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Determination of 1-nitropyrene with enzyme-linked immunosorbent assay versus high-performance column switching technique

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Abstract

An enzyme-linked immunosorbent assay (ELISA) on microplates and a HPLC coupled-column switching method were compared for the determination of the nitroarene compound 1-nitropyrene in airborne particulate organic matter collected at a busy intersection over a period of 2 months. After purification of the sample extract with silica, in the multidimensional chromatographic method nitroarenes were separated on a RP_{18} precolumn from matrix constituents followed by on-line reduction to corresponding aminoarenes with a Pt catalyst on alumina and a further separation of 1-aminopyrene on a second RP_{18} column. Methanol–water (70:30, v/v) was the mobile phase used. With ELISA, a six-fold overestimation was obtained for untreated samples. After clean-up it was lowered to ~1.6-fold overestimation which was mainly caused by cross-reaction of 2-nitropyrene and 2-nitrofluoranthene.

Keywords: Diesel soot; 1-Nitropyrene

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are mainly introduced into the environment as a result of incomplete combustion or pyrolysis. In the past, a derivative class of the PAHs, the nitro-PAHs (NPAHs) have received special attention because of their potential mutagenic and carcinogenic activity. One of the most abundant NPAHs in the environment is 1-nitropyrene (1-NP). The main source of 1-NP is the emission of diesel-engined vehicles [1]. In the last decade, it could be shown that inhalation of high levels of diesel soot produces lung tumors in rats while it is still unclear whether this is caused by the nonextractable core, adsorbed organics or both [2]. In view of this harmful potential it seems indicated to monitor the part of diesel soot in ambient particulate matter. However, diesel soot is difficult to distinguish from small carbonaceous particles resulting from other sources of combustion. Therefore, several investigators suggested the use of a diesel soot marker, especially the use of 1-NP as a benchmark compound [1,3]. The concentration of nitropyrenes in diesel exhaust matter is approximately 20 to 30-fold higher than in the exhaust of gasoline engines [4–6]. The objective of this paper is to present some results on the usefulness of two different analytical methods for a large scale and rapid quantitative determination of 1-NP in airborne particulate matter; (1) immunoassay and (2) multidimensional HPLC.

Nowadays, immunological techniques are increasingly accepted tools in environmental science. Im-

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munoassays for chemical contaminants were mainly developed for screening of aquatic and soil contamination. The vast majority of these methods was applied to pesticides that are generally hydrophilic, nonvolatile, and stable in water. However, the spectrum of commercial immunochemical test kits was extended also to other trace contaminants like PAHs, polychlorinated biphenyls (PCBs), and BTX (benzene, toluene, xylenes) that are lipophilic and in some cases highly volatile [7–11]. Until now, applications of immunological methods for the determination of priority pollutants on air particulates are very rare. Roda et al. [12] reported on the determination of benzo[*a*]pyrene and the correlation with chemical and atmospheric parameters. However, in this study results obtained by the immunoassay were not compared with an independent chromatographic method. Scheepers et al. [13,14] applied an immunoassay for the detection of metabolites of parent and NPAHs in urine samples from persons occupationally exposed to diesel exhaust. The monoclonal antibodies were generated in mice with 6-aminobenzo[*a*]pyrene covalently coupled to bovine serum albumin. As published by Gomes and Santella [15] the affinity of these antibodies was highest for 1-aminopyrene (1-AP), pyrene and benzo[*a*]pyrene. Compared to 1-NP, corresponding cross-reactivity with these compounds was one or two orders of magnitude higher. Haas et al. [16] developed an immunoassay for nitrofluoranthenes with an absolute detection limit at the nanogram-level. Application to real samples was not reported.

