Development of an untargeted DNA adductomics method by ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry

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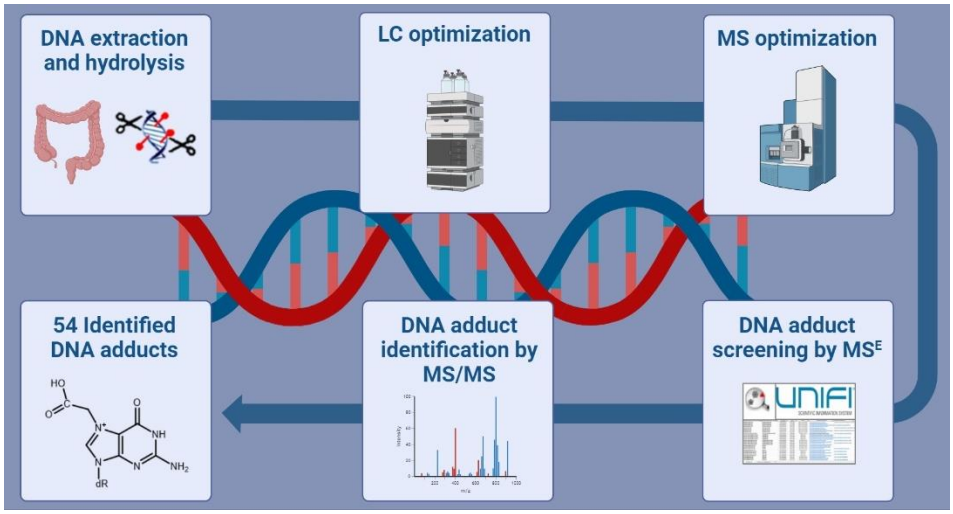
■ ABSTRACT

Background: Genotoxicants originating from inflammation, diet, and environment can covalently modify DNA, possibly initiating the process of carcinogenesis. DNA adducts have been known for long, but the old methods allowed to target only a few known DNA adducts at a time, not providing a global picture of the "DNA adductome". DNA adductomics is a new research field, aiming to screen for unknown DNA adducts by high resolution mass spectrometry (HRMS). However, DNA adductomics presents several analytical challenges such as the need for high sensitivity and for the development of effective screening approaches to identify novel DNA adducts.

Results: In this work, a sensitive untargeted DNA adductomics method was developed by using ultra-high performance liquid chromatography (UHPLC) coupled via an ESI source to a quadrupole-time of flight mass spectrometric instrumentation. Mobile phases with ammonium bicarbonate gave the best signal enhancement. The MS capillary voltage, cone voltage, and detector voltage had most effect on the response of the DNA adducts. A low adsorption vial was selected for reducing analyte loss. Hybrid surface-coated analytical columns were tested for reducing adsorption of the DNA adducts. The optimized method was applied to analyse DNA adducts in calf thymus, cat colon, and human colon DNA by performing a MS^E acquisition (all-ion fragmentation acquisition) and screening for the loss of deoxyribose and the nucleobase fragment ions. Fifty-four DNA adducts were tentatively identified, hereof 38 never reported before.

Significance: This is the first untargeted DNA adductomics study on human colon tissue, and one of the few untargeted DNA adductomics studies in the literature reporting the identification of such a high number of unknowns. This demonstrates promising results for the application of this sensitive method in future human studies for investigating novel potential cancer-causing factors.



■ INTRODUCTION

DNA adductomics, a new -omics science exploring the modifications of DNA from endogenous or exogenous genotoxicants, has developed over the past few years [1]. The exposure of human DNA to genotoxic chemicals induces the formation of covalent DNA adducts which, if not repaired, can lead to gene mutations, ultimately increasing the risk of cancer [2]. The measurement of DNA adducts is of fundamental importance in assessing the potential effect of exposure to endogenous and exogenous carcinogens from inflammation, diet, and environment – and in understanding their mechanisms of action[1].

Several analytical methods, such as immunochemical methods, ^{32}P-postlabeling techniques, and LC-MS, have been used for DNA adduct analysis, and LC-MS is now considered to be the gold standard technique. However, older MS instruments are limited in terms of both sensitivity and selectivity allowing only monitoring of targeted DNA adducts, not providing a comprehensive profiling of the "DNA adductome" [3]. With modern high resolution mass spectrometry (HRMS), providing high sensitivity and accurate compound mass, DNA adducts can be identified with high confidence. HRMS, together with ultra-high performance (UHP)LC, have opened new horizons in the screening of unknown DNA adducts and led to the development of this new research field [4].

DNA adductomics by UHPLC-HRMS presents new challenges, demanding new sample preparation protocols, chromatographic methods, data acquisition, and data analysis approaches. The major analytical challenge in DNA adductomics is still the need for high sensitivity and selectivity, as DNA adducts are present at trace levels and in a very complex matrix [3,4]. Indeed, while selective extraction of the analytes of interest can be performed by targeted methods, this is not possible for untargeted analyses, resulting in severe matrix effects and reduced sensitivity. Another challenge in DNA adductomics is the screening and identification of both known and novel DNA adducts. Whereas traditional -omics sciences rely on extensive existing databases, spectral libraries, and software support, DNA adductomics is in a developmental phase. Recently, a DNA adduct database [5], a mass spectral library [6], and new software [7–10] have been developed for DNA adduct profiling. A few studies reported the development of untargeted DNA adductomics strategies for identification of unknowns [9,11–13].

In this present work, we focus on screening for unknown DNA adducts, primarily to initiate studies related to colorectal cancer (CRC). Previous analyses of colon tissues with older techniques reported DNA adducts coming from red meat intake, alcohol intake, and smoking [14]. Although the studies showed promising results, the association of CRC with these exposures is only partially understood[15], and more advanced methods for profiling the colon epithelial DNA adductome would add new insight.

We report here a sensitive UHPLC-HRMS based method for this purpose. The chromatographic and mass spectrometric conditions of a UHPLC coupled to a quadrupole-time of flight MS (Vion-qTOF) via an ESI source were optimized by using a mixture of DNA adduct reference standards in order to increase the sensitivity of the instrumental analysis. In addition, we implemented an MS^E acquisition (all-ion fragmentation acquisition) for the untargeted screening of DNA adducts. Finally, we applied the developed method for the analysis of DNA adducts in calf thymus, cat colon, and human colon DNA.

■ MATERIALS AND METHODS

Chemicals and materials

Milli-Q ultra-pure water (Merck Life Sciences, Søborg, Denmark), methanol optima LC-MS grade from Thermo Fisher Scientific (Waltham, MA), and formic acid, acetic acid, ammonium acetate, ammonium formate, and ammonium bicarbonate from Merck (St. Louis, MO) were used for the UHPLC analysis.

The following DNA adducts and nucleosides, as reference standards, were purchased from Toronto Research Chemicals: 2′-deoxyadenosine (dA); 2'-deoxyguanosine (dG); thymidine (dT); 2'-deoxyuridine (dU); 2'-deoxy-N^6-methyladenosine (N^6-Me-dA); 5-methyl-2'-deoxycytidine (5-Me-dC); O^6-methyl-2'-deoxyguanosine (O^6-Me-dG); 2'-deoxy-N3-methyluridine (3-Me-dU); 3-methylthymidine (3-Me-dT); N^4,5-dimethyldeoxycytidine (N^4,5-DiMe-dC); N^2-ethyl-2′-deoxyguanosine (N^2-Et-dG); N^6-(2-hydroxyethyl)-2'-deoxyadenosine (N^6-(2-OH-Et)-dA); 8-oxo-2'deoxyguanosine (8-Oxo-dG); etheno-2'-

deoxy-β-D-adenosine (1,N^6-ε-dA); 3,N^4-etheno-2'-deoxycytidine (3,N^4-ε-dC); 3-(2-deoxy-β-D-erythro-pentofuranosyl)pyrimido[1,2-a]purin-10(3H)-one (M1-dG); 3-(2-Deoxy-β-D-erythro-pentofuranosyl)-3,5-dihydropyrimido[1,2-a]purine-6,10-dione (6-Oxo-M1-dG); γ-Hydroxy-1,N2-propano-2'-deoxyguanosine (Acr-1I-dG); α-Methyl-γ-hydroxy-1,N2-propano-2'-deoxyguanosine (mixture of diastereomers) (Cro-1I-dG) (Cro-1II-dG); N-(2'-deoxyguanosin-8-yl)-4-aminobiphenyl (8-(N'-ABP)-dG); and N2-(deoxyguanosin-8-yl)-2-amino-3,8-dimethylimidazo[4,5-f] quinoxaline (8-(N'-MeIQx)-dG). The following nucleoside reference standards were purchased from Merck**:** adenosine, deoxycytidine (dC), uridine. Stock solutions of the DNA adduct standards were dissolved at 1 or 0.5 mg mL^{-1} in methanol, or a mixture of water and methanol. A stock solution was prepared containing all the standards at 20 µg mL^{-1}. The working solutions were diluted with water to concentrations ranging from 100 ng mL^{-1} to 1 pg mL^{-1}.

