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Quantitative Liquid Chromatography-Nanoelectrospray Ionization-High Resolution Tandem Mass Spectrometry Analysis of Acrolein-DNA Adducts and Etheno-DNA Adducts in Oral Cells from Cigarette Smokers and Non-smokers

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Abstract

Cigarette smoking is an important source of human exposure to toxicants and carcinogens and contributes significantly to cancer morbidity and mortality worldwide. Acrolein, a widespread environmental pollutant, is present in relatively high amounts in cigarette smoke and can react directly with DNA to form DNA adducts, which serve as important biomarkers for the assessment of exposure to acrolein and its potential role in smoking related cancer. Etheno-DNA adducts are promutagenic DNA lesions that can derive from exogenous chemicals as well as endogenous sources, including lipid peroxidation. In this study, we developed a combined method for the quantitation of (6*R*/*S*)-3-(2′-deoxyribos-1′-yl)-5,6,7,8,-tetrahydro-6-hydroxypyrimido[1,2-*a*]purine-10(3*H*)-one (α-OH-Acr-dGuo), (8*R*/*S*)-3-(2′-deoxyribos-1′-yl)-5,6,7,8,-tetrahydro-8-hydroxypyrimido[1,2-*a*]purine-10(3*H*)-one (γ-OH-Acr-dGuo), 1,N^6-etheno-dAdo (εdAdo) and 3,N^4-etheno-dCyd (εdCyd) adducts in oral rinse and cytobrush DNA from smokers and non-smokers by liquid chromatography-nanoelelctrospray ionization-high resolution tandem mass spectrometry (LC-NSI-HRMS/MS). For oral rinse samples, there was a statistically significant difference between the levels of α-OH-Acr-dGuo, γ-OH-Acr-dGuo, εdAdo and εdCyd in smokers (12.1 ± 17.9, 163 ± 227, 182 ± 568, and 194 ± 400 adducts/10^9 nucleotides, respectively) and non-smokers (1.85 ± 2.08, 5.95 ± 4.23, 7.69 ± 11.7, and 6.07 ± 10.9 adducts/10^9 nucleotides, respectively). For cytobrush samples, there was a statistically significant difference between the levels of γ-OH-Acr-dGuo and εdAdo in smokers (259 ± 540, and 82.9 ± 271 adducts/10^9 nucleotides, respectively) and non-smokers (7.37 ± 5.09, and 16.2 ± 30.2 adducts/10^9 nucleotides, respectively), but not for α-OH-Acr-dGuo and εdCyd. Our results demonstrate that oral mucosa

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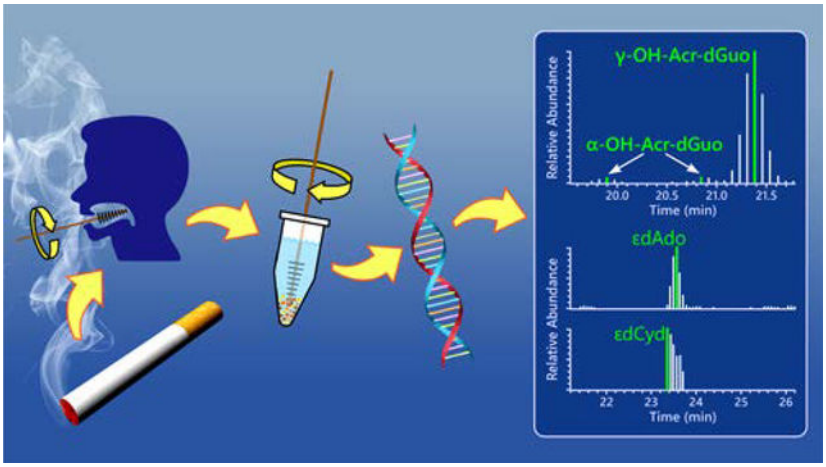
Supporting Information

Protocols for [^{15}N]DNA generation and isolation from bacteria. Further details of artifact control studies.

cells are an excellent source of material for evaluating DNA adducts to be used as biomarkers of tobacco smoke exposure and molecular changes potentially related to cancer.

Graphical Abstract



Introduction

There are 1.1 billion tobacco smokers in the world[1] and 32 million adult cigarette smokers in the U.S.[2] Therefore, smoking continues to be a major source of human exposure to relatively high amounts of toxicants and carcinogens resulting in substantially increased incidences of oral cavity cancer, lung cancer, and at least 14 other types of cancer as well as numerous other diseases.[3, 4] A major goal of our research program is to develop biomarkers which can be used to determine individual risk for cancer in people who use tobacco products, as an avenue to early detection and prevention of cancer. DNA adduct levels have the potential to become biomarkers of cancer risk in smokers because they are central to the carcinogenic process.[5] While certain exposure biomarkers such as total nicotine equivalents and total NNAL are related to cancer risk, DNA adducts may increase the specificity of the relationship by providing an integration of exposure, absorption, distribution, metabolism and DNA repair.[6, 7] However, there are major challenges involved in the quantitative analysis of DNA adducts in accessible human tissues. Oral cells are one readily available source of material for assessing DNA adducts and molecular changes potentially associated with cancer.[5]

The study reported here focuses on two pairs of exocyclic oral cell DNA adducts. The first pair is formed from acrolein – (6*R*/*S*)-3-(2′-deoxyribos-1′-yl)-5,6,7,8,-tetrahydro-6-hydroxypyrimido[1,2-*a*]purine-10(3*H*)-one (α-OH-Acr-dGuo, **1**) and (8*R*/*S*)-3-(2′-deoxyribos-1′-yl)-5,6,7,8,-tetrahydro-8-hydroxypyrimido[1,2-*a*]purine-10(3*H*)-one (γ-OH-Acr-dGuo, **2**). The second pair is produced in DNA as a result of lipid peroxidation - 1,N^6-etheno-dAdo (εdAdo, **3**) and 3,N^4-etheno-dCyd (εdCyd, **4**)[8–16] (Figure 1).

Acrolein, with a mean level in mainstream smoke of American brands of 177 μg per cigarette using the Health Canada smoking conditions,[17] is one of the most toxic compounds

in cigarette smoke, but there is scant evidence for its carcinogenicity.[18–20] Acrolein is considered by IARC as “not classifiable as to its carcinogenicity in humans (Group 3)” based on inadequate evidence for its carcinogenicity in laboratory animals.[20] Nevertheless, data from humans indicates that acrolein forms DNA adducts at similar mutational hotspots in the *TP53* gene as found in smoking-related lung cancers, recalling observations with benzo[*a*]pyrene diol epoxide.[21–23] These studies, along with its ease of reactivity with DNA to form the moderately mutagenic adducts **1** and **2**, have rekindled interest in the possible carcinogenic effects of acrolein. The etheno adducts **3** and **4** are promutagenic DNA lesions that can be formed as a result of lipid peroxidation, a consequence of the oxidative damage and inflammation produced by cigarette smoking, alcohol consumption, exposure to $PM_{2.5}$, and related causes of the inflammatory response.[10–14]

