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Biomonitoring of Carcinogenic Heterocyclic Aromatic Amines in Hair: A Validation Study

Erin E. Bessette[1], **Isil Yasa**[1], **Deborah Dunbar**[1], **Lynne R. Wilkens**[2], **Loic Le Marchand**[2], and **Robert J. Turesky**[1,*]

[1]Division of Environmental Health Sciences, Wadsworth Center, New York State Department of Health, Empire State Plaza, Albany, New York 12201

[2]Cancer Research Center of Hawaii, University of Hawaii, 1236 Lauhala St., Honolulu, Hawaii, 96813

Abstract

A facile method was established to measure heterocyclic aromatic amines (HAAs) accumulated in human hair and rodent fur. The samples were digested by base hydrolysis, and the liberated HAAs were isolated by tandem solvent/solid-phase extraction. Quantification was done by liquid chromatography/tandem mass spectrometry, using a triple stage quadrupole mass spectrometer in the selected reaction monitoring mode. In a pilot study of 12 human volunteers, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) was detected in hair of six meat-eaters at levels ranging from 290 to 890 pg/g hair. 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC) were below the limit of quantification (LOQ) (50 pg/g hair) in hair from meat-eaters and six vegetarians. PhIP was detected in the hair from one vegetarian, and at level just above the LOQ (65 pg/g hair), indicating PhIP exposure occurs primarily through meat consumption. The levels of PhIP in hair samples from two meat-eaters varied by less than 24% over a 6-month interval, signifying that the exposure to PhIP and its accumulation in hair are relatively constant over time. In a controlled feeding study, female C57BL/6 mice were given these HAAs in their drinking water for 1 month, at six daily dose concentrations ranging from 0, 0.080 to 800 μg/kg body weight. PhIP was detected in fur of mice at all doses, whereas AαC and MeIQx were detected in fur at dosages ≥0.8 μg AαC/kg body weight and ≥8 μg MeIQx/kg body weight. There was a strong positive relationship between dosage and each of the HAAs accumulated in fur and their DNA adducts formed in liver and colon (p-values <0.0001); however, the levels of HAA in fur did not correlate to the levels of DNA adducts after adjustment of dose. Thus, hair appears to be a promising long-lived biomarker with by which we can assess the exposure to PhIP, a potential human carcinogen.

CORRESPONDING AUTHOR FOOTNOTE, Robert J. Turesky, Ph.D., Division of Environmental Health Sciences, Wadsworth Center, New York State Department of Health, Phone: 518-474-4151, Fax: 518-473-2095. Rturesky@wadsworth.org.

Supporting Information Available: The LC-ESI/MS/MS chromatograms of HAAs spiked into hair samples of a vegetarian at a level of 800 pg/g hair (Figure S-1); the LC-ESI/MS/MS chromatograms of HAAs accumulated in rodent fur following subchronic treatment with HAAs at a dose of 80 μg HAA/day/kg body wt (Figure S-2); the MS^3 product ion spectrum of guanyl-C8-AαC and [$^{13}C_6$]-guanyl-C8-AαC in liver of mice treated subchronically with 800 μg AαC/day/kg body wt (Figure S-3); the MS^3 product ion spectrum of guanyl-C8-PhIP and [$^{13}C_6$]-guanyl-C8-PhIP in liver of mice treated subchronically with 800 μg PhIP/day/kg body wt (Figure S-4); the MS^3 product ion spectrum of guanyl-C8-MeIQx and guanyl-C8-[^2H_3C]-MeIQx in liver of mice treated subchronically with 800 μg MeIQx/day/kg body wt (Figure S-5); the reconstructed ion mass chromatograms of the LC-ESI/MS/MS^3 traces of DNA adducts of dG-C8-AαC in colon of untreated and treated with 80 μg/day/kg (Figure S-6); the reconstructed ion mass chromatograms of the LC-ESI/MS/MS^3 traces of DNA adducts of dG-C8-PhIP in colon of untreated and treated (80 μg/day/kg (Figure S-7),; and the reconstructed ion mass chromatograms of the LC-ESI/MS/MS^3 traces of DNA adducts of dG-C8-MeIQx in colon of untreated and treated (800 μg/day/kg (Figure S-8). This material is available free of charge via the Internet at http://pubs.acs.org.

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INTRODUCTION

More than 20 heterocyclic aromatic amines (HAAs) are formed in meats cooked well-done (1). Many HAAs are carcinogenic and induce tumors at multiple sites, including lung, forestomach, liver, blood vessels, colorectum, prostate, and the female mammary gland, during long-term feeding studies of rodents (1). PhIP is the most mass-abundant carcinogenic HAA formed in meats and poultry cooked well-done: its concentration can range from several parts-per-billion (ppb) up to 500 ppb (2,3). MeIQx and AαC are two other prominent carcinogenic HAAs formed in cooked meats (2,3).

Considerable research has been devoted to determining the etiological role of HAAs in human cancers (1). Biomarkers of HAA exposure and genetic damage are required if we are to assess the human health risk posed by these genotoxicants (4). Several short-term biomarkers of PhIP and MeIQx have been established, including the unaltered HAAs and their metabolites in urine (5–8). However, these biomarkers are transient and only capture the preceding 24 h of exposure to HAAs. For individuals who chronically but intermittently consume cooked meats, urinary HAA biomarkers can be undetected, and these individuals can be misclassified. Thus, long-lived biomarkers of HAAs are required for any reliable assessment of HAA exposure, for epidemiological investigations. There have been several reports on putative HAA-blood protein adducts of PhIP (9,10) and HAA-DNA adducts of PhIP or MeIQx in humans (9,11–13), but the identity of the adducts, and information on the kinetics of adduct formation and persistence over time, have yet to be determined. There is an urgent need to establish and validate stable, long-term biomarkers of HAAs for use in population studies.

Various drugs and contaminants have been reported to accumulate in the hair of humans and the fur of animals. Human hair and animal fur have served as matrices for biomonitoring of chemicals such as nicotine, other drugs and narcotics, and hormones (14–16). However, those exposures are at levels exceeding the levels of HAAs by at least three orders of magnitude. Studies with experimental laboratory animals have shown that ^{14}C-labeled PhIP accumulates in melanin-rich tissues, including fur (17). Alexander and coworkers (17,18) established a method to quantitate PhIP in mouse fur and human hair by gas chromatography-negative ion chemical ionization mass spectrometry, and recently Kobayashi established a method to quantitate PhIP in human hair by LC-ESI/MS (19,20). There are important drawbacks to both methods: they are labor-intensive, they require elaborate purification procedures, and they require large quantities of hair - from 200 mg up to several grams – for assay.

In this article, we describe a facile tandem solvent/solid-phase extraction (SPE) method to isolate PhIP, MeIQx, and AαC from human hair and rodent fur. The quantification of HAAs is done by liquid chromatography with electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS), using a triple stage quadrupole mass spectrometer in the selected reaction monitoring (SRM) mode and the stable isotope dilution method. The method employs 50 mg of hair for assay and has a limit of quantification (LOQ) of each HAA at 50 parts per trillion (ppt). We have also explored the dose-response relationship between the accrual of HAAs in fur and genetic damage (DNA adduct formation) in liver and colon of female C57BL/6 mice, following subchronic exposure to these HAAs over a 10,000-fold dose range.

EXPERIMENTAL PROCEDURES

Caution: PhIP, MeIQx and AαC are potential human carcinogens and should be handled with caution in a well-ventilated fume hood with the appropriate protective clothing.

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Materials and Methods

PhIP and 1-[^2H_3C]-PhIP (97% isotopic purity), MeIQx and 3-[^2H_3C]-MeIQx (97% isotopic purity), and AαC were purchased from Toronto Research Chemicals (Toronto, ON, Canada). [4b,5,6,7,8,8a-$^{13}C_6$]-AαC (isotopic purity >99%) was a kind gift from Dr. D. Doerge, National Center for Toxicological Research, Jefferson, AR. [$^{13}C_{10}$]dG (isotopic purity >99%) was purchased from Cambridge Isotopes (Andover, MA). DNase I (Type IV, from bovine pancreas), alkaline phosphatase (from *Escherichia coli*), and nuclease P1 (from *Penicillium citrinum*) were purchased from Sigma (St. Louis, MO). Phosphodiesterase I (from *Crotalus adamanteus* venom) was purchased from GE Healthcare (Piscataway, NJ). Hypersep™ filter SpinTips C-18 (20 mg) were acquired from Thermo Scientific (Palm Beach, FL). Oasis MCX resins (30 mg) were purchased from Waters (New Milford, MA). ACS reagent-grade formic acid (88%) was purchased from J.T. Baker (Phillipsburg, NJ). Soluene 350 was purchased from PerkinElmer (Waltham, MA). All solvents used were high-purity B & J Brand from Honeywell Burdick and Jackson (Muskegon, MI).