The following products for DNA extraction and hydrolysis were purchased from Merck: Ribonuclease A from bovine pancreas for molecular biology; Proteinase K from *Tritirachium album* BioUltra, for molecular biology; Sodium dodecyl sulfate (SDS) BioUltra, for molecular biology; Phenol:chloroform:isoamyl alcohol mixture (25:24:1, *v/v/v*) BioUltra, for molecular biology; Deoxyribonucleic acid sodium salt from calf thymus, Type I; Deoxyribonuclease I from bovine pancreas, Type IV (DNaseI); Phosphodiesterase I from *Crotalus atrox* (Western Diamondback Rattlesnake), Type IV (PDEI); Alkaline Phosphatase from bovine intestinal mucosa (AP); Tris(hydroxymethyl)aminomethane hydrochloride (Tris HCl); ethylenediaminetetraacetic acid (EDTA); sodium chloride (NaCl); and magnesium chloride hexahydrate $MgCl_2$ $6H_2O$. Ethanol was purchased from Thermo Fisher Scientific (Waltham, MA).

DNA from calf thymus

DNA from calf thymus was hydrolyzed before analysis. In brief, 0.5 mg of DNA was dissolved in 1 mL of incubation buffer (10mM Tris-HCl and 5 mM $MgCl_2$, adjusted at pH 7). Five hundred units of DNaseI were added and the sample was incubated overnight in a mixer at 37°C. The next day, an additional 500 units of DNaseI were added to the sample, together with 0.01 units of PDEI and 100 units of AP. The sample was incubated overnight in a mixer at 37°C. The next day, two volumes of cold methanol were added to the sample to precipitate the proteins. The supernatant was collected and evaporated. The sample was dissolved with a total of 300 µL of H_2O:MeOH (90:10, *v/v*) and transferred to an injection vial.

DNA from cat colon

A colon sample was obtained from a cat that was euthanized at the owner's request at the University Hospital for Companion Animals, University of Copenhagen. The owner signed an informed consent allowing that the cat be used for teaching and research purposes following euthanasia. The colon epithelial tissue was scraped off the resected colon and 0.5 g was ground in liquid nitrogen. The sample was dissolved in 2.5 mL of the DNA digestion buffer (50 mM TRIS HCl, 10 mM EDTA and 100 mM NaCl, adjusted at pH 8). SDS 300 µL 10%, and 250 units of proteinase K were added to the sample and incubated overnight at 37°C. The next day, 250 units of RNase were added and incubated for two hours at 37°C. DNA was extracted from the sample with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1, *v/v/v*) and mixed by inversion. After centrifugation at 1600 g for 10 min, the upper phase was collected. Two volumes of cold EtOH were added, and the vial was inverted for DNA precipitation. After centrifugation at 1600 g for 10 min, the supernatant was discarded and the DNA was washed with 3 mL of EtOH:H_2O (70:30, *v/v*). The sample was inverted again, followed by centrifugation at 1600 g for 5 min. The supernatant was discarded, and the sample was air dried at room temperature for 10-15 min. The DNA was dissolved in 10mM Tris-HCl, 5 mM $MgCl_2$, adjusted to pH 7, and left overnight at 4°C. The following day, the DNA-extraction yield (0.89 mg) and purity ($OD_{260/280}$=1.87 $OD_{260/230}$=2.12) were assessed by UV spectroscopy using a Nanodrop spectrophotometer. The equivalent of 0.5 mg of DNA was used for the subsequent DNA-hydrolysis procedure, as described for DNA from calf thymus.

DNA from human colon

Human colonic tissue was obtained from 15 patients recruited at Hvidovre Hospital in Copenhagen (Denmark) from January to November 2021. Patients with hereditary colorectal cancer, sporadic colorectal cancer, ulcerative colitis, and other conditions, who were scheduled for whole or partial resection of their colon were included in the study. The research protocol was approved by the Municipal Ethics Committee of the Capital Region of Denmark (H-19045171) and all participants provided written informed consent to participation. The colon tissue was collected at Hvidovre Hospital and non-tumorous tissue was transferred to the Department of Nutrition, Exercise and Sports (NEXS) at the University of Copenhagen for further analysis. Approximately 0.25-0.50 g of colon tissues were ground in liquid nitrogen. The procedure for DNA extraction was the same as for cat colon tissues. The DNA-extraction yield (0.254-1.899 mg) and purity ($OD_{260/280}$ = 1.7-1.98, $OD_{260/230}$ =1.91-2.25) were assessed by UV. The equivalent of 0.25 mg of DNA was used for the subsequent DNA-hydrolysis procedure. DNA was dissolved in 1.3 mL of incubation buffer (10mM Tris-HCl and 5 mM $MgCl_2$, adjusted at pH 7). Two hundred fifty units of DNaseI were added and the sample was incubated overnight in a mixer at 37°C. The next day, an additional 250 units of DNaseI were added to the sample, together with 0.004 units of PDEI and 50 units of AP. The sample was incubated overnight in a mixer at 37°C. The next day, two volumes of cold methanol were added to the sample to precipitate the proteins. The supernatant was collected and evaporated. The sample was dissolved with a total of 200 µL of H_2O:MeOH (90:10, *v/v*). A global pool was made by mixing 5 µL of each sample.

UHPLC-HRMS method optimization

The analysis was performed on an H class Acquity UHPLC coupled to a Vion-IMS-qTOF (Waters, Milford, MA) via an ESI source. The UHPLC system was equipped with a quaternary pump and an autosampler thermostated at 10°C. A C18 HSS T3 column (100 × 2.1 mm, 1.8 µm particle size) (Waters) was used at a flow of 0.4 mL min^{-1} at 50°C.

Different mobile phases were compared to improve chromatographic separation and sensitivity, and to decrease in-source fragmentation and adduct formation. Details of the mobile phase comparison experiment are provided in the Supplementary Material (Table S1). The best chromatographic condition used 10 mM NH_4HCO_3 as mobile phase A, and MeOH with 10 mM NH_4HCO_3 as mobile phase B. Several MS spectrometric parameters and acquisitions were evaluated to optimize the sensitivity and to decrease the in-source fragmentation of DNA adducts. Different values of capillary voltage, sampling cone voltage, source temperature, desolvation temperature, desolvation gas, collision energy, mass range, profile or automatic mode in the quadrupole isolation, and detector voltage were tested as summarized in the Supplementary Material (Table S2). The optimal tuning parameters of the Vion-IMS-qTOF were: capillary voltage 0.5 kV; sampling cone voltage 20 V; source temperature 110 °C; desolvation temperature 600 °C; desolvation gas 800 (L/h); collision energy 6 eV; cone gas 50 (L/h). The detector voltage was set to 3000V. The Vion-IMS-Q-TOF was operated in MS^E acquisition mode and samples were acquired in positive polarity mode. For both the low and high energy trace, the scan range was 50−1000 m/z and the scan time 0.4 s. For the high energy trace, optimization of the collision energy was performed to obtain an informative fragmentation pattern, finally choosing a mass energy ramp ranging from 20 to 50 eV.

Finally, in order to reduce eventual adsorption processes of DNA adducts, low adsorption injection vials and low adsorption columns were tested by analysing the mix of DNA adduct reference standards at concentrations between 1 and 100 ng mL^{-1}. Five different injection vials - LC-MS certified clear glass Vial, TruView Vial and Quan Recovery Max Peak (all Waters), Low Adsorption Vial (Supelco, Merck, St. Louis, MO), and Reduced Surface Activity RSA-Pro Vial (Microsolv, Greater Wilmington, NC) - were compared by performing 14 repeated injections over a period of 24 hrs. The Low Adsorption Vial gave the least adsorption and the highest stability of the signal over time and was chosen for the subsequent column comparison. A hybrid surface coated, low adsorption Premier C18 HSS T3 column (100 × 2.1 mm, 1.8 µm particle size) (Waters) was compared with the HSS T3 column used in previous experiments and a hybrid surface coated low adsorption Premier C18 BEH (ethylene bridge hybrid) column (100 × 2.1 mm, 1.8 µm particle size) (Waters) was compared with a regular C18 BEH column (100 × 2.1 mm, 1.8 µm particle size)

(Waters). The latter provided the best results and was used for analysis of real samples.