Several previous studies have quantified these DNA adducts by mass spectrometry and related techniques in oral cells or saliva. Nath *et al.* analyzed gingival tissue DNA by a ^{32}P-postlabelling-HPLC method and found significantly higher levels of adduct **2** in smokers than in non-smokers (1360 vs 460 adducts/10^9 unmodified DNA bases).[24] Bessette *et al.* quantified several DNA adducts in buccal cell DNA from smokers and reported levels of **1** and **2** greater than 500 adducts per 10^9 unmodified DNA bases.[25] Chen and Lin analyzed human saliva for adducts **1** plus **2** and reported a mean of 1040 adducts/10^9 nucleotides; in the same analysis, they quantified **3** and **4** with mean levels of 990 ± 500 and 720 ± 490 adducts/10^9 nucleotides, respectively.[13] Li *et al.* reported levels of adduct **2** of 1500 adducts/10^9 nucleotides and 2000 adducts/10^9 nucleotides in smokers and non-smokers, respectively, showing a non-significant difference.[26]

Artifact formation can occur easily in analyses of adducts **1** and **2** due to the ubiquitous occurrence of acrolein in H_2O, laboratory reagents, and other common sources. In previous studies, we and others have developed methods to avoid artifact formation, for example by including glutathione as a scavenger during analysis.[27, 28] In this study, we developed an artifact-free method for simultaneous quantitation by liquid chromatography-nanoelelctrospray ionization-high resolution tandem mass spectrometry (LC-NSI-HRMS/MS) of adducts **1–4** in oral cells of smokers and non-smokers.

Experimental Section

Chemicals and Enzymes

Acr-dGuo and [$^{13}C_{10}{}^{15}N_5$]Acr-dGuo (comprising both adducts **1** and **2**) were prepared as described[29] by reaction of acrolein with fully labeled dGuo (Toronto Research Chemicals); εdAdo, [$^{13}C_5$]εdAdo (labeled with ^{13}C in the five carbons of the deoxyribose ring), εdCyd and [$^{13}C^{15}N_2$]εdCyd (labeled with ^{13}C in position 5, and with ^{15}N in positions 4 and 6) were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Reagents for DNA isolation were obtained from Qiagen (Germantown, MD). Calf thymus DNA, micrococcal nuclease and phosphodiesterase II were purchased from Worthington Biochemical Co. (Lakewood, NJ). Alkaline phosphatase and other solvents were obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI).

Study Design and Oral Cell Collection

Subjects were recruited through the Tobacco Research Programs, University of Minnesota, at the Delaware Clinical Research Unit, and at the Minnesota State Fair through the University of Minnesota research and outreach programs. The study was approved by the University of Minnesota Institutional Review Board. Study participants were adults (more than 18 years old) who were non-smokers or smokers of at least 10 cigarettes/day. Eligible volunteers were asked to brush their teeth with a pre-pasted disposable toothbrush and to wait 15 min between brushing and collecting oral rinse samples. While waiting, participants were asked to take a breathalyzer test to measure their blood alcohol concentration, a carbon monoxide breath test to confirm their smoking status, and to complete a set of self-report questionnaires about their smoking, medication and alcohol use, demographics, and medical history. Oral rinse samples were collected by vigorously swishing 10 mL of sterile normal saline from cheek to cheek for at least 30 sec, then spitting the rinse into a sterile collection tube. After 15 min, cheek cell samples were collected by using a soft-bristled cytobrush to brush the oral mucosa inside the right cheek from top to bottom approximately 30 times. A separate cytobrush was used for collecting cells from the left cheek. The cells were collected by shaking each brush in its own separate 15 mL sterile polypropylene tube containing 5 mL of sterile saline solution. After the collection, oral rinse and cytobrush samples were centrifuged at 2,000*g* for 15 min to pellet the cells and the pellets were transferred to 1.5 mL centrifuge tubes. For this transfer, we used 200 μL of sterile saline solution for the oral rinse samples and 100 μL each for the cytobrush cell pellets obtained from each subject's right and left cheek, which were then combined. The samples were stored at −20 °C until DNA isolation and analysis. In this study, we analyzed oral rinse and cheek cell samples from 19 smokers and 20 non-smokers.

DNA Isolation from Oral Cells

DNA isolation was performed using the DNA purification from oral rinse and cytobrush protocols from Qiagen (Qiagen, Valencia, CA) with some modifications. Briefly, samples were thawed and centrifuged at 15,000*g* for 2 min to pellet the oral cells. After pouring off the supernatant, oral rinse and cytobrush cells were lysed using 1000 μL and 300 μL of cell lysis solution containing 10 mM glutathione (GSH), respectively, and incubated for 20 min at room temperature. GSH was used to inhibit the artifactual formation of Acr-dGuo (**1** and **2**), εdAdo (**3**) and εdCyd (**4**). This was followed by a 15 min treatment at room temperature with 10 μL of proteinase K for oral rinse samples and 2 μL of proteinase K for cytobrush samples, and a 30 min treatment at 37 °C with 6 μL of RNase for oral rinse samples and 2 μL of RNase for cytobrush samples. Then, 340 μL and 100 μL of protein precipitation solution was added to oral rinse and cytobrush cells, respectively, and the samples were centrifuged at 16,000 *g* for 10 min. DNA was precipitated by pouring the oral rinse supernatant into a 5 mL tube containing 1460 μL of 100% ice-cold 2-propanol and the cytobrush supernatant into a 1.5 mL tube containing 450 μL of 100% ice-cold 2-propanol. The precipitated oral rinse DNA pellet was washed with 1000 μL of 70% ice-cold 2-propanol and then 1000 μL of 100% ice-cold 2-propanol, and the cytobrush DNA with 300 μL of 70% ice-cold 2-propanol and then 300 μL of 100% ice-cold 2-propanol. DNA was dried and stored at −20 °C until analysis.

DNA Hydrolysis and Sample Purification

The isolated DNA (0.5 – 50 μg) was dissolved in 200 μL of 10 mM sodium succinate/5 mM $CaCl_2$ buffer (pH 7.0) containing 5mM GSH to which 10 fmol of [$^{13}C_{10}{}^{15}N_5$]Acr-dGuo ([$^{13}C_{10}{}^{15}N_5$]**1** and **2**), [$^{13}C_5$]εdAdo ([$^{13}C_5$]**3**), and [$^{13}C^{15}N_2$]εdCyd ([$^{13}C^{15}N_2$]**4**) were added as internal standards. To monitor the possible artifactual formation of Acr-dGuo (**1** and **2**), εdAdo (**3**) and εdCyd (**4**), some samples were spiked with 5 μg of [^{15}N]DNA isolated from cultures of *E. Coli* grown in ^{15}N-containing media. Protocols for [^{15}N]DNA generation and isolation from bacteria are presented in the Supporting Information. A buffer blank (200 μL) was prepared each time and used as a negative control to evaluate possible contamination; a sample of calf thymus DNA (20 μg in 200 μL of buffer) was also included in each set of samples as a positive control. The mixture was heated at 100 °C for 30 min and cooled to room temperature. Then, the DNA was enzymatically hydrolyzed by incubation with 7.5 units of micrococcal nuclease and 0.045 units of phosphodiesterase II at 37 °C overnight. Then, 15 units of alkaline phosphatase (from calf intestine) were added, and the mixture was incubated at 37 °C overnight. The resulting mixtures were partially purified by centrifugal filtration (Ultracel 10K, Millipore). A 10 μL aliquot was removed for dGuo quantitation, and the remaining hydrolysate was purified using a solid-phase extraction (SPE) cartridge [Strata-X, 33 μm, 30 mg/1 mL (Phenomenex, Torrance, CA)] activated with 3 mL of MeOH and 3 mL of H_2O containing 0.1 mM GSH. After the samples were applied, the cartridges were washed with 6 mL of H_2O containing 0.1 mM GSH and 1 mL 5% CH_3OH in H_2O containing 0.1mM GSH, and the analytes were eluted into high recovery glass vials (Thermo Scientific, San Jose, CA) with 1 mL 35% CH_3OH in H_2O containing 0.1 mM GSH. Prior to elution, 0.65 μL of 100 mM GSH was added in each vial. The eluants were evaporated to dryness and stored at 4 °C.

