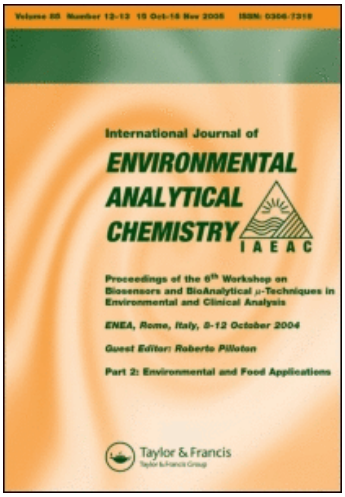

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The study of properties of HPLC determination of polycyclic aromatic nitrogen heterocycles

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The study of properties of HPLC determination of polycyclic aromatic nitrogen heterocycles

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The study of the separation of polycyclic aromatic nitrogen heterocycles (PANHs) by reverse-phase liquid chromatography with an octadecyl stationary phase is presented. The retention behaviour of a mixture of PANHs was studied under different chromatographic conditions. A mixture of phosphate buffer/acetonitrile was used as mobile phase in isocratic and gradient modes. The effect of different pH mobile phase in the range from 2.5 to 6.5 has been investigated to describe retention changes of PANHs as a function of their acid/base properties. Different concentrations of phosphate buffer as a component of the mobile phase were used to study the effect of ionic strength. Very good RP-HPLC separation of 24 PANHs and 16 EPA polycyclic aromatic hydrocarbons (PAHs) was obtained without a pre-separation step in a test mixture and the extract of a real soil sample. Limits of detection of PANHs obtained by two detection techniques, ultraviolet-diode array detection (UV-DAD) and fluorescence detector (FD), are compared. The proposed method is tested with a real soil sample.

Keywords: Polycyclic aromatic nitrogen heterocycles; Azaarenes; PANHs; PAHs; Reverse-phase liquid chromatography

1. Introduction

Polycyclic aromatic nitrogen heterocycles (PANHs), frequently called 'azaarenes' are classified as *N*-heterocycles where one or more carbon atoms in the polycyclic aromatic hydrocarbons structure are replaced by nitrogen [1]. The majority of the PANHs identified in most samples contain one nitrogen atom, but also compounds with two and three nitrogen atoms have been found [2]. PANHs are widespread in the environment, analogously to the polycyclic aromatic hydrocarbons (PAHs) [3]. They are released into the environment from diverse sources such as incomplete combustion of fossil fuels and organic material in the presence of nitrogen-containing substances; spills or effluents of several industrial activities like oil drilling, refining, and storage; coal tar distillation; and wood preservation. Coal products (combustion gases,

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creosote, etc.) are the main source of these compounds [4, 5]. PANHs and PAHs are present in the urban atmosphere because of a growing amount of automobile waste gases, where the concentrations are at the $ng\,m^{-3}$ level [1, 3, 6]. Tobacco smoke and food, namely food processed at high temperatures, for example by grilling, smoking, and frying [4, 7, 8], or products of the thermal degradation of organic compounds, are also a significant source of PANHs and PAHs [9]. When using fuels with a high concentration of these compounds, high NO_x emissions are produced [5]. Other sources of these compounds, having a direct effect particularly on benthic organisms, are sediments in rivers and lakes. Sediments from sewage-disposal plants are contaminated with PAHs and PANHs. Therefore, it is impossible to use those sediments for composting in the agriculture [10]. Sediments in rivers flowing through industrial cities contain 128–2451 $ng\,g^{-1}$ of PANHs. The concentrations of individual PANHs in sediments show strong seasonal variations [10].

Environmental pollution by these compounds has been of increasing concern. The rain can dissolve PANHs containing aerosols, and so the PANHs are accessible for the living organisms, especially for the aquatic organisms [10]. PANHs are also semivolatile and therefore can enter the lungs [1, 3]. Both PAHs and PANHs as well as their transformation products are well known as biologically active environmental pollutants with mutagenic and carcinogenic properties [6]. Many studies have used various tests to demonstrate these dangerous properties (Ames test, Mutatox test, and others) [7, 9, 11]. Therefore, the monitoring of these organic pollutants is an important issue in all industrial countries [4].

A relatively high number of studies have focused on the determination of PANHs in the last years. Many of these studies have used gas chromatography (GC) or liquid chromatography (LC) systems [2, 6, 8, 12–16]. Liquid chromatography systems with fluorescence detection (HPLC-FD) are often used for routine analysis of PANHs and other organic pollutants in many laboratories.

Due to the similar properties of PAHs and PANHs, it is often necessary to pre-separate and clean up individual fractions. Östman *et al.* [2, 17] have developed a clean-up method for PANHs using silica open columns and acid/base extraction. Similar methods are used very often [7, 8]. Ruckmick *et al.* [5] have used semi-preparative normal-phase liquid chromatography (NPLC) to obtain fractions of compounds present in non-distillable coal derived liquids. Rivera *et al.* [7] have used another clean-up procedure with open columns for the determination of PAHs, PANHs and heterocyclic aromatic amines in charcoal-grilled meat. These clean-up methods can be used for separation and determination of azaarenes in PAHs mixtures. These methods are in most cases time-consuming and may be a potential source of contamination and/or loss of sample and/or loss of analytes. In many cases, they also consume a high volume of solvents.

