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Clean-up and analysis of carbazole and acridine type polycyclic aromatic nitrogen heterocyclics in complex sample matrices

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Abstract

A HPLC method for the analysis of polycyclic aromatic nitrogen heterocyclics (PANHs) in complex sample matrices is presented. It isolated and separated carbazole and acridine type PANHs with an absolute recovery of between 79–98%. Open column chromatography is used as an initial step to isolate a PANH fraction. By applying normal-phase liquid chromatography using a dimethylaminopropyl silica stationary phase and utilising back-flush technique it was possible to separate the PANH fraction into two fractions containing acridine type and carbazole type PANHs, respectively. The method applied on a sample of solvent refined coal heavy distillate (SRC II HD). A number of 3–5 ring acridines and carbazoles were identified with GC–electron impact MS and quantified with GC–nitrogen–phosphorous detection. Polycyclic aromatic hydrocarbons (PAHs) were determined in the SRC II HD sample by automated on-line clean-up and analysis of the obtained PAH fraction with coupled LC–GC–flame ionization detection. There was no overlap between the PANH and the PAH fractions with this method, and carbazoles and acridines were efficiently separated. © 1997 Elsevier Science B.V.

Keywords: Coal; Polycyclic aromatic nitrogen heterocyclics; Polynuclear aromatic hydrocarbons; Acridines; Carbazoles

1. Introduction

Polycyclic aromatic nitrogen heterocyclics (PANHs) with a single endocyclic nitrogen heteroatom, can be divided into two classes: acridines (containing a pyridine ring) and carbazoles (containing a pyrrole ring). A source of PANHs is pyrosynthesis during incomplete combustion of nitrogen-containing organic matter. PANHs have thus been detected in urban air particulate matter [1], tobacco smoke [2] and automobile exhaust [3]. Other sources of PANHs are petroleum products such as crude oil [4] and high boiling petroleum distillates [5] as well as coal tar [6] and shale oil [7]. In the bottom sludge of stored solvent-refined coal liquids, carbazoles were found to the most abundant class of compounds [8].

A number of PANHs are known to be mutagenic and/or carcinogenic [9–12]. PANHs with two and three rings are mostly classified as mutagenic, whereas compounds with four or more aromatic rings are probably carcinogenic [13]. These biological effects makes PANHs interesting from both an occupational and an environmental point of view. In general, acridines have a higher mutagenic activity than carbazoles, their mutagenic activity is comparable to polycyclic aromatic hydrocarbons (PAHs) [14]. The concentration of PANHs detected in en-

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vironmental samples is lower compared to the corresponding parent PAHs. In urban air particulate matter, the concentration of PANHs is one- to three-orders of magnitude less than those of the parent PAHs [15]. Due to their biological activity, it is of interest to develop methods of analysis in order to identify and quantify PANHs in the environment.

A PANH fraction from environmental samples consists of a complex mixture of compounds, due to the large amount of isomers. This cause problems with co-eluting peaks when using chromatographic separation techniques. Thus, chemical analysis of PANHs require a group separation of acridine- and carbazole-type compounds in order to facilitate identification as well as quantification. Acridine-type PANHs are slightly basic due to the unshared electron pair of the nitrogen not participating in the aromatic delocalisation. In carbazole-type PANHs the unshared electron pair is incorporated into the aromatic π orbitals giving these PANHs weak acidic properties. Chromatographic methods that takes advantage of the difference in acid–base properties, ability to form hydrogen bonds and polarity can be used for separation between the two groups. However, a problem with chromatography of PANHs, regarding both open column chromatography and HPLC methods, is the incomplete resolution of the two groups of PANHs when a wide range of molecular masess is considered. This is especially the case when regarding shielded acridines.

PANHs are not as well investigated as PAHs, although a number of analytical methods have been published. Most of the methods used acid–base extraction and open column chromatography [1,4,16–19], but there are also some HPLC methods for separation of PANHs from PAHs and as well as for separation of different PANHs classes [15,20–24]. The drawbacks of most the methods are: overlap of the two PANH classes, only PANHs with two and three aromatic rings are included in most of the investigations and the analytical procedures are rather time consuming.

This paper presents a fast HPLC method for the isolation and class separation of acridines and carbazoles with 3–5 aromatic rings. GC–MS was used for identification of the PANHs while quantitative analysis was performed with GC utilising nitrogen selective detection. PAHs were determined using on-line coupled LC–GC [25]. The method is applied on a solvent refined coal sample (SRC).

