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Critical Review

High-performance liquid chromatography of nitrated polycyclic aromatic hydrocarbons

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Summary of Contents

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Josef Cvačka obtained his BSc degree in chemistry in 1993 at the Faculty of Science, Charles University, Prague, Czech Republic. He then graduated in 1995 with an MSc degree in analytical chemistry from the Department of Analytical Chemistry, Charles University. His diploma thesis dealt with the optimisation of HPLC separation of azodyes. At present he is a PhD student in the same Department. He is interested in the development of new HPLC methods for the determination of biologically-active organic substances at trace concentration levels. His thesis will deal with the HPLC determination of nitrated polycyclic aromatic hydrocarbons in waters using chemiluminescence detection.

Introduction

It is nearly two decades since Jager[1] and Pitts *et al.*[2] discovered independently that polycyclic aromatic hydrocarbons (PAHs; a list of abbreviations is given at the end of the paper) can undergo atmospheric reactions with nitrogen oxides to form nitro derivatives. Nitrated PAHs (NPAHs) are also directly emitted by diesel and petrol engines, and therefore their concentrations are raised in cities with heavy traffic and are further increased during smog episodes. So far, these compounds have been found also in carbon black and photocopier toners, fly ash, exhaust emissions from waste incineration plants, products of coal combustion, natural and waste waters, sediments, cigarette smoke and some foodstuffs (see reviews[3–6]). Aromatic systems of NPAHs found in various matrices have typically from two to five rings and one, two or three nitro groups. Samples contain a number of isomers, but the abundances of individual compounds in various types of sample are different. In the air the most volatile NPAHs, such as nitrobiphenyls and nitronaphthalenes occur predominantly in the gas phase, whereas 'heavier' isomers (nitro derivatives of pyrene, fluoranthene, anthracene, chrysene and others) are associated mainly with the particulate phase. In complex vehicle exhaust samples, nitropyrenes, nitroanthracenes, nitrophenanthrenes, nitrofluoranthenes, nitrofluorenes, nitronaphthalenes and others are found. The common concentrations of NPAHs in the atmosphere are at pg m^{-3} levels and diesel exhaust particles contain ng g^{-1} levels of NPAH isomers. The concentrations of dinitro-PAHs are approximately 10 times lower in both types of sample. Extensive efforts have been made to model NPAH formation and decay in the atmosphere.[7–10]

NPAHs are of particular interest because of their genotoxicity. Early investigators[11] pointed out that extracts of diesel and air particulate matter exhibit strong direct mutagenicity when tested in the Ames *Salmonella typhimurium* assay. Non-substituted PAHs, which are abundant in such samples, are mutagenic only after metabolic activation. It was shown that the main portion of direct-acting mutagenicity of diesel and air particulates is associated with NPAHs.[2,11,12] Some NPAHs, such as the dinitropyrenes, are among the most potent mutagens ever tested.[13,14] According to the International Agency for Research on Cancer,[15] some NPAHs are possibly carcinogenic to humans. Biological properties of NPAHs are discussed in detail elsewhere.[13,16,17]

Various methods for the determination of NPAHs have been reviewed by Moreira and co-workers.[3,6] Owing to the complexity of environmental samples and to the need to distinguish among isomers of NPAHs with different biological properties, chromatographic techniques have mainly been used. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been employed in more than 90% of all analyses.[6] The main advantage of GC is the higher separation efficiency, which allows the separation of a larger number of compounds. Several hundred thousand theoretical plates can be generated with capillary GC compared with HPLC (up to

20 000 theoretical plates using conventional equipment).[18] Moreover, the combination of GC with mass spectrometry (MS) provides an excellent and powerful tool for the identification of individual compounds. The limitations of the GC are connected with the low volatility and instability of some compounds. It was found that partial decomposition of NPAHs occurs not only in the injector,[19] but also in the column[20] and in the GC–MS interface.[20] This effect made the identification and quantification of isomers difficult, especially at the low levels common in environmental samples. This problem is overcomed by using HPLC, usually carried out at room temperature. HPLC is able to separate both small, volatile molecules and large, unstable molecules, as illustrated by the example of the separation of 22 NPAHs (Fig. 1). Reliable results can be obtained with relatively simple, inexpensive LC equipment, so that HPLC is often employed in the determination of NPAHs. Chemiluminescence and partially fluorescence and electrochemical detection are comparable in terms of selectivity and sensitivity to GC detection techniques such as NPD or NICIMS and NIAPIMS. Moreover, sample clean-up is very simple in many HPLC analyses. The continuing use of LC methods for the determination of NPAHs is documented by the increase in the number of published papers on this topic from one in 1980 to nine in 1996. Different types of sample have been analysed using HPLC: atmospheric (34%) and vehicle exhaust (29%) samples, biological materials (16%), foodstuffs (7%), reaction mixtures (5%) and others (see Table 1).

Sample preparation

The preparation of samples depends not only on the nature of the sample but also on the HPLC detection technique which is going to be used. Generally, the more specific the detector, the simpler is the sample preparation. Therefore, the use of electrochemical, fluorescence or chemiluminescence detection allows the preparation of samples to be simplified compared with the use of a universal spectrophotometric detector.

Atmospheric and diesel or gasoline exhaust samples are treated in a similar way. The first step, extraction of particulate materials on glass-fibre filters or of gaseous substances trapped on polyurethane foam, is performed by Soxhlet or ultrasonic extraction. Time-consuming Soxhlet extraction, usually performed for 12–24 h, is increasingly being replaced by ultrasonication for 15–30 min. The recoveries with ultrasonic extraction are in the range 90–95%.[21] Dichloromethane is the most common solvent for Soxhlet extraction and benzene–ethanol (4 + 1)[22] or toluene[18] have also been used. Ultrasonication is performed with benzene–ethanol (3 + 1),[23–26] dichloromethane[27,28] or 40% dichloromethane in hexane.[29] Supercritical fluid extraction can also efficiently extract NPAHs from complex matrices, but it is employed only rarely. The highest recoveries are obtained using pure $CHClF_2$ or CO_2 modified with 10% of toluene.[30] The extract is filtered through a membrane filter and concentrated, usually in a rotary evaporator under reduced pressure. The remaining organic solvent is removed under a gentle stream of inert gas, usually nitrogen. There is the possibility of direct injection of the redissolved extract into the HPLC system,[31] but, owing to the complexity of such types of matrices, further clean-up or fractionation of the sample is usually necessary.

