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On-line coupling of immunosorbent and liquid chromatographic analysis for the selective extraction and determination of polycyclic aromatic hydrocarbons in water samples at the ng l^{-1} level

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Abstract

A method employing immunoaffinity chromatography for the selective extraction of polycyclic aromatic hydrocarbons (PAHs) from water was developed. An immunosorbent (IS) based on the immobilization of anti-fluorene antibodies on silica was coupled on-line with LC and applied to the selective extraction of PAHs from water samples. The procedure involves the use of a solubilizer to limit unwanted adsorption on vessels or tubing. Results show that a compromise has to be found between a good solubilization and limitation of the elution from the IS due to the addition of this modifier. The contribution of the selective antigen–antibody interaction to the retention mechanism is demonstrated. The on-line method was optimized for the six more volatile PAHs, and was also applied to the 16 PAHs included in the US Environmental Protection Agency priority list. The sensitivity of the fluorescence detection associated to the selectivity of the extraction sorbent allows one to detect PAHs between 2 and 10 ng l^{-1} from a sample volume as low as 20 ml. The presence of several PAHs at 20 ng l^{-1} in surface water was confirmed by spectral identification using diode array detection coupled in series with a fluorescence detector.

Keywords: Extraction methods; Water analysis; Environmental analysis; Immunoadsorbents; Polynuclear aromatic hydrocarbons

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants that mainly result from incomplete combustion of various materials, in particular petroleum fuels. Their determination in water is difficult as their levels of concentration in water are low due to their low solubility in water. In the European Union (EU) regulations for the monitoring of the quality of surface water used for drinking purposes, six PAHs are listed and the sum of their concentrations must not exceed 0.2 μg l^{-1} with a concentration limit of 0.02 μg l^{-1} for benzo[*a*]pyrene [1]. Moreover, 16 compounds of this class have been listed by the US Environmental Protection Agency (EPA) as priority pollutants because of their carcinogenic properties. The 2–3 rings are both volatile and also hydrophobic, the logarithmic water–octanol partition coefficient values (log K_{ow}) are between 3.3 and 5.2. The 4–6 rings are very hydrophobic (log $K_{ow} \geq 5$), they tend to stick everywhere, leading to losses during sampling and storage. To carry out a reliable analysis, powerful analytical methods have thus to be set up.

Reversed-phase liquid chromatography (RPLC) with fluorescence detection was shown to be an

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excellent technique for the measurement of PAHs in environmental matrices and was used for the development of certified standard reference materials [2]. For the determination at the ng l^{-1} level, a preconcentration step is necessary and owing to the trend for decreasing the use of organic solvents in laboratory, solid-phase extraction (SPE) is preferred over liquid–liquid extraction (LLE). However in order to avoid the adsorption of PAHs on sample containers or connection tubing, an organic solvent [3–10] or a surfactant [7–9] should be added before extraction.

Classical extraction sorbents used for the extraction of PAHs from water are C_8 [5], C_{18} [3,4,6–10] or polymers of styrene–divinylbenzene [7,8]. In most cases, the sorbent is packed in cartridges or disks. Using these devices, the SPE step is entirely dissociated from the separation step. Automated on-line systems allowing the coupling of the SPE with LC have also been used [9,10]. The use of other sorbents developing more specific interactions such as Chromspher π, Boos Glass, Boos Silica or Blue Pearls that are mainly based on π–π interactions have also been reported [9]. The retention of PAHs on these sorbents is mainly based on hydrophobic interactions. Many other compounds can then be co-extracted with PAHs rendering difficult the detection and quantification of the target pollutants at low levels of concentration. Therefore, there is an interest in developing more selective sorbents in order to simplify the extraction procedure and to minimize the matrix effects.

In recent years, extraction sorbents based on immunoaffinity involving analyte–antibody interactions have been developed. Antibodies produced against a target compound are immobilized on a solid-phase to obtain an immunosorbent (IS) that is used as a classical extraction sorbent. The first commercial ISs were introduced for the clean-up of samples for the determination of aflatoxins. Other ISs have been described in the literature for the analysis of single pesticides such as carbendazim, chlortoluron, atrazine or terbutylazine [11–13]. Because of the unavoidable cross-reactivity of antibodies against small molecules, antibodies are also able to trap compounds from the same structural family as the one of the antigen. By coupling the antibody cartridge with LC, we can then achieve a selective extraction and a quantitative analysis of several compounds from the same family. Our group took advantage of this selective extraction technique to extract a whole group of structurally related compounds such as triazine and phenylurea pesticides [14–20] including their degradation products. This technique was applied to the extraction of these pollutants from waters [14–16,20], foodstuffs [17,18], soils and sediments [19].

These ISs have been employed both in off-line and on-line procedures including LC with UV–diode array detection (DAD) and LC with atmospheric pressure chemical ionization (APCI) MS. Due to the selectivity of the extraction, chromatograms present a clear baseline, so that the sample volume can be as low as 20 ml for the determination of several phenylureas and triazines at the ng l^{-1} level using IS/APCI-MS [20,21].

To develop an immunosorbent for the selective extraction of PAHs in water, antibodies against fluorene have been synthesized. Moreover, in order to simplify and to automate the procedure, an on-line device was used. This system is particularly well adapted to the most volatile PAHs that are difficult to extract using an off-line procedure if an evaporation step is required before the injection because losses of these compounds can occur [5,6]. Fluorene is one of the most soluble and volatile PAHs. This immunosorbent was then produced to selectively extract fluorene from highly contaminated waters but also other volatile PAHs such as naphthalene, acenaphthene, phenanthrene, anthracene and fluoranthene that are the less hydrophobic PAHs among the 16 priority ones, containing 2–3 aromatic rings, and that can be detected in waters in spite of their low solubility. Nevertheless, the structural similarity within this group of pollutants shows the possibility to use this sorbent for the extraction of the 16 priority PAHs.

The objective of this work was to evaluate the performance of an on-line method combining extraction with an anti-fluorene IS specially designed for the trapping of the 2–3 rings PAHs and LC with both UV DAD and fluorescence detection. This required study of (i) the efficiency of the IS for the trapping of the various PAHs, (ii) the effect of the necessary addition of organic solvents or surfactants in water samples before extraction on breakthrough

volumes and recoveries, (iii) the selectivity of the IS for the extraction of PAHs from real samples, (iv) the possibility of the method for the quantification at the ng l^{-1} level using fluorescence detection with confirmation by UV spectrum using UV DAD. The advantage of coupling an on-line selective extraction and the combination of both fluorescence and UV DAD is necessary for confirmation of the identity of the analytes in unknown samples.

