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Identification of Carcinogen DNA Adducts in Human Saliva by Linear Quadrupole Ion Trap/Multistage Tandem Mass Spectrometry

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Abstract

DNA adducts of carcinogens derived from tobacco smoke and cooked meat were identified, by liquid chromatography-electrospray ionization/multi-stage tandem mass spectrometry (LC-ESI/MS/MS^n), in saliva samples from 37 human volunteers on unrestricted diets. The *N*-(deoxyguanosin-8-yl) (dG-C8) adducts of the heterocyclic aromatic amines 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP); 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC); 2-amino-3,8-dimethylmidazo[4,5-*f*]quinoxaline (MeIQx); and the aromatic amine, 4-aminobiphenyl (4-ABP) were characterized and quantified, by LC-ESI/MS/MS^n, employing consecutive reaction monitoring at the MS^3 scan stage mode with a linear quadrupole ion trap (LIT) mass spectrometer (MS). DNA adducts of PhIP were found most frequently: dG-C8-PhIP was detected in saliva samples from 13 of 29 ever-smokers and in saliva samples from 2 of 8 never-smokers. dG-C8-AαC and dG-C8-MeIQx were identified solely in saliva samples of 3 current smokers, and dG-C8-4-ABP was detected in saliva from 2 current-smokers. The levels of these different adducts ranged from 1 to 9 adducts per 10^8 DNA bases. These findings demonstrate that PhIP is a significant DNA-damaging agent in humans. Saliva appears to be a promising biological fluid in which to assay DNA adducts of tobacco and dietary carcinogens, by selective LIT MS techniques.

Introduction

The covalent modification of DNA by chemical mutagens is recognized as the initiating step in chemical carcinogenesis (1). DNA adduct measurement is an important endpoint, both for cross-species extrapolation of the biologically effective dose and for the human risk

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Supporting Information Paragraph: Calibration curves of DNA adducts (Figure S-1) are shown in Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

assessment of exposure to chemical carcinogens (2,3). Identification and quantification of chemical-specific adducts in the target tissue are the most relevant findings for risk assessment (3). However, the opportunity to measure carcinogen DNA adducts in human tissues is often precluded by the unavailability of biopsy samples. Thus, accessible biological fluids, such as blood (4), urine (5), exfoliated bladder epithelial cells in urine (6), or exfoliated mammary epithelial cells in milk of lactating women (7,8), have served as surrogate matrices in which to assess exposure to chemicals, or their metabolites, or formation of DNA adducts. The identification of DNA adducts clearly demonstrates exposure to the biologically active metabolite; however, the adduct must correlate with cancer risk, if it is considered valid as a biomarker of health risk (9).

The oral cavity is the portal of entry for carcinogens that are ingested in the diet or inhaled through smoking. Epithelial buccal cells and leukocytes are the principal mammalian cells present in saliva (10,11). Cells of the oral cavity have been employed to measure biomarkers of genetic damage, molecular, and cellular changes following exposure to tobacco smoke or arsenic, as well as to assess the efficacy of chemopreventive agents (11-17). The expression of mRNA of several cytochrome P450 (P450) enzymes, including P450s 1A1 and 1A2, which bioactivate carcinogens, has been detected in buccal cells (18,19). When placed in primary culture, buccal cells were shown to bioactivate aflatoxin B_1 (18), benzo[a]pyrene (B[a]P) (20), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (21), to metabolites that bound to DNA or protein. More recently, the P450 1A2 protein was detected in human salivary glands, by immunohistochemical methods (22). P450 1A2 is a principal enzyme involved in the bioactivation of heterocyclic aromatic amines (HAAs) and the aromatic amine 4-aminobiphenyl (4-ABP) (23,24).

Both ^{32}P-postlabeling (25-27) and immunohistochemical techniques (28,29) have been employed to screen for DNA adducts in cells of the oral cavity. Several of the studies reported differences in total DNA adduct levels between smokers and non-smokers; however, the complexity of the adduct profiles and the inability to identify specific DNA adducts precluded any interpretation on the principal DNA damaging agents and their significance in the risk of development of oral cancer or cancers of other organs (25-27,30). It is noteworthy that high levels of DNA adducts were detected in the oral cavity of nonsmokers: many of these lesions could be attributed to genotoxicants in the diet.

Electrospray ionization (ESI) is a soft-ionization technique employed to detect non-volatile and thermally labile compounds (31). The on-line coupling of liquid chromatography with ESI-tandem mass spectrometry can be used to provide structural information on DNA adducts, and the incorporation of stable, isotopically labeled internal standards allows precise and accurate quantification of DNA adducts (32,33). While the levels of sensitivity of recent vintage MS instruments are sufficient to detect carcinogen DNA adducts at trace levels in human tissues, there is still a need to establish DNA adduct biomarkers in surrogate tissues or fluids that can be obtained non-invasively, to permit assessment of DNA damage posed by chemical carcinogens.

We recently employed a linear quadrupole ion trap mass spectrometer (LIT MS) to screen for DNA adducts of environmental, dietary, and endogenous genotoxicants, by data-dependent constant neutral loss scanning, followed by triple-stage mass spectrometry from oral cells of smokers (34). Cyclic DNA adducts of acrolein, a highly reactive α,β-unsaturated aldehyde that is formed endogenously through lipid peroxidation, and that also arises in cigarette smoke (35), were identified. However, DNA adducts of other tobacco-related or meat-derived carcinogens, that include benzo[a]pyrene (B[a]P), 4-ABP, 2-AαC, PhIP, and MeIQx (34), were not detected. In this present study, we have screened for DNA adducts of the above tobacco and meat-associated carcinogens in saliva samples of subjects,

employing liquid chromatography-electrospray ionization/multi-stage tandem mass spectrometry (LC-ESI/MS/MS^n) at the MS^3 scan stage with the LIT MS. DNA adducts of all of these carcinogens, except for B[a]P, were identified in a number of human saliva samples. Human saliva appears to be a highly promising biological fluid with which we can monitor exposure to various carcinogens, through detection of their DNA adducts.

