Adenine derivatization for LC-MS/MS epigenetic DNA modifications studies on monocytic THP-1 cells exposed to reference particulate matter

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Abstract

The aim of this study was to explore the impact of three different standard reference particulate matter (ERM-CZ100, SRM-1649, and SRM-2975) on epigenetic DNA modifications including cytosine methylation, cytosine hydroxymethylation, and adenine methylation. For the determination of low levels of adenine methylation, we developed and applied a novel DNA nucleobase chemical derivatization and combined it with liquid chromatography tandem mass spectrometry. The developed method was applied for the analysis of epigenetic modifications in monocytic THP-1 cells exposed to the three different reference particulate matter for 24 h and 48 h. The mass fraction of epigenetic active elements As, Cd, and Cr was analyzed by inductively coupled plasma mass spectrometry. The exposure to fine dust ERM-CZ100 and urban dust SRM-1649 decreased cytosine methylation after 24 h exposure, whereas all 3 p.m. increased cytosine hydoxymethylation following 24 h exposure, and the epigenetic effects induced by SRM-1649 and diesel SRM-2975 were persistent up to 48 h exposure. The road tunnel dust ERM-CZ100 significantly increased adenine methylation following the shorter exposure time. Two-dimensional scatters analysis between different epigenetic DNA modifications were used to depict a significantly negative correlation between cytosine methylation and cytosine hydroxymethylation supporting their possible functional relationship. Metals and polycyclic aromatic hydrocarbons differently shapes epigenetic DNA modifications.

Keywords: Cytosine methylation; Cytosine hydroxymethylation; Adenine methylation; Particulate matter; LC-MS/MS; Chemical derivatization

1 Introduction

Exposure to particulate matter (PM) is associated with an increasing prevalence and exacerbation of respiratory diseases and cardiovascular risks [1,2]. The cellular damages induced by PM include the generation of reactive oxygen species (ROS), which can severely disturb the balance between antioxidants and free radicals, which can oxidize macromolecules, namely DNA, lipids, and proteins. PM itself can cause many adverse effects, and additionally, numerous chemicals that are adsorbed on the surface of the particles can act as harmful pollutants. A very prominent class of such pollutants are polycyclic aromatic hydrocarbons (PAHs), such as benzo[a]pyrene (B[a]P), pyrene,

fluoranthene and others. PAHs can be metabolized by cytochromes p450 (CYPs) enzymes resulting in dione-derivates or diol epoxide-derivates. These chemically activated substances can then form PAH-glutathione (PAH-GSH) adducts or PAH-DNA adducts which can cause G-T transversion [3,4]. Investigations of fine PM from different combustion and non-combustion sources showed that diesel exhaust particles had the highest toxicological potential compared with other PM such as biomass and coal combustion or gasoline exhaust PM [5]. A comparison between particles of different size collected in traffic area showed that fine particles (<2.5 μm) were more cytotoxic than coarse particles (2.5–10 μm) [6]. The results of the mentioned studies show that the PM toxicity depends on multiple physico-chemical characteristics like particle size and chemical identity.

Previous studies on PM induced-effects mainly focused on cytotoxicity, genotoxicity, and oxidative-stress *in vivo* or *in vitro*. In contrast, the impact of PM exposure on epigenetic DNA modifications is studied to a much lesser extent. Cytosine methylation (5 mC) and cytosine hydroxymethylation (5 hmC) are widely known as two important epigenetic DNA markers because they have a significant impact on gene regulation [7]. The abnormal alterations of 5 mC and 5 hmC levels are linked to different pathologies, e.g. cancer, and can be used as diagnostic or prognostic indicators [8–10]. However, much less knowledge is available for other epigenetic changes of nucleobases like adenine. Recently, Sun et al. reported that N6-methyladenine may be a potential epigenetic marker associated with gene expression in eukaryotes [11]. Until now, the research about epigenetic DNA modifications caused by PM exposure has mainly focused on cytosine methylation and cytosine hydroxymethylation. For instance, Janssen et al. found that global cytosine hypomethylation levels in placental tissues were associated with residential PM exposure [12]. Bellavia and co-workers compared the effects of different sizes of PM collected from downtown streets (fine <2.5 μm; coarse 2.5–10 μm) on blood DNA cytosine methylation and found decreased levels of 5 mC level after exposure to fine PM [13]. Nys et al. showed that both ambient PM_{10} and $PM_{2.5}$ had negative associations with 5-hmdC levels in human buccal cells [14].

The impact of PM on adenine methylation is still unknown and understanding the effects of PM exposure on complex epigenetic DNA modifications and their endogenous relations will help to understand how PM affects cell metabolism and functions. Since the expected concentration levels of methylated adenine in eukaryote cell DNA are very low, a sensitive and reliable analytical method for their quantification is an indispensable tool. One possibility to improve the detection sensitivity is the chemical derivatization prior to chromatographic separation and mass spectrometric detection. This was shown in recent works where chemical derivatization combined with LC-MS/MS was applied for the analysis of cytosine derivatives including 5-methylcytosine and 5-hydroxymethylcytosine. For instance, those methods used derivatives containing a bromoacetone functional group, which specifically reacts with cytosine on the 4-N and 3-N position forming penta-cylic derivatives [15–17]. Guo et al. used a different derivative containing an anhydride group, which reacts with the primary amine group on the 4-N position of cytosine [18]. After derivatization both the retention behavior of the derivatives during liquid chromatography and the ionization efficiency of derivatives in the MS-source were significantly improved. The mentioned methods did not use internal standards, even though it is widely accepted that the use of internal standards with similar physical and chemical properties as the target analytes is indispensable for a reliable quantification in LC-MS/MS. We used 2-bromo-4′-phenylacetophenone (BPAP) as reagent for the derivatization of 2′-deoxycytidine (dC), 5-methyl-2′-dexoycytidine (5-mdC), and 5-hydroxymethyl-2′-deoxycytidine (5-hmdC). BPAP also reacts with adenine (Ade) and N6-methyladenine (6 mA) forming 3-N derivatives (minor product) and 9-N derivatives (major product, used for quantification) [19]. To further improve the reliability of the method, substituted internal standards were synthesized by using 2-bromo-2′-acetonaphthone (BAN) reagent for the derivatization of 5-mdC, Ade, and 6 mAde. The LC-MS methods were validated and used for the analysis of epigenetic DNA modifications in THP-1 cells. THP-1 cells were chosen because they are human monocytic cells and they offer a very broad application range for example as a cancer cell model in *in vitro* studies [20], to study the macrophage differentiation processes [21], or to explore the macrophage physiological-related activities *in vitro* [22].

