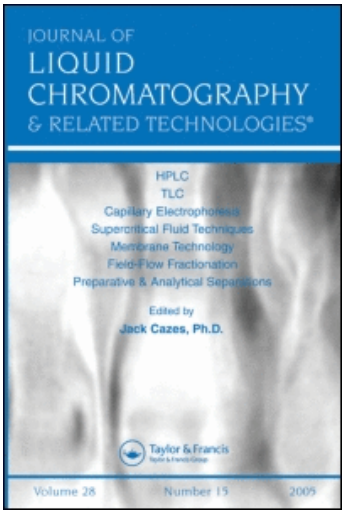

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Abstract: The development and validation of a reversed-phase high performance liquid chromatographic method with fluorescence and UV detection for the determination in sediments of 16 polyaromatic hydrocarbons (PAHs) listed as priority pollutants by EPA is described. The analytes are extracted by ultrasonification and purified by solid-phase extraction (SPE) with C_{18} mini-column cartridges. The factors influencing the recovery ratio such as sediment characteristics, composition, and flow rate of the sample loaded on the cartridge, and volumes of acetone necessary to elute the analytes from the cartridges are discussed. In the defined conditions the recoveries are always over 80%. A Waters PAH C_{18} column with an acetonitrile-water gradient elution was employed for the separation of the purified samples. The linearity of the data and the limits of quantification and detection are determined for each molecule. The method is successfully applied to the quantitative analysis of 16 PAHs in marine, brackish, and river sediments.

Keywords: Environmental analysis, US EPA PAHs, Extraction method, SPE, RP-HPLC, Sediment

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INTRODUCTION

Polynuclear aromatic hydrocarbons are the most contaminating xenobiotic pollutants in the natural environment because of their carcinogenic and mutagenic properties.[1] They are produced by the combustion of a wide class of substances such as saturated and unsaturated hydrocarbons, fossil fuels, peptides, and carbohydrates.[2]

An important group of polycyclic aromatic compounds are the polycyclic aromatic hydrocarbons (PAHs) with at least two fused benzenic rings and no element other than carbon and hydrogen. These compounds may reach the aquatic environment in domestic and industrial effluents. They are also introduced into the natural environment during direct contamination by crude oil or refinery products.[3]

PAHs present characteristics of lipophilicity, low water solubility, adsorption to marine particles, and accumulation into the sediments, which makes them a dangerous group of chemicals.[4] This fact prompted US Environmental Protection Agency (US EPA) to include PAHs molecules in the priority pollutant list.[5–7] Nevertheless, although hundreds of PAHs exist in the environment, only 16 PAHs, i.e., naphthalene (Naph), acenaphthylene (Ac), acenaphtene (Ace), fluorene (Flu), phenanthrene (Phe), anthtracene (Anth), fluoranthene (Fluo), pyrene (Pyr), benzo[a]anthracene (BaAn), chrysene (Chr), benzo[b]fluoranthene (BbFl), benzo[k]fluoranthene (BkFl), benzo[a]pyrene (BaPy), dibenzo[a,h]anthracene (DiAn), benzo[g,h,i]-perylene (BePe), and indeno[1,2,3-cd]pyrene (InPy), have been chosen as representative by US EPA and must be routinely analyzed in environmental samples. In fact, analysis of PAHs in marine sediments is particularly important because they are considered as pollution indicators, since they give a view of the spatial distribution of the pollutants.[2,8]

At the present time, the analytical techniques most often used for PAHs detection and quantification in solid matrices include:

a) Extraction by soxhlet[9] or mechanical shaking.[10] These procedures have been, for many years, the standard method for preparing a solvent extract of solid matrices containing PAHs. Nevertheless, the extraction times are long and a great quantity of toxic and very volatile solvents (such as ether, dichloromethane, toluene, etc.) is necessary. Supercritical fluid extraction[11,12] (SFE) or microwave-assisted solvent extraction[13] constitute an interesting but expensive methodology and complicated equipments are required.

