

Methodology for Quantitative Determination of Polycyclic Aromatic Hydrocarbons in Protozoa Cultures

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Abstract. Two simple and sensitive analytical methods based on matrix solid phase dispersion (MSPD) and solid phase extraction (SPE), both followed by high performance liquid chromatography (HPLC) with fluorescence detection (FL) were developed for determining the high molecular weight polycyclic aromatic hydrocarbons (PAH) benzo(a)anthracene, benzo(b)fluoranthene and benzo(a)pyrene, in liquid cultures of the ciliate protozoan *Colpoda cucullus*. The first method employed MSPD technique for extraction of the analytes from a very small amount of microorganisms and insoluble organic matter. The second method used SPE to extract analytes from the liquid medium. Validation parameters indicated good linearity ($r^2 > 0.99$) and precision (inter-day RSDs $< 7\%$) for both methods. Recovery values were $>90\%$ for MSPD and 70% for SPE with limits of detection between 0.02 and $0.03 \mu\text{g/g}$ for MSPD-HPLC and between 0.03 and $0.04 \mu\text{g/L}$ for SPE-HPLC. The developed methodology was successfully used for the quantitative determination of PAH in microorganism cultures for the first time.

Keywords: polycyclic aromatic hydrocarbons, matrix solid phase dispersion, solid phase extraction, high performance liquid chromatography-fluorescence, protozoan, *Colpoda cucullus*.

Resumen. Dos métodos analíticos simples y sensibles, basados en las técnicas de dispersión de matriz en fase sólida (DMFS), extracción en fase sólida (EFS) y cromatografía de líquidos de alta resolución (CLAR) con detección de fluorescencia, fueron desarrollados para la determinación de los hidrocarburos aromáticos policíclicos (HAP) de alto peso molecular: benzo(a)antraceno, benzo(b)fluoranteno y benzo(a)pireno en cultivos líquidos del protozoario ciliado *Colpoda cucullus*. El primer método empleó a la DMFS para la extracción de los analitos de cantidades muy pequeñas de microorganismos y también de la materia orgánica insoluble. El segundo método empleó a la EFS para extraer a los analitos del medio de cultivo líquido. Los parámetros de validación de ambos métodos indicaron buena linealidad ($r^2 > 0.99$) y precisión (RSDs $< 7\%$). Los recobros obtenidos fueron $>90\%$ con el método DMFS y 70% con el método EFS, con límites de detección entre 0.02 y $0.03 \mu\text{g/g}$ para DMFS-CLAR y entre 0.03 y $0.04 \mu\text{g/L}$ para EFS-CLAR. La metodología analítica se aplicó por primera vez de manera muy exitosa para la determinación cuantitativa de los HAP en cultivos de microorganismos.

Palabras clave: hidrocarburos aromáticos policíclicos, dispersión de matriz en fase sólida, extracción en fase sólida, cromatografía de líquidos de alta resolución-fluorescencia, protozoarios, *Colpoda cucullus*.

Introduction

Abundant research has demonstrated that microorganisms, such as bacteria, fungi, algae and protozoa, can be suitable bio-indicators or biosensors of organic and inorganic pollutants in soil and aquatic habitats [1-9]. The use of protozoa in toxicity and bioaccumulation bioassays for pollutants have particular advantages over other microorganisms because: 1) the lack of a cell wall in the vegetative state and their delicate external membranes allow a faster interaction and/or diffusion of substrates into the cell, 2) the high reproduction rates allow results in bioassays within 24 h [10], and 3) protists are eukaryotic and their reactions to environmental change can be related to those of metazoan organisms more convincingly than those of prokaryotes [11]. For these reasons, different groups of aquatic and soil protozoa, have been successfully used as test organisms for pollutants.

