52)*	0.30 ± 0.50	*3.03*	*0 (-2.44)*
Set C	5% raw wild rice flour	**2.44 ± 0.23**	***3.61***	***1.28***	3.89 ± 0.65	*10.70*	*0 (-2.91)*	1.13 ± 0.31	*2.50*	*0 (-0.24)*	0.03 ± 0.04	*0.17*	*0 (-0.11)*
	5% wild rice hulls	**11.37 ± 0.51**	***19.32***	***3.43***	**6.66 ± 0.21**	***10.44***	***2.89***	1.15 ± 0.09	*1.57*	*0.73*	0.01 ± 0.01	*0.03*	*0.00*
	5% oat hulls	**4.22 ± 0.21**	***5.80***	***2.64***	**1.93 ± 0.07**	***2.46***	***1.40***	1.29 ± 0.33	*2.88*	*0 (-0.30)*	0.11 ± 0.09	*0.46*	*0 (-0.24)*
	plain	0.89 ± 0.06	*1.14*	*0.65*	0.62 ± 0.12	*1.13*	*0.12*	0.72 ± 0.11	*1.15*	*0.29*	0.02 ± 0.02	*0.08*	*0 (-0.04)*
Set D	10% raw wild rice flour	5.21 ± 0.50	*8.74*	*1.68*	3.44 ± 0.73	*9.74*	*0 (-2.87)*	2.57 ± 0.40	*4.89*	*0.25*	0.11 ± 0.06	*0.31*	*0 (-0.09)*
	10% oat hulls	2.52 ± 1.21	*8.83*	*0 (-3.78)*	2.35 ± 0.10	*3.11*	*1.59*	1.70 ± 0.24	*2.93*	*0.46*	0.10 ± 0.02	*0.19*	*0.02*
	plain	1.28 ± 0.28	*2.50*	*0.06*	0.92 ± 0.26	*2.25*	*0 (-0.42)*	0.61 ± 0.08	*0.92*	*0.31*	detected	NA	NA
Set E	5% cooked corn flour	2.59 ± 0.46	*4.97*	*0.21*	1.47 ± 0.25	*2.82*	*0.12*	1.99 ± 0.28	*3.47*	*0.50*	0.07 ± 0.04	*0.20*	*0 (-0.06)*
	10% cooked	4.10 ± 0.79	*8.89*	*0 (-0.69)*	2.51 ± 0.37	*5.06*	*0 (-0.03)*	0.20 ± 0.30	*1.12*	*0 (-0.72)*	0.17 ± 0.10	*0.56*	*0 (-0.22)*

Sample		Norharman			Harman			PhIP			AαC		
		*ng HAA/g raw beef ± SD***	*upper 95% CI ****	*lower 95% CI ****	*ng HAA/g raw beef ± SD*	*upper 95% CI*	*lower 95% CI*	*ng HAA/g raw beef ± SD*	*upper 95% CI*	*lower 95% CI*	*ng HAA/g raw beef ± SD*	*upper 95% CI*	*lower 95% CI*
	corn flour												
	10% wild rice hulls	19.56 ± 2.46	*44.10*	*0 (-4.97)*	12.47 ± 1.60	*32.10*	*0 (-7.16)*	1.47 ± 0.36	*2.80*	*0.14*	0.11 ± 0.07	*0.35*	*0 (-0.13)*
	plain	1.29 ± 0.46	*3.26*	*0 (-0.68)*	0.62 ± 0.22	*1.57*	*0 (-0.33)*	0.44 ± 0.13	*0.92*	*0 (-0.03)*	0.07 ± 0.12	*0.50*	*0 (-0.36)*
Set F	corn bran extract	4.48 ± 1.77	*17.18*	*0 (-8.23)*	2.76 ± 1.02	*11.28*	*0 (-5.75)*	3.53 ± 0.76	*9.45*	*0 (-2.39)*	0.07 ± 0.13	*0.52*	*0 (-0.37)*
	plain	1.49 ± 0.52	*3.88*	*0 (-0.90)*	0.98 ± 0.09	*1.44*	*0.51*	1.54 ± 0.23	*2.76*	*0.31*	0.04 ± 0.06	*0.24*	*0 (-0.16)*

*Samples with **values in bold** have confidence intervals that do not overlap with the confidence interval of the corresponding plain sample.*
**n=2 for all samples*
***SD = standard deviation*
****CI = confidence interval. Values represent the upper and lower boundaries of the 95% CI range, with units of ng HAA/ g raw beef.*

Table 8: MeIQx content of selected treatments

Sample		*ng MeIQx/g raw beef ± SD**	*upper 95% CI **	*lower 95% CI **
Set A	5% raw corn flour	*1.64 ± 0.11*	*2.22*	*1.05*
	5% cooked wild rice flour	*0.41 ± 0.18*	*1.05*	*0 (-0.23)*
	plain	*0.75 ± 0.11*	*1.24*	*0.25*

**SD = standard deviation*
***CI= confidence interval. Values represent the upper and lower boundaries of the 95% CI range, with units of ng HAA/g raw beef*

Although we took steps to minimize all factors potentially increasing the variability of HAA formation between cooking events (using meat from the same muscle group of a single animal, creating a standardized thawing procedure, cooking with an electric instead of a gas or charcoal grill, blending of patties cooked on different spots of the grill etc.), the values for the blank samples already show some variability between individual HAA values from different cooking events (see Figure 15).

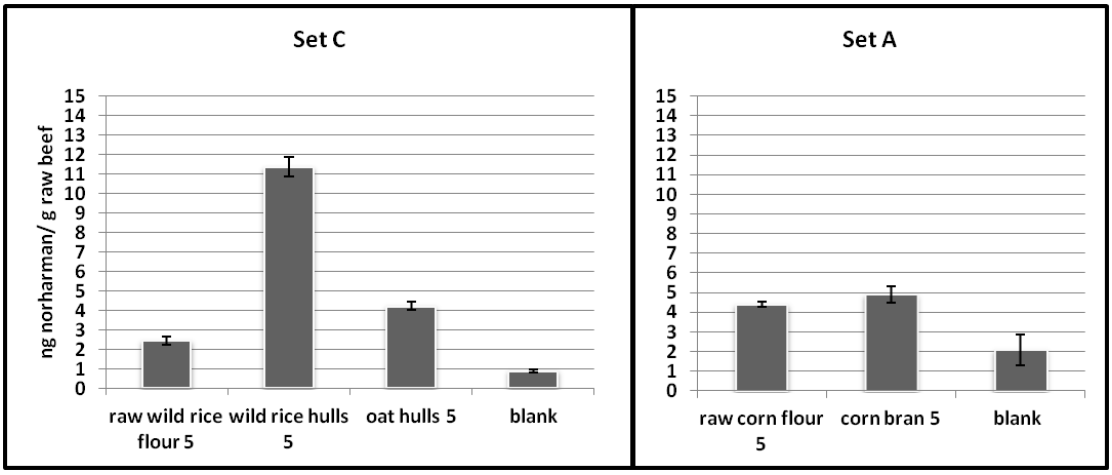

Figure 15: Norharman concentrations from two cooking sets. Note differing blank values.

Multiple factors could contribute to these differences, but inconsistent cooking conditions (for example, different maximum temperatures) between cooking events are assumed to be primarily responsible. Therefore, direct comparison of HAA concentrations from cereal treatments is only valid for other treatments cooked in the same set. Calculated 95%

confidence intervals were relatively broad, and most treatment samples overlapped the confidence interval of their respective plain sample. This is a reflection of the uncertainty created by only analyzing samples in duplicate, but additionally, confidence intervals for standard addition data are generally broader than confidence intervals for other data because standard addition is an extrapolation technique. Comparison of HAA treatment values between different sets can only be done as percentages of their respective blanks, thus adjusting for cooking variability.

4.4.1. Effects of wild rice materials

Beef patties cooked with 5% cooked wild rice flour had slightly less MeIQx than their respective plain sample (Table 8). Wild rice has been shown to decrease thiobarbituric acid reactive substances (TBARS) in beef patties during 48 h of storage at -18 °C (*145*). The TBARS assay measures malondialdehyde equivalents (secondary lipid oxidation products) in a sample, and is therefore not a direct assessment of radical-scavenging ability. Wild rice and wild rice hull extract also showed antioxidant capabilities (assessed by TBARS) in raw ground beef during storage. A major contributor to this activity was suggested to be phytic acid (*146*), which suppresses lipid oxidation by chelating metal ions, not scavenging radicals. Therefore, phytic acid would not affect MeIQx formation by scavenging pyrazine radicals. Later work published by this group, however, identified several phenolic compounds with antioxidant capabilities (as assessed with TBARS) in wild rice extracts, including vanillin, *m*-hydroxybenzaldehyde, and syringaldehyde (*147*), which would all have potential pyrazine radical-scavenging capabilities.

In contrast, concentrations of the HAAs norharman and harman, with potential major formation routes not involving radicals, were increased with wild rice treatments. For example, addition of 10% wild rice hulls (Set E in Table 7) resulted in 15- and 20-fold increases in norharman and harman, respectively, for this treatment compared to its control. Addition of 5%

wild rice hulls produced significantly greater norharman and harman levels (13- and 11-fold increases over the control). Wild rice flour also tended to increase norharman and harman levels, but not to the same extent as the hulls. Wild rice contains 0.194 g tryptophan/100 g dry matter (*148*), a precursor for norharman and harman. For comparison, whole yellow corn flour, which did not produce as dramatic an increase in norharman and harman relative to its blank compared to wild rice materials (Figure 16), contains only 0.055 g tryptophan/100 g dry matter (*148*). Although the formation pathways for harman and norharman are not fully understood yet, adding additional tryptophan to the cooking system via wild rice materials apparently dominates any potential HAA-reducing effects of other wild rice constituents such as trapping Maillard reaction intermediates (if mechanisms such as the Pictet-Spengler condensation are considered) or trapping radicals (if radicals from tryptophan pyrolysis are involved in β-carboline formation).

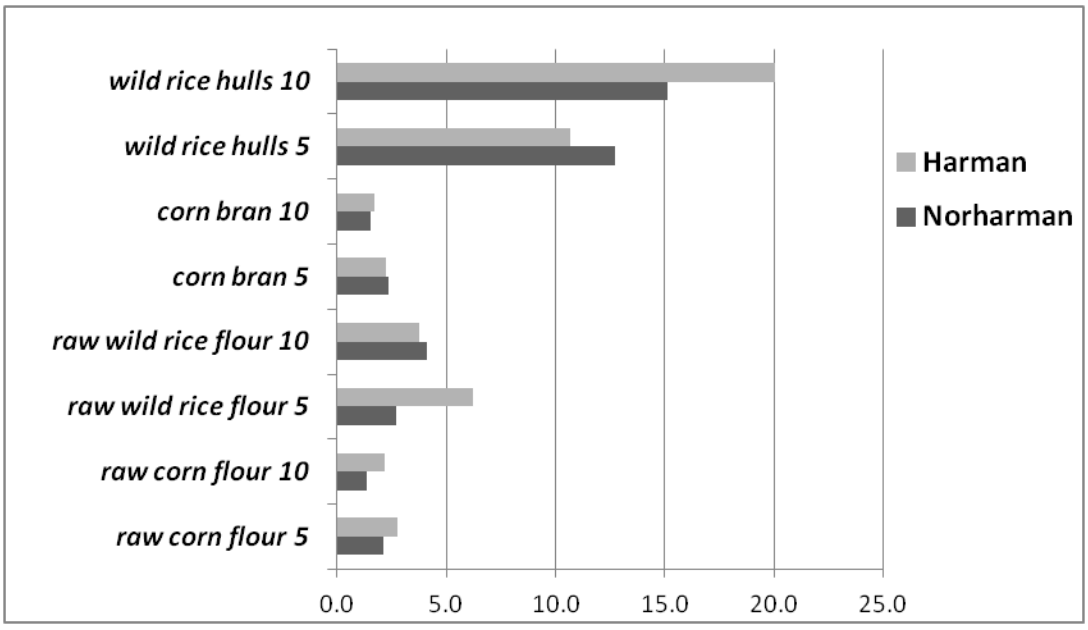

Figure 16: Relative norharman and harman contents of wild rice and corn treatments. Values are presented as ratios of the heterocyclic aromatic amine concentrations of treatment samples to heterocyclic aromatic amine contents of the respective blanks.

PhIP formation was also promoted with wild rice treatments. Wild rice contains substantial amounts of phenylalanine (0.78 g/100 g dry solids) (*148*), which has been demonstrated to be a precursor of PhIP formation (*77*). Although it has been suggested that free radicals may be involved in PhIP formation, these results suggest that increased precursor concentration is a stronger determiner of PhIP production than the presence of antioxidants.

4.4.2. Effects of corn materials

We had hypothesized that corn, because of its high levels of hydroxycinnamic acids, would be most effective at scavenging radicals and reducing HAA formation out of the cereals used in this study. Again, norharman and harman, where the involvement of radicals in their formation mechanism is still only theoretical, were not reduced in the corn treatments relative to the blanks. However, the levels formed were only in the range of ~1-3.5 times the blank, in contrast to wild rice materials. This is most likely due to the lower tryptophan content in corn compared to wild rice.

The effect of corn materials on PhIP formation was unclear; the corn bran samples had PhIP levels similar to blank levels, but the corn flour samples appeared to tend towards increased levels. Compared to wild rice flour, the phenylalanine content of corn flour is lower (0.38 g/100 g dry solids) (*148*), but the content of some antioxidants such as the hydroxycinnamic acids is much higher (*131*). Many research groups have successfully applied antioxidants to PhIP reduction (*109, 111, 117*). However, it is unclear from our results whether the lower PhIP formation in burgers containing corn samples compared to burgers containing wild rice samples is due to higher antioxidant concentration or lower phenylalanine concentration, or both (Figure 17).

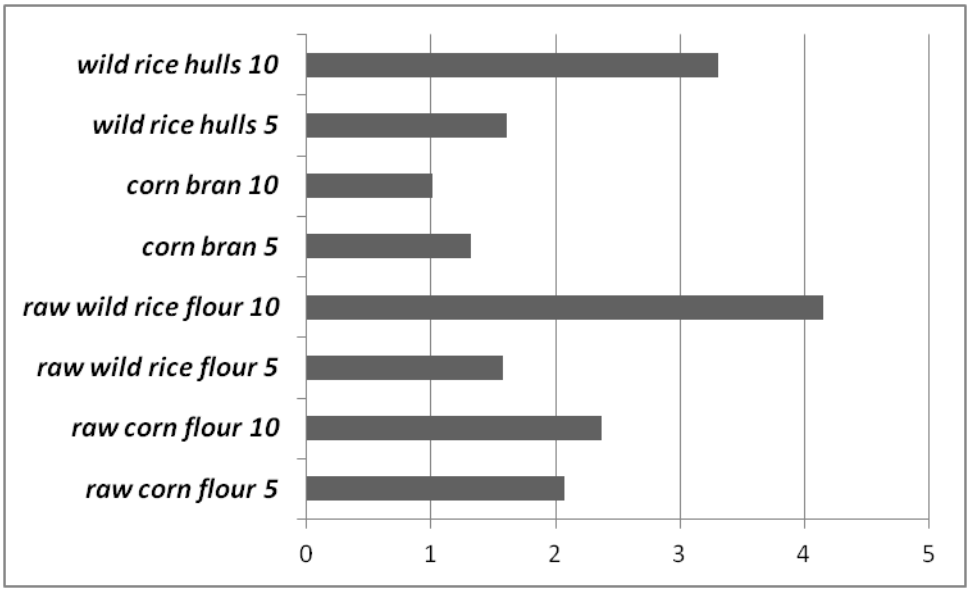

Figure 17: Relative PhIP contents of wild rice and corn treatments. Values are presented as ratios of the heterocyclic aromatic amine concentrations of treatment samples to heterocyclic aromatic amine contents of the respective blanks.

We were unable to quantify the IQ- and IQx-type derivatives, which are the HAAs where radical scavenging has been linked to reduced formation in the past (*104, 149*), in most corn-based treatments (except for 5% cooked corn flour). MeIQx content of the burger containing 5% cooked corn flour was not reduced relative to the blank. The inability of the analytical procedures used in this study to detect and quantify the IQ/IQx derivatives is unfortunate, especially for the corn samples since corn contains abundant amounts of radical-scavenging antioxidants. Thus, this analytical set-up prevented us from definitely testing our hypothesis.

4.4.3. Effects of oat hulls

Beef patties cooked with 5% oat hulls formed significantly greater amounts of norharman and harman compared to plain beef (4.5 and 2.9X the plain levels, respectively). This may be due to tryptophan levels in oats (0.204 g/100 g dry solids, although this value is from oat grain and not the hulls) (*148*). However, unlike wild rice hulls, increased levels (10% vs. 5%) of oat hulls did not appear to produce relatively more norharman and harman levels, indicating that although tryptophan is a determining factor of β-carboline formation, it is only one

important constituent to consider in the system. While higher amounts oat hulls add more tryptophan to the reaction system it can get depleted in other precursors possibly required for the β-carboline formation such as aldehydes or Strecker aldehyde precursors, respectively. PhIP levels for oat hull-treated samples were 1.7 and 2.5 times the blank for the 5 and 10% treatment levels, respectively, which may reflect the addition of phenylalanine through the oat material (0.75 g/ 100 g dry oat flour solids) (*148*). AαC contents formed in all replicates from these treatment sets were quite low, leading to high coefficients of variation (standard deviation as a percentage of the mean) and limiting valid treatment effect analysis for this compound.

