Journal of Chromatography A, 1262 (2012) 138–147

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Extraction and analysis of polycyclic aromatic hydrocarbons and benzo[a]pyrene metabolites in microalgae cultures by off-line/on-line methodology based on matrix solid-phase dispersion, solid-phase extraction and high-performance liquid chromatography☆

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ARTICLE INFO

Article history:
Received 22 May 2012
Received in revised form 10 August 2012
Accepted 4 September 2012
Available online 16 September 2012

Keywords:
Matrix solid-phase dispersion
Solid-phase extraction
On-line methods
HPLC
PAH
Microalgae cultures

ABSTRACT

This paper describes the development and validation of an analytical methodology to determine the presence of four PAHs: benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene and benzo[a]pyrene in cultures of the green microalgae *Selenastrum capricornutum*. The metabolites of benzo[a]pyrene (BaP), 4,5-dihydrodiol benzo[a]pyrene, 9,10-dihydrodiol benzo[a]pyrene, 3-hydroxy benzo[a]pyrene and 9-hydroxy benzo[a]pyrene were also included. The methodology consisted of three parts: (1) separation of liquid media from biomass samples by centrifugation of pure cultures, (2) off-line extraction of analytes from biomass by a miniaturized matrix solid phase dispersion (MSPD) method and from liquid media by a solid phase extraction (SPE) method and (3) on-line SPE preconcentration and analysis of the MSPD and SPE extracts, separately, by high performance liquid chromatography with fluorescence detection (HPLC-FD). The off-line/on-line (MSPD/SPE–HPLC-FD) method was validated over a concentration range of 20–200 pg mg^{-1} obtaining good linearity ($r^2 > 0.9912$) and precision values measured as relative standard deviation (RSD) < 5%, recovery values were in the range of (40–66%) and the limits of detection (LODs) ranged from 2 to 6.5 pg mg^{-1}. The off-line/on-line (SPE/SPE–HPLC-FD) method was validated over a concentration range of 5–120 pg mL^{-1}; $r^2 > 0.9913$ and RSD < 7.36%, recovery values were in the range of 38–74% and LODs ranged from 0.8 to 2.3 pg mL^{-1}. This methodology was applied to samples from cultures exposed to BaP at 5 ng mL^{-1} with different exposure times (0.75, 1.5, 3, 6, 24 and 48 h). The analytical methodology was suitable for measuring the very low amounts of residual BaP and metabolites produced in bioassays. Results showed that some of the metabolites favored by the microalgae are the dihydrodiols. The microalgae cultures were able to decrease the BaP level in the liquid medium below the United States Environmental Protection Agency (USEPA) limit (<0.2 ng mL^{-1}).

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are compounds consisting of two or more fused aromatic rings. They are formed by the incomplete combustion of organic materials from anthropogenic (smoke, automobile and industrial emissions, petroleum refineries, etc.) and natural activities (forest fires, active volcanoes activities, etc.). PAHs are persistent, widely distributed in the environment and toxic, even at low concentrations. Compounds having more than four rings can be carcinogenic and/or teratogenic. For these reasons, this group of contaminants is monitored continuously by the environmental agencies such as the USEPA and European Environment Agency [1,2].

PAHs can be removed from polluted environmental samples by several processes including adsorption, volatilization, photolysis, chemical degradation and microbial degradation; the last is the most important. Many publications have reported that a wide variety of bacteria and fungi are capable of degrading PAHs such as anthracene, phenanthrene and benzo[a]pyrene to polar metabolites [2–6]. Several microalgae species also have been reported to degrade certain PAHs such as naphthalene, fluoranthene and pyrene [2]. However, references describing PAHs degradation by microalgae are less numerous than those describing degradation by bacteria or fungi. The green microalgae *Selenastrum capricornutum* is commonly used in such assays due to its ability to survive in both eutrophic and oligotrophic environments and grow easily and

☆ Presented at the 38th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Anaheim, CA, USA, 16–21 June 2012.