Coupled-column HPLC (multidimensional HPLC, column switching techniques) is a very efficient technology as preconcentration, separation and, if necessary, analyte derivatization can be combined and automatized. Generally, two different stationary phases are involved. For the determination of nitroarenes several methods were applied which made use of different separation and reduction columns, detectors and mobile phases [17–19]. Based on results from Veigl et al. [18] in the present study a modified column switching technique was applied for the determination of 1-NP in air particulate extracts. Further, attempts were made to correlate the observed 1-NP concentrations with the amount of soot in sampled particulate matter.

2. Experimental

2.1. Chemicals

All chemicals were obtained from either Merck (Darmstadt, Germany) or Aldrich (Steinheim, Germany). Goat anti-rabbit IgG–horseradish peroxidase conjugate (goat anti-rabbit IgG–POD) was from Pierce (Rockford, USA). The NIST SRM 1650, a diesel exhaust particulate matter with a certified 1-NP content, was obtained from Promochem (Wesel, Germany). All water used was ultrapure and obtained by reversed osmosis including UV-treatment (Milli-RO 5 Plus, Milli Q_{185} Plus; Millipore, Eschborn, Germany). All solvents used were HPLC-grade.

2.2. Air particulate sampling

Airborne particulates were collected from a busy intersection in central Munich over a 24-h period on glass fiber filters (∅ 47 mm; MN 85/90 BF, Macherey-Nagel, Düren, Germany and GF/C, Whatman, Maidstone, UK) using two low-volume samplers (Type GS 050/3-C, equipped with an open face sampler head Type K4, Derenda, Berlin, Germany) in parallel; one filter for the determination of 1-NP in airborne particulate matter, the second for soot determination. Air was sampled at a flow-rate of 2.3 $m^3\ h^{-1}$. Filter samples were taken over a period of 2 summer months (July and August, 1995). Loaded filters used for the determination of 1-NP were extracted in 15 ml of dichloromethane by sonication for 30 min. The organic solvent was removed under a gentle stream of N_2 and the remaining residue was redissolved in 1 ml of methanol. The crude extracts were directly measured with the HPLC column switching system or after dilution with pure water (1:1, v/v) with ELISA. In a later stage of the study, the residue was redissolved in 200 μl of cyclohexane, followed by a short clean-up procedure as outlined below. Determination of soot in airborne particulate matter was done with a thermochemical method which measures nonextractable carbon detecting the evolved carbon dioxide by means of coulometric titration as described elsewhere [20].

2.3. Clean-up procedure

A short clean-up procedure was performed with a disposable glass column (50 mm×8 mm I.D.), filled with 0.5 g of silica (Lichroprep Si 60) and conditioned with 5 ml of cyclohexane. After addition of sample, another 5 ml of cyclohexane were applied and corresponding eluate containing mainly the PAHs was discarded completely. The solvent was changed to a mixture of cyclohexane–methylene chloride (5:1, v/v) and seven 1-ml portions were added. Only the last 3 ml were collected, evaporated to dryness and redissolved in methanol for HPLC or ELISA analysis or in toluene for GC–HRMS, respectively.

2.4. HPLC column switching method for quantification of 1-NP

The HPLC system consisted of two pumps, models S1000 and S1300, S3400 fluorometric detector (λ_{ex}=360 nm, λ_{em}=430 nm), S4110 column oven (80°C, Sykam, Gilching, Germany) and Promis II autosampler (Spark, Emmen, Netherlands). The following columns were used:

1. Column 1 (C1): 25×4 mm I.D. RP_{18} 5 μm endcapped (Lichrospher, Merck, 50995)
2. Column 2 (C2): 250×4 mm I.D. RP_{18} 5 μm endcapped (Lichrospher, Merck, 50936)
3. Column 3 (C3): 30×4 mm I.D. packed with Pt catalyst on alumina (Merck, 8.18829).