4.4.4. Effects of corn bran extract

We chose to investigate the effect of adding whole cereal materials to beef patties because this approach mirrors the current use of cereal materials in other cooked beef products such as meatloaf and, if successful, would likely be a consumer-acceptable HAA-mitigation strategy. However, from a scientific standpoint, using whole cereal materials adds complexity and potential confounding factors to the already-challenging area of HAA reduction by altering moisture retention and migration during cooking (*43, 150*), and contributing Maillard reaction precursors (amino-group containing compounds including amino acids and reducing sugars) and specific amino acids required for HAA formation (such as tryptophan) to the cooking system. Reductions or increases in HAA levels could be attributed to any of these factors or to interplay of some or all of them. Thus, we prepared a corn bran extract to selectively investigate the effects of cereal grain antioxidants on HAA formation with minimal interference from added amino acids and reducing sugars. Although our extraction protocol probably did not eliminate 100% of the reducing sugars or amino acids, their levels should be dramatically reduced compared to the addition of the whole grains. Additionally, alkaline hydrolysis of the corn bran following the organic solvent extraction liberated the ester-linked hydroxycinnamic acids such as

ferulic acid and its derivatives. The radical scavenging activity of ferulic acid should not be dramatically changed by being ester-linked to cell wall polymers as compared to the free acid because its antioxidant activity depends on the phenolic hydroxyl group, not the carboxyl group. However, the free acids can more easily migrate in the matrix. The extract was adsorbed to cellulose and incorporated into burgers. The purpose of this method of extract addition was a) to match the moisture content level of the extract-treated burger to the whole material treatments and b) to mimic the distribution of antioxidants that is achieved by adding the flours. Cellulose was chosen over starch because the majority of polysaccharides in corn bran are non-starch cell wall polysaccharides, and cellulose is the second major (after arabinoxylans) cell wall polysaccharide of corn bran. An extract mimicking a 10% addition of the whole material (corn bran) was used in the cooking experiment.

Norharman, harman, PhIP, and AaC formed at levels ranging from 2-3 times the HAA formation in the blank. Thus, major differences between the addition of 10% corn bran and the extract thereof to the beef patties was not observed. This increase is certainly surprising given the fact that most amino acids should have been excluded from the extract added to the burger. In the UV chromatograms from the unspiked corn bran extract samples, the MeIQx peak was very small in one replicate (but not quantifiable due to co-elution) and undetectable in the second replicate. Although no firm conclusions can be drawn from this single experiment, it is possible that the extract's phenolic acids liberated from the corn cell walls during saponification had suppressed MeIQx formation by scavenging radicals.

4.4.5. Moisture retention and formation of heterocyclic aromatic amines

Several research groups have discussed the relationship of moisture-holding capacity of meat and HAA formation during cooking. Increased moisture retention is associated with

increased temperature at the meat cooking surface, because less energy is used to evaporate moisture and is therefore available to heat the surface (*150*). Increased surface temperature alone would promote increased HAA formation. However, the situation is complicated by the fact that HAA precursors (amino acids, creatine, and reducing sugars) are transported to the high-temperature cooking surface during moisture evaporation, so moisture retention decreases the concentration of HAA precursors at the meat surface. Increased moisture retention in fried beef patties has been associated with decreased HAA formation in previous studies (*43, 85, 150*). In contrast, Smith et al. (*151*) did not find a correlation between HAA formation and moisture retention in grilled beef steaks.

Beef patties fried with cereal flours lost less moisture per gram of raw beef than either plain patties or patties prepared with bran or hull fractions (Table 9). This effect is likely due to the presence of starch in the cereal flour. Starch granules absorb water and expand during heat-moisture treatment (gelatinization). Samples prepared with cooked flour tended to lose less moisture than samples prepared with raw flour. Starch granules in the cooked flour were completely gelatinized and were therefore likely able to absorb more moisture during the cooking process than the raw starch granules, which may not have had enough time to completely gelatinize.

Table 9: Cooking loss during frying

Treatment	5% addition		10% addition	
	g of cooking loss/ g of raw beef	*percent ratio of HAAs formed in sample to blank**	*g of cooking loss/ g of raw beef*	*ratio of HAAs formed in sample to blank**
raw wild rice flour	0.49	332.8	0.46	402.7
cooked wild rice flour	0.48	212.6	0.47	335.3
wild rice hulls	0.55	852.5	0.51	1383.5
raw corn flour	0.49	220.2	0.46	178.3
cooked corn flour	0.46	251.7	0.45	287.3
corn bran	0.54	182.8	0.49	142.2
blank	0.56 g of cooking loss/ g beef (average)			

**Sum of norharman, harman, PhIP, and AαC formed in treatment sample as a percent ratio to sum of these HAAs formed in the respective blank. 100=equivalent to blank.*

Because previous research reported a reduction in norharman and PhIP formation in fried beef patties via incorporation of raw potato starch (*43*), we compared the effects of raw and cooked cereal flour treatments on HAA formation. No clear trend was observed in HAA formation with cooked versus raw flour.

Chapter 5. Conclusions and future research directions

The principal source of HAAs, a class of potent mutagenic compounds formed during high heat cooking of proteinaceous foods, in human diets is overcooked meats. Reduction of HAA formation during cooking is a proactive approach to limiting HAA intakes. We hypothesized that the addition of cereal materials to beef patties would reduce HAA formation during cooking due to the presence of radical-scavenging antioxidants in the cereals. The formation mechanisms of HAAs are assumed to involve free radicals, although mechanistic work supporting the involvement of radicals in HAA formation has only been reported for the IQx-type HAAs.

In preparation for testing our hypothesis, we optimized a previously validated HAA extraction method and developed an HPLC separation method. We then quantified HAA concentrations in burgers cooked with 5 and 10% raw wild rice flour, wild rice hulls, cooked wild rice flour, raw corn flour, corn bran, cooked corn flour, and oat hulls. We also prepared a corn bran extract and added it to burgers using cellulose as a carrier. Contrary to our hypothesis, we observed no difference or else an increase in norharman, harman, PhIP, and AαC levels of treated samples relative to plain samples. For the patties cooked with whole cereal materials, the addition of HAA precursors (especially tryptophan and phenylalanine) to the cooking matrix via the cereals may have promoted increased HAA formation. However, this should not have been the case for the patties prepared with corn bran extracts since the extraction method should have excluded most amino acids.

We were unable to effectively analyze the IQx-type HAAs due to matrix interference in the UV chromatograms, thus our hypothesis with regard to these HAAs could neither be proven nor rejected.

Although the IQx-type HAAs were the major targets of our hypothesis, the results for the other HAAs were somewhat unexpected and suggested several follow-up research lines. First, the need for additional basic mechanistic work investigating the formation of HAAs is imperative to identify valid HAA reduction strategies. Specifically, antioxidants have been heavily prescribed for general HAA reduction without solid mechanistic justification, and some studies, including this one, have not observed HAA reductions when antioxidant-rich treatments are applied. It is possible that the HAA reductions observed in some studies were a result of a non-antioxidant effect, such as precursor blocking, or that HAA formation suppression via antioxidants only applies to some HAA classes (such as the IQx-type HAAs) and not to others. In addition, it is possible that more than one pathway exists for the formation of some HAAs, such as PhIP. In this case, adding crude extracts may suppress one pathway but simultaneously increase another pathway.

Thus, activity-guided fractionation offers a promising strategy for expanding our understanding of the mechanism of reduced HAA formation observed with many plant-based antioxidant sources. This method takes advantage of a full repertoire of extraction and fractionation techniques to trace and identify the active (in this case, HAA-suppressing) compounds of a crude extract. Once these compounds are identified, their mode(s) of action(s) can be investigated.

Model systems may provide an environment that facilitates an activity-guided fractionation approach by providing a way to reduce variability between cooking events. As demonstrated in this study, it is difficult to control variability in the cooking event, even with a carefully designed experimental set-up. Although models systems (for example, heating beef juice in an oil bath or temperature-controlled heating block instead of cooking beef patties on a grill) do not fully reflect real-life situations , they may provide a way to reduce variability.

Additionally, if extracts are tested, an even, reproducible distribution of these extracts in a beef patty or on a steak is difficult to achieve, but a beef juice model system could easily incorporate extracts. Finally, the activity-guided approach requires an assay that is fast to perform. Although HAA analysis will always be time-consuming, using a model system will reduce analysis time. However, a beef juice model system has at least one serious disadvantage; the moisture content is very different than in, for example, a burger or a steak. Since moisture content affects HAA formation, the active compounds identified in an activity-guided fractionation process based on a model system must be tested in real meat products.

Finally, the results from this study also highlight the necessity of a sensitive, and most importantly, selective detection method, such as LC-MS/MS or fluorescence, to study the entire range of HAAs. For the non-fluorescing HAAs, LC-MS/MS is the best option for accurate identification and quantification.

References

1. Jägerstad, M.; Skog, K. I.; Arvidsson, P.; Solyakov, A., Chemistry, formation and occurrence of genotoxic heterocyclic amines identified in model systems and cooked foods. *Z. Lebensm. Unters. Forsch.* **1998,** 207, 419-427.
2. Turesky, R. J., Formation and biochemistry of carcinogenic heterocyclic aromatic amines in cooked meats. *Toxicol. Lett.* **2007,** 168, 219-227.
3. Gibis, M., Effect of oil marinades with garlic, onion, and lemon juice on the formation of heterocyclic aromatic amines in fried beef patties. *J. Agric. Food Chem.* **2007,** 55, 10240-10247.
4. Gibis, M., Optimized HPLC method for analysis of polar and nonpolar heterocyclic amines in cooked meat products. *J. AOAC Int.* **2009,** 92, 715-724.
5. Widmark, E., Presence of cancer-producing substances in roasted food. *Nature* **1939,** 143, 984.
6. Seppilli, A.; Sforzolini, G. S., Carcinogenic polycyclic hydrocarbons in meat cooked on a grill. *Boll. Soc. Ital. Biol. Sper.* **1963,** 39, 110-112.
7. Lijinsky, W.; Shubik, P., Benzo(a)pyrene and other polynuclear hydrocarbons in charcoal-broiled meat. *Science* **1964,** 145, 53-54.
8. Nagao, M.; Honda, M.; Seino, Y.; Yahagi, T.; Sugimura, T., Mutagenicities of smoke condensates and the charred surface of fish and meat. *Cancer Lett.* **1977,** 2, 221-226.
9. Ames, B. N.; McCann, J.; Yamaski, E., Methods for detecting carcinogens and mutagens with the *Salmonella*/ mammalian microsome mutagenicity test. *Mutat. Res.* **1975,** 31, 347-364.
10. Matsumoto, T.; Yoshida, D.; Mizusaki, S.; Okamoto, H., Mutagenic activity of amino acid pyrolyzates in *Salmonella typhimurium* TA 98. *Mutat. Res.* **1977,** 48, 279-286.
11. Matsumoto, T.; Yoshida, D.; Mizusaki, S.; Okamoto, H., Mutagenicities of the pyrolyzates of peptides and proteins. *Mutat. Res.* **1978,** 56, 281-288.
12. Commoner, B.; Vithayathil, A. J.; Dolara, P.; Nair, S.; Madyastha, P.; Cuca, G. C., Formation of mutagens in beef and beef extract during cooking. *Science* **1978,** 201, 913-916.
13. Murkovic, M., Analysis of heterocyclic aromatic amines. *Anal. Bioanal. Chem.* **2007,** 389, 139-146.
14. Tsuda, M.; Negishia, C.; Makino, R.; Sato, S.; Yamaizumi, Z.; Hirayama, T.; Sugimura, T., Use of nitrite and hypochlorite treatments in determination of the contributions of IQ-type and non-IQ-type heterocyclic amines to the mutagenicities in crude pyrolyzed materials. *Mutat. Res.* **1985,** 147, 335-341.
15. Felton, J. S.; Knize, M. G.; Wood, C.; Wuebbles, B. J.; Healy, S. K.; Stuermer, D. H.; Bjeldanes, L. F.; Kimble, B. J.; Hatch, F. T., Isolation and characterization of new mutagens from fried ground beef. *Carcinogenesis* **1984,** 5, 95-102.
16. Skog, K.; Solyakov, A.; Jagerstad, M., Effects of heating conditions and additives on the formation of heterocyclic amines with reference to amino-carbolines in a meat juice model system. *Food Chem.* **2000,** 68, 299-308.
17. Yoshida, D.; Matsumoto, K.; Yoshimura, R.; Matsuzaki, T., Mutagenicity of amino-α-carbolines in pyrolysis products of soybean globulin. *Biochem. Biophys. Re. Commun.* **1978,** 83, 915-920.
18. Matsumoto, T.; Yoshida, D.; Tomita, H., Determination of mutagens, amino-α-carbolines in grilled foods and cigarette smoke condensate. *Cancer Lett.* **1981,** 12, 105-110.

19. Yoshida, D.; Matsumoto, K., Isolation of 2-amino-9H-pyrido[2,3-b]indole and 2-amino-3-methyl-9H-pyrido[2,3-b]indole as mutagens from pyrolysis product of tryptophan. *Agric. Biol. Chem.* **1979,** 43, 1155-1156.
20. Yoshida, D.; Nishigata, H.; Matsumoto, T., Pyrolytic yields of 2-amino-9H-pyrido[2,3-b]indole and 3-amino-1-methyl-5H-pyrido[4,3-b]indole as mutagens from proteins. *Agric. Biol. Chem.* **1979,** 43, 1769-1770.
21. Sugimura, T.; Kawachi, T.; Nagao, M.; Yahagi, T.; Seino, Y.; Okamoto, T.; Shudo, K.; Kosuge, T.; Tsuji, K.; Wakabayashi, K.; Iitaka, Y.; Itai, A., Mutagenic principle(s) in tryptophan and phenylalanine pyrolysis products. *Proc. Japan Acad.* **1977,** 53, 58-61.
22. Sharma, R. K.; Chan, W. G.; Seeman, J. I.; Hajaligol, M. R., Formation of low molecular weight heterocycles and polycyclic aromatic compounds (PACs) in the pyrolysis of α-amino acids. *J. Anal. Appl. Pyrolysis* **2003,** 66, 97-121.
23. Arvidsson, P.; Van Boekel, M. A. J. S.; Skog, K.; Solyakov, A.; Jägerstad, M., Formation of heterocyclic amines in a meat juice model system. *J. Food Sci.* **1999,** 64, 216-221.
24. Pfau, W.; Skog, K., Exposure to β-carbolines norharman and harman. *J. Chromatogr. B.* **2004,** 802, 115-126.
25. Yamaguchi, K.; Shudo, K.; Okamoto, T.; Sugimura, T.; Kosuge, T., Presence of 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole in broiled beef. *Gann* **1980,** 71, 745-746.
26. Yamaizumi, Z.; Shiomi, T.; Kasai, H.; Nishimura, S.; Takahashi, Y.; Nagao, M.; Sugimura, T., Detection of potent mutagens, Trp-P-1 and Trp-P-2, in broiled fish. *Cancer Lett.* **1980,** 9, 75-83.
27. Yamamoto, T.; Tsuji, K.; Kosuge, T.; Okamoto, T.; Shudo, K.; Takeda, K.; Iitaka, Y.; Yamaguchi, K.; Seino, Y.; Yahagi, T.; Nagao, M.; Sugimura, T., Isolation and structure determination of mutagenic substances in L-glutamic acid pyrolysate. *Proc. Japan Acad.* **1978,** 54(B), 248-250.
28. Melo, A.; Viegas, O.; Eça, R.; Petisca, C.; Pinho, O.; Ferreira, I. M. P. L. V. O., Extraction, detection, and quantification of heterocyclic aromatic amines in Portuguese meat dishes by HPLC/diode array. *J. Liq. Chrom. & Rel. Technol.* **2008,** 31, 772-787.
29. Gross, G. A.; Grüter, A., Quantitation of mutagenic/carcinogenic heterocyclic aromatic amines in food products. *J. Chromatogr.* **1992,** 592, 271-278.
30. Skog, K. I., Carcinogenic heterocyclic amines in model systems and cooked foods: a review on formation, occurence and intake. *Food Chem. Toxicol.* **1998,** 36, 879-896.
31. Wakabayashi, K.; Tsuji, K.; Kosuge, T.; Takeda, K.; Yamaguchi, K.; Shudo, K.; Iitaka, Y.; Okamoto, T.; Yahagi, T.; Nagao, M.; Sugimura, T., Isolation and structure determination of a mutagenic substance in L-lysine pyrolysate. *Proc. Japan Acad.* **1978,** 54(B), 569-571.
32. Yokota, M.; Narita, K.; Kosuge, T.; Wakabayashi, K.; Nagao, M.; Sugimura, T.; Yamaguchi, K.; Shudo, K.; Itaka, Y.; Okamoto, T., A potent mutagen isolated from a pyrolyzate of L-ornithine. *Chem. Pharm. Bull.* **1981,** 29, 1473-1475.
33. Nukaya, H.; Watanabe, H.; Ishida, H.; Tsuji, K.; Suwa, Y.; Wakabayashi, K.; Nagao, M.; Sugimura, T.; Kosuge, T., Isolation and structural determination of a mutagenic substance in creatine pyrolyzate. *Chem. Pharm. Bull.* **1991,** 39, 533-535.
34. Jägerstad, M.; Skog, K.; Grivas, S.; Olsson, K., Formation of heterocyclic aromatic amines using model systems. *Mutat. Res.* **1991,** 259, 219-233.
35. Dvorak, Z., Creatine as an indicator of net muscle proteins. *J. Sci. Food Agric.* **1981,** 32, 1033-1036.
36. Kasai, H.; Yamaizumi, Z.; Wakabayashi, K.; Nagao, M.; Sugimura, T.; Yokoyama, S.; Miyazawa, T., Potent novel mutagens produced by broiling fish under normal conditions. *Proc. Japan Acad.* **1980,** 56(B), 278-283.

