Potential sources of carcinogenic heterocyclic amines in Chinese mutton shashlik

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ABSTRACT

Heterocyclic amines (HAs) are formed in muscle meat during its cooking under ordinary conditions. In this work, we measured the concentration of 15 kinds of heterocyclic aromatic amines (HAAs) in mutton shashlik which is one of the most popular dishes in China and provided the basis for efforts to quantify dietary exposure to these compounds. Five mutton shashliks bought in market were determined and the concentrations of Harman, Norharman, DMIP, PhIP and A α C were 0.40–1.36, 0.07–0.50, 0.73–65.07, 0.90–25.62, and 0.39–2.13 ng/g, respectively. The mimic experiments have been carried out in an oven to investigate the influence of time, temperature, flavours, and area of heating surface on the formation of HAAs in ground mutton roasted. The levels of HAAs in cooked meat are highly dependent on the cooking time and temperature and increased with the area of heating surface. Meanwhile, flavours have a different impact because of their various characters.

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1. Introduction

Mutton, which has rich nutrition and special flavour, is a type of flavoured food in China. Mutton provides high levels of zinc and iron. Also it is a great source of high quality protein, unsaturated fat, trace elements and B Vitamins which are essential for metabolic reactions in the body. Now there is a popular cookery of mutton in China that we call the food mutton shashlik which can be ordered in most restaurants. High temperature, the essential condition of this cookery, can lead to the formation of heterocyclic amines (HAs), especially heterocyclic aromatic amines (HAAs), in heated meat (Bermudo, Ruiz-Calero, Puignou, & Galceran, 2005; Borgen & Skog, 2004; Busquets, Bordas, Toribio, Puignou, & Galceran, 2004; Martín-Calero, Ayala, González, & Afonso, 2007). Although only trace amounts (ng/g) of these compounds are formed, they are still considered a risk factor for human cancer due to their frequent intake.

HAAs were first isolated from charred meat and fish (Nagao, Honda, Seino, Yahagi, & Sugimura, 1977). Since 1977, more than 20 HAAs have been isolated and identified from cooked muscle foods (Ohgaki et al., 1984; Övervik, Kleman, Berg, & Gustafsson, 1989; Wakabayashi, Nagao, Esumi, & Sugimura, 1992; Wakabayashi & Sugimura, 1998). Over the years, many researchers have suggested that the formation of HAAs is a result of the condensation of free amino acids, creatine, creatinine, monosaccharides, disaccharides, or dipeptides, which all may act as precursors of

HAAs during cooking at high temperature (Jägerstad, Grivas, Olsson, & Negishi, 1984; Pais, Salmon, Knize, & Felton, 1999; Skog & Jägerstad, 1990; Solyakov, Skog, & Jägerstad, 1999). The International Agency for Research on Cancer (IARC, 1993) has recorded some of these HAAs in monographs. 2-Amino-3-methylimidazo[4,5-f]quinoline (IQ) is classified as a probable human carcinogen (class 2A) and 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), 2-amino-3,4-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-9H-pyrido[2,3-b]indole (A α C), 2-amino-3-methyl-9H-pyrido[2,3-b]indole (MeA α C), 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2), 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole (Glu-P-1), and 2-aminodipyrido[1,2-a:3',2'-d]imidazole (Glu-P-2) are classified as possible carcinogens (class 2B).

Many purification techniques have been described (Toribio, Busquets, Puignou, & Galceran, 2007; Toribio, Puignou, & Galceran, 2000; Vollenbröker & Eichner, 2000), mainly followed by chromatographic methods including liquid chromatography with ultraviolet (LC/UV) (Vollenbröker & Eichner, 2000), electrochemical (LC/ECD) (Bermudo et al., 2005; Martín-Calero, Pino, Ayala, González, & Afonso, 2007) or fluorescence detection (Martín-Calero et al., 2007), or gas chromatography with nitrogen–phosphorus selective detection (GC/NPD) (Kataoka & Kijima, 1997). However, in recent years a large number of works based on the use of either liquid chromatography or gas chromatography in combination with mass spectrometry (MS) have been published (Barceló-Barrachina, Moyano, & Galceran, 2004; Barceló-Barrachina, Santos, Puignou, & Galceran, 2005; Barceló-Barrachina et al., 2006; Casal, Mendes,

