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1 **A VALIDATED UHPLC-MS/MS METHOD FOR SIMULTANEOUS**
2 **QUANTIFICATION OF 9 EXOCYCLIC DNA ADDUCTS INDUCED BY**
3 **8 ALDEHYDES**

4
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23 **Abstract**

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

36
37 **Keywords:** aldehydes; cancer; oxidative stress; adductomic; exposure biomarkers; analytical
38 method validation; ultrahigh performance liquid chromatography -electrospray ionization-
39 tandem mass spectrometry.

40

41 1. INTRODUCTION

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

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

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

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

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

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

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

91 and AA. In the same way, Croto, Acro and HNE were the best representatives of α - β
92 unsaturated aldehydes. Structures of aldehydes and the corresponding deoxyguanosine
93 adducts are listed in Table 1. The method was then validated according to the European
94 Medicines Agency guideline on bioanalytical method validation [10]. This method was
95 developed to meet some requirements: minimize biological samples size, labor, consumable
96 materials and analysis time. Moreover, relative levels of the different adducts can be
97 compared in a single experiment. It also aims to establish profiles of exocyclic DNA adducts
98 and may serve for adductomic approaches.
99

100 2. EXPERIMENTAL

101 2.1 Chemical Hazards 102

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

106 107 2.2 Chemicals and Enzymes

108 2'-Deoxyguanosine (dG) was obtained from Alfa Aesar (Karlsruhe, Germany). [¹³C₁₀,
109 ¹⁵N₅] dG was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Acrolein,
110 crotonaldehyde, acetaldehyde, formaldehyde (37% in water), glyoxal solution (40% in
111 water), pyruvaldehyde solution (40% in water), 4-hydroxynonenal-dimethyl acetal (HNE-
112 DMA), 1,1,3,3-tetraethoxypropane, sodium cyanoborohydride (NaBH₃CN), calf thymus
113 DNA, alkaline phosphatase grade I from calf intestine, phosphodiesterase I from *Crotalus*
114 *adamanteus* venom, deoxyribonuclease II type V from bovine spleen and nuclease P1 from
115 *Penicillium Citrinum* were all purchased from Sigma-Aldrich (Stenheim, Germany).
116 Phosphodiesterase II SPH was obtained from Worthington Biochemical (Lakewood, NJ,
117 USA). Potassium phosphate monobasic (KH₂PO₄) was procured from Fluka (Steihem,
118 Germany). LC-MS grade water, methanol, glacial acetic acid and acetonitrile were all
119 obtained from VWR (Kelsterbach, Germany). The DNA extraction kit Nucleobond[®] CB 100
120 was purchased from Macherey-Nagel (Duren, Germany). HNE solution was prepared from
121 HNE-DMA following the instructions of the supplier company (Sigma-Aldrich). MDA
122 solution was obtained by hydrolysis of 1,1,3,3-tetraethoxypropane.

123

124 **2.3 Chromatography**

125 Each UHPLC systems used for DNA adducts purification and MS/MS detection were
126 equipped with 2 Nexera X2 LC-30AD pumps and 2 DGU-20A_{5R} degassing units (Shimadzu,
127 Kyoto, Japan). For purification and quantification of synthesized standards, the instrument
128 was equipped with a 1.2 mL loop manual injector (Rheodyne, Rohert Park, CA, USA), a
129 Varian ProStar column oven (Varian, Walnut Creek, CA, USA) and a SPD-20A prominence
130 UV/Vis detector (Shimadzu, Kyoto, Japan). When coupled to the mass spectrometer, the
131 UHPLC system was equipped with a CTO-20AC prominence oven and a Nexera X2 SIL-
132 30AC autosampler (Shimadzu, Kyoto, Japan). The analytical column used for standards
133 purification and quantification was an Atlantis[®] T3 OBD[™] Prep column 100Å, 10 x 250
134 mm, 5 µm (Waters, Milford, MA, USA). The mobile phases were A, water and B, pure
135 methanol. The column temperature was kept at 50 °C and the flow rate was 2 mL/min. The
136 different elution gradient systems adapted to purify and quantify synthesized adducts are
137 represented in *Supplementary data* table S-3.

138 For analytes separation, the analytical column was a reversed phase Acquity C₁₈ UPLC[®] HSS
139 C18 SB 1.0 x 150 mm, 1.8 µm (Waters, Ireland) with the following solvents: mobile phase A,
140 0.1% acetic acid in water and mobile phase B, 0.1% acetic acid in methanol/acetonitrile
141 (50/50). The column temperature was kept at 50 °C and the flow rate was maintained at 0.2
142 mL/min using an injection volume of 2.5 µL. The elution pump gradient ramped from 5% B,
143 up to 80% B at 2.5 min, which was held for 1.5 min, and then re-equilibrated to the starting
144 conditions for 3 min thus, the run to run time was 7 min.

