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Enhanced sensitivity for the determination of ambiphilic polyaromatic amines by LC–MS/MS after acetylation

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Abstract

A new method for the analysis of aminonitropyrenes and diaminopyrenes was developed for urine and hemoglobin samples using LC–MS/MS. A good separation by LC was only achieved after derivatization of the amino group, which also increased sensitivity to a limit of detection (LOD) of 0.1 pg (on column) for diaminopyrene and 5 pg for aminonitropyrene using electrospray ionization (ESI). Compared to a derivatization with pentafluorobenzoyl chloride yielding only one sensitive MS/MS transition, acetylation offers the advantages of a higher selectivity with two sensitive MS/MS transitions and the possibility of a direct detection of acetylated aminonitropyrenes and diaminopyrenes formed metabolically in vivo. Acetylated diaminopyrene was detected in urine and after hydrolysis of the corresponding hemoglobin adducts followed by acetylation in blood samples of rats after administration of dinitropyrene but not in controls. A method based on GC–MS with negative chemical ionization of the electrophore labelled metabolites was non-selective since only one major ion $[M-HF]^-$ was formed and some isobaric peaks were observed preventing unequivocal analyte identification at concentrations close to the LOD.
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1. Introduction

Many polyaromatic amines and nitroaromatics are suspected or known human carcinogens [1] and dinitropyrenes are among the most potent mutagens tested [2]. The only relevant source of human exposure to dinitropyrenes seems to be diesel engine exhaust since dinitropyrenes have not been observed in tobacco smoke or fried food despite of the use of sensitive methods. After resorption, polyaromatic amines and nitroaromatics are metabolically activated to nitroso intermediates or hydroxylamines forming covalent adducts with hemoglobin [3,4] or with DNA [5]. Complete enzymatic reduction of nitroarenes leads to amines [6]. Sensitive and selective quantitation methods for biomarkers of human exposure to diesel engine emissions are required for a refined risk assessment. Mainly gas chromatography coupled to negative chemical ionization mass spectrometry (GC–NCI-MS) after derivatization has been used for the detection of polyaromatic amines in aqueous matrices, e.g. in wastewater or after release from hemoglobin adducts by alkaline hydrolysis [7,8].

In aqueous matrices like urine and blood, hardly any sample workup is required when using a method based on liquid chromatography coupled to triple quadrupole mass spectrometry (LC–MS/MS) resulting in faster and more reproducible methods as compared to GC–MS methods [9]. In addition, the use of LC–MS/MS offers highly selective analyte identification in complex matrices [10]. Although compounds with basic amino groups should be effectively ionized by electrospray ionization (ESI), only few LC–MS methods have been published for primary aromatic amines [11,12]. Some methods are described for the measurement of heterocyclic aromatic amines found as food contamination based on LC–MS/MS [13]. Furthermore, the use of atmospheric pressure chemical ionization (APCI) and especially atmospheric pressure photoionization (APPI) sources used for more lipophilic compounds like steroids may be an alternative ionization method for aromatic amines as previously described [14,15]. In combination with a derivatization of the ambiphilic diaminopyrenes and aminonitropyrenes, difficult chromatographic behavior such as peak tailing on common

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reversed-phase LC columns can be overcome as described for biogenic amines using 7-fluoro-4-nitrobenzoxy-diazole for derivatization [16]. Additionally ion-pairing compounds may be useful to get a well resolved separation, but ion-pairing reagents often suppress the ionization of the analytes in ESI as described for heptafluorobutyric acid [17]. The acetylation of amines is a well known metabolic pathway and many acetylated amines were detected in urine of humans exposed to corresponding amines. Therefore, sensitivity and precision were tested for ESI, APCI, and APPI sources with or without derivatization and a method for the quantification of diaminopyrene or aminonitropyrene as acetylated derivatives in urine and after alkaline hydrolysis from hemoglobin of rats treated with dinitropyrene was developed.

2. Experimental

2.1. Instrumentation

LC–MS/MS was carried out with an Agilent 1100 autosampler and an Agilent 1100 LC system (Agilent Technologies, Waldbronn, Germany) coupled to an API 3000 triple stage quadrupole mass spectrometer equipped with a Turbo Ionspray (TIS), APCI or APPI source (Applied Biosystems, Darmstadt, Germany). For GC–MS measurements, a GC-MSD (5973) equipped with a NCI source and a temperature programmable inlet with solvent vent function was used (Agilent).

2.2. Reagents

1,3-, 1,6-, and 1,8-Diaminopyrene (DAP) were obtained from Tokyo Kasai Kogyo Co. (Tokyo, Japan). 1,6- and 1,8-Aminonitropyrene (ANP) were a kind gift of Dr. Basu (Department of Chemistry, University of Connecticut, Storrs, CT, USA). A mixture of dinitropyrene isomers was synthesized as described by Hashimoto and Shudo [18]. 1-Aminopyrene (AP) was from Aldrich (Taufkirchen, Germany) and d_9-1-nitropyrene was from Dr. Ehrenstorfer (Augsburg, Germany).

2.3. Synthesis of d_9-1-aminopyrene

d_9-1-Aminopyrene (d_9-1-AP) was prepared by reduction of d_9-1-nitropyrene (20 mg) with hydrazine hydrate (400 μL) and Pd/C (10 mg) in 50 mL ethanol (96%) at 90 °C for 2 h followed by purification with a SepPak C18 (300 mg, Waters, Eschborn, Germany) solid phase extraction (SPE) cartridge. The reaction mixture was diluted with four volumes of water and applied to the SPE cartridge, which had been prewashed with acetonitrile and water. After application of the reaction mixture, the cartridge was washed with water and d_9-1-aminopyrene was eluted with ethanol (96%, 3 mL). The identity of d_9-1-aminopyrene was confirmed based upon LC-UV and LC–MS/MS analyses with ESI using 1-aminopyrene as reference. Retention times, UV spectra, and product ion spectra taking into account a mass shift of 9 amu were identical.

