Published in final edited form as:

Chem Res Toxicol. 2007 February ; 20(2): 263–276. doi:10.1021/tx0601713.

Novel LC-ESI/MS/MSⁿ 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

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Abstract

An accurate and sensitive liquid chromatography-electrospray ionization/multi-stage mass spectrometry (LC-ESI/MS/MSⁿ) technique has been developed for the characterization and quantification of 2'-deoxyguanosine (dG) adducts of the dietary mutagen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). PhIP is an animal and potential human carcinogen that occurs in grilled meats. Following enzymatic digestion and adduct enrichment by solid-phase extraction (SPE), PhIP—DNA adducts were analyzed by MS/MS and MSⁿ scan modes on a 2-D linear quadrupole ion trap mass spectrometer (QIT/MS). The major DNA adduct, *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (dG-C8-PhIP), was detected in calf thymus (CT) DNA modified *in vitro* with a bioactivated form of PhIP and in the colon and liver of rats given PhIP as part of the diet. The lower limit of detection (LOD) was 1 adduct per 10⁸ DNA bases, and the limit of quantification (LOQ) was 3 adducts per 10⁸ DNA bases in both MS/MS and MS³ scan modes, using 27 μg of DNA for analysis. Measurements were based on isotope dilution with the internal standard, *N*-(deoxyguanosin-8-yl)-2-amino-1-(trideutero)methyl-6-phenylimidazo[4,5-*b*]pyridine (dG-C8-[²H₃C]-PhIP). The selected reaction monitoring (SRM) scan mode in MS/MS was employed to monitor the loss of deoxyribose (dR) from the protonated molecules of the adducts ([M + H - 116]⁺). The consecutive reaction monitoring (CRM) scan modes in MS³ and MS⁴ were used to measure and further characterize product ions of the aglycone ion (BH₂⁺) (Guanyl-PhIP). The MS³ scan mode was effective in eliminating isobaric interferences observed in the MS/MS scan mode and resulted in an improved signal-to-noise (S/N) ratio. Moreover, the product ion spectra obtained by the MSⁿ scan modes provided rich structural information about the adduct and were used to corroborate the identity of dG-C8-PhIP. In addition, an isomeric dG-PhIP adduct was detected *in vivo*. This LCESI/MS/MSⁿ method is the first reported application on the use of the MS³ scan mode for the analysis of DNA adducts *in vivo*.

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²Unpublished results.

Introduction

Heterocyclic aromatic amines (HAAs¹) are a class of experimental animal carcinogens and potential human carcinogens that form during the preparation of grilled meats, poultry, and fish under common household cooking conditions (1,2). Among the more than 20 mutagenic HAAs that have been discovered to date, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is the most mass-abundant HAA formed in beef and chicken (2,3). Studies also suggest that PhIP is present in tobacco smoke condensate (4), beer, and wine (5). The concentration of PhIP formed in meat staples is variable and depends on the type of meat and the method of cooking. The highest concentrations of PhIP, up to 500 parts per billion (ppb), generally occur in meats and poultry that are cooked to the well-done stage (2,3).

The covalent modification of DNA by chemical mutagens is regarded as the initiating step in chemical carcinogenesis (6). PhIP, similar to many other genotoxicants, must undergo metabolic activation to covalently adduct to DNA. The two-phase bioactivation of PhIP begins with oxidation by cytochrome P450 enzymes, primarily by cytochrome P450 1A2 (P450 1A2) in humans, to form the *N*-hydroxylated intermediate, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (HONHPhIP) (7). Next, phase II enzymes, such as acetyltransferases or sulfotransferases, convert HONH-PhIP to esters that further undergo heterolytic cleavage to produce the reactive nitrenium ion, which adducts to DNA. The bioactivation of HONH-PhIP by sulfotransferases has been reported to be particularly important in the mutagenesis of this compound in mammalian cells (8). Several presumed PhIP—DNA adducts have been detected in the tissues of experimental laboratory animals by ³²P-postlabeling (9) or *in vitro* by fluorescence (10) or MS techniques (11,12); however, only *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (dG-C8-PhIP), obtained from the reaction of *N*-acetoxy-PhIP with dG or DNA *in vitro*, has been extensively characterized by ¹H NMR and MS (11-14).

The measurement of DNA adducts has been used for cross-species extrapolation of the biologically effective dose and for human carcinogenic risk assessments of exposure to chemical carcinogens (15,16). In addition to ³²P-Postlabeling, several other biochemical and analytical techniques have been employed for the detection and quantification of DNA adducts; however, each of these methods has its own specific limitations. ³²P-Postlabeling is the most widely used of these methods because of its sensitivity and ability to detect many different classes of DNA adducts (17). However, this technique requires the handling of highly radioactive phosphorus, lacks the incorporation of an internal standard for the determination of adduct recoveries and compensation for labeling efficiencies, and provides no structural information about the adduct (18). Moreover, the complex profiles and the abundance of lesions detected by ³²P-postlabeling of DNA from human subjects exposed to numerous genotoxicants give rise to even greater uncertainty in the identity of the adduct in question (19). HPLC coupled with electrochemical (20) or fluorescence (21) detection has been used to measure oxidative DNA lesions or polycyclic aromatic hydrocarbon adducts, respectively, but these detection methods are not applicable to all types of DNA adducts. Internal standards are usually not incorporated into the assay, and structural information on the nature of the DNA adduct is again lacking. Immunohistochemistry (IHC) has been successfully used to monitor different classes of carcinogen-DNA adducts *in vivo* (22), but its use always entails an uncertainty about the specificity of the antibody and its potential to recognize an epitope other than the DNA adduct, leading to a false-positive finding.

Several different MS methods have been used for the detection of DNA adducts. Accelerator mass spectrometry (AMS) is a highly sensitive technique (23), but it requires the dosing of subjects with a radiolabeled substrate, which is not feasible in population-based studies. An AMS technique that detects DNA adducts following derivatization with [¹⁴C]-labeled agents

is currently under development for use in human biomonitoring studies, but inefficient DNA adduct labeling is a potential issue (24). Gas chromatography-negative ion-chemical ionization/mass spectrometry (GC-NICI/MS) has been used to successfully measure several classes of DNA adducts, following chemical derivatization (25,26). Because only one ion is usually monitored, the structural information provided about the adduct is limited. The dG-C8 adducts of several arylamines and PhIP also have been measured indirectly, following alkaline hydrolysis of the lesion with concomitant formation of the parent amines, which were measured by GCNICI/MS (27). However, this approach does not appear amenable to the measurement of dG- N^2 adducts of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (8-MeIQx), which are important lesions formed *in vivo* (28,29).

Over the past decade, atmospheric pressure ionization (API) techniques have been used to detect nonvolatile and thermally labile DNA adducts (18,26,30). The on-line coupling of HPLC with ESI-tandem mass spectrometry (LC-ESI/MS/MS) has been used to analyze a range of carcinogen—DNA adducts with diverse structures (18,26,30-37). LC-ESI/MS with triple quadrupole tandem mass spectrometry (TQ/MS/MS) has been most commonly used for the quantification of DNA adducts. For trace analysis detection, the TQ/MS/MS system is operated in the selected reaction monitoring (SRM) scan mode. In this mode, the protonated adducts ($[M + H]^+$) are selectively transmitted by the first mass analyzer (Q_1) and are subjected to collision-induced dissociation (CID), typically with argon gas, in the second quadrupole (Q_2). These collision conditions generally result in the loss of deoxyribose $[M + H - 116]^+$ to form the protonated base adducts $[BH_2]^+$, which are selectively transmitted through the third quadrupole (Q_3). The SRM scan mode is particularly effective for precise quantification because of the rapid duty cycle of TQ/MS/MS. Higher collision energy conditions result in more extensive fragmentation of the aglycone BH_2^+ ion and can be used to obtain additional structural information about the adducts (18). However, the full product ion scan mode, by TQ/MS/MS, has rarely been employed for the characterization of DNA adducts *in vivo* (29) because the slow scan rate and the insensitivity of the scan mode preclude facile detection of adducts at trace levels. Thus, the analyst must rely solely on the characteristic retention time (t_R) and a single SRM transition ($[M + H - 116]^+$) as criteria for analyte identification.

During the past 15 years, quadrupole ion trap mass spectrometry (QIT/MS), developed and patented by Paul and coworkers in 1960 (38), has emerged as a powerful tool, with a variety of bioanalytical applications including proteomics and analyses of dietary and environmental contaminants (39,40). QIT/MS, which is a tandem-in-time technique, differs from TQ/MS/MS, which is a tandem-in-space technique, primarily in the mass filters used. Although the TQ/MS/MS filters out all ions except the designated ions of interest, the QIT/MS permits ion storage and sequential ejection of ions in the order of mass through adjustment of the strength of the quadrupole field holding the ions. This scan filter enables consecutive reaction monitoring (CRM), or multi-stage scan events (MS^n), to be performed by QIT/MS, eliminating isobaric interferences that remain with the SRM scan mode of the TQ/MS/MS. Moreover, the rapid-scanning capacity of the instrument permits the routine acquisition of full product ion spectra, enabling extensive mass spectral characterization of the analytes. However, the ion-trapping capacity of the popular 3-D QIT/MS can be adversely affected by space charge effects, which occur with an influx of a high density of ions; such effects can result in ion—ion interactions that distort the quadrupole field, mass resolution, and mass scale shifts, leading to a loss in sensitivity (41). Moreover, the low (ca. 5%) trapping efficiencies, when ions are injected into the trap from an external ESI source (42), can restrict the use of the 3-D trap for quantitative measurements. Recent investigations on a newly developed 2-D QIT/MS have reported improvements in ion storage volume and trapping efficiencies, with reduced space—charge effects (41), and increased sensitivity due to radial ion ejection and dual detection

(43). The 2-D QIT/MS has been advertised to have sensitivity and reproducibility superior to those characteristics of some 3-D QIT/MS instruments (43-45).

In this investigation, we have explored the scanning capabilities of 2-D QIT/MS for the quantification of PhIP—DNA adducts both *in vitro* and in experimental animals fed a PhIP-containing diet. The LC-ESI/MS analysis of PhIP—DNA adducts has never been reported in experimental, laboratory animals. Our goal was to establish a robust SPE procedure to enrich PhIP—DNA adducts and to employ 2-D QIT/MS and its MSⁿ scan capabilities for both the corroboration and quantification of PhIP—DNA adducts. We report the first use of the MS³ scan mode for the quantification and the MS³ and MS⁴ scan modes for the characterization of dG-C8-PhIP *in vivo* at levels as low as 1 adduct per 10⁸ DNA bases. The high sensitivity of the 2-D QIT/MS also enabled us to characterize a previously unreported isomeric dG-PhIP—DNA adduct *in vivo*.

Experimental Procedures

Caution

PhIP and several of its derivatives are carcinogenic to rodents, and they should only be handled in a well-ventilated fume hood with the appropriate protective clothing.

