Comprehensive REVIEWS in Food Science and Food Safety

Heterocyclic Aromatic Amines in Cooked Meat Products: Causes, Formation, Occurrence, and Risk Assessment

Monika Gibis

Abstract: Meat products are sources of protein with high biological value and an essential source of other nutrients, such as vitamins and minerals. Heating processes cause food to become more appetizing with changes in texture, appearance, flavor, and chemical properties by the altering of protein structure and other ingredients. During heat treatment, heterocyclic aromatic amines (HAAs), potent mutagens/carcinogens, are formed due to the Maillard reaction. The HAAs are classified in at least 2 groups: thermic HAAs (100 to 300 °C) and pyrolytic HAAs (>300 °C). This review focuses on the parameters and precursors which affect the formation of HAAs: preparation, such as the marinating of meat, and cooking methods, including temperature, duration, and heat transfer, as well as levels of precursors. Additionally, factors are described subject to pH, and the type of meat and ingredients, such as added antioxidants, types of carbohydrates and amino acids, ions, fat, and other substances inhibiting or enhancing the formation of HAAs. An overview of the different analytical methods available is shown to determine the HAAs, including their preparation to clean up the sample prior to extraction. Epidemiological results and human daily intake of HAAs obtained from questionnaires show a relationship between the preference for very well-done meat products with increased HAA levels and an enhanced risk of the incidence of cancer, besides other carcinogens in the diet. The metabolic pathway of HAAs is governed by the activity of several enzymes leading to the formation of DNA adducts or HAA excretion and genetic sensitivity of individuals to the impact of HAAs on human cancer risk.

Keywords: antioxidants, β-carbolines, heterocyclic aromatic amines, meat, precursors

Introduction

Individuals have been exposed to a range of toxic substances throughout human history. These can be naturally occurring mutagens, which are mainly found in plant substances, or process-induced mutagens, which can arise during manufacture, such as from heating. Nitrosamines, polycyclic aromatic hydrocarbons, and heterocyclic aromatic amines are typical heat-induced compounds (Ferguson 2010; Oostindjer and others 2014). Widmark (1939) reported for the first time that extracts of roasted horse meat induced cancer in the mammary glands of mice when multiple-swabbed on the back. The heterocyclic aromatic amines (HAAs) that are the focus of this article belong to the process-induced mutagens that cannot be detected in unheated products (Skog and others 1998b). In general, HAAs are formed from heated products which contain sources of nitrogenous compounds, mainly heated foods of animal origin, such as proteins and creatine (Skog and others 1998b). The formation of HAAs is principally dependent on the temperature, heat transfer, and heating conditions used (Murkovic 2004a; Sugimura and others 2004). The structures of HAAs which are formed at temperatures between 100 and 300 °C are called thermic HAAs, IQ-type HAAs (imidazoquinoline or imidazoquinoxaline) or aminoimidazoazaarenes (Jägerstad and others 1998). Above 300 °C, pyrolysis of proteins and individual amino acids occurs and HAAs are formed which are known as pyrolytic HAAs or non-IQ-type HAAs (Jägerstad and others 1998). Another classification of HAAs subdivides them into non-polar and polar HAAs (IQ-type compounds) due to their chemical properties. The names, abbreviations, properties, mutagenicity (Ames test), and molecular structures of HAAs are shown in Table 1.

Occurrence of Heterocyclic Amines

Besides the important formation factors of cooking temperature and duration of the heat treatment, thermic HAAs occur in almost all heated foods of animal origin, such as meat and fish, because creatine, the precursor needed for their formation, is contained in these products (Jägerstad and others 1998; Murkovic 2000). 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) has been detected not only in products of animal origin, but also in wine, beer (Manabe and others 1993), and smoked cheese (Skog and others 1994; Naccari and others 2009). The source is possibly environmental, since PhIP can occur in burning

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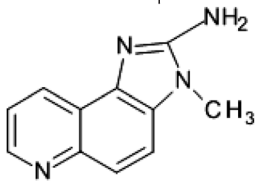
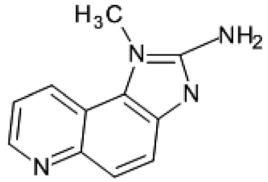
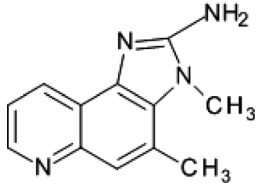
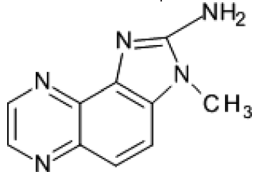
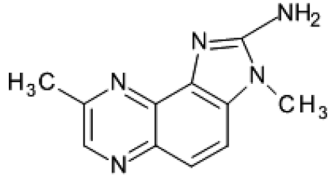
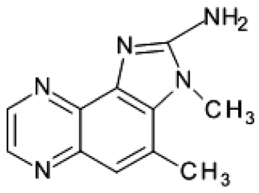
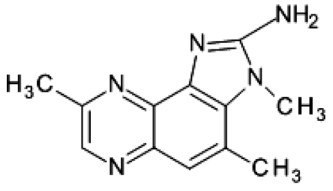
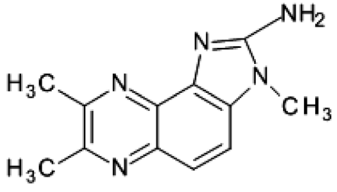
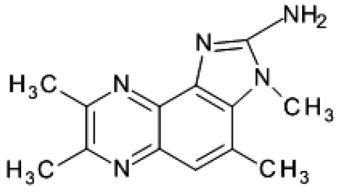
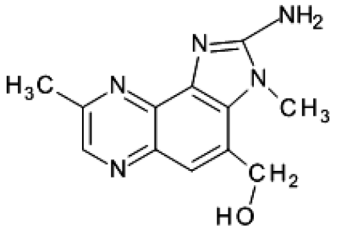
Table 1–Chemical structure, name, and molecular weight of 30 HAAs; some of their properties and indication of their mutagenicity (Ames assay in the presence of S-9 mix using *Salmonella* Typhimurium strains TA98, TA100 and TA1538.

Structure	Abbreviation/Chemical name CAS No	Molecular weight (g/mol) Properties[a]	Mutagenicity ($\times 10^3$rev/μg)[b]
2-Amino-imidazoquinolines			
	IQ 2-Amino-3-methylimidazo[4,5-f]quinoline 76180-96-6	192.2 polar pKa 5.86 ± 0.40[c]	433 (TA98) 7 (TA100) 300 (TA1538)
	Iso-IQ 2-Amino-1-methylimidazo[4,5-*f*]quinoline 102408-25-3	192.2 polar pKa 5.99 ± 0.40[c]	na
	MeIQ 2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline 77094-11-2	212.3 polar pKa 6.22 ± 0.40[c]	661 (TA98) 30 (TA100) 859 (TA1538)
2-Amino-imidazoquinoxalines			
	IQx 2-Amino-3-methylimidazo[4,5-*f*]quinoxaline 108354-47-8	199.3 polar pKa 1.96 ± 0.50[c]	75.4 (TA98) 1.5 (TA100) 103 (TA1538)
	8-MeIQx 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline 77500-04-0	213.3 polar pKa 2.20 ± 0.50[c]	145 (TA98) 14 (TA100) 84.7 (TA1538)

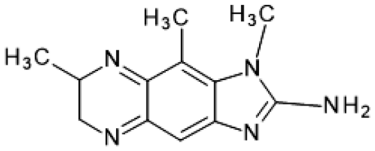
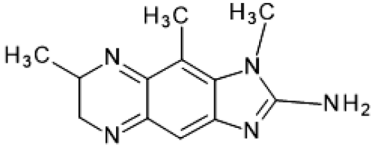
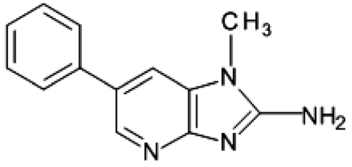
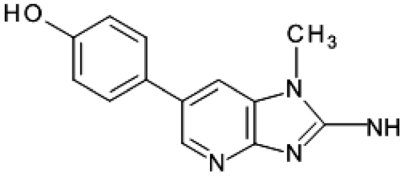
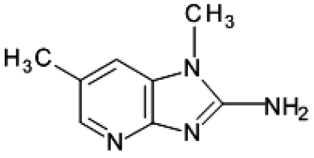
(*Continued*)

Table 1–Continued.

Structure	Abbreviation/Chemical name CAS No	Molecular weight (g/mol) Properties[a]	Mutagenicity (x 10^3rev/μg)[b]
	4-MeIQx 2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoxaline 108354-48-9	213.3 polar pKa 2.32 ± 0.50[c]	1162 (TA98) 51 (TA100) 1042 (TA1538)
	4,8-DiMeIQx 2-Amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline 95896-78-9	227.3 polar pKa 2.56 ± 0.50[c]	183 (TA98) 8 (TA100) 225 (TA1538)
	7,8-DiMeIQx 2-Amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline 92180-79-5	227.3 polar pKa 2.49 ± 0.50[c]	163 (TA98) 9.9 (TA100) 189 (TA1538)
	TriMeIQx 2-Amino-3,4,7,8-tetraimethylimidazo[4,5-*f*]quinoxaline 132898-07-8	241.3 polar pKa 2.85 ± 0.50[c]	na
	4-CH_2OH-8-MeIQx 2-Amino-4-hydroxymethyl-3,8-dimethylimidazo[4,5-*f*]quinoxaline 153954-29-1	243.3 polar pKa 2.17 ± 0.50[c] 13.52 ± 0.50[d]	99 (TA98) 2.6 (TA100)

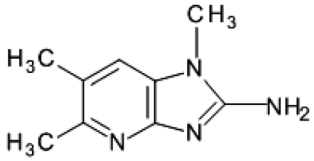
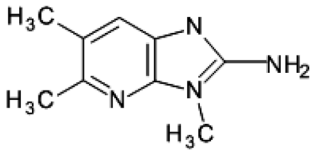
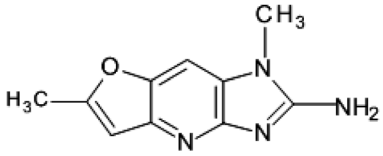
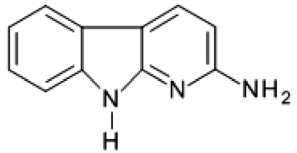
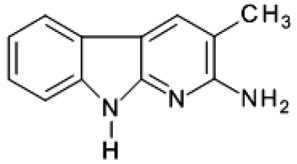
(*Continued*)

Table 1–Continued.

Structure	Abbreviation/Chemical name CAS No	Molecular weight (g/mol) Properties[a]	Mutagenicity (×10³rev/μg)[b]
	7-MeIgQx 2-Amino-1,7-dimethyl-1*H*-imidazo[4,5-*g*]quinoxaline 934333-16-1	213.2 polar pKa 3.95 ± 0.50[c]	na
	7,9-MeIgQx 2-Amino1,7,9-tridimethyl-1*H*-imidazo[4,5-*g*]quinoxaline 156243-39-9	227.3 polar pKa 4.65 ± 0.50[c]	0.67 (TA98)
2-Amino-imidazopyridines 	PhIP 2-Amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine 105650-23-5	224.3 polar pKa 7.72 ± 0.30[c]	1.8 (TA98) 0.12 (TA100) 2.9 (TA1538)
	4′OH-PhIP 2-Amino-1-methyl-6-(4′hydroxyphenyl)-imidazo[4,5-*b*]pyridine 126861-72-1	240.6 polar pKa 7.79 ± 0.30[c] 8.27 ± 0.15[d]	0.002 (TA98)
	DMIP 2-Amino-1,6-dimethylimidazo[4,5-*b*]pyridine 132898-04-5	162.2 polar pKa 8.16 ± 0.30[c]	0.008 (TA1538)

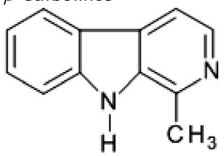
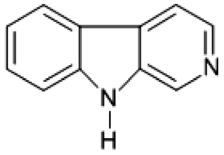
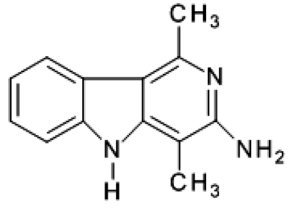
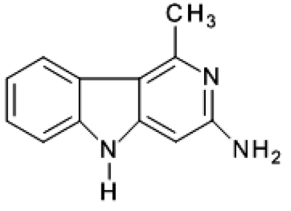
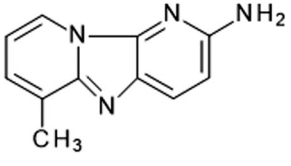
(*Continued*)

Table 1–Continued.

Structure	Abbreviation/Chemical name CAS No	Molecular weight (g/mol) Properties[a]	Mutagenicity (×10³rev/μg)[b]
	1,5,6 TMIP 2-Amino-1,5,6-trimethylimidazo[4,5-*b*]pyridine 161091-55-0	176.2 polar pKa 8.66 ± 0.30[c]	100 (TA1538)
	3,5,6 TMIP 2-Amino-3,5,6-trimethylimidazo[4,5-*b*]pyridine 57667-51-3	176.2 polar pKa 8.36 ± 0.30[c]	na
	IFP 2-Amino-1,6-dimethyl-furo[3,2-*e*]imidazo[4,5-*b*]pyridine 357383-27-8	202.3 polar pKa 7.52 ± 0.40[c]	~10 (TA1538)
α-Carbolines 	AαC 2-Amino-9*H*-pyrido[2,3-*b*]indol 26148-68-5	183.2 nonpolar pKa 6.79 ± 0.30[c] 14.87 ± 0.40[d]	0.3 (TA98) 0.02 (TA100)
	MeAαC 2-Amino-3-methyl-9*H*-pyrido[2,3-*b*]indol 68006-83-7	197.2 nonpolar pKa 7.08 ± 0.30[c] 15.25 ± 0.40[d]	0.2 (TA98) 0.12 (TA100)

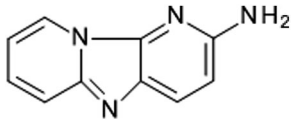
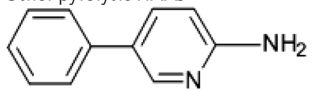
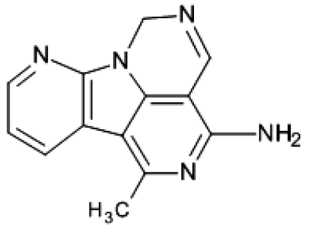
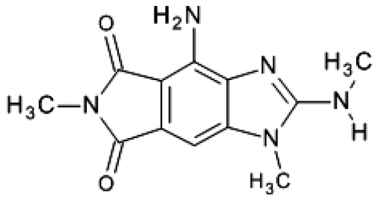
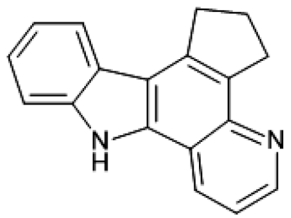
(*Continued*)

Table 1–Continued.