Diverse biological responses have been shown by protozoan in studies related to their exposure to heavy metals [12-18]. In these cases, bioaccumulation seemed to be an important mechanism of resistance [19], and soil ciliates seemed to be quite resistant compared to ciliates from other habitats [20]. The body of literature regarding the interactions of the most important organic pollutants with protozoa is much smaller

than that available on inorganic pollutants. Despite the limited research in this area, an inhibitory effect was observed in a number of soil protozoa populations when they were exposed to the organochlorine pesticides DDT and Lindane [21, 22]. Likewise, it has been shown that some agricultural formulations containing the organophosphorus pesticides malathion and diazinon affect cell activities on the soil ciliate *Colpoda inflata* [23] and the marine ciliate *Euplotes crassus* [24]. In addition, nitric oxide (NO) production in *Paramecium primaurelia* was shown to be sensitive to μM concentrations of diazinon [25]. Moreover, the alteration of some biological functions in free-living coast ciliates has been related to pollution from crude oil, a common anthropogenic source of polycyclic aromatic hydrocarbons (PAH) [26]. However, despite the above mentioned alterations in ciliate function, it has also been reported that the number of some cultivatable protozoa was higher when they were harvested from (PAH)-polluted soils than when coming from unpolluted soils [27]; indeed, more than 90% of ciliate 18S rRNA sequences found in soils polluted with PAH belonged to the class *Colpodea* [28].

Even if there are some interesting reports on this subject, there is not enough information to fully understand the interactions of protozoa and the most common environmental organic pollutants. Bioassays in the laboratory are indispensable as a

first approach to study not only toxicity or alteration of biological functions in protozoa, but also bioconcentration as a sign of resistance. Case studies in this area have been reported for biodegradation, as in the case of PAH degradation by microalgae, bacteria and fungi, a subject that has been extensively documented [29]. However, it is remarkable that modern, simple and validated analytical methods to determine organic pollutants in microorganisms and their specific and diverse culture media were difficult to find in the literature, despite being very useful tools to detect resistance and biomarkers for organic xenobiotics in protozoa.

To date, only a few papers dealing with the development of modern analytical methodology to determine the concentration of some endogenous organic compounds in microorganisms have been published. A headspace solid-phase microextraction (SPME) method coupled to gas chromatography was used for the quantification of poly (3-hydroxybutyrate) in bacterial biomass harvested from a fermentation process [30]. In another work, mycrocystins were extracted from algal dietary supplements (tablets of *Chlorella* and *Spirulina*) with methanol or 5% acetic acid aqueous solution followed by a solid-phase extraction (SPE) cleanup; the extracts were then analyzed by liquid chromatography-UV detection and by two other bioanalytical methods [31]. These compounds were also quantified in blooms and cyanobacterial strain cultures by matrix solid-phase dispersion and liquid chromatography-mass spectrometry [32].

Notably, dangerous organic xenobiotics, such as PAH, have not been analyzed in unicellular organisms, despite the fact that PAH have been determined in many other environmental matrices (e.g., air, water, soil, sediments, biota) and food using a variety of analytical instruments and extraction techniques [33-42]. Therefore, MSPD, a suitable technique for the preparation, extraction and fractionation of solid, semi-solid and/or highly viscous biological samples [38], was used in this work to develop a miniaturized and reliable method for the selective extraction of three priority and persistent PAH (benzo(a)anthracene, benzo(b)fluoranthene and benzo(a)pyrene) from *Colpoda cucullus*, a soil protozoan that has high potential to be bioindicator and biosensor of organic pollution [43]. The analysis of extracts was performed by HPLC with fluorescence detection. The developed MSPD-HPLC method was subsequently applied to the analysis of biomass collected from PAH-exposed cultures of *Colpoda cucullus*. In addition, a SPE method was developed for the extraction of remaining PAH from the liquid culture media. The MSPD-HPLC and SPE-HPLC methods allowed the determination of studied PAH at trace concentration levels in the three different components of the culture: biomass, liquid media and solid (insoluble) organic matter.

