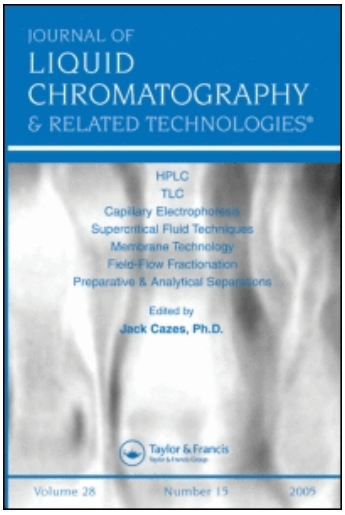

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Computer-Assisted Method for the Threshold Optimization of Fluorescence Detection Conditions in HPLC of Polycyclic Aromatic Hydrocarbons

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COMPUTER-ASSISTED METHOD FOR THE THRESHOLD OPTIMIZATION OF FLUORESCENCE DETECTION CONDITIONS IN HPLC OF POLYCYCLIC AROMATIC HYDROCARBONS

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Abstract

The computer assisted method of λ_{ex}, λ_{em} optimization in programmed fluorescence detection of PAHs included in EPA norm has been investigated. Optimization procedure utilises the preference of the threshold methods. Finding of optimum detection conditions was done in the reduced parametric space (λ_{ex}, λ_{em}). This space consisted of points with guaranteed minimal detection sensitivities of tested PAHs. The relative detection sensitivities of PAHs, elution characteristics, different natural fluorescences, chromatographic resolution of hardly separable pairs of peaks and also the reproducibilities of peak heights and

retention times under gradient conditions were taken into consideration. The results of optimization indicate that it is difficlut to find acceptable compromise for fluoranthene and chrysene. By shifting of emmision wavelength the sensitivities of chrysene and fluoranthene changed in the mutually opposite direction. Therefore developed program used five steps. Fluoranthene, pyrene and benzo(a)anthracene, chrysene were measured at different detection conditions. If there is a specific need to analyse most dangerous PAHs then the program can be adjusted accordingly.

Introduction

The sensitivity of chromatographic analysis (observed peak height in a chromatogram) is an important aspect of the chromatographic process. The detection limit is often defined as the concentration of the analyte that will provide a signal-to-noise ratio of 3 : 1 [1]. The minimum detectable amount of the analyte is determined by the detector noise, detector sensitivity towards the analyte of interest (defined as signal output per unit mass concentration of analyte in eluent) and also by the performance of the chromatographic system [2]. Analytical chemist is generally interested in the minimum detectable concentration. It can be increased by the injection of larger sample volume, preconcentration on a precolumn as well as the selection of optimal detection conditions [3].

Sixteen polycyclic aromatic hydrocarbons (PAHs, according to EPA 610 norm) are commonly detected in HPLC by UV and fluorescence detection. Because of rather different fluorescence detection characteristics (optimal λ_{exc}, λ_{emis}) of tested PAHs, the programmable fluorescence detectors are used to satisfy the acceptable signal intensity of all components [4]. Many combinations of λ_{exc}, λ_{emis} were published in the literature [5-7].

The aim of this paper is to discuss the optimization procedure which utilises the fluorescence spectra of individual PAHs for the optimization of λ_{exc}, λ_{emis} in programmed fluorescence detection. Procedure reflects the differences between natural fluorescences, elution characteristics and chromatographic separation of tested PAHs.

Experimental

Instruments

Reversed phase HPLC was performed on a Waters Assoc. model 510 pumps with a Supelco LC-PAH column (length = 150 mm, inner diameter = 4.6 mm) and a

Tab. 1. The Composition of the Gradient Mobile Phase

Time [min]	w(A) [%]	w(B) [%]
0	100	0
1	100	0
15	0	100
25	0	100

A = 50 % v/v acetonitrile, B = acetonitrile, flow rate = 1.3 ml/min

Waters Assoc. programmable fluorescence detector. Fluorescence spectra were measured by LS-50, Perkin Elmer luminiscence spectrophotometer (2% filter, 5 nm both slits, spectra measured 3 times and averaged) . The composition of the gradient mobile phase is listed in Tab. 1.

