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Quantitation of Carcinogenic Heterocyclic Aromatic Amines and Detection of Novel Heterocyclic Aromatic Amines in Cooked Meats and Grill Scrapings by HPLC/ESI-MS

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A tandem solid-phase extraction method was used to isolate carcinogenic heterocyclic aromatic amines (HAAs) from cooked meats. The following 10 HAAs were identified by HPLC/ESI-MS/MS: 2-amino-9*H*-pyrido[2,3-*b*]indole (2-AαC), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (8-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-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-1,7,9-trimethylimidazo[4,5-*g*]quinoxaline (7,9-DiMeI*g*Qx), and 2-amino-1-methylimidazo[4,5-*b*]quinoline (IQ[4,5-*b*]); the latter HAA has not previously been reported in cooked meats. The concentrations of these HAAs ranged from <0.03 to 15 ppb in cooked meats and poultry, to 75 ppb in cooked beef extract, and to 85 ppb in grill scrapings. The product ion scan mode was used to confirm the identities of these HAAs. Six other compounds were detected that appear to contain the *N*-methylimidazoquinoxaline skeleton on the basis of their product ion spectra, and these compounds are probable isomers of IQx, 8-MeIQx, and DiMeIQx. A number of known HAAs and novel HAAs of unknown genotoxic potential are formed at appreciable levels in cooked meats.

KEYWORDS: Heterocyclic aromatic amines; mutagens; LC/MS

INTRODUCTION

More than 20 mutagenic heterocyclic aromatic amines (HAAs) have been isolated from various cooked meats, fish, and poultry (*1*, *2*). HAAs have also been detected in tobacco smoke condensate and diesel exhaust (*3*, *4*). These compounds are formed through the reaction of creatinine with fragmented hexoses and pyrazine or pyridine derivatives that occur during the cooking of foods (*5*−*11*). Other HAAs, the pyrolysis-type HAAs, are formed directly through thermal decomposition of proteins or amino acids (*1*, *2*). HAAs are potent bacterial mutagens (*1*, *12*) that have been reported to induce tumors in multiple organs, including the colon, prostate, and mammary glands, in rodent bioassays (*1*). Some epidemiological studies have reported elevated incidences of colorectal and breast cancer in individuals who frequently consume grilled meats, with the highest risk observed for individuals who consume well-done meats (*13*, *14*). These findings suggest that exposure to HAAs may contribute to human cancers, although the link between HAA exposure and cancer risk is not firmly established (*15*).

The formation of HAAs in cooked meats is dependent upon the type of animal flesh and the temperature and duration of cooking, which may lead to concentrations of HAAs that vary by >100-fold (*2*, *10*, *11*, *16*). Therefore, reliable data on the exposure of HAAs are required to determine the potential role of these compounds in human cancers. The detection and quantitation of HAAs, which are usually present in cooked foods at the low parts per billion (ppb) level, remain analytical challenges. In early analyses of HAAs in cooked meats, multiple chromatographic steps were required to isolate HAAs from complex food matrices, and bacterial mutagenicity assays were used as a means of tracking the biologically active fractions (*12*, *17*). Once the mutagenic components were purified by HPLC, the structures were elucidated by NMR and mass spectrometry (*12*). The corroboration of the identities of the HAAs was accompanied by unambiguous chemical syntheses. Subsequently, more refined analytical methods were established for the quantitation of HAAs, such as HPLC-UV with photodiode array or fluorescence detection, where analyte identification was based on the characteristic UV spectra of the HAAs (*5*, *7*, *8*). However, in some meats that were well-cooked, interfering components coextracted with the HAAs and precluded the unambiguous identification of the HAAs by UV

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spectroscopy. Moreover, the estimation of the HAA content was uncertain in some products because the internal standards employed did not adequately compensate for the different recoveries of various HAAs (*5*, *8*). Because of these limitations, quantitative mass spectrometry (MS) methods were established using either gas chromatography (GC/MS) or high-performance liquid chromatography (HPLC/MS). Optimal sensitivity by GC/MS was achieved in the negative ion chemical ionization (NICI) mode, following chemical derivatization, and some HAAs were detected in cooked beef at <1 ppb by GC/NICI-MS (*18*). Several nonpolar HAAs were also detected in grilled meats by GC/MS with electron impact ionization in the selective ion monitoring (SIM) scan mode (*19*). Early HPLC/MS studies used thermospray ionization in the SIM scan mode (*20*−*22*). More recent studies have employed atmospheric pressure chemical ionization (APCI) with the selective reaction monitoring (SRM) scan mode (*8*, *10*, *11*) or electrospray ionization (ESI) with SRM scan mode (*8*, *23*). The incorporation of isotopically labeled HAA internal standards to compensate for differences in recovery of the various HAAs throughout the extraction processes has resulted in quantitative and reproducible analyses. However, corroboration of the identities of these carcinogenic HAAs in cooked meats by acquisition of full product ion spectra has rarely been reported in these analyses (*8*, *10*).

We recently discovered a previously unreported HAA, an isomer of 8-MeIQx, in the urine of subjects who consumed grilled meats and subsequently identified this compound in cooked meat (*24*). This finding has prompted us to re-examine cooked meats for other HAAs that may have remained undetected in previous analyses (*12*, *17*, *24*). In this study, we simplified the isolation procedure (*5*) and employed both SRM and product ion spectra scan modes of the triple-quadrupole MS to quantify known HAAs and to characterize novel, putative HAAs that are present in cooked meats. Our findings show that a number of previously unreported HAAs are formed in meats and poultry prepared under typical household cooking practices.

MATERIALS AND METHODS

Caution: *HAAs are carcinogenic to rodents and should be handled carefully*.

Chemicals. The following chemicals were purchased from Toronto Research Chemicals (Downsview, ON, Canada): IQ and trideuterated 3-[$^2H_3$C]-IQ (isotopic purity >99%), 2-amino-1-methylimidazo[4,5-*b*]-quinoline (IQ[4,5-*b*]), 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (8-MeIQx) and trideuterated 3-[$^2H_3$C]-8-MeIQx (isotopic purity >97%), 2-amino-3,7-dimethylimidazo[4,5-*f*]quinoxaline (7-MeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and trideutrated 1-[$^2H_3$C]-PhIP (isotopic purity >99%), 2-amino-9*H*-pyrido[2,3-*b*]indole (2-AαC), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), and 5-chloro-4-nitro-2,1,3-benzoselenediazole. 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), 3-[$^2H_3$C]-4,8-DiMeIQx, and 3-[$^2H_3$C]-7,8-DiMeIQx (isotopic purities >99%) were synthesized as previously described (*21*). [4b,5,6,7,8,8b-$^{13}C_6$]-2-AαC (isotopic purity >99%) was a kind gift of Dr. D. Doerge, NCTR. 2-Amino-3,4-dimethylimidazo-[4,5-*f*]quinoxaline (4-MeIQx) was a kind gift of Dr. M. Knize, Lawrence Livermore National Laboratory, Livermore, CA, and 2-amino-1,7,9-trimethylimidazo[4,5-*g*]quinoxaline (7,9-DiMe*Ig*Qx) was kindly provided by Dr. K. Wakabayashi, National Cancer Center Research Institute, Tokyo, Japan. Oasis mixed mode cation exchange (MCX) LP extraction (500 mg) and microLC vials were purchased from Waters Corp. (Milford, MA). Extrelut-20 resin was obtained from EMD Chemicals (Gibbstown, NJ). Trideuterated 1-[$^2H_3$C]-creatinine was purchased from Sigma/Aldrich (Milwaukee, WI). Bacterial grade, Difco beef extract paste was purchased from Becton Dickinson and Co. (Sparks, MD).