Chemicals

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and isotopically labeled 2-amino-1-(trideuteromethyl)-6-phenylimidazo[4,5-*b*]pyridine ([²H₃C]-PhIP; >99% isotopic purity) were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Salmon sperm DNA was obtained from Fisher (Pittsburgh, PA). Phosphodiesterase I (from *Crotalus adamanteus* venom) was purchased from GE Healthcare (Piscataway, NJ). All solvents used were HPLC-grade from Fisher or Sigma-Aldrich (St. Louis, MO) or high-purity B & J Brand from Honeywell Burdick and Jackson (Muskegon, MI). ACS reagent grade HCO₂H (88%) was purchased from J.T. Baker (Phillipsburg, NJ). Isolute C18-(EC) (25 mg of resin in 1 mL polypropylene cartridge holder) SPE cartridges containing additionally washed polyethylene frits were purchased from Biotage (Charlottesville, VA). All other chemicals and enzymes used were purchased from Sigma-Aldrich.

Preparation of the dG-C8-PhIP and dG-C8-[²H₃C]-PhIP Standards

Both the unlabeled and isotopically labeled dG-C8-PhIP standards were prepared according to previously published methods (11,13). Briefly, the nitro derivative of PhIP (46) was reduced to HONH-PhIP with hydrazine and Pd/C. HONH-PhIP was immediately treated with acetic anhydride in the presence of dG, forming *N*-acetoxy-PhIP or *N*-acetoxy-[²H₃C]-PhIP, the reactive intermediates involved in the reaction with dG (11,13). The UV spectral data of the isotopically labeled internal standard was identical to that of authentic dG-C8-PhIP, which was synthesized and characterized by ¹H NMR (11,13). The chemical isotopic purity of dG-C8-[²H₃C]-PhIP exceeded 99%. Concentrations of the unlabeled and labeled adducts were determined by UV spectroscopy.

PhIP-Modified Calf Thymus DNA

[³H]-PhIP-modified CT DNA was generously provided by F. F. Kadlubar (NCTR, Jefferson, AR) and prepared as previously described (13). The extent of PhIP-modification was estimated at 1 adduct per 10⁶ DNA bases, and the dG-C8-PhIP adduct was determined to represent >90% of the total bound material following enzymatic digestion of the DNA and analysis by HPLC, on the basis of liquid scintillation counting (13).

LC-ESI/MS Analyses

Mass spectral data were acquired on either a Finnigan LTQ 2-D linear ion trap mass spectrometer (LTQ) (Thermo Electron, San Jose, CA) or a Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer (TSQ) (Thermo Electron). Both mass spectrometers were equipped with an Ion Max electrospray ionization source operated in positive ionization mode and used Xcalibur software (version 1.4; Thermo Electron) for system operation and data manipulation. Data from on-line analyses were acquired in centroid mode. Chromatography for each instrument was performed on an 1100 Series capillary LC system (Agilent Technologies, Palo Alto, CA) equipped with an Aquasil C₁₈ column (1 mm i.d. × 250 mm, 5 μm particle size; Thermo Electron). All of the samples were prepared in Waters (Milford, MA) borosilicate total recovery capillary LC vials unless otherwise specified. Samples were stored at 5 °C in an Agilent autosampler throughout the analyses. The flow rate was set to 50 μL/min using mixtures of (A) 9:1 H₂O/CH₃CN (high-purity B & J Brand solvents) with 0.1% HCO₂H and (B) CH₃CN with 0.1% HCO₂H and a gradient starting at 0% B for the first minute followed by a linear gradient to 100% B over 9 min, held at 100% B for 3 min, back to starting conditions (0% B) over 1 min, and a 6 min period to allow the column to re-equilibrate to the starting conditions (0% B). Individual MS instrument parameters were optimized by infusing the dG-C8-PhIP adduct (1 μg/mL) with a syringe pump into the MS source through a mixing tee at a flow rate of 5 μL/min with LC solvent (1:1 A/B solvents) flowing at 50 μL/min and are described below.

LTQ MS Parameters

Samples (8 μL) were injected through an 8-μL injection loop into a six-port switching valve injector (Rheodyne) that diverted the column eluent to waste for the first 4 min of the gradient. Representative optimized instrument tune parameters were as follows: capillary temperature 285 °C; source spray voltage 3.5 kV; source current 2.8 μA; sheath gas setting 60; sweep gas setting 5; capillary voltage 40 V; tube lens voltage 110 V; and in-source fragmentation 15 V. These instrument tune parameters were re-optimized as necessary for maximal signal and sensitivity. Helium was used as the collision damping gas in the ion trap, set at a pressure of 1 mTorr. One μscan was used for data acquisition, and the maximum injection time was 100 ms. The automatic gain control (AGC) settings were full MS target 30,000 and MSⁿ target 10,000. The instrument method used for the acquisition of on-line data consisted of a single segment that contained four different scan events. The first and second scan events in the MS/MS scan mode produced the aglycone ion BH₂⁺ adducts [M + H - 116]⁺ from the protonated molecules dG-C8-PhIP ([M + H]⁺ at *m/z* 490) and dG-C8-[²H₃C]-PhIP ([M + H]⁺ at *m/z* 493), under the following conditions (scanning from *m/z* 240 to 400 Da): isolation width 3.0 *m/z*; normalized collision energy 25; activation Q 0.4, and activation time 30 ms. The third and fourth scan events, in the MS³ scan mode, generated second-generation product ions from the aglycone ion adducts BH₂⁺ under the following conditions (scanning from *m/z* 140 to 400 Da): isolation width 1.5 *m/z*; normalized collision energy 35; activation Q 0.35; and activation time 30 ms. Further characterization of ions present in the MS³ product ion spectra with the MS⁴ scan mode was performed using the following conditions (scanning from *m/z* 90 to 380 Da): isolation width 3.0 *m/z*; normalized collision energy 45; activation Q 0.45; and activation time 30 ms.

TSQ MS Parameters

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 15 V. Data acquisition in the SRM scan mode, monitoring the loss of dR from the protonated molecule (dG-C8-PhIP: [M + H]⁺ *m/z* 490 → 374 [M + H - 116]⁺ and dG-C8-[²H₃C]-PhIP [M + H]⁺ *m/z* 493 → 377 [M + H - 116]⁺), was

performed using the following conditions: collision energy 25; peak width (in Q1 and Q3) m/z 0.7 Da; scan width m/z 0.7 Da; scan time 0.1 s; and collision gas Argon at 1.5 mTorr. The same MS parameters were used for the constant neutral loss (CNL) scan mode experiments, monitoring the loss of 116 Da from the protonated ions ($[M + H - 116]^+$; loss of dR), scanning from m/z 350 to 550 at a rate of 100 Da/s.

Sources of Ion Suppression

The water, organic solvents, and consumable items, including SPE cartridges and individual SPE components, were investigated for the presence of contaminants capable of provoking ion suppression effects. The details of the ion expression experiments and data are provided in the Supporting Information.

PhIP-Modified DNA and Calibration Curves

The PhIP-modified CT DNA at a level of adduct modification of 1 adduct per 10^6 DNA bases was diluted in series, using salmon sperm DNA as a diluent, to levels of adduct modification of 1, 0.3, and 0.1 adducts per 10^7 DNA bases (assuming $50 \mu\text{g}$ of DNA/mL = 1 absorbance unit at 260 nm). Quantitative LC-ESI/MS/MSⁿ measurements were conducted following enzymatic DNA digestion and SPE as described below, using dG-C8-[²H₃C]-PhIP as an internal standard, and were quantified by means of an external calibration curve. Intra-day and inter-day accuracy and precision of the method were determined by triplicate analyses of the PhIP-modified DNA at all levels of adduct modification and by triplicate analyses of the calibration samples at all concentrations on multiple days. These experiments were performed over a time span of 6 months.

External calibration curves were produced in triplicate by the addition of a fixed amount of dG-C8-[²H₃C]-PhIP as the internal standard, with varying amounts of unlabeled dG-C8-PhIP. We used two different sets of calibrants, based upon the LOD and LOQ of adduct detection, which were set at 3σ and 10σ SD units, respectively, above the background signal of unmodified DNA (47). The calibration samples used for quantification of PhIP—DNA modified at ≥ 3 adducts per 10^8 DNA bases contained (all listed quantities are on-column amounts) 25 pg of internal standard, which corresponds to 5 adducts of dG-C8-[²H₃C]-PhIP per 10^7 DNA bases for $27 \mu\text{g}$ of DNA and 0, 0.5, 1.5, 5, 15, 30, and 50 pg of the dG-C8-PhIP calibrant, corresponding to 0 and 1–100 adducts per 10^8 DNA bases. The calibration samples used for PhIP—DNA modified at 1 adduct per 10^8 DNA bases contained (all listed quantities are the on-column amounts) 5 pg of internal standard, which corresponds to 1 adduct of dG-C8-[²H₃C]-PhIP per 10^7 DNA bases for $27 \mu\text{g}$ of DNA and 0, 0.5, 1, 2, 3.5, 5 pg of the dG-C8-PhIP calibrant, corresponding to 0 and 1–10 adducts per 10^8 DNA bases. Calibration curves were generated in both MS/MS and MS³ scan modes. The calibration curve in the MS/MS scan mode was plotted as the amount ratio of unlabeled dG-C8-PhIP relative to dG-C8-[²H₃C]-PhIP on-column (amount (in pg) of the unlabeled standard divided by the fixed amount of the internal standard (in pg)) against the integrated peak area ratio of unlabeled dG-C8-PhIP to dG-C8-[²H₃C]-PhIP (peak area of the ion at m/z 374 divided by the peak area of the ion at m/z 377). The calibration curve generated for the MS³ scan mode plotted the ratio of unlabeled dG-C8-PhIP relative to dG-C8-[²H₃C]-PhIP on-column (amount (in pg) of unlabeled dG-C8-PhIP divided by the fixed amount of the internal standard (in pg)) against the integrated peak area ratio of unlabeled dG-C8-PhIP to dG-C8-[²H₃C]-PhIP (combined peak areas of the major fragment ions at m/z 357, 329, 304, and 250 divided by the combined peak areas of the ions m/z 360, 332, 307, and 253). The peak width of the extracted ions was 1 Da. Linear regression was performed using the method of least-squares.

DNA Digestion Conditions

The enzymatic digestion conditions used for the hydrolysis of DNA to the 2'-deoxynucleosides are a modification of the procedure described by Lin and co-workers (13). DNA (50 $\mu\text{g}/50\ \mu\text{L}$ for the 1 adduct per 10^6 DNA bases PhIP—DNA or 100 $\mu\text{g}/100\ \mu\text{L}$ for all other levels of PhIP—DNA adduct modification) was used for the digestions. In the experiments examining the kinetics of the enzymatic DNA digestion, dG-C8-[$^2\text{H}_3\text{C}$]-PhIP was added to the DNA solutions prior to enzyme digestion at an amount equivalent to 5 adducts per 10^6 bases (370 pg dG-C8-[$^2\text{H}_3\text{C}$]-PhIP/50 μg DNA). For all other studies, dG-C8-[$^2\text{H}_3\text{C}$]-PhIP was added to the DNA solutions at an amount equivalent to 1 or 5 adducts per 10^7 DNA bases. The DNA samples were digested as follows, using enzyme concentrations based upon 1 mg DNA/1 mL 5 mM bis-tris-HCl buffer (pH 7.1) containing 10 mM MgCl_2 . DNase I (Type IV from bovine pancreas; 2542 U/mL in 0.15 M NaCl; 254.2 U/mg DNA) was added, and the mixture was incubated for 1.5 h. Next, nuclease P₁ (from *Penicillium citrinum*; 100 U/mL in 1 mM ZnCl_2 ; 4 U/mg DNA) was added, and the incubation was continued for a further 3 h at 37 °C. Alkaline phosphatase (from *E. coli*; 24 U/mL in 1 mM MgCl_2 ; 2 U/mg DNA) and phosphodiesterase I (from *Crotalus adamanteus* venom; 1.7 U/mL in 110 mM Tris-HCl at pH 8.9, containing 110 mM NaCl, 15 mM MgCl_2 , and 50% glycerol; 0.0714 U/mg DNA) were added last, and the incubation was continued for an additional 3 h at 37 °C. Cold $\text{C}_2\text{H}_5\text{OH}$ (200 proof) was added to the hydrolysis mixture (3 $\times$ the total volume), and the $\text{C}_2\text{H}_5\text{OH}$ /DNA solution was stored at $-80\ ^\circ\text{C}$ until sample analyses were performed.