2.4. Derivatization

After optimization of derivatization time and temperature, analytical standards (50 μg) were acetylated by addition of 200 μL acetic anhydride at 60 °C for 30 min. Pentafluorobenzoylation was performed by addition of 100 μL pentafluorobenzoyl chloride to a solution of 50 μg analyte in 200 μL acetonitrile. Solvent was evaporated under a gentle stream of nitrogen, standards were dissolved in 1 mL of acetonitrile and stored at −20 °C. Working solutions were prepared by dilution with LC buffer solution and acetonitrile (1:1, v/v).

For GC–NCI-MS, samples were conjugated with pentafluoropropionic anhydride in the presence of triethylamine in toluene at 50 °C for 15 min. The reaction was stopped by the addition of water and ammonia (5%). The organic phase was dried with sodium sulfate and either evaporated to dryness or directly injected.

2.5. General optimization of LC–MS/MS parameters

For diaminopyrene isomers, aminonitropyrene isomers, 1-aminopyrene and d_9-1-aminopyrene, ionization and fragmentation patterns were examined by infusion experiments into the different sources with or without prior derivatization. MS parameters such as declustering potential (DP) or collision energy (CE) were optimized with the “quantitative optimization” function of analyst 1.3.1 or 1.3.2. A syringe pump was used to provide a constant analyte infusion into the LC eluent (300 μL/min) via a T-connection. Analyte concentrations were chosen in the range of 1–100 ng/μL to obtain a constant signal in the Q1 scan mode. Since severe signal distortion was observed for non-derivatized diaminopyrene during LC, several different stationary phases and mobile phase buffers were tested. The conditions used for certain applications are given in further detail as indicated. LODs, LOQs, and instrument precision were determined using diluted standard solutions.

2.6. GC–NCI-MS analysis

A method for the analysis of aromatic amines released from hemoglobin adducts was applied with minor modifications [19]. Using an injection volume of 20 μL and solvent vent by helium (temperature gradient: 105 °C for 0.62 min; 1600 °C/min until 320 °C), separation was performed on a HP5-MS column (30 m × 0.25 mm × 0.25 μm) with helium as carrier gas at a flow rate of 1 mL/min (oven temperature: 90 °C for 1 min; 25 °C/min until 320 °C; 320 °C for 5 min). Ions were detected in the single ion monitoring mode with a NCI source temperature of 240 °C and methane as reactand gas. The following ions were monitored: d_9-1-aminopyrene m/z 352 $[M-H–F]^-$, 1-aminopyrene m/z 343 $[M-H–F]^-$, diaminopyrene m/z 504 $[M-H–F]^-$, aminonitropyrene m/z 408 $[M]^-$ and m/z 358 $[M-H–CF_3]^-$.

Table 1
MS/MS transitions for LC–MS/MS analysis with ESI of acetylated analytes in biological matrix

Compound	Transition *m/z*	DP (V)	CE (V)
Doubly acetylated DAP (quantifier)	317.3 → 217.1	50	50
Doubly acetylated DAP (qualifier)	317.3 → 232.3	50	40
Singly acetylated DAP (quantifier)	275.2 → 217.2	30	30
Singly acetylated DAP (qualifier)	275.2 → 232.2	30	30
Singly acetylated ANP (quantifier)	305.3 → 216.1	60	50
Singly acetylated ANP (qualifier)	305.3 → 246.3	60	35
1-AP (quantifier)	260.1 → 218.1	71	33
1-AP (qualifier)	260.1 → 201.1	71	47
d_9-1-AP (quantifier)	269.1 → 227.1	71	33
d_9-1-AP (qualifier)	269.1 → 210.1	71	47

DAP: diaminopyrene, ANP: aminonitropyrene, 1-AP: 1-aminopyrene, DP: declustering potential, CE: collision energy.

2.7. Administration of dinitropyrene to rats

Rats were dosed with a dinitropyrene mixture (3.5 mg/kg b.w.) in tricaprylin by gavage. Urine was collected from 0 to 12 h after treatment and stored at −20 °C until further analysis. Prior to LC–MS/MS analysis, proteins and particles were removed by centrifugation (128 000 × *g*, 30 min, 4 °C) and 10 μL of the supernatants were injected after addition of d_9-1-aminopyrene as internal standard (10 pg/10 μL final concentration). Analytes (acetylated DAP and acetylated ANP) were identified using *m/z* 275.2 → *m/z* 217.2 and *m/z* 305.3 → *m/z* 216.1 as quantifier and *m/z* 275.2 → *m/z* 232.2 and *m/z* 305.3 → *m/z* 246.3 as qualifier (Table 1). A calibration curve from 0.1 to 50 pg/10 μL and from 1 to 500 pg/10 μL for diaminopyrene and aminonitropyrene, respectively (d_9-1-aminopyrene 10 pg/10 μL) was used for comparison of retention times and quantitation. Urine from control animals was spiked with standards to evaluate recovery and precision.

For the determination of hemoglobin adducts, animals dosed with dinitropyrene (20 mg/kg b.w.) were sacrificed by CO_2 asphyxiation and blood samples were collected by cardiac puncture 24 h after treatment. Red blood cells were separated from plasma by centrifugation, hemoglobin was prepared according to the method of Riedel et al. [20] and stored at −20 °C until further analysis. After the addition of d_9-1-aminopyrene (1 ng/10 μL), alkaline hydrolysis was carried out by sonicating hemoglobin (100 mg) dissolved in 1N NaOH (0.01% SDS) (5 mL) for 1 h at room temperature. After centrifugation (5 min, 1500 × *g*), samples were applied to OASIS MCX SPE cartridge (150 mg, 6 cm^3) prewashed with acetonitrile (2 mL), 0.1N HCl (2 mL) and 1N HCl (2 mL). The cartridges were washed with 1N HCl (2 mL), methanol (2 mL), and acetonitrile (2 mL) and analytes were eluted with 5% aqueous NH_3 (25%, v/v) in acetonitrile (5 mL). Toluene and water (1 mL each) were added and the mixture was vigorously shaken for 1 min. For acetylation, the organic phase was dried with anhydrous Na_2SO_4, mixed with 0.5 M triethylamine in toluene (200 μL) and acetic anhydride (200 μL), and the reaction was carried out at 60 °C for 30 min. Excess acetic anhydride was allowed to react with water (1 mL) and prior to vigorous mixing, 5% aqueous NH_3 (25%, v/v) in water (1 mL) was added. The organic phase was evaporated to dryness by vacuum centrifugation, the sample was dissolved in 200 μL LC buffer (50% 5 mM ammonium acetate pH 3 in acetonitrile, v/v) and stored at −20 °C until LC–MS/MS analysis.