2. Experimental

2.1. Chemicals

HPLC-grade acetonitrile was obtained from Mallinckrodt Baker (Deventer, Netherlands) and methanol from Carlo Erba (Milan, Italy). LC-quality water was prepared by purifying demineralized water in a Milli-Q filtration system (Millipore, Bedford, MA, USA). Other chemicals were purchased from Prolabo, Merck, SDS and Fluka. The various PAHs were supplied by Mallinckrodt Baker. Stock solutions of selected solutes were prepared by weighing and dissolving them in methanol or acetonitrile and stored at 4°C. The phosphate-buffered saline (PBS) solution consisted of a 0.01 *M* sodium phosphate buffer containing 0.15 *M* NaCl (pH 7.4) and 0.2% azide.

2.2. Apparatus

LC analyses were performed with a Varian LC System Workstation including a Varian Star 9010 solvent-delivery system, a Model 9065 Polychrom DAD system and a Varian 9075 fluorescence detector. Immunopre-column and analytical column switching was accomplished with two Rheodyne (Berkeley, CA, USA) valves. Conditioning of the immunopre-column and percolation of samples were performed using two Milton-Roy pumps according to the scheme described in Fig. 1. This system of preconcentration was totally constituted by stainless steel tubing.

2.3. Stationary phases and columns

The analytical column was a 250×3 mm I.D. column prepacked with the PAH16-Plus silica from Mallinckrodt Baker. Preconcentrations were made through experimental stainless steel pre-columns (30×4.6 mm I.D.) prepacked with 220 mg of the immunosorbent based on anti-fluorene antibodies bonded onto glutardialdehyde-activated silica particles of 50 nm pore size (Mallinckrodt Baker). Polyclonal anti-fluorene antibodies were supplied by Professor F. Le Goffic. These antibodies were produced by the injection in an animal of an immunizing agent obtained by coupling the fluorenyl-methyloxy-carbonyl-*o*-succinimide to a carrier protein, i.e., bovine serum albumin, and by following an immunization procedure similar to that previously described [14]. Their immobilization on the activated silica sorbent was achieved as previously reported [14]. The synthesis of the anti-atrazine immunosorbent used in this study have been previously described [19]. Other preconcentrations on non-selective sorbents were carried out on pre-columns prepacked with styrene–divinylbenzene copolymer PRP-1, 20×2 mm I.D., 5–10 μm (Hamilton, Reno, NV, USA) and with C_{18} silica (ODS) column, 10×2 mm I.D., 10 μm (Upchurch Scientific, Oak Harbor, WA, USA).

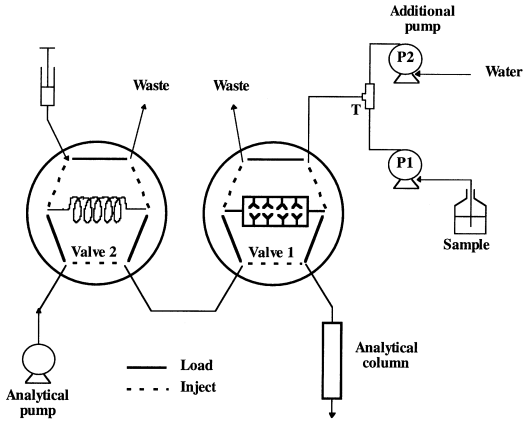

Fig. 1. Simplified scheme of the experimental set-up used for the on-line preconcentration on immunosorbent.

2.4. LC conditions

The gradient used for the separation of PAHs is as follows: a mixture of acetonitrile–water (50:50) from 0 to 5 min, 50% to 100% of acetonitrile from 5

to 20 min and 100% of acetonitrile up to 35 min, the flow-rate used was 0.5 ml min^{-1}. UV detection was set at 215 or 249 nm. The fluorescence program was as follows: λ_{ex}=220 nm and λ_{em}=340 nm from 0 to 12 min, λ_{ex}=240 nm and λ_{em}=340 nm from 12 to 16.5 min, λ_{ex}=240 nm and λ_{em}=440 nm from 16.5 to 20 min, λ_{ex}=280 nm and λ_{em}=398 nm from 20 to 23.5 min, λ_{ex}=238 nm and λ_{em}=416 nm from 23.5 to 27 min and λ_{ex}=296 nm and λ_{em}=420 nm from 27 to 35 min.

2.5. Immunoaffinity procedure

In the on-line system, a pre-column packed with the sorbent used for the extraction step takes place at the loop position of a six-port switching valve according to Fig. 1. The first step of the procedure is achieved in the load position and consists in conditioning the sorbent. For the immunopreconcentration, the immunosorbent is conditioned with 6 ml of PBS and then 6 ml of LC-grade water. For the preconcentration on ODS or PRP-1, the sorbents are conditioned with 6 ml of acetonitrile, 6 ml of methanol and 6 ml of water. The sample is percolated through the pre-column at a flow-rate of 2 ml min^{-1}. By switching the valve 1, compounds trapped on the sorbent are eluted on-line from the pre-column to the analytical column by an acetonitrile–water gradient used for the analytical separation at a flow-rate of 0.5 ml min^{-1}. When the immunosorbent is not in use, it is stored at 4°C in a solution of PBS (containing 0.2% of azide) after a washing step using 70% of methanol (10 ml).

3. Results and discussion

3.1. On-line coupling of the immunosorbent with LC

Few studies have been devoted to the on-line preconcentration of PAHs in aqueous samples. Vera-Avila and Covarrubias have shown that it was necessary to add 5% of acetonitrile in the sample for the extraction on ODS of the least hydrophobic PAHs, i.e., naphthalene, and 23% of acetonitrile for the others in order to limit their adsorption on the flasks [10]. In contrast, for the extraction of the PAHs on ODS or π–π interaction based sorbents, Brouwer et al. have investigated the addition of various surfactants [9]. Although the antigen–antibody interactions are not only based on hydrophobic interactions the addition of an organic solvent results in a decrease of the interactions. As a consequence, breakthrough will occur due to insufficient retention which can be measured by incomplete extraction recoveries.

3.1.1. Recoveries of extraction and solubilization of PAHs

This study focused on the selective extraction of PAHs from aqueous matrices such as industrial effluent or waste waters with emphasis on the first six PAHs included in the priority list, i.e., naphthalene, acenaphthene, fluorene, phenanthrene, anthracene and fluoranthene.