The final optimized conditions were used for the analyses of the hydrolyzed DNA from calf thymus, cat colon and human colon, using the following gradient: 0-1 min (5% B), 1−21 min (0−99% B), followed by a 2 min wash at 99% B and 2 min equilibration at 5% B. The mass spectrometer was periodically cleaned, and externally calibrated every 2 weeks using the calibration solution Major Mix (Waters). Lock mass correction was applied continuously during the runs by infusing 15 µL min^{-1} of 100 ng mL^{-1} leucine-enkephalin (Waters) for 0.5s every 5 minutes. Three technical replicates were performed for each condition evaluated. For the evaluation of the chromatographic conditions, the three replicates were run after washing with the mobile phase for 1 hour and running two blanks for assuring column conditioning. Each different additive was evaluated at increasing concentrations on the same day. To avoid instrumental variability, the best concentration of each additive was chosen and compared with the others on the same day. To minimize column passivation in the evaluation of the mass spectrometric conditions and for vial comparisons, a highly concentrated DNA adduct mix was injected continuously over 4 hours before the runs. The different chromatographic columns were compared on the same day. Injection volume for all analyses was 5 µL.

Data analysis and DNA adduct identification

Raw data files obtained for the optimization of the chromatographic and mass spectrometric parameters were acquired by UNIFI software (version 1.9.4.053) (Waters), and transformed into .mzML format using the MSconvert tool (version 3.0.19199) (https://proteowizard.sourceforge.io/projects.html) [16]. The converted files were then imported into MZmine (version 2.53) (http://mzmine.github.io) for further analysis [17]. Values such as peak area, full width at half maximum (FWHM), and the asymmetry factor, were extrapolated from the analyzed chromatographic runs. Briefly, the Targeted Peak Detection module was used for integrating the peaks related to the adducts and in-source fragments of the DNA adduct standards. Peak integration was checked and manually corrected when necessary. The RANSAC alignment was used for aligning the chromatographic runs acquired under the same chromatographic conditions. The Join Aligner was used for aligning runs acquired with different chromatographic conditions, so retention time could be omitted from the alignment. Parameters used for data analysis in MZmine are reported in the Supplementary Material (Table S3). Retention time, peak area of every single adduct, and ratio of $[M+H]^+$ over any other adduct or in-source fragment, FWHM, asymmetry factor, peak capacity, and resolution were extrapolated as reported elsewhere [18], and used for the comparison. See Supplementary Material S3 for details on the calculations.

Raw data files related to the analysis of DNA from calf thymus, cat colon, and human colon were acquired, pre-processed and analyzed by UNIFI software (version 1.9.4.053) (Waters) in four steps. The first step was uploading into UNIFI a comprehensive database created by La Barbera *et al*. [5], and matching the features extracted in MS1. If the features matched an entry in the database, they were referred to as known, and otherwise as unknown.

The second step consisted in a search of the loss of -dR within the low energy trace (MS1). In particular, $[M-dR+H]^+$ was included in the list of possible adducts, even though it is formally an in-source fragment. The features reporting both $[M-dR+H]^+$ and $[M+H]^+$ or $[M+Na]^+$ or $[M+K]^+$ were manually investigated by checking the extracted ion chromatogram (XIC) and were validated if the chromatographic peaks were aligned.

The third step consisted in a search for the ion fragments related to the unmodified nucleobases adenine, cytosine, guanine, thymine, and uracil in the high energy trace (MS2). The known features were manually investigated in the XIC if they reported a nucleobase fragment that matched with their identity, e.g. ethyl-dA was manually investigated if it showed adenine as a fragment. For the unknown features, only the features with intensity higher than 1E4 reporting the nucleobase fragments were manually investigated. The features were validated if the chromatographic peaks of the precursor and fragments were aligned.

The final and fourth step was running a targeted MS2 acquisition with a collision energy ramp of 20-50 eV of the features selected in step 2 and 3. The

fragmentation spectra of the selected compounds were manually investigated and matched, when possible, with the spectral library produced by Villalta *et al.* [6], with MzCloud spectral library, or with the *in-silico* predicted fragments reported in the DNA adduct database [5]. The parameters for the data analysis with UNIFI are reported in the Supplementary Material (Table S4). Identification confidence levels were associated with the DNA adducts based on the work of Schymanski *et al.* [19] and the Metabolomics Standards Initiative [20]: level 1) if the compounds matched the retention time and MS2 spectra of a reference standard; level 2) if the compounds matched with the MS2 spectra reported in a spectral library; level 3) if the compounds showed the typical fragmentation pattern of a DNA adduct (i.e. loss of -dR and nucleobase fragment).

■ RESULTS AND DISCUSSION

In recent years, UHPLC-HRMS has become the gold standard for DNA adduct analysis, especially because it provides reliable identification of DNA adducts and can perform untargeted DNA adductomics [4]. Measurements of as little as 1 DNA adduct in 10^{10} unmodified nucleotides have been accomplished by targeted methods [21], but untargeted methods do not reach similar levels of sensitivity [22]. To develop a sensitive untargeted method, we therefore optimized several chromatographic and mass spectrometric parameters. The formation of adducts such as $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[2M+H]^+$, $[2M+Na]^+$, and high levels of the in-source fragment $[M\text{-}dR+H]^+$, was detected from 25 DNA adducts. The aim of this study was therefore to improve the response of the DNA adducts by increasing $[M+H]^+$, while simultaneously decreasing the other adducts and the $[M\text{-}dR+H]^+$ fragment.

Optimization of Chromatographic Conditions

Several mobile phases have been used in the past for the analysis of DNA adducts. These include water (H_2O) as mobile phase A, and methanol (MeOH) or acetonitrile (ACN) as mobile phase B, both phases either with or without the addition of acetic acid (CH_3COOH), formic acid (HCOOH), or ammonium acetate (CH_3COONH_4) [12,22–24]. However, to the best of our knowledge, there is no study showing a comprehensive comparison of the commonly used chromatographic conditions for the analysis of different classes of DNA adducts. Only one study reports the comparison of ammonium acetate, formate ($HCOONH_4$) and bicarbonate (NH_4HCO_3) for the analysis of 4 acetaldehyde DNA adducts, showing the last to be the most promising additive [25]. This was confirmed in another study, where HILIC was used for detecting 2 acrolein DNA adducts [26]. Based on this, we tested chromatographic conditions using different concentrations of HCOOH, CH_3COOH, CH_3COONH_4, $HCOONH_4$ and NH_4HCO_3, in the mobile phases (Table S1). ACN as phase B was excluded because MeOH showed much higher ionization efficiency in preliminary experiments. The mobile phase evaluation was carried out only in positive polarity mode, as most of the DNA adducts showed better ionization in positive mode. Each additive was evaluated at different concentrations and then the best concentration of each additive was compared with the others. Although the intensities of $[M+H]^+$ and $[M\text{-}dR+H]^+$ were changed by using different mobile phases, the ratio between $[M+H]^+$ and $[M\text{-}dR+H]^+$ was almost constant, and was therefore not the discriminant parameter for the mobile phase evaluation. Increasing HCOOH concentration lowered the intensity of $[M+H]^+$ and [M-dR+H] and raised the intensity of $[M+Na]^+$ for most compounds, so a concentration of 0.05% HCOOH was chosen for further comparisons (Figure S1). There was no particular trend with the concentration of CH_3COOH but the highest signal was obtained at 0.3% (Figure S2), which also showed less $[M+Na]^+$, $[M+K]^+$, $[2M+H]^+$ and $[2M+Na]^+$, and therefore was chosen as the best condition. The area of $[M+H]^+$ was increased by decreasing the concentration of CH_3COONH_4 for all compounds other than thymine, Me-dC, and M1-dG (Figure S3). An increase in the formation of $[M+C_2H_6N_2+H]^+$, possibly due to a contaminant such as acetamidine [27], was detected for some of the compounds. Therefore, 0.5 mM CH_3COONH_4 was chosen as the best condition. In the case of $HCOONH_4$, most of the compounds reached a maximum signal at 5mM $HCOONH_4$ (Figure S4), which was chosen as the best condition. Finally, NH_4HCO_3 showed very heterogeneous behavior across all compounds (Figure S5). Therefore, 10mM NH_4HCO_3 was chosen, giving

