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Quantitation of 13 Heterocyclic Aromatic Amines in Cooked Beef, Pork, and Chicken by Liquid Chromatography−Electrospray Ionization/Tandem Mass Spectrometry

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The concentrations of heterocyclic aromatic amines (HAAs) were determined, by liquid chromatography−electrospray ionization/tandem mass spectrometry (LC-ESI-MS/MS), in 26 samples of beef, pork, and chicken cooked to various levels of doneness. The HAAs identified were 2-amino-3-methylimidazo[4,5-*f*]quinoline, 2-amino-1-methylimidazo[4,5-*b*]quinoline, 2-amino-1-methylimidazo[4,5-*g*]quinoxaline (I*g*Qx), 2-amino-3-methylimidazo[4,5-*f*]quinoxaline, 2-amino-1,7-dimethylimidazo[4,5-*g*]quinoxaline (7-MeI*g*Qx), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, 2-amino-1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*]pyridine, 2-amino-1,6,7-trimethylimidazo[4,5-*g*]quinoxaline, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline, 2-amino-1,7,9-trimethylimidazo[4,5-*g*]quinoxaline, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-9*H*-pyrido[2,3-*b*]indole, and 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole. The concentrations of these compounds ranged from <0.03 to 305 parts per billion (micrograms per kilogram). PhIP was the most abundant HAA formed in very well done barbecued chicken (up to 305 μg/kg), broiled bacon (16 μg/kg), and pan-fried bacon (4.9 μg/kg). 7-MeI*g*Qx was the most abundant HAA formed in very well done pan-fried beef and steak, and in beef gravy, at concentrations up to 30 μg/kg. Several other linear tricyclic ring HAAs containing the I*g*Qx skeleton are formed at concentrations in cooked meats that are relatively high in comparison to the concentrations of their angular tricyclic ring isomers, the latter of which are known experimental animal carcinogens and potential human carcinogens. The toxicological properties of these recently discovered I*g*Qx derivatives warrant further investigation and assessment.

KEYWORDS: Heterocyclic aromatic amines; food mutagens; food carcinogens; LC-ESI-MS/MS

INTRODUCTION

Heterocyclic aromatic amines (HAAs) are potent bacterial mutagens, and carcinogens in experimental animals, that occur in well-done grilled meats (*1, 2*). Since the discovery of HAAs three decades ago (*3*), more than 20 HAAs have been identified in cooked meats (*4*). Several HAAs, including 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC) and 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), also arise in tobacco smoke condensate and diesel exhaust (*5, 6*). HAAs in high doses induce tumors at multiple sites of rodents during long-term feeding studies: included are tumors of the liver, colorectum, prostate, and female mammary gland (*4*). A number of these tissues in humans are capable of bioactivation of HAAs to genotoxic species (*7*). Moreover, several epidemiological studies have reported that frequent consumption of well-done cooked beef containing HAAs leads to an increased risk of development of cancers in these tissues (*8, 9*). These observations have led to the hypothesis that HAAs play a causal role in colorectal and possibly other common forms of human cancers (*10–12*).

The concentrations of HAAs in cooked meats can vary by more than 100-fold range. The amounts of HAAs formed are dependent upon the type of meat, method of cooking, and the temperature and duration of cooking (*1, 2*). Quantitative measurements of HAAs in foods are essential, if we are to accurately estimate exposures and human cancer risks (*11*). Many studies reported on the estimates of HAAs in foods

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employed HPLC coupled with UV photodiode array or fluorescence detection for measurement (*13*). This approach has been successfully used to estimate the concentrations of known HAAs in a variety of cooked meats. However, the known HAAs have been reported to account for only about 30% of the total mutagenicity attributed to this class of genotoxicants in well-done cooked beef (*1, 14*). We recently employed liquid chromatography−electrospray ionization/tandem mass spectrometry (LC-ESI-MS/MS) to characterize HAAs in urine of meat eaters; in that study, we discovered 2-amino-1,7-dimethylimidazo[4,5-*g*]quinoxaline (7-MeI*g*Qx) (*15, 16*), an isomer of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (8-MeIQx), which is a potent bacterial mutagen and rodent carcinogen (*4*). Thereafter, we identified 7-MeI*g*Qx in fried ground beef patties and also characterized, by LC-ESI-MS/MS, other putative HAAs that contain an imidazoquinoxaline skeleton (*15, 17*). Thus, a number of previously uncharacterized HAAs of undetermined genotoxic potential are shown to be present in cooked meats.

A food frequency questionnaire (FFQ) has been devised to assess exposure to HAAs and other genotoxicants in cooked meats and these toxicants' potential causal role in dietary-linked cancers (*11*). A meat-cooking practices module and a database were established to provide estimates on the amounts of 8-MeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), and PhIP, three of the principal HAAs formed in various types of meat cooked by a number of methods and to varying degrees of doneness. Photographs depicting these meat samples were incorporated into the FFQ (*18, 19*) to serve as a means to gauge an individual's preference of meat "doneness" and to estimate the HAA intake. Our recent discovery of 7-MeI*g*Qx and other previously unreported HAAs (*15–17*) prompted us to reexamine several of the commonly eaten meat staples listed in this FFQ to determine the concentrations of 7-MeI*g*Qx and other presumed HAAs containing the 2-amino-1-methylimidazo[4,5-*g*]quinoxaline (I*g*Qx) structure, relative to the amounts of known carcinogenic HAAs formed in these foods. Our findings demonstrate that 7-MeI*g*Qx is one of the most abundant HAAs formed in cooked meat sample types of this database. Several other HAAs containing the I*g*Qx skeleton (*17, 20*) also occur in cooked meats and contribute to the daily burden of HAA exposure. Studies on the toxicological properties of 7-MeI*g*Qx and other linear tricyclic ring HAAs are warranted.

EXPERIMENTAL PROCEDURES

Caution. Heterocyclic aromatic amines are carcinogenic. They should be handled in a well-ventilated hood with extreme care and use of appropriate protective clothing and equipment.

Chemicals. The following chemicals were purchased from Toronto Research Chemicals (Downsview, Ontario, Canada): 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3-methylimidazo[4,5-*f*]quinoline (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); 8-MeIQx, 3-[$^2H_3$C]-8-MeIQx (isotopic purity ~96%); PhIP and trideuterated 1-[$^2H_3$C]-PhIP (isotopic purity >99%); AαC; MeAαC; and 5-chloro-4-nitrobenzo[1,2,5]-selenadiazole. [4b,5,6,7,8,8a-$^{13}C_6$]-2-AαC (isotopic purity >99%) was a kind gift from Dr. D. Doerge, NCTR, Jefferson, AR. 2-Amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), trideuterated 3-[$^2H_3$C]-4,8-DiMeIQx (isotopic purity >99%), and 3-[$^2H_3$C]-IQx (isotopic purity >99%) were synthesized as previously described (*17, 21*). Trideuterated 1-[$^2H_3$C]-IQ[4,5-*b*] was synthesized from 1-[$^2H_3$C]creatinine (>99% isotopic purity) and 2-aminobenzaldehyde (*22, 23*). 7-MeI*g*Qx and trideuterated 1-[$^2H_3$C]-7-MeI*g*Qx (>99% isotopic purity) were synthesized as previously described with [$^2H_3$C]I used for the methylation step, instead of CH_3I (*15*). 2-Amino-1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*]pyridine (IFP) was kindly provided by Dr. M. Knize, Lawrence Livermore National Laboratory, Livermore, CA. 2-Amino-1,7,9-trimethylimidazo[4,5-*g*]quinoxaline (7,9-DiMeI*g*Qx) was kindly provided by Dr. K. Wakabayashi, National Cancer Center Research Institute, Tokyo, Japan. CNBr, pyruvaldehyde (40%), hydrazine hydrate (50% v/v), 1-[$^2H_3$C]creatinine, [$^2H_3$C]I, [$^2H_3$C]-NH_2-HCl (all >99% isotopic purity), and palladium/carbon (10%) were purchased from Sigma-Aldrich (Milwaukee, WI). 4-Fluoro-5-nitrobenzene-1,2-diamine was purchased from Maybridge through Ryan Scientific Inc. (Isle of Palms, SC). Oasis MCX LP extraction (500 mg) and borosilicate glass total recovery capLC vials were purchased from Waters (Milford, MA). Extrelut-20 resin was obtained from EMD Chemicals (Gibbs Town, NJ). A Baker C_{18} cartridge (1 g) and Whatman glass-backed silica preparative TLC plates (1000 μm) were purchased through Krackeler Scientific, Inc. (Albany, NY).