2. Experimental

2.1. Chemicals

The acridine-type reference substances were purchased from Promochem (Wesel, Germany) and the carbazole-type reference substances were kindly provided by Professor M. Zander. Hexane and methyl-*tert*.-butyl ether (MTBE) (HPLC-grade, Rathburn, Walkerburn, UK), were used as mobile phase components without further purification. All other solvents (analytical grade, Merck, Darmstadt, Germany) were distilled in an all glass apparatus prior to use. The solvent refined coal, was obtained from National Institute of Standards and Technology (Washington, DC, USA).

2.2. Open column chromatography

Open column chromatography was performed on a 60×6 mm glass column slurry packed with silica-gel (Kieselgel 60, 35–70 mesh, Merck). The silica was heated to 450°C for 24 h and subsequently deactivated with 10% (w/w) of distilled water. The deactivated silica was stored in cyclohexane until used.

2.3. Liquid chromatography

The HPLC-system consisted of a Waters 590 programmable solvent delivery module using a flow-rate of 2.0 ml/min, a Waters automatic valve station for switching the column flow and a Hitachi 655A UV detector monitoring the effluent at 254 nm. The investigated bonded phases were aminopropylsilica (Nucleosil 5 NH_2, 5 μm, 200×4 mm), diol-propylsilica (Nucleosil 7 OH, 7 μm, 250×4 mm), nitrophenylpropylsilica (Nucleosil 5 NO_2, 5 μm, 200×4 mm), cyanopropylsilica (Nucleosil 5 CN, 5 μm, 200×4 mm), phenylpropylsilica (Nucleosil 5 C_6H_5, 5 μm, 200×4 mm), dimethylamino-propylsilica (Nucleosil 5 $N(CH_3)_2$, 5 μm, 200×4 mm), vinyl pyrrolidone polymer (γPVP 5 μm, 250×4 mm), 2-(1-pyrenyl)-ethyldimethyl-silyl (PYE, Cos-

mosil, 5 μm, 150×4.6 mm) diaminopropylsilica (5 μm, 250×4 mm) and triaminopropylsilica (5 μm, 250×4 mm). All Nucleosil bonded phases were purchased from Macherey–Nagel (Düren, Germany). The PYE column was purchased from Nacalai Tesque, Japan, while the diaminopropylsilica, triaminopropylsilica and γPVP silica columns were purchased from ES Industries (Berlin, NJ, USA). A PC computer based laboratory data system (ELDS Win Pro, Chromatography Data Systems, Svartsjö, Sweden) was used for registering of the detector signals from the HPLC and GC systems and also for operating the HPLC system switch valves.

2.4. Gas chromatography

The GC system was a Varian 3700 (Varian, Walnut Creek, CA, USA) equipped with a split/splitless injector, a nitrogen–phosphorus detection (NPD) system and a capillary column (DB-5, 5% phenylmethylpolysiloxane gum, 30 m×0.32 mm, d_f=0.10 μm, J&W Scientific, Folsom, CA, USA) using hydrogen as carrier gas for the separation. Temperature programmed analysis was applied using an initial temperature of 70°C which was kept for 3 min. This was followed by a linear temperature increase of 10°C/min up to 300°C which then was kept for 5 min. The temperatures of injector and detector were 300°C.

2.5. Gas chromatography–mass spectrometry

The GC–MS consisted of a Varian 3400 gas chromatography system coupled to an Incos 50 quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) operating in electron impact (EI) mode with an ionisation potential of 70 eV. A temperature of 310°C was maintained in the GC–MS transfer line. All GC parameters were the same as described in Section 2.4 with the exception that helium was used as carrier gas.

2.6. Liquid chromatography–gas chromatography

On-line coupled LC–GC described elsewhere was applied in order to analyse PAHs [25]. The HPLC part of the system consisted of a Varian 9001 HPLC pump, a Marathon autosampler (Spark Holland, Emmen, Netherlands) equipped with a 100 μl injection loop and a Hitachi L4000 UV detector (Merck–Hitachi, Germany) monitoring the effluent at 254 nm. The HPLC column was a nitropropyl modified silica (Nucleosil 5 NO_2, 5 μm, 75×4 mm). HPLC-grade pentane (Rathburn) was used as mobile phase at a flow of 1 ml/min. The outlet of the UV detector was switched either to solvent waste or to a loop interface equipped with a 500 μl stainless-steel loop. The loop interface was connected to the GC system by fused-silica tubing.