Analysis of Acr-dGuo (1 and 2), εdAdo (3), and εdCyd (4) by LC-NSI-HRMS/MS

Samples were dissolved in 20 μL of H_2O for LC-NSI-HRMS/MS analysis. The analysis was carried out on an Orbitrap Fusion Lumos instrument (Thermo Scientific, San Jose, CA) interfaced with a UPLC system (Ultimate 3000 RSLCnano UPLC, Thermo Scientific, Waltham, MA) using nanoelectrospray ionization. The UPLC was equipped with a 5 μL loop and the separation was performed using a capillary column (75 μm ID, 20 cm length, 15 μm orifice) prepared by hand packing a commercially available fused-silica emitter (New Objective, Woburn MA) with Luna C_{18} bonded separation media (Phenomenex, Torrance, CA). The mobile phase consisted of 2 mM NH_4OAc and CH_3CN. The gradient started at 1% CH_3CN for 6 min at a flow rate of 0.9 μL/min, increased to 13% CH_3CN in 20 min at a flow rate of 0.3 μL/min and then to 30% CH_3OH in 1 min, holding at this composition for 4 min. The gradient was then returned to 1% CH_3CN in 1 min and the system was re-equilibrated at this mobile phase composition for 1 min at a flow rate of 0.9 μL/min before the next injection. The source temperature was set at 300 °C and the spray voltage was static at 2200 V. The maximum injection time was 400 ms and the normalized automatic gain control (AGC) was set at 50%. The precursor ions were isolated by the quadrupole with an isolation width of *m/z* 1.5 and fragmented by higher energy collisional dissociation (HCD) at 20% for Acr-dGuo (**1** and **2**), [$^{13}C_{10}{}^{15}N_5$]Acr-dGuo ([$^{13}C_{10}{}^{15}N_5$]**1** and **2**), εdAdo (**3**) and [$^{13}C_5$]εdAdo ([$^{13}C_5$]**3**) and 30% for εdCyd (**4**) and [$^{13}C^{15}N_2$]εdCyd ([$^{13}C^{15}N_2$]**4**) and the

fragment ions were detected by the Orbitrap detector at a resolution of 60,000. The ion transitions monitored with accurate mass extracted were: *m/z* 324.1 → *m/z* 208.0829 ($[M + H]^+ \rightarrow [BH]^+$) for adducts **1** and **2**, *m/z* 339.1 → *m/z* 218.0849 for [$^{13}C_{10}{}^{15}N_5$]**1** and **2**, *m/z* 276.1 → *m/z* 160.0618 for adduct **3**, *m/z* 281.1 → *m/z* 160.0618 for [$^{13}C_5$]**3**, *m/z* 252.1 → *m/z* 136.0505 for adduct **4** and *m/z* 255.1 → *m/z* 139.0480 for [$^{13}C^{15}N_2$]**4**. To monitor the artifactual formation of these adducts in the [^{15}N]DNA added to the samples before the enzymatic hydrolysis, the following ion transitions were included in the method: *m/z* 329.1 → *m/z* 213.0681 for [$^{15}N_5$]Acr-dGuo ([$^{15}N_5$]**1** and **2**), *m/z* 281.1 → *m/z* 165.0469 for [$^{15}N_5$]εdAdo ([$^{15}N_5$]**3**), and *m/z* 255.1 → *m/z* 139.0416 for [$^{15}N_3$]εdCyd ([$^{15}N_3$]**4**).

Calibration curves were prepared using standard solutions of adducts **1** and **2**, [$^{13}C_{10}{}^{15}N_5$]**1** and **2**, **3**, [$^{13}C_5$]**3**, **4**, and [$^{13}C^{15}N_2$]**4**). A constant amount each of [$^{13}C_{10}{}^{15}N_5$]**1** and **2**, [$^{13}C_5$]**3** and [$^{13}C^{15}N_2$]**4** (200 amol/μL) was mixed with different amounts of adducts **1–4** (5–500 amol/μL).

Quantitation of dGuo was performed on a HPLC (Agilent Technologies, Palo Alto, CA) system with a UV detector operated at 254 nm. A 0.5 mm × 250 mm capillary Luna 5 μm C_{18} column (Phenomenex, Torrance, CA) was used. The elution program was a gradient from 5% to 22% CH_3OH in H_2O in 20 min at a flow rate of 10 μL/min, increased to 80% CH_3OH over 5 min, and then returned to 5% CH_3OH in 5 min followed by 5 min re-equilibration. Calibration curves (1–100 μg/mL dGuo) were prepared and run after each batch of samples.

Method Validation

The limit of detection (LOD) was established using standard solutions of adducts **1–4**. The limit of quantitation (LOQ), accuracy and precision of the method were determined by analyzing calf thymus DNA enriched with different amounts of Acr-dGuo (**1** and **2**), εdAdo (**3**) and εdCyd (**4**) standards (0.1, 0.4, 1, 2, and 4 fmol for adduct **1**; 0.1, 0.3, 1, 3, and 6 fmol for adduct **2**; 0.1, 0.6, 1, 3, and 6 fmol for adducts **3** and **4**). Each sample was analyzed in triplicate. Background levels of adducts **1–4** in calf thymus DNA were determined by analyzing three non-spiked samples; these amounts were subtracted from each amount detected in the spiked samples. The LOQ was defined as the lowest Acr-dGuo (**1** and **2**), εdAdo (**3**) and εdCyd (**4**) amount added to calf thymus DNA that produced a coefficient of variation lower than 20%. Accuracy was determined by comparing added and measured amounts of the adducts at each level. Precision was determined as intra-day and inter-day coefficients of variation (% CV) for the triplicate samples analyzed on three separate days.

In addition to the method validation studies, we investigated intra-day and inter-day variation of DNA adduct levels obtained by oral rinse. Samples were collected from three non-smoking subjects on the same day at three time points (9:30 am, 12:30 pm, 3:30 pm) and on three separate days, and analyzed for adducts **1–4.**

Statistical Analysis

Statistical analyses were performed using SigmaPlot 12.5 (Systat Software, San Jose, CA, https://systatsoftware.com/products/sigmaplot/). The non-parametric Mann-Whitney U test

was used to compare (**1** and **2**), εdAdo (**3**) and εdCyd (**4**) levels between smokers and non-smokers. Statistical significance was set at $p<0.05$.

