

Development of a targeted adductomic method for the determination of polycyclic aromatic hydrocarbon DNA adducts using online column-switching liquid chromatography tandem mass spectrometry

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Abbreviations: PAH, polycyclic aromatic hydrocarbon; B[b]F, benzo[b]fluoranthene; DB[a,l]P, dibenzo[a,l]pyrene; DB[a,h]A, dibenzo[a,h]anthracene; B[a]P, benzo[a]pyrene; B[b]FDE, (\pm)-*anti*-benzo[b]fluoranthene-9,10-dihydrodiol-11,12-epoxide; B[b]FDE-N²-dG, 12-(deoxyguanosine-N²-yl)-9,10,11-trihydroxy-9,10,11,12-tetrahydrobenzo[b]fluoranthene; B[b]FDE-N⁶-dA, 12-(deoxyadenosine-N⁶-yl)-9,10,11-trihydroxy-9,10,11,12-tetrahydrobenzo[b]fluoranthene; B[b]FDE-N⁴-dC, 12-(deoxycytidine-N⁴-yl)-9,10,11-trihydroxy-9,10,11,12-tetrahydrobenzo[b]fluoranthene; DB[a,l]PDE, (\pm)-*anti*-dibenzo[a,l]pyrene-11,12-dihydrodiol-13,14-epoxide; DB[a,l]PDE-N²-dG, 14-(deoxyguanosine-N²-yl)-11,12,13-trihydroxy-11,12,13,14-tetrahydrodibenzo[a,l]pyrene; DB[a,l]PDE-N⁶-dA, 14-(deoxyadenosine-N⁶-yl)-11,12,13-trihydroxy-11,12,13,14-tetrahydrodibenzo[a,l]pyrene; DB[a,l]PDE-N⁴-dC, 14-(deoxycytidine-N⁴-yl)-11,12,13-trihydroxy-11,12,13,14-tetrahydrodibenzo[a,l]pyrene; DB[a,h]ADE, (\pm)-*anti*-dibenz[a,h]anthracene-3,4-dihydrodiol-1,2-epoxide; DB[a,h]ADE-N²-dG, 1-(deoxyguanosine-N²-yl)-2,3,4-trihydroxy-1,2,3,4-tetrahydrodibenz[a,h]anthracene; DB[a,h]ADE-N⁶-dA, 1-(deoxyadenosine-N⁶-yl)-2,3,4-trihydroxy-1,2,3,4-tetrahydrodibenz[a,h]anthracene; DB[a,h]ADE-N⁴-dC, 1-(deoxycytidine-N⁴-yl)-2,3,4-trihydroxy-1,2,3,4-tetrahydrodibenz[a,h]anthracene; B[a]PDE, (\pm)-*anti*-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide; B[a]PDE-N²-dG, 10-(deoxyguanosine-N²-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene; B[a]PDE-N⁶-dA, 10-(deoxyadenosine-N⁶-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene; B[a]PDE-N⁴-dC, 10-(deoxycytidine-N⁴-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene; PAHDE-dN, PAH-dihydrodiol epoxide-2'-deoxynucleoside; ESI, electrospray ionisation; LC-MS/MS, liquid

chromatography-tandem mass spectrometry (mass spectrometry/mass spectrometry); SRM, selected reaction monitoring; CNL, constant neutral loss; CID, collision induced dissociation.

Abstract

Human exposure to polycyclic aromatic hydrocarbons (PAHs) from sources such as industrial or urban air pollution, tobacco smoke and cooked food is not confined to a single compound, but instead to mixtures of different PAHs. The interaction of different PAHs may lead to additive, synergistic or antagonistic effects in terms of DNA adduct formation and carcinogenic activity resulting from changes in metabolic activation to reactive intermediates and DNA repair. The development of a targeted DNA adductomic approach using liquid chromatography-tandem mass spectrometry (LC-MS/MS) incorporating software based peak picking and integration for the assessment of exposure to mixtures of PAHs is described. For method development PAH modified DNA samples were obtained by reaction of the *anti*-dihydrodiol epoxide metabolites of benzo[a]pyrene, benzo[b]fluoranthene, dibenzo[a,l]pyrene (DB[a,l]P) and dibenz[a,h]anthracene, with calf thymus DNA *in vitro* and enzymatically hydrolysed to 2'-deoxynucleosides. Positive LC-electrospray ionisation (ESI)-MS/MS collision induced dissociation product ion spectra data showed that the majority of adducts displayed a common fragmentation for the neutral loss of 116u (2'-deoxyribose) resulting in a major product ion derived from the adducted base. The exception was the DB[a,l]P dihydrodiol epoxide adduct of 2'-deoxyadenosine which resulted in major product ions derived from the PAH moiety being detected. Specific detection of mixtures of PAH adducted 2'-deoxynucleosides was achieved using online column-switching LC-MS/MS in conjunction with selected reaction monitoring (SRM) of the $[M+H]^+$ to $[M+H-116]^+$ transition plus product ions derived from the PAH moiety for improved sensitivity of detection and a comparison was made to detection by constant neutral loss scanning. In conclusion different PAH DNA adducts were detected by employing SRM $[M+H-116]^+$ transitions or constant

neutral loss scanning. However for improved sensitivity of detection optimised SRM transitions relating to the PAH moiety product ions are required for certain PAH DNA adducts for the development of targeted DNA adductomic methods.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) represent a group of structurally related organic compounds, formed primarily by the incomplete combustion of organic matter, which are ubiquitously occurring environmental contaminants ¹⁻⁶. Benzo[*a*]pyrene (B[*a*]P) has been extensively used as a model compound for studying the mechanism of biological action and assessment of exposure to PAHs ⁷. However human exposure to PAHs is not confined to a single compound but instead occurs as complex mixtures of different PAHs ⁴. Examples of human sources of exposure to PAHs include industrial or urban air pollution, automobile exhaust emissions, tobacco smoke, coal tar, as well as charred or grilled food and cereals ^{2, 3, 8}. Experimental animal studies have demonstrated the mutagenic and carcinogenic properties of a number of different PAHs and also provided evidence for differences in their carcinogenic potency ⁹⁻¹¹. The tumour initiating activity of PAHs is related to their ability to form DNA adducts following metabolic activation to reactive intermediates. One activation pathway is mediated by the combined action of the cytochrome P450 enzymes, predominantly CYP 1A1 and CYP 1B1 with microsomal epoxide hydrolase (EHPX1) which results in the formation of the ultimate electrophilic reactive species, a PAH-dihydrodiol epoxide. ¹² This can react with the exocyclic amino groups of guanine (N²) adenine (N⁶) and cytosine (N⁴) in DNA (**Figure 1**) ¹³⁻¹⁵. In addition planar PAHs can cause induction of P450 enzymes by binding the cytosolic aryl hydrocarbon receptor. This complex dimerises with the aryl hydrocarbon nuclear translocator, which on entering the nucleus binds to xenobiotic response elements upstream of cytochrome P450 genes resulting in their transcription. ¹⁶ The interaction of different PAHs may lead to additive, synergistic or antagonistic effects, resulting in changes in processes such as metabolism and DNA repair with the consequence of an

altered carcinogenic outcome when compared to exposure to a single PAH.¹⁷⁻²² Furthermore weakly or non-carcinogenic PAHs present in mixtures can alter the DNA adduct formation activity of a single PAH in animals.²³ The simultaneous determination of multiple PAH DNA adducts via a targeted DNA adductomics approach would allow for the profiling and assessment of exposure to mixtures of PAHs and potentially provide insight into the mechanism of cancer induction.