   Finally, one of the alternatives for PAHs extraction is the utrasonification of the solid environmental matrice.[2,10] This technique permits short extraction times and small solvent volumes.

b) Purification of the investigated molecules from the extract, generally by solid-phase extraction (SPE). Different sorbents such as Florisil,[14,15]

alumina,[16] silica,[17] cyclohexyl-bonded silica[18] or octadecyl-bonded silica (C_{18} stationary phase)[10,18] are available for this purpose. C_{18} mini-column cartridges present the advantage of being suitable for the isolation of a large diversity of organic pollutants.[19]

c) Identification by gas chromatography (GC) with flame ionization (FID) or mass spectrometry detectors (MS) mass spectrometry[20,21] or analysis by reversed-phase high performance liquid chromatography (RP-HPLC) with fluorescence and spectrophotometric (UV) detection.[2,22]

Because of its sensibility and rapidity, the combination ultrasonic extraction, purification with C_{18} SPE mini-column cartridges and PAHs separation with RP-HPLC is attractive.

In a preceding paper we described a chromatographic technique for the detection of PAHs in the sediments of the urban sewage area of the littoral ecosystem of Marseille (France).[2] This method could be improved for recovery yields, accuracy, and detection rapidity, by using a method which enables measurement of PAHs in soils.[10] In the present article, the procedure has been devised for the determination of PAHs in river, brackish, and marine sediments. This method is divided into three steps; ultrasonic solvent extraction, SPE with C_{18} mini-column cartridges for purification, and analysis by RP-HPLC with fluorescence and UV detection.

EXPERIMENTAL

Chemicals

SDS (Peypin, France) supplied acetone and methanol (HPLC grade purity solvents) used for the extraction steps. For chromatographic analysis, acetonitrile and water of HPLC grade and 0.2 μm membrane filters were purchased from Carlo Erba (Val de Reuil, France). Bond Elut Jr C_{18} mini-column cartridges containing 1 g of adsorbent (Varian, Harbor City, CA, USA) were employed for the clean-up of the studied polycyclic aromatic hydrocarbons in the extraction solutions from sediments. A standard mixture with the 16 EPA priority pollutants PAHs in 1 mL of dichloromethane was obtained from PolyScience (Cat No: 6719M, Niles, IL, USA).

Standard Solution and Calibration

A mixture of 16 PAHs (stock standard solution), 200 μg/L of each substance, was dissolved in methanol by dilution into a 25 mL flask of the standard mixture. Working standard solutions (0.06, 0.8, 8, 48, 100, 200, and 400 μg/L) were generated by dilution in methanol of the stock solution. All

these solutions may be stored 6 months at ambient temperature, but only in the dark to prevent the eventual photochemical degradation of polynuclear molecules. When fluorescence and UV detectors were switched on, external calibration curves were constructed for each investigated molecule by injection of the seven working standard solutions. The integrated peak areas were used to quantify the 16 PAHs.

Instruments

HPLC analyses were performed at 21°C, using a degassed mobile phase and a high performance liquid chromatograph, Waters 2475 Alliance (Milford, MA, USA), equipped with an automatic injector. The LC system was coupled with a Waters 2475 multi wavelength fluorescence detector. A Waters 2487 UV detector was also used. Data acquisition and processing were carried out on Millennium[32] (Waters) chromatography software. Separation of the 16 selected PAHs was performed on an analytical Waters PAH C_{18}, 250 × 4 mm i.d., 5 μm column (Milford, MA, USA), which was connected to a 20 × 4 mm pre-column containing a similar coating (YMC, Schermbeck, Germany, Art: YP99S050204).

HPLC Analysis

The flow rate was 1 mL/min and the injection volume 25 μL. Two solvents were used: acetonitrile and water. The starting conditions were 65% acetonitrile–35% water. The elution profile for separating PAHs molecules was: 0–25 min, 65–100% acetonitrile (linear gradient); 25–40 min, 100% acetonitrile (isocratic); 40–41 min, 100–65% acetonitrile (linear gradient). This composition was maintained isocratically during 20 min before a new injection. All the chromatograms were recorded only during the thirty-five first minutes. Fluorescence detection was used at programmed wavelengths, based on settings suggested in the norm NF ISO 13877 and concerning the HPLC analysis of PAHs in industrially polluted soils:[2,16] 0–8.8 min excitation wavelength of 280 nm and emission wavelength of 340 nm (detection of: naphthalene, acenaphtene, fluorene, phenanthrene); 8.8–10 min, excitation wavelength of 253 nm and emission wavelength of 402 nm (detection of anthracene); after 10 min, excitation wavelength of 305 nm and emission wavelength of 430 nm (detection of: fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]-fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene).