Synthesis of DNA Adduct Standards

N-(deoxyguanosin-8-yl)-PhIP (dG-C8-PhIP) (21,22), *N*-(deoxyguanosin-8-yl)-MeIQx (dG-C8-MeIQx) (23,24), and *N*-(deoxyguanosin-8-yl)-2-amino-9*H*-pyrido[2,3-*b*]indole (dG-C8-AαC) (25) were prepared by reaction of their *N*-acetoxy-HAA derivatives with dG or [$^{13}C_{10}$] dG (5 mg) in 100 mM potassium phosphate buffer (pH 8.0). In the case of dG-C8-MeIQx, the isotopically labeled internal standard was prepared with *N*-acetoxy-3-[^2H_3C]-MeIQx and unlabeled dG.

Human Hair Collection

Volunteers from Albany, New York, were recruited and asked whether they eat meat on a regular basis ("meat-eaters"; n=6) or not ("vegetarians", n=6). The clippings of hair from volunteers were obtained when the subjects went to their local barbers to get their hair cut during the time period March 2008 – March 2009. The subjects were non-smokers and did not use hair dyes. The hair samples (0.25 – 2.0 g) were collected in plastic zip-lock bags and stored at −20 °C until processed. The samples were rendered anonymous (designated as meat-eater or vegetarian). This study was approved by the Institutional Review Board at the Wadsworth Center.

Animal Studies: Bioaccumulation of HAAs in Fur and DNA Adduct Formation in Liver and Colorectal Tissue

Female C57BL/6 mice were purchased from Jackson Labs (Bar Harbor, ME) and were used in compliance with guidelines established by the National Institutes of Health Office of Laboratory Animal Welfare. Animals were housed four per cage with corn cob bedding and were given Prolab RMH 3500 (PMI Nutrition International LLC, W. Fisher & Son, Inc., Somerville, NJ) and water *ad libitum*. The diet was autoclaved at 121 °C for 12 min. The animals (n = 4 per cage) were allowed to acclimate for 6 days prior to the start of dosing. The three HAAs were administered in their drinking water. Each of five cages was provided with a different dose, and the sixth cage was given undosed water. The hydrochloride salts of PhIP, MeIQx, and AαC, each at a concentration of 2.5 mg/mL, were prepared in autoclaved H_2O, diluted to concentrations for each HAA at 5×10^{-6}, 5×10^{-7}, 5×10^{-8}, 5×10^{-9}, 5×10^{-10} g/mL (weight of non-protonated amine) and stored in glass watering bottles. On the basis of daily water consumption of 4 mL (26) and an average body weight of 25 g/mouse, the estimated daily dose exposures of the HAAs were: 800, 80, 8, 0.8, and 0.08 μg/kg body weight. The cage of untreated, control animals received autoclaved water. New solutions of HAAs were prepared every 7 days for the duration of the dosing study, which was conducted for 28 days. Quantitative

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analysis of HAAs in the water samples by LC-ESI/MS/MS was conducted to confirm that the HAAs were stable over time (data not shown).

On day 29, all 24 mice were euthanized with CO_2, followed by cervical dislocation. The backs of the mice were humidified with isopropanol:H_2O (7:3), and the fur was clipped down to the skin, using electric clippers with a 40-gauge blade. We elected not to shave the fur on the back of the mice prior to dosing of the HAAs because previous studies (17,18), reported that the regrowth of fur on the shaved backs of mice did not occur uniformly and that the rate of growth varied greatly between animals. A time-course study in mice showed that all of the ^{14}C-labeled-PhIP was cleared from the hair roots at 7-days post-treatment and was present in the distal portion of the hair shafts 4 weeks after the exposure (27). Hence, the bulk of the hair shaft retrieved from mice exposed to HAAs in our study is expected to contain significant levels of HAAs (vide infra). The livers were quickly excised, rinsed in cold saline, and snap frozen in a dry-ice / isopropanol bath. The colon (ileo-cecal junction to the rectum) was cut longitudinally, and the excised tissues were washed in PBS, to remove fecal contents, and then soaked 15–20 minutes in PBS containing 1.5 mM EDTA, 3,000 U heparin/L, DTT (80 mg/L) and phenylmethanesulphonyl fluoride (40 mg/L) prior to scraping of the mucosal epithelial cell layer, which was quick frozen in a dry-ice / isopropanol bath.

Isolation of HAAs from Human Hair and Rodent Fur

Since the growth cycles of individual hair follicles are asynchronous (16) and can lead to variable uptake of HAAs, hair shafts and fur samples were finely minced with professional electric hair clippers to a length of 1 – 2 mm, to obtain a within-sample homogenous blend. Aliquots of human hair (50 mg) or rodent fur (10 mg) were washed with 0.1 N HCl (1 mL) by vortexing for 30 s in an Eppendorf tube, followed by centrifugation and removal of the supernatant. This washing procedure was repeated three times. Thereafter, CH_3OH (1 mL) was added to the hair/fur samples and the same washing procedure was conducted three times. The hair/fur samples were allowed to dry in a ventilated hood for 30 min to remove residual CH_3OH. Thereafter, 1N NaOH (1 mL) and HAA internal standards (50 pg in 50 μL H_2O) were added. The hair matrix was digested by heating at 80 °C for 1 h (human hair) or 2 h (rodent fur). Upon cooling, the HAAs were isolated from the digested hair matrix, by solvent extraction with ethyl acetate (2 × 5 mL). The organic fraction was processed by SPE, employing a Waters Oasis MCX resin (30 mg) and the solvent conditions used for the isolation of HAAs from human urine (28). The purified extracts were resuspended in 0.1% HCO_2H:CH_3OH (1:1) (30 μL) and assayed by LC-ESI/MS/MS (*vide infra*). The efficacy of the washing procedure to remove any HAAs that may reside on the external surface of the hair/fur shaft was assessed by spiking vegetarian hair or control fur of untreated rodents with 1000 pg of unlabeled HAAs prior to conducting the washing procedure. The ensuing LC/MS measurements of the base-hydrolyzed hair samples revealed that >95% of the added HAAs were removed by this washing procedure (data not shown).

Spectrophotometic Characterization of Melanin in Hair and Fur

Hair (5 mg) and fur (1 mg) were digested in a mixture of Soluene 350: H_2O (9:1 v/v, 1 mL), by heating at 95 °C for 1 h (hair) or 2 h (fur). Upon cooling, the spectra were recorded over the wavelength range of 400 to 800 nm with an Agilent 8453 Model UV/VIS spectrophotometer. The absorbance at 500 nm represents the total amount of melanin (eumelanin and pheomelanins), and the A_{650}/A_{500} ratio reflects the proportion of eumelanin to total melanin in the hair sample (29). The estimate of melanin was based on the absorbance of 100 μg/mL of melanin (eumelanin) corresponding to 0.99 at 500 nm (29).

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Isolation of Liver and Colon DNA

Livers (500 mg) or colon epithelia (entire sample) were homogenized in 3 volumes of TE buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0). The nuclear pellet was obtained by centrifugation at 2500 g for 10 min at 4 °C and was diluted with 1 mL TE buffer, followed by digestion with RNase A (37.5 μL, 10 mg/mL) for 2 h at 37°C. Proteinase K (35 μL, 20 mg/mL) and SDS (0.5% w/v) were added, and the incubation continued for 2 h at 37°C. DNA was isolated by phenol/chloroform extraction (30).

Enzyme Digestion and SPE Enrichment of DNA Adducts

The enzymatic digestion conditions used for the hydrolysis of DNA to 2′-deoxynucleosides were previously described (21,22), and employed DNase I for 1.5 h, followed by incubation with nuclease P1 for 3 h, and lastly incubated with alkaline phosphatase and phosphodiesterase for 18 h. Thereafter, 3 volumes of cold C_2H_5OH (200 proof) were added, to remove protein and salts. The supernatant containing adducts was concentrated and processed by SPE as previously described (31).

LC-ESI/MS/MS Analysis of HAAs in Hair and Fur

Chromatography was performed with an Agilent 1100 Series capillary LC system (Agilent Technologies, Palo Alto, CA) equipped with an Aquasil C18 column (0.5 × 250 mm, 5 μm particle size) from Thermo Scientific (Bellefonte, PA). Analytes were separated on a gradient. The A solvent contained 0.1% HCO_2H, 10% CH_3CN in H_2O, and the B solvent contained 0.1% HCO_2H, 5% H_2O in CH_3CN. The flow rate was set at 12 μL/min, starting at 100% A and holding for 1 min, followed by a linear gradient to 100% B at 30 min. The gradient was reversed to the starting conditions over 1 min and a post-run time of 8 min was required for re-equilibration. The mass spectral data were obtained on a Finnigan™ Quantum Ultra Triple Stage Quadrupole MS (TSQ/MS) with an Ion Max electrospray ionization source operated in positive ionization mode (Thermo Fisher, San Jose, CA). The instrument operations and and data manipulations were controlled by Xcalibur version 2.07 software.