Syntheses. Supporting Information on the method of synthesis accompanies this paper. Trideuterated 1-[$^2H_3$C]-IQ[4,5-*b*] was prepared by the reaction of 1-[$^2H_3$C]-creatinine with 2-aminobenzaldehye as previously reported (*25*). 2-Amino-1,8-dimethylimidazo[4,5-*f*]quinoxaline (1-Iso-8-MeIQx) was prepared from 5-chloro-4-nitro-2,1,3-benzoselenediazole with 3-nitrobenzene-1,2,4-triamine as the key intermediate (*26*) with minor changes as previously reported (*24*). The synthesis of 2-amino-1-methylimidazo[4,5-*f*]quinoxaline (1-Iso-IQx) was done in the same manner as that of 1-Iso-8-MeIQx except that glyoxal was used instead of methylglyoxal for the reaction with 3-nitrobenzene-1,2,4-triamine to form the 5-nitroquinoxalin-6-amine intermediate (*24*). Following the reaction of 2-aminoimidazo[4,5-*f*]-quinoxaline with CH_3I, the isomeric derivatives IQx and 1-Iso-IQx were separated by HPLC and desalted by solid-phase extraction (*24*).

1-Iso-IQx: (0.3 mg) ^{1}H NMR (DMSO-d_6) δ 8.80 (d, J = 1.75 Hz, 1H, 8-H or 7-H), 8.72 (d, J = 1.75 Hz, 1H, 7-H or 8-H), 7.77 (d, J = 8.77 Hz, 1H, 4-H), 7.66 (J = 8.77 Hz, 1H, 5-H), 6.64, 2H, NH_2), 4.12 (3H, *N*1-CH_3); EI-MS (relative intensity), *m*/*z* 199.1 ($M^{\bullet+}$ 100%), 198.7 (72%), 184.0 (5%), 183.0 (7%), 171.0 (27%).

IQx: (0.6 mg) ^{1}H NMR (DMSO-d_6) δ 8.80 (d, J = 1.75 Hz, 1H, 8-H or 7-H), 8.74 (d, J = 1.75 Hz, 1H, 7-H or 8-H), 7.83 (d, J = 8.77 Hz, 1H, 4-H), 7.60 (J = 8.77 Hz, 1H, 5-H), 6.64, 2H, NH_2), 3.67 (3H, *N*3-CH_3); EI-MS (relative intensity), *m*/*z* 199.1 ($M^{\bullet+}$ 100%), 198.7 (55%), 184.0 (12%), 183.0 (7%), 171.0 (27%).

The prominent downfield shift of the resonance signal of the *N*1-CH_3 group of 1-Iso-IQx relative to the signal of the *N*3-CH_3 group of IQx is consistent with the corresponding resonance signals of the *N*1-CH_3 and *N*3-CH_3 groups of 1-Iso-8-MeIQx and 8-MeIQx, respectively (*24*). The 2D-NOESY spectrum also showed peaks arising from nuclear Overhauser effects (NOEs) for 1-Iso-IQx between the *N*1-CH_3 and the NH_2 group and very weak signals between the *N*1-CH_3 and H-4 and H-5 atoms, whereas the 2D-NOESY spectrum of IQx revealed a strong NOE between the *N*3-CH_3 and the NH_2 group, as well as with the *N*3-CH_3 and H-4 atom. The 2D-NOESY spectra were used to differentiate the sites of methylation of the two different isomers.

Cooking Procedures. Patties of freshly ground beef (<20% fat content) and boneless, skinless, chicken breast patties (100−110 g, 9−10 cm diameter, thickness of 1.10−1.20 cm) were purchased from a local supermarket in Little Rock, AR. The meats were fried on a flat, metal grill at a local cafeteria in Jefferson, AR, where the cooking surface varied between 150 and 180 °C. The temperature was estimated with a Fluke 714 thermocouple calibrator (Tulsa, OK). Other ground beef samples were pan-fried in a metal skillet at a surface temperature ranging from 180 to 200 °C, pan-fried at 300 °C (*27*), or barbecued over charcoal on a grill where the cooking surface temperature varied between 230 and 300 °C. The duration of cooking time was dependent upon the experiment and is stated in the respective tables or figure caption.

Tandem Solvent Solid-Phase Extraction of HAAs from Cooked Meat. Grilled meat samples (4 g) were spiked with isotopically labeled HAAs (4 ng). Beef extract paste and meat scrapings (1 g) were spiked with 10 ng of internal standards. The samples were homogenized in 1 N NaOH (16 mL) and mixed thoroughly with an Extrelut-20 resin (12 g) as previously described (*5*). Instead of using PRS and C_{18} cartridges for tandem purification as done previously, both cartridges were replaced with an Oasis MCX LP extraction cartridge (500 mg), which was connected in series to the Extrelut-20 resin. The use of the MCX cartridge instead of the PRS and C_{18} cartridges permitted the recovery of all HAAs in one fraction (*28*) rather than separation of the polar and apolar HAAs in two fractions (*5*). As a result, only one analysis by HPLC/ESI-MS was required. The HAAs were eluted from the Extrelut-20 resin by washing with CH_2Cl_2/toluene (95:5) (70 mL) and adsorbed onto the MCX cartridge. The MCX cartridge was washed with 0.1 N HCl (5 mL), followed by CH_3OH (5 mL), and then the HAAs were eluted from the MCX cartridge with 5% NH_4OH in CH_3OH (5 mL). Samples were evaporated to dryness under a stream of nitrogen gas, followed by centrifugation in vacuo at 37 °C. The purified extracts were dissolved in mobile phase HPLC buffer (2 or 8 μL). A full description of the elution scheme and the method of sample concentration are provided in the Supporting Information.

Method Validation and Performance. The accuracy of the method was determined with fried meat (cooked at 150−180 °C, 10 min per side) or barbecued poultry (cooked at 230−300 °C, 10 min per side) that was spiked with 1 ng of the respective HAAs per gram of cooked meat. The accuracy of the method for the detection of HAAs was defined as the %[(estimated value − nominal value)/target value], where the nominal value was the concentration of HAAs found in cooked meats and the target value was the concentration of HAAs found in cooked meat plus spiked HAAs (1 ng/g of cooked meat). The recovery of HAAs was determined by adding 4 ng of isotopically labeled HAAs to the purified meat extracts (4 g) just prior to LC/ESI-MS analysis and comparing the response to samples where the standards were added prior to the extraction process. Ion suppression was assessed by tandem extraction of cooked meat (4 g) without the addition of any internal standards. The residues were resuspended in HPLC mobile phase buffer (30 μL) containing 4 ng of isotopically labeled internal standards. Ion suppression was determined by the diminution in signal of this HAA-fortified extract relative to the signal of pure standards. A volume of 2 μL of purified extract was assayed for all quantitative measurements. The confirmation of HAAs was achieved by the product ion scan mode or by monitoring several characteristic transition ions in the SRM scan mode (vide infra). In meat samples where there were low concentrations of HAAs, either the extracts were concentrated or 8 μL of extract was assayed.

Quantitation was done by comparison to a calibration curve prepared by injecting 266 pg of internal standards (2 μL) containing unlabeled HAAs at nine calibrant levels ranging from 2.66 to 4000 pg injected on column (the equivalent of 0.01−15 ppb of HAA in cooked meat). For grilled scrapings, for which the amounts of HAAs exceeded 15 ppb, the amount of extract analyzed was decreased (0.2 μL) so that the amount of signal measured was within the response of the calibration curve. Because isotopically labeled internal standards were not available for IQx, 7,9-DiMeI*g*Qx, and MeAαC, the labeled 3-[$^2H_3$C]-8-MeIQx, 1-[$^2H_3$C]-PhIP, and [$^{13}C_6$]-2-AαC were used as internal standards, respectively. The coefficient of determination (r^2) of all HAA calibration curves exceeded 0.998.