A sample of 10 ml of LC-grade water spiked at 1 μg l^{-1} with a mixture of the six PAHs was percolated on the anti-fluorene IS. Various amounts of acetonitrile were added in order to limit the adsorption of the compounds on the glass bottles or the tubing of the on-line set-up. Table 1 presents the obtained recoveries. Using 5% of acetonitrile in the water sample, recoveries of extraction from this IS are between 24 and 60% depending of the compounds. By increasing the amount of acetonitrile up to 10%, better recoveries of extraction are obtained, particularly for the more hydrophobic compounds. This higher amount of organic solvent allows a better solubilization of the PAHs that are extracted in higher amount by the IS. A percentage of 25% of acetonitrile is too high and, in spite of a better

Table 1
Recoveries (%) obtained after the preconcentration of 10 ml of LC-grade water containing various amounts of acetonitrile and spiked at 1 μg l^{-1} with a mixture of PAHs on anti-fluorene IS

Compounds	Anti-fluorene IS		
	5%, n=2	10%, n=3	25%, n=5
Naphthalene	24±1	15±1	6±1
Acenaphthene	41±1	27±1	8±1
Fluorene	36±0	42±2	11±1
Phenanthrene	44±1	61±2	14±1
Anthracene	60±2	73±1	16±2
Fluoranthene	46±2	58±2	22±2

Table 2
Effect of the percolated volume on the recoveries of extraction (%): preconcentration of LC-grade water spiked with 10 ng of each PAH on the anti-fluorene IS

Compound	Percolated volume				S.D.
	10 ml	20 ml	50 ml	100 ml	20 ml (n=5)
Naphthalene	15	9	4	2	0
Acenaphthene	27	16	8	4	3
Fluorene	42	35	15	9	3
Phenanthrene	61	56	27	18	3
Anthracene	73	47	25	14	5
Fluoranthene	58	60	32	29	6

solubilization of the compounds in the sample than when using 10% of acetonitrile, low recoveries are obtained due to an insufficient retention of all the PAHs on the IS. For a 10-ml volume of sample, the addition of 10% of acetonitrile is then a good compromise between an efficient solubilization and the retention of the solutes on the IS.

3.1.2. Breakthrough volumes

In order to reach low levels of concentration, it is important to concentrate large volumes of sample. It is then important to evaluate the volume of sample that can be percolated on the IS. In a SPE process, a breakthrough of the compounds which are present in the sample at a trace level is due to an insufficient retention of the solutes. The retention of the solutes on the IS depends on the affinity of the antibodies towards the different compounds. Increasing volumes of sample spiked with constant amounts of PAHs and containing 10% of acetonitrile were percolated on the IS. Recoveries of extraction are reported in Table 2. Up to 20 ml, extraction recoveries are satisfactory, they are still between 35 and 60% except for the two-ring aromatic compounds, i.e., naphthalene and acenaphthene. Those two compounds are well solubilized with 10% of acetonitrile but the affinity of the antibodies for them is low and breakthrough occurs. The optimized conditions for these two compounds consist in the percolation of 10 ml of sample containing 5% of acetonitrile. For the four other compounds, the best conditions consist in the percolation of 20 ml of sample, with 10% of acetonitrile.

To limit the breakthrough due to the addition of acetonitrile other modifiers were used. Different percentages of tetrahydrofurane and isopropanol were tested. Difficulties concerning the regeneration of the immunosorbent have occurred when using tetrahydrofurane. With regards to isopropanol, its effect on the breakthrough was tested on an other immunosorbent and has shown to be higher than that of acetonitrile: lower recoveries were obtained when the same amounts of solvent were added. Table 3 presents recoveries of extraction obtained on the

Table 3
Recoveries obtained after the preconcentration of 20 ml of LC-grade water spiked at 0.5 μg l^{-1} with a mixture of PAHs on ODS and on anti-fluorene IS (n=2, S.D.=1–6%)

Compounds	ODS				Anti-fluorene IS			
	ACN		Tween 20	Brij-35	ACN		Tween 20	Brij-35
	10%	25%	0.1%	$3\cdot10^{-4}$ M	10%	25%	0.1%	$3\cdot10^{-4}$ M
Naphthalene	42	10	61	70	9	4	15	15
Acenaphthene	60	18	85	56	16	5	15	26
Fluorene	64	27	83	50	35	5	30	53
Phenanthrene	62	34	80	47	56	8	29	65
Anthracene	57	37	75	39	47	9	29	46
Fluoranthene	54	57	90	51	60	14	15	57

ACN=Acetonitrile.

anti-fluorene IS when percolating 20 ml of LC-grade water containing 10 or 25% of acetonitrile and also two different types of surfactant used as solubilizer.

The use of Brij-35 was reported in a study using on-line preconcentration of PAHs on ODS silica or sorbents developing specific π–π interactions [9]. Tween 20 is used as a solubilizer in the biological field and particularly in immunoaffinity-based methods [22,23]. Concentrations of Tween 20 between 0.05 and 0.2% were tested, the conditions giving the highest recoveries correspond to the use of 0.1%. Those recoveries are reported in Table 3. They are lower than those obtained using 10% of acetonitrile. The concentration of Brij-35 was fixed at $3 \cdot 10^{-4}$ *M* according to a previous study [9]. Under these conditions, the recoveries are higher for the antigen, i.e., fluorene, similar for the other compounds to those obtained with 10% acetonitrile.

In order to evaluate the potential of this IS for the extraction of PAHs from water, similar experiments were carried out on C_{18} silica (ODS) and PRP-1. Recoveries of extraction obtained on ODS after the preconcentration of 20 ml of samples containing the various solubilizers are also reported in Table 3. For a sample volume of 20 ml, the use of 25% of acetonitrile results in a large breakthrough and low recoveries of extraction are obtained. In contrast, the use of 10% of acetonitrile is a good compromise for the good solubilization and a sufficient retention of the solutes on the two sorbents. With regard to less hydrophobic PAHs, i.e., naphthalene and acenaphthene, 5% of acetonitrile should be better as it was already mentioned. In contrast, better recoveries are obtained with Tween 20 as solubilizer than with Brij-35. However, when using Tween 20, the resulting chromatogram has shown the presence of many interfering compounds introduced by the surfactant and co-extracted on ODS that render the analysis of PAHs difficult.