2.5. Enzyme linked immunosorbent assay (ELISA)

The indirect competitive 1-NP-ELISA was done as described elsewhere [21]. Briefly, microtiter plates (96 F bottom wells with high binding capacity, Greiner, Frickenhausen, Germany) were coated with a nitropyrenebutyric acid–bovine serum albumin conjugate (NPBA–BSA). The plates were covered with adhesive plate sealers to prevent evaporation. After 15–18 h the plates were washed with PBS-Tween using an automatic plate washer (Easy Washer 812 SW1, SLT Labinstruments, Crailsheim, Germany). Plate sites not occupied by the NPBA–BSA conjugate were blocked with blocking buffer (PBS containing 2% casein) at room temperature. Plates were then washed as before. Sample or standard (50 μl/well) and diluted rabbit antiserum (dilution 1:30 000 in PBS; 100 μl/well) were added and incubated at room temperature with agitation for 30 min. After washing, goat anti-rabbit IgG–horseradish peroxidase conjugate was added and the plates were again incubated with agitation at room temperature for 60 min and washed as before. Then, substrate solution was added. Finally, the enzyme reaction was stopped with sulphuric acid and the absorbance was read at 450 nm (Easy Reader 340 ATC, SLT Labinstruments). All determinations were performed at least in triplicate. The calibration curve for 1-NP was linear in the 0.1 to 10 μg/l (0.4 n*M* to 40 n*M*) range with a center point (IC_{50}) at 1.5 μg/l (6 n*M*) and a limit of detection (LOD) (S/N=3) of 0.02 μg/l (80 p*M*) in acetonitrile–water (10:90, v/v). The average coefficients of variation were 4.9 and 7.8% over the range of the standard curve for intra-assay (on the same plate, n=12) and inter-assay (same number of aliquots were run on three different plates) precision, as was determined with low (0.1 μg/l), medium (1 μg/l) and high (10 μg/l) 1-NP concentrations.

2.6. GC–HRMS

GC–HRMS analyses were carried out on a HP 5890 Series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany) connected to a VG AutoSpec mass spectrometer at a resolution of 10 000 (VG Analytical, Manchester, UK) using a DB-5-MS column (60 m×0.25 mm I.D., 0.1 μm film thickness, J&W Scientific, Folsom, USA) and helium as carrier gas. The temperature of the splitless injector and the GC–MS interface was held at 270°C. NPAH-analysis started at 90°C with a heating rate of 10°C min^{-1} up to 200°C, followed by 6°C min^{-1} to 300°C, held for 40 min. EI-ionization of the sample was performed using an electron energy of 40 eV with a trap current of 800 μA. The two electric fields of the mass spectrometer were operated at a voltage of 8000 V. The mass spectrometer was operated in the selected ion monitoring (SIM) mode with the mass of the molecular ion M^+ of 247.0633. The identification of 1-NP, 2-NP and 2-nitrofluoranthene (2-NF) was

performed with reference compounds by comparison of retention times.

3. Results and discussion

3.1. Multidimensional HPLC method

It was first reported by Sigvardson and Birks [22] that 1-NP and 1-AP show different retention times. Precolumn reduction led to a retardation of 1-AP on the analytical column whereas with postcolumn reduction 1-NP was retarded. Brinkman suggested that the difference in retention times observed for the nitro- and amino-analogues could be used for the separation of these compounds [23]. Finally, a multicolumn HPLC method for the determination of 1-NP via on-line reduction to 1-AP and fluorescence detection was reported by Veigl et al. [18]. On a first column, packed with a pyrenebutyric acid amide stationary phase, the NPAHs were strongly retained. The corresponding fraction was then transferred onto a RP_{18} column and NPAHs were separated followed by postcolumn, in-line reduction to the respective amino-PAHs. The applicability of this method was demonstrated with diesel particulate extracts. Based on this method, in the present investigation a coupled-column switching technique was used connecting a precolumn (C1), reduction column and the analytical column (C2) via two automatic six-port switching valves (V_1, V_2). Configuration and positions are outlined in Fig. 1 and Table 1. Liquid chromatography was carried out isocratically at a temperature of 80°C and a flow-rate of 1 ml min^{-1} (Fig. 2a and b).

