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Enrichment technique in an automated liquid microchromatograph with a capillary mixer

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Abstract

An automatic device based on a microcolumn liquid chromatograph with a capillary mixer and a laboratory-made fluorescence microcell is described. The device provides adjustment of the sample matrix in such a way that owing to the increase in the analyte capacity factor the sample is enriched on the analytical column. The on-line arrangement of the precolumn, mixer, analytical column and fluorescence detector enables trace amounts of the analyte in the sample to be treated. In the mixer the sample volume eluting from the precolumn (or the sample resulting from an off-line extraction) is mixed with a solvent of low elution strength, then the enrichment of the sample takes place on the analytical column. The device is characterized by a high degree of automation and high reproducibility of the measured data (R.S.D. = 0.8%) with zero losses of the analyte during the enrichment process. The applicability of the system was verified on the examples of determination of six polycyclic aromatic hydrocarbons in an organic extract (acetonitrile) and of the determination of trace amounts of fluoranthene (tens of nanograms per litre) in tap water.

1. Introduction

Typical cases of the determination of trace amounts of analytes in samples are environmental samples and biological samples containing drugs, biologically active substances, ions, toxic substances etc. Trace amounts of the studied substances are often accompanied by further substances present in the sample matrix. The amounts of samples available for analysis are often limited. An important role is played by efficient separation and miniaturization of all parts of the analyser. Both of these requirements are represented by microcolumn liquid chromatography [1–5]. Application of an enrichment technique prior to the determination of trace amounts of analytes is usually necessary. In connection with liquid chromatography, the enrichment by solid-phase extraction is most frequently used [6–8]. Packed precolumns then work as enrichment units [7–11]. Trace determinations require highly sensitive and often selective detection. Automation is important from the point of view of the speed and accuracy of operations performed, mainly with serial analyses.

This paper describes an analytical system connecting the above-mentioned demands for the determination of trace analytes: microcolumn liquid chromatography + enrichment unit + fluorescence detection + automation (the analytical system is controlled by a computer). An

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important element of the whole system is the application of a capillary mixer adjusting the sample matrix so that the analyte capacity factor is increased. Hence, enrichment is carried out not only on the precolumn but also on the head of the analytical column.

2. Theory

Injection on to the analytical column in elution chromatography is limited by the volume V_{max}. The injected volume V_{max} causes a permissible dispersion of the eluated analyte zone [12,13]. V_{max} is proportional to the analytical column dead volume V_{Mcol}, the analyte capacity factor k_{col} and the value of $1/\sqrt{n_{col}}$, where n_{col} is the number of theoretical plates of the analytical column. If there is a sample of volume $V > V_{max}$, direct injection leads to volume overloading of the column. However, injection of the volume V is possible if the analyte capacity factor k_{col} in the sample matrix is increased. This can be performed by application of a mixer [14]. Alternate injection of the sample volume segment and the volume segment of a solvent of low eluting strength (non-eluting solvent) results in mixing of both liquids. This results in the required increase in k_{col}, application of the volume V to the analytical column and, hence, enrichment of the analyte on the column.

Connection of a sorption precolumn with the analytical column is an important means of enrichment of trace amounts of the analyte from the sample. The precolumn can be loaded maximally with a volume V_B which represents the breakthrough volume [7,11]:

$$V_B = V_{Mpre}(1 + k_{pre})\left(1 - \frac{2}{\sqrt{n_{pre}}}\right)$$

where V_{Mpre} is the precolumn dead volume, k_{pre} the analyte capacity factor in the sample matrix in the case of injection on the precolumn and n_{pre} the number of theoretical plates of the precolumn. An increase in k_{pre} leads to an increase in V_B and, hence, to enrichment of the analyte on the precolumn. If the analyte is desorbed from the precolumn in a volume $V_{DES} > V_{max}$, the situation is identical with that as for $V > V_{max}$. Application of a mixer between the precolumn and the analytical column ensures the adjustment of the matrix of the desorbed volume V_{DES} and the possibility of injection on to the analytical column. V_{DES} is mixed with the volume of the non-eluting solvent V_{NS} with a resulting capacity factor k_Σ. The equation