Structure	Abbreviation/Chemical name CAS No	Molecular weight (g/mol) Properties[a]	Mutagenicity (×10³rev/μg)[b]
β-Carbolines 	Harman 1-methyl-9*H*-pyrido[4,3-*b*]indole 486-84-0	182.2 nonpolar pKa 8.62 ± 0.30[c] 15.87 ± 0.40[d]	co-mutagenic
	Norharman 9*H*-pyrido[4,3-*b*]indole 244-63-3	168.2 nonpolar pKa 7.85 ± 0.10[c] 15.44 ± 0.30[d]	co-mutagenic
γ-Carbolines 	Trp-P-1 3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole 62450-06-0	211.3 nonpolar pKa 10.88 ± 0.10[c] 16.02 ± 0.40[d]	39 (TA98) 1.7 (TA100)
	Trp-P-2 3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole 62450-07-1	197.2 nonpolar pKa 10.59 ± 0.30[c] 15.59 ± 0.40[d]	104.2 (TA98) 1.8 (TA100)
δ-Carbolines 	Glu-P-1 2-Amino-6-methyldipyrido[1,2-a:3′2′-*d*]imidazole 67730-11-4	198.3 nonpolar pKa 6.33 ± 0.30[c]	49 (TA98) 3.2 (TA 100)

(*Continued*)

Table 1–Continued.

Structure	Abbreviation/Chemical name CAS No	Molecular weight (g/mol) Properties[a]	Mutagenicity (x 10^3rev/μg)[b]
	Glu-P-2 2-Amino-dipyrido[1,2-a:*3′2′-d*]imidazole 67730-10-3	184.3 nonpolar pKa 5.80 ± 0.30[c]	1.9 (TA 98) 1.2 (TA100)
Other pyrolytic HAAs			
	Phe-P-1 2-Amino-5-phenylpyridine 33421-40-8	170.2 nonpolar pKa 6.32 ± 0.13[c]	na
	Orn-P-1 4-Amino-6-methyl-1*H*-2,5,10,10*b*-tetraazafluoranthene 78859-36-6	237.3 nonpolar pKa 9.52 ± 0.20[c]	na
	Cre-P-1 4-Amino.1,6-dimethyl-2-methylamino-1*H*,6*H*-pyrrolo-[3,4-*f*]benzimidazole-5,7-dione 133883-91-7	259.3 nonpolar pKa 4.83 ± 0.20[c]	19 (TA98) 0.4 (TA100)
	Lys-P-1 1,2,3,8-Tetrahydro-cyclopenta[*c*]pyrido [3,2-*a*]carbazole 69477-66-3	258.3 nonpolar pKa 6.87 ± 0.20[c] 15.83 ± 0.20[d]	na

[a] Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994–2015 ACD/Labs).
[b] References: Sugimura and others 2004; Wyss and Kaddurah-Daouk 2000; na, not analyzed.
[c] Most basic (temperature 25 °C).
[d] Most acidic (temperature 25 °C).

processes. Heterocyclic aromatic amines are similarly detected in rain water and cigarette smoke condensate (Xu and others 2010; Liu and others 2013). Individual HAAs were also detected in particles from diesel exhaust fumes (Kataoka 1997). Over 25 mutagenic HAAs have been isolated and identified since 1977 (Alaejos and Afonso 2011) (Table 1). However, the concentrations detected in food vary widely, as will be discussed later.

The HAAs occurring most often in meat are PhIP, MeIQx, 4,8-DiMeIQx, IQ, MeIQ, and AαC (Skog and others 1998a, b). The PhIP concentrations in most studies are between 1 and 70 ng/g meat (Gross and Grueter 1992; Alaejos and Afonso 2011). Concentrations of MeIQx up to 23 ng/g can be detected (Skog and others 1995). As a rule, concentrations of up to 6 ng/g meat are found (Skog and others 1998b). 4,8-DiMeIQx is mostly detected only in the lower concentration range of around 1 ng/g meat. In some studies, IQ could not be detected at all (Skog and others 1998b). Table 2 to 4 show an overview of the occurrence of HAAs in beef patties, chicken breasts, and pork using different preparation methods.

While meat and meat products are frequently studied, fewer studies were found about processed meat (Table 5). However, IQ levels of 3.8 to 10.5 ng/g were recorded in fried bacon (Johansson and Jägerstad 1994). IQ and MeIQ could also be detected in grilled sausages (Gerbl and others 2004). The HAA levels of bacon prepared in different ways were investigated in a study (Sinha and others 1998). Levels of 0.4 to 4.3 ng MeIQx/g and 0 to 4.8 ng PhIP/g could be determined with pan-frying. MeIQx levels of 0 to 4 ng/g with ovenroasting differed only a little from frying. However, significantly higher PhIP levels of 1.4 to 30.3 ng/g were detected for oven roasting (Sinha and others 1998). Further studies show that high HAA concentrations can be found in bacon (Table 5). The use of various preparation methods affects the formation of HAAs differently for different meat products. The grill sausages investigated were pan-fried or grilled (Abdulkarim and Smith 1998). Along with different heating times, the heating temperature was also varied. With both cooking methods, along with norharman and harman, MeIQx and PhIP could also be detected (Table 5). In offal products (beef liver, lamb kidney, and beef tongue), which were thermally processed, HAAs were found in concentrations only near the detection limit, except for norharman and harman. Both β-carbolines were found in concentrations below 2 ng/g; only DMIP, MeIQx, and 4,8-DiMeIQx were detectable in cooked kidney and tongue up to 0.25 ng/g (Khan and others 2009). The reason for the low contents of IQ-type HAAs may be the lack of the precursor creatine (Harris and others 1997). The wide-ranging variation of precursors observed in studies with meat from various animal species (Zimmerli and others 2001; Skog and Solyakov 2002; Sun and others 2010; Puangsombat and others 2012; Zaidi and others 2012), clearly affected the formation of HAAs (Liao and others 2010, 2011b; Gibis and Weiss 2015).

Also the pH-value is known to influence the Maillard reactions (Cremer and Eichner 2000) and thereby also the formation of HAAs. This was shown that the content of HAAs in pork increased about 22% on average (MeIQx – 33%; 4,8-DiMeIQx – 17%; and harman – 9%) in the PSE (pale, soft, and exudative) meat samples with lower pH values when grilled than the normal muscle at a core temperature of 95 °C (Polak and others 2009b). At the lower core temperature of 70 °C, no remarkable difference in HAA formation could be observed between PSE meat and normal meat (Table 4). PSE is associated with pale color, low pH, and a high drip loss causing by preslaughter stress and genetics of pigs with a fast *post mortem* glycolysis (Polak and others 2009b). In a study investigating normal muscle meat of different animal species, the measured pH values of the uncooked meat of the different animals (range of pH: 5.56 to 6.12) showed no significant linear correlation to the HAA levels, except for PhIP with a very weak correlation ($r = 0.27$, $p < 0.05$) (Gibis and Weiss 2015). Additionally, the duration of aging influenced the HAA concentrations for both PSE and normal pork. Significantly higher levels were observed after longer aging of pork (Polak and others 2009b) and beef (Polak and others 2009a).

Meat from horses showed threefold higher glucose levels than beef (Rossier 2003), which reduced the content of HAAs (Gibis and Weiss 2015). Similar results were observed in pork containing high and low levels of glucose (Olsson and others 2002). Contrarily, chicken had very low glucose levels, but a similar content of creatine, which increased, in particular, the concentration of PhIP by about a factor of 10 (Gibis and Weiss 2015). The same decrease in concentrations of PhIP, and less in levels of other HAAs, was observed in a model system by the addition of saccharides (Skog and Jägerstad 1990).

Formation of Heterocyclic Aromatic Amines

Formation of imidazoquinolines and imidazoquinoxalines

The pyridines and pyrazines formed from hexoses and amino acids, respectively, in the Maillard reaction via the Strecker degradation serve as building blocks for the IQ compounds. The reaction is depicted in Figure 1A. The creatine cyclizes to creatinine during heating and reacts in an aldol reaction with the pyridine or pyrazine derivatives, respectively, to generate imidazoquinoline and imidazoquinoxaline (Skog and Jägerstad 1993). The aldehydes arising, together with creatinine, also play an important role in the formation of the imidazole rings of the polar HAAs. The 2 parts can be joined to each other via a Strecker aldehyde to a Schiff base. The mechanism was confirmed for IQx, MeIQx, and 4,8-DiMeIQx by using ^{14}C-labeled glucose (Skog and Jägerstad 2005).

Formation of PhIP

Phenylalanine, reducing sugars and creatinine could be detected in PhIP formation. It could be shown in a model trial with radioactively labeled carbon in the phenylalanine molecule that the phenyl ring was completely built into the PhIP molecule (Zöchling and Murkovic 2002). Further trials showed that creatinine forms a part of the imidazole ring. The authors were able to identify the following reaction steps in PhIP formation in a model trial: First, phenylacetaldehyde is formed from phenylalanine via the Strecker degradation. The phenylacetaldehyde formed reacts in an aldol reaction with creatinine to form an intermediate product. In the subsequent condensation reaction, PhIP arose from this substance (Zöchling and Murkovic 2002). The mechanism of the reaction is shown in Figure 1B. The formation of formaldehyde from phenylacetaldehyde and phenylalanine, and the combination of both formaldehyde and ammonia in the generation of PhIP from phenylacetaldehyde and creatinine were reported in the reaction pathways that produce PhIP (Zamora and others 2014). In the presence of oxidized lipid, other amino acids competed with phenylalanine for the lipid, and amino acid degradation products were formed, among which α-keto acids seemed to play a role in these reactions (Zamora and others 2013b). However, unoxidized lipids did not contribute to PhIP formation (Zamora and others 2012).

Table 2–Occurrence of HAAs in ground beef patties using different heating conditions.

Cooking procedure	Cooking time (min)	Temperature (°C)	PhIP (ng/g)[b]	MeIQx (ng/g)[b]	4,8-Di-MeIQx (ng/g)[b]	Norharman (ng/g)[c]	Harman (ng/g)[c]	Others (ng/g)[bc]	Reference
Fried	4–20	150	nd–1.8	nd–0.6	nd	1.1	na		(Knize and others 1994)
		190	nd–9.8	0.1–1.3	0.4	0.15	na		
		230	1.3–32	0.4–7.3	nd	1.6	na		
Fried	12	198	4.9	4.3	1.3	na	na	AαC (21)	(Thiébaud and others 1995)
		277	68	16	4.5				
Fried	8	165	0.08	0.2	nd	na	na		(Johansson and others 1995b)
		200	1.5	1.6	0.4				
Fried	5–7	150	0.01	nd	nd	na	na		(Skog and others 1995)
		225	1.1	2.2	0.8				
Fried	–	–	67.5	16.4	4.5	na	na		(Knize and others 1997a)
Grilled	–	–	50	2	nd				
Barbecued	6 + 6	200	2.35	0.27	nd	1.87	0.61	Trp-P-2 (1.7)	(Abdulkarim and Smith 1998)
(15%fat)	3.5 + 3.5	240	0.10	0.80	nd	2.17	0.88	Trp-P-2 (1.5)	
Fried	3 + 3	150	0.43	nd	0.31	0.96	0.31		
(15% fat)	5 + 5	190	0.57	0.84		2.0	0.83		
	7.5 + 7.5	230	0.25	1.00		5.65	1.7		
Fried	12–20	175	0.9–6.2	0.5–0.8	0.8–0.9	na	na	IQ(0.7-1.3), MeIQ(0.1-0.3)	(Balogh and others 2000)
		200	4.0–25.4	1.5–4.2	0.9–4.5			IQ(1.7-4.4), MeIQ(0.5-2.1)	
		225	13.3–1.4	3.5–5.8	3.0–4.8			IQ(2.8-5.3), MeIQ(2-3.5)	
Fried	–	–	nd–1.5	nd–1.3	nd	na	na		(Zimmerli and others 2001)
Fried	3	230	0.2	1.0	nd	2.1	1.5		(Jautz and others 2008)
	4.5	230	0.9	2.0	0.4	5.0	3.5		
	6	230	3.8	4.8	3.0	10.4	8.9		
Fried/	10	180	0.7	1.0	0.2	na	na	[d]IQ (0.1/0.05), IFP (0.1/nd)	(Ni and others 2008)
Oven-broiled	10	186	nd	nd	nd			IQ[4,5-b] (0.3/nd), IgQx (0.5/nd), 7-MeIgQx (2.4/0.3), 6,7-DiMeIgQx (0.2/0.05), 7,9-DiMeIgQx (0.7/nd)	
Fried/	15	189	2.7	3.0	0.6	na	na	[d]IQ (0.1/0.06), IFP (0.6/nd) IQ[4,5-b] (0.3/0.4), IgQx	
Oven-broiled	15	189	0.06	0.02	0.02			(1.5/0.03), 7-MeIgQx (9.5/0.1), 6,7-DiMeIgQx (0.3/nd), 7,9-DiMeIgQx (2.2/0.02)	
Fried/	20	191	2.9	3.7	0.7	na	na	[d]IQ (0.2/0.1), IFP (0.7/0.14) IQ[4,5-b] (0.3/0.15), IgQx	
Oven-broiled	20	191	1.23	0.38	0.1			(1.8/0.3), 7-MeIgQx (11.7/0.3), 6,7-DiMeIgQx (0.4/nd), 7,9-DiMeIgQx (3.0/0.12)	
Fried[a]	4.5	230	2.6	4.9	1.8	13.5	21.4		(Gibis 2009)
Fried[a]	2-3	~190	0.1–0.2	0.2–1.7	nd–0.2	0.2–0.9	0.7–1.7		(Gibis and Weiss 2010)

[a]Fried/grilled on a double-plate grill; [b]nd not detected; [c]na not analyzed; [d]HAA ng/g (fried/oven-broiled).

Table 3–Occurrence of HAAs in chicken breast.

Cooking procedure	Cooking time (min)	Cooking temperature (°C)	PhIP (ng/g)[c]	MeIQx (ng/g)[bc]	4,8-DiMeIQx (ng/g)[bc]	Norharman (ng/g)[b]	Harman (ng/g)[c]	Others (ng/g)	Reference
Pan-fried	14–36	197–221	12–70	1–3	1–4	na	na		(Sinha and others 1995)
Barbecued	10–43	177–260	27–480	nd–9	nd–2	na	na		
Boiled	9–17	79–86	6–150	nd-3	nd	na	na		
Pan-fried	16	175	0.7	na	na	na	na		(Persson and others 2002)
	18	200	10.5	na	na	na	na		
	12	225	29.7	na	na	na	na		
Pan-fried	12–34	140–225	<0.1–38.2	0.1–1.8	0.1–0.6	0.5–6.9	0.3–7.5		(Solyakov and Skog 2002)
Roasted	24–40	175–240	nd	nd	nd	<0.1–3.3	<0.1–1.7		
Deep-fat fried	11	160	<0.1[a]	<0.1[a]	<0.1[a]	0.5	0.3		
Boiled	38	200	nd	nd	0.1[a]	<0.1[a]	<0.1[a]		
Pan-fried	14–36	197–211	8.8–48.5	0.6–2.3	0.8–3.6	na	na	IQ(0.1-0.2), IQx(0.1-0.2), IFP(2.7-15.9), IQ[4,5-b]([a]-0.2), IgQx(0.3-0.9), 7-MeIgQx(1.5-8.7), 6.7-DiMeIgQx ([a] -0.1), 7.9-DiMeIgQx(0.2-0.9), AαC([a] -0.1), MeAαC[a]	(Ni and others 2008)
Oven-broiled	9–17	179–186	5.6–72.0	0.1–2.8	0.1–2.0	na	na	IQ(0.2-0.3), IQx[a], IFP(0.4-11.3), IQ[4,5-*b*] ([a]-0.3), IgQx(0.2-0.4), 7-MeIgQx(1.0-11.1), 6.7-DiMeIgQx([a]-0.2), 7.9-DiMeIgQx(0.1-1.8), AαC(0.2-9,4), MeAαC([a]-1.3)	
Fried[d]	5	220	2.4	2.1	0.11	0.07	0.09		(Gašperlin and others 2009)
Grilled[e]	18	220	0.3	0.2	0.13	0.11	0.05		
Fried[d]	4.5	230	3.8	0.2	nd	0.8	0.9		(Gibis 2009)
Pan-fried	5 + 5	180	18.3	1.8	1.1	1.4	2.8	IQ(1.8), AαC(0.2), MeAαC[a]	(Liao and others 2010)
Deep-fat fried	10	180	2.2	0.8	0.4	5.4	12.3	AαC(0.3), MeAαC[a]	
Charcoal- grilled	10 + 10	200	31.1	1.2	3.6	32.2	31.7	Trp-P-2(3.6), Trp-P-1 (1.5), AαC(5.6), MeAαC(1.6)	
Roasted	20	200	0.04	nd	nd	3.1	0.7	AαC[a], MeAαC[a]	

[a] Traces (≤0.05 ng/g).
[b] na, not analyzed.
[c] nd, not detected.
[d] Fried on a double-plate grill.
[e] Grilled on an infrared grill.