Aimed to comprehensively analyze epigenetic DNA modifications, the levels of 5-mdC, 5-hmdC, and 6 mA in THP-1 cells after exposure to three reference PM, fine dust ERM-CZ100, urban dust SRM-1649, and diesel SRM-2975 were determined. To further explore the need of specific positive controls for distinct DNA modifications, the epigenetic modifier 5-aza-2′-deoxycytidine (5-azadC), and the oxidizer tert-butyl hydroperoxide (TBHP) were used. Inductively coupled plasma mass spectrometry (ICP-MS) analysis of epigenetically relevant metals as Arsenic, Chromium and Cadmium [23] was performed to depict the role of different metal content in the reference materials.

2 Materials and methods

2.1 Reagents

2′-Deoxycytidine (dC) was purchased from Alfa Aesar (Kandel, Germany). 5-Methyl-2′-deoxycytidine (5-mdC) and 5-hydroxymethyl-2′-deoxycytidine (5-hmdC) were obtained from Jena Bioscience (Jena, Germany). Adenine (Ade), guanine (Gua), cytosine (Cyt), thymine (Thy), triethylamine (TEA), acetic acid, fine dust ERM-CZ100 (CZ100, ERM® certified reference material), BAN, BPBP, 5-azadC, and TBHP were bought from Sigma-Aldrich (Taufkirchen, Germany). Diesel PM SRM2975 (diesel PM, NIST® SRM® 2975) and urban dust SRM1649 (UD 1649, NIST® SRM® 1649) were from the National Institute of Standards and Technology (Gaithersburg, USA). N6-methyladenine (6 mA)

was obtained from Cayman Chemical Company (Michigan, USA). Dimethyl sulfoxide (DMSO) was purchased from Biomol (Hamburg, Germany). Formic acid was bought from VWR international (Leuven, Belgium). Monocytic THP-1 cells were from ECACC (No. 88081201). DNA Degradase Plus™ and DNA degradase™ were obtained from Zymo Research (Freiburg, Germany). Acetonitrile (ACN) and methanol (LC-MS grade) were from ChemSolute (Munich, Germany). LC-MS-grade water was generated by a Milli-Q Reference System from Merck Millipore (Burlington, Massachusetts, US). Nitrogen was provided by a liquid nitrogen tank (Linde, Munich, Germany).

2.2 Cell culture, particle exposure, and DNA extraction

THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI) medium (containing 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, and 1% penicillin/streptomycin solution) at 37 °C with 5% CO_2 in a humidified atmosphere. PM suspensions (1 mg/mL) were freshly prepared in medium without FBS by using an ultrasonic bath for 20 min directly before exposure. 5-azadC and tert-butyl hydroperoxide (1 mg/mL, TBHP) stock solutions were prepared in water and used as positive controls. The prepared suspensions were added into the medium to reach a final exposure concentration of 200 μg/mL for ERM-CZ100 and SRM1649, 40 μg/mL for SRM2975, 2.3 μg/mL (10 μM) of 5-aza-2′-deoxycytidine, or 9 μg/mL (10 μM) of tert-butyl hydroperoxide. THP-1 cells (0.12 million cells/cm^2 in T-75 flasks, 24 h prior to exposure) were treated with these PM suspensions or the positive control substances for 24 or 48 h. The established concentrations of PM caused 90% cell viability after 24 h exposure according to concentration-response pre-tests performed beforehand. Finally, THP-1 cells were harvested and washed with 10 mL of phosphate buffer solution. For DNA extraction, we followed a protocol published by Miller et al. [24] Quantity and purity of DNA were determined on a NanoDrop™ One/One^C (Thermo Scientific, Germany). Following 24 h and 48 h THP-1 exposures, approximately 300 μg and 500 μg of DNA were extracted, respectively, and three independent analytical runs per condition were performed.

2.3 Epigenetic DNA modifications

2.3.1 Liquid chromatography-mass spectrometry

LC-MS/MS measurements were carried out on an Agilent 1100 HPLC system consisting of a degasser, a binary pump, an autosampler, and a column compartment (Agilent Technologies, USA). Mass spectrometry was performed on a QTrap 4000 equipped with an ESI source (Sciex, USA). A Kinetex® C18 (2.6 μm, 100 Å, 100 × 2.1 mm i.d., Phenomenex, USA) analytical column was used for the analysis of methylated and hydroxymethylated cytosine. Mobile phase A was water. Mobile phase B was acetonitrile. Gradient elution with a flow of 250 μl/min was applied as follows: 0 min 65% A; in 7 min−50% A; from 8-17 min to 10% A; from 18-23 min to 65% A (equilibration). The eluent between 3 and 7 min was infused into the MS, the other gradient fractions were eluted to the waste. A Kinetex® F5 (2.6 μm, 100 Å, 100 × 3.0 mm i.d., Phenomenex, USA) was used for the analysis of adenine methylation. Mobile phase A was 0.1% acetic acid. Mobile phase B was acetonitrile. Gradient elution with a flow of 250 μl/min was used as follows: 0 min 85% A; in 18 min−50% A; from 19-28min to 10% A; from 29 to 34 min 85% A (equilibration). Only the elution fraction from 9 to 18 min was infused into the MS.