If there is a difference in recoveries on ODS between the addition of Tween 20 or Brij-35, the inverse is observed on the immunosorbent. This different effect of the surfactants on the IS and on ODS can be explained by the fact that the retention of PAHs is not based on the same mechanisms on the IS than on ODS. Tween 20 seems to have a strong effect on the affinity of the antibodies for PAHs and decreases the breakthrough volume.

Better recoveries could be expected by the addition of 25% of acetonitrile by using a more hydrophobic sorbent such as a styrene–divinylbenzene copolymer PRP-1. A pre-column packed with PRP-1 was used for the preconcentration of 20 ml of surface water spiked with a mixture of compounds including the six PAHs. A similar experiment was carried out on the IS. The resulting chromatograms obtained with UV and fluorescence detection are presented in Fig. 2a Fig. 2b for the PRP-1 sorbent and Fig. 2c Fig. 2d for the IS. The comparison of the chromatograms shows the difference in quality of the coupling of these two sorbents with the analysis using a C_{18} silica column and an acetonitrile gradient as described in Section 2.4. Broad peaks are obtained with PRP-1. This is due to the strong interactions between the PAHs and this sorbent associated to an insufficient elution strength of the mobile phase in the beginning of the gradient (50% of acetonitrile during 5 min), the strong interactions being confirmed by high recoveries of 60–85% for the six PAHs obtained for the percolation of 20 ml of water containing 10% of acetonitrile on PRP-1. The shape of the peaks when using the IS demonstrates the good quality of the coupling between this immunoaffinity-based sorbent and the analytical column packed with C_{18} silica.

As a conclusion to this section, for the more hydrophobic PAHs, i.e., fluorene, phenanthrene, anthracene and fluoranthene, recoveries obtained on ODS and on the IS are quite similar when using 10% of acetonitrile or Brij-35. For the naphthalene and acenaphthene, recoveries are better on ODS, than on the IS. This lower retention on the anti-fluorene antibody can be explained by the difference in structure between the PAH antigen, fluorene, and these two compounds. An anti-naphthalene antibody is under study to evaluate its ability to extract those two compounds. Nevertheless, these results have proven that the use of 10% of acetonitrile or Brij-35 for a sample volume of 20 ml is a good compromise for a good solubility of the compounds in the sample and a good retention on the IS.

3.1.3. Optimization of the on-line coupling

To limit the breakthrough of the PAHs on the IS due to the addition of the modifier in the sample, the concentration of this solubilizer has to be decreased

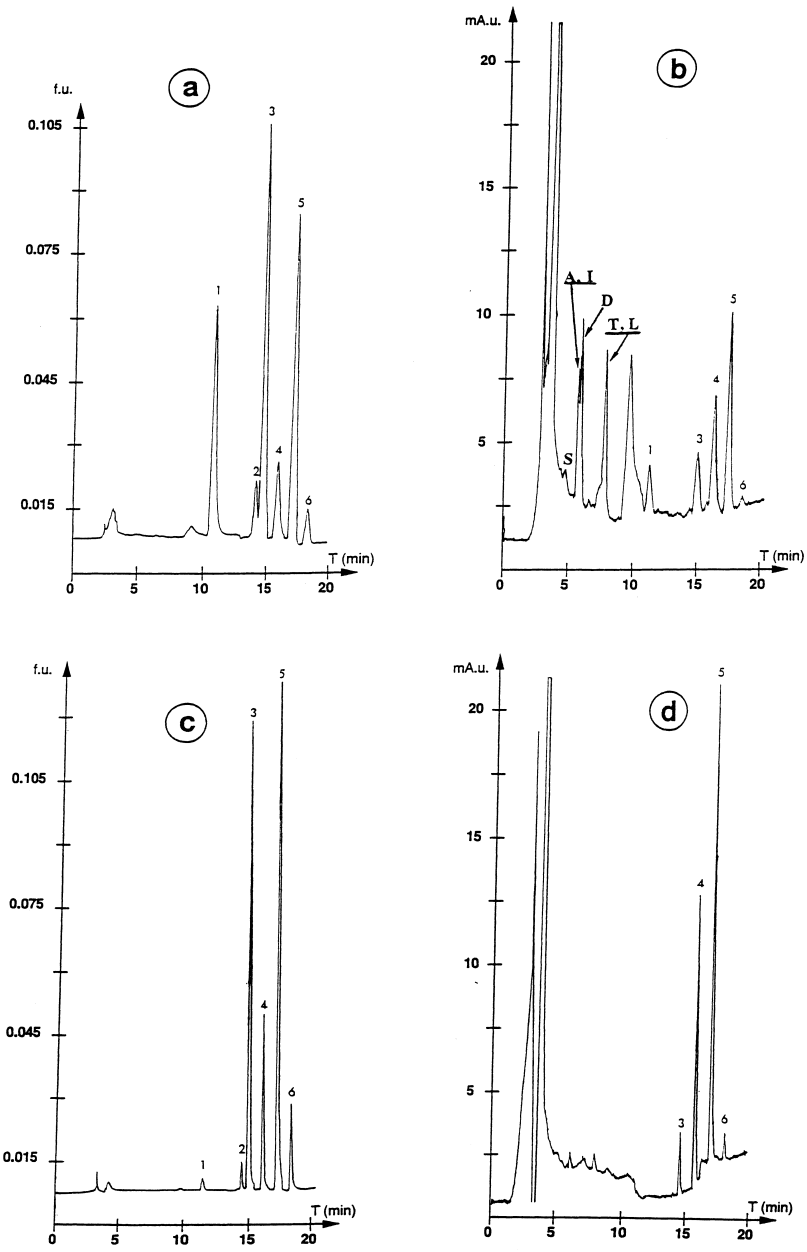

Fig. 2. Quality of the on-line coupling with LC: preconcentration of 20 ml of surface water containing 10% of acetonitrile and spiked at 0.5 μg l^{-1} with a mixture of phenylureas, triazines and PAHs on PRP-1 (a, b) and on the anti-fluorene IS. (c, d). UV detection at 249 nm (b, d) and fluorescence detection (a, c). Phenylureas and triazines: (S) simazine, (A) atrazine, (T) terbutylazine, (I) isoproturon, (D) diuron, (L) linuron. PAHs: (1) naphthalene, (2) acenaphthene, (3) fluorene, (4) phenanthrene, (5) anthracene, (6) fluoranthene.