Results

Development of the Analytical Procedure

We developed a method for the quantitation of Acr-dGuo (**1** and **2**), εdAdo (**3**) and εdCyd (**4**) adducts in human oral cells. The protocol is outlined in Figure 2.

Artifactual formation of adducts **1–4** can occur during DNA isolation, hydrolysis and SPE clean-up steps, potentially leading to an overestimation of the adducts in DNA samples. In a previous study, we developed an artifact-free method for the analysis of adducts **1** and **2** in lung tissue DNA and GSH was chosen as scavenger to inhibit the artifact during sample analysis.[30] In the study reported here, we modified the method to simultaneously analyze adducts **1–4** in human oral cells. We carefully monitored and controlled the artifactual formation of adducts **1–4** and tested different GSH concentrations during each step of the analysis. Consistent with our previous study,[30] there was no difference in the amounts of adducts **1** and **2** measured in oral cell DNA extracted with 5 or 10mM GSH, or no scavenger present, indicating minimal artifactual formation during DNA isolation and purification. However, we observed artifactual formation of adducts **3** and **4** during this step. Adduct **3** levels in DNA extracted with 10mM GSH were ~ 50% and ~ 70% lower than levels measured in DNA extracted with 5 mM GSH and no scavenger present, respectively. Adduct **4** levels in DNA extracted with 10mM GSH were ~ 30% and ~ 50% lower than levels measured in DNA extracted with 5 mM GSH and no scavenger present, respectively.

When the scavenger was omitted during the hydrolysis and SPE steps, the amounts of adducts **1–4** measured were significantly higher and highly variable, indicating artifactual formation during these two steps. Most of the artifact of adduct **2** was formed during the SpeedVac drying step, while adduct **1** was formed to a much lesser extent during both the hydrolysis and drying steps. The artifactual formation of adducts **3** and **4** was observed mainly during the hydrolysis step. Therefore, we tested different GSH concentrations in calf thymus DNA during DNA hydrolysis (0.5, 5, and 10 mM GSH) and used 0.1 mM GSH during the SPE step, as reported in our previous study.[30] There was no significant difference in the levels of adducts **1–4** measured in DNA treated with 0.5, 5 or 10 mM GSH; however, we measured slightly lower levels of adducts **3** and **4** when using 5 mM GSH during the hydrolysis step. Aminoguanidine (AG, 0.5 and 5 mM) was also tested as a scavenger to inhibit artifactual formation of adducts **3** and **4** during both the hydrolysis and SPE clean-up steps, but it was ineffective. Therefore, GSH (5mM) was chosen as scavenger for the DNA hydrolysis step. As previously reported for adducts **1** and **2,**[30] to further investigate and ensure an efficient inhibition of the artifactual formation of adducts **3** and **4** by using GSH, we analyzed purified dAdo and dCyd following the same protocol. Commercial dAdo and dCyd contained trace amounts of adducts **3** and **4**, respectively, and were purified by HPLC before use. We measured the levels of **3** and **4** artificially formed during sample analysis. The results showed that by using 5 mM GSH during DNA hydrolysis and 0.1 mM GSH during the SPE step, the artifactual formation of adducts **3** and **4** was less than 25% of the

levels measured in human oral cell samples. It should be noted that this artifact monitoring method provides the maximum levels formed during the sample work-up since real DNA samples contain other matrix components to further scavenge acrolein and other aldehydes and therefore lower artifact formation, as shown below by good intra- and inter-day CV% for positive controls. Further details of the method development and validation are presented in the Supporting Information.

The LC-NSI-HRMS/MS method used here was based on the method previously developed[30] for the analysis of adducts **1** and **2** in lung tissue DNA, with some modifications. The analysis was carried out on an Orbitrap Fusion Lumos instrument and the gradient was modified to make the run time shorter (33 min). Fragmentation ions for adducts **3** and **4** and internal standards [$^{13}C_5$]**3** and [$^{13}C^{15}N_2$]**4**, were included. Using HCD fragmentation in the LC-NSI-HRMS/MS system, the product ion spectra of adducts **3** and **4** had several major ions. Because the highest signal intensities in the spectra of both adducts **3** and **4** were *m/z* 160.0618 and *m/z* 136.0505, respectively, these ions were selected for quantitative analysis.

An LOD of 2 amol on-column for adducts **1** and **2**, and 1 amol on-column for adducts **3** and **4** was achieved using standard solutions of adducts **1–4**. The concentration range for the calibration curves and validation experiments were chosen to cover the range of the levels of adducts **1–4** found in human oral cell samples. The calibration curves for adducts **1–4** showed good linearity within the low concentration range ($R^2 > 0.99$). The assay accuracy was calculated as a percentage of added amount of adducts **1–4** to 20 μg of calf thymus DNA and the average accuracy was 101% for adduct **1**, 99% for adduct **2**, 108% for adduct **3** and 105% for adduct **4** ($n = 5$), and good linearity was observed across the tested concentration ranges (Figure 3).

The assay precision and the LOQ were determined by analyzing 20 μg of calf thymus DNA ($n = 12$). The intra-day and inter-day CV were both within 20% (Table 1) and an assay LOQ of 1 adduct/10^9 nucleotides for adducts **1–4** was achieved based on a CV of 3% for adduct **1**, 16% for adducts **2** and **4**, and 6% for adduct **3**.

Quantitation of Acr-dGuo (1 and 2), εdAdo (3), and εdCyd (4) in Human Oral Cells

The method was applied to the analysis of Acr-dGuo (**1** and **2**), εdAdo (**3**), and εdCyd (**4**) in oral cells from 19 smokers and 20 non-smokers. Typical extracted ion chromatograms for the quantitation of adducts **1–4** in human oral cells using LC-NSI-HRMS/MS are presented in Figure 4.

DNA was isolated from oral rinse and cytobrush samples and analyzed for adducts **1–4**. [^{15}N]DNA isolated from cultures of *E. Coli* grown in ^{15}N-containing media was spiked in some samples to monitor the artifactual formation of these adducts during the DNA hydrolysis and SPE steps. As shown in Figure 4, we did not observe artifactual formation of adducts **1–4** in [^{15}N]DNA, further confirming that there was minimal artifactual formation of adducts **1–4** in oral cell DNA during sample analysis.

The levels of adducts **1–4** in oral cell DNA from smokers and non-smokers are presented in Tables 2 (oral rinse) and 3 (cytobrush) for the same subjects. Adducts **1** and **4** were not detected in the majority of cytobrush samples and in oral rinse samples from non-smokers.

For oral rinse samples, there was a statistically significant difference between smokers and non-smokers for adducts **1–4** (p=0.001 for adduct **1** and p<0.001 for adducts **2–4**); for cytobrush samples, there was a statistically significant difference between smokers and non-smokers for adducts **2** (p<0.001) and **3** (p=0.009). We did not observe a consistent difference in any of the adduct levels between male and female smokers or male and female non-smokers and there was at most a weak correlation between adducts **1–4** levels in oral rinse and cytobrush samples from the same subjects.