Chemicals

The standards of 16 polycyclic aromatic hydrocarbons naphthalene (1), acenaphthylene (2), acenaphthene (3), fluorene (4), phenanthrene (5), anthracene (6), fluoranthene (7), pyrene (8), benzo(a)anthracene (9), chrysene (10), benzo(b)fluoranthene (11), benzo(k)fluoranthene (12), benzo(a)pyrene (13), dibenz(a,h)anthracene (14), benzo(g,h,i)perylene (15), indeno(c,d)pyrene (16) ,all over 98% of purity, were purchased from Supelco, USA. The acetonitrile for gradient was from Merck, Germany. Standard solutions of individual polycyclic aromatic hydrocarbons were prepared by dissolving of the analytes in acetonitrile (1 $mg.dm^{-3}$). Ten microliters of standard solution (200 μg/ml of each PAH) was injected to the chromatograph.

Optimization program

Optimization program was written in Turbo Pascal 7.0 and runs on PC-AT, 486 DX, 33 MHz. Program works from the lowest wavelength to the highest (step 1 nm).

Theory

The goal of the optimization of detection conditions of a group of components with the different spectral characteristics is to obtain the areas of all peaks in a chromatogram as maximal as possible. The area of Gaussian peak A_i can be related to the peak height at the maximum $h_{max,i}$ by the following equation [3]:

$$A_i = h_{\max,i} \cdot \sigma_i \cdot \sqrt{2 \cdot \pi} \quad (1)$$

where σ_i is the standard deviation of *i-th* Gaussian peak i. If the fluorescence intensity ϕ is the quantity measured by a detector, then assuming small enough absorbances at the exciting wavelength, $h_{max,i}$ can be expressed in terms of the excitation beam intensity ϕ_0, quantum yield ξ, the proportionality factor *k* including the geometry of the fluorescence spectrometer, molar absorptivity ε, the cell path length *l* and a concentration of the analyte $c_{max,i}$.

$$h_{\max,i} = \phi_{\max,i} = \phi_0 \cdot \xi_i \cdot \left(2.3 \cdot \varepsilon_i \cdot l \cdot c_{\max,i}\right) \quad (2)$$

where $\phi_{max,i}$ is the fluorescence intensity at the peak maximum. Thus eq. (1) can be transformed to the following expression:

$$A_i = \phi_{\max,i} \cdot \sigma_i \cdot \sqrt{2 \cdot \pi} \quad (3)$$

It follows from eq (3), that the area of the Gaussian peak depends linearly on ϕ max,i .

Optimization criterion

To design the suitable optimization criterion, the following facts (concerning to the tested PAHs) should be taken into account.

- different fluorescence spectra,
- different natural fluorescences,
- retention order in chromatographic separation,
- chromatographic resolution of "critical" pairs of peaks,
- reproducibilities of retention times under gradient conditions.

The optimal combination of λ_{ex} and λ_{em} for the individual component can be easily found from it's excitation and emmision fluorescence spectra. However ,the same task applied to the set of components is much more complicated. If the natural fluorescence of component is high, detection can be easy at non-optimum wavelengths, whereas if the natural fluorescence is low, it may be desirable to approach the highest sensitivity possible.

Theoretically, each component could be measured at its own optimal detection conditions. However this is only the theoretical possibility. The feasible number of program steps is limited both by the reproducibility of retention times under gradient conditions and chromatographic resolution of "critical" pairs of peaks. Therefore the compromise between the sensitivity of detection and the number of program steps should be found.

The optimization criterion should reflect the above mentioned realities. In our work it was defined by the following way. At first, to evaluate the error of

experimental measurements, the linearity relationship between the fluorescence intensity $\phi_{i,j}$ corresponding to particular emission wavelengths $\lambda_{em,i}$ as read out from the emission spectra and the fluorescence intensities $\phi_{ex,j}$ read out from the excitation spectrum at the corresponding excitation wavelengths $\lambda_{ex,j}$ was verified for all tested PAHs.

$$\phi_{i,j} = f(\phi_{ex,j}) \tag{4}$$

The meanings of ϕ_i, $\phi_{ex,j}$, $\lambda_{em,i}$ and $\lambda_{ex,j}$ are shown on the excitation and emission spectra of benzo(b)fluoranthene in Fig. 1, 2.