Warzecha *et al.* [8] have developed a method using a combination of normal phase semi-preparative liquid chromatography (NPLC), and reverse-phase liquid chromatography (RPLC) for the determination of groups of azaarenes. This method was used for the separation of a limited number of azaarenes per sample (a standard mixture of nine azaarenes), although the group of PANHs is much larger. The separation of PANHs in NPLC was performed using silica gel modified with different types of polar groups. Low long-term stability and lower separation efficiency were the reasons for replacement of NPLC by RPLC separation, especially with C18 stationary phases. Mobile phases mainly based on acetonitrile/aqueous buffer were used in RPLC to set the best conditions for PANHs separation [8, 18, 19].

Successful separation of a large mixture of PANHs in the presence of other organic pollutants requires a detailed study of PANH behaviour in the selected chromatographic system. We report in this article the possibility of using RPLC with a C18 stationary phase for the separation and determination of individual PANHs and PAHs in composite samples. The effect of the mobile-phase composition, based on acetonitrile/phosphate buffer, was studied. The method was applied to a real soil sample.

2. Experimental

2.1 *Reagents and chemicals*

Separate stock solutions in acetonitrile were made for each compound. PANHs (figure 1) were Aldrich products excluding benz[*a*]acridine, benz[*c*]acridine, dibenz[*ai*]acridine, dibenz[*aj*]acridine, dibenz[*ah*]acridine, dibenz[*ch*]acridine, and 7-H-dibenzo[*cg*]carbazole, which were from Dr Ehrenstorfer GmbH (Germany). The standard solution of 16 EPA PAHs in hexane was a Dr Ehrenstorfer GmbH (Germany) product. Acetonitrile used as mobile phase was provided by Sigma-Aldrich (St. Louis, MO). Sodium hydrogen phosphate p.a. was from Lachema (Brno, Czech Republic) and potassium hydrogen phosphate p.a. was from Serva (Hauppauge, NY). Water used in the laboratory was prepared using Simplicity 185 equipment (Millipore, Molsheim, France). Buffer solutions were filtered through a 45 μm Nylon 66 filter (Supelco, Bellafonte, PA) before use in the HPLC system.

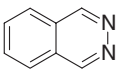
2.2 *Soil-sample preparation*

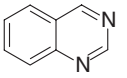
Soil samples taken from a depth of 20–30 cm were air-dried at ambient temperature and, after removal of coarse materials (sticks, grits), sieved through a 2 mm sieve. Five grams of each soil sample was Soxhlet-extracted with a 125 mL mixture of acetonitrile and methanol (80 : 20, v/v) [20]. The extract was concentrated to 5 mL and filtered using a syringe filter (Econofilter, 0.45 μm RC, Agilent Technologies, Santa Clara, CA). The filtered extract was concentrated to 1 mL under a stream of nitrogen and analysed by HPLC.

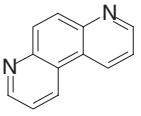
2.3 *Chromatographic separation and detection*

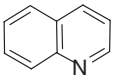
The chromatographic apparatus consisted of an HP 1100 series liquid chromatograph (Agilent Technologies, Inc., Palo Alto, CA) equipped with a model 1100 DAD detector and HP 1046 fluorescence detector. Chromatographic columns with bonded C18 groups were used to study the separation of a mixture of PANHs and PAHs: Ace 5 C18 (250 × 4.6 mm i.d., 5 μm). The column dead time was determined using the retention time of uracile.

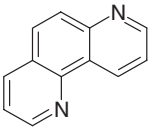
The temperature was thermostatically equilibrated at 30°C, to obtain a satisfactory reproducibility and peak broadening. After a series of measurements, the column was treated with a mixture of acetonitrile and water while slowly increasing the ratio of