$$\frac{V_{DES} + V_{NS}}{V_{max}} = \frac{k_\Sigma + 1}{k_{col} + 1}$$

describes the possibility of sampling $V_{DES} > V_{max}$ provided that the non-linear dependence of k_Σ on φ_Σ (the volume fraction of the solvent of higher elution strength in the sample matrix). The chromatographic system often used with a reversed phase is characterized by the logarithmic dependence $\log k_\Sigma = f(\varphi_\Sigma)$. This is the important condition so that V_{DES} could be injected completely with the enrichment factor V_{DES}/V_{max}.

3. Experimental

3.1. Apparatus, fluorescence microcell

A schematic diagram of the laboratory-made microcolumn liquid chromatograph [15,16] (Institute of Analytical Chemistry, Brno, Czech Republic) is shown in Fig. 1. The apparatus is controlled by a PMD 85-2 computer (Tesla, Bratislava, Slovak Republic) and connected to a PU 4027 fluorescence detector (Philips, Cambridge, UK) additionally equipped with an FSA emission filter (418-nm cut-off filter) of an FS 950 Fluoromat fluorimeter (Kratos, Ramsey, NJ, USA).

We used a fluorescence flow microcell of our own design with a volume of 0.34 μl, which suits the microcolumn system. It consists of a fused-silica capillary (0.25 mm I.D.). The illuminated part of the microcell is 7 mm long.

3.2. Chemicals

For preparation of the mobile phases and solutions of polycyclic aromatic hydrocarbons

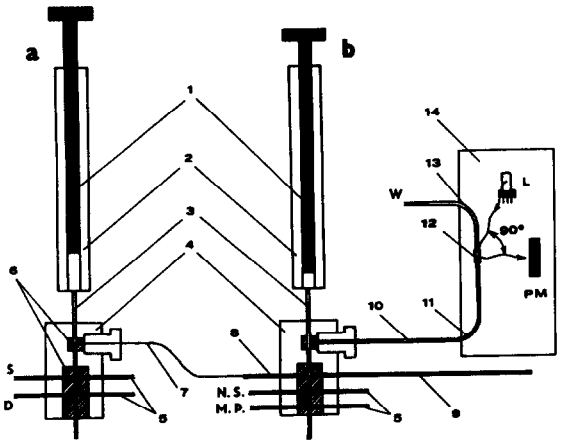

Fig. 1. Connection of the enrichment unit (a) with the analytical unit (b). 1 = Piston; 2 = glass syringe (80 μl); 3 = injection needle (0.3 mm I.D. × 0.5 mm O.D.) provided with a side outlet (0.15 mm I.D.) and closed at the end; 4 = liquid distribution block; 5 = mobile phase and sample inlets; 6 = PTFE seals; 7 = connecting capillary (2.6 μl); 8 = precolumn (50 × 0.31 mm I.D.); 9 = store capillary; 10 = capillary mixer (100 × 0.25 mm I.D.); 11 = analytical column (115 × 0.25 mm I.D.); 12 = fluorescence microcell; 13 = mobile phase waste; 14 = fluorimeter; S = sample; D = solvent for desorption from the precolumn; N.S. = non-eluting solvent; M.P. = mobile phase; W = waste; L = xenon lamp; PM = photomultiplier.

(PAHs), acetonitrile (analytical-reagent grade) and methanol (for HPLC) (Merck, Darmstadt, Germany) were used. Six PAH standards were selected: anthracene, fluoranthene and chrysene (Lachema, Brno, Czech Republic) and pyrene, benzo[*b*]fluoranthene and benzo[*b*]pyrene (Supelco, Belefonte, PA, USA). Stock standard solutions of the PAHs were prepared in acetonitrile at a concentration of 300 mg/l and stored in a refrigerator.

3.3. *Microcolumn, mixer, precolumn, adsorbents, packing*

The analytical column and the mixer were made of two parts of a fused-silica capillary (0.25 mm I.D.) and were connected together by fixing into a glass capillary of larger diameter (0.5 mm I.D.). The length of the mixer capillary was 100 mm and that of the microcolumn 115 mm. At the end of the microcolumn a glass capillary (1 mm × 10 μm I.D.) with a piece of a glass-wool was fixed so that leakage of the sorbent from the microcolumn was prevented. The end of the microcolumn was connected directly with the microcell.