2.3.2 Nucleobases methylation and hydroxymethylation

DNA (30 μg) was dissolved in 60 μL of tris-EDTA (TE) buffer containing 1 μL of DNA degradase plus™ and 2 μL of reaction buffer for cytosine modification studies and in 0.5 μL of DNA degradase and 2.5 μL of reaction buffer (Zymo research, Germany) for adenine methylation studies, respectively. The mixture was kept at 37 °C for 24 h at 800 rpm on a Thermo mixer C (Eppendorf, Germany). Afterwards, the DNA hydrolysate was heated at 70 °C for 20 min to deactivate the enzymes. DNA hydrolysate was dried under a gentle nitrogen flow in a Vapotherm basis mobil I (Barkey, Germany) at room temperature. For cytosine methylation and hydroxymethylation studies 10 μL of BPAP solution (10 mg/mL) and 50 μL of ACN containing 0.04% of glacial acetic acid were added to hydrolysated DNA, and kept at 80 °C for 4 h at 850 rpm on a ThermoMixer C. Then the mixture was moved to a fridge and stored at −20 °C for 10 min. After centrifugation at 10 000 rpm for 10 min in a Heraeus™ Biofuge Pico® Centrifuge (Thermo Scientific, Germany), 30 μL of supernatant was mixed with 20 μL of internal standard solution (Section 1.2, supporting information) for the measurement of 5-hmdC. Another 3 μL of supernatant was diluted in a total volume of 300 μL ACN, and 30 μL of diluted supernatant was mixed with 20 μL of internal standard solution (Section 1.2, supporting information) for the measurement of dC and 5-mdC. Each sample was injected three times into the LC-MS system with an injection volume of 10 μL. Cytosine methylation and hydroxymethylation were calculated based on mole values as follows [25]:

$$\textit{Cytosine methylation \%} = \text{5-mdC}/(\text{5-mdC} + \textit{dC}) \times 100\% \quad (1)$$

$$\textit{Cytosine hydroxymethylation \%} = \text{5-hmdC}/(\text{5-mdC} + \textit{dC}) \times 100\% \quad (2)$$

For adenine methylation studies, 100 µL of 88% formic acid was added to hydrolysated DNA and kept at 20 °C for 2 h at 800 rpm. Thereafter the mixture was dried under a gentle nitrogen flow at room temperature and dissolved in 10 µL of DMSO. The solution was mixed with 50 µL of BPAP solution (4 mg/mL), 10 µL of TEA solution (2 mg/mL), and kept at 80 °C for 11 h at 1500 rpm on a ThermoMixer C. Afterwards the reaction solution was stored in a fridge at −20 °C for 10 min. The mixture was centrifuged at 10 000 rpm for 10 min in a Heraeus™ Biofuge Pico® Centrifuge after adding 30 µL of internal standard solution (Section 1.4, supporting information). Then 60 µL of supernatant was taken into a glass vial for the measurement of 6 mA. Another 3 µL of supernatant was diluted in a total volume of 300 µL ACN. 70 µL of this acetonitrile solution was mixed with 30 µL internal standard solution (Section 1.4, supporting information) for the measurement of Ade. Each sample was injected three times into the LC-MS system with an injection volume of 10 µL. Adenine methylation was calculated based on mole values as follows [26]:

$$Adenine\ methylation\ \% = 6\mathrm{mA}/Ade \times 100\% \tag{3}$$

2.4 ICP-MS

The Standard Reference NIST Materials SRM-1649, ERM-CZ100 and SRM-2975 have been weighed and acidified with a mixture of nitric acid (69%) and hydrogen peroxide (30%). Subsequently the acidified samples have been digested by a Microwave speedwave ENTRY (Berghof, Germany) and diluted to a final concentration of 13% of HNO_3. All the samples have been filtered through a 0.45 µm syringe filter and analyzed by an Agilent 7700 Series ICP-MS. Calibration standard curves of 1, 10, 100 and 300 µg/L for arsenic, cadmium and chromium have been used for the quantification. The calibration standard lines have been prepared from the initial calibration verification standard (Agilent, USA): 10 ppm for arsenic, cadmium and chromium in a matrix of 5% of nitric acid. In all samples 20 µg/L of scandium and rhodium have been spiked and were used as internal standards. For every samples 4 technical repetitions have been performed. The detection limits for arsenic, cadmium and chromium were estimated as <0.005 µg/L, <0.0017 µg/L, and 0.0069 µg/L, respectively.

2.5 Statistical analysis

The statistical data was processed by SPSS 20.0 software (SPSS, Inc). For clustered columns, the analysis of variance was calculated between the control and exposed groups. For two-dimensional scatters, linear regression, coefficient of determination, and p-value were evaluated between different epigenetic modifications. Data difference was considered to be significant when p-values were less than 0.05.