We investigated intra-day and inter-day variation of adducts **1–4** obtained by oral rinse samples collected from three non-smoking subjects on the same day at 3 time points (9:30 am, 12:30 pm, 3:30 pm) and on 3 separate days. Adduct **1** was not detected in any of the samples analyzed in this experiment. The overall intra-individual variation was 21% for adduct **2**, 27% for adduct **3**, and 24% for adduct **4**. The overall inter-day variation was 61% for adduct **2**, 56% for adduct **3**, and 63% for adduct **4**.

Discussion

We present a validated LC-NSI-HRMS/MS method for the combined analysis of Acr-dGuo (**1** and **2**), εdAdo (**3**), and εdCyd (**4**) in oral cells of smokers and non-smokers. The accuracy, precision, and detection limits of the new method were established thus providing a path for the quantitation of these important DNA modifications. This is apparently the first application of HRMS for quantitative analysis of oral cell DNA adducts. The results of this study clearly demonstrate the effect of cigarette smoking on levels of adducts **2** and **3**, which were significantly higher in smokers than non-smokers in samples collected by both oral rinse and cytobrush. Levels of adducts **1** and **4** were also significantly higher in smokers vs. non-smoker DNA samples obtained by oral rinse but not in the cytobrush DNA samples.

The 27-fold difference of adduct **2** between smokers' and non-smokers' oral rinse samples and the 36-fold difference of adduct **2** between smokers' and non-smokers' cytobrush samples were highly significant (p<0.001). These results contrast with our previous studies of acrolein-DNA adducts using similar mass spectrometry based methods in which we found no significant differences when comparing these adduct levels in lung tissue or leukocytes from smokers vs. non-smokers.[28, 30] The data from these studies are summarized in Table 4 and compared with the data reported here.

We also observed that the levels of adduct **2** in oral cell DNA from smokers were significantly higher than in lung or leukocyte DNA. These results as well as the differences between smokers and non-smokers in levels of adduct **2** undoubtedly reflect the fact that the oral cavity is the first site of exposure to cigarette smoke. Levels of acrolein – averaging 177 μg per cigarette in one study, and a range of 139–213 μg per cigarette in another, both under Health Canada smoking conditions, and 30.8–82.6 μg per cigarette under ISO conditions – are very high.[17, 31] Acrolein reacts directly with DNA to form adducts **1** and **2**; no

metabolism is required.[8] Clearly, adduct **2** in oral cell DNA is an exposure biomarker for acrolein. The extent to which it may also be a biomarker of effect requires further study.

Glutathione is important in the detoxification of acrolein. Thus, cigarette smokers have 4–5 times higher levels of urinary 3-hydroxypropyl mercapturic acid, the major end product of glutathione detoxification of acrolein, than non-smokers, and levels of this metabolite rapidly decrease after smoking cessation.[32, 33] We have previously speculated that glutathione detoxification of acrolein is efficient in smokers, thus controlling levels of adduct **2** in lung and leukocyte DNA such that they did not differ from non-smokers, but that was not observed here.[28, 30] Cellular glutathione concentrations have been reported to be higher in human lung tissue (11.6 ± 3.0 nmol/mg protein) than in human oral mucosa (1 – 4.80 nmol/mg protein).[34–36] The higher glutathione concentrations in lung tissue compared to oral tissue may play some role in the lower levels of adduct **2** in lung DNA compared to oral mucosa DNA, but further studies are needed.

Levels of adduct **2** in oral cells collected both by oral rinse and cytobrush were higher than those of adduct **1**. These results are consistent with most other studies that have quantified these adducts by mass spectrometry in various tissues, but differ from our results in the analysis of DNA from human lung, in which comparable amounts of adducts **1** and **2** were found.[13, 24, 26, 28–30, 37] Amino acids and proteins can facilitate the formation of adduct **2** compared to **1.**[37] It is unclear whether this mechanism is involved in the differential levels of adducts **1** and **2** in lung versus oral cells or leukocytes, or whether other mechanisms facilitate formation of adduct **1** in human lung.

As noted in the Introduction, several previous studies have investigated levels of acrolein adducts **1** and **2** in oral tissues and saliva. The amounts reported were generally consistent with those found here. The study by Nath *et al.* using gingival tissue reported significantly higher levels of adduct **2** in smokers than non-smokers, consistent with our results, while that of Li *et al.* found no difference in levels of adduct **2** in salivary DNA from smokers compared to non-smokers.[24, 26] The other studies did not present comparative data.[13, 25] A recent study using immunochemical methods without apparent artifact prevention reported levels of adduct **2** considerably higher than in any of these studies.[38]

Levels of adduct **3** were also significantly higher in smokers than in non-smokers, for samples collected by both oral rinse and cytobrush, while those of adduct **4** were higher only in the oral rinse samples. Our data are somewhat lower compared to the only previous report of these adduct levels in the oral cavity, based on data from human saliva. Thus, Chen and Lin found levels of 99 ± 50 and 72 ± 49 adducts **3** and **4**, per 10^8 normal nucleotides, respectively, in human salivary DNA samples from healthy volunteers; smoking status was not reported.[13]

Lipid peroxidation and related oxidative processes as well as inflammation are closely associated with cigarette smoking and lead to the formation of etheno-DNA adducts. Thus, cigarette smoking consistently produces elevated levels of 8-*iso*-PGF2α, an F2 isoprostane and biomarker of oxidative damage and PGE-M, a biomarker of inflammation, among others.[39–41] The elevated levels of adduct **3** and **4** observed in the oral cells of smokers in

this study are consistent with these previous studies. However, there was quite substantial variation in the levels of adduct **3** and **4** in both smokers and non-smokers, suggesting that other factors influencing the inflammatory response likely contribute, and further studies are needed. As mentioned above, during sample collection, participants were asked to take a breathalyzer test to measure their blood alcohol concentration and to provide some personal information, including alcohol consumption. Alcohol can be one of the factors that influences levels of adducts **3** and **4** and that may have contributed to the high variation observed in this study, but our relatively small sample size prevents further analysis of this variable.

Inter-subject variations of adducts **1–4** were large and far greater than the intra-subject variations which ranged from 27 – 63%, depending on the adduct and the day measured. This indicates that the inter-subject variations were due to inter-individual differences in exposure and endogenous factors affecting the DNA adduct levels.

The classical initiation promotion and co-carcinogenicity models of tobacco carcinogenesis were established by decades of research demonstrating that cigarette smoke contains both types of activities, and that initiating and promoting/co-carcinogenic activities were both required for its carcinogenicity.[42] Thus, tumor initiators such as benzo[*a*]pyrene and other polycyclic aromatic hydrocarbons as well as tumor promoters and co-carcinogens combined to demonstrate the effects of tobacco smoke as a complete carcinogen in both rodent inhalation and skin painting studies. The results presented here potentially recapitulate the initiation/promotion model of tobacco carcinogenesis in the human oral mucosa by demonstrating elevated DNA adduct formation in cigarette smokers by a genotoxic compound (acrolein) and products of inflammation (adducts **3** and **4**). The weakness in this argument is that acrolein, while highly toxic, is a relatively weak mutagen and has only limited or no demonstrated carcinogenicity.[9, 20] In contrast to acrolein, oral cell DNA adducts of mutagenic oral cavity carcinogens such as *N'*-nitrosonornicotine and dibenzo[*a,l*]pyrene could provide the genotoxic stimulus to act together with etheno-DNA adducts and other inflammatory/oxidative damage processes to result in oral cavity cancer as observed in smokers.[43–45]

There was a poor correlation between adduct levels obtained by cytobrush and mouthwash from the same individuals. This undoubtedly reflects the methods of collection. Mouthwash will capture saliva and perhaps certain cells that are being shed whereas collection with a cytobrush will likely provide a more homogeneous cell population. The contribution of bacterial DNA to the data reported here is also unknown.