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Fernandes, Oliveira, & Ferreira, 2004; Toribio, Moyano, Puignou, & Galceran, 2002; Turesky, Taylor, Schnackenberg, Freeman, & Holland, 2005; Zhang et al., 2008), especially liquid chromatography coupled to mass spectrometry (LC/MS) have been widely used for HAAs analysis in these complex samples (Toribio et al., 2002; Barceló-Barrachina et al., 2004, 2006; Turesky et al., 2005). LC/MS is favoured because it provides both qualitative and quantitative information, without the requirement of analytes derivatization. Moreover, the use of narrow-bore reversed-phase UPLC columns in combination with electrospray ionisation (ESI) sources and tandem MS (MS/MS) instruments has allowed an improvement in the sensitivity and also a reduction in the analysis time (Barceló-Barrachina et al., 2006).

Our objectives were to estimate the content of HAAs in mutton shashlik and provide the guide to cooking practices. The influence of time, temperature, spices and heated area of meat on the formation of HAAs in mutton was investigated. The studied samples were prepared according to the information on dietary practices. After being validated in cooking experiments, it may be possible to predict the formation of heterocyclic amines in our cooking and to design safer cooking equipment and procedures. Accurate determination of HAAs in the samples was performed using the solid-phase extraction method described by Vollenbröker and Eichner (2000) and liquid chromatography coupled to tandem mass spectrometry (UPLC–ESI–MS/MS).

2. Experimental

2.1. Chemicals

The compounds studied were 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (4,8-DiMeIQx), 2-amino-3,7,8-trimethylimidazo[4,5-f]quinoxaline (7,8-DiMeIQx), 2-amino-3,4,7,8-tetramethylimidazo[4,5-f]quinoxaline (TriMeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-1,6-dimethylimidazo[4,5-b]pyridine (DMIP), 2-amino-9H-pyrido[2,3-b]indole (AαC), 2-amino-3-methyl-9H-pyrido[2,3-b]indole (MeAαC), 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2), 2-amino-6-methyldipyrro[1,2-a:3',2'-d]imidazole (Glu-P-1), 2-aminodipyrro[1,2-a:3',2'-d]imidazole (Glu-P-2), 1-methyl-9H-pyrido[3,4-b]indole (Harman), and 9H-pyrido[3,4-b]indole (Norharman) and were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). HAAs methanolic stock standard solutions of 10 µg/ml were prepared and used for further dilutions. TriMeIQx was used as an internal standard in the experiment. Standards and samples were passed through a 0.2 µm nylon filter (Pall, USA) before injection into the UPLC system.

Methanol, acetonitrile, hexane, ethanol, and dichloromethane were of HPLC grade, and were all provided by Fisher (New Jersey, USA). Ultrapure water was obtained from a Milli-Q Plus 185™ coupled to an Elix 5™ system (Millipore, Schwalbach, Germany). Sodium hydroxide used for the sample treatment was obtained from BeiHua (Beijing, China) and chemicals for mobile phase preparation (ammonium acetate and acetic acid) were obtained from GFS Chemicals (Columbus, USA), and all were of analytical grade. Mobile phases were also filtered with a 0.2 µm nylon filter (Pall, USA). LiChrolut EN SPE cartridges (200 mg, 3 ml) were supplied by Merck (Darmstadt, Germany).