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http://dx.doi.org/10.1016/j.chroma.2012.09.015

rapidly even at contaminated sites [7–13]. There are some reports of biodegradation of heavy PAHs by green microalgae; most of these have been focused on the behavior of benzo[a]pyrene (BaP), the most common marker of carcinogenicity, although BaP is not the only carcinogenic PAH. Although most studies have monitored only the disappearance of PAHs from the culture medium or biomass, a few studies also have shown that some microalgae are capable of metabolizing BaP to compounds with high polarity such as dihydrodiols, hydroxides and quinones [8,14,15]. The analysis and identification of these metabolites is difficult due to their instability to light, temperature, pH and air [16], as well as their low levels of production in exposed cultures. Their concentrations are usually below the detection limits of the classical analytical methodologies that are commonly used to perform this type of bioassay. For this reason, it is necessary to develop rapid and sensitive methods to determine the very low levels of the PAH-metabolites that are formed in environmental or microbiological culture samples and that are potentially more toxic and carcinogenic than the parents PAHs [17,18]. A better understanding of the biodegradation process is also needed.

The main problem for the extraction of trace levels of organic contaminants from complex samples, such as the cultures of microorganisms, is the limited amount of sample available. As mentioned above, the most common extraction technique utilized in biodegradation assays is the classic liquid–liquid extraction (LLE), which often consumes large amounts of samples and reagents and presents various other inconveniences as well such as many extractions and clean-up steps and the generation of considerable amounts of wastes. Alternative sorbent-based extraction techniques, such as solid phase extraction (SPE) for the treatment of liquid samples or matrix solid phase dispersion (MSPD) for the treatment of solid samples, can provide better results than traditional LLE. SPE allows high concentration factors because it uses a small volume of samples and it is extremely versatile in that it can be applied to a wide range of samples and compounds [19,20]. On the other hand, MSPD is a sample preparation strategy that is widely applied to solid, semisolid or viscous samples because it uses very small amounts of sample and consequently this technique is well-suited for miniaturization. Some works report the application of MSPD to less than 50 mg of sample and 100 mg of sorbent. In both, SPE and MSPD extraction, the most commonly used sorbent is silica C-18 [21–25].

Even though SPE and MSPD are able to achieve high concentration factors and provide low detection limits to the whole methodology, for example 1000, when 1 L of water sample is preconcentrated to 1 mL by SPE, sometimes even lower detection limits are required for the accurate measurement of small concentrations of trace contaminants in the order of ppb or ppt, as is the case of PAH metabolites. To accomplish this, the extract can be preconcentrated before its introduction into the analytical system. On-line methodologies are very useful when the sample amount is limited, the analytes are labile or a very high sensitivity is necessary. The use of on-line SPE has made possible the development of faster and more sensitive methods by reducing sample preparation times and increasing sample throughput. Conditioning, washing and elution steps can be performed automatically, and most of systems also allow the treatment of one sample while another is being analyzed [26–29].

The aim of this work was to develop and validate a new and very sensitive methodology for the extraction of PAHs and metabolites from *S. capricornutum* liquid culture components (liquid-media and biomass), using SPE and MSPD, respectively. Extraction was followed by on-line preconcentration and HPLC analysis of the extracts using the high sensitivity provided by fluorescence detection. While the instrumental limits of detection of the studied compounds ranged from 120 to 200 pg mL^{-1}, this off-line/on-line methodology made it possible to achieve detection limits on the range of 2.0–6.5 pg mg^{-1} for biomass and 0.8–2.3 pg mL^{-1} for liquid media, allowing monitoring of the changing concentrations of analytes over time, for both PAHs and the metabolites produced in exposure assays. The level of BaP selected for the exposure test (5 ng mL^{-1}) was close to its solubility in water, 2.3 ng mL^{-1} [30], a concentration representative of that found in the contaminated environmental waters. This is a novel application because a very low concentration of PAHs and metabolites can be analyzed from a small sample amount, in an average time of 45 min. Therefore, the developed methodologies allow a better study of the degradation kinetics of PAHs in microalgae cultures than those commonly used in laboratory biodegradation assays. Previous works applied several liquid–liquid extraction steps, decolorize and evaporate the extracts. These procedures can easily be done by the use of SPE or MSPD. Likewise, some works used in bioassays more than a hundred-fold greater concentrations (100–1200 ng mL^{-1}) than the saturated solubility values, possibly because their methods did not have adequate limits of detection [12,14,31].