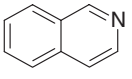
2-phthalazine 

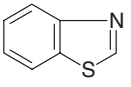
3-quinazoline 

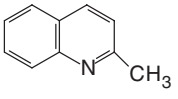
4-4,7-phenanthroline 

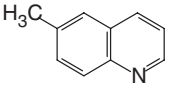
5-quinoline 

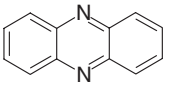
6-1,7-phenanthroline 

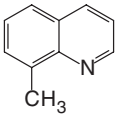
7-isoquinoline 

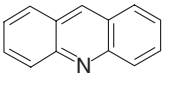
8-benzothiazole 

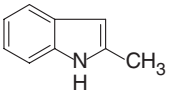
9-2-methylquinoline 

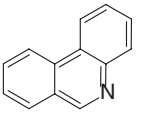
10-6-methylquinoline 

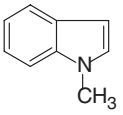
11-phenazine 

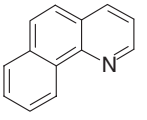
12-8-methylquinoline 

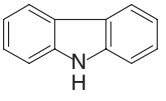
13-acridine 

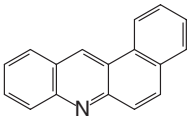
14-2-methylindole 

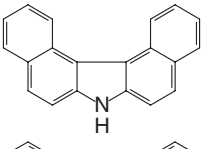
15-phenanthridine 

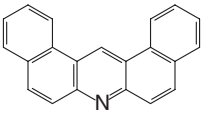
16-1-methylindole 

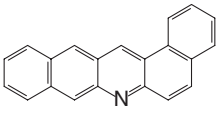
17-benzo[*h*]quinoline 

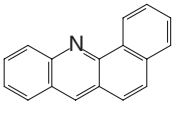
18-carbazole 

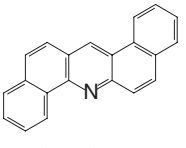
19-benz[*a*]acridine 

20-7-H-dibenzo[*cg*]carbazole 

21-dibenz[*aj*]acridine 

22-dibenz[*ai*]acridine 

23-benz[*c*]acridine 

24-dibenz[*ah*]acridine 

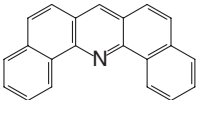
25-dibenz[*ch*]acridine 

Figure 1. PANHs used in the study.

acetonitrile up to 100% (v/v). To obtain reproducible results, appropriate column conditioning with selected initial mobile phase (up to 30 hold-time volumes) after column cleaning was performed.

2.4 *Quantification*

Quantitative measurements were performed on the basis of the absolute calibration curve method in the range from 172 fg injection^{-1} to 2.5 mg injection^{-1}. Limits of detection (LOD) for UV (231 nm) and fluorimetric detection were calculated for all separated compounds from calibration curves according to the Graham method [21]. Obtained values were compared with published PANHs limits of detection.

3. Results and discussion

3.1 *Effect of organic modifier content*

The effect of the acetonitrile content in the acetonitrile/10 mmol L^{-1} phosphate buffer mobile phase was investigated. Acetonitrile content was changed from 20 to 50% (v/v) in five different mobile phases at pH 2.5, 3.5, 4.5, 5.5, and 6.5, respectively, corresponding to the main region of aqueous pK_a of the acid/base equilibrium of the target nitrogen heterocycles. The stationary/mobile phase equilibrium was tested with the stability of column pressure, detector baselines, and retention times of analytes, before each analysis. A volume of mobile phase equal to 15 column void volumes was used to flush the chromatography column to equilibrate the new separation conditions.

In the mobile phase with low contents of acetonitrile (20%, v/v), the total peak's overlap was observed for carbazole/benzo[*h*]quinoline and isoquinoline/benzothiazole. Generally, the increasing content of acetonitrile in the mobile phase decreases the retention time values of individual PANHs. The shape of acetonitrile dependence curves differs for individual PANHs. The most significant changes in resolution were observed when the pH of the aqueous part of the mobile phase was 3.5 (figure 2). A low effect of the organic modificator content on the retention order was observed when the pH of the aqueous part of the mobile phase was 6.5 (figure 3). Therefore, it may be summarized that the best separation of the tested mixture was achieved using 30–35% (v/v) of acetonitrile in the mobile phase, at pH 6.5.

3.2 *Effect of mobile phase pH*

The effect of the pH of the acetonitrile/phosphate buffer mobile phase on retention of individual PANHs was investigated. The pH value of the mobile phase aqueous part was changed from 2.5 to 6.5, with acetonitrile content ranging from 20 to 50% (v/v).

The group of tested solutes can be divided into three subgroups according to the variability of retention times in the investigated pH range, corresponding to the pK_a of individual PANHs. Retention times of the first subgroup of PANHs (indole, 1-methylindole, 2-methylindole, benzothiazole, phenazine, carbazole) with a pK_a lower than 3.0 [22] were practically unchanged with the mobile phase pH. Retention times