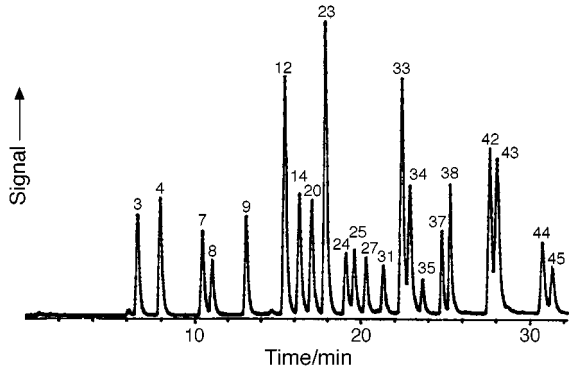


Fig. 1 An example of the separation of 22 NPAH standards. Peaks: 3 = 6-NQ; 4 = 5-NQ; 7 = 5-N-6-MQ; 8 = 8-N-7-MQ; 9 = 1,8-DNN; 12 = 1,5-DNN; 14 = 1-NN; 20 = 2-NN; 23 = 2-NB; 24 = 1-N-2-MN; 25 = 2,7-DNF; 27 = 4-NB; 28 = 3-NB; 31 = 2-NF; 33 = 9-NPH; 34 = 9-NA; 35 = 3-NPH; 37 = 1,6-DNP; 38 = 1-NP; 42 = 4-N-p-T; 43 = 6-NC; 44 = 3-NPer; 45 = 6-NBaP. Separation conditions: Alltech ODS column (250 × 2.1 mm id); 31 min gradient from 24 to 80% acetonitrile in water at 35 °C; spectrophotometric detection at 254 nm. Reprinted from ref. 54 with courtesy of Elsevier Science.

Simple clean-up was applied to atmospheric and diesel exhaust samples[24,25] prior to HPLC with chemiluminescence detection. The benzene–ethanol (3 + 1) extract was purified by extraction with 5% NaOH, 20% H_2SO_4 and water. After the benzene phase was evaporated to dryness, the residue was dissolved in ethanol and NPAHs were reduced by refluxing with NaHS. The reaction mixture was extracted with benzene. Several drops of a saturated solution of ascorbic acid were added and mixture was evaporated to dryness. The residue was dissolved in 0.2 ml acetonitrile containing ascorbic acid and injected into the HPLC system.

Fractionation of the sample allows one to simplify the matrix and hence to remove interfering compounds. Schleibinger *et al.*[21] compared different fractionation procedures for atmospheric particulate samples. It was found that relatively high recoveries are achieved using solid-phase extraction (87–95%), column chromatography (87–91%) or preparative HPLC (83–92%), whereas low values are obtained using TLC (55–60%). The most popular fractionation technique is solid-phase extraction on silica gel Sep-Pak cartridges (Waters, Milford, MA, USA). The common preparation scheme is as follows. An extract dissolved in the minimum volume of dichloromethane is applied to the top of the cartridge. The cartridge is then eluted stepwise with 3 ml of hexane, 6 ml of dichloromethane and finally 3 ml of methanol. The hexane eluate contains aliphatic hydrocarbons and PAHs and this fraction is discarded. Dichloromethane elutes oxy- and nitro-PAHs and this fraction is further dried under nitrogen, the residue is dissolved in the mobile phase or methanol and an aliquot of the solution is injected on to the HPLC column. Methanol elutes more polar compounds from the sample. The recovery of such a preparation scheme for NPAHs was reported to be 95%.[32] A similar fractionation procedure involves a Bond Elut silica (Analytical International, Harbor City, CA, USA) cartridge.[33] Elution is performed with 6 ml of cyclohexane followed by 6 ml of dichloromethane and an average recovery of about 85% for NPAHs was achieved. Different organic solvents are used for fractionation on an alumina Sep-Pak column.[23] This column is eluted with benzene (discarded), followed by trichloromethane. The trichloromethane fraction is evaporated to dryness, the residue is dissolved in methanol and an aliquot of the solution is injected into the HPLC system. TLC fractionation on silica gel has been used in sample preparation for dinitropyrene analysis.[34] After the TLC separation with toluene–hexane (5 + 1), the plates were dried and appropriate regions were extracted with methanol.

Fractionation can also be performed using HPLC.[29] The extract purified on silica gel is separated on amino-modified silica gel (μBondapak-NH_2, Waters) with 10% dichloromethane in pentane. The fraction eluting between 40 and 70 ml is collected and concentrated and the residue is dissolved in methanol. The sample preparation procedure can also involve chemical reduction of NPAHs to APAHs, when fluorescence or chemiluminescence detection is used. Reduction is performed

Table 1 Selected HPLC methods for the determination of NPAHs. AS, air and air particulate sample; CB, carbon black; RM, reaction mixture; VES, vehicle exhaust sample; DCM, dichloromethane; PBA, pyrenebutyric acid amide on LiChrosorb Si 100