Table 4–Occurrence of HAAs in pork.

Material	Cooking procedure	Cooking time (min)	Cooking temperature (°C)	PhIP (ng/g)[a]	MeIQx (ng/g)[a]	4,8-DiMeIQx (ng/g)[a]	Norharman (ng/g)[b]	Harman (ng/g)[a b]	Others (ng/g)[b]	Reference
Pork chop	Fried	8–9.5	150–225	nd–4.8	nd–2.6	nd–1.1	na	na		(Skog and others 1995)
Pork chop	Fried	5–15	175	nd	nd–3.8	nd	na	na		(Sinha and others 1998)
Pork patties (70 g)										
RN$^-$ allele	Fried	3 + 3	200	1.9	1.9	0.4	na	na		(Olsson and others 2002)
Non RN$^-$ allele	Fried			0.2	1.5	0.2	na	na		
Pork chops RN$^-$ allele pH 5.32	Pan-fried	3 + 3	160–200	0.05–0.1	0.1–0.2	nd	0.6–1.7	1.1–0.7	IQx (0.1–0.2)	(Olsson and others 2005)
Non RN$^-$ allele pH 5.58	Pan-fried			0.1–3.3	0.1–0.8	nd	nd	nd	IQx (nd)	
Pork patties	Boiled	8–16	100	nd	0.4–1.0	nd	na	na		(Shin 2005)
	Broiled	12–19	177–225	nd–2.7	1.2–1.6	0.3–0.7	na	na		
	Pan-Fried	9–21	177–225	0.3—10–5	0.6–5.0	0.3–1.7	na	na		
Pork steak[c]	Pan-fried		220	0.8–3.1	0.6–4.6	0.2–1.0	0.2–0.5	0.9–0.2		(Polak and others 2009b)
pH 5.48			core temp							
pH 5.60			70–95	0.8–2.7	0.9–3.1	0.2–0.9	0.2–0.5	0.9–0.2		
Pork top loin	Pan–fried	8 + 8	204	1.8	1.1	1.2	na	na	IQx (nd)	(Puangsombat and others 2012)
	Baked	70		2.2	0.2	0.9	na	na	IQx (nd)	
Pork patty	Fried (50 g)	5	180	18.4	3.2	0.7	na	na		(Zhang and others 2013)
Pork meat-ball				5.3	1.0	0.3	na	na		
Pork strip				5.4	1.0	0.3	na	na		
Pork loin	Pan-fried		204 core temp 77	13.1	7.6	1.6	na	na		(Vangnai and others 2014)
Pork patties (80 g) pH 5.56	Grilled[d]	2.7	220	2.3	1.1	0.5	1.1	0.5		(Gibis and Weiss 2015)

[a] na not analysed,
[b] nd not detected.
[c] *M. longissimus dorsi*
[d] fried on a double-plate grill

Table 5–Comparison of the content of the most common HAAs in cooked bacon and processed meat.

Preparation method	Heating condition		HAA[a]					Reference
	Time (min)	Temp. (°C)	MeIQx (ng/g)	4,8-DiMeIQx (ng/g)	PhIP (ng/g)	Norharman (ng/g)	Harman (ng/g)	
Bacon fried	12–16	170	0.9–27	0.5–2.4	nd–52	nd–22	nd–30	(Gross and others 1993)
Bacon micro-waved (600 W)	3		0.1	nd	nd	3.3	nd	
Bacon pan-fried	5	150	2.8	3.4	0.2	na	na	(Johansson and Jägerstad 1994)
Bacon pan-fried	4–16	176–177	0.4–4.3	<0.2	<0.2–4.8	na	na	(Sinha and others 1998)
Bacon oven-broiled	4–7	175–185	0.2–4	<0.2	1.4–30.3	na	na	
Bacon micro-waved	1.8–3.3		<0.2–1.5	<0.2	<0.2–3.1	na	na	
Bacon pan-fried	1.5	230	8.1	4.5	28.4	na	na	(Guy and others 2000)
Bacon grilled	1.5	230	1.6	0.9	5.0	na	na	
Bacon pan-fried	16.1	176	3.0	0.7	4.9	na	na	(Ni and others 2008)
Bacon oven-broiled	7.5	175	2.6	5.2	15.9	na	na	
Bacon pan-fried	3	204	4.0	3.6	6.9	na	na	(Puangsombat and others 2012)
Bacon pan-fried	3–6	160	1.5–4.9	nd	0.1–1.1	5–14.1	0.4–1.7	(Gibis and others 2015)
	2–3	210	2.4–5.6	nd	1.3–2.6	13.7–19.9	1.3–1.6	
Falun sausage fried	5	160	0.6	nd	nd	na	na	(Johansson and Jägerstad 1994)
Sausage fried	6	160	0.7	0.2	0.1	na	na	
Ham pan-fried	5–19	175	<0.2–1.8	<0.2	<0.2–0.3	na	na	(Knize and others 1997b)
Hot dogs pan-fried	4–18	175–177	nd	nd	nd	na	na	(Sinha and others 1998)
Hot dogs oven-broiled	3–10	180–185	nd	nd	nd	na	na	
Hot dogs grill/barbecued	5–15	232–252	nd	nd	nd	na	na	
Pork sausage fried	6–15	150–230	nd–0.7	nd	nd–1.1	nd–0.8	nd–3.1	(Abdulkarim and Smith 1998)
Pork sausage barbecued	12/7	200/240	0.35/0.8	nd	0.1/1.3	6.1/1.16	0.57/4.2	
Sausage fried	9	175–200	<0.2	<0.5	<0.1	0.3	0.3	(Busquets and others 2004)
Pork sausage patties fried	21	179	5.1	0.7	0.2	<0.3	<0.3	(Ni and others 2008)
Pizza topping salami oven-baked	12–20	230	nd–2.6	nd	nd–0.4	107.4–186.1	11.4–24.7	(Gibis and Weiss 2013)
	10–12	250	nd–0.2	nd	0.1–0.3	143.2–146.1	15.3–21.0	
Pizza topping ham oven-baked	12–20	230	0.2–3.1	0.5–2.1	0.2–0.8	4.5–10.3	2.5–4.8	
	10–12	250	0.1–0.2	0.5–0.6	0.3–0.5	5.6–7.0	4.3–4.9	

[a]na, not analyzed; nd, not detected.

Table 6–Effect of different antioxidant ingredients on the formation of HAAs in fried or grilled beef products, inhibition of HAA formation (%) and increase of HAA levels compared to the controls without antioxidant components as indicated by +; na indicates not analyzed; nd indicates not detected.

Product	Ingredient	Concentration	Cooking time (min)	Cooking temp. (°C)	PhIP (%)	MeIQx (%)	4,8-DiMeIQx (%)	Norharman (%)	Harman (%)	Others (%)	Reference
Beef patty	Cherry tissue	11.5%	8 + 8	170	93	62	81	na	na	IQ(72), MeIQ(50)	(Britt and others 1998)
Beef steak	Rosemary	Spread[a]	20	180	75	38	39	na	na	IQ(72), MeIQ(64)	(Murkovic and others 1998)
	Garlic	Spread[a]			54	71	78	na	na	IQ(32), MeIQ(40)	
	Sage	Spread[a]			100	40	100	na	na	IQ(100), MeIQ(77)	
	Thyme	Spread[a]			75	61	100	na	na	IQ(74), MeIQ(61)	
Beef patty	Oleoresin rosemary	1%	10 + 10	225	44	30	77	na	na	IQ(72), MeIQ(87)	(Balogh and others 2000)
		10%			45	12	68	na	na	IQ(72), MeIQ(72)	
	Vitamin E	1%			69	48	79	na	na	IQ(86), MeIQ(79)	
		10%			72	26	71	na	na	IQ(88), MeIQ(64)	
Beef patty (100 g; 15.4% fat)	Minced garlic	4.5%	10 + 10	225	34	13	24	na	na		(Shin and others 2002a)
		18.2%,			71	62	62	na	na		
	Sulfur[b] Comp.	0.17 mM			51	28	46	na	na		
		0.67 mM			82	66	81	na	na		
		1.01 mM			90	70	81	na	na		
Beef patty (90 g)	Virgin olive oil fresh	40 g	5 + 5	200	60	5	nd	50	40		(Persson and others 2003a)
	Stored for 1 year				75	65	nd	80	0		
Beef patty	Grape-seed	0.5%	10 + 10	210	1	26	100	48	+3493	IQ(100), MeIQ(17), AαAC(29)	(Ahn and Gruen, 2005b)
		1%			25	64	100	64	+6949	IQ(100), MeIQ(34), AαAC(43)	
	Pine bark	0.5%			37	47	100	55	28	IQ(100), MeIQ(100), AαAC(+11)	
		1%			36	62	100	61	32	IQ(100), MeIQ (100), AαAC(29)	
	Oleoresin rosemary	0.5%			39	6	24	49	23	IQ(100), MeIQ(26), AαAC(100)	
		1%			58	23	100	100	55	IQ(100), MeIQ(100), AαAC(100	
	BHA/BHT	0.02%			12	+3	15	52	27	IQ(100), MeIQ(4), AαAC(17)	
Beef patty	Grape- seed	0.1%	6 + 6	210	72	67	66	na	na		(Cheng and others 2007)
	Apple	0.1%			69	59	63	na	na		
	Elderberry	0.1%			45	6	19	na	na		
	Pineapple	0.1%			13	27	18	na	na		
Beef patty	Carvacrol	1%		200 core temp 70	78	72	nd	na	na	MeIQ (58)	(Friedman and others 2009)
Beef patty (100 g)	Minced garlic	4.8%	5 + 5	220	16	51	nd	6	6	Trp-P-1(15), Trp-P-2, Glu-P-1, & Glu-P-2 (100), AαAC (+39)	(Jung and others 2010)
		13%			83	100	nd	96	88	Trp-P-1(100), Trp-P-2, Glu-P-1, & Glu-P-2 (100), AαAC (100)	
Beef patty (70 g)	Hibiscus marinade[c]	0.2%	2.7	230	18	36	nd	+1	+2		(Gibis and Weiss 2010)
		0.8%			64	56	nd	+66	+33		

(*Continued*)

Table 6–Continued.

Product	Ingredient	Concentration	Cooking time (min)	Cooking temp. (°C)	PhIP (%)	MeIQx (%)	4,8-DiMeIQx (%)	Norharman (%)	Harman (%)	Others (%)	Reference
Beef patty (100 g)	Galangal	0.2%	5 + 5	204	19	18	na	na	na		(Puangsombat and others 2011)
	Fingerroot	0.2%			37	31	na	na	na		
	Turmeric	0.2%			38	41	na	na	na		
	Cumin	0.2%			6	1	na	na	na		
	Coriander seeds	0.2%			6	3	na	na	na		
	Rosemary	0.2%			36	50	na	na	na		
Beef patty (70 g, frozen)	Grape-seed[d]	0.2%	2.7	230	50	12	nd	+3	+6		(Gibis and Weiss 2012)
		0.8%			71	93	nd	+25	+55		
	Rosemary extract[e]	0.12%			35	16	nd	+34	+55		
		1.5%			91	54	nd	+50	3		
Beef patty (30 g, 6.2×1.2 cm)	Ascorbic acid	0.02 mM	3 + 3	200	19	17	14	na	na		(Wong and others 2012)
	Niacin				19	19	15	na	na		
	Pyrido xamine				43	42	38	na	na		
Beef steak (1.4 cm thick)	Beer	50%	3 + 3	180	50	>99	>98	na	na	IQ(>97)	(Viegas and others 2012)
	Beer + spices[f]				91	>76	64	na	na	IQ(>97)	
	Wine				+1	>76	70	na	na	IQ(>97)	
	Wine + spices[f]				32	33	64	na	na	IQ(72)	
	DA[g] Beer + spices[f]				61	52	>98	na	na	IQ(>97)	
	DA[g] Wine + spices[f]				77	51	>98	na	na	IQ(>97)	
Beef patty (62.5 g, 10 mm thick)	Soy lecithin[h]	1% L	2.7	220	+20	47	nd	+2	+21		(Natale and others 2014)
		5% L			+90	26	nd	+8	+16		
	Soy lecithin[h] + GSE[i]	1% L + 0.1% GSE			50	47	nd	1	+24		
		5% L + 0.1% GSE			70	37	nd	55	47		
		1% L + 0.2% GSE			10	42	nd	2	+21		
		5% L + 0.2% GSE			+40	26	nd	+20	+24		
	Pure GSE[i]	0.1%			50	37	nd	3	13		
		0.2%			20	32	nd	+23	+5		

[a] Spices spread on surface.
[b] Organosulfur compounds: diallyl disulfide, dipropyl disulfide, diallyl sulfite, allyl methyl sulfide, allyl mercaptan, cysteine, cysteine.
[c] Plant extracts, .5 g of marinade used on each side of the patty.
[d] Grape-seed or Hibiscus extract in water-in sunflower oil emulsion (marinade) (emulsifier E472c: citric acid esters of mono and diglycerides); 1.5 g of marinade used on each side.
[e] Rosemary extract in sunflower oil marinade; 1.5 g of marinade used on each side.
[f] Marinade containing ginger (2.8 %), garlic (2.9 %), rosemary (0.4 %), thyme (0.25 %), and red chili pepper (0.1 %, w/v).
[g] DA, dealcoholized wine or beer.
[h] Soy lecithin containing 0.18 % DL-α-tocopherols homogenized at 22500 psi to liposomes (L) used as marinade (10 g, marinating time 3 h at 8 °C) compared to control (rapeseed oil refined).
[i] Pure or in liposomes incorporated GSE (grape-seed extract).