Acenaphtylene and indeno[1,2,3-cd]pyrene were determined by UV at 316 nm due to a lack of fluorescence. In fact, this wavelength constituted a good compromise to record these two compounds with good selectivity and correct sensitivity in the ultraviolet spectra.[23,24]

Extraction of PAHs in Sediments

The sediment samples were collected and set in polyethylene containers then immediately transported to the laboratory without any exposure to light. They were oven dried at 35°C and ground with an agate mortar before PAHs extraction.

A sediment sample of 1 to 2 g was introduced into a 50 mL flask, and 20 mL of acetone were added. The flask was ultrasonicated in a sonication bath for 30 min. A P. Selectra ultrasonic cleaning bath (P. Selectra, Barcelone, Spain) with a mean frequency of 40 KHz and a power of 200 W was used for this purpose. The mixture was vigorously shaken then decanted for half an hour.

Solid Phase Purification of the Extract

Ten mL of the liquid extract was collected into a 10 mL flask after filtration of the overlaying solvent with a 9.0 cm GF/C glass microfibre filter provided by Whatman International (Maidstone, UK). 25 mL of water was added to the filtrate. The C_{18} cartridge was prewetted before using with 5 mL of acetone and 5 mL of a solvent constituted with 40% of acetone in water. The extraction solution was then percolated through the octadecylsilica cartridge at a flow rate below 2 mL/min. After the sample loading, the cartridge was air dried for 15 min and the analytes were eluted and removed from the cartridge with 3×1 mL of acetone. Before each elution the mini-column had to be impregnated for 10 min with acetone. Two mL of methanol was aspired through the sorbent bed in order to complete the PAHs recuperation.

The purified eluate was collected in a 5-mL volumetric flask and was adjusted with methanol before RP-HPLC analysis.

RESULTS AND DISCUSSION

Analytical Separation

Different conditions of elution gradient and flow rates were tried with the waters PAH C_{18} (250×4 mm) column, but finally the conditions described in this paper were the most satisfying for the separation of the 16 PAHs. Indeed, they allow a complete separation of the analyzed compounds in less than 30 min. Figure 1 shows sharp and well resolute peaks. Chromatograms A and B clearly demonstrate that the elution times increase with the molecular sizes. In Table 1 are recorded the elution times and the capacity factors of all the investigated compounds. HPLC flow rate was optimized to reach a good selectivity of the different molecules. Indeed, an increase of flow rate would diminish the peak quality and excessively increase the