General Methods. Mass spectra of synthetic derivatives were obtained on a Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Electron, San Jose, CA). Typical instrument tuning parameters used were as follows: source spray voltage 3.0 kV, N_2 sheath gas setting 10, auxiliary gas setting 0, capillary temperature 350 °C, and tube lens offset 80; the in-source collision-induced dissociation (CID) offset was 10 V. The collision energy was set at 28 V for all compounds in the MS/MS scan mode. Argon, set at 1.5 mTorr, was used as the collision gas. Full-scan or product ion spectra (100−300 Da) were acquired with these MS parameters at a scan rate of 200 Da/s. Analyses were conducted in the positive ionization mode. 1D NMR spectra were collected on a Bruker 500 MHz DRX spectrometer (Bruker BioSpin Corp., Billerica, MA) using solutions in DMSO-d_6. 2D spectra were collected on a Bruker 600 MHz DRX Spectrometer, equipped with a cryoprobe. HPLC separations were done with an Agilent 1100 HPLC system equipped with a photodiode array detector (Palo Alto, CA) equipped with a Rheodyne 7725i (Rhonert Park, CA) manual injector. The accurate mass measurement data of the products of syntheses were obtained at the Mass Spectrometry Center in the School of Chemical Sciences, University of Illinois, Urbana–Champaign. The MS instrument was a Q-tof Ultima API (Waters Corp., Milford, MA).

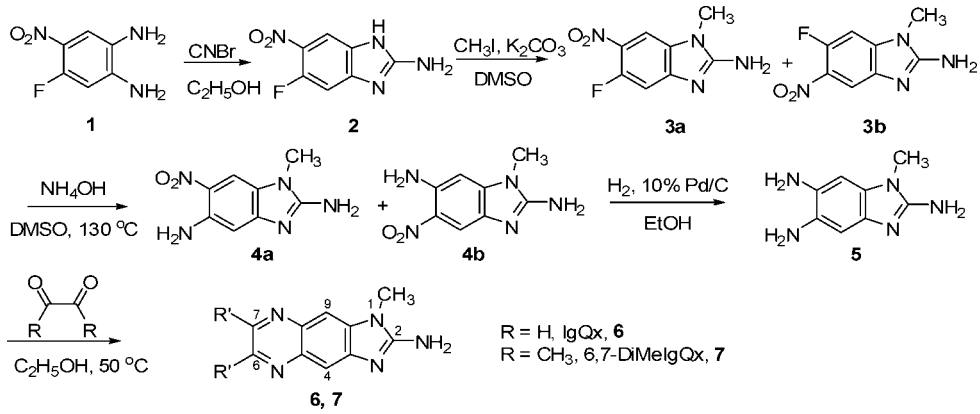
Syntheses. *2-Amino-1-methylimidazo[4,5-g]quinoxaline (IgQx) and 2-Amino-1,6,7-trimethylimidazo[4,5-g]quinoxaline (6,7-DiMeIgQx).* The syntheses of I*g*Qx, 6,7-DiMeI*g*Qx, and their trideuterated internal standards were done by modification of a method described for the synthesis of 7-MeI*g*Qx (**Scheme 1**) (*15*).

*5-Fluoro-6-nitro-1H-benzimidazole-2-amine (**2**).* CNBr treatment of the commercially available 4-fluoro-5-nitrobenzene-1,2-diamine (**1**) gave a 94% yield of 5-fluoro-6-nitro-1*H*-benzimidazole-2-amine (**2**), as previously reported (*15*).

*5-Fluoro-1-methyl-6-nitro-1H-benzo[d]imidazole-2-amine (**3a**) and 6-Fluoro-1-methyl-5-nitro-1H-benzo[d]imidazole-2-amine (**3b**).* Compound **2** (75 mg, 0.38 mmol) in anhydrous DMSO (4 mL), containing anhydrous K_2CO_3 (330 mg, 3.06 mmol), was methylated by the addition of CH_3I (27 μL, 0.42 mmol) over 10 min with stirring. The reaction was continued overnight at room temperature with stirring. Then, water (50 mL) was added, and the mixture was applied to a Baker C_{18} SPE cartridge (1 g). The cartridge was washed with water, and the desired product was eluted with CH_3OH. The eluent was concentrated, and the residue was purified by flash chromatograpy with $CH_3OH-CH_2Cl_2$ (starting from 1:100 to 1:30 with 0.5% NH_4OH) to give a light yellow solid mixture of **3a** and **3b** (38 mg, 0.18 mmol, 48%). Major species (tentatively **3a**): ^{1}H NMR (DMSO-d_6) δ 7.99 (d, J_{HF} = 6.8 Hz, 1H, H-7), 7.41 (br, 2H, 2-NH_2), 7.13 (d, J_{HF} = 13.1 Hz, 1H, H-4), 3.60 (s, 3H, *N*1-CH_3). Minor species (tentatively **3b**): ^{1}H NMR (DMSO-d_6) δ 7.81 (d, J = 6.7 Hz, 1H, H-4), 7.44 (d, J_{HF} = 12.1 Hz, 1H, H-7), 7.05 (br, 2H, 2-NH_2), 3.60 (s, 3H, *N*1-CH_3). HRMS ESI-MS *m/z* for $[M + H]^+$: $C_8H_8FN_4O_2$, calculated, 211.0631; found, 211.0628.

*1-Methyl-6-nitro-1H-benzo[d]imidazole-2,5-diamine (**4a**) and 1-Methyl-5-nitro-1H-benzo[d]imidazole-2,6-diamine (**4b**).* The substituted amines **4a** and **4b** were obtained by treatment of a mixture of the isomers **3a** and **3b** (8 mg, 0.038 mmol) in DMSO (1.2 mL) with NH_4OH (26% v/v) (0.4 mL) for 5 days at 130 °C. After the mixture was cooled, water (100 mL) was added, and the solution was applied to a Baker C_{18} SPE cartridge (1 g). The product was collected as described above.

Scheme 1. Chemical Synthesis of IgQx and 6,7-DiMeIgQx



The eluent was concentrated and purified by preparative TLC to give an isomeric mixture of **4a** and **4b** (5.7 mg, 0.028 mmol, 74%). In order to distinguish the two isomers, homonuclear ROESY experiments were conducted to identify through-space dipolar interactions between protons in spatial proximity. ROE cross-peaks were observed between the *N*1-CH_3 signal and the 2-NH_2 and H-7 resonances for both **4a** and **4b**. The identification of the phenyl NH_2 signal was independently verified by an ROE cross-peak to H-7 in compound **4a** and to H-4 in compound **4b**. ^{1}H NMR (DMSO-d_6): **4a**, δ 7.589 (s, 1H, H-7), 7.22 (br, 2H, 2-NH_2), 7.06 (br, 2H, 5-NH_2), 6.49 (s, 1H, H-4), 3.41 (s, 3H, *N*1-CH_3); **4b**, δ 7.593 (s, 1H, H-4), 7.13, (br, 2H, 6-NH_2), 6.749, (br, 2H, 2-NH_2), 6.494 (s, 1H, H-7), 3.38 (s, 3H, *N*1-CH_3). HRMS ESI-MS *m*/*z* for $[M + H]^+$: $C_8H_{10}N_5O_2$, calculated, 208.0834; found, 208.0826.