The ³²P postlabelling method has been the most widely used method for the detection of PAH DNA adducts in animal and human tissues offering the advantage that only small amounts of the DNA sample are required for analysis and also that it allows multiple adducts to be detected²⁴⁻²⁶. For human DNA analysis, mixtures of adducts are detected as a “diagonal radioactive zone” which represents a smear of radioactivity on thin layer chromatography plates.²⁷ Immunological methods employing antibodies recognising PAH DNA adducts, in combination with fluorescence or chemiluminescence as well as immunohistochemical detection, have also been used for the screening of PAH DNA adducts in human tissues^{5, 26, 28, 29}. The main disadvantage of these methods is that they lack specificity. Due to sufficient gains in sensitivity from the advancement of instrumentation, liquid chromatography (LC)-electrospray ionisation (ESI)-mass spectrometry (MS) now represents a viable alternative method for the detection of DNA adducts, with the advantage of providing structural information of the adduct under investigation³⁰. LC-tandem mass spectrometry (MS/MS) methods using selected reaction monitoring (SRM) have been described for the detection of B[a]P derived DNA adducts in *in vitro* treated human cells, mouse liver and human lung tissues with accurate quantitation achieved using stable isotope internal standards³¹⁻³⁵. From numerous publications it is evident that the majority of adducted 2'-deoxynucleosides show a common fragmentation in

positive ESI-MS/MS, this being the neutral loss of 116 u following collision induced dissociation (CID), which corresponds to the loss of the 2'-deoxyribose³⁰. Selective detection of these adducted 2'-deoxynucleosides may be achieved by SRM of the $[M+H]^+$ to $[M+H-116]^+$ transition. The potential of using mass spectrometry for the screening of DNA adducts by monitoring a large range of such transitions has recently been exploited by Kanaly *et al.* allowing the detection of multiple DNA adducts simultaneously. This has led to the concept of producing an adductome map of all DNA adducts in a sample. This approach holds great potential for gaining further insight into DNA adduct profile differences between human populations exposed to mixtures of environmental genotoxins³⁶⁻³⁸.

In the present study we describe the development of a targeted DNA adductomic approach using an online column-switching LC-MS/MS methodology for the assessment of exposure to mixtures of PAHs. For method development PAH modified DNA samples were obtained by the reaction of the dihydrodiol epoxide metabolites of B[a]P, benzo[b]fluoranthene (B[b]F) dibenzo[a,l]pyrene (DB[a,l]P), and dibenz[a,h]anthracene (DB[a,h]A) with calf thymus DNA *in vitro*.

Experimental Procedures

Chemicals

(±)-*Anti*-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (B[*a*]PDE) was purchased from the National Cancer Institute (NCI) Chemical Carcinogen Reference Standard Repository (Midwest Research Institute, Kansas City, Missouri). (±)-*Anti*-benzo[*b*]fluoranthene-9,10-dihydrodiol-11,12-epoxide (B[*b*]FDE)³⁹⁻⁴³, (±)-*anti*-dibenzo[*a,l*]pyrene-11,12-dihydrodiol-13,14-epoxide (DB[*a,l*]PDE)⁴⁴ and (±)-*anti*-dibenz[*a,h*]anthracene-3,4-dihydrodiol-1,2-epoxide (DB[*a,h*]ADE)^{40, 45, 46} were synthesised according to methods described previously. **Caution:** *PAH-dihydrodiol epoxides are mutagens and carcinogens. Protective clothing should be worn and appropriate safety procedures followed when working with these compounds.* Calf thymus DNA, deoxyribonuclease I (from bovine pancreas), snake venom phosphodiesterase I (from *Crotalus adamanteus*), alkaline phosphatase (from *E.coli*) and mass spectrometry grade formic acid were purchased from Sigma (Poole, UK). All other reagents (analytical grade), tetrahydrofuran (extra dry) and HPLC grade acetonitrile were purchased from Fisher Scientific (Loughborough, UK). HPLC grade water, 18.2 MΩcm output quality was obtained from Maxima purification equipment (Elga, High Wycombe, UK).

Reaction of calf thymus DNA with different PAH-dihydrodiol epoxides

To four 25 mg aliquots of calf thymus DNA (1mg/mL) dissolved in 0.1 M K₃PO₄ buffer, pH 7.4 were added 1 mg/mL solutions of the individual *anti*-PAH-dihydrodiol epoxides dissolved in tetrahydrofuran, B[*a*]PDE (5.0 mL), B[*b*]FDE (1.0 mL), DB[*a,l*]PDE (1.0 mL) and DB[*a,h*]ADE (1.0 mL). The DNA solutions were incubated

at 37 °C in the dark for 24 h and then extracted 8 times with equal volumes of water-saturated diethyl ether followed by 4 times with water-saturated isoamyl alcohol. The adducted calf thymus DNA was precipitated by the addition of 0.8 volumes of ice-cold isopropanol. Following mixing by inversion the DNA was pelleted by centrifugation at 4,000 rpm for 20 min. The supernatant was discarded and the DNA pellet was washed with 5.0 mL of ethanol followed by 5.0 mL ethanol/HPLC grade water (70:30, v/v) and centrifuged at 4,000 rpm for 10 min at each washing step. The pellet was air dried and dissolved in 40.0 mL of HPLC grade water. The concentration of each PAH-dihydrodiol epoxide adducted calf thymus DNA sample was calculated by determining the absorbance at 260 nm (GeneQuant spectrophotometer, Biochrom, Cambridge, UK) and assuming that one absorbance unit equals 50 µg/mL for double stranded DNA. The samples were stored at -20°C.

Enzymatic hydrolysis of *in vitro* modified DNA for LC-ESI-MS/MS analysis

Each of the *in vitro* modified DNA samples (1.0 mg) was dried down using a centrifugal vacuum evaporator. The dried DNA samples were dissolved in 990 µL of 50 mM BisTris, 0.1 mM EDTA, pH 7.1 buffer plus 10 µL of 1 M MgCl₂ and incubated with 100 µL of deoxyribonuclease I (2 mg/mL dissolved in 0.15 M NaCl, 10 mM MgCl₂) at 37°C for 6 h. The samples were further incubated with 60 µL of snake venom phosphodiesterase I from *Crotalus adamanteus* (0.001 U/µL dissolved in 0.11 M Tris-HCl, 0.11 M NaCl, 15 mM MgCl₂, pH 8.9) and 26.2 µL of alkaline phosphatase from *E. coli* (0.305 U/µL) at 37°C for 15 h. The hydrolysed DNA samples were centrifuged at 14,000 rpm for 5 min. The supernatants were transferred to new tubes and evaporated to dryness using a centrifugal vacuum evaporator (Speed vac plus SC210A, Savant, Farmingdale, US). The dried samples were dissolved in 1.0

mL of HPLC grade water/methanol (80:20, v/v) to give a 1 µg/µL final concentration of hydrolysed DNA.

Preparation of PAH-dihydrodiol epoxide modified DNA mixture

The hydrolysed B[a]PDE modified calf thymus DNA sample (1 µg/µL) was diluted 1:5 with hydrolysed control calf thymus DNA (1 µg/µL). The four different hydrolysed PAH-dihydrodiol epoxide modified calf thymus DNA samples (100 µg of each) were combined and following the addition of the [¹⁵N₅]B[a]PDE-N²-dG internal standard (10 pmol) were evaporated to dryness. The dried sample was dissolved in 100 µL of HPLC grade water/methanol (80:20, v/v) to give a solution of 1 µg/µL concentration for each hydrolysed PAH-dihydrodiol epoxide modified DNA.