The microcolumn was packed with reserved-phase Separon SGX C_{18} of particle diameter 5 μm (Tessek, Prague, Czech Republic) by a suspension technique from CCl_4 at a pressure 20 MPa.

The capillary precolumn (46 × 0.31 mm I.D.) was packed with the same material and by the same technique as for the analytical column; the particle diameter was 10 μm.

3.4. *Description and function of microchromatograph*

Fig. 1 shows the connection of two laboratory-made microchromatographs: a microchromatograph as an enrichment unit [(a) and Fig. 1] and a microchromatograph as an analytical separation unit [(b) in Fig. 1]. The microcolumn chromatograph has been described previously [15]. The syringe (2) of the microchromatograph with a piston (1) and a needle (3) (Fig. 1) serves for sampling and injection of the mobile phases and the sample. The liquids are pushed out of the needle via a side opening (0.15 mm I.D.) as the end of the needle is blind. The needle passes through the PTFE seal in the liquid distribution block (4). Its movement is controlled by a stepper. The liquid is sucked into a syringe when the side opening in the needle is connected with the inlet to the reservoir (5). The liquid is pushed out of the syringe when the liquid distribution block is adjusted in such a way so that the needle side opening points directly to the mixer and the microcolumn [(b) in Fig. 1] or to the precolumn [(a) in Fig. 1].

3.5. *Sample processing*

Let us consider treating a trace amount of an analyte with enrichment on the precolumn. The sample from the syringe (2) of the enrichment unit [(a) in Fig. 1] is injected on to the precolumn (8). The analyte is retained and the remaining part of the matrix leaves by a store capillary (9). The analyte is desorbed with a solvent of high elution strength and gradually

fills part of the store capillary. The store capillary retains the sample volume desorbed from the precolumn before sampling to the mixer and analytical column. Then the solution of analyte, or a group of analytes, is ready for processing in the analytical separation unit [(b) in Fig. 1].

The sample is injected automatically according to a given programme in the following sequence:

(1) sucking of a sufficient volume (20–50 μl) of the non-eluting solvent into the syringe (2b);

(2) injection of the first volume segment of the non-eluting solvent into the mixer (10);

(3) sucking of the first volume segment of the sample (extract) from the store capillary (9) to the syringe (3b); the needle volume is 2 μl;

(4) injection of the first volume segment of the sample + the second volume segment of the non-eluting solvent into the mixer (10) simultaneously.

The cycle of sample injection with the non-eluting solvent according to steps 3 and 4 is repeated as many times as necessary until the total volume of the extract from the store capillary (9) is transported together with the non-eluting solvent to the mixer and the analytical column. Elution with the mobile phase then follows.

The same injection method can be used in the analysis of an extract resulting from off-line extraction of a sample with a complex matrix. There is the possibility of treating a much higher extract volume in the analytical separation unit in comparison with direct injection of an aliquot of the extract on to the analytical column.

4. Results and discussion

Experiments were designed in such a way that the theoretical assumptions for sample enrichment could be verified practically. Reproducibility of the measured data was established, in addition to the recovery characteristics for the automated microcolumn chromatograph with the enrichment unit. The practical applicability of the device was verified on the example of determination of trace amounts of fluoranthene in tap water and six selected PAHs in the organic extract.

4.1. Chromatographic system

PAHs were selected as test substances with respect to their high affinity for the hydrophobic surface of the stationary phase used. The advantage consists in the application of sensitive fluorescence detection for the determination of these substances. The occurrence of PAHs in the environment needs to be carefully checked owing to their toxicity [17–20]. In most of the experiments fluoranthene (molecular mass 202.26 g/mol) was used. Its concentration in water indicates contamination of the environment by PAHs. The maximum excitation and emission wavelengths of fluoranthene in acetonitrile were found to be $\lambda_{ex} = 350$ nm and $\lambda_{em} = 467$ nm, which are very close to the values in the literature [21].