145

146 **2.4 Mass spectrometric conditions**

147 The separation system interfaces with a triple quadrupole MS (LCMS–8030Plus, Shimadzu).
148 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 NaBH₃CN, and it was allowed to stand for 30 min at room
168 temperature. A second aliquot of NaBH₃CN was then added, and the mixture was incubated
169 at 37 °C for 30 min. This procedure was repeated once more. The addition of NaBH₃CN
170 leads to the formation of N²-ethyl-dG, N²-methyl-dG and 5,6-dihydro-M₁dG, 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

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

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

183 Isotopically labeled standards were synthesized at small scale by incubation of 1 mg/mL of
184 [¹³C₁₀, ¹⁵N₅]dG with aldehydes under constant stirring at 37 °C. For each labeled adduct
185 synthesis, three solvents were tested and the choice between PB, dimethylformamide and
186 DMSO was done with respect to the best yield and purity of adducts synthesis
187 (*Supplementary data* table S-5). Similarly to above, immediately after incubation, labeled
188 adducts of AA, FA and MDA were reduced by the addition of NaBH₃CN to their stable
189 forms [¹³C₁₀, ¹⁵N₅]N²-ethyl-dG, [¹³C₁₀, ¹⁵N₅]N²-methyl-dG and 5,6-dihydro-[¹³C₁₀,
190 ¹⁵N₅]M₁dG, respectively. Labeled standards were purified on the same LC-UV systems as
191 their homologues. The fractions containing each labeled adduct were combined and
192 concentrated under vacuum using a Speedvac® to obtain a final volume of approximately 200
193 μL. The absence of unlabeled adducts was checked on LC-MS/MS and was consistent with
194 the initial purity of [¹³C₁₀, ¹⁵N₅]dG. The final concentration of each labeled adduct was
195 determined by comparing its chromatographic peak area to the one of the corresponding
196 unlabeled adduct at the concentration of 100 ng/mL.

197

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 K₂EDTA 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/H₃PO₄, 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/H₃PO₄ 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 NaBH₃CN 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 NaBH₃CN 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

223 centrifuged again at 10000 rpm for 10 min at 4 °C. The supernatant was discarded and the
224 DNA pellet was dried on Speedvac® then dissolved in 50 µL of ultrapure water. To all
225 aliquots, 25 µL of IS mixture was added. Calibration standards were prepared by spiking
226 DNA with a mixture the synthesized DNA adducts to reach concentration levels of 0.25, 0.5,
227 1, 2.5, 5, 10, 25, 50, 100, 175 and 250 ng/mL for each adduct and 25 µL a mixture of internal
228 standards (IS) prepared at 0.32 ng/mL of labeled HNEdG and 1.6 ng/mL of all the other
229 labeled adducts. For determining the inter- and intra-day precision and accuracy, QC samples
230 were similarly prepared at 4 levels as recommended by the EMA guideline [10], the lower
231 limit of quantification (LLOQ) QC (5 ng/mL for GxdG, cMGdG and CEEdG and, 0.25 ng/mL
232 for the other 6 adducts), low QC (10 ng/mL for GxdG, cMGdG and CEEdG and, 0.4 ng/mL for
233 the other 6 adducts), medium QC (2.5 ng/mL for MDAdG and 80 ng/mL for the other 8
234 adducts) and high QC (80 ng/mL for MDAdG, and 200 ng/mL for the 8 remaining adducts).
235 Then, the DNA was enzymatically hydrolyzed following a procedure adapted from Genies *et*
236 *al.* [11]. A mixture of enzymes containing phosphodiesterase II (0.05 U), bovine spleen
237 deoxyribonuclease II (5 U) and *Penicillium Citrinum* nuclease P1 (1 U) in 10 µL of adequate
238 buffer (200 mM succinic acid, 100 mM CaCl₂, pH 6) was added to DNA. This solution was
239 immediately incubated under gentle stirring for 2h at 37 °C. The next step was addition of
240 calf intestine alkaline phosphatase (4.6 U), *Crotalus adamanteus* venom phosphodiesterase I
241 (0.03 U) and 14 µL of pH 8 buffer (500 mM Tris-HCl, 1 mM EDTA). Again, the mixture
242 was incubated under gentle stirring for 2h at 37 °C. The reaction was stopped by the addition
243 of 8 µL of 0.1 M HCl, 13.5 µL of 5 mM NaCl and cold ethanol to ensure the precipitation of
244 enzymes. The mixture was centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant was
245 transferred into a new tube and evaporated to dryness. The hydrolyzed DNA was
246 reconstituted in 20 µL of HPLC grade water and transferred to HPLC vials containing low
247 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 occurring 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.