HAA Analysis in Cooked Meat by HPLC/ESI-MS/MS. The chromatography of HAAs was conducted with a capillary HPLC system from LC Packings/Dionex (Amsterdam, The Netherlands) and consisted of an UltiMate quaternary pump and a Famos autosampler. The HAAs were separated with an Aquasil C_{18} reversed-phase column (3 μm particle size, 1 × 150 mm) from Thermo Electron Corp. (Bellefonte, PA) with a precolumn (1 × 10 mm) containing C_8 reversed-phase resin from Supelco (Bellefonte, PA). The flow rate was set to 50 μL/min with a linear gradient over 20 min, starting from 0.1% HCO_2H to a solvent containing 90% CH_3CN/9.9% H_2O/0.1% HCO_2H.

HAA detection and quantitation were done by ESI-MS/MS with a Micromass/Waters Quattro Ultima triple-quadrupole mass spectrometer (Manchester, U.K.). Quantitative analysis was conducted in positive ionization mode using the SRM transitions $[M + H]^+ \rightarrow [M + H - 15]^{\bullet+}$ for IQ, IQ[4,5-*b*], IQx, 8-MeIQx, 4,8-DiMeIQx, 7,9-DiMeI*g*Qx, and PhIP (loss of $CH_3^{\bullet}$) and $[M + H]^+ \rightarrow [M + H - 18]^{\bullet+}$ (loss of $CD_3^{\bullet}$) for the respective trideuterated internal standards (*10*, *11*, *29*). For 2-AαC, 2-[$^{13}C_6$]-AαC, and MeAαC, the SRM transition was $[M + H]^+ \rightarrow [M + H - 44]^+$, which is attributed to the loss of NH_3 followed by HCN. The dwell time for each transition was set at 0.1 s. The capillary voltage was set at 3.5 kV, the cone voltage was set at 50 V, and hexapoles 1 and 2 were set at 18 and 1 V, respectively. The collision energy was optimized for each HAA with values between 29 and 32 eV. The source and desolvation temperatures were 120 and 350 °C, respectively. The N_2 cone gas flow rate was set at 95 L/h, and the desolvation gas flow rate was 500 L/h. Argon was used as the collision gas and set at a pressure of 2.5 mTorr. Product ion spectra of the analytes were obtained on the protonated molecules $[M + H]^+$ scanning from *m*/*z* 100 to 250 at a scan speed of 150 Da/s using the same acquisition parameters.

RESULTS

Identification of HAAs; Performance and Validation of the Analytical Method. The structures of the HAAs investigated in this study are presented in **Figure 1**. HAAs were readily detected in all cooked meat products, and typical SRM traces of the HPLC/ESI-MS/MS chromatograms showing the isotopically labeled internal standards and HAAs formed in barbecued chicken and grill scrapings of fried beef are presented in **Figure 2**. (SRM traces of the HPLC/ESI-MS/MS chromatograms of fried ground beef and beef extract are available as Supporting Information.) A number of known HAAs are present in the cooked chicken and scrapings of pan-fried beef. In addition, six other prominent peaks (labeled peaks 1−6) are also detected in cooked meats and pan-fried beef scrapings, which have the transition $[M + H \rightarrow M + H - CH_3^{\bullet}]^+$ common with IQ- and IQx-type structures (*10*, *11*, *30*). The characterization of these peaks by tandem mass spectrometry is discussed below.

The performance and validation of the analytical method was conducted with pan-fried ground beef patties and barbecued poultry because these matrcies are different, which may influence the performance of the method. All of these HAAs, which range from polar (IQ- and MeIQx-types) to apolar molecules (PhIP, 2-AαC, and MeAαC), were recovered from cooked beef and chicken at sufficient levels to estimate accurately the concentration of HAAs at <0.1 ppb (**Table 1**). Lower levels of recovery were noted for several of the apolar HAAs, which may be partially attributed to the resuspension of the purified extracts in mobile phase HPLC buffer (0.1% HCO_2H) rather than CH_3CN. The apolar HAAs were less soluble in mobile phase buffer than the polar HAAs and appeared to be retained to the glass wall of the capLC vials. Resuspension of the purified extract in CH_3CN resulted in higher recoveries of PhIP and 2-AαC derivatives, but injection of this organic solvent onto the HPLC column resulted in significant peak broadening and poor resolution, particularly for the most polar HAAs.

The estimates of HAA content, accuracy, precision (% RSD), and degree of ion suppression are reported in **Table 1**. The requirement for each HAA to be quantitated against its corresponding internal standards for accurate measurements was apparent for IQx, 7,9-DiMeI*g*Qx, and MeAαC, which were quantified using 3-[$^2H_3$C]-8-MeIQx, 1-[$^2H_3$C]-PhIP, and [$^{13}C_6$]-2AαC, respectively, as surrogate internal standards. The accuracies and precision of estimates of these HAAs are less consistent than those values obtained for the other HAAs that were measured with their corresponding internal standards (**Table 1**). The limit of quantitation (LOQ), assuming a signal-to-noise ratio (S/N) of 10 for pure standards, approaches 1 pg of HAA on column. However, the LOQ is increased in cooked meat samples because of the elevated and variable background noise of the different SRM transitions and the effects of ion suppression, which decreased the response of HAA signals by as much as 30−65%. The severity of the background noise and the diminution of the HAA signals were dependent upon the type of meat and method of cooking. Thus, reliable quantitation of HAAs in grilled meats required the detection of ∼5−10 pg of analyte, and the LOQ of HAAs in grilled meats approached 30 parts per trillion (ppt).

HAA Content in Cooked Meats, Grill Scrapings, and Poultry and Kinetics of HAA Formation in Grilled Beef. The estimated HAA content in cooked meat samples, percent loss of meat weight during cooking, and cooking parameters are reported in **Table 2**. The kinetics of HAA formation as a function of time was measured in ground beef fried at a temperature of 150−180 °C. After 3 min, the concentrations of HAAs formed were relatively modest, but increased during the time of cooking (**Figure 3**). The meat patties fried for 10 min