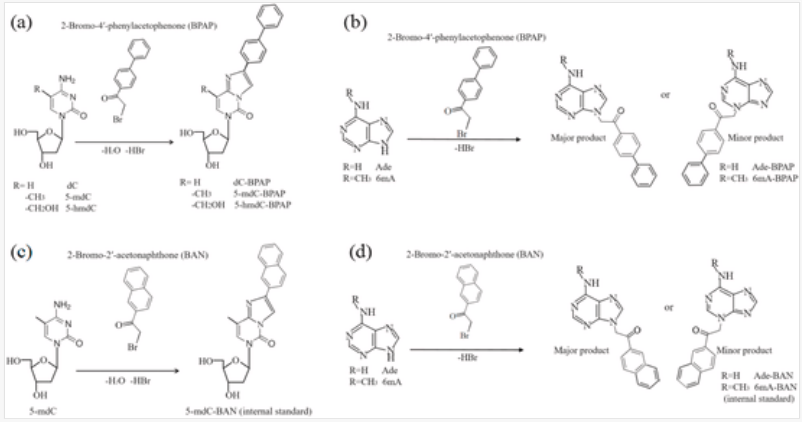
3 Results

3.1 Method development

The proposed reactions and mechanisms for chemical derivatization of cytosine and adenine are shown in Fig. 1 and – more detailed - in Fig. S1, supporting information. Chemical derivatizations of dC, 5-mdC, and 5-hmdC (Fig. 1a) were optimized concerning the following parameters: reaction time, concentration of BPAP, volume of acetic acid, and reaction temperature (Section 1.1, supporting information, and Fig. 2). The production of dC and 5-mdC derivatives reached an equilibrium after 4 h with 15 mg/mL of BPAP, which were then chosen as final reaction time and BPAP concentration. The generation of dC and 5-hmdC derivatives was most effective at 80 °C which was selected as the reaction temperature. If the volume of acetic acid was higher than 0.1 µL, the amount of 5-hmdC derivative decreased more than 80%. Finally, 0.02 µL of acetic acid was selected. In order to study adenine methylation levels, a novel chemical derivatization of 6 mA was developed. BPAP was used to react with adenine and 6 mA to generate Ade-BPAP and 6 mA-BPAP derivatives (Fig. 1b). Adenine chemical derivatization was then optimized considering the reaction time, the concentration of BPAP, the concentration of TEA, and the reaction temperature (Section 1.3, supporting information, and Fig. 3). The production of the adenine derivative reached an optimum at 11 h, 4 mg/mL, and 80 °C, which were selected as the optimized conditions. The adenine derivative was in equilibrium between 0.05 and 4 mg/mL of TEA. If the concentration of TEA was higher than 4 mg/mL, the production of the adenine derivative decreased about 20%. Finally, 2 mg/mL of TEA was selected. Similar to the chemical derivatization of dC, the reaction between 5-mdC and BAN, used to produce the internal standard 5-mdC-BAN, was also optimized in terms of reaction time, temperature and concentration of acetic acid and BAN (Fig. 1c). The resulting conditions were 2 h, 15 mg/mL of BAN, 0.1 µL of acetic acid, and 80 °C (Fig. S2, supporting information). The reaction time, temperature and concentrations of TEA and BAN for adenine and 6 mA derivatives, used as internal standards (Fig. 1d), were also optimized. Optimum conditions were 11 h, 2 mg/mL of BAN, 2 mg/mL of TEA, and 80 °C (Fig. S3, supporting information).

alt-text: Fig. 1

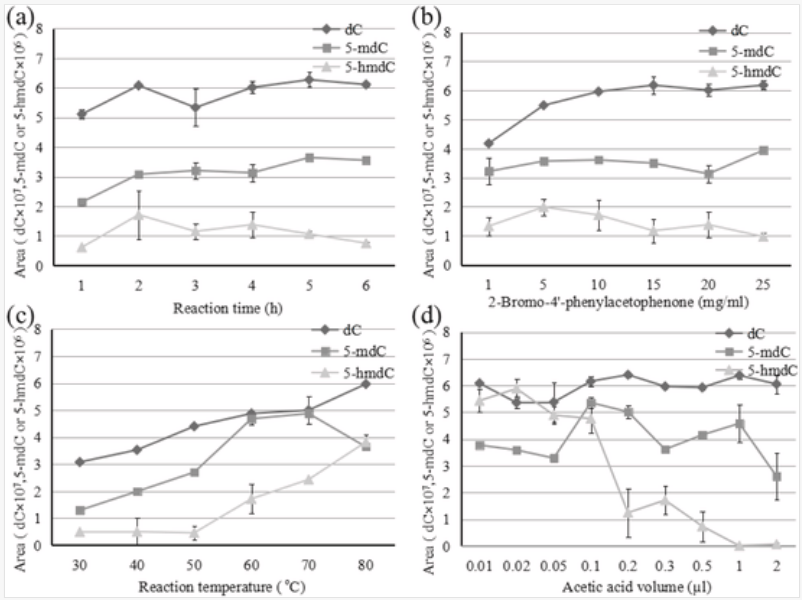
Fig. 1



Proposed reactions of (a) target analytes dC-BPAP, 5-mdC-BPAP, and 5-hmdC-BPAP (b) target analytes Ade-BPAP and 6 mA-BPAP (c) internal standard 5-mdC-BAN (d) internal standard Ade-BAN and 6 mA-BAN.

alt-text: Fig. 2

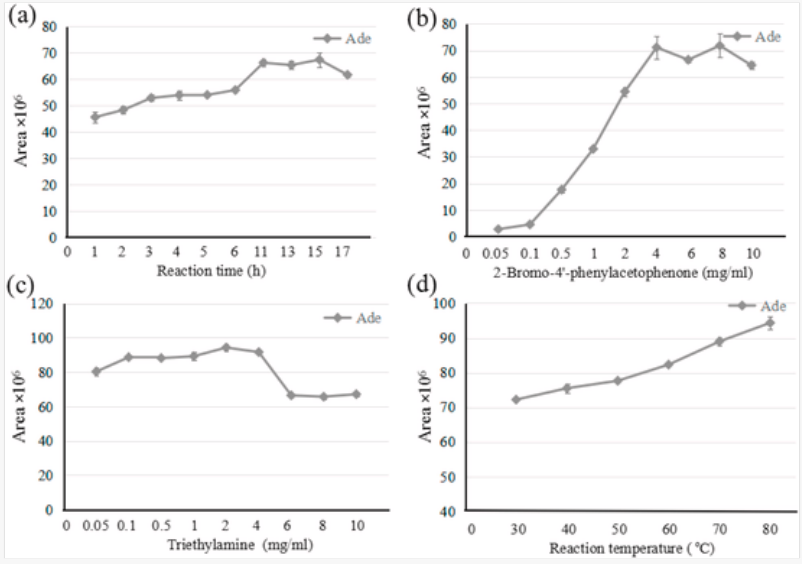
Fig. 2



Optimization of target analyte reactions between dC, 5-mdC, 5-hmdC, and BPAP, mean ± standard error (n = 3). (a) Reaction time with 20 mg/mL of BPAP solution, 0.3 μL of glacial acetic acid, and 80 °C; (b) Different concentrations of BPAP at constant 0.3 μL of glacial acetic acid, 4 h, and 80 °C; (c) Different temperatures with constant 10 mg/mL of BPAP, 0.3 μL of glacial acetic acid, and 4 h; (d) Different volumes of acetic acid with constant 10 mg/mL of BPAP, 4 h, and 80 °C.

alt-text: Fig. 3

Fig. 3



Optimization of target analyte reactions between Ade and BPAP, mean ± standard error (n = 3). (a) Reaction time at constant 6 mg/mL of BPAP, 6 mg/mL of TEA, and 80 °C; (b) Different concentrations of BPAP with 6 mg/mL of TEA for 11 h, and 80 °C; (c) Different concentrations of TEA at constant 4 mg/mL of BPAP for 11 h, and 80 °C; (d) Different reaction temperatures at constant 4 mg/mL of BPAP, 2 mg/mL of TEA, and 11 h.