As it was expected the function *f* in eq. (4) was linear for all tested compounds. The intercepts a_i of the equation

$$\phi_{i,j} = a_i + b_i \cdot \phi_{ex,j} \tag{5}$$

were negligible as expected, while the quality of this model expressed by the correlation coefficients (near 1.0 for all tested compounds) is considered as acceptable. Therefore, the eq. (5) can be written as

$$\phi_{i,j} = b_i \cdot \phi_{ex,j} = \frac{b_i'}{\phi_{ex,max}} \cdot \phi_{ex,j} \tag{6}$$

where $\phi_{ex,max}$ is the maximal fluorescence intensity in an excitation spectrum (from which also the $\phi_{ex,j}$ values are read out). The maximum possible fluorescence intensity ϕ_{max} can be obtained by the measurement at the main maxima of excitation and emission spectra. Dividing eq. (6) by ϕ_{max} writing in appropriate fashion

$$\frac{\phi_{i,j}}{\phi_{max}} = \frac{b_i'}{\phi_{max}} \cdot \frac{\phi_{ex,j}}{\phi_{ex,max}} \tag{7}$$

and introducing new symbols gives

$$\phi_{norm} = b_i'' \cdot \phi_{ex,norm} \tag{8}$$

According to eq. (8) the fluorescence intensity $\phi_{i,j}$ normalised with respect to ϕ_{max} i.e. ϕ_{norm}, is directly proportional to $\phi_{ex,norm}$ i.e. to the fluorescence intensity $\phi_{ex,j}$ normalised to $\phi_{ex,max}$. The proportionality factor - slope b_i'' depends on the choice of $\lambda_{em,i}$ (see Fig. 2). Investigation on the relationship between b_i'' and $\phi_{em,i}$ read out from the emission spectra at the different $\lambda_{em,i}$ values manifested that the slope b_i'' can be written as

$$b_i'' = \frac{\phi_{em,i}}{\phi_{em,max}} = \phi_{em,norm} \tag{9}$$

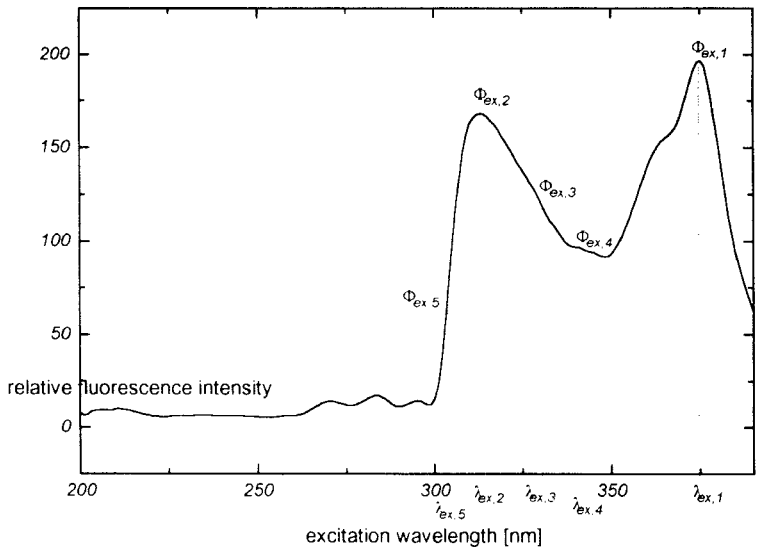

Fig. 1. Excitation spectrum of Benzo(b)fluoranthene measured at optimum emmision wavelength 445 nm..