Determination of PAHDE-dN LC-MS/MS CID product ion spectra

The LC-MS/MS consisted of a Waters Alliance 2695 separations module with a 100 µL injection loop connected to a Micromass Quattro Ultima Pt. (Micromass, Waters Ltd., Manchester, UK) tandem quadrupole mass spectrometer with an electrospray interface. The temperature of the electrospray source was maintained at 110°C and the desolvation temperature at 350°C. Nitrogen was used as the desolvation gas (650 L/h) and the cone gas was set to zero. The capillary voltage was set at 3.20 kV. The cone and RF1 lens voltages were 42 V and 25 V, respectively.

The mass spectrometer was tuned by using a 2'-deoxyguanosine (10 pmol/µL) standard solution dissolved in 0.1% formic acid/acetonitrile (70:30, v/v) introduced by continuous infusion at a flow rate of 10 µL/min with a Harvard model 22 syringe pump (Harvard Apparatus Ltd., Edenbridge, UK).

An aliquot of each enzymatically hydrolysed PAH-dihydrodiol epoxide modified calf thymus DNA sample was injected onto a Synergi Fusion-RP 80A C18, 4 μ m, 30 \times 2.0 mm column attached to a KrudKatcher disposable pre-column (0.5 μ m) filter (Phenomenex, Macclesfield, UK). The column was eluted using a gradient with solvent A, 0.1% formic acid and solvent B, acetonitrile at a flow rate of 120 μ L/min. The following gradient was used: 0 min–15%B, 10 min–15%B, 20 min–35%B, 30 min–35%B, 30.1 min–100%B (200 μ L/min), 35 min–100%B (200 μ L/min), 35.1 min–15%B and 45 min–15%B. The collision gas was argon (indicated cell pressure 2.0×10^{-3} mbar) and the collision energy set at 12 eV. The scan time was set to 0.5 s and the resolution was one or two m/z units at peak base. LC-MS/MS product ion spectra were obtained in positive electrospray ionisation mode MS/MS CID for the molecule ion $[M+H]^+$ for each PAH-dihydrodiol epoxide-2'-deoxynucleoside (PAHDE-dN): B[*b*]FDE-N²-dG (C₃₀H₂₇N₅O₇) m/z 570.2; B[*b*]FDE-N⁶-dA (C₃₀H₂₇N₅O₆) m/z 554.2; B[*b*]FDE-N⁴-dC (C₂₉H₂₇N₃O₇) m/z 530.2; DB[*a,l*]PDE-N²-dG (C₃₄H₂₉N₅O₇) m/z 620.2; DB[*a,l*]PDE-N⁶-dA (C₃₄H₂₉N₅O₆) m/z 604.2; DB[*a,l*]PDE-N⁴-dC (C₃₃H₂₉N₃O₇) m/z 580.2; DB[*a,h*]ADE-N²-dG (C₃₂H₂₉N₅O₇) m/z 596.2; DB[*a,h*]ADE-N⁶-dA (C₃₂H₂₉N₅O₆) m/z 580.2; DB[*a,h*]ADE-N⁴-dC (C₃₁H₂₉N₃O₇) m/z 556.2; B[*a*]PDE-N²-dG (C₃₀H₂₇N₅O₇) m/z 570.2; B[*a*]PDE-N⁶-dA (C₃₀H₂₇N₅O₆) m/z 554.2; B[*a*]PDE-N⁴-dC (C₂₉H₂₇N₃O₇) m/z 530.2. The mass spectral data were acquired in continuum mode over a period of 20 to 30 min and processed using MassLynx version 4.1 (Micromass, Waters Ltd.).

Determination of PAHDE-dN adducts using online column-switching LC-MS/MS

The online column switching valve system consisted of an automated switching valve (motorised two-position six-port valve, Waters Ltd., Hertfordshire, UK) connected to pump A incorporating an autosampler (Waters Alliance 2695 separations module) and an isocratic pump B (Gynotek GmbH, Germering, Germany). Pump A was connected via the switching valve to the trap column, Synergi Fusion-RP 80A C18, 4 μ m, 30 \times 2.0 mm column attached to a KrudKatcher disposable pre-column (0.5 μ m) filter and Pump B was connected via the switching valve to the analytical column, Synergi Fusion-RP 80A C18 (4 μ m, 250 \times 2.0 mm) column attached to a Synergi Fusion-RP 80A C18 (4 μ m, 4.0 \times 2.0 mm) guard column and KrudKatcher disposable pre-column (0.5 μ m) filter. The outlet of the analytical column was directly connected to the mass spectrometer. The configuration of the online column switching valve system is summarised in Figure 2.

Sample loading: An aliquot of the PAH-dihydrodiol epoxide modified calf thymus DNA mixture (1 μ g/ μ L of hydrolysed DNA) containing 1 pmol of the [$^{15}\text{N}_5$]B[a]PDE-N²-dG stable isotope internal standard was injected onto the trap column using pump A with the switching valve in position 1. The impurities on the trap column were eluted to waste using a gradient with solvent A, 0.1% formic acid and solvent B, acetonitrile at a flow rate of 120 μ L/min. The following gradient was used: 0 min–15%B, 10 min–15%B, 20 min–28%B, 35 min–28%B, 35.1 min–100%B (200 μ L/min), 40 min–100%B (200 μ L/min), 40.1 min–15%B and 75 min–15%B. Concurrently isocratic flow at 120 μ L/min via the analytical column, which was eluted with 0.1% formic acid/acetonitrile (70:30, v/v), was maintained to the mass spectrometer by means of pump B.

Sample elution: At 22.0 min the switching valve was switched to position 2 allowing the purified PAHDE-dN adducts to be back-flushed from the trap column onto the analytical column and then subsequent elution into the mass spectrometer. The isocratic flow was maintained by pump B at flow rate of 120 $\mu\text{L}/\text{min}$ with 0.1% formic acid/acetonitrile (70:30, v/v) for 13.0 min. Concurrently the flow from pump A bypassed the trap column and was diverted directly to waste. At 35.0 min the switching valve was switched back to position 1 and the configuration of the online column switching system reverted back to initial conditions as described for the sample loading above. The total run time was 75 min.

Mass spectrometry: A Waters Micromass Quattro Ultima Pt tandem quadrupole mass spectrometer with an electrospray interface was used. The temperature of the electrospray source was maintained at 110°C and the desolvation temperature at 350°C. Nitrogen gas was used as the desolvation gas (650 L/h) and the cone gas (25 L/h). The capillary voltage was set at 3.20V. The cone and RF1 lens voltages were 42 V and 25 V, respectively. The collision gas was argon (indicated cell pressure 2.0×10^{-3} mbar) and the collision energy set at 12 eV.

SRM analysis: The hydrolysed DNA samples were analysed in positive ESI-MS/MS SRM mode for the $[\text{M}+\text{H}]^+$ ion to base $[\text{B}+\text{H}_2]^+$ or PAH moiety fragment m/z transitions: B[b]FDE-N²-dG, 570 to 454; B[b]FDE-N⁶-dA, 554 to 438; B[b]FDE-N⁴-dC 530 to 414; DB[a,l]PDE-N²-dG, 620 to 504, 620 to 353; DB[a,l]PDE-N⁶-dA, 604 to 488, 604 to 335; DB[a,l]PDE-N⁴-dC, 580 to 464, 580 to 353; DB[a,h]ADE-N²-dG, 596 to 480; DB[a,h]ADE-N⁶-dA, 580 to 464; DB[a,h]ADE-N⁴-dC, 556 to 440; B[a]PDE-N²-dG, 570 to 454; B[a]PDE-N⁶-dA, 554 to 438, 554 to 285; B[a]PDE-N⁴-

dC, 530 to 414, 530 to 285; [¹⁵N₅]B[a]PDE-N²-dG internal standard, 575 to 459. The dwell time was set to 100 ms and the resolution was two *m/z* units at peak base.

