A VALIDATED UHPLC-MS/MS METHOD FOR SIMULTANEOUS QUANTIFICATION OF 9 EXOCYCLIC DNA ADDUCTS INDUCED BY 8 ALDEHYDES

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Abstract

Human exposure to aldehydes is implicated in several diseases including cancer. These strong electrophilic compounds can react with nucleophilic sites in DNA to form reversible and irreversible modifications. These modifications, if not repaired, can contribute to pathogenesis. The aim of our study was to provide a mass spectrometry (MS)-based profiling method for identifying potential biomarkers of aldehydes exposure. We have developed and validated a highly sensitive method using ultra high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UHPLC-ESI-MS/MS) for the simultaneous quantitation of 9 exocyclic DNA adducts derived from 8 main exogenous and endogenous aldehydes, namely formaldehyde, acetaldehyde, acrolein, crotonaldehyde, malondialdehyde, 4-hydroxy-2-nonenal, glyoxal and methylglyoxal. Finally, we applied the established method to quantify adducts in genomic DNA isolated from the blood of a smoker and a non-smoker blood samples in order to demonstrate its applicability.

Keywords: aldehydes; cancer; oxidative stress; adductomic; exposure biomarkers; analytical method validation; ultrahigh performance liquid chromatography -electrospray ionization-tandem mass spectrometry.

1. INTRODUCTION

Aldehydes are widespread in the environment. Exposure to aldehydes can occur through inhalation of outdoor and indoor emissions but also through food ingestion. Aldehydes are also found in tobacco smoke, automobile exhaust, and other emissions due to industrial processes and combustion of fossil fuels, wood, plastics, kerosene, cotton, biogenic and biomass [1]. Aldehydes can also be produced by overheating frying oils and cooking [1,2]. The indoor aldehydes concentrations are usually 2 – 10 times higher than the outdoor ones. Home's sources of aldehydes include building materials, hardwood, plywood, laminate floorings, adhesives, paints and solvents, smoking, household products, and the use of un-vented fuel-burning appliances, like gas stoves or kerosene space heaters [3]. In addition, aldehydes occur as intermediates of metabolic activation of a wide range of xenobiotics, including alcohol, therapeutic agents [4], environmental carcinogens [5] and amino acids [6]. They are also produced endogenously by biosynthesis of lipids [7] and by oxidative stress-induced lipid peroxidation. The main process is likely to be the so-called β-cleavage reaction of lipid alkoxy-radicals [2]. Therefore, aldehydes represent a major component of the exposome.

The International Agency for Research on Cancer (IARC) classifies formaldehyde as carcinogenic for humans. Acetaldehyde is also possibly carcinogenic (category 2B) but recognized as a carcinogen when associated with alcoholic beverages (category 1). Acrolein, crotonaldehyde, malondialdehyde and methylglyoxal are not classifiable (category 3). Nevertheless, most of them can damage DNA by reaction with exocyclic amino group of DNA bases, resulting in the formation of promutagenic lesions that increase the risk of cancer development [8].

Among the aldehydes produced by lipid peroxidation, 4-hydroxy-2-nonenal and malondialdehyde can also form exocyclic DNA adducts. Thus, they are considered to

contribute to the mutagenic and carcinogenic effects associated to oxidative stress and consequently, the development of cancer [9].

The future challenge is ultimately to identify and validate the aldehyde-bases adducts as biomarkers associated with both endogenous and environmental aldehydes exposures and cancer risk.

Since DNA adducts play an important role in aldehydes genotoxicity and occur at very low concentrations *in vivo*, a sensitive and accurate method for quantification of these adducts is required for the analysis of small quantities of DNA in human samples. Recently, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has become a golden standard method for the quantification of modified nucleosides such as DNA adducts. The two main advantages of the development of LC-MS/MS methods are *i)* the ability to detect all the exocyclic adducts with a gain in sensitivity, compared to the previous reference method *i.e.* ^{32}P-Postlabeling and *ii)* the proposition of screening methods to identify any DNA adduct.

In this work, we focused on the first advantage. Thus, we developed and validated a novel sensitive method using isotope dilution ultrahigh performance liquid chromatography - electrospray ionization- tandem mass spectrometry (UHPLC-ESI-MS/MS), for simultaneous detection and quantification of 9 exocyclic DNA adducts derived from 8 main exogenous and endogenous aldehydes. These aldehydes, namely formaldehyde (FA), acetaldehyde (AA), acrolein (Acro), crotonaldehyde (Croto), malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), glyoxal (Gx) and methylglyoxal (MG), were selected because they were representative of poly-unsaturated fatty acids (PUFAs) peroxidation [1] and we thus expected to observe changes in relative concentrations with oxidative stress variations. The leading compounds of dicarbonyl aldehydes were MDA, Gx and MG. The main saturated aldehydes from PUFAs peroxidation but the highest contaminants in air (indoor and outdoor) were FA

and AA. In the same way, Croto, Acro and HNE were the best representatives of α-β unsaturated aldehydes. Structures of aldehydes and the corresponding deoxyguanosine adducts are listed in Table 1. The method was then validated according to the European Medicines Agency guideline on bioanalytical method validation [10]. This method was developed to meet some requirements: minimize biological samples size, labor, consumable materials and analysis time. Moreover, relative levels of the different adducts can be compared in a single experiment. It also aims to establish profiles of exocyclic DNA adducts and may serve for adductomic approaches.

2. EXPERIMENTAL

2.1 Chemical Hazards

Aldehydes are volatile, highly reactive compounds, and known carcinogens and mutagens. Caution should therefore be exercised while handling these compounds; they should be handled within a fume hood using appropriate personal protection equipment (PPE).

2.2 Chemicals and Enzymes

2'-Deoxyguanosine (dG) was obtained from Alfa Aesar (Karlsruhe, Germany). [$^{13}C_{10}$, $^{15}N_5$] dG was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Acrolein, crotonaldehyde, acetaldehyde, formaldehyde (37% in water), glyoxal solution (40% in water), pyruvaldehyde solution (40% in water), 4-hydroxynonenal-dimethyl acetal (HNE-DMA), 1,1,3,3-tetraethoxypropane, sodium cyanoborohydride ($NaBH_3CN$), calf thymus DNA, alkaline phosphatase grade I from calf intestine, phosphodiesterase I from *Crotalus adamenteus* venom, deoxyribonuclease II type V from bovine spleen and nuclease P1 from *Penicillium Citrinum* were all purchased from Sigma-Aldrich (Stenheim, Germany). Phosphodiesterase II SPH was obtained from Worthington Biochemical (Lakewood, NJ, USA). Potassium phosphate monobasic (KH_2PO_4) was procured from Fluka (Steihem, Germany). LC-MS grade water, methanol, glacial acetic acid and acetonitrile were all obtained from VWR (Kelsterbach, Germany). The DNA extraction kit Nucleobond® CB 100 was purchased from Macherey-Nagel (Duren, Germany). HNE solution was prepared from HNE-DMA following the instructions of the supplier company (Sigma-Aldrich). MDA solution was obtained by hydrolysis of 1,1,3,3-tetraethoxypropane.