Materials and Methods

Caution: AαC, 4-ABP, B[a]P, MeIQx, PhIP, and their derivatives are carcinogens, and they should only be handled in a well-ventilated fume hood with the appropriate protective clothing.

Chemicals. MeIQx, 3-[^2H_3C]-MeIQx, and PhIP were purchased from Toronto Research Chemicals (Toronto, ON, Canada). 2-Nitro-9*H*-pyrido[4,5-*b*]indole was a kind gift from Dr. D. Miller (National Center for Toxicological Research,(Jefferson, AR). (±)-*r*-7,*t*-8-Dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene ((±) (anti)B[a]PDE)) was purchased from the NCI Chemical Carcinogen Reference Standards Repository, Midwest Research Institute (Kansas City, MO). 4-Nitrobiphenyl was purchased from Aldrich (Milwaukee, WI). Calf thymus (CT) DNA, deoxyguanosine (dG), DNase I (Type IV, bovine pancreas), alkaline phosphatase (from *E. coli*), and nuclease P1 (from *Penicillium citrinum*) were purchased from Sigma (St. Louis, MO). [$^{13}C_{10}$]-dG was purchased from Cambridge Isotopes (Andover, MA). Phosphodiesterase I (from *Crotalus adamanteus* venom) was from GE Healthcare (Piscataway, NJ). All solvents used were high-purity B & J Brand from Honeywell Burdick and Jackson (Muskegon, MI). ACS reagent-grade formic acid (88%) was purchased from J.T. Baker (Phillipsburg, NJ). HyperSep™ filter SpinTips C18 (20 mg) were from Thermo Scientific (Palm Beach, FL). Oragene-DNA saliva kits were from Genotek Inc. (Ontario, Canada).

Preparation of the DNA adducts standards

N-(Deoxyguanosin-8-yl)-PhIP (dG-C8-PhIP) (36,37), *N*-(deoxyguanosin-8-yl)-MeIQx (dG-C8-MeIQx) (38,39), and *N*-(deoxyguanosin-8-yl)-AαC (dG-C8-AαC) (40) 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). *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-4-ABP) was prepared by reaction of *N*-hydroxy-4-ABP with pyruvonitrile, followed by reaction with dG or [$^{13}C_{10}$]-dG (41). For the case of dG-C8-MeIQx, the internal standard was prepared by reaction of the *N*-acetoxy derivative of 3-[^2H_3C]-MeIQx with dG. The (±)-*anti*-B[a]PDE-derived dG adducts, 10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (dG-N^2-B[a]P) were prepared by reaction of (±)-*anti*-B[a]PDE with dG as described (42). The adducts were purified by solid phase extraction (SPE), followed by HPLC purification (38,39), and were isolated as a mixture of unresolved isomers. The isotopic purity of the [$^{13}C_{10}$]-dG labeled internal standards exceeded 99%, whereas the isotopic purity of the dG-C8-[^2H_3C]-MeIQx adduct was 96.5%.

Tritium-labeled PhIP-modified, tritium-labeled 4-ABP-modified and tritium-labeled B[a]P-modified calf thymus DNA

[^{3}H]-PhIP-modified CT DNA was prepared as described (36). The extent of [^{3}H]-PhIP modification was estimated at 1 adduct per 10^6 unmodified DNA bases. [^{3}H]-4-ABP-modified CT DNA (62 adducts per 10^8 unmodified DNA bases) (43), and [^{3}H]-B[a]P-modified CT DNA (111 adducts per 10^8 unmodified DNA bases) (44) were provided by Dr. F. Beland (National Center for Toxicological Research, Jefferson, AR).

Isolation of DNA from saliva

Saliva (~4 mL) was collected in Oragene-DNA kits, and stored at -20 or -80 °C prior to isolation of DNA. The DNA was isolated following the manufacturer's instructions. However, further purification of the DNA was required for the efficient enzymatic hydrolysis of DNA to its mononucleosides (unpublished observations). The purified salivary DNA was further processed by proteinase K treatment, and then treated with RNase A and RNase T1, followed by extraction with phenol and chloroform (45). The DNA was precipitated in 9:1 C_2H_5OH: 3M sodium acetate buffer (pH 6.0), followed by washing of the DNA filament with C_2H_5OH:H_2O (7:3). The amount of DNA recovered from saliva samples ranged from 9 to 51 μg.

Enzyme Digestion and Solid Phase Extraction (SPE) of DNA adducts

Isotopically labeled internal standards were added to the DNA solution prior to enzyme digestion, at a level of 10 adducts per 10^8 bases. The enzymatic digestion conditions used for the hydrolysis of DNA (9 – 51 μg) in 5 mM Bis-Tris-HCl buffer (pH 7.1, 50 μL) employed DNAse I for 1.5 h, followed by incubation with nuclease P1 for 3 h, and then treatment with alkaline phosphatase and phosphodiesterase for 18 h (37). The DNA digest solution was diluted with high-purity water (200 μL; Burdick and Jackson), and the adducts were purified by SPE, using HyperSep™ filter SpinTips. The DNA digest extracts were applied to a SpinTip, which was placed on a vacuum manifold and prewashed with CH_3OH containing 0.1% HCO_2H (0.5 mL), followed by 10% CH_3OH in 0.1% HCO_2H (0.5 mL). The SpinTip was then washed with 10% CH_3OH in 0.1% HCO_2H (2 × 0.25 mL), to remove non-modified 2′-deoxynucleosides. Then, the desired adducts were eluted with CH_3OH containing 0.1% HCO_2H (0.3 mL) into silylated glass insert capillary LC vials (Microliter Analytical Supplies, Suwanee, GA). Samples were evaporated to dryness by vacuum centrifugation and then reconstituted in 1:1 DMSO/H_2O (20 μL).