A chromatographic system with a reversed phase was selected at this type represents about 80% of all applications in liquid chromatography. Using the analytical microcolumn with Separon SGX C_{18} reversed phase, the dependence of the capacity factor k of fluoranthene on the content of acetonitrile in the acetonitrile–water mobile phase was studied. For a volume fraction φ of acetonitrile in the mobile phase in range 0.8–0.4, the logarithmic dependence $\log k = \log k_0 - m\ \varphi$ (correlation coefficient $r = 0.9913$) was found. The values of k and φ are presented in Table 1. For the given range of $\varphi \approx 0.8$–0.4 the values of the constants are $k_0 = 1200$ and $m = 3.67$. With a decrease in φ below 0.4 the values of $\log k$ will increase with a higher slope m than for the measured interval $\varphi \approx 0.8$–0.4. The dependence of $\log k = f(\varphi)$ is an important assumption for analyte enrichment due to addition of a non-eluting solvent to the sample matrix.

The capillary mixer ensures adjustment of the sample matrix (organic extract) by mixing with a non-eluting solvent (water). The function of the mixer is fulfilled by a fused-silica capillary (100 × 0.25 mm I.D.). The capacity of the mixer enables a volume segment of the sample (extract) of 1 μl

Table 1
Dependence of capacity factor of fluoranthene on the acetonitrile content in the mobile phase.

Vol. fraction of CH_3CN (φ)	0.80	0.70	0.60	0.55	0.50	0.45	0.40
k	1.7	3.1	7.0	9.7	16.0	27.5	52.7

to be mixed with a volume segment of the non-eluting solvent. Injection of 1 μl of the extract is the optimum because it fills only the space of the injection needle (3b) of the syringe (2b) (Fig. 1). In the needle the extract is mixed minimally, it does not enter the space of the syringe (2b) and, hence, the analyte losses are eliminated. The volume segment of the non-eluting solvent can be increased as desired. An increase in this volume leads to a decrease in the peak width. With perfect mixing of segments [(3 × 1-μl segments of the sample in 80% CH_3CN) + (3 × 3-μl segments of the non-eluting solvent in 20% CH_3CN) = 12 μl], the contribution to band broadening by the sample volume is negligible and the peak width is 12.1 mm for the experimental conditions used. However, with the real capillary mixer the peak width experimentally measured for the same total sample volume (12 μl) is 13.9 mm. Hence the influence of the total sample volume on band broadening is evident and the volume segments are incompletely mixed. An increase in the volume segment of the non-eluting solvent from 1 to 3 μl leads to only a 4% decrease in the peak width. Therefore, 1 μl of the non-eluting solvent and thus a ratio of segments of 1:1 (μl) were used. A change of the mixer geometry could ensure, if necessary, more perfect mixing of segments.

4.2. Reproducibility, recovery

The reproducibility of the measured data characterizes the precision and the reliability of the tested device. Experiments were performed under the following conditions: injection of 30 μl of 30 μg/l fluoranthene in 40% CH_3CN, desorption from the precolumn with 20 μl of CH_3CN, injection of 9 μl of the extract from the store capillary into the mixer and the analytical column and elution with 80% acetonitrile at a flow-rate of 10 μl/min. The results for seven measurements of the peak area are presented in Table 2. The reproducibility is represented by the value of the relative standard deviation (R.S.D. = 0.8%), calculated according to the equation R.S.D. = $(\sigma_{abs}/\overline{hw}) \cdot 100$, where σ_{abs} is the absolute standard deviation and $\overline{hw}$ is the average value of the product peak height · width. The R.S.D. for tens to hundreds of ppt of fluoranthene in the sample does not exceed 5%. Automation of all the steps during the treatment of the sample contributes to a high accuracy of measurement of the experimental data.