2.2. Instrument and MS conditions

Chromatographic separation of HAAs was performed on a Waters Acquity ultra-performance liquid chromatography system,

equipped with a quaternary pump system (Milford, MA, USA), using an Acquity BEH C8 column (100 mm × 2.1 mm i.d., 1.7 µm particle size) (Waters, Milford, MA, USA). Optimum separation was achieved with a binary mobile phase at a flow rate of 0.3 ml/min. Solvent A: acetonitrile; solvent B: 30 mM acetic acid/ammonium acetate (pH 4.5). The gradient elution programme was 0–0.5 min, 20% A; 0.5–2.0 min, 20–35% A; 2.0–2.5 min, 35–45% A; 2.5–3.0 min, 45–70% A; 3.0–4.5 min, 70% A; and 4.5–6.0 min, return to initial conditions, and equilibration of the column. The sample volume injected was 2 µl.

The UPLC system was coupled to a Quattro Premier XE triple quadrupole mass spectrometer (Micromass, Milford, MA, USA) using the electrospray ionisation source. For all compounds, the MS instrument was operated in the positive mode and the data were acquired in multiple-reaction monitoring (MRM). Source working conditions were as follows: cone voltage, 40 V; capillary voltage, 3.0 kV; source temperature, 100 °C; desolvation temperature, 400 °C; cone gas flow rate, 50 l/h; desolvation gas flow rate, 800 l/h. Nitrogen (99.9% purity) and argon (99.9999% purity) were used as cone and collision gases, respectively. The MRM transitions as well as the individual collision energy voltages applied for the analysis are summarised in Table 1. The dwell time established for each transition was 100 ms, and the inter-scan delay was set at 10 ms. Data acquisition was carried out by MassLynx v4.0 software.

2.3. Sample treatment

One of the aims of this study was to know the contents and concentration of HAAs formed under various normal cooking conditions. For this reason, the mutton was processed at cooking conditions similar to the normal habits previously. But the temperature of cooking of mutton shashlik was hard to be controlled when using the charcoal grilling. So we choose the oven instead to mimic the domestic cooking conditions because of adjustable time and temperature.

Raw mutton was purchased at a local supermarket in Beijing and frozen until use, then thawed at 4 °C overnight prior to cooking. Skin, fat parts, and bones were removed from lamb leg before cooking. The mutton samples were cut into cubes (10 mm × 10 mm × 10 mm), sliced (50 mm × 10 mm × 10 mm) and minced. The cooking method selected was roasting in oven and a Teflon-coated frying pan (260 mm × 260 mm) was used. In our experiments, common flavours (oil, salt, sugar, ground white pepper powder, cumin powder, paprika powder, and five spices powder) were purchased from a local grocery store and they were mixed to the mutton. The raw mutton (weight 40 ± 0.5 g) was roasted in oven at a selected temperature and time. The samples for every conditional experiment were roasted in duplicate to obtain exact results. Finally, the samples were smashed by the chopper and stored at –18 °C until analysis.

To extract the analytes, the following purification method was used (Vollenbröker & Eichner, 2000). First 1 g of sample was extracted in 10 ml of methanolic NaOH (methanol/1 N NaOH 3:7) in a centrifuge tube, and after addition of the internal standard the supernatant of the centrifuged mixture was applied to the LiChrolut EN cartridge for SPE. After washing with 3 ml of methanolic NaOH (methanol/0.1 N NaOH 55:45), 2 × 0.7 ml of hexane, 2 × 0.7 ml of ethanolic hexane (ethanol/hexane 1:4), 3 ml of methanolic NaOH (methanol/0.1 N NaOH 55:45) again and finally 2 × 0.7 ml of hexane, again the extract was obtained by elution with 3 ml of ethanolic methylene chloride (ethanol/methylene chloride 1:9). Then the eluent was evaporated under nitrogen and the residue was dissolved in 100 µl of acetonitrile. Before injection into UPLC/MS/MS system the solution was passed through a 0.2 µm nylon filter (Pall, USA). The limits of quantifica-

Table 1
SRM transitions and limit of quantitation (LOQ) of method.