LC/MS Parameters

Chromatography was performed with an Agilent 1100 Series capillary LC system (Agilent Technologies, Palo Alto, CA) equipped with an Aquasil C18 column (0.32 × 250 mm) from Thermo Fisher (Bellafonte, PA). Samples (6 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 an LTQ MS (ThermoElectron, San Jose, CA), and 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 220 °C; source spray voltage 1.5 kV; source current 2.8 μA; no sheath gas, sweep gas or auxiliary gas was employed; capillary voltage 32 V; tube lens voltage 110 V; and in-source fragmentation 10 V.

We employed the LIT MS in the tandem MS/MS scan mode to monitor the loss of deoxyribose from the protonated molecules of the adducts ($[M + H - 116]^+$), followed by the consecutive reaction monitoring can mode at the MS^3 scan stage, to characterize the product ions of the aglycone adducts $[BH_2]^+$. The top two or three most abundant ions produced at the MS^3 scan stage were used for quantitative measurements. The ions monitored in MS > MS^2 > MS^3 scan modes were as follows: dG-C8-PhIP (*m*/*z* 490.1 > 374.1 > 250.2, 329.2, 357.2); [$^{13}C_{10}$]-dG-C8-PhIP (*m*/*z* 500.1 > 379.1 > 251.2, 333.3, 362.2); dG-C8-MeIQx (*m*/*z* 479.1 > 363.1 > 239.2, 318.3, 346.3); dG-C8-[^2H_3C]-C8-MeIQx (*m*/*z* 482.1 > 366.1 > 242.2, 321.2, 349.3); dG-AαC (*m*/*z* 449.1 > 333.1 > 288.2, 316.3); [$^{13}C_{10}$]-dG-AαC (*m*/*z* 459.1 > 338.1 > 292.3, 321.2); dG-C8-4-ABP (*m*/*z* 435.1 > 319.1 > 208.2, 274.2, 302.2);

[$^{13}C_{10}$]-dG-C8-4-ABP (*m*/*z* 445.1 > 324.1 > 210.2, 278.2, 307.2), dG-*N*^{2}-B[a]P (*m*/*z* 570.1 > 454.1 > 257.2, 285.2, 303.1); and [$^{13}C_{10}$]-dG-*N*^{2}-B[a]P (*m*/*z* 580.1 > 459.1 > 257.2, 285.2, 303.1).

For DNA adducts of HAAs and 4-ABP, the normalized collision energies were set at 32 and 40, and the isolation widths were set at 3.0 and 1.0 Da, respectively, for the MS^2 and MS^3 scan modes. The activation Q was set at 0.35 and the activation time was 5 ms, for both scan modes. For dG-*N*^{2}-B[a]P, the isolation widths were set at 5.0 Da for both MS^2 and MS^3 scan modes. The activation Q was set for 0.35 and the activation times were 20 ms for MS^2 and 5 ms for MS^3. Helium was used as the collision damping gas in the ion trap and was set at a pressure of 1 mTorr. 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. With these MS parameters, about 12 scans were acquired for each adduct and its corresponding internal standard.

Subjects

Both men and women were participants in this study. Some subjects were residents of New York City or the immediate vicinity; other subjects resided in Albany, New York. The subjects were either current-smokers, former-smokers, or never-smokers. All the subjects were on unrestricted diets. This study was approved by the Institutional Review Boards at the Wadsworth Center and the Albert Einstein College of Medicine.

Accuracy and Performance of the Analytical Method

Calibration curves were constructed with unlabeled DNA adducts added to 15 μg of DNA digest (45 nmol of deoxynucleoside), which was assayed by LC-ESI/MS/MS^3, following SPE. The range in the level of spiking, reported as adducts per 10^8 DNA bases (and average amount of adduct in pg) per 15 μg of DNA was: 0 (0 pg), 0.3 (0.06 pg), 0.6 (0.12 pg), 1.0 (0.21 pg), 3.0 (0.62 pg), 6.0 (1.2 pg) and 10 (2.1 pg). The internal standards were added at a level equivalent to 10 adducts per 10^8 bases for dG-C8-MeIQx and dG-C8-PhIP (2.2 pg); 7 adducts per 10^8 bases for dG-C8-ABP (1.4 pg); and 13 adducts per 10^8 bases (2.7 pg) for dG-C8-AαC. The calibration curves were done in triplicate for each calibrant level, 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 0.98 (Supporting Information, Figure S-1). The dissociation efficiency of the dG-*N*^{2}-B[a]P adduct was poor, and the recovery of total ion counts, when going from MS^2 to MS^3 stage scan mode with the LIT MS was very low in comparison to the other adducts investigated (unpublished observations). The poor sensitivity prevented the acquisition of a complete calibration curve for the dG-*N*^{2}-B[a]P adduct. Therefore, the estimates of B[a]P adducts in DNA were based upon peak area and assumed that the response of the adduct was equal to the response of the internal standard, with injection of the equivalent of 10 adducts per 10^8 bases (2.6 pg of [$^{13}C_{10}$]-dG-*N*^{2}-B[a]P).

The accuracy of the method and the limit of quantification (LOQ) values of the DNA adducts were determined with DNA isolated from saliva from a non-smoker, that contained non-detectable levels of DNA adducts (<3 adducts per 10^9 DNA bases). CT-DNA modified with PhIP, 4-ABP, and B[a]P with defined levels of dG-C8-PhIP (36), dG-C8-ABP (43), and dG-*N*^{2}-B[a]P (44) was diluted with salivary DNA from the volunteer, to achieve a level of carcinogen DNA modification of 0, 1, 3, or 10 adducts per 10^8 DNA bases in 50 μg of DNA.

Results

Method Development and Validation

PhIP, 4-ABP, AαC, MeIQx, and B[a]P were selected for screening, because these carcinogens arise in tobacco smoke (35,46-48) and/or are formed in cooked meats (49). These compounds undergo metabolic activation by cytochrome P450 enzymes to produce electrophiles that react with DNA (32,44). The structures of these carcinogens and their DNA adducts are shown in Figure 1. We employed the LIT MS in tandem MS/MS to monitor the loss of deoxyribose from the protonated molecules of the adducts ([M +H-116]$^+$), followed by the consecutive reaction monitoring scan mode at the MS^3 stage, to characterize and measure product ions of the aglycone adducts [BH$_2$]$^+$.