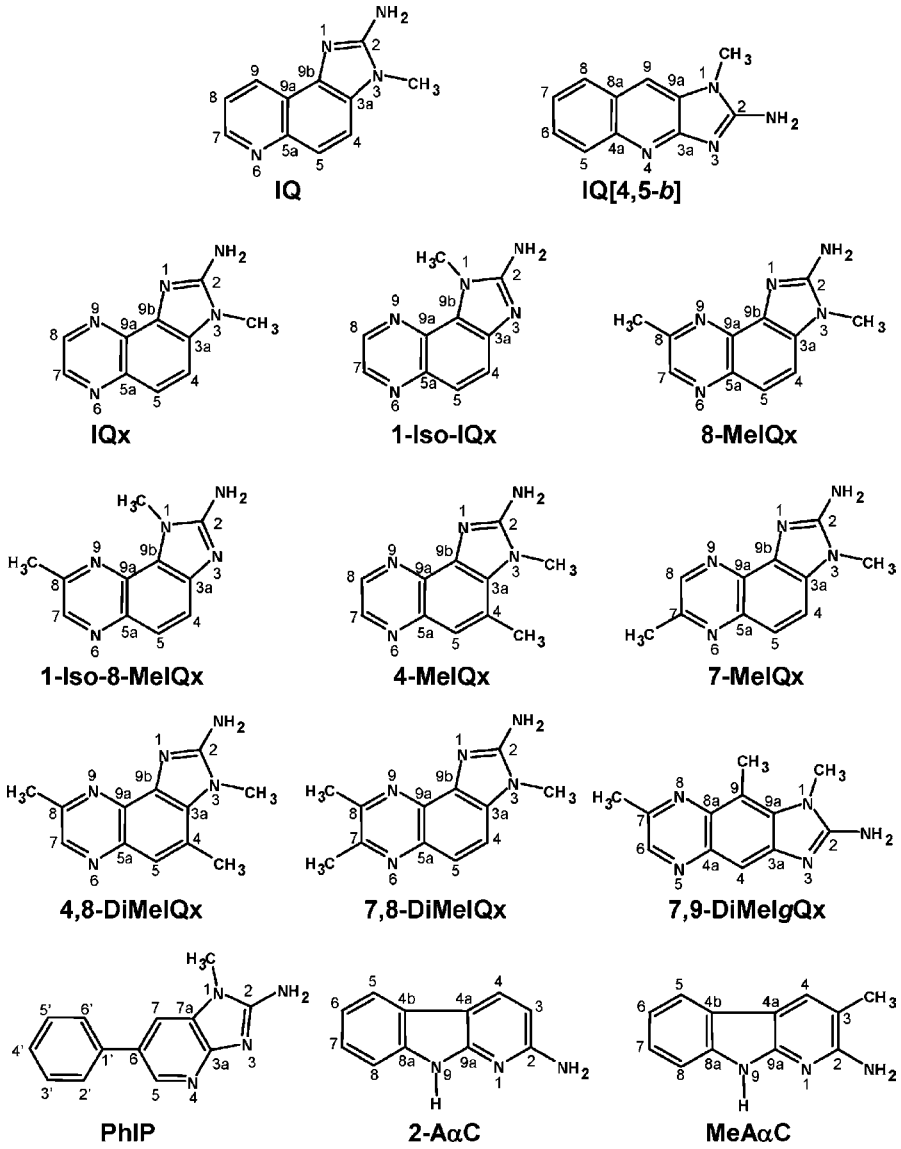


Figure 1. Chemical structures of HAAs investigated in this study.

Table 1. Summary of Performance of Analytical Method for HAA Determination in Cooked Meats (Picograms of HAA per Gram of Meat)[a]

	IQ	IQ[4,5-*b*]	IQx	8-MeIQx	4,8-DiMeIQx	7,9-DiMeIgQx	PhIP	2-AαC	MeAαC
					Fried Beef				
amount	<30	<30	115 ± 14	1450 ± 51	175 ± 20	94 ± 61	161 ± 10	<30	<30
spiked amount	1070	1070	734	2450	1210	1050	1200	1220	402
% accuracy	107	107	73	100	104	96	104	122	40
% RSD	3.1	5.1	8.3	2.7	6.5	15	15	19	10
% recovery	54 ± 24	20 ± 4	ND[b]	51 ± 19	43 ± 12	ND	31 ± 15	20 ± 12	ND
% ion suppression	49 ± 15	27 ± 9	ND	36 ± 18	35 ± 16	ND	23 ± 16	24 ± 22	ND
					Barbecued Chicken				
amount	118 ± 12	118 ± 18	<30	335 ± 65	276 ± 47	90 ± 26	9990 ± 61	8690 ± 1290	225 ± 82
spiked amount	1130	1270	538	1330	1250	1230	10900	9700	706
% accuracy	101	115	54	99	98	114	87	100	48
% RSD	8.5	9.5	30	4.5	4.2	10.2	2.3	16.5	28.3
% recovery	80 ± 18	67 ± 5	ND	50 ± 16	51 ± 8	ND	50 ± 12	34 ± 10	ND
% ion suppression	65 ± 7	27 ± 2	ND	61 ± 5	66 ± 3	ND	38 ± 9	24 ± 16	ND

[a] Values are estimates of HAA content in meat without and with spiking of HAAs (1000 pg/g of meat). Meat samples were cooked for 10 min at 150–180 °C per side and then assayed for HAA content. Average ± SD ($N = 6$ independent extractions). LOQ < 30 pg of HAA/g of meat. [b] Not determined.

were well-done but quite palatable. Under these cooking conditions, 8-MeIQx was the most prominent HAA, followed by a novel, previously unreported isomer of MeIQx (vide infra). Cooking beef at 300 °C resulted in higher concentrations of HAAs, and PhIP was the principal HAA (**Table 2**). Peaks were also detected in cooked meat products with SRM transitions at t_R values that corresponded to IQ, IQx, 7,8-DiMeIQx, IQ[4,5-*b*], 7,9-DiMeI*g*Qx, and the pyrolysis-type mutagens 2-AαC and MeAαC (**Figure 2**). The grill scrapings obtained from ground beef patties fried at 150−180 °C contained some of the highest

