Statistical tools for the optimization of a highly reproducible method for the analysis of polycyclic aromatic hydrocarbons in sludge samples

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Statistical tools for the optimization of a highly reproducible method for the analysis of Polycyclic Aromatic Hydrocarbons in sludge samples

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The aim of this study is to develop and optimize an analytical method of 14 priority PAHs based on Accelerated Solvent Extraction (ASE) coupled to RP-HPLC / fluorescence detection. Statistical tools were used to demonstrate the influence of the parameters during the optimization steps. The final parameters were selected to provide analytical errors statistically as low as possible. First, couples of excitation/emission detection wavelengths were tested and some were finally selected to provide errors lower than 2 %. It was then demonstrated that PAH extraction efficiencies are not statistically influenced by the ASE parameters. It was also found that the ASE extraction from sludge samples provide statistically similar results to those obtained with traditional Soxhlet extraction, but with lower reproducibility error. After optimization, the accuracy of the method was validated with a certified sludge. In conclusion, an optimized analytical procedure has been proposed to monitor PAHs during lab-scale experiments requiring highly repeatable and accurate results from low sample volume contaminated by PAHs at trace levels.

Keywords: Accelerated Solvent Extraction; RP-HPLC ; Statistical tools; PAHs; Sewage sludge.

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Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are widely distributed in the environment, especially in atmosphere particles, soils, sediments, and sewage sludge. Their widespread distribution is due to numerous anthropogenic and natural sources of production. Mainly, the PAHs are formed by incomplete combustion of organic solids, petroleum, coke or fossil fuels (1-2). More than 74 PAHs have been identified but only 16 are currently monitored by the US Environmental Protection Agency (EPA) and the Environmental Commission of the European Community (3). Most of these 16 priority PAHs are suspected to present toxic, carcinogenic, and/or mutagenic properties at low concentrations. Since the PAHs are highly hydrophobic, they are readily adsorbed onto the suspended particles of primary and secondary sludge in wastewater treatment plants (WWTPs) (4). Such contaminated sludge cannot therefore be recycled by spreading on agricultural soils because of the potential toxic effects and the high persistence of PAHs in the environment. Consequently, the fate of PAHs during sludge treatment has become over the last ten years a significant subject of study for the WWTP managers. However, the lack of a standardized procedure for PAH analysis in sewage sludge is highly prejudicial for inter-laboratory studies.

Several PAH analytical methods have already been described in the literature by registered laboratories and governmental agencies (5-10). Since 1986, the US Environmental Protection Agency (EPA) has proposed standard methodologies for the extraction and the analysis of PAHs in sewage sludge (6). However, the proposed methods require high sludge quantities, from 10 to 100 g dry weight, and the validation of a method requiring lower amounts of samples (down to 1 g dry weight) is desirable. In addition, practical reproducibility errors of these methods are high - from 21 % to 44 % - and the PAH concentrations provided by registered laboratories may vary up to 300 % (11). The high variability of the results is likely due to the large

variety of PAH extraction/analysis procedures involved (7). In this study, two steps are considered for the optimization of the PAH analytical method: the first is the PAH extraction from dried sample of sludge, and the second is the PAH quantification from the extracts.

Many PAH extraction methods have already been described in the literature: Soxhlet, methanolic saponification, ultrasonication, microwaves and, more recently, Accelerated Solvent Extraction (ASE) and Supercritical Fluid Extraction (SFE). All these methods provide similar extraction yields but not same analytical errors (9-10; 12). In the present work, two of these methods are studied: (i) the first is the Soxhlet extraction, which is considered as the reference but requires long extraction time (6-24 hours) and high solvent volumes (150-250 ml). (ii) The second is the Accelerated Solvent Extraction (ASE), an automated method providing high reproducibility and enhanced security. In addition, the ASE only needs short time of extraction (20 minutes) and low solvent amount (20 ml). Despite a relatively important investment, the extraction of PAHs by ASE is especially recommended for intensive use in lab-scale assays (13-14). Among the parameters, only few experimental values have been reported for the temperature, static time, pressure, sample amount and solvent mixture composition (Table 1). Recent results showed that the ASE extraction yields are closely similar to the other PAH extraction method efficiencies in case of contaminated soils and sediments (9-10; 12-15). Such results need to be statistically demonstrated in case of the sludge because of the high specific interactions between the organic matrix, the solvent, and the PAHs.

*** [TABLE 1]**

After extraction, PAH concentrations of the extracts are determined either by Gas Chromatography coupled with Flame Ionization Detector, or Mass Spectrometry (GC/FID or GC/MS), or by Reverse Phase High-Performance Liquid Chromatography

coupled with Photodiode array or Fluorescence Detector (RP-HPLC/PDA or RP-HPLC/FLU). Since 1970, the latter method has provided good results considering the high specific detection of the PAHs within complex samples. Indeed, the excitation and emission wavelengths are highly specific for each molecular formula (17). However, many different PAH detection wavelengths have been reported in the literature (Table 2). The main disadvantage of the RP-HPLC/FLU method consists in the resolution of the peaks and highest PAH separation efficiencies are generally found with a C_{18} column presenting a selective polymeric phase (Bakerbond C18 Widepore, Supelcosil LC-PAH) (17). Two other parameters also influence the PAH separation by RP-HPLC: the elution temperature and the length of the solvent gradient. The separation efficiency increases significantly with the lowest temperature and the longest solvent gradient (17; 19). Several experimental values are reported in Table 2. Since numerous analytical conditions are available in literature, the detection wavelengths, the elution temperature, and the length of the gradient need to be tested to optimize the accuracy and the reproducibility of the analytical method.