during the percolation in the pre-column. This can be achieved by the use of an additional pump as previously described [9]. The idea is to add a high amount of organic solvent or modifier in the sample containers to prevent the adsorption of the PAHs and to use an additional pump to reduce the amount of

modifier during the percolation through the pre-column. This system is described in Fig. 1: the sample containing the modifier is pumped via pump 1 (P1) and further diluted by addition of water pumped with the pump 2 (P2). Then if the final percolated volume of sample is higher than for the experiments previously described due to the dilution, the amount of modifier in the pre-column is decreased. The effect of such a dilution via an additional pump was studied using Brij-35 and authors have reported that higher extraction recoveries are obtained on ODS [9]. The concentration of the surfactant in the sample being higher than the critical micellar concentration (CMC, 10^{-4} M), Brij-35 forms micelles that allow the solubilization of PAHs. By decreasing the concentration of Brij-35 below the CMC value down to $8.6 \cdot 10^{-5}$ M just before the pre-column, micelles are disrupted immediately and do not continue to act as modifiers in the pre-column, allowing a higher retention of PAHs on the sorbent.

In our case, when using Brij-35 as modifier, the dilution does not allow a larger retention of PAHs on the immunosorbents. This can be seen by comparing recoveries obtained by the direct percolation of 20 ml of $3 \cdot 10^{-4}$ M of Brij-35 (Table 3) to recoveries obtained when 10 ml of sample are diluted up to 35 ml to decrease the concentration of Brij-35 to $8.6 \cdot 10^{-5}$ M (Table 4). The micelles are disrupted but the final percolated volume is too high (35 ml instead of 20 ml) and then breakthrough of the solutes still occurs. To limit the percolated volume, only 5 ml of water was added to the 15 ml of sample, the concentration of Brij-35 being then about $2.2 \cdot 10^{-4}$ M for a final volume of 20 ml (Table 4). The comparison of the results of Table 3 (20 ml of sample at $3 \cdot 10^{-4}$ M of Brij-35) shows that if the concentration of the surfactant is still higher than the CMC, the recoveries of extraction on the IS can not be increased. Therefore, dilution of the sample containing Brij-35 does not allow to improve the recoveries. Similar experiment were carried out with acetonitrile as modifier. Even if a large volume is percolated on the IS, 35 ml (Table 4) instead of 20 ml (Table 3), the decrease of the content in acetonitrile down to 3% allows one to obtain better recoveries for the two more polar PAHs, i.e., naphthalene and acenaphthene. But, when the dilution is used, the sample volume of 10 ml instead of 20 ml will not allow to obtain the same limit of detection. Moreover, the solubilization of the three more hydrophobic PAHs is not sufficient and recoveries are lower than without dilution. The comparison of the dilution effect with the same sample volume of 10 ml (Table 1 compared with Table 4) shows that the dilution is not interesting when 10% is used. To limit the risk of an insufficient solubilization of the more hydrophobic PAHs, the dilution system was applied when 25% of acetonitrile are added to the sample. In this case, even if a larger volume is percolated on the IS, 35 ml (Table 4) instead of 20 ml (Table 3), the decrease of the content in acetonitrile down to 7% allows to obtain better recoveries for all the PAHs, the dilution limiting considerably the breakthrough. However, to evaluate the interest of the use of dilution, these recoveries obtained for a sample volume of 10 ml have to be compared to recoveries of Table 1 also obtained by percolating 10 ml of sample. This comparison shows that the use of 25% of acetonitrile further diluted with water does not allow to obtain better recoveries than when 10% of acetonitrile were added without further dilution. For this study concerning these six PAHs, the dilution is not necessary. However, this system has shown that in some cases it allows to limit the breakthrough of the solutes and can be interesting for the study when

Table 4
Recoveries of extraction (%) obtained after the percolation of samples spiked with PAHs, containing various amounts of modifier on anti-fluorene IS and using an additional pump to dilute the modifier ($n=2$, S.D.=1–6%)

	Brij-35		ACN	
V sample/C	10 ml	15 ml	10 ml	10 ml
	$3 \cdot 10^{-4}$ M	$3 \cdot 10^{-4}$ M	10%	25%
V added water	25 ml	5 ml	25 ml	25 ml
V percolated/C	35 ml	20 ml	35 ml	35 ml
	$8.6 \cdot 10^{-5}$ M	$2.2 \cdot 10^{-4}$ M	3%	7%
Naphthalene	12	14	12	9
Acenaphthene	15	28	30	17
Fluorene	30	46	35	48
Phenanthrene	26	50	29	59
Anthracene	15	35	24	38
Fluoranthene	26	45	15	41

V=Volume. C=Content (concentration or percentage) of modifier in sample or after dilution.

low sample volume are sufficient to reach low limits of detection.

3.2. Selective and non-selective interactions

3.2.1. Retention by selective interactions on the IS

We have shown that PAHs are very hydrophobic and tend to stick everywhere, therefore non-selective interactions can certainly occur with the protein-based sorbent in addition to the selective interaction that takes place on the recognition sites of the antibodies [24]. To evaluate the contribution of the non-selective interactions on the retention of the PAHs on the IS, an anti-atrazine immunosorbent was used. This immunosorbent possesses similar characteristics as the anti-fluorene IS (220 mg of activated silica bonded with a similar amount of antibodies). Its specificity towards the triazine group has been previously described [16,19].

Recoveries of extraction obtained with these two immunosorbents after the percolation of 20 ml of LC-grade water spiked at 0.5 $\mu g\ l^{-1}$ with PAHs and containing 10% of acetonitrile or Brij-35 are presented in Table 5. For the two modifiers, the retention of PAHs on anti-fluorene IS is better than on the anti-atrazine IS showing a somewhat higher specificity of the anti-fluorene antibodies towards the six PAHs. Nevertheless, PAHs are so hydrophobic that they are partially retained by anti-atrazine IS with non-specific interactions. Those non-specific interactions are also involved in the retention of PAHs on anti-fluorene IS but the retention is better due to the addition of the specific antigen–antibody interactions in the retention mechanism.