Compounds	Matrix	Sample Preparation	Stationary phase	Mobile phase	Detection	Ref.
1-NN, 2-NN, 1,5-DNN, 1,8-DNN	RM	–	ODS LiChrosorb, 250 × 4 mm id, 5 μm	MeOH–water (75 + 25)	Spectrophotometric at 254 nm	63
2-NB, 3-NB, 4-NB, 1-NN, 2-NN, 2-NF, 9-NA, 1,3-DNP, 1,3,6-TNP, 2,7-DNF and others	VES	Soxhlet extraction with DCM, filtration, evaporation, silica gel fractionation, drying, dissolution in mobile phase	ODS Ultrasphere, 250 × 4 mm id, 5 μm	5% propanol in 0.05 M monochloroacetic acid–sodium acetate buffer (pH 3.8), 50 °C	Electrochemical, TLC flow cell with glassy carbon working electrode at −0.6 V *vs*. Ag/AgCl	23
2-NN, 9-NA, 1-NP	AS	Ultrasonic extraction with DCM, evaporation, drying, dissolution in DCM, silica gel fractionation, evaporation, dissolution in MeOH	ODS Nucleosil, 250 × 4 mm id, 10 μm	MeCN–MeOH–acetic acid–sodium acetate buffer (pH 4.0) (3 + 4 + 3)	Electrochemical, wall-jet flow cell with glassy carbon working electrode at −0.65 V *vs*. Ag/AgCl	28
1-NN, 2-NF, 4-NB, 1-NP, 2,7-DNF, 1,3-DNP and others	VES	Ultrasonic extraction with DCM, filtration, evaporation, drying, silica gel fractionation, drying, dissolution in MF	Two ODS Alltech, 500 × 1 mm id, 10 μm	Monochloroacetic acid–sodium acetate buffer (pH 4.7–MeCN (3 + 7)	Electrochemical, TLC flow cell with glassy carbon working electrode at −0.6 V *vs*. Ag/AgCl	27
1-NP, 6-NBaP	AS, VES	Soxhlet extraction with benzene–EtOH (8 + 2), evaporation, drying, dissolution, reduction with $NaBH_4$–$CuCl_2$, extraction with benzene, evaporation, dissolution in MeCN	ODS Zorbax, 250 × 4.6 mm id	MeCN–water (65 + 35) buffered with NH_3 or Bu_4NPO_4	Fluorescence after off-line reduction; 365/430 nm (1-NP); 430/497 nm (6-NBaP)	22
1-NN, 2-NN, 2-NF, 9-NA, 1-NP and others	VES	Soxhlet extraction with DCM, evaporation, dissolution in MeOH–H_2SO_4, dilution with MeOH	ODS Spherisorb, 150 × 6 mm id, 3 μm	MeOH–1 mM H_2SO_4 (85 + 15)	Fluorescence after on-line electrochemical reduction	31
2-NF, 9-NA, 1-NP, 1,3-DNP, 7-NBaA, 6-NC	AS, VES	Soxhlet extraction with DCM, evaporation, fractionation on silica gel, evaporation, dissolution, fractionation on $–NH_2$ silica gel, evaporation, dissolution in MeOH	ODS Zorbax, 250 × 4.6 mm id	MeOH–water gradient	Fluorescence after on-line reduction on column packed with Zn–silica	29
1-NP	VES	Reflux with toluene, filtration, evaporation, dimethyl sulfoxide–cyclohexane separation, fractionation on silica gel column, evaporation, dissolution in MeOH	Column switching system with PBA (250 × 4 mm id, 7 μm) and ODS (Seibersdorf) (250 × 4 mm id, 5 μm)	MeOH–water	Fluorescence after on-line reduction on a column packed with Rh–Pt catalyst on alumina (354/433 nm)	60
1-NP, 1,3-DNP, 1,6-DNP, 1,8-DNP, BaP	AS	Ultrasonic extraction with benzene–ethanol (3 + 1), filtration, extraction with 5% NaOH, 20% H_2SO_4, water, evaporation, dissolution in MeOH, reduction with NaHS, extraction with benzene, evaporation, dissolution in MeCN	Column switching system with two ODS columns for simultaneous determination BaP and NPAHs	Imidazole–perchloric acid buffer (pH 7.6)–MeCN (1 + 1) for NPAHs, water–MeCN (1 + 3) for BaP	TCPO–H_2O_2 chemiluminescence after off-line reduction	24
2-NFA, 1-NP, 2-NP, 4-NP, 6-NC	AS	Ultrasonic extraction with benzene–ethanol (3 + 1), filtration, evaporation, dissolution in MeCN	Column switching system with two ODS separation columns (Cosmosil $5C_{18}$AR, 250 × 4.6 mm id, 5 μm and Cosmosil $5C_{18}$MS, 250 × 4.6 mm id, 5 μm), reduction column [Zn–glass beads (1 + 1)] and ODS concentration column (Cosmosil $5C_{18}$MS)	(1) Imidazole–perchloric acid buffer (pH 6.7)–MeCN (1 + 4) (2) Imidazole–perchloric acid buffer (pH 7.6)–MeCN (1 + 1) (3) 10 mM ascorbic acid	TCPO–H_2O_2 chemiluminescence after on-line reduction	26

continued over—

Table 1—*continued*

Compounds	Matrix	Sample Preparation	Stationary phase	Mobile phase	Detection	Ref.
1-NP, 1,3-DNP, 1,6-DNP, 1,8-DNP	AS	Ultrasonic extraction with benzene–ethanol (3 + 1), filtration, extraction with 5% NaOH, 20% H_2SO_4, water, evaporation, dissolution in EtOH, reduction with NaHS, extraction with benzene, evaporation, dissolution in MeCN	ODS Cosmosil, 250 × 4.6 mm id	10 mM imidazole buffer (pH 7.6)–MeCN (1 + 1)	TCPO–H_2O_2 chemiluminescence after off-line reduction	25
1-NN, 2-NF, 9-NA, 1-NP, 6-NC, 3-NPer	CB	Soxhlet extraction with toluene, evaporation, dissolution in DCM	ODS Zorbax, 250 × 4.6 mm id	MeCN–TRIS hydrochloride buffer (pH 6.5) (78 + 22)	TCPO–H_2O_2 chemiluminescence after on-line reduction on column packed with Zn and glass beads	41

on the crude extract[22] in cases when no further fractionation is to be performed, or on one of the fractions.[35] Sample preparation can also be incorporated into the chromatographic system. Tejada *et al.*[36] injected directly an extract dissolved in 50% methanol in dichloroethane without further purification. They used a multi-column HPLC system with two ODS columns. On the first column, NPAHs were separated from other types of compounds and transferred to the second analytical column. The advantage of such sample treatment is the very high recovery. Tejada *et al.*[36] reported a 102% recovery for 1-NP.

An interesting approach to the fractionation of complex environmental samples is bioassay-directed fractionation.[37–39] This is a potent technique for selecting bioactive compounds based on the Ames mutagenicity assay performed on individual fractions. Biologically active fractions are subjected to further analysis.