*2-Amino-1-methylimidazo[4,5-g]quinoxaline (**6**) and 2-Amino-1,6,7-trimethylimidazo[4,5-g]quinoxaline (**7**).* A mixture of **4a** and **4b** isomers (2 × 20 mg, 0.097 mmol) was added to two tubes of ethanol (6 mL) and reduced with H_2 at atmospheric pressure, using 10% Pd/C (10 mg) as a catalyst. The reaction mixtures were stirred for 1.5 h to give the unstable triamine, 1-methyl-1*H*-benzo[*d*]imidazole-2,5,6-triamine (**5**) (100% yield, on the basis of LC-ESI-MS infusion $[M + H]^+$ at *m*/*z* 178.1). After filtration of the reaction mixtures through a Celite pad, the filtrates were concentrated *in vacuo* to ≈2 mL, followed by the addition of glyoxal (40%, 12.5 μL, 0.11 mmol) or 2,3-butanedione (97%, 9.6 μL, 0.11 mmol). The solutions were heated to 50 °C for 1 h. After concentration, the residues were purified by a preparative silica TLC plate using 15% CH_3OH in CH_2Cl_2 in the presence of 0.5% NH_4OH to give compound **6** (16 mg, 0.080, 82%) and compound **7**(21 mg, 0.092 mmol, 95%), respectively. Compound **6**: ^{1}H NMR (DMSO-d_6) δ 8.70 (d, J = 1.9 Hz, 1H, H-6), 8.68 (d, J = 1.9 Hz, 1H, H-7), 7.70 (s, 1H, H-9), 7.62 (s, 1H, H-4), 7.35 (br, 2H, 2-NH_2), 3.68 (s, 3H, *N*1-CH_3). An ROE cross-peak was observed between the *N*1-CH_3 signal and the H-9. ROE cross-peaks were also observed between H-6 and H-4 and between H-7 and H-9. HRMS ESI-MS *m*/*z* calculated for $[M + H]^+$: $C_{10}H_{10}N_5$, calculated, 200.0936; found, 200.0934. Compound **7**: ^{1}H NMR (DMSO-d_6) δ 7.53 (s, 1H, H-9), 7.47 (s, 1H, H-4), 7.18 (br, 2H, 2-NH_2), 3.60 (s, 3H, *N*1-CH_3), 2.60 (s, 3H, 7-CH_3), 2.52 (s, 3H, 6-CH_3). An ROE cross-peak was observed between the *N*1-CH_3 signal and the H-9, and ROE cross-peaks were observed between 6-CH_3 and H-4, as well as between 7-CH_3 and H-9. HRMS ESI-MS *m*/*z* for $[M + H]^+$: $C_{12}H_{14}N_5$, calculated, 228.1249; found, 228.1253.

Cooking of Meat Samples. The meat types (beef, pork, and chicken) and cooking methods were previously reported in detail (*2, 18, 19*). The cooked samples, which were stored at −80 °C after cooking, were blind coded, and identities and methods of cooking were not revealed until all of the quantitative analyses of HAAs in meat samples had been completed. We assayed selected samples from the FFQ representing the meats most frequently consumed in the United States and the cooking methods most commonly used: pan frying, oven broiling, grilling/barbecueing, or roasting.

Two types of chicken were assayed: skinless, boneless chicken breasts and breasts with skin and bones. The bones (from whole chicken and breasts with skin and bones) were removed manually, and edible parts (flesh and skin) of the cooked chicken were finely minced to form a composite sample for the specific chicken type, method of cooking, and degree of doneness. The cooking levels for skinless, boneless chicken breast samples that were grilled/barbecued were just done, well done, and very well done. The internal temperature of the chicken at 80 °C was designated as just done; the internal temperature was at least 5 °C higher for well done and at least 10 °C higher for very well done. The pan-fried chicken breasts (without skin and bones) were cooked in a Teflon-coated frying pan with 2 tablespoons of cooking oil. Oven-broiled chicken breasts (with or without skin and bones) were cooked in a commercial gas range broiler, with the meat placed 12.5 cm away from the heat source. Grilled/barbecued chicken breasts (with or without skin and bones) were prepared on a home propane gas barbecue unit with ceramic briquettes (Sunbeam Model 44M39, $27^1/_2$ in. × 15 in., 44000 BTU).

Beef was purchased from a supermarket: hamburger patties of freshly ground lean beef (15% fat, 1.5−2.0 cm thick × 10 cm diameter), steaks (top loin, New York strip, USDA choice steaks, 2.6−3.3 cm thick), and roasts (eye round roast, 4−6 lbs). Hamburger patties and steaks were pan-fried, oven-broiled, or grilled/barbecued, while roasts were cooked in an oven. The hamburger patties and steaks were cooked to four levels of doneness: rare, medium, well done, and very well done. The degree of doneness for the varying types of beef products was primarily defined by internal temperature: the internal temperature of 60 °C as rare, 70 °C as medium, 80 °C as well done, and 90 °C as very well done. Hamburger patties and steaks were pan-fried in a Teflon-coated frying pan without added oil. Oven-broiled hamburger patties and steaks were cooked in a commercial gas range broiler with the meat placed 12 cm away from the heat source. Grilled/barbecued patties and steaks were prepared on the Sunbeam gas barbecue unit with ceramic briquettes. Roast beef was cooked, in a gas oven heated to 160 °C, to three levels of doneness: rare, medium, and well done. Drippings were collected from two roasts and combined. Gravy was made from the crusty drippings after skimming off all the fat except one-quarter cup (56 g). In a saucepan, $^1/_4$ cup of flour, $1^3/_4$ cups of water, and $^1/_4$ cup of fat with all the drippings were mixed. This mixture was cooked until thick and bubbly (*18*).

Tandem Solvent−Solid-Phase Extraction (SPE) of HAAs from Cooked Meat. The extraction procedure followed the method that was developed by Gross (*24*) and then modified by Turesky (*17*). The cooked meat samples (2 g) were homogenized with 1 N NaOH (4 mL/g of meat, spiked with isotopically labeled internal standards at 2.5 ng/g of meat), and mixed thoroughly with an Extrelut-20 resin (3 g of resin/g of meat). The mixture was placed into an empty polypropylene cartridge (50 mL) containing a 200 μm pore size frit and connected to a Waters Oasis MCX LP extraction cartridge (150 mg). The MCX resin was prewashed with 5% NH_4OH in CH_3OH (2 mL), followed by 2% CH_3CO_2H in CH_3OH (2 mL) prior to connection to the Extrelut-20 resin. The HAAs were eluted from the Extrelut-20 resin with 5% toluene in CH_2Cl_2 (50 mL) and adsorbed onto the MCX cartridge. The Extrelut-20 resin was discarded, and the MCX cartridge was then sequentially washed with toluene−CH_2Cl_2 (5:95, 2 mL), 0.01 N HCl in

H_2O-CH_3OH (40:60, 2 mL), and CH_3OH (2 mL), followed by H_2O (2 mL) and $NH_4OH-CH_3OH-H_2O$ (2:15:85, 2 mL). Then, the HAAs were eluted from the resin with 2 mL of 5% NH_4OH in CH_3OH and collected into Eppendorf tubes. The eluent was concentrated to dryness by vacuum centrifugation (Savant ISS110 Speedvac concentrator). The residue was resuspended in 40 μL of DMSO−0.1% HCO_2H (1:1) with a final concentration of internal standards at 125 pg/μL (assuming 100% recovery). Two microliters of the extract was quantified for the HAA content.

Characterization and Quantification of HAAs in Meat Samples by LC-ESI-MS/MS. Chromatography of HAAs was performed with an Agilent Technologies 1100 series capillary LC system (Palo Alto, CA) equipped with an Aquasil C18 reversed-phase column (3 μm particle size, 1 × 200 mm) from Thermo Electron Corp. (Bellefonte, PA) with a Javelin precolumn (1 × 10 mm) containing the C18 reversed-phase resin. The A solvent was 1 mM $CH_3CO_2NH_4$−0.1% HCO_2H−10% CH_3CN−89.9% H_2O. The B solvent contained 0.1% HCO_2H−4.9% H_2O−95% CH_3CN. The flow rate was set to 40 μL/min. The solvent composition was isocratic at 4% B for 10.5 min, followed by a linear gradient to 15% B at 15 min; it was then increased to 100% B at 25 min and held at 100% B for 7 min, followed by reequilibration to starting conditions.

The MS analyses were performed on a Finnigan TSQ Quantum Ultra triple quadrupole (TSQ) mass spectrometer employing the MS parameters described in General Methods. Quantitative analyses were conducted in the selective reaction monitoring (SRM) scan mode. The transitions monitored (0.02 s dwell time per transition) and approximate chromatographic retention times (t_R) were as follows: $[M + H]^+ > [M + H - 15]^{+\bullet}$ (loss of $CH_3^\bullet$) for IQ (199 > 184, t_R = 9.4 min), I*g*Qx (200 > 185, t_R = 12.0 min), IQx (200 > 185, t_R = 12.9 min), 7-MeI*g*Qx (214 > 199, t_R = 19.8 min), 8-MeIQx (214 > 199, t_R = 20.7 min), IFP (203 > 188, t_R = 21.60 min), IQ[4,5-*b*] (199 > 184, t_R = 21.6 min), 6,7-DiMeI*g*Qx (228 > 213, t_R = 22.5 min), 4,8-DiMeIQx (228 > 213, t_R = 23.2), 7,9-DiMeI*g*Qx (228 > 213, t_R = 23.8 min), and PhIP (225 > 210, t_R = 24.6 min) and $[M + H]^+ > [M + H - 18]^{+\bullet}$ (loss of $CD_3^\bullet$) for the respective trideuterated internal standards. The transition $[M + H]^+ > [M + H - 17]^+$ (loss of NH_3) was used for AαC (184 > 167, t_R = 25.5 min), MeAαC (198 > 167, t_R = 27.0 min), and [$^{13}C_6$]AαC (190 > 173).