Enzyme Kinetics of PhIP—DNA Hydrolysis

The enzymatic DNA digestion was performed on PhIP—DNA that was modified at 1 adduct per 10^6 DNA bases as described above, except that the incubation time following the addition of alkaline phosphatase and phosphodiesterase I was extended to 24 h total, and aliquots of the digestion mixture were removed at various time points for TQ/MS/MS analysis. Aliquots from the digestion mixtures were removed at $t = 0$ (before the addition of any enzyme), $t = 1.5\ \text{h}$ (1.5 h after the addition of DNase), $t = 4.5\ \text{h}$ (3 h after the addition of nuclease P₁), $t = 7.5\ \text{h}$ (3 h after the addition of alkaline phosphatase and phosphodiesterase I), and at later time points. At the designated time points, 8 μL aliquots were removed from the samples (equivalent to 8 μg of DNA), the enzymes were pelleted in $\text{C}_2\text{H}_5\text{OH}$, the supernatant was removed and dried by vacuum centrifugation, and the dried residues were resuspended in 1:1 DMSO/ H_2O (32 μL). LC-ESI/MS/MS analyses were performed on the TSQ in the SRM scan mode using 8 μL injections of the total sample (equivalent to 3 pg of dG-C8-PhIP and 15 pg of dG-C8-[$^2\text{H}_3\text{C}$]-PhIP).

SPE DNA Adduct Enrichment Procedure

The proteins from the $\text{C}_2\text{H}_5\text{OH}$ /DNA digest solution were precipitated by centrifugation at 15,000g for 5 min. The DNA-adduct containing supernatant was removed and dried by vacuum centrifugation. Samples were purified by SPE using Isolute C18(EC) 25 mg SPE cartridges containing additionally washed polyethylene frits. High-purity B & J Brand solvents were used throughout the SPE adduct enrichment procedure. The SPE cartridges were first conditioned/activated with CH_3OH ($3 \times 1\ \text{mL}$). The DNA digest samples were loaded next in 5% CH_3OH in 0.1% HCO_2H (1 mL). This was followed by a single wash with water (1 mL). The nonmodified 2'-deoxynucleosides were removed by the addition of 10% CH_3OH (1 mL). The desired PhIP-dG adducts were eluted with CH_3OH (1 mL) into total recovery capillary LC vials. The CH_3OH eluent was evaporated to dryness by vacuum centrifugation and reconstituted in 1:1 DMSO/ H_2O (30 μL).

Animal Studies and ^{32}P -Postlabeling

PhIP-treated rat colon and liver DNA samples were obtained from a study conducted by Dr. H. Schut (University of Toledo College of Medicine, Toledo, OH), which was approved by the Institute of Animal Care and Utilization Committee (IACUC) at the University of Toledo College of Medicine. Briefly, female Sprague—Dawley rats (6–7 weeks old) were treated for 21 days with 0.04% (w/w) PhIP in their diet (AIN-76A) and were sacrificed on day 22 by means of sodium pentobarbital solution (180 mg/kg, i.p.), followed by the removal of blood from the abdominal veins and the retrieval of organs. Untreated female, Sprague—Dawley rats (6–7 weeks old) were obtained from Charles River Laboratories (Raleigh, NC). The DNA was isolated by a salt precipitation procedure as previously described (48). The ^{32}P -postlabeling assay was conducted with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (approximately 4,000 Ci/mmol), using the intensification (limiting [ATP]) version of the assay (49). PhIP—DNA adducts, following enzymatic digestion and labeling, were separated from nonmodified bis-phosphate nucleotides by multidimensional polyethyleneimine—cellulose TLC, as previously reported (48). The ^{32}P -postlabeled PhIP—DNA adducts were visualized by autoradiography, and radioactive spots (adducts) were quantified by Cerenkov counting, where the extent of adduct formation was expressed as relative adduct labeling (RAL), taking into account the intensification factors of the individual adducts (48,49).

Statistical Analyses

The dG-PhIP adduct levels are presented as the means $\pm$ standard deviations ($N = 3$). The unpaired Student's t -test was performed to determine whether the samples assayed in MS/MS and MS³ were significantly different using Graphpad (San Diego, CA).

Results

Product Ion Spectra of dG-C8-PhIP by QIT/MS

Solutions of dG-C8-PhIP and dG-C8- $[\text{}^2\text{H}_3\text{C}]$ -PhIP were infused into the source and analyzed by LC-ESI/MS/MSⁿ on the LTQ. In the full scan mass spectrum, a single ion corresponding to the protonated molecule $[\text{M} + \text{H}]^+$ was detected at m/z 490 for dG-C8-PhIP and at m/z 493 for dG-C8- $[\text{}^2\text{H}_3\text{C}]$ -PhIP.² The product ion spectra (MS/MS) of dG-C8-PhIP displayed a prominent fragment ion as the base peak at m/z 374 $[\text{M} + \text{H} - 116]^+$; this is the characteristic aglycone ion $[\text{BH}_2]^+$, guanyl-C8-PhIP (11), and arises from a loss of dR with the back-transfer of a hydrogen from the sugar moiety to the base.² The second-generation product ions (MS³) gave characteristic mass fragmentations of the guanyl-C8-PhIP base $[\text{BH}_2]^+$ (Figure 1). Some of the pathways of mass fragmentation are similar to those reported for dG-C8-PhIP (11) and other dG-C8-HAA and dGC8-arylamine adducts (Scheme 1) by TQ/MS/MS analysis (29,50, 51) or by QIT/MS (52); however, several unique product ions are present as well. The proposed structures of the fragment ions and neutral losses are tentative; confirmation will require exact mass measurements or the use of stable isotopes. The principal product ion of the aglycone $[\text{BH}_2]^+$ occurred at m/z 357 $[\text{BH}_2 - 17]^+$ and arises from the loss of NH_3 from either the N1 amide or N² amino group of guanine (53), whereas the ion at m/z 329 $[\text{BH}_2 - 45]^+$ occurs either from a concerted loss of NH_3 and CO or from a loss of $\text{H}_2\text{N-C(OH)}$ because of the cleavage of the N1–C2 and C5–C6 bonds of guanine. The product ion at m/z 250 $[\text{BH}_2 - 124]^+$ (loss of $\text{C}_4\text{H}_4\text{N}_4\text{O}$) is attributable to the cleavage of the N7–C8 and C4–N9 bonds of the guanine base

Supporting Information Available: Experimental details and discussion on the investigations into sources of ion suppression; ion suppression effects of the dG-C8-PhIP standard provoked from organic solvents of differing purities (Figure 1) and from individual components of isolate C18(EC) SPE cartridges containing additionally washed polyethylene frits (Figure 2); representative calibration curve of dG-C8- $[\text{}^2\text{H}_3\text{C}]$ PhIP at 5 adducts per 10^7 DNA bases with unlabeled dG-C8-PhIP present at a level equivalent to 1 to 100 adducts per 10^8 DNA bases (Figure 3); comparison of product ion spectra (MS³) from the dG-C8-PhIP standard and dG-C8-PhIP enzymatically released from PhIP-modified CT DNA (3 adducts per 10^8 DNA bases) (Figure 4). This material is available free of charge via the Internet at <http://pubs.acs.org>.

with retention of the CN moiety of guanine to the protonated PhIP molecule. The second-generation productions at m/z 357 $[\text{BH}_2 - 17]^+$ (loss of NH_3) and m/z 332 $[\text{BH}_2 - 42]^+$ (loss of H_2NCN) underwent fragmentation at MS^4 to produce fragment ions at m/z 329 and 304, respectively, indicative of the loss of CO. The expulsion of the CO group from guanine was not observed in the MS^n spectra of several C8-substituted alkylaniline dG adducts acquired by QIT-MS (52). The second-generation product ions of dG-C8- $[\text{}^2\text{H}_3\text{C}]$ -PhIP gave the same mass fragmentation pattern and same relative intensities as for the unlabeled standard, but all of the masses were shifted to +3 Da.

Enzyme Kinetics for the Recovery of dG-C8-PhIP from DNA

The enzymatic DNA digestion protocol used in these studies was previously reported to recover dG-C8-PhIP from DNA (13). We examined the kinetics of the DNA digestion in order to determine the shortest incubation period and the minimum amount of enzyme necessary for the complete recovery of dG-C8-PhIP from DNA, in part to minimize potential adduct decomposition observed as a result of the prolonged incubation (11). The enzyme hydrolysis conditions reported by Lin and co-workers (13) required a total of 13.5 h of incubation using the same amount of enzyme used in our study. Our results show that the recovery of dG-C8-PhIP following DNA digestion is quantitative after 3 h, following the addition of alkaline phosphatase and phosphodiesterase I (total incubation time = 7.5 h) (Figure 2). In our study, the ratio of dG-C8-PhIP to dG-C8- $[\text{}^2\text{H}_3\text{C}]$ -PhIP and the response in the MS signals were relatively constant over 24 h (total incubation time = 28.5 h), indicating that the internal standard and dG-C8-PhIP released from the PhIP-modified DNA were stable toward the digestive enzymes over time.

The optimized enzyme digestion conditions described above were employed to examine DNA adducts in the colon from rats chronically treated with PhIP. DNA adduct formation was monitored in the CNL scan mode by TQ/MS/MS, using the transition $[\text{M} + \text{H}]^+ \rightarrow [\text{M} + \text{H} - 116]^+$ (loss of dR), which is a common pathway of fragmentation for many deoxynucleo-side adducts (18,26). The LC-ESI/MS/MS chromatogram of colon DNA from a rat exposed to PhIP at 2 adducts per 10^7 bases (RAL), estimated by ^{32}P -postlabeling (*vide infra*), showed the presence of two peaks at m/z 490. The major peak at t_R 10.17 min corresponds to the protonated molecule $[\text{M} + \text{H}]^+$ dG-C8-PhIP, and a minor peak detected at t_R 9.18 min is a presumed dG-PhIP isomer (Figure 3). There was no evidence for a ring-opened guanine adduct of dG-C8-PhIP at m/z 383, as previously observed in CT DNA modified with a large excess of *N*-acetoxy-PhIP (11), or spirobisguanidino-PhIP derivatives (54), as previously observed when other enzyme hydrolysis conditions were employed. Our findings show that the currently employed enzyme conditions do not decompose the dG-C8-PhIP adduct and that ring-opened adducts are not detected in the PhIP-treated rat colon DNA. Thereafter, we sought to quantify and further characterize both the dG-C8-PhIP and the putative isomeric dG adduct by QIT/MS, as described below.