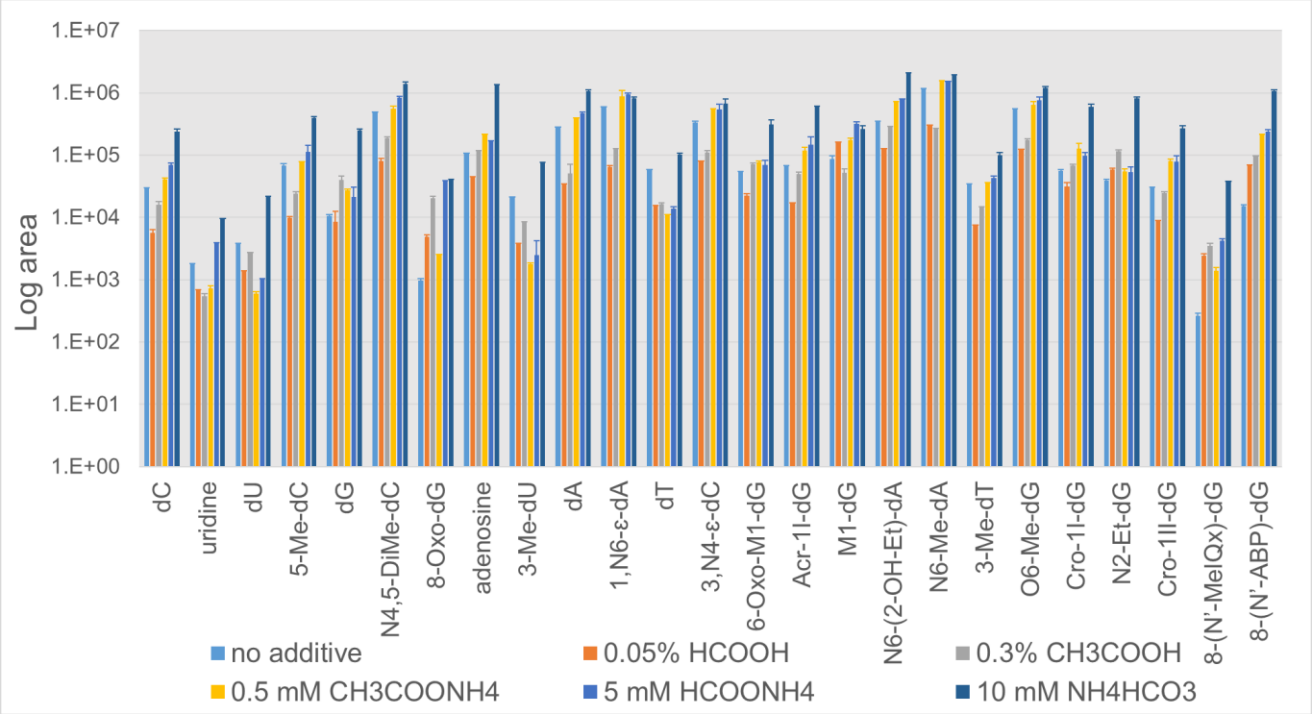


Figure 1: Log of the area of the most abundant adduct ion of the DNA adduct standards, analyzed using the following additives in the mobile phases: no additive, 0.05% HCOOH, 0.3% CH_3COOH, 5mM CH_3COONH_4, 5mM $HCOONH_4$, and 10mM NH_4HCO_3

the highest average signal among the DNA adducts. In conclusion, 0.05% HCOOH, 0.3% CH_3COOH, 5mM CH_3COONH_4, 5mM $HCOONH_4$, and 10mM NH_4HCO_3 were chosen for further comparison.

As shown in Figure 1, the best mobile phase for the majority of the DNA adducts was 10mM NH_4HCO_3, showing an increase of up to 2 orders of magnitude compared to the other conditions. This was mainly due to the ability of NH_4HCO_3 to suppress the formation of $[M+Na]^+$ and $[M+K]^+$ to a much greater extent than the other additives. Both the number of DNA adducts forming Na^+ and K^+ adducts and the area of the Na^+ and K^+ ions were lower than in the other conditions (Figures S1-S5). Under almost all conditions, uridine, dU and 8-Oxo-dG, together with 3-Me-dU, dT, and 3-Me-dT, showed only $[M+Na]^+$ and very little $[M+H]^+$, due to their acidic properties [28,29]. Nucleosides that can exist in multiple tautomeric hydroxy forms, such as thymine, uracil, and 8-oxo-dG, easily lose their proton. Uracil- and thymine-derived adducts also showed a better response in negative polarity (data not shown), suggesting the need for separate acquisition and optimization in future work. The chromatographic parameters did not affect performance for any of the mobile phases. All tested conditions gave optimal retention, peak shape, resolution, and reproducibility. (Figures S6, S7, S8, Table S5).

In order to further reduce the formation of $[M+Na]^+$ or $[M+K]^+$, a final comparison was performed using different mobile phase bottles, i.e. common glass bottles vs low density polyethylene (LDPE) bottles (Waters). A slight reduction of $[M+Na]^+$ and a clear reduction of $[M+K]^+$ was found for most of the adducts when using the LDPE bottles. However, this did not improve the sensitivity of the method as the intensity of the $[M+H]^+$ remained constant (Figure S9).

Optimization of Mass Spectrometric Conditions

Once the best chromatographic condition was chosen, a comprehensive mass spectrometric optimization was carried out to increase the intensity of the signal and reduce the loss of -dR from the DNA adducts. The parameters investigated included ESI related parameters (capillary and sampling cone voltage, source temperature, desolvation gas flow and temperature), collision energies (the minimum required to ensure the transmission of the ions in qTOF), acquisition mode related parameters (mass range, the use of automatic or manual profile mode, i.e. automatically or manually optimizing the quadrupole parameters), and detector parameters (detector

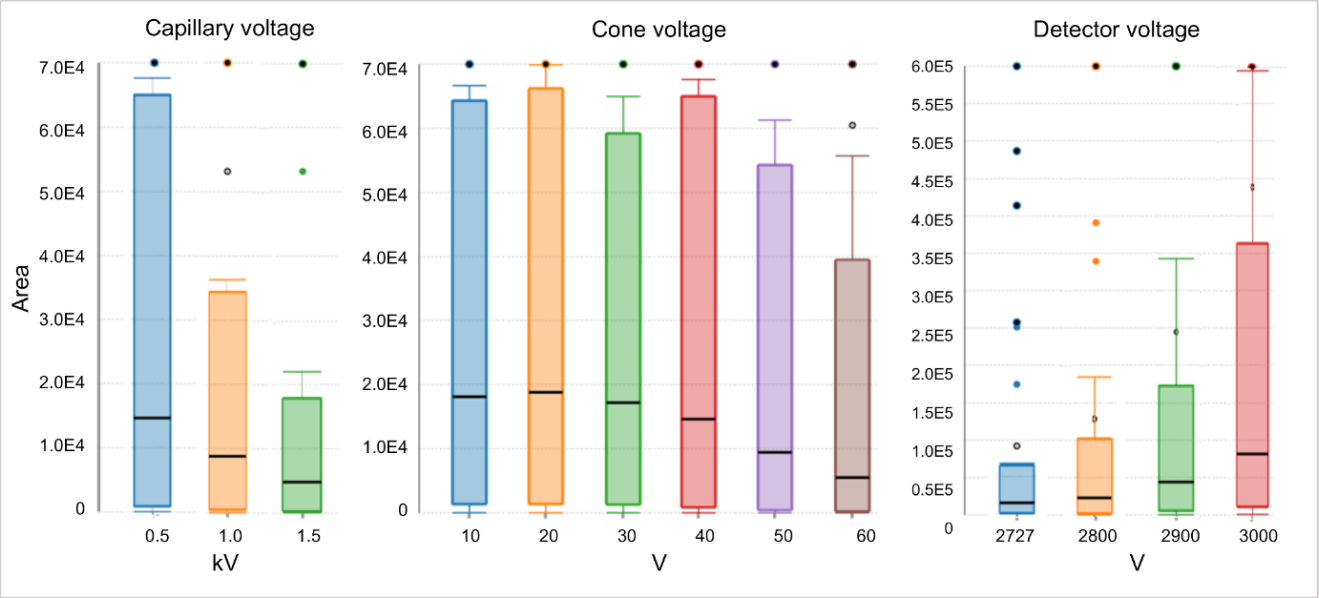


Figure 2: Box-and-Whisker plots showing the ditribution of the peak area of the DNA adduct standards at different values of capillary, sampling cone, and detector voltage (Graph created in www.goodcalculators.com).