In summary, this study establishes a highly reliable LC-NSI-HRMS-MS method for the quantitation of DNA adducts **1–4** in human oral cell DNA. The results clearly demonstrate elevated levels of acrolein-DNA adduct γ-OH-Acr-dGuo (**2**) and εdAdo (**3**) in oral cells from smokers vs. non-smokers independent of the method of collection while levels of α-OH-Acr-dGuo (**1**) and εdCyd (**4**) were higher in smokers compared to non-smokers only in the samples collected by mouthwash. These results provide potentially important leads for the use of oral cell DNA adducts as biomarkers of tobacco smoke exposure and biological effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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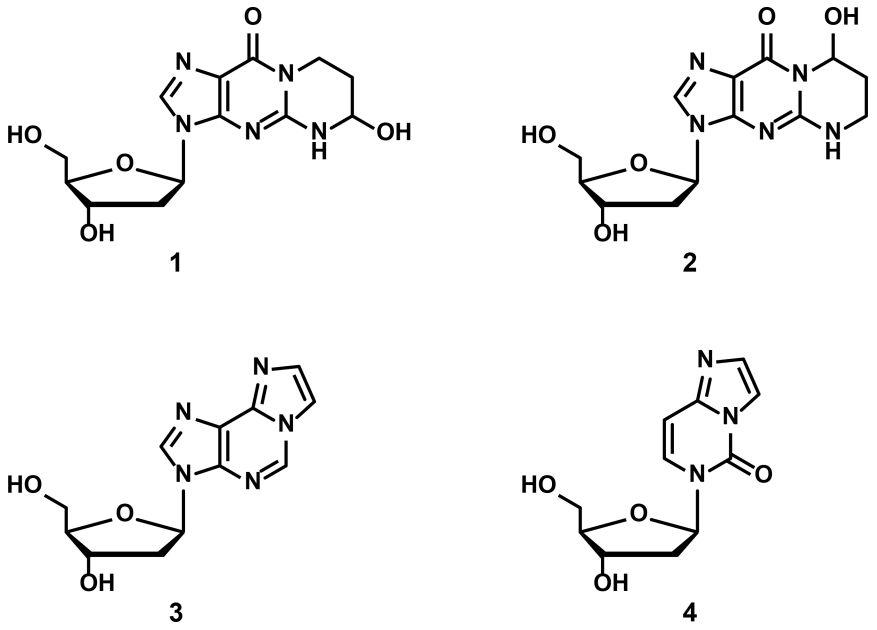

Figure 1.
Structures of α-OH-Acr-dGuo (**1**), γ-OH-Acr-dGuo (**2**), εdAdo (**3**), and εdCyd (**4**).

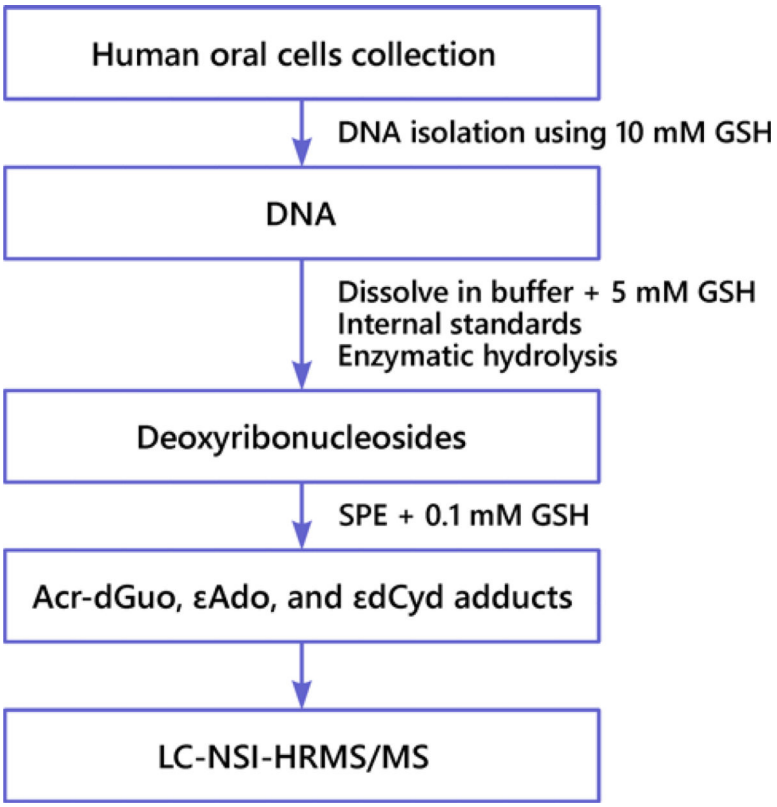

Figure 2.
Scheme of the LC-NSI-HRMS/MS method for analysis of Acr-dGuo (**1** and **2**), εdAdo (**3**) and εdCyd (**4**) in human oral cells.