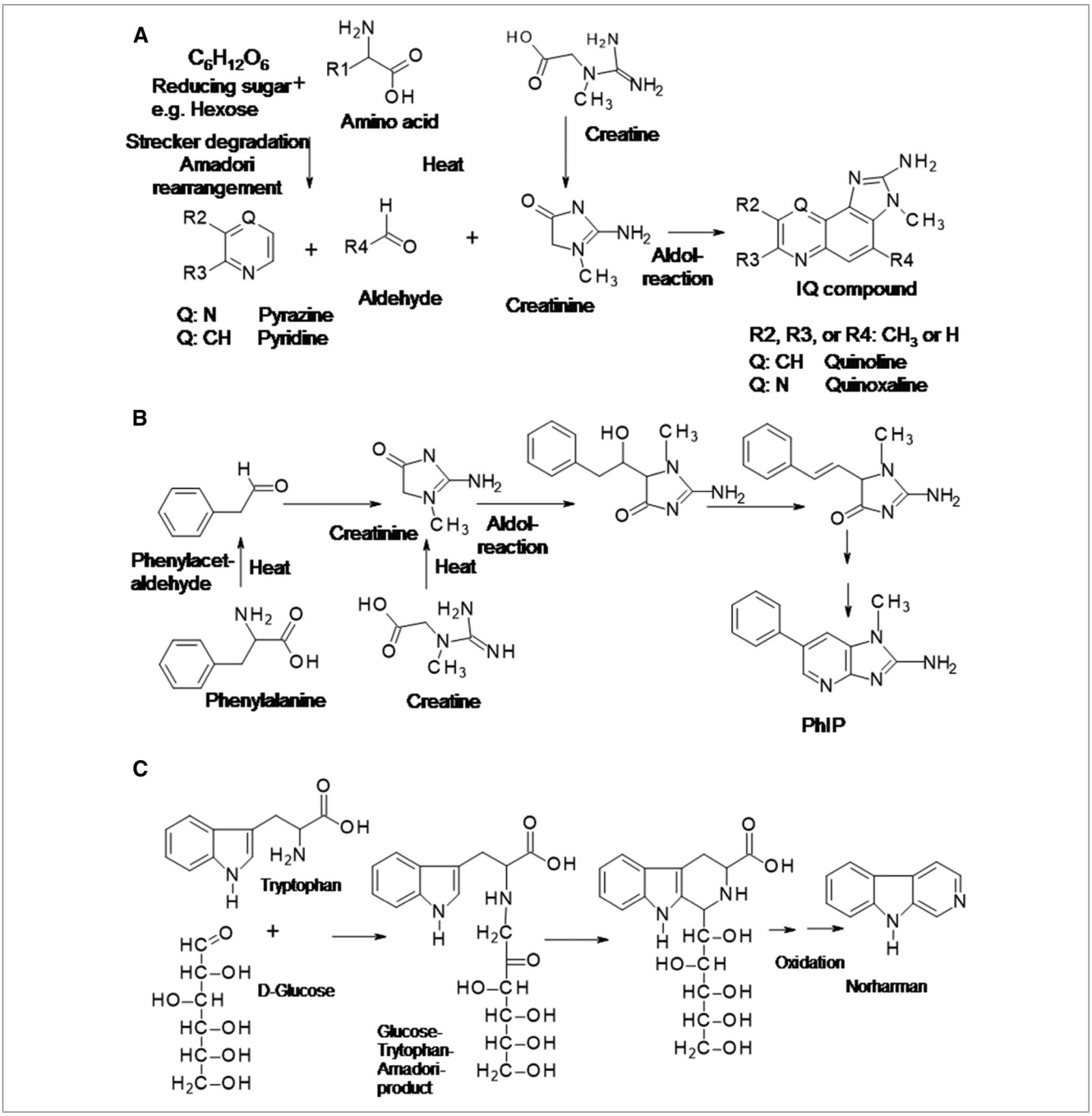


Figure 1–Formation of heterocyclic amines: (A) mechanism of thermophilic HAAs with creatine, sugar, and amino acids (modified) (Jägerstad and others 1983), (B) mechanism of formation of PhIP without sugar (modified) (Zöchling and Murkovic 2002), and (C) formation of norharman as β-carboline (modified) (Roenner and others 2000).

Formation of β-carboline

The reaction of the formation of β-carboline norharman is shown in Figure 1C (Roenner and others 2000). A similar reaction is postulated for both β-carbolines (norharman and harman). Both substances have no free amino group, which causes no mutagenicity in the Ames test (Pfau and Skog 2004). In comparison to other carbolines (pyrolytic HAAs), the reaction of norharman and harman could be formed at lower temperatures and they occurred in cooked meat, fish, and meat extract (Ziegenhagen and others 1999) as well as in processed meat, particularly such pizza toppings as salami and cooked ham (Gibis and Weiss 2013). Tryptophan, in particular, was found as a precursor and glucose enhanced the formation (Pfau and Skog 2004). Under the same condition with an excess of tryptophan, harman and norharman were found at levels 70 and 20 times higher, respectively (Skog and others 2000). The other carbolines are normally formed as typical pyrolysis products of amino acids above a temperature of 300 °C (Jägerstad and others 1998).

Precursors of Heterocyclic Aromatic Amines

Creatine and creatinine

Creatine, as a nonproteinogenic amino acid, occurs in the form of creatine phosphate in the muscle of vertebrates and serves as an energy store. Heating transforms it into the physiologically inert cyclization product creatinine (Murkovic and others 1999). The resulting creatinine is needed to form the imidazole ring. If no creatinine is available, no imidazoquinoline and imidazoquinoxaline are formed. By contrast, the formation of nonpolar HAAs is independent of the presence of creatinine (Murkovic 2004b). The longer the heating time, the more creatine is converted to creatinine. The higher the addition of creatine/creatinine during the heat treatment of meat and fish, the higher is the mutagenicity of the products (Felton and Knize 1991). A promoting indication that creatine is significantly involved in the formation of thermic HAAs is that there are only small amounts of creatine in protein-rich foods, such as cheese, beans, liver, and shrimp. Only very small amounts of mutagenic activity could be detected in these products after preparation (Cheng and others 2006; Chen and others 1990). The molar ratios of total creatine/glucose was found from 0.89 up to 9.84 for beef, pork, mutton, chicken, duck, and goose (Liao and others 2011b) and 1.2 up to 12 for veal, beef, pork, lamb, horse, turkey, and ostrich, respectively, except for vension and chicken with ratios up to 60.7 (Gibis and Weiss 2015) in meat from different animals. Trials with a model system showed that creatinine forms a part of the PhIP molecule (Zöchling and Murkovic 2002). It was shown in a further trial that beef with a high creatine level (1.5 mg/g) demonstrated a higher mutagenic activity than beef with a low creatine level (Jackson and others 1994).

Free amino acids

Free amino acids are, similar to creatine, necessary for the formation of HAAs. However, several different amino acids can form the same mutagens, for example, threonine, glycine, lysine, alanine, and serine all lead to MeIQx formation (Jägerstad and Skog 1991). Several amino acids were heated in the presence of creatinine and glucose in model trials and all the amino acids showed mutagenic activity (Johansson and others 1995a). Other authors showed that some amino acids had less mutagenic activity with dry heating. According to this study, the amino acids serine and threonine had the highest mutagenic activity (Overvik and others 1989). Which mutagens are formed depends on the amino acids present. PhIP is formed from the amino acid phenylalanine (Manabe and others 1992, 1993). Other authors determined, using ^{13}C NMR spectrometry, that a ^{13}C-labeled phenylalanine molecule is built into the structure of the PhIP molecule (Murkovic and others 1999). In model studies, phenylalanine, as a known precursor for PhIP, is highest in the chicken model system, and the compound is also formed from tyrosine and isoleucine, which are similarly highest in chicken (Skog and others 1998b). However, no correlation between the amount of phenylalanine and the levels of PhIP was observed in pork (Olsson and others 2002). MeIQx, by contrast, arose from the amino acids glycine, threonine, alanine and lysine (Johansson and Jägerstad 1996; Johansson and others 1995a). In the model, the amino acids threonine, alanine, and lysine took part in the formation of 4,8-DiMeIQx (Johansson and others 1995a). The amino acids glycine, alanine, and lysine were responsible for the formation of 7,8-DiMeIQx. With the exception of L-cysteine, which reduced the mutagenicity in the Ames test, all amino acids increased the mutagenic activity (Lee and others 1994). When 4-oxo-2-nonenal, a compound of oxidization of fat, was present, only the addition of methionine, glycine, and serine significantly increased the amount of PhIP formed ($p < 0.05$), whereas cysteine, lysine, tryptophan, histidine, tyrosine, and alanine reduced ($p < 0.05$) PhIP significantly (Zamora and others 2013b). The generation of PhIP is also associated with the capacity of 4-oxo-2-nonenal to constitute the Strecker degradation of phenylalanine to phenylacetaldehyde (Zamora and others 2013a). Amino acids also formed HAA amino acid adducts, for example, the PhIP adduct with glycine was formed easily within 5 min by heating at 200 °C, which is probably based on the dehydration condensation of the amino group of PhIP and carboxyl group of glycine (Kataoka and others 2012). The content of free amino acids as the sum of all encoded and nonencoded amino acids is not highly correlated with the formation of HAAs in grilled beef; but derivatives of amino acid, such as creatinine, and the amino acids alanine, phenylalanine, and lysine are related to the formation of aminoimidazoarenes and PhIP, while the other amino acids do not participate in the formation of HAAs (Szterk and Waszkiewicz-Robak 2014). Similar findings were observed in pork, beef, and chicken, only lysine, tyrosine, phenylalanine, proline, isoleucine, and aspartic acid showed significantly positive correlations to levels of PhIP, while no significant correlation between any amino acid and contents of other HAAs was found (Gibis and Weiss 2015).

Carbohydrates

The presence of reducing sugars plays an important role in the formation of HAAs. Along with creatine, creatinine, and amino acids, reducing sugars such as glucose, fructose, ribose, erythrose, and lactose are also necessary for the formation of these mutagens (Skog and Jägerstad 1993). Glucose was necessary for forming mutagenic activity in the Ames test both in aqueous model systems and with the heating of meat samples (Skog and others 1992). The sugars occurring naturally in meat are present in approximately half the molar quantity of creatine and amino acids. If the sugar concentration is increased beyond the naturally occurring ratio, a reduction is observed in HAA formation (Skog and Jägerstad 1990). The same was observed for glucose, fructose, sucrose, and lactose as for sugar, however, the monosaccharides showed the most distinct inhibitory effects (Skog and Jägerstad 1990). Some authors found higher ratios of total creatine/glucose with values from 0.89 up to 9.84 (Liao and others 2011b) and up to 60.7, respectively, (Gibis and Weiss 2015) for animals of different species. These ratios of the precursors to each other also play a key role in the formation of HAAs. Higher levels were found for MeIQx by adding lactose in model systems (molecular ratio lactose/amino acids/creatinine – 2:1:1) with 5% water content (Dennis and others 2015). The inhibition of the formation of the mutagenic compounds by an excess of sugars is proposed to be an effect of Maillard reaction products, which may block the formation of imidazoquinoxalines by attacking creatine (Skog and Jägerstad 1990). Therefore, the reason is due to the formation of Maillard products which changes the reaction path and results in a reduction in HAA concentration (Shin and others 2002c). A significant reduction in mutagenicity and in the content of MeIQ, PhIP, DiMeIQx, IQ, IQx, and norharman was attained in grilled chicken that was marinated with honey containing glucose and fructose in the same ratio and a small amount of sucrose (Hasnol and others 2014; Shin and Ustunol 2004). It was also observed in a study with pork, from pigs as carriers of the RN allele, containing increased concentrations of residual glycogen that this fact caused about 90% lower levels of PhIP and 50% lower of total mutagenic HAAs in cooked meat compared with cooked meat from normal pigs (Olsson and others 2002).

Different oligosaccharides, such as fructooligosaccharide, galactooligosaccharide, isomaltooligosaccharide, or inulin, reduced the HAA formation in cooked meat products (Shin and others 2003, 2004; Persson and others 2004) . The addition of oligosaccharides affected the mass transfer of the precursors to the surface, which results in higher water-holding capacity as well as thereby a lower weight loss of cooked products and reduction of mass transfers of precursors (Persson and others 2004).

Physical Factors

Heating time and heating temperature

The formation of HAAs depends on the heating time and the heating temperature depending on the used cooking method. The heating temperature has the largest effect on HAA formation. The formation of mutagenic substances begins at around 125 °C (Jägerstad and others 1998; Skog and others 1998b). However according to Ahn and Gruen (2005a), few or no HAAs were detected at temperatures (160 °C) and a heating time of 15 min in ground beef as a model systems with glass test tubes. Only after increasing the heating temperature and time, the HAA concentration rose. At 180, 200, and 220 °C, significant concentrations of polar HAAs (IQ, MeIQ, MeIQx, 4,8-DiMeIQx, and PhIP) and nonpolar HAAs (harman, norharman, and AαC) were determined after 20 min. By contrast, neither MeIQ nor 4,8-DiMeIQx could be detected at 180 °C and a cooking time of 10 min. The MeIQ concentrations detected were under 2 ng/g at all 3 temperatures (Ahn and Gruen 2005a). The HAA concentrations in the trials performed increased only after about 5 min, but then disproportionally. This observation could be explained by the surface temperature being under 100 °C in the first few minutes of the heating process. The temperature range that is required for the formation of the mutagenic substances was only reached after about 5 min (Ahn and Gruen 2005a).

It could be shown in further experiments (Bordas and others 2004) that the concentration of HAAs was increased with an increase in the temperature and an extension of the cooking time. A 4,8-DiMeIQx concentration of 1.2 ng/g was detected in an aqueous model system with meat flavor extract (bouillon, extract/water 1:2) after heating for 1 h at 175 °C in an oven. With the same temperature and a doubling of the heating time, a threefold quantity of 4,8-DiMeIQx was detected. In this meat flavor model system in dry conditions, changing the heating times and temperatures from 100 °C for 1 h to 2 h, 150 °C for 30 min to 1 h, and 200 °C for 10 min up to 30 min, respectively, made the effect of temperature and time on HAA formation clear. While a MeIQx concentration of 3.5 ng/g was found at 100 °C for both times, at 150 °C, there was a concentration of around 4 ng/g for both heating times. After increasing to 200 °C, the concentration increased from 3.7 to 10.7 ng/g for the longer heating time (Bordas and others 2004). A significant increase of PhIP concentration was already observed at 100 °C after 2 h compared to a heating time of 1 h (Bordas and others 2004).

The results showed that HAA formation depends on temperature, time, and cooking methods. Further studies are shown in Table 2 to 4 concerning cooked meat samples (beef patties, chicken breasts, and pork) under different cooking methods and at various heating conditions.

Heat and mass transfer in cooked meat products

The objectives of cooking meat products are to warrant microbial safety and to produce flavor in meat. Some chemical and physical alterations take place during the cooking of meat, such as protein denaturation, melting of fat, water evaporation, changes in texture and shape, water transport of solutes, and formation of crust on the surface (Kondjoyan and others 2014). The denaturation of meat proteins during heating results in shrinkage, hardening, and the release of juice. First, the denaturation of the myosin takes place at approximately 53 to 58 °C, second, collagen shrinks and denatures at approximately 70 to 74 °C similar as sarcoplasmic proteins, and third actin denaturation at about 80 to 82 °C results in a decrease in water-holding capacity with the release of serum (Bertram and others 2006). Free amino acids and sugars in the outer parts of the meat react via the Maillard reaction during frying and form a variety of reaction products, which are important for the color and flavor of cooked meat; amino acids containing sulfur are known as precursors for the final flavor (Shahidi and others 2014).