voltage). The results of the mass spectrometric optimization are shown in terms of both the intensity of the most abundant ion for each DNA adduct, and the ratio between that ion and the DNA adduct after the loss of –dR (Figure S10). Increasing the capillary and sampling cone voltages resulted in a remarkable decrease of the signal and increase of the loss of -dR. A minimum value was therefore chosen for the capillary voltage, and a value of 20 eV was chosen for the cone voltage. An increase in the signal was seen when the desolvation gas temperature was increased. The mass range did not affect the signal greatly, and an automatic profile was better than manual. Changing the source temperature, the desolvation gas flow, and the collision energy did not produce any particular improvement. The signal was strongly affected by the detector voltage, which was optimal at 3000 V and increased the signal by up to 10 times. In conclusion, minimizing capillary and sampling cone voltages, and raising detector voltage within the range allowed by the instrument caused the most remarkable increase in the signal (Figure 2). A partial reduction of -dR loss was achieved by reducing capillary and sampling cone voltages (Figure S10).

Reduction of adsorption processes

Many other factors, besides the ionization efficiency in the source, can affect the sensitivity of the method. For compounds present in traces such as DNA adducts, adsorption processes can occur on several surfaces, such as the injection vials, the metal surfaces of the chromatographic system and the column. Several precautions were taken for controlling adsorption processes in the optimization process. However, the results often showed a discrete change in the intensity over continuous injection series. To explore this issue, 5 different injection vials and 4 different columns were evaluated. Four different low-adsorption injection vials were compared with a standard glass vial (LC-MS certified). The standard glass vial displayed the highest instability, with a clear signal decrease after the first hour for several of the DNA adducts (Figure S11). The RSA-Pro vial gave the highest signal for most of the compounds, but the signal tended to increase after the first hour, and then decrease after 4 hours of injections. The other three vials showed a quite stable signal over time, generally up to 16 hours. However, the Quan Recovery vial gave a much lower signal for M1-dG and O^6-Me-dG compared to the other vials, whereas the TruView vial showed a lower signal for 8-(N'-ABP)-dG and no signal for 8-(N'-MeIQx)-dG, suggesting that the TruView vial is not suitable for the analysis of bulky DNA adducts. In addition, the reproducibility was evaluated by comparing the relative standard deviation of the DNA adduct areas in three replicates at 14 different time points, showing the

Low Adsorption vial to be the most reproducible (Figure S12). In conclusion, the vial showing an acceptable behavior in terms of intensity, stability and reproducibility for DNA-adduct analysis is the Low Adsorption vial.

The chromatographic column chosen for developing this method, HSS T3, has recently been marketed as Premier HSS T3, where a hybrid organic-inorganic surface, based on an ethylene-bridged siloxane chemistry has been applied on the metal surfaces for reducing the adsorption of nucleotides [30]. In addition, a C18 BEH (ethylene bridge hybrid) column, which is resistant to higher pH, has also been marketed as Premier BEH by following the same principle. Since the basic pH of the mobile phase containing 10 mM NH_4HCO_3 (pH 9.4) could compromise the performance of silica-based columns such as HSS T3, we compared the HSS T3 *vs* HSS T3 Premier by using 10mM NH_4HCO_3 acidified at pH 7.4 as mobile phase, and the BEH *vs* BEH Premier by using 10mM NH_4HCO_3 (pH 9.4). When comparing the HSS T3 *vs* HSS T3 Premier at pH 7.4, some of the most acidic DNA adducts, such as uridine, 3-Me-dU, dU, and 8-Oxo-dG showed a remarkable improvement in the Premier HSS T3. This can be explained by a reduction of the adsorption of acidic negatively charged analytes on the metal oxide layer of the 'native' HSS T3, which is positively charged at pH≤7 [30] (Figure S13). An increase of the signal was shown also for 8-(N'-MeIQx)-dG and 8-(N'-ABP)-dG, but the mechanism of the interaction is unknown. When comparing the BEH vs BEH Premier at pH 9.4, no significant difference was shown, which can be explained by the fact that the metal oxide layer of the 'native' BEH does not interact with DNA adducts because it is not charged at pH≥7. Most of the DNA adducts showed larger area in the HSS T3 Premier compared to the BEH, however, the chromatographic separation was remarkably worse because of the mobile phase acidification (Figure S14). In conclusion, BEH with 10mM NH_4HCO_3 (pH 9.4) was chosen for further analysis. Other low adsorption non-silica based columns, designed for reducing the adsorption of more basic compounds, such as DNA adducts, should be investigated in future studies.

The current method allowed to detect DNA adduct standards in the range 0.2-522.5 fmol (Table S6), showing higher sensitivity compared to other untargeted methods [31] and reaching a LOD appropriate for the detection of the amount of DNA adducts commonly present in biological samples [32].

Acquisition mode and identification approach

Several approaches have been employed in the past for screening of DNA adducts, often by monitoring the loss of -dR and the unmodified nucleobase fragment ions. Early DNA adductomics primarily utilized triple quadrupole (QqQ) instrumentation to perform neutral loss screening [31], whereas more recent studies have taken advantage of HRMS [4], which allows performing different types of acquisition modes such as data dependent acquisition (DDA) - neutral loss triggered [22], wide selected ion monitoring tandem mass spectrometry (Wide-SIM/MS2) [33], and data independent acquisition (DIA) [9]. Whereas DDA selects specific precursor ions for fragmentation resulting in clean MS2 spectra, DIA fragments the entire range of ions, requiring elaborate data analysis software for the investigation of the spectra [9] and a supplementary MS2 targeted acquisition for confirming the identity of the compound. However, the selective approach of DDA carries the risk of losing the fragmentation of the least abundant compounds [4], which is an issue for compounds present as traces in complex biological matrices such as DNA adducts. To facilitate detection and fragmentation of a high number of features, MS^E acquisition, which is equivalent to a broadband DIA, otherwise known as all-ion fragmentation (AIF), was therefore chosen in this study. The data were screened for DNA adducts in a four-step approach (Figure 3). Firstly, the features were extracted from the raw data and matched with our comprehensive DNA adduct database. The features matching with the database were addressed as known since the database reports a collection of DNA adducts already found in previous studies [5]. The unknown features were set to a maximum of 10000. The second step was to monitor the in-source fragment $[M\text{-}dR+H]^+$ in the low energy trace (MS1) for both known and unknown features. Due to in-source fragmentation, a pseudo-MS^3 fragmentation was performed and a third step of screening introduced, monitoring the fragment