37. Hargraves, W. A.; Pariza, M. W., Purification and mass spectral characterization of bacterial mutagens from commercial beef extract. *Cancer Res.* **1983,** 43, 1467-1472.
38. Galceran, M. T.; Pais, P.; Puignou, L., Isolation by solid-phase extraction and liquid chromatographic determination of mutagenic amines in beef extracts. *J. Chromatogr. A* **1996,** 719, 203-212.
39. Knize, M. G.; Happe, J.; Healy, S. K.; Felton, J. S., Identification of the mutagenic quinoxaline isomers from fried ground beef. *Mutat. Res.* **1987,** 178, 25-32.
40. Felton, J. S.; Knize, M. G.; Shen, N. H.; Andresen, B. D.; Bjeldanes, L. F.; Hatch, F. T., Identification of the mutagens in cooked beef. *Environ. Health Perspect.* **1986,** 67, 17-24.
41. Kasai, H.; Yamaizumi, Z.; Shiomi, T.; Yokoyama, S.; Miyazawa, T.; Wakabayashi, K.; Nagao, M.; Sugimura, T.; Nishimura, S., Structure of a potent mutagen isolated from fried beef. *Chem. Lett.* **1981**, 485-488.
42. Borgen, E.; Solyakov, A.; Skog, K., Effects of precursor composition and water on the formation of heterocyclic amines in meat model systems. *Food Chem.* **2001,** 74, 11-19.
43. Persson, E.; Sjoeholm, I.; Nyman, M.; Skog, K., Addition of various carbohydrates to beef burgers affects the formation of heterocyclic amines during frying. *J. Agric. Food Chem.* **2004,** 52, 7561-7566.
44. Pais, P.; Salmon, C. P.; Knize, M. G.; Felton, J. S., Formation of mutagenic/carcinogenic heterocyclic amines in dry-heated model systems, meats, and meat drippings. *J. Agric. Food Chem.* **1999,** 47, 1098-1108.
45. Turesky, R. J.; Bur, H.; Huhnh-Ba, T.; Aeschbacher, H. U.; Milon, H., Analysis of mutagenic heterocyclic amines in cooked beef products by high-performance liquid chromatography in combination with mass spectrometry. *Food Chem. Toxicol.* **1988,** 26, 501-509.
46. Skog, K.; Knize, M. G.; Felton, J. S.; Jägerstad, M., Formation of new heterocyclic amine mutagens by heating creatinine, alanine, threonine and glucose. *Mutat. Res.* **1992,** 268, 191-197.
47. Pais, P.; Moyano, E.; Puignou, L.; Galceran, M. T., Liquid chromatography-electrospray mass spectrometry with in-source fragmentation for the identification and quantification of fourteen mutagenic amines in beef extracts. *J. Chromatogr. A* **1997,** 775, 125-136.
48. Bordas, M.; Moyano, E.; Puignou, L.; Galceran, M. T., Formation and stability of heterocyclic amines in a meat flavour model system Effect of temperature, time and precursors. *J. Chromatogr. B* **2004,** 802, 11-17.
49. Kim, I. S.; Wakabayashi, K.; Kurosaka, R.; Yamaizumi, Z.; Jinno, F.; Koyota, S.; A., T.; Nukaya, H.; Takahashi, M., Isolation and identification of a new mutagen, 2-amino-4-hydroxymethyl-3,8-dimethylimidazo[4,5-f]quinoxaline, from beef extract. *Carcinogenesis* **1994,** 15, 21-26.
50. Nukaya, H.; Koyota, S.; Jinno, F.; Ishida, H.; Wakabayashi, K.; Kurosaka, R.; Kim, I. S.; Yamaizumi, Z.; Ushiyama, H., Structural determination of a new mutagenic heterocyclic amine, 2-amino-1,7,9-trimethylimidazo[4,5-g]quinoxaline (7,9-DiMeIgQx), present in beef extract. *Carcinogenesis* **1994,** 15, 1151-1154.
51. Ni, W.; Mcnaughton, L.; LeMaster, D. M.; Sinha, R.; Turesky, R., Quantitation of 13 heterocyclic aromatic amines in cooked beef, pork, and chicken by liquid chromatography-electrospray ionization/tandem mass spectrometry. *J. Agric. Food Chem.* **2008,** 56, 68-78.
52. Turesky, R.; Bessette, E. E.; Dunbar, D.; Liberman, R. G.; Skipper, P. L., Cytochrome P450-mediated metabolism and DNA binding of 2-amino-1,7-dimethylimidazo[4,5-*g*]quinoxaline and its carcinogenic isomer 2-amino-3,8-dimethylimidazo[4,5-*f*] quinoxaline in mice. *Chem. Res. Toxicol.* **2011,** 25, 410-421.

53. Felton, J. S.; Knize, M. G.; Shen, N. H.; Lewis, P. R.; Andresen, B. D.; Happe, J.; Hatch, F. T., The isolation and identification of a new mutagen from fried ground beef: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). *Carcinogenesis* **1986,** 7, 1081-1086.
54. Wakabayashi, K.; Kurosaka, R.; Kim, I.-S.; Nukaya, H.; Ushiyama, H.; Ochiai, M.; Fukutome, K.; Nagaoka, H.; Sugimura, T.; Nagao, M., Identification of three new mutagenic heterocyclic amines and human exposure to known heterocyclic amines. *Special Publication - Royal Society of Chemistry* **1994,** 151, 352-357.
55. Khan, M. R.; Busquets, R.; Santos, F. J.; Puignou, L., New method for the analysis of heterocyclic amines in meat extracts using pressurised liquid extraction and liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* **2008,** 1194, 155-160.
56. WHO, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. **2012,** 1-105.
57. Carmichael, P. L.; Stone, E. M.; Grover, P. L.; Gusterson, B. A.; Phillips, D. H., Metabolic activation and DNA binding of food mutagens and other environmental carcinogens in human mammary epithelial cells. *Carcinogenesis* **1996,** 17, 1769-1772.
58. Nauwelaers, G. B., Erin E.; Gu, Dan; Tang, Yi-Jin; Rageul, Julie; Fessard, Valerie; Yuan, Jian-Min; Yu, Mimi C.; Langouet, Sophie; Turesky, Robert J., DNA Adduct Formation of 4-Aminobiphenyl and Heterocyclic Aromatic Amines in Human Hepatocytes. *Chem. Res. Toxicol.* **2011,** 24, 913-925.
59. Sugimura, T.; Wakabayashi, K.; Nakagama, H.; Nagao, M., Heterocyclic amines: Mutagens/carcinogens produced during cooking of meat and fish. *Cancer Sci.* **2004,** 95, 290-299.
60. Adamson, R. H.; Takayama, S.; Sugimura, T.; Thorgeirsson, U. P., Induction of hepatocellular carcinoma in nonhuman primates by the food mutagen 2-amino-3-methylimidazo[4,5-*f*]quinoline. *Environ. Health Perspect.* **1994,** 102, 190-193.
61. Nowell, S.; Coles, B.; Sinha, R.; MacLeod, S.; Ratnasinghe, D. L.; Stotts, C.; Kadlubar, F. F.; Ambrosone, C. B.; Lang, N. P., Analysis of total meat intake and exposure to individual heterocyclic amines in a case-control study of colorectal cancer: contribution of metabolic variation to risk. *Mutat. Res.* **2002,** 506-507, 175-185.
62. Le Marchand, L.; Hankin, J. H.; Pierce, L. M.; Sinha, R.; Nerurkar, P. V.; Franke, A. A.; Wilkens, L. R.; Kolonel, L. N.; Donlon, T.; Seifried, A.; Custer, L. J.; Lum-Jones, A.; Chang, W., Well-done red meat, metabolic phenotypes and colorectal cancer in Hawaii. *Mutat. Res.* **2002,** 506-507, 205-214.
63. Anderson, K. E.; Sinha, R.; Kulldorff, M.; Gross, M.; Lang, N. P.; Barber, C.; Harnack, L.; DiMagno, E.; Bliss, R.; Kadlubar, F. F., Meat intake and cooking techniques: associations with pancreatic cancer. *Mutat. Res.* **2002,** 506-507, 225-231.
64. Stidl, R.; Sontag, G.; Koller, V.; Knasmüller, S., Binding of heterocyclic aromatic amines by lactic acid bacteria: Results of a comprehensive screening trial. *Mol. Nutr. Food Res.* **2008,** 52, 322-329.
65. Orrhage, K.; Sillerström, E.; Gustaffson, J.-A.; Nord, C. E.; Rafter, J., Binding of mutagenic heterocyclic amines by intestinal and lactic acid bacteria. *Mutation Res.* **1994,** 311, 239-248.
66. Funk, C.; Braune, A.; Grabber, J. H.; Steinhart, H.; Bunzel, M., Model studies of lignified fiber fermentation by human fecal microbiota and its impact on heterocyclic aromatic amine adsorption. *Mutation Res.* **2007,** 624, 41-48.
67. Borosky, G. L., Carcinogenic carbocyclic and heterocyclic aromatic amines: a DFT study concerning their mutagenic potency. *J. Mol. Graphics Modell.* **2008,** 27, 459-465.
68. Murray, S.; Lake, B. G.; Gray, S.; Edwards, A. J.; Springall, C.; Bowey, E. A.; Williamson, G.; Boobis, A. R.; Gooderham, N. J., Effect of cruciferous vegetable consumption on heterocyclic aromatic amine metabolism in man. *Carcinogenesis* **2001,** 22, 1413-1420.

69. Laser Reuterswärd, A.; Skog, K.; Jägerstad, M., Mutagenicity of pan-fried bovine tissues in relation to their content of creatine, creatinine, monosaccharides and free amino acids. *Food Chem. Toxicol.* **1987,** 25, 755-762.
70. Ahn, J.; Gruen, I. U., Heterocyclic amines: 1. Kinetics of formation of polar and nonpolar heterocyclic amines as a function of time and temperature. *J. Food Sci.* **2005,** 70, C173-C179.
71. Knize, M. G.; Dolbeare, F. A.; Carroll, K. L.; Moore, D. H.; Felton, J. S., Effect of cooking time and temperature on the heterocyclic amine content of fried beef patties. *Food Chem. Toxicol.* **1994,** 32, 595-603.
72. Kondjoyan, A.; Chevolleau, S.; Greve, E.; Gatellier, P.; Sante-Lhoutellier, V.; Bruel, S.; Touzet, C.; Portanguen, S.; Debrauwer, L., Modelling the formation of heterocyclic amines in slices of *longissimus thoracis* and *semimambranosus* muscles subjected to jets of hot air. *Food Chem.* **2010,** 123, 659-668.
73. Chiu, C. P.; Chen, B. H., Stability of heterocyclic amines during heating. *Food Chem.* **2000,** 68, 267-272.
74. Skog, K.; Solyakov, A.; Jägerstad, M., Effects of heating conditions and additives on the formation of heterocyclic amines with reference to amino-carbolines in a meat juice model system. *Food Chem.* **2000,** 68, 299-308.
75. Laser Reuterswärd, A.; Skog, K.; Jägerstad, M., Effects of creatine and creatinine content on the mutagenic activity of meat extracts, bouillons and gravies from different sources. *Food Chem. Toxicol.* **1987,** 25, 747-754.
76. Pfau, W.; Rosenvold, K.; Young, J. F., Formation of mutagenic heterocyclic aromatic amines in fried pork from Duroc and Landrace pigs upon feed supplementation with creatine monohydrate. *Food Chem. Toxicol.* **2006,** 44, 2086-2091.
77. Zöchling, S.; Murkovic, M., Formation of the heterocyclic aromatic amine PhIP: identification of precursors and intermediates. *Food Chem.* **2002,** 79, 125-134.
78. Jägerstad, M.; Laser Reuterswärd, A.; Olsson, R.; Grivas, S.; Nyhammar, T.; Olsson, K.; Dahlqvist, A., Creatin(in)e and Maillard reaction products as precursors of mutagenic compounds: Effects of various amino acids. *Food Chem.* **1983,** 12, 255-264.
79. Jägerstad, M.; Olsson, K.; Grivas, S.; Negishi, C.; Wakabayashi, K.; Tsuda, M.; Sato, S.; Sugimura, T., Formation of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline in a model system by heating creatinine, glycine and glucose. *Mutation Res.* **1984,** 126, 239-244.
80. Skog, K.; Jägerstad, M., Incorporation of carbon atoms from glucose into the food mutagens MeIQx and 4,8-DiMeIQx using ^{14}C-labelled glucose in a model system. *Carcinogenesis* **1993,** 14, 2027-2031.
81. Overvik, E.; Kleman, M.; Berg, I.; Gustaffson, J.-A., Influence of creatine, amino acids, and water on the formation of the mutagenic heterocyclic amines found in cooked meat. *Carcinogenesis* **1989,** 10, 2293-2301.
82. Wang, P.; Odell, G., Formation of pyrazines from thermal treatment of some amino-hydroxy compounds. *J. Agric. Food Chem.* **1973,** 21, 868-870.
83. Skog, K.; Jägerstad, M., Effects of glucose on the formation of PhIP in a model system. *Carcinogenesis* **1991,** 12, 2297-2300.
84. Skog, K.; Jägerstad, M., Effects of monosaccharides and disaccharides on the formation of food mutagens in model systems. *Mutat. Res.* **1990,** 230, 263-272.
85. Skog, K.; Jagerstad, I. M.; Reutersward, A. L., Inhibitory effect of carbohydrates on the formation of mutagens in fried beef patties. *Food Chem. Toxicol.* **1992,** 30, 681-688.
86. Kato, T.; Michikoshi, K.; Minowa, Y.; Kikugawa, K., Mutagenicity of cooked hamburger is controlled delicately by reducing sugar content in ground beef. *Mutation Res.* **2000,** 471, 1-6.

87. Choi, S.; Ko, J., Analysis of cyclic pyrolysis products formed from amino acid monomer. *J. Chromatogr. A* **2011,** 1218, 8443-8455.
88. Milic, B.; Djilas, S.; Vlaovic, D., Effect of temperature on the kinetics of free radical formation during the pyrolysis of some amino acids. *Analyst* **1987,** 112, 779-781.
89. Djilas, S.; Milic, B.; Canadanovic-Brunet, J. M., ESR spectral study of free radical formation during the pyrolysis of hydroxyamino acids. *J. Anal. Appl. Pyrolysis* **1994,** 28, 157-161.
90. Shulman, G.; Simmonds, P. G., Thermal composition of aromatic and heteroaromatic amino acids. *Chem. Commun.* **1968**, 1040-1042.
91. Chiavari, G., Pyrolysis-gas chromatography/mass spectrometry of amino acids. *J. Anal. Appl. Pyrolysis* **1992,** 24, 123-137.
92. Herraiz, T.; Galisteo, J.; Chamorro, C., L-tryptophan reacts with naturally occurring and food-occurring phenolic aldehydes to give phenolic tetrahydro-*β*-carboline alkaloids: Activity as antioxidants and free radical scavengers. *J. Agric. Food Chem.* **2003,** 51, 2168-2173.
93. Herraiz, T., Tetrahydro-β-carboline-3-carboxylic acid compounds in fish and meat: possible precursors of co-mutagenic β-carbolines norharman and harman in cooked foods. *Food Addit. Contam.* **2000,** 17, 859-866.
94. Murkovic, M.; Weber, H.; Geiszler, S.; Fröhlich, K.; Pfannhauser, W., Formation of the food associated carcinogen 2-amino-1methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in model systems. *Food Chem.* **1999,** 65, 233-237.
95. Pearson, A. E.; Chen, C.; Gray, J. I.; Aust, S. D., Mechanism(s) involved in meat mutagen formation and inhibition. *Free Rad. Biol. Med.* **1992,** 13, 161-167.
96. Milic, B.; Djilas, S.; Canadanovic-Brunet, J. M., Synthesis of some heterocyclic amino-imidazoazarenes. *Food Chem.* **1993,** 46, 272-276.
97. Namiki, M.; Hayashi, T., Development of novel free radicals during the amino-carbonyl reaction of sugars with amino acids. *J. Agric. Food Chem.* **1975,** 23, 487-491.
98. Hayashi, T.; Ohta, Y.; Namiki, M., Electron spin resonance spectral study on the structure of the novel free radical products formed by the reactions of sugars with amino acids or amines. *J. Agric. Food Chem.* **1977,** 25, 1282-1287.
99. Hayashi, T.; Namiki, M., On the mechanism of free radical formation during browning reaction of sugars with amino compounds. *Agric. Biol. Chem.* **1981,** 45, 933-939.
100. Milic, B.; Piletic, M. V., The mechanism of pyrrole, pyrazine and pyradine formation in non-enzymic browning reaction. *Food Chem.* **1984,** 13, 165-180.
101. Roberts, R. L.; Lloyd, R. V., Free radical formation from secondary amines in the Maillard reaction. *J. Agric. Food Chem.* **1997,** 45, 2413-2418.
102. Hofmann, T., Studies on radical intermediates in the early stage of the nonenzymatic browning reaction of carbohydrates and amino acids. *J. Agric. Food Chem.* **1999,** 47, 379-390.
103. Kato, T.; Harashima, T.; Moriya, N.; Kikugawa, K.; Hiramoto, K., Formation of the mutagenic/carcinogenic imidazoquinoxaline-type heterocyclic amines through the unstable free radical Maillard intermediates and its inhibition by phenolic antioxidants. *Carcinogenesis* **1996,** 17, 2469-2476.
104. Kikugawa, K., Involvement of free radicals in the formation of heterocyclic amines and prevention by antioxidants. *Cancer Lett.* **1999,** 143, 123-126.
105. Lee, H.; Lin, M.; Lin, S., Characterization of the mutagen 2-amino-3-methylimidazo[4,5-*f*]quinoline prepared from a 2-methylpyridine/creatinine/acetylformaldehyde model system. *Mutagenesis* **1994,** 9, 157-162.
106. Vitaglione, P.; Fogliano, V., Use of antioxidants to minimize the human health risk associated to mutagenic/carcinogenic heterocyclic amines in food. *J. Chromatogr. B* **2004,** 802, 189-199.