The effect of temperature and time on HAA formation was described in the previous section. However, the cooking method used also has a big effect on the formation of mutagenic activity. Authors found in different studies that no amounts of HAAs or mutagenicity occurred with gentle cooking methods, such as stewing, steaming, and boiling as temperatures are below 120 °C (Persson and others 2002; Joshi and others 2015) (Table 2 and 3). With dry-heating methods, such as frying in a pan, significantly higher mutagen levels occur than with oven-roasting (Table 2 to 4). In addition to the temperatures and heating time, the methods differed between the type of heat transfer, such as convection, conduction, or radiation, and the surrounding media, such as metal, water, fat, or air, which result in different heat transfer coefficients (Houšová and Topinka 1985; Pan and Singh 2002; Zorrilla and Singh 2003; Kondjoyan and others 2013). It could be shown in model trials that the preparation of hamburgers in a pan led to a much higher mutagenic activity and HAA concentrations than oven-roasting. A comparison of the results of preparation in a deep-fat fryer, a convection oven, and on a contact grill showed clear differences (Persson and others 2002) (Table 2 and 3). MeIQx levels after preparation on a contact grill are around 10 times higher than with the other methods. With preparation in a deep-fat fryer or a convection oven, lower levels of HAAs were formed. The differences are explained by the significantly better heat transfer in methods with direct contact, such as grilling or frying in a pan (heat transfer coefficient of pan 150, air 40 Wm^2/K, and up to 10000 Wm^2/K for boiling water), than in methods without direct contact (air convection 30 to 40 Wm^2/K) (Pan and Singh 2002; Sprague and Colvin 2011). There is an indirect heat application in ovenroasting as the heat has to be transferred by convection. There is also an indirect heat transfer using microwave methods, as the energy here is transferred in the form of radiation (Haskaraca and others 2014; Singh and Heldman 2014). That is the reason why preparations of meat products using a microwave pretreatment result in very low HAA concentrations compared to only charcoal-grilled or deep-fried chicken or beef samples (Felton and others 1994; Jinap and others 2013). However, Barrington and others (1990) showed a high mutagenic activity in 1 beef steak sample which were microwave-cooked for 5 to 7 min per side in contrast to lower times. This treatment most probably resulted in high water loss. The highest HAA levels occurred when frying on a contact grill (Pan and others 2000). The reason for this is the direct contact with the heating medium, which results in a higher conductive heat transfer.

Kinetics of HAA formation

The kinetics of HAA formation was studied by using model systems prepared with precursors, such as creatinine, glucose, dipeptides, and free amino acids, analogously to levels in beef (Arvidsson and others 1997) and beef juices as a model system, which were obtained from roasted beef (Arvidsson and others 1999). The HAAs are stable at ambient temperature, but they are disposed to degradation at higher temperatures (Jackson and Hargraves 1995; Arvidsson and others 1997). Degradation occurred at 100 °C in solutions of standard HAAs and increased significantly with temperatures at 200 to 225 °C. In this connection, PhIP was found to be the most disposed to degradation, followed by MeIQx, 4,8-DiMeIQx, and IQx (Chiu and Chen 2000). However, degradation in meat juice systems or meat was different because the formation can balance more due to various parameters, such as heat transfer, mass transfer, vaporization of water, and crust formation, which complicate the kinetics calculations. Besides the generation reactions, the kinetics of HAAs also included a subsequent degradation of HAA compounds. The formation of HAAs ([HAA] levels in ng/g, t for time) is supposed to follow a first-order reaction equation with a rate constant given by the Arrhenius equation (Arvidsson and others 1997, 1999; Tran and others 2002):

$$\frac{\partial\,[HAA]}{\partial t} = A \cdot e^{\left(-\frac{E_a}{RT}\right)} \tag{1}$$

$$k = \frac{k_b\,T}{h} \cdot e^{\left(\frac{\Delta S}{R}\right)} \cdot e^{\left(-\frac{\Delta H}{RT}\right)}, \tag{2}$$

where E_a is the activation energy, R is the gas constant (8.3145 J/(mol·K)), and A is the unknown exponential prefactor. For the reaction mechanism, the modulation of Eq. 1 was used in the Eyring Eq. 2 for the determination of the temperature-dependence (T in K) of rate constant of the formation where k is the rate constant, k_b is the Boltzmann constant (1.381×10^{-23} J/K), h is the Planck constant (6.626×10^{-34} J s), and ΔH is the activation enthalpy (Arvidsson and others 1997, 1999). The activation entropy ΔS calculated showed that the rate-limiting step for the formation of PhIP follows rather a monomolecular reaction, whereas for all the IQx-type compounds the formation probably follows a bimolecular reaction of pseudo-first order (1 of the 2 reactants being in large excess) for MeIQx, 4,8-DiMeIQx, and IQx (Jägerstad and others 1998). The limiting step of the rate could be the reaction between creatinine, aldehyde, and pyrazine (Arvidsson and others 1997, 1999). Using numerical methods with different mathematical and computational modeling of pan-frying can show the predictive values of the transport of water and the temperature distribution in patties, as well as the associated formation of HAAs, for turning once and multi-turning (Sprague and Colvin 2011). The modeling of the formation of HAAs in slices of beef (*musculus longissimus thoracis* and *semimembranosus*) subjected to jets of hot air depicted the influence of extreme dehydration (low water activity) obtained, which slowed the formation of IQx, MeIQx, and, particularly, 4,8-DiMeIQx compared with superheated steam treatments. By contrast, a reverse effect was found for PhIP levels, which increased 1.4- to 5.5-fold (Kondjoyan and others 2010a; 2010b). In this connection, the knowledge is important that many reactions favoring at various environmental conditions can simultaneously proceed. In model systems with high temperature around 225 °C or long cooking times, formation and degradation of the HAA in particular PhIP were reported (Chiu and Chen 2000; Arvidsson and others 1997). Additionally there is known from model studies that dry conditions (freeze dried meat juice) resulted in an increased formation of PhIP, DMIP, TMIP, and IFP, but in the wet system (meat juice), MeIQx and 4,8 DiMeIQx was favored (Borgen and others 2001).

Chemical Factors

Fat content and lipid oxidation products

No clear statement could be made on the role of fat in the development of mutagenic activity. The fat content of the products affected the formation of mutagenic substances, but it is not clear whether this was due to physical or chemical influences (Johansson and Jägerstad 1993; Hwang and Ngadi 2002).

Moreover, the precursors being necessary for HAA formation, such as creatine/creatinine, free amino acids, and carbohydrates, are present almost exclusively in lean meat, and a higher fat content results in a dilution of the precursors (Knize and others 1985) leading to a reduction of the mutagenic activity in the Ames test (Chen and others 1990). However, it has also been shown that a higher fat content in meat results in a shorter time needed to reach a fixed meat surface temperature due to a more effective heat transfer and thereby an increased formation (Abdulkarim and Smith 1998). Furthermore, if the fat is oxidized the HAA formation is enhanced that was shown in different studies (Johansson and Jägerstad 1993; Zamora and others 2012). Nonetheless, many scientists agree that there is an optimal fat content for the formation of the highest possible HAA concentration in a product.

Regarding beef patties, HAAs such as MeIQx, PhIP, norharman, harman, and Trp-P-2 were found at higher levels with about 5% fat than those with 15% fat after frying at 150, 170, and 190 °C (Abdulkarim and Smith 1998). In beef patties with 15% and 30% fat content, which were cooked to a core temperature of 100 °C on a propane grill using a cooking time from 10 up to 26 min (weight loss from 46% to 55%), the low-fat patties had the higher levels of PhIP, but lower levels of AαC than the high-fat patties (Knize and others 1997c).

Lipids had increased the yield of pyrazines and Strecker aldehydes in model trials (Arnoldi and others 1990). A possible reason for this is fat oxidation. Oxidized fats led to radicals which supported the formation of HAAs by this oxidation (Johansson and Jägerstad 1993). The content of HAAs in meat, including pan residue frying with different frying fats, or oils (butter, margarine, margarine fat phase, liquid margarine, rapeseed oil, and sunflower seed oil), was significantly lower after frying in sunflower seed oil or margarine than after frying with the other fats (Johansson and others 1995b). The variations in generation of MeIQx and DiMeIQx could be stated with regard to the concentrations of antioxidants, such as vitamin A, vitamin E, and tocopherols/tocotrienols, and status of oxidation (peroxide and anisidine value) (Johansson and others 1995b) as antioxidants can reduce the formation while the oxidized fat can increase it. Other researchers reported that fats also have an enhancing influence on the yield of HAAs in model systems after the addition of iron ions (Fe^{2+} or Fe^{3+}), probably by free radicals formed during thermally induced lipid oxidation. In this model system, the level of MeIQx formed was nearly doubled, probably due to iron-catalyzed lipid peroxidation and, thus, formation of free radicals (Felton and others 2000).

Most authors assume that the heat transfer is improved with an increase in the fat content to an optimal fat–water ratio (Hwang and Ngadi 2002) leading to a shortening of the cooking time. In this study using high fat meat emulsions, the authors observed that the activation energy of HAA formation was reduced at high-fat

levels due to a more thermodynamically favored reaction (Hwang and Ngadi 2002). An increase in the fat content over the optimal ratio results in a decreased HAA concentration (Persson and others 2008; Abdulkarim and Smith 1998).

Moisture content and a_w-value

Water serves as a reaction and transport medium during the heating of meat and meat products. With water, the precursor substances creatine/creatinine, amino acids, glucose, and so on, can be transported to the surface of the product (Persson and others 2002). At the surface, these substances are exposed to higher temperatures and so contribute to the formation of HAAs (Overvik and others 1989). The formation of mutagenic substances could be significantly reduced by reducing water vaporization during the cooking process (Persson and others 2004). The mutagenic activity is reduced with a reduction of the water content and an increase in the fat content; the capacity of the transport medium declines and the precursors become diluted (Knize and others 1985).

An increase in water-holding capacity could, thus, be achieved by the addition of water-binding substances into minced meat (Persson and others 2003b, 2004; Wang and others 1982). The authors showed that with the addition of a tripolyphosphate plus sodium chloride mixture (Persson and others 2003b), soy protein (Wang and others 1982) or carbohydrates (Shin and others 2003, 2004; Persson and others 2004) to minced meat products, water was bound, leading to a reduction in HAA formation. Wrapping hamburgers with carrageenan had a similar effect (Schoch 2003). The extreme dehydration obtained with the hot-air jets resulted in a low water activity and slowed down the formation of IQx, MeIQx and, particularly, 4,8-DiMeIQx compared with superheated steam treatments (Kondjoyan and others 2010a,b). But the reverse effect was detected for PhIP concentrations which increased (Kondjoyan and others 2010b). In pan-fried bacon, the heat treatment of bacon with water activity around 0.93 caused a high content of MeIQx (Gibis and others 2015).

Antioxidants and reducing agents

Next to physical influences (temperature, time, and preparation method), added substances, such as nitrite, antioxidants, or spices, also showed an inhibiting effect on HAA formation as summarized for beef products as an example in (Table 6). Both main components such as lipids and proteins may be oxidized in a series of radical reactions that include steps of initiation, propagation, and termination with simultaneous formation of free radicals (Weiss and others 2010). The antioxidants did not only inhibit fat oxidation. It is known that free radicals may be involved in the mechanism of HAA generation and Maillard reaction. The antioxidants showed that they would scavenge the free radicals and reduce HAA formation, and this could inhibit the radical reactions in HAA formation, which was shown in an electron paramagnetic resonance experiment (Kikugawa 1999). Depending on the substances added, HAA formation could be stimulated or inhibited. When nitrite was added to meat products, several HAAs, such as Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, and AαC, were transformed under acidic conditions (pH 2) into their hydroxy derivatives in model systems and so lost their mutagenic activity (Furihata and Matsushima 1986). Nitrite is known as an additive with properties as a reducing agent, at low pH values, that can reduce the concentration of non-IQ-type HAAs in model systems (Tsuda and others 1985; Shin 2005). Nitrite can also react with the amino acid cysteine causing the formation of an antioxidant, and free radicals can, thus, be blocked (Shin 2005). The generation of IQ-compounds is reduced by the addition of tocopherol (vitamin E) (Balogh and others 2000; Lan and others 2004). Tocopherol is a naturally occurring antioxidant. The inhibition is due to the direct blocking of free radicals (Shin 2005). In addition, the breakdown products of tocopherol react with precursors, which are then no longer available for the formation of mutagens. Ascorbic acid or sodium ascorbate as a naturally occurring antioxidant and reductone, respectively, could also similarly bring about an HAA reduction (Kato and others 2000; Kikugawa and others 2000; Dundar and others 2012; Wong and others 2012). A moderate inhibitory effect (approximately 20%) on the formation of PhIP, 4,8-DiMeIQx, and MeIQx was found for water-soluble vitamins, such as niacin and ascorbic acid; whereas pyridoxamine reduced the concentrations of all 3 HAAs by approximately 40% (Wong and others 2012). This study showed that pyridoxamine reduced the level of PhIP significantly by trapping the phenylacetaldehyde and reacted with the latter to form a pyridoxamine-phenylacetaldehyde adduct which was confirmed by using LC–ESI–MS/MS and NMR spectroscopy (Wong and others 2012). Rosemary extract reduced the content of HAAs in fried beef patties (Puangsombat and Smith 2010; Damašius and others 2011; Gibis and Weiss 2012). An inhibiting effect of the tomato carotenoid fraction on the formation of imidazoquinolines (IQx, MeIQx, and DiMeIQx) was reported in model systems containing freeze-dried bovine meat juice (Vitaglione and others 2002). Using carotenoid extract at a concentration of 1000 mg/kg, inhibitions of 13% of MeIQx and of 5% of 4,8-DiMeIQx in the meat juice model system were observed. The effect of the main tomato flavonoid, quercetin, gave an inhibition of MeIQx formation between 9% and 57% with a maximum effect of 67% at 10 mg/kg (Vitaglione and others 2002).

Butylated hydroxyanisole (BHA) is a synthetic antioxidant. It was seen in a study that the HAA level could be lowered by 40% with BHA (Weisburger 2005). A few years later, this inhibitory effect was confirmed and it was discovered that other phenolic antioxidants, such as epigallocatechin gallate and sesamol (Oguri and others 1998), had a positive effect on the reduction of HAA formation (Weisburger and others 1994).

Extracts of vegetables and fruit could also lead to a reduction in mutagenic activity (Britt and others 1998). Next to pure antioxidants, extracts with polyphenols present in plants were particularly effective (Ahn and Gruen 2005b; Cheng and others 2007; Gibis and Weiss 2012; Liao and others 2011a). The levels of different HAAs, such as MeIQx or PhIP, can be lowered by heating model systems with polyphenols, such as quercetin, rutin, catechin, catechin gallate, and *n*-propyl gallate (Arimoto-Kobayashi and others 2003; Ahn and Gruen 2005b). The results indicated that phenols having 2 hydroxy groups at meta positions of the aromatic ring were the most efficient inhibitors. The presence of alkyl or carboxylic groups as additional substituents in the aromatic ring reduced the inhibitory effect slightly (Arimoto-Kobayashi and others 2003). On the other hand, the introduction of additional hydroxy and amino groups mostly cancelled the inhibitory effect, which was also mostly absent in ortho and para dihydroxy derivatives; in complex phenols, the presence of several rings with opposite effects produced a reduced inhibitory effect (Arimoto-Kobayashi and others 2003). An isotope-labeling study showed that all fragments contained had phenylalanine as the origin. The reaction employed phenylacetaldehyde and epigallocatechin gallate, which further confirmed the ability of epigallocatechin gallate to generate adducts with phenylacetaldehyde, which may reduce the ability

of PhIP formation (Cheng and others 2009). In addition, extracts containing anthocyanins showed a similar inhibitory effect on the formation of HAAs (Gibis and Weiss 2010).