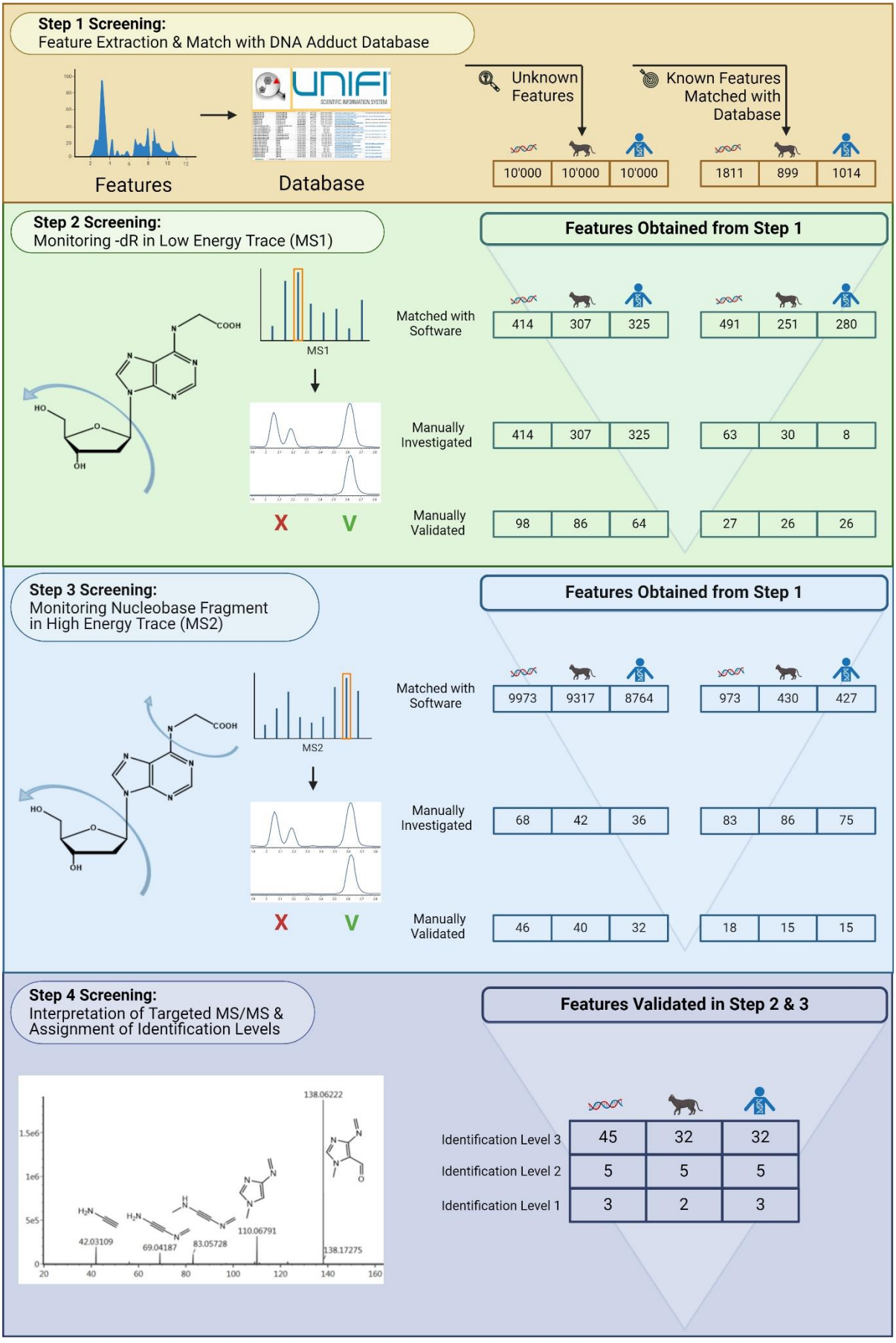


Figure 3: Summary of the selected features and tentatively identified DNA adducts in DNA from calf thymus, cat colon and human colon (Created with BioRender.com).

ions belonging to the unmodified nucleobase in the high energy trace (MS2). Since some DNA adducts did not show in-source fragmentation, features obtained from both step 2 and 3 were selected for further validation. We considered monitoring the loss of -dR in the high energy trace (MS2), but the software reported the loss for almost the totality of the features. These artifacts are due to the unselective fragmentation of the MS^E acquisition, and the inability of the used software to perform peak picking on both low and high energy trace before aligning fragments to precursors. This also affected the screening for the nucleobase fragments as shown by the high number of matches, but in this case, the *m/z* of the unmodified nucleobases could be easily extracted from the XIC and compared with the precursor ions by manual investigation. Manual investigation of the XIC was performed for both step 2 and 3 since it is necessary to avoid false positives. However, it is extensively time consuming and could be avoided by the development of more advanced software to reconstruct the parent-fragment ion relationships and obtain pseudo-MS/MS spectra from DIA acquisitions. The software currently available, such as MS-DIAL[34], allow such a reconstruction only for a limited number of features matching with a reference library, not allowing the screening for novel undiscovered compounds.

After the screening of the DNA adducts in step 2 and 3, the features were further fragmented through targeted MS2, leading to the tentative identification of 54 DNA adducts in DNA from calf thymus, cat colon, and human colon (Table S7). Three of them were identified at level I, 5 at level II, and 46 at level III. Eight of the DNA adducts identified at level III matched with our DNA adduct database [5] and 38 were unknowns. These were classified as DNA adducts due to the loss of -dR or the nucleobase fragment in targeted MS2, but their structures were not elucidated.

The identification of such a high number of unknown DNA adducts shows the great potential of this method and identification workflow for performing untargeted DNA adductomics to find new genotoxins in our exposome; it also highlights the need for the development of better bioinformatic tools to facilitate DNA adduct screening in DIA.

DNA adducts in DNA from calf thymus, cat colon and human colon

The optimized untargeted method was applied for the identification of DNA adducts in DNA from calf thymus, cat colon, and human colon from 15 patients with hereditary CRC, sporadic CRC or other conditions such as inflammatory bowel disease, leading to the tentative identification of 53, 39 and 40 DNA adducts, respectively (Table S7). The DNA adducts that were assigned an identity, i.e. that matched with our database [5], were found in all three samples but in different amounts. On the contrary, some of the unknowns were unique either in DNA from calf thymus or colon tissues. Several expected DNA adducts were identified including 8-Oxo-dG, dU, deoxyxanthosine (dX), deoxyinosine (dI), 5-Me-dC, and 5-hydroxy-methyl-dC (5-OH-Me-dC). 8-Oxo-dG is one of the most studied DNA adducts, and it derives from the oxidative damage to DNA caused by reactive oxygen species (ROS), which occurs endogenously as part of normal metabolism [35]. dU, dX, and dI are a result of deamination of dG, dA, and dC, respectively. This occurs spontaneously as a result of several mechanisms such as simple hydrolysis, interaction with nitric oxide-derived species or ROS during inflammation, and by the activity of deaminase enzymes [36,37]. 5-Me-dC and 5-OH-Me-dC are a result of epigenetic modification processes [38]. High abundance of these adducts is therefore expected.

Other adducts, which have also been found previously in multiple studies, were also identified, such as multiple isomers of carboxy-methyl-dG (carboxy-Me-dG), carboxy-ethyl-dG (carboxy-Et-dG), carboxy-hydroxyethyl-dG (carboxy-OH-Et-dG), hydroxy-methyl-dG (OH-Me-dG), and hydroxyethyl-dG (OH-Et-dG). Carboxy-Me-dG and carboxy-Et-dG have the same accurate mass as the adducts glyoxal-dG and Me-glyoxal-dG. However, in both cases and in both isomers, the loss of CO_2 in the fragmentation pattern strongly suggests the identities reported here. Glyoxal-dG has been found to be unstable and is partially transformed to N^2-carboxy-Me-dG [39]. The compound may therefore derive mainly from rearrangements of adducts formed by glyoxal, which is widely used in industrial processes, is present in cigarette smoke and food, and it is a common byproduct of the ubiquitous glycolysis pathway [39].

Me-glyoxal is also a byproduct of glycolysis and reacts with dG to form N^2-carboxy-Et-dG [40]. 1-Carboxy-Et-dG and N^6-Carboxy-OH-Et-dG have been shown to result from exposure to acrylamide, a carcinogen widely used for industrial purposes, present in cigarette smoke, and deriving from starchy foods baked, roasted or fried at high temperature [41,42]. The formation of OH-Me-dG, results from the exposure to formaldehyde; this is an endogenously formed carcinogen, but also derives from many external sources [43]. Finally, the formation of OH-Et-dG has been found to derive from the industrial chemical ethylene oxide, nitrosamines, and alcohol exposure [44,45]. Due to the presence of these adducts in commercial calf thymus DNA, in cat colon and human colon DNA, and across multiple samples and studies found in the literature, we assume that they are not deriving from a specific exposure, but they are most likely due to endogenous processes, or a combination of multiple exposures.

In the only reported multi-targeted DNA adductomics study on colon tissues from CRC patients, the features were matched with an in-house database and 17 DNA adducts were tentatively identified, including O^6-Carboxy-Me-dG, carboxy-Et-dG, OH-Et-dG, oxo-dG, and other similar DNA adducts [46]. The DNA adducts detected in this and our study partially derive from exposures that are considered risk factors for CRC such as alcohol, smoking, or endogenous processes such as inflammation. However, a wide range of DNA adducts deriving from sources potentially increasing CRC risk [14], such as lipid peroxidation, heterocyclic aromatic amines, or polycyclic aromatic hydrocarbons present in cooked red meat and tobacco, were not observed. The prevalence of small and polar DNA adducts in our study, as well as in other recent DNA adductomics studies in humans [46], might derive from the inefficiency of the analytical method to extract and detect the totality of DNA adducts due to their extremely different chemical properties, especially bulky, apolar DNA adducts, which are unstable in aqueous solution. In addition, we cannot rule out that some of the small, polar adducts are partially caused by procedures related to DNA isolation and hydrolysis. The fact that we observe many of the same adducts in calf thymus DNA as in colon tissues calls for caution, and further studies will have to show to what extent artifacts play a role [47]. Improvement in the sample preparation to reduce matrix effect, better extract bulky DNA adducts, and control artifact formation, might provide a higher and more reliable number of DNA adducts. Nonetheless, our current method, which is based on a 25-minutes chromatographic run with no sample pre-concentration, allowed us to identify a number of DNA adducts that is higher or comparable to other studies, where sample pre-concentration or fractionation, and nano-ESI-HPLC were used [12,22,31,33,48,49]. In addition, this is the first untargeted study on colon tissues, and one of the few detecting and validating such a high number of unknowns when compared with other untargeted methods applied to other samples [12,13,50], showing the current MS^E-based method and identification workflow to be highly promising for performing untargeted DNA adductomics. The detected unknown DNA adducts show the potential of DNA adductomics in exploring new possible exposures and improve understanding of the causes of CRC.