A modified Varian 3700 gas chromatograph was used as the GC system. It was equipped with a FID system and a column system consisting of a fused-silica retention gap (3 m×0.53 mm) in series with a pre-column (2 m×0.32 mm) coated with 0.25 mm 5% phenylmethylsiloxane gum (DB-5, J&W Scientific). This was connected by an all glass Y-splitter to a solvent vapour exit (a 0.2 m×0.53 mm fused-silica line) and a 28 m×0.32 mm fused-silica capillary column coated with 0.25 mm 5% phenylmethylsiloxane gum (DB-5, J&W Scientific). Hydrogen was used as carrier gas. During injection the temperature was kept at 55°C. 2 min after completed injection, a linear temperature increase of 20°C/min was applied until a temperature of 155°C was reached. After 1 min delay a linear temperature increase of 7°C/min was applied until final temperature of 300°C was reached. This temperature was then held for 10 min.

Calibration of the LC–GC system was made by the use of a solution of anthracene in cyclohexane injected on the LC. Reversal of the HPLC column mobile phase flow was set to the start of elution of anthracene (2.90 min). Concurrent solvent evaporation injection time was selected as the end of the elution of the reversely eluted anthracene peak (5.80 min). The injection time on to the GC for the backflush fraction trapped in the loop interface was found to be 3.25 min at a loop transfer pressure of 0.90 kg/cm^2 and an injection temperature of 55°C. Solvent ventilation time of the early solvent vapour exit was set to 0.30 min.

3. Results and discussion

A scheme of a complete clean-up procedure for

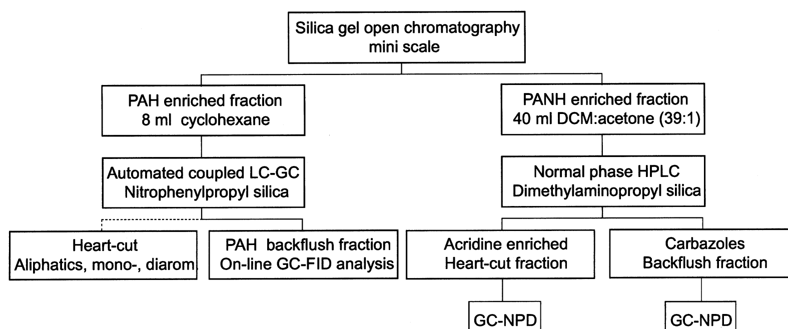

Fig. 1. Clean-up scheme for the analysis of acridines, carbazoles and PAHs.

the analysis of acridines, carbazoles and PAHs is presented in Fig. 1. Open column chromatography was used as a pre-separation step in order to obtain a PAH and a PANH containing fraction. Subsequent quantitative analysis of PANH was performed using GC–NPD. Identification of the carbazole- and acridine-type compounds in the PANH fraction was done by the use of GC–MS. On-line coupled LC–GC was applied in order to isolate and analyse the PAH fraction [25].

3.1. Pre-separation

Open column chromatography on deactivated silica was used as an initial step to isolate a PANH enriched fraction. A pre-fraction that contained paraffins, olefins, mono- and dicyclic aromatics and PAHs was eluted using 8 ml cyclohexane. By subsequent elution with 40 ml methylene chloride–acetone (39:1) a PANH enriched fraction was obtained. When using this method there was no overlap of sterically hindered PANH into the PAH fraction. This is otherwise a common problem in many of the published analytical methods based on open column chromatography and HPLC [24]. Deactivation of the silica gel with water was necessary to eliminate irreversible adsorption, particularly regarding non-shielded acridines.

3.2. Group separation of PANHs

Separation of acridines and carbazoles on open silica columns is difficult, due to overlap of sterical hindered acridines into the carbazole-fraction. In order to develop a complete group separation of 3–5 ring carbazoles and acridines based on normal-phase HPLC a number of bonded HPLC-phases, were investigated regarding their separation ability with respect to acridines and carbazoles. The columns were operated in normal-phase mode using isocratic elution. The investigated bonded phases were: γPVP, diol-, nitro-, cyano-, phenyl- amino-, diamino-, triamino-, dimethylaminopropyl- and 2-(1- pyrenyl)-ethyldimethylsilica.