Also the technology of marinating plays an important role for the inhibition of HAA formation. Marinating with nano-sized liposomes containing grape-seed extract (approximately 100 nm) as a marinade showed no further inhibition of HAA formation compared to the pure grape-seed extract (Natale and others 2014) (Table 6). This treatment with a liposomal encapsulation system as a marinade in comparison to the fried beef patties without grape-seed extract showed an inhibition only for MeIQx, while the β-carbolines showed increased levels (Natale and others 2014). This observation shows that the application form also plays a key role in the marinating of meat, because liposomes may diffuse very quickly into the meat, due to the very small particle size, where they can interact with other meat components, such as fat or proteins, and no release of the polyphenols may occur (Gibis and others 2014). Consequently, no polyphenols are available on the surface for the inhibition reaction with HAA precursors released. The same grape-seed extract in a water in oil emulsion as marinade (droplet size 1 μm) remained as oil film on the surface and clearly reduced the content of MeIQx and PhIP (Gibis and Weiss 2012) (Table 6).

Organosulfur compounds

Organosulfur compounds are another class of substances that may be effective in inhibiting nonenzymatic browning reaction (Cheng and others 2006). These compounds, such as glutathione, L-cysteine, L-cystine, and deoxyalliin, inhibited the formation of mutagens in the model system (Shin and others 2002c) and in fried ground beef patties (Shin and others 2002b), as well as minced garlic (Shin and others 2002a) or marinades containing garlic, onion, and lemon juices which inhibited the HAA formation (Gibis 2007) (Table 6). It has been suggested that sulfur amino acids containing thiol groups greatly inhibit nonenzymatic browning of heated amino acid–glucose mixtures. The related mechanisms are proposed that aldehydes and ketones are reacted with carbonyl groups due to thiol-containing compounds reducing carbonyl groups or with double bonds in brown products to form colorless materials (Friedman and Molnar-Perl 1990). Garlic as a plant of the *Allium* genus contains a variety of organic sulfur compounds, such as allicin, diallyl disulfide, diallyl sulfide, and dipropyl disulfide (Jung and others 2010) or acetyl cysteine, cysteine, and glutathione in meat juice model systems (Schoch 2003), which could inhibit HAA formation.

The most well-studied compound containing sulfur in relation to the inhibition of HAA formation is sodium bisulfite ($NaHSO_3$). Addition of sodium bisulfite was demonstrated to inhibit the formation of HAAs in canned foods significantly (Krone and others 1986).

Analysis of HAAs

Sample preparation

In complex matrices such as meat and fish products, HAAs are only present in pbb concentrations and the efficiency of sample preparation is affected by the matrices. The sensitive and precise quantification of these compounds is complicated. Some authors described that the planar aromatic ring system of the blue dye, such as copper phthalocyanine trisulfonate, which is linked to polymeric carbohydrates such as cotton or rayon, interacts especially with the planar structure of the molecules consisting of 3 or more fused-rings such as HAAs or polyaromatic hydrocarbons (Hayatsu and others 1983). Sorbents, such as blue rayon (Wu and others 1995; Kataoka and Pawliszyn 1999), blue cotton (Kurosaka and others 1992; Nukaya and others 1994; Wakabayashi and others 1995), or blue chitin (Bang and others 2002, 2004), were used. These separation methods based on the adsorption of HAAs are highly selective (Skog 2004). However, the most common sample preparation for HAAs was a tandem solid-phase extraction method (Gross 1990) and was further optimized (Gross and Grueter 1992). In this method, the HAA extraction is applied using diatomaceous earth with an organic solution, such as dichloromethane (Gross and Grueter 1992), or ethyl acetate (Santos and others 2004). Then, for the sample preparation, the extract was divided into a polar and nonpolar fraction using a tandem solid-phase extraction with PRS (propylsulfonic acid) and C_{18} cartridges as a cation exchanger and a clean-up cartridge for hydrophobic impurities, respectively. The replacement of PRS and C_{18} solid phase extraction using a mix-mode OASIS MCX cartridge or a change in elution solvents and order allowed the collection of the HAAs in 1 fraction (Messner and Murkovic 2004; Turesky and others 2005). Mix-mode materials have a dual functionality consisting of copolymers and silica gel particles which have the ability of hydrophobic and ion exchange interactions, respectively. In addition to these isolation methods, supercritical carbon dioxide with methanol (10%) applying a pressure of 414 bar at 55 °C allowed the isolation of HAAs from solid material with high recoveries of the quinolines and quinoxalines (Thiebaud and others 1994). By contrast, pure supercritical carbon dioxide resulted in an inefficient isolation of HAAs (Thiebaud and others 1994). A combined acetone extraction using a phenylsulfonic acid silica cartridge for HAA isolation was used by other authors (Calbiani and others 2007). Solid-phase microextraction (SPME) permitted the simultaneous extraction and concentration of analytes, such as HAAs, from gaseous, aqueous, and solid matrices (Cárdenes and others 2004, 2006; Martín-Calero and others 2007). The principle of this HAA method is the equilibrium between the analytes, sample matrix and organic polymer-coated fiber, which resulted in time-saving and reduction of organic solutions (Cardenes and others 2006), and for determination of nonpolar HAAs (Martín-Calero and others 2007). In particular, the samples should be frozen to inhibit interference when the samples contain a high fat content. Other isolation methods are reported in a review by Toribio and others (2000), but many methods have the disadvantages that they are very time- and cost-consuming, and in most methods, only the polar HAAs can be determined. A very fast extraction method is the single-drop microextraction: a simple direct immersion technique combined with nanocellulose and multiwalled carbonanotubes plus ionic liquid was used for the extraction and determination of MeIQx (Ruiz-Palomero and others 2014). In another study, the extraction was performed by using hollow-fiber membrane liquid-phase microextraction, which contained 2.5-cm lengths of porous polypropylene fibers impregnated with organic solvent to facilitate simultaneous extraction from an alkaline aqueous sample into a low-volume acidic acceptor phase (Cooper and others 2014).

Analytical methods to determine HAAs. The determination method of HAAs was performed using electrophoretic or chromatographic techniques with diverse detection systems. The most frequently applied technique for HAA separation is high-performance liquid chromatography (HPLC) combined with ultraviolet (UV) and fluorescence detection or UV and mass spectrometry (MS), which are based, in most cases, on the sample preparation method described by Gross and colleagues (Gross and

Grueter 1992; Gross and others 1992). Combining HPLC with capillary electrophoresis with field-amplified sample injection, coupled to a mass spectrometer used in either the MS or tandem MS (MS/MS) mode, was evaluated for 16 HAAs (Sentellas and others 2003).

Liquid chromatography–mass spectrometry (LC-MS) and liquid chromatography–tandem mass spectrometry (LC-MS/MS) are the important methods for the identification and quantification of most HAAs and were used in the following ionization techniques: thermospray (Gross and Fay 1995), electrospray (ESI) (Johansson and others 1995a; Turesky and others 2005; Ni and others 2008), or combined (Fay and others 1997). Sixteen HAAs were sensitively and reproducibly identified and quantified with ultra-high-performance liquid chromatography (UHPLC) and MS/MS in the very short time of 2 min for routine analysis of meat extracts (Barcelo-Barrachina and others 2006) and the HAAs in human urine after hydrolysis of metabolites can be quantified (Fu and others 2014). Due to the option of oxidation of HAAs, they can be detected using an electrochemical or coulometric electrode array detector (Holder and others 1996; Krach and Sontag 2000). The LC-MS technique containing a quadrupole time-of-flight MS was used as a detector for 9 water-soluble HAAs in aerosols (Samy and Hays 2013). Recently, highly sensitive analyses of UPLC-MS/MS as very fast and cost-saving methods were developed for high sample throughput (Pais and Knize 2000; Murkovic 2007; Lomas and Layne 2012; Fu and others 2014).

Methods using gas chromatography (GC) are only possible after using a derivatization step, such as silylation, because the HAAs are nonvolatile substances (Kataoka and others 2002; Casal and others 2004). Nitrogen–phosphorus (Kataoka and others 2002) or MS detectors (Liu and others 2013; Xu and others 2010) are used in current GC detection methods.

A combination of GC or HPLC coupling with a MS as a highly sensitive and selective detector was sometimes performed for the determination of HAAs. A precise quantification method in HAA analysis using MS as detector is attained using deuterated internal standards (Edmonds and others 1986; Yamaizumi and others 1986; Holdert and others 1997; Guy and others 2000). Another, less popular, method is capillary electrophoresis (Viberg and others 2006; Barcelo-Barrachina and others 2007) or thin-layer chromatography (Jautz and Morlock 2006; Jautz and others 2008) using diode array, electrochemical, fluorescence, or MS detection. A highly selective and sensitive technique is also the determination of HAAs using an enzyme-linked immunosorbent assay (Vanderlaan and others 1989).

Metabolism of Heterocyclic Aromatic Amines

Metabolization of heterocyclic aromatic amines

Most carcinogenic substances do not act mutagenically directly upon their intake. They are only activated in the organism with the help of metabolic enzymes. This also applies to HAAs, which only act mutagenically after enzyme activation (Felton and others 1999). The HAAs are resorbed in the small intestine, transported to the liver and activated there (Lynch and others 1995).

N-oxidation by cytochrome P 450 enzymes is an initial step in the metabolic activation of HAA compounds (Sasaki and others 2002). The most important reaction is necessary for activation of the following acetylation reaction. An oxidation of the amino group by means of cytochrome P 450 1A2 follows more a predicted one-electron transfer than with the prior suggested two-electron transfer mechanism (Sasaki and others 2002). An N-acetoxy amine is formed by an O-acetylation from the resulting N-hydroxyl ion. This reaction step is catalyzed by N-acetyltransferases (NATs) or N-hydroxy-HAA metabolites are esterified by sulfotransferases (SULTs), L-seryl-tRNA, and L-prolyl-tRNA synthetases, and other ATP-dependent enzymes (Turesky and Le Marchand 2011). The resulting esters are unstable and undergo degradation reaction. The resulting N-acetoxy amine is a very reactive molecule, which can form a nitrenium ion (Garcia-Closas and Sinha 2000) of the exocyclic amine group, which is the critical metabolite implicated in toxicity and DNA damage (Turesky and Le Marchand 2011). The nitrenium ion is able to combine with DNA and can cause mutations. These mutations can then cause tumors. IQ, MeIQ, MeIQx, PhIP, Trp-P-2, Glu-P-1, and MeAαC could demonstrably form adducts with the guanidine bases occurring in DNA (Wakabayashi and Sugimura 1998). Figure 2 shows the major pathway of metabolism of MeIQx as a prototype of HAAs, including the activation paths by nitrosation with nitric oxide under inflammatory conditions. Some HAAs, such as IQ and MeIQx, can undergo nitrosation with nitric oxide at neutral pH conditions to form 2-nitrosoamino-3-methylimidazo[4,5-*f*]quinoline and 2-nitrosoamino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline, respectively. These N-nitroso-IQ compounds are transformed to very reactive diazonium compounds that may generate covalent DNA adducts with the DNA bases, such as guanine (Lakshmi and others 2005). The mechanism shown (Figure 2) for the N-acetyltranferases(NAT2)-catalyzed activation of N-nitroso-MeIQx has been suggested (Zenser and others 2009). There are large differences of P 450s between human and other species, such as rodents, in terms of the regioselectivity and catalytic activity of HAA oxidation, which affects the toxicological characteristics (Turesky and others 2002). Several epidemiological studies proposed a role for NAT2 activity in the genetic susceptibility of humans for various types of cancer (Le Marchand and others 2002; Butler and others 2008). The data support the hypothesis that, particularly in individuals who smoke and have genetic susceptibility with the rapid phenotypes for both NAT2 and cytochrome (CYP1A2) as well as an increased intake of meat carcinogens have a higher risk for the incidence of colorectal cancer (Le Marchand and others 2002). Human NAT2 catalyzes O-acetylation of N-hydroxyl derivatives of IQ, MeIQ, and PhIP, and a number of polymorphic genotypes of human NAT1 and NAT2, respectively, are known. In particular, the genotype NAT2 (NAT2-4 wild allele) as a fast acetylator (Sugimura and others 2004) has often been associated with a higher risk of colorectal cancers (Ishibe and others 2002; Le Marchand and others 2002). In contrast to carbon hydroxylation is the inactivation step, the resulting compounds undergo different reactions catalyzed by phase II enzymes, such as sulfotranferase, glucuronosyl transferase, or gluthatione transferase, and the metabolites formed are excreted with the urine (Figure 2).

Biomarkers of HAAs for human biomonitoring. Different biomarkers of HAAs were investigated for possible use in human biomonitoring studies. Long-term biomarkers for HAAs are especially required which can be applied in epidemiology studies, because HAAs become mutagenic after metabolic activation by N-hydroxylation of the exocyclic amine group to generate an intermediate, the arylnitrenium ion, which is the hazardous ion forming DNA adducts (Turesky and Le Marchand 2011). The usage of a protein carcinogen adduct as a biomarker requires that this adduct correlates to the intake, damage of DNA, and cancer risk. The acid-labile HAA serum albumin adducts in humans were discussed as possible biomarkers and as a dosimeter for the intake