273

274

2.8.2 Carry-over

275 Carry-over was assessed by injecting methanol after the highest calibration level for each

276 adduct. Analytes peaks should not exceed 20% of the ones at LLOQ and 5% for the IS.

277

278

2.8.3 Lower limit of quantification

279 The lower limit of quantification (LLOQ) is the lowest concentration of analyte in a sample

280 which can be quantified. Knowing that aldehydes DNA adducts are naturally present in

281 DNA, the peaks areas of the lowest levels of the DNA calibration curves were considered for

282 regression analysis and the LLOQ was defined as the concentration of lowest calibrator with

283 an acceptable accuracy and precision (<20%).

284

285

2.8.4 Accuracy and precision

286 For determination of accuracy and precision within-run (intraday) and between-run (inter-

287 day), QC samples were analyzed in 4 levels (LLOQ QC, low QC, medium QC and high QC)

288 against the calibration curves. To determine within-run accuracy, a minimum of 5 samples

289 per QC were analyzed in a single run and the obtained concentrations were compared with

290 the nominal value (as percentage). The within-run precision was expressed by the coefficient

291 of variation (CV) between the repeated individual measures of analyte. Whereas, QC samples

292 from at least 3 runs done on at least 2 different days were analyzed and similar calculation as

293 above evaluated the between-run accuracy and precision. Acceptance criteria for both within

294 and between-run were $\pm 20\%$ for the LLOQ QC and $\pm 15\%$ for the other QC levels.

295

296 **2.8.5 Matrix effects**

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

306

307 **2.8.6 Linearity**

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

317

318 **2.9 Application of the validated method**

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

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

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

332 With mean MW of DNA = 6490 g/mol, the obtained results were multiplied by 10⁷, and the
333 levels of adducts were expressed as adducts per 10⁷ nucleotides.

334

335 3. RESULTS

336

337 3.1.Method validation

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

341

342 3.1.1. Selectivity

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

350

351 3.1.2. Carry-over

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

355

356 3.1.3. Lower limit of quantification

357 The calibrator at a concentration of 0.25 ng/mL was the LLOQ of the six following adducts:
358 AcrodG, CrotodG, reduced AAdG, reduced FAdG, HNEdG and reduced MDAdG
359 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 $\pm 20\%$ 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 $\pm 20\%$ for LLOQ and $\pm 15\%$ 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 $\pm 14\%$ for all adducts except reduced FAdG, for
373 which IS-normalized MF varied of $\pm 41\%$. 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 [$^{13}\text{C}_{10}$, $^{15}\text{N}_5$]FAdG to establish reduced AAdG calibration curve knowing
380 that, its corresponding IS was still used to identify the adduct. Similarly, [$^{13}\text{C}_{10}$, $^{15}\text{N}_5$]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 $\pm 15\%$ 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 $\pm 20\%$. These
386 values were similar for all adducts because LLOQ was selected to meet this $\pm 20\%$
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 CEEdG, 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 CEEdG were
409 detected but not quantified in the sample since their levels were below the LLOQ.

410 In the DNA of non-smoker blood, AcrodG and reduced AAdG were not detected while,
411 reduced FAdG, HNEdG, and GxdG were detected but not quantifiable. In contrast, the levels
412 of CEEdG, cMGdG and reduced MDAdG were higher than in smoker blood DNA (41.9, 26.9
413 and 4.3 adducts per 10^7 normal nucleotides, respectively).
414

415 4. DISCUSSION

416 Given the implication of exocyclic DNA adducts in biomonitoring risk assessment, many
417 UHPLC-ESI-MS/MS methods have been developed for their simultaneous detection and
418 quantification. Churchwell *et al.* developed a method for simultaneous quantification of 4
419 adducts including the non-reduced adduct of MDA [13], whereas Yin *et al.* succeeded to
420 separate and quantify dG, dC and dA adducts of acrolein and their isomers [14]. Similarly,
421 Zhang *et al.* developed a method to quantify both diastereomers of CrotoG [15]. Sixteen
422 DNA adducts were simultaneously analyzed in human lung biopsy specimens, most of them
423 were ethenoadducts with one exocyclic adduct corresponding to non-reduced adduct of MDA
424 [16]. All these methods were using stable isotope labeled standards of each adduct as internal
425 standard but, to our knowledge, none of them was fully validated according to international
426 guidelines.