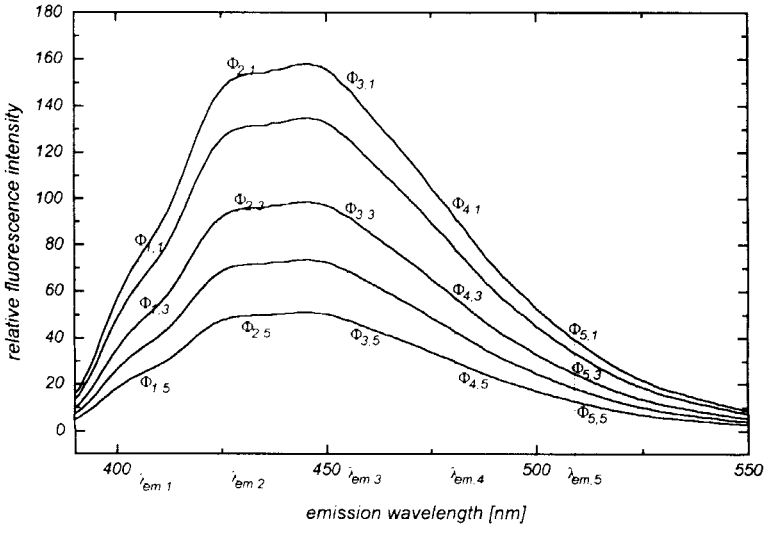

Fig. 2. Emission spectra of Benzo(b)fluoranthene (c = 0.0985 mg/ml) measured at λ_{ex1}, λ_{ex2}, λ_{ex3}, λ_{ex4}.

where $\phi_{em,max}$ is the maximal fluorescence intensity in a corresponding emission spectrum. Combining eq. (8) and (9) gives the following equation for ϕ_{norm}

$$\phi_{norm} = \phi_{ex,norm} \cdot \phi_{em,norm} \tag{10}$$

The values of all these quantities lie in the interval <0;1>. According to eq. (10), the optimization criterion was designed by the following expression:

$$K_k = 1 \qquad if \qquad \phi_{norm(\lambda_{exc},\lambda_{emis}),k} \geq P_k \tag{11}$$

$$K_k = 0 \qquad if \qquad \phi_{norm(\lambda_{exc},\lambda_{emis}),k} < P_k \tag{12}$$

$$OC = \sum_k K_k \tag{13}$$

where k designates the component (PAH) and P_k is a threshold value of fluorescence intensity over which the sensitivity of detection is acceptable. Optimization criterion OC is an integer (number of components which passed the threshold condition), defined as a sum of boolean expressions K_k.

Results and discussion

Before any optimization the goal of the process should be defined unambiguously. In this case several facts should be taken into consideration. The sensitivity of fluorescence detection can be increased by the programming of excitation and emission wavelengths. One possible way is to determine the minimum number of λ_{exc}, λ_{emis} combinations and satisfy the acceptable sensitivity of detection for all tested solutes. The choice of optimal λ_{exc}, λ_{emis} combinations should be based on finding conditions at which the largest number of compounds can be analysed at sufficiently low detection limits. Therefore the differences between natural fluorescences are considered. Threshold values P_k, over which the absolute fluorescence intensities are acceptable, were chosen with respect to the different natural fluorescences of tested PAHs (see Table 2).

As it can be seen from Table 2, acenaphthylene displays a very low natural fluorescence in comparison with other PAHs, then it was excluded from optimization process. It can be detected by UV absorption much more successfully. The optimal λ_{exc}, λ_{emis} indeno(c,d)pyrene was far from all other PAHs then it should be measured at its own maximum 300 / 500 nm. Table 2 shows the differences between natural fluorescences of tested PAHs. Benzo(k)fluoranthene displays highest natural fluorescence (approximately 15 x higher than naphthalene fluoranthene and indeno(c,d)pyrene which display the lowest fluorescence). The fluorescences of other PAHs (except anthracene,

Table 2. Comparison of natural fluorescences of tested PAHs measured at their own optimal λ_{exc}, λ_{emis}