2.3 Chromatography

Each UHPLC systems used for DNA adducts purification and MS/MS detection were equipped with 2 Nexera X2 LC-30AD pumps and 2 DGU-20A_{5R} degassing units (Shimadzu, Kyoto, Japan). For purification and quantification of synthesized standards, the instrument was equipped with a 1.2 mL loop manual injector (Rheodyne, Rohert Park, CA, USA), a Varian ProStar column oven (Varian, Walnut Creek, CA, USA) and a SPD-20A prominence UV/Vis detector (Shimadzu, Kyoto, Japan). When coupled to the mass spectrometer, the UHPLC system was equipped with a CTO-20AC prominence oven and a Nexera X2 SIL-30AC autosampler (Shimadzu, Kyoto, Japan). The analytical column used for standards purification and quantification was an Atlantis® T3 OBD™ Prep column 100Å, 10 x 250 mm, 5 µm (Waters, Milford, MA, USA). The mobile phases were A, water and B, pure methanol. The column temperature was kept at 50 °C and the flow rate was 2 mL/min. The different elution gradient systems adapted to purify and quantify synthesized adducts are represented in *Supplementary data* table S-3.

For analytes separation, the analytical column was a reversed phase Acquity C_{18} UPLC® HSS C18 SB 1.0 x 150 mm, 1.8 µm (Waters, Ireland) with the following solvents: mobile phase A, 0.1% acetic acid in water and mobile phase B, 0.1% acetic acid in methanol/acetonitrile (50/50). The column temperature was kept at 50 °C and the flow rate was maintained at 0.2 mL/min using an injection volume of 2.5 µL. The elution pump gradient ramped from 5% B, up to 80% B at 2.5 min, which was held for 1.5 min, and then re-equilibrated to the starting conditions for 3 min thus, the run to run time was 7 min.

2.4 Mass spectrometric conditions

The separation system interfaces with a triple quadrupole MS (LCMS–8030Plus, Shimadzu). The electrospray ionization source was set in the positive ion mode as follows: drying gas

149 flow, 10 L/min; nebulizer gas flow, 3 mL/min; ion spray voltage, -4500 V; heat block
150 temperature, 280 °C and desolvation line temperature, 150 °C. Nitrogen was used either for
151 nebulization and desolvation. High purity Argon was used as collision gas.
152 Each standard was individually verified by full scan mass spectrometry and analyzed for at
153 least three times in selected ion monitoring (SIM) mode with an injection volume of 1 µL.
154 The initial products corresponding to each standard were characterized by their *m/z*
155 (precursors $[M+H]^+$). Collision energies were set at 10, 20 and 30 V for product ion scan of
156 $[M+H]^+$. A multiple reaction monitoring (MRM) mode was then adopted for optimization of
157 standards transitions. The two most intensive mass transitions were selected for each standard
158 useful for its identification and quantification and one transition for each internal standard.
159 The main fragment used for the quantification for all adducts corresponded to mass loss of
160 116 amu and 121 amu corresponding to loss of deoxyribose (dR) and labeled dR,
161 respectively. Mass transitions and optimized MRM parameters are detailed in Table 2.
162

163 **2.5 Preparation of DNA Adducts Standards**

164 Adducts standards were synthesized according to previous studies (*Supplementary data* table
165 S-1). Briefly, dG was incubated with aldehyde in phosphate buffer (PB) under gentle stirring.
166 In the case of FA, AA and MDA adduct synthesis, the solution was reduced immediately
167 after incubation by the addition of $NaBH_3CN$, and it was allowed to stand for 30 min at room
168 temperature. A second aliquot of $NaBH_3CN$ was then added, and the mixture was incubated
169 at 37 °C for 30 min. This procedure was repeated once more. The addition of $NaBH_3CN$
170 leads to the formation of N^2-ethyl-dG, N^2-methyl-dG and 5,6-dihydro-M_1dG, the most stable
171 adducts of AA, FA, and MDA, respectively.
172 Retention times of dG and all adducts are shown in *Supplementary data* table S-2 following
173 the purification performed on LC-UV according to different LC methods shown in

Supplementary data table S-3. The fractions containing each adduct were combined, followed by concentration under vacuum using a Speedvac®.

The amount of each synthesized adduct was quantified using LC-UV with the same chromatographic conditions as their purification except for cMGdG and HNEdG quantified on LC methods D and G in *Supplementary data* table S-3, respectively. Knowing the retention times of adducts, peaks areas were noted and the amount of each purified adduct was calculated according to the equation $C_i=A_i/\varepsilon_i k$ (A: adduct peak area; ε: adduct extinction coefficient; k: instrument response constant). Further quantification details and calculation are provided in *Supplementary data* text 1 and *Supplementary data* table S-4.

Isotopically labeled standards were synthesized at small scale by incubation of 1 mg/mL of [$^{13}C_{10}$, $^{15}N_5$]dG with aldehydes under constant stirring at 37 °C. For each labeled adduct synthesis, three solvents were tested and the choice between PB, dimethylformamide and DMSO was done with respect to the best yield and purity of adducts synthesis (*Supplementary data* table S-5). Similarly to above, immediately after incubation, labeled adducts of AA, FA and MDA were reduced by the addition of $NaBH_3CN$ to their stable forms [$^{13}C_{10}$, $^{15}N_5$]N^2-ethyl-dG, [$^{13}C_{10}$, $^{15}N_5$]N^2-methyl-dG and 5,6-dihydro-[$^{13}C_{10}$, $^{15}N_5$]M_1dG, respectively. Labeled standards were purified on the same LC-UV systems as their homologues. The fractions containing each labeled adduct were combined and concentrated under vacuum using a Speedvac® to obtain a final volume of approximately 200 µL. The absence of unlabeled adducts was checked on LC-MS/MS and was consistent with the initial purity of [$^{13}C_{10}$, $^{15}N_5$]dG. The final concentration of each labeled adduct was determined by comparing its chromatographic peak area to the one of the corresponding unlabeled adduct at the concentration of 100 ng/mL.

198 **2.6 Samples preparation**

199 Two human blood samples, from a smoker and a non-smoker, were used for the application
200 of the method on real samples. For both donors, 5 mL of venous blood was withdrawn into a
201 BD Vacutainer® spray-coated K_2EDTA tube then, immediately frozen at -80 °C. The DNA
202 was extracted from whole blood using Macherey-Nagel kit (Macherey-Nagel, Nucleobond®
203 CB 100). The DNA was purified using the NucleoBond® AXG 100 column. Following the
204 supplier procedure, the sample was loaded into the column and washed three times with a
205 100mM Tris/H_3PO_4, pH 6.3 buffer containing 15% (v/v) of ethanol and 1.15 M KCl, then, the
206 DNA was eluted from the column with a 100mM Tris/H_3PO_4 pH 8.5 buffer containing 15%
207 (v/v) ethanol and 1M KCl and then, isopropanol was added for DNA precipitation. After a
208 washing step with 70% ethanol in water, the DNA was reduced with $NaBH_3CN$ as described
209 in section 2.5 then, precipitated with 5 M NaCl and cold ethanol. Following centrifugation,
210 the supernatant was discarded and the DNA pellet was dried on Speedvac® then reconstituted
211 in adjusted volume of ultrapure water to obtain a final concentration of 1 mg/mL measured
212 by UV using a Nanodrop® spectrometer.