The injection volume was 10 μL, LC separation was accomplished on an Atlantis C18 column (100 mm × 2 mm, 5 μm, Waters, Eschborn, Germany). Gradient elution with a flow of 350 μL/min and 5 mM ammonium acetate pH 3 (solvent A) and acetonitrile (solvent B) was carried out using the following conditions: 5–30% B in 1 min, 30–60% B in 12 min, 60–98% B in 2 min, 98% B for 0.5 min, 98–5% B in 1 min, 5% B for 3.5 min. Electrospray ionization in the positive ion mode was carried out using nitrogen as nebulizing and curtain gas (NEB 10, CUR 8), a source temperature of 450 °C and an ion spray voltage of 4000 V. Two specific transitions for each analyte with dwell times of 200 ms were monitored in the MRM mode using nitrogen as collision gas (CAD 4) (Table 1).

3. Results and discussion

3.1. Gas chromatography–mass spectrometry

A method established for the analysis of other aromatic amines released from hemoglobin adducts was slightly modified [19]. Using a solvent vent by helium enabled the injection of a larger volume (20 μL) yet providing narrow peaks and thus high signal to noise ratios. The HP5-MS column used, showed reduced signal tailing compared to other DB5 columns. This might be attributed to its neutral properties, whereas other DB5 columns frequently show acidic characteristics [21,22]. Compared to GC columns especially designed for amine separation like Rtx®-5 Amine [23], HP5-MS can be used at higher oven temperatures, which is of advantage for the analysis of high-molecular-weight chemicals with low volatility. The source temperature of 240 °C was unusually high for NCI but a reduced signal tailing in the source was achieved (data not shown). For diaminopyrene, 1-aminopyrene, and d_9-1-aminopyrene the most intense ions were $[M-H–F]^-$ ions. For aminonitropyrene, however, *m/z* 408 $[M]^-$ and *m/z* 358 $[M-H–CF_3]^-$ were more abundant. Both fragmentations are typical for fluorinated compounds [12]. A typical GC–NCI-MS chromatogram is given in Fig. 1. GC–MS analysis resulted in low LODs below 2 pg on column.

3.2. Liquid chromatography–mass spectrometry

3.2.1. Derivatization

Derivatization of primary amines may improve the LC properties of the analytes due to a better resolution in combination with reduced tailing of the signals [24]. Additionally,

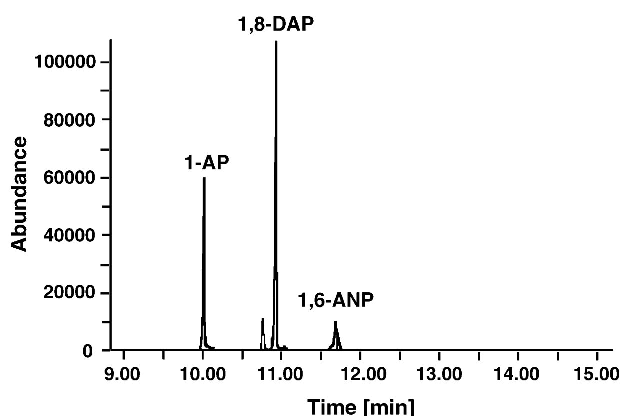

Fig. 1. GC–NCI-MS chromatogram for analyte standards after pentafluoropropionylation, 20 μL injected, concentration of all analytes: 100 fg/μL.

formed product ions can be used for unequivocal identification if a tandem mass spectrometer is used as detector [25]. These benefits may result in an increase of sensitivity [25,26]. The conjugation with pentafluorobenzoylchloride was selected since the pentafluorobenzoyl moiety enables the use of APCI in combination with an electron-capture ionization mechanism known for highest sensitivity in GC-NCI–MS and LC-APCI–MS/MS [27,28].

Acetylation of hydroxy, amino, and acidic C–H groups is a well known biotransformation pathway and acetylated amines are frequently observed in urine of humans exposed to amines and nitroaromatics [6]. Therefore, instrumental method development for acetylated amines would be of advantage. Derivatization conditions for acetylation were optimized to 60 °C and 30 min due to the formation of triply acetylated diaminopyrenes at higher temperatures or decreased derivatization efficiency for aminonitropyrene at lower temperatures.

3.2.2. Ionization and fragmentation

3.2.2.1. Ionization. Precursor ions for 1-aminopyrene, aminonitropyrene, diaminopyrene, and their acetylated and pentafluorobenzoylated derivatives after ESI, APCI or APPI are summarized in Table 2 . In the positive ion mode, protonation was the major ionization mechanism for 1-AP, ANP, and DAP and their acetylated derivatives with ESI, APCI, and APPI except for DAP. An unusual $[M^{\bullet}]^+$ as most abundant precursor ion was observed for DAP in ESI which may be explained by an electrolytic oxidation due to charge balance requirements [29] or doubly charged dimers. In the negative ion mode, deprotonated precursor ions were observed with all sources applied.