Results and Discussion

Pretreatment of sample

Tests to optimize the isolation of microbes from the culture medium were conducted by centrifugation of 15 mL of the

liquid culture at different speeds (1100, 1500 and 3500 rpm) for 2 min. After centrifugation, tubes were allowed to rest for 5 min and then several aliquots of residue and supernatant were collected for observation in the optical microscope. The results obtained from these assays showed that at low speed centrifugation, the sedimentation of solid matter was favored and the protozoa remained suspended in the supernatant. By comparison, the two highest-speed centrifugations trailed both, microorganisms and solid organic matter to the bottom of the tube, leaving the supernatant free from these two components. From these results, a protocol for separating the three main components of the culture was designed. The first step consisted of a low speed centrifugation (at 1100 rpm) for 2 min to eliminate the suspended solid material. The second step was a subsequent centrifugation of the collected supernatant (containing the microorganisms) at the highest speed (3500 rpm) for 8 min to allow the complete sedimentation of biomass, leaving the liquid medium free from microorganisms. Finally, the air-dried biomass and the clean liquid medium were analyzed by the MSPD-HPLC and SPE-HPLC methods, respectively.

Optimization of MSPD conditions

Chromabond C18-PAH silica was selected as the dispersant phase to perform MSPD extraction because its lipophilic character allowed appropriate retention of the compounds of interest. A sample/sorbent ratio of 1:20 was used with only 5 mg of sample and 100 mg of C18 sorbent. This ratio was shown to be satisfactory even though it was very different from the typical 1:1 or 1:4 reported in literature for other MSPD applications [38]. A very small amount of sample was used because it represented the maximum dry biomass obtained from 60 mL of the liquid culture from each bioassay. Acetonitrile was selected as elution solvent because it led to the quantitative desorption of analytes from the MSPD cartridge. However, it was necessary to perform a clean-up before analyte elution, because interferences contained in non-cleaned extracts affected detection and integration of the peak corresponding to B(a)A in the chromatogram (see Fig. 1a). Pure water and water-acetonitrile eluents were assayed for elimination of unwanted co-eluted compounds having a more polar nature than PAH. The optimized elution sequence was performed as follows: 1) 3 mL of deionized water, 2) 3 mL of a poorly-eluting acetonitrile-water mixture (30:70 v/v) and 3) 500 μ L of pure acetonitrile. This sequence provided recovery values >91% for all analytes and a good detection level. In figure 1b a chromatogram of MSPD extract from protozoa samples after application of the optimized cleanup sequence is shown. It is noted that unwanted co-eluted matrix components are no longer present in this chromatogram. Likewise, peaks having the same retention times that analytes were not observed in chromatograms from non spiked samples (chromatograms not shown). Partial (fractional) and global (accumulated) recoveries obtained from the assays using the optimized sequence are included in Table 1. As observed, breakthrough of analytes does not occur during the cleanup steps, whereas a practically complete elution is achieved with