213

214 **2.7 Preparation of calibration standards and quality control samples**

215 Working solutions were prepared daily for validation experiments. Stock solutions were
216 stored at -80 °C.

217 Calibration standards and quality control (QC) samples were prepared in 1 mg/mL calf
218 thymus DNA solution. Aliquots of 50 µg of a single batch of calf thymus DNA solution were
219 reduced by addition of $NaBH_3CN$ as described above. Then, the pH was neutralized by
220 addition of 10 µL of 0.1 M NaOH and ice-cold ethanol was added to the solution and mixed
221 well by inversion until the DNA was visible and then centrifuged at 10000 rpm for 10 min at
222 4 °C. The obtained DNA pellet was washed with 1 mL of ethanol/water (70:30 v/v) and

centrifuged again at 10000 rpm for 10 min at 4 °C. The supernatant was discarded and the DNA pellet was dried on Speedvac® then dissolved in 50 µL of ultrapure water. To all aliquots, 25 µL of IS mixture was added. Calibration standards were prepared by spiking DNA with a mixture the synthesized DNA adducts to reach concentration levels of 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 175 and 250 ng/mL for each adduct and 25 µL a mixture of internal standards (IS) prepared at 0.32 ng/mL of labeled HNEdG and 1.6 ng/mL of all the other labeled adducts. For determining the inter- and intra-day precision and accuracy, QC samples were similarly prepared at 4 levels as recommended by the EMA guideline [10], the lower limit of quantification (LLOQ) QC (5 ng/mL for GxdG, cMGdG and CEdG and, 0.25 ng/mL for the other 6 adducts), low QC (10 ng/mL for GxdG, cMGdG and CEdG and, 0.4 ng/mL for the other 6 adducts), medium QC (2.5 ng/mL for MDAdG and 80 ng/mL for the other 8 adducts) and high QC (80 ng/mL for MDAdG, and 200 ng/mL for the 8 remaining adducts). Then, the DNA was enzymatically hydrolyzed following a procedure adapted from Genies *et al.* [11]. A mixture of enzymes containing phosphodiesterase II (0.05 U), bovine spleen deoxyribonuclease II (5 U) and *Penicillium Citrinum* nuclease P1 (1 U) in 10 µL of adequate buffer (200 mM succinic acid, 100 mM $CaCl_2$, pH 6) was added to DNA. This solution was immediately incubated under gentle stirring for 2h at 37 °C. The next step was addition of calf intestine alkaline phosphatase (4.6 U), *Crotalus adamenteus* venom phosphodiesterase I (0.03 U) and 14 µL of pH 8 buffer (500 mM Tris-HCl, 1 mM EDTA). Again, the mixture was incubated under gentle stirring for 2h at 37 °C. The reaction was stopped by the addition of 8 µL of 0.1 M HCl, 13.5 µL of 5 mM NaCl and cold ethanol to ensure the precipitation of enzymes. The mixture was centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant was transferred into a new tube and evaporated to dryness. The hydrolyzed DNA was reconstituted in 20 µL of HPLC grade water and transferred to HPLC vials containing low volume inserts for analysis on LC-MS/MS. DNA in the remaining pellet was redissolved in

248 ultrapure water and quantified by UV spectroscopy on Nanodrop® for evaluation of
249 hydrolysis yield.
250
251 **2.8 Method validation**
252 The developed method was validated, according to the European Medicines Agency (EMA)
253 guideline on bioanalytical method validation [11], in terms of selectivity, carry-over, lower
254 limit of quantification (LLOQ), inter- and intra-day accuracy and precision, matrix effects
255 and linearity.
256 Since aldehydes DNA adducts are naturally occuring in DNA, matrix-matched calibration
257 with internal standard correction was selected for the quantification in the current work. The
258 analytic standards were spiked into a single batch of reduced calf thymus DNA as described
259 above. A sufficient quantity of homogenous reduced DNA was used for all validation
260 solutions to avoid variability of the subsamples results due to inhomogeneity.
261
262 **2.8.1 Selectivity**
263 The first step in analytical validation is to assess selectivity of the method. The latter should
264 be able to differentiate the analytes of interest and IS from endogenous components in the
265 matrix. An IS should be used and should preferably be a related standard with a retention
266 time close to that of the analyte. It must be added to the fraction to be analyzed at the
267 beginning of the experiment and must have an appropriate form, particularly suitable for MS
268 detection. The analyte shall elute at the characteristic retention time that is typical for the
269 corresponding calibration standard under the same experimental conditions. Stable isotope
270 labeled internal standards using ^{15}N or ^{13}C meets all these requirements. Identification
271 criteria, specified in European Commission Decision 2002/657/EC, consist also in the
272 presence of 4 identification points: the precursor ion and 2 daughter ions.

2.8.2 Carry-over

Carry-over was assessed by injecting methanol after the highest calibration level for each adduct. Analytes peaks should not exceed 20% of the ones at LLOQ and 5% for the IS.

2.8.3 Lower limit of quantification

The lower limit of quantification (LLOQ) is the lowest concentration of analyte in a sample which can be quantified. Knowing that aldehydes DNA adducts are naturally present in DNA, the peaks areas of the lowest levels of the DNA calibration curves were considered for regression analysis and the LLOQ was defined as the concentration of lowest calibrator with an acceptable accuracy and precision (<20%).

2.8.4 Accuracy and precision

For determination of accuracy and precision within-run (intraday) and between-run (inter-day), QC samples were analyzed in 4 levels (LLOQ QC, low QC, medium QC and high QC) against the calibration curves. To determine within-run accuracy, a minimum of 5 samples per QC were analyzed in a single run and the obtained concentrations were compared with the nominal value (as percentage). The within-run precision was expressed by the coefficient of variation (CV) between the repeated individual measures of analyte. Whereas, QC samples from at least 3 runs done on at least 2 different days were analyzed and similar calculation as above evaluated the between-run accuracy and precision. Acceptance criteria for both within and between-run were ± 20% for the LLOQ QC and ± 15% for the other QC levels.

2.8.5 Matrix effects

Matrix effects were investigated by calculating the ratio between the slopes of analytes in matrix-matched calibration curves and the slopes of analytes-only calibration curves. For that reason, calibration standards at same concentration levels were prepared by spiking 50 µL water with 25 µL of IS mixture and 25 µL of different standards stock solutions. The calibration curves were obtained by plotting peak area of each analyte versus standard concentrations. Therefore, the ratio of both slopes expressed the matrix factor (MF). Besides, the IS-normalized MF was also calculated by dividing the MF of the analyte by the MF of the IS which was done in the same way as above but by plotting peak area of each analyte to its corresponding labeled IS.

2.8.6 Linearity

In order to determine the linearity of the method, calibration solutions in DNA were prepared in a range of 0.25 ng/mL to 250 ng/mL (levels are listed in section 2.7). Each day, three calibration curves of each adduct in DNA were plotted by injecting the calibrators in triplicate for each curve. This was done for at least three days. The calibration curves were obtained by plotting peak area ratio of each analyte to its corresponding labeled IS versus standard concentrations. The back calculated concentrations of the calibration standards must be within ± 15% of the nominal value, with the exception for the LLOQ for which it must be within ± 20%. At least 75% of the calibration standards, with a minimum of six calibration standard levels, should fulfill this criterion.

2.9 Application of the validated method

Two human blood samples, from a smoker (30 cigarettes per day) and a non-smoker, were obtained from Tabacology Unit at CHU UCL Namur asbl in Belgium in order to check the

full applicability of the validated method. Both donors were females aged of 55 years old. Ethical approval for the blood collection was obtained from the ethical committee of CHU UCL Namur Godinne (NUB: B039201316167). Blood samples were collected from non-smokers and from smokers before smoking cessation at their first visit to the unit. After sample treatment detailed in section 2.6, a 50 µg aliquot of each extracted genomic DNA was enzymatically hydrolyzed after addition of 25 µL of IS mixture and processed as previously described in section 2.7. QC samples were run between samples injections to verify sensitivity and instrumental performance. The level of DNA adducts was calculated following Eq. 1 [12].