Representative optimized instrument tune parameters used were as follows: capillary temperature 275 °C; source spray voltage 4.0 kV; sheath gas setting 35; tube lens offset 95; capillary offset 35; and source fragmentation 10 V. The spray voltage was set at 1500 V; the in-source fragmentation was −10 V; and the capillary temperature was 300 °C. The sheath gas was set at 3 arbitrary units. Quantitative measurements were done by selected reaction monitoring (SRM). The peak and scan widths (in Q1 and Q3) were set at 0.7 Da. The following transitions and collision energies were used for the quantification of HAAs: PhIP and [^2H_3C]-PhIP: 225.1 → 210.1 and 228.1 → 210.1 @ 33 eV; MeIQxx and [^2H_3C]-MeIQx: 214.1 → 199.1 and 217.1 → 199.1 @ 31 eV; AαC and [$^{13}C_6$]-AαC: 184.1 → 167.1 and 190.1 → 173.1 @ 28 eV. The dwell time for each transition was 10 ms. Argon was used as the collision gas and was set at 1.5 mTorr. Product ion spectra were acquired on the protonated molecules $[M + H]^+$ scanning from *m/z* 100 to 500 at a scan speed of 500 amu/s, using the same acquisition parameters.

LC-ESI/MS/MS Analysis of HAAs in Rodent Chow

The fur of the untreated, control rodents was found to contain PhIP (*vide infra*). Therefore, HAAs were assayed in the rodent chow, to determine if the chow was a potential source of contamination. The rodent chow (2 g) was dissolved in 1 N NaOH (10 mL), and 5 ng of HAA internal standards were added. The HAAs were isolated by tandem SPE (3). The chow was mixed to a homogenous paste and interspersed with the Extrelut-20 resin (12 g), which was then placed into a cartridge connected in series to a Waters Oasis MCX resin (150 mg). The resin was washed with dichloromethane:toluene (95:5), and the HAAs were recovered from

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the MCX resin as previously described (3). The quantitative LC-ESI/MS/MS measurements were conducted with the TSQ/MS, employing the same conditions described above for measurements of HAAs in hair samples.

LC-ESI/MS/MS^3 Analyses of HAA-DNA Adducts

Chromatography was performed with the Agilent 1100 Series capillary LC system described above and equipped with an Aquasil C18 column (0.32 × 250 mm, 5 μm particle size). Samples (2 μL) were injected, and analytes were separated with a gradient. The solvent conditions were held at 100% A (solvent composition: 0.01% HCO_2H and 10% CH_3CN) for 2 min, followed by a linear gradient to 100% B (solvent composition: 95% CH_3CN containing 0.01% HCO_2H) over 30 min at a flow rate of 6 μL/min. The MS instrumentation was a Finnigan™ LTQ mass spectrometer (Thermo Fisher, San Jose, CA). The Xcalibur Version 2.07 software was used for data manipulations. Analyses were conducted in the positive ionization mode and employed an ADVANCE nanospray source from Michrom Bioresource Inc. (Auburn, CA). Representative optimized instrument tuning parameters were as follows: capillary temperature 225 °C; source spray voltage 3.5 kV; source current 0.5 μA; no sheath gas, sweep gas or auxiliary gas was employed; capillary voltage 40 V; tube lens voltage 110 V; and in-source fragmentation 10 V. One μscan was used for data acquisition. The automatic gain control settings were full MS target 30,000 and MS^n target 10,000, and the maximum injection time was 50 ms. The MS/MS scan mode produced the aglycone ion BH_2^+ adducts $[M + H - 116]^+$ or $[M + H - 121]^+$ from the protonated dG-HAA adducts or $[^{13}C_{10}]$dG-HAA adducts, respectively. The MS^3 product ion spectra of the aglycones were acquired from *m/z* 125 to 400. The normalized collision energies were set at 24 and 34, and the isolation widths were set at 4.0 and 1.5 Da, respectively, for the MS^2 and MS^3 scan modes. The activation Q was set at 0.35 and the activation time was 30 ms for both transitions. Helium was used as the collision damping gas in the ion trap and was set at a pressure of 1 mTorr. The quantification of DNA adducts was done at the MS^3 scan stage.

Quantitation of DNA Adducts

DNA adducts were quantitated at the MS/MS3 scan stage, employing either the top 2 or 3 most abundant product ions of the BH_2^+ adducts. All mouse colon samples were spiked with internal standards at a level of 3.0 adducts per 10^8 DNA bases, and the amount of DNA employed for analyses was on average 50 μg. Liver samples from the control and low-dose treated mice (0.08, 0.8, and 8 μg HAA/kg body weight), employed 100 μg DNA for assay and were spiked with internal stndards at a level of 3.0 adducts per 10^8 DNA bases. The high-dose samples (80 and 800 μg HAA/kg body weight) employed 25 μg DNA and were spiked with internal standards at 3.0 adducts per 10^7 DNA bases. External calibration curves were employed to estimate DNA adduct levels. Internal standards (9.1×10^{-15} mol/2 μL, 4.1 – 4.4 pg/20 μL), corresponding to a level of internal standard at 3 adducts per 10^8 DNA bases for 100 μg DNA (303 nmol deoxynucleosides), and unlabeled HAAs at eight calibrant levels (0, 0.15, 0.44, up to 44 pg/20 μL, corresponding 0, 0.1, 0.3, up to 30 adducts per 10^8 bases for the equivalent of 100 μg of DNA) were assayed. The calibration curves were done in triplicate with 2 μL of the calibrants, and the data were fitted to a straight line (area of response of the adduct/internal standard versus the amount of adduct/internal standard) using ordinary least-squares with equal weightings. The coefficient of determination (r^2) values of the slopes exceeded 0.99. The accuracy and precision of the method was previously reported for PhIP (22). The limit of detection (LOD) and limit of quantification (LOQ) (32) of the adducts were, respectively ~ 1 and 3 adducts per 10^9 DNA bases, when 50 μg DNA were used for analysis.

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Quantitative Measurement of HAAs in Human Hair, and Performance of the Method

External calibration curves were used for quantitation of HAAs in human hair and rodent fur. The curves were conducted in triplicate employing a fixed amount of [^2H_3C]-PhIP, [^2H_3C]-MeIQx or [$^{13}C_6$]-AαC (50 pg) and 0, 2.5, 5, 10, 15, 20, or 40 pg of unlabeled HAAs. For the determination of HAA levels in rodent fur exposed to the three highest dose levels of HAAs, the calibrants for HAAs included 100, 500, and 5,000 pg unlabeled HAAs. The coefficient of determination (r^2) values of the slopes of the calibration curves exceeded 0.99. The within-day and between-day precisions for PhIP were calculated in triplicate or quadruplicate as described (33), with hair samples from two subjects. The measurements were repeated on three separate days. The LOQ was defined to be the lowest concentration required to give a signal equal to the average intensity of the blank signal plus 10 times the standard deviation (SD) of the blank (32). The blank was a hair sample of a vegetarian.

Statistical Methods

The intraclass correlation coefficient (ICC), the proportion of the overall variance attributed to between sources, was used as a measure of reproducibility (34) and the % coefficient of variation (%CV), the SD divided by the mean × 100%, was used as a measure of precision. The dose-response relationship was examined in a linear regression of HAA value on dose, where the doses were coded consecutively from 0 to 5; the Wald test for the slope parameter was used to test significance. Log transformation of HAA values, as log(x+1), was used in the computation of the ICC and the regressions, to better meet model assumptions. The Pearson's correlation coefficient, unadjusted as well as adjusted for dose, was used to assess the correlation between different HAA levels in fur and DNA adducts in colon and liver, as well as levels of a particular HAA (e.g., PhIP) across tissues (34). P-values <0.05 are considered statistically significant.

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RESULTS

HAA Biomarkers

The chemical structures of HAAs and the DNA adducts employed as biomarkers in this study are shown in Figure 1.

LC-ESI/MS/MS Analysis of PhIP in Human Hair

The chromatograms of the SRM traces of PhIP and [^2H_3C]-PhIP isolated from hair samples of a vegetarian and a meat-eater are presented in Figure 2. A peak corresponding to PhIP (t_R 15.7 min) is readily discerned in the chromatogram of the meat-eater. The small peak observed in the chromatogram of the vegetarian is attributed to the isotopic impurity of [^2H_3C]-PhIP, which contained PhIP at a level of 3% contamination. The product ion spectrum of the analyte obtained from the hair sample of the meat-eater is in excellent agreement with the spectrum of the synthetic standard and confirms the identity of the analyte as PhIP. The amount of PhIP was estimated at 675 pg/g hair for the meat-eater. MeIQx and AαC were not detected in any of the hair samples obtained from any of the meat-eaters or vegetarians (<50 pg/g hair, data not shown).

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Performance of the Analytical Method

The accuracy and precision of the analytical method were determined by spiking hair samples of a vegetarian with HAAs, prior to base hydrolysis, at a concentration of 0, 100, 400 or 800 ppt (Table 1). All of the HAAs were detected in the spiked hair samples (Supporting Information, Figure S-1). The mean estimates at each spiked dose of PhIP, AαC and MeIQx were within 11% of the target value, other than the estimate of MeIQx, at the lowest level of spiking, which was within 30%. The average accuracy across the spiked doses of all three

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HAAs was within 10% of the target value, and the precision, defined (%)CV, was <10%, at spiking levels of 400 ppt and above, and <20% for 100 ppt.