LC-ESI/MS/MSⁿ Analysis of dG-C8-PhIP

The signal of response of dG-C8-PhIP was highly sensitive by 2-D linear QIT/MS. The LOD of dG-C8-PhIP by 2-D QIT/MS in the MS^3 scan mode was <50 fg (100 amol), for column dimensions of 1×250 mm, a $5\text{-}\mu\text{m}$ particle size, and a flow rate of $50\text{ }\mu\text{L}/\text{min}$.² The LC-ESI/MS/MSⁿ traces of the dG-C8-PhIP standard (5 pg on column) are shown in Figure 4. Impurities present in the water used for the mobile phase greatly contributed to the background chemical noise. The background signals in the full scan and MS/MS scan modes were considerably lower with high-purity B & J Brand water than with high-purity grade water from the NanoDiamond organic-free water purification system (Barnstead International). In CRM, there was a significant improvement in S/N as the MS^n scan modes increased from full scan mode MS (mass chromatogram monitoring at m/z 490 $[\text{M} + \text{H}]^+$) to higher-stage MS/MS and MS^3 scan

modes. In addition, the S/N ratios of dG-C8-PhIP were comparable in the reconstructed total-ion-current chromatograms of the MS³ scan mode, irrespective of the use of high-purity B & J Brand grade water or NanoDiamond organic-free water as the mobile phase, indicative of the enhanced and selective scanning capabilities offered by the MSⁿ scan modes of the 2-D QIT/MS. Moreover, the high dissociation efficiency of the 2-D QIT/MS resulted in a recovery of >90% of the total ion counts of the dG-C8-PhIP adduct when going from MSⁿ to MSⁿ⁺¹ stage scan modes. The high dissociation efficiency of the LTQ has been previously reported for other analytes (41).

Sources of Ion Suppression

During the course of SPE method development to remove unmodified deoxynucleosides, we observed a >95% decrease in the signal intensity of the dG-C8-PhIP adduct spiked in the DNA digest and purified by SPE. Numerous studies have examined the mechanisms and potential sources of ion suppression of various analytes, focusing primarily on the biological matrices as the sources of suppression (55–57). Our initial investigations suggested that the consumables used for processing the samples, rather than the biological matrix, were the major contributors to the diminution of the signal. Following a rigorous investigation into all of the consumable items used for sample preparation, we found the solvent purity (Supporting Information, Figure 1) and the Isolute C18(EC) SPE cartridges (Supporting Information, Figure 2), in particular the polyethylene frits used in the SPE cartridges, to be responsible for the majority of the observed ion suppression in our samples. Experiments conducted to identify sources of ion suppression and their results are presented in Supporting Information. Currently, the response in the signal of the dGC8-PhIP or dG-C8-[²H₃]-C8-PhIP adduct that is processed through the DNA digest (1–100 adducts per 10⁸ bases) and the SPE procedure is ~30–50% relative to the pure standard.

Analysis of PhIP Adducts in DNA

We employed LC-ESI/MS/MSⁿ with the 2-D QIT/MS for the quantification of the dGC8-PhIP adduct in several different PhIP-modified DNA samples: (1) CT DNA modified *in vitro* through the reaction with *N*-acetoxy-PhIP (11,13) and (2) rat liver and rat colon DNA isolated from experimental laboratory animals fed a PhIP-containing diet. Quantification of the DNA adduct was based on isotope dilution with the deuterated internal standard, dGC8-[²H₃C]-PhIP, using an external calibration curve. Quantification was done in both MS/MS and MS³ scan modes.

Method Validation and Performance (Analysis of PhIP-Modified Calf Thymus DNA)

A representative calibration curve used for the estimation of PhIP-modified DNA at g3 adducts per 10⁸ DNA bases (the LOQ) and spiked with dGC8-[²H₃C]-PhIP (25 pg injected on column) at a level of 5 adducts per 10⁷ DNA bases (for 27 μg equivalent of DNA on column) is shown in Supporting Information (Figure 3). The MS/MS and MS³ calibration curves were indistinguishable ($P = 0.94$; the values for the slope and y-intercept were 1.073 ± 0.015 (slope) and -0.021 ± 0.014 (y-intercept), and 1.075 ± 0.018 (slope) and -0.025 ± 0.016 (y-intercept), for MS/MS and MS³, respectively; for both curves, the precision r^2 exceeded 0.995). The MS scanning parameters and AGC settings employed for the analytical scan cycle of the LTQ were optimized so that 20 scans were recorded across all 4 peaks, that is, MS/MS and MS³ scans for dG-C8-PhIP and dG-C8-[²H₃]-PhIP, at all calibrant levels. These MS parameters and scan settings provided quantitative and accurate estimates of dG-C8-PhIP over a 100-fold range in levels of DNA adduct modification.

The dG-C8-PhIP-adducted CT DNA (1 adduct per 10⁶ bases) was serially diluted with unmodified DNA to adduct levels of 1, 3, and 10 adducts per 10⁸ DNA bases and was then quantified by both MS/MS and MS³ scan modes. The LC-ESI/MS/MSⁿ chromatograms of dG-

C8-PhIP-modified CT DNA at levels of 0, 1, and 3 adducts per 10^8 DNA bases and dG-C8- $[^2\text{H}_3\text{C}]$ -PhIP added to CT DNA at 1 adduct per 10^7 DNA bases are shown in Figure 5. The background signals (chemical noise) of the dGC8-PhIP adduct were estimated in nonmodified DNA at 0.4 ± 0.2 and 0.2 ± 0.1 adducts per 10^8 bases in MS/MS and MS^3 scan modes, respectively (mean, $\pm$ between-day S.D., $N = 16$ independent measurements over 4 days). The LOD and LOQ of dG-C8-PhIP in modified DNA were determined to be ~ 1 and ~ 3 adducts per 10^8 DNA bases, respectively (Table 1) (47). The signal of dG-C8-PhIP is discernible at a level of 1 adduct per 10^8 DNA bases. In the MS/MS scan mode, the response is 2-fold above the background chemical noise level. In CRM, the specificity of the detection of dG-C8-PhIP increases from the MS/MS stage to the MS^3 stage, and there is an ~ 5 -fold improvement in the S/N. Moreover, high quality MS^3 product ion spectra were obtained on dG-C8-PhIP at a level of modification of 3 adducts per 10^8 DNA bases (Supporting Information, Figure 4). The product ion spectra are in excellent agreement with the spectrum for the synthetic dG-C8-PhIP, and they corroborate the identity of the adduct.

The performance of the method is summarized in Table 1. The results show intra-day accuracy and precision in both MS/MS and MS^3 scan modes from triplicate DNA samples prepared on the same day. Inter-day accuracy and precision from repetitive analyses of the DNA samples performed over a period of 6 months are also shown in Table 1. The estimates of dGC8-PhIP obtained in both MS/MS and MS^3 scan modes were in excellent agreement and on average, within 10%, of the target value.

Analysis of dG-C8-PhIP and Isomeric dG-PhIP Adducts in Rat Liver and Rat Colon DNA by QIT/MS and ^{32}P -Postlabeling

Rat colon and liver DNA isolated from untreated animals and experimental laboratory rats fed PhIP as part of the diet were measured by LC-ESI/MS/MSⁿ. The estimates of adduct formation were compared to the values obtained by ^{32}P -postlabeling (Table 2). The level of PhIP modification was estimated by ^{32}P -postlabeling at $\sim 20 \pm 3$ adducts in 10^8 DNA bases for colon DNA and $\sim 3.0 \pm 0.6$ adducts in 10^8 DNA bases for liver DNA samples. The ^{32}P -postlabeling profiles obtained by 2-D TLC of treated rat liver and colon DNA digests reveal two principal spots (Figure 6, panels A and C, respectively). The synthetic bisphosphate-dG-C8-PhIP adduct has the same R_f value as the more slowly migrating lesion (11).² The second spot, which represents the major lesion, may be either an incompletely digested oligonucleotide containing the dG-C8-PhIP adduct (58) or the minor isomeric dG-PhIP adduct (described below), which is labeled at high efficiency by polynucleotide kinase. There are no spots visible by 2-D TLC of the untreated rat liver and colon DNA following ^{32}P -postlabeling (Figure 6, panels B and D, respectively).

The dG-C8-PhIP adduct (t_R 9.9 min) is the major peak observed in the LC-ESI/MS/MSⁿ chromatograms of the rat liver and colon DNA samples (Figure 7). A second peak (t_R 9.1 min) with the same nominal mass as that of dG-C8-PhIP is also observed in the mass chromatograms of the rat colon and liver DNA samples. The response of the signal of the isomeric dGPhIP adduct was $\sim 3\%$ of the signal for dG-C8-PhIP in both tissues for these samples. The base peaks in the product ion spectra of both adducts were observed at m/z 374 [BH_2^+] in the MS/MS scan mode.² The estimates of dG-C8-PhIP, determined by LC-ESI/MS/MSⁿ, are 15- to 20-fold greater than the estimates of total adducts estimated by ^{32}P -postlabeling (Table 2).

The identity of dG-C8-PhIP was corroborated by acquisition of the product ion spectra (MS^3) of guanyl-C8-PhIP [BH_2^+] at m/z 374 (Figure 8). Both dG-C8-PhIP and the isomeric dGPhIP adduct in colon DNA of rats underwent further characterization by multi-stage MS^n product ion spectra (Figure 8). The MS^3 stage product ion spectra of the [BH_2^+] ions of both adducts displayed fragment ions at m/z 357 [$\text{BH}_2 - 17^+$], due to the loss of NH_3 , as the base peaks. A second common fragment ion was observed at m/z 332 [$\text{BH}_2 - 42^+$] and is attributed

to the expulsion of NH_2CN or $\text{HN}=\text{C}=\text{NH}$ from the N1–C2 and N3–C4 bonds of guanine (53). For guanyl-C8-PhIP, additional prominent ions in the MS^3 stage product ion spectrum were also observed at m/z 329, 304, and 250 (Scheme 1). In the MS^3 stage product ion spectrum of the isomeric dGPhIP adduct, a minor fragment ion was observed at m/z 225, which corresponds to the nominal mass of the protonated PhIP molecule.