Only two of the investigated columns were able to completely separate acridines and carbazoles: the dimethylaminopropylsilica and aminopropylsilica stationary phases. These columns were thus further investigated. Tables 1 and 2 contain retention data of

Table 1
Capacity factors (k') for dimethylaminopropylsilica

Compound	% MTBE in hexane			
	10	20	33	50
Benzo[*h*]quinoline	1.7	1.4	1.1	0.9
Benz[*c*]acridine	1.7	1.4	1.1	0.9
Acridine	2.5	2.1	1.7	1.2
Dibenz[*c,h*]acridine	2.5	2.1	1.7	1.2
Dibenz[*a,h*]acridine	3.3	2.7	2.3	1.8
Phenanthridine	3.6	2.9	2.3	1.8
10-Azabenz[*a*]pyrene	4.0	3.3	2.6	2.0
Benz[*a*]acridine	4.6	3.5	2.6	2.0
Dibenz[*a,j*]acridine	6.9	5.0	3.6	2.4
Dibenz[*a,i*]acridine	7.9	5.7	4.0	2.7
Carbazole	29.4	17.0	10.1	6.1
Benzo[*b*]carbazole	60.2	33.8	19.7	11.3
Benzo[*a*]carbazole	84.4	47.5	27.9	16.5
Benzo[*c*]carbazole	97.6	54.9	32.0	18.7

MTBE in percentage of the mobile phase. Mean value of 3 measurements. The relative standard deviation was less than 5%.

Table 2
Capacity factors (k') for aminopropylsilica

Compound	% MTBE in hexane			
	10	20	33	50
Benz[*c*]acridine	1.9	1.2	0.9	0.7
Dibenz[*c*,*h*]acridine	2.4	1.6	1.2	0.9
Benzo[*h*]quinoline	2.6	1.6	1.2	0.9
Dibenz[*a*,*h*]acridine	3.2	2.0	1.4	1.0
10-Azabenz[*a*]pyrene	5.5	3.2	2.2	1.7
Acridine	6.1	3.2	2.2	1.7
Benz[*a*]acridine	8.7	4.5	2.9	2.0
Phenanthridine	8.9	4.8	3.3	2.3
Dibenz[*a*,*j*]acridine	11.2	5.5	3.8	2.3
Dibenz[*a*,*i*]acridine	13.7	6.6	4.0	2.6
Carbazoles	15.3	6.8	4.0	2.6
Benzo[*b*]carbazole	27.6	11.7	6.1	3.5
Benzo[*a*]carbazole	29.8	12.2	6.9	3.5
Benzo[*c*]carbazole	42.3	17.8	9.3	5.3

MTBE in percentage of the mobile phase. Mean value of 3 measurements. The relative standard deviation was less than 5%.

fourteen PANH standard compounds on the two stationary phases and shown the separation ability of acridines and carbazoles with different polarity of the mobile phase. The capacity ratio (k') is tabulated for a mobile phase composition of 10, 20, 33 and 50% MTBE in hexane. It is important that the separation include nitrogen heterocyclic compounds with more than just two or three aromatic rings, since PANHs with higher ring numbers have been detected in a number of different samples [1,9].

Complete separation of PANHs with 3–5 rings into carbazoles and acridines was achieved by applying isocratic elution on the dimethylaminopropylsilica stationary phase. A mobile phase consisting of hexane–MTBE (1:1) gave a total analysis time of less than 18 min. By reversing the flow just prior to the start of the elution of carbazole, the earliest eluting carbazole type component, this group could be eluted as one single peak in back-flush mode (Fig. 2). Acridine type compounds were isolated in a heart-cut fraction, ranging from benzo[*h*]quinoline the earliest eluting component to dibenz[*a*,*i*]acridine, the latest eluting component (Fig. 2 Table 1).

When using the aminopropylsilica column it was only possible to separate 3–5 ringed acridines from carbazoles when using a mobile phase containing 10% or less MTBE in hexane. This resulted in a total analysis time of 50 min. If more MTBE was mixed into the mobile phase, dibenzo[*a*,*i*]acridine and carbazole obtained the same capacity factor and thus coeluted, Table 2. Thus, the dimethylaminopropylsilica phase is preferable to use for the group separation of acridines and carbazoles due to higher degree of separation and much shorter analysis time, due to favourable retention mechanism.