427 Thus, we selected 8 aldehydes representatives of PUFAs [1] and synthesized for each
428 compounds the corresponding adducts to 2'dG as described in many earlier studies. In
429 parallel, their $^{13}\text{C}_{10},^{15}\text{N}_5$ labeled homologues were prepared in the same manner but in a
430 smaller scale. Synthesized adducts are chemically stable when present in DNA; however
431 those of AA, FA and MDA are considered unstable once DNA is hydrolyzed into 2'-
432 deoxynucleosides. For this reason, a reduction step using NaBH_3CN was essential to stabilize
433 these adducts to allow their quantification [17].

434 The choice of column, the LC solvent, the type and concentration of buffer are all critical
435 aspects for good chromatography, but also for improving MS sensitivity [14,18]. One of the
436 most critical point in the method development is the separation of the DNA adducts from the
437 four non-modified nucleosides. Indeed, even if a MS detection is employed, the typical
438 difference in concentrations between normal nucleosides and DNA adducts ranged between
439 10^6 and 10^9 . In this situation a slight overlap in chromatographic peaks corresponding to

440 DNA adduct with the ones of non-modified nucleosides will result in a drastic ion
441 suppression of DNA adducts. The development of specific analytical protocols for the
442 quantification of exocyclic DNA-adducts via LC-ESI-MS/MS has evolved rapidly in recent
443 years and generated significant scientific progress [13]. Most of the methods for the
444 separation of DNA adducts from aldehydes in the literature use reversed phase
445 chromatography on C18 column. Methods using a sub-2 μm particles UHPLC column [15]
446 allow quick separation whereas over-2 μm HPLC columns lead to longer analysis times,
447 usually around 30 min [13,19,20]. Yin *et al.* [14] have developed a sensitive approach for
448 accurate quantification of CrotoDg adducts using stable isotope dilution UHPLC-MS/MS
449 analysis on a C18 column (2.1 mm i.d. x 50 mm, 1.8 μm). The limits of detection (LODs,
450 S/N=3) and the limits of quantification (LOQs, S/N =10) were estimated around 50 amol and
451 150 amol, respectively. Using this method, both diastereomers of CrotoDg adducts were
452 detected in untreated human cell line with a frequency of 2.5-20 adducts per 10^8 nucleotides.
453 Some authors introduce online column switching, capillary separation, and nanoESI in order
454 to increase the sensitivity of the methods. For example, these improvements have been
455 successfully used to determine DNA adducts derived as a result of oxidative stress and lipid
456 peroxidation [21] and enable detection limits of ~ 1 adduct in 10^9 nucleotides using 1–10 μg
457 of DNA [22]. Another example is reported by Churchwell *et al.* [13] for the simultaneous
458 analysis of four different lipid peroxidation and oxidative stress derived DNA adducts in
459 DNA hydrolysates of 100 μg or less using on-line sample preparation coupled with LC-MS.
460 This method leads to the quantification of DNA adducts at levels below one adduct in 10^8
461 normal nucleotides in untreated rat and normal human liver tissue. Singh *et al.* [19] have
462 developed a sensitive online column-switching LC-MS/MS method that allowed the dose-
463 dependent detection of reduced AAdG in DNA exposed to cannabis cigarette smoke. In a
464 previous study, non-reduced FAdG in nasal DNA of rats exposed to [$^{13}\text{C}_2$] 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.

490 Finally, we chose to use reversed phase chromatography and selected the Acquity UPLC®
491 HSS C₁₈ SB (1.0 x 150 mm, 1.8 μm) column for the separation of DNA adducts formed from
492 8 aldehydes. This UHPLC column is a C18 grafted non-encapped low-coverage high
493 strength synthetic silica allowing a run-to-run analysis time of 7 min with a good separation
494 of DNA-adducts from normal nucleosides (figure 2). We tried to focus on developing a
495 method for the quick simultaneous analysis of DNA adducts so it can be dedicated to analyze
496 a large number of samples. We also set up the separation to combine several peaks
497 corresponding to isomers of the same adduct in one peak in order to obtain a better
498 sensitivity. The elution gradient was adjusted to minimize analysis time and obtain the
499 optimal sensitivity for all adducts. A co-elution of adducts from different aldehydes was not a
500 problem since there was no isobaric compounds and thus each MRM transition was specific
501 of a DNA adduct. The peak shape of some adducts was not symmetrical, we attribute this to a
502 partial co-elution of diastereomers since these adducts contain chiral carbons. The only two
503 DNA adducts isomers separated were those from CE₂G.