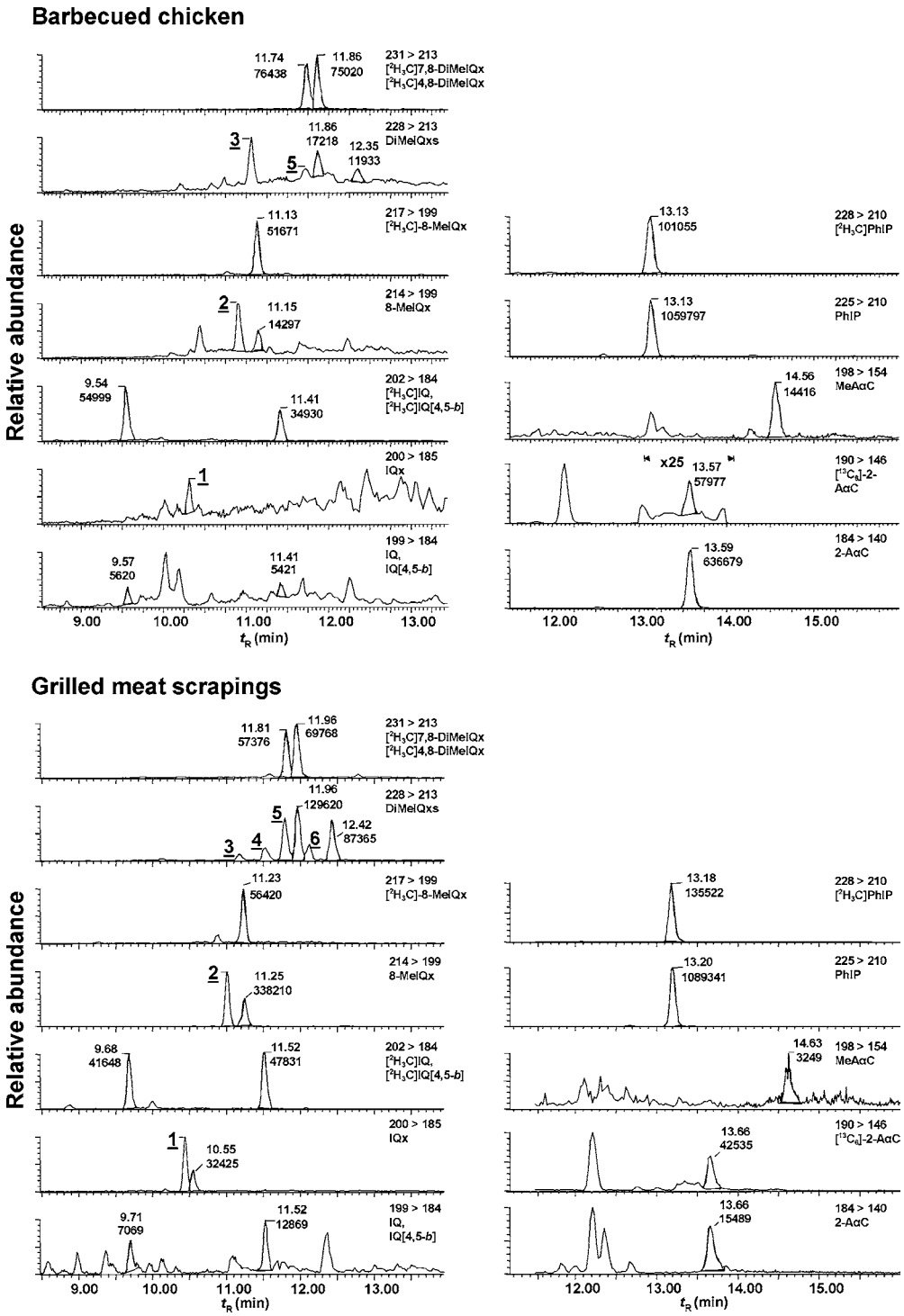


Figure 2. HPLC/ESI-MS/MS analysis of HAAs formed in barbecued chicken and scrapings of pan-fried meat. (Analyses of fried ground beef and beef extract are available as Supporting Information.) Transitions and approximate t_R values monitored are 199 > 184 IQ (9.54 min) and IQ[4,5-*b*] (11.41 min) and 202 > 184 3-[$^2H_3$]IQ and IQ[4,5-*b*]; 200 > 185 IQx (10.55 min); 214 > 199 8-MeIQx (11.15 min) and 217 > 199 3-[$^2H_3$]-8-MeIQx; 228 > 213 7,8-DiMeIQx (11.74 min), 4,8-DiMeIQx (11.86 min), 7,9-DiMe*Ig*Qx (12.35 min) and 231 > 213 3-[$^2H_3$C]-7,8-DiMeIQx, and 3-[$^2H_3$C]-4,8-DiMeIQx); 225 > 210 PhIP (13.13 min) and 228 > 210 1-[$^2H_3$]-PhIP; 184 > 140 2-AαC (13.59 min) and 190 > 146 2-[$^{13}C_6$]-AαC; 198 > 154 MeAαC (14.56 min). Peaks of known HAAs and labeled internal standards are shaded, and approximate t_R values and peak area are reported. Unshaded peaks labeled 1–6 appear to be isomeric HAA derivatives, and product ion spectra of the compounds are shown in **Figures 4** and **5**.

concentrations of IQ- and MeIQx-type HAAs, whereas the highest concentrations of the pyrolysis-type HAAs, 2-AαC and MeAαC, were found in barbecued beef and chicken that were cooked well-done at elevated temperatures, consistent with previous data (*5*, *11*, *16*). As previously reported (*12*), cooked, bacterial grade meat extract also contained relatively high concentrations of some HAAs.

Confirmation of Known HAAs and Characterization of Novel HAAs by the Full-Scan Product Ion Spectra Scan Mode. In regulatory MS, stringent criteria are required for confirmation of analyte identity. The acquisition of the full-scan electron impact or product ion spectrum is the method of choice, followed by a limited mass scan over the portion of the spectrum that contains the characteristic structural information

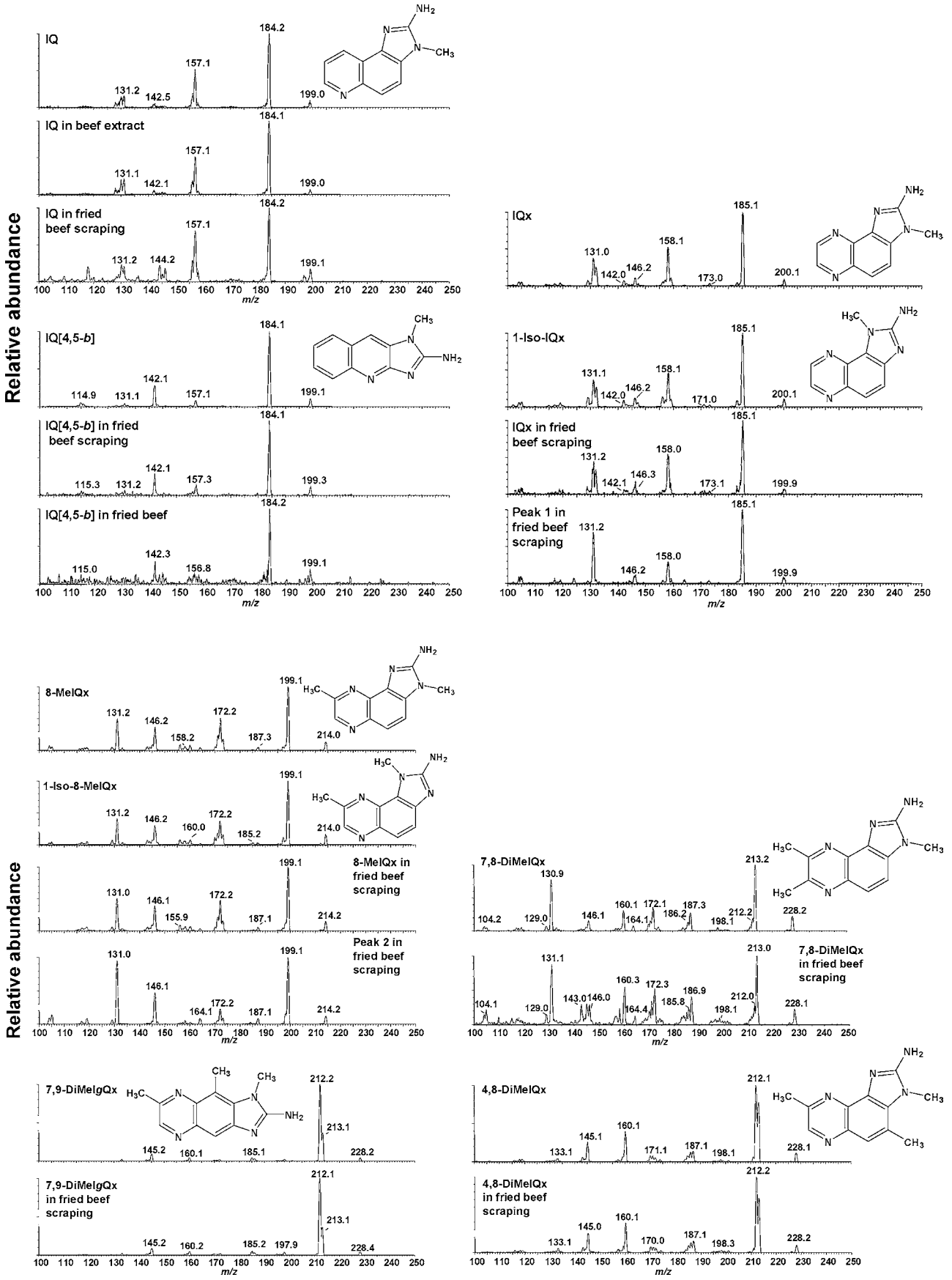
Table 2. Estimates of HAAs in Cooked Meats and Poultry (Picograms of HAA per Gram of Meat)[a]

type of cooked meat	IQ	IQ[4,5-*b*]	Iso-IQx (peak 1)	IQx	Iso-MeIQx (peak 2)	8-MeIQx	4,8-Di-MeIQx	7,9-Di-MeIgQx	PhIP	2-AαC	MeAαC	temp (°C)/ min per side	meat wt loss (%)
pan-fried beef 1	40 ± 8	31 ± 2	349 ± 21	257 ± 71	3790 ± 602	3720 ± 85	588 ± 32	549 ± 121	426 ± 27	<30	<30	190/12	53
pan-fried beef 2	260 ± 43	210 ± 48	1420 ± 288	390 ± 80	13800 ± 2230	5310 ± 715	1430 ± 345	1020 ± 345	15200 ± 2900	3320 ± 900	143 ± 60	300/6	32
pan-fried beef 3	<30	NA[c]	NA	115 ± 4	NA	1450 ± 51	175 ± 20	84 ± 61	161 ± 10	<30	<30	150–180/10	42
pan-fried beef scrapings[b]	1940 ± 95	3300 ± 404	12500 ± 2570	6380 ± 450	119000 ± 19400	62600 ± 3420	15000 ± 176	7670 ± 3690	82500 ± 560	2890 ± 596	757 ± 244	150–180	
barbecued beef 1	36 ± 5	46 ± 8	154 ± 39	<30	1180 ± 145	527 ± 69	119 ± 39	63 ± 16	2190 ± 304	2800 ± 188	88 ± 20	230–300/10	34
barbecued beef 2	129 ± 22	172 ± 8	1070 ± 80	200 ± 150	6490 ± 790	1600 ± 95	431 ± 17	569 ± 40	13900 ± 440	7750 ± 621	285 ± 170	230-300/10	51
beef extract	75200 ± 230	97 ± 9	5700 ± 168	3060 ± 192	27600 ± 814	37800 ± 611	6204 ± 280	10900 ± 2970	1820 ± 72	415 ± 29	<30	not known	
barbecued chicken	118 ± 12	NA	NA	<30	NA	335 ± 65	276 ± 47	90 ± 26	10000 ± 60	8700 ± 1290	225 ± 82	230–300/10	33

[a] Iso-IQx, peak 1; Iso-MeIQx, peak 2; 3-[$^2H_3$C]-8-MeIQx was used as an internal standard for estimations of both putative HAAs. Average ± SD (N = 3 or 6). LOQ < 30 pg of HAA/g of meat. [b] Pan-fried scrapings were collected from beef fried at 150–180 °C over 10 min from the kinetics study described in the caption of **Figure 3**. [c] Not assayed.