*** [TABLE 2]**

The aim of this study is the optimization and the validation of an analytical method of 14 of the 16 priority PAHs, as described below (the acenaphtene and the acenaphthylene compounds were removed because of their low fluorescent properties). This method was developed in order to monitor PAHs in lab-scale experiments using low-contaminated sludge samples. Thus, the purpose of this study is to obtain the highest reproducibility of the analysis in spite of low PAH concentrations and low sample volumes (200-300 ml). Statistical tools were used to demonstrate the effect of each parameter. The optimization of the analytical method was carried out in three steps: (i) First, the PAHs analysis by RP-HPLC and the fluorescence detection were optimized in order to obtain repeatability and

reproducibility errors lower than 2 %. (ii) Then, the influence of the ASE extraction parameters was evaluated. The PAH extraction efficiencies by ASE were compared to those obtained by the classical Soxhlet method. (iii) Finally, the optimized method was validated by the determination of the PAH recoveries in spiked sludge and in certified material.

The 14 studied PAHs are as follow : Na, naphthalene; Fl, fluorene; Ph, phenanthrene; An, anthracene; Flu, fluoranthene; Py, pyrene; BaA, benzo(a)anthracene; Ch, chrysene; BbF, benzo(b)fluoranthene; BkF, benzo(k)fluoranthene; BaP, benzo(a)pyrene; DB, dibenzo(ah)anthracene; BP, benzo(ghi)perylene; Ind, indeno(123cd)pyrene.

Experiment

Chemicals. All chemicals were of analytical grade. The solvents were provided by J.T.Baker-Mallinkrodt (Noisy le Sec, France) with purity over 98% for acetone, acetonitrile, hexane, methanol and toluene. The borosilicate glassware and the experimental apparatus were previously rinsed with a mixture of acetonitrile:acetone (50:50).

The 10 mg/l standard solution of the 16 priority PAHs was prepared by Dr-Ehrenstorfer-Schäfers laboratory (Augsburg, Germany, PAH Mix-9, purity over 98%). 10 to 1000-fold dilutions of the standard solution were prepared in acetonitrile, and the diluted solutions were stored at - 20°C.

The certified sludge (CRM n°088 - PAH in dried sewage sludge) was provided by Promochem (Molsheim, France) with the following certified PAH concentrations ($mg.kg^{-1}$ of dry weight): Pyrene, 2.16 ± 0.09; Benzo(a)Anthracene, 0.93 ± 0.09;

Benzo(a)pyrene, 0.94 ± 0.09; Benzo(b)Fluoranthene, 1.17 ± 0.08; Benzo(k)Fluoranthene, 0.57 ± 0.05; Indeno(123cd)Pyrene, 0.81 ± 0.06.

Sludge Sample Preparation. A long-term PAH-contaminated sludge was used as a stock mixture during the optimization steps. The sludge corresponded to a mixture of primary and secondary sludge (50:50, v:v). Prior to PAH extraction, 300 ml of the sludge mixture was centrifuged (20 000 g, 25 min.). The supernatant was stored at -20°C for further Solid-Phase Extraction. The pellet was grounded with 4 mm glass beads, dried in a ventilated oven (60 hours - 40°C), sieved on a 2 mm mesh size and stored at -20°C for further ASE or Soxhlet extraction.

Liquid Chromatography procedure. The analytical system was composed of a sampler injector (Waters 717plus Autosampler), a solvent degasser (Waters Inline Degasser), a peristaltic pump system (Waters600 Controller) and a programmable fluorimetric detector (JASCO FP-1520). Excitation and emission wavelengths were changed according to the elution time of each PAH. The C_{18} column was provided by Bakerbond (PAH 16-Plus Bakerbond^{TM} : 5 micron, 3x250 mm, 120 Å). The column temperature was maintained at 25°C by immersion in a regulated water bath. The elution sequence was as follows (flow rate of 0.3 ml/min.) : 5 min. of isocratic elution (acetonitrile:water, 60:40), 30 min. of linear gradient from 60 to 100 % acetonitrile, 30 min. of isocratic elution (acetonitrile-100%) and 30 min. of isocratic rinsing of the column by a mixture of acetonitrile:water (60:40).