The quantification of HAAs was performed with an external calibration curve using 125 pg/μL of internal standards containing unlabeled HAAs at nine calibrant levels ranging from 0 to 1250 pg injected on the column; these levels were equated to 0, 0.025, 0.05, 0.3, 0.5, 1.0, 2.5, 5.0, 10.0, and 25 μg/kg of HAAs in cooked meat. Due to the high concentrations of 7-MeI*g*Qx and PhIP found in some meat samples, the concentration of the calibrants in the upper range was increased to 100 μg/kg for 7-MeI*g*Qx and 300 μg/kg for PhIP. Since isotopically labeled internal standards were not available for 7,9-DiMeI*g*Qx, MeAαC, and IFP, [$^2H_3$C]-6,7-DiMeI*g*Qx, [$^{13}C_6$]-AαC, and [$^2H_3$C]-IQ[4,5-*b*] were used as the respective internal standards. The coefficient of determination (r^2) of all HAA calibration curves exceeded 0.995.

RESULTS

Identification of Known HAAs and Detection of Novel HAAs in Cooked Meat by LC-ESI-MS/MS. The chemical structures of 14 HAAs formed in cooked meat and investigated in this study are depicted in **Figure 1**. Thirteen out of the 14 HAAs were identified in our cooked beef, pork, and poultry samples. MeIQ was the only HAA not detected in these cooked meat samples, consistent with findings of previous studies (*18, 19*). A representative LC-ESI-MS/MS analysis of HAAs formed in ground beef patties pan-fried to the well-done level at 191 °C is presented in **Figure 2**. Eleven of the HAAs under study were detected in this fried meat sample. The carboline derivatives AαC and MeAαC were below the limit of detection (LOD) in all cooked beef samples, consistent with their occurrence only in fried beef cooked at elevated temperatures (>250 °C) (*1, 17*). 7-MeI*g*Qx (**Figure 2**, t_R = 19.8 min) was the principal HAA

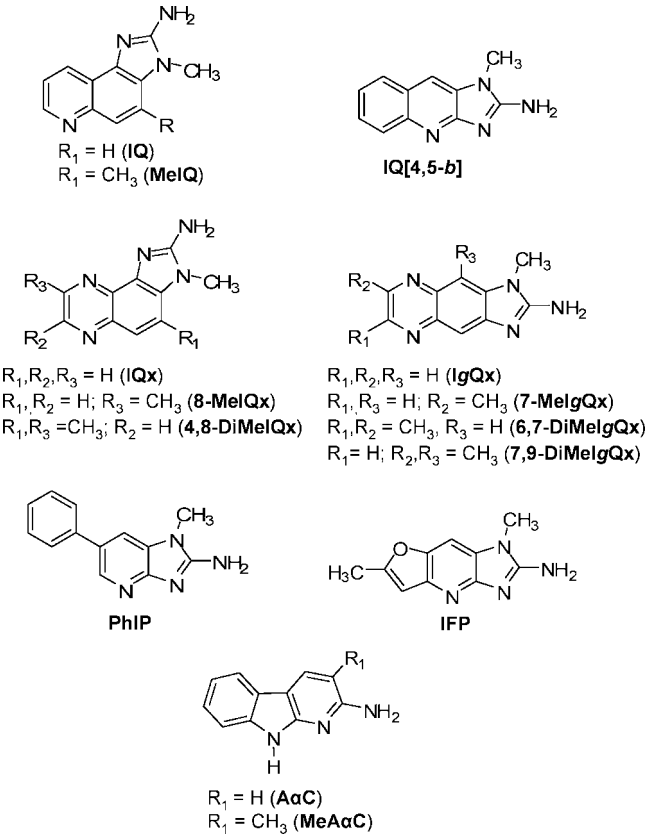


Figure 1. Chemical structures of HAAs investigated in this study.

formed in fried ground beef patties. Two novel HAAs, which appeared to contain the I*g*Qx skeleton, on the basis of their ESI product ion spectra (*15, 17*), were identified. These two HAAs were thought to be I*g*Qx (t_R = 12.0 min) and 6,7-DiMeI*g*Qx (t_R = 22.5 min).

Syntheses of 6-MeI*g*Qx and 6,7-DiMeI*g*Qx. We undertook the chemical syntheses of I*g*Qx and 6,7-DiMeI*g*Qx to determine whether either compound was formed in cooked beef. The synthesis is depicted in **Scheme 1**. The key intermediates were the isomeric compounds 1-methyl-6-nitro-1*H*-benzo[*d*]imidazole-2,5-diamine (**4a**) and 1-methyl-5-nitro-1*H*-benzo[*d*]imidazole-2,6-diamine (**4b**), both of which underwent reduction to form 1-methyl-1*H*-benzo[*d*]imidazole-2,5,6-triamine (**5**). The triamine readily condensed with glyoxal or 1,3-butadione to form the desired I*g*Qx (**6**) and 6,7-DiMeI*g*Qx (**7**) homologues. The incorporation of [$^2H_3$C]I in the reaction scheme was done to prepare the stable, isotopically labeled compounds for internal standards.

Quantification of HAAs in Cooked Meats. The tandem SPE method employed to isolate HAAs from cooked meats (*25*) was originally developed for the analysis of HAAs by HPLC with UV and fluorescence detection (*24*). We adapted the method by replacing the individual propylsulfonic acid (PRS) and C18 resins with an Oasis MCX LP extraction cartridge, which contains a mixture of hydrophobic and cation-exchange resins (*17*). The use of the MCX resin permitted the recovery of all of the HAAs in one eluent. We previously validated this method by LC-ESI-MS/MS measurements of six prevalent HAAs spiked to pan-fried beef and barbecued chicken cooked well done at a level of 1 μg/kg. The accuracy of the method was on average within 15% of the target value with coefficients of variation ≤15%, based upon isotopic dilution with internal standards. The lower limit of quantification (LOQ) approached 0.030 μg/kg (*17*).

Many HAAs were readily detected in all cooked meat products of the FFQ database, except for those samples cooked