Constant neutral loss analysis: ESI-MS/MS constant neutral loss (CNL) scanning was performed from *m/z* 500 to 650 monitoring the neutral loss of 116 u following CID. The scan time was set to 0.5 s and the resolution was two *m/z* units at peak base. The data were acquired in centroid mode.

Data analysis: Raw data files were transformed into netCDF files using the Databridge utility within MassLynx (version 4.1). Peak picking and integration was performed with XCMS software version 1.18.0.⁴⁷ Unless stated otherwise, default parameter settings were used. Peaks were extracted using the “matchedFilter” method. For CNL data the signal to noise ratio cut-off (snthresh) was 5, full width at half maximum (fwhm) of matched filtration gaussian model peak was 35 s, step size for profile generation (step) was 0.2 *m/z*, number of merged steps prior to filtration (steps) was 5 and minimum *m/z* difference for peaks with overlapping retention time was 2. For SRM data the default settings were altered in the following parameters: snthresh = 1.4, fwhm = 35, step = 1, steps = 1. All subsequent data processing steps were the same for both data types. Peak grouping was performed with the bandwidth (bw) set so 30 s and the width of overlapping *m/z* slices to 1 *m/z*. Non-linear retention time correction was performed using the following parameters: family = symmetric and span = 0.5. In a repeated peak grouping step the bandwidth was reduced to 10 s. The resulting peak list was imported into Microsoft Office Excel. Peaks eluting with retention times earlier than 30 min and later than 65 min were excluded and the peak areas normalised to the signal of the [¹⁵N₅]B[a]PDE-N²-dG internal standard before

being plotted as bubble charts. The x -axis represents the retention time and the y -axis represents the m/z ratio for each PAH DNA adduct detected. The size of the bubble is directly proportional to the peak area for each adduct.

For comparison peak area integrations were also performed manually for CNL extracted ion chromatograms (m/z window = 1 Da) or SRM transitions within the MassLynx chromatogram window using the mean smoothing method, window size (scans) set to ± 3 and the number of smooths set to 2.

Results

PAHDE-dN LC-MS/MS CID product ion spectra

The calf thymus DNA samples treated separately with four different PAH-dihydrodiol epoxides, were enzymatically hydrolysed to 2'-deoxynucleosides and subjected to analysis using LC-ESI-MS/MS CID for the predicted $[M+H]^+$ molecular ion of the exocyclic amino group PAH-dihydrodiol epoxide adduct for each 2'-deoxynucleoside. The PAHDE-dN adducts eluted between 20 and 30 min following analysis by LC-ESI-MS/MS CID. The product ion spectra for each PAHDE-dN are shown in **Figure 3**. The different PAHDE-dN exhibited a common CID fragmentation pathway. Distinct product ions resulting from the $[M+H]^+$ precursor ions were observed for each PAHDE-dN corresponding to the PAH adducted base ($[(\text{PAHtriol}+\text{base})+\text{H}_2]^+$) following cleavage of the glycosidic bond and neutral loss of the 2'-deoxyribose group. For the majority of the PAHDE-dN except for DB[a,l]PDE-N⁶-dA, the $[(\text{PAHtriol}+\text{base})+\text{H}_2]^+$ product ion was one of the major ions detected. Product ions corresponding to the loss of one H₂O molecule from the $[(\text{PAHtriol}+\text{base})+\text{H}_2]^+$ ion were also observed. No product ions resulting from the neutral loss of 17 u corresponding to -NH₃ were observed, thus confirming that PAH-dihydrodiol epoxide adduct formation for each 2'-deoxynucleoside was by reaction with the exocyclic -NH₂ group at position N² for guanine, N⁶ for adenine and N⁴ for cytosine.

The spectrum for each PAHDE-dN contained product ions derived from the PAH moiety. Product ions corresponding to $[\text{PAHtriol}]^+$ as well as a $[\text{PAHdiol}]^+$ due to loss of H₂O were observed. A product ion corresponding to the loss of CO from the $[\text{PAHdiol}]^+$ ion was also observed. The relative intensity of these product ions to the $[(\text{PAHtriol}+\text{base})+\text{H}_2]^+$ product ion was considerably higher for DB[a,l]PDE-N⁶-dA when compared to the other PAHDE-dN. All the spectra also contained product ions

that corresponded to the unadducted base ($[\text{Base}+\text{H}_2]^+$) at m/z 152 [guanine+ H_2] $^+$, m/z 136 [adenine+ H_2] $^+$, and m/z 112 [cytosine+ H_2] $^+$. The fragmentation pathway of the different PAHDE-dN is summarised in **Figure 4**.

Online column-switching LC-MS/MS analysis of PAHDE-dN adducts

Initial experiments involved optimisation of the mobile phase conditions for the separation of unmodified 2'-deoxynucleosides from PAHDE-dN on the trap column. A gradient separation with a mobile phase consisting of 0.1% formic acid and acetonitrile was found to give the optimal separation with the different PAHDE-dN eluting between 24 to 32 min from the trap column. The online column-switching valve system was configured as shown in **Figure 2** and the valve was switched to position 2 at 22 min following injection of the sample, which allowed for the unmodified 2'-deoxynucleosides plus any other matrix impurities that may be present to be eluted to waste, while PAHDE-dN were back-flushed onto the analytical column for analysis using the mass spectrometer.

The enzymatically hydrolysed PAH-dihydrodiol epoxide modified calf thymus DNA samples were analysed using SRM with transitions derived from the LC-MS/MS CID product ion spectra described above. Typical online column-switching LC-MS/MS SRM ion chromatograms are shown in **Figure 5**. Multiple peaks corresponding to structural stereoisomers were detected for each PAHDE-dN adduct since the calf thymus DNA was treated with racemic mixtures of PAH-dihydrodiol epoxides. No observable peaks were detected in enzymatically hydrolysed control calf thymus DNA that was not exposed to any of the PAH-dihydrodiol epoxides. The relative proportion of PAH-dihydrodiol epoxide adducts formed with the different 2'-deoxynucleosides was estimated from the peak areas for each adduct. For B[b]FDE

adduct formation with dG (98.7%) > dC (0.8%) > dA (0.5%). For DB[*a,l*]PDE adduct formation with dG (66.0%) > dA (30.4%) > dC (3.7%). For DB[*a,h*]ADE adduct formation with dG (99.3%) > dA (0.6%) > dC (0.1%). For B[*a*]PDE adduct formation with dG (99.4%) > dA (0.6%) > dC (0.07%).