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

510 Our method is the first one to quantify simultaneously 9 DNA adducts from aldehydes by
511 using calibration in DNA using the stable isotope dilution. Indeed, all the published
512 calibration curves were using calibrators prepared in water. LLOQ was determined for
513 Acro₂G at 0.25 ng/mL corresponding to a level of 2.4 adducts per 10⁷ normal nucleotides.
514 Liu *et al.* quantified Acro₂G 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 MDAAdG 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 CEAdG (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

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

552 Adducts levels in human samples were reported in earlier studies to be in higher levels
553 comparing to our LLOQ. The type of tissue sample can have a real influence on adducts
554 amounts. Indeed, the pathological conditions contribute to adduction. For example, levels of
555 28-51 AcrodG adducts per 10^7 normal nucleotides [26] and 2.4-5.6 HNEdG per 10^7 normal
556 nucleotides were found in DNA of brain tissues from Alzheimer's disease subjects and age-
557 matched controls; the mean level of AcrodG adducts was about 0.9 ± 0.1 adducts per 10^7
558 normal nucleotides in DNA samples of extracted human leukocytes [14] and a level of 1
559 reduced AAdG per 10^7 normal nucleotides was quantified in human liver DNA [17]. In
560 addition, 0.2–0.4 CrotodG per 10^7 normal nucleotides were detected in untreated human
561 MRC5 cells [15] and CEdG was detected in WM-266-4 human melanoma cells at a
562 frequency of one lesion per 10^7 nucleotides, and in human breast tumor at levels 3–12
563 adducts per 10^7 dG [29].

564

565 5. CONCLUSION

566 For the first time ever, we have described the development and the validation, according
567 to international guidelines, of an analytical method on UHPLC-ESI-MS/MS to quantify
568 simultaneously 9 exocyclic DNA adducts derived from 8 aldehydes from DNA extracted
569 calibrators and QCs. After synthesis, identification and quantitation of these adducts and their
570 $^{13}\text{C}_{10}, ^{15}\text{N}_5$ isotopes homologues, calibration curves were established ranging from 0.25
571 (LLOQ) to 250 ng/mL (ULOQ) of adducts in both matrices water and DNA in the aim to
572 describe the response of the instrument with regard to analyte concentration. Quality control
573 samples were prepared and analyzed to prove the method within-run, and between-run
574 accuracy and precision. Absence of carry-over was also checked. The method is able to
575 differentiate the 9 analytes of interest and their homologues IS using identification criteria
576 specified by European Commission Decision 2002/657/EC. The method meets all the
577 requirements listed in the EMA guidelines. This is essential to ensure the acceptability of the
578 performance and the reliability of analytical results. This validated method has been proved
579 to be applicable on real samples and may be extended to the quantification of other adducts in
580 the future. It can furthermore be used in adductomic approaches specially to assess the DNA
581 damages that can be attributed to oxidative stress. These adducts are generated from
582 aldehydes obtained from external environment as well as from endogenous oxidative stress
583 related pathway. They have been proposed to be involved in the mutagenicity of oxidative
584 stress and could be used as biomarkers of the latter. The validated UHPLC-MS/MS method
585 was found to be sensitive enough and accurate for low-level quantification of 9 exocyclic
586 DNA adducts derived from 8 main exogenous and endogenous aldehydes, namely
587 formaldehyde, acetaldehyde, acrolein, crotonaldehyde, malondialdehyde, 4-hydroxy-2-
588 nonenal, glyoxal and methylglyoxal.

589

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594

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598

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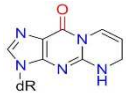

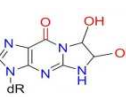


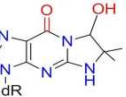

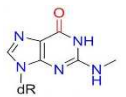
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
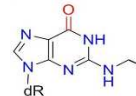
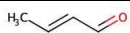
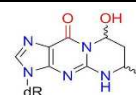
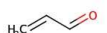
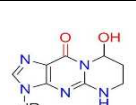
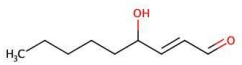

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691 Tables

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- <i>a</i>]purin-10(3 <i>H</i>)-one-deoxyguanosine	Reduced MDAdG	
Glyoxal	Gx		3-(2'-deoxy-β-D-erythro-pentofuranosyl)-5,6,7-trihydro-6,7-dihydroxyimidazo[1,2- <i>a</i>]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		N²-ethyl-deoxyguanosine	Reduced AAdG	
Crotonaldehyde / 2 x acetaldehyde	Croto / 2 x AA		α-R/S-methyl-γ-hydroxy-1,N²-propano- deoxyguanosine	CrotodG	
Acrolein	Acro		α-R/S-hydroxy-1,N²-propano-deoxyguanosine γ-hydroxy-1,N²-propano-deoxyguanosine	AcrodG	
4-hydroxy-2- nonenal	HNE		4-hydroxy-2-nonenal-deoxyguanosine	HNEdG	