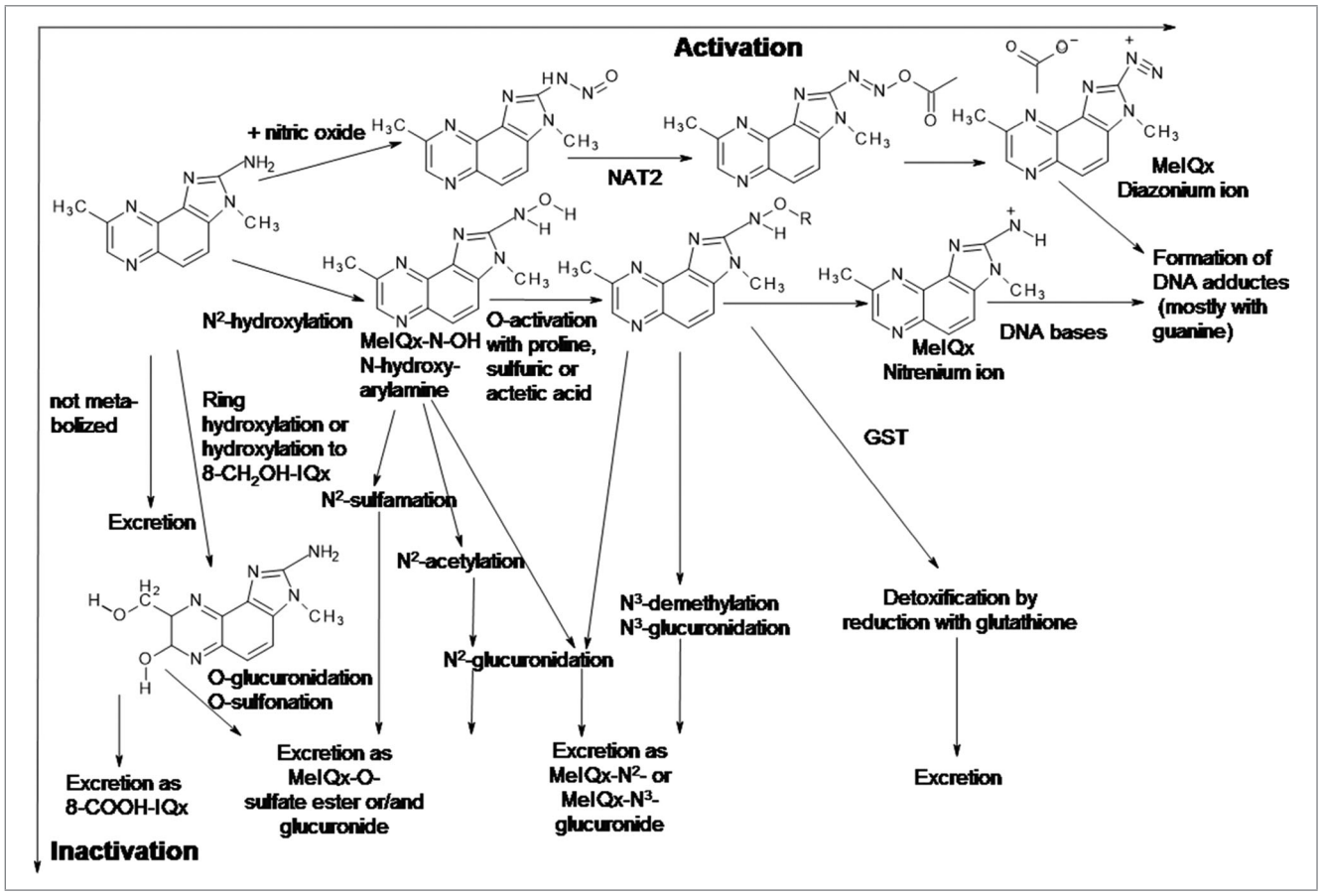


Figure 2–Possible metabolism of MeIQx as prototype of HAAs. Activation reactions of HAAs, such as nitrosation by nitric oxide and N-hydroxylation, are drawn from left to right. Inactivation reactions are vertically arranged (modified) (Wyss and Kaddurah-Daouk 2000; Turesky and Le Marchand 2011); NAT, N-acetyltransferase, GST, glutathione S-transferase, –R is –C=OCH_3 for acetylation, $-SO_3^{2-}$ for sulfonylation, $-PO^{3-}$ for phosphorylation, and O-prolyl for prolylation.

of HAAs. However, the levels of these adducts were reported to be below the limit of MeIQx detection (29 attomol/mg) (Lynch and others 1993). The HAAs interact with blood proteins, such as hemoglobin and serum albumin. However, the levels of IQ, MeIQx, and PhIP bound to hemoglobin are very low in experimental laboratory animals because only about 0.01% of the dose was found, and the levels of adducts of HAAs with serum albumin of rats, other rodents, and humans are only several times higher and the adduct linked to cysteine of serum albumin HAA adduct (Turesky and Le Marchand 2011), for example, MeIQx (Lynch and others 1993) and PhIP (Chepanoske and others 2004). Unmetabolized PhIP is discussed as a long-term biomarker of HAAs because ^{3}H-labeled PhIP accumulates in hair without metabolization (Alexander and others 2002). However, PhIP analyzed as a biomarker in hair should take the melanin content of hair into account for the correct exposure assessment of PhIP, because the concentration in hair is dependent on the melanin levels (Turesky and others 2013). This biomarker can confirm a long-lasting intake to PhIP in epidemiology studies, but it does not represent DNA damage. The formation of DNA adducts is assumed to be the first stage in induced carcinogenesis. It was reported that HAA adducts were mainly formed at the C-8 position of guanine in DNA and could also be used as biomarkers (Turesky and Vouros 2004). PhIP-DNA adducts have been frequently detected by immunohistochemistry or ^{32}P-postlabeling, and MS methods were used to identify HAA adducts *in vitro* and *in vivo*, for example, in tissue of humans, such as the mammary tissue of women in the United States, indicating that PhIP may be associated as a causal agent of human breast cancer (Gu and others 2012). In addition, HAA adducts were identified in the colon, pancreas, and mucosa (Turesky and Vouros 2004; Jamin and others 2007; Turesky 2007).

HAA Mutagenesis and Carcinogenesis

Mutagenicity

After metabolic activation with S-9 mix, HAAs can be assigned to the group of the most strongly mutagenic compounds (Sugimura 1997). The demonstration of mutagenicity was made by means of short-term bacterial tests. These mutagenicity tests also enabled the detection of biologically active substances in complex foods (Ames and others 1975). Substances that are detectable with the Ames test have genotoxic potential (Sugimura and others 2004). The mutagenicity of HAA compounds are depicted in Table 1. Trp-P-1, Trp-P-2, Glu-P-1, and Glu-P-2 are, after metabolic activation, strong mutagens for the *Salmonella* Typhimurium TA98, TA100, and TA1538 strains used in the Ames test (Table 1) (Sugimura and others 2004). After the addition of S9 mix, which is necessary for metabolic activation, the IQ compounds MeIQ, IQ, 4,8-DiMeIQx, MeIQx, Glu-P-1, and Trp-P-1 acted as significantly stronger mutagens than AαC, MeAαC, and PhIP. In addition, the IQ compounds showed a significantly stronger mutagenic activity than aflatoxin B1 and benzo(a)pyrene (Sugimura 1997). The relative mutagenic potentials of MeIQ,

MeIQx, and PhIP vary with the test assay. In *Salmonella* Typhimurium strain TA98, the relative potencies were MeIQ > MeIQx > PhIP; however, they were PhIP ≥ MeIQ > MeIQx in a Chinese hamster ovary system (US HHS PHS 2002) or IQ > MeIQ > Trp-P-1 ≥ MeIQx >> PhIP in human-derived hepatoma (HepG2) cells (Knasmuller and others 1999). The relative mutagenicity is therefore difficult to assess and the effect of intake of certain amounts of HAAs are difficult to predict. Norharman, belonging to the ß-carbolines, showed no mutagenic activity in the Ames test (Nagao and others 1977). However, norharman, similarly to harman, could strengthen the mutagenic activity of the other HAAs and acted co-mutagenically (Wakabayashi and others 1982). Aniline and norharman (β-carboline) formed aminophenylnorharman by using a S9 mix of rat liver or *in vivo* in rodents; hydroxyaminophenylnorharman was generated and finally converted to an acetoxy derivative, which can form DNA adducts and induce mutations (Ohnishi and others 2001; Totsuka and others 2004). Co-mutagenic activity can also be perceived with other aromatic amines such as o-toluidine (Hada and others 2001).

Carcinogenicity

The carcinogenic effect of HAAs in animal trials with rats and mice could be demonstrated. In the trials, the animals were fed concentrations of 0.03% or 0.04% HAAs in their food over a period of 2 years. After several months, feeding with IQ, MeIQ, and MeIQx resulted in the formation of tumors in all the trial animals (Ohgaki and others 1987). The tumors appeared mainly in the animals' livers or intestines. Of the control rats who received normal food, only 4% of the animals developed tumors. After feeding with 0.06% MeIQx for several weeks, tumors appeared in the rats' livers, lungs, and lymph nodes (Ohgaki and others 1987). A dose of 10 to 20 mg IQ per kg body weight in the food of nonhuman primates similarly caused carcinoma formation after several months (Adamson and others 1990, 1996). Based on animal trials, the International Agency for Research on Cancer has classified IQ as a possible carcinogen (class 2A) and 8 other HAAs (MeIQ, MeIQx, PhIP, AαAC, MeAαAC, Trp-P-1, Trp-P-2, and Glu-P-2) as probable carcinogens (class 2b) (IARC 1993).

However, it is difficult to prove a connection between HAA intake and the development of different types of tumors in humans. The quantity of HAAs ingested by humans is much smaller than the quantities fed to trial animals. In this context, the present results of animal long-term studies cannot be used to set a specific threshold value for the human intake of HAAs. Nevertheless, epidemiological studies in the last decade have shown an increased risk of incidence of different cancers for humans who prefer and eat high amounts of very well-done meat.

Risk Assessment

Daily intake of heterocyclic aromatic amines

The quantity of mutagenic substances ingested is very difficult to estimate. The information about daily HAA intake can be very different in epidemiological studies. Alongside different eating habits of people, the type of preparation and the frequency of meat consumption also play an important role.

Although the levels in heated foods were low, a connection between meat consumption and the development of colorectal carcinomas could be determined in different epidemiological studies (Le Marchand and others 2002; Nowell and others 2002). The results of studies in different countries showed that it is difficult to define a standard value for the quantity of ingested HAAs. The daily intake of the major HAAs per person in various countries found in different studies is shown in Table 7. In American studies, the HAA intakes calculated were often remarkably higher than, for example, in Sweden. One reason for this could also be that much more grilled or fried meat in general is eaten in United States than in Sweden. In China, the daily HAA intake is estimated at 50 ng/d (Table 7). The HAA intake in Spain at 606 ng/d per person is clearly higher in comparison, which can be explained by the preparation of meat, such as grilling, barbecuing, and pan-frying, and the highest frequency of meat consumption in Europe (Busquets and others 2004).

In a study from the United States, the authors used consumption data from 3 consecutive days of 3563 participants (Layton and others 1995) which was collected from an USDA sponsored random survey of the U.S. population during 1989. It was preferred to investigate the average daily HAA intake with the food in order to be able to estimate the intake of PhIP, MeIQx, DiMeIQx, IQ, and AαC. The HAA concentrations used to calculate the daily HAA intake were taken mainly from databases. In addition, data from the literature containing the different heating times and preparation methods (including grilling, frying, and roasting) were used. On this basis, the estimated daily average HAA intake in the United States was determined to be 26 ng/kg body weight and per day (Layton and others 1995).

In a New Zealand study, data on prepared meat with known HAA concentrations were taken from the literature and compared with the eating habits of New Zealanders. The estimated intake is around 1000 ng/d per person (Thomson and others 1996). Lower values were obtained in a later New Zealand study which was similarly concerned with the connection between meat consumption and HAA intake. The consumption quantities of different sorts of meat, the modes of preparation, and the degree of browning were established by means of a questionnaire. From this, a total HAA intake of 164 ng/d per person was calculated (Norrish and others 1999). In a Swedish study, 267 women and 277 men in Stockholm between the ages of 50 and 75 years were asked about their eating habits. The frequency of consumption of grilled or cooked meat and fish with known HAA levels was recorded with the help of a questionnaire (Augustsson and others 1997, 1999). In order to be able to take the preferred, individual degree of browning into account, the test participants were shown photos of each product with 4 different degrees of browning. Using this information, the questionnaire produced a mean HAA intake of 160 ng/d per person (Augustsson and others 1997). After several analytical investigations of various meat and fish dishes from restaurants, HAA intake in Switzerland was calculated to be up to 400 ng/d per person (Zimmerli and others 2001). Data for more than 25000 U.S. citizens on their meat consumption and preparation methods were evaluated in a U.S. cross-sectional study. The calculated daily HAA intake is about 420 ng/d per person (Keating and Bogen 2001). The HAA levels in the study were only roughly estimated. In addition, the food was not prepared using the usual household methods, whereby significantly higher and not "everyday" concentrations arose.

With the help of a questionnaire, 344 participants in Germany were asked about their frequency of consumption of 7 types of meat and fish, taking into account the preparation methods. In order to record the preferred degree of browning, photos of samples with different degrees of browning, from light to very heavy browning, were attached to the questionnaire. The mean daily HAA intake was 103 ng/d per person in this study (Rohrmann and Becker 2002). In a sensory test of another study, some panelists

Table 7–Estimated dietary daily intake of major HAAs (ng per person and day) reported in various studies.

Country	Subjects	MeIQx	DiMeIQx[a]	PhIP	MeIQ	Other HAAs	Total HAAs	Reference
U.S.A.	3563	182	56	1162		364 AαAc, 21 IQ	1820 (2415)[c]	(Layton and others 1995)[b]
Sweden	544	72	16	72			160	(Augustsson and others 1997)
U.S.A.	673	33–45	4	286–458			323–507	(Byrne and others 1998)[d]
U.S.A.	374	20–33	1.5–2.2	78–110			100–145	(Sinha and others 2001)[e]
Switzerland	–[f]	72–85	13–78	124–156	0–39	0–39 IQ,	209–397	(Zimmerli and others 2001)[f]
Japan	39035	9.8–11	0.7–6.3	39–47	5.6–9.8	2.8–4.9 Trp-P-1	58–77	(Kobayashi and others 2002)[a,g]
U.S.A.	537	94–135	6.5–11	161–218			261–364	(Nowell and others 2002)[h]
Germany	385 (344)[i]	34	2	63			103	(Rohrmann and Becker 2002)[i]
U.S.A.	21780	98–147	20–33	644–889		176–219 MeIQ and Trp-P-1	938–1288	(Keating and Bogen 2004)[j]
Spain	3221	29	14–15	344			606 (934)[k]	(Busquets and others 2004)
Singapore	497	29	13	5.8		2.2 IFP and 7,8-DiMeIQx	50	(Wong and others 2005)[l]
Sweden	15174	22–256	8–90	29–475			59–821	(Ericson and others 2007)[m]
Germany	25540	17–22	3–3.8	41–45			61–71	(Rohrmann and others 2009)[n]
Malaysia	600	312	16[a]	195	30		554	(Jahurul and others 2010)[o]
Croatia	94	65–85		164–2968			327–4363	(Klapec and Periš 2014)[p]

[a] 4,8-DiMeIQx and 7,8-DiMeIQx.
[b] Mean intake based on 70 kg body weight.
[c] Included minor occurring HAAs (Glu-P-1, Glu-P-2, Trp-P-1, Trp-P-2, MeIQ, MeAaC).
[d] Questionnaire assessing HCA intake in each cohort ($n = 216, 226, 231$ participants) separately.
[e] Case-control study of cases ($n = 146$), colorectal adenomas.
[f] Sum of IQ, 7,8-DiMeIQx, MeIQ, 4,8-DiMeIQx, MeIQx, and PhIP; total meat (ready to eat) about 130 g/person/d (calculated).
[g] Food frequency questionnaire study of 39035 participants.
[h] Case-control study with 157 cases (colorectal cancer) and 380 controls.
[i] Questionnaire assessing HAA intake of 385 participants (plus repeat 344).
[j] Continuing survey of food intakes by individuals (CSFII) databases (U.S.) with 21780 records.
[k] Sum of DMIP, Glu-P-2, IQ, MeIQx, MeIQ, Glu-P-1, 7,8-DiMeIQx, 4,8-DiMeIQx, norharman, harman, Trp-P-2, Trp-P-1, PhIP, AαC, MeAαC.
[l] Food-frequency questionnaires completed by 497 randomly sampled Chinese men and women aged 20–59 year.
[m] Malmö diet and cancer cohort ($n = 15174$), (low intake – 175 °C and high intake – 200 °C).
[n] Prospective cohort study (25540 participants) (cases of colorectal cancer $n = 516$ and controls ($n = 3699$).
[o] Food-frequency questionnaire of 600 participants.
[p] Questionnaire of 94 Croatian females (low intake and high intake); total HAA intake = (Σ intake MeIQx and PhIP) · 1.249.