Application of 20 μl of crude methanol extract was done via an autosampler followed by the retardation of 1-NP on the precolumn using methanol–water (70:30, v/v) as mobile phase. Early eluting polar interferences were discarded. In a second step, 1-NP and compounds with similar retention times on the RP_{18} precolumn were transferred to the reduction column where 1-NP was catalytically reduced to 1-AP. In comparison to other species coeluted from the precolumn, 1-AP had a short retention on the RP_{18} analytical column and was first observed in the chromatogram. In the meantime the precolumn was washed with methanol–water (90:10, v/v) to elute highly hydrophobic compounds. After 32 min the precolumn was reconditioned with methanol–water (70:30, v/v) for 5 min. Initially, reduction was carried out with a mixture of zinc powder and silica in combination with acetonitrile–Tris–HCl buffer (78:22, v/v) as mobile phase as described earlier [22]. Because of the consumption of powdered zinc caused by the organic solvent a loss of catalytic activity with increasing number of chromatographic runs was found. In contrast, quite stable activity over months was observed when using a Pt catalyst on alumina at

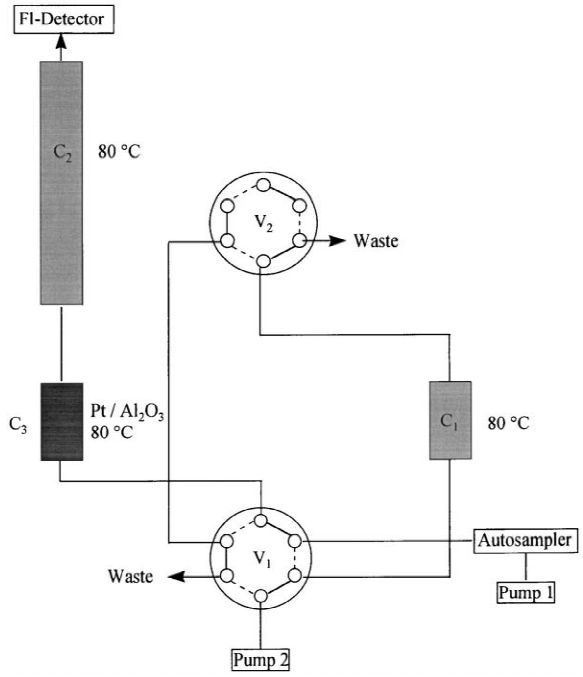

Fig. 1. Schematic diagram of the HPLC system. Abbreviations and operating instructions are described in the text. Inject: dotted line; Load: solid line.

Table 1
Time program

Step	Time (min)	Position of switching valve 2	Position of switching valve 1
1	0	Inject	Load
2	0.75	Inject	Inject
3	1.5	Load	Load
4	32	Inject	Load