3.2.2. Selective extraction of PAHs from real samples

A mean to evaluate the selectivity of the IS is to apply it to the extraction of PAHs from real samples and to study its effect on the matrix. Fig. 2 corresponds to the analysis of surface water spiked with a mixture of triazines, phenylureas and PAHs and preconcentrated on the non-specific sorbent PRP-1 (a, b) and on the anti-fluorene IS (c, d). UV detection at 249 nm (Fig. 2b Fig. 2d) was chosen because it allows the detection of five PAHs among the six present in the sample but also the detection of triazines and phenylureas when they are extracted. All these compounds can be identified on the chromatogram corresponding to the preconcentration on the non-selective PRP-1 sorbent (Fig. 2b). In contrast, only PAHs can be identified on the chromatogram corresponding to the extraction on anti-fluorene antibodies (Fig. 2d). Triazines and phenylureas have not been recognized by these antibodies. This can only be explained by the selectivity of the IS that only allows the extraction of the PAHs from this sample, because the hydrophobicity of the selected triazines and phenylureas is low enough (log K_{ow} between 2 and 3.5) to prevent high non-specific interactions on the IS. For more hydrophobic compounds present in real samples, one can expect them to be retained on the IS by non-selective interactions to a certain extent. However, recoveries will be

Table 5
Selectivity of the IS towards the PAHs: preconcentration of 20 ml of LC-grade water containing 10% of acetonitrile or $3 \cdot 10^{-4}$ *M* of Brij-35 and spiked with 0.5 $\mu g\ l^{-1}$ with the mixture of PAHs on the anti-atrazine and anti-fluorene ISs

Compounds	Recoveries (%)			
	Brij-35		Acetonitrile	
	Fluorene IS	Atrazine IS	Fluorene IS	Atrazine IS
Naphthalene	15	10	9	7
Acenaphthene	26	21	19	13
Fluorene	53	24	35	15
Phenanthrene	65	36	55	32
Anthracene	46	34	47	30
Fluoranthene	57	43	55	50

higher for PAHs due to the additive contribution of selective interactions.

To better illustrate this selectivity, anti-fluorene IS was applied to the selective extraction of the PAHs from a dirty urban run-off water. A sample of 20 ml of water containing Brij-35 and spiked with PAHs at 0.5 μg l^{-1} was preconcentrated on the IS (Fig. 3c and d) and on ODS (Fig. 3a and b) that allows a better coupling with LC than PRP-1. UV detection (Fig. 3a and c) shows the influence of the matrix of

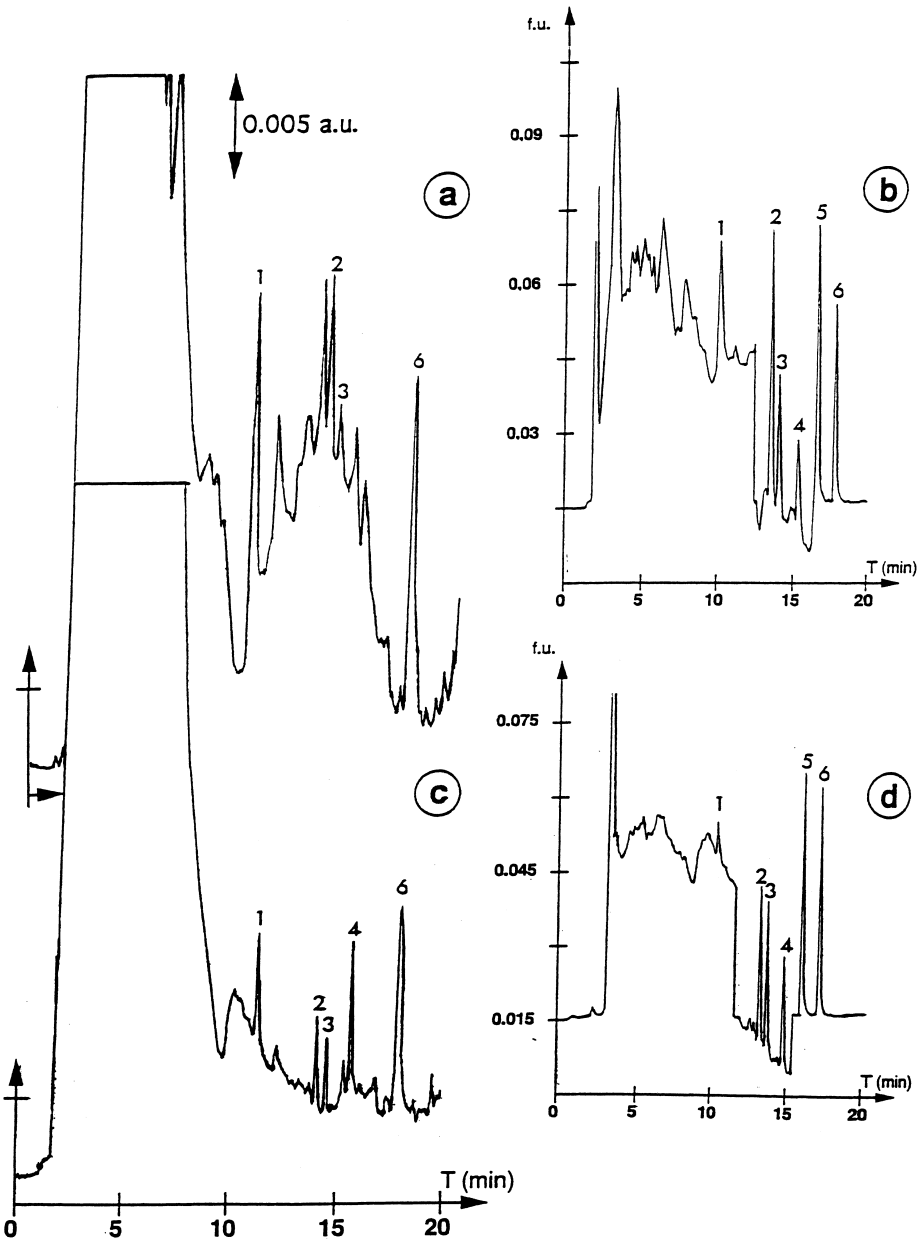

Fig. 3. Preconcentration of 20 ml of urban run-off water containing $3 \cdot 10^{-4}$ *M* of Brij-35 and spiked at 0.5 μg l^{-1} with PAHs on C_{18} silica (a, b) and on the anti-fluorene IS (c, d). UV detection at 215 nm (a, c) and fluorescence detection (b, d). Compounds: see Fig. 2.

the sample. On the chromatogram obtained using ODS (Fig. 3a), a large hump due to the co-extraction of many interfering compounds is observed and renders difficult the detection and quantification of these PAHs in this highly contaminated water. In comparison, the use of the IS allows the selective extraction of PAHs that can be easily quantified. The use of the IS is particularly interesting for the quantification of the compounds 2, 3 and 4 (Fig. 3c). To increase the sensitivity of the method, fluorescence detection was used for the two extraction sorbents and resulting chromatograms are presented in Fig. 3b and d. Fluorescence detection is more selective but there are still interfering compounds in the beginning of the chromatogram obtained using ODS (Fig. 3b) that have not been extracted by the IS (Fig. 3d). The selectivity of the extraction step coupled to the sensitivity of the detector allows one to reach low concentration levels in contaminated water and to confirm the presence of these pollutants in the sample.