107. Zöchling, S.; Murkovic, M.; Pfannhauser, W., Effects of industrially produced flavours with pro- and antioxidative properties on the formation of the heterocyclic amine PhIP in a model system. *J. Biochem. Biophys. Methods* **2002,** 53, 37-44.
108. Murkovic, M.; Steinberger, D.; Pfannhauser, W., Antioxidant spices reduce the formation of heterocyclic amines in fried meat. *Z. Lebensm. Unters. Forsch.* **1998,** 207, 477-480.
109. Balogh, Z.; Gray, J. I.; Gomaa, E. A.; Booren, A. M., Formation and inhibition of heterocyclic aromatic amines in fried ground beef patties. *Food Chem. Toxicol.* **2000,** 38, 395-401.
110. Gibis, M.; Weiss, J., Antioxidant capacity and inhibitory effect of grape seed and rosemary extract in marinades on the formation of heterocyclic amines in fried beef patties. *Food Chem.* **2012,** 134, 766-774.
111. Cheng, K.-W.; Wu, Q.; Zheng, Z.; Peng, X.; Simon, J. E.; Chen, F.; Wang, M., Inhibitory effect of fruit extracts on the formation of heterocyclic amines. *J. Agric. Food Chem.* **2007,** 55, 10359-10365.
112. Puangsombat, K.; Jirapakkul, W.; Smith, J., Inhibitory activity of Asian spices on heterocyclic amines formation in cooked beef patties. *J. Food Sci.* **2011,** 76, T174-T180.
113. Quelhas, I.; Petisca, C.; Viegas, O.; Melo, A.; Pinho, O.; Ferreira, I. M. P. L. V. O., Effect of green tea marinades on the formation of heterocyclic aromatic amines and sensory quality of pan-fried beef. *Food Chem.* **2010,** 122, 98-104.
114. Shin, H. S.; Ustonol, Z., Influence of honey-containing marinades on heterocyclic aromatic amine formation and overall mutagenicity in fried beef steak and chicken breast. *J. Food Sci.* **2006,** 69, 147-153.
115. Gibis, M.; Weiss, J., Inhibitory effect of marinades with hibiscus extract on formation of heterocyclic aromatic amines and sensory quality of fried beef patties. *Meat Sci.* **2010,** 85, 735-742.
116. Friedman, M.; Zhu, L.; Feinstein, Y.; Ravishankar, S., Carvacrol facilitates heat-induced inactivation of *Escherichia coli* O157:H7 and inhibits formation of heterocyclic amines in grilled ground beef patties. *J. Agric. Food Chem.* **2009,** 57, 1848-1853.
117. Britt, C.; Gomaa, E.; Gray, J. I.; Booren, A. M., Influence of cherry tissue on lipid oxidation and heterocyclic aromatic amine formation in ground beef patties. *J. Agric. Food Chem.* **1998,** 46, 4891-4897.
118. Damasius, J.; Venskutonis, P. R.; Ferracane, R.; Fogliano, V., Assessment of the influence of some spice extracts on the formation of heterocyclic amines in meat. *Food Chem.* **2011,** 126, 149-156.
119. Awney, H.; Sindi, H., The effect of rosemary on the mutagenic activity of heterocyclic amines extracted from common food consumed in Saudi Arabia. *Int. J. Food Sci. Nutr.* **2010,** 61, 192-203.
120. Persson, E.; Graziani, G.; Ferracane, R.; Fogliano, V.; Skog, K., Influence of antioxidants in virgin olive oil on the formation of heterocyclic amines in fried beefburgers. *Food Chem. Toxicol.* **2003,** 41, 1587-1597.
121. Awney, H., The effect of green tea and olive oil on the mutagenic activity of heterocyclic amines extracted from common food consumed in Saudi Arabia. *Int. J. Food Sci. Nutr.* **2011,** 62, 295-302.
122. Viegas, O.; Amaro, L. F.; Ferreira, I. M. P. L. V. O.; Pinho, O., Inhibitory effect of antioxidant-rich marinades on the formation of heterocyclic aromatic amines in pan-fried beef. *J. Agric. Food Chem.* **2012,** 60, 6235-6240.
123. Messner, C.; Murkovic, M., Evaluation of a new model system for studying the formation of heterocyclic amines *J. Chromatogr. B* **2004,** 802, 19-26.

124. Cheng, K.-W.; Chen, F.; Wang, M., Inhibitory activities of dietary phenolic compounds on heterocyclic amine formation in both chemical model system and beef patties. *Mol. Nutr. Food Res.* **2007,** 51, 969-976.
125. Huang, D.; Ou, B.; Prior, R. L., The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005,** 53, 1841-1856.
126. Cheng, K.-W.; Wong, C.; Cho, C.; Chu, I.; Sze, K.; Lo, C.; Chen, F.; Wang, M., Trapping of phenylacetaldehyde as a key mechanism responsible for naringenin's inhibitory activity in mutagenic 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine formation. *Chem. Res. Toxicol.* **2008,** 21, 2026-2034.
127. Cheng, K.-W.; Wong, C.; Chao, J.; Lo, C.; Chen, F.; Chu, I.; Che, C.; Ho, C. T.; Wang, M., Inhibition of mutagenic PhIP formation by epigallocatechin gallate *via* scavenging of phenylacetaldehyde. *Mol. Nutr. Food Res.* **2009,** 53, 716-725.
128. Bin, Q.; Peterson, D. G.; Elias, R. J., Influence of phenolic compounds on the mechanisms of pyrazinium radical generation in the Maillard reaction. *J. Agric. Food Chem.* **2012,** 60, 5482-5490.
129. Bunzel, M., Chemistry and occurrence of hydroxycinnamate oligomers. *Phytochemistry Rev.* **2010,** 9, 47-64.
130. Ralph, J.; Quideau, S.; Grabber, J. H.; Hatfield, R. D., Identification and synthesis of new ferulic acid dehydrodimers present in grass cell-walls. *J. Chem. Soc. Perkin Trans. 1* **1994**, 3485-3498.
131. Bunzel, M.; Ralph, J.; Marita, J. M.; Hatfield, R. D.; Steinhart, H., Diferulates as structural components in soluble and insoluble cereal dietary fibre. *J. Sci. Food Agric.* **2001,** 81, 653-660.
132. Oguri, A.; Suda, M.; Totsuka, Y.; Sugimura, T.; Wakabayashi, K., Inhibitory effects of antioxidants on formation of heterocyclic amines. *Mutat. Res.* **1998,** 402, 237-245.
133. Janoszka, B.; Blaszczyk, U.; Warzecha, L.; Strozyk, M.; Damasiewicz-Bodzek, A.; Bodzek, D., Clean-up procedures for the analysis of heterocyclic aromatic amines (aminoazaarenes) from heat-treated meat samples. *J. Chromatogr. A* **2001,** 938, 155-165.
134. Bang, J.; Nukaya, H.; Skog, K., Blue Chitin columns for the extraction of heterocyclic amines from cooked meat. *J. Chromatogr. A* **2002,** 977, 97-105.
135. Santos, F. J.; Barcelo-Barrachina, E.; Toribio, F.; Puignou, L.; Galceran, M. T.; Persson, E.; Skog, K.; Messner, C.; Murkovic, M.; Nabinger, U.; Ristic, A., Analysis of heterocyclic amines in food products: interlaboratory studies. *J. Chromatogr. B* **2004,** 802, 69-78.
136. Jautz, U.; Morlock, G., Validation of a new planar chromatographic method for quantification of the heterocyclic aromatic amines most frequently found in meat. *Anal. Bioanal. Chem.* **2007,** 387, 1083-1093.
137. Jautz, U.; Gibis, M.; Morlock, G., Quantification of heterocyclic aromatic amines in fried meat by HPTLC/UV-FLD and HPLC/UV-FLD: A comparison of two methods. *J. Agric. Food Chem.* **2008,** 56, 4311-4319.
138. Casal, S.; Mendes, E.; Fernandes, J. O.; Oliveira, M. B. P. P.; Ferreira, M. A., Analysis of heterocyclic aromatic amines in foods by gas chromatography-mass spectrometry as their tert.-butyldimethylsilyl derivatives. *J. Chromatogr. A* **2004,** 1040, 105-114.
139. Olsson, V.; Solyakov, A.; Skog, K.; Lundstrom, K.; Jagerstad, M., Natural variations of precursors in pig meat affect the yield of heterocyclic amines - Effects of RN genotype, feeding regime, and sex. *J. Agric. Food Chem.* **2002,** 50, 2962-2969.
140. *Chemische Analytik: Verfahren der Standardaddition*; DIN 32633; Deutsches Institüt für Normung: Berlin, 1998.
141. Knezevic, A. StatNews #73: Overlapping confidence intervals and statistical significance. http://www.cscu.cornell.edu/news/statnews/stnews73.pdf (July 29),

142. Polyakova, Y.; Koo, Y. M.; Row, K. H., Application of ionic liquids as mobile phase modifier in HPLC. *Biotechnol. Bioproc. E.* **2006,** 11, 1-6.
143. Martin-Calero, A.; Tejral, G.; Ayala, J. H.; Gonzalez, V.; Afonso, A. M., Suitability of ionic liquids as mobile-phase additives in HPLC with fluorescence and UV detection for the determination of heterocyclic aromatic amines. *J. Sep. Sci.* **2010,** 33, 182-190.
144. Toribio, F.; Moyano, E.; Puignou, L.; Galceran, M. T., Comparison of different commercial solid-phase extraction cartridges used to extract heterocyclic amines from a lyophilised meat extract. *J. Chromatog. A* **2000,** 880, 101-112.
145. Minerich, P. L.; Addis, P. B.; Epley, R. J.; Bingham, C., Properties of wild rice/ground beef mixtures. *J. Food Sci.* **1991,** 56, 1154-1157.
146. Wu, K.; Zhang, W.; Addis, P. B.; Epley, R. J.; Salih, A. M.; Lehrfeld, J., Antioxidant properties of wild rice. *J. Agric. Food Chem.* **1994,** 42, 34-37.
147. Asamarai, A. M.; Addis, P. B.; Epley, R. J.; Krick, T. P., Wild rice hull antioxidants. *J. Agric. Food Chem.* **1996,** 44, 126-130.
148. Composition of Foods Raw, Processed, Prepared USDA National Nutrient Database for Standard Reference, Release 24. In Agriculture, U. D. o., Ed. 2011.
149. Kikugawa, K.; Kato, T.; Hiramoto, K.; Takada, C.; Tanaka, M.; Maeda, Y.; Ishihara, T., Participation of the pyrazine cation radical in the formation of mutagens in the reaction of glucose/glycine/creatinine. *Mutat. Res.* **1999,** 444, 133-144.
150. Persson, E.; Sjoholm, I.; Skog, K., Effect of high water-holding capacity on the formation of heterocyclic amines in fried beefburgers. *J. Agric. Food Chem.* **2003,** 51, 4472-4477.
151. Smith, J. S.; Ameri, F.; Gadgil, P., Effect of marinades on the formation of heterocyclic amines in grilled beef steaks. *J. Food Sci.* **2008,** 73, T100-T105.

Appendix A: Chemical list

Chemical name	Hazard symbol				Manufacturer
	Health	Fire	Reactivity	Personal Protection	
2-Amino-9H-pyrido[2,3-b]indole (AαC)	2	1	1	D	Toronto Research Chemicals (North York, ON, Canada)
Acetone	2	3	0	H	Fisher Scientific (Waltham, MA, USA)
Acetonitrile	2	3	0	H	Fisher Scientific (Pittsburgh, PA, USA)
Ammonium acetate	2	1	1	B	Sigma Aldrich (St. Louis, MO, USA)
Ammonium hydroxide (33%)	3	0	0	C	Sigma Aldrich (St. Louis, MO, USA)
1-Butyl-3-methylimidazolium tetrafluoroborate (BMIm-BF_4)	2	1	0	C	Sigma Aldrich (St. Louis, MO, USA)
3,4,8-Trimethyl-3H-imidazo[4,5-f]quinoxalin-2-amine (4,8-DiMeIQx)	2	1	1	D	Toronto Research Chemicals (North York, ON, Canada)
Ethanol	2	3	0	H	Decon Labs, Inc. (King of Prussia, PA, USA)
Ethyl acetate	2	3	0	G	Sigma Aldrich (St. Louis, MO, USA)
1-Methyl-9H-pyrido[3,4-b]indole (harman)	0	0	0	B	Toronto Research Chemicals (North York, ON, Canada)
Hydrochloric acid (37%)	3	0	2	D	Sigma Aldrich (St. Louis, MO, USA)
3-Methyl-3H-imidazo[4,5-f]quinolin-2-amine (IQ)	0	1	0	F	Toronto Research Chemicals (North York, ON, Canada)
3,8-Dimethyl-3H-Imidazo[4,5-f]quinoxalin-2-amine (MeIQx)	1	0	0	K	Toronto Research Chemicals (North York, ON, Canada)
Methanol	2	3	0	H	Sigma Aldrich (St. Louis, MO, USA)
9H-Pyrido[3,4-b]indole (norharman)	1	1	0	B	Toronto Research Chemicals (North York, ON, Canada)
Orthophosphoric acid	2	0	0	E	Sigma Aldrich (St. Louis, MO, USA)
2-Amino-1-methyl-6-	2	1	0	E	Toronto Research

Chemical name	Hazard symbol				Manufacturer
	Health	Fire	Reactivity	Personal Protection	
phenylimidazo[4,5-b]pyridine (PhIP)					Chemicals (North York, ON, Canada)
Sodium chloride	0	0	0	E	Sigma Aldrich (St. Louis, MO, USA)
Sodium hydroxide	3	0	2	J	Fisher Scientific (Waltham, MA, USA)
Sodium sulfate	1	0	0	A	Sigma Aldrich (St. Louis, MO, USA)
Triethylamine	3	3	0	B	Sigma Aldrich (St. Louis, MO, USA)

Appendix B: Equipment list

Instrument	Model	Manufacturer
Analytical balance	AB135-S/FACT	Mettler Toledo (Columbus, OH, USA)
Analytical balance	AUX 220	Shimadzu (Columbia, MD, USA)
Balance	AQT 1500	Adam Equipment, Inc (Danbury, CT, USA)
Blender	Osterizer Galaxie	Oster (Fort Lauderdale, Fl, USA)
Centrifuge	J2-MC	Beckman (Fullerton, CA, USA)
Centrifuge	Dynac 0101	Clay Adams (now BD Diagnostic Systems) (Franklin Lakes, NJ, USA)
Electric grill	25331 Super Sear Nonstick Indoor Searing Grill	Hamilton Beach (Southern Pines, NC, USA)
Electric grill	Q140	Weber (Palatine, IL, USA)
Freeze-dryer	Freeze Dryer 8	LabConco (Kansas City, MO, USA)
Freeze-dryer	FreeZone	LabConco (Kansas City, MO, USA)
Freeze-dryer	VirTis Freezemobile	SP Scientific (Warminster, PA, USA)
HPLC system	D-7000 data interface L-7100 pump L-7250 autosampler L-7300 column oven L-7455 diode array detector L-7485 fluorescence detector	Hitachi (Santa Clara, CA, USA)
Karl Fischer coulometric titrater	Aquatest CMA	Photovolt (Indianapolis, IN, USA)
Magnetic stirrer		Chemglass (Vineland, NJ, USA)
Meat mixer	Double Action	Leland Southwest (Fort Worth, TX, USA)
Microcentrifuge	Biofuge 15	Heraeus Instruments (Buckinghamshire, England)
Mill	FitzMill Homiloid hammer mill	The Fitzpatrick Company (Elmhurst, IL, USA)
pH meter	pH 340	Corning (Corning, NY, USA)
Rapid Visco Analyzer	RVA-4	Newport Scientific (Warriewood, NSW, Australia)
Roto-Evaporator	RotoVapor R-124	Büchi (Flawil, Switzerland)
Roto-Evaporator	Rotovapor RII	Büchi (Flawil, Switzerland)
Shaker	Orbital Shaker 980001	VWR Scientific Products (Radnor, PA, USA)
Sonicator	2200	Branson Ultrasonics (Danbury, CT, USA)
Spectrofluorimeter	FP6200	Jasco (Easton, MD, USA)
Vacuum manifold	unknown	unknown

Instrument	Model	Manufacturer
Vortex	Vortex Genie 2	Fisher Scientific (Waltham, MA, USA)
Water distillation apparatus	AG-2	Corning Glass Works (Corning, NY, USA)

Appendix C: Heterocyclic aromatic amine standard curves

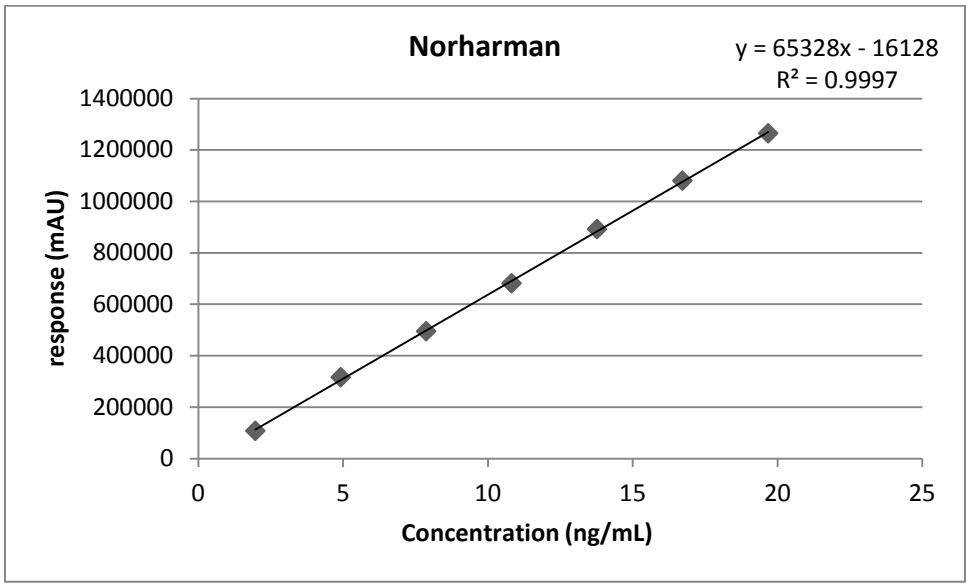

Figure 18: Norharman standard curve, concentration range 2 - 20 ng/mL, fluorescence detection (354 nm excitation, 450 nm emission).