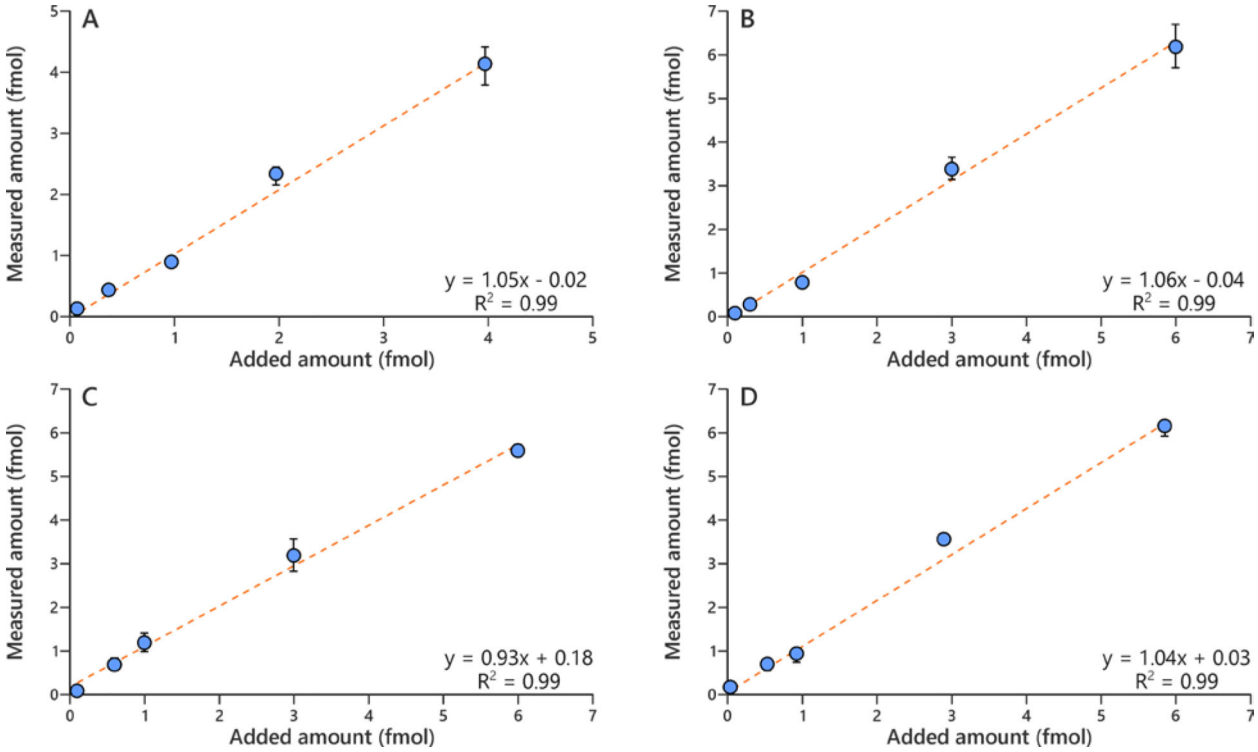

Figure 3.

Relationship between added amount and measured amount of (A) α-OH-Acr-dGuo (**1**), (B) γ-OH-Acr-dGuo (**2**)(C), εdAdo (**3**), and (D) εdCyd (**4**) in human oral cells.

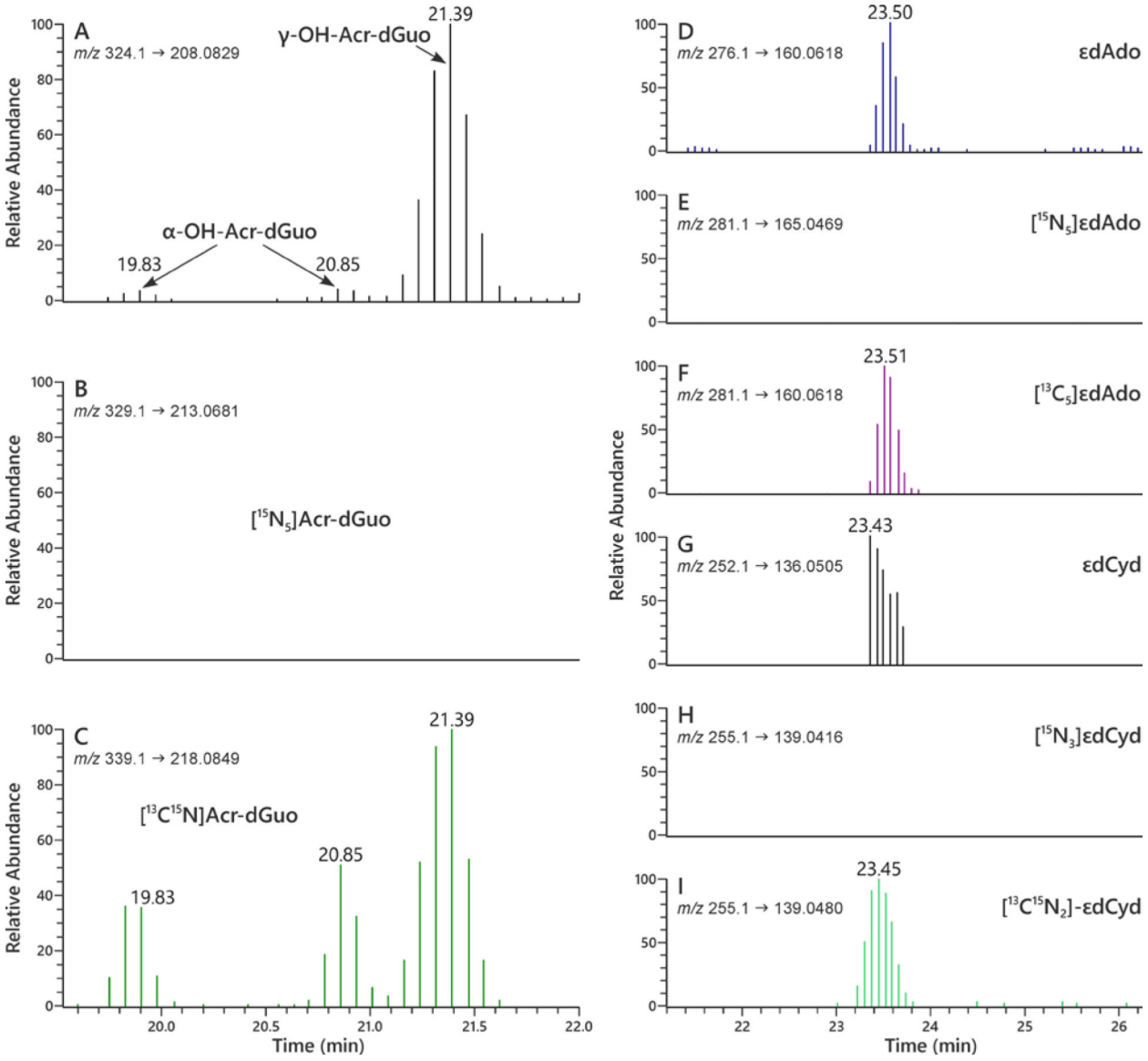

Figure 4.

LC-NSI-HRMS/MS chromatograms obtained upon analysis of human oral cell DNA obtained from an oral rinse sample for Acr-dGuo (**1** and **2**), εdAdo (**3**), and εdCyd (**4**). SRM was carried out at (A) *m/z* 324.1 → *m/z* 208.0829 for adducts **1** and **2**, (B) *m/z* 329.1 → *m/z* 213.0681 for spiked ^{15}N bacterial DNA adducts [$^{15}N_5$]**1** and **2**, and (C) *m/z* 339.1 → *m/z* 218.0849 for internal standard adducts [$^{13}C_{10}{}^{15}N_5$]**1** and **2**; SRM was carried out at (D) *m/z* 276.1 → *m/z* 160.0618 for adduct **3**, (E) *m/z* 281.1 → *m/z* 165.0469 for spiked ^{15}N bacterial DNA adduct [$^{15}N_5$]**3**, and (F) *m/z* 281.1 → *m/z* 160.0618 for internal standard adduct [$^{13}C_5$]**3**; SRM was carried out at (G) *m/z* 252.1 → *m/z* 136.0505 for adduct **4**, (H) *m/z* 255.1 → *m/z* 139.0416 for spiked ^{15}N bacterial DNA adduct [$^{15}N_3$]**4**, and (I) *m/z* 255.1 → *m/z* 139.0480 for internal standard adduct [$^{13}C^{15}N_2$]**4.** In each case, the transition

monitored represents loss of the deoxyribose ring with transfer of a proton to the base, e.g. ($[M + H]^+ \rightarrow [BH]^+$).

Table 1.

Assay precision determined by analyzing calf thymus DNA (n = 12).

Adduct	Calf Thymus DNA adducts/10^9 nucleotides	CV%	
		Intra-day	Inter-day
α-OH-Acr-dGuo (**1**)	12.1 ± 1.3	8	12
γ-OH-Acr-dGuo (**2**)	12.6 ± 2.3	12	9
εdAdo (**3**)	21.8 ± 5.7	11	12
εdCyd (**4**)	36.9 ± 3.5	10	9

Table 2.