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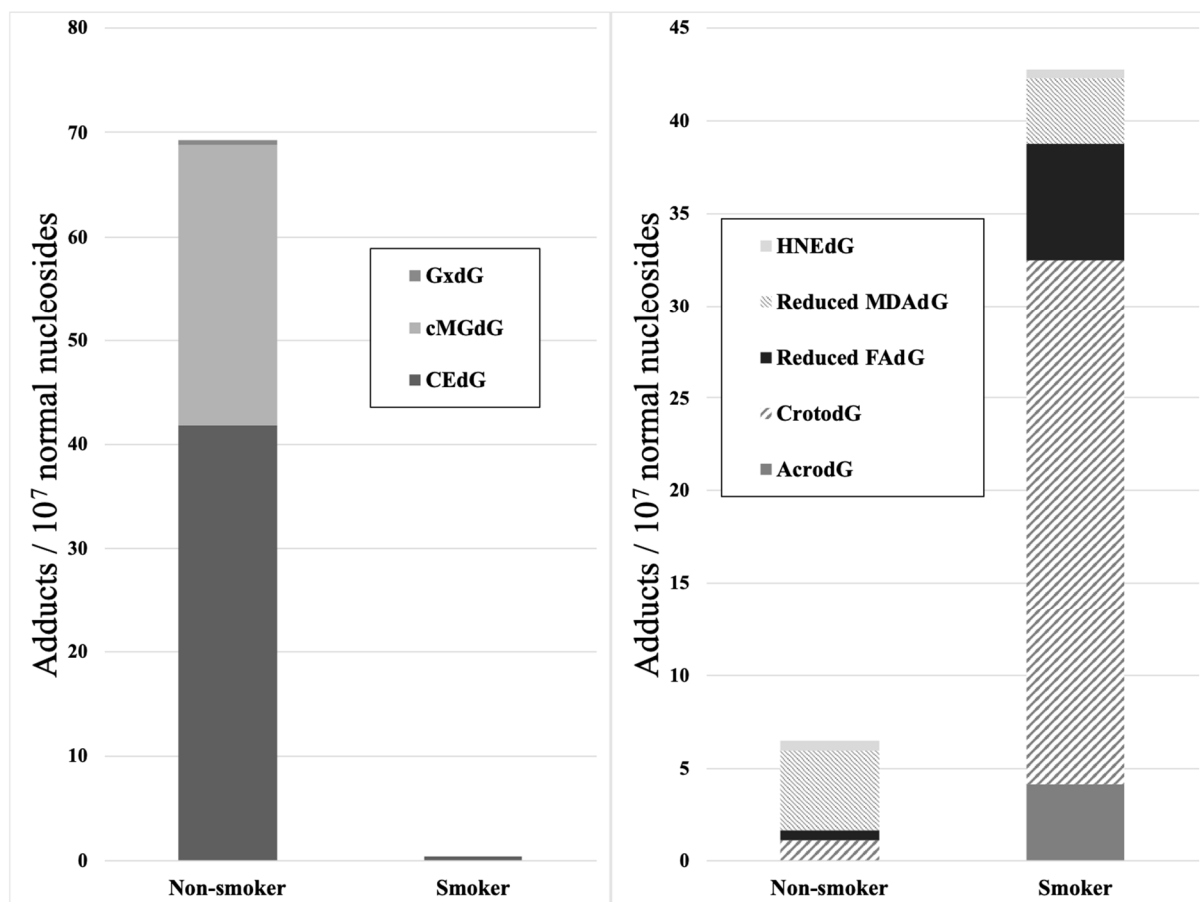
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695 **Table 2:** Mass transition and optimized MRM parameters for each DNA adduct