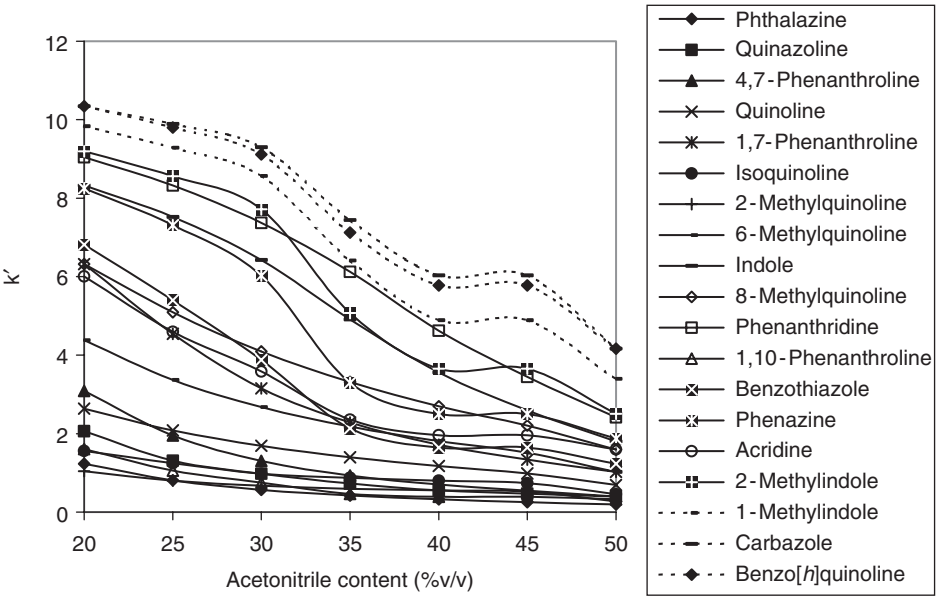


Figure 2. Effect of acetonitrile content in the mobile phase (pH 3.5) on retention times of PANHs.

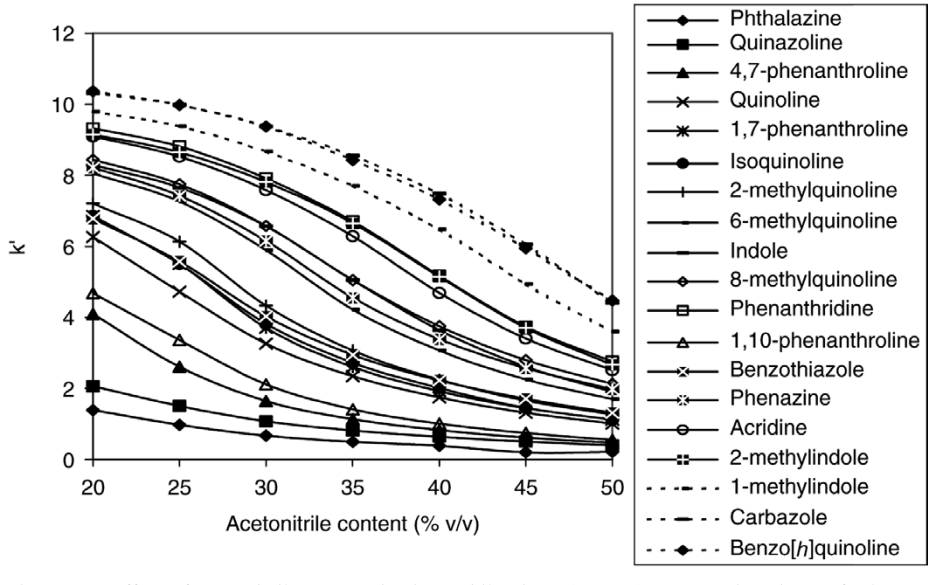


Figure 3. Effect of acetonitrile content in the mobile phase (pH 6.5) on retention times of PANHs.

of the second subgroup of PANHs (benzo[*h*]quinoline, phenanthridine, quinazoline, 1,7-phenanthroline, 4,7-phenathroline, phtalazine) with a pK_a within the range of 3.2 and 4.7 were effected especially in the pH range from 2.5 to 3.5. Maximum changes in the retention times were observed for the third subgroup of PANHs

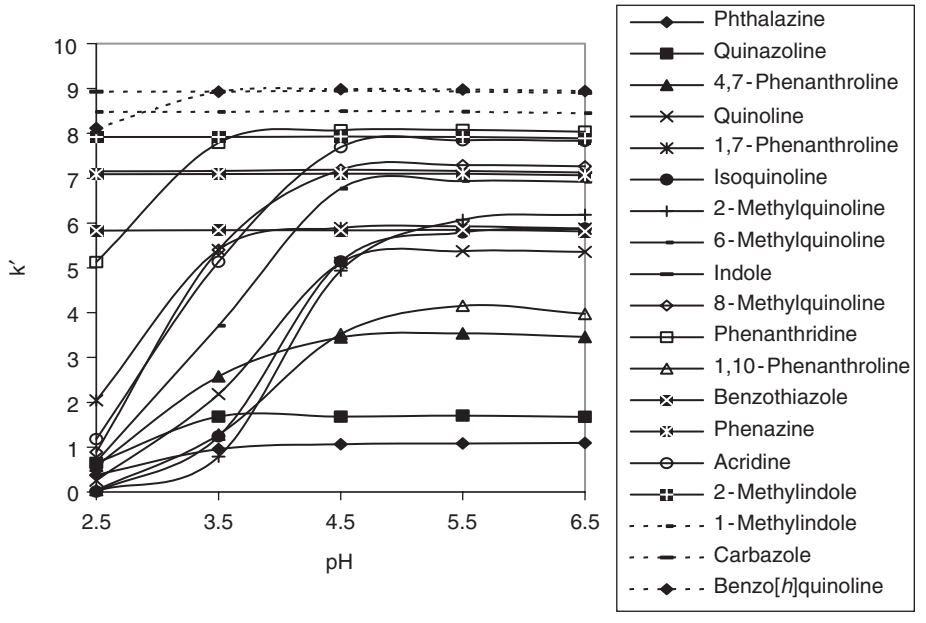


Figure 4. Effect of buffer pH on retention time of PANHs in the mobile phase with 20% (v/v) of acetonitrile.