Both dG-PhIP adducts were characterized by the MS^4 stage product ion spectra (Figure 8), and both spectra show many of the same product ions, but the isomeric dG-PhIP adduct underwent more extensive fragmentation. The m/z 357 $[\text{BH}_2 - 17]^+$ (loss of NH_3) of both adducts underwent further fragmentation of the guanine moiety at MS^4 to produce ions at m/z 329 $[\text{BH} - 17]^+ \rightarrow [\text{BH}_2 - 17 - 28]$ (loss of CO), m/z 315 $[\text{BH}_2 - 17]^+ \rightarrow [\text{BH}_2 - 17 - 42]^+$ (loss of $\text{HN}=\text{C}=\text{NH}$), and m/z 302 $[\text{BH}_2 - 17]^+ \rightarrow [\text{BH}_2 - 17 - 55]^+$ (loss of C_2HNO) (Scheme 1). These proposed pathways of fragmentation were previously reported for dG-C8 adducts of IQ (50) and 8-MeIQx (29). In the case of the isomeric dG-PhIP, the MS^4 stage product ion spectrum of the m/z 225 ion produced a major fragment ion at m/z 210 (loss of CH_3^+) as the base peak and a minor fragment ion at m/z 147 (loss of C_6H_6). This spectrum is identical to the MS/MS product ion spectrum of PhIP.² The neutral fragments generated in the MS^3 and MS^4 stage mass spectra of the isomeric dG-PhIP adduct, many of which are in common with those generated from dG-C8-PhIP, suggest that the site of adduction is not the exocyclic N^2 , endocyclic N1, or the O^6 atoms of guanine. In the absence of the accurate mass measurements or stable isotopes at selected atoms of guanine (53) that would be needed for elucidation of pathways of fragmentation, the exact structure of the isomeric dG-PhIP adduct cannot be determined.

Discussion

We report here the first use of both MS/MS and MS^3 scan modes for the detection, characterization, and quantification of dG-C8-PhIP with a 2-D QIT/MS, at levels of ≥ 1 adduct per 10^8 DNA bases. High quality MS^3 product ion spectra were obtained for dG-C8-PhIP at levels of 3 adducts per 10^8 DNA bases; these spectra permitted the unambiguous identification of the adduct (Supporting Information, Figure 4). Moreover, the duty cycle of the 2-D QIT/MS is sufficiently rapid that quantitative measurements and adduct characterizations can be performed simultaneously, in MS/MS and MS^n scan models. The dG-C8-PhIP adduct has been detected, by ^{32}P -postlabeling, in numerous tissues of experimental laboratory animals (9). However, to the best of our knowledge, this is the first LC/MS study conducted to corroborate the structure of the adduct formed *in vivo*.

In addition to dG-C8-PhIP, a minor isomeric dG-PhIP adduct was detected in colon and liver tissues of experimental rats; this adduct had previously been detected, by TQ/MS/MS, in CT DNA modified at high levels with *N*-acetoxy-PhIP (11). The aglycone $[\text{BH}_2]^+$ ion of this isomeric dG-PhIP adduct does not undergo fragmentation at the guanine base with retention of the N^2 amino or cyanamide groups of guanine on the protonated PhIP moiety, at either the MS^3 or the MS^4 stage. We previously identified isomeric dG-C8 and dG- N^2 adducts of IQ and 8-MeIQx (59) and found these aforementioned fragmentation pathways to be characteristic of the dG- N^2 adducts of IQ and 8-MeIQx (adduction between the C-5 atom of the HAA heteronucleus and the N^2 amino group of dG) by TQ/MS/MS (29,50) and QIT/ MS^n .² The absence of those fragment ions in the MS^n product ion spectra of the isomeric dG-PhIP adduct suggests that the N^2 atom of dG is not the site of PhIP adduction. The MS^n product ion spectra also suggest that adduction does not occur at the O^6 or N1 atoms of guanine (Figure 8).

Guanyl-*N*7 intermediates have been proposed to be involved in the formation of dG-C8 arylamine DNA adducts (60). The initial bond formation occurs as a hydrazo linkage between the NH_2 position of the arylamine and the N7 atom of guanine, followed by rearrangement to

the more stable dG-C8 adduct; alternatively, the adduct can undergo depurination. Quaternary N7-dG adducts of aflatoxin B₁ and methylating agents arise *in vivo*, but they are unstable and result in spontaneous depurination of DNA to produce the corresponding DNA base and abasic site ($t_{1/2} = 24\text{--}160\text{ h in vivo}$) (61,62). Indeed, PhIP has been reported to induce DNA strand breakages; an N7 guanine adduct could account for such breakage (63). We have observed that the isomeric dG-PhIP adduct undergoes depurination more rapidly than dG-C8-PhIP.² On the basis of the current mass spectral data, we cannot exclude as plausible structures either a dG-N7 adduct or an uncommon dG-C8 adduct (64) formed with a carbenium ion intermediate derived from a reactive N-O-ester of PhIP. The exact structure of the isomeric dG adduct and its potential role in the genotoxicity of PhIP remain to be determined.

The data obtained by LC-ESI/MS/MSⁿ and ³²P-postlabeling are in qualitative agreement: the DNA adduct levels are about 5-fold higher in the colon than in the liver (Table 2). The adduct profiles are consistent with the target tissue specificity of PhIP, which induces tumors in the colon, but not the liver, of rats (1). Glutathione transferase A1 and UDP-glucuronosyl transferase 1A1 are phase II enzymes that are actively involved in the detoxification of HONH-PhIP. Higher levels of these enzymes are expressed in the liver than in the colon (65,66), which may provide greater protection for the liver against genotoxic PhIP metabolites.

However, the quantitative measurements of both DNA adduct detection methods are not in good agreement. The estimates of dG-C8-PhIP obtained by ³²P-postlabeling analysis are 15- to 20-fold lower than the estimates obtained by LC-ESI/MS/MSⁿ. The discrepancy in quantitative measurements by these two different methods is not surprising. The enzyme hydrolysis conditions used to measure PhIP—DNA adducts by LC-ESI/MS/MSⁿ and ³²P-postlabeling are vastly different. The DNA used for the ³²P-postlabeling studies (48,49) was digested with monococcal nuclease (MN) and spleen phosphodiesterase (SPD), whereas the enzymatic digestion scheme employed for samples analyzed by LC-ESI/MS/MSⁿ used a cocktail of four enzymes. Two independent laboratories have reported that enzymatic digestion of PhIP—DNA is incomplete with MN and SPD and that subsequent treatment with nuclease P1 is required to recover most of the oligomeric adduct as 3'-phospho-dG-C8-PhIP (58,67). In addition, the ATP-deficient, or intensification, conditions employed for our ³²P-postlabeling study used a 200:1 ratio of 3',5'-bisphospho-2'-deoxyribonucleotides to ³²P-ATP; although these conditions enhance the labeling of some bulky DNA adducts, including dG-C8-PhIP, it is unlikely that the labeling of dG-C8-PhIP was quantitative on the basis of the $>10^6$ mol excess of nonmodified 2'-deoxyribonucleotides (48). Finally, the recovery of the postlabeled, bisphosphate dG-C8-PhIP adduct, by TLC in an interlaboratory validation study, was reported to be only 5–10% (68), which can account for much of the discrepancy in the estimates of dG-C8-PhIP determined by ³²P-postlabeling and LC-ESI/MS/MSⁿ methods. We previously reported that DNA adducts of IQ formed in the livers of non-human primates are underestimated by about 5-fold, when determined by ³²P-postlabeling measurements, relative to estimates obtained by TQ/MS/MS (69). The accurate quantification of DNA adducts at trace levels by ³²P-postlabeling, for which the extent of enzyme hydrolysis and adduct labeling efficiency are both uncertain, is difficult, and it underscores the value of employing internal standards for accurate and reproducible quantification of DNA adducts by LC/MS methods.

Many of the studies on quantitative measurement of DNA adducts *in vivo* have been done by TQ/MS/MS and employ the SRM scan mode in order to attain the sensitivity required to detect DNA adducts formed at trace levels. However, the structural information obtained by SRM is limited because the CID conditions are optimized to monitor the transition $[M + H - 116]^+$ (loss of dR), and other product ions are generally not observed (18). The in-source CID offset can be set at a voltage sufficiently high to cause deribosylation of dG-C8-PhIP (or other adducts) prior to Q₁; therefore, essentially an MS³ scan event can be performed to obtain the product ions arising from the aglycone BH₂⁺ ion in Q₃ (11). The full product ion scan mode

in Q₃ provides rich structural information about the adduct, but it is accompanied by a significant loss in instrument sensitivity and is not routinely used for the trace analysis of DNA adducts. We successfully acquired in-source CID product ion spectra of isomeric dG-C8 and dG-N² adducts of MeIQx formed in the liver of rats treated with 8-MeIQx (29); however, 1 mg of DNA was required for analysis. Such a large quantity of DNA is rarely available from human biopsy samples for biomonitoring studies. The in-source CID technique for adduct fragmentation has also been performed in the selected ion monitoring (SIM) scan mode, using a single quadrupole to acquire both [M + H]⁺ and [BH₂]⁺ ions of the dG-C8 adduct of 4-aminobiphenyl (4-ABP) in rat tissues (31). This scan technique can be used for quantification and confers one qualifier ion for corroboration of structure; however, the SIM scan mode is generally less selective and sensitive than the SRM scan mode.

Both 3-D and 2-D QIT/MS are capable of MSⁿ scan events. Several recent reports describe the quantification of DNA adducts of α,β -unsaturated aldehydes (37,70) as well as qualitative characterizations of DNA adducts derived from aldehydes (71), nitrosamines (72), benzo[a]pyrene (73), and alkylanilines (52,74) by 3-D QIT/MS. However, only the MS/MS scan mode was used for data quantification; there was no report on the use of the MS³ scan mode for either the quantification or characterization of DNA adducts *in vivo*. The capacity of the 2-D QIT/MS to employ MSⁿ scan events for both the quantification and acquisition of high-quality full product ion spectra of DNA adducts formed at trace physiological levels is an important advance in DNA adduct detection techniques.

The LOD of dG-C8-PhIP on the QIT/MS with the LTQ is ~1 adduct per 10⁸ DNA bases, when 27 μ g of DNA are used for analysis with a 1 mm i.d. column at a flow rate of 50 μ L/ min. The performance of the analytical method was investigated for accuracy and precision over a time period of up to 6 months, using 3 independent measurements, at levels of adduct modification ranging from 1 to 100 adducts per 10⁸ DNA bases. The accuracy of the quantification was, within average, 10% of the target values, and the inter-day %CV values were between 6 and 9% at levels of adduct modification above the LOD. The precision of the assay includes not only the variation in MS instrument parameters and SPE processing but also the variation in the efficiency of enzyme hydrolysis of PhIP-modified DNA. We chose to do triplicate determinations for the samples, rather than conducting 5–10 measurements for improved precision because our goal is to measure PhIP—DNA adducts in human specimens, for which limiting quantities of DNA (generally <100 μ g) will preclude more than 2–3 replicate measurements. Thus, the performance of the analytical method reported here is representative of real world measurements. The sensitivity, accuracy, and precision of dG-C8-PhIP measurements, on 2-D QIT/MS, are comparable to the MS measurements of several different classes of carcinogen adducts at similar levels of modification of DNA, using either a TQ/MS/MS or single quadrupole MS instrument with narrow bore HPLC columns (29,31,72,75,76).