Table 8–Epidemiological human studies investigating consumption of well-done meat or intake of major HAAs and cancer risk (without indication - $p_{trend} < 0.05$; N-acetyltransferase (NAT1) or (NAT2); sulfotransferase (SULT)).

Study	Material/Content of HAAs	Odds ratio OR / relative risk RR (95 % CI)	Cancer type	Number of subjects (age); cancer cases	Reference
Case-control	Very well-done meat	OR 4.36 (2.08–9.60)	Colon/rectum	380 controls (20–88) and 157 cases	(Nowell and others 2002)
	High content of MeIQx	OR 4.09 (1.94–9.08)			
Case-control	AhR GA/AA or NAT1 rapid acetylator & intake of red meat	OR 1.60 (1.23–2.09)	Colon/rectum	1002 polyp cases and 1493 controls	(Shin and others 2008)
	AhR GA/AA or NAT1 rapid acetylator & intake of MeIQx	OR 1.48 (1.14–1.92)			
	AhR GA/AA or NAT1 rapid acetylator & intake of PhIP	OR 1.39 (1.11–1.74) $p_{trend} = 0.055$			
	AhR GA/AA or NAT1 rapid acetylator & intake of DiMeIQx	OR 1.37 (1.10–1.71) $p_{trend} = 0.115$			
Case-control	Total HAAs	OR 0.99 (0.21–4.81)	Colon/rectum	117 cases and 238 controls	(Kobayashi and others 2009)
	MeIQx, PhIP, DiMeIQx	no significant OR			
Cohort study	High intake of PhIP	RR 1.47 (1.13–1.93)	Colon/rectum	(25540 participants) 516 cases and 3966 controls	(Rohrmann and others 2009)
	High intake of MeIQx or DiMeIQx	no significant RR			
Case-control	High red meat consumption	OR 2.02 (1.06–3.83) $p_{trend} = 0.38$	Colon/rectum	158 cases and 649 controls	(Ferrucci and others 2009)
	High intake of MeIQx	OR 1.90 (1.05–3.42) $p_{trend} = 0.07$			
	High intake of pan-fried meat	OR 1.72 (0.96–3.07)			
Case control	High consumption of red meat	OR 1.4 (1.2–1.6)	Colon/rectum	2543 cases (1881 adenomas/ 622 hyperplastic polyp) and 3764 controls	(Fu and others 2011)
	High intake of MeIQx	OR 1.4 (1.2–1.6)			
	High intake of PhIP	OR 1.3 (1.1–1.5)			
	High intake of DiMeIQx	OR 1.3 (1.1–1.5)			
Case-control	High intake of PhIP	OR 1.81 (1.24–2.64)	Colon/rectum	413 cases and 796 controls	(Barbir and others 2012)
	High intake of MeIQx	OR 1.45 (0.99–2.12)			
	High intake of DiMeIQx	OR 1.35 (0.94–1.93)			
	High intake of MeIQx & slow SULT1A1 activity	OR 7.94 (2.20–28.68)			
Case-control	High intake of MeIQx & NAT1 activity (rs6586714)	OR 1.43 (1.11–1.85)	Colon/rectum	1205 cases and 1387 controls	(Gilsing and others 2012)
Case-control	High intake of MeIQx	OR 1.87 (1.44–2.44)	Colon/rectum	1645 controls and 1062 cases	(Helmus and others 2013)
	High intake of DiMeIQx	OR 1.68 (1.29–2.17)			
	Red meat-derived mutagenic activity	OR 1.77, (1.36–2.30)			
Case-control	Fast NAT2 activity & high intake of DiMeIQx	OR 1.70 (1.06–2.75)	Colon/rectum	1016 cases and 1355 controls	(Voutsinas and others 2013)
	Fast NAT2 activity & high intake of MeIQx	OR 1.91 (1.16–3.16)			
	Fast NAT2 activity & high intake of PhIP	OR 2.14 (1.31–3.49)			
Case-control	High consumption of red meat	OR 1.8 (1.5–2.2)	Lung	1903 cases and 2073 controls	(Lam and others 2009)
	High consumption of processed meat	OR 1.7 (1.4–2.1)			
	High intake of PhIP	OR 1.5 (1.2–1.8)			
	High intake of MeIQx	OR 1.4 (1.2–1.7)			
	High intake of DiMeIQx	OR 1.0 (0.8–1.2), $p_{trend} = 0.09$			
	High mutagenic activity	OR 1.4 (1.1–1.7)			
Case-control	High consumption of well-done red meat	OR 1.52 (0.93–2.46)	Prostate	531 advanced & 195 localized cases and 527 controls (40-79)	(John and others 2011)
	Higher consumption of hamburgers	OR 1.79 (1.10–2.92)			
	Medium-high intake of PhIP	OR 1.42 (0.98–2.04)			
	MeIQx, DiMeIQx	no significant OR			

(*Continued*)

Table 8–Continued.

Study	Material/Content of HAAs	Odds ratio OR / relative risk RR (95 % CI)[a]	Cancer type	Number of subjects (age); cancer cases	Reference
Case-control	High intake of MeIQx	OR 6.07 (2.24–16.4)	Bladder	884 cases and 878 controls	(Lin and others 2012)
	High intake of PhIP	OR 2.67 (1.2–5.96)			
	High intake of DiMeIQx	OR 3.44 (1.5–7.89)			
	Mutagenic activity	OR 6.32 (2.51–15.94)			
	High red meat consumption & unfavorable genotypes (≥6)	OR 5.09 (2.89–8.96)			
Case control	High consumption of barbecued meat	OR 1.35 (1.01–1.79)	Renal	1192 cases and 1175 controls	(Daniel and others 2011)
	High intake of MeIQx, PhIP, DiMeIQx	No significant OR			
Case-control	High intake of MeIQx, PhIP, DiMeIQx	No significant OR	Breast	2686 cases and 3508 controls	(Mignone and others 2009)
Cohort study	High intake of meat mutagens or high consumption of meat	No significant RR	Breast	120755 post-menopausal women, 3818 cases of invasive breast cancer (during 8 years)	(Kabat and others 2009)

[a]Categorized by quartiles among controls Please include is footnote under the table.

preferred the darker and very well-done beef patties, because they liked a well-done appearance and roasted flavor, which resulted in an increase of HAA levels by factors of 8.5 for MeIQx, 2 for PhIP, 4.5 for norharman, and 2.4 for harman between the lightest grilled (for 120 s) and darkest product grilled (for 180 s) (Gibis and Weiss 2010). This shows that the preference of color and roasted flavor plays a central role in the personal daily intake of HAAs. As mentioned previously, the frequency of meat consumption and, above all, the types of preparation result in different intake levels of individual HAAs in different countries. Eating habits play also an important role, whereby the contribution of individual foods to the HAA intake was different (Rohrmann and others 2009). Most of the daily intake of HAAs is of PhIP, followed by MeIQx (Augustsson and others 1997; Keating and Bogen 2004).

In a study obtained from food-frequency questionnaires of 497 randomly sampled Chinese men and women from Singapore, the assessed mean exposure to HAAs was approximately 50 ng/d; this was 50% higher among younger (20 to 39 year-olds) compared with older individuals (40 to 59 year- olds) (Wong and others 2005). In this assessment, PhIP, MeIQx, and 4,8-DiMeIQx were the most abundant HAAs detected, and concentrations of total HAAs ranged from <0.10 to 6.77 ng/g. The highest contents were found for Chinese-style roasted pork. Generally, 7 meat-cooking methods contributed 90.1% of this intake, namely, pan-fried fish, pork, and chicken; deep-fried chicken and fish; roasted/barbecued pork; and grilled minced beef (Wong and others 2005). The data in Table 7 confirmed that there is a large range (50 to 1820 ng/d per person) of exposure to HAAs between the different countries. One reason is the different frequency of meat consumption, for example Greece had approximately a 30% lower frequency of meat consumption than Spain (BVDF 2011), and the also different preparation methods. Also the consumer preference of color and roasted flavor plays a key role in the human exposure of HAAs. Compared to data of meat consumption, the food-frequency questionnaires with images of different degrees of browning of each preparation methods give additional information of the HAA-intake, which was included in the some intake studies (Augustsson and others 1997; Rohrmann and Becker 2002; Rohrmann and others 2009).

Epidemiological data

Risk evaluation. The findings on the mechanisms of action of HAAs and mutagenicity and carcinogenicity tests have caused international associations which are concerned with estimating the risks of potentially carcinogenic substances to try to classify HAAs according to their risk. In addition, efforts are being made to issue nutritional, preparation, and other recommendations to minimize risks.

Most meat is consumed fried, grilled, or roasted, whereby the HAA intake is related to the intake of meat. Many studies associate high intake of meat, mainly red meat and processed meat, with a high risk of an incidence of cancers, particularly colorectal cancer. However, there is evidence also that this risk may not be a function of meat as such, but may reflect high fat intake and/or carcinogens generated through various home or restaurant cooking and processing methods (Ferguson 2010). The cancer risk may be modified by certain genotypes (Barbir and others 2012). The key to the connection between meat and cancer is not the meat itself, but the intake of carcinogenic substances, such as HAAs, polyaromatic hydrocarbons or nitrosamines (Gevaart-Durkin and de Peyster 2014). But not only carcinogenic substances in meat but also other factors such as physical activities, or smoking which

matters to the multifactoriality of the cancer development. Also the combination of HAAs and with other ingredients such as fibers showed the evidence that a diet high in dietary fiber sources may reduce potentially carcinogenic effects of HAAs through changing their absorption and excretion (Ferguson 2010). Assessing this risk, however, is exceedingly difficult. The carcinogenic effect has been confirmed in animal trials. However, significantly higher doses of HAAs were administered in these trials than are usually ingested with the human diet. The intake of thermal mutagens with food is low and is about 5 to 10 μg per meal (Skog and others 1998b). Absorption, metabolization, and excretion of HAAs are further important factors in estimating the risk (Turesky 2007). On the one hand, further carcinogenic and tumor promoters are also ingested along with HAAs in food, which can possibly enhance the carcinogenic effects of food components, such as heme iron, or free radicals (Sugimura and others 2004; Irigaray and Belpomme 2011). Otherwise, protective substances, such as antioxidants, are also ingested alongside the toxic substances. These can then combine with other food components and, thus, have a positive bioactive effect (Forte and others 2008; Rahman and others 2014).

It is difficult to establish a connection between food components and various cancers as carcinogenesis is a very complex process occurring over long time periods. Besides HAAs, other carcinogenic compounds such as polycyclic aromatic hydrocarbons in red meat and in addition to nitrosamines are present in processed meat. However, human case control studies with a large number of participants have shown an increasing risk of the incidence of different types of cancer in many cases, particularly colorectal cancer and the high intake of MeIQx, PhIP, and DiMeIQx as well as a high consumption of red meat or an increasing mutagenic activity (Table 8). Nonetheless, the assumption that a connection between the consumption of meat and processed meat and an increased risk of cancer exists is strengthening. It is suggested in a personal recommendation by the World Cancer Research Fund that not more than 500 g of red meat, including meat products, should be consumed per week, and very little if any of this should be processed meat (WCRF/AICR 2007). Red meat refers to beef, pork, lamb, and goat (from domesticated animals), including that contained in processed foods. The consumption of fish, poultry, and game is preferable to that of pork, beef, and lamb. This recommendation was issued because the consumption of the latter kinds, in contrast to other types of meat, is now considered to be connected with an increased risk of various tumors. Above all, no charred meat or meat grilled directly over flames should be consumed. Carcinogenic HAAs are produced with grilling and their intake should be minimized by less frequent consumption of grilled products. Gentler preparation methods are boiling, stewing, steaming, and microwaving (Felton and others 1994; Rahman and others 2014). It has already been determined that the individual genetic disposition for a fast or slow metabolization of HAAs in the body may play a role in the development of colorectal cancer. Colorectal adenoma risk was positively associated with PhIP, MeIQx, and DiMeIQx (P trend = 0.006, 0.022, and 0.045, respectively) intake (Barbir and others 2012). The phenotypes of sulfotransferases (SULT1A1) also modified the effect of MeIQx on colorectal adenoma risk ($P > 0.01$) such that the association of MeIQx intake with colorectal adenoma was stronger for slow than for normal metabolizing phenotypes (Barbir and others 2012). It was determined in studies that there is an increased risk of developing breast, lung and others types of cancer with the consumption of red, strongly roasted meat. However, some researchers could not show a connection between HAA intake and the development of prostate cancer (Norrish and others 1999). Nevertheless, they determined that there is an increased risk of cancer for people who prefer to eat well-roasted meat. Sinha and others (2000) investigated the effect of different HAAs on the development of lung cancer in a further study. MeIQx was the only HAA to have an increased effect on the risk of lung cancer, while no effect could be detected for the other HAAs investigated. In addition, they determined that an increase in MeIQx intake of 10 ng results in an increase of 5% in the risk of colorectal adenomas. A study carried out in the United States on the connection between meat consumption and the development of breast cancer came to the conclusion that HAA intake had no effect on the risk of cancer (Delfino and others 2000). Although not all studies have led to consistent results, epidemiological studies showed divergent results on pork and poultry (Joshi and others 2015). The whole meal composition of the dishes, the cooking method of meat and lifestyle as physical activities and fiber intake are very important for the effect of meat containing HAAs or other carcinogenic compounds (Ferguson 2010). However, there is now close consensus that an increased risk of cancer with high consumption of red meat and meat products. The evidence that red meat and processed meat is a cause of colorectal cancer is now more convincing than in the mid-1990s (WCRF/AICR 2007). For this reason, the IARC has now classified the consumption of red meat as probably carcinogenic to humans (Group 2A), and processed meat as carcinogenic to humans (Group 1), based on sufficient evidence in humans that the consumption of processed meat causes colorectal cancer (Bouvard and others 2015). The experts concluded that each 50 g portion of processed meat eaten daily increases the risk of colorectal cancer by 18% (WHO-IARC 2015).

Conclusions

Meat develops a typical and desirable meat aroma and taste after cooking. In addition, the main objective of heat processing is to assure that the product is microbiologically safe. However, harmful process contaminants, such as mutagenic and/or carcinogenic HAAs, are formed in the Maillard reaction during the cooking process. Various compounds are formed and others destroyed, including beneficial nutrients. This overview concerning different investigations in this research field enables us to learn more about the formation, metabolism, reaction mechanisms, and factors affecting the generation of HAAs. It may be possible to find future approaches for further inhibition of their formation. The studies presented here are of importance to meat product manufacturers and consumers and may also persuade the industry that a proper assessment of the human intake of HAAs in cooked meat is necessary, with more and better detailed information about preparation methods, knowledge about the reactions of precursors, food structure, and sensory preferences of consumers. Thus, consolidated knowledge is required to reduce human exposure to HAAs and, consequently, to minimize the risk of the incidence of cancer related to these compounds. The epidemiological results show that the evidence concerning red meat and processed meat is stronger than 20 years ago and that they are a convincing cause of colorectal cancer. The evidence for a high intake of HAAs has not resulted in consistent findings, but some case-control studies show an increased relative risk, or odds ratio, for a high intake of HAAs, or the preference for very well-done meat products, could cause colorectal cancer. Additional studies are needed to bridge this gap. HAA formation in food should be taken into account by food

manufacturers when food processes are designed concerning food safety and quality.

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