No	compound	λ_{exc} / λ_{emis}	relative fluorescence intensity (normalized to compound 12)	concentration [mg/ml]
1	Naphthalene	282 / 340	0.06	0.0985
2	Acenaphthylene	246 / 332	0.0008	0.0990
3	Acenaphthene	258 / 336	0.35	0.0985
4	Fluorene	234 / 312	0.34	0.0990
5	Phenanthrene	246 / 383	0.21	0.0980
6	Anthracene	250 / 406	0.75	0.0985
7	Fluoranthene	282 / 456	0.05	0.0985
8	Pyrene	331 / 395	0.18	0.0985
9	Benzo(a)anthracene	284 / 392	0.29	0.0990
10	Chrysene	270 / 381	0.27	0.0985
11	Benzo(b)fluoranthene	301 / 445	0.14	0.0985
12	Benzo(k)fluoranthene	310 / 420	1.00	0.0985
13	Benzo(a)pyrene	373 / 406	0.59	0.0985
14	Dibenz(a,h)anthracene	298 / 402	0.24	0.0985
15	Benzo(g,h,i)perylene	385 / 413	0.15	0.0985
16	Indeno(c,d)pyrene	300 / 500	0.07	0.0990

benzo(a)pyrene) are approximately on the same level (natural fluorescences of benzo(b)fluoranthene, benzo(g,h,i)perylene and pyrene is slightly lower). The data from Table 2 were utilised in the choice of threshold P_k values. Low natural fluorescence of components were compensated by adequately high level of P_k in optimization. By this way the optimum was shifted closer to conditions which favoured compounds with lower natural fluorescence.

Optimization procedure

It follows from eq. (11), (12) and (13) that the sets of points with the different values of optimization criterion *OC* can be appointed in the three-dimensional space (λ_{exc}, λ_{emis}, *OC*). The value of *OC* (at the certain co-ordinates of parametric space) indicates the number of components which passed the threshold condition. The illustrative example of such "overlapping " plot (only of four components - because of the complexity of plot for all sixteen PAHs) is shown in Fig. 3.

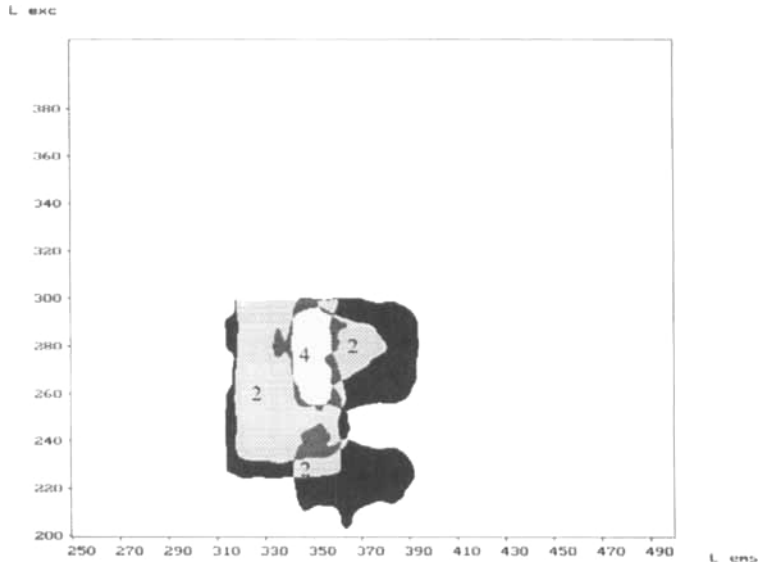

Fig. 3. " Overlapping" plot for naphthalene, acenaphthene, acenaphtylene and phenanthrene. Threshold was 0.25 for all components.

This kind of plot still contains a lot of redundant information and it's not lucid. Each component can generally be located in the points with the different value of optimization criterion *OC*. The "overlapping" plot can considerably be simplified in the next step of optimization . In this procedure each component is located to the points with the highest value of criterion *OC* (to the points with the highest number of other components which passed threshold condition together with this component) and deleted from the points with lower value of *OC*. After this procedure each component is situated in the set of points with one value of *OC* only. Optimal λ_{ex}, λ_{em} combinantion for the given set of components (included in the above set of points) then lies in the point with the highest minimum value of ϕ_{norm} for the given set of components.