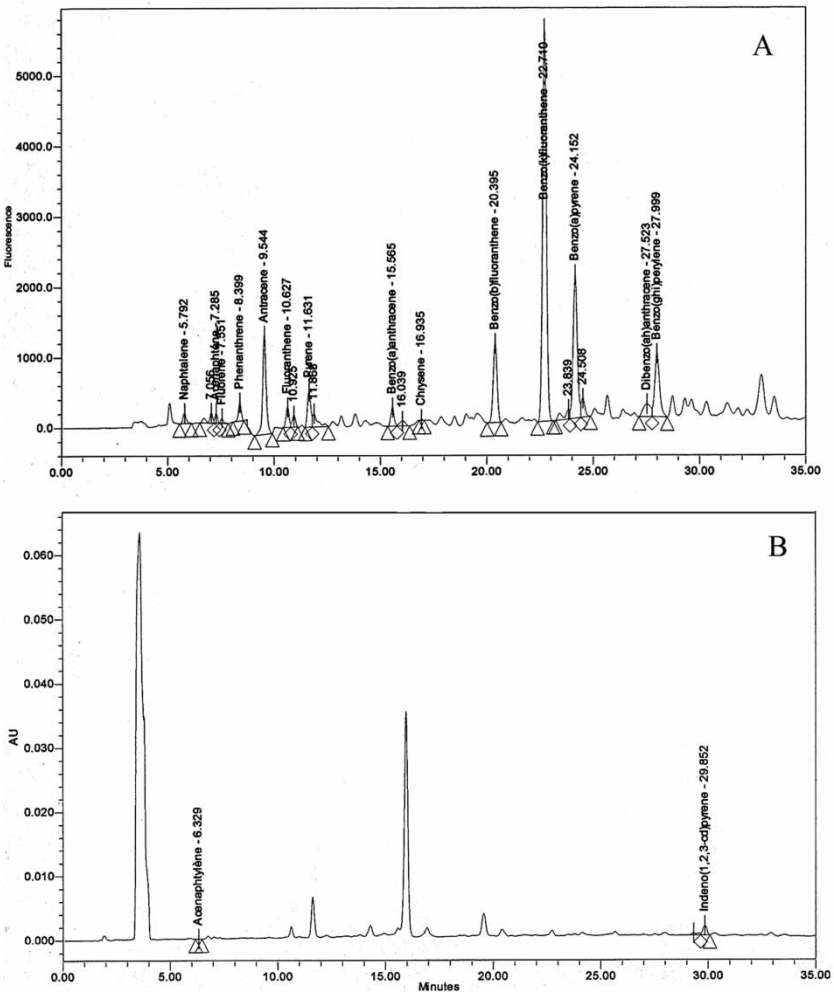

Figure 1. HPLC analysis of PAHs in a marine sediment. Concentrations (μg/kg-dry sediment): Naph: 493, Ac: 43, Ace: 111, Flu: 141, Phe: 1872, Anth: 728, Fluo: 3962, Pyr: 3940, BaAn: 2150, Chr: 212, BbFl: 2097, BkFl: 1144, BaPy: 2430, DiAn: 844, BePe: 1994, InPy: 1452. Conditions: Waters PAH C_{18} column (250 × 4.6 mm i.d., 5 μm). Volume injected, 25 μL. Temperature, 21°C. Flow-rate, 1 mL/min. Eluents: water and acetonitrile. Gradient, linear from 35 to 100% of acetonitrile in 25 min then isocratic elution with 100% acetonitrile. Chromatogram A: Fluorescence detection. Chromatogram B: UV detection.

pressure without a significant gain in analysis time. YMC 20 × 4 mm guard column allows injection of relatively polluted environmental extracts without any deterioration of the analytical column whose life-time is improved.

Table 1. Elution order, name, capacity factor, linearity data, limit of detection, limit of quantification of the 16 US EPA PAHs

Elution order	Name	Capacity factor (k′)[a]	Linearity data (r)	Limit of detection, LD (μg/kg-dry sediment)	Limit of quantification, LQ (μg/kg-dry sediment)
1	Naphthalene	0.62	0.9996	15	50
2	Acenaphthylene	0.77	0.9996	10	40
3	Acenaphthene	1.03	0.9995	10	40
4	Fluorene	1.11	0.9997	10	40
5	Phenanthrene	1.35	0.9997	15	50
6	Anthracene	1.67	0.9998	10	30
7	Fluoranthene	1.97	0.9996	10	30
8	Pyrene	2.25	0.9996	10	40
9	Benzo[a]anthracene	3.35	0.9996	10	40
10	Chrysene	3.74	0.9996	15	40
11	Benzo[b]fluoranthene	4.70	0.9999	10	40
12	Benzo[k]fluoranthene	5.35	0.9997	10	40
13	Benzo[a]pyrene	5.75	0.9992	10	30
14	Dibenzo[a,h]anthracene	6.69	0.9997	10	30
15	Benzo[ghi]perylene	6.82	0.9996	10	30
16	Indeno[1,2,3-cd]pyrene	7.35	0.9994	15	50

[a]$k' = (tr - to)/to$.

Limits of Detection, Quantification, and Linearity

The 25 μL injection volume allows correct sensitivity without drawling peaks. The wavelengths of fluorescence detection are issued from the French norm concerning the PAHs analysis in soils,[16] and were already used to detect PAHs in marine polluted sediments.[2] The UV detector works at 316 nm and enables the monitoring of PAHs whose fluorescence detection remains a problem (Figure 1B). UV detection is less selective than fluorescence detection, which must be privileged.