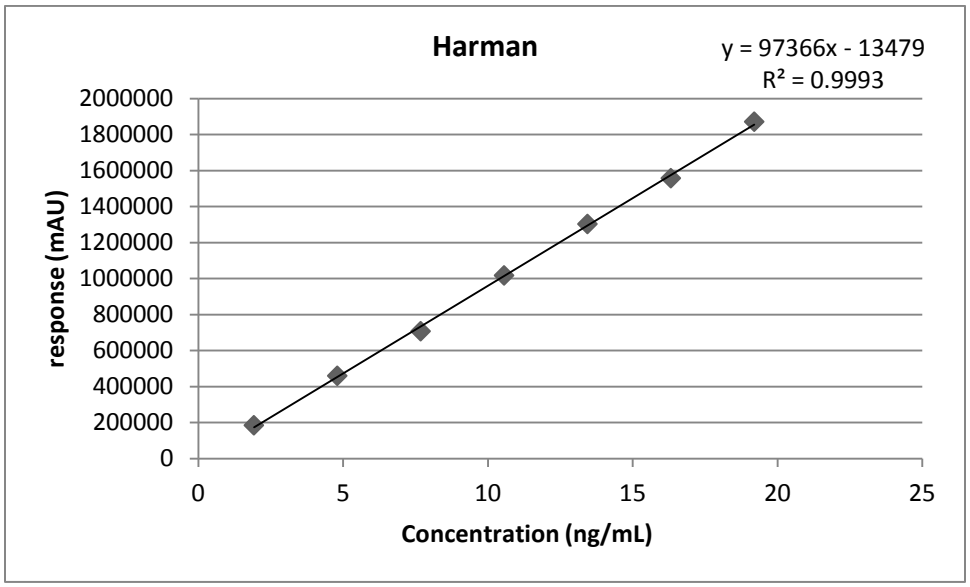

Figure 19: Harman standard curve, concentration range 2 - 19 ng/mL, fluorescence detection (354 nm excitation, 450 nm emission).

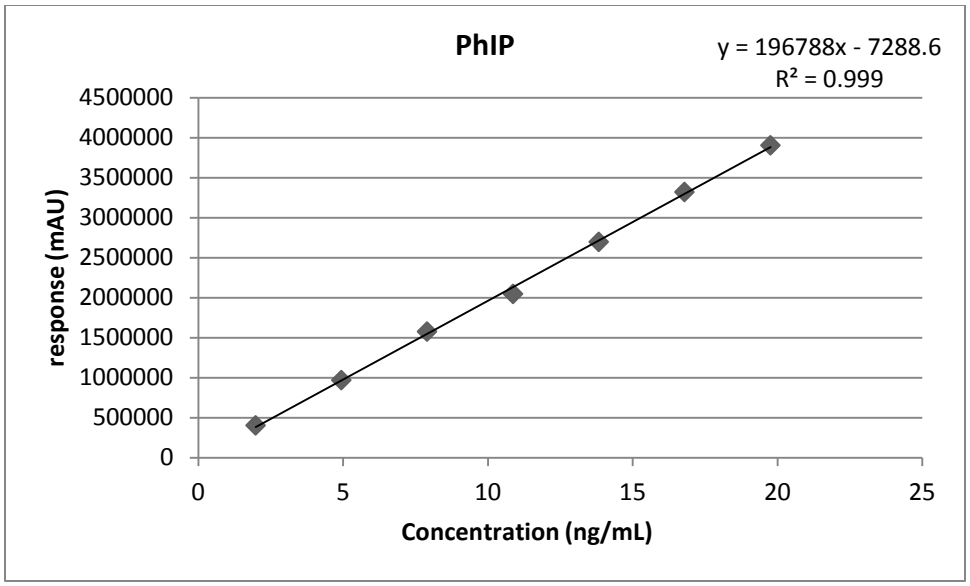

Figure 20: PhIP standard curve, concentration range 2 - 20 ng/mL, fluorescence detection (337 nm excition, 395 nm emission).

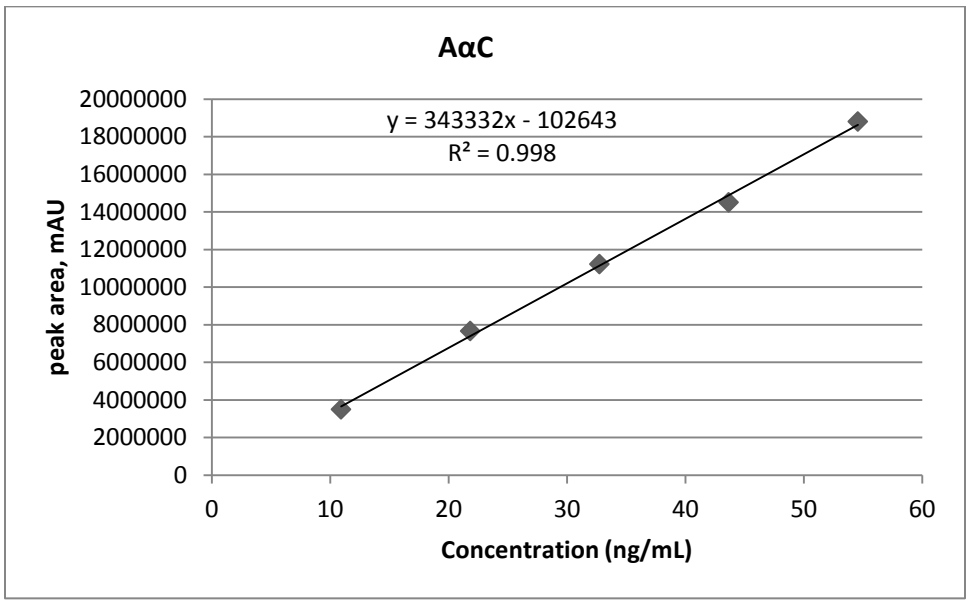

Figure 21: AαC standard curve, concentration range 11 - 55 ng/mL, fluorescence detection (353 nm excition, 404 nm emission).

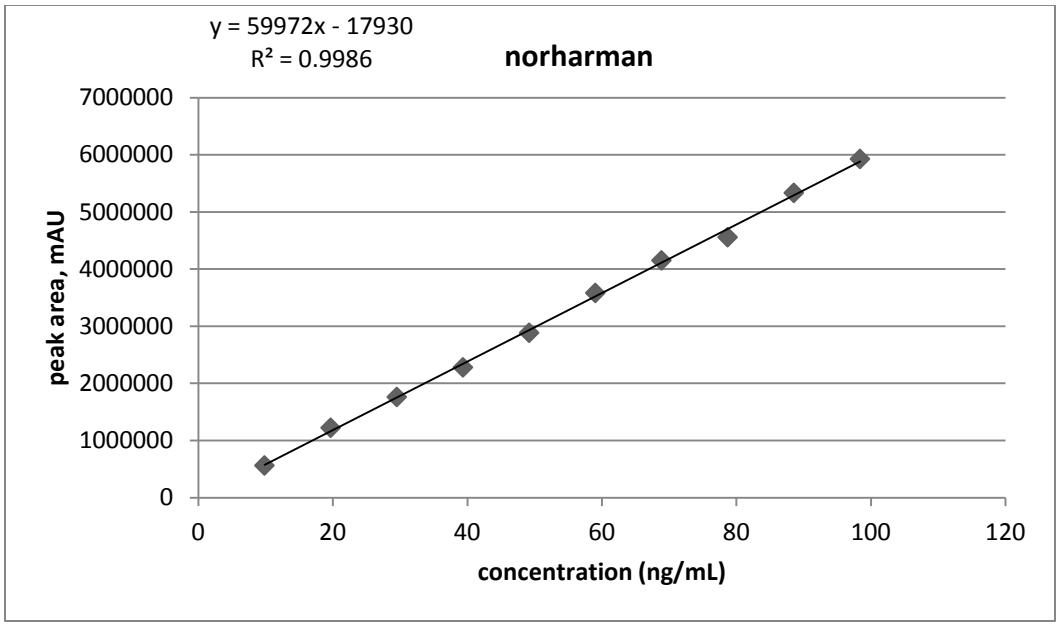

Figure 22: Norharman standard curve, concentration range 9.8 - 98 ng/mL, fluorescence detection (354 nm excitation, 450 nm emission).

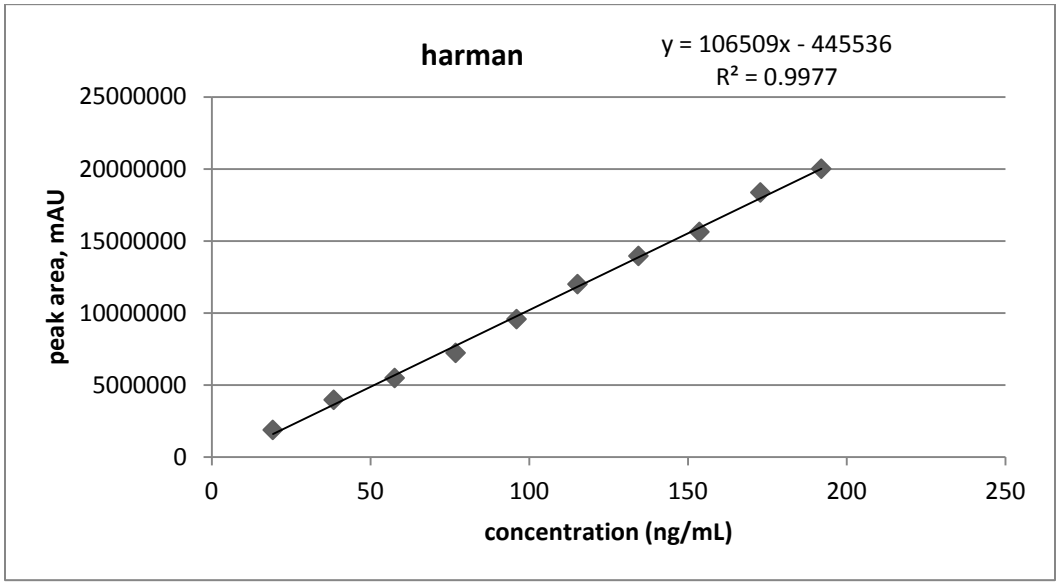

Figure 23: Harman standard curve, concentration range 19.2 - 192 ng/mL, fluorescence detection (354 nm, 450 nm emission).

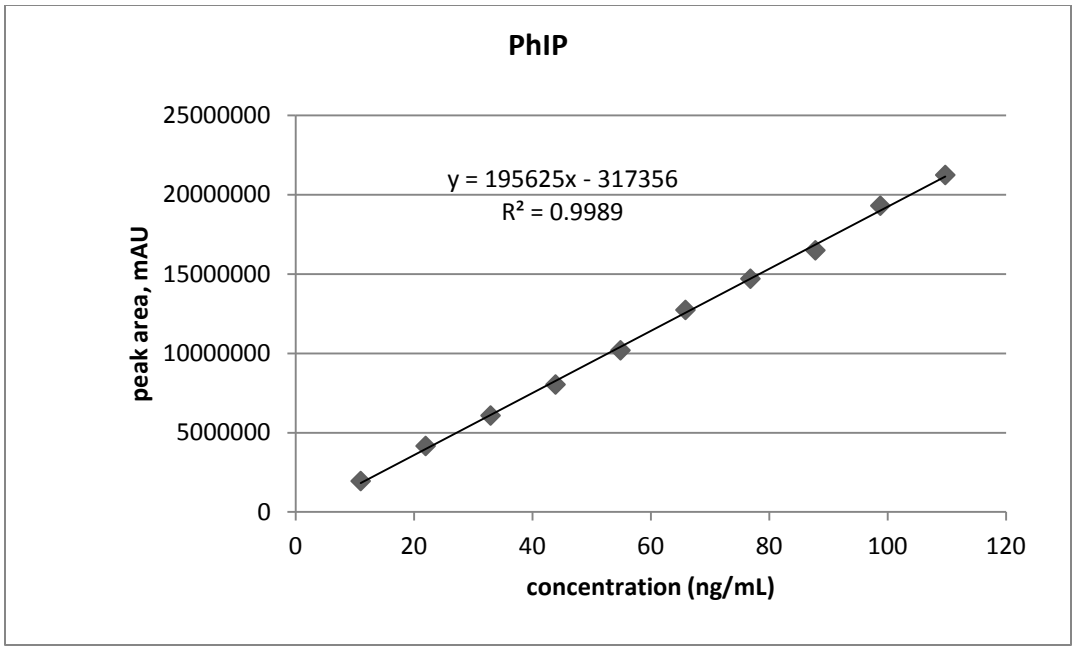

Figure 24: PhIP standard curve, concentration range 10.9 – 109 ng/mL, fluorescence detection (337 nm excitation, 395 nm emission).

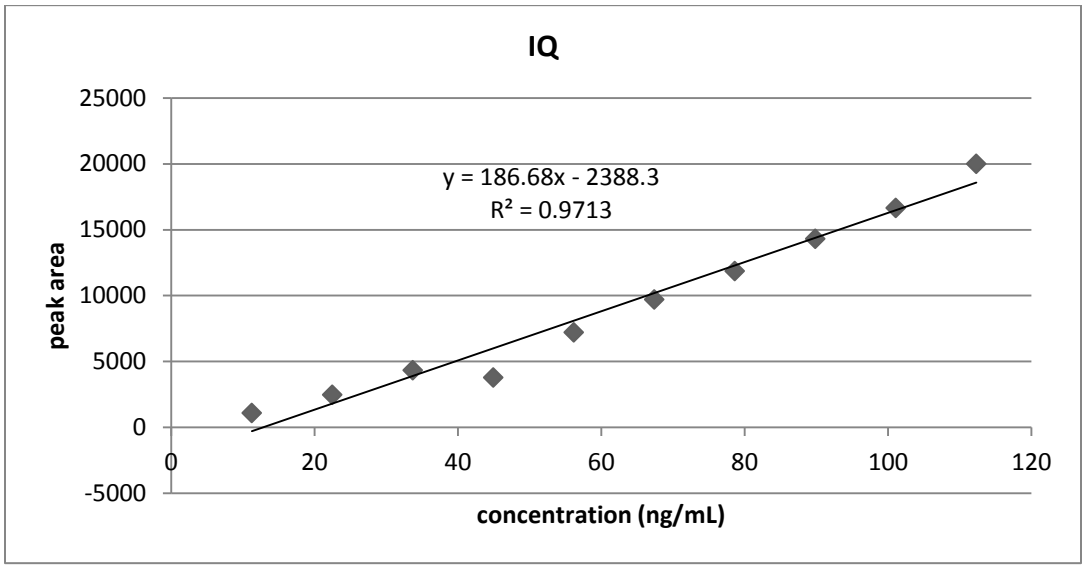

Figure 25: IQ standard curve, concentration range 11.2 - 112 ng/mL, UV absorption (270 nm).

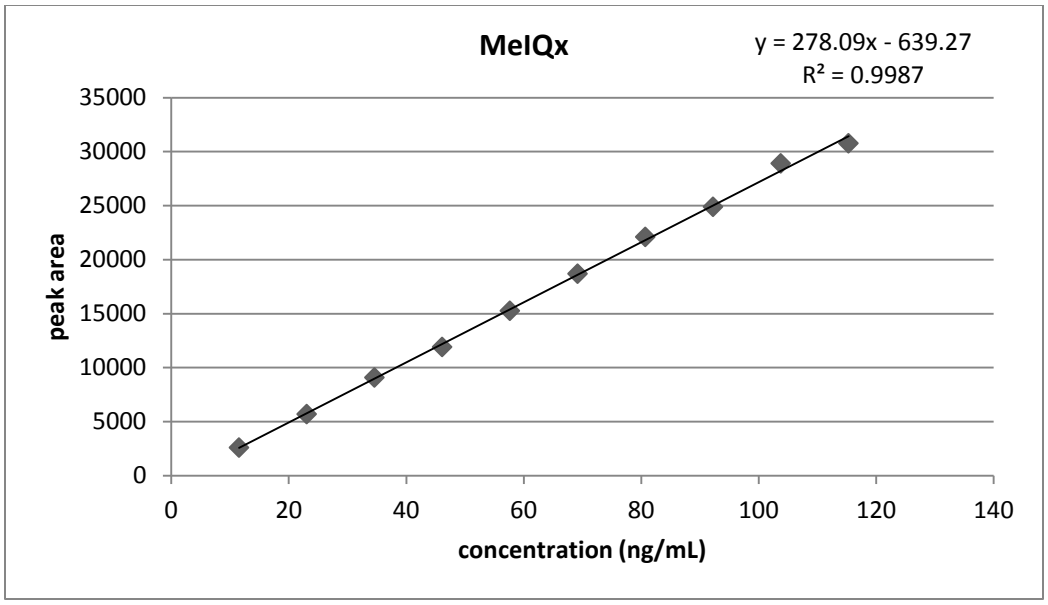

Figure 26: MeIQx standard curve, concentration range 11.5- 115 ng/mL, UV absorption (268 nm).