■ CONCLUSIONS

In this study, a sensitive UHPLC-HRMS untargeted method was developed for the detection and identification of DNA adducts. The optimization of the chromatographic conditions showed the mobile phases containing 10mM NH_4HCO_3 with a BEH C18 column to be the best condition in terms of signal enhancement of a mixture of DNA adducts used as reference standards. The capillary voltage, the sampling cone voltage, and the detector voltage highly affected the response of the DNA adducts. Additionally, a low adsorption vial was selected for the highest stability of the signal over time. These optimised analytical conditions were used for the analysis of DNA from calf thymus, cat colon, and human colon from CRC patients using MS^E acquisition, where the DNA adducts were screened by monitoring the loss of -dR and the ions belonging to the unmodified nucleobases. The 25-minutes method led to the identification of 54 DNA adducts, of which 38 have never been reported before, showing good promise for the application of this untargeted method to provide a comprehensive profiling of the DNA adductome in human tissues. Improved sensitivity of the analytical method by optimizing the sample preparation, and the

development of new screening bioinformatic tools will further enhance this technology for translation into cancer research.

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References

[1] S. Balbo, R.J. Turesky, P.W. Villalta, DNA Adductomics, Chem Res Toxicol 27 (2014) 356–366.

[2] L.A. Loeb, C.C. Harris, Advances in Chemical Carcinogenesis: A Historical Review and Prospective, Cancer Res 68 (2008) 6863.

[3] N. Tretyakova, P.W. Villalta, S. Kotapati, Mass spectrometry of structurally modified DNA., Chem Rev 113 (2013) 2395–436.

[4] P. Villalta, S. Balbo, The Future of DNA Adductomic Analysis, Int J Mol Sci 18 (2017) 1870.

[5] G. La Barbera, K.D. Nommesen, C. Cuparencu, J. Stanstrup, L.O. Dragsted, A Comprehensive Database for DNA Adductomics, Front Chem 10 (2022) 1–11.

[6] S.J. Walmsley, J. Guo, A. Tarifa, A.P. DeCaprio, M.S. Cooke, R.J. Turesky, P.W. Villalta, Mass Spectral Library for DNA Adductomics, Chem Res Toxicol 37 (2023) 310.

[7] C.W. Hu, Y.J. Chang, W.H. Chang, M.S. Cooke, Y.R. Chen, M.R. Chao, A Novel Adductomics Workflow Incorporating FeatureHunter Software: Rapid Detection of Nucleic Acid Modifications for Studying the Exposome, Environ Sci Technol 58 (2024) 75–89.

[8] K.J. Murray, E.S. Carlson, A. Stornetta, E.P. Balskus, P.W. Villalta, S. Balbo, Extension of Diagnostic Fragmentation Filtering for Automated Discovery in DNA Adductomics, Anal Chem 93 (2021) 5754–5762.

[9] P.F.M. Sousa, G. Martella, K.M. Åberg, B. Esfahani, H. V. Motwani, nLossFinder—A Graphical User Interface Program for the Nontargeted Detection of DNA Adducts, Toxics 9 (2021) 78.

[10] S.J. Walmsley, J. Guo, P. Murugan, C.J. Weight, J. Wang, P.W. Villalta, R.J. Turesky, Comprehensive Analysis of DNA Adducts Using Data-Independent wSIM/MS2Acquisition and wSIM-City, Anal Chem 93 (2021) 6491–6500.

[11] V. Guidolin, E.S. Carlson, A. Carrà, P.W. Villalta, L.A. Maertens, S.S. Hecht, S. Balbo, Identification of New Markers of Alcohol-Derived DNA Damage in Humans, Biomolecules 11 (2021) 1–20.

[12] A. Stornetta, P.W. Villalta, S.S. Hecht, S.J. Sturla, S. Balbo, Screening for DNA Alkylation Mono and Cross-Linked Adducts with a Comprehensive LC-MS3 Adductomic Approach, Anal Chem 87 (2015) 11706–11713.

[13] N. Ragi, S.J. Walmsley, F.C. Jacobs, T.A. Rosenquist, V.S. Sidorenko, L. Yao, L.A. Maertens, C.J. Weight, S. Balbo, P.W. Villalta, R.J. Turesky, Screening DNA Damage in the Rat Kidney and Liver by Untargeted DNA Adductomics, Chem Res Toxicol 37 (2024) 340–360.

[14] B. Tudek, E. Speina, Oxidatively damaged DNA and its repair in colon carcinogenesis, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 736 (2012) 82–92.

[15] N. Murphy, V. Moreno, D.J. Hughes, L. Vodicka, P. Vodicka, E.K. Aglago, M.J. Gunter, M. Jenab, Lifestyle and dietary environmental factors in colorectal cancer susceptibility, Mol Aspects Med 69 (2019) 2–9.

[16] M.C. Chambers, B. MacLean, R. Burke, D. Amodei, D.L. Ruderman, S. Neumann, L. Gatto, B. Fischer, B. Pratt, J. Egertson, K. Hoff, D. Kessner, N. Tasman, N. Shulman, B. Frewen, T.A. Baker, M.Y. Brusniak, C. Paulse, D. Creasy, L. Flashner, K. Kani, C. Moulding, S.L. Seymour, L.M. Nuwaysir, B. Lefebvre, F. Kuhlmann, J. Roark, P. Rainer, S. Detlev, T. Hemenway, A. Huhmer, J. Langridge, B. Connolly, T. Chadick, K. Holly, J. Eckels, E.W. Deutsch, R.L. Moritz, J.E. Katz, D.B. Agus, M. MacCoss, D.L. Tabb, P. Mallick, A cross-platform toolkit for mass spectrometry and proteomics, Nat Biotechnol 30 (2012) 918–920.

[17] T. Pluskal, S. Castillo, A. Villar-Briones, M. Orešič, MZmine 2: Modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data, BMC Bioinformatics 11 (2010).