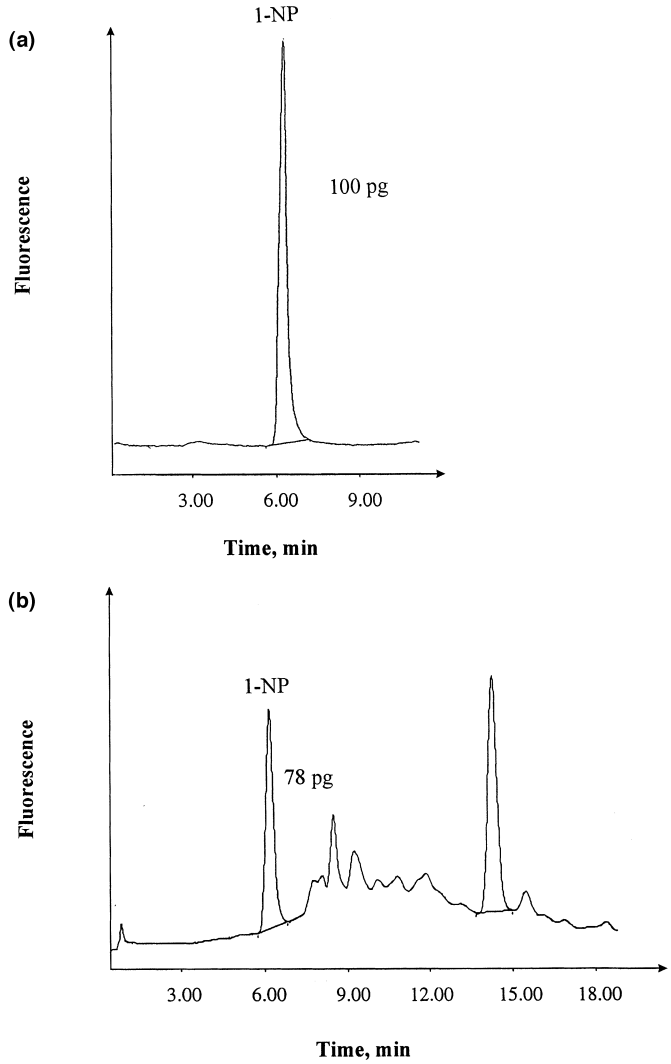

Fig. 2. (a) Multicolumn HPLC chromatogram of 1-nitropyrene standard solution. Conditions as Table 1. (b) Multicolumn chromatogram of an airborne particulate extract. Conditions as in Table 1.

high temperature and methanol–water as mobile phase, as described elsewhere [24]. Reduction rate was greater than 97% as was calculated by comparison of the plots of peak areas vs. concentration after injection of several dilutions of 1-NP or 1-AP (40 fmol to 4 pmol on column). Relative standard deviation ($n=3$) of each peak height was less than 2%. Over the observed concentration range from 40 fmol to 8 pmol a linear calibration graph was obtained ($r=0.998$). The LOD was determined to be 20 fmol ($S/N=3$). Overall recovery was $101.1\pm0.8\%$ ($n=3$) as was found with SRM 1650 (certified value for 1-NP: 19 ± 2 μg/g).

Separation of 1-NP and 2-NP was not achieved with the described system, even by gradient elution. 2-NP is formed by atmospheric transformation and was not identified in direct emissions [25,26]. However, an equimolar amount of 2-NP shows only 2% of the 1-NP signal at 360/430 nm. Because the amount of 2-NP in airborne particulate matter is

Table 2
1-Nitropyrene concentration in urban particulate matter [26]

City	1-Nitropyrene (pg m^{-3})
Claremont (CA, USA)	3–10
Torrance (CA, USA)	3–60
Detroit (MI, USA)	16–30
Hamilton (Ontario, Canada)	12
Gothenburg (Sweden)	3.8–20
Santiago (Chile)	28–110
Beijing (China)	29.8–229

comparable to or lower than the amount of 1-NP this compound should not interfere with the determination of 1-NP in the multidimensional chromatographic method and therefore, the 2-NP signal was ignored [27,28].

After a period of 2 months and a daily sampling, about sixty samples were analyzed. With multidimensional HPLC 1-NP concentrations were obtained in the range of 11 pg m^{-3} to 106 pg m^{-3}. The results are in agreement with measurements at urban places