After derivatization with pentafluorobenzoylchloride, the only compound related ions observed for the monofunctional molecules 1-aminopyrene and aminonitropyrene were *m*/*z* 167 $[C_6F_5^{\bullet}]^-$ and, with lower intensity, $[M-H]^-$ after negative ionization. The dissociation could not be prevented by reducing the declustering potential. No $[M-PFB]^-$ was observed as precursor ion which has been described as dissocia-

Table 2
Observed precursor ions and fragments for the analytes of interest (diaminopyrene, DAP; aminonitropyrene, ANP; 1-aminopyrene, 1-AP) after electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI)

	Compound of interest	Experiment	Observed precursor ions	*m*/*z*	Observed fragments	*m*/*z*
Primary amines	1,3-, 1,6- and 1,8-DAP	ESI	$[M^{\bullet}]^+$	232	$[M-28]^+$	204, 176
	1,3-DAP	APCI, APPI	$[M+H]^+$	233	$[M-NH_3+H]^+$, $[M-NH_3–CHNH_2+H]^+$	216, 189
	1,6- and 1,8-DAP	APCI, APPI	$[M+H]^+$	233	$[M-NH_2+H]^+$, $[M-NH_3–CHNH_2+H]^+$	217, 189
	1,6- and 1,8-ANP	ESI, APCI, APPI	$[M+H]^+$	263	$[M-NH_3+H]^+$, $[M-HNO_2+H]^+$, $([M-NH_3–CHNH_2+H]^+)$	246, 216, (189)
	1-AP	ESI, APCI, APPI	$[M+H]^+$	218	$[M-NH_3+H]^+$, $[M-NH_3–CHNH_2+H]^+$	201, 189
Acetylated amines	1,3-DAP	ESI, APCI, APPI	$[M+H]^+$	317	$[M-NH_3COCH_3–COCH_2+H]^+$, $[M-NH_3–CHNH_2+H]^+$, $([M-COCH_3–COCH_2+H]^+)$	216, 189, (232)
	1,6- and 1,8-DAP	ESI, APCI, APPI	$[M+H]^+$	317	$[M-NH_2COCH_3–COCH_2+H]^+$, $[M-NH_3–CHNH_2+H]^+$, $([M-COCH_3–COCH_2+H]^+)$	217, 189 (232)
	1,6- and 1,8-ANP	ESI, APCI, APPI	$[M+H]^+$	305	$[M-HNO_2–COCH_2+H]^+$, $[M-NO_2–CHNHCOCH_2+H]^+$, $([M-NH_2COCH_3+H]^+)$	216, 189, (246)
	1-AP	ESI, APCI, APPI	$[M+H]^+$	260	$[M-COCH_2+H]^+$, $[M-NHCOCH_3+H]^+$	218, 202
PFB amines	1,3-, 1,6- and 1,8-DAP	ESI, APCI, APPI	$[C_6F_5^{\bullet}]^-$, $([M-H]^-)$	167, (619)	$[C_6F_5^{\bullet}]^-$ $[M-HC_6F_5–H]^-$	167, 451
	1,6- and 1,8-ANP	ESI, APCI, APPI	$[C_6F_5^{\bullet}]^-$, $([M-H]^-)$	167, (455)	$[C_6F_5^{\bullet}]^-$	167
	1-AP	ESI, APCI, APPI	$[C_6F_5^{\bullet}]^-$, $([M-H]^-)$	167, (410)	$[C_6F_5^{\bullet}]^-$	167

tive electron capture atmospheric pressure chemical ionization MS for hydroxylated polyaromatic hydrocarbons [28]. Deprotonation and electron capture are competing mechanisms and deprotonation may predominate when acidic hydrogen atoms are present [30]. This is in accordance with the occurrence of $[M-H]^-$ for secondary amines produced by pentafluorobenzoylation of polyaromatic amines.

3.2.2.2. Fragmentation. For aromatic amines such as 4-chloroaniline, 1-aminopyrene, aminonitropyrene or diaminopyrene, a typical constant neutral loss (CNL) of 18 amu (CNL of NH_3) was observed upon collision induced fragmentation experiments using a triple quadrupole mass spectrometer as described for 4-aminobiphenyl [12]. In complex samples from biological matrix, this transition might be confused with the unspecific loss of water or hydroxyl radical observed for many oxygen containing substances [31]. In addition to the loss of 17 or 18 amu after collision induced dissociation with nitrogen, a fragment ion $[M-28]^+$ corresponding to a loss of C–NH_2 typical for aromatic amines with a relatively low intensity was observed for diaminopyrene (Table 2). The observed differences in fragmentation patterns of diaminopyrene isomers with and without derivatization (Table 2) and after pentafluorobenzoylation (data not shown) in the positive ion mode may be explained by different abilities for resonance stabilization of the resulting cation due to *o*- or *p*-positioned substituents. In the negative ion mode, the only observed yet very sensitive ion produced by collision induced fragmentation for monofunctional amines after pentafluorobenzoylation was m/z 167 $[C_6F_5^{\bullet}]^-$. For the bifunctional amine diaminopyrene, a loss of 168 amu $[M-H\text{–}C_6F_5H]^-$ was additionally observed.

None of the fragments of derivatized analytes might be confused with an unspecific loss of H_2O yielding a more unequivocal and specific identification in complex biological matrix. For diaminopyrene isomers, acetylation yielded two sensitive SRM transitions to be used for quantification (quantifier) and confirmation of analyte identity (qualifier) whereas only one sensitive transition was observed without derivatization (Fig. 2).

Based upon results from “quantitative optimization” during infusion experiments, analyses of acetylated and primary amines in the positive ion mode, and that of pentafluorobenzoylated amines in the negative ion mode were more sensitive after “quantitative optimization” compared to vice versa ionization mode.

3.2.3. Liquid chromatography

Strong peak tailing upon liquid chromatography of primary amines is a well known phenomenon, which has resulted in the development of new LC materials such as polar modified phases [32]. Several LC columns (Synergi Hydro, Atlantis C18, XTerra C18, Luna C8, Symmetry Shield C8, YMC Aqua, Hypersil BDS C8, ReproSil-Pur 120 CN and Reprogel) were tested. Traditional C18 columns and CN columns showed unacceptable peak shapes for diaminopyrene. Ultra pure, extensively endcapped or polar modified columns showed an acceptable chromatographic separation and peak shapes only during the first few separations for diaminopyrene. This might be attributed to a decomposition of silica based LC column material by the LC buffers used which results in chemically modified silanol groups [33]. The use of polymeric type LC columns improved chromatographic behavior of diaminopyrene in relation to aminonitropyrene and 1-aminopyrene but lower theoretical plate numbers reduced resolution and may be responsible for peak widths of up to 2 min.