The chromatogram of tested PAHs is shown in Fig. 4. Chromatographic resolution of "critical" groups of peaks (acenaphthene, fluorene), (benzo(a)anthracene, chrysene), (benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene) were not sufficient for the non-problematic switching of λ_{ex}, λ_{em}. The ability to quantitate the peaks will be affected by retention time variability.

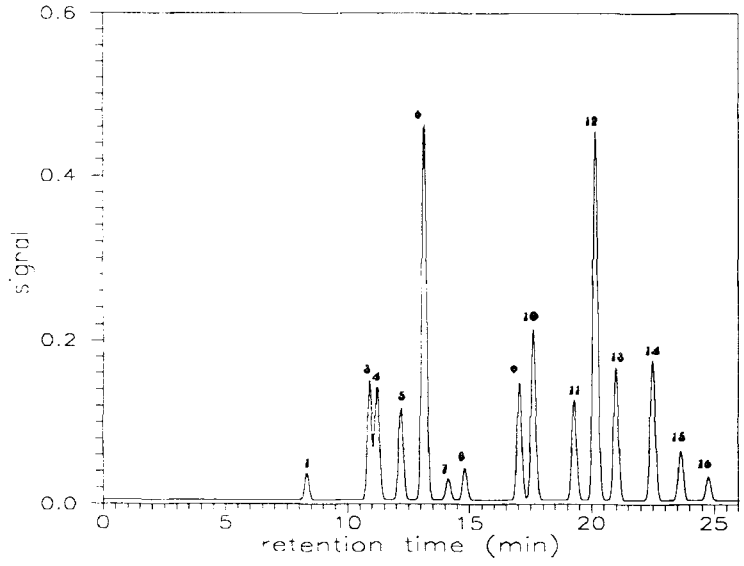

Fig. 4. The chromatogram of PAHs tested mixture (c = 200 μg/ml of each PAH) measured at detection conditions of program No. 1. For the conditions see experimental. For the numbering of peaks see Table 2.

According to this fact, above groups would be optimized together and maximum possible number of wavelengths switching is nine. The threshold values P_k were chosen with respect to the natural fluorescences of tested PAHs. Table 3 shows the results of optimization.

In program No. 1. the maximum possible number of λ_{ex}, λ_{em} switches were used. In other words program No. 1 represented maximal overall sensitivity of detection (optimized with above approach) of tested PAHs which could be achieved using separation system described in this work. Naphthalene, phenanthrene, anthracene and indeno(c,d)pyrene were measured at their ideal conditions. With fluoranthene having relatively low sensitivity some of the sensitivity of adjacent component in a chromatogram pyrene was sacrified by choosing compromise condition that favoured fluoranthene.

This approach was applied also for benzo(b)fluoranthene which displays also low natural fluorescence in comparison with benzo(k)fluoranthene and benzo(a)pyrene. The high sensitivity of benzo(k)fluoranthene results in a

Table 3. The Results of Optimization. For Numbering of PAHs see Table 2.

Program No.	component No.	λ_{ex} / λ_{em}	ϕ_{norm}
1	1	282 / 340	1.0
	3	237 / 323	0.65
	4		0.63
	5	246 / 383	1.0
	6	250 / 406	1.0
	7	271 / 410	0.89
	8		0.31
	9	266 / 384	0.75
	10		0.83
	11	281 / 428	0.91
	12		0.63
	13		0.68
	14	290 / 413	0.81
	15		0.79
	16	300 / 500	1.0
2	1	282 / 340	1.0
	3		0.62
	4		0.45
	5	252 / 376	0.91
	6		0.75
	7	268 / 406	0.73
	8		0.39
	9		0.66
	10		0.025
	11	285 / 424	0.90
	12		0.75
	13		0.66
	14		0.62
	15		0.73
	16	300 / 500	1.0
3	1	282 / 340	1.0
	3		0.62
	4		0.45
	5	252 / 376	0.91
	6		0.75
	7	268 / 390	0.11
	8		0.74
	9		0.69
	10		0.77
	11	285 / 424	0.90
	12		0.75
	13		0.66
	14		0.62
	15		0.73
	16	300 / 500	1.0