(quinoline, 2-methylquinoline, 6-methylquinoline, 8-methylquinoline, isoquinoline, acridine, 1,10-phenanthroline) with a pK_a over 4.9, especially in the pH range from 2.5 to 5.0. These pH retention trends were similar in all mobile phases irrespective of the acetonitrile content (within the range of 20–50%, v/v), as shown in figures 4 and 5.

The transport of most compounds through the column is relatively fast at low pH values of the mobile phase. Therefore, a separation of these compounds in isocratic conditions is difficult. At a pH greater than 5.5, a minimum overlap of peaks was found. In the case of individual mixtures, especially in the presence of other analytes, the variation of pH in the range of 3.5–4.5 offers an effective tool for efficient changes of resolution by changing the retention times of PANHs. Since the PAH retention is independent of the pH mobile phase in the pH range 2.5–7.5, the effective control of PANHs and PAHs resolution can be achieved by changes in PANHs retentions.

3.3 *Effect of ionic strength*

The effect of ionic strength was investigated in the mobile phase containing 20% (v/v) of acetonitrile in the range between 5 and 20 mmol dm^{-3} at pH 4.1, where the retention times of most tested compounds were significantly affected, and at pH 6.0, where retention times were not significantly affected. Very small retention changes were found using the mobile phase at pH 4.1 for acridine, 2-methylquinoline, 6-methylquinoline, 8-methylquinoline, isoquinoline, 1,10-phenanthroline, and phenanthridine (figure 6). The small effect of ionic strength on retention times of PANHs may be significant when

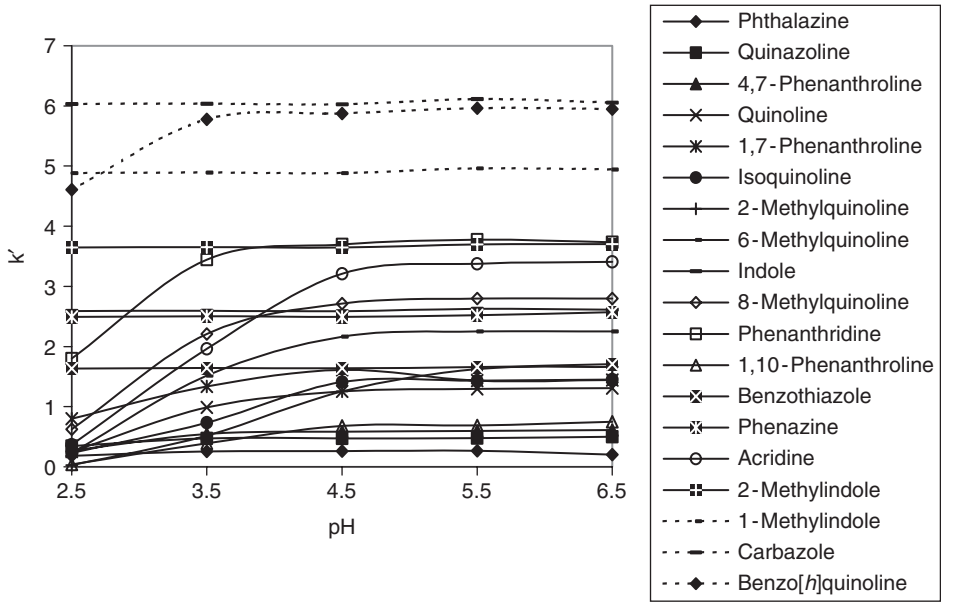


Figure 5. Effect of buffer pH on retention time of PANHs in the mobile phase with 45% (v/v) of acetonitrile.