Relative adduct level = [DNA adducts concentration (ng/mL)/MW of DNA adducts (g/mol)] / [DNA concentration (ng/mL)/Mean MW of DNA (g/mol)] (equation I)

With mean MW of DNA = 6490 g/mol, the obtained results were multiplied by 10^7, and the levels of adducts were expressed as adducts per 10^7 nucleotides.

3. RESULTS

3.1.Method validation

The validation of the developed method has succeeded in terms of selectivity, carry-over, limit of quantification, inter- and intra-day accuracy and precision, matrix effects and linearity.

3.1.1. Selectivity

The method is able to differentiate the analytes of interest and IS from endogenous components in the matrix since a stable isotope labeled form for each analyte was used as an IS, which is particularly suited for MS detection. It was added to the DNA matrix at the beginning of the experiment before hydrolysis step and had the same retention time than the corresponding analyte. In addition, analytes were identified by the presence of 4 confirmation points: the precursor ion and 2 daughter ions figured in a transition of quantification and a second transition of confirmation per analyte.

3.1.2. Carry-over

No significant chromatographic peaks greater than 20% of the LLOQ and 5% for the IS response were detected when analyzing adducts and IS in methanol injection after ULOQ injection. These results confirm the absence of carry-over.

3.1.3. Lower limit of quantification

The calibrator at a concentration of 0.25 ng/mL was the LLOQ of the six following adducts: AcrodG, CrotodG, reduced AAdG, reduced FAdG, HNEdG and reduced MDAdG corresponding to 1.2, 1.1, 1.3, 1.4, 0.9 and 1.3 adducts per 10^7 normal nucleotides,

360 respectively. GxdG, cMGdG and CEdG were quantified starting at a concentration of 5
361 ng/mL corresponding to 23.7 and 22.7 adducts per 10^7 normal nucleotides the first and the
362 two last adducts, respectively. Both accuracy and precision values did not exceed ± 20% at
363 the LLOQ.
364
365 **3.1.4. Accuracy and precision**
366 Accuracy and precision were determined for each adduct per QC in a single run and between
367 3 different runs. They were all within ± 20% for LLOQ and ± 15% for other samples (as
368 shown in *Supplementary data* tables S-6 and S-7 for inter- and intra-day evaluations,
369 respectively).
370
371 **3.1.5. Matrix effects**
372 The CV of IS-normalized MF was within ± 14 % for all adducts except reduced FAdG, for
373 which IS-normalized MF varied of ± 41 %. Therefore, we selected to quantify adducts
374 against DNA matrix calibration standards spiked with the same amount of IS mixture.
375
376 **3.1.6. Linearity**
377 Linearity was assessed through three runs of validation, in each, one calibration curve
378 in DNA was established per analyte, plotted to its corresponding labeled IS. However, we
379 selected the reduced [$^{13}C_{10}$, $^{15}N_5$]FAdG to establish reduced AAdG calibration curve knowing
380 that, its corresponding IS was still used to identify the adduct. Similarly, [$^{13}C_{10}$, $^{15}N_5$]CrotodG
381 was used to establish GxdG and cMGdG linearity and their labeled homologues helped to
382 determine adducts retention times. All DNA calibration curves were linear weighted by 1/C
383 and the back calculated concentrations of the calibration standards, with a minimum of six
384 levels, were within ± 15% of the nominal value (maximal values are shown in *Supplementary*

385 *data* tables S-8), with the exception for the LLOQ for which it was within ±20%. These
386 values were similar for all adducts because LLOQ was selected to meet this ±20%
387 requirement. Linearity was obtained over a range of 0.25 ng/mL to 250 ng/mL for AcrodG,
388 CrotodG, reduced AAdG and reduced FAdG. Whereas, for reduced MDAdG, it covered a
389 range of 0.25 ng/mL to 100 ng/mL. For GxdG, cMGdG and CEdG, linearity was determined
390 from 5 ng/mL to 250 ng/mL.
391
392 **3.2. Application of the validated method**
393 The developed method was applied to determine simultaneously the concentration of the
394 studied adducts in extracted genomic DNA from a smoker blood *versus* a non-smoker blood.
395 The cigarette smoke contains many aldehydes known to link covalently DNA bases for which
396 DNA adduct can be considered as biomarker. The exocyclic DNA adducts were identified in
397 the samples by the presence of a parent ion and 2 daughter ions for each adduct. In addition,
398 the retention time of each adduct in the sample was compared to the retention time of its
399 labeled IS and to that in calibration standards. QC samples analyzed between human samples
400 confirmed instrument performance.
401 Adducts at the LLOQ and above were quantified against calibration curve in DNA and the
402 relative adduct level (RAL) was calculated using equation I. A significant difference in
403 adducts levels (figure 1) was remarkable between the two samples.
404 Except for cMGdG, GxdG and reduced AAdG, all adducts were detected in the smoker blood
405 DNA. The levels of AcrodG and reduced FAdG were 4.1 and 6.3 adducts per 10^7 normal
406 nucleotides, respectively. Comparing to non-smoker DNA, CrotodG occurred at a higher
407 level of 28.3 adducts per 10^7 normal nucleotides on the contrary to reduced MDAdG present
408 at a level of 3.5 adducts per 10^7 normal nucleotides. However, HNEdG and CEdG were
409 detected but not quantified in the sample since their levels were below the LLOQ.

In the DNA of non-smoker blood, AcrodG and reduced AAdG were not detected while, reduced FAdG, HNEdG, and GxdG were detected but not quantifiable. In contrast, the levels of CEdG, cMGdG and reduced MDAdG were higher than in smoker blood DNA (41.9, 26.9 and 4.3 adducts per 10^7 normal nucleotides, respectively).

4. DISCUSSION

Given the implication of exocyclic DNA adducts in biomonitoring risk assessment, many UHPLC-ESI-MS/MS methods have been developed for their simultaneous detection and quantification. Churchwell *et al.* developed a method for simultaneous quantification of 4 adducts including the non-reduced adduct of MDA [13], whereas Yin *et al.* succeeded to separate and quantify dG, dC and dA adducts of acrolein and their isomers [14]. Similarly, Zhang *et al.* developed a method to quantify both diastereomers of CrotodG [15]. Sixteen DNA adducts were simultaneously analyzed in human lung biopsy specimens, most of them were ethenoadducts with one exocyclic adduct corresponding to non-reduced adduct of MDA [16]. All these methods were using stable isotope labeled standards of each adduct as internal standard but, to our knowledge, none of them was fully validated according to international guidelines.

Thus, we selected 8 aldehydes representatives of PUFAs [1] and synthesized for each compounds the corresponding adducts to 2'dG as described in many earlier studies. In parallel, their $^{13}C_{10}$,$^{15}N_5$ labeled homologues were prepared in the same manner but in a smaller scale. Synthesized adducts are chemically stable when present in DNA; however those of AA, FA and MDA are considered unstable once DNA is hydrolyzed into 2'-deoxynucleosides. For this reason, a reduction step using $NaBH_3CN$ was essential to stabilize these adducts to allow their quantification [17].