The estimates and LOQ values of the DNA adducts were determined with CT-DNA modified with [^{3}H]-PhIP, [^{3}H]-ABP and [^{3}H]-B[a]P, with known levels of dG-C8-PhIP (36), dG-C8-4-ABP (43), and dG-*N*^{2}-B[a]P (44) and diluted with salivary DNA from a volunteer who harbored non-detectable levels of adducts (<3 adducts per 10^9 bases). The level of carcinogen DNA modification was established at 0, 1, 3, or 10 adducts per 10^8 DNA bases in 50 μg of DNA. The spiking of salivary DNA with carcinogen-modified CT-DNA enabled us to determine the efficiency of enzyme hydrolysis, the accuracy of the method, and the recovery of DNA adducts. The estimates of DNA adducts are presented in Table 1. The estimates of dG-C8-PhIP were within 10% of the target value for all levels of spiking. The level of dG-C8-ABP was underestimated by about 30% at all levels of spiking, whereas the estimate of the dG-*N*^{2}-B[a]P was ~60% greater than the target value. The target value of dG-C8-PhIP in [^{3}H]-PhIP-modified CT-DNA was determined by liquid scintillation counting of dG-C8-[^{3}H]-PhIP, isolated by HPLC, after enzymatic digestion of the DNA (36); the target values of dG-C8-4-ABP and dG-*N*^{2}-B[a]P were based upon the levels of adducts determined by triple stage quadrupole tandem mass spectrometry (TSQ/MS/MS), in an independent laboratory (43,44). We previously estimated, by TSQ/MS/MS (50), the amount of dG-*N*^{2}-B[a]P adduct in this B[a]P-modified CT-DNA at a level of 15 ± 2.1 adducts per 10^8 DNA bases (mean ± SD, n = 3): the target value was 10 adducts per 10^8 bases. The response of the dG-*N*^{2}-B[a]P adduct obtained by LIT MS at the MS^3 scan stage was about 10-fold weaker than the responses observed for the other DNA adducts investigated. The inter-laboratory estimates of the levels of these DNA adducts are quite comparable, if we consider that the assays employed different sets of internal standards, different enzymes and DNA digestion conditions, and different MS instruments for analyses. Our data reveal that potential constituents co-purified with the human salivary DNA samples do not impair the hydrolysis of the DNA or the subsequent analysis of DNA adducts.

The reconstructed ion chromatograms of the LC-ESI/MS/MS^3 traces of salivary DNA adducts from the volunteer, with and without addition of CT-DNA modified with PhIP and 4-ABP, diluted to a level of 1 adduct per 10^8 bases, are shown in Figure 2. Neither adduct is detected in the unspiked DNA sample (Figure 2A); however, both adducts are readily measured in the salivary DNA sample spiked with the CT-DNA modified with PhIP and 4-ABP (Figure 2B). The LC-ESI/MS/MS^3 traces of dG-C8-MeIQx and dG-C8-AαC adducts spiked at a level of 1 adduct per 10^8 bases, in the same salivary DNA sample prior to enzyme digestion and SPE, were also readily measured (unpublished observations). On the basis of the level of the signal of the DNA adducts to the background signal (51), the LOQ values of all the adducts, except for dG-*N*^{2}-B[a]P, are ~5 – 10 adducts per 10^9 DNA bases, and the LOQ value of dG-*N*^{2}-B[a]P was ~50 adducts per 10^9 bases, when 15 μg of DNA are injected on the column.

Analysis of Carcinogen DNA Adducts in Human Saliva

Salivary DNA samples were obtained from 37 volunteers (Table 2): 19 subjects were male and 18 were female. The ages ranged from 32 to 84 years. The subjects were categorized as current-smokers, former-smokers, or never-smokers, on the basis of self-report. Some demographics of the volunteers and the estimates of DNA adducts measured are reported in Table 2. Representative reconstructed ion chromatograms of the LC-ESI/MS/MS^3 traces of carcinogen DNA adducts found in saliva from two current-smokers are depicted in Figures 3A and 3B. The dG-C8 adducts of PhIP, MeIQx, AαC, and 4-ABP were identified in both saliva samples.

The MS^3 scan stage mode was employed both for quantification of the adducts and for characterization of the product ion spectra of the aglycone adducts $[BH_2]^+$. The product ion spectra of the salivary DNA adducts and the respective internal standards, are shown in Figure 4. The MS^3 product ion spectra provide rich structural information about these adducts and corroborate their identities. The proposed pathways of mass fragmentation of the DNA adducts have been previously reported (32,34,37,52): the product ion spectra of the salivary DNA adducts were in excellent agreement with the spectra of the synthetic adducts.

The dG-C8-PhIP adduct was detected most frequently; it was found in 15 subjects, at levels that ranged from 1 to 9 adducts per 10^8 DNA bases. dG-C8-AαC and dG-C8-MeIQx were detected in saliva samples of 3 current-smokers, followed by dG-C8-4-ABP, which was identified in saliva samples from 2 current-smokers.

Pilot exposure analyses

Subjects were sorted into two groups based on the concentration of PhIP adducts (not detected versus detected). Logistic regression models were used for testing association between PhIP adducts and other variables. Because of the limited sample size, covariates including age, race, gender and lung cancer status were first tested. After univariate logistic analyses covariates, including gender and lung cancer status, were not included because they are not likely to be confounder ($p>0.25$). The multivariate logistic model examined parameters, including age, race, tobacco smoking status [never, former (quit x one year), current] and cumulative dose (pack years), quit years (recent <10 years, or greater), as well as dietary factors such as meat, grilled meat, and fruit and vegetable intake and amount, along with alcohol intake and quantity. No parameters were clearly significant predictors of the most commonly detected DNA adduct (PhIP), although the multivariate model suggested some borderline relationships between PhIP adduct detectability and smoking (current $p=0.062$, former $p=0.060$), and current alcohol intake ($p=0.078$).