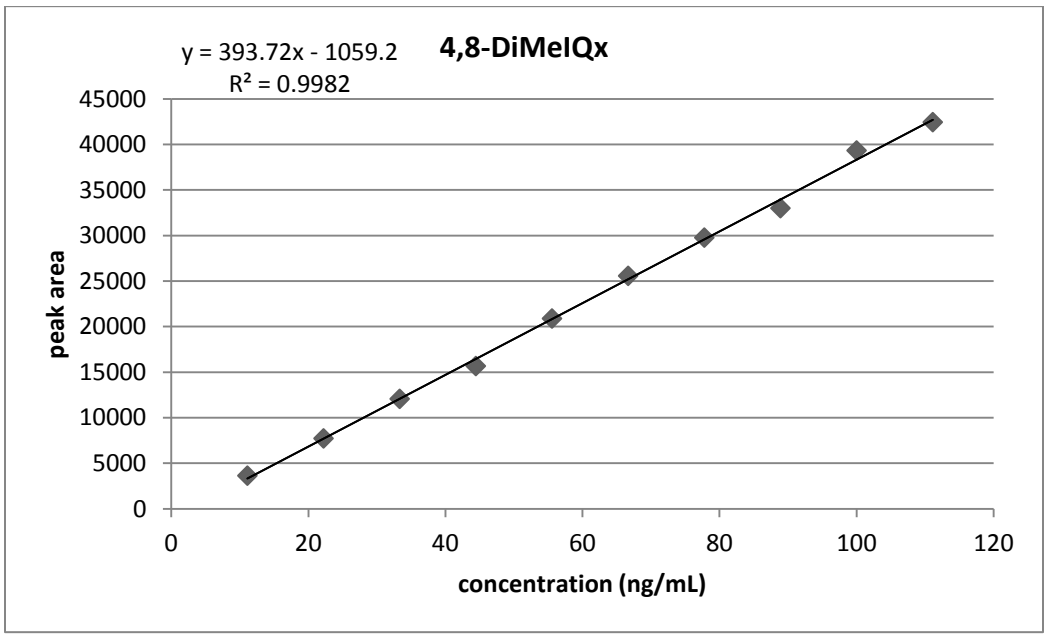

Figure 27: 4,8-DiMeIQx standard curve, concentration range 11.1 – 111 ng/mL, UV absorption (270 nm

Appendix D: Standard addition calculation example

Reference: *Chemische Analytik: Verfahren der Standardaddition, 1998, DIN 32633, Deutsches Institut für Normung*

A. Calculation of harman concentration in patties fried with 10% cooked corn flour

Key data:

- Patties prepared by weighing **6.30 g** of cooked corn flour and adding raw beef to make **60 g**
- Net post-frying weight of three patties was **108.02 g**
- Beef extraction begins with addition of 50 g of 1 M sodium hydroxide solution to 20 g of cooked material, for 70 g of basic mixture. 10 g aliquots of this mixture are removed and extracted, so **HAAs recovered in one extraction replicate come from (20/7) g of cooked material**.
- Spiking volume: **135 µL**
- Concentration of harman in spike: **76.8 ng/mL**
- Dissolution volume for final extraction residue: **135 µL**

Table 10: Harman peak areas

	harman peak area	**concentration of harman in extract from spike (ng/mL)**
Replicate 1, unspiked	6005096	0
Replicate 2, unspiked	6101934	0
Replicate 1, spiked	12795087	76.8
Replicate 2, spiked	11032824	76.8

Calculation steps:

Plot peak area vs. harman concentration from spike. Draw a linear regression curve and extrapolate to the x-intercept.

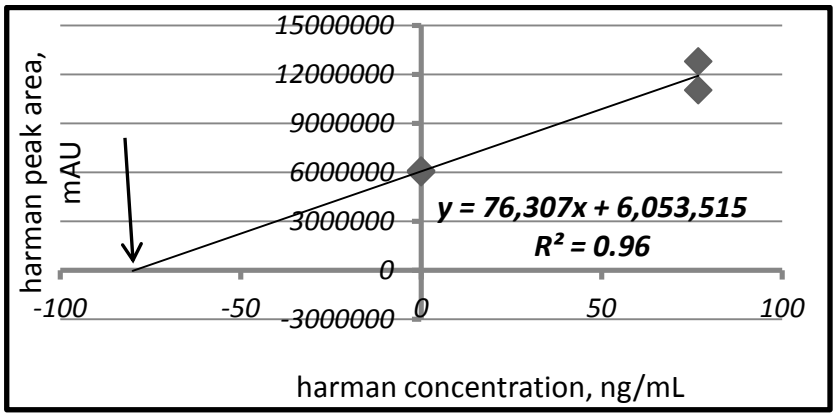

Figure 28: Standard addition plot for harman

Use the linear regression equation to solve for the absolute value of the x-intercept:

$$|x| = \frac{6{,}053{,}515}{76{,}307} = \mathbf{79.33}\ \frac{\boldsymbol{ng\ harman}}{\boldsymbol{mL\ extract}}$$

This value represents the concentration of harman in the unspiked injected extract.

Convert this value to units of ng harman/ g cooked material:

$$\frac{\left(79.33\,\frac{\text{ng harman}}{\text{mL extract}}\right)(0.135\ \text{mL extract})}{\left(\frac{20}{7}\,g\ cooked\ material\right)} = \mathbf{3.748}\ \frac{\textbf{ng harman}}{\textbf{g cooked material}}$$

Convert this value to ng harman/ g raw beef:

$$\left(\frac{3.748\ \text{ng harman}}{\text{g cooked material}}\right)\left(\frac{108.02\ \text{g cooked material}}{180\ \text{g raw material}}\right)\left(\frac{60\ \text{g raw material}}{53.7\ \text{g raw beef}}\right)$$

$$= \frac{\mathbf{2.51\ ng\ harman}}{\mathbf{g\ raw\ beef}}$$

B. Calculation of standard deviations

$\overline{\boldsymbol{y}}_{\boldsymbol{unspiked}}$ = average peak area of unspiked sample

$$= (6005096 + 6101934)/(2) = \mathbf{6053515}$$

$\overline{\boldsymbol{y}}_{\boldsymbol{spiked}}$ = average peak area of spiked sample

$$= (12795087 + 11032824)/(2) = \mathbf{11913955}$$

$\boldsymbol{s_{y\ unspiked}}$ = standard deviation of peak areas from unspiked samples =

$$\sqrt{\frac{(6005096-6053515)^2+(6101934-6053515)^2}{1}} = \mathbf{68474}$$

$\boldsymbol{s_{y\ spiked}}$ = standard deviation of peak areas from spiked samples

$$= \sqrt{\frac{(12795087-11913955)^2+(11032824-11913955)^2}{1}} = \mathbf{1246108}$$

$\boldsymbol{s_{y\ overall}}$ = overall standard deviation of peak areas in data set = $\sqrt{\frac{s^2_{y\ unspiked}+s^2_{y\ spiked}}{2}}$

$$= \sqrt{\frac{(68474)^2+(1246108)^2}{\mathbf{2}}} = \mathbf{882460}$$

$\boldsymbol{s_{concentration}}$ = standard deviation converted to concentration

$$= \frac{s_{y\ overall}}{\text{slope of standard addition line equation}} = \frac{882460}{76307}$$

$$= \mathbf{11.56}\ \mathbf{ng\ harman}/\mathbf{mL\ extract}$$

Convert this value to ng harman/ g raw beef:

$$\left(\frac{\left(11.56\frac{\text{ng harman}}{\text{mL extract}}\right)(0.135\text{ mL extract})}{\left(\frac{20}{7}\text{g cooked material}\right)}\right)\left(\frac{108.02\text{ g cooked material}}{180\text{ g raw material}}\right)\left(\frac{60\text{ g raw material}}{53.7\text{ g raw beef}}\right)$$

$$= \frac{\mathbf{0.37\ ng\ harman}}{\mathbf{g\ raw\ beef}}$$

Harman concentration in this sample is therefore: $\mathbf{2.51 \pm 0.37\ \ ng\ harman/g\ raw\ beef}$

C. Calculation of 95% confidence intervals

Variables defined:

- $\boldsymbol{n}$ = number of parallel determinations = $\mathbf{2}$
- $\boldsymbol{x_a}$ = concentration of harman from *spike* = $\mathbf{76.8\ ng/mL}$

- $\boldsymbol{x_{calc}}$ = calculated concentration of harman in unspiked $extract$ = **79.33 ng harman/mL extract**
- $\boldsymbol{f}$ = degrees of freedom = $(2n) - 2 = \mathbf{2}$
- $\boldsymbol{z_t}$ = factor from t − distribution
- $\boldsymbol{b_a}$ = slope from standard addition line equation

2. Equation for 95% confidence interval for a value determined by standard addition:

$$a = \frac{z_{t\,(f,0.05)} \times s_{y_{overall}}}{b_a} \times \sqrt{\frac{1}{2n} + \frac{2 \times \left(\frac{x_a}{2} + x_{calc}\right)^2}{n \times x_a^2}}$$

3. Solve equation:

$$a = \frac{4.303 \times 882460}{76307} \times \sqrt{\frac{1}{2(2)} + \frac{2 \times \left(\frac{76.8}{2} + 79.33\right)^2}{2 \times (76.8)^2}} = \mathbf{80.24}\ \frac{\textbf{ng harman}}{\textbf{mL extract}}$$

4. Convert to ng harman/g raw beef:

$$\left(\frac{\left(80.24\,\frac{\text{ng harman}}{\text{mL extract}}\right)(0.135\text{ mL extract})}{\left(\frac{20}{7}\text{ g cooked material}\right)}\right)\left(\frac{108.02\text{ g cooked material}}{180\text{ g raw material}}\right)\left(\frac{60\text{ g raw material}}{53.7\text{ g raw beef}}\right)$$

$$= \frac{\mathbf{2.54\ ng\ harman}}{\mathbf{g\ raw\ beef}}$$

5. 95% confidence interval for harman concentration in beef patties cooked with 10% cooked corn flour:

2.51 ± 2.54 ng harman/ g raw beef

D. Calculation of recovery rates

$$\text{recovery rate} = \left(\frac{\text{slope of standard addition curve for harman}}{\text{slope of calibration curve for harman}}\right) \times 100 = \left(\frac{76307}{106509}\right) \times 100 =$$

71.6%

Appendix E: MeIQx content of selected samples

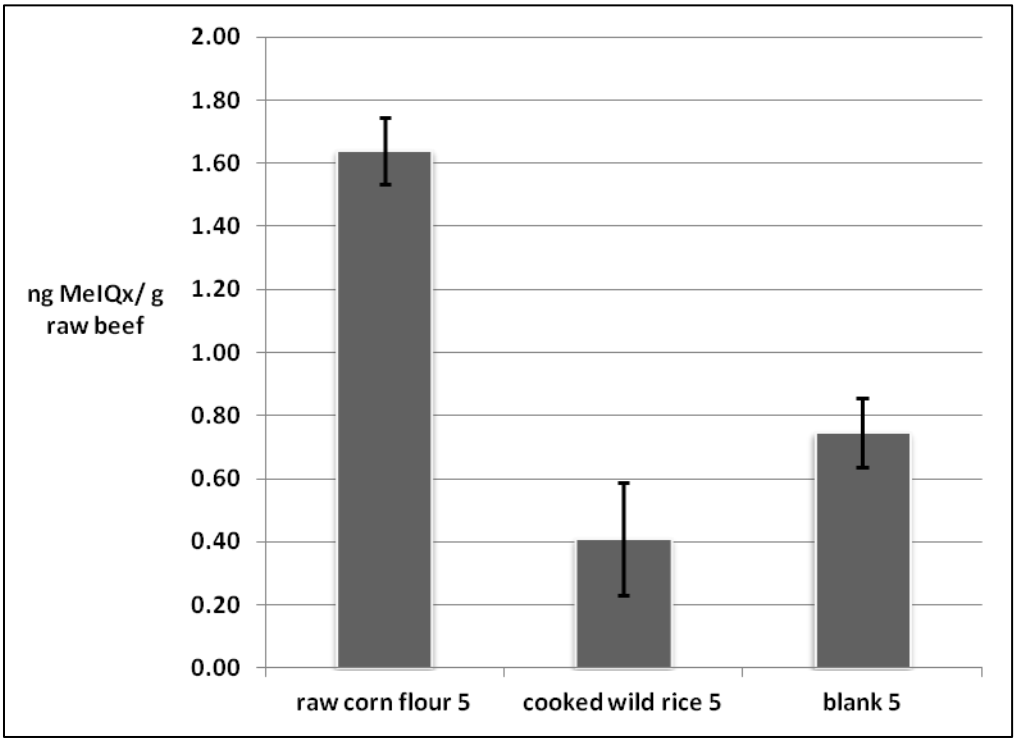

Figure 29: MeIQx content of selected samples

Appendix F: Chromatograms

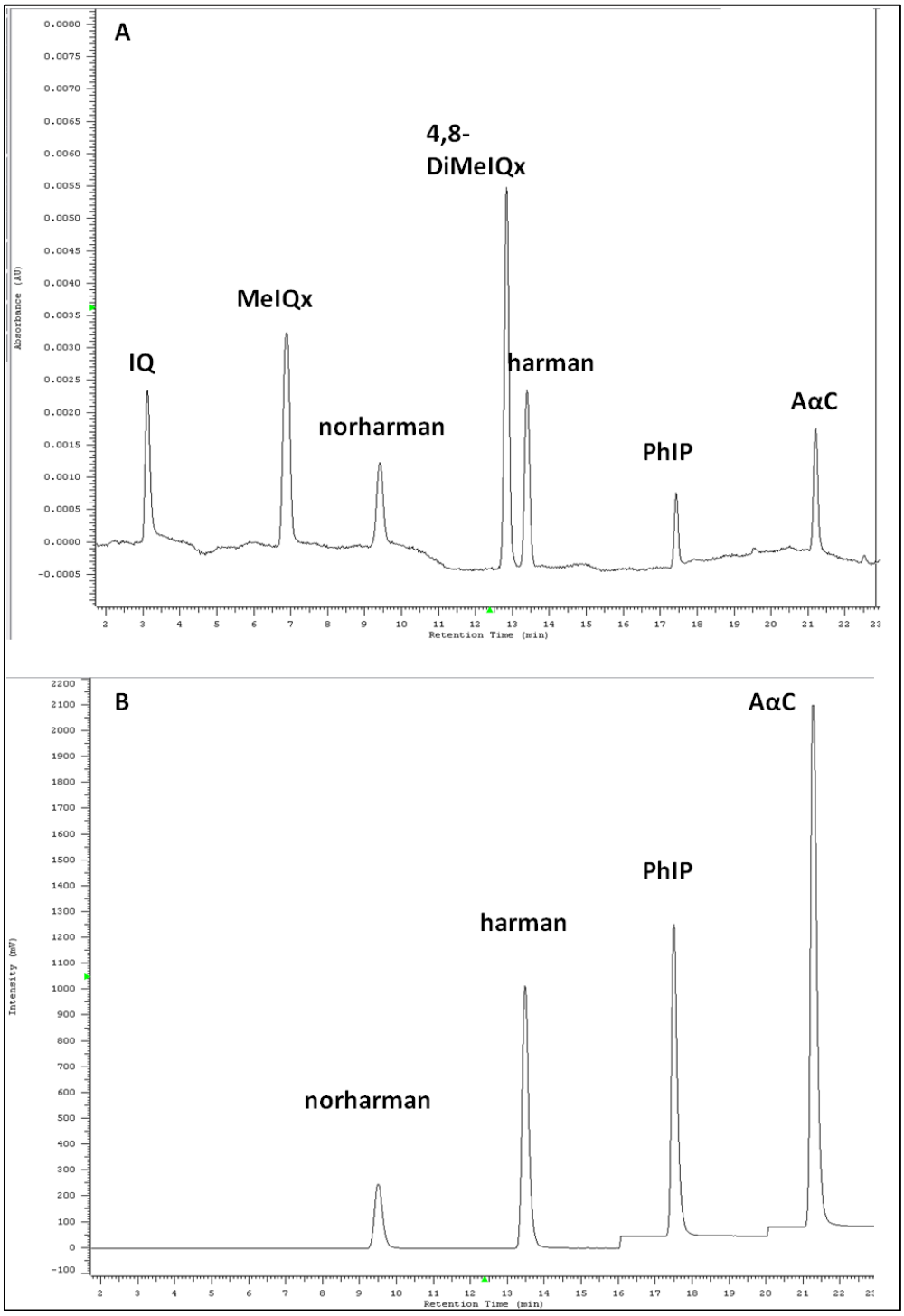

Figure 30: Separation of heterocyclic aromatic amine standard solutions. A = UV absorption (268 nm), B = fluorescence detection.

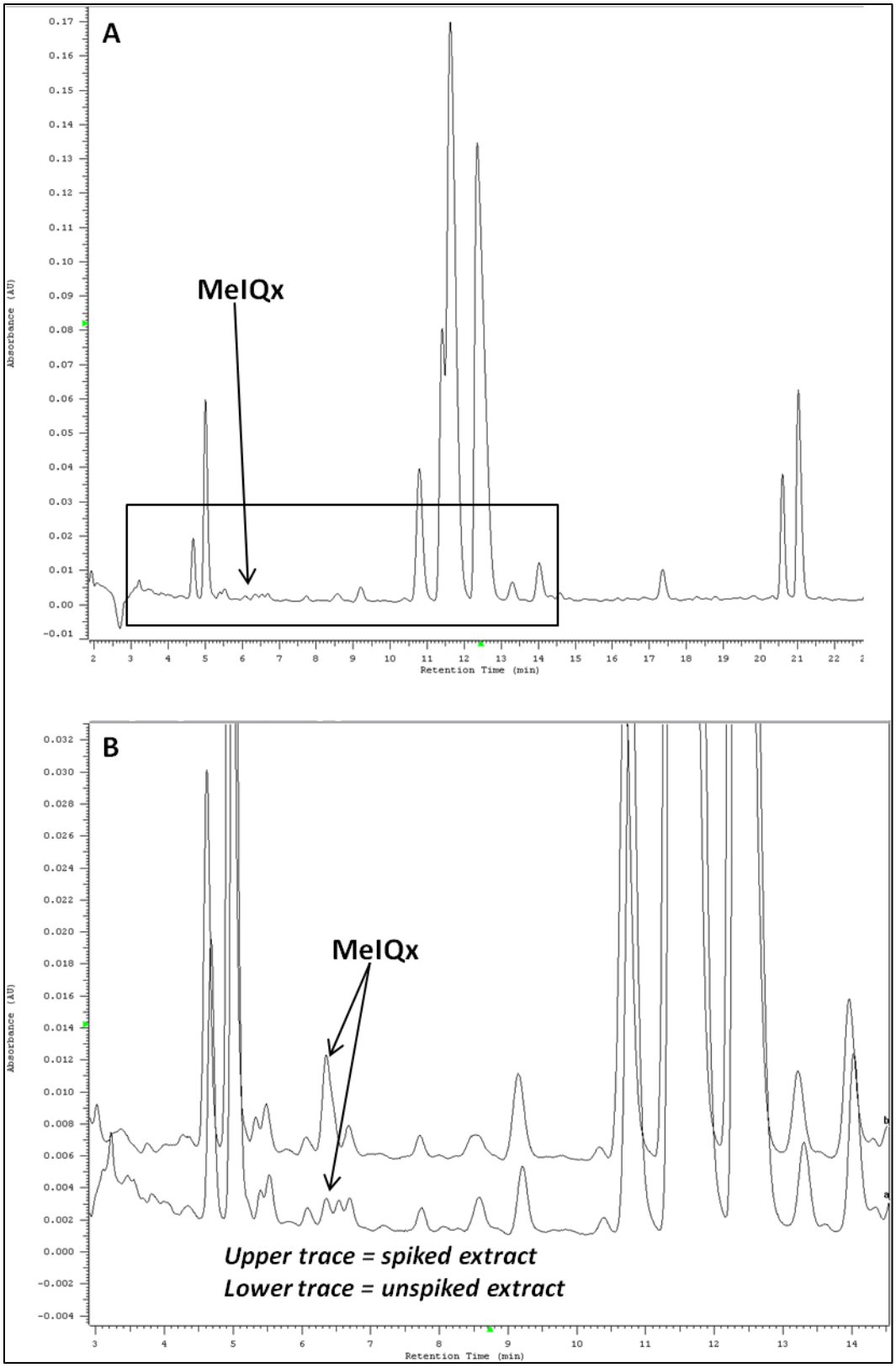

Figure 31: Extract of plain beef patties from Set A (Table 8), UV absorption (268 nm). A = complete chromatogram, B = close-up of selection.