In humans, putative PhIP—DNA adducts have been detected in colon tissue by ³²P-postlabeling or by alkaline hydrolysis of DNA followed by analysis of the released PhIP by GC-NICI/MS (27). Adducts in mammary glands have been detected by IHC (77), adducts in exfoliated breast epithelial cells by ³²P-postlabeling (78), and adducts in lymphocytes by acid hydrolysis and analysis of released PhIP by LC/MS (79) at adduct levels ranging from 1 to >10 adducts per 10⁸ DNA bases. Collectively, these findings reveal that PhIP—DNA adducts do form in human tissues even though the concentration of PhIP in the diet is generally at a low ppb level. However, data on the adduct structure(s) obtained by the biochemical and analytical methods employed in all of these studies are ambiguous. On the basis of these reported levels of adduct modification in some human DNA samples, the level of sensitivity we have attained with the 2-D QIT/MS should be sufficient to definitively characterize and measure intact dG-C8-PhIP from human DNA samples. These investigations are currently underway in our laboratory.

Ion suppression is an important contributing factor in the reproducibility and accuracy problems associated with trace analyses of complex matrices (57). The ESI process is particularly susceptible to ion suppression (55-57), which occurs at the LC/MS interface when a coeluting component from the LC adversely influences the ionization or transmission of the analyte of interest into the vapor phase (57). We found that the impurity of the solvents and the polyethylene frits used in the SPE cartridges were major contributors to the ion suppression effects of dG-C8-PhIP. The diminution in the signal of dG-C8-PhIP in SPE-processed samples is not attributable to the deterioration in performance of the QIT/MS. If interfering components coeluted from the LC with dG-C8-PhIP, producing space charge effects and a decrease in sensitivity of the QIT/MS, the AGC would have adjusted the ionization time, and a different number of scans across the peaks would have been observed. However, the number of scans across the peak of dG-C8-PhIP as a pure standard, in calibration curves, or in SPE-purified samples from DNA digests was constant.

The development of an on-line adduct enrichment technique (31) or the employment of low-flow analysis, using nanospray ESI/MS, could circumvent some of the ion suppression effects (80), further improving the sensitivity of detection of dG-C8-PhIP or other DNA adducts. Indeed, recent studies have reported the detection and quantification of DNA adducts of IQ and 4-ABP at several adducts per 10⁹ DNA bases, assaying less than 10 µg of DNA by means of capillary chromatography and nanospray TQ/MS/MS (36,50).

Our goal is to use the QIT/MSⁿ, in conjunction with other MS techniques, to measure HAA-DNA adducts in human DNA samples as part of a battery of methodologies to be used in the risk assessment of PhIP and other HAAs in the etiology of human cancers. The powerful scanning capabilities of the 2-D QIT/MS provide a unique tool for the unambiguous identification of DNA adduct biomarkers *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

This research was funded by the Wadsworth Center, New York State Department of Health, and National Institutes of Health research Grant R21 ES014438. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS, NIH.

1 Abbreviations

8-MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
 IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline
 PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
 [²H₃C]-PhIP, 2-amino-1-(trideutero)methyl-6-phenylimidazo[4,5-*b*]pyridine
 4-ABP, 4-aminobiphenyl
 dG, 2'-deoxyguanosine
 dG-C8-PhIP, *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
 dG-C8—[²H₃C]-PhIP, *N*-(deoxyguanosin-8-yl)-2-amino-1-(trideutero)methyl-6-phenylimidazo[4,5-*b*]pyridine
 HONH-PhIP, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
 dR, deoxyribose
 AGC, automatic gain control
 AMS, accelerator mass spectrometry
 CID, collision-induced dissociation
 CNL, constant neutral loss

CRM, consecutive reaction monitoring
 CT DNA, calf thymus DNA
 GC-NICI/MS, gas chromatography-negative ion chemical ionization/mass spectrometry
 HAA, heterocyclic aromatic amine
 IHC, immunohistochemistry
 LCESI/MS, liquid chromatography-electrospray ionization/mass spectrometry
 LC-ESI/MS/MSⁿ, liquid chromatography-electrospray ionization/multi-stage mass spectrometry
 LC-ESI/MS/MS, liquid chromatography-electrospray ionization/tandem mass spectrometry
 LOD, limit of detection
 LOQ, limit of quantification
 MN, monococcal nuclease
 MSⁿ, multi-stage MS scan event
 LTQ, Finnigan LTQ 2-D linear ion trap mass spectrometer
 ppb, parts-per-billion
 QIT/MS, quadrupole ion trap mass spectrometry
 RAL, relative adduct labeling
 SIM, selected ion monitoring
 S/N, signal-to-noise
 SPE, solid phase extraction
 SPD, spleen phosphodiesterase
 SRM, selected reaction monitoring
 TQ/MS/MS, triple quadrupole tandem mass spectrometry
 TSQ, Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer

References

- (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]
- (2). Felton, JS.; Jagerstad, M.; Knize, MG.; Skog, K.; Wakabayashi, K. Contents in Foods, Beverages and Tobacco. In: Nagao, M.; Sugimura, T., editors. *Food Borne Carcinogens Heterocyclic Amines*. John Wiley & Sons, Ltd.; Chichester, England: 2000. p. 31-71.
- (3). Sinha R, Rothman N, Brown ED, Salmon CP, Knize MG, Swanson CS, Rossi SC, Mark SD, Levander OA, Felton JS. High concentrations of the carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) occur in chicken but are dependent on the cooking method. *Cancer Res* 1995;55:4516–4519. [PubMed: 7553619]
- (4). Manabe S, Tohyama K, Wada O, Aramaki T. Detection of a carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, in cigarette smoke condensate. *Carcinogenesis* 1991;12:1945–1947. [PubMed: 1934275]
- (5). 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]
- (6). Miller EC. Some current perspectives on chemical carcin—genesis in humans and experimental animals: Presidential address. *Cancer Res* 1978;38:1479–1496. [PubMed: 348302]
- (7). Butler MA, Iwasaki M, Guengerich FP, Kadlubar FF. Human cytochrome P-450PA (P450IA2), the phenacetin O-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and N-oxidation of carcinogenic arylamines. *Proc. Natl. Acad. Sci. U.S.A* 1989;86:7696–7700. [PubMed: 2813353]
- (8). Wu RW, Panteleakos FN, Kadkhodayan S, Bolton-Grob R, McManus ME, Felton JS. Genetically modified Chinese hamster ovary cells for investigating sulfotransferase-mediated cytotoxicity and mutation by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. *Environ. Mol. Mutagen* 2000;35:57–65. [PubMed: 10692228]
- (9). Schut HA, Snyderwine EG. DNA adducts of heterocyclic amine food mutagens: implications for mutagenesis and carcinogenesis. *Carcinogenesis* 1999;20:353–368. [PubMed: 10190547]

- (10). Marsch GA, Goldman EN, Fultz E, Shen NH, Turteltaub KW. Heterogeneous DNA adduct formation in vitro by the acetylated food mutagen 2-(acetoxyamino)-1-methyl-6-phenylimidazo-[4,5-b]pyridine: a fluorescence spectroscopic study. *Chem Res. Toxicol* 1995;8:659–670. [PubMed: 7548748]
- (11). Rindgen D, Turesky RJ, Vouros P. Determination of in vitro formed DNA adducts of 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine using capillary liquid chromatography/electrospray ionization/tandem mass spectrometry. *Chem. Res. Toxicol* 1995;8:1005–1013. [PubMed: 8605282]
- (12). Crosbie SJ, Murray S, Boobis AR, Gooderham NJ. Mass spectrometric detection and measurement of N²-(2'-deoxyguanosin-8-yl)PhIP adducts in DNA. *J. Chromatogr., B* 2000;744:55–64.
- (13). Lin D-X, Kaderlik KR, Turesky RJ, Miller DW, Lay JO Jr. 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]
- (14). Frandsen H, Grivas S, Andersson R, Dragsted L, Larsen JC. Reaction of the N²-acetoxy derivative of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) with 2'-deoxyguanosine and DNA. Synthesis and identification of N²-(2'-deoxyguanosin-8-yl)-PhIP. *Carcinogenesis* 1992;13:629–635. [PubMed: 1576716]
- (15). Poirier MC, Beland FA. DNA adduct measurements and tumor incidence during chronic carcinogen exposure in animal models: Implications for DNA adduct-based human cancer risk assessment. *Chem. Res. Toxicol* 1992;5:749–755. [PubMed: 1489923]
- (16). La DK, Swenberg JA. DNA adducts: biological markers of exposure and potential applications to risk assessment. *Mutat. Res* 1996;365:129–146. [PubMed: 8898994]
- (17). Randerath K, Randerath E, Agrawal HP, Gupta RC, Schurda ME, Reddy MV. Postlabeling methods for carcinogen-DNA adduct analysis. *Environ. Health Perspect* 1985;62:57–65. [PubMed: 3910421]
- (18). Turesky RJ, Vouros P. Formation and analysis of heterocyclic aromatic amine-DNA adducts in vitro and in vivo. *J. Chromatogr., B* 2004;802:155–166.
- (19). Randerath E, Miller RH, Mittal D, Avitts TA, Dunsford HA, Randerath K. Covalent DNA damage in tissues of cigarette smokers as determined by ³²P-postlabeling assay. *J. Natl. Cancer Inst* 1989;81:341–347. [PubMed: 2915370]
- (20). Floyd RA, Watson JJ, Wong PK, Altmiller DH, Rickard RC. Hydroxyl free radical adduct of deoxyguanosine: sensitive detection and mechanisms of formation. *Free Radical Res. Commun* 1986;1:163–172. [PubMed: 2577733]
- (21). Jankowiak R, Small GJ. Fluorescence line-narrowing spectroscopy in the study of chemical carcinogenesis. *Anal. Chem* 1989;61:1023A–1031A. [PubMed: 2729598]
- (22). Santella RM. Immunological methods for detection of carcinogen-DNA damage in humans. *Cancer Epidemiol., Biomarkers Prev* 1999;8:733–739. [PubMed: 10498391]
- (23). Turteltaub KW, Felton JS, Gledhill BL, Vogel JS, Southon JR, Caffee MW, Finkel RC, Nelson DE, Proctor ID, Davis JC. Accelerator mass spectrometry in biomedical dosimetry: relationship between low-level exposure and covalent binding of heterocyclic amine carcinogens to DNA. *Proc. Natl. Acad. Sci U.S.A* 1990;87:5288–5292. [PubMed: 2371271]
- (24). Farmer PB, Brown K, Tompkins E, Emms VL, Jones DJ, Singh R, Phillips DH. DNA adducts: Mass spectrometry methods and future prospects. *Toxicol. Appl. Pharmacol* 2005;207:293–301. [PubMed: 15990134]
- (25). Giese RW, Vouros P. Methods development toward the measurement of polyaromatic hydrocarbon-DNA adducts by mass spectrometry. *Res. Rep. Health Eff. Inst* 1993;1–25. [PubMed: 8267901]
- (26). Koc H, Swenberg JA. Applications of mass spectrometry for quantitation of DNA adducts. *J. Chromatogr., B* 2002;778:323–343.
- (27). 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 ³²P-postlabeling. *Chem. Res. Toxicol* 1994;7:733–739. [PubMed: 7696526]