The differences in retention of these solutes on these two columns can be explained in terms of the two methyl substituents added on the di-

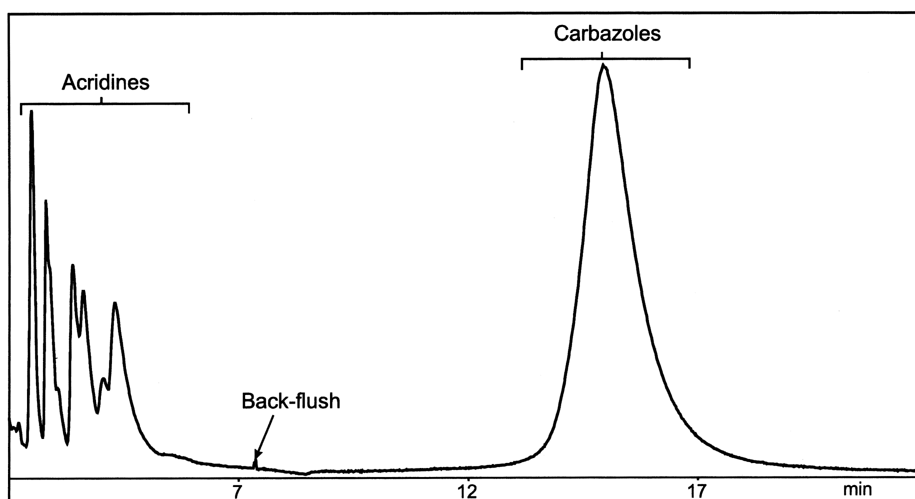

Fig. 2. Clean-up of carbazoles and acridines in the SRC sample, using the dimethylaminopropylsilica column.

Table 3
Absolute recovery of eight carbazole-type PANHs for the complete clean-up procedure

Compound	Recovery (%)	R.S.D. (%)
Carbazole	93	10
Benzo[*def*]carbazole	91	9
Benzo[*a*]carbazole	98	9
Benzo[*b*]carbazole	89	8
Benzo[*c*]carbazole	94	7
Dibenzo[*a,i*]carbazole	85	12
Dibenzo[*c,g*]carbazole	94	12
Dibenzo[*b,g*]carbazole	89	10

Mean value of 5 measurements.

methylaminopropyl silica. These substituents decrease the retention of acridines compared to the aminopropylsilica by eliminating the hydrogen bonding between the free electron pair on the nitrogen atom of the solute and the hydrogens of the amino group on the stationary phase. The retention of carbazoles is increased on the dimethylaminopropylsilica compared to aminopropylsilica (Tables 1 and 2), since the methyl substituents increase the electron density on the amino group by an inductive effect. This result in a stronger hydrogen bonding interaction between the hydrogen bonded to the nitrogen atom of the carbazoles and the free electron pair of the amino group. These differences in retention behaviour on the two bonded phases, increase the separation ability of carbazoles and acridines on the dimethylpropylsilica stationary phase [26].

In order to determine the absolute recovery for the overall method five replicate measurements of PANH reference substances were made, Tables 3 and 4. For the carbazole-type compounds the recovery was between 85–98%. For the acridine-type compounds the recovery was slightly lower, between 79–92%. The lower recovery for the latter compound group was attributed to adsorption of the slightly basic acridines on both the open column and HPLC column.

Table 4
Absolute recovery of nine acridine-type PANHs for the complete clean-up procedure

Compound	Recovery (%)	R.S.D. (%)
Benzo[*h*]quinoline	80	5
Acridine	81	3
Phenanthridine	84	9
Benz[*c*]acridine	81	8
Benz[*a*]acridine	89	11
10-Azabenzo[*a*]pyrene	81	8
Dibenz[*a,h*]acridine	79	11
Dibenz[*a,j*]acridine	85	9
Dibenz[*a,i*]acridine	92	8

Mean value of 5 measurements.