3.2 MS characterization

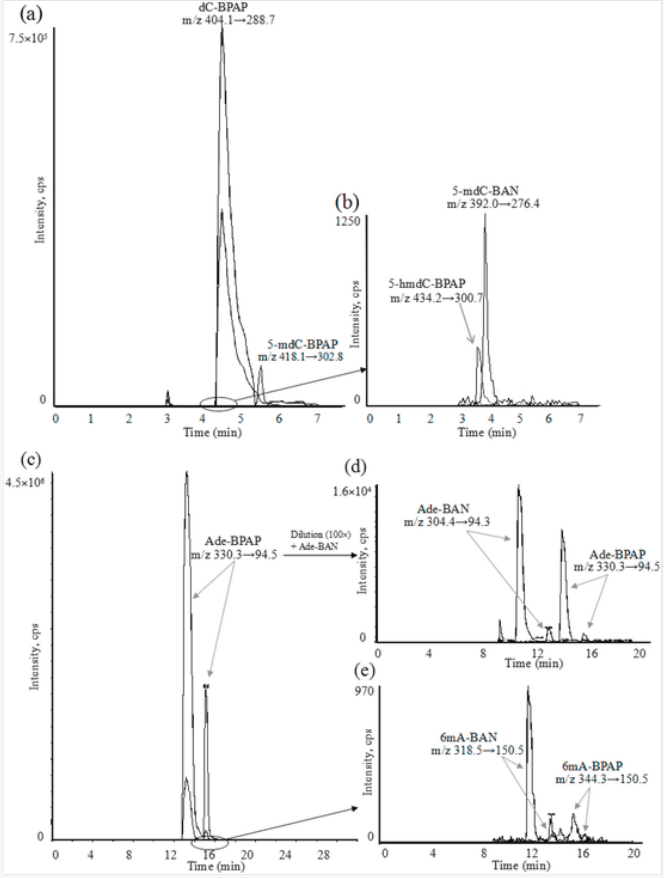
The parameters for mass spectrometric detection of each standard and its respective derivative were optimized, and the quantification was performed using multiple reaction monitoring (MRM) in the positive ion mode. For dC and its derivatives, the most intensive transition was the loss of deoxyribose moieties from nucleosides. For 5-hmdC and 5-hmdC derivatives, the fragment ions at m/z 142.2 and 318.9 were not stable and easily lost a water molecule further generating new fragment ions at m/z 124.1 and 300.7, respectively. For Ade and 6 mA, the typical fragment ions were at m/z 94.1 and m/z 119.1. Similarly, the derivatives had the most sensitive fragment ions at m/z 136.2 and m/z 150.4 which were [Ade+H]+and $[6\text{ mA} + H]^+$. For each analyte, two transitions were selected for the detection using MRM. The first transition was used for quantification and the second one was used for qualification. Tuning parameters are summarized in Table S1, supporting information. Representative mass spectra obtained by collision induced fragmentation are shown in Figs. S4 and S5, supporting information.

3.3 Method validation

Methods were validated considering linearity, accuracy, precision, and the instrument limits of detection and quantification (Section 1.5, supporting information). Calibration curves showed good linearity with $R^2 > 0.99$. Three concentration levels (low, med, and high) were selected to evaluate accuracy and precision. Comparison between derivatized and non-derivatized target molecules showed that both the chromatographic retention of the target analytes during liquid chromatography and the detection performance of the target analytes by MS were greatly improved after derivatization (Tables S2 and S3, supporting information). The methods were applied to the analysis of THP-1 cell samples after exposure experiments. Typical MRM traces are shown in Fig. 4. Ade and 6 mA formed two derivatives with BPAP and BAN, respectively, showing two peaks for Ade-BPAP, Ade-BAN, 6 mA-BPAP, and 6 mA-BAN in Fig. 4c–e. The chromatograms underline the capacity of the analytical methods to detect and quantify the low concentration of the target analytes.

alt-text: Fig. 4

Fig. 4



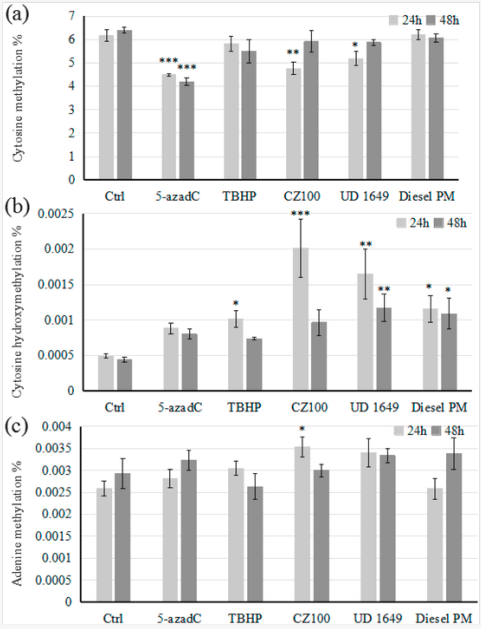
MRM-traces of (a) target analytes dC-BPAP, 5-mdC-BPAP, 5-hmdC-BPAP, and internal standard 5-mdC-BAN (b) target analyte 5-hmdC-BPAP and internal standard 5-mdC-BAN (c) target analytes Ade-BPAP and 6 mA-BPAP, and internal standard 6 mA-BAN (d) target analyte Ade-BPAP and internal standard Ade-BAN (e) target analyte 6 mA-BPAP and internal standard 6 mA-BAN. The analytes were determined in control samples.