Carbon black is a complex matrix containing NPAHs. Jin *et al.*[40] compared dichloromethane, benzene, toluene and chlorobenzene as organic solvents for Soxhlet extraction of carbon black. Chlorobenzene was found to extract the highest mass of organic compounds. For spectrophotometric detection,[40] further fractionation of the extract on a silica gel cartridge is necessary. The cartridge is eluted successively with hexane, dichloromethane and methanol; the dichloromethane fraction is concentrated, dried, diluted in methanol and analysed by HPLC. When chemiluminescence detection is used,[41] it is possible to inject the evaporated and redissolved toluene extract without further purification.

Soil and sediment samples can be extracted with dichloromethane[42,43] or benzene.[44] Maggard *et al.*[42] compared Soxhlet and blender extraction with dichloromethane for extraction of NPAHs from soils. It was found that the blender procedure gives a higher recovery (> 90%) than Soxhlet extraction (> 80%). The extract can be further purified using gel permeation chromatography.[43] After evaporation, the residue is dissolved in acetonitrile and injected on to the HPLC column.

There is very little information about the preparation of water samples. The papers available deal with degradation or stability study of nitropyrenes.[44,45] The concentrations of NPAHs are relatively high and therefore simple sample preparation is performed. Water samples are extracted three times by simple liquid–liquid extraction with benzene–ethanol (4 + 1)[45] or ethyl acetate.[44] The extracts are dried and the residue is dissolved in a solvent and analysed by HPLC.

Foodstuff samples are extracted ultrasonically with benzene–ethanol (4 + 1)[46] or acetonitrile;[47] Soxhlet extraction with acetonitrile[47] is also possible. The extract is then cleaned using size-exclusion chromatography on an SX-3 column with cyclohexane–ethyl acetate (1 + 1)[47] or fractionated into neutral, acidic and basic fractions.[46] NPAHs present in the neutral fraction are reduced to APAHs and analysed by HPLC with fluorescence detection.

Urine samples[48] are purified using solid-phase extraction on octadecyl-modified silica gel Sep-Pak cartridges (Waters). The sample is applied to a cartridge pre-washed with methanol and water. After washing the cartridge with water, NPAHs are eluted with methanol. The recovery is about 89%.

The recovery of the sample preparation procedure is considerably lower than 100%, and therefore an internal standard should be used for accurate quantification of NPAHs. Surprisingly, an internal standard has been used in only a few analyses. Murahashi *et al.*[24] and Hayakawa *et al.*[25] used 2-fluoro-7-nitrofluorene as an internal standard in analysis of air particulates for 1-NP, 1,3-DNP, 1,6-DNP and 1,8-DNP. As NPAHs are not photochemically stable, it is recommended to carry out all manipulations with sample and standards in the dark.

Chromatographic systems, stationary and mobile phases

Most analyses are performed in the reversed-phase mode, utilising octadecyl-modified silica as the stationary phase. Mobile phases consist of acetonitrile or methanol and water or buffer solution. Both isocratic and gradient elution are employed in NPAHs separations. Grosse-Rhode *et al*. described the chromatography of NPAHs on several chemically modified copolymer[49] and anthryl-modified silica[50] stationary phases. A cyano column with hexane–propan-2-ol mobile phases has also been used.[39,51] Normal-phase chromatography is not common in NPAH analysis; these systems are employed to ensure better compatibility with the detection method used.[38] Some studies of the retention of NPAHs in normal-phase chromatography have been reported.[52,53] Greibrokk *et al.*[53] found a silica column together with dichloromethane–hexane mobile phases to be very useful for separations of NPAH positional isomers. Pirkle-type chiral stationary phases[52] do not facilitate the separation of NPAHs.

Retention indices[54] and retention data[49,53,55] have been published for different stationary and mobile phases. Fu *et al.*[52] discussed some relationships between the structure of NPAHs and retention order. The advantages of computer assistance in new chromatographic method development were demonstrated using the DryLab G simulation program.[54,56]

Almost all separations are carried out using conventional columns of 4.0 or 4.6 mm id. Narrow-bore or microbore columns facilitate a several-fold decrease in mobile phase consumption and improvements in sample mass-detection sensitivity, but have been employed only rarely,[27,54,55,57] so far,

in the LC of NPAHs, and then mostly for theoretical studies.[54,55,57] A column of 10 mm id has also been used in a system including fraction collection for off-line MS detection.[38]

Analyses of real samples are usually performed using relatively simple experimental arrangements of HPLC equipment. Column-switching techniques, however, are also employed.[18,26,58–61] These systems, which are sometimes complex, are constructed in different ways. The use of two columns with different properties[18,60] allows the incorporation of a sample pre-treatment step in the HPLC analysis and thus saves time. A system consisting of an initial column containing a reducing agent for conversion of NPAHs to APAHs, with a second analytical column,[58,60] provides another example of column switching. Column-switching systems can also be hybridized into two subsystems with different conditions for parallel NPAH and PAH analysis.[59]

Detection techniques

As shown in Fig. 2, different detection techniques are used in the HPLC of NPAHs. In the analysis of real samples, sensitivity of detection is one of the most important factors. Fig. 3 depicts the detection limits of NPAHs for all techniques used in HPLC systems. It can be seen that the most sensitive technique appears to be chemiluminescence, with DLs between 100 fg and 10 pg. Fluorescence is also very sensitive, with DLs of 1–10 pg. Slightly higher DLs are obtained when on-line conversion of NPAHs to APAHs is less than 100%, *e.g.*, when an electrochemical reductor is employed (see below). Amperometric detectors, operated in the reductive mode, provide DLs from tens to hundreds of picograms, depending on the experimental arrangement. The few references dealing with the LC–MS of NPAHs provide little information concerning sensitivity. Under negative ion chemical ionization many substances are detected at picogram levels, but some NPAHs are not detected even at microgram levels.

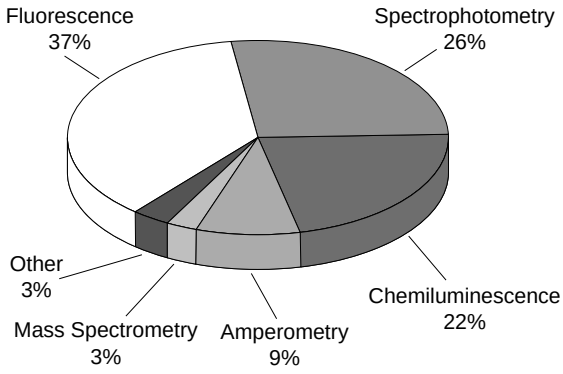


Fig. 2 Detection techniques used in the HPLC analysis of NPAHs.