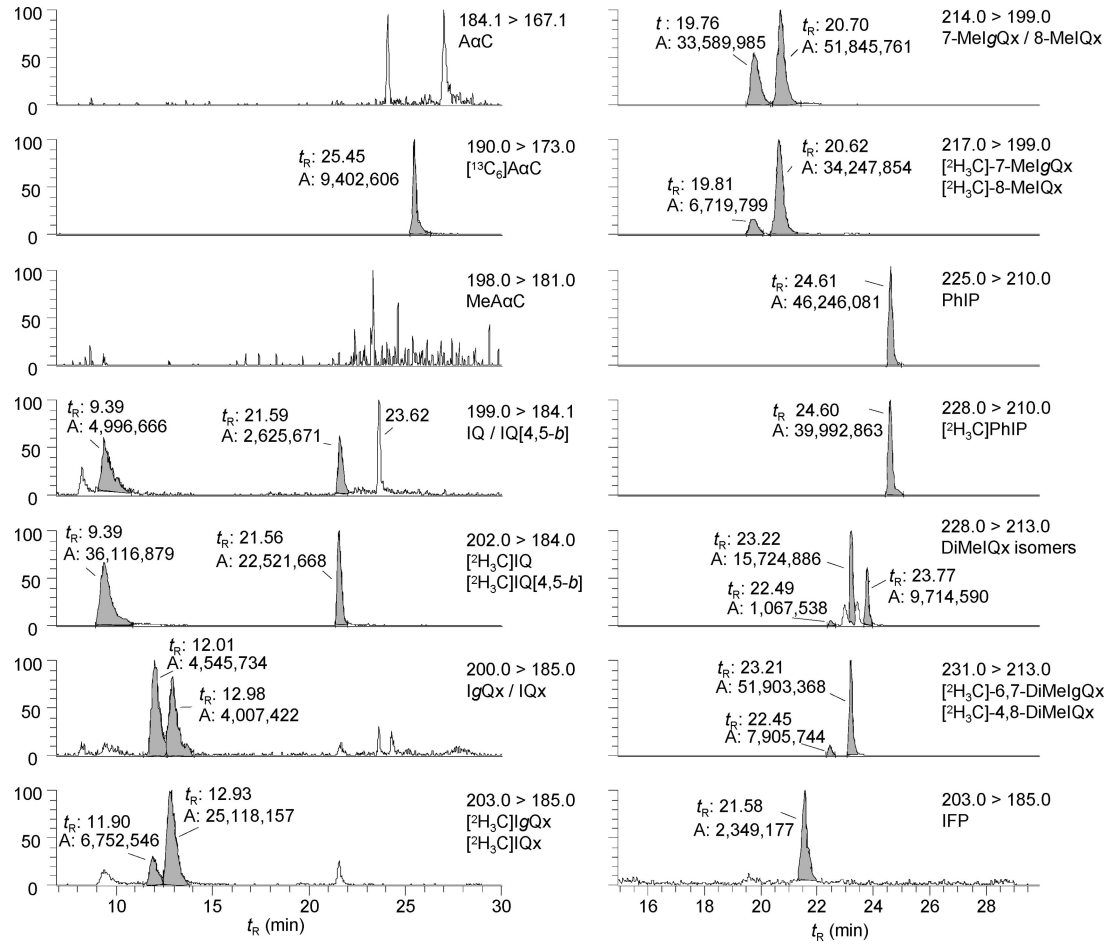


Figure 2. LC-ESI-MS/MS analysis of HAAs formed in pan-fried beef cooked well done. The approximate t_R values are IQ (9.4 min), I*g*Qx (12.0 min), IQx (12.9 min), 7-MeI*g*Qx (19.8 min), 8-MeIQx (20.70 min), IFP (21.6 min), IQ[4,5-*b*] (21.6 min), 6,7-DiMeI*g*Qx (22.5 min), 4,8-DiMeIQx (23.2 min), 7,9-DiMeI*g*Qx (23.8 min), PhIP (24.6 min), AαC (25.5 min), and MeAαC (27.0 min). Peaks of HAAs and isotopically labeled internal standards are shaded, and approximate t_R values and peak area are reported.

at low temperature (100 °C); for the latter, the amounts of all HAAs were below 1 μg/kg (data not shown). The estimates of HAAs in cooked pork, chicken, beef, and beef gravy are reported in **Tables 1**−**4**. The relative levels of HAAs formed in several of these cooked meat samples are presented in **Figures 3** and **4**.

HAA Content in Pork Meats. Sausage patties pan-fried very well done contained 8-MeIQx at a concentration of 5.1 μg/kg. Low amounts of PhIP (0.2 μg/kg) were also detected. The estimates of these HAAs are comparable to the values previously determined by HPLC with UV/fluorescence detection (*19*). However, the recently discovered 7-MeI*g*Qx was the most abundant HAA, at an estimated concentration of 8.4 μg/kg (**Figure 3A**). Three other linear tricyclic ring HAAs, I*g*Qx, 6,7-DiMeI*g*Qx, and 7,9-DiMeI*g*Qx, were detected at concentrations that ranged between 0.2 and 1.8 μg/kg. Pan-fried and oven-broiled bacon strips contained high amounts of PhIP, consistent with the findings of previous reports (*19, 26*). 7-MeI*g*Qx was the second most abundant HAA formed in cooked bacon, followed by 8-MeIQx. Pork chops pan-fried very well done contained only trace quantities of HAAs; 7-MeI*g*Qx was the most abundant, at 0.4 μg/kg. The remaining HAAs were formed at concentrations below 1.0 μg/kg in all cooked pork meats (**Table 1**).

HAA Content in Cooked Chicken. PhIP was the principal HAA formed in all types of chicken samples prepared by barbecuing, oven broiling or pan frying. PhIP was formed in very well done barbecued chicken at a concentration of 305 μg/kg (**Figure 3B** and **Table 2**), consistent with the estimates of PhIP obtained by HPLC with UV/fluorescence detection (*2*). Other prominent HAAs formed in barbecued chicken cooked very well done were AαC (40 μg/kg), IFP (26 μg/kg), 7-MeI*g*Qx (11.3 μg/kg), 8-MeIQx (7.7 μg/kg), and MeAαC (6.1 μg/kg).

HAA Content in Cooked Beef. 7-MeI*g*Qx was the major HAA formed in hamburgers and steak pan-fried at a surface temperature of 191 °C for all levels of doneness (**Figure 4** and **Tables 3** and **4**). Increasing the time of cooking and degree of well doneness increased the HAA content. Other HAAs identified at concentrations above 1 μg/kg in hamburgers or steak were I*g*Qx, 6,7-MeI*g*Qx, 7,9-DiMeI*g*Qx, 8-MeIQx, 4,8-DiMeIQx, IFP, and PhIP. As previously reported, oven broiling of hamburgers produced much lower concentrations of HAAs than did pan frying (*18*).

HAA Content in Roast Beef Gravy. Roast beef was reported to contain low amounts of 8-MeIQx, 4,8-DiMeIQx, and PhIP (<1 μg/kg) at all degrees of doneness, but the gravy prepared from well-done drippings of roast beef contained 8-MeIQx (7.1 μg/kg) and PhIP (4.1 μg/kg) (*18*). Our estimates of both HAAs in gravy, by quantitative LC-ESI-MS/MS (**Table 4**), agree with those published values (*18*); markedly, the amount of 7-MeI*g*Qx formed in gravy exceeded the amounts of 8-MeIQx and PhIP, by 3.6- and 8.7-fold, respectively, in the present study.

Confirmation of Previously Characterized and Recently Discovered HAAs by Product Ion Spectra Scan Mode. The identities of the 13 HAAs were confirmed by acquiring full-scan product ion spectra of the analytes; these spectra proved

Table 1. HAAs in Cooked Pork Meat Products[a]

pork sample, cooking method, doneness	temp (°C)		time of cooking (min)		HAA content (μg/kg)												
	surface	internal			IQ	I*g*Qx	IQx	7-MeI*g*Qx	8-MeIQx	IFP	IQ[4,5-*b*]	6,7-DiMeI*g*Qx	4,8-DiMeIQx	7,9-DiMeI*g*Qx	PhIP	AαC	MeAαC
pork chops, pan-fried, very well done	176	90	15	av	ND	ND	ND	0.37	ND	ND	0.16	ND	ND	ND	ND	ND	ND
				SD				0.03			0.02						
sausage patties, pan-fried, very well done	179	99	21	av	0.07	1.80	0.72	8.37	5.07	0.32	0.10	0.17	0.72	1.25	0.23	ND	ND
				SD	0.00	0.11	0.04	0.38	0.01	0.02	0.02	0.01	0.03	0.05	0.02		
bacon, oven-broiled, very well done	175	NM	7.2	av	ND	2.98	0.68	9.08	2.61	0.41	0.11	0.24	0.52	0.77	15.91	0.26	ND
				SD		0.37	0.13	0.37	0.06	0.04	0.02	0.01	0.02	0.11	0.78	0.04	
bacon, pan-fried, very well done	176	NM	16.1	av	ND	1.05	0.49	3.46	3.00	0.16	0.05	0.08	0.72	0.43	4.90	ND	ND
				SD		0.10	0.04	0.38	0.21	0.02	0.06	0.01	0.03	0.05	0.81		

[a] Mean ± SD of three independent analyses. ND = not detected (<0.03 μg/kg). NM = not measured.