The reproducibility of the method was assessed by measuring PhIP levels in hair samples of two meat-eaters per day across 3 days. The results are summarized in Table 2. The within-day and between-day precision values (%)CV in estimates of PhIP were ≤8.1% and 12.9%, respectively. The ICC for log PhIP was 0.93 for within-day levels, where the 6 days were treated as independent, and 0.92 for between-day levels, with the daily mean values used for each subject. Thus, the analytical method is accurate and precise; the intra- and inter-day estimates for the levels of PhIP in hair are highly reproducible.

The variation in the levels of PhIP in hair of these two subjects collected from three different haircuttings collected over a 6-month interval are reported in Table 3. The levels of PhIP accumulated in hair were fairly constant overtime: the mean values were within 24% of one another. The average levels for the three different time points were not statistically different (p=0.83), when analysis of variance techniques were used after adjustment for subject level means. Additionally, the ICC was 0.83 among month levels, using the daily mean for each subject, indicating good agreement over the 6-month interval. The PhIP level was log-transformed in the analyses above, so that it would better meet model assumptions.

Estimate of HAAs in Human Hair and Fur of Female C57BL/6 Mice

The hair samples from six meat-eaters, six vegetarians, and the fur of four female C57BL/6 control mice fed standard chow were assayed for HAAs (Table 4). PhIP was identified in hair samples of all meat-eaters. The levels of PhIP were below the LOQ in the hair samples of five of the vegetarians, whereas the amount of PhIP in the hair of the one vegetarian who tested positive was just above the LOQ (65 pg/g). The level of PhIP in hair of meat-eaters ranged from 290 to 890 pg/g hair, or from 9.1 to 64 ng/g melanin, when adjusted for melanin content.

PhIP was unexpectedly detected in the fur of all four control mice, at levels ranging from 680 to 750 pg/g fur, or from 7.9 – 9.4 ng/g melanin (Table 4). MeIQx and AαC were not detected (<50 pg/g fur). The mouse chow, which had been sterilized by autoclaving at 121 °C for 15 min, was found to contain PhIP (0.9 ± 0.1 ppb); however, MeIQx and AαC were below the LOQ (<0.1 ppb). Assuming that the mice consume 4 g of chow per day (26) and that the bioavailability of PhIP is 100%, then the exposure to PhIP via the diet corresponds to about 144 ng of PhIP/day/kg body weight; this dose is ~2-fold greater than the lowest dose of PhIP given in the water supply during the subchronic study. However, all mice in the study, experimental and control, were exposed to this background level of PhIP.

Relationships between the Levels of HAAs Accumulated in Fur and DNA Adduct Formation in Liver and Colon of Female C57 Black Mice, following Subchronic Dosing of HAAs

PhIP was detected in fur of mice treated at all doses (0.08 – 800 μg of HAA/day/kg body weight), whereas greater doses of AαC (≥0.8 μg/day/kg body weight) and MeIQx (≥8 μg/day/kg body weight) were required to accumulate these HAAs in fur at levels above the LOQ. The LC-ESI/MS/MS chromatograms of HAAs in rodent fur at the dose corresponding to the LOQ are presented in Supporting Information, Figure S-2.

There was a strong positive dose-response relationship between dosage and each of the HAAs accumulated in fur, with each of the three p-values for the slopes <0.0001 (Figure 3). The mean PhIP levels in fur were significantly higher than in the mean MeIQx levels in fur at all doses, and they were greater than the mean AαC levels in fur at all but one dose (8×10^{-7} g/kg body wt), with p-values <0.0001. When the mean background level of PhIP in fur, attributed to the PhIP contamination in the animal feed, was subtracted from all values, the resulting mean PhIP

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levels in fur were still significantly higher than the mean MeIQx levels in fur at the doses of 8×10^{-8} and 8×10^{-7} g/kg body wt, and significantly higher than the mean AαC level in fur at the dose of 8×10^{-8} g/kg body wt ($p < 0.0001$). However, at the higher doses, the resulting mean PhIP levels in fur were significantly lower than the mean levels for MeIQx and AαC (p values <0.0001). Therefore, PhIP appears to have a stronger binding affinity to fur than does either AαC or MeIQx at lower doses, but not at higher doses.

The between-animals variation in the estimates of HAAs accumulated in hair was large, up to 50% for some doses, but the precision in measurements of HAAs in fur within-animals was on average <10% (%)CV. This wide range in HAAs accumulated in fur may be attributed to the variable rate of metabolism of HAAs between animals: the slower the rate of hepatic metabolism of HAAs, the higher the levels of unaltered HAAs in the blood stream that can bind to pigments and proteins in the growing cells at the base of the hair follicle (16). A large between-animals variation in the rate of urinary excretion of PhIP and its metabolites has been previously observed within the first 6 h of dosing of C57BL/6 mice, at exposure levels to PhIP that were similar to those employed in our study (35), demonstrating that significant between-animal differences exist in the rates of PhIP metabolism.

The dG-C8 adducts of PhIP, MeIQx, and AαC are the principal lesions of these HAAs (36) (Figure 1). On the basis of DNA adduct formation, AαC was the most potent liver genotoxicant among the three HAAs investigated. The LC-ESI/MS/MS^3 chromatograms of liver DNA adducts in the untreated and exposed mice at the dose concentration required to produce DNA adducts at or above the LOQ are presented in Figure 4. The product ion spectra of the adducts and internal standards are in excellent agreement (Supporting Information, Figure S-3). dG-C8-AαC adduct was formed at 3.9 ± 0.6 adducts per 10^9 DNA bases, a level which was above the LOQ, at a subchronic dose concentration of 8 µg AαC/day/kg body weight. dG-C8-MeIQx and dG-C8-PhIP were formed in liver at levels above the LOQ at doses ≥80 µg HAA/day/kg body weight. The mean AαC-DNA adduct levels in liver was significantly higher than the mean PhIP- and MeIQx-DNA adduct levels in liver, at doses ≥80 µg HAA/kg/day body wt and higher (p-values <0.03 at 80 µg/kg body wt and <0.0001 at 800 µg/kg body wt), indicating that, in terms of bioactivation, AαC is the most efficient among these three compounds. DNA adducts of AαC and PhIP were detected in colorectum at doses ≥80 µg HAA/day/kg body wt; MeIQx adducts were not detected in colorectum at any dose (Figures S-6 – S-8) At the highest dose (800 µg HAA/day/kg body wt), the mean level for PhlP-DNA adducts was significantly higher than for AαC adducts ($p < 0.0001$).

The levels of HAA accumulated in fur and the levels of HAA-DNA adducts formed in liver and colon showed a clear dose-response relationship (Figure 3); however, the extent of accrual of HAAs in fur was not correlated with the level of adduct formation, when adjusted for dose. For example, the correlation r values (r's) were 0.75 and 0.80 between the levels of PhIP accumulated in fur and levels of PhIP-DNA adducts in liver and colon, respectively, without adjustment for dose, but the correlations were −0.07 and 0.25 after adjustment for dose. It should be noted that the adjusted correlations assume that the relationship between the HAA levels in fur and DNA adduct levels is similar across dose. The small sample size within dose ($n = 4$) does not allow for good estimation of correlation within dose. However, there is little evidence that the four mice within each dose rank similarly on the HAA and DNA adducts. Similarly, the r values were 0.66 and 0.70 between the levels of AαC found in fur and AαC-DNA adduct levels in liver and colon, respectively, before adjustment, and −0.19 and 0.28 after adjustment. The r value between MeIQx levels in fur and liver adducts was 0.67 before adjustment and −0.18 after adjustment. None of the adjusted correlations were significant. Therefore, HAA accumulation in fur is a poor correlate for DNA adduct formation in liver or colorectum.

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DISCUSSION

A rapid and facile tandem solvent/SPE clean-up method has been devised to isolate PhIP, MeIQx, and AαC from hair. Because of the high sensitivity of the TSQ MS instrument, only 50 mg of hair are required for chemical analysis. This extraction procedure is much less labor intensive and time-consuming than two other previously reported isolation methods, which required 0.2 – 2 g of hair for assay (17,19,37). All three methods employ base hydrolysis to digest the hair matrix and liberate the HAAs. We observed that the base hydrolysis digestion conditions previously used (100 °C for 45 min) resulted in the complete decomposition of AαC, in our study. Moreover, a significant amount of H-D isotopic exchange occurred for [$^{2}H_{3}C$]-MeIQx and [$^{2}H_{3}C$]-PhIP: the levels of H-D exchange were as high as 20%. Lowering the temperature of hydrolysis to 80 °C, in the present study, preserved AαC, and there was no detection of H-D exchange for either [$^{2}H_{3}C$]-MeIQx or [$^{2}H_{3}C$]-PhIP, even if the time of hydrolysis was increased to 3 h (data not shown).

In our human pilot study, PhIP was detected in hair samples of all six meat-eaters, but only detected in hair of one out of six vegetarians: the level of PhIP in that hair sample was just above the LOQ (65 pg/g). These preliminary data suggest that the exposure to PhIP occurs primarily through consumption of cooked meats or poultry, and that non-meat derived sources of exposure to PhIP (38,39) are probably negligible. MeIQx and AαC were not detected in hair from any of the meat-eaters or vegetarians. The extent of exposure to PhIP, MeIQx or AαC is unknown for these subjects; however, PhIP is recognized to be the most-mass abundant HAA formed in meats and poultry cooked well-done (2,3). Our data and those data previously published (19,40) reveal that a very high percentage of meateaters have quantifiable amounts of PhIP in their hair.