Both detectors permit recording the 16 US PAHs with very good limits of detection and quantification (Table 1). These limits were calculated by using the European norm NF ENV 13005.[25] The linearity of calibration curves is high for each molecule and the regression coefficients (r) are generally over 0.992.

Ultrasonic Extraction

The ultrasonic extraction method is efficient and rapid. It needs very little solvent, 20 mL of acetone for 1–2 g of sediment. Furthermore, acetone is relatively less toxic than other solvents used to extract PAHs, such as dichloromethane or toluene.[10,16] In this methodology, the conditions of extraction are rigorously defined (traceability) contrary to methods using Soxhlet apparatus, where the number of extraction cycles can never be known exactly. It needs, however, to introduce a certified sample for each serial of analysis in order to check that the power of the ultrasonic bath doesn't diminish with time.

Solid Phase Purification and Recovery Tests

Marine sediment samples can be strongly polluted and are capable of damaging the analytical column. That is why a purification step of extracts by SPE is necessary. This technique is more modern and advantageous than traditional elimination methods of interfering molecules by liquid phase extractions.

The determination of recovery yields was performed by adding definite quantities of standard PAHs to uncontaminated sediments. Characteristics of these sediments are presented in Table 2. The spiked samples are purified by percolation through C_{18} mini-column cartridges, then analyzed by HPLC. The results are summarized in Table 3. For each molecule, rate or recovery is always equal or superior to 80% and mean recoveries are around 90% for sediments A and B.

Nevertheless, lower values of recoveries are observed for BaPy and DiAn in the brackish sediment A.

Table 2. Characteristics of sediments used for recovery tests

Characteristics	Sediment A	Sediment B
Medium	Brackish Lagoon	Marine Harbour
Total oganic carbon (%)	2.6	0.4
Grain size determination		
Fraction:		
Over 2 mm (%)	6.3	13.0
2 mm–500 μm (%)	2.2	30.1
500–250 μm (%)	4.1	14.3
250–163 μm (%)	5.1	5.2
163–63 μm (%)	13.3	9.7
Under 63 (%)	75.3	40.7
Under 2 μm (%)	5.7	1.0

This is quite logical, since this last sample contains a rather important level of organic carbon and particulate matter inferior to 63 μm and capable to retain strongly heavy and complex molecules such as these two PAHs.

Table 3. Recoveries of PAHs with two spiked sediments

PAHs	Concentrations (μg/kg-dry sediment)	Recoveries[a] (%)	
		Sediment A	Sediment B
Naphthalene	199–400	89 ± 3	87 ± 3
Acenaphthylene	196–393	92 ± 4	93 ± 3
Acenaphthene	198–398	85 ± 3	85 ± 2
Fluorene	196–402	88 ± 3	88 ± 1
Phenanthrene	198–396	97 ± 3	94 ± 4
Anthracene	198–402	82 ± 3	89 ± 2
Fluoranthene	196–394	93 ± 3	93 ± 1
Pyrene	200–396	92 ± 2	93 ± 2
Benzo[a]anthracene	199–400	87 ± 3	91 ± 1
Chrysene	200–394	91 ± 2	92 ± 2
Benzo[b]fluoranthene	200–402	94 ± 2	91 ± 2
Benzo[k]fluoranthene	200–401	90 ± 2	89 ± 3
Benzo[a]pyrene	196–401	80 ± 1	87 ± 4
Dibenzo[a,h]anthracene	197–394	80 ± 2	86 ± 3
Benzo[ghi]perylene	198–396	90 ± 1	90 ± 2
Indeno[1,2,3-cd]pyrene	200–402	92 ± 4	89 ± 1
Mean recoveries (%)		89 ± 5	90 ± 2

[a]Mean ± standard deviation (n = 5).