Discussion

Saliva is a rich source of DNA (53), and is easily obtained, and routinely used for genetic analyses (45) and evaluation of DNA damage (17). The oral cavity is directly exposed to carcinogens present in tobacco smoke and in the diet. Over the past 20 years, cells of the oral cavity have been screened by ^{32}P-postlabeling or immunohistochemical methods (25-29), for DNA adducts. A plethora of lesions have been detected; however, the identities of these presumed DNA adducts have never been confirmed by MS techniques. The objective of our study was to determine whether DNA adducts derived from several prototypical carcinogens present in tobacco smoke or cooked meat could be detected in saliva of subjects on unrestricted diets, by application of selective LIT MS scanning techniques.

Our findings demonstrate that LC-ESI/MS/MS^3 with the LIT MS can be used to screen for carcinogen-DNA adducts in human saliva. An important advantage of the LIT MS over

triple stage quadrupole mass spectrometer (TSQ MS) instruments, which have been more commonly used for the quantification of DNA adducts (32,33), is the ability of the LIT MS to acquire MS^3 product ion spectra. We have used this multi-stage tandem scanning technique for structural characterization and identification of aglycone adducts $[BH_2]^+$ of several different carcinogen DNA adducts, in tissues of experimental animals and humans (34,37,54). We also employed LIT MS at the MS^3 scan stage for quantitative measurements of dG-C8-PhIP (37). The intra-day and inter-day precision values (co-efficient of variation %) were <10% at the LOQ: this level of performance is close to the precision achieved by TSQ MS instruments (37). However, the slow duty cycle of the sequence of scanning events of the LTQ MS, which is termed the μscan, and which includes the time periods for the initial pre-scan event, the ion injection, isolation, activation and mass analysis, restricts the number of scans acquired. The paucity of scans can lead to an insufficient number of data points acquired for each peak and result in imprecise measurements, when multiple DNA adducts are measured in a single scan segment. The injection time of the LIT MS, the most time-consuming event of the μscan, can be reduced, so as to shorten the duty cycle and augment the number of scans acquired across the peaks. However, the sensitivity of response can decrease concomitantly, if the duration of the injection time is too brief. A too-brief injection time can adversely affect the quality and reproducibility of the product ion spectra, and the quantitative estimates become less precise. Indeed, we have observed a reduction in the precision of adduct measurements (Table 1), when the number of DNA adducts and internal standards assayed in the same scan segment is increased from 1 to 4 or 5 adducts, even though ~12 scans were acquired for each adduct and its internal standard. In contrast to the extensive spectral data acquired by LIT MS at the MS^3 scan stage for analyte characterization, the criteria used for DNA adduct identification using TSQ MS instruments are generally limited to a monitoring of the loss of deoxyribose ($[M+H]^+ \rightarrow [M+H-116]^+$) in the selected reaction monitoring (SRM) mode combined, with the characteristic t_R of the adduct (32,33). Product ion spectra can be obtained with the TSQ MS in the tandem MS/MS mode (39,55); however, the slow duty cycle of the TSQ MS in the full-scan mode, typically of the order of 0.1% (depending upon the monitored *m*/*z* range) results in a drastic reduction in sensitivity (56). Hence, the product ion spectra of DNA adducts are not routinely acquired in biomonitoring studies with TSQ MS instruments. However, the duty cycle of the TSQ MS is extremely rapid in the SRM mode, and the high level of sensitivity of the SRM mode allows accurate and precise quantitation, when multiple analytes are measured simultaneously (56). Thus, both LIT MS and TSQ MS instruments have important applications in the biomonitoring of DNA adducts.

In the present study, we have identified dG-C8-PhIP adduct in saliva of about 45% of the ever-smokers. DNA adducts of AαC, MeIQx, and 4-ABP were also detected, but at lower frequency. Firm conclusions about the source(s) of exposure to PhIP and other dietary carcinogens cannot be made as the concentrations of HAAs in cooked meats can vary over a 100-fold range (49,57): we did not detect a relationship between PhIP adduct formation and self-reported frequent grilled meat or total meat intake. The approximation of tobacco usage through self-reported smoking history is also likely to lead to uncertainty in the estimate of carcinogen exposure, but there may be a relationship among PhIP adduct formation, tobacco exposure and alcohol intake, albeit of borderline statistical significance in this pilot study. Moreover, both well-done meat consumption (58) and tobacco usage (59) induce the levels of expression of P4501A2, which bioactivate HAAs and 4-ABP (60) and can lead to elevated levels of DNA adducts.

The diet is considered to be the major source of exposure to PhIP. The amount of PhIP formed in cooked meats can range from less than 1 part-per-billion (ppb) up to 500 ppb (49), depending upon the type of meat, the cooking temperature and the method of cooking (57,61). AαC and MeIQx also form in cooked meats, and generally occur at <10 ppb levels.

To our knowledge, 4-ABP has not been reported in cooked meats. PhIP, AαC, and 4-ABP also arise in mainstream tobacco smoke. The levels of PhIP have been reported to range from 11 to 23 ng/cig (47), whereas AαC can arise at levels as high as 258 ng/cig (48). The levels of 4-ABP occur at 0.1 to 4.3 ng/cig (46,62,63). There are no reports of MeIQx formation in tobacco smoke (47). On the basis of studies with model systems, creatinine, a constituent of muscle, is thought to be an essential precursor for the formation of PhIP (64). However, PhIP has been detected in incineration ash, and in airborne and diesel-exhaust particles (65), in addition to tobacco smoke (47). These findings suggest that PhIP is a ubiquitous environmental contaminant. The mechanisms of PhIP formation during combustion remain to be determined.

The high frequency of detection of dG-C8-PhIP in salivary DNA is noteworthy. PhIP has also been detected with high frequency in hair samples of omnivores (66-68), and a high percentage of mammary gland (69) and prostate gland biopsy (70) samples have tested positive for putative PhIP-DNA adducts, by immunohistochemical techniques. The presumed dG-C8-PhIP adduct was also detected, by ^{32}P-postlabeling, in about 50% of the exfoliated mammary epithelial cells sampled from milk of lactating mothers (8). These data demonstrate that PhIP can damage DNA in multiple tissues of humans.