Extraction procedures. **Solid Phase Extraction (SPE).** The PAHs were extracted from the liquid phase (supernatant) by Solid-Phase Extraction (SPE). The affinity column was provided by Supelco^{TM} (Supelclean ENVI-18). The extraction was performed according to the Supelco^{TM} procedure. The sample was passed three times

through the column. The PAHs were eluted with 6ml of a mixture of toluene:methanol (10:1). The sample was then evaporated under nitrogen flow to dryness and the residue was dissolved in 2 ml of acetonitrile. **Accelerated Solvent Extraction (ASE).** The extraction from dried sludge samples was performed with an ASE-200 system (DIONEX^{TM}). The extraction solvent consisted of a mixture of hexane:acetone (50:50). The ASE cells were filled as follow (from bottom to the top) : a filter of glass fiber (Diameter 19 mm, Whatmann^{TM}), 1 g of Alumina (Sigma^{TM}), 1 g or 0,5 g of dried sludge sample and 1.5 g of Hydromatrix (Varian^{TM}). After extraction, the sample was evaporated under nitrogen flow to dryness. The residue was then dissolved in 5ml of acetonitrile, and was immediately analyzed (no storage). **Soxhlet extraction.** The Soxhlet extraction procedure was based on EPA method 8310 (6). The method was previously optimized and validated internally on certified material. The Soxhlet was filled with 0.5 g of sludge sample and 120 ml of hexane:acetone (50:50). The PAH extraction was performed at 50°C during 16 hours. The extract was first evaporated under vacuum in a Rotavapor (Buchi^{TM}) at 40°C, and then evaporated to dryness under gentle nitrogen flow. The residue was dissolved in 5 ml of acetonitrile determined by weighing, and immediately analyzed (no storage).

Experimental plans and statistical analysis. Three independent half-experimental plans were performed in order to optimize the ASE extraction parameters, and to reduce the number of extractions by grouping by 2 variables (26). If one group of variable statistically influenced the extraction efficiency, each variable was then tested separately. In the first half-plan experiment, four parameters possessing a low or a high level were studied : the temperature, 100°C or 120°C ; the number of cycle, 2 or 3 ; the static time of extraction, 5 or 8 min. ; the composition of the solvent mixture (hexane:acetone), 50:50 or 25:75. The second half-plan was performed in order to reduce the sludge amount (lab-scale requirement): 1g or 0.5g. The third half-plan was

performed on two parameters: the solvent volume, 60 or 90 % of the cell and the gas purging time, 60 or 100 sec.

These experimental plans were carried out by assuming that all parameters were independent between the plans. The results were compared with a statistical test of multiple variances (ANOVA). Each extraction assay was repeated three times and the averages were compared by one factor-ANOVA test (26). The efficiencies of the Soxhlet and ASE extractions were compared by a t-test under a Student law at 5 %. The hypothesis of normality and independence between the assays was formulated to apply the ANOVA and the t statistical tests. The acceptance of a null hypothesis at 5% indicated that the tested averages were statistically similar at 95 % (no significant difference between the two statistical populations).

Results and Discussion

Optimization of the RP-HPLC - Fluorescence detection. Amongst the parameters influencing the resolution of the detected peaks, the temperature of the RP-HPLC column significantly influence the PAH elution time and the peak separation efficiency (data not shown). According to previous works, the elution temperature was fixed at its lowest level for the best peak resolution (17; 19). Since a water bath was used as a regulator of the column temperature, the temperature was regulated at 25°C because of ambient air limitation. The other RP-HPLC elution parameters were optimized according to the values reported in Table1. It was observed that an increase of the elution gradient from 5 min. to 30 min. contribute to enhance the peak resolution. Similar results were observed with a decrease of the solvent flow rate from 0.5 $ml.min^{-1}$ to 0.3 $ml.min^{-1}$ (data not shown). A longer elution gradient and a slower flow rate resulted in an extension of the analysis time from 35 min. to 90 min. The

Figure 1 reports the chromatograms obtained under these conditions. The PAH peaks are readily identified either in the standard solution or in the sludge extract. The chromatograms exhibit only few interfering peaks due to the high specificity of the fluorescence detection, in contrast with mass-spectrometry detection chromatograms (24). The identification of the peaks was additionally confirmed with a photodiode array detector (PDA) by comparison of the experimental peak spectrum and the spectra reported in the literature (28) (data not shown).

*** [FIGURE 1]**

In contrast with methods based on a high response of the detection system to reach the lowest detection limits (12, 21-22, 25), well-separated peaks were here necessary to reduce the errors of peak integration. Indeed, the optimization of the fluorescence detection was based on the reduction of the repeatability errors, which corresponded to the relative standard deviation of three analysis of the same sample and was highly dependent on the peak sharpness. Since the test of all excitation/emission wavelengths found in the literature would have been highly complex and unrealizable, only the most common wavelengths were tested and were definitely selected when the repeatability error reached a value lower than 2 % (see Table 3). Thus, a pair of excitation/emission wavelengths was found to provide highly repeatable results for the analysis of each PAH either in the standard solution (>25 repetitions) or in the sludge sample extracts (4 repetitions). Moreover, it was observed that the highest areas of the peaks did not systematically correspond to the most repeatable results. The analytical errors varied more according to the signal stability than of the intensity of the response. The selected excitation/emission wavelengths did not correspond to the lowest detection limit, as normally defined (12, 21-22, 25).