Figure 3. Kinetics of HAA formation over time in beef fried at 150–180 °C. Each time point represents three independent measurements of three fried beef patties combined prior to extraction of HAAs.

and then multiple ion or reaction monitoring when insufficient quantities of analyte is available, such as in trace analyses investigations (*31*). Many of the reported studies on the analyses of HAAs in cooked meats have used characteristic t_R values for peak identification and on-line UV spectral data for confirmation of analyte identity (*2*, *5*−*9*). In some investigations, the amounts of HAAs were below the LOD or interfering substances precluded the acquisition of spectral data to confirm the identities of the analytes. In this study, we confirmed the identities of HAAs in cooked meats (HAA content > 0.03 ppb) by monitoring several characteristic fragment ions in SRM scan mode (data not shown) and by acquisition of full-scan product ion spectra, which provided rich structural information (*10*, *29*) for confirmation of the identities of these analytes. The product ion spectra of known HAAs identified in cooked meats and grilled scrapings, putative HAAs peaks 1 and 2, and the spectra of reference compounds are presented in **Figure 4**. The fragment ions of the respective analytes closely match those observed in the product ion spectra of the reference compounds. The proposed mechanisms of fragmentation of these HAAs with triple-quadrupole MS and quadrupole ion trap MS systems have been previously reported (*10*, *24*, *29*). Investigations on 8-MeIQx, 3-[$^2H_3$]-8-MeIQx, and 2-[^{14}C]-8-MeIQx with a triple-quadrupole MS have shown that the most facile cleavage of MeIQx-type molecules occurs at the *N*-methylimidazole moiety to produce a $[M + H - CH_3^{\bullet}]^+$ radical species with many of the other fragment ions presumably derived from this radical species (*10*, *24*). Moreover, most of the secondary fragment ions occur through a breakage of the pyrazinyl ring and not the *N*-methylimidazole moiety, which remains intact under these CID conditions (*10*, *24*). For the pyrolysis HAA, 2-AαC, prominent fragment ions are observed in the product ion spectrum at *m/z* 185 $[M + H - NH_3 + H_2O]^+$, *m/z* 167 $[M + H - NH_3]^+$, and *m/z* 140 $[M + H - NH_3 - HCN]^+$. The same fragment ions are observed for the methylated homologue MeAαC; prominent fragment ions are observed at *m/z* 199 $[M + H - NH_3 + H_2O]^+$, *m/z* 181 $[M + H - NH_3]^+$, and *m/z* 154 $[M + H - NH_3 - HCN]^+$. Fragmentation also occurs at the methyl group to form the radical ion $[M + H - CH_3^{\bullet}]^+$ at *m/z* 183. Further fragmentation of the pyridinyl-amine ring is also observed for MeAαC. On the basis of precursor scan analyses and product ion spectra measurements at elevated in source CID conditions, the fragment ion at *m/z* 127 $[M + H - NH_3 - (HCN)_2]^+$ is derived from the fragment ions at *m/z* 154 $[M + H - NH_3 - HCN)]^+$, which is formed from the 3-methylpyridine[2,3-*b*]indole charged species at *m/z* 181 $[M + H - NH_3]^+$. The fragment ion at *m/z* 128 $[M + H - NH_3 - C_2H_2 - HCN]^+$ occurs through the intermediate fragment ion at *m/z* 155 $[M + H - NH_3 - C_2H_2]^+$, which is also derived from the 3-meth-


Relative abundance
IQ
IQ in beef extract
IQ in fried beef scraping
IQ[4,5-b]
IQ[4,5-b] in fried beef scraping
IQ[4,5-b] in fried beef
IQx
1-Iso-IQx
IQx in fried beef scraping
Peak 1 in fried beef scraping
8-MeIQx
1-Iso-8-MeIQx
8-MeIQx in fried beef scraping
Peak 2 in fried beef scraping
7,8-DiMeIQx
7,8-DiMeIQx in fried beef scraping
7,9-DiMeIgQx
7,9-DiMeIgQx in fried beef scraping
4,8-DiMeIQx
4,8-DiMeIQx in fried beef scraping
m/z

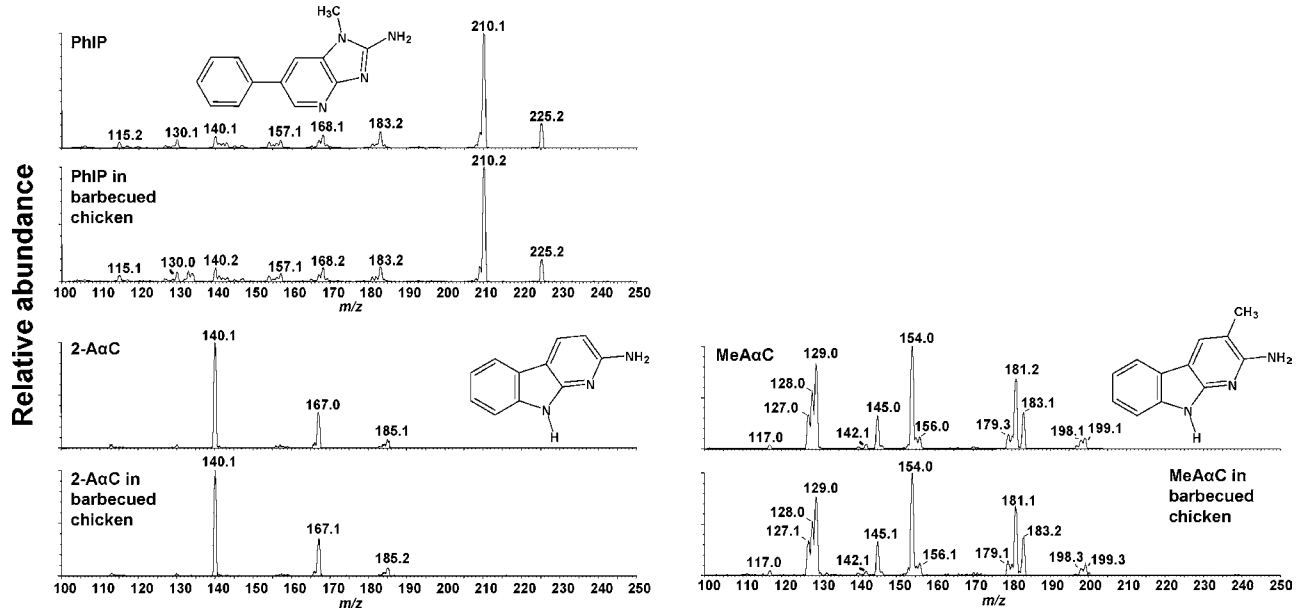


Figure 4. Product ion spectra $[M + H]^+$ of synthetic HAAs, analytes, and putative HAA. Peaks 1 and 2 formed in cooked meats and scrapings of meat fried at a surface temperature of 150–180 °C. For 2-AαC and MeAαC, water ion clusters $[M + H - NH_3 + H_2O]^+$ *(24, 29)* are observed in the spectra, at *m/z* 185 and 199, respectively.

ylpyridine[2,3-*b*]indole charged species at *m/z* 181. The secondary fragment ion at *m/z* 129 $[M + H - CH_3^{\bullet} - (HCN)_2]^+$ occurs through the demethylated charged radical species at *m/z* 183 $[M+H - CH_3^{\bullet}]^+$, whereas the fragment ion at *m/z* 144 is derived from the water cluster ion at *m/z* 199 $[M + H - NH_3 + H_2O]^+$.