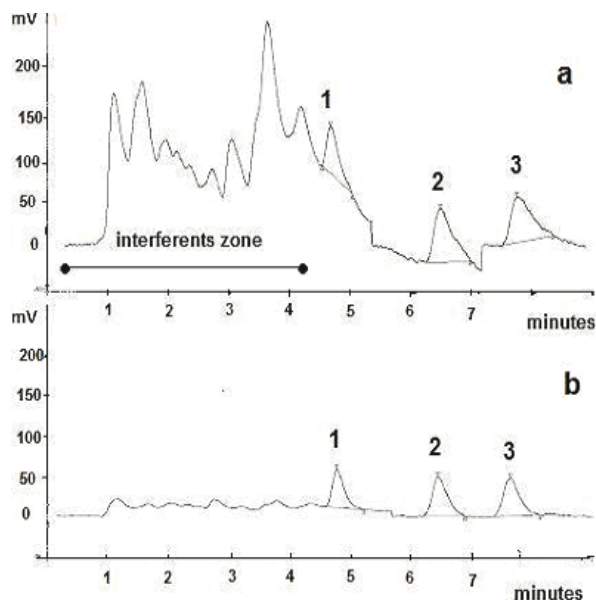


Fig. 1. HPLC chromatograms of MSPD extracts from protozoa samples: (a) without cleanup, (b) after application of the optimized cleanup sequence. Chromatographic conditions: 5 μm C18 column (150 mm × 4.6 mm i.d.) and isocratic elution with methanol at a flow rate of 1 mL/min. Injected volume: 20 μL. Fluorescence detection: 1. B[a]A λ_{ex} = 284 nm, λ_{em} = 405 nm; 2. B[b]F, λ_{ex} = 254 nm λ_{em} = 430 nm, γ 3. B[a]P λ_{ex} = 263 nm, λ_{em} = 410 nm. Samples spiked at 0.3 μg/g.

only 500 μL of pure acetonitrile. The “not analyzed fractions” in Table 1, were either cloudy and/or colored fractions resulting from the initial water washing of the MSPD column.

Optimization of SPE conditions

A simple SPE-methodology with 250 mg of C18 packed in cartridges was optimized for the isolation of PAH from the culture liquid medium, using a sample volume of 15 mL. A preliminary assay was performed by loading the cartridge with purified and deionized water fortified at 1 μg/L of each analyte. As expected, the effluent did not contain the analytes because they are very hydrophobic compounds and were strongly retained on the C18 phase. Their elution from the SPE-cartridge was

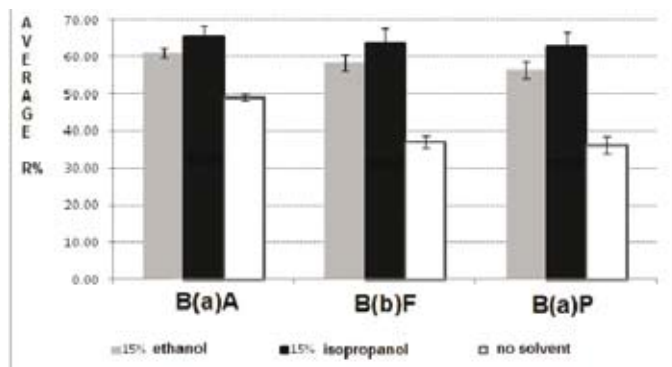


Fig. 2. Comparative SPE average percent recoveries of PAH in centrifuged liquid culture and liquid culture added with two different solvents. Cartridges packed with 250 mg of C18. Elution sequence: 1) 5 mL of water, 2) 5 mL of acetonitrile-water mixture (30:70 v/v) and 3) 4 mL of acetonitrile. Sample volume = 15 mL. Sample fortified at 1 μg/L; n = 3.

achieved with 5 mL of pure acetonitrile but recoveries obtained from this assay were low for all compounds (36 to 49%). It is known that losses of highly hydrophobic compounds, such as PAH, are often due to their strong tendency to adsorb on the walls of vessels that are in contact with their aqueous solutions. To improve recoveries, the solubility of analytes in the aqueous sample was increased by addition of a suitable organic solvent [44]. Thus, ethanol and isopropanol (15%) were tested separately. The recoveries obtained after SPE elution with acetonitrile are presented in Fig. 2. It can be observed that isopropanol provided the best recoveries (63 to 66%) when compared to ethanol (56 to 61%). Recoveries were still not very high, but a higher content of organic modifier was not assayed to avoid breakthrough of the analytes from the cartridge during the loading step. Nevertheless, recoveries obtained with 15% isopropanol allowed an appropriately low limit of detection for the analysis of samples from bioassays.

Considering these preliminary results, all assays for optimization of PAH extraction from real samples (centrifuged culture medium free from microorganisms) were performed with addition of 15% isopropanol to the sample. The same cleaning eluents were used in both MSPD and SPE, but taking into account that the amount of SPE sorbent was larger than the MSPD sorbent, a 5 mL volume of each solvent was used instead

Table 1. Optimization of the MSPD elution sequence for PAH in air-dried biomass.