*** [TABLE 3]**

In the same way, the minimum and maximum PAH concentrations of the calibration curves were determined for repeatability errors exceeding 2 %. High errors were encountered for the lowest concentrations or in the case of saturation of the fluorescence detector. The upper and lower limits of the calibration curves were determined as follow : 250 – 10000 $\mu g.l^{-1}$ (Naphthalene); 10 – 1000 $\mu g.l^{-1}$ (Fluorene, Anthracene, Benzo(a)Anthracene, Benzo(k)fluoranthene, Benzo(a)Pyrene, Benzo(ghi)perylene, Indeno(123cd)pyrene), 25 – 1000 $\mu g.l^{-1}$ (Phenanthrene, Fluoranthene, Chrysene, Benzo(b)fluoranthene, Dibenzo(ah)anthracene). The minimum values were ten times higher than the detection limits (1 - 1.5 $\mu g.l^{-1}$), but provided higher repeatable results in comparison with results found in the literature (8, 10, 12, 21-22).

In addition, the calibration error corresponding to the comparison of a standard solution at 100 $\mu g.l^{-1}$ with the theoretical calibration curve was also tested (Table 3). It was found that the calibration error was always lower than 2 % and the calibration curve was valid for more than 100 sludge sample analysis.

In conclusion, this study demonstrated for the first time that the analysis of PAHs from sludge extracts by RP-HPLC and fluorimetric detection provide highly accurate and repeatable values and is reliable during time. Therefore, only a limited number of injections (2-3) is needed to estimate the PAH concentration from sludge extracts.

Optimization of the PAH extraction from sewage sludge samples. PAH extraction from the liquid phase (Solid Phase Extraction). PAH extraction from the liquid phase was performed by Solid-Phase Extraction. Using a 100 $\mu g.l^{-1}$ standard solution, the PAH recoveries in spiked aqueous samples were mostly satisfactory with about 90 % to 100 % of PAH recovery except for the fluorene (75 %), chrysene (33 %), dibenzo(ah)anthracene (32 %), benzo(ghi)perylene (28 %), benzo(a)pyrene (65 %) and indeno(123cd)Pyrene (63 %). Moreover, the PAH extraction by SPE presented

high repeatability errors (>20 %). In addition, the PAH concentrations in the liquid phase remain always negligible whatever the sludge sample. The soluble fraction of PAHs represents less than 1 percent of the total amount found in contaminated sludge. The low PAH levels in the aqueous phase result from their low solubility in water and their very strong adsorption onto the sludge organic matrix, as previously reported (4). Consequently, the PAH concentration from the liquid phase can always be considered as negligible. **PAH extraction from the solid phase (Accelerated Solvent Extraction).** The PAH extraction from the solid phase was performed by Accelerated Solvent Extraction (ASE). Some parameters were suspected to influence the PAH extraction efficiencies, such as the cell pressure, the temperature, the number of cycles, the sample amount, the purging time, the flush rate of the extraction cell and the solvent mixture composition (hexane:acetone). Thus, three independent experimental plans were implemented in order to optimize the PAH extraction yields. The extraction pressure was first fixed at 100 bars. This value corresponds to the upper limit for the PAH extraction from sludge sample, according to DIONEX^{TM} recommendations.

In the first experimental plan, four ASE parameters were tested. A low and a high value were defined for each parameter as, respectively, 100°C and 120°C for the temperature, 2 and 3 for the number of cycle of extraction, 5 min. and 8 min. for the static extraction time, 25:75 and 50:50 of hexane:acetone for the solvent mixture composition. Each low and high level was tested and the results were statistically compared by a multiple analysis of variance (ANOVA) (see Table 4). In the first plan, no significant difference was observed between the assays. Consequently, these ASE parameters have no influence on the PAH extraction efficiencies. Similar results were previously observed in contaminated soils or sediments (9-10 ; 12 ; 14-15). Therefore, the ASE extraction yields seem not to be influenced by the sample matrix because of

the strong operating conditions (high temperature and pressure), and these results should be valid for any kind of sample. Moreover, this method is suitable for the analysis of the PAHs except the naphthalene, which was not recovered after the sample evaporation because of highly volatile properties (Table 4).

*** [TABLE 4]**

The next two experimental plans were performed in order to test the influence of secondary parameters. The results are reported in Table 4. The differences of absolute values measured between the experimental plans are explained by the actual low homogeneity of the fresh sludge stock mixture. However, statistical conclusions are independent and remain valid for each half-experimental plan. The main objectives of the second and third plans were to reduce the sample amount, the extraction time, and the solvent consumption. Thus, in the second experimental half-plan, no significant difference was observed between 0.5 g and 1 g of sludge sample, except for the fluorene. The volume of sludge sample can therefore be reduced to a minimal level of 0.5g. Since two repetitions (2×0.5g) of the PAH extraction require approximately 300 ml of fresh sludge, this amount of sludge is compatible with lab-scale experiments. The third experimental half-plan was performed to reduce the solvent consumption by decreasing the flush rate of the extraction cell (90% to 60%). The extraction time was also reduced by decreasing the final purge time from 100 sec. to 60 sec. As same as for the other factors, the PAH extraction efficiencies were not influenced by these parameters (Table 4).