- (28). Turesky RJ, Box RM, Markovic J, Gremaud E, Snyderwine EG. Formation and persistence of DNA adducts of 2-amino-3-methylimidazo[4,5-f]quinoline in the rat and nonhuman primates. *Mutat. Res* 1997;376:235–241. [PubMed: 9202760]
- (29). 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]
- (30). Singh R, Farmer PB. Liquid chromatography-electro-spray ionization-mass spectrometry: the future of DNA adduct detection. *Carcinogenesis* 2006;27:178–196. [PubMed: 16272169]
- (31). Doerge DR, Churchwell MI, Marques MM, Beland FA. Quantitative analysis of 4-aminobiphenyl-C8-deoxyguanosyl DNA adducts produced in vitro and in vivo using HPLC-ES-MS. *Carcinogenesis* 1999;20:1055–1061. [PubMed: 10357788]
- (32). Beland FA, Churchwell MI, Von, Tungeln LS, Chen S, Fu PP, Culp SJ, Schoket B, Gyorffy E, Minarovits J, Poirier MC, Bowman ED, Weston A, Doerge DR. High-performance liquid chromatography electrospray ionization tandem mass spectrometry for the detection and quantitation of benzo[a]-pyrene-DNA adducts. *Chem. Res. Toxicol* 2005;18:1306–1315. [PubMed: 16097804]
- (33). Beland FA, Churchwell MI, Doerge DR, Parkin DR, Malejka-Giganti D, Hewer A, Phillips DH, Carmichael PL, Gamboa, da CG, Marques MM. Electrospray ionization-tandem mass spectrometry and ³²P-postlabeling analyses of tamoxifen-DNA adducts in humans. *J. Natl. Cancer Inst* 2004;96:1099–1104. [PubMed: 15265972]
- (34). Gamboa da CG, Churchwell MI, Hamilton LP, Von, Tungeln LS, Beland FA, Marques MM, Doerge DR. DNA adduct formation from acrylamide via conversion to glycidamide in adult and neonatal mice. *Chem. Res. Toxicol* 2003;16:1328–1337. [PubMed: 14565774]
- (35). Ruan Q, Kim HY, Jiang H, Penning TM, Harvey RG, Blair IA. Quantification of benzo[a]pyrene diol epoxide DNA-adducts by stable isotope dilution liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom* 2006;20:1369–1380. [PubMed: 16557497]
- (36). Ricicki EM, Soglia JR, Teitel C, Kane R, Kadlubar F, Vouros P. Detection and quantification of N-(deoxyguanosin-8-yl)-4-aminobiphenyl adducts in human pancreas tissue using capillary liquid chromatography-microelectrospray mass spectrometry. *Chem. Res. Toxicol* 2005;18:692–699. [PubMed: 15833029]
- (37). Liu X, Lovell MA, Lynn BC. Detection and quantification of endogenous cyclic DNA adducts derived from trans-4-hydroxy-2-nonenal in human brain tissue by isotope dilution capillary liquid chromatography nanoelectrospray tandem mass spectrometry. *Chem. Res. Toxicol* 2006;19:710–718. [PubMed: 16696574]
- (38). Paul W. Electromagnetic traps for charged and neutral particles (Nobel lecture). *Agnew. Chem., Int. Ed. Engl* 1990;29:739–748.
- (39). Cooks RG, Glish GL, McLuckey SA, Kaiser RE. Ion trap mass spectrometry. *Chem. Eng. News* 1991;69:26–41.
- (40). Andreu V, Pico Y. Liquid chromatography-ion trap-mass spectrometry and its application to determine organic contaminants in the environment and food. *Curr. Anal. Chem* 2005;1:241–265.
- (41). Schwartz JC, Senko MW, Syka JEP. A Two-dimensional quadrupole ion trap mass spectrometer. *J. Am. Soc. Mass Spectrom* 2002;13:659–669. [PubMed: 12056566]
- (42). Quarmby ST, Yost RA. Fundamental studies of injection and ion trapping of electrosprayed ions on a quadrupole ion trap. *Int. J. Mass Spectrom* 1999;190/191:81–102.
- (43). Thermo Electron. Thermo Electron Corporation; San Jose, CA: 2006. Radial Ejection and Dual Detection on the Finnigan LTQ Linear Ion Trap Mass Spectrometer. PSB 117.
- (44). Bonilla LE, Thakur R, Guzzetta A, Jaffe JD. Radically improved bottom-up protein identification using a Finnigan LTQ ion trap mass spectrometer. *JASCO Appl. Notes* 2006;328
- (45). Riter LS, Gooding KM, Hodge BD, Julian RK Jr. Comparison of the Paul ion trap to the linear ion trap for use in global proteomics. *Proteomics* 2006;6:1735–1740. [PubMed: 16475232]
- (46). Turesky RJ, Lang NP, Butler MA, Teitel CH, Kadlubar FF. Metabolic activation of carcinogenic heterocyclic aromatic amines by human liver and colon. *Carcinogenesis* 1991;12:1839–1845. [PubMed: 1934265]

- (47). 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 acquisition and data quality evaluation in environmental chemistry. *Anal. Chem* 1980;52:2242–2249.
- (48). Schut HA, Herzog CR. Formation of DNA adducts of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in male Fischer-344 rats. *Cancer Lett* 1992;67:117–124. [PubMed: 1483260]
- (49). Randerath E, Agrawal HP, Weaver JA, Bordelon CB, Randerath K. ³²P-postlabeling analysis of DNA adducts persisting for up to 42 weeks in the skin, epidermis and dermis of mice treated topically with 7,12-dimethylbenz[a]anthracene. *Carcino-genesis* 1985;6:1117–1126.
- (50). Soglia JR, Turesky RJ, Paehler A, Vouros P. Quantification of the heterocyclic aromatic amine DNA adduct N-(deoxyguanosin-8-yl)-2-amino-3-methylimidazo[4,5-f]quinoline in livers of rats using capillary liquid chromatography/microelectrospray mass spectrometry: a dose-response study. *Anal. Chem* 2001;73:2819–2827. [PubMed: 11467522]
- (51). Chiarelli MP, Lay JO Jr. Mass spectrometry for the analysis of carcinogen-DNA adducts. *Mass Spectrom. Rev* 1992;11:447–493.
- (52). Li L, Chiarelli MP, Branco PS, Antunes AM, Marques MM, Goncalves LL, Beland FA. Differentiation of isomeric C8-substituted alkyraniline adducts of guanine by electrospray ionization and tandem quadrupole ion trap mass spectrometry. *J. Am. Soc. Mass Spectrom* 2003;14:1488–1492. [PubMed: 14652195]
- (53). Gregson JM, McCloskey JA. Collision-induced dissociation of guanine. *Int. J. Mass Spectrom. Ion Processes* 1997;165/166:475–485.
- (54). Brown K, Guenther EA, Dingley KH, Cosman M, Harvey CA, Shields SJ, Turteltaub KW. Synthesis and spectroscopic characterization of site-specific 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine oligodeoxyribonucleotide adducts. *Nucleic Acids Res* 2001;29:1951–1959. [PubMed: 11328879]
- (55). King R, Bonfiglio R, Fernandez-Metzler C, Miller-Stein C, Olah T. Mechanistic investigation of ionization suppression in electrospray ionization. *J. Am. Soc. Mass Spectrom* 2000;11:942–950. [PubMed: 11073257]
- (56). Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal. Chem* 2003;75:3019–3030. [PubMed: 12964746]
- (57). Jessome LL, Volmer DA. Ion suppression: a major concern in mass spectrometry. *LC-GC* 2006;24:498–510.
- (58). Pfau W, Brockstedt U, Sohren KD, Marquardt H. ³²P-post-labelling analysis of DNA adducts formed by food-derived heterocyclic amines: evidence for incomplete hydrolysis and a procedure for adduct pattern simplification. *Carcinogenesis* 1994;15:877–882. [PubMed: 8200090]
- (59). Turesky RJ, Rossi SC, Welti DH, Lay JO 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 N2 atoms of guanine. *Chem. Res. Toxicol* 1992;5:479–490. [PubMed: 1391614]
- (60). Humphreys WG, Kadlubar FF, Guengerich FP. Mechanism of C8 alkylation of guanine residues by activated arylamines: evidence for initial adduct formation at the N7 position. *Proc. Natl. Acad. Sci. U.S.A* 1992;89:8278–8282. [PubMed: 1518858]
- (61). Groopman JD, Busby WF Jr, Wogan GN. Nuclear distribution of aflatoxin B1 and its interaction with histones in rat liver in vivo. *Cancer Res* 1980;40:4343–4351. [PubMed: 7438068]
- (62). Hecht SS. Biochemistry, biology, and carcinogenicity of tobacco-specific N-nitrosamines. *Chem. Res. Toxicol* 1998;11:559–603. [PubMed: 9625726]
- (63). Pfau W, Martin FL, Cole KJ, Venitt S, Phillips DH, Grover PL, Marquardt H. Heterocyclic aromatic amines induce DNA strand breaks and cell transformation. *Carcinogenesis* 1999;20:545–551. [PubMed: 10223180]
- (64). Enya T, Kawanishi M, Suzuki H, Matsui S, Hisamatsu Y. An unusual DNA adduct derived from the powerfully mutagenic environmental contaminant 3-nitrobenzanthrone. *Chem. Res. Toxicol* 1998;11:1460–1467. [PubMed: 9860488]