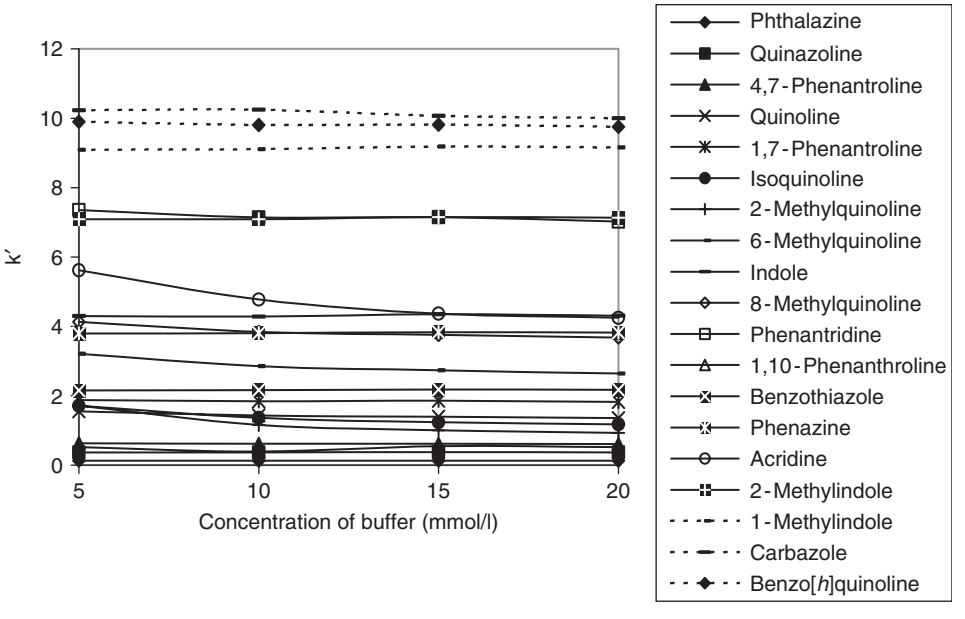


Figure 6. Effect of ionic strength on retention time of PANHs in the mobile phase pH 4.1 and 20% of acetonitrile.

the analysed sample contains different amounts of electrolyte, originating from the sample preparation.

3.4 *Separation of PANHs and PAHs mixture*

Gradient elution was used for the separation of 24 PANHs and 16 PAHs in the test mixture. Different slopes with several steps of acetonitrile (A) and phosphate buffer (B) mobile phase gradient composition can be used according to the required separation of analytes in real samples. An example of a very good resolution of all analytes in the tested mixture can be seen in figure 7. The gradient of acetonitrile used for the elution was as follows: 0–15 min: 30–35%A; 15–25 min: 35–45%A; 25–90 min: 45–70%A; 90–110 min: 70–85%A. Isocratic elution with 85%A was used in the last step of analysis (110–140 min). The flow-rate gradient of mobile phase was applied