3.4 Epigenetics modification in THP-1 cells

The background cytosine methylation in controls are about 6.3%. After 24 h exposure to CZ100 and UD 1649, the cytosine methylation significantly decreased to about 4.8% and 5.2%, respectively (Fig. 5a). However, the cytosine hypomethylation was restored after 48 h exposure. All 3 p.m. significantly increased cytosine hydroxymethylation by about 4-fold, 3.3-fold, and 2.4-fold of control levels after 24 h exposure to CZ100, UD 1649, and diesel PM, respectively (Fig. 5b). Even after 48 h exposure, increased cytosine hydroxymethylation was observed after exposure to UD 1649 and diesel PM with 2.7-fold and 2.5-fold of control levels (Fig. 5b). Significantly increased adenine methylation levels were observed after 24 h exposure to CZ100 while adenine methylation level was restored following 48 h exposures (Fig. 5c). Correlation analysis between different epigenetic modifications were performed, and a significantly negative correlation was found between cytosine methylation and cytosine hydroxymethylation (R^2 = 0.1776, p-value<0.001, Fig. 6a). In contrast, no significant relationships were found between cytosine methylation and adenine methylation (Fig. 6b).

alt-text: Fig. 5

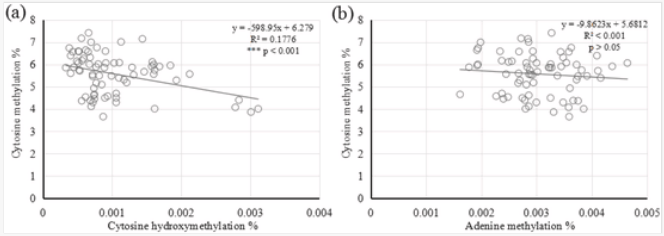
Fig. 5



Percentages of cytosine methylation (a) cytosine hydroxymethylation (b) adenine methylation (c) in untreated cells (Ctrl) and following 24 h or 48 h THP-1 exposures to 5-azadC (5-aza-2′-deoxycytidine), TBHP (tert-butyl hydroperoxide), CZ100 (ERM-CZ100), UD 1649 (urban dust SRM-1649), Diesel PM (diesel PM SRM-2975). Data are shown as mean ± standard error (n = 6), * = p-value < 0.05, ** = p-value < 0.01,*** = p-value < 0.001.

alt-text: Fig. 6

Fig. 6



Two-dimensional scatters between different epigenetic modifications based on six independent experiments for each exposure.

3.5 ICP-MS

The certified mass fraction values of elements are available for SRM-1649, ERM-CZ100, and SRM-2975, however the most epigenetic active elements As, Cd and Cr were not analyzed in SRM-2975 particles (diesel PM). The average mass concentration of As, Cd and Cr in the used reference PM samples was analyzed by ICP-MS and the results are shown in Table 1. As expected diesel PM SRM-2975 do not contain high content of metals, however a non-negligible concentration of Cr was found. The fine dust ERM-CZ100 contains the largest amount of chromium while the urban dust particles SRM-1649 are characterized for having the largest amount of arsenic. With respect to the total amount of considered epigenetic active elements, no significant differences can be observed between CZ-100 and UD-1649, however ERM CZ-100 contains higher total amount of elements with respect to the other tested reference PM.

alt-text: Table 1

Table 1

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Mass fraction values (mg/Kg) of As, Cd, and Cr as evaluated by ICP-MS in the present study and as reported in CZ-100 and UD-1649 NIST certificates, or in Ball et al. for diesel PM SRM-2975 [27]. *, not measured in our study.

	CZ-100	UD-1649	Diesel PM
As	8.58 ± 0.17	78.07 ± 1.42	1.24 ± 0.02

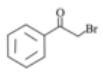
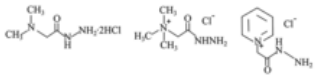
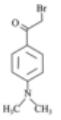
Cd	0.97 ± 0.02	27.52 ± 0.14	0.08 ± 0.01
Cr	238.13 ± 6.03	145.02 ± 1.57	4.22 ± 0.01
Co*	14.3	16.4 ± 0.4	0.1 ± 0.1
Cu*	462	223 ± 7	0.9 ± 0.6
Ni*	58 ± 7	166 ± 7	0.5 ± 0.7
Zn*	1240	1680 ± 40	16 ± 4
Fe*	38144	29800 ± 700	0.9
Total	≈40200	≈32200	≈24

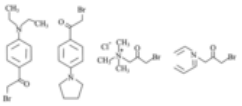
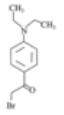
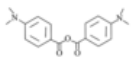
4 Discussion

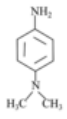
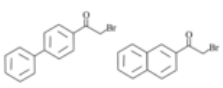
In the current study, we explored the impact of three standard reference PM on epigenetic DNA modifications (cytosine methylation, cytosine hydroxymethylation, and adenine methylation). A novel chemical derivatization method was developed for the analysis of 6 mA. To improve the retention behaviour of adenine and 6 mA in reversed phase liquid chromatography, we used BPAP to generate Ade-BPAP and 6 mA-BPAP derivatives in order to introduce a hydrophobic biphenyl group. The limits of detection (LOD) of all target analytes was also significantly improved compared to those found without derivatization. For instance, the LOD for 5-mdC and 5-hmdC decreased 2.5- and 5-fold respectively after derivatization, compared to the detection without proceeding derivatization (Table S2, supporting information) due to the improved ionization efficiency of the target analytes in the MS-source.