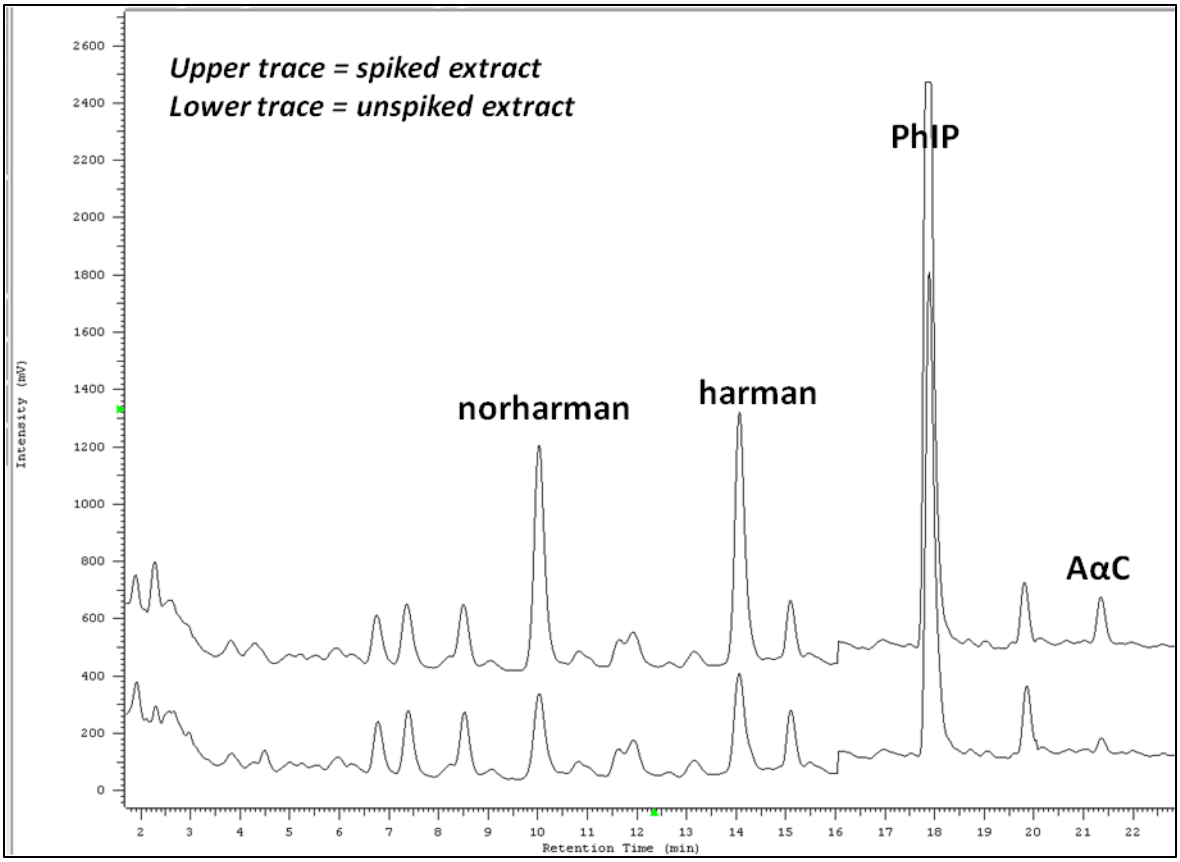

Figure 32: Extract of plain fried beef patties, fluorescence detection.

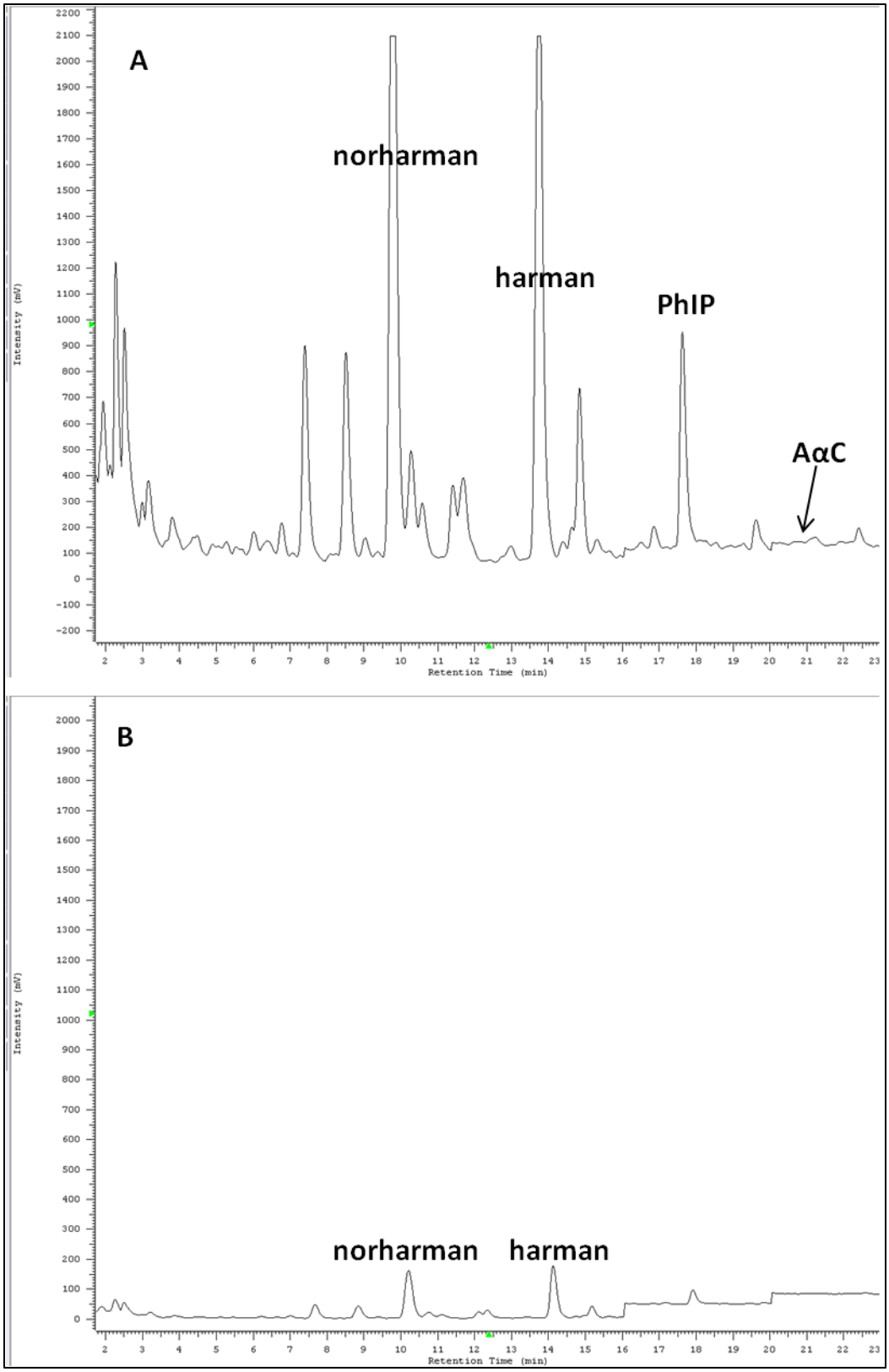

Figure 33: Extract of beef patties fried with 10% wild rice hulls, unspiked (fluorescence detection). A = original extract, B = diluted extract.

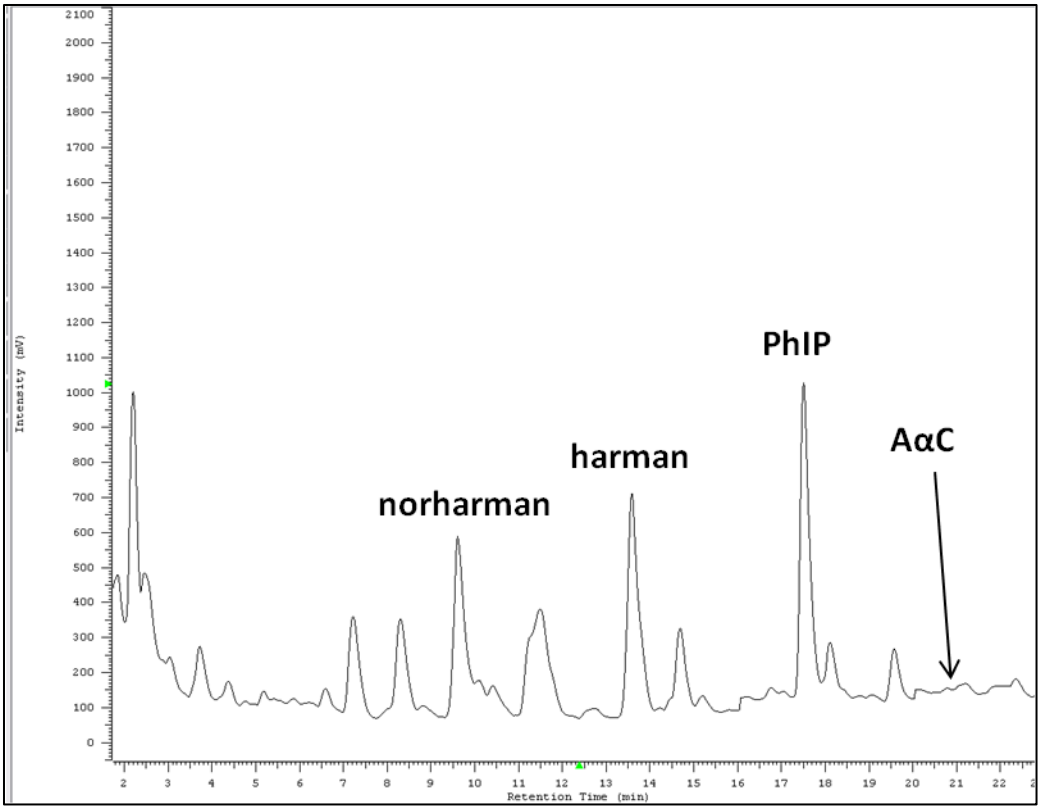

Figure 34: Extract of beef patties fried with 10% raw wild rice flour, unspiked, fluorescence detection.

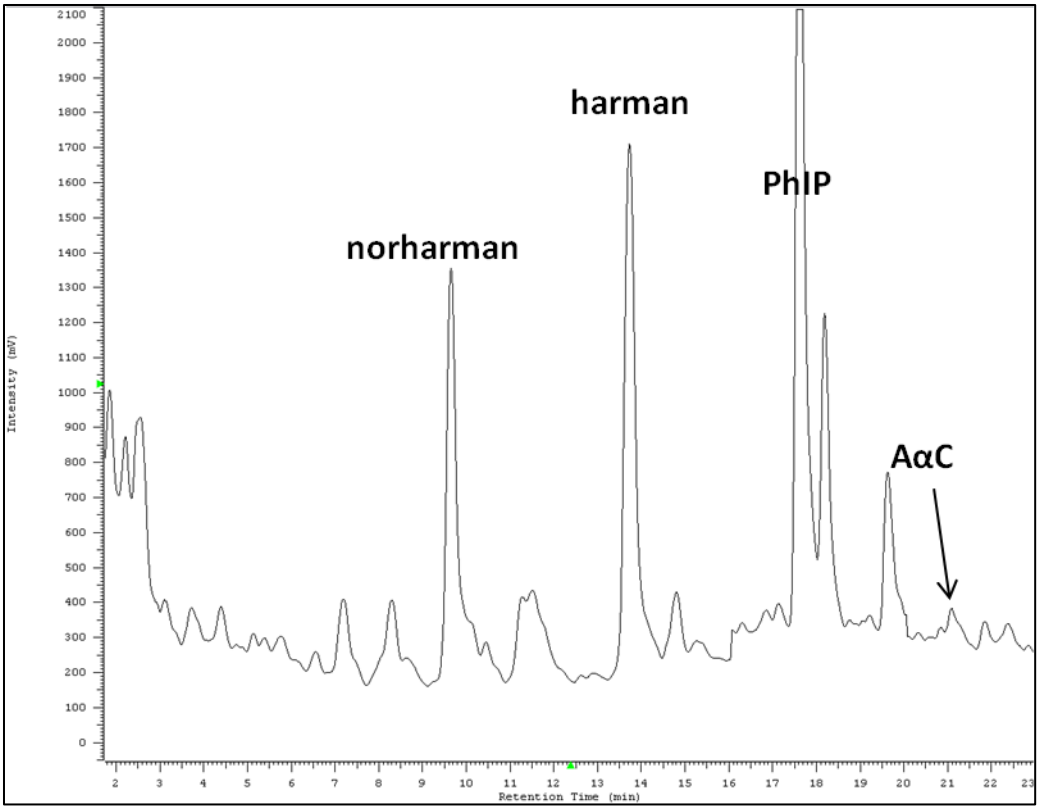

Figure 35: Extract of beef patties fried with 10% cooked wild rice flour, unspiked, fluorescence detection.

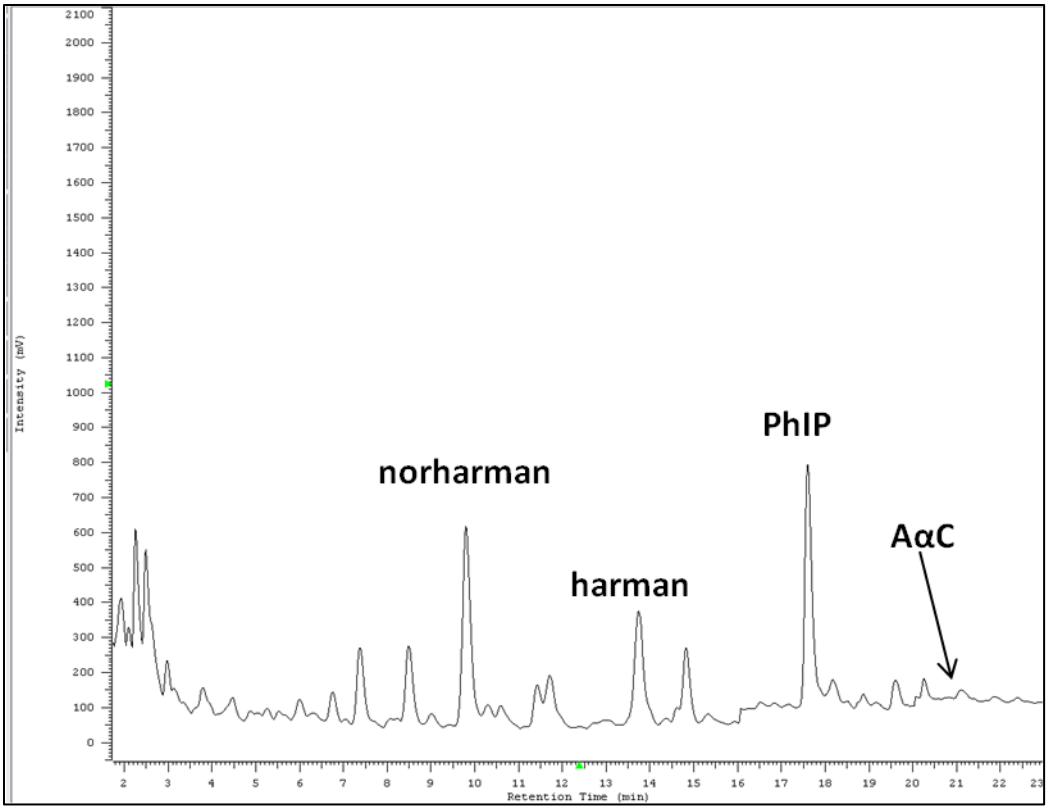

Figure 36: Extract of beef patties fried with 10% oat hulls, unspiked, fluorescence detection.