The C_{18} mini-column cartridges used contain 1 g of sorbent. These cartridges can not trap PAH molecules when they are dissolved in pure acetone, that is the reason why some water (60%, v/v) must be added to the extract in order to improve PAH retention on C_{18} stationary phase. The influence of acetone percentage on recoveries of the artificially spiked sediment B (200–400 μg/kg of each PAH–dry basis) may be observed in Figures 2 and 3. The recovery yields dramatically decrease over 40% of

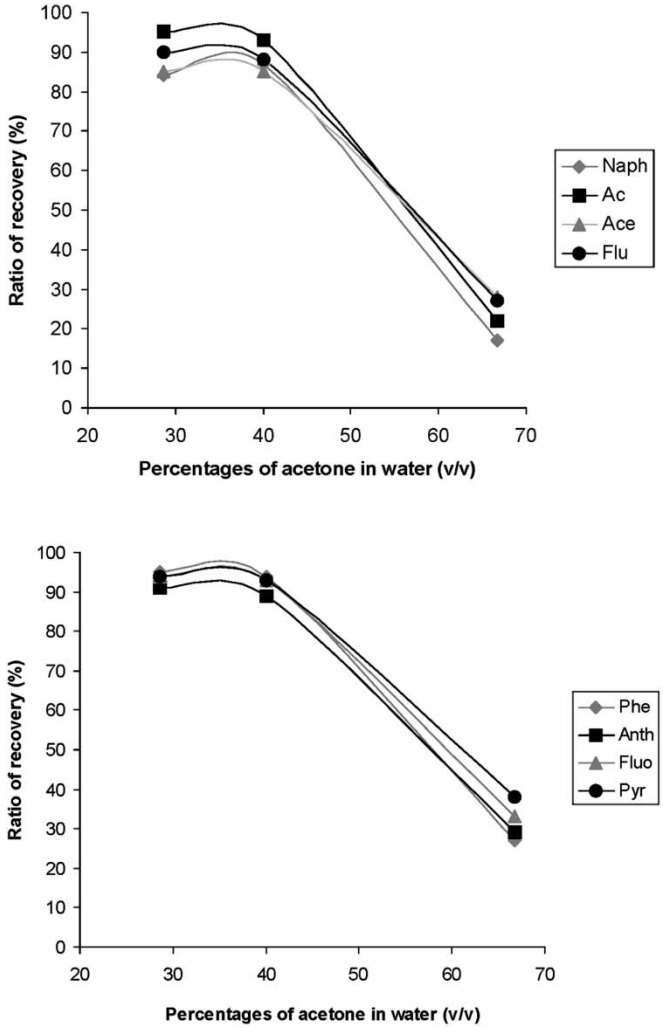

Figure 2. Effect of the percentage of acetone on the recovery of Naph, Ac, Ace, Flu, Phe, Anth, Fluo, of Pyr. These graphs were obtained by addition of successively 5, 15, and 25 mL of water with 10 mL of unpurified organic extract.