3.3. Analysis of solvent refined coal

In order to demonstrate the complete method, a solvent refined coal heavy distillate (SRC II) was investigated. Analysis of PANHs as well as PAHs was performed. A 0.5 mg amount of SRC II was applied on an open silica column and the PAH fraction eluted with 8 ml cyclohexane. The PANH fraction was subsequently eluted with 40 ml methylene chloride–acetone (39:1). This pre-fractionation step isolated the carbazole and acridine fraction from the bulk of the complex SRC II sample matrix. The PANH enriched fraction evaporated precisely to dryness in a rotary evaporator under reduced pressure at a temperature of 35°C. It was then dissolved in 100 μl cyclohexane, prior to injection onto the HPLC system equipped with a dimethylaminopropylsilica column. Separation of the two compound classes were performed with back-flush technique described above and showed in Fig. 2. Acridine type compounds were collected as a heart-cut fraction between 0.5 to 6 min. Carbazole type compounds were collected as a single back-flush peak between 12 to 18 min. The two fractions were analysed with GC–EI-MS in order to establish the identity of the present compounds. A large number of PANH compounds were identified. This included isomers of carbazoles, alkylated carbazoles, benzocarbazoles, alkylated benzocarbazoles and benzoquinolines as well as acridines, alkylated acridines, benzoacridines and alkylated benzoacridines. Figs. 3 and 4 shown the GC–NPD traces of the carbazole and acridine fractions respectively with the identification of the peaks noted in the chromatograms.

Quantitative analysis were performed using GC–NPD. Benzo[*def*]carbazole and 10-azabenz[*a*]pyrene had been added as internal standards to the sample prior to the open column chromatography pre-separation. Five replicate analyses were performed of the solvent refined coal sample and the concentration of

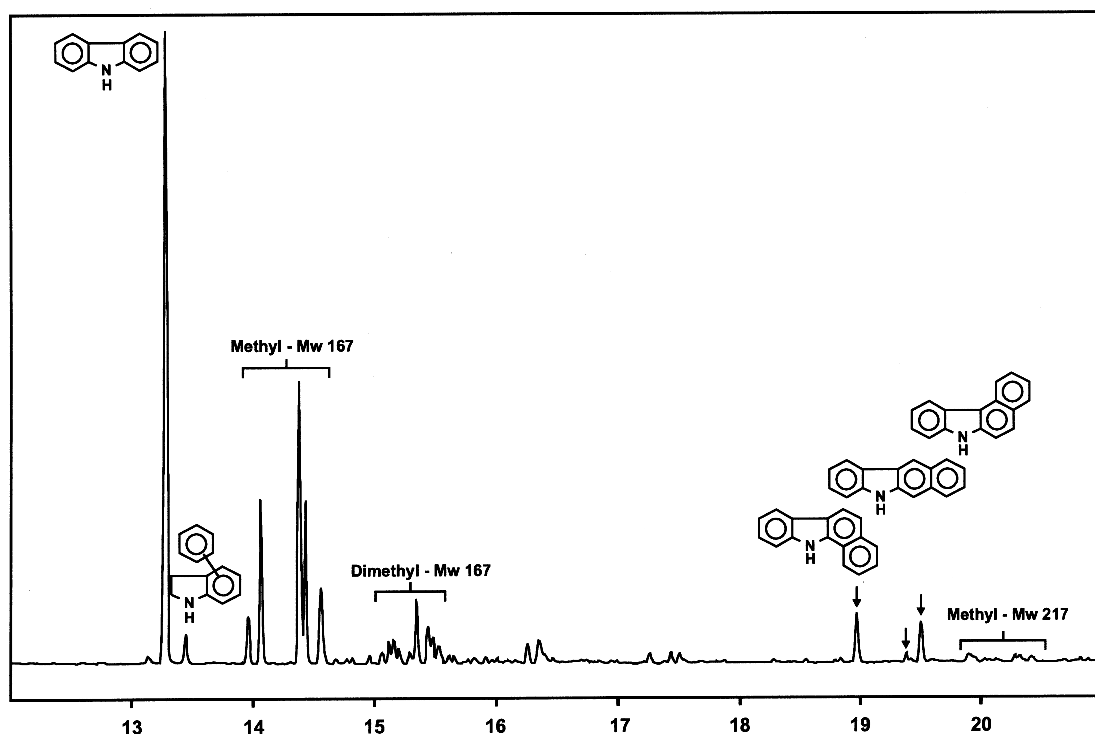

Fig. 3. GC–NPD chromatogram of the carbazoles fraction from the SRC sample, isolated with back-flush technique on dimethylaminopropylsilica column.

twenty-two acridine- and twenty-eight carbazole-type PANHs are listed in Tables 5 and 6, respectively. The precision of the method was high with respect to both acridines and carbazoles.