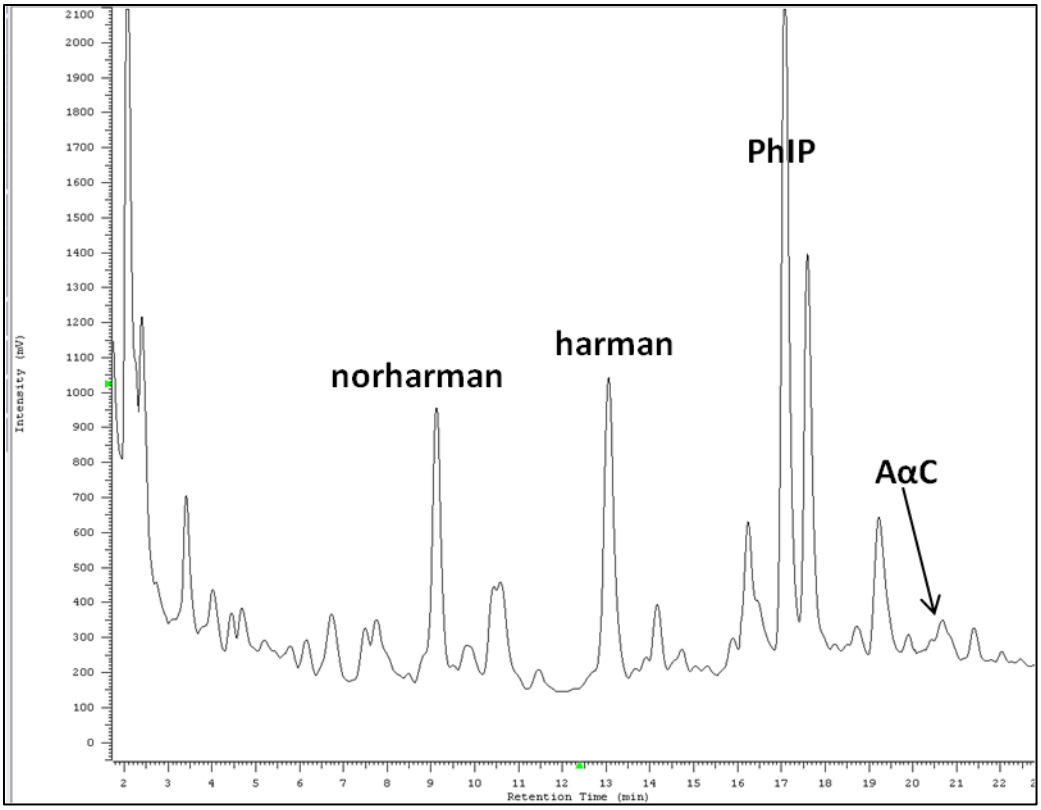

Figure 37: Extract of beef patties fried with 10% raw corn flour, unspiked, fluorescence detection.

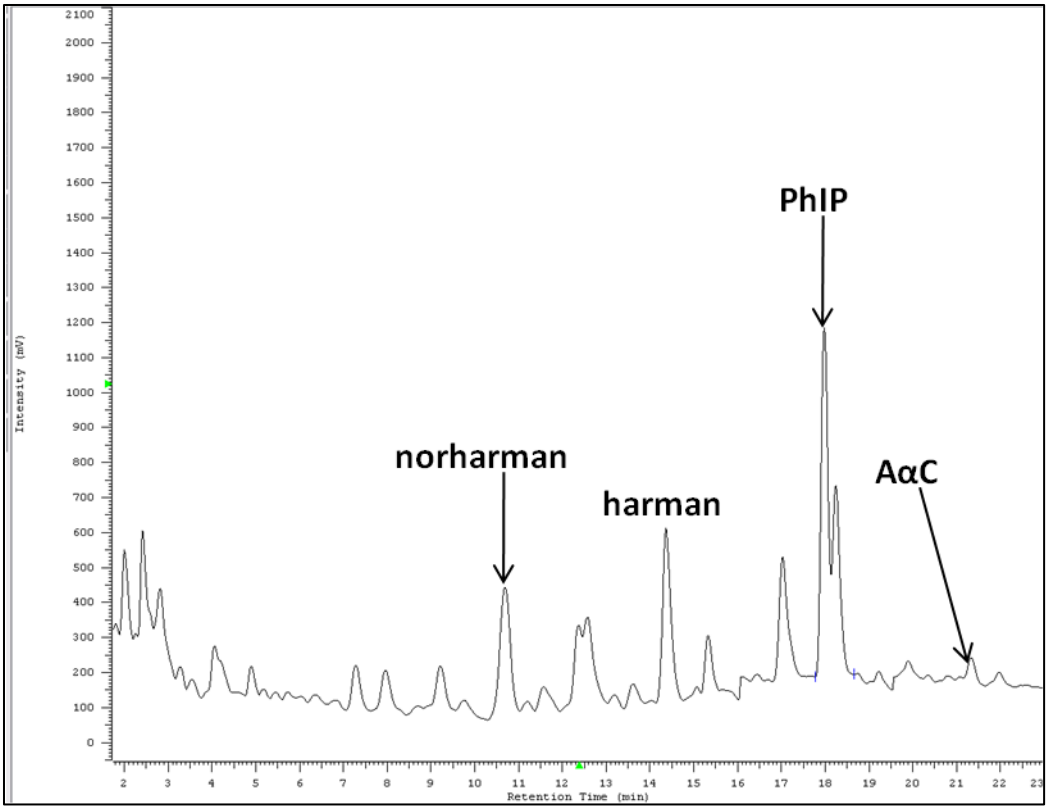

Figure 38: Extract of beef patties fried with 10% cooked corn flour, unspiked, fluorescence detection.

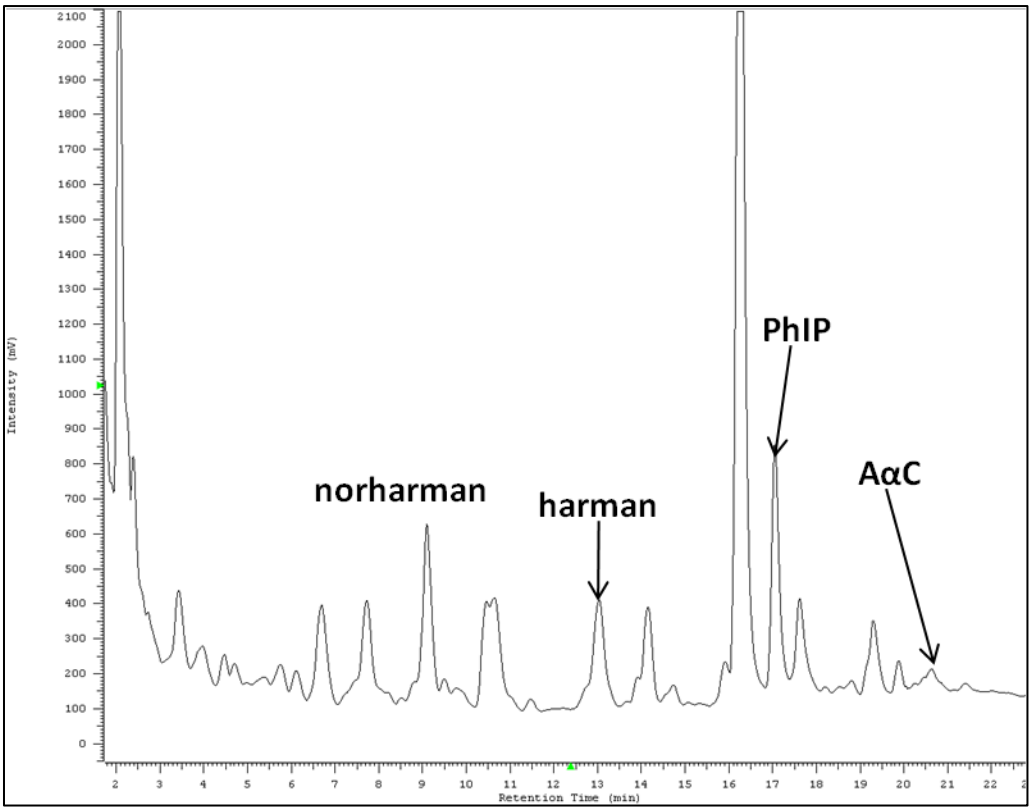

Figure 39: Extract of beef patties fried with 10% corn bran, unspiked, fluorescence detection.

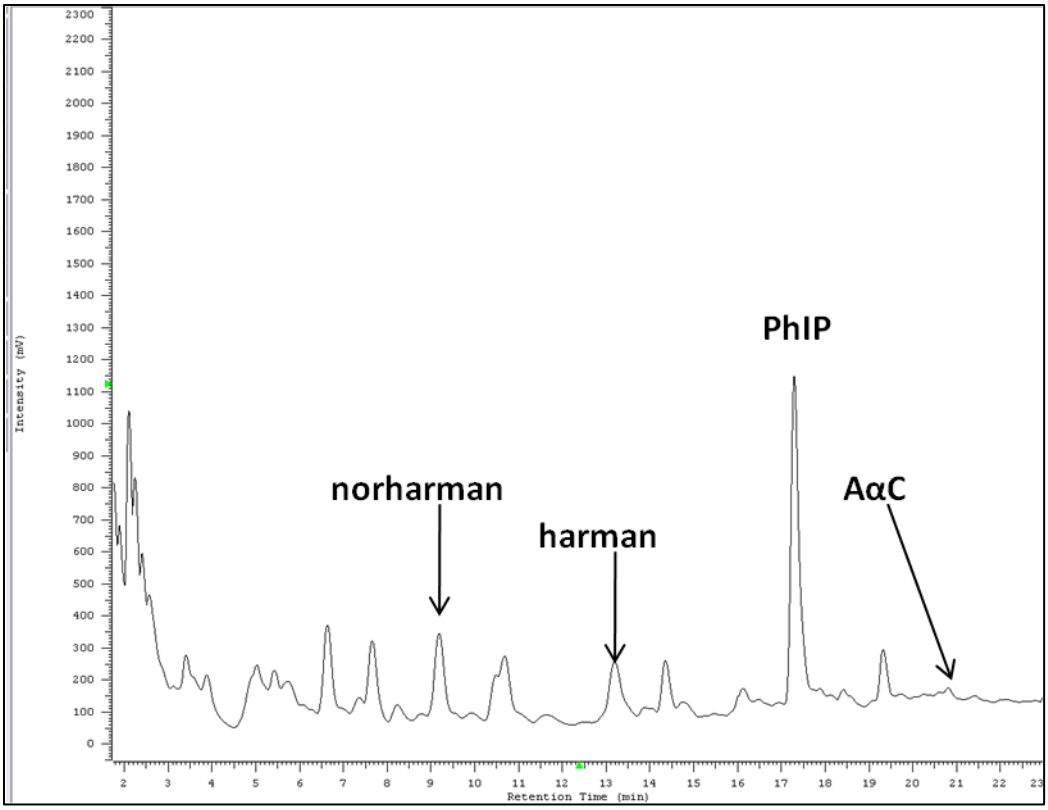

Figure 40: Extract of beef patties fried with corn bran extract, unspiked, fluorescence detection

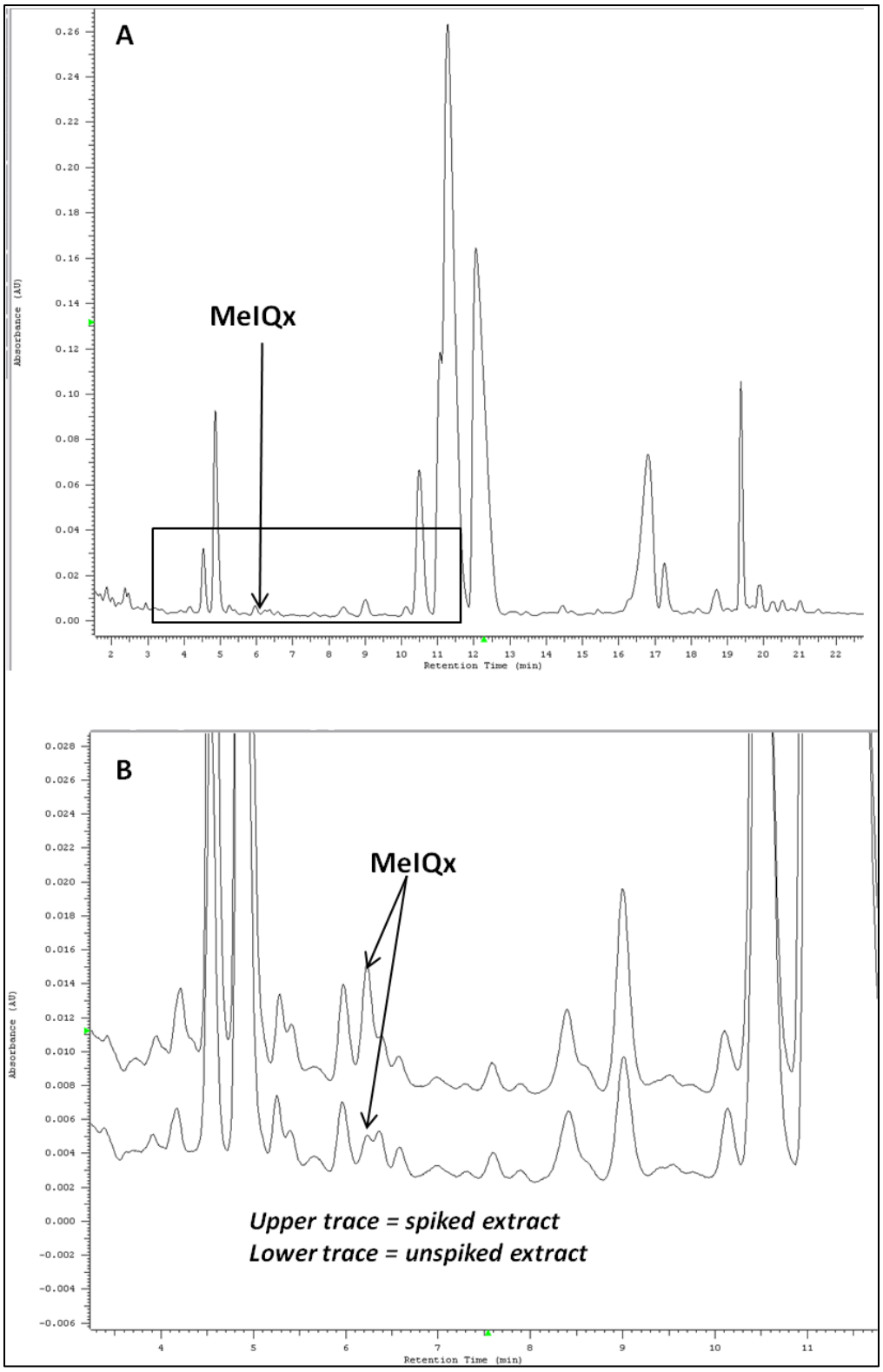

Figure 41: Extract of beef patties fried with 5% raw corn flour, UV absorption (268 nm). A = complete chromatogram, B = close-up of selection.

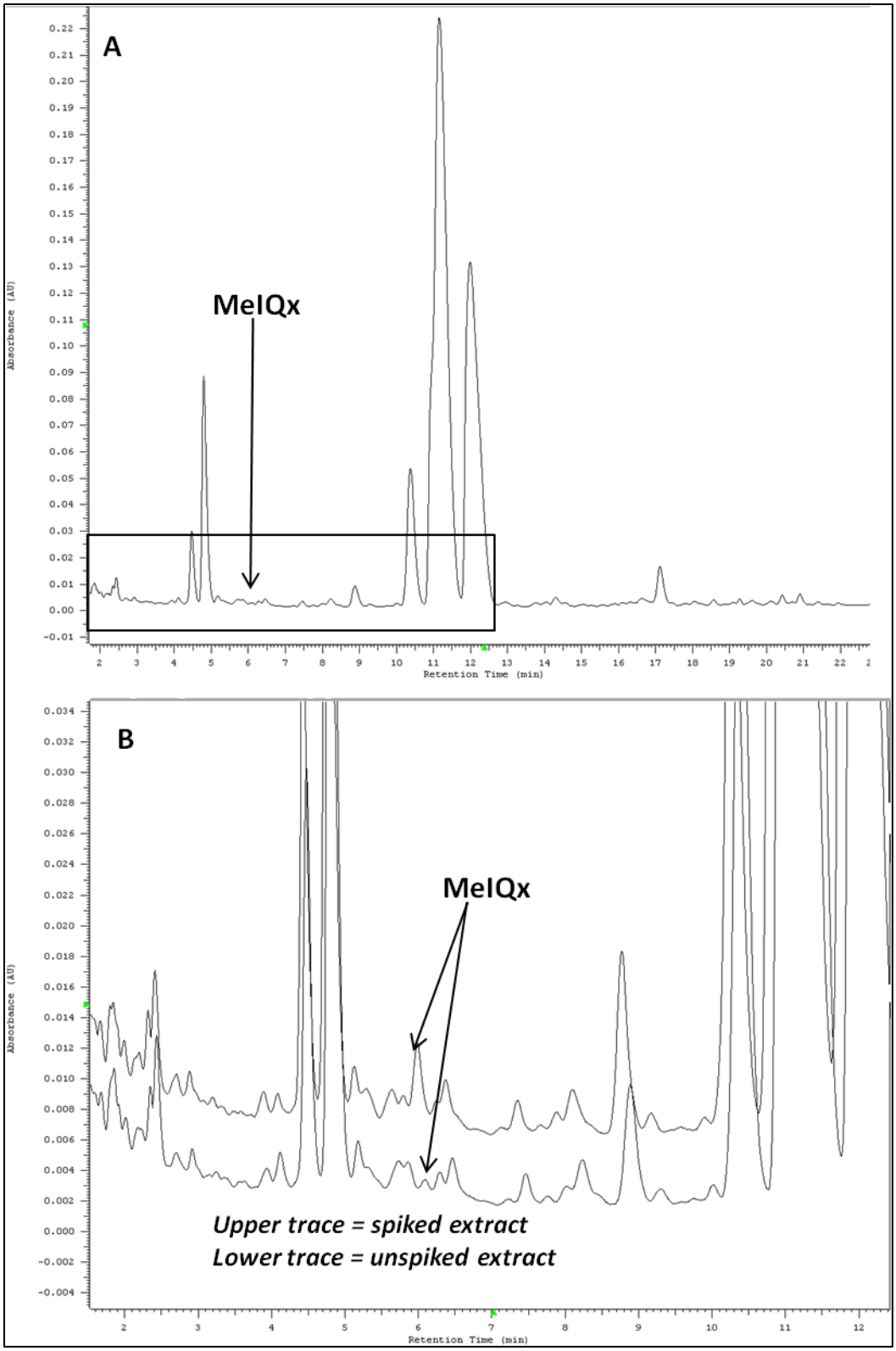

Figure 42: Extract of beef patties fried with 5% cooked wild rice flour, UV absorption (268 nm). A = complete chromatogram, B = close-up of selection