Comparison of different mass spectrometric operating modes for the analysis of PAHDE-dN adduct mixtures

In order to compare different mass spectrometric operating modes the mixture of the enzymatically hydrolysed PAH-dihydrodiol epoxide modified calf thymus DNA samples was analysed using optimised SRM transitions for the most abundant product ions which included transitions relating to the product ions derived from the PAH moiety (**Figure 6A**), SRM transitions resulting from the neutral loss of 116 u corresponding to the loss of 2'-deoxyribose (**Figure 6B**) and CNL scanning for the neutral loss of 116 u (**Figure 6C**). The data obtained were processed using automated peak picking and integration via XCMS software. The results were compared with manual integration for validation. Both sets of peak integrations were comparable (not shown), therefore proving that the less time-consuming automated data handling is a viable method for DNA adductomic analyses. The mass spectrometer signal response (peak area) for each DNA adduct was corrected using a [¹⁵N₅]B[*a*]PDE-N²-dG stable isotope internal standard which was previously synthesised.³³ Previous analysis of the [¹⁵N₅]B[*a*]PDE-N²-dG standard using isocratic elution revealed the presence of two stereoisomer peaks. However the current gradient elution employed did not resolve the two peaks and a single peak was observed.³³ The PAH DNA adduct analysis data were visualised as an adductomic map where the *x*-axis represents the retention time and the *y*-axis represents the *m/z* ratio for each PAH DNA adduct detected. The size

of the bubble on the map is directly proportional to the peak area for each adduct detected which has been normalised to the peak area of the $[^{15}\text{N}_5]\text{B}[a]\text{PDE-N}^2\text{-dG}$ stable isotope internal standard. Comparison of the DNA adductomic map profiles of adducted 2'-deoxynucleoside peaks detected using SRM transitions resulting from the neutral loss of 116 u (**Figure 6B**) and CNL (**Figure 6C**) showed the presence of additional ions which were also undetected in SRM transitions optimised for the most abundant product ions (**Figure 6A**). The most prominent additional ion was detected at m/z 640 (retention time 37.4 min, (labelled x)) following CNL analysis (**Figure 6C**). The use of optimised SRM transitions for the most abundant product ions resulted in a greater signal response for the detection of $\text{DB}[a,l]\text{PDE-N}^6\text{-dA}$ and $\text{DB}[a,l]\text{PDE-N}^4\text{-dC}$ adducts when compared to detection using SRM transitions resulting from the neutral loss of 116 u and CNL (**Figure 6A**).

Discussion

Humans in the course of their everyday lives are exposed to mixtures of PAHs which may interact with each other and ultimately lead to alterations in their individual carcinogenic activity. The ability to simultaneously detect multiple PAH derived DNA adducts would allow for the improved assessment of exposure and potentially offer a mechanistic insight into the carcinogenic process following exposure to mixtures of PAHs. The importance of determining multiple PAH DNA adducts rather than single adducts was highlighted by Beland *et al.* who reported that for mice fed a diet containing coal tar, the B[a]PDE-N²-dG adduct determined by LC-M/MS only represented a small proportion of the total adduct burden in lung DNA as determined by ³²P postlabelling. A similar observation was made for the analysis of human lung DNA samples. For only 1 out of 26 DNA samples showing the presence of PAH DNA adducts as determined by ³²P-postlabelling was the B[a]PDE-N²-dG adduct detected by LC-MS/MS, indicating that the other adducts are derived from different PAHs ³¹. These findings highlight the fact that humans are exposed to mixtures of PAHs. Therefore the development of a targeted DNA adductomic approach for the assessment of exposure to mixtures of PAHs would be highly advantageous. Kanaly *et al.* have proposed a DNA adductome approach using a series of SRM transitions totalling 374 for monitoring the neutral loss of 2'-deoxyribose. The diverse structural nature of DNA adducts, ranging from small hydrophilic modifications such as 8-oxo-7,8-dihydro-2'-deoxyguanosine adducts to larger (bulky) more hydrophobic PAH adducts, represents a chromatographic challenge for the adequate separation of such adducts in a single chromatography run. ^{36, 37} An improved approach would be to develop more focused methods for different chemical classes of DNA adducts providing better chromatographic separation and sensitivity. Examples include

recently developed LC-MS/MS methods for the detection of different oxidative DNA damage adducts and hydrophobic DNA adducts.⁴⁸⁻⁵¹ In the present study we investigated the use of online column-switching LC-MS/MS methodology for the detection of PAH derived DNA adducts which have characteristic highly hydrophobic properties.

Initial experiments focused on obtaining DNA adduct product ion spectra for calf thymus DNA samples reacted *in vitro* with the different *anti*-PAH-dihydrodiol epoxide metabolites of B[b]F, DB[a,l]P, DB[a,h]A and B[a]P. LC-ESI-MS/MS CID analysis was performed for the predicted $[M+H]^+$ molecular ion of the exocyclic amino group PAH-dihydrodiol epoxide adduct for each 2'-deoxynucleosides following enzymatic hydrolysis of the PAH-dihydrodiol epoxide modified DNA. The *anti*-PAH-dihydrodiol epoxide adducts of B[b]F, DB[a,l]P and DB[a,h]A and B[a]P showed a common fragmentation with one of the major product ions detected corresponding to the adducted base resulting from the cleavage of the glycosidic bond and the neutral loss of 2'-deoxyribose. In contrast the DB[a,l]PDE-N⁶-dA adduct showed a different fragmentation pattern with negligible formation of the adducted base product ion. Instead ions corresponding to the PAH moiety dominated the product ion spectrum. This may be explained by differences in the structural conformation of DB[a,l]PDE adducts which are derived from a parent PAH that demonstrates a considerable molecular distortion due to atomic overcrowding in the fjord region⁵²

The data obtained from the product ion spectra were used to select specific SRM transitions for the different PAHDE-dN adducts that in turn were used to develop an online column-switching LC-MS/MS for the analysis of an enzymatically hydrolysed

PAH-dihydrodiol epoxide modified DNA mixture. Online column-switching confers the advantage that such an enzymatically hydrolysed DNA sample can be analysed directly by LC-MS/MS without the requirement for off-line pre-purification of the DNA adduct from the unmodified 2'-deoxynucleosides as well as removing any other impurities that may be present. The PAHDE-dN adducts are retained on the trap column whilst unmodified 2'-deoxynucleosides and any hydrophilic adducted 2'-deoxynucleosides are eluted to waste, after which the switching valve diverts the flow from the trap column into the analytical column, which is connected to the mass spectrometer. This approach not only provides the advantage of increased sample throughput but also a substantial reduction in matrix effects and concomitant ionisation suppression. Matrix effects may arise by competition for charge, or increased droplet viscosity and surface tension from high concentrations of co-eluting, interfering compounds for the formation of gas phase ions in the electrospray source.

⁵³ In general, similar SRM results monitoring for $[M+H-116]^+$ transitions were obtained when compared to CNL scanning for the neutral loss of the 2'-deoxyribose. However, CNL afforded the detection of additional minor ions including a more prominent unidentified ion at m/z 640. The identity of the majority of these extra ions may be attributed to solvent adducts and not related to ^{13}C isotope $[M+1]^+$ ions, as the automated peak picking software was set to exclude these peaks. Therefore careful attention is required for the interpretation of CNL data. The advantage of CNL is that it allows for the screening of unknown adducts. However, in the case of PAH DNA adducts this approach does not allow for the monitoring of all DNA adducts that are formed as exemplified by DB[a,l]PDE-N⁶-dA. Significant detection of DB[a,l]PDE-N⁶-dA was only possible using SRM transitions for the product ions derived from the PAH moiety which were the most abundant. It is generally found that anti-dihydrodiol

epoxides of planar PAHs such as benzo[a]anthracene and B[a]P react preferentially with 2'-deoxyguanosine^{14, 54} whereas those derived from non-planar PAHs such as benzo[c]phenanthrene and DB[a,l]P also react to a major extent with 2'-deoxyadenosine.^{14, 55, 56} Accordingly, the major adduct observed for treatment of calf thymus DNA with the *anti*-dihydrodiol epoxides of the planar PAHs, B[b]F, DB[a,h]A and B[a]P was derived from the reaction with guanine. In contrast treatment with DB[a,l]PDE resulted in significant formation of adenine adducts. More than one peak was observed for each adduct which corresponded to different diastereoisomers resulting from treatment of calf thymus DNA with racemic mixtures of PAH-dihydrodiol epoxides.^{14, 56} The addition of the [¹⁵N₅]B[a]PDE-N²-dG stable isotope internal standard allowed for the determination of the relative level of each DNA adduct and also correction of any variations of the mass spectrometer signal response between analyses. Ideally stable isotope internal standards are required for each adduct to allow accurate quantitation of the absolute levels of the different DNA adducts which is difficult to achieve given the multi-step routes quite often involved in their chemical syntheses and the resulting limited yields producing amounts being inadequate to permit application in routine analysis. A possible compromise would be to use three stable isotope labelled internal standards eluting at different sections of the chromatographic run, thus accounting for regional differences in matrix effects along the chromatogram.