The power of the product ion scan mode for the identification and characterization of HAAs in complex cooked food matrices is illustrated for 7,8-DiMeIQx in grilled meat scrapings (**Figure 2**). An interfering peak (peak 5) eluted 3 s prior to the t_R of 3-[$^2H_3$]-7,8-DiMeIQx (11.81 min), and the unknown analyte and unlabeled 7,8-DiMeIQx were not resolved. The product ion spectrum of 7,8-DiMeIQx was acquired at the tailing portion of peak 5. The pattern of the background-subtracted spectrum matches the spectrum of the reference 7,8-DiMeIQx compound with some minor interfering peaks attributed in part to peak 5 (vide infra). Accurate measurements on the concentration of 7,8-DiMeIQx in cooked meats were not possible because of the coelution of 7,8-DiMeIQx with peak 5 and the common SRM transition, which precluded separation of the respective analytes by different mass transitions. The use of the product ion scan mode was essential to identify 7,8-DiMeIQx and to demonstrate the presence of two compounds in peak 5.

Two prominent analytes, labeled peaks 1 and 2 that elute at t_R values of 10.44 and 11.01 min in grilled meat scrapings (**Figure 2**), were observed to have the same nominal masses as protonated IQx (*m/z* 200) and 8-MeIQx (*m/z* 214), respectively, and eluted just prior to these known HAAs. The product ion spectra of peaks 1 and 2 are presented in **Figure 4**, along with those of IQx and 8-MeIQx isolated from cooked meats, the synthetic reference compounds, and the synthesized *N*1-CH_3 isomers of IQx and 8-MeIQx. The base peak fragment ions observed at *m/z* 185 for IQx, 1-Iso-IQx, and peak 1 and at *m/z* 199 for 8-MeIQx, 1-Iso-8-MeIQx, and peak 2 can be attributed to cleavage of the *N*-methylimidazole group $[M + H - CH_3^{\bullet}]^+$ by analogy with the reference HAA compounds. The subsequent loss of two HCN moieties from the pyrazinyl ring of the IQx- and 1-Iso-IQx-*N*-demethyl radical species results in product ions at *m/z* 158 $[M + H - CH_3^{\bullet} - HCN]^+$ and *m/z* 131 $[M + H - CH_3^{\bullet} - (HCN)_2]^+$ (*10*, *24*). These same types of fragment ions are also observed in the spectrum of peak 1. In the case of the protonated 8-MeIQx- and 1-Iso-8-MeIQx-*N*-demethyl radical species, further fragmentation occurs principally at the pyrazinyl ring of the MeIQx skeleton to form product ions at *m/z* 172 $[M + H - CH_3^{\bullet} - HCN]^+$, *m/z* 158 $[M + H - CH_3^{\bullet} - CH_3CN]^+$, *m/z* 146 $[M + H - CH_3^{\bullet} - C_3H_3N]^+$, and *m/z* 131 $[M + H - CH_3^{\bullet} - HCN - CH_3CN]^+$ (*10*, *24*, *29*). A number of these product ions are also observed in the spectrum of peak 2. The similarities in these product ion spectra suggest that the chemical structures of these analytes are related to IQx-type HAAs. We initially postulated that peaks 1 and 2 may be the *N*1-methyl isomers of IQx and 8-MeIQx. However, both synthetic 1-Iso-IQx and 1-Iso-8-MeIQx derivatives eluted at different t_R values from the respective analytes in cooked meats, excluding these isomers as plausible structures. The 4-MeIQx and 7-MeIQx isomers were other plausible structures for peak 2; however, both compounds also had different t_R values compared to the analyte in cooked meats. Linear tricyclic ring isomers of IQx and 8-MeIQx are possible structures for peaks 1 and 2. Structural elucidation by NMR spectroscopy is required after large-scale isolation of these molecules from cooked meats.

Four other peaks (peaks 3−6) elute at t_R values between 11.15 and 12.15 min (fried meat scrapings, **Figure 2**), with protonated ions $[M + H]^+$ at *m/z* 228, which correspond to the nominal mass of protonated DiMeIQx derivatives ($C_{11}H_{10}N_5$, 227.1). The full-scan product ion spectra of peaks 3−6 are presented in **Figure 5**, and the spectra resemble those of known HAAs as well. The spectra of peaks 3, 5, and 6 display prominent fragment ions at *m/z* 213 and 212, which are attributed to $[M + H - CH_3^{\bullet}]^+$ and $[M + H - CH_4]^+$. The fragment ion at *m/z* 212 is detected prominently in MeIQx derivatives that contain a methyl group attached to C-4 but not the C-7 or C-8 atoms of the heterocyclic skeleton (*10*, *24*, *29*). The fragment ion at *m/z* 212 is postulated to occur through loss of $CH_3^{\bullet}$ from the *N*3 atom of the MeIQx molecule, followed by the preferential loss of $H^{\bullet}$ from the 4-methyl group, which is *epi* to the nitrogen radical (or loss of $CH_3^{\bullet}$ from the *N*1 atom of the linear tricyclic ring 7,9-DiMeIgQx derivative, followed by loss of $H^{\bullet}$ from the 9-methyl group) (*10*, *24*, *29*). However, peaks 3, 5, and 6 undergo less fragmentation than angular tricyclic ring MeIQx homologues under these CID conditions, and the spectra more

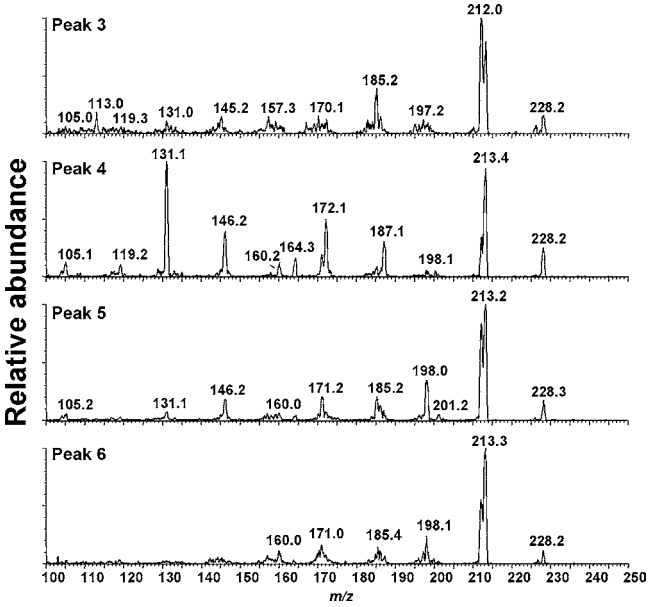


Figure 5. Product ion spectra $[M + H]^+$ of putative HAA peaks 3–6 isolated from fried meat scrapings.

closely resemble those of linear type ring-structured HAAs, such as 7,9-DiMeI*g*Qx or PhIP, which undergo less fragmentation under these CID conditions. In contrast to these compounds, peak 4 undergoes extensive fragmentation under these CID conditions, and the product ion spectrum resembles those of the angular tricyclic ring MeIQx derivatives. The small contribution of the fragment ion at *m*/*z* 212 $[M + H - CH_4]^+$ and the prominent fragment ion at *m*/*z* 131 $[M + H - CH_3^{\bullet} - C_4N_2H_6]^+$ in the product ion spectrum of peak 4 are also noted in product ion spectra of MeIQx and DiMeIQx derivatives containing a CH_3 group attached to the C-7 or C-8 atoms but not the C-4 or C-5 atoms of the heterocyclic skeleton (*10*, *24*). The product ion spectra of these analytes suggest that previously unreported putative HAAs of both angular and linear tricyclic ring structures are present in cooked meats.