The bioactivation of PhIP, other HAAs, and 4-ABP is catalyzed by several enzymes present in different organs of the body. The liver is the most metabolically active organ in the biotransformation of HAAs and 4-ABP to genotoxicants. The carcinogenic *N*-hydroxy metabolites form principally by action of P450 1A2 (23,60) in the liver, and can reach the oral cavity through systemic circulation (71), followed by phase II activation in cells of the oral cavity. However, P450s 1A1, 1A2, or 1B1, that are expressed in the buccal cells or salivary glands of the oral cavity (18), can also directly bioactivate PhIP and the other procarcinogens noted above (72,73). Moreover, saliva contains peroxidases (74), which can catalyze the bioactivation of all these compounds (75).

The vast majority of DNA isolated from saliva with the Oragene kit is of human origin, with a median bacterial content of 11.8% (http://www.dnagenotek.com/pdf_files/PDWP002_BacterialContent.pdf). Epithelial buccal cells and leukocytes are the two principal mammalian cell types found in saliva (10,11). Like other epithelial cell types, buccal cells constantly and rapidly generate. For healthy oral epithelia, the time frame from new cell production to exfoliation of the buccal cell from the mucosal surface is estimated to be between 5 and 12 days (76). The leukocytes, which originate mainly from the gingival crevice (77), and then migrate into the oral cavity, are predominantly short-lived neutrophils and other granulocytes. Given the short lifespans of both buccal and leukocyte cell types, we believe that the DNA adducts present in saliva are likely to occur from recent exposures to carcinogens. Studies will be required to determine whether adducts are formed in both cell-types or whether they preferentially form in one type.

In summary, human saliva appears to be a promising fluid in which we can monitor DNA adducts of tobacco and meat-associated carcinogens, through the use of selective LIT MS methods. The high percentage of samples that are positive for dG-C8-PhIP is striking. Future studies that examine kinetics of PhIP-DNA adduct formation in cells of the oral cavity of humans exposed to defined amounts of PhIP, combined with studies that can unravel the myriad of plausible enzymes that contribute to PhIP adduct formation in oral cells will be required before this biomarker can be exploited in human population studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

4-ABP	4-aminobiphenyl
MeIQx	2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
A*α*C	2-amino-9*H*-pyrido[2,3-*b*]indole
B[a]P	benzo[a]pyrene
(±) (anti)B[a]PDE)	(±)-*r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene
CT-DNA	calf thymus DNA
dG	deoxyguanosine
ESI	electrospray ionization
HAA	heterocyclic aromatic amine
LC-ESI/MS/MS^n	liquid chromatography-electrospray ionization/multi-stage tandem mass spectrometry
LIT MS	linear quadrupole ion trap mass spectrometer

LOQ	limit of quantification
MS	mass spectrometer
ppb	part-per-billion
SPE	solid phase extraction
SRM	selected reaction monitoring
TSQ MS	triple stage quadrupole mass spectrometer
TSQ/MS/MS	triple stage quadrupole/tandem mass spectrometry
dG-C8-4-ABP	*N*-(deoxyguanosin-8-yl)-4-ABP
dG-C8-MeIQx	*N*-(deoxyguanosin-8-yl)-MeIQx
dG-C8-AαC	*N*-(deoxyguanosin-8-yl)-AαC
dG-C8-PhIP	*N*-(deoxyguanosin-8-yl)-PhIP
dG-N^2-B[a]P	10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene

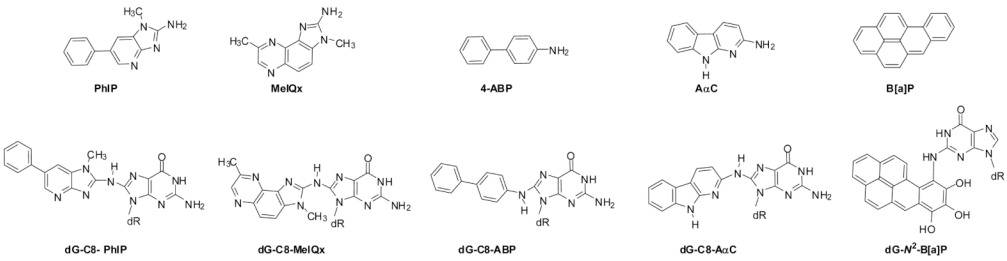

Figure 1.
Chemical structures of tobacco and meat carcinogens and their DNA adducts monitored in human saliva.

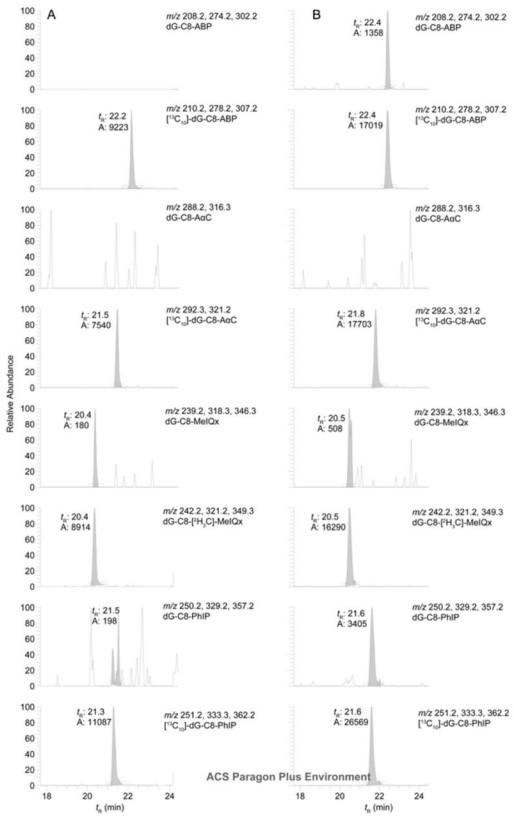

Figure 2.