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		

697 **Figures**

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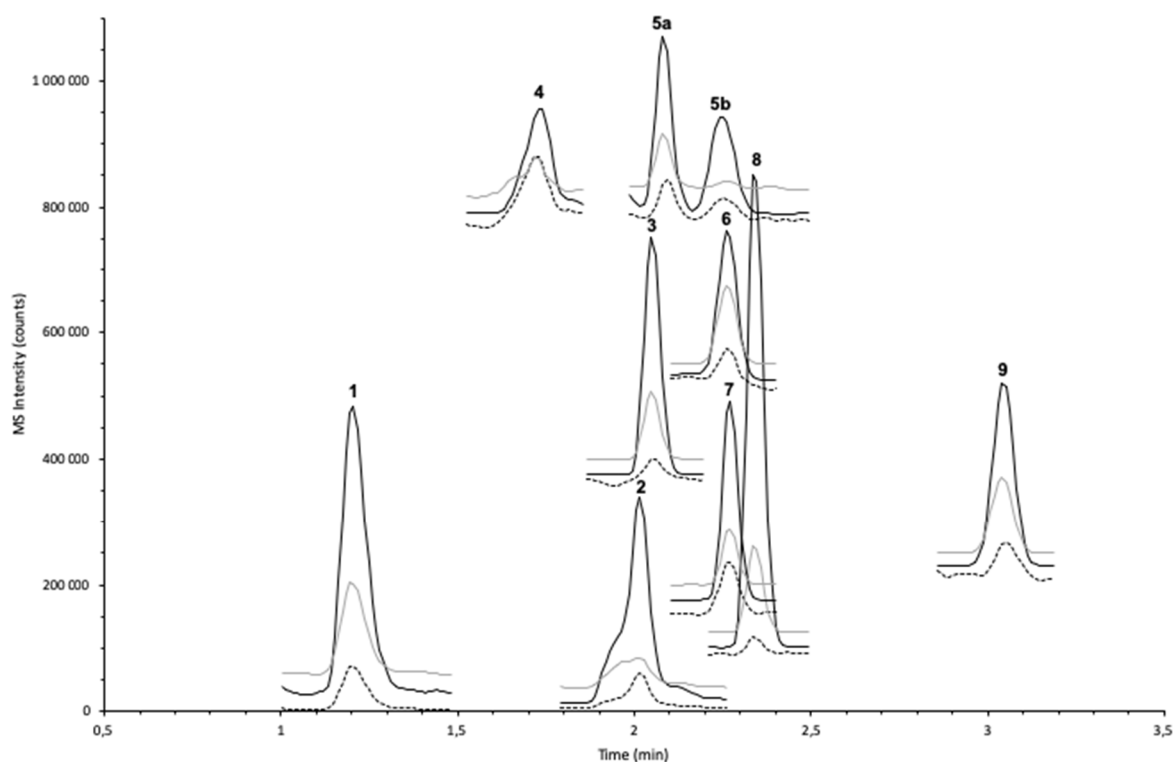


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701 **Figure 1:** DNA adducts levels pattern of genomic DNA isolated from both a smoker and a
702 non-smoker blood samples. The levels of adducts detectable but lower than the LLOQ
703 were arbitrary set to 0.5 adduct / 10⁷ normal nucleosides.

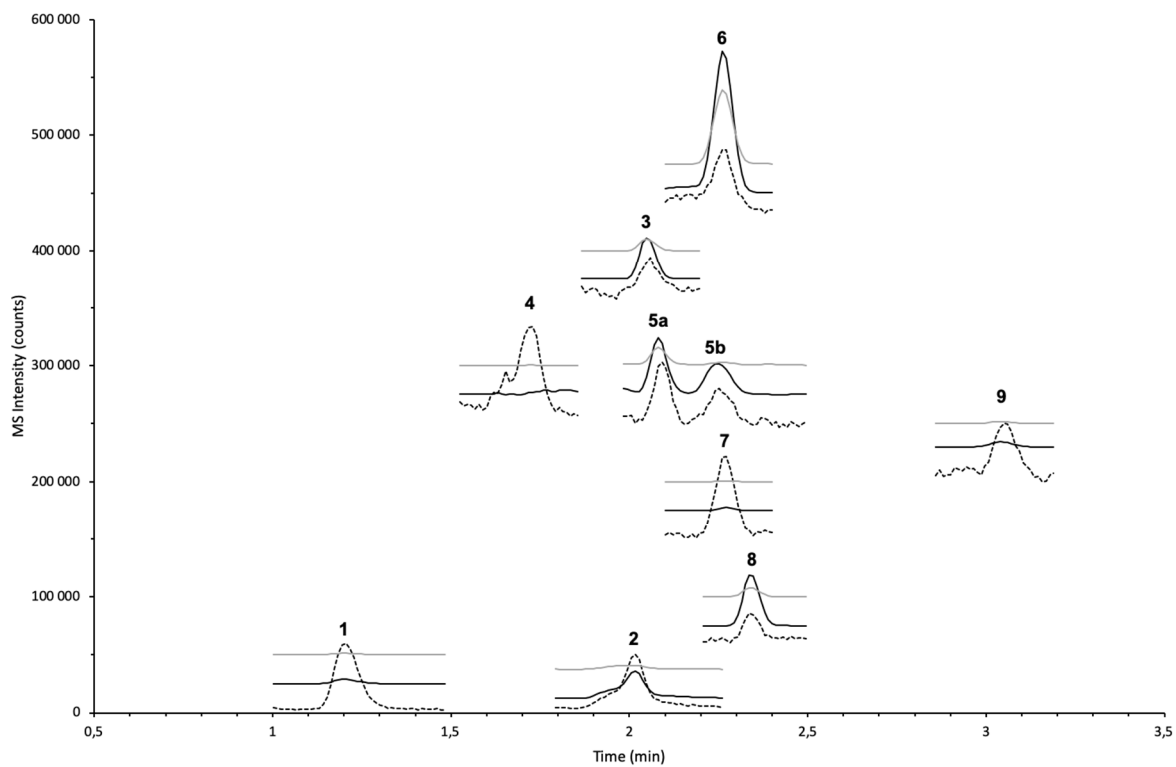
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707 **Figure 2:** Chromatogram of a DNA calibrator at 10 ng/mL. For each peak, the dotted lines
708 correspond to the $^{15}\text{N}_5, ^{13}\text{C}_{10}$ internal standards, the black and the grey lines correspond to
709 the first and the second 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.
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714 **Figure 3:** Chromatogram of a DNA adducts from a smoker's blood. For each peak, the