The choice of column, the LC solvent, the type and concentration of buffer are all critical aspects for good chromatography, but also for improving MS sensitivity [14,18]. One of the most critical point in the method development is the separation of the DNA adducts from the four non-modified nucleosides. Indeed, even if a MS detection is employed, the typical difference in concentrations between normal nucleosides and DNA adducts ranged between 10^6 and 10^9. In this situation a slight overlap in chromatographic peaks corresponding to

DNA adduct with the ones of non-modified nucleosides will result in a drastic ion suppression of DNA adducts. The development of specific analytical protocols for the quantification of exocyclic DNA-adducts via LC-ESI-MS/MS has evolved rapidly in recent years and generated significant scientific progress [13]. Most of the methods for the separation of DNA adducts from aldehydes in the literature use reversed phase chromatography on C18 column. Methods using a sub-2 µm particles UHPLC column [15] allow quick separation whereas over-2 µm HPLC columns lead to longer analysis times, usually around 30 min [13,19,20]. Yin *et al.* [14] have developed a sensitive approach for accurate quantification of CrotodG adducts using stable isotope dilution UHPLC-MS/MS analysis on a C18 column (2.1 mm i.d. x 50 mm, 1.8 µm). The limits of detection (LOD_s, S/N=3) and the limits of quantification (LOQs, S/N =10) were estimated around 50 amol and 150 amol, respectively. Using this method, both diastereomers of CrotodG adducts were detected in untreated human cell line with a frequency of 2.5-20 adducts per 10^8 nucleotides.

Some authors introduce online column switching, capillary separation, and nanoESI in order to increase the sensitivity of the methods. For example, these improvements have been successfully used to determine DNA adducts derived as a result of oxidative stress and lipid peroxidation [21] and enable detection limits of ~1 adduct in 10^9 nucleotides using 1–10 µg of DNA [22]. Another example is reported by Churchwell *et al.* [13] for the simultaneous analysis of four different lipid peroxidation and oxidative stress derived DNA adducts in DNA hydrolysates of 100 µg or less using on-line sample preparation coupled with LC-MS. This method leads to the quantification of DNA adducts at levels below one adduct in 10^8 normal nucleotides in untreated rat and normal human liver tissue. Singh *et al.* [19] have developed a sensitive online column-switching LC-MS/MS method that allowed the dose-dependent detection of reduced AAdG in DNA exposed to cannabis cigarette smoke. In a previous study, non-reduced FAdG in nasal DNA of rats exposed to [$^{13}CD_2$] formaldehyde

465 was quantified by a highly sensitive nano-UPLC-MS/MS method with 20 amol limit of
466 detection on a C18 analytical column switching [20]. Notwithstanding the increase in
467 sensitivity, the main drawback of these techniques is a long analysis time (up to 50 min).
468 Yin *et al.* [14] reported the effect of additives in mobile phase for the detection of the
469 acrolein-derived DNA adducts by LC-ESI-MS/MS using reversed phase chromatography. In
470 their report, the optimization of additive species in the mobile phase enhanced the ESI-MS
471 intensities by 2.3-8.7 times. Zhang *et al.* [15] also reported the influence of additive for the
472 CrotodG adducts. In these two methods, ammonium bicarbonate seemed to be the best choice
473 for separation of stereoisomers of acrolein and crotonaldehyde adducts in comparison to
474 formic acid and three volatile ammonium buffers. In our method, our mass spectrometer gave
475 the best S/N ratio for acetic acid (0.1% v/v) that was not reported in the cited works.
476 Recently, hydrophilic interaction chromatography (HILIC) has attracted attention, as an
477 alternative to reversed-phase chromatography, because this chromatographic mode is capable
478 of separating hydrophilic and polar organic compounds [23]. In addition to the ability for
479 separation of hydrophilic compounds, the mobile phases used in HILIC separation are
480 generally organic solvent-rich ones, and such solvent composition is suitable to improve the
481 ionization efficiency in the ESI process by enhancing the efficiency of desolvation [24].
482 Three commercially available stationary phases, possessing different polar functional groups
483 (aminopropyl, dihydroxypropyl, and carbamoyl), were examined for the separation of four
484 normal deoxynucleosides together with acrolein and crotonaldehyde DNA adducts. The
485 improvement of sensitivity for a variety of compounds in HILIC-ESI-MS/MS has been
486 reported compared to reversed phase LC-ESI-MS/MS [25]. Unfortunately, the gradient
487 needed to last for at least 20 min to obtain a good separation and elute dG [23]. Another
488 drawback in the use of HILIC is the long time it takes to equilibrate the column after the
489 gradient. This leads to long run-to-run times not compatible with large number of samples.

Finally, we chose to use reversed phase chromatography and selected the Acquity UPLC® HSS C_{18} SB (1.0 x 150 mm, 1.8 µm) column for the separation of DNA adducts formed from 8 aldehydes. This UHPLC column is a C18 grafted non-endcapped low-coverage high strength synthetic silica allowing a run-to-run analysis time of 7 min with a good separation of DNA-adducts from normal nucleosides (figure 2). We tried to focus on developing a method for the quick simultaneous analysis of DNA adducts so it can be dedicated to analyze a large number of samples. We also set up the separation to combine several peaks corresponding to isomers of the same adduct in one peak in order to obtain a better sensitivity. The elution gradient was adjusted to minimize analysis time and obtain the optimal sensitivity for all adducts. A co-elution of adducts from different aldehydes was not a problem since there was no isobaric compounds and thus each MRM transition was specific of a DNA adduct. The peak shape of some adducts was not symmetrical, we attribute this to a partial co-elution of diastereomers since these adducts contain chiral carbons. The only two DNA adducts isomers separated were those from CEdG.

As usually observed for nucleosides, the most intensive transition was the loss of deoxyribose moiety and its labeled homologue for all adducts and internal standards, respectively. These transitions were chosen for adducts quantification by injecting 2.5 µL of samples or calibration standards starting from 50 µg of DNA. In previous developed methods, 15 µL were injected from 20 µg of DNA solution [14]; these conditions were improved to 2 µL of 5 µg DNA solution [15].

Our method is the first one to quantify simultaneously 9 DNA adducts from aldehydes by using calibration in DNA using the stable isotope dilution. Indeed, all the published calibration curves were using calibrators prepared in water. LLOQ was determined for AcrodG at 0.25 ng/mL corresponding to a level of 2.4 adducts per 10^7 normal nucleotides. Liu *et al.* quantified AcrodG starting 0.02 ng/mL knowing that their method detected only

515 one adduct using a calibration curve in water [26]. This study reported a matrix effect of 80%
516 evaluated on few samples. During the validation of our method, by adding the DNA adducts
517 at the beginning of the sample preparation procedure, the matrix effects and extraction
518 recoveries were evaluated on calibration curves were found to be between 14% and 41%,
519 respectively, highlighting the necessity to quantify from DNA extracted calibrators.
520 Therefore, matrix-matched calibration standards were used to quantify adducts. Similarly,
521 comparing to previous studies, our validated method can appear as less sensitive than other
522 methods. Again, this is due to the calibration realized in DNA instead of water as previously
523 reported. LLOQ of CrotodG, HNEdG, Reduced AAdG, Reduced FAdG and Reduced
524 MDAdG were 1.1, 0.9, 1.3, 1.4 and 1.3 adducts per 10^7 normal nucleotides, respectively,
525 corresponding to 0.25 ng/mL for each adduct which is at least in the same order of magnitude
526 than previous studies if the instrumental sensitivity is taken into account, meaning that the
527 concentration factor due to the time consuming sample concentration (on-line or off-line) is
528 neglected [13,15,19,20,26]. We had to raise the LLOQ for the 3 remaining adducts GxdG
529 (LLOQ = 5 ng/mL corresponding to 23.7 adducts per 10^7 normal nucleotides), cMGdG and
530 CEdG (LLOQ = 5 ng/mL corresponding to 22.7 adducts per 10^7 normal nucleotides) since
531 they were present in DNA at higher concentration thus lowering accuracy and precision.
532 Therefore, that means that higher levels of adducts might be present in DNA for these
533 adducts.
534 The applicability of the method was assessed from 2 human blood samples. For these two
535 samples the DNA was extracted and analyzed using our method (Figures 3 and 4). The
536 objective here was to prove that the method can be applied on real samples and to check if the
537 adduct levels quantified here are similar to some relevant ones in the literature. This will
538 validate the possibility to apply the method on larger sample sets to conclude about the
539 contradictory state between smoker and non-smoker DNA adducts profiles in further