3.3. Quantification, limit of detection and confirmation using UV DAD and fluorescence detection in series

For all the experiments previously described, standard deviation (S.D.) for several replicates were between 1–6% for 10 ml or 20 ml of samples containing 10% of acetonitrile as solubilizer showing a high repeatability of the system as it was already mentioned with other ISs [14,19–21]. After the elution by the mobile phase, a washing step using 10 ml of a mixture containing methanol–water (70:30) was used to wash the immunopre-column. Ten ml of the PBS solution was then used for the regeneration of the antibodies. That ensures a reproducible behavior of the immunosystem as shown by the examples of repeatability given in Tables 1 and 2. Although experiments have been optimized for the 2–3 rings compounds, we have evaluated the performance of the whole system IS–LC–UV DAD–fluorescence detection with the 16 priority PAHs. Our knowledge on on-line preconcentration technique has shown us that quantification is reproducible with incomplete recoveries, provided calibration curves are constructed with spiked solutions using the whole on-line system with strictly similar experimental conditions. Therefore, this type of calibration means, it is not necessary to know or measure recoveries. Fig. 4 shows the calibration curves obtained after the percolation of 20 ml of water containing 10% of acetonitrile spiked between 20 and 500 ng l^{-1} on the anti-fluorene IS. These curves were constructed using measurements of peak areas obtained by fluorescence detection. A good linearity characterized by correlation coefficients higher than 0.997 for 14 among the 16 PAHs was obtained. Acenaphthylene and indeno[1,2,3-*cd*]pyrene are not detected because of their low fluorescence. These results indicate that constant recoveries of extraction are obtained for the whole calibration range for the 14 compounds. The recoveries of extraction of the more hydrophobic compounds are between 15 and 30% for the percolation of 20 ml of water containing 10% of acetonitrile. Those recoveries were not optimized and the conditions of solubilization for these compounds necessitate higher amount of modifier. However, in spite of these low recoveries, quantification at a concentration level lower than 0.02 μg l^{-1} can be carry out in contaminated water. The chromatogram corresponding to the analysis of a surface water spiked at 20 ng l^{-1} with the 16 PAHs (Fig. 4) and obtained using fluorescence detection shows that the association of the selectivity of the extraction using anti-fluorene IS and the sensitivity of the detection allows the easy detection of all the 14 PAHs included in the priority list at concentration levels between 0.3 ng l^{-1} (as for benzo[*k*]fluoranthene) and 12 ng l^{-1} in surface water (as for pyrene and benzo[*ghi*]perylene) with a signal-to-noise ratio of 3.

Fig. 5 shows the chromatogram of the analysis previously described in Fig. 4 and obtained by UV detection using two different wavelengths, 234 and 249 nm. The UV characteristics of PAHs do not allow the quantification of these compounds at low levels of concentration as it is done using the fluorescence detector but, even if the signal is low, the UV spectrum of each detectable peak can be compared to a library of spectra that allows to confirm the presence of the pollutants in the water. As an example, a selection of three spectra is presented in Fig. 5. Peaks corresponding to the retention times of anthracene, pyrene and chrysene were obtained and DAD has allowed one to identify

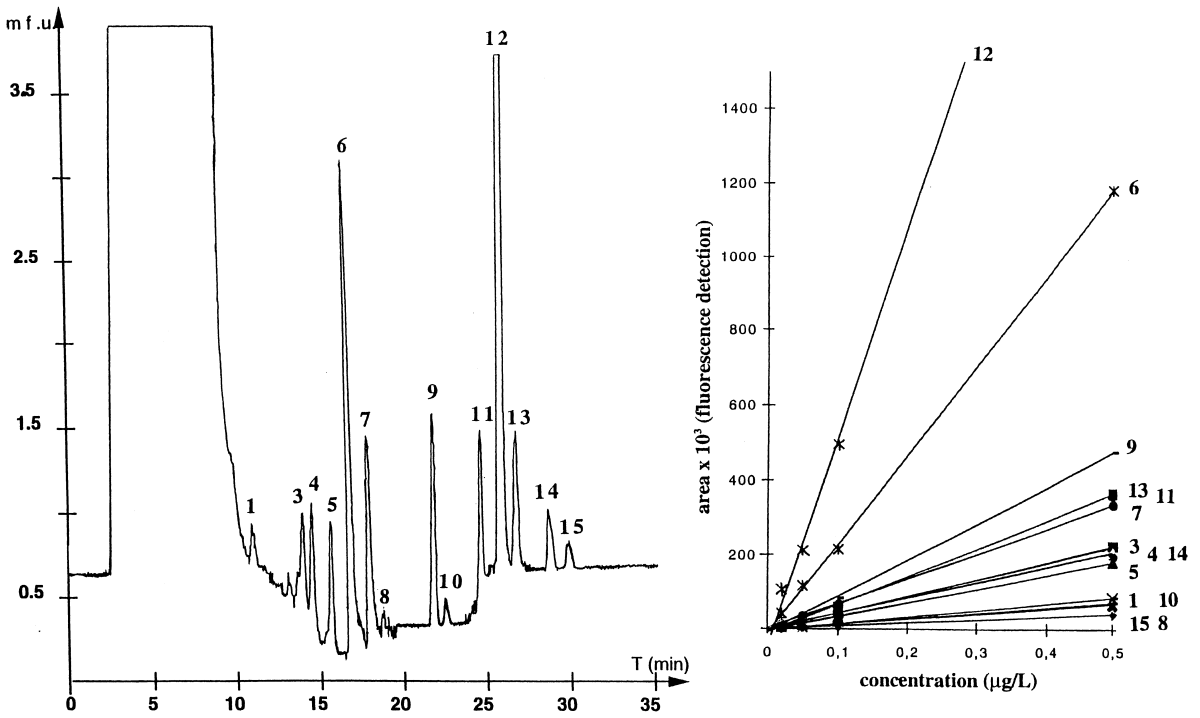

Fig. 4. Preconcentration of 20 ml of surface water containing 10% of acetonitrile and spiked at 20 ng l^{-1} with PAHs on anti-fluorene IS. Fluorescence detection. Calibration curves obtained by preconcentrating LC-grade water containing 10% of acetonitrile and spiked at concentration levels between 20 and 500 ng l^{-1} with PAHs. Compounds: (1) naphthalene, (2) acenaphthylene, (3) acenaphthene, (4) fluorene, (5) phenanthrene, (6) anthracene, (7) fluoranthene, (8) pyrene, (9) benz[*a*]anthracene, (10) chrysene, (11) benzo[*b*]fluoranthene, (12) benzo[*k*]fluoranthene, (13) benzo[*a*]pyrene, (14) dibenz[*ah*]anthracene, (15) benzo[*ghi*]perylene.

these compounds by comparing the spectra to spectra of PAHs included in a library.