predominance of this peak in the group. Nine switches of excitation and emmision wavelenghts during analysis is relatively high number. In some cases (fluorescence detector does not allow the programming of such number of switches) it is desirable to reduce this number in spite of decreasing the sensitivity of detection. Therefore we examined this possibility. The initial groups of components used in program No. 1 (see Table 3) were gradually overlapped and detection of them were optimized. In program No. 2, naphthalene, acenaphthene and fluorene were optimized together. According to low natural fluorescence of naphthalene, this group was measured at naphthalene's ideal detection condition. The values of ϕ_{norm} for acenaphthene and fluorene listed in Table 3 were similar to the program No. 1. Detection conditions for phenanthrene and anthracene were shifted to the optimum of phenanthrene, because of high natural sensitivity of anthhracene. Program No. 2 and No. 3 differed in the detection conditions for the group of fluoranthene, pyrene, benzo(a)anthracene and chrysene only. Fluoranthene displayed low natural fluorescence, therefore it should be favoured in compromise conditions. The results of optimization show that it is hard to find acceptable compromise for fluoranthene and chrysene. By shifting of emmision wavelength the sensitivities of chrysene and fluoranthene changed in the mutually opposite direction. Therefore, if there was a need to analyse both fluoranthene and chrysene, they would not be measured at the same detection condition. Finally, group, which consisted of benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene and benzo(g,h,i)perylene. Under the compromise conditions for this group benzo(b)fluoranthene response was 90 % its ideal conditions response, with benzo(k)fluoranthene 75 % of its ideal, for benzo(a)pyrene and dibenzo(a,h)anthracene around 65 % and or benzo(g,h,i)perylene almost 73 %. Indeno(c,d)pyrene was measured at their own ideal λ_{ex}, λ_{em}. Chromatograms of tested PAHs measured at above conditions are shown in Fig. 4 - 6. In order to establish the reproducibility of the method (peak heights), seven replicate injections of a standard PAH mixture were made. Results are listed in Table 4.

The toxicological characteristics of tested PAHs show that the health risks of individual PAHs are not at the same level. PAHs are known to be carcinogenic as a result of oxidative reactions in the body [8]. According to literature [9-11], PAHs can be classified into 4 groups. Naphthalene, acenaphthylene, acenaphthene, fluorene (group A) are weak carcinogenic resp. non-carcinogenic. Phenathrene, anthracene, fluoranthene, pyrene, chrysene and benzo(g,h,i)perylene (group B) are moderately active, benzo(b), benzo(k)fluoranthene, dibenzo(a,h)anthracene

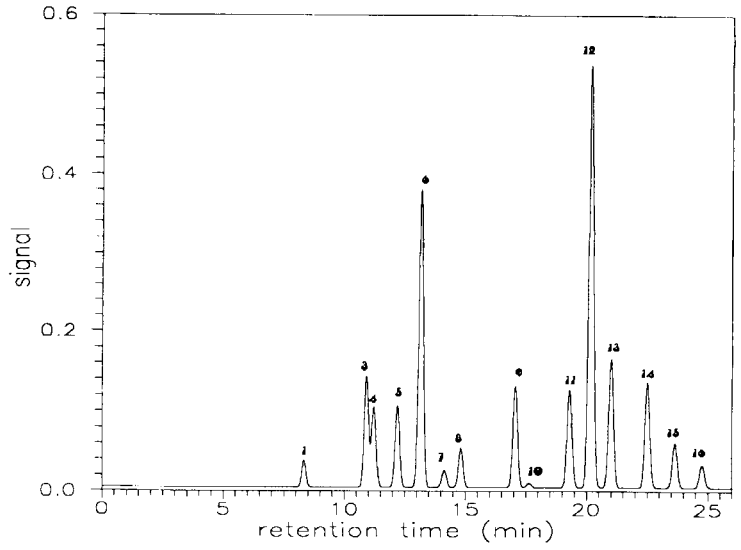

Fig. 5. The chromatogram of PAHs tested mixture (c = 200 μg/ml of each PAH) measured at detection conditions of program No. 2.