Trifluoroacetic acid (0.1%), 5 mM ammonium acetate pH 2, pH 2.5, pH 3, and pH 3.5, 0.1% formic acid, water, 5 mM ammonium bicarbonate pH 10.5, 50 mM ammonium acetate pH 3 were used as polar mobile phase constituents, acetonitrile and acetonitrile with isopropanol as unpolar mobile phase constituents. Chromatographic behavior of aminonitropyrene and 1-aminopyrene was sufficient using most of these buffers but diaminopyrenes could not be separated with acceptable peak shapes.

An improved chromatographic behavior could be achieved for acetylated and pentafluorobenzyolated diaminopyrenes. The formation of amides which do not interact with silanol residues to the same extent as primary amines may explain this effect. In comparison to acetylation, the large substituent C_6F_5 makes the molecule more lipophilic resulting in the lowest LOD since C_6F_5 shields the secondary amine more effectively to be less prone to unwanted silanol–amine interactions on silica based LC columns than an amide with a smaller substituent. In addition, the more lipophilic analyte elutes at a higher organic solvent rate which results in a more efficient solvent evaporation with better ionization efficiency, and therefore, a higher sensitivity.

3.3. Comparison of different analytical techniques

LODs and precision for the LC–MS/MS measurement of standard solutions of derivatized and non-derivatized analytes using ESI, APCI, and APPI were compared to those obtained by GC–NCI-MS (Table 3). In analyses of unmodified diaminopyrenes in concentrations of 50 pg/10 μL, peak intensities were similar to those observed with acetylated diaminopyrenes. Due to a more than linear decrease in sensitivity observed below concentrations of 10 pg/10 μL as described previously [15], the signal to noise ratio of acetylated diaminopyrene isomers was significantly higher resulting in lower LODs (Fig. 3). The lowest LODs with LC–MS/MS were obtained detecting pentafluorobenzoylated standards with APCI. As described above, dissociation of the pentafluorobenzoylated precursor ion readily occurs in different sources with the lowest dissociation in APCI giving the lowest LOD.

After analyte derivatization with pentafluorobenzoyl chloride in biological matrix, an intensive, but tailing signal was observed in the SRM transitions of all analytes. This intensive and strongly tailing signal coeluted with the analytes,

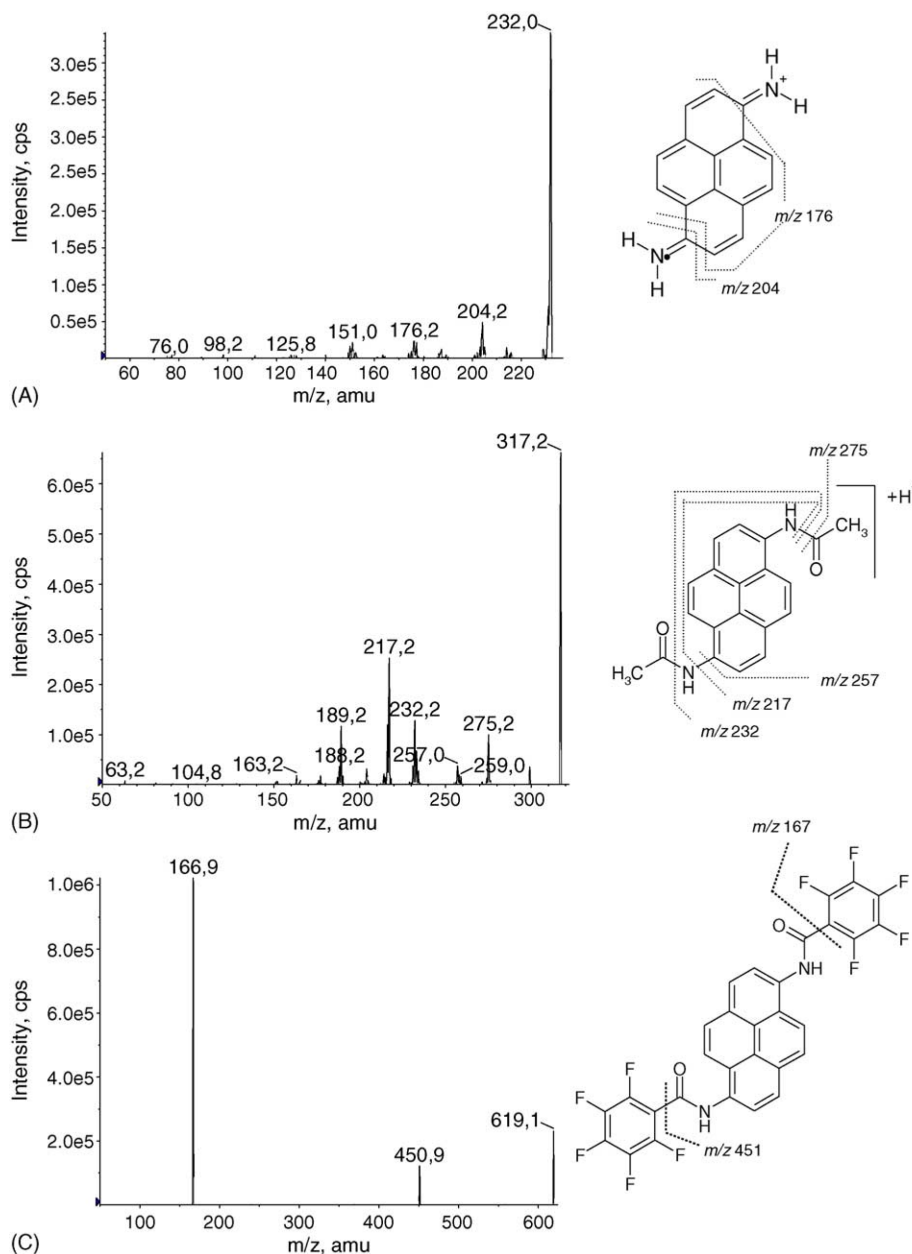

Fig. 2. ESI product ion spectra of 1,6-diaminopyrene: (A) without derivatization; (B) after acetylation; (C) APCI product ion spectrum of 1,6-diaminopyrene after pentafluorobenzolyation.

which resulted in a decrease in signal to noise ratio as well as a distortion of the analyte peak shape. The signal could not be identified by precursor ion scan or Q1 scan experiments and the elimination was not achieved by further sample clean-up steps after derivatization. Again, this indicated the lack of selectivity of the fragment ion *m/z* 167. Therefore, pentafluorobenzoylation was neglected for the analysis of biological samples.