4. Conclusions

We have demonstrated that it is possible to use the anti-fluorene IS for the selective extraction of PAHs from real water. PAHs are very hydrophobic and, then, their retention on the IS is based on the selective antigen–antibody interaction but also to a contribution of non-selective hydrophobic interactions. However, the selectivity of the IS allows to limit the effect of the matrix and low levels of concentration were reached in surface water. The sensitivity of fluorescence detection coupled to the selectivity of the IS permitted the quantification of PAHs between 10 ng l^{-1} and 20 ng l^{-1} for a low sample volume of 20 ml that is rather difficult to obtain using non-selective sorbents in dirty matrices. The presence of the PAHs can also be confirmed at low levels of concentration using DAD for the comparison of spectra. The optimization of the on-line system was done for six of the most volatile PAHs that are difficult to extract using an off-line procedure. However, for the more hydrophobic PAHs, the conditions of solubilization have to be modified to increase their extraction recoveries. An IS more specific to these compounds and based on anti-pyrene antibodies is also under study to increase the potential of the method for the 4–6 aromatic rings PAHs. Concerning the less hydrophobic compounds from this group, i.e., naphthalene and acenaphthene, anti-naphthalene antibodies are also under study. One can expect that, by mixing these antibodies with the anti-fluorene ones, good re-

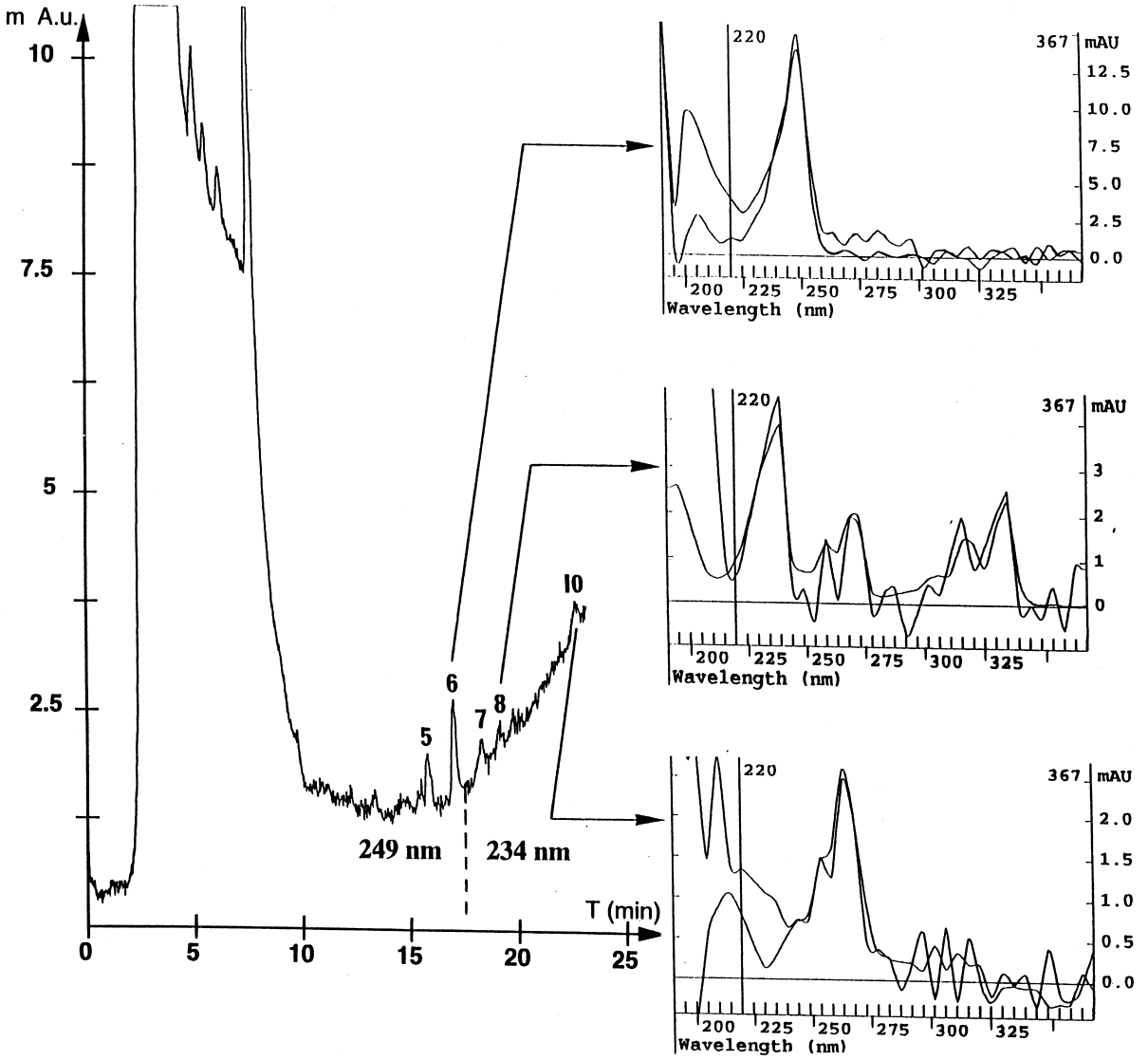

Fig. 5. Preconcentration of 20 ml of surface water containing 10% of acetonitrile and spiked at 20 ng l^{-1} with PAHs on anti-fluorene IS. UV detection at 234 and 249 nm. The inserts represent the match of UV spectra. Compounds: see Fig. 4.

coveries of extraction will be obtained for the six volatile PAHs.

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