Levels Acr-dGuo (**1** and **2**), εdAdo (**3**), and εdCyd (**4**) in oral rinse DNA from smokers (A) and non-smokers (B).

A. Smokers Oral rinse sample	**DNA (μg)** [a]	**α-OH-Acr-dGuo (1)**	**γ-OH-Acr-dGuo (2)**	**εdAdo (3)**	**εdCyd (4)**
		adducts/10^9 nucleotides			
1	13.1	4.01	91.6	5.84	3.06
2	17.9	5.19	170	9.76	4.77
3	8.23	67.5	211	52.3	37.4
4	24.9	ND [b]	254	17.4	9.89
5	2.51	ND	ND	12.2	6.91
6	16.9	51.9	365	48.7	45.9
7	9.74	ND	10.8	9.32	736
8	6.36	5.06	1020	15.9	21.8
9	5.52	22.9	74.9	12.5	ND
10	7.31	ND	82.2	15.6	46.3
11	17.2	6.58	140	2490	15.9
12	4.23	ND	87.8	307	1510
13	4.35	14.5	167	337	791
14	6.92	8.43	82.9	39.9	387
15	22.1	4.59	51.8	ND	ND
16	30.8	18.6	170	14.8	23.6
17	11.2	7.83	14.9	45.9	31.6
18	33.5	ND	14.9	12.5	3.06
19	26.3	ND	89.1	9.69	5.35
Mean ± S.D. [c]	14.2 ± 9.49	12.1 ± 17.9	163 ± 227	182 ± 568	194 ± 400
B. Non-smokers Oral rinse sample	**DNA (μg)** [a]	**α-OH-Acr-dGuo (1)**	**γ-OH-Acr-dGuo (2)**	**εdAdo (3)**	**εdCyd (4)**
		adducts/10^9 nucleotides			
1	1.54	ND [b]	ND	ND	ND
2	5.15	ND	10.1	3.74	12.7
3	9.81	ND	10.6	6.49	5.07
4	14.3	2.11	8.66	3.86	3.83
5	11.8	3.18	13.8	3.97	4.32
6	13.1	ND	ND	11.8	10.6
7	18.1	ND	7.21	6.64	5.96
8	18.9	ND	ND	55.9	50.2
9	20.9	ND	4.29	3.79	3.41
10	18.6	ND	8.31	5.58	5.01
11	31.2	ND	1.32	1.42	ND
12	30.4	ND	4.52	2.48	ND
13	9.48	ND	5.75	7.63	ND

14	23.3	ND	3.32	3.29	ND
15	6.12	ND	5.86	5.56	ND
16	27.3	ND	0.99	1.44	ND
17	9.32	ND	ND	3.69	ND
18	5.87	ND	ND	3.95	ND
19	14.8	ND	14.5	4.84	ND
20	23.3	ND	4.84	8.58	ND
Mean ± S.D.[c]	15.6 ± 8.61	1.85 ± 2.08	5.95 ± 4.23	7.69 ± 11.7	6.07 ± 10.9

[a] calculated on the basis of the dGuo quantified by HPLC-UV

[b] ND: not detected

[c] calculated using ½ LOQ of the adduct for ND values

Table 3.

Levels Acr-dGuo (**1** and **2**), εdAdo (**3**), and εdCyd (**4**) in cytobrush DNA from smokers (A) and non-smokers (B). There is a spacing problem in part B

A. Smokers Cytobrush sample	**DNA (μg)** [a]	**α-OH-Acr-dGuo (1)**	**γ-OH-Acr-dGuo (2)**	**εdAdo (3)**	**εdCyd (4)**
			adducts/10^9 nucleotides		
1	1.59	ND [b]	51.7	15.1	ND
2	2.65	38.9	1560	44.9	ND
3	1.88	ND	35.7	18.7	ND
4	1.64	ND	314	1200	nd [c]
5	2.17	ND	ND	12.1	ND
6	1.08	34.8	302	58.7	108
7	3.99	ND	15.6	9.24	ND
8	0.59	44.9	1950	58.2	ND
9	2.09	ND	146	16.3	32.9
10	0.66	ND	216	47.5	ND
11	2.44	ND	20.5	14.9	ND
12	2.86	ND	34.6	12.2	351
13	2.94	ND	83.2	9.05	14.1
14	2.16	ND	12.1	14.8	30.1
15	2.81	ND	20.8	11.9	ND
16	2.39	ND	23.5	11.8	49.2
17	3.02	ND	76.2	7.21	16.2
18	3.81	ND	29.5	10.7	ND
19	4.72	ND	19.7	6.33	ND
Mean ± S.D. [d]	2.27 ± 0.94	11.8 ± 13.1	259 ± 540	82.9 ± 271	36.9 ± 79.9
B. Non-smokers Cytobrush sample	**DNA (μg)** [a]	**α-OH-Acr-dGuo (1)**	**γ-OH-Acr-dGuo (2)**	**εdAdo (3)**	**εdCyd (4)**
			adducts/10^9 nucleotides		
1	0.83	ND [b]	ND	ND	ND
2	2.83	ND	ND	9.19	ND
3	3.71	ND	16.5	8.91	ND
4	0.85	ND	ND	19.3	ND
5	1.76	ND	ND	11.3	ND
6	2.59	ND	10.8	14.4	33.3
7	1.31	ND	ND	11.4	1060
8	1.39	ND	ND	11.6	ND
9	1.44	ND	ND	10.7	ND
10	1.97	ND	11.3	7.41	ND
11	5.06	ND	ND	5.07	ND
12	9.52	ND	ND	4.62	ND

13	2.59	ND	ND	7.98	ND
14	3.58	ND	ND	11.4	ND
15	3.49	ND	ND	6.62	ND
16	5.26	ND	ND	5.12	ND
17	4.56	ND	ND	143	246
18	5.83	ND	ND	4.06	ND
19	2.92	ND	ND	7.69	ND
20	4.33	ND	ND	5.94	ND
Mean ± S.D. [d]	3.29 ± 2.10	ND	7.37 ± 5.09	16.2 ± 30.2	72.2 ± 238

[a] calculated on the basis of the dGuo quantified by HPLC-UV

[b] ND: not detected

[c] nd: not determined

[d] calculated using ½ LOQ of the adduct for ND values

Table 4.

Comparison of acrolein-DNA adducts with previous studies.

	α-OH-Acr-dGuo (1) adducts/10^9 nucleotides		γ-OH-Acr-dGuo (2) adducts/10^9 nucleotides	
DNA Sample Type	**Smokers**	**Non-smokers**	**Smokers**	**Non-smokers**
Lung[30]	8.6 ± 2.7	10.3 ± 4.7	19.9 ± 14.3	14.7 ± 6.4
Leukocyte[28]	ND	ND	7.0 ± 2.5	9.7 ± 5.5
Oral rinse	12.1 ± 17.9	1.85 ± 2.08	163 ± 227	5.95 ± 4.23
Cytobrush	1.8 ± 13.1	ND	259 ± 540	7.37 ± 5.09