HAP	% average recovery (1 mL fractions) n = 3									
	water			ACN-water 30:70 (v/v)			ACN**		%R global	%RSD
	1	2	3	1	2	3	1	2		
B[a]A	—	—	*	*	*	*	98	*	98	3.6
B[b]F	—	—	*	*	*	*	101	*	101	3.6
B[a]P	—	—	*	*	*	*	91	*	91	4.1

*not detected; — not analyzed; **500 μL fractions; ACN = acetonitrile; RSD = relative standard deviation; spiked level = 15 μg/g; sample amount = 5mg.

of 3 mL. The following elution sequence was then applied in triplicate assays to the SPE column after sample percolation: 1) 5 mL of water, 2) 5 mL of acetonitrile-water mixture (30:70, v/v) and 3) 5 mL of acetonitrile. To evaluate possible analyte breakthrough and recoveries, 1 mL fractions were collected and analyzed by HPLC at each step. Table 2 presents the global and partial average recoveries obtained from independent assays and the relative standard deviations (RSDs). From this data, it can be observed that analytes were not lost in the cleaning steps, reaching total elution after 4 mL of acetonitrile, thereby it was the final elution volume. Global recoveries for all compounds were close to 70%.

Method performance

The MSPD-HPLC and SPE-HPLC method performances were evaluated with spiked dried biomass and centrifuged liquid culture samples respectively. Table 3 shows the results from the validation of both methods. The curves "peak area vs. concentration" showed adequate linearity (correlation coefficients higher than 0.99) within the range 0.1-2 µg/g for MSPD-HPLC and 0.1-1 µg/L for SPE-HPLC. Accuracy was determined from the curves of recovered amount vs. added amount of PAH. The slope in these equations is of particular interest because it represents the average recovery of each compound (>90% for MSPD and 70% for SPE). Table 3 also shows the method precision (<4% for MSPD and 7% for SPE), determined in terms of reproducibility by running five analysis of each sample

spiked with PAH at 1 µg/L over five different days. The limits of detection (LODs) were 0.02-0.03 µg/g for MSPD-HPLC and 0.03-0.04 µg/L for SPE-HPLC, at a signal-to-noise (S/N) ratio of 3. The limits of quantification (LOQs) were 0.07-0.10 µg/g for MSPD-HPLC and 0.10-0.13 µg/L for SPE-HPLC, at a signal-to-noise (S/N) ratio of 10. These LODs and LOQs can still be 10-fold reduced by evaporation of the extract; however, this was not necessary for the aims of the present work.

Application of the MSPD-HPLC and SPE-HPLC methods to the analysis of PAH in protozoa and liquid culture

The two developed methods MSPD-HPLC and SPE-HPLC were applied to PAH exposed protozoa cultures at two exposure times (2 and 5 h). The biomass and liquid medium were separated by differential centrifugation as previously described. The air-dried biomass was analyzed by the MSPD-HPLC method and the liquid medium by SPE-HPLC. Insoluble organic matter was also isolated for quantification of the adsorbed PAH fraction. In this case, the MSPD-HPLC method was used. In this way, the overall distribution of PAH in microbial cells, liquid medium and insoluble organic matter was determined.

PAH adsorption on the surface of glassware was also examined. For this test, control cultures composed of medium without protozoa, spiked at the same concentration (1 µg/L) and exposed for the same times (2 and 5 h) as bioassays were prepared and analyzed by SPE-HPLC. The glass vessels used

Table 2. Optimization of the SPE elution sequence for PAH in centrifuged liquid culture with 15% isopropanol.

HAP	% Average Recovery (1 mL fractions) n = 3								Global %R	RSD (%)
	water		ACN-water (30:70, v/v)		ACN					
	1-4	5	1-5	1	2	3	4	5		
B[a]A	—	*	*	52	13	9	0.2	*	74	4.0
B[b]F	—	*	*	47	15	8	0.8	*	71	5.8
B[a]P	—	*	*	41	19	9	0.9	*	70	6.3

*not detected; — no analyzed; RSD = relative standard deviation; ACN = acetonitrile; spiked level = 1 µg/L; sample volume = 15 mL.