Electrospray ionization of acetylated diaminopyrene gave highest sensitivity (Table 3) due to the fact that the analyte is protonated in the liquid phase during ESI and no transfer to the gas phase is required as for APCI and APPI. This is supported by the highest sensitivity for electrospray ionization obtained with an acidic buffer (5 mM ammonium acetate, pH 3). In contrast, APCI and APPI were more sensitive using pure water as polar solvent. Because of a more efficient transfer to the gas phase due to its lower polarity, acetylated aminonitropyrene was detected with highest sensitivity by APPI. The higher ESI sensitivity of acetylated diaminopyrenes compared to aminonitropyrene is explained by the presence of two basic secondary amino groups making protonation efficient for diaminopyrene. ESI sensitivity of aminonitropyrene however, is decreased by the electron withdrawing capacities of the nitro group. This method resulted in LODs of 0.1 and 5 pg on column and a precision of 5% and 7% for diaminopyrene and aminonitropyrene isomers,

Table 3
Comparison of LODs (pg/10 μL on column for S/N = 3), precision and linearity for instrumental analysis of standards

Derivatization	ESI						APCI					APPI					GC/MS
	None		Acetylation		PFBCl		None		Acetylation		PFBCl	None	Acetylation		PFBCl		PFPA
1,6-DAP																	
LOD	n.d.		0.1		n.d.		5		1		n.d.	n.d.	1		n.d.		
Precision (%) at (× pg)	n.d.		5	30	n.d.		n.d.		8	10	n.d.	n.d.	4 ($n=6$)	10	n.d.		
Linearity (R^2)	n.d.		0.3–1000	(0.997)	n.d.		n.d.		1–1000	(1.0)	n.d.	n.d.	1–1000	(0.996)	n.d.		
1,8-DAP																	
LOD	n.d.		0.1		0.3		n.d.		1		1	n.d.	1		1		<1
Precision (%) at (× pg)	n.d.		4	30	3 ($n=4$)	30	n.d.		16	10	n.d.	n.d.	9 ($n=6$)	10	7 ($n=6$)	30	
Linearity (R^2)	n.d.		0.3–1000	(0.996)	0.3–100	(0.992)	n.d.		1–1000	(1.0)	n.d.	n.d.	1–1000	(0.997)	1–300	(0.999)	
1,3-DAP																	
LOD	n.d.		0.5		1		5		1		0.1	n.d.	1		n.d.		
Precision (%) at (× pg)	n.d.		5	30	5 ($n=4$)	30	n.d.		12	10	n.d.	n.d.	8 ($n=6$)	10	n.d.		
Linearity (R^2)	n.d.		0.3–1000	(0.996)	1–300	(0.993)	n.d.		1–1000	(1.0)	n.d.	n.d.	1–1000	(0.991)	n.d.		
1,6-ANP																	
LOD	n.d.		5		n.d.		0.1		1		0.1	0.2	n.d.		n.d.		
Precision (%) at (× pg)	n.d.		10	30	n.d.		4	10	7	100	n.d.	8	n.d.		n.d.		
Linearity (R^2)	n.d.		1–1000	(0.979)	n.d.		0.1–1000	(0.999)	1–1000	(0.993)	n.d.	n.d.	n.d.		n.d.		
1,8-ANP																	
LOD	5		5		1		0.3		1		n.d.		0.3		1		<1
Precision (%) at (× pg)	8	20	7	30	2 ($n=4$)	30	7	10	5	100	n.d.		11 ($n=6$)	10	4 ($n=6$)	30	
Linearity (R^2)	5–100	(0.997)	1–1000	(0.969)	1–300	(0.998)	0.3–1000	(0.999)	1–1000	(0.992)	n.d.		0.3–300	(0.993)	3–300	(0.998)	
1-AP																	
LOD	5	(2.1)	1		n.d.		1		0.3		0.1	n.d.	1		n.d.		<1
Precision (%) at (× pg)	12	20	5	30	n.d.		11	10	7	10	n.d.	n.d.	7 ($n=6$)	10	n.d.		
Linearity (R^2)	5–100	(0.996)	1–1000	(0.995)	n.d.		1–1000	(0.999)	0.3–1000	(1.0)	n.d.	n.d.	1–300	(0.997)	n.d.		
d_9-1-AP																	
LOD	n.d.		n.d.		0.3		1		1		n.d.	n.d.	1		0.3		
Precision (%) at (× pg)	n.d.		n.d.		3 ($n=4$)	30	12	10	10	10	n.d.	n.d.	6 ($n=6$)	10	2 ($n=6$)	30	
Linearity (R^2)	n.d.		n.d.		0.3–300	(0.997)	3–1000	(0.999)	1–1000	(1.0)	n.d.	n.d.	1–300	(0.997)	0.3–1000	(0.990)	

If not specifically indicated, precision was determined by 20 consecutive injections of the same sample. Linearity was determined in duplicate. For each source and analyte, optimized conditions were used as described. DAP: diaminopyrene, ANP: aminonitropyrene, 1-AP: 1-aminopyrene, n.d.: not determined.