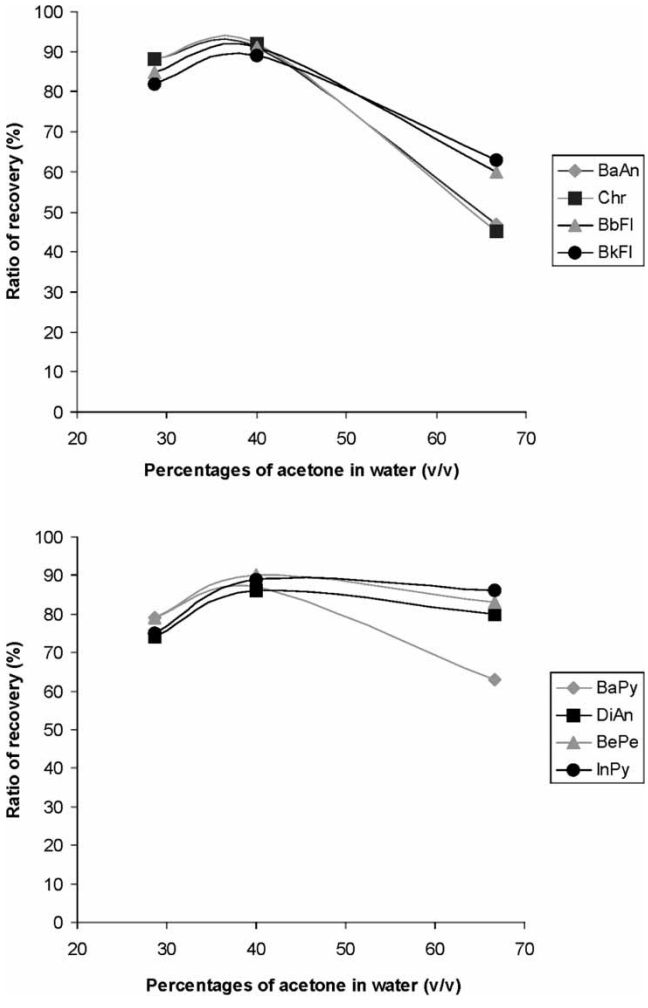

Figure 3. Effect of the percentage of acetone on the recovery of. These graphs were obtained by addition of successively 5, 15, and 25 mL of water with 10 mL of unpurified organic extract.

acetone for the whole PAHs, except for very heavy molecules, which are strongly fixed on the C_{18} phase: InPy, BePe, and DiAn.

For these three compounds, and also BaPy, a clear diminution of recoveries is recorded due to a weak solubility in the mobile phase when water concentration reaches about 60% (Figure 3).

The first step of the reversed-phase SPE is the clean up of the mini-colums with a water miscible solvent (5 mL of acetone) to activate the C_{18} chains. Then, the cartridges are pre-conditioned and equilibrated with 5 mL of an

acetone-water mixture (40%–60%, v/v) containing the same proportions of acetone-water as the extract.

Often, in publications, flow rates of liquid sample on C_{18} cartridges range from 2 to 30 mL/min.[18]

With high flow rates, the preparation times of samples may be shorter. However, when the flow rates are too important, equilibration processes can't be correctly obtained in the cartridge. This phenomenon induces a less efficient retention on the C_{18} sorbent. This is the reason an extraction manifold is used (Varian, Harbor City, CA, USA, Art: 12 234102), which allows a percolation under vacuum and an adjustment of the liquid phase flow-rate. A flow rate not exceeding 2 mL/min was chosen in order to get the best extraction recoveries. After percolation of the extract the cartridges are air dried for 15 min to eliminate residual water in the sorbent, which could disturb the correct desorption of the 16 PAHs.

Elution of PAHs from the SPE Cartridge

Several sorts of solvents may be used to recover PAHs contained in the C_{18} cartridge. In a previous work, 3 mL of ethanol and 5 mL of diethyl ether were utilized for this purpose.[2] Nevertheless, mean recoveries obtained in these conditions (75%) may be improved. The recoveries are substantially improved by using 3 × 1 mL of acetone and by impregnation of the stationary phase with the solvent for 10 min between each elution.

In Figure 4 are reported the ratio of recovery of the four PAHs, which are the most retained by the C_{18} sorbent, BaPy, DiAn, BePe, and InPy, versus the number of 1 mL fractions of eluted acetone.

It may be seen that 2 fractions of 1 mL acetone represent the minimal volume for a quantitative recuperation of all the PAHs, but 3 fractions of

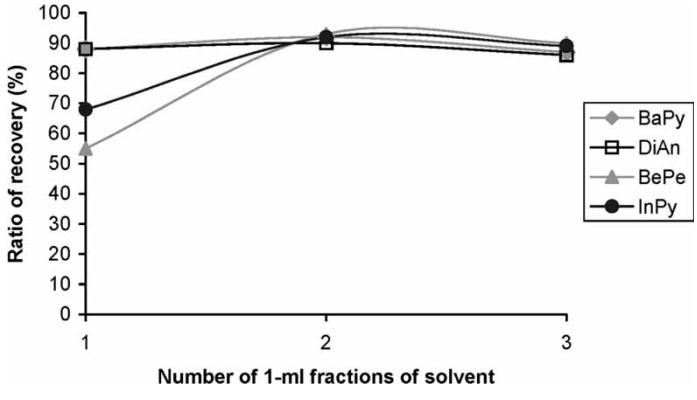

Figure 4. Attainable recovery of BaPy, DiAn, BePe, and InPy with different volumes of acetone used as eluting solvent.