Analyte	Precursor ion (<i>m/z</i>)	Quantitation		Confirmation		LOQ (pg/g)
		Collision energy (eV)	Product ion (<i>m/z</i>)	Collision energy (eV)	Product ion (<i>m/z</i>)	
MeAαC	198	181	20	154	28	20
AαC	184	167	24	140	30	24
PhIP	225	210	25	183	32	12
Trp-P-1	212	195	25	168	30	37
Trp-P-2	198	154	30	181	25	62
Harman	183	115	30	168	25	40
Norharman	169	115	30	142	25	49
4,8-DiMeIQx	228	213	25	212	34	25
7,8-DiMeIQx	228	131	35	213	25	28
Glu-P-1	199	184	25	172	25	20
MeIQ	213	198	25	197	35	13
MeIQx	214	199	26	131	36	102
IQ	199	157	30	184	24	200
Glu-P-2	185	158	24	131	26	22
DMIP	163	148	20	147	30	40

Fig. 1. The amount of Harman, Norharman, MeIQx, PhIP, and DMIP in roasted mutton influenced by cooking time and temperature.

tion are shown in Table 1, which are similar to those obtained previously using other UPLC–MS/MS systems (Barceló-Barrachina et al., 2006).

3. Results and discussion

3.1. The influence of cooking temperature and cooking time

The HAAs formation as a function of time and temperature was measured in ground mutton roasted at a temperature of 100–250 °C at every 25 °C for 5, 10, 15, 20, 25 and 30 min, respectively. The mutton cubes (40 g) without flavours were roasted on the Teflon-coated frying pan in the oven. Both time and temperature were found to have a strong impact on the formation of HAAs (Fig. 1). Seven HAAs (PhIP, Harman, Norharman, MeIQx, DMIP, 7,8-DiMeIQx, and MeIQ) were found, and 7,8-DiMeIQx and MeIQ were detected at a concentration of 0.67 and 1.36 ng/g, respectively only in sample roasted at 250 °C for 30 min. According to the data in Fig. 1, Harman and Norharman were detected in most samples, because the excess creatinine can improve the formation of Harman and Norharman in mutton (Jägerstad, Skog, Arvidsson, & Solyakov, 1998), and the amounts of them increased continuously by prolonging the cooking time or by heightening the cooking temperature. No MeIQx was found at 100 °C roasting even for 30 min or less than 15 min at any temperature. PhIP was not

detected if the cooking time was less than 15 min at any temperature and increased greatly for 30 min. DMIP was only detected when the cooking temperature exceeded 175 °C and when the cooking time was longer than 20 min. According to the data it could be concluded the levels of HAAs in cooked mutton are highly dependent on the cooking time and temperature and increased as the time was prolonged from 5 to 30 min and the temperature was heightened from 100 to 250 °C. These aminoimidazole-azaarenes, also known as thermic mutagens, are extremely mutagenic due to their formation at the much lower ordinary cooking temperature (<300 °C) (Skog, 1993).

3.2. The influence of area of heating surface

The mutton samples (cubes, slice and minced meat, respectively) without flavours were roasted in oven at 250 °C for 30 min. The content and levels of HAAs are shown in Fig. 2. The size order of surface areas were minced sample, cube sample, and sliced sample. The amount of HAAs in the roasted sliced mutton was the smallest of all and it increased by amplifying the area of heating surface. DMIP and PhIP were increased sharply in cube and minced samples compared with the sliced mutton and the levels of these even reached 110.7 and 120.37 ng/g in minced mutton. The AαC and IQ were synthesised in the roasted minced mutton because of extreme cooking conditions, and the latter is a probable human carcinogen which should be avoided in our cooking.