In conclusion, the ASE parameters can be chosen with a high degree of freedom according to the experiment requirements, such as a low consumption of solvent, a reduced amount of sample, or a short time of analysis. In this study, the ASE parameters were selected to monitor further the PAHs during lab-scale experiments and the final parameters are as follow : temperature of 120°C, 2 cycles of extraction, 5

min. of static time, hexane:acetone (50:50), flush rate of 60%, 60 sec. of purging time and 0.5 g of sample in the extraction cell. The time of extraction does not exceed 20 min.

Comparison of the optimized PAH extraction method (ASE) and the Soxhlet reference method. Considered as the reference method, the PAH extraction by Soxhlet was compared to the previously optimized ASE method. The statistical results are reported in Table 5. It appeared that the PAH recoveries by ASE are from 94 % to 115 % compared to the Soxhlet concentrations. According to the statistical t-test, no difference was observed between the two extraction methods. This result confirms the accuracy of the optimized ASE method. Moreover, similar results between ASE and Soxhlet extractions were already reported with contaminated soils and sediments (9; 14-16). The ASE and soxhlet extraction methods are therefore highly comparable whatever the sample matrix.

The reproducibility errors of the ASE and the Soxhlet methods were also calculated by three analysis of the same sludge sample. The Soxhlet method presented the highest reproducibility errors from 5 % to 9 % with an average of 7.5 %. In comparison, the ASE method provided reproducibility errors lower than 2 % for the same sludge sample. This result was confirmed for more than 80 extractions of sludge sample (Table 6). Therefore, the ASE extraction is statistically more reproducible than the Soxhlet extraction.

*** [TABLE 5]**

*** [TABLE 6]**

Validation of the analytical method with a certified contaminated sewage sludge. The PAH losses during the extraction step were firstly determined to confirm the accuracy of the values. A standard solution of the 14 studied PAHs (from Naphthalene

to Indeno(123cd)Pyrene) was added in a fresh sludge sample. The spiked sludge was then analyzed by the optimized method and the spiked values were determined and compared to the non-spiked sludge. The total recoveries are presented in the Table 7. It appeared that all added PAHs were recovered and only the naphthalene was totally lost during the evaporation step. The highly volatile Fluorene was also partially lost during the PAH extraction with about 10% of losses. Nevertheless, the proposed method presents no significant losses for the other PAHs.

*** [TABLE 7]**

The accuracy of the analytical method was finally validated by the determination of the PAH concentrations in certified sludge material (CRM088 - Bureau of Reference EUR n°15039) (8). The certified concentrations resulted from the sludge analysis by 11 international laboratories using major analytical techniques (GCFID-GCMS-LCFLU). The measured values were in the range of the referenced concentrations (Table 8). The method described in the present paper seems to extract more the lowest PAHs than the highest (+ 20.5 % versus –11.2 %), but measured concentrations are included in the standard deviation of the certified values.

*** [TABLE 8]**

Conclusion

The Polycyclic Aromatic Hydrocarbons present high hydrophobic properties and their monitoring in long-term contaminated environment is particularly complex because of their strong interactions with the organic compounds. In this study, an analytical method was optimized in order to monitor 14 priority PAHs during lab-scale experiments. Low PAH concentrations, low sample volume, and a high reproducibility of the analysis were the main analytical constraints. In a first step, it was shown that the separation efficiency of the PAH peaks was strongly dependent of the elution

temperature, as low as possible, and of the length of the solvent mixture gradient, as long as possible. After optimization, the fluorescence PAH detection and quantification provided high accurate and repeatable results (errors lower than 2 %).In a second step, the PAH extraction by ASE from sewage sludge was optimized. None of the ASE parameters has a significant effect on the PAH extraction efficiencies. Thus, the operating conditions can be fixed according to the own practical constraints, such as a low consumption of solvent and a short time of extraction. Although the PAH extraction by ASE presented similar results to the reference method of Soxhlet, the ASE method presents statistically the highest reproducibility. In addition, the accuracy of the optimized method was validated on certified reference sludge with results statistically similar to the certified concentrations. However, the naphthalene was not recovered after the evaporation step and cannot be analyzed by this method. In conclusion, the optimized method was successful according to the high accuracy, the high reproducibility, and the high reliability during time and is consequently suitable for an intensive use during lab-scale experiments.

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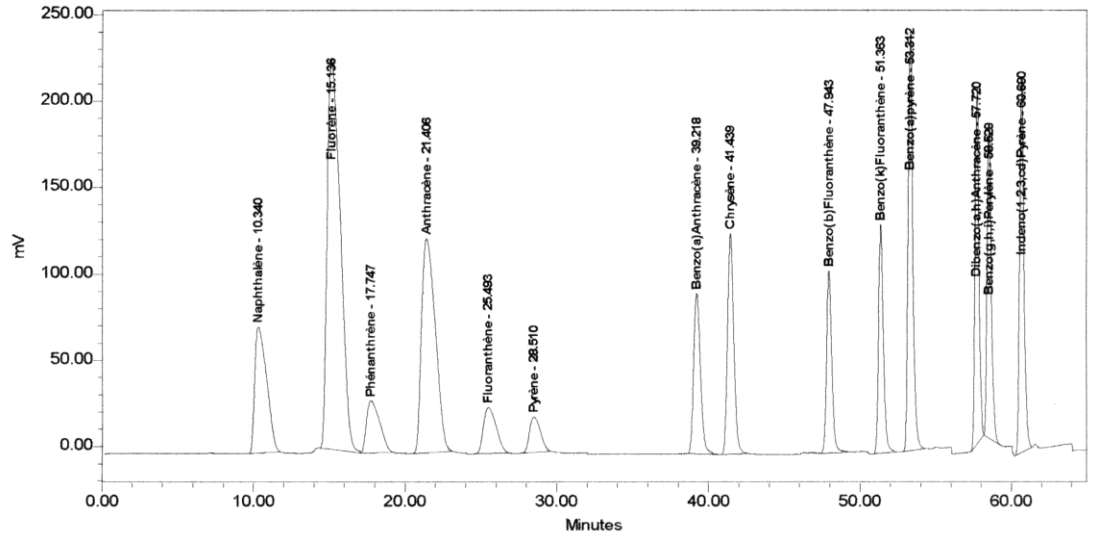
Table 1: Summary of the Accelerated Solvent Extraction parameters found in the literature *(DCM = dichloromethane; "-" = not communicated).*