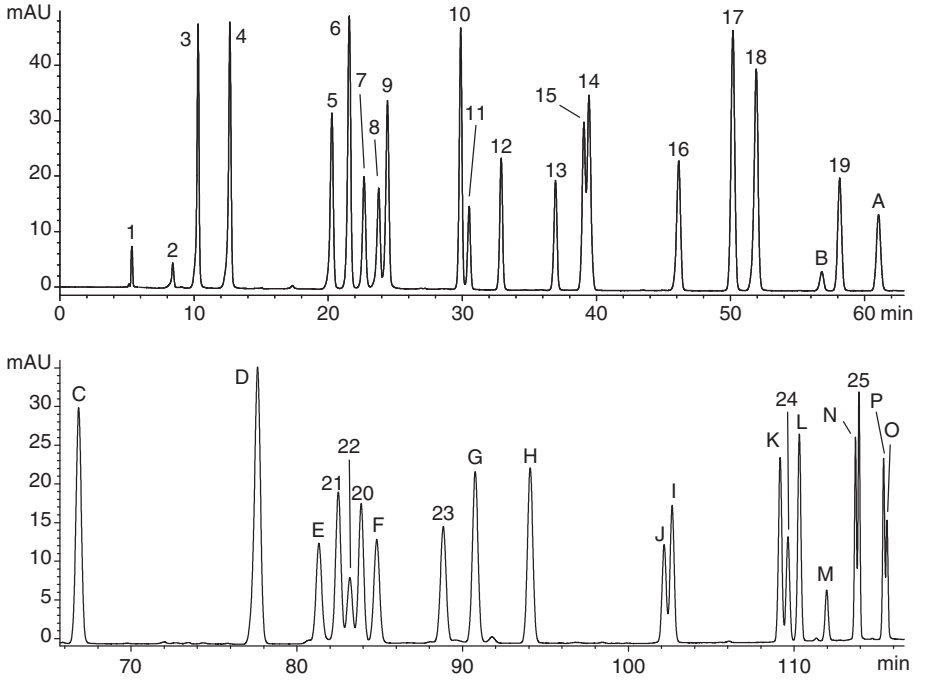


Figure 7. Separation of tested compounds.
Mobile phase: acetonitrile/phosphate buffer pH 6.00. **Flow rate:** 0–90 min: 0.5 $cm^3\,min^{-1}$; 90–110 min: 0.5–0.9 $cm^3.\,min^{-1}$; 100–140 min: 0.9 $cm.\,min^{-1}$.Content of acetonitrile: 0–15 min: 30–35%; 15–25 min: 35–45%; 25–90 min: 45–70%; 90–110 min:70–85%; 110–140 min: 85% **Columns:** 2× Ace 5 C18 (250 × 4.6 mm i.d, 5 µm) in tandem connection. Column temperature: 30°C. Detection: UV 231 nm. **1**- uracil. **PANHs**: **2**- phthalazine, **3**- quinazoline, **4**- 4,7-phenanthroline, **5**- quinoline, **6**- 1,7-phenanthroline, **7**- isoquinoline, **8**- benzothiazole, **9**- 2-methylquinoline, **10**- 6-methylquinoline, **11**- phenazine, **12**- 8-methylquinoline, **13**- acridine, **14**- 2-methylindole, **15**- phenanthridine, **16**- 1-methylindole, **17**- benzo[*h*] quinoline, **18**- carbazole, **19**- benz[*a*]acridine, **20**- 7-H-dibenzo[*cg*]carbazole, **21**-dibenz[*aj*]acridine, **22**-dibenz[*ai*]acridine, **23**-benz[*c*]acridine, **24**- dibenz[*ah*]acridine, **25**- dibenz[*ch*]acridine. **PAHs**: A- naphthalene, **B**- acenaphthylene, **C**- acenaphthene, **D**- fluorene, **E**- phenanthrene, **F**- anthracene, **G**- fluoranthene, **H**- pyrene, **I**- benz[*a*]anthracene, **J**- chrysene, **K**- benzo[*b*]fluoranthene, **L**- benzo[*k*]fluoranthene, **M**- benzo[*a*]pyrene, **N**- dibenz[*ah*]anthracene, **O**- benzo[*ghi*]perylene, **P**- indeno[1,2,3-cd]pyrene.

Table 1. Limits of PANH detection.

Compounds	DAD-LOD (ng injection^{-1})	R^2	DAD-LOD[a] (ng injection^{-1})	FD-LOD (ng injection^{-1})	R^2	FD-LOD[b] (ng injection^{-1})
Phthalazine	4.00E+00	0.996		No flo.	–	
Quinazoline	2.29E+00	0.999		3.97E+00	0.997	
4,7-Phenanthroline	2.26E+00	0.999		2.47E−02	0.985	
Quinoline	2.14E+00	0.999		1.27E+01	0.969	5.00E+00
1,7-Phenanthroline	5.00E+00	0.994		6.92E−02	0.983	
Isoquinoline	4.49E+00	0.987		3.45E−01	0.994	
Benzothiazol	2.82E+00	0.998		1.46E+00	0.999	
2-Methylquinoline	5.08E+00	0.994		1.52E+00	0.999	
6-Methylquinoline	2.08E+00	0.999		2.98E+00	0.991	
Phenazine	2.03E+00	0.999		2.98E+02	0.946	
8-Methylquinoline	2.10E+00	0.999		1.04E+00	0.972	
Acridine	1.13E+00	0.999		7.35E−03	0.991	2.50E+00
2-Methylindole	1.78E+00	0.999		2.76E−02	0.999	
Phenanthridine	2.09E+00	0.999		8.40E−03	0.994	1.00E+00
1-Methylindole	1.12E+00	0.999		3.79E−03	0.992	
Benzo[*h*]quinoline	1.11E+00	0.999		3.93E−02	0.753	1.50E+00
Carbazole	1.11E+00	0.999		8.08E−03	0.923	
Benz[*a*]acridine	9.43E−01	0.999	5.00E+00	7.60E−03	0.962	5.00E+00
Dibenz[*aj*]acridine	9.73E−01	0.999	3.00E−01	2.09E−03	0.996	2.30E−02
Dibenz[*ai*]acridine	2.00E+00	0.999		3.21E−01	0.993	
Benz[*c*]acridine	1.17E+00	0.999		6.00E−03	0.996	1.10E−02
7-*H*-Dibenzo[*cg*]carbazole	1.22E+00	0.999		5.84E−05	0.995	
Dibenz[*ah*]acridine	8.75E−01	0.961		2.93E−03	0.998	9.50E−03
Dibenz[*ch*]acridine	9.50E−01	0.967	4.00E−01	1.11E−03	0.988	6.20E−03

[a]Literature value [8, 23].
[b]Literature value [4, 9].

simultaneously to accelerate the elution of the latest eluted solutes (see figure 7). As can be seen, satisfactory separation with good resolution was achieved for the tested compounds without preliminary separation of PANH and PAH groups.

3.5 *Limits of detection*

As is well known, detection limits for the PANHs obtained by the fluorescence detection are generally lower in comparison with the detection limits obtained by other common detection techniques. The same effect was found for the majority of PANHs in our study comparing FLD and UV-DAD detection (see table 1). The LOD values obtained with fluorimetric detection are comparable with those published for GC-FID and GC-MS techniques. Published values for individual PANHs range between 0.5 and 3.0 ng injection^{-1} for GC-FID, and between 0.01 and 0.14 ng injection^{-1} for GC-MS [1, 16, 23].