The average daily exposure of PhIP in the USA is about 6 ng/kg body wt (41), an exposure that is ~20-fold lower than the dose of PhIP consumed by mice via the contaminated chow. Thus, a higher percentage of the PhIP dose appears to be bound to human hair than mouse fur, when dose exposure is expressed as ng/kg body weight (Table 4). The calculated half-life of PhIP in plasma of C57BL/6 mice dosed at 40 ng/kg body weight was estimated as 1.1 h (35), and the plasma half-life of PhIP in humans consuming a comparable dose of PhIP in well-done cooked beef was calculated as 3.6 h (5). The slower clearance of PhIP in humans may be reflective of the higher amounts of PhIP available for incorporation in human hair than as opposed to mouse fur. The exposure, based on body surface area (ng/cm^2), can also be used to extrapolate the PhIP dose and its bioaccumulation in hair between species. The conversion of the dose from body weight to surface area translates to an approximate exposure of PhIP via the contaminated chow at about 0.076 ng PhIP/cm^2 for mice versus an average dose of 0.023 ng PhIP/cm^2 for a human weighing 70 kg (42). When the exposure to PhIP is expressed as surface area, the levels of PhIP accumulated in rodent fur and human hair (pg/g hair or ng/g melanin) are closer than when dose is expressed as ng/kg body weight (Table 4).

The subchronic dosing studies in the mouse revealed that the minimal concentrations of AαC and MeIQx required to detect their presence in fur at levels above the LOQ were, respectively, about 10-fold and 100-fold higher than the dose of PhIP (Figures 3). Neither MeIQx nor AαC was detected in human hair samples, indicating that the levels of dietary exposure to these HAAs (41) are below the threshold levels at which their accumulation in hair can be detected. Therefore, we would not expect the biomonitoring of PhIP in human hair to be a reliable surrogate biomarker to predict the exposure to other HAAs. However, the levels of PhIP in hair, when adjusted for melanin content, were reported to correlate with the individual intake of two other HAA dietary carcinogens, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline and the tryptophan pyrolysate Trp-P-1 (but not MeIQx), that were estimated from a food frequency questionnaire (FFQ), in a pilot study conducted in Tokyo (43).

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The levels of PhIP measured in hair of subjects in our pilot study are within the same range of the levels of PhIP previously detected in hair of subjects in Norway (60 – 7500 pg/g hair) (40) and in Japan (180 – 3600 pg/g hair) (20). The wide range of PhIP accumulated in hair is due to several variables. The different concentration of PhIP in the diet constitutes one critical variable. The pharmacokinetics and metabolism of PhIP represents another important variable. There are large inter-individual differences in the hepatic P450 1A2 protein content, which can vary by more than 50-fold (44); this enzyme accounts for approximately 70% of the metabolism of PhIP in humans (5). Thus, the amount of unmetabolized PhIP in the blood stream that reaches the hair follicle, following first-pass metabolism, can widely range and may affect the levels of PhIP accrued in hair. As noted earlier, the pigmentation of hair also affects the amount of PhIP incorporated into hair. PhIP has a high binding affinity for eumelanin (45), a pigment which is a more predominant in black hair than in lighter-colored hair (29). Indeed, the levels of PhIP accumulated in fur of mice with black pigmentation are far greater than the levels accumulated in mice with brown-, grey- or yellow-pigmented fur (45). Thus, hair pigments and the level of eumelanin are likely to affect the extent of PhIP incorporation into human hair, and the accumulation of PhIP in hair of subjects with gray hair, which has less melanin content than colored hair, may be an age-dependent issue that also requires consideration.

Beyond the 12 subjects from the pilot study, none of whom used hair dye, we assayed the PhIP content in hair samples from several hair-dye users. However, the peak attributed to PhIP was low in intensity, and isobaric interferences prevented us from obtaining reliable estimates of the PhIP content (data not shown). Approximately 25% of the male population and 42% of the female population have been reported to use hair dyes in the United States, Europe, and Japan (46), with permanent hair dye being the most commonly used hair-dye product. The aromatic amine 4-aminobiphenyl (4-ABP) is present in some permanent hair dyes (47), and oxidation products of 4-ABP, such as 4-nitrobiphenyl, hydrazo-, azo- azoxy, or mixed dimers are formed (47), under the oxidizing conditions used to develop the desired dye colors (46,48). A significant portion of the PhIP bound in the hair-shaft also may undergo similar oxidation reactions during the dye development process and would escape detection with our current analytical technique. The pretreatment of dyed-hair samples with reducing agents can cause mixed dimers and nitro derivatives to revert back to their parent amines (47). This chemical treatment of hair dye samples may be utilized to develop a robust and quantitative assay to measure for PhIP in dyed-hair samples. Surprisingly, PhIP was detected in many dyed-hair samples, when assayed by LC-ESI/MS in the selected ion monitoring mode (19); this mode of analysis is less specific than the SRM mode employed in our study. The nature of the type of hair-dye treatments used by those subjects was not reported (19,20).

We examined the relationship between the level of HAAs accumulated in rodent fur and the extent of DNA adduct formation in liver and colon, target tissues of DNA damage by these genotoxicants. The relationship between HAAs present in hair and DNA adducts is tenuous: the accumulation of HAAs in fur represents a portion of the dose of the unmetabolized compounds, while DNA adducts represent a portion of the dose that undergoes metabolic activation. Moreover, the levels of HAA-DNA adducts formed in liver and colon are influenced by the relative amounts and activities of phase I and phase II enzymes expressed in these organs that metabolize HAAs; the balance between the enzymes involved in bioactivation and those involved detoxication, in these two organs, are likely to be different among PhIP, MeIQx, and AαC. DNA repair processes and cell turnover will also influence the level of DNA adducts. The DNA adducts are assumed to be at or approaching a steady-state level after subchronic dosing for 28 days (49). For the mouse model, the HAA hair biomarker poorly predicts the extent of DNA damage in either liver or colorectum, when adjusted for dose.

In summary, a facile and robust method has been established to quantitate PhIP in human hair. This hair biomarker represents an integrated exposure to PhIP over a time period of weeks to

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months. Biomonitoring the levels of PhIP in hair may be a superior means by which to assess dietary exposures than is the conventional FFQ (50), which is especially problematic when exposure to the compound of interest spreads over a range of food items at varying levels of concentrations. The accuracy of the FFQ is particularly challenging in the assessment of levels of PhIP formation, a process highly dependent on the type of meat cooked, as well as the method, temperature, and duration of cooking; these variable parameters can lead to differences of PhIP (or other HAA concentrations) by more than 100-fold (3,51). Studies that examine the interrelationship among dietary exposure to PhIP, P450 1A2 activity, and the melanin content of hair are required in order for us to determine how these factors affect the level of PhIP accumulated in hair, and the reliability of this hair biomarker as an accurate measure of dietary intake of PhIP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

4-ABP, 4-aminobiphenyl
MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
AαC, 2-Amino-9*H*-pyrido[2,3-*b*]indole
PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
dG, 2′-deoxyguanosine
dG-C8-MeIQx, *N*-(deoxyguanosin-8-yl)-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
dG-C8-AαC, *N*-(deoxyguanosin-8-yl)-2-amino-9*H*-pyrido[2,3-*b*]indole
dG-C8-PhIP, *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
dR, deoxyribose
CID, collision-induced dissociation
FFQ, food frequency questionnaire
HAA, heterocyclic aromatic amine
ICC, intraclass correlation coefficient
LC-ESI/MS/MS, liquid chromatography-electrospray ionization/tandem mass spectrometry
LC-ESI/MS/MS^n, liquid chromatography-electrospray ionization/multi-stage mass spectrometry
LOD, limit of detection
LOQ, limit of quantification
LTQ, Finnigan™ LTQ 2-D linear ion trap mass spectrometer
ppt, parts per trillion
QIT/MS, quadrupole ion trap mass spectrometry
SPE, solid phase extraction
SRM, selected reaction monitoring
TSQ/MS, Finnigan™ Quantum Ultra Triple Stage Quadrupole MS

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Reference List

1. Sugimura T, Wakabayashi K, Nakagama H, Nagao M. Heterocyclic amines: Mutagens/carcinogens produced during cooking of meat and fish. Cancer Sci 2004;95:290–299. [PubMed: 15072585]

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2. Felton JS, Knize MG, Bennett LM, Malfatti MA, Colvin ME, Kulp KS. Impact of environmental exposures on the mutagenicity/carcinogenicity of heterocyclic amines. Toxicology 2004;198:135–145. [PubMed: 15138037]

3. Ni W, McNaughton L, LeMaster DM, Sinha R, Turesky RJ. Quantitation of 13 heterocyclic aromatic amines in cooked beef, pork, and chicken by liquid chromatography-electrospray ionization/tandem mass spectrometry. J. Agric. Food Chem 2008;56:68–78. [PubMed: 18069786]

4. Sinha R. An epidemiologic approach to studying heterocyclic amines. Mutat. Res 2002;506–507:197–204.

5. Boobis AR, Lynch AM, Murray S, de la Torre R, Solans A, Farré M, Segura J, Gooderham NJ, Davies DS. CYP1A2-catalyzed conversion of dietary heterocyclic amines to their proximate carcinogens is their major route of metabolism in humans. Cancer Res 1994;54:89–94. [PubMed: 8261468]

6. Kidd LC, Stillwell WG, Yu MC, Wishnok JS, Skipper PL, Ross RK, Henderson BE, Tannenbaum SR. Urinary excretion of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in White, African-American, and Asian-American men in Los Angeles County. Cancer Epidemiol. Biomarkers Prev 1999;8:439–445. [PubMed: 10350440]

7. Stillwell WG, Turesky RJ, Sinha R, Tannenbaum SR. N-oxidative metabolism of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) in humans: excretion of the N^2-glucuronide conjugate of 2-hydroxyamino-MeIQx in urine. Cancer Res 1999;59:5154–5159. [PubMed: 10537291]

8. Kulp KS, Knize MG, Fowler ND, Salmon CP, Felton JS. PhIP metabolites in human urine after consumption of well-cooked chicken. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci 2004;802:143–153.