Matrix	Sample Amount	Solvent Mixture	T° (°C)	Pressure (bars)	Static Time	References
Soil, Sludge	20 g	hexane:acetone (50 :50)	100	100 to 140	5 min.	[6]
Sludge	1 g	hexane:acetone (50 :50)	100	100	8 min.	[8]
Sediment	0.3 g	hexane:acetone (50 :50)	100	140	5 min.	[9]
Soil	7 g	DCM:acetone (50 :50)	100	140	5 min.	[10]
Soil, Sludge	20 g	hexane:acetone:toluene (10 : 5 : 1)	100	*138*	10 min.	[12]
Soil	-	hexane:acetone (50 :50)	100	140	5 min.	[14]
Sediment, soil, sludge	-	DCM:acetone (50 :50)	100	140	5 min	[15]
Soil	7 g	hexane:acetone or DCM:acetone (50 :50)	70 to 200	90 to 140	5 to 16 min.	[16]

Table 2 : Summary of the elution parameters and the fluorescence wavelengths found in the literature for the PAH analysis by RP-HPLC - Fluorescence detection *("-" : not communicated). The gradient of elution was performed from acetonitrile/water (60% - 40 % v/v) to acetonitrile (100%).*

References		[6]	[7]	[12]	[17]	[18]	[19]	[20]	[21]	[22]	[23]	[24]	[25]
Gradient time	(min.)	25	-	30	30	5	25	5	25	-	25	5	16.5
Flow rate	(ml/min)	0.5	-	1	2	-	0.5	2	1	1	-	0.5	1
Excitation /Emission Wavelengths	*Na*	280/ 389	-	220/ 330	280/ 340	-	280/ 330	-	280/ 340	-	280/ 340	280/ 340	-
	Fl		-	225/ 315	249/ 362	320/ 404		-		-			-
	Ph		-	244/ 370	250/ 400	-	250/ 370	-	250/ 376	259/ 370		295 /380	265/ 350
	An		-		285/ 450	-	250/ 405	260/ 430		252/ 405		280/ 430	
	Flu		268/ 462	237/ 460	333/ 390	-	280/ 450		286/ 460	284/ 460	280/ 410		265/ 430
	Py		-	237/ 385	285/ 385	320/ 404	270/ 390			336/ 398			
	BaA		-	277/ 376	260/ 360	257/ 407	265/ 380	285/ 385	-	-			-
	Ch		-		295/ 425	269/ 361			-	368/ 384			-
	BbF		234/ 420	255/ 420	296/ 405	290/ 409	290/ 430		305/ 403	-		285 /460	-
	BkF		298/ 424			284/ 427		305/ 405		-			-
	BaP		268/ 398							378/ 406			-
	DB		-	300/ 415		-	290/ 410	-		-			-
	BP		234/ 420			-		300/ 455	305/ 425	-			295/ 460
	Ind		302/ 500	250/ 495	300/ 500	303/ 500	300/ 500			-	305/ 500		-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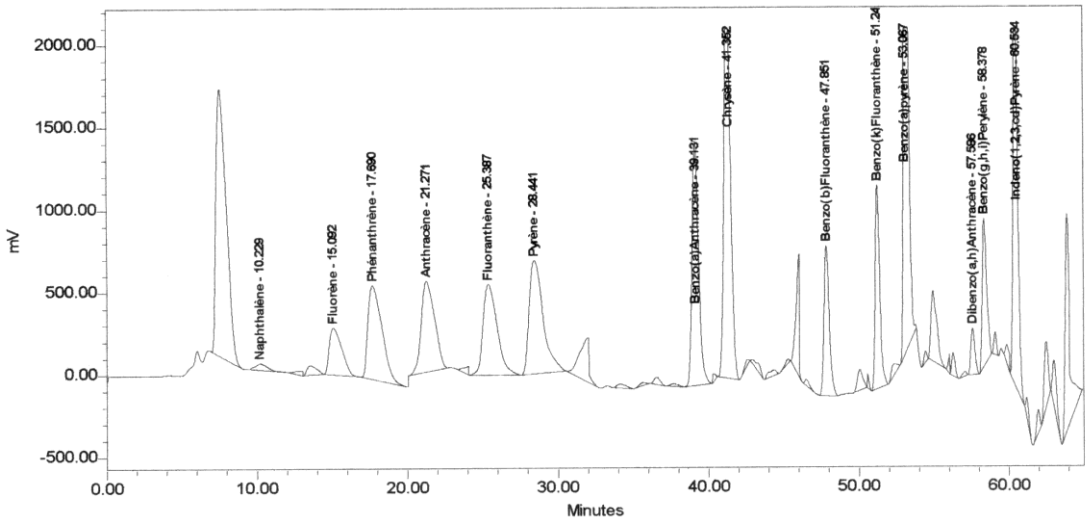
200.00
150.00
100.00
50.00
0.00
Naphthalène - 10.340
Fluorène - 15.136
Phénanthrène - 17.747
Anthracène - 21.406
Fluoranthène - 25.493
Pyrène - 28.510
Benzo(a)Anthracène - 39.218
Chrysène - 41.439
Benzo(b)Fluoranthène - 47.943
Benzo(k)Fluoranthène - 51.383
0.00
10.00
20.00
30.00
40.00
50.00
60.00
Minutes