It should be noted that the work presented here focuses on the formation of DNA adducts following metabolic activation of PAHs to dihydrodiol epoxides and reaction with the exocyclic amino groups of different 2'-deoxynucleosides. There is the possibility for other types of PAH DNA adducts to be formed via alternate metabolic

pathways. For example, for B[a]P an alternate route for activation involves metabolism by cytochrome P450 and/or peroxidases that leads to the formation of radical cations following one electron oxidation. These can form adducts at the C-8 or N-7 positions of guanine as well as the N-3 or N-7 positions of adenine. These adducts are very unstable compared to those formed with the exocyclic amino group of guanine and adenine and are rapidly lost from the DNA by depurination.⁵⁷ Another pathway involves dihydrodiol dehydrogenase (an aldo-keto reductase) which results in the oxidation of benzo[a]pyrene-7,8-dihydrodiol to a catechol. This catechol undergoes auto-oxidation to form a reactive *o*-quinone, benzo[a]pyrene-7,8-quinone, that reacts with DNA to form both stable and depurinating adducts⁵⁸. Obviously the depurinating adducts would not be detected by the current approach since it relies on cleavage of the glycosidic bond. However, we have obtained some preliminary product ion spectra showing that the reaction of benzo[a]pyrene-7,8-quinone with 2'-deoxyguanosine results in adducts having a major product ion corresponding to the adducted base formed by the neutral loss of 2'-deoxyribose (Dr. Gonalo Gamboa da Costa, National Center for Toxicological Research, Arkansas, US, personnel communication). Furthermore, consideration should be given to the complex chemical nature of environmental contaminants that also contain nitrated PAHs such as the nitroketone 3-nitrobenzanthrone, which is an extremely potent mutagen present in the exhaust of diesel engines and in airborne particulate matter. We have recently shown that the 3-nitrobenzanthrone adducts of 2'-deoxyguanosine generate major product ions corresponding to the adducted base resulting from the neutral loss of 2'-deoxyribose and would be amenable to detection by the current approach.⁵⁹

The newly developed methodology will initially be applied to *in vitro* studies where cells will be dosed with mixtures of PAHs in the presence or absence of cancer chemopreventative agents. However, improvements in sensitivity of the online column-switching LC-ESI-MS/MS approach for the detection of PAH DNA adducts are required to make it applicable for analysis of environmentally exposed human samples, where sample quantity is limited and total PAH adduct levels in white blood cell DNA have been reported to be as low as 1-36/10⁸ unmodified DNA bases using the ³²P postlabelling technique ⁵. Increased sensitivity can be obtained by the use of capillary or nano LC coupled to micro- or nanoelectrospray ionisation mass spectrometry. The recently developed microfluidic based technique of LC/chip-MS/MS should allow for increased sensitivity. The LC/chip methodology has been applied to the detection of 2'-deoxyguanosine adducts formed by 4-aminobiphenyl with a detection limit of 20 amol and requiring only 1.25 µg of DNA. ⁶⁰ An alternative approach would be to utilise the new generation of high resolution mass spectrometers which can be operated in full scan mode, and therefore would not require MS/MS optimisation for each DNA adduct and show comparable sensitivity to triple quadrupole instruments making them highly amenable for DNA adduct screening. These instruments provide high mass accuracy coupled with very rapid scan times, offering the potential to perform non-targeted analysis without prior knowledge of the DNA adduct identity. ^{61, 62}

In conclusion different PAH DNA adducts can be detected by employing SRM [M+H-116]⁺ transitions or by CNL scanning. However optimised SRM transitions for the PAH moiety product ion are required to detect certain PAH DNA adducts with sufficient sensitivity. For the development of DNA adductomic approaches, we

propose the use of optimised SRM transitions to achieve high sensitivity and PAH DNA adduct coverage. CNL should be incorporated into the method development to map for unexpected product ions. An online column-switching LC-MS/MS method for the profiling of exposure to PAH mixtures has been developed. The implementation of online column-switching and software-based peak picking and integration makes the method suitable for high throughput analyses. The method will allow the assessment of additive, synergistic or antagonistic effects of mixtures of different PAHs with regards to the formation of DNA adducts.

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Figure Legends

Figure 1 Structures of the PAH-dihydrodiol epoxides and PAHDE-dN adducts.

Figure 2 Schematic representation of the online column-switching system.

Figure 3 Typical LC-MS/MS CID product ion spectra for the $[M+H]^+$ 2'-deoxynucleoside adducts formed following the treatment of calf thymus DNA with PAH-dihydrodiol epoxides (A) B[b]FDE-N²-dG m/z 570 (10 μ g) (B) B[b]FDE-N⁶-dA m/z 554 (30 μ g) (C) B[b]FDE-N⁴-dC m/z 530 (40 μ g) (D) DB[a,l]PDE-N²-dG m/z 620 (10 μ g) (E) DB[a,l]PDE-N⁶-dA m/z 604 (10 μ g) (F) DB[a,l]PDE-N⁴-dC m/z 580 (10 μ g) (G) DB[a,h]ADE-N²-dG m/z 596 (10 μ g) (H) DB[a,h]ADE-N⁶-dA m/z 580 (30 μ g) (I) DB[a,h]ADE-N⁴-dC m/z 556 (50 μ g) (J) B[a]PDE-N²-dG m/z 570 (10 μ g) (K) B[a]PDE-N⁶-dA m/z 554 (30 μ g) (L) B[a]PDE-N⁴-dC m/z 530 (50 μ g). The values in brackets represent the amount of enzymatically hydrolysed DNA that was analysed on column. The trap column was eluted using a gradient with solvent A, 0.1% formic acid and solvent B, acetonitrile at a flow rate of 120 μ L/min.

Figure 4 Summary of the positive ESI-MS/MS CID product ions formed for the different PAHDE-dN adducts.

Figure 5 Typical online column-switching LC-MS/MS SRM ion chromatograms for the analysis of 2'-deoxynucleoside adducts following the treatment of calf thymus DNA with (A) B[b]FDE(10 μ g) (B) DB[a,l]PDE (10 μ g) (C) DB[a,h]ADE (10 μ g) and (D) B[a]PDE(5 μ g). The values in brackets represent the amount of enzymatically

hydrolysed DNA that was analysed. The SRM transitions monitored for each PAHDE-dN adduct are shown on the figure. The trap column was eluted using a gradient with solvent A, 0.1% formic acid and solvent B, acetonitrile at a flow rate of 120 μ L/min. The analytical column was eluted isocratically with 0.1% formic acid/acetonitrile (70:30, v/v) at a flow rate of 120 μ L/min.