Quantitative analysis of PAH was performed with on-line LC–GC using FID [25]. Fig. 5 shows the GC–FID trace of the isolated and separated PAH fraction with the identity of a number of peaks marked in the chromatogram. GC–EI-MS was used to ascertain the identity of the PAH compounds. 2,2′-Binaftyl was used as internal standard and added prior to the open column chromatography pre-separation. Table 7 shows the concentrations and the coefficients of variation for fifteen individual PAHs in the SRC II sample. As can be seen the concentration of PAHs was much higher than carbazoles and acridines. The concentration of the three ringed PAH compound phenanthrene was more than 200-times higher than acridine and more than 10-times higher than carbazole.

In conclusion: a method of analysis of PANHs has been developed that separates acridine- and carbazole-type PANHs. This includes PANH compounds containing four and five aromatic rings. The method exhibits a high precision with a relative standard deviation ≤15% in most cases. The accuracy of the overall method, as calculated from analysis of reference substances, was also shown to be good with absolute recovery of 80–100% and relative standard deviation in the range of 10%.

The limit of detection of the method using off-line LC clean-up and GC–NPD, is sufficient to be applied for PANH analysis in order to investigate the occurrence of acridine and carbazole type compounds in ambient air, using stationary high volume samplers. Quantitative analyses of PANHs at the low ng/m^3 level using personal sampling require a developed method using coupled LC–GC–NPD. A method for sampling and analysis of PAH in urban air, using personal sampling and analysis with coupled LC–GC–FID [25,27], will be modified for this purpose in our continuous work. Possible matrix

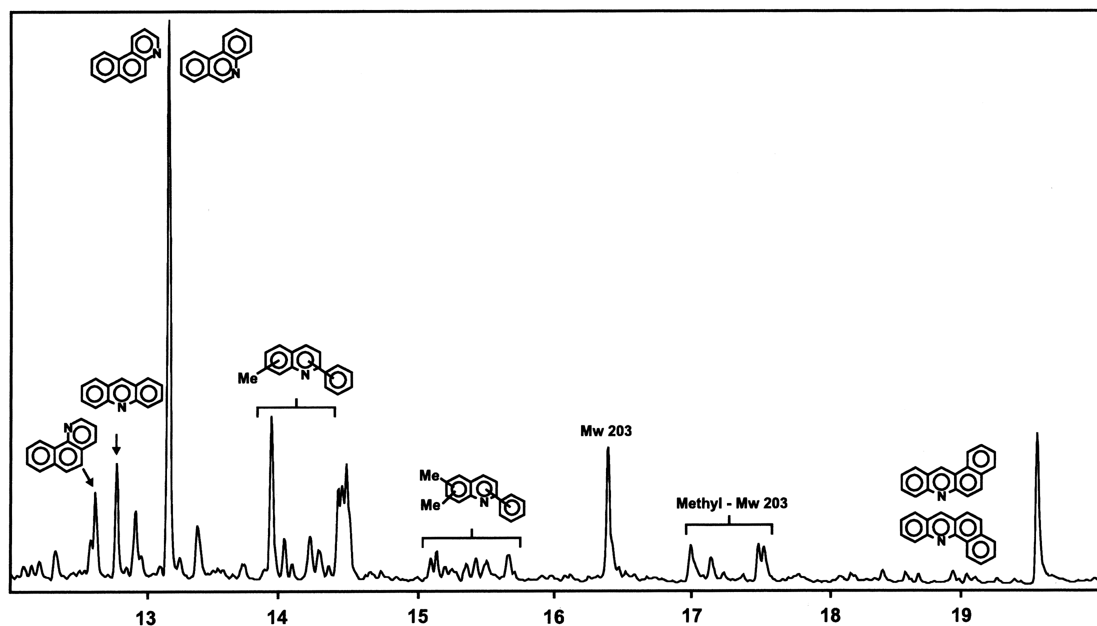

Fig. 4. GC–NPD chromatogram of the acridines fraction from the SRC sample, isolated with heart-cut technique on dimethylaminopropyl silica column.

Table 5
Concentration of twenty-two acridine-type PANHs detected in a solvent refined coal heavy distillate

Compound	Concentration (μg/g)
Benzo[*h*]quinoline	240
Acridine	340
Phenanthridine+benzo[*f*]quinoline	2000
Methylsubstituted M_r 179 (6 compounds)	170–800
Dimethylsubstituted M_r 179 (6 compounds)	110–200
M_r 203	880
Methylsubstituted M_r 203 (4 compounds)	100–300
Benz[*c*]acridine	28
Benz[*a*]acridine	140

Mean value of 5 measurements. The range of the relative standard deviation was 8–19%.