The term recovery is connected here with evaluation of the possible losses during the process of enrichment on the precolumn. The same volume (20 μl) of the same sample (30 μg/l fluoranthene in 40% CH_3CN) was injected

Table 2
Reproducibility of measurements using the automatic microcolumn LC system with the enrichment unit

Parameter	Measurement No.						
	1	2	3	4	5	6	7
Peak height (mm)	154	152	153.5	152	160.5	153	151
Peak width (mm)[a]	6.50	6.50	6.55	6.55	6.20	6.60	6.55
Height · width (mm^2)	1000	988	1005	995	995	1009	990

[a] Peak width at half-height.

(1) directly onto the analytical column (via the mixer) and (2) with enrichment on the precolumn (for measurement conditions, see above). By comparison of the fluoranthene peak areas (see Ratio 2/1 in Table 3), we conclude that during the process of enrichment on the precolumn analyte losses do not occur.

4.3. Example of determination of fluoranthene and PAHs

For the given microcolumn chromatographic system with the fluorescence microcell, a minimum detectable amount, defined by the peak height which is twice the baseline noise, of 8.0 pg of fluoranthene in an injection of 20 μl of a 3 μg/l solution of fluoranthene in 45% methanol was found.

The content of fluoranthene was determined in a sample of tap water, using chromatographic conditions based on the ASTM standard method [22]. A 1-ml sample of water (city of Brno) was adjusted by adding 0.82 ml of methanol to a concentration of 45% methanol in the matrix. The conditions of sample treatment are given in Fig. 2. By the method of standard additions with substraction of the blank, a concentration 120 ng/l of fluoranthene in the original sample of water was found (Fig. 2). The minimum detectable amount found of 8.0 pg of fluoranthene with injection and precolumn loading of up to 800 μl for 45% methanol in the sample matrix determines the detection limit to be 10 ng/l of fluoranthene.

An example of the determination of six selected PAHs in the organic extract (acetonitrile) is presented in Fig. 3. By changing the excitation

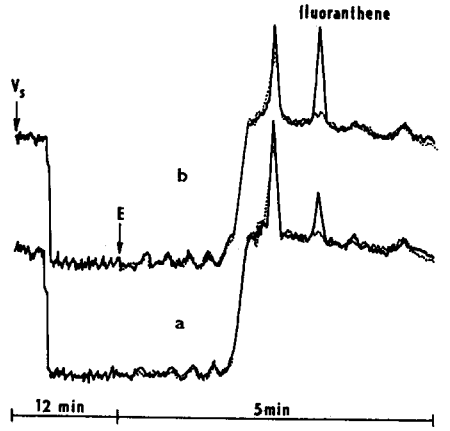

Fig. 2. Determination of fluoranthene in tap water (city of Brno). Mixer, analytical column and precolumn, see Fig. 1. Sampling: 200 μl of water in 45% methanol into the precolumn. Desorption from the precolumn: 19 μl of methanol. V_s = sampling of 14 μl of methanolic extract and 15 μl of distilled water into the mixer and analytical column. E = elution with 90% methanol, flow-rate 5 μl/min. Water analysis (a) without addition of fluoranthene, concentration determined 120 ng/l; (b) with addition of fluoranthene (100 ng/l). Dotted line indicates blank.