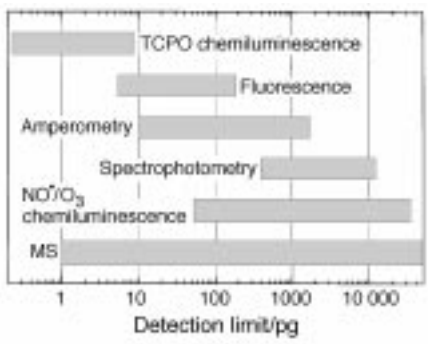


Fig. 3 Detection limits of HPLC detection techniques for NPAHs.

Spectrophotometric detection

Spectrophotometric detectors are almost universal, because they are simple, cheap and reliable. Therefore, they are used widely in LC. Unfortunately, their sensitivity is not high enough for the trace analysis of NPAHs. Nevertheless, spectrophotometric detectors are very convenient for use in cases where the demands on sensitivity are not so high. These detectors are useful for the testing of new columns and mobile phases[49,53] and for evaluating the effects of mobile phase composition and temperature.[55] Investigations of retention mechanisms[62] and the relationships between structure and retention order[52] are carried out spectrophotometrically.

Spectrophotometric detection is also suitable for the analysis of reaction mixtures and for determination of reaction products. This detection technique has been used for the rapid determination of 1-NN, 2-NN, 1,5-DNN, 1,8-DNN and naphthalene[63] and methyl derivatives of 4-NB[64] in reaction mixtures.

Microsomal incubation products of 6-NBaP have been separated and detected by spectrophotometry at 254 nm.[65] The stability of 1-NP and 1,6-DNP in environmental water samples and soil suspensions was examined by HPLC measurements of their degradation products.[45] Spectrophotometric detection was also used in a study of the biodegradation of 1-NP.[44]

A spectrophotometric detector has been used for the confirmation of the presence of nitropyrenes in carbon blacks and toners[51] and for separation of 2-NF and other mutagenic compounds from sterilised soil.[42]

Diode-array detectors can provide additional information about substances by the on-line recording of UV/VIS spectra. This approach has been applied in the determination of 1-NP in diesel particulate extracts[18] and in the analysis of carbon black.[40,66] Carbon black samples were examined for nine NPAHs.[40] Several peaks had retention times close to those of standard samples of these compounds, but only one (3-NFO) had a UV/VIS spectrum matching its standard.

Electrochemical detection

Electrochemical detection is based on electrochemical reaction of the determinand at the electrode surface and requires several conditions to be fulfilled. The mobile phase must be electrically conductive to support charge transfer between the mobile phase and electrode, and the mobile phases and samples must be free of oxygen when working at negative potentials. Oxygen reduction in the chromatographic system can give rise to high background current levels, which limits the useful working potential range. To avoid the presence of oxygen, stainless-steel tubing must be used throughout with no Teflon tubing, and mobile phases must be de-gassed. Several approaches have been used for mobile phase de-gassing: Galceran and Moyano[28] de-gassed the mobile phase with helium for 2 h at 50 °C and helium pre-saturated with the deoxygenated mobile phase was also bubbled throughout the chromatographic process; Rappaport *et al.*[32] continuously heated the mobile phase under nitrogen in a flask fitted with a reflux condenser. Also, a special oxygen scrubber column[29] or a porous graphite guard cell, operated at a very low potential,[31] can be inserted between the pump and the injector. Glassy carbon electrodes are sensitive to passivation by heavy metals, so that chemicals for mobile phase preparation must be of very high purity. Even when using very pure chemicals, repolishing of the electrode is recommended monthly[27] or weekly.[32]

Electrochemical cells used for NPAH detection are of the thin-layer[27,29,32] or wall-jet[28] type, each with a three electrode system, *i.e.*, working, auxiliary and reference electrodes.

Typical detectors contain a glassy carbon[27,28,32] or a gold/mercury[29] working electrode with an Ag/AgCl reference electrode, or a porous graphite working electrode[67] with a palladium-based modified H_2/H^+ reference electrode. Almost all electrochemical detectors are operated in the constant reductive amperometric mode with the working electrodes typically in the range between −500 and −650 mV *versus* the reference electrode. Attempts to use electrochemical detectors in the differential-pulse mode were not very successful.[29] Measurement of hydrodynamic voltammograms can help to confirm the analytes' identity.[28,29,32]

Chromatographic separations with electrochemical detection are usually carried out in the isocratic mode, but measurement in the gradient mode is also possible.[29]

Nitro-substituted and oxygenated PAHs have been determined in atmospheric samples from Barcelona[28] with DLs of 200–1600 pg for 2-NN, 9-NA and 1-NP. Ang *et al.*[67] described the determination of nitro- and oxy-derivatives of PAHs in ambient air particulates in Singapore with DLs at subnanogram levels. Rappaport *et al.*[32] looked for 16 NPAHs in diesel exhaust (2-ANFO, 7-NFCA, 2,7-DNFO, 3-N-9-F, 2,7-DNF, 1-NN, 2-NB, 2-NN, 3-NB, 4-NB, 2-NF, 1,3,6-TNP, 9-NA, 1,3-DNP, 1-NP, 4-NFA). Confirmation of the identity of these compounds was based on comparisons of hydrodynamic voltammograms. Using this approach, the presence of 1-NP was confirmed and its concentration quantified. The DLs were 10–100 pg for most substances. A further increase in sensitivity can be achieved by using microbore LC.[27] Compared with a similar system employing a conventional column,[32] sensitivities in the micro-HPLC system (0.91 μl thin-layer flow cell, two 500 × 1 mm id columns, injection valve and connections of minimum internal dead volume) were 3–7 times higher. Column efficiencies in this system for NPAHs varied between 26 000–30 000 theoretical plates. MacCrehan *et al.*[29] compared amperometric, differential-pulse amperometric and fluorescence detection techniques for the analysis of air and diesel particulate matter samples; 1-NP was determined by amperometric detection with a DL of 60 pg. A large-area porous graphite working electrode[31] not only allowed the determination of NPAHs in the reductive amperometric mode, but also the high degree of electrochemical conversion to APAHs can be used in subsequent fluorescence detection.