Table 6
Concentration of twenty-eight carbazole-type PANHs detected in solvent refined coal heavy distillate

Compound	Concentration (μg/g)
Carbazole	7300
Benzo[*x*]indole	300
Methylsubstituted M_r 167 (5 compounds)	460–3000
Dimethylsubstituted M_r 167 (9 compounds)	150–1000
Benzo[*a*]carbazole	860
Benzo[*b*]carbazole	200
Benzo[*c*]carbazole	1000
Methylsubstituted M_r 217 (9 compounds)	80–230

Mean value of 5 measurements. The range of the relative standard deviation was 3–25%.

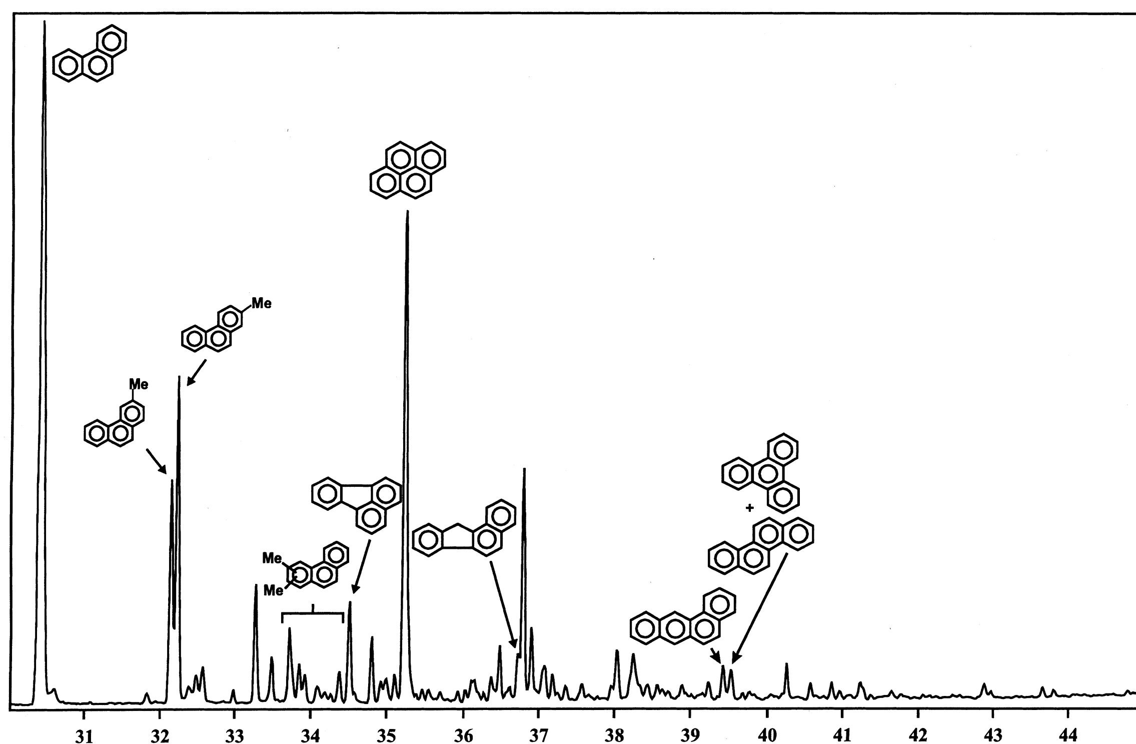

Fig. 5. LC–GC–FID chromatogram of the PAH fraction from the SRC sample, isolated with back-flush technique on coupled LC–GC with nitropropylsilica column.

Table 7
Concentration of eighteen PAHs detected in solvent refined coal heavy distillate

Compound	Concentration (mg/g)
Phenanthrene	80
Anthracene	3.6
3-Methylphenanthrene	22
2-Methylphenanthrene	34
9+4-Methylphenanthrene	3.6
1-Methylphenanthrene	4.2
Dimethylphenanthrene (7 isomers)	2.8–15
Fluoranthene	9.3
Pyrene	50
Benzo[*a*]fluorene	4.2
Benz[*a*]anthracene	3.6
Chrysene+triphenylene	2.9

Mean value of 5 measurements. The range of the relative standard deviation was 5–13%.

effect due to polar interferences from environmental samples can be reduced by using pre-separation with open column chromatography on deactivated silica.

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