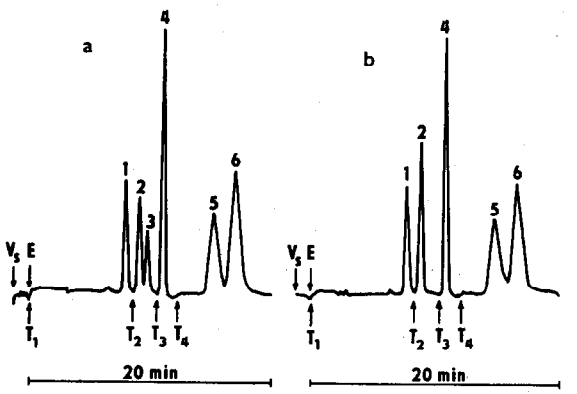

Fig. 3. Determination of six PAHs in acetonitrile. Mixer and analytical column, see Fig. 1. V_s = sampling of 6 μl of PAH solution and 7 μl of water into the mixer and analytical column; E = isocratic elution with 70% acetonitrile. Concentrations: 1 = anthracene, 100; 2 = fluoranthene, 200; 3 = pyrene, 200; 4 = chrysene, 60; 5 = benzo[*b*]fluoranthene, 60; 6 = benzo[*a*]pyrene, 60 μg/l. Emission cut-off filter, 370 nm. (a) Time programming of ($\lambda_{ex}/\lambda_{em}$): $T_1 = 0$ min (350/398 nm); $T_2 = 8.5$ min (330/430 nm); $T_3 = 10.6$ min (264/385 nm); $T_4 = 12.4$ min (300/428 nm). (b) The same conditions as in (a) but $T_2 = 8.5$ min (354/467 nm).

Table 3
Comparison of peak areas in cases 1 and 2 (see text)

Measurement No.	Peak height · width (mm^2)		Ratio 2/1
	Case 1	Case 2	
1	640	630	0.984
2	645	658	1.020
3	652	645	0.989

and emission wavelengths λ_{ex} and λ_{em}, the sensitivity and selectivity of determination can be influenced (the pyrene peak in Fig. 3b was eliminated).

By injection of 6 μl of acetonitrile extract, sample enrichment on the analytical column is obtained. With the fluoranthene sample in 100% acetonitrile we have a maximum volume of 0.4 μl, which is the volume that could be applied directly to the analytical column with the allowed 20% decrease in the separation efficiency. Connection of the mixer with the analytical column, therefore, represents a fifteen times higher enrichment of fluoranthene on the column in case of the acetonitrile extract or the possibility of a fifteen times higher increase in the precolumn volume in case of the sample of water.

4.4. *Analyte losses*

Analyte losses by sorption on the surface of parts of the apparatus or on the walls of the vessels may represent a difficult problem [17,20,23]. This was also observed in our experiments. We prepared solutions (0.5 ml) of 300 μg/l of fluoranthene in acetonitrile–water mixtures of various composition. We injected 3 μl of these solutions on to the microcolumn via the mixer and the peak area was evaluated. Its decrease with decreasing concentration of acetonitrile in the matrix demonstrates that in the determination of trace amounts this effect has to be taken into account (Fig. 4). The peak height of fluoranthene did not change for up to 2 min after sample preparation in the glass vessel. Addition of a solvent with a high elution strength to the sample matrix is often necessary to prevent analyte losses and then a system mixer is essential to ensure the required enrichment.

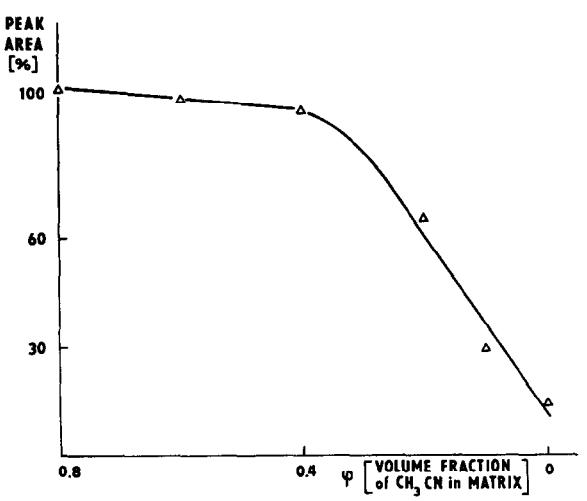

Fig. 4. Adsorption of fluoranthene on the glass surface of the sample flask. Abscissa = content of acetonitrile (φ) in the sample matrix; ordinate = peak area of fluoranthene (%).

5. Conclusions

The application of a microcolumn liquid chromatograph connected on-line with a mixer, enrichment unit and fluorescence microcell has been demonstrated. The device is characterized by automated operation with high reproducibility of the measured data (R.S.D. = 0.8%). The procedure for sample treatment ensures elimination of analyte losses during the process. The analyser permits analyte enrichment from the sample both on the precolumn and on the analytical column connected with the capillary mixer. As a result, the degree of analyte enrichment increases. The applicability of the proposed device was shown by the examples of the determination of trace amounts of fluoranthene in tap water and of six selected PAHs in an acetonitrile extract.

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