9. Dingley KH, Curtis KD, Nowell S, Felton JS, Lang NP, Turteltaub KW. DNA and protein adduct formation in the colon and blood of humans after exposure to a dietary-relevant dose of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. Cancer Epidemiol. Biomarkers Prev 1999;8:507–512. [PubMed: 10385140]

10. Magagnotti C, Orsi F, Bagnati R, Celli N, Rotilio D, Fanelli R, Airoldi L. Effect of diet on serum albumin and hemoglobin adducts of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in humans. Int. J. Cancer 2000;88:1–6. [PubMed: 10962432]

11. Friesen MD, Kaderlik K, Lin D, Garren L, Bartsch H, Lang NP, Kadlubar FF. Analysis of DNA adducts of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in rat and human tissues by alkaline hydrolysis and gas chromatography/electron capture mass spectrometry: validation by comparison with ^{32}P-postlabeling. Chem. Res. Toxicol 1994;7:733–739. [PubMed: 7696526]

12. Totsuka Y, Fukutome K, Takahashi M, Takashi S, Tada A, Sugimura T, Wakabayashi K. Presence of N^2-(deoxyguanosin-8-yl)-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (dG-C8-MeIQx) in human tissues. Carcinogenesis 1996;17:1029–1034. [PubMed: 8640908]

13. Magagnotti C, Pastorelli R, Pozzi S, Andreoni B, Fanelli R, Airoldi L. Genetic polymorphisms and modulation of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-DNA adducts in human lymphocytes. Int. J Cancer 2003;107:878–884. [PubMed: 14601045]

14. DuPont RL, Baumgartner WA. Drug testing by urine and hair analysis: complementary features and scientific issues. Forensic Sci. Int 1995;70:63–76. [PubMed: 7860037]

15. Nakahara Y, Takahashi K, Kikura R. Hair analysis for drugs of abuse. X. Effect of physicochemical properties of drugs on the incorporation rates into hair. Biol. Pharm. Bull 1995;18:1223–1227. [PubMed: 8845810]

16. Gratacos-Cubarsi M, Castellari M, Valero A, Garcia-Regueiro JA. Hair analysis for veterinary drug monitoring in livestock production. J Chromatogr. B Analyt. Technol. Biomed. Life Sci 2006;834:14–25.

17. Alexander J, Reistad R, Hegstad S, Frandsen H, Ingebrigtsen K, Paulsen JE, Becher G. Biomarkers of exposure to heterocyclic amines: approaches to improve the exposure assessment. Food Chem. Toxicol 2002;40:1131–1137. [PubMed: 12067575]

18. Hegstad S, Reistad R, Haug LS, Alexander J. Eumelanin is a major determinant for 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) incorporation into hair of mice. Pharmacol. Toxicol 2002;90:333–337. [PubMed: 12403055]

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19. Hashimoto H, Hanaoka T, Kobayashi M, Tsugane S. Analytical method of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in human hair by column-switching liquid chromatography-mass spectrometry. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci 2004;803:209–213.
20. Kobayashi M, Hanaoka T, Hashimoto H, Tsugane S. 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) level in human hair as biomarkers for dietary grilled/stirfried meat and fish intake. Mutat. Res 2005;588:136–142. [PubMed: 16289877]
21. Lin D, Kaderlik KR, Turesky RJ, Miller DW, Lay JO Jr, Kadlubar FF. Identification of N-(Deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine as the major adduct formed by the food-borne carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, with DNA. Chem. Res. Toxicol 1992;5:691–697. [PubMed: 1446011]
22. Goodenough AK, Schut HA, Turesky RJ. Novel LC-ESI/MS/MS^n method for the characterization and quantification of 2'-deoxyguanosine adducts of the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine by 2-D linear quadrupole ion trap mass spectrometry. Chem Res. Toxicol 2007;20:263–276. [PubMed: 17305409]
23. Turesky RJ, Rossi SC, Welti DH, O.Lay J Jr, Kadlubar FF. Characterization of DNA adducts formed in vitro by reaction of *N*-hydroxy-2-amino-3-methylimidazo[4,5-*f*]quinoline and *N*-hydroxy-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline at the C-8 and N^2 atoms of guanine. Chem. Res. Toxicol 1992;5:479–490. [PubMed: 1391614]
24. Paehler A, Richoz J, Soglia J, Vouros P, Turesky RJ. Analysis and quantification of DNA adducts of 2-amino-3,8- dimethylimidazo[4,5-f]quinoxaline in liver of rats by liquid chromatography/electrospray tandem mass spectrometry. Chem. Res. Toxicol 2002;15:551–561. [PubMed: 11952342]
25. Frederiksen H, Frandsen H, Pfau W. Syntheses of DNA-adducts of two heterocyclic amines, 2-amino-3-methyl-9H-pyrido[2,3-b]indole (MeA{alpha}C) and 2-amino-9H-pyrido[2,3-b]indole (A{alpha}C) and identification of DNA-adducts in organs from rats dosed with MeA{alpha}C. Carcinogenesis 2004;25:1525–1533. [PubMed: 15059926]
26. Williams, CSF. Practical Guide to Laboratory Animals. St. Louis: C.V. Mosby Company; 1976.
27. Hegstad S, Ingebrigtsen K, Reistad R, Paulsen JE, Alexander J. Incorporation of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine into hair of mice. Biomarkers 2000;5:24–32.
28. Holland RD, Taylor J, Schoenbachler L, Jones RC, Freeman JP, Miller DW, Lake BG, Gooderham NJ, Turesky RJ. Rapid biomonitoring of heterocyclic aromatic amines in human urine by tandem solvent solid phase extraction liquid chromatography electrospray ionization mass spectrometry. Chem. Res. Toxicol 2004;17:1121–1136. [PubMed: 15310244]
29. Ozeki H, Ito S, Wakamatsu K, Thody AJ. Spectrophotometric characterization of eumelanin and pheomelanin in hair. Pigment Cell Res 1996;9:265–270. [PubMed: 9014213]
30. Gupta RC, Reddy MV, Randerath K. 32P-postlabeling analysis of non-radioactive aromatic carcinogen–DNA adducts. Carcinogenesis 1982;3:1081–1092. [PubMed: 7139866]
31. Bessette EE, Goodenough AK, Langouet S, Yasa I, Kozekov ID, Spivack SD, Turesky RJ. Screening for DNA adducts by data-dependent constant neutral loss-triple stage mass spectrometry with a linear quadrupole ion trap mass spectrometer. Anal. Chem 2009;81:809–819. [PubMed: 19086795]
32. MacDougall D, Amore FJ, Cox GV, Crosby DG, Estes FL, Freeman DH, Gibbs WE, Gordon GE, Keith LH, Lal J, Langner RR, McClelland NI, Phillips WF, Pojasek RB, Sievers RE. Guidelines for data acquistion and data quality evaluation in environmental chemistry. Anal. Chem 1980;52:2242–2249.
33. Mottier P, Hammel YA, Gremaud E, Guy PA. Quantitative high-throughput analysis of 16 (fluoro) quinolones in honey using automated extraction by turbulent flow chromatography coupled to liquid chromatography-tandem mass spectrometry. J Agric. Food Chem 2008;56:35–43. [PubMed: 18078314]
34. Snedecor, GW.; Cochran, WG. Statistical Methods. Ames, IA: The Iowa State University Press; 1967.
35. Turteltaub KW, Vogel JS, Frantz CE, Shen N. Fate and distribution of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in mice at a human dietary equivalent dose. Cancer Res 1992;52:4682–4687. [PubMed: 1511434]
36. Turesky RJ, Vouros P. Formation and analysis of heterocyclic aromatic amine-DNA adducts in vitro and in vivo. J Chromatogr B Analyt. Technol. Biomed. Life Sci 2004;802:155–166.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

37. Hegstad S, Lundanes E, Reistad R, Haug LS, Becher G, Alexander J. Determination of the food carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in human hair by solid-phase extraction and gas chromatography-mass spectrometry. Chromatographia 2000;52:499–504.