Table 2. HAAs in Cooked Chicken[a]

chicken sample, cooking method, and degree of doneness	temp (°C)		time of cooking (min)		HAA content (μg/g)												
	surface	internal			IQ	I*g*Qx	IQx	7-MeI*g*Qx	8-MeIQx	IFP	IQ[4,5-*b*]	6,7-DiMeI*g*Qx	4,8-DiMeIQx	7,9-DiMeI*g*Qx	PhIP	AαC	MeAαC
chicken breast with skin and bone, barbecued, well done	191[b]	83	63	av	0.13	0.24	ND	2.76	0.63	1.09	0.36	0.18	0.32	0.55	19.07	4.09	0.47
				SD	0.02	0.02		0.68	0.14	0.01	0.06	0.01	0.06	0.11	1.09	0.43	0.07
chicken without skin and boneless, barbecued, well done	260	93	40	av	0.64	0.80	ND	7.71	1.98	5.97	ND	ND	1.42	1.12	78.52	5.38	0.66
				SD	0.09	0.13		0.28	0.05	0.77			0.02	0.10	4.83	0.03	0.06
chicken without skin and boneless, barbecued, very well done	260	99	43	av	0.91	ND	ND	11.28	7.70	25.56	ND	ND	5.52	2.89	304.71	40.36	6.12
				SD	0.11			2.38	1.15	1.66			0.48	0.79	10.89	6.98	0.76
chicken without skin and boneless, oven-broiled, just done	86	80	9	av	0.07	0.20	ND	1.02	0.13	0.39	ND	ND	0.11	0.08	5.60	0.18	ND
				SD	0.04	0.09		0.06	0.01	0.02			0.01	0.01	0.09	0.03	
chicken without skin and boneless, oven-broiled, well done	79	91	14	av	0.15	0.44	ND	3.64	0.82	2.61	0.34	0.24	0.52	0.55	31.83	2.54	0.36
				SD	0.09	0.07		0.24	0.04	0.18	0.01	0.01	0.02	0.07	3.65	0.17	0.02
chicken without skin and boneless, oven-broiled, very well done	83	98	17	av	0.30	0.29	ND	11.10	2.81	11.33	ND	ND	1.98	1.81	71.96	9.43	1.31
				SD	0.00	0.03		0.32	0.20	0.62			0.33	0.13	4.17	1.27	0.25
chicken without skin and boneless, pan-fried, just done	197	76	14	av	0.18	0.25	0.07	1.48	0.58	2.66	ND	0.06	0.78	0.24	8.77	ND	ND
				SD	0.17	0.03	0.00	0.14	0.02	0.10		0.01	0.03	0.06	0.03		
chicken without skin and boneless, pan-fried, well done	202	82	28	av	0.11	0.62	0.10	5.07	1.25	5.63	0.24	0.22	1.57	0.63	19.47	ND	ND
				SD	0.01	0.05	0.01	0.13	0.07	0.25	0.01	0.01	0.23	0.04	0.39		
chicken without skin and boneless, pan-fried, very well done	211	90	36	av	0.21	0.89	0.21	8.65	2.34	15.88	ND	ND	3.61	0.90	48.54	0.07	ND
				SD	0.01	0.19	0.02	0.42	0.08	0.64			0.19	0.10	2.83	0.01	

[a] Mean ± SD of three independent analyses. ND = not detected (<0.03 μg/kg). [b] 500 °C during flashing.

Table 3. HAAs in Cooked Beef[a]

beef sample, cooking method, doneness	temp (°C)		time of cooking (min)		HAA content (μg/g)												
	surface	internal			IQ	I*g*Qx	IQx	7-MeI*g*Qx	8-MeIQx	IFP	IQ[4,5-*b*]	6,7-DiMeI*g*Qx	4,8-DiMeIQx	7,9-DiMeI*g*Qx	PhIP	AαC	MeAαC
ground beef																	
oven-broiled, rare	180	59	6	av	ND	ND	ND	ND	ND	ND	0.26	ND	ND	ND	ND	ND	ND
				SD							0.06						
oven-broiled, medium	186	68	10	av	0.05	ND	ND	0.31	ND	ND	0.12	0.05	ND	ND	ND	ND	ND
				SD	0.01			0.04			0.01	0.00					
oven-broiled, well done	189	81	15	av	0.06	0.03	ND	0.10	0.02	ND	0.39	ND	0.01	0.02	0.06	ND	ND
				SD	0.09	0.01		0.02	0.01		0.55		0.00	0.00	0.04		
oven-broiled, very well done	191	90	20	av	0.10	0.30	0.06	1.35	0.38	0.14	0.15	ND	0.10	0.12	1.23	0.11	ND
				SD	0.18	0.02	0.01	0.05	0.20	0.01	0.09		0.00	0.02	0.30	0.11	
pan-fried, medium	180	69	10	av	0.10	0.50	0.10	2.40	1.00	0.10	0.30	0.20	0.20	0.71	0.70	ND	ND
				SD	0.10	0.00	0.00	0.20	0.10	0.00	0.10	0.10	0.00	0.10	0.10		
pan-fried, well done	189	81	15	av	0.10	1.50	0.40	9.50	3.00	0.60	0.40	0.30	0.60	2.20	2.70	ND	ND
				SD	0.00	0.30	0.00	1.50	0.20	0.00	0.30	0.10	0.00	0.10	0.10		
pan-fried, very well done	191	90	20	av	0.20	1.80	0.40	11.70	3.70	0.70	0.30	0.40	0.70	3.00	2.90	ND	ND
				SD	0.20	0.10	0.00	0.40	0.30	0.00	0.10	0.10	0.00	0.20	0.10		
steak																	
pan-fried, medium	186	70	16	av	0.20	0.76	0.16	4.05	1.43	0.34	0.16	0.23	0.33	0.62	1.77	ND	ND
				SD	0.09	0.05	0.01	0.53	0.06	0.03	0.02	0.04	0.01	0.09	0.04		
pan-fried, well done	189	84	26	av	0.19	1.55	0.31	12.31	3.17	1.26	0.49	0.28	0.73	2.92	5.08	ND	ND
				SD	0.01	0.06	0.01	0.81	0.08	0.08	0.14	0.04	0.01	0.12	0.25		
pan-fried, very well done	191	93	33	av	0.28	4.03	0.69	23.65	6.50	4.03	0.16	0.68	2.06	6.31	12.46	ND	ND
				SD	0.03	0.02	0.05	1.73	0.23	0.14	0.00	0.08	0.04	0.29	0.17		

[a] Mean ± SD of three independent analyses. ND = not detected (<0.03 μg/g).

Table 4. HAA Formation in Roast Beef Gravy[a]

roast beef gravy, degree of doneness	temp (°C)		time of cooking (min)		HAA content (μg/g)												
	oven	internal			IQ	I*g*Qx	IQx	7-MeI*g*Qx	8-MeIQx	IFP	IQ[4,5-b]	6,7-DiMeI*g*Qx	4,8-DiMeIQx	7,9-DiMeI*g*Qx	PhIP	AαC	MeAαC
oven-roasted, rare	160	53	96	av	ND	0.44	0.13	3.78	1.10	0.21	0.15	0.23	0.23	0.73	0.30	ND	ND
				SD		0.03	0.02	0.26	0.05	0.01	0.05	0.01	0.00	0.06	0.01		
oven-roasted, medium	160	64	120	av	0.21	0.34	0.13	2.74	1.08	0.11	0.11	ND	0.21	0.50	0.06	ND	ND
				SD	0.11	0.00	0.02	0.07	0.08	0.01	0.01		0.01	0.04	0.01		
oven-roasted, well done	160	86	182	av	0.32	2.77	0.78	29.52	8.43	3.24	0.04	0.90	1.40	6.09	3.41	ND	ND
				SD	0.03	0.10	0.03	2.81	0.16	0.29	0.01	0.05	0.10	0.64	0.17		

[a] Mean ± SD of three independent analyses. ND = not detected (<0.03 μg/g).

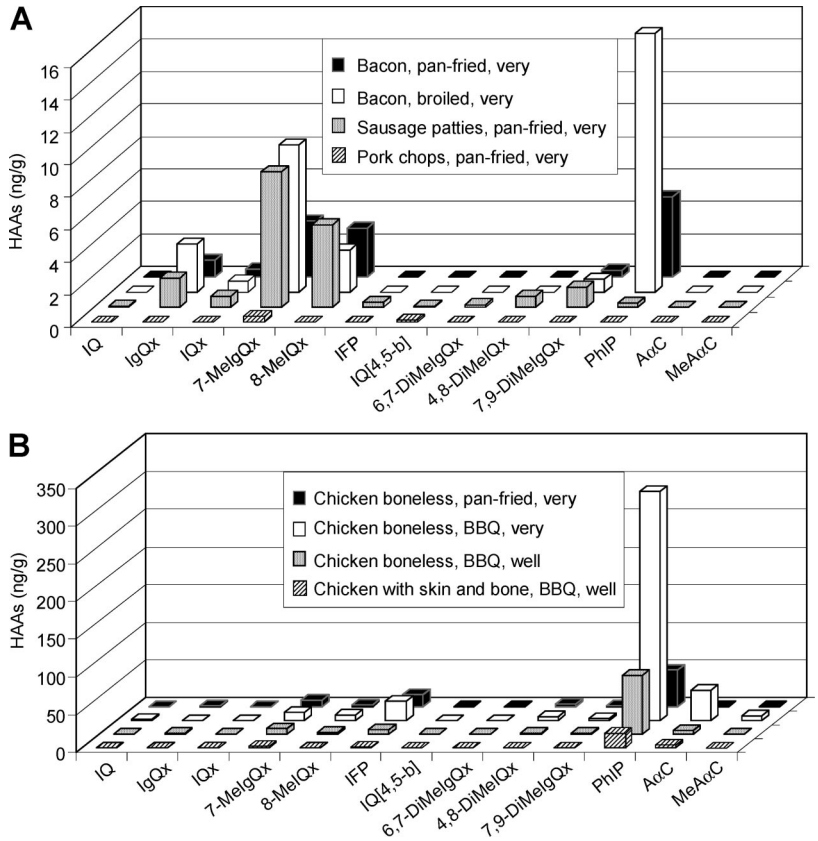


Figure 3. HAA formation in (**A**) cooked bacon, sausage patties, and pork chops and (**B**) pan-fried and barbecued chicken prepared at a range of temperatures and doneness.