A gold/mercury thin layer electrochemical cell,[29] operated in the differential-pulse mode under gradient elution conditions, gave very poor results. Although several NPAHs were identified, there were experimental difficulties with coordinating the gradient-elution and the base-potential programs.

Fluorescence detection

Most trace HPLC analyses of NPAHs are accomplished using fluorescence detection. Because of the strong electron-withdrawing effect of the nitro group, NPAHs themselves are not fluorescent. Therefore, it is necessary to convert NPAHs into a fluorescent species, and this is done mostly by reduction of the nitro group into an amino group. APAHs are strongly fluorescent, so that the sensitivity of determination is very high. Table 2 gives the optimum excitation and emission wavelengths for 35 amino analogues of NPAHs.

Two approaches are used with NPAH reduction. This reaction can be performed before an HPLC analysis (off-line methods) or directly in a chromatographic system (on-line methods). On-line methods require more complicated HPLC equipment, involving a reduction column[29,33,36,47,54,56,60,68] or a large-area porous graphite electrode operated at a negative potential.[23,31,68] Off-line methods are time-consuming, laborious and difficult to automate for routine analyses. Both on-line and off-line methods of reduction are almost equally employed in the HPLC analysis of NPAHs.

Different chemical reagents have been used for off-line reduction. The reaction can be accomplished using (i) sodium tetrahydroborate with copper(II) chloride[22,34] or (ii) copper(I) chloride[21] as a catalyst at room temperature for 3–16 h, (iii) aqueous sodium hydrosulfide[24,69–71] for 1–1.5 h or (iv) zinc powder in hydrochloric acid.[72,73] Other metal powders (cadmium, copper and platinum) have also been tested,[68] but apart from zinc only cadmium–copper (1 + 1) has a high reduction efficiency. Kinouchi *et al.*[46] used specific nitroreductase purified from *Bacteroides fragilis* for reduction of NPAHs. APAHs can be further derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate.[43]

Several reducing materials for the on-line reduction of NPAHs have been tested. Columns packed with zinc powder or mixtures of zinc powder with silica[29,54] or glass beads[68] have limited lifetimes of a few days. Zinc is consumed by the reaction and small voids are formed in the packing, so that the chromatographic efficiency decreases. Zinc columns operate at room temperature and reduction yields are typically greater than 99%. A certain amount of supporting electrolyte or buffer is necessary in the mobile phase to facilitate the reaction.[29,41] Other types of reduction columns employed in NPAH analysis are based on catalytic reactions. The packing is not consumed during the reaction and these columns have a very long, almost indefinite, lifetime.[36] Catalytic reduction columns perform best when they are maintained at elevated temperatures, typically at 60–80 °C.[36] Noble metals on a solid support are the main catalysts reported. A 'three-way' catalyst,[36] designed to reduce hydrocarbons in automobile exhaust emission, is active only in methanol–water mobile phase. No reduction was observed in acetonitrile–water mixtures. Pt–Rh on alumina[36,60,74] was

Table 2 Excitation and emission wavelengths for fluorescence detection of reduced NPAHs

Compound	$\lambda_{ex}/\lambda_{em}$/nm
2-Nitrobiphenyl	227/394[54]
3-Nitrobiphenyl	232/399[54]
4-Nitrobiphenyl	285/367[54]
2,2′-Dinitrobiphenyl	228/372[54]
1-Nitronaphthalene	243/429[36]
1-Nitro-2-methylnaphthalene	244/414[54]
2-Nitronaphthalene	234/403[36]
1,3-Dinitronaphthalene	247/420[54]
1,5-Dinitronaphthalene	231/390[36]
1,8-Dinitronaphthalene	229/417[54]
1,3,6,8-Tetranitronaphthalene	264/417[54]
1-Nitrofluorene	285/370;[36] 290/365[21]
2-Nitrofluorene	285/370[54]
2,7-Dinitrofluorene	292/387[54]
2-Nitro-9-fluorenone	290/365[54]
3-Nitro-9-fluorenone	245/407[54]
2,7-Dinitro-9-fluorenone	232/387[54]
2-Nitroanthracene	260/495[36]
9-Nitroanthracene	263/505[36]
9-Methyl-10-nitroanthracene	267/534[36]
9,10-Dinitroanthracene	264/495[54]
9-Nitrophenanthrene	247/430;[54] 345/430[21]
1-Methyl-9-nitrophenanthrene	254/440[36]
1-Nitropyrene	360/430;[36] 345/433[21]
4-Methyl-3-nitropyrene	355/420[36]
1,3-Dinitropyrene	395/445[36]
1,6-Dinitropyrene	369/442[36]
1,8-Dinitropyrene	395/454[36]
1,3,6-Trinitropyrene	396/460[36]
1,3,6,8-Tetranitropyrene	397/465[36]
3-Nitrofluoranthene	300/530;[36] 295/515[21]
8-Nitrofluoranthene	300/550[36]
6-Nitrochrysene	273/437;[36] 345/430[21]
6-Nitrobenzo[*a*]pyrene	420/475[36]
3-Nitroperylene	227/540[54]

observed to be more active and to have a higher reductive capacity than the 'three-way' catalyst. The degree of conversion for 1-NP and other NPAHs approaches 100%.[36] The use of a Pt catalyst supported on alumina has also been reported.[33] Other catalyst materials were found to be inferior to these. When tested in methanol–water mobile phases, commercially available Rh on alumina also reduced 1-NP, but neither Pd on alumina nor Rh metal powder worked.[36]

Several systems[23,31,68] utilize electrochemical reduction of NPAHs to amino derivatives. The extent of conversion using this technique is not very high and it depends on the flow rate of the mobile phase.[31] Murayama and Dasgupta[31] reported an approximately 5% reductive conversion, whereas, in another system,[68] values of about 13–14% were found.