38. Manabe S, Kurihara N, Wada O, Tohyama K, Aramaki T. Formation of PhIP in a mixture of creatinine, phenylalanine and sugar or aldehyde by aqueous heating. Carcinogenesis 1992;13:827–830. [PubMed: 1586994]

39. Manabe S, Suzuki H, Wada O, Ueki A. Detection of the carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in beer and wine. Carcinogenesis 1993;14:899–901. [PubMed: 8504483]

40. Reistad R, Nyholm SH, Huag LS, Becher G, Alexander J. 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in human hair as biomarker for dietary exposure. Biomarkers 1999;4:263–271.

41. Bogen KT, Keating GA. U.S. dietary exposures to heterocyclic amines. J. Expo. Anal. Environ. Epidemiol 2001;11:155–168. [PubMed: 11477514]

42. Handbook of Toxicology. Taylor & Francis, Inc.; 2001.

43. Kobayashi M, Hanaoka T, Tsugane S. Validity of a self-administered food frequency questionnaire in the assessment of heterocyclic amine intake using 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) levels in hair. Mutat. Res 2007;630:14–19. [PubMed: 17392018]

44. Turesky RJ, Constable A, Richoz J, Varga N, Markovic J, Martin MV, Guengerich FP. Activation of heterocyclic aromatic amines by rat and human liver microsomes and by purified rat and human cytochrome P450 1A2. Chem. Res. Toxicol 1998;11:925–936. [PubMed: 9705755]

45. Hegstad S, Reistad R, Haug LS, Alexander J. Eumelanin is a major determinant for 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) incorporation into hair of mice. Pharmacol. Toxicol 2002;90:333–337. [PubMed: 12403055]

46. Reisch MS. Flush with color: Continuing progress in hair dye chemistry satisfies diverse desires of young and old. Chem. Eng. News 2003;81:25–27.

47. Turesky RJ, Freeman JP, Holland RD, Nestorick DM, Miller DW, Ratnasinghe DL, Kadlubar FF. Identification of aminobiphenyl derivatives in commercial hair dyes. Chem. Res. Toxicol 2003;16:1162–1173. [PubMed: 12971805]

48. Corbett JF, Menkart J. Hair Coloring. Cutis 1973;12:190–197.

49. Frantz CE, Bangerter C, Fultz E, Mayer KM, Vogel JS, Turteltaub KW. Dose-response studies of MeIQx in rat liver and liver DNA at low doses. Carcinogenesis 1995;16:367–373. [PubMed: 7859370]

50. Kristal AR, Peters U, Potter JD. Is it time to abandon the food frequency questionnaire? Cancer Epidemiol. Biomarkers Prev 2005;14:2826–2828.

51. Sinha R, Rothman N, Salmon CP, Knize MG, Brown ED, Swanson CA, Rhodes D, Rossi S, Felton JS, Levander OA. Heterocyclic amine content in beef cooked by different methods to varying degrees of doneness and gravy made from meat drippings. Food Chem. Toxicol 1998;36:279–287. [PubMed: 9651044]

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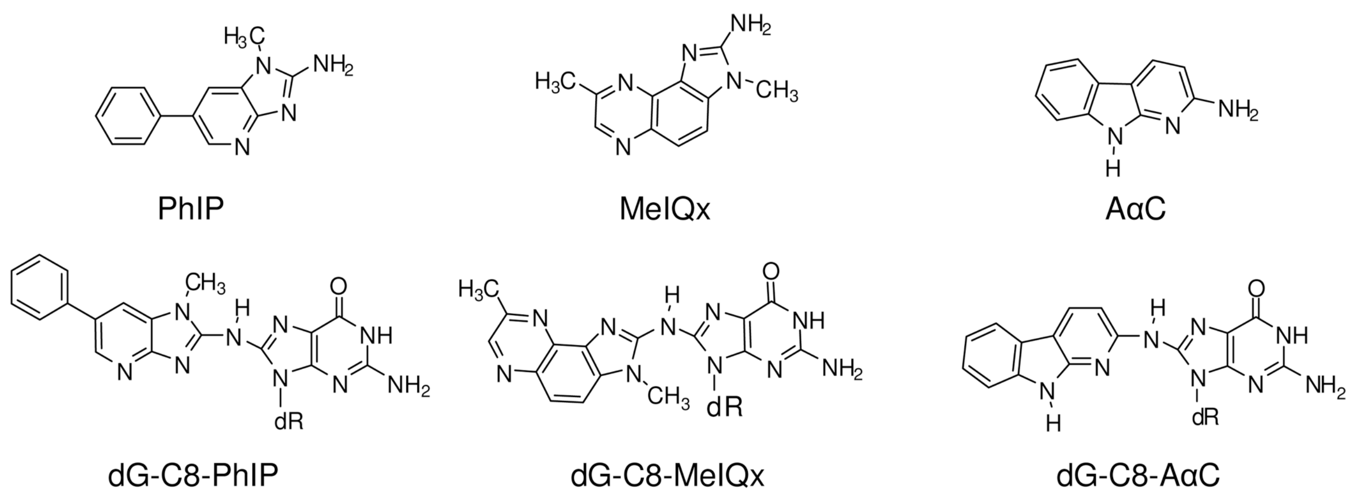

Figure 1.
Chemical structures of HAAs and principal DNA adducts employed as biomarkers

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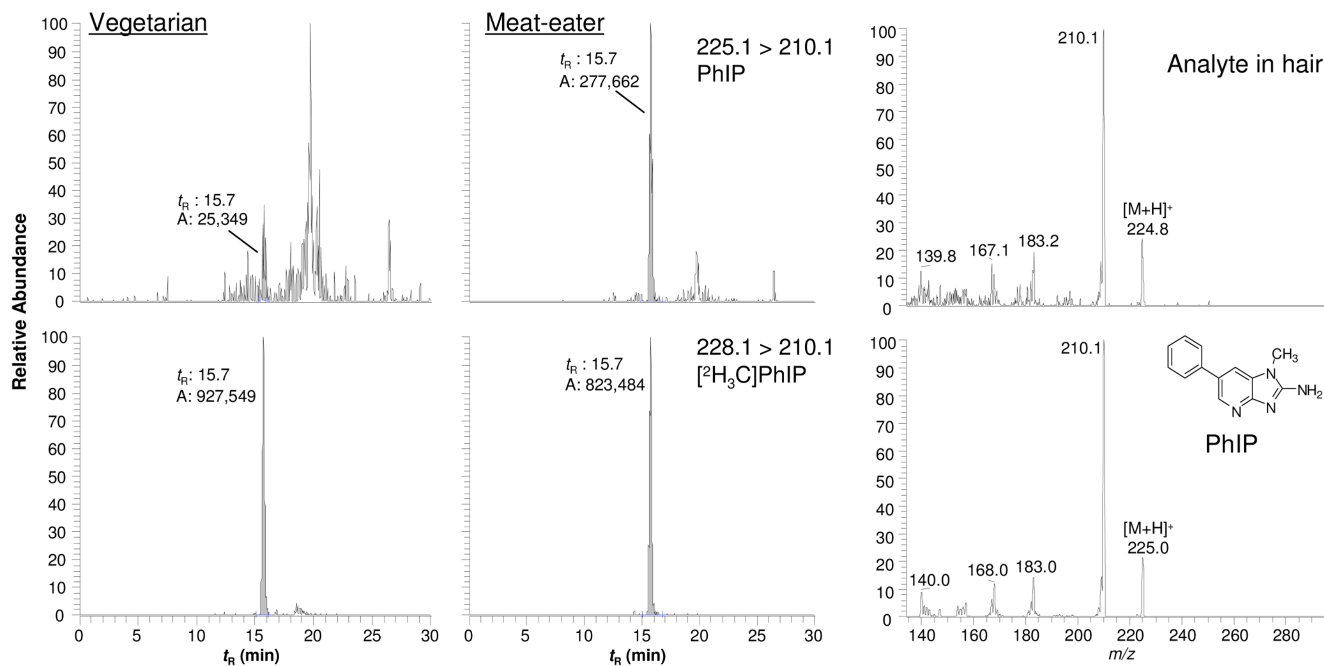

Figure 2.

The SRM traces of PhIP and [$^{2}H_{3}C$]-PhIP isolated from hair of a vegetarian (left panel) and meat-eater (middle panel) with the associated area counts (A) and t_R of the analytes. The relative abundances on the respective Y-axes of these plots are normalized to 100%. The product ion spectra of PhIP and the analyte isolated from hair of the meat-eater are presented in the right panel.