Table 3. Validation parameters for MSPD-HPLC (a) and SPE-HPLC (b) methods.

	Method	B[a]A	B[b]F	B[a]P
Calibration ^a	a (0.1-2 µg/g)	y = 0.908x + 0.034	y = 0.957x + 0.125	y = 0.941x + 0.026
	b (0.1-1 µg/L)	y = 0.744x + 0.049	y = 0.727x + 0.040	y = 0.700x + 0.021
(r ²) ^b	a	0.999	0.996	0.994
	b	0.991	0.989	0.991
RSD% ^c	a (1 µg/g)	3.3	4.0	3.6
	b (1 µg/L)	6.0	6.3	7.4
LOD ^d	a (µg/g)	0.03	0.02	0.03
	b (µg/L)	0.04	0.03	0.04

^aadded vs. recovered amount curve.

^bcorrelation coefficient; ^c relative standard deviation; ^d limit of detection.

for the control cultures were identical to those used for cultures with organisms. Table 4 presents the quantities of PAH found in the three different parts of each culture (liquid medium, protozoa microorganism and insoluble organic matter). In this table, the amounts found in the control cultures are also presented and the adsorbed percentage of PAH on glass vessels is calculated from the total added amount. It was found that PAH adsorption on the glass walls was statistically significant (between 58 and 71%) and increased with exposure time. The total amount of analytes quantified in the exposed cultures was therefore close to the amount (not glass-adsorbed) found in the controls, indicating the correct balance of available PAH between the three parts of the culture (protozoa + medium + solid organic matter). The average recovery of PAH calculated from each part of the culture is shown in Fig. 3, where it can be observed that an increase in the amount of PAH in the protozoa occurred when the exposure time increased. As expected, increasing exposure time caused a decline of PAH amount in the liquid medium. In solid organic matter, the quantity of PAH was small but not negligible because it fluctuated between 1.2 and 2.7%. Again, there was an increase in PAH levels in the solid organic matter, with increasing exposure time.

These results demonstrate that the developed analytical methods are very reliable because they made it possible to quantify analytes with good accuracy and sensitivity in all constituents of the microorganism's culture. Additionally, the obtained global balances of PAH agreed very well with the available analyte quantities in the culture, as determined from the concentrations measured in controls. Moreover, the MSPD-HPLC method seemed to be very robust because it could also be applied to the analysis of insoluble organic matter. For all the above reasons, the great utility of these analytical tools for doing this kind of biological testing was well demonstrated. It is important to realize that this is the first work reporting results of PAH exposure assays with microorganisms with such a level of detail.

The results obtained from these bioassays suggested that *C. cucullus* can retain the studied PAH and that their retention increased with exposure time. However, results are not yet sufficient to understand if this retention is the result of bioaccumulation or simple external adsorption. More research is also

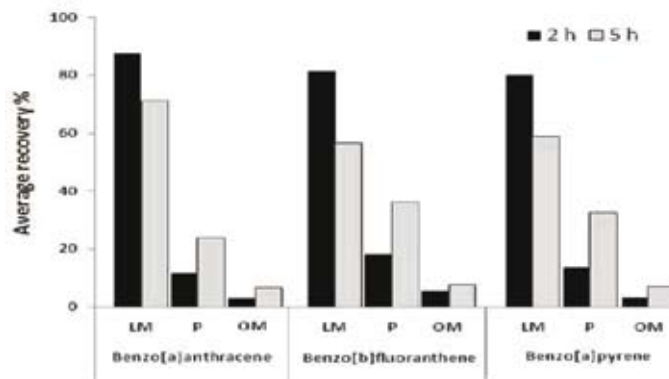


Fig. 3. Average recovery ($n = 3$) of PAH found in the three different parts of the culture (liquid medium (LM), protozoa (P) and insoluble organic matter (OM)) at two exposure times.

needed to elucidate protozoan behavior when in contact with organic pollutants such as PAH. The MSPD-HPLC and SPE-HPLC developed analytical methods will greatly contribute to the possibility of implementing these organisms as bioindicators of pollution.