experiments. Except for reduced AAdG, cMGdG and GxdG, all analytes were present in smoker DNA. In non-smoker DNA, AcrodG and reduced AAdG were not detected; HNEdG, GxdG and reduced FAdG were detected but not quantifiable. However, the adduct levels of CrotodG and reduced FAdG were lower for smokers than non-smokers while CEdG and reduced MDAdG levels were higher. Besides this, only non-smoker blood DNA contained cMGdG and GxdG. These differences in both DNA adducts profiles may be attributed to large subject-to-subject variability exposure to complex mixtures, or other confounding factors [26]. Our results are contradictory to previous works on smokers and non-smokers samples. Zhang *et al.* has reported no relationship in AcrodG levels to self-reported time since cessation of smoking [27]. AcrodG level was 4.1 adducts per 10^7 nucleotides in our study which was higher than the previously reported levels of 0.3 ± 0.1 per 10^7 nucleotides for both smokers and non-smokers DNA [28].

Adducts levels in human samples were reported in earlier studies to be in higher levels comparing to our LLOQ. The type of tissue sample can have a real influence on adducts amounts. Indeed, the pathological conditions contribute to adduction. For example, levels of 28-51 AcrodG adducts per 10^7 normal nucleotides [26] and 2.4-5.6 HNEdG per 10^7 normal nucleotides were found in DNA of brain tissues from Alzheimer's disease subjects and age-matched controls; the mean level of AcrodG adducts was about 0.9 ± 0.1 adducts per 10^7 normal nucleotides in DNA samples of extracted human leukocytes [14] and a level of 1 reduced AAdG per 10^7 normal nucleotides was quantified in human liver DNA [17]. In addition, 0.2–0.4 CrotodG per10^7 normal nucleotides were detected in untreated human MRC5 cells [15] and CEdG was detected in WM-266-4 human melanoma cells at a frequency of one lesion per 10^7 nucleotides, and in human breast tumor at levels 3–12 adducts per 10^7 dG [29].

5. CONCLUSION

For the first time ever, we have described the development and the validation, according to international guidelines, of an analytical method on UHPLC-ESI-MS/MS to quantify simultaneously 9 exocyclic DNA adducts derived from 8 aldehydes from DNA extracted calibrators and QCs. After synthesis, identification and quantitation of these adducts and their $^{13}C_{10}$,$^{15}N_5$ isotopes homologues, calibration curves were established ranging from 0.25 (LLOQ) to 250 ng/mL (ULOQ) of adducts in both matrices water and DNA in the aim to describe the response of the instrument with regard to analyte concentration. Quality control samples were prepared and analyzed to prove the method within-run, and between-run accuracy and precision. Absence of carry-over was also checked. The method is able to differentiate the 9 analytes of interest and their homologues IS using identification criteria specified by European Commission Decision 2002/657/EC. The method meets all the requirements listed in the EMA guidelines. This is essential to ensure the acceptability of the performance and the reliability of analytical results. This validated method has been proved to be applicable on real samples and may be extended to the quantification of other adducts in the future. It can furthermore be used in adductomic approaches specially to assess the DNA damages that can be attributed to oxidative stress. These adducts are generated from aldehydes obtained from external environment as well as from endogenous oxidative stress related pathway. They have been proposed to be involved in the mutagenicity of oxidative stress and could be used as biomarkers of the latter. The validated UHPLC-MS/MS method was found to be sensitive enough and accurate for low-level quantification of 9 exocyclic DNA adducts derived from 8 main exogenous and endogenous aldehydes, namely formaldehyde, acetaldehyde, acrolein, crotonaldehyde, malondialdehyde, 4-hydroxy-2-nonenal, glyoxal and methylglyoxal.

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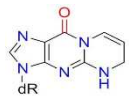
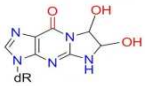
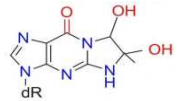
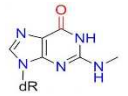
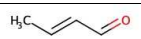
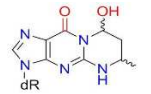
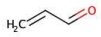
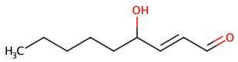
690

691 Tables

692 **Table 1:** Aldehydes and adducts names and structures

Aldehydes	Aldehydes abbrev.	Aldehydes structure	Adducts names	Adducts abbrev.	Adducts structure
Malondialdehyde	**MDA**		**5,6-dihydro-3-(2-deoxy-β-D-*erythro*-pentafuranosyl)pyrimido[1,2-α]purin-10*(3H)*-one-deoxyguanosine**	**Reduced MDAdG**	
Glyoxal	**Gx**		**3-(2'-deoxy-β-D-*erythro*-pentofuranosyl)-5,6,7-trihydro-6,7-dihydroxyimidazo[1,2-*a*]purine-9-one**	**GxdG**	
Methylglyoxal	**MG**		**N^2-(1-carboxyethyl)-2'-deoxyguanosine**	**CEdG**	
			1,N^2-(1,2-dihydroxy-2-methyl)ethano-2'-deoxyguanosine	**cMGdG**	
Formaldehyde	**FA**		**N^2-methyl-deoxyguanosine**	**Reduced FAdG**	

Acetaldehyde	**AA**		**N^2-ethyl-deoxyguanosine**	**Reduced AAdG**	
Crotonaldehyde / 2 x acetaldehyde	**Croto / 2 x AA**		**α-R/S-methyl-γ-hydroxy-1,N^2-propano-deoxyguanosine**	**CrotodG**	
Acrolein	**Acro**		**α-R/S-hydroxy-1,N^2-propano-deoxyguanosine γ-hydroxy-1,*N2*-propano-deoxyguanosine**	**AcrodG**	
4-hydroxy-2-nonenal	**HNE**		**4-hydroxy-2-nonenal-deoxyguanosine**	**HNEdG**	

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695 **Table 2:** Mass transition and optimized MRM parameters for each DNA adduct

Aldehydes	Adducts	Q1	Q3	CE (eV)	Retention time (min)
Acrolein	AcrodG	324.1	208.1	-11	2.02
			190.2	-28	
	[$^{13}C_{10}$, $^{15}N_5$]AcrodG	339	218	-11	
Crotonaldehyde	CrotodG	338	222.1	-13	2.26
			178.1	-27	
	[$^{13}C_{10}$, $^{15}N_5$]CrotodG	353	232	-13	
Acetaldehyde	Reduced AAdG	296.1	180.2	-11	2.27
			163.1	-31	
	Reduced [$^{13}C_{10}$, $^{15}N_5$]AAdG	311	190	-11	
Formaldehyde	Reduced FAdG	282.1	166	-10	2.06
			149.1	-32	
	Reduced [$^{13}C_{10}$, $^{15}N_5$]FAdG	297	176	-10	
4-Hydroxy-2-nonenal	HNEdG	424	308.2	-13	3.05
			152.2	-27	
	[$^{13}C_{10}$, $^{15}N_5$]HNEdG	439	318	-13	
Malondialdehyde	Reduced MDAdG	306.1	190.1	-11	2.34
			135.1	-39	
	Reduced [$^{13}C_{10}$, $^{15}N_5$]MDAdG	321	200	-11	
Glyoxal	GxdG	326.1	210	-11	1.21
			164.2	-29	
	[$^{13}C_{10}$, $^{15}N_5$]GxdG	341	220	-11	
Methylglyoxal	cMGdG	340.2	224.1	-10	1.72
			152.1	-32	
	[$^{13}C_{10}$, $^{15}N_5$]cMGdG	355	234	-10	
	CEdG	340.1	224	-12	2.08 and 2.39
			177.9	-18	
	[$^{13}C_{10}$, $^{15}N_5$]CEdG	355	234	-12	