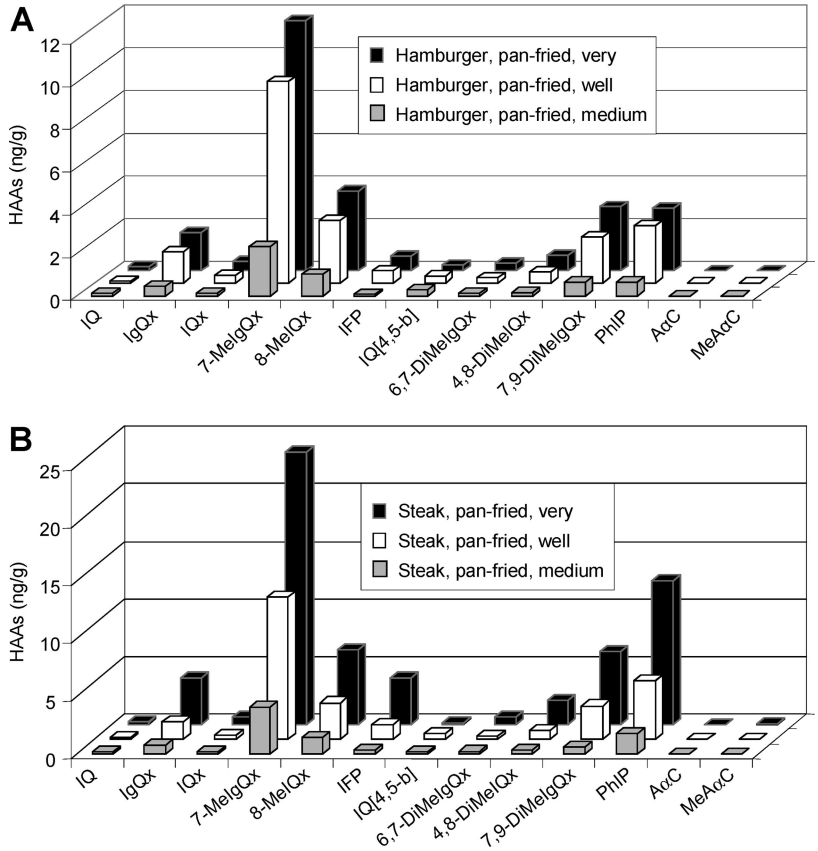


Figure 4. HAA formation in (**A**) pan-fried ground beef patties and (**B**) pan-fried beef steak prepared at a range of doneness.

to be in excellent agreement with the spectra of the synthetic compounds. Many of the ESI product ion mass spectra have been published previously (*16, 17*). The product ion spectra of two newly discovered HAAs, I*g*Qx and 6,7-DiMeI*g*Qx, and the

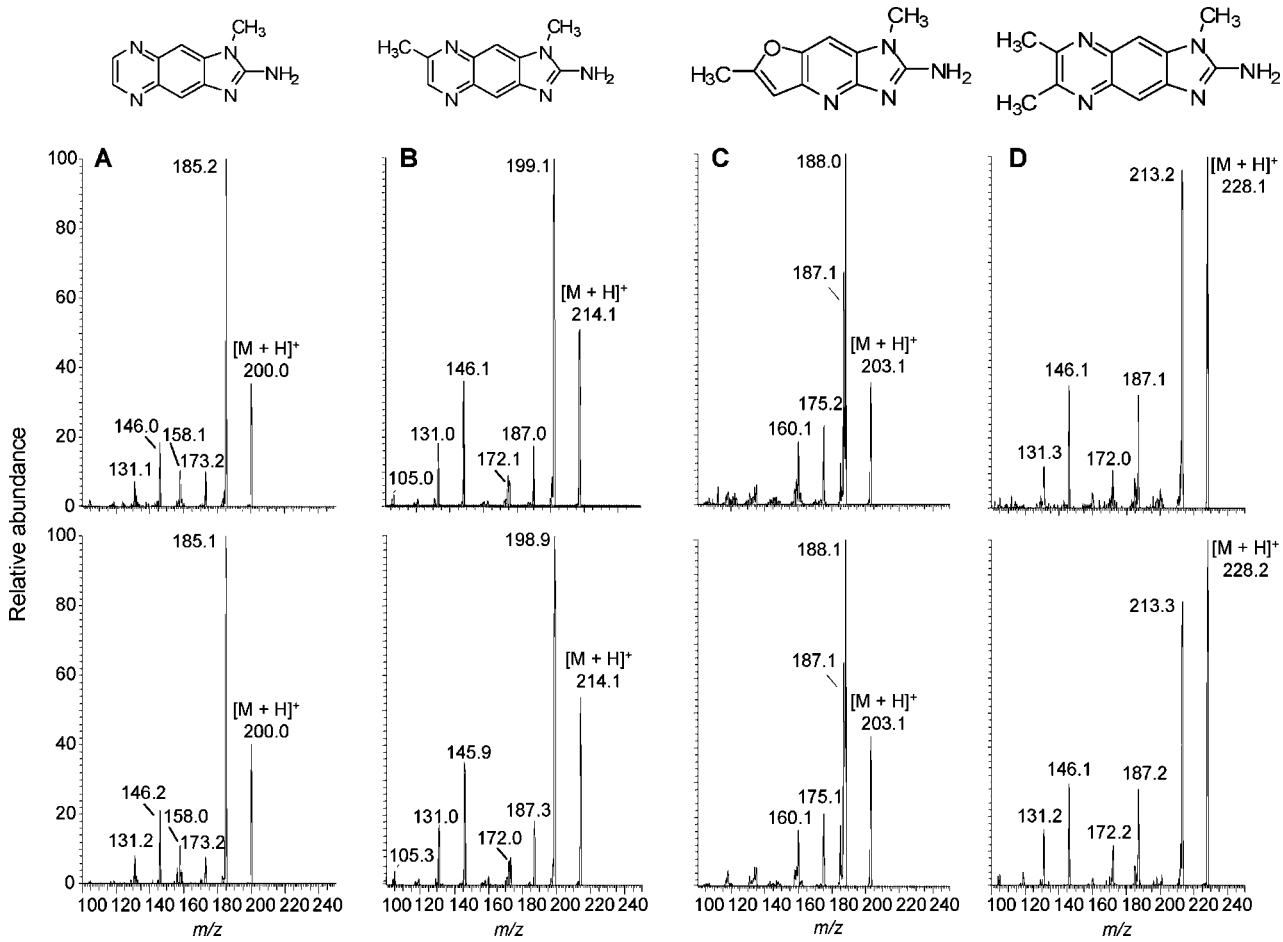


Figure 5. LC-ESI-MS product ion spectra of (**A**) I*g*Qx, (**B**) 7-MeI*g*Qx, (**C**) IFP, and (**D**) 6,7-DiMeI*g*Qx formed in pan-fried meat cooked very well done (upper panels) and the respective synthetic compounds (lower panels).

spectrum of 7-MeI*g*Qx, and IFP, the only HAA investigated in this study that contains an oxygen atom in the 5-membered heterocyclic ring (*27*), and the synthetic reference compounds, are presented in **Figure 5**. The product ion spectra corroborate the identities of these analytes in the cooked meat samples.