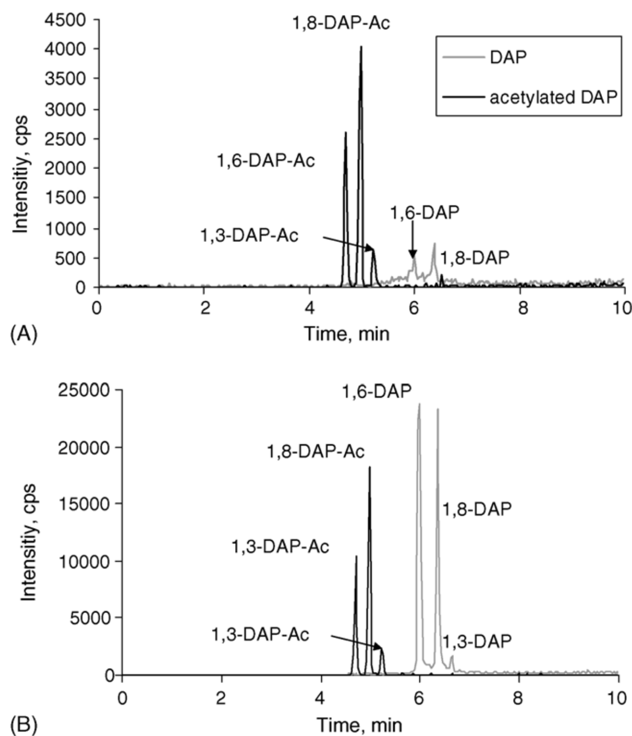

Fig. 3. Comparison of LC–MS/MS chromatograms with ESI for acetylated or non-acetylated diaminopyrene isomers (gradient elution with ammonium bicarbonate (5 mM, pH 10.5) and acetonitrile on a Waters XTerra® MS C_{18} column 50 mm × 2.1 mm, 2.5 μm); (A) 10 pg and (B) 50 pg of each isomer were injected directly or after dervatization.

respectively, and was used for the determination for urinary levels of *N*-acetylated diaminopyrene isomers and the levels of hemoglobin adducts in rats treated with dinitropyrene.

GC–NCI-MS analysis of pentafluoropropionylated standards resulted in low LODs (below 2 pg on column) and was more sensitive than LC–MS/MS determination with ESI for the analytes of concern (Table 3). Since negative chemical ionization is a specific ionization and detection technique for electrophoric compounds in comparison to electron impact ionization, low noise levels and high sensitivity can be achieved [27]. Sample preparation is comparable to the LC–MS/MS method used, since sample clean-up as well as derivatization are necessary for the measurement of diaminopyrene in biological fluids. However, a direct and sensitive measurement of acetylated amines without further derivatization is not possible by GC–NCI-MS. The limit of detection in spiked hemoglobin was lower than that of the LC–MS/MS method (40 pg/200 mg versus 300 pg/200 mg hemoglobin) for diaminopyrene isomers. However, at concentrations close to the LOD, many peaks were present in single ion monitoring chromatograms in biological samples (data not shown). Consequently, compound identification relied exclusively on retention times, but for basic compounds priming effects of the GC column may result in irreproducible retention times [23]. Therefore, identification of dinitropyrene derived metabolites upon GC–MS measurements could not be unequivocally achieved at low concentrations.

3.4. Application to biological samples

Since the formation of aminonitro- or diaminopyrene from dinitropyrene in vivo has not been directly demonstrated, a proof of concept experiment with rats was performed. Urine and blood samples were analyzed with the most specific and sensitive LC–MS/MS method based on ESI after acetylation after treatment of rats with dinitropyrene (3.5 and

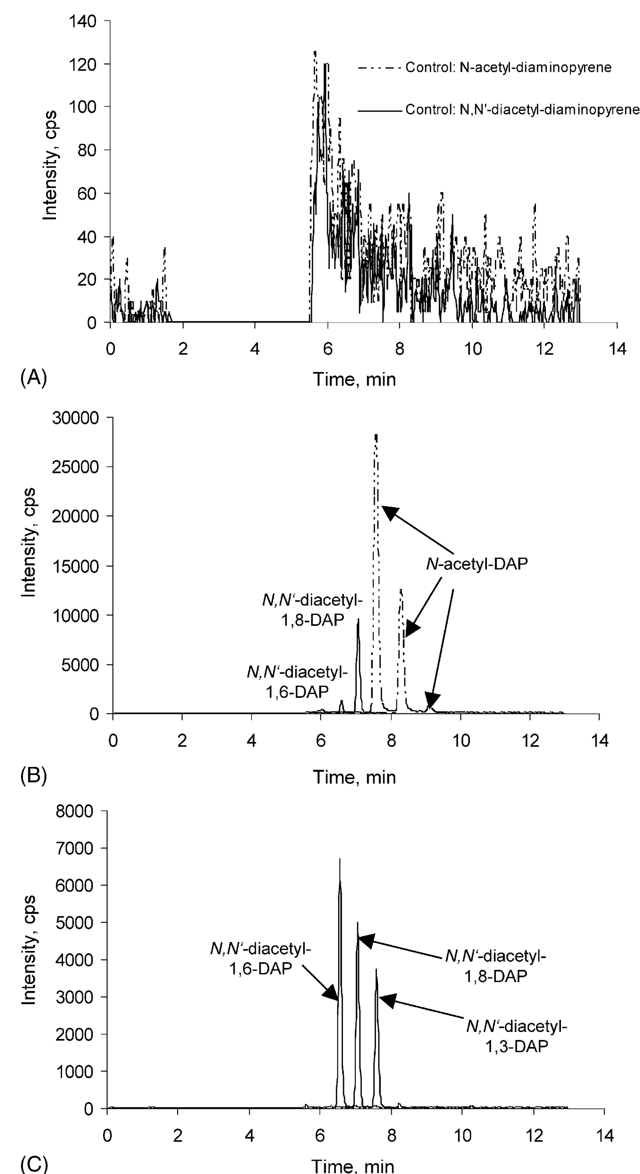

Fig. 4. Urine (0–12 h) of a control rat (A) and a rat treated with a mixture of dinitropyrene isomers by gavage (B) as well as a standard injection of *N*,*N*′-diacetyl-diaminopyrene isomers (C). The LC–MS/MS chromatogram with ESI (B) shows *N*,*N*′-diacetyl-diaminopyrene isomers or *N*-acetyl-diaminopyrene isomers (gradient elution with ammoniumacetate (5 mM, pH 3) and acetonitrile on a Waters Atlantis™ C_{18} column 100 mm × 2.1 mm, 5 μm).