696

Figures

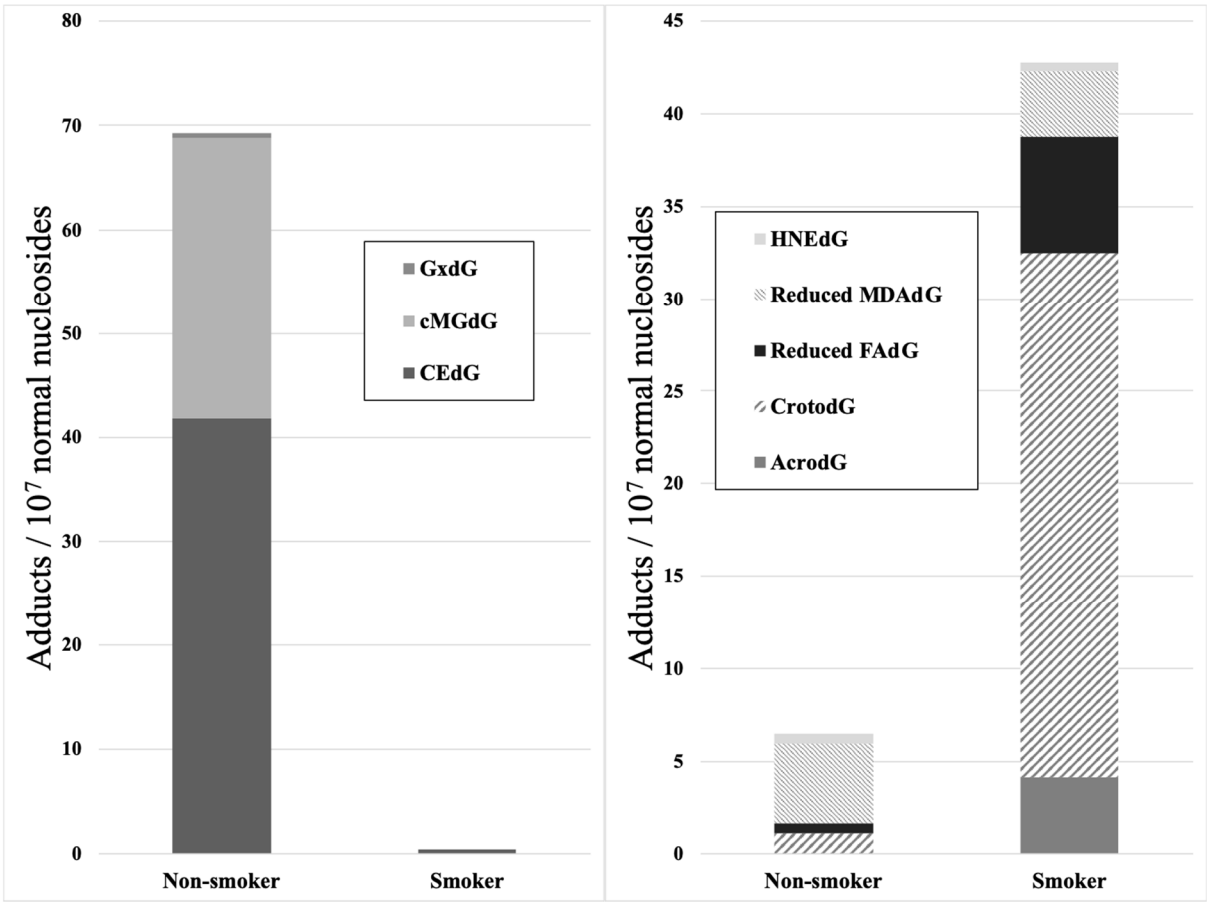
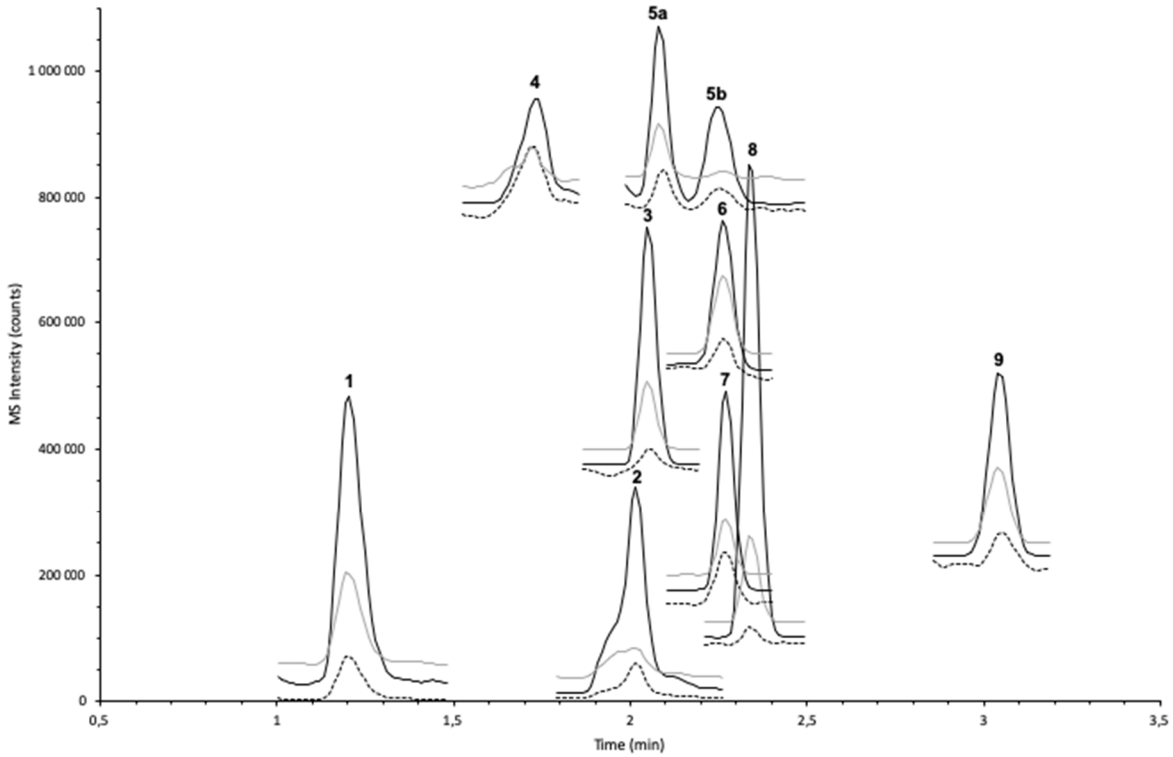


Figure 1: DNA adducts levels pattern of genomic DNA isolated from both a smoker and a non-smoker blood samples. The levels of adducts detectable but lower than the LLOQ were arbitrary set to 0.5 adduct / 10^7 normal nucleosides.