Until now, different derivative reagents have been used to analyze cytosine-related modifications in DNA and RNA. For instance, very low LODs for 5-mdC and 5-hmdC were obtained after derivatization with 2-bromo-1-(4-diethylamino-phenyl)-ethanone resulting in 0.04 fmol and 0.06 fmol, respectively [17]. Similarly, LODs values like 0.10 fmol for 5-mdC and 0.06 fmol for 5-hmdC were calculated by using 2-Bromo-1-(4-dimethylamino-phenyl)-ethanone [16]. In our study we used BPAP and BAN, and we reached LODs of 0.33 and 0.23 fmol for 5-mdC and 5-hmdC, respectively (Table 2). The observed difference can be attributed to both different derivatizing reagents and different instruments. In previous studies, the investigation of adenine methylation levels was mainly based on N6-methyl-2′-deoxyadenosine by using LC-MS/MS with LOD values ranging from 0.42 fmol to 20 fmol [28–30]. However, N6-methyl-2′-deoxyadenosine is expensive and a suitable internal standard for N6-methyl-2′-deoxyadenosine is difficult to obtain. In contrast, N6-methyladenine (6 mA) is cheap and thus adenine methylation studies based on N6-methyladenine evaluation are an ideal alternative approach. A disadvantage of using 6 mA is its poor retention in LC (on both reverse and normal phase chromatography) and its low mass spectrometric intensity. In this study, we could develop a novel derivatization method for 6 mA prior to LC leading to significantly improved retention behavior and increased mass spectrometric detectability. The LOD achieved (0.67 fmol) is the lowest reported detection limit for 6 mA applying LC-MS/MS. With the aim to increase the reliability of the quantification, suitable internal standards were synthesized (5-mdC-BAN for cytosine modification analysis; Ade-BAN and 6 mA-BAN for adenine modification) and successfully applied in our study.

Summary for the used derivative reagents for cytosine-related modifications.

Targets	Derivative reagent	LOD (dC, 5-mdC,5-hmdC, Ade, and 6mA)	Reference
cytidine; dC; 5-methylcytidine; 5-mdC;	 2-bromoacetophenone	15.3 fmol (dC) 22.7 fmol (5-mdC)	[15]
5-fodC; 5-cadC;	 Girard's D, T, P	no	[50]
5-mdC; 5-hmdC; 5-fodC; 5-cadC;		0.10 fmol (5-mdC) 0.06 fmol (5-hmdC)	[16]

Targets	Derivative reagent	LOD (dC, 5-mdC,5-hmdC, Ade, and 6mA)	Reference
5-methylcytidine; 5-hydroxymethylcytidine; 5-formylcytidine; 5-carboxycytidine;	 2-bromo-1-(4-diethylamino-phenyl)-ethanone 2-bromo-1-(4-1-pyrrolidinylphenyl)-ethanone 3-Bromoacetonyltrimethylammonium bromide ω-bromoacetonylpyridinium bromide	no	[51]
5-mdC; 5-hmdC; 5-fodC; 5-cadC; 5-methylcytidine; 5-hydroxymethylcytidine; 5-formylcytidine; 5-carboxycytidine;	 2-bromo-1-(4-diethylamino-phenyl)-ethanone	0.04 fmol (5-mdC) 0.06 fmol (5-hmdC)	[17]
5-mdC; 5-hmdC; 5-fodC; 5-cadC;	 4-(dimethylamino) benzoic anhydride	2.24 fmol (5-mdC) 2.53 fmol (5-hmdC)	[18]

Targets	Derivative reagent	LOD (dC, 5-mdC,5-hmdC, Ade, and 6mA)	Reference
cytidine 5′-mono- phosphate; 2′-deoxycytidine 5′-monophosphate; 5-methylcytidine 5′-monophosphate; 5-methyl-2′-deoxycytidine 5′-monophosphate;	 N,N-Dimethyl-p-phenylenediamine	no	[52]
dC; 5-mdC; 5-hmdC; Ade; 6mA;	 BPAP and BAN	0.22 fmol (dC) 0.33 fmol (5-mdC) 0.23 fmol (5-hmdC) 1.48 fmol (Ade) 0.67 fmol (6mA)	Current study

The application of optimized LC-MS methods including the novel chemical derivatization allowed a comprehensive evaluation of epigenetic DNA modifications induced by reference PM in monocytic THP-1 cells.

Global DNA hypomethylation is associated with aging and cancer [31], and it is therefore suggested to be a useful biomarker for carcinogenesis [32–34]. In the present study, we found that at non-cytotoxic conditions, both fine dust ERM-CZ100 and urban dust SRM-1649 significantly induced a transient cytosine hypomethylation and increased the levels of cytosine hydroxymethylation to a similar extent, while diesel SRM-2975 was irreversibly acting only on cytosine hydroxymethylation levels. These reference PM samples contain various epigenetically active chemical components such as the analyzed metals arsenic, cadmium and chromium, and also other elements and organic compounds like PAHs and nitro-PAHs (Table 1, Table S4 and material certificates). It was previously shown that exposure to arsenic induces a depletion of S-adenosylmethionine (SAM) and inhibits DNA methyltransferase gene expression (DNMT1 and DNMT3A) leading to DNA hypomethylation in HaCaT keratinocytes and a decreased enzymatic activity of DNA methyltransferase in rat liver TRL 1215 cells [35,36]. Cadmium is considered to be an epigenetic carcinogen perturbing DNA methylation levels via indirect mechanisms such as ROS generation and DNA repair inhibition while chromium inhibits histone-remodelling and alters gene expression via its genotoxic effects [37,38]. While higher levels of metal content in urban dust and fine dust materials can explain the higher efficacy in inducing hypomethylation, the higher content of nitro-PAHs in diesel SRM-2975 particles could explain its efficient and irreversible induction of cytosine hydroxymethylation. In fact, despite a 5 fold lower tested concentration of diesel PM with respect to the cell treatments with fine and urban dust PMs, the extremely high concentrations of nitro-PAHs, as reported in the material certificates, are differentiating diesel PM from urban dust and fine dust PM. This is related to freshly collected diesel combustion particles, not exposed to any atmospheric photo-oxidation reactions. *In vitro*, an increased amount of 5-hmdC after exposure to traffic-related fine PM and its organic extracts was also observed in neuroblastoma SH-SY5Y cells [39]. Interestingly, the authors showed that the induced DNA hydroxymethylation was oxidative-stress mediated and was involved in neuronal pathology. However, the oxidizer TBHP used in our study only reversibly induced increased 5-hmdC levels. A recent *in vivo* study in mice exposed to traffic-related PM (1 h daily for 3 months) showed decreased 5-hmdC levels in lung and liver but no effects in the kidney, and no significant differences on 5 mC levels in all analyzed internal organs [40]. In the latter study the 5mC/5-hmC ratio in the lung DNA of exposed mice increased suggesting that the loss of 5-hmC can be an early event in carcinogenesis [40]. Even if 5-hmdC levels depend on many factors, intensive studies showed that 5-hmdC is a stable epigenetic modification, and aberrant changes of 5-hmdC levels have been associated with diseases such as cancer and Rett syndrome [41,42]. An increase of 5-hmdC was, for example, found in the peripheral blood of patients with non-cancerous prostate diseases while decreased levels of 5-hmdC were observed in human colorectal carcinoma tissues, with respect to tumor-adjacent normal tissues, and in hepatocellular carcinoma tissues as well [16,43–45]. In the present study, we found a significant negative correlation between cytosine methylation and hydroxymethylation indicating a crosstalk between the two epigenetic mechanisms. In fact, TET proteins play a pivotal role in epigenetic processes through modification of 5 mC to 5-hmC which has a functional link with DNA elimination during genome rearrangements [46,47].