Other Putative HAAs in Cooked Meats. One peak (**Figure 2**, t_R = 23.6 min), which displayed the same SRM transition (199 > 184) as did IQ and IQ[4,5-*b*], was found in 17 out of 26 meat samples. Moreover, the product ion spectra displayed prominent fragment ions at *m*/*z* 184, 157, and 131, as do the product ion spectra of several isomers of IQ (*23*). There are several plausible structures for this presumed isomer of IQ; additional studies are required to unambiguously determine its identity. If we assume an ionization efficiency comparable to that of IQ, this compound occurs in cooked meat at concentrations ranging from 0.2 to 1.0 μg/kg. Two putative isomers of DiMeIQx $[M + H]^+$ at *m*/*z* 228 occurred in many pan-fried beef samples cooked well done or very well done: one isomer eluted just prior to 4,8-DiMeIQx (t_R = 23.2 min), and the second compound eluted just before 7,9-DiMeI*g*Qx (t_R = 23.8 min) (**Figure 2**). The product ion spectra of these analytes are suggestive of structures that contain the amino-*N*-methylimidazo[4,5-*f*]quinoxaline or amino-*N*-methylimidazo[4,5-*g*]quinoxaline ring structure (*17*). The identities of these compounds remain to be elucidated.

DISCUSSION

The HAA content of hamburger patties, beef steak, roast beef gravy, pork, and chicken cooked by common household methods, to varying degrees of doneness, has been determined by quantitative LC-ESI-MS/MS using the stable, isotope dilution method. Ten known HAAs and three recently characterized HAAs were identified; these latter compounds were postulated to be linear tricyclic ring isomers of IQx, 8-MeIQx, and 4,8-DiMeIQx (*17*). The syntheses of 7-MeI*g*Qx, I*g*Qx, and 6,7-DiMeI*g*Qx were undertaken to determine whether these compounds were formed in cooked beef (**Scheme 1**) (*15*). The t_R values of the reference compounds, the trideuterated internal standards, and the analytes in cooked meats were identical. Moreover, the product ion spectra of the analytes and of the respective reference compounds were in excellent agreement (**Figure 5**) (*15*). Thus, these three HAAs, along with 7,9-DiMeI*g*Qx, which was previously identified in beef extract (*20*), represent a new class of linear HAAs, which can arise at appreciable concentrations in cooked meats.

The most important finding of our study is the detection of 7-MeI*g*Qx in cooked beef at high levels relative to the concentrations of other HAAs. 7-MeI*g*Qx arises in cooked beef at higher levels than do 8-MeIQx, 4,8-DiMeIQx, and PhIP, which have been recognized as three of the most mass-abundant HAAs formed in cooked beef (*1, 28*). The sum of the masses of 7-MeI*g*Qx and the three other I*g*Qx-ring-structured HAAs exceeds 50% of the total HAA content of hamburgers and steak cooked well done and very well done and the content of gravy obtained from very well done roast beef. Moreover, these I*g*Qx-structured HAAs represent a significant proportion of the total daily intake of HAAs in bacon.

In general, the HAA content of all of the meat samples increased with degree of doneness, but the individual HAAs measured were not produced to the same extent by each cooking method and doneness level. Barbecuing or pan frying produced larger amounts of HAAs than did oven broiling, a result in agreement with earlier findings on HAA formation as a function of temperature and method and duration of cooking (*1, 2, 17–19, 29*). The amounts of HAAs determined, by LC-ESI-MS/MS, in various cooked meat samples of the FFQ are in good agreement to those estimates previously obtained by HPLC with UV/fluorescence detection (*2, 18, 19*). The average concentrations of 8-MelQx, PhIP, and 4,8-DiMelQx in a high-temperature quality control (high QC) cooked ground beef sample were [mean (coefficient of variation)] 7.2 (0.36), 10.9 (0.24), and 1.66 (0.40) ng/g, respectively, determined by HPLC with UV/fluorescence detection. The average concentrations of 8-MelQx, PhIP, and 4,8-DiMelQx from five batches of this high QC meat sample, estimated by LC-ESI-MS/MS, were 6.0 (0.04), 7.8 (0.08), and 1.8 (0.12) ng/g, respectively. The amount of 7-MeI*g*Qx estimated in this high QC meat sample was 31.0 (0.16) ng/g. The precision of the LC-ESI-MS/MS assay is superior to the precision of the HPLC with UV/fluorescence detection because the former's use of stable, istopically labeled internal standards accurately compensates for recovery of the analytes from complex food matrices (*17*).

The estimates of PhIP, 8-MeIQx, and 4,8-DiMeIQx in cooked meat samples, measured by HPLC with UV/fluorescence, have been incorporated into a meat-cooking module within the FFQ to assess dietary intake of these HAAs (*11*). Epidemiological studies investigating the risk of development of colorectal and prostate cancers have examined meat consumption, and the investigators have proposed that some of these HAAs are potential causal agents of cancer (*11, 12*). Our examination of these same meat samples, by LC-ESI-MS/MS, has shown that 10 additional HAAs are present at variable concentrations in many of these meat staples; clearly, some of these additional compounds should be taken into account when the causal role of HAAs in the risk of developing cancer is being assessed.

Short-term bacterial mutagenesis assays, such as the Ames assay, have been an effective screening tool for the identification of many mutagenic HAAs in complex food matrices. However, the mutagenic potencies of HAAs vary over a >1000-fold range in bacterial assays (*4*), and only HAAs either possessing high mutagenic activity or present at great abundance have been successfully isolated and characterized from cooked meats when bacterial mutagenicity assays are employed for screening (*28, 30*). HAAs containing the I*g*Qx ring structure, such as 7-MeI*g*Qx and 7,9-DiMeI*g*Qx, are ≈1000-fold weaker bacterial mutagens than their respective angular tricyclic ring isomers (*15*). Consequently, the I*g*Qx molecules present in cooked meats are not easily detected in the Ames assay. Screening for HAAs by LC-ESI-MS/MS-based methods instead of biological assays has enabled us to identify novel I*g*Qx-structured HAAs and other putative compounds in cooked beef (*17*).

While bacterial mutation assays have served as a valuable tool to detect HAAs of high genotoxic potency in cooked meat, the mutation assays do not reliably predict carcinogenic potency in mammals (*31*). The large differences in the ranges of potency of HAAs observed in bacterial mutation assays (*4*) are not observed in mammalian cell assays (*32–34*). Moreover, the total doses of HAAs required to induce tumors in 50% of the animals tested in carcinogen bioassays fall generally within a factor of 10 (*4*). The genotoxic potency of 7-MeI*g*Qx (*15, 20*) is comparable to the potencies of the aromatic amines 4-aminobiphenyl (4-ABP) and 2-napthylamine (2-NA) in the Ames *Salmonella typhimurium* tester strains TA98 and YG1024 (frameshift-specific) or YG1029 (primarily point mutation-specific) (*35, 36*). Both 4-ABP and 2-NA are bladder carcinogens in the experimental dog model, and both compounds are recognized human urinary bladder carcinogens (*37*). Given that the concentrations of 7-MeI*g*Qx and its homologues in cooked meats are high in comparison to the concentrations of angular tricyclic ring isomers, some of which are strong experimental animal carcinogens (*4*), studies on the toxicological properties of 7-MeI*g*Qx and other linear tricyclic HAAs are warranted.

ABBREVIATIONS USED

IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; IQ[4,5-*b*], 2-amino-1-methylimidazo[4,5-*b*]quinoline; I*g*Qx, 2-amino-1-methylimidazo[4,5-*g*]quinoxaline; IQx, 2-amino-3-methylimidazo[4,5-*f*]quinoxaline; 7-MeI*g*Qx, 2-amino-1,7-dimethylimidazo[4,5-*g*]quinoxaline; 8-MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; IFP, 2-amino-1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*]pyridine; 6,7-DiMeI*g*Qx, 2-amino-1,6,7-trimethylimidazo[4,5-*g*]quinoxaline; 4,8-DiMeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline; 7,9-DiMeI*g*Qx, 2-amino-1,7,9-trimethylimidazo[4,5-*g*]quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; AαC, 2-amino-9*H*-pyrido[2,3-*b*]indole; MeAαC, 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole; CID, collision-induced dissociation; FFQ, food frequency questionnaire; LC-ESI-MS/MS, liquid chromatography−electrospray ionization/tandem mass spectrometry; MS, mass spectrometry; SPE, solid-phase extraction; SRM, selected reaction monitoring.

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