Figure 6 DNA adductomic maps and typical online column-switching LC-MS/MS total ion chromatograms for the analysis of 2'-deoxynucleoside adducts in a mixture consisting of a combination of calf thymus DNA modified with different PAH-dihydrodiol epoxides using (A) SRM transitions for the most abundant product ions (B) SRM transitions resulting from the neutral loss of 116 u and (C) CNL scanning for the neutral loss of 116 u. An equivalent of 10 μ g of hydrolysed DNA was analysed for each PAH-dihydrodiol epoxide treatment. The trap column was eluted using a gradient with solvent A, 0.1% formic acid and solvent B, acetonitrile at a flow rate of 120 μ L/min. The analytical column was eluted isocratically with 0.1% formic acid/acetonitrile (70:30, v/v) at a flow rate of 120 μ L/min.

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Figures

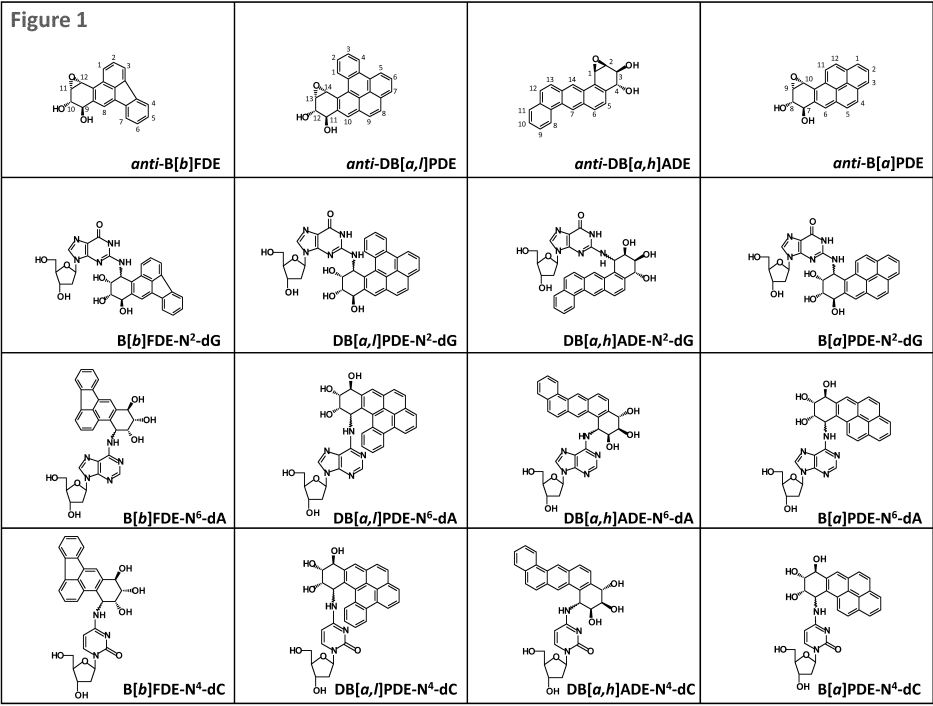


Figure 1.

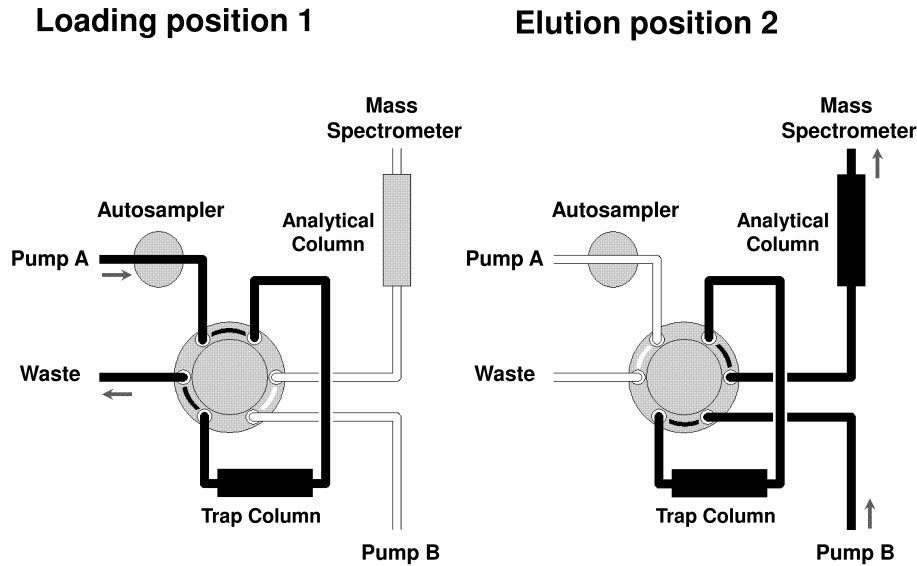


Figure 2

Figure 2.