A reversibly increased level of 6 mA was observed only following fine dust ERM-CZ100 exposures and no effects were observed after exposure with the demethylating agent 5-aza-2′-deoxycytidine, or the oxidizing agent tert-butyl hydroperoxide, asking for specific adenine methylation related positive controls. Furthermore, we did not observe a correlation between cytosine and adenine methylation levels indicating that the two methylation processes are controlled by different mechanisms. The mechanism of adenine methylation modifications influenced by PM is not studied yet although some studies have shown associations between adenine methylation and different exogenous factors like environmental stress on mouse brains and the development of stress-induced neuropathology [48,49].

In summary, exposure to different PM reference materials, with distinct chemical composition, results in dissimilar epigenetic changes. Metal-rich PM exposures (ERM-CZ100 and SRM-1649) reversibly decreased cytosine methylation levels and increased adenine methylation content while all tested PM induced cytosine hydroxymethylation as observed, with smaller magnitude, following the treatments with the oxidizer TBHP. Oxidative stress is largely recognized as the major cause of many chronic diseases and plays a pivotal role in the pathogenesis of environmental lung diseases. The crosslinks between oxidative stress and epigenetic mechanisms need to be further investigated as well as the role of the chemical composition of different airborne PM in inducing genotoxicity and immunotoxicity.

5 Conclusions

In this study, we explored the impact of three reference PM on epigenetic DNA modifications in monocytic THP-1 cells. LC-MS/MS methods with preceding chemical derivatization of target analytes were optimized and a novel chemical derivatization for N6-methyladenine was developed. The derivatization significantly improved the retention behavior of the target analytes during chromatographic separation and increased the sensitivity of these targets during mass spectrometric detection. The methods were validated and subsequently applied for the quantification of epigenetic DNA modifications in monocytic THP-1 cellular DNA. Cell exposure to fine dust ERM-CZ100 and urban dust SRM-1649 decreased global cytosine methylation levels, while all tested PM increased cytosine hydroxymethylation. The two epigenetic processes were significantly negatively associated. A reversible increase of adenine methylation was observed after exposure to fine dust ERM-CZ100, which was independent compared to the observed cytosine methylation changes. Further studies are needed to investigate the epigenetic impact of PM exposure on human health, especially considering the different elemental/organic carbon content of PM as well as its metal content. An emphasis

of such studies should be the examination of PM originating from break wear or abrasion particles from e-cars at emission sources.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2021.114127.

Uncited references

[50–52].

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The corrections made in this section will be reviewed and approved by a journal production editor. The newly added/removed references and its citations will be reordered and rearranged by the production team.

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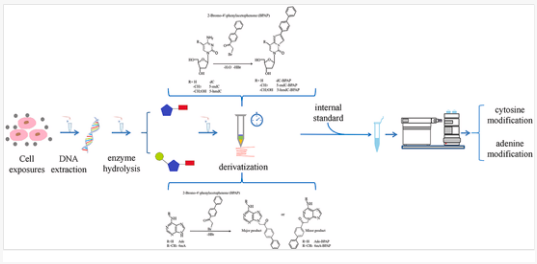
Graphical abstract

alt-text: Image 1



Highlights

- Development and validation of derivatization methods for epigenetic biomarkers.
- The epigenetic relevant elements As, Cd and Cr were analyzed by ICP-MS.
- NIST reference PM induced *in vitro* epigenetic changes in monocytic cells.

Appendix A Supplementary data

The following is the Supplementary data to this article:

Multimedia Component 1

Multimedia component 1

alt-text: Multimedia component 1

Queries and Answers

Q1

Query: Please confirm that the provided emails “dibucchianico@helmholtz-muenchen.de, lintelmann@helmholtz-muenchen.de” are the correct address for official communication, else provide an alternate e-mail address to replace the existing one, because private e-mail addresses should not be used in articles as the address for communication.

Answer: I confirm that the provided email are the correct address.

Q2

Query: Please ensure that affiliations, affiliation addresses, present addresses, and corresponding author's information are complete and accurate (street address, city, state if applicable, ZIP code if applicable, province if applicable, country, e-mail address, and telephone and fax numbers with country codes) and that all abbreviations are spelled out.

Answer: Information are correct.

Q3

Query: Have we correctly interpreted the following funding source(s) and country names you cited in your article: TEA, United States; China Scholarship Council, China; HFG, United States; Helmholtz Association, Germany; Thermo Scientific, United States; ACN, Australia?

Answer: Helmholtz Virtual Institute of Complex Molecular Systems in Environmental Health (HICE), Germany.
Helmholtz Association of German Research Centers (HFG), Germany.
aeroHEALTH Helmholtz International Lab, Germany and Israel.
China Scholarship Council, China.

Q4

Query: Uncited references: This section comprises references that occur in the reference list but not in the body of the text. Please position each reference in the text or, alternatively, delete it. Any reference not dealt with will be retained in this section. Thank you.

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Q5

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Q6

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