A.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1500.00
1000.00
500.00
0.00
-500.00
Naphthalène - 10.229
Fluorène - 15.092
Phénanthrène - 17.680
Anthracène - 21.271
Fluoranthène - 25.387
Pyrène - 28.441
Benzo(b)Fluoranthène - 47.951
0.00
10.00
20.00
30.00
40.00
50.00
60.00
Minutes

B.

Table 3 : Summary of the repeatability and reproducibility errors according to the excitation/emission wavelengths (PAH fluorescence detection). *The study was performed with injections (20 µl) of a standard solution (100 µg/l of each PAH) according to the optimized HPLC conditions. " * " = finally selected excitation/emission wavelengths (25 repetitions); ND=not detected.*

	Excitation/ Emission Wavelengths	Repeatability error (%) (maximum)	Calibration error (%)	Repeatibility error on sludge sample (%)
			Average (10 repetitions)	Average (4 repetitions)
Na	272 / 334	4.7	-	-
	280 / 330 *	**0.7 (1.4)**	1.3	0.4
Fl	**266 / 312 ***	**0.4 (1.5)**	1.5	0.4
Ph	295 / 380	4.1	-	-
	250 / 370 *	**0.7 (1.6)**	1.2	0.4
An	**250 / 400 ***	**0.4 (1.3)**	1.1	0.8
Flu	260 / 430	N.D.	-	-
	365 / 460	N.D.	-	-
	280 / 430 *	**0.6 (1.5)**	0.8	0.6
Py	236 / 394	4.7	-	-
	320 / 404	2.8	-	-
	270 / 394	N.D.	-	-
	280 / 430	N.D.	-	-
	260 / 410 *	**1.1 (1.9)**	0.95	0.7
BaA	268 / 384	4.4	-	-
	280 / 430 *	**0.5 (1.6)**	0.8	0.5
Ch	**268 / 384 ***	**0.6 (1.3)**	1.1	0.1
BbF	292 / 460	7.5	-	-
	234 / 420 *	**0.4 (1.9)**	0.6	0.2
BkF + BaP	292/460	11.3	-	-
	292/430	5.3	-	-
	300/430	4.2	-	-
	270 / 400 *	**0.6 (1.6)**	0.8	0.8
DB + BP	285/460	2.8	-	-
	300/500	ND	-	-
	285/400	ND	-	-
	300 / 407 *	**0.5 (0.9)**	1	0.8
Ind	285/460	2.3	-	-
	300 / 500 *	**0.8 (2)**	0.6	0.4

Table 4 : Experimental optimization half-plans applied in the case of the ASE PAH extraction. The low and high levels were defined for 4 parameters (*plan 1* : Temperature 100°C-120°C, Static time 5min.– 8 min., Cycles 2 – 3, hexane:acetone 25:75 - 50:50), 1 parameter (*plan 2* : sample amount 0.5 g – 1 g) and 2 parameters (*plan 3*: Purge time 60 sec.-100 sec., Flush rate 60 % - 90 %). Multiple analysis of variance (ANOVA - 1 factor) were performed between the assays and H_0 (none statistical difference) was confirmed at 95 % for F factor lower than 2.665 (1st plan), lower than 9.55 (second plan) and 4.07 (third plan). *below detection limit. **not measured due to temporary detector failure.