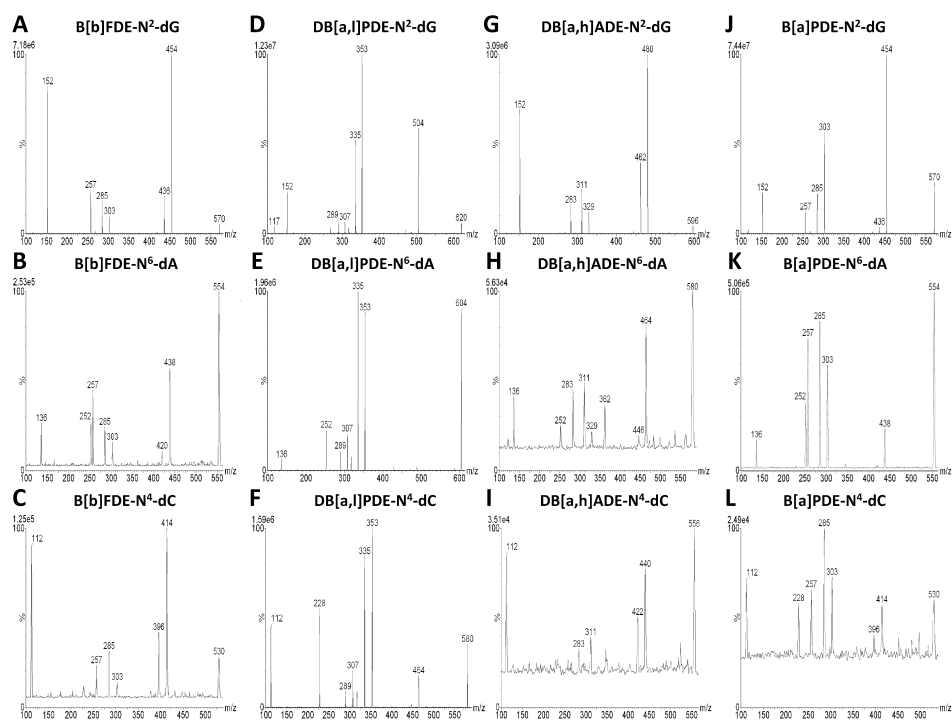


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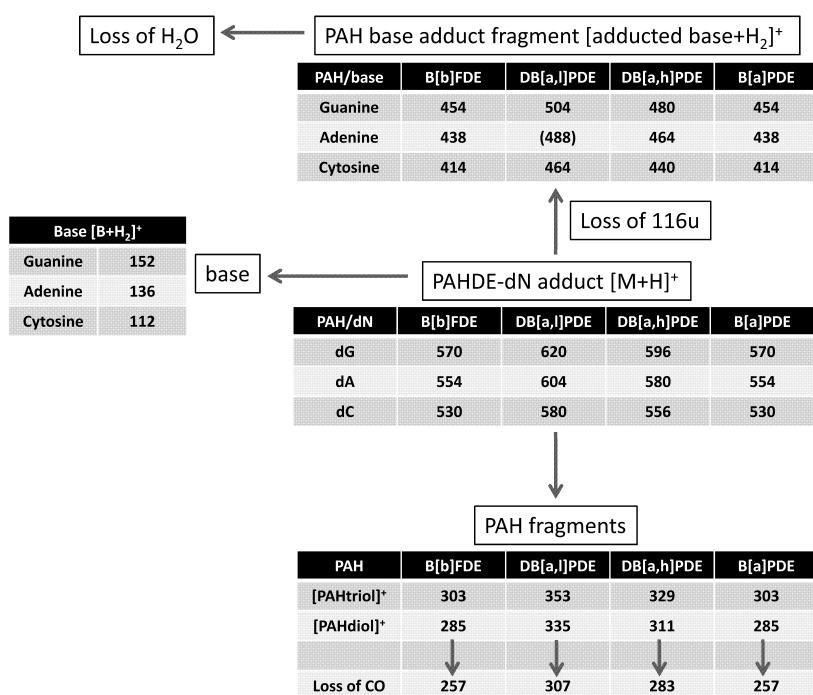


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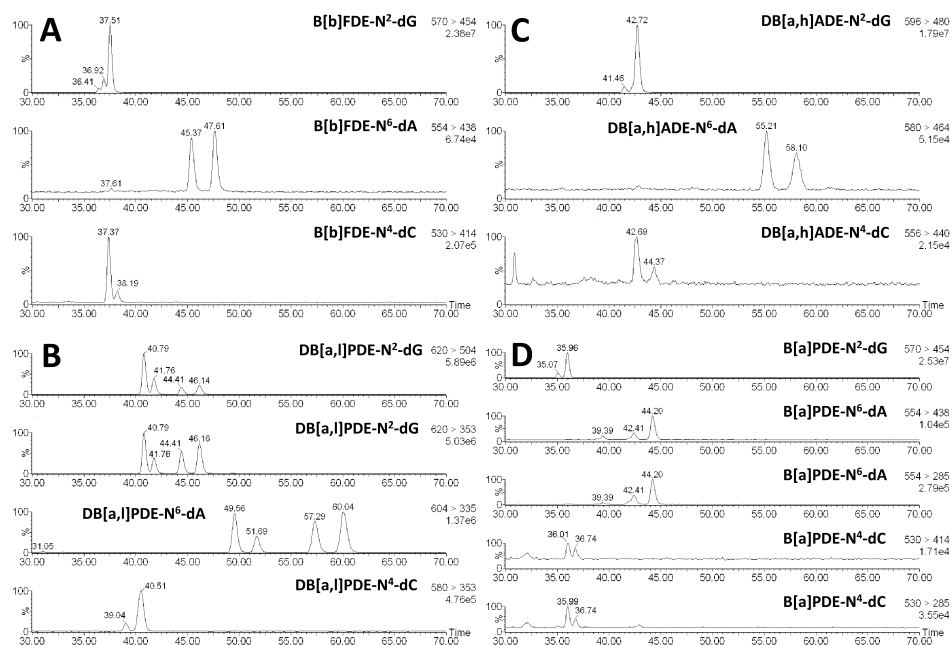


Figure 5

Figure 5.

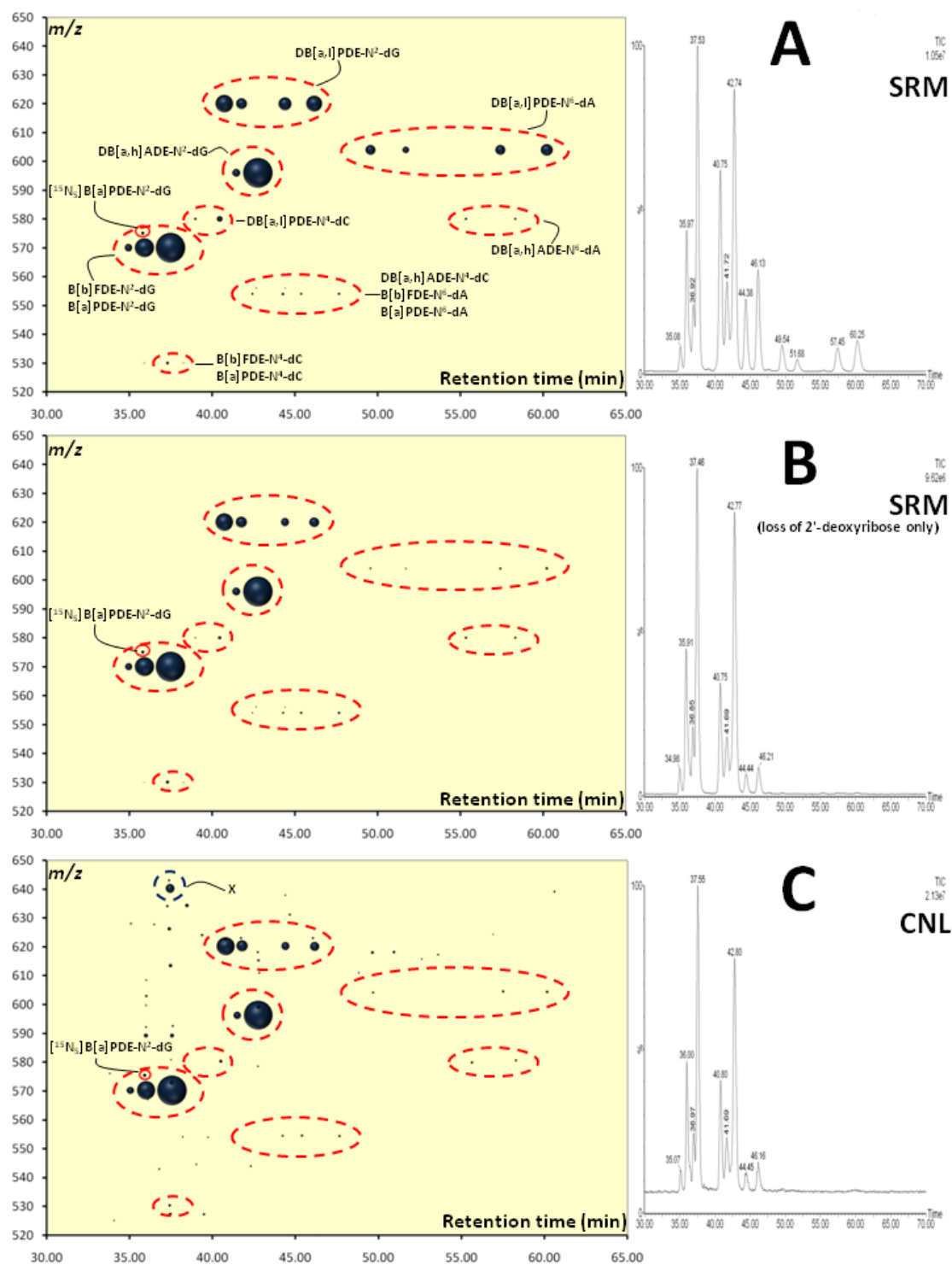


Figure 6.