The reductive column can be placed either before[29,47,54] or after[33,68,75] the analytical column. Electrochemical reduction is performed in the post-column arrangement.[23,31,68] More sophisticated equipment employs a reductive column in a column-switching system.[36,60]

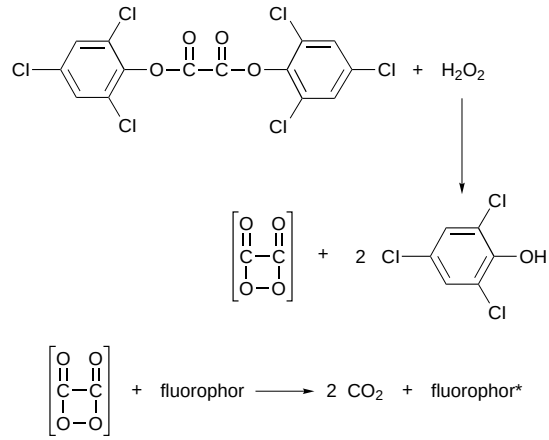
Fluorescence detection has been employed in the analyses of various types of samples. Concentrations of 1-NP, 1,3-DNP, 1,6-DNP and 1,8-DNP were measured in atmospheric dust particles[76] and in the air[24] in Japan; 1-NP, 1,6-DNP and 1,8-DNP were detected in an ambient aerosol at an urban site and suburban site in Michigan.[34] Levels of 1-NP, 2-NF and 3-NFA were quantified in suspended particulate matter in Berlin.[21] Ten mononitro-PAHs were identified in atmospheric samples collected in Paris.[74] Atmospheric samples were also analysed for 1-NP,[35] 1-NP and 3-NFA,[69] 1-NP and 6-NBaP.[22] Hisamatsu *et al.*[71] identified mutagenes formed by photochemical reaction of pyrene with nitrogen dioxide. The following substances were determined in vehicle engine exhausts: 1-NP, 6-NBaP and 9-NA in diesel particulate extract,[33] 1,3-DNP, 1,6-DNP and 1-NP in sooty emissions of cars,[23] 9-NA and other NPAHs in exhausts from diesel engines;[31] 1-NP in particulate emissions from vehicles.[36] Concentrations of 1-NP, 2-NF, 9-NA, 7-NBaA and 6-NBaP were determined in NIST Standard Reference Material SRM1650.[29] Fluorescence detection has also been used in analyses of sediments and soils (1-NP, 9-NA, 2-NN, 6-NC, 6-NBaP)[43] and foodstuffs (1-NP).[46,47] 1-NP and its metabolites were determined in biological samples[68] and amounts of 1-NP were also measured in leaves from trees growing under various traffic conditions.[70]

Chemiluminescence detection

At present, TCPO–H_2O_2 chemiluminescence is the most sensitive HPLC detection technique in NPAH analysis. The DLs are typically 1–2 orders of magnitude lower than those which can be achieved in the same chromatographic system using fluorescence detection. Moreover, the high degree of selectivity allows the direct determination of NPAHs in complex matrices with minimum sample preparation. The exceptional selectivity of chemiluminescence detection is documented by the chromatogram in Fig. 4, obtained by direct injection of a crude extract from airborne particulates after off-line reduction.

This detection approach is based on reaction between corresponding amino derivatives of NPAHs (fluorophore) with a mixture of bis(2,4,6-trichlorophenyl)oxalate (TCPO) and hydrogen peroxide,[77] as shown in the reaction scheme.

The high sensitivity of detection is due to a combination of high efficiences in the excitation step and high chemiluminescence quantum yields. As no light source is used, a low background is obtained. A basic catalyst in the mobile phase is necessary to accomplish the reaction. Several basic buffers have been tested.[23,41] Tris(hydroxymethyl)aminomethane hydrochloride[41] or imidazole–perchloric acid[23–26,59,78,79] buffers



appeared to be the most suitable for this purpose. Acetonitrile is a typical organic modifier for this type of analysis.

The conversion of NPAHs into APAHs is performed as in fluorescence analysis. Off-line reduction with NaHS,[24,25,78–81] an on-line catalyst[58] or Zn–glass bead[26,41] column and electrochemical reduction[23] at glassy carbon working electrode have been used. The chemiluminescence radiation can be detected by commercially available chemiluminescence detectors[23–26,78] or by fluorescence detectors with the light source turned off.[41,58]

Various samples have been analysed for NPAHs by means of chemiluminescence. Diurnal concentrations of 1,3-DNP, 1,6-DNP, 1,8-DNP, 1-NP and benzo[*a*]pyrene were measured in the air of Kanazawa city.[24,25,80] Murahashi *et al.*[59] published a method for the simultaneous determination of NPAHs and PAHs in airborne particulates with column switching equipment. Another column switching system designed by Murahashi *et al.*[26] is suitable for the sensitive determination of 1-NP, 2-NP, 4-NP, 2-NFA and 6-NC in airborne particulates. Concentrations of nitrated pyrenes and their derivatives were measured in sooty emission from cars;[23] mono- and dinitro-PAHs were determined in diesel exhaust particle extracts.[58] Dinitropyrenes and nitropyrenes were determined in emission particulates from vehicles with diesel and gasoline engines.[78,79] NPAHs were also detected in carbon black.[41]

A different type of chemiluminescence detector, originally designed for a GC system**,** has been evaluated in conjunction with HPLC. Details of this NO·–O_3 gas-phase detector[57,82] and the interface[83] are described elsewhere. Unfortunately, this

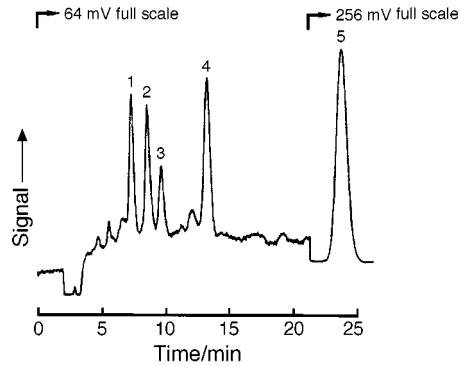


Fig. 4 Chromatogram of benzene–ethanol extract from airborne particulates after off-line reduction. Peaks: 1 = 1,6-DNP; 2 = 1,8-DNP; 3 = 1,3-DNP; 4 = 2-fluoronitrofluorene (internal standard); 5 = 1-NP. Separation conditions: Cosmosil ODS column 250×4.6 mm id); 10 mM imidazole buffer (pH 7.6) – acetonitrile (1 + 1 v/v) mobile phase; TCPO chemiluminescence detection. Reprinted from ref. 25 with courtesy of the American Chemical Society.

detector is much less sensitive (10–30 ng in ref. 57, 50 pg in ref. 54) than the TCPO–H_2O_2 chemiluminescence detector. Together with the commercial unavailability of the detector, this is probably the main reason why no practical applications have been published.