Conclusions

The developed MSPD-HPLC and SPE-HPLC methods were shown to be suitable for the determination of benzo(a)anthracene, benzo(b)fluoranthene and benzo(a)pyrene in different constituents of *C. cucullus* liquid cultures. These methods provided good linearity ($r^2 > 0.99$) and precision (inter-day RSDs $< 7\%$). The recovery values were $>90\%$ for MSPD and 70% for SPE with limits of detection between 0.02 and $0.03 \mu\text{g/g}$ for MSPD-HPLC and between 0.03 and $0.04 \mu\text{g/L}$ for SPE-HPLC. The developed MSPD and SPE extraction methods proved to be very simple and economical in comparison with conventional methods (for example, classic liquid-liquid extraction), and the MSPD technique was applied for the first time to the extraction of organic contaminants at trace levels from a low amount of protozoa cells. In addition, the MSPD-HPLC method was

Table 4. Average amount of PAH determined in each component of the culture at 2 exposure times and the percentage adsorbed on glass material (spiked amount = 60 ng).

compound	t. exp. (h)	Average PAH amount (ng)				
		protozoa*	liquid medium**	organic matter	control	% glass vessels
B[a]A	2	2.7 ± 0.3	22 ± 1.5	0.7 ± 0.06	25 ± 1.2	58
	5	3.9 ± 0.4	12 ± 0.8	1.1 ± 0.09	16 ± 1.0	71
B[b]F	2	3.9 ± 0.4	18 ± 1.2	1.2 ± 0.08	22 ± 1.5	62
	5	7.2 ± 0.5	11 ± 0.9	1.6 ± 0.20	20 ± 1.2	67
B[a]P	2	3.0 ± 0.3	17 ± 0.4	0.7 ± 0.05	22 ± 0.8	66
	5	6.3 ± 0.5	11 ± 0.8	1.4 ± 0.1	19 ± 1.0	69

*Dried biomass = 5 mg; **total sample volume = 60 mL.

very robust because it could also be used to determine PAH in insoluble organic matter present in the culture medium. Application of both methods in real bioassays was easy and fast giving results indicating that the three studied persistent pollutants could be retained by protozoa. However, this observation should be corroborated with further biological experimentation using these analytical tools.

Experimental

Chemicals and materials

Benzo(a)anthracene (B[a]A), benzo(b)fluoranthene (B[b]F) and benzo(a)pyrene (B[a]P) with purity $\geq 99\%$ were supplied by Chem. Service (West Chester, PA, USA). Stock solutions (100 mg/L) were prepared in acetonitrile and kept at 4°C until used. Working diluted standards of various concentrations were prepared from these stock solutions. HPLC-grade methanol and acetonitrile were purchased from EM Science Merck (Gibbstown, NJ, USA). Deionized water was obtained from a MilliQ water purification system (Millipore, Bedford, MA, USA). CHROMABOND C18-PAH silica (particle diameter 40 μm) was acquired from Macherey-Nagel (PA, U.S.A.).

Cultures and bioassays

Colpoda cucullus cells, kindly supplied by Dr. V. M. Luna Pabello from the National University of Mexico, were grown in a barley medium, previously bacterized with *E. coli*. The cell culture was maintained in logarithmic growth by daily re-isolations in Petri dishes (ϕ 10 cm). Cultures were harvested after 24 h incubation at 28°C (environ 700 cells/mL, measured by microscope counting) and used in the SPE or MSPD method development assays. Bioassays were made in 60 mL of this medium spiked at 1 $\mu\text{g/L}$ of each PAH at exposure times of 2.0 and 5.0 h. Exposure media were allowed to settle for 10 min before introducing the protozoan. In addition, a reference culture medium without microorganisms was prepared at each different exposure time to evaluate PAH adsorption on the glass containers. All experiments were made in triplicate. After exposure to pollutants, the biomass, insoluble organic matter and liquid medium were separated from the culture and PAH concentrations were determined in each part independently.