Reconstructed ion chromatograms of the LC-ESI/MS/MS^3 traces of DNA adducts present in a saliva of a never-smoker with (A) non-detectable DNA adducts, and (B) LC-ESI/MS/MS^3 traces of DNA adducts in saliva of the same subject, after spiking with PhIP- and 4-ABP-carcinogen-modified CT-DNA, at a level of 1 adduct per 10^8 bases. The dG-C8 adducts of MeIQx and AαC, and their internal standards, were also monitored. The peak observed for dG-C8-MeIQx (t_R 20.5 min) is attributed to the isotopic impurity of the dG-C8-[$^2H_3$C]-MeIQx internal standard (96.5% purity), which is contaminated with the unlabeled adduct at a level of 3.5%. The retention time (t_R) and area are reported.

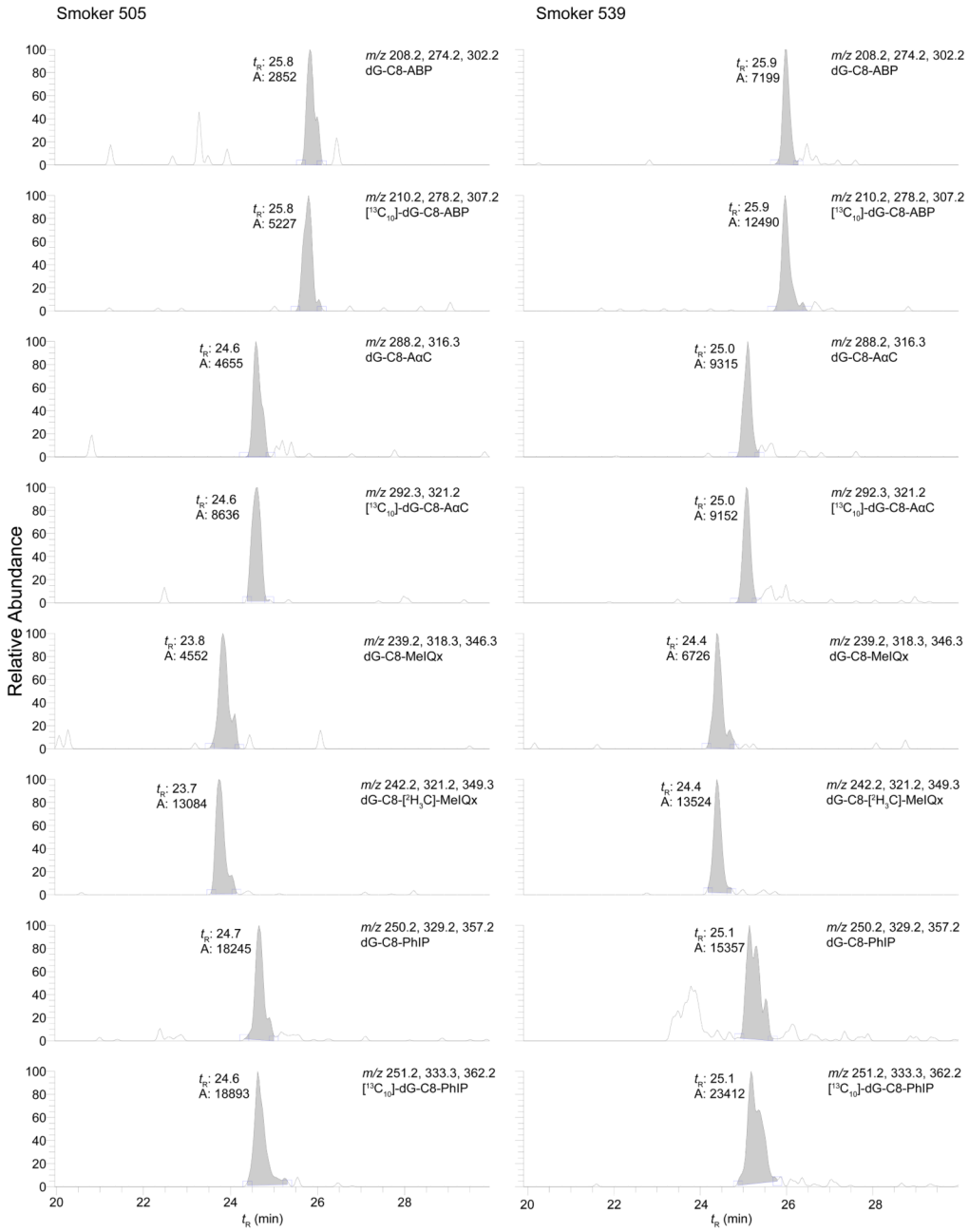

Figure 3.

Reconstructed ion chromatograms of the LC-ESI/MS/MS^3 traces of DNA adducts present in saliva samples from two current-smokers. All DNA adducts were present at levels above the LOQ.

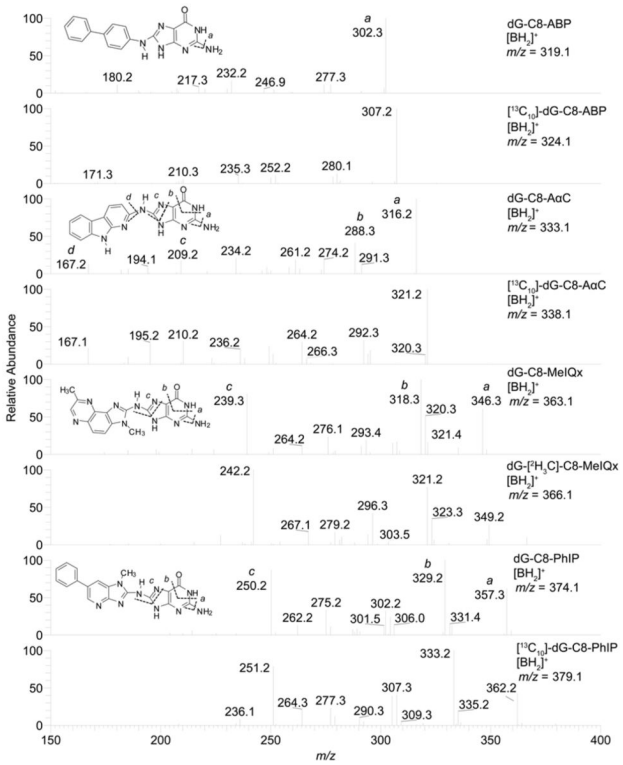

Figure 4.