2 mL are better. However, 2 mL methanol is needed to complete the elution. Furthermore, methanol in the final extract prevents a destabilization of the HPLC column when a sample is injected.

Applications

Marine Sediments

Two samples of marine sediments were analyzed with the described method. The first sample (C) is relatively uncontaminated and is furnished by the General Association of Environmental Analytical Laboratories: A.G.LA.E[26] "l'Association Générale des Laboratoires d'Analyse de l'Environnement". The second one (D) is collected from a Mediterranean polluted harbor and is supplied by the Interprofessional Office of Analytical Studies: B. I. P. E. A[27] "Bureau Interprofessionnel d'Etudes Analytiques". These two samples contain known PAHs concentrations and constitute a material of reference for this study. The results obtained with our HPLC method have been compared with the theoretical values in Table 4. For sediment C, mean deviation between the observed levels and reference values is 8.7%. Sediment D presents a mean deviation a little more important (19.2%). A more efficient grinding (under 80 μm) achieved by A. G. L. A. E explains for a great part this difference. Sediment D is much more heterogeneous. It also contains a lot of pollutants, which diminish the accuracy of the HPLC analysis. However, for both samples, the results are always comparable with those obtained by other laboratories.

River Sediment

The river sediment provided by A. G. L. A. E. (sediment E) shows weak deviations with the theoretical values (Table 5). The mean recorded deviation is 7.5%. These very good results are explained by the fine granulometry of this material, associated with low concentrations of pollutants other than PAHs. For the sediments C and D, the BePe concentrations are the most distant, and slightly higher in comparison with the referential values. This fact can be explained by a very good recovery for this compound with the C_{18} mini-column cartridges (90%). Thus, the methodology described here authorizes the recovery of particularly heavy PAHs, which are often difficult to succeed with more conventional methods.

CONCLUSIONS

The 16 PAHs of US Environmental Protection Agency present in marine, brackish, and river sediments may be easily extracted with ultrasonic waves

Table 4. Results found with two marine sediments used as references

	Poorly polluted harbor (sediment C)		Highly polluted harbor (sediment D)	
PAHs	Reference values (μg/kg-dry sediment)	HPLC/fluorescence UV[a] (μg/kg-dry sediment)	Reference values (μg/kg-dry sediment)	HPLC/fluorescence UV[a] (μg/kg-dry sediment)
Anthracene	64	64	420	378
Fluoranthene	320	298	1866	2197
Benzo[a]anthracene	150	138	968	1301
Benzo[b]fluoranthene	158	149	1370	1439
Benzo[k]fluoranthene	101	91	614	740
Benzo[a]pyrene	140	135	1205	1478
Dibenzo[a,h]anthracene	53	63	256	247
Benzo[ghi]perylene	118	136	926	1408
Indeno[1,2,3-cd]pyrene	109	99	911	974

[a]Mean of 10 replicate analyses.

Table 5. Results found with a river sediment used as reference

PAHs	Highly polluted river (sediment E)	
	Reference values μg/kg-dry sediment	HPLC/fluorescence UV[a] μg/kg-dry sediment
Anthracene	581	541
Fluoranthene	3050	3070
Benzo[a]anthracene	1508	1345
Benzo[b]fluoranthene	1700	1635
Benzo[k]fluoranthene	1174	1145
Benzo[a]pyrene	1594	1725
Dibenzo[a,h]anthracene	700	736
Benzo[ghi]perylene	1370	1599
Indeno[1,2,3-cd]pyrene	1409	1274

[a]Mean of 4 replicate analyses.

using acetone as solvent then purified on Bond Elut Jr C_{18} mini-column. The method is not very expensive, for it needs weak quantities of solvents. The chemical reagents are not very harmful and the ratio of recoveries of the studied compounds is high. HPLC analysis by acetonitrile water gradient elution allows a rapid separation of the different molecules. This technique is highly selective, sensitive, and accurate and can be used for a large range of PAHs concentrations. Finally, the mentioned technique could be used for other environmental samples for example seaweeds, fishes, and mollusks with very few modifications.

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