- (65). Lin D-X, Meyer DJ, Ketterer B, Lang NP, Kadlubar FF. Effects of human and rat glutathione-S-transferase on the covalent binding of the N-acetoxy derivatives of heterocyclic amine carcinogens in vitro: a possible mechanism of organ specificity in their carcinogenesis. *Cancer Res* 1994;54:4920–4926. [PubMed: 8069858]
- (66). Strassburg CP, Nguyen N, Manns MP, Tukey RH. UDP-glucuronosyltransferase activity in human liver and colon. *Gastroenterology* 1999;116:149–160. [PubMed: 9869613]
- (67). Fukutome K, Ochiai M, Wakabayashi K, Watanabe S, Sugimura T, Nagao M. Detection of guanine-C8-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine adduct as a single spot on thin-layer chromatography by modification of the ³²P-postlabeling method. *Jpn. J. Cancer Res* 1994;85:113–117. [PubMed: 8144391]
- (68). Phillips DH, Castegnaro M. Standardization and validation of DNA adduct postlabelling methods: report of interlaboratory trials and production of recommended protocols. *Mutagenesis* 1999;14:301–315. [PubMed: 10374998]
- (69). Gangl ET, Turesky RJ, Vouros P. Determination of in vitro- and in vivo-formed DNA adducts of 2-amino-3-methylimidazo[4,5-f]quinoline by capillary liquid chromatography/microelectrospray mass spectrometry. *Chem. Res. Toxicol* 1999;12:1019–1027. [PubMed: 10525280]
- (70). Liu X, Lovell MA, Lynn BC. Development of a method for quantification of acrolein-deoxyguanosine adducts in DNA using isotope dilution-capillary LC/MS/MS and its application to human brain tissue. *Anal. Chem* 2005;77:5982–5989. [PubMed: 16159131]
- (71). Stout MD, Jeong YC, Boysen G, Li Y, Sangaiah R, Ball LM, Gold A, Swenberg JA. LC/MS/MS method for the quantitation of trans-2-hexenal-derived exocyclic 1,N(2)-propanodeoxyguanosine in DNA. *Chem. Res. Toxicol* 2006;19:563–570. [PubMed: 16608168]
- (72). Lao Y, Villalta PW, Sturla SJ, Wang M, Hecht SS. Quantitation of pyridyloxobutyl DNA adducts of tobacco-specific nitrosamines in rat tissue DNA by high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry. *Chem. Res. Toxicol* 2006;19:674–682. [PubMed: 16696570]
- (73). Bhattacharya S, Barbacci DC, Shen M, Liu JN, Casale GP. Extraction and purification of depurinated benzo[a]pyrene-adducted DNA bases from human urine by immunoaffinity chromatography coupled with HPLC and analysis by LC/quadrupole ion-trap MS. *Chem. Res. Toxicol* 2003;16:479–486. [PubMed: 12703964]
- (74). Jones CR, Sabbioni G. Identification of DNA adducts using HPLC/MS/MS following in vitro and in vivo experiments with arylamines and nitroarenes. *Chem. Res. Toxicol* 2003;16:1251–1263. [PubMed: 14565767]
- (75). Doerge DR, Churchwell MI, Fang JL, Beland FA. Quantification of etheno-DNA adducts using liquid chromatography, on-line sample processing, and electrospray tandem mass spectrometry. *Chem. Res. Toxicol* 2000;13:1259–1264. [PubMed: 11123967]
- (76). Beland FA, Churchwell MI, Von Tungeln LS, Chen S, Fu PP, Culp SJ, Schoket B, Gyorffy E, Minarovits J, Poirier MC, Bowman ED, Weston A, Doerge DR. High-performance liquid chromatography electrospray ionization tandem mass spectrometry for the detection and quantitation of benzo[a]-pyrene-DNA adducts. *Chem. Res. Toxicol* 2005;18:1306–1315. [PubMed: 16097804]
- (77). Zhu J, Chang P, Bondy ML, Sahin AA, Singletary SE, Takahashi S, Shirai T, Li D. Detection of 2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine-DNA adducts in normal breast tissues and risk of breast cancer. *Cancer Epidemiol., Biomarkers Prev* 2003;12:830–837. [PubMed: 14504191]
- (78). Gorlewska-Roberts K, Green B, Fares M, Ambrosone CB, Kadlubar FF. Carcinogen-DNA adducts in human breast epithelial cells. *Environ. Mol. Mutagen* 2002;39:184–192. [PubMed: 11921188]
- (79). Magagnotti C, Pastorelli R, Pozzi S, Andreoni B, Fanelli R, Airoidi 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]
- (80). Gangl ET, Annan MM, Spooner N, Vouros P. Reduction of signal suppression effects in ESI-MS using a nanosplitting device. *Anal. Chem* 2001;73:5635–5644. [PubMed: 11774901]

Figure 1. Product ion spectrum of dG-C8-PhIP by 2-D QIT/MS. MS³ product ion spectrum of the aglycone guanylyl-C8-PhIP $[BH_2]^+$ at m/z 374.

Scheme 1.

Proposed Pathways of Fragmentation of Guanyl-C8-PhIP at MS³ and Prominent Product Ions Observed at the MS⁴

Figure 2. Kinetics of the enzymatic digestion of PhIP-modified CT DNA (1 adduct per 10⁶ DNA bases) monitoring the ratio of dG-C8-PhIP/dG-C8-[²H₃C]-PhIP (5 adducts per 10⁶ DNA bases) by TQ/MS/MS, using SRM scan mode.

Figure 3.

Analysis of PhIP—DNA adducts *in vivo*, using the CNL scan mode of the TSQ to monitor the loss of dR ($[M + H - 116]^+$). The mass chromatograms show the extracted ion at m/z 490 $[M + H]^+$ for dG-C8-PhIP. (A) PhIP-treated rat colon DNA. (B) Untreated rat DNA.

Figure 4.

Influence of solvent purity on the S/N of the dG-C8-PhIP standard (5 pg on column). (A) HPLC-grade CH_3CN and distilled and deionized H_2O (Barnstead NANOpure Diamond) as the mobile phase. (B) High-purity B & J Brand CH_3CN and water as the mobile phase. The top panels represent the protonated ion $[M + H]^+$ at m/z 490 (peak width 1 Da) extracted from the full scan MS monitoring from m/z 100 to 1000 Da; the panels in the middle represent the reconstructed total-ion current chromatograms of the MS/MS spectrum of the protonated ion $[M + H]^+$ at m/z 490, scanning from m/z 240 to 550 Da; and the lower panels represent the reconstructed total-ion current chromatograms of the MS^3 spectrum of the aglycone ion $[\text{BH}_2]^+$ at m/z 374, scanning from m/z 185 to 550 Da.

Figure 5.

LC-ESI/MS/MSⁿ chromatograms of dG-C8-PhIP-modified CT DNA at levels of (from top to bottom) 0, 1, and 3 adducts per 10⁸ DNA bases and dG-C8-[²H₃C]-PhIP (bottom panel) added to DNA at 1 adduct per 10⁷ DNA prior to enzymatic digestion. (A) MS/MS scan mode. (The ions at *m/z* 374 and 377 were extracted for dG-C8-PhIP and dG-C8-[²H₃C]-PhIP, respectively.) (B) MS³ scan mode. (The ions at *m/z* 250, 304, 329, and 357 were extracted for guanylyl-C8-PhIP, and the ions at *m/z* 253, 307, 332, and 360 were extracted for guanylyl-C8-[²H₃C]-PhIP.)

Figure 6. ^{32}P -postlabeling TLC profiles of colon and liver DNA of rats treated with PhIP as part of the diet (0.04% (w/w) for 21 days. Autoradiography was done for 5 h at -80°C for the PhIP-treated rat samples and for 16 h at -70°C for the untreated rat samples. (A) PhIP-treated rat liver DNA. (B) Untreated rat liver DNA. (C) PhIP-treated rat colon DNA. (D) Untreated rat colon DNA. The more slowly migrating lesion has the same R_f as bisphospho-dG-C8-PhIP (11).

Figure 7.

LC-ESI/MS/MSⁿ chromatograms of PhIP-modified DNA *in vivo* following enzymatic digestion of (from top to bottom) the colon of an untreated rat and the liver and colon of rats exposed to PhIP as part of the diet (0.04% (w/w) for 21 days, and dG-C8-[²H₃C]-PhIP (bottom panel) added to the rat DNA samples at 5 adducts per 10⁷ DNA prior to enzymatic digestion. (A) MS/MS scan mode. (B) MS³ scan mode. The y-scale of the isomeric dG-PhIP adduct is magnified 5×. In the MS/MS scan mode, the ions at *m/z* 374 and 377 were extracted for dG-C8-PhIP and dG-C8-[²H₃C]-PhIP, respectively. In the MS³ scan mode, the ions at *m/z* 250, 304, 329, and 357 were extracted for guanyl-C8-PhIP, and the ions at *m/z* 253, 307, 332, and 360 were extracted for guanyl-C8-[²H₃C]-PhIP.

Figure 8.

Product ion mass spectra of dG-C8-PhIP and the unknown dG-PhIP isomer, following enzymatic digestion of rat colon DNA. (A) dG-C8-PhIP (from left to right): MS³ of the aglycone ion [BH₂]⁺ at m/z 374 and MS⁴ of the ion at m/z 357. (B) Unknown dG-PhIP isomer (from left to right): MS³ of the aglycone ion [BH₂]⁺ at m/z 374 and MS⁴ of the ion at m/z 357 and MS⁴ of the ion at m/z 225.

Table 1

Quantification of dG-C8-PhIP in Modified DNA: Intra-day and Inter-day Values (Mean and RSD(%), $N = 3$ Independent DNA Samples)
^a

	level of adduct modification	intra-day variation		MS ³	inter-day variation		MS ³
		MS/MS			MS/MS		
day 1	100 adducts per 10 ⁸ DNA bases RSD (%)	111	118 ^b		109	112	
day 2		0.6(%)	3.1(%)		6.2(%)	7.4(%)	
day 3		108	110				
day 1	10 adducts per 10 ⁸ DNA bases RSD (%)	9.1(%)	8.4(%)				
day 2		109	108				
day 3		9.1(%)	8.4(%)				
day 1	3 adducts per 10 ⁸ DNA bases RSD (%)	10.6	10.6		10.8	10.6	
day 2		11.3(%)	6.1(%)		9.90%	7.10%	
day 3		10.5	10.4				
day 1	1 adduct per 10 ⁸ DNA bases RSD (%)	16.1(%)	13.5(%)				
day 2		11.2	10.7				
day 3		3.6(%)	1.8(%)				
day 1		3.7	3.3		3.3	3.1	
day 2		1.4(%)	1.5(%)		7.7(%)	9.0(%)	
day 3		3.1	3.2				
day 4		5.2(%)	2.2(%)				
day 5		3.2	3.0				
day 6		2.3(%)	9.3(%)				
day 1		3.2	3.1				
day 2		6.3(%)	1.9(%)				
day 3		3.2	2.7				
day 4		4.7(%)	5.9(%)				
day 5		3.3	3.2				
day 6		4.5(%)	5.9(%)				
day 1	1 adduct per 10 ⁸ DNA bases RSD (%)	1.4	0.8		1.1	1.0	
day 2		16.7(%)	32.1(%)		17.7(%)	20.8(%)	
day 3		1.1	1.2				
day 4		17.7(%)	9.1(%)				
day 5		1.2	1.0				
day 6		19.0(%)	15.8(%)				
day 1		1.0	0.8				
day 2		9.0(%)	35.1(%)				
day 3		1.0	1.0				
day 4		3.9(%)	1.5(%)				
day 5		1.1	1.0				
day 6		11.6(%)	7.6(%)				

^aThe background signal of nonmodified DNA was 0.4 ± 0.2 adducts and 0.2 ± 0.1 adducts per 10⁸ DNA bases in MS/MS and MS³ scan modes (mean $\pm$ between day S.D., $N = 16$ independent measurements conducted over 4 different days).

^bMS/MS and MS³ values are statistically different ($P < 0.04$).

Table 2Estimates of PhIP—DNA Adducts in Rat Colon and Liver by QIT/MS and ³²P-postlabeling^a

tissue	QIT/MS estimates (mean ± SD) (adducts per 10 ⁸ DNA bases)			³² P-postlabeling (mean ± SD) (RAL, adducts per 10 ⁸ DNA bases)
	MS ⁿ	dG-C8-PhIP	dG-PhIP isomer	total adducts
colon	2	N.D. ^b	N.D.	N.D.
(untreated)	3	N.D.	N.D.	
colon	2	329 ± 22	8.6 ± 2.0	20 ± 3
(PhIP-treated)	3	361 ± 22	7.7 ± 1.6	
liver	2	N.D.	N.D.	N.D.
(untreated)	3	N.D.	N.D.	
liver	2	69.5 ± 2.1	3.2 ± 0.6	3.0 ± 0.6
(PhIP-treated)	3	69.9 ± 0.2	3.6 ± 1.0	

^aN = 3 analyses from independent DNA samples.^bN.D. = not detected.