705



706

707 **Figure 2:** Chromatogram of a DNA calibrator at 10 ng/mL. For each peak, the dotted lines
708 correspond to the $^{15}N_5$,$^{13}C_{10}$ internal standards, the black and the grey lines correspond to
709 the first and the second MRM transitions in the table 1. Peaks: 1. GxdG, 2. AcrodG, 3.
710 Reduced FAdG, 4. cMGdG, 5a. and 5b. CEdG, 6. CrotodG, 7. Reduced AAdG, 8.
711 Reduced MDAdG, 9. HNEdG.

712

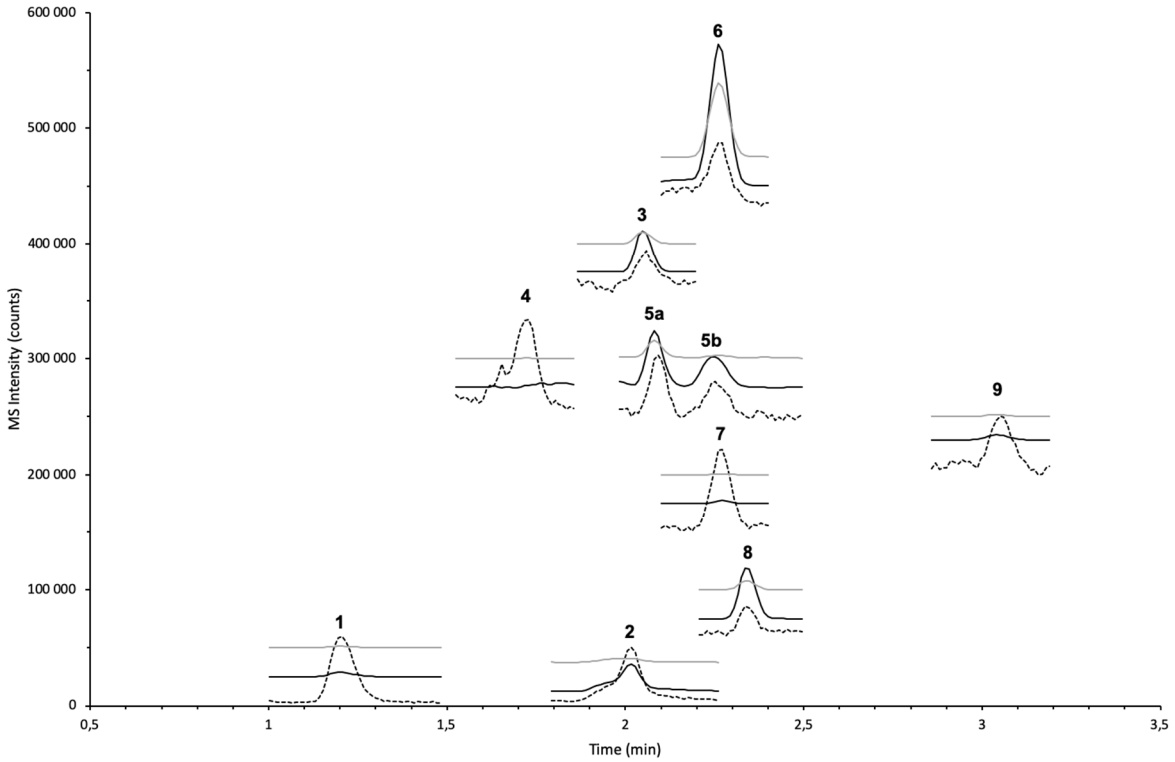
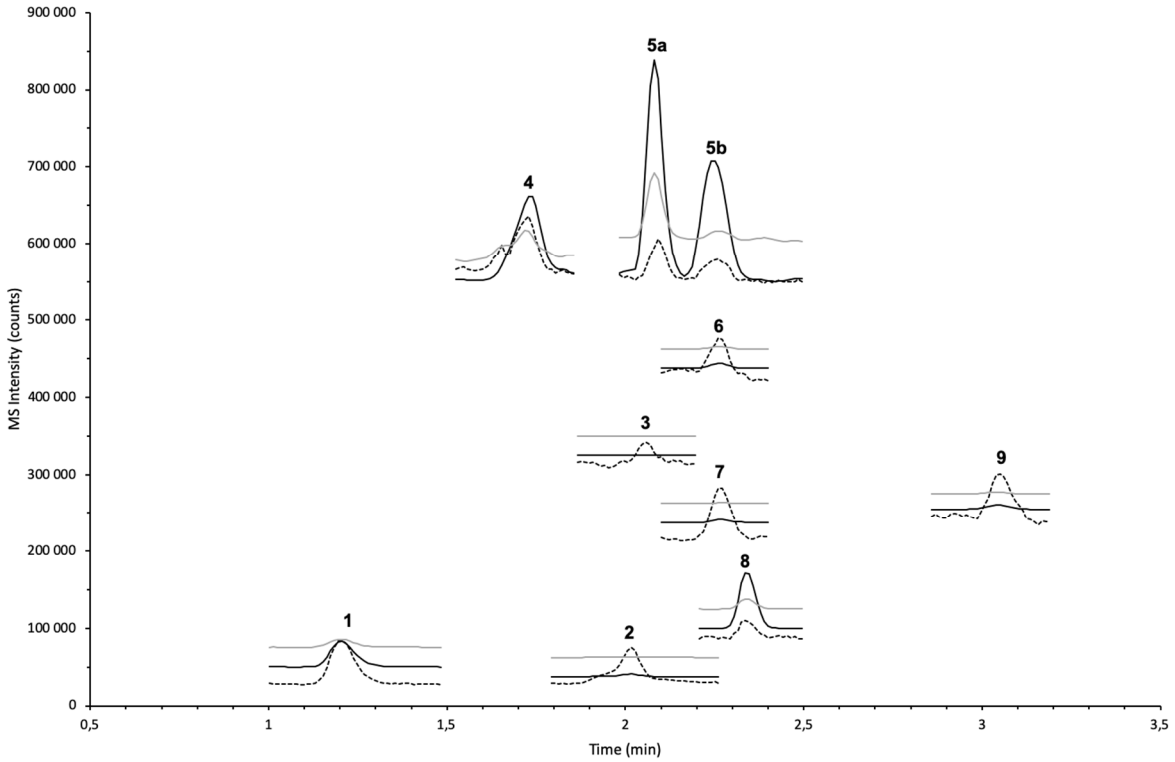


Figure 3: Chromatogram of a DNA adducts from a smoker's blood. For each peak, the dotted lines correspond to the $^{15}N_5$,$^{13}C_{10}$ internal standards, the black and the grey lines correspond to the first and the second MRM transitions in the table 1. Peaks: 1. GxdG, 2. AcrodG, 3. Reduced FAdG, 4. cMGdG, 5a. and 5b. CEdG, 6. CrotodG, 7. Reduced AAdG, 8. Reduced MDAdG, 9. HNEdG.



720

721 **Figure 4:** Chromatogram of a DNA adducts from a non-smoker's blood. For each peak, the

722 dotted lines correspond to the $^{15}N_5$,$^{13}C_{10}$ internal standards, the black and the grey lines

723 correspond to the first and the second MRM transitions in the table 1. Peaks: 1. GxdG, 2.

724 AcrodG, 3. Reduced FAdG, 4. cMGdG, 5a. and 5b. CEdG, 6. CrotodG, 7. Reduced

725 AAdG, 8. Reduced MDAdG, 9. HNEdG.

726