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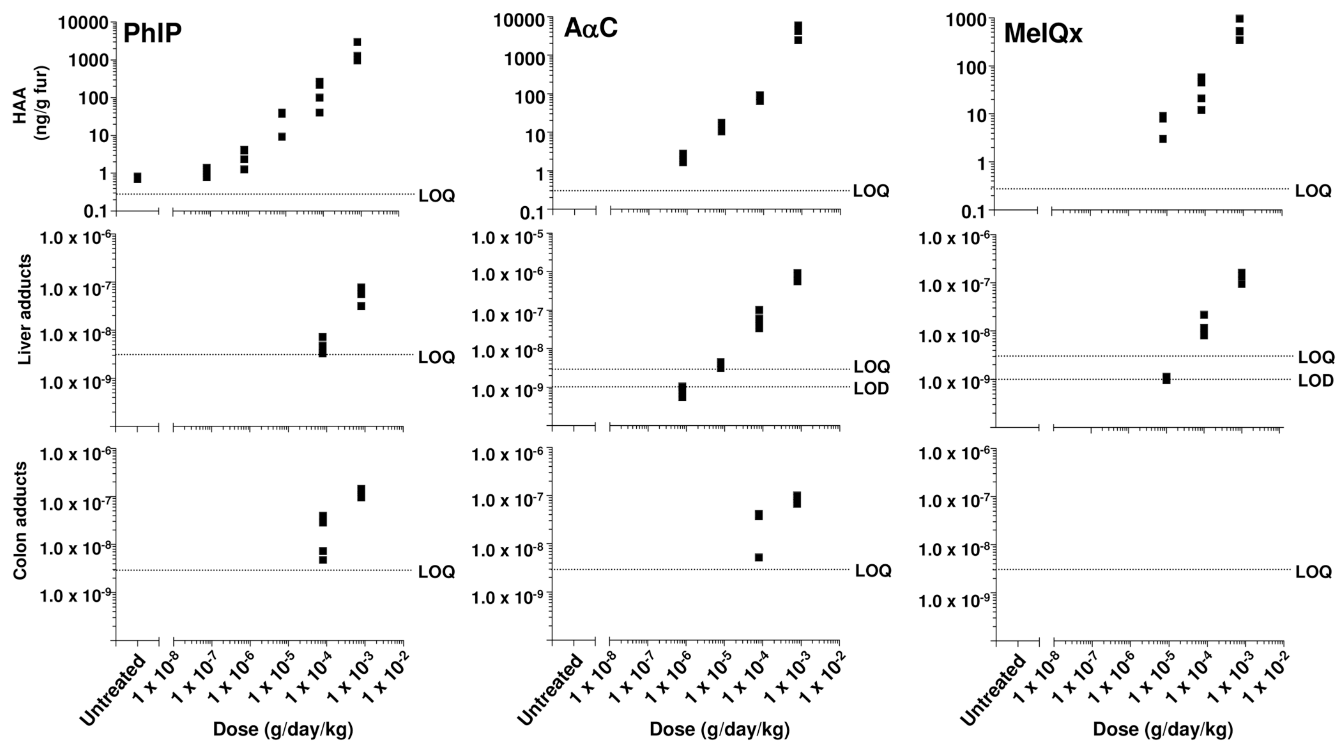

Figure 3.
Dose-response curves for PhIP, AαC, and MeIQx accumulation in mouse fur, and DNA adduct formation in liver and colon, as a function of dose, following subchronic feeding with HAAs for 28 days. MeIQx adducts were not detected in the colon.

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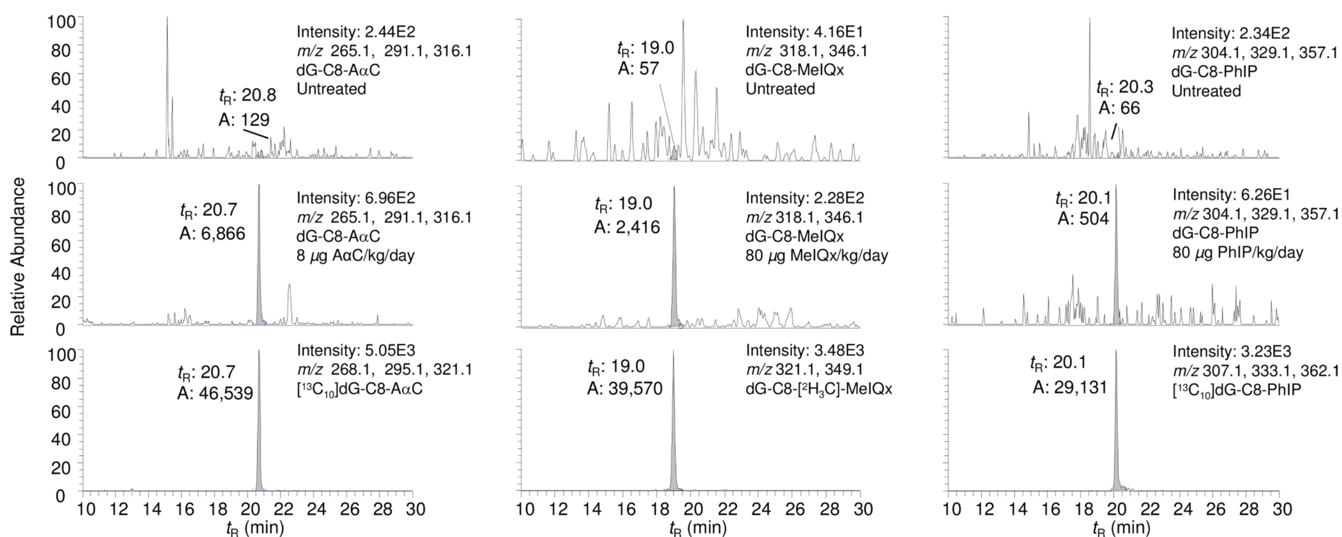

Figure 4.

Reconstructed ion mass chromatograms of the LC-ESI/MS/MS^3 traces of DNA adducts of dG-C8-AαC, dG-C8-PhIP, dG-C8-MeIQx, and their internal standards in liver DNA of untreated mice and in mice exposed to the lowest dose (AαC (8 µg/day/kg), MeIQx (80 µg/day/kg), and PhIP (80 µg/day/kg)) required to induce adduct formation at or above the LOQ. The area counts (A), t_R, and ion intensity of the analytes are noted.

Table 1

Accuracy and Precision of the Method: Estimates of HAAs (pg/g) Spiked into Hair Samples of a Vegetarian

Spiking level	PhIP	AαC	MeIQx
Unspiked	<50	<50	<50
100 ppt			
AVG	109	109	131
SD	12.8	5.7	24.4
400 ppt			
AVG	444	376	378
SD	6.6	20.9	29.8
800 ppt			
AVG	816	848	857
SD	30.8	49.4	43.1

N = 3 independent measurements, mean ± SD

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Table 2

Within-Day and Between-Day Estimates and Precisions of PhIP Measurements in Human Hair

		PhIP (pg/g hair)					
Subject		**Day 1**	**Day 2**	**Day 3**	**Overall**	**CV(%) within-day**	**CV(%) between-day**
1	Mean	522	514	483	508		
	SD	21	65	10	38		
	RSD(%)	4.1	12.6	2.1	7.4	8.1	7.8
2	Mean	896	765	712	802		
	SD	39	35	66	94		
	RSD(%)	4.3	4.6	9.2	11.7	6.1	12.9

n = 3 or 4 replicates per day

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Table 3

PhIP Levels in Hair of Two Subjects Over a 6-Month Interval

	PhIP (pg/g hair)						
		Subject 1				Subject 2	
	Month 1	Month 3	Month 6		Month 1	Month 3	Month 6
AVG	545	407	520	**AVG**	766	669	855
SD	11	32	14	**SD**	35	34	14

n = 3 or 4 independent measurements

The average levels across the three months were not statistically different ($p=0.83$), using analysis of variance techniques after adjustment for subject level means.

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Table 4

PhIP Content in Human Hair and Mouse Fur and Binding as a Function of Melanin Content

Subject	Hair Color	Melanin content (mg/g hair)	A_{650}/A_{500}	PhIP (pg/g hair)	PhIP (ng/g melanin)
Vegetarian 1	Light brown	8.2	0.27	<50	N.D.
Vegetarian 2	Brown	11.8	0.27	<50	N.D.
Vegetarian 3	Light brown	7.4	0.20	<50	N.D.
Vegetarian 4	Dark brown	12.8	0.28	<50	N.D.
Vegetarian 5	Light blonde	8.3	0.08	<50	N.D.
Vegetarian 6	Black	38.6	0.28	65 ± 5.8	1.7 ± 0.2
Meat-Eater 1	Black-gray	14.4	0.37	890 ± 40	64 ± 2.8
Meat-Eater 2	Brown	14.0	0.32	520 ± 21	37 ± 1.5
Meat-Eater 3	Brown - red	12.0	0.32	290 ± 14	24 ± 1.2
Meat-Eater 4	Black	54.8	0.33	490 ± 120	9.1 ± 2.2
Meat-Eater 5	Brown	12.5	0.32	790 ± 15	63 ± 1.2
Meat-Eater 6	Black	not assayed	not assayed	430 ± 27	not assayed
Mouse 1	Black	80.0	0.30	750 ± 65	9.4 ± 0.8
Mouse 2	Black	88.0	0.33	770 ± 75	8.8 ± 0.9
Mouse 3	Black	90.0	0.34	710 ± 165	7.9 ± 1.8
Mouse 4	Black	83.0	0.36	680 ± 180	8.2 ± 2.2

n = 3 independent measurements, mean ± SD

Mice ingested PhIP in contaminated chow (144 ng/kg body wt); humans estimated to ingest PhIP at 5.5 ng/kg body wt (40).

N.D. = Not detected