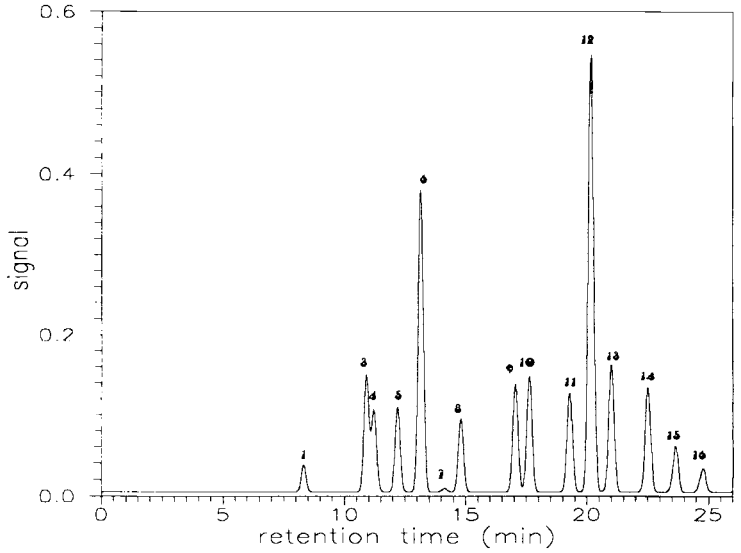

Fig. 6. The chromatogram of PAHs tested mixture (c = 200 μg/ml of each PAH) measured at detection conditions of program No. 3.

Table 4. Reproducibility (% RSD) of Detector Response (peak heights) and retention times for Seven Replicate Injections of PAH mixture (conc. of each PAH was 200 μg/ml).

compound	% *RSD* of retention time	% *RSD* of peak height
Naphthalene	1.1	2.2
Acenaphthylene	1.4	-
Acenaphthene	2.1	1.2
Fluorene	1.6	1.1
Phenanthrene	1.8	0.7
Anthracene	1.1	0.9
Fluoranthene	1.0	2.1
Pyrene	0.9	1.5
Benzo(a)anthracene	1.6	1.1
Chrysene	3.0	1.0
Benzo(b)fluoranthene	2.0	0.8
Benzo(k)fluoranthene	1.5	0.9
Benzo(a)pyrene	1.1	0.7
Dibenz(a,h)anthracene	0.7	1.2
Benzo(g,h,i)perylene	1.4	0.5
Indeno(c,d)pyrene	1.2	0.4

and indeno(c,d)pyrene are strong carcinogens and benzo(a)pyrene is the most dangerous carcinogen. If there was a specific need to analyse most dangerous PAHs from the tested mixture then the program could have been adjusted accordingly.

For example benzo(a)anthracene and chrysene have the similar natural fluorescences, but chrysene is only moderately active carcinogen and benzo(a)anthracene is strong carcinogen. This could be taken into consideration in optimization. Afterwards conditions were shifted closer to the ideal conditions of benzo(a)anthracene (275 / 386 nm). Under these conditions benzo(a)anthracene response was 92 % of its ideal conditions and chrysene 66 % of its ideal.

Conclusion

Presented work discusses the method of λ_{exc}, λ_{emis} optimization in programmed fluorescence detection. The procedure utilises the preference of the threshold optimization. Parametric space was reduced to the points with guaranteed minimal detection sensitivities of tested PAHs. The relative detection sensitivity of each PAH, elution characteristics, different natural fluorescences, chromatographic resolution of "critical" pairs of peaks and also the reproducibilities of retention times under gradient conditions were taken into account. The threshold values P_k were chosen with respect to the differences between the natural fluorescences of PAHs. By this way PAHs with lower natural fluorescence were measured at such compromise conditions that favoured them (naphthalene, fluoranthene, benzo(b)fluoranthene). If there is a specific need to analyse especially the most dangerous PAHs then the toxicological data should be taken into consideration.

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