MS^3 product ion spectra of DNA adducts identified in saliva DNA samples of current-smokers and MS^3 product ion spectra of the corresponding internal standards (after background subtraction), from upper to lowest panel: dG-C8-4-ABP; [$^{13}C_{10}$]-dG-C8-4-ABP; dG-C8-AαC; [$^{13}C_{10}$]-dG-C8-AαC; dG-C8-MeIQx; dG-C8-[$^{2}H_3C$]-MeIQx; dG-C8-PhIP; and [$^{13}C_{10}$]-dG-C8-PhIP.

Table 1

Estimates of DNA Adducts in Human Salivary DNA Mixed with Carcinogen-Modified CT-DNA

Target Adduct Level per 10^8 DNA bases	dG-N^2-B[a]P	dG-C8-PhIP	dG-C8-4-ABP	Independent measurements
1.0	N.A.[a]	1.0 ± 0.1	0.8 ± 0.3	5
3.0	N.A.	2.9 ± 1.1	1.9 ± 0.4	6
10.0	15.7± 1.0	11.7 ± 1.2	6.0 ± 0.8	3

[a]Not assayed (N.A.), the LOQ value for dG-N^2-B[a]P was 5 adducts per 10^8 DNA bases

Table 2

Levels of Carcinogen DNA Adducts in Human Saliva

							Adducts per 10^8 DNA Bases			
Subject	**Gender**	**Race**[a]	**Age**	**Self-reported smoking history**[b]	**Cig/day**	**DNA assayed (μg)**[c]	**dG-C8-PhIP**	**dG-C8-MeIQx**	**dG-C8-AαC**	**dG-C8-4-ABP**
27	F	H	51	never	0	51	2.7	N.D.[d]	N.D.	N.D.
11	M	H	57	current	30	47	N.D.	N.D.	N.D.	N.D.
23	M	W	Unknown	current	10	44	8.5	N.D.	N.D.	N.D.
19	F	W	63	current	20-40	42	4.5	1.5	N.D.	N.D.
37	M	W	53	never	0	36	N.D.	N.D.	N.D.	N.D.
26	M	W	34	current	20	35	7.8	N.D.	N.D.	N.D.
17	F	B	48	current	10	34	2.0	N.D.	N.D.	N.D.
33	F	W	43	never	0	33	N.D.	N.D.	N.D.	N.D.
36	F	W	49	never	0	33	1.1	N.D.	N.D.	N.D.
28	M	W	53	never	0	29	N.D.	N.D.	N.D.	N.D.
3	F	H	75	20 yr	5	29	N.D.	N.D.	N.D.	N.D.
21	M	W	75	current	20-40	27	1.2	N.D.	N.D.	N.D.
1	M	H	84	20 yr	3	27	N.D.	N.D.	N.D.	N.D.
9	M	B	68	5 yr	14	27	N.D.	N.D.	N.D.	N.D.
34	F	W	55	never	0	26	N.D.	N.D.	N.D.	N.D.
25	M	W	Unknown	current	20	26	7.7	N.D.	N.D.	N.D.
20	F	W	63	current	20-40	26	6.5	5.1	9.2	5.8
30	F	H	44	never	0	26	N.D.	N.D.	N.D.	N.D.
24	M	W	Unknown	current	20	26	2.4	N.D.	N.D.	N.D.
14	F	H	69	30 yr	20	24	N.D.	N.D.	N.D.	N.D.
15	F	H	48	current	3	24	9.7	3.5	5.4	5.5
12	F	W	63	current	40	21	N.D.	N.D.	N.D.	N.D.
29	F	W	74	never	0	21	N.D.	N.D.	N.D.	N.D.
13	F	B	48	7 yr	30	20	N.D.	N.D.	N.D.	N.D.
35	F	W	43	10 yr	10	20	2.8	N.D.	N.D.	N.D.
8	M	H	78	20 yr	40	20	N.D.	N.D.	N.D.	N.D.
16	M	W	62	19 yr	40-60	19	2.0	N.D.	N.D.	N.D.

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							Adducts per 10^8 DNA Bases			
Subject	**Gender**	**Race[a]**	**Age**	**Self-reported smoking history[b]**	**Cig/day**	**DNA assayed (μg)[c]**	**dG-C8-PhIP**	**dG-C8-MeIQx**	**dG-C8-AαC**	**dG-C8-4-ABP**
32	M	H	47	<20 yr	1 time only	18	N.D.	N.D.	N.D.	N.D.
31	F	W	87	<20 yr	1	18	N.D.	N.D.	N.D.	N.D.
22	M	B	Unknown	17 yr	10	17	1.8	N.D.	N.D.	N.D.
10	M	B	65	current	10	16	N.D.	N.D.	N.D.	N.D.
5	F	B	58	current	10-20	14	N.D.	N.D.	N.D.	N.D.
2	M	H	80	current	3	13	N.D.	N.D.	N.D.	N.D.
7	M	H	45	current	20	13	N.D.	N.D.	N.D.	N.D.
6	M	B	70	< 3 months	10	12	N.D.	N.D.	N.D.	N.D.
18	F	B	50	3 yr	20-40	12	1.8	N.D.	N.D.	N.D.
4	M	H	50	< 1 yr	20	9	N.D.	N.D.	N.D.	N.D.

[a] W = White, B = Black, H = Hispanic

[b] Reported time since quitting

[c] Samples with the highest DNA content were assayed first, in batches of 5 or 6 samples.

[d] N.D. = not detected, < 5 adducts per 10^9 DNA bases.