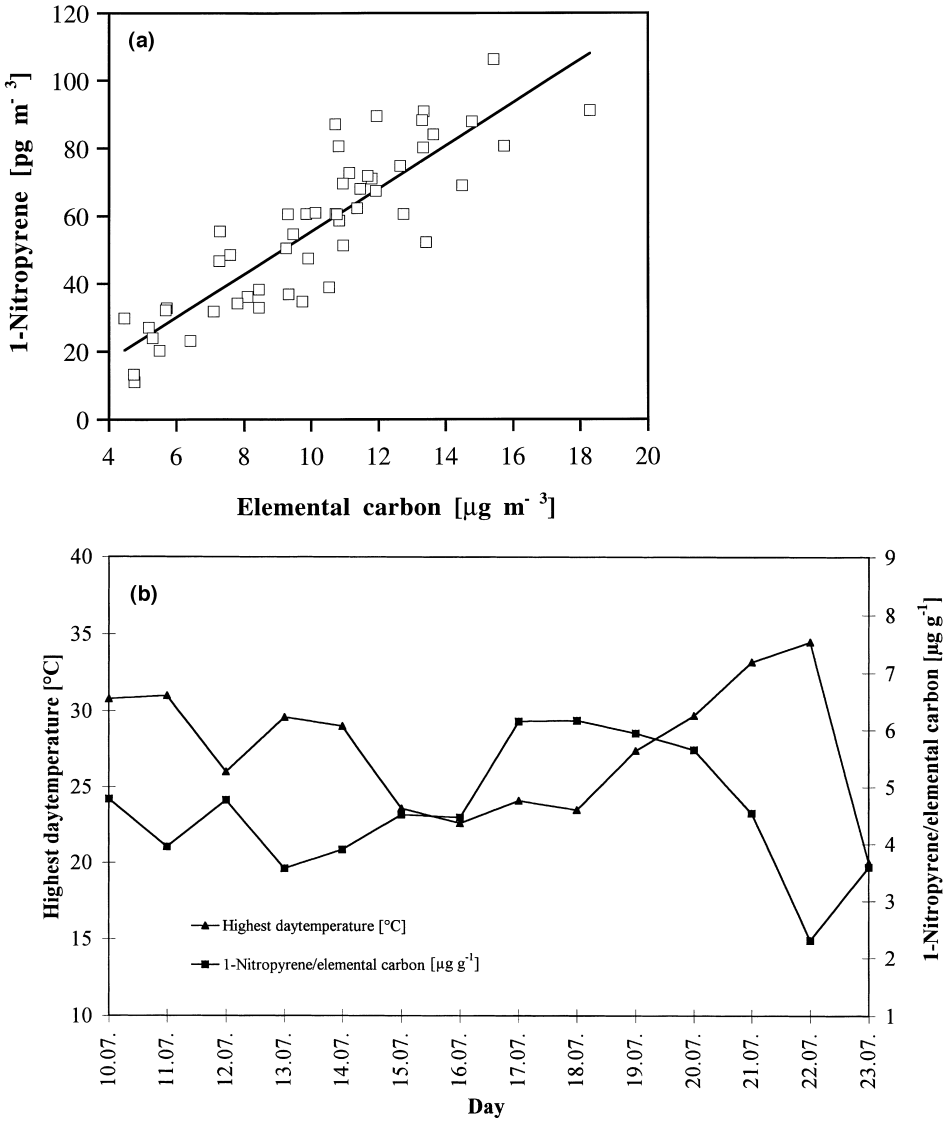

Fig. 3. (a) Comparison of 1-nitropyrene and elemental carbon concentration in urban particulate matter ($r=0.87$, $n=53$). (b) 1-Nitropyrene/elemental carbon-ratio in comparison with the highest day temperature.

reported by other groups (Table 2). We observed a highly significant correlation between 1-NP and soot concentrations (Fig. 3a) but surprisingly, the 1-NP/elemental carbon-ratio was not constant. It was markedly influenced by the ambient temperature (Fig. 3b). Higher temperature leads to a lower ratio and vice versa. Therefore, we speculate that at higher temperatures 1-NP is decomposed or desorbed and distributed in the gas phase. Seasonal variations of the concentrations of dinitropyrenes and 1-NP were also reported by Hayakawa et al. [29].