DISCUSSION

The different scan modes of triple-quadrupole MS have been employed for the quantitation of carcinogenic HAAs and the detection of unknown putative HAAs in cooked meats (*10*, *11*, *23*) and in urine of humans following consumption of cooked meats (*24*). In this study, we used the highly sensitive SRM scan mode for the detection and quantitation of 10 known HAAs in cooked meats with a LOQ of <1 ppb. The product ion scan mode was used to corroborate the identities of these HAAs and to characterize unknown analytes that appear to contain related HAA ring structures. Both HPLC/ESI-MS/MS (*11*, *21*, *23*, *29*) and GC/MS (*18*, *19*) analyses of HAAs have provided structural information about these analytes that reinforces existing data on HAA estimates in cooked meats obtained by HPLC-UV detection, particularly at the low parts per billion level, where UV detection was not sufficiently sensitive to acquire characteristic spectra for confirmation of the analytes' identities (*5*, *8*, *9*, *32*). We confirmed the identities of the HAAs 8-MeIQx, 4,8-DiMeIQx, and PhIP that have been previously reported in cooked meats (*2*, *9*−*12*, *21*) by acquistion of the analytes' product ion specta. 7,8-DiMeIQx, IQ, and IQx, which have rarely been reported in cooked beef because the amounts are below the LOD when analyzed by HPLC-UV detection (*9*), were also identified. The pyrolysis-type HAAs, 2-AαC and MeAαC, were also identified in fried ground beef; however, these compounds were most prominent in well-done barbecued beef and poultry prepared under high temperatures (225−300 °C). Some of the highest concentrations of HAAs were detected in scrapings of fried meat, which are commonly consumed with meat as gravy, and consistent with previous findings (*10*, *24*, *29*). The presence and relative amounts of many of these HAAs measured in beef and poultry prepared under different types of cooking practices (pan-fried vs barbecue) and temperatures are largely consistent with estimates reported in previous investigations (*2*, *5*−*11*, *16*).

The linear tricyclic ring HAAs, IQ[4,5-*b*] and 7,9-DiMeI*g*Qx, were also detected at appreciable levels in cooked meats. This is the first report on the identification of IQ[4,5-*b*] and 7,9-DiMeI*g*Qx in beef and poultry cooked under common household conditions; 7,9-DiMeI*g*Qx has been previously identified in cooked beef extract (*12*). IQ[4,5-*b*] is synthesized by reaction of creatinine with 2-aminobenzaldehyde at elevated temperature (*25*), which may be the route of formation of this HAA in cooked meats. Recently, another structurally related linear tricyclic ring HAA, 2-amino-(1,6-dimethylfuro[3,2-*e*]imidazo-[4,5-*b*])pyridine, was identified in cooked meats (*33*), suggesting that other HAAs with linear ring structures may be present in cooked meats.

In this study, we also detected six novel analytes that appear to contain the IQx heterocyclic skeleton on the basis of the product ion spectra of the protonated molecules. The two most prevalent compounds (peaks 1 and 2) have the same nominal molecular weights as IQx and 8-MeIQx, respectively. The *N*1-CH_3 isomers of IQx and 8-MeIQx were excluded as structures on the basis of differences in t_R values and product ion spectra. 4-MeIQx and 7-MeIQx were also excluded as plausible structures for the 8-MeIQx isomer on the basis of these criteria. The accurate mass measurements of the protonated ions of peak 2 and 8-MeIQx in grilled meats were observed at *m*/*z* 214.108 and at *m*/*z* 214.109 for synthetic 8-MeIQx (data not shown, calculated $C_{11}H_{12}N_5$ $[M + H]^+$ *m*/*z* 214.1087) (*24*). The same principal fragment ions in the product ion spectra of these grilled meat derivatives were within 2 millimass units of each other and within 2 millimass units of those corresponding fragment ions of synthetic 8-MeIQx (*24*). Thus, peak 2 is an isomer of 8-MeIQx, and peak 1 is a probable isomer of IQx. The other four remaining analytes (peaks 3−6) are likely to be isomers of DiMeIQx; both angular tricyclic ring and linear tricyclic ring structures are suggested on the basis of the respective product ion spectra. The assignments of the nitrogen atoms within the heterocyclic rings, the ring junctions, and the sites of methylation will require large-scale purification of these novel HAAs from cooked meats in sufficient amounts for analysis by NMR spectroscopy. Some of these unknown HAAs are present in grilled meat scrapings at concentrations >100 ppb. The high concentrations of these compounds in grilled meat scrapings, which are devoid of the bulk mass of the cooked meats, may facilitate their isolation and purification for further spectroscopic analyses.

Early investigations on the isolation and identification of HAAs from grilled meats used chromatographic separations that required bacterial mutagenicity assays as a means of monitoring biologically active fractions (*12*, *17*). Because the mutagenic potency of HAAs varies by >10000-fold in bacterial assays (*34*), only HAAs of high mutagenic activity or HAAs found in large abundance were detected (*12*, *17*), whereas HAAs of moderate genotoxic potency or present in low abundance may have remained undetected. The known HAAs estimated in some

meats that were cooked well-done were reported to account for <30% of the total mutagenic activity (*17*, *32*). The novel HAAs detected in this current study may be responsible for some of these uncharacterized mutagens in cooked meats. Interestingly, the extracted LC/MS ion chromatograms of HAAs in cooked beef and bacon strips following purification by tandem solid-phase extraction revealed a prominent analyte at *m*/*z* 214 (the nominal molecular weight of protonated 8-MeIQx) that also eluted immediately prior to 8-MeIQx (*6*, *22*). However, those analyses were conducted in the SIM scan mode, and the product ion spectrum of the peak was not acquired. That analyte may be the newly discovered isomer of 8-MeIQx that we have described in this study.

Minor changes in chemical structure can profoundly influence the biological activities and potencies of HAAs in mutagenicity assays (*35*, *36*). However, the differences in carcinogenic potency of a number of HAAs of diverse chemical structures tested thus far in rodent bioassays are within 10-fold (*1*, *34*). Because HAAs in grilled meats are believed to contribute to the etiology of several common types of human cancers such as colon, prostate, and breast (*1*), it is important to elucidate the structures of these new compounds and assess their genotoxic properties.

ABBREVIATIONS USED

2-AαC, 2-amino-9*H*-pyrido[2,3-*b*]indole; MeAαC, 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole; 1-Iso-8-MeIQx, 2-amino-1,8-dimethylimidazo[4,5-*f*]quinoxaline; 4-MeIQx, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoxaline; 7-MeIQx, 2-amino-3,7-dimethylimidazo[4,5-*f*]quinoxaline; 8-MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; IQx, 2-amino-3-methylimidazo[4,5-*f*]quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; 7,9-DiMeI*g*Qx, 2-amino-1,7,9-trimethylimidazo[4,5-*g*]quinoxaline; 4,8-DiMeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline; 7,8-DiMeIQx, 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline; CID, collision-induced dissociation conditions; CNL, constant neutral loss; DEP/EI-MS, direct exposure probe/electron ionization mass spectrometry; ESI-MS/MS, electrospray ionization tandem mass spectrometry; GC/NICI-MS, gas chromatography/negative ion chemical ionization mass spectrometry; HAAs, heterocyclic aromatic amines; LOD, limit of detection; LOQ, limit of quantitation; MCX, mixed mode cation resin/C18 reversed phase resin; ppt, parts per trillion; NOESY, two-dimensional nuclear Overhauser exchange spectroscopy; NOEs, nuclear Overhauser effects; SIM, selected ion monitoring; SRM, selected reaction monitoring.

Supporting Information Available: Description of the spectroscopic conditions of analyses of synthetic intermediates of 1-Iso-IQx and 1-Iso-8-MeIQx, and tandem solid phase isolation procedure of HAAs from cooked meats and grill scrapings. This material is available free of charge via the Internet at http://pubs.acs.org.

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