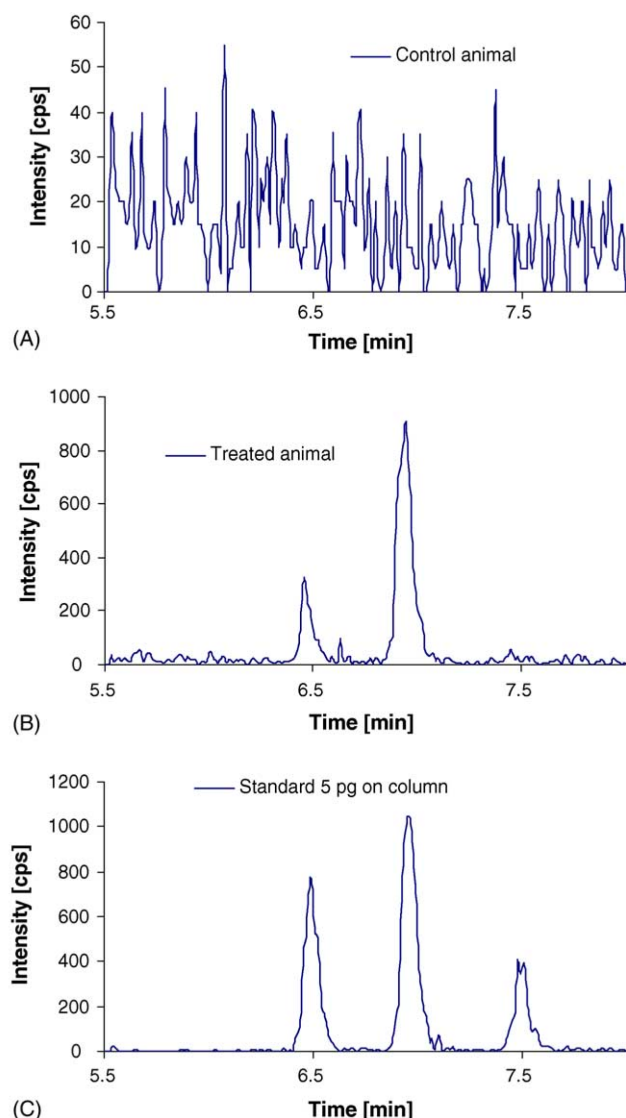

Fig. 5. LC–MS/MS chromatogram with ESI for acetylated diaminopyrenes released from hemoglobin of a control rat (A), of a rat dosed with 20 mg/kg b.w. dinitropyrene (B), and for a *N*,*N′*-diacetyl-diaminopyrene standard (C).

20 mg/kg b.w., respectively). In urine samples, mono- and bis-*N*-acetylated diaminopyrenes could be detected without derivatization (Fig. 4). This clearly shows that a reductive metabolism of dinitropyrene followed by acetylation occurs after treatment of rats with dinitropyrene by gavage. After acetylation, diaminopyrene released from hemoglobin after a single oral administration of dinitropyrene could also be determined with this method (Fig. 5).

The detection of acetylated diaminopyrene in urine samples was achieved with only one sample preparation step of ultracentrifugation. Compared to rats which are poor acetylators humans are good acetylators. Therefore, the detection of acetylated diaminopyrene in urine samples of humans highly exposed to diesel engine emissions, i.e. tunnel workers or miners after a specific sample enrichment procedure may be possible. In addition, the hemoglobin adducts formed in rats after a single dose without a possible accumulation indicates that the method should be useful for human biomonitoring. Since human hemoglobin has a biological half life of 60 days and no repair mechanisms are known for hemoglobin adducts, continuous exposure results in an accumulation of hemoglobin adducts. In combination with high exposure to diesel engine emissions and a sample enrichment procedure, the detection in humans may also be possible for a more realistic dinitropyrene dose compared to 20 mg/kg b.w. As an exposure assessment for dinitropyrene is not available at present, it is not possible to calculate a LOD necessary for the detection of diaminopyrene in humans. On the other hand, the data clearly demonstrate that the analytical method is applicable to mechanistic studies in animal models and in vitro studies.

4. Conclusion

The method presented here combines the following positive effects: (I) Acetylation improved peak shapes and reproducibility in LC of diaminopyrenes resulting in high signal to noise ratios. In spite of the use of several different LC column materials, non-derivatized diaminopyrene isomers show unfavorable characteristics for liquid chromatography. (II) Competing ionization mechanisms in ESI result in $[M^{\bullet}]^+$ besides $[M+H]^+$ precursor ions for diaminopyrene. Only one characteristic fragmentation pathway for diaminopyrene occurred with high efficiency. Acetylation resulted in only one ionization mechanism, yielding one, efficiently generated, precursor ion. (III) Compared to the fragmentation of non-derivatized primary amines or pentafluorobenzoylated amines, acetylation allows for unequivocal analyte identification in biological matrix by specific retention times and the use of two specific transitions which can be used as quantifier and qualifier and are distinguishable from an unspecific loss of water. Therefore, acetylation resulted in increased sensitivity and selectivity above all in biological matrix.

In the present work, acetylated diaminopyrenes in urine as well as hemoglobin conjugates of dinitropyrene metabolites have been detected in vivo after exposure of rats to dinitropyrenes for the first time. In addition, the presented method can be applied for toxicokinetic studies. The LC–MS/MS method of these compounds may be also applicable to human biomonitoring. However, for the generation of robust quantitative data optimization steps for sample preparation/enrichment are required.

In general, the derivatization method combined with LC coupled to ESI-MS/MS is applicable to other aromatic amines measured, i.e. in human biomonitoring as shown for 1-aminopyrene. For aminonitropyrene, however, no derivatization is necessary, due to sufficient chromatographic behavior, unequivocal fragmentation patterns and high detection sensitivity using APCI or APPI. The discrepancy between diaminopyrene and aminonitropyrene isomers shows,

that de novo testing for sensitivity enhancement with or without derivatization and for the optimum source is required for each substance.

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