3.3. The influence of flavours

The sample cubes with different flavours were roasted in oven at 250 °C for 20 min. The flavours including salt, sugar, soy oil, white pepper powder, cumin powder, red pepper powder, and five spice powder were used in ordinary cooking of mutton shashlik in China. The effects of various flavours treatments on the HAAs formation at different cooking conditions are presented in Fig. 3. Every flavour could induce the synthesis of HAAs and different flavours may produce specific kind of HAAs. Five spice powder and red pepper powder induced more types of HAAs and the former resulted in producing MeAαC, PhIP, and 4,8-DiMeIQx, also increasing the amount of DMIP greatly. Salt, the pre-requisite flavour in cooking of mutton shashlik, induced synthesised Trp-P-2, MeIQ, IQ, Glu-P-2, and DMIP, but the increasing extent was small. There was a little effect on the content and concentration of HAAs when

Fig. 2. The amount of HAAs in roasted mutton influenced by cooking area of heating surface.

Fig. 3. The amount of HAAs in roasted mutton influenced by flavours.

Table 2

Quantitation of HAAs (ng/g extract) in five mutton shashliks bought in market using UPLC-ESI-MS/MS method.

Analyte	Concentration (ng/g)				
	Charcoal grilled mutton shashlik		Electric grilled mutton shashlik		
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
A α C	0.50	1.36	0.40	0.15	n.d.
PhIP	0.31	0.79	0.50	0.07	0.18
Harman	6.27	65.07	4.64	0.73	2.59
Norharman	5.85	25.62	11.41	0.90	2.52
DMIP	0.87	1.18	0.39	0.58	2.13

using sugar, soy oil, and white pepper powder, respectively. The amounts of these were augmented largely by using the powder flavours. This has been explained as the result of increased transport of water-soluble precursors to the surface where the reactions occur (Jägerstad et al., 1998; Persson, Sjöholm, & Skog, 2003). This phenomenon has been noted with carbohydrates that reduce cooking loss such as polysaccharides, starches, gums, and fibres that were shown to effectively decrease HAAs formation and mutagenicity.

3.4. Determination of mutton shashlik from market

Five mutton shashliks were bought in market. Two samples were grilled on charcoal and the other three samples were grilled on electric tube. The results are shown in Table 2. Harman, Norharman, DMIP, and PhIP were determined in all the samples and there was no A α C in sample 5. Due to different cooking conditions and sample shape the concentrations of Harman, Norharman, DMIP, PhIP, and A α C were 0.40–1.36, 0.07–0.50, 0.73–65.07, 0.90–25.62, and 0.39–2.13 ng/g. The amounts of HAAs in samples grilled on charcoal were higher than those of that grilled on electric tube because the temperature of charcoal's flame was higher than electric tube. In 1993, the International Agency for Research and Cancer, IARC, judged PhIP to be possible human carcinogens. Neither Harman nor Norharman is mutagenic but instead strengthens the genotoxicity of other HAAs (Sugimura, Nagao, & Wakabayashi, 1982).

4. Conclusions

The analysis of five mutton shashliks bought in market in Beijing showed that a wide variety of HAAs was formed during the cooking process. Harman, Norharman, DMIP, PhIP, and A α C were detected in levels of 0.40–1.36, 0.07–0.50, 0.73–65.07, 0.90–25.62, and 0.39–2.13 ng/g, respectively. The amounts of heterocyclic aromatic amines in samples grilled on charcoal were much higher than those of that grilled on electric tube because the temperature of charcoal's flame was higher than electric tube. The high levels of HAAs were mutagens to lead to cancer. Moreover the influence factors, cooking temperature, cooking time, area of heating surface, and flavours, on the formation of HAAs in ground mutton roasted in the oven were investigated. According to the data it could be concluded that the levels of HAAs in cooked mutton were highly dependent on cooking time and temperature and increased as the time was prolonged from 5 to 30 min and the temperature was heightened from 100 to 250 °C. Meanwhile HAAs increased with the area of heating surface, and most powder flavours improved their formation. Sugar, soy oil, and white pepper powder have little effect on them. These analytical results will provide useful values to ordinary cookery for Chinese people and to epidemiologists for the determination of representative exposure of the Chinese population to HAAs.

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