3.2. Immunochemical method

The NPAHs are a group of at least 200 different compounds, and approximately 450 compounds have been identified in diesel emissions, in addition to the products of atmospheric reactions with NO_x, O_3, and OH-radicals [30,31]. The antiserum used should be fairly specific for 1-NP. Therefore, the nitro function was preserved in the hapten which was synthesized for the immunogen preparation. However, absolute specificity of an antibody for a single compound is difficult to achieve as cross-reactivity (CR) is an intrinsic property of an antibody. Generally, CR is expressed as the concentration of a compound (IC_{50}) needed to displace 50% of the antibody from bound target analyte or target analyte–protein conjugate. It is calculated according to the formula:

$$CR_{50}[\%] = \frac{\text{Target analyte concentration at 50\% antibody binding}}{\text{Concentration of the cross-reacting compound at 50\% antibody binding}}$$

The cross-reactivity pattern of the antiserum that was used in the investigation is outlined in Fig. 4 [21]. For parent PAHs, corresponding CR_{50} values on a molar basis were well below 1%. These compounds will not constitute a serious problem in the ELISA. Therefore, the nitro group plays an important role in analyte recognition by the antibody. Especially the 1,6- and 1,8-dinitropyrenes could interfere even at very low levels. Other nitroarenes which are formed in the atmosphere and found in airborne particulates, such as 2-NP, 4-NP and 2-NF may cause interferences if present at concentrations comparable to 1-NP [19]. Based on these data, with the ELISA an overestimation of 1-NP in crude

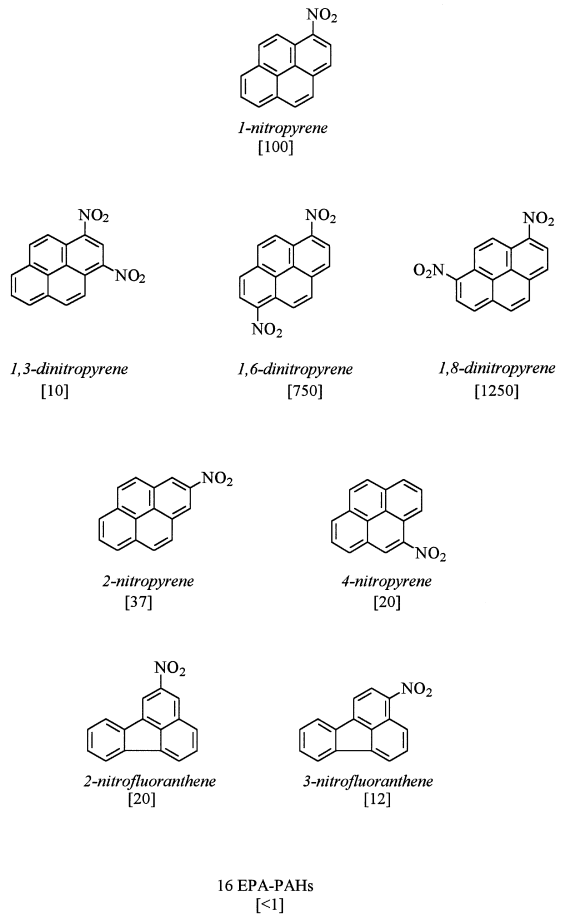

Fig. 4. Chemical structures of target analyte and compounds with cross-reactivities higher than 10% in the 1-NP-ELISA. Number in brackets corresponds to the percent cross-reactivity based on the IC_{50} value [21].

methanol extracts of ambient particulate organic matter was very likely.

Initially, we observed an about six-fold overestimation compared to the 1-NP concentration as measured by the HPLC method. We supposed that this was at least partly caused by the presence of dinitropyrenes as well as other cross-reacting species. As a consequence, a short clean-up was applied to remove these interferences. Only those eluate fractions were collected which contained the 1-NP as was proven before with 1-NP calibrating solutions. After applying this clean-up to the airborne particulate extracts, the overestimation rate was reduced to only about 60% (Fig. 5).