Mass spectrometric detection

Analysis using mass spectrometry is very convenient and provides considerable information about the nature of the analyte. Although some LC–MS instruments are on the market, this method is not yet well established. Moreover, the equipment is expensive so that only a few analytical procedures have been described so far.

LC–MS with a nebulizer and a moving belt was used to characterize 2-NF, its metabolites and related compounds.[48] The interface affects the separation only slightly. The equipment, operating in the electron ionization mode, gives acceptable mass spectra down to 65 ng of injected substance and the responses are linear in the range 0.2–1.2 μg.

A particle beam interface was employed to identify hydrocarbons, PAHs and NPAHs in extracts of fossil fuel combustion emissions.[84] The best results for NPAHs were obtained under negative ion chemical ionization. The DLs determined in the FIA–MS mode for 1,8-DNN, 9-NA, 3-NFO, 2,2′-DNB, 1-NP, 2,7-DNF, 2,7-DNFO were at the picogram level. Some compounds, such as 1-NN and 2-NN, were not detectable even at microgram levels. The calibration curves exhibited good linearity over a range of greater than two orders of magnitude.

Mass spectrometry is also very useful off-line, when selected fractions from HPLC runs are analysed. Using this approach, more than 50 NPAHs have been tentatively identified in an extract of diesel exhaust particulates.[38]

Standards and reference materials

Precise and accurate determinations of NPAHs require the use of appropriate standards and certified reference materials. The Institute for Reference Materials and Measurements (IRMM) prepared a series of eight nitropolyarenes (1-NP, 1-NN, 2-NN, 9-NA, 6-NC, 3-NFA, 6-NBaP, 2-NMNF) with a purity better then 99% as certified reference materials.[85] J. T. Baker[86] supply 23 mono-NPAHs and nine di- and tri-NPAHs as neat reference materials in 95–99% purity, Dr. Ehrenstorfer[87] offers 42 mono-, di- and trinitro-PAHs (Dr. Ehrenstorfer Reference Materials). AccuStandard Inc. sell 17 mono-NPAHs and 12 di- and trinitro-PAHs as neat substances or solutions in toluene (100 μg ml^{-1}).[88] Standard reference materials SRM 1587 (nitrated polycyclic aromatic hydrocarbons in methanol; 2-NF, 9-NA, 3-NFA, 1-NP, 7-NBaA, 6-NC, 6-NBaP), SRM1596 (dinitropyrene isomers and 1-nitropyrene in methylene chloride; 1-NP, 1,3-DNP, 1,6-DNP, 1,8-DNP) and SRM1650 (diesel particulate matter, with certified content of 1-NP) are available from NIST.[89]

A review[90] dealing with the synthesis of nitropolyarenes is useful for cases where the requisite isomer is not commercially available.

Appendix

Abbreviations

APAHs	Amino-polycyclic aromatic hydrocarbons
2-ANFO	2-Acetamido-3-nitro-9-fluorenone
DL	Detection limit
2,2′-DNB	2,2′-Dinitrobiphenyl
2,7-DNF	2,7-Dinitrofluorene
2,7-DNFO	2,7-Dinitro-9-fluorenone
1,5-DNN	1,5-Dinitronaphthalene
1,8-DNN	1,8-Dinitronaphthalene
1,3-DNP	1,3-Dinitropyrene
1,6-DNP	1,6-Dinitropyrene
1,8-DNP	1,8-Dinitropyrene
FIA	Flow-injection analysis
GC	Gas chromatography
HPLC	High-performance liquid chromatography
MS	Mass spectrometry
9-NA	9-Nitroanthracene
2-NB	2-Nitrobiphenyl
3-NB	3-Nitrobiphenyl
4-NB	4-Nitrobiphenyl
7-NBaA	7-Nitrobenzo[*a*]anthracene
6-NBaP	6-Nitrobenzo[*a*]pyrene
6-NC	6-Nitrochrysene
2-NF	2-Nitrofluorene
3-N-9-F	3-Nitro-9-fluorene
2-NFA	2-Nitrofluoranthene
3-NFA	3-Nitrofluoranthene
4-NFA	4-Nitrofluoranthene
7-NFCA	7-Nitrofluorene-1-carboxylic acid
3-NFO	3-Nitro-9-fluorenone
NICIMS	Negative ion chemical ionization mass spectrometry
NIAPIMS	Negative ion atmospheric pressure ionization mass spectrometry
2-NMNF	2-Nitro-7-methoxynaphtho-(2,1-b)furan
5-N-6-MQ	5-Nitro-6-methylquinoline
8-N-7-MQ	8-Nitro-7-methylquinoline
1-NN	1-Nitronaphthalene
2-NN	2-Nitronaphthalene
4-N-p-T	4-Nitro-*p*-terphenyl
1-NP	1-Nitropyrene
2-NP	2-Nitropyrene
4-NP	4-Nitropyrene
NPD	Nitrogen–phosphorus detector
3-NPer	3-Nitroperylene
3-NPH	3-Nitrophenanthrene
9-NPH	9-Nitrophenanthrene
5-NQ	5-Nitroquinoline
6-NQ	6-Nitroquinoline
NPAHs	Nitrated polycyclic aromatic hydrocarbons
PAHs	Polycyclic aromatic hydrocarbons
1,3,6-TNP	1,3,6-Trinitropyrene
TCPO	Bis(2,4,6-trichlorophenyl) oxalate
UV/VIS	Ultraviolet/visible

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