[18] G. La Barbera, M. Antonelli, C. Cavaliere, G. Cruciani, L. Goracci, C.M. Montone, S. Piovesana, A. Laganà, A.L. Capriotti, Delving into the Polar Lipidome by Optimized Chromatographic Separation, High-Resolution Mass Spectrometry, and Comprehensive Identification with Lipostar: Microalgae as Case Study, Anal Chem 90 (2018) 12230–12238.
[19] E.L. Schymanski, J. Jeon, R. Gulde, K. Fenner, M. Ruff, H.P. Singer, J. Hollender, Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence, Environ Sci Technol 48 (2014) 2097–2098.
[20] L.W. Sumner, A. Amberg, D. Barrett, M.H. Beale, R. Beger, C.A. Daykin, T.W.-M. Fan, O. Fiehn, R. Goodacre, J.L. Griffin, T. Hankemeier, N. Hardy, J. Harnly, R. Higashi, J. Kopka, A.N. Lane, J.C. Lindon, P. Marriott, A.W. Nicholls, M.D. Reily, J.J. Thaden, M.R. Viant, Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI), Metabolomics 3 (2007) 211–221.
[21] P.W. Villalta, J.B. Hochalter, S.S. Hecht, Ultrasensitive High-Resolution Mass Spectrometric Analysis of a DNA Adduct of the Carcinogen Benzo[a]pyrene in Human Lung, Anal Chem 89 (2017) 12735–12742.
[22] A. Carrà, V. Guidolin, R.P. Dator, P. Upadhyaya, F. Kassie, P.W. Villalta, S. Balbo, Targeted High Resolution LC/MS3 Adductomics Method for the Characterization of Endogenous DNA Damage, Front Chem 7 (2019) 658.
[23] S. Balbo, S.S. Hecht, P. Upadhyaya, P.W. Villalta, Application of a High-Resolution Mass-Spectrometry-Based DNA Adductomics Approach for Identification of DNA Adducts in Complex Mixtures, Anal Chem 86 (2014) 1744–1752.
[24] L.Y. Hemeryck, C. Rombouts, E. De Paepe, L. Vanhaecke, DNA adduct profiling of in vitro colonic meat digests to map red vs. white meat genotoxicity, Food and Chemical Toxicology 115 (2018) 73–87.
[25] R. Yin, S. Liu, C. Zhao, M. Lu, M.S. Tang, H. Wang, An ammonium bicarbonate-enhanced stable isotope dilution UHPLC-MS/MS method for sensitive and accurate quantification of acrolein-DNA adducts in human leukocytes, Anal Chem 85 (2013) 3190–3197.
[26] H. Murakami, R. Horiba, T. Iwata, Y. Miki, B. Uno, T. Sakai, K. Kaneko, Y. Ishihama, N. Teshima, Y. Esaka, Progress in a selective method for the determination of the acetaldehyde-derived DNA adducts by using HILIC-ESI-MS/MS, Talanta 177 (2018) 12–17.
[27] J. Abián, F. Sáchez-Baeza, E. Gelpí, D. Barceló, On the origin of some controversial ions (m/z 59, 60, 77, and 119) in the thermospray reagent plasma from ammonium acetate, J Am Soc Mass Spectrom 5 (1994) 186–193.
[28] A. Liguori, A. Napoli, G. Sindona, Survey of the proton affinities of adenine, cytosine, thymine and uracil dideoxyribonucleosides, deoxyribonucleosides and ribonucleosides, J. Mass Spectrom 35 (2000) 139–144.
[29] C. von Sonntag, Nucleobases, Nucleosides and Nucleotides, in: Free-Radical-Induced DNA Damage and Its Repair, Springer, Berlin, Heidelberg, 2006: pp. 211–334.
[30] M. DeLano, T.H. Walter, M.A. Lauber, M. Gilar, M.C. Jung, J.M. Nguyen, C. Boissel, A. V. Patel, A. Bates-Harrison, K.D. Wyndham, Using Hybrid Organic–Inorganic Surface Technology to Mitigate Analyte Interactions with Metal Surfaces in UHPLC, Anal Chem 93 (2021) 5773–5781.
[31] M.S. Cooke, C.W. Hu, Y.J. Chang, M.R. Chao, Urinary DNA adductomics – A novel approach for exposomics, Environ Int 121 (2018) 1033–1038.
[32] F. Bishehsari, L. Zhang, R.M. Voigt, N. Maltby, B. Semsarieh, E. Zorub, M. Shaikh, S. Wilber, A.R. Armstrong, S.S. Mirbagheri, N.Z. Preite, P. Song, A. Stornetta, S. Balbo, C.B. Forsyth, A. Keshavarzian, Alcohol Effects on Colon Epithelium are Time-Dependent, Alcohol Clin Exp Res 43 (2019) 1898–1908.
[33] J. Guo, P.W. Villalta, R.J. Turesky, Data-Independent Mass Spectrometry Approach for Screening and Identification of DNA Adducts, Anal Chem 89 (2017) 11728–11736.
[34] H. Tsugawa, T. Cajka, T. Kind, Y. Ma, B. Higgins, K. Ikeda, M. Kanazawa, J. Vandergheynst, O. Fiehn, M. Arita, MS-DIAL: data-independent MS/MS deconvolution for comprehensive metabolome analysis, Nat Methods 12 (2015) 523–526
[35] M. Dizdaroglu, P. Jaruga, Mechanisms of free radical-induced damage to DNA, Free Radical research 46 (2012) 382–419.
[36] B. Pang, J.L. McFaline, N.E. Burgis, M. Dong, K. Taghizadeh, M.R. Sullivan, C.E. Elmquist, R.P. Cunningham, P.C. Dedon, Defects in purine nucleotide metabolism lead to substantial incorporation of xanthine and hypoxanthine into DNA and RNA, Proceedings of the National Academy of Sciences 109 (2012) 2319–2324.
[37] Y.W. Kow, Repair of deaminated bases in DNA, Free Radic Biol Med 33 (2002) 886–893.

[38] S. Kumar, V. Chinnusamy, T. Mohapatra, Epigenetics of Modified DNA Bases: 5-Methylcytosine and Beyond, Front Genet 9 (2018).
[39] H. Wang, H. Cao, Y. Wang, Quantification of N2-Carboxymethyl-2'-deoxyguanosine in Calf-thymus DNA and Cultured Human Kidney Epithelial Cells by Capillary HPLC-Tandem Mass Spectrometry Coupled with Stable Isotope-dilution Method, Chem Res Toxicol 23 (2010) 74.
[40] M. Frischmann, C. Bidmon, J. Angerer, M. Pischetsrieder, Identification of DNA adducts of methylglyoxal, Chem Res Toxicol 18 (2005) 1586–1592.
[41] J.J. Solomon, J. Fedyk, F. Mukai, A. Segal, Direct Alkylation of 2'-Deoxynucleosides and DNA following in Vitro Reaction with Acrylamide, Cancer Research 45 (1985) 3465-3470.
[42] G.G. Da Costa, M.I. Churchwell, L.P. Hamilton, L.S. Von Tungeln, F.A. Beland, M.M. Marques, D.R. Doerge, DNA Adduct Formation from Acrylamide via Conversion To Glycidamide in Adult and Neonatal Mice, Chem Res Toxicol 16 (2003) 1328–1337.
[43] K. Lu, S. Craft, J. Nakamura, B.C. Moeller, J.A. Swenberg, Use of LC-MS/MS and stable isotopes to differentiate hydroxymethyl and methyl DNA adducts from formaldehyde and nitrosodimethylamine, Chem Res Toxicol 25 (2012) 664–675.
[44] E.M. Tompkins, D.J.L. Jones, J.H. Lamb, D.A. Marsden, P.B. Farmer, K. Brown, Simultaneous detection of five different 2-hydroxyethyl-DNA adducts formed by ethylene oxide exposure, using a high-performance liquid chromatography/electrospray ionisation tandem mass spectrometry assay, Rapid Communications in Mass Spectrometry 22 (2008) 19–28.
[45] N. Tretyakova, M. Goggin, D. Sangaraju, G. Janis, Quantitation of DNA adducts by stable isotope dilution mass spectrometry, Chem Res Toxicol 25 (2012) 2007–2035.
[46] L.Y. Hemeryck, A.I. Decloedt, J. Vanden Bussche, K.P. Geboes, L. Vanhaecke, High resolution mass spectrometry based profiling of diet-related deoxyribonucleic acid adducts, Anal Chim Acta 892 (2015) 123–131.
[47] H. Chen, S. Krishnamachari, J. Guo, L. Yao, P. Murugan, C.J. Weight, R.J. Turesky, Quantitation of Lipid Peroxidation Product DNA Adducts in Human Prostate by Tandem Mass Spectrometry: A Method That Mitigates Artifacts, Chem Res Toxicol 32 (2019) 1850–1862.
[48] V. Guidolin, E.S. Carlson, A. Carrà, P.W. Villalta, L.A. Maertens, S.S. Hecht, S. Balbo, Identification of New Markers of Alcohol-Derived DNA Damage in Humans, Biomolecules 11 (2021) 1–20.
[49] J. Guo, P.W. Villalta, C.J. Weight, R. Bonala, F. Johnson, T.A. Rosenquist, R.J. Turesky, Targeted and Untargeted Detection of DNA Adducts of Aromatic Amine Carcinogens in Human Bladder by Ultra-Performance Liquid Chromatography-High-Resolution Mass Spectrometry, Chem Res Toxicol 31 (2018) 1382–1397.
[50] J. Guo, J.S. Koopmeiners, S.J. Walmsley, P.W. Villalta, L. Yao, P. Murugan, R. Tejpaul, C.J. Weight, R.J. Turesky, The Cooked Meat Carcinogen 2-Amino-1-methyl-6-phenylimidazo[4,5- b]pyridine Hair Dosimeter, DNA Adductomics Discovery, and Associations with Prostate Cancer Pathology Biomarkers, Chem Res Toxicol 35 (2022) 703–730.