	Experimental plan n° 1		***Experimental plan n°2***		***Experimental plan n°3***	
	Average ($\mu g.l^{-1}$)	F Factor (H_0<2.665)	Average ($\mu g.l^{-1}$)	F Factor (H_0<9.55)	Average ($\mu g.l^{-1}$)	F Factor (H_0<4.07)
Naphthalene	*	*	*	*	*	*
Fluorene	166 ±12	1.035	165 ±10	10.77	321 ±12	0.257
Phenanthrene	497 ±45	1.662	504 ±90	4.8	123 ±3	0.229
Anthracene	97 ±7	0.707	70 ±6	2.5	130 ±4	0.158
Fluoranthene	658 ±48	0.526	394 ±33	2.2	643 ±14	0.064
Pyrene	734 ±56	0.702	506 ±53	3.1	883 ±12	0.033
Chrysene	**	**	**	**	**	**
Benzo(a)anthracene	195 ±15	0.696	123 ±9	1.3	203 ±8	0.148
Benzo(b)fluoranthene	301 ±25	0.729	198 ±19	4.0	318 ±9	0.078
Benzo(k)fluoranthene	130 ±10	0.776	80 ±6	2.0	116 ±3	0.047
Benzo(a)pyrene	250 ±20	0.656	160 ±15	2.2	219 ±5	0.046
Dibenzo(ah)anthracene	44 ±3.5	0.756	32 ±4	5.1	55 ±2	0.073
Benzo(ghi)perylene	154 ±14	0.997	117 ±18	9.1	190 ±8	0.145
Indeno(123cd)pyrene	77 ±10	0.242	24 ±6	0.4	44 ±11	0.282

Table 5 : PAH recoveries of the ASE extraction compared to the Soxhlet extraction used as reference method. A t test (Student law) was performed for the statistical comparison. Hypothesis H_0 of none difference was confirmed at 95 % for t values lower than 3.182.

PAH:	*Fl*	*Ph*	*An*	*Flu*	*Py*	*BaA*	*Ch*	*BbF*	*BkF*	*BaP*	*DB*	*BP*	*Ind*
Recovery (%)	98.3	100.4	111.1	94.4	98.1	110.5	105.6	108.2	106.6	108.9	106.4	115.8	97.1
t value (<3.182)	1.280	0.948	0.779	1.507	0.772	0.690	0.905	0.753	0.623	1.724	1.239	1.528	2.304

Table 6 : Errors of reproducibility calculated during PAH monitoring of lab-scale experiments (ASE extraction and RP-HPLC/fluorescence detection). The presented values are the average of more than 80 measurements of the reproducibility errors. The reproducibility error was calculated after three extraction-analysis of the same sludge sample.

PAH:	*Fl*	*Ph*	*An*	*Flu*	*Py*	*BaA*	*Ch*	*BbF*	*BkF*	*BaP*	*DB*	*BP*	*Ind*
Average of errors of reproducibility (%)	**1.83**	**1.75**	**1.53**	**1.45**	**1.68**	**1.32**	**1.72**	**1.47**	**1.24**	**1.31**	**1.86**	**1.65**	**1.61**
Concentration ($mg.kg_{dry\ weight}^{-1}$)	0.49	3.52	0.92	10.74	10.69	3.79	4.47	4.94	2.53	4.23	0.75	2.81	4.10

Table 7 : Measured and expected concentrations of contaminated sludge spiked with 50 µg.l^{-1} of the 14 PAHs standard mixture.

PAH:	*Fl*	*Ph*	*An*	*Flu*	*Py*	*BaA*	*Ch*	*BbF*	*BkF*	*BaP*	*DB*	*BP*	*Ind*
Measured Concentration (µg.l^{-1})	60.4	163	84.7	332	360	171	164	197	139	189	87.8	148	208
Expected Concentration (µg.l^{-1})	66.6	160	82.6	329	355	165	171	196.9	140.1	190.4	83.1	144	208.1
Recovery (%)	**90.8**	**102.1**	**101.3**	**101**	**101.5**	**103.5**	**95.6**	**99.9**	**99.8**	**99.3**	**105.6**	**102**	**99.9**

Table 8 : Measured and certified concentrations of the material CRM088.

	Certified concentrations		**Measured Concentrations**		**Difference**
	Min – **Average** – Max ($mg / kg_{dry\ dry\ weight}$)	rsd (%)	Average ($mg / kg_{dry\ dry\ weight}$)	rsd (%)	
Pyrene	1.76 – **2.16** – 2.70	4.2	**2.604**	4.9	+ 20.5 %
Benzo(a)anthracene	0.65 – **0.93** – 1.14	9.7	**0.931**	4.2	+ 0.1 %
Benzo(b)fluoranthene	0.99 – **1.17** – 1.39	7.7	**1.169**	4.5	- 0.1 %
Benzo(k)fluoranthene	0.41 – **0.57** – 0.71	8.8	**0.52**	4.8	- 8.8 %
Benzo(a)pyrene	0.62 – **0.91** – 1.22	9.9	**0.803**	4.2	- 11.8 %
Indeno(123cd)pyrene	0.57 – **0.81** – 0.98	7.4	**0.719**	4.7	- 11.2 %

Figure 1 : Elution chromatogram of the 16 priority PAHs standard solution (**A**) and the contaminated-sludge extract obtained after Accelerated Solvent Extraction (**B**). *injection 20µl, gradient (30 min.) of acetonitrile:water (60:40 to 100:0) , flow rate 0.3 ml.min, temperature 25°C, fluorescence program : 0 min., 280/330, 13 min., 266/312, 17 min., 250/370, 20 min., 250/400 ; 24 min., 280/430 ; 27.5 min., 260/410 ; 32 min., 280/430 ; 40.2 min., 268/384 ; 46 min, 234/420 ; 50.5 min., 270/400 ; 56 min., 300/407 ; 60 min., 300/500.*