3.6 *Determination of PANHs and PAHs in real soil samples*

The HPLC method developed was used for the determination of PANHs and PAHs contents in soil samples collected in the vicinity of an oil factory and selected surrounding points of human settlement in the region of Central Moravia,

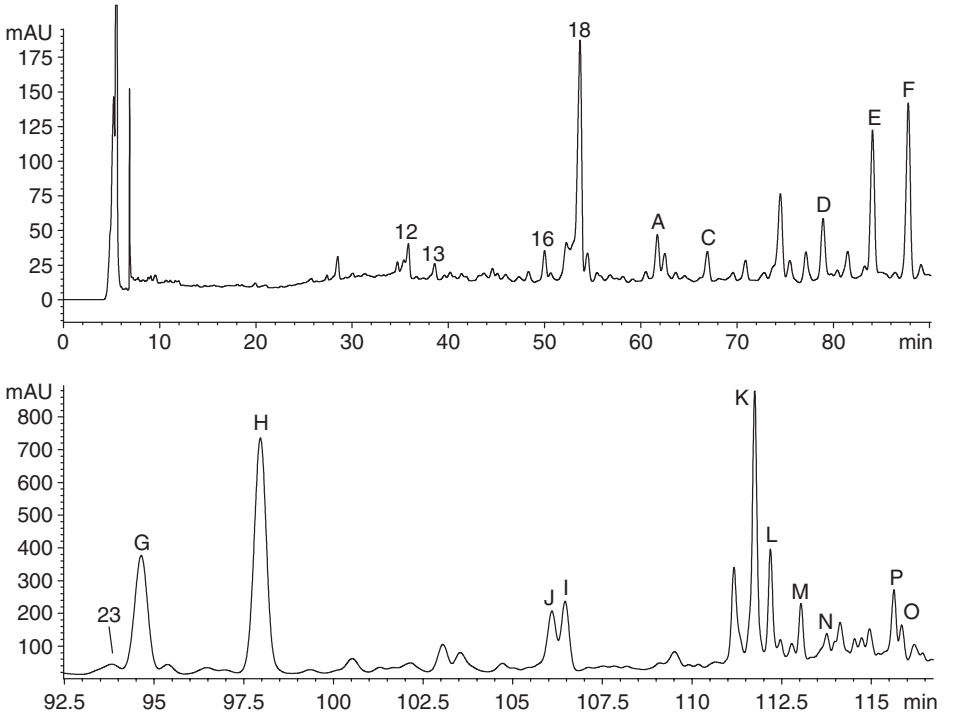


Figure 8. Chromatogram of a soil sample extract. Peak markers as in figure 7.

Czech Republic. Sampling points were chosen from the standard sampling net used for long-lasting PAHs monitoring. An example of a chromatogram of a soil sample extract collected near the oil factory is shown in figure 8. Results of analysis of soil sample extracts are shown in table 2. The concentration of individual PANHs identified in the soil samples is on average three times lower than the average concentration of PAHs.

4. Conclusions

We have investigated the HPLC retention behaviour of PANHs in a C18 stationary phase depending on the content of organic modifier, pH of the mobile phase, and ionic strength. The resolution and retention order of individual PANHs are directly proportional to the acetonitrile/phosphate buffer ratio, pH mobile phase, pK_a of individual PANHs, and mostly independent of the ionic strength in the region under investigation. The small effect of ionic strength allows a higher variability in concentration of buffer used for the mobile-phase preparation. The possibility of changing the mobile phase pH allows the separation conditions of HPLC determination of PANHs to be controlled in particular environmental samples also containing PAHs. We have developed an HPLC method for separation and simultaneous determination of 24 PANHs and 16 EPA–PAHs without a pre-separation step. Low detection limits were achieved. The developed method was successfully used for the determination of

Table 2. Contents of the PAHs and PANHs in soil samples.

Site	Valašské Meziříčí (ng g^{-1} of soil)	Juřinka (ng g^{-1} of soil)	Mšenovice (ng g^{-1} of soil)	Příluky (ng g^{-1} of soil)
2-Methylquinoline				7
8-Methylquinoline	118			23
acridine	61	21	22	11
1-Methylindole	103			
Carbazole	185		5	
Benz[*c*]acridine	162			
Acenaphthylene	716			
Phenanthrene	438	11	47	19
Anthracene	753		7	4
Fluoranthene	792	21	17	9
Pyrene	1398	16	90	4
Chrysene	846	16	97	26
Benz[*a*]anthracene	611	12	52	15
Benzo[*b*]fluoranthene	123	21	100	42
Benzo[*k*]fluoranthene	363	4	25	9
Benzo[*a*]pyrene	598	17	43	20
Dibenz[*ah*]anthracene	77		9	
Indeno[*1,2,3-c,d*]pyrene	421	14	30	17
Benzo[*ghi*]perylene	270	11	26	15

PANH and PAH contents in soil samples collected near an oil factory and in spiked soil samples in experiments focused on the study of distribution equilibrium of PANHs in the soil–water interphase [24].

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