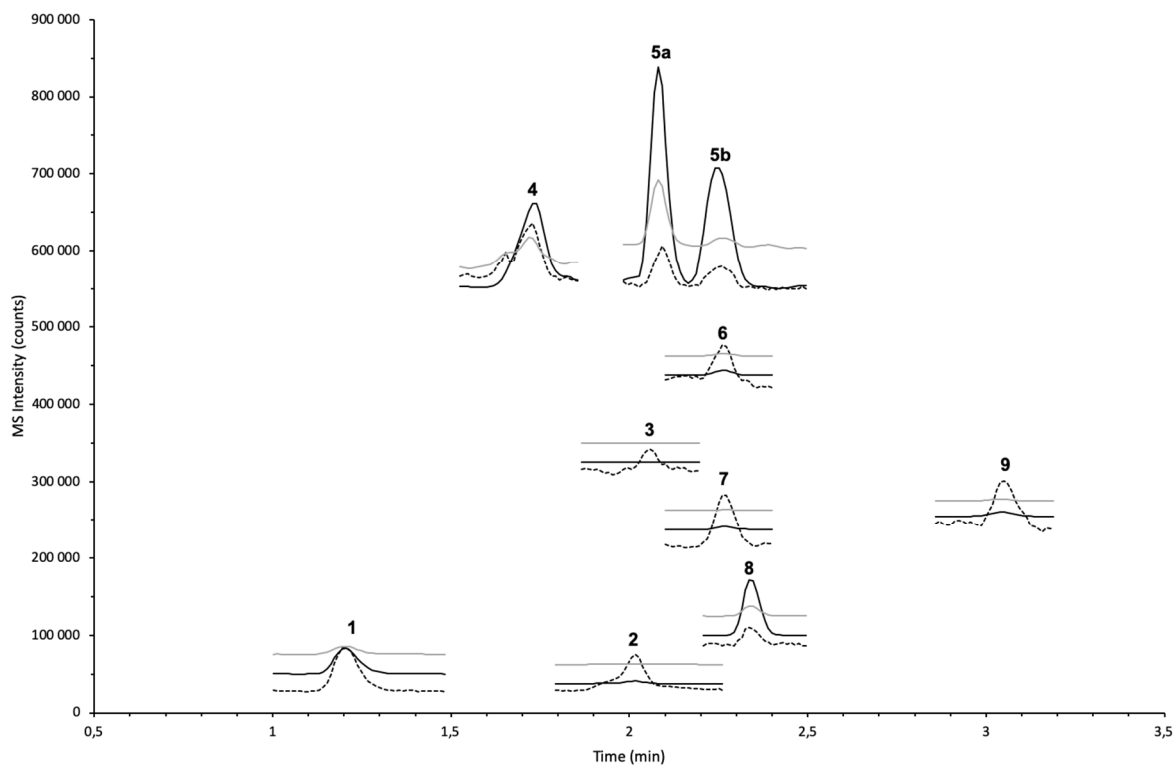
715 dotted lines correspond to the $^{15}\text{N}_5, ^{13}\text{C}_{10}$ internal standards, the black and the grey lines

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

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

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

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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 $^{15}\text{N}_5, ^{13}\text{C}_{10}$ 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.

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