In view of this overestimation we analyzed the

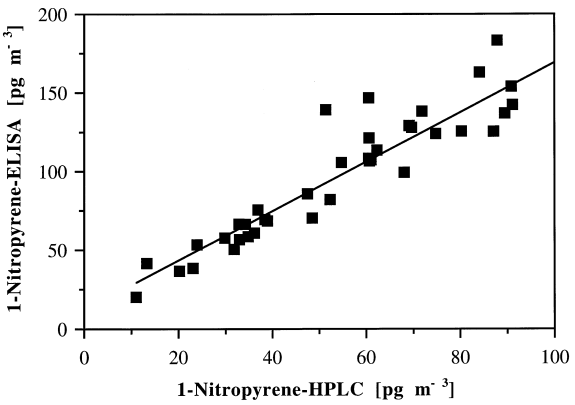

Fig. 5. Comparison of HPLC- and ELISA-1-NP determination in purified airborne particulate extracts. HPLC data were correlated to the amount of 1-NP equivalents as calculated from the 1-NP calibration curve ($r=0.93$, $n=42$).

eluate from clean-up with GC–HRMS (Fig. 6). Besides 1-NP, the corresponding fraction contained higher amounts of both 2-NF and 2-NP. Taking into account the cross-reactivity of these substances (Fig. 4) it can be assumed that the remaining overestimation is caused by these two compounds generated in the atmosphere by photochemical reactions. Because of these interferences, the 1-NP-ELISA as a fast and cost-effective screening technique for monitoring emissions caused by diesel-engined vehicles should not be used at locations far away from main traffic routes. The closer the sampling location to the source the higher is the accuracy of the 1-NP concentration when measured with the ELISA [32].

4. Conclusions

Current efforts have demonstrated that there is a high correlation between 1-NP and soot concentration in ambient particulate organic matter and thus, supporting the use of 1-NP as a marker for diesel emissions. Especially for studies on a larger scale, analytical determination of this nitroarene can constitute an alternative for the measurement of diesel soot as it can be faster and more cost-effective. After sample extraction and a short clean-up procedure, the described multidimensional HPLC method allows quantification of 1-NP at the picogram-level with high sample throughput. With the ELISA a six-fold overestimation was obtained for untreated samples. After pretreatment it was reduced to ~1.6-fold overestimation. Apart from the additional clean-up step the ELISA still constitutes a cost-effective alternative method for large-scale screening as multiple samples can be determined in parallel with no need for expensive equipment. However, the results can be considered only as semiquantitative, since cross-reacting nitroarenes such as 2-NF and 2-NP which are nonrelated to diesel exhaust emissions but generated photochemically during the

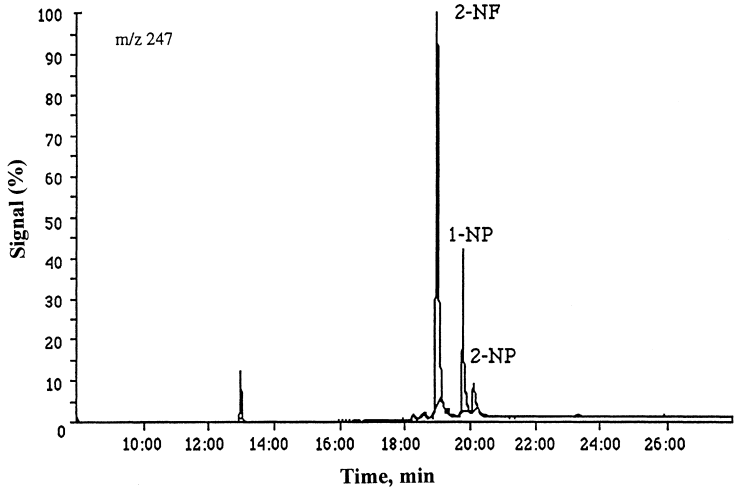

Fig. 6. GC–HRMS chromatogram of the 1-NP fraction after clean-up of an urban air particulate extract.

transport of the particles in the atmosphere were shown to interfere with the immunological method. If it were possible to produce antibodies with increased selectivity for 1-NP then the application range of ELISA would be extended and the accuracy of the results would be enhanced.

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