Chromatographic analysis and detection

A Varian model 9012 liquid chromatographic pump (Palo Alto, CA, USA) and a Varian model Pro Star 363 fluorescence detector (B[a]A $\lambda_{\text{ex}} = 284$ nm, $\lambda_{\text{em}} = 405$ nm; B[b]F $\lambda_{\text{ex}} = 254$ nm, $\lambda_{\text{em}} = 430$ nm; B[a]P $\lambda_{\text{ex}} = 263$ nm, $\lambda_{\text{em}} = 410$ nm) were employed for HPLC analysis. Manual injection was performed using a Rheodyne model 7125 injection valve with a 20 μL loop. Quantitative measurements of peak areas were provided by the Varian Star workstation version 4.5. Separation was carried out on a 5 μm RES ELUT C18 stainless steel Varian

column (150 mm \times 4.6 mm i.d.) connected to a guard column (13 mm \times 4.6 mm i.d.) packed with a 10 μm Nucleosil C18 stationary phase from Phenomenex (Torrance, CA, EUA). Isocratic elution with methanol as the mobile phase was used at a flow rate of 1 mL/min.

Sample preparation

Collection

Microorganisms were concentrated and isolated from the liquid culture medium by a differential centrifugation procedure with, 1) centrifugation of 60 mL of culture for 2 min at 1100 rpm (organic matter collected in the bottom of the centrifugation tube) and 2) re-centrifugation of the supernatant for 8 min at 3000 rpm (microorganisms collected in the bottom of the centrifugation tube). The protozoan material was then air-dried for the MSPD procedure and the resulting liquid medium (free from microorganisms and insoluble organic matter) was treated by SPE.

MSPD procedure

Five milligrams of dried cells were placed in an agate mortar and gently blended with 100 mg of C18-PAH silica (preconditioned with 2 mL acetonitrile) to obtain a homogeneous mixture. This mixture was introduced into a 1 mL polypropylene cartridge with a polyethylene frit in the bottom, tightly compressed and covered with another polyethylene frit. To remove interferences from the MSPD cartridge, a wash with 3 mL of deionized water was performed, followed with 3 mL of an acetonitrile-water mixture (30:70 v/v). Finally, analytes were eluted from the cartridge with only 500 μL of acetonitrile. This extract (20 μL) was then injected into the HPLC-FL system.

SPE procedure

A simple off-line SPE method for isolation of PAH from the liquid medium was optimized by loading 15 mL of sample mixed with 15% isopropanol onto a 250 mg Chromabond C18-PAH (Macherey-Nagel, PA, USA) silica cartridge preconditioned with a 4 mL volume of acetonitrile, 5 mL of acetonitrile-water mixture (30:70 v/v) and 5 mL of water. After vacuum drying, the inverse elution sequence was applied to the cartridge by successively passing through 5 mL of water, 5 mL of an acetonitrile-water mixture (30:70 v/v) and 4 mL of acetonitrile. This procedure allowed PAH to be eluted in the acetonitrile fraction free from polar interferences. This extract (20 μL) was directly analyzed by HPLC-FL.

Recovery studies and method validation

Linear calibration curves were prepared for the MSPD-HPLC method at six spiked levels (0.1, 0.2, 0.3, 0.6, 1.0 and 2.0 $\mu\text{g/g}$) on 5 mg of protozoan biomass and for the SPE-HPLC method

(0.1, 0.2, 0.3, 0.7, 0.9 and 1.0 µg/L) on a 15 mL volume of the liquid culture medium. Three replicates were analyzed for each level. Method linearity was evaluated from the curve “peak area vs. concentration” using linear regression analysis and from the curve “added amount vs. recovered amount”, where the slope × 100 represents average recovery. Precision was obtained from the analysis of five extractions performed over five different days on protozoan samples spiked at 1.0 µg/g and with liquid medium spiked at 1.0 µg/L. Method detection limits (LODs) and quantification limits (LOQs) at a signal to noise ratio of 3 and 10, respectively, were determined.

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