2. Experimental

2.1. Materials and reagents

The PAHs standards (benzo[a]anthracene (BaA), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF) and benzo[a]pyrene (BaP)) were purchased from Chem Service (West Chester, PA, USA) with a certified purity >99%. BaP metabolite standards (3-hydroxy benzo[a]pyrene (3-OH BaP), 9-hydroxy benzo[a]pyrene (9-OH BaP), 4,5-dihydrodiol benzo[a]pyrene and 9,10-dihydrodiol benzo[a]pyrene) were acquired from the NCI Chemical Carcinogen Reference Standards Repository (Kansas, MO, USA). Individual standard solutions were prepared by appropriate dilution of the standards in acetonitrile at 1.0 mg L^{-1}. A stock solution of a mixture of eight analytes at 500 ng mL^{-1} was prepared in acetonitrile and stored in the dark at 4 °C. Water (18.2 MΩ cm^{-1} resistivity) was obtained from a Millipore Simplicity UV (Bedford, MA, USA) deionizer. Silica CHROMABOND C18-PAH (particle diameter 45 μm) from MACHEREY-NAGEL (Bethlehem, PA, USA) was used as a sorbent to perform SPE and MSPD. Nucleosil C18 (particle diameter 10 μm) was purchased from Phenomenex (San Francisco, CA, USA) and was used to pack the SPE precolumn of the on-line concentration system. HPLC-grade solvents such as methanol, acetonitrile, isopropanol and the following reagents were purchased from J.T. Baker (Phillipsburg, NJ, USA): ascorbic acid ($C_6H_8O_6$), sodium nitrate ($NaNO_3$), calcium chloride dehydrate ($CaCl_2{\cdot}2H_2O$), magnesium sulphate heptahydrate ($MgSO_4{\cdot}7H_2O$), dibasic potassium phosphate (K_2HPO_4), monobasic potassium phosphate (KH_2PO_4), nitric acid (HNO_3), sulfuric acid (H_2SO_4), sodium lauryl sulfate (SDS), sodium heptane sulfate and sodium chloride (NaCl). All substances were reactive grade. The culture medium ingredient peptone proteose was acquired from MCD LAB (Tlalnepantla, MEX, México). All the glassware was cleaned before each experiment with an aqueous solution of HNO_3 3% (v/v) overnight. Afterwards it was rinsed with distillated water and finally with 1 mL of 100% ACN.

2.2. Algal cultures

A culture of the microalgae *S. capricornutum* was obtained from The Culture Collection of Algae (UTEX) at The University of Texas (Austin, TX, USA). The stock culture was maintained in 150 mL Bristol's medium supplemented with 1 g L^{-1} of proteose peptone in a 250 mL Erlenmeyer flask under axenic conditions at room temperature. A 16/8 h light/dark photoperiod was generated by T5 14 W cool-white fluorescent lamps. The cultures were aerated by

orbital agitation at 100 rpm using a THERMO SCIENTIFIC Model 2346 (Dubuque, IA, USA) shaker. Under these conditions, the algal cells reached the mid-exponential growth phase (7–10 days) with a cell number of (2–6) × 10^6 cells mL^{-1}. The cell number was counted using a Neubauer haemocytometer with improved rulings (Boeco, Germany). Microalgae were harvested after 7–10 days (in mid-growth phase) and isolated from 15 mL of liquid culture medium by centrifugation for 20 min at 3900 rpm, using a SIGMA 2–5 centrifuge Laborzentrifugen (Osterode am Harz, Germany). The pellet of cells thus obtained was then air-dried and used for the MSPD procedure, while the supernatant (i.e., the liquid culture medium) was treated by the SPE method. Reserve cultures were maintained for longer periods on solid culture medium (Bristol's medium + 1 g L^{-1} of proteose peptone + 1.5% of agar).

2.3. Apparatus

The selected PAHs and metabolites were analyzed on a Varian model 210 liquid chromatograph, coupled to a model 363 fluorescence detector (FD) (Palo Alto, CA, USA) using LC Workstation Multi-instrument Version 6.20 software. This HPLC system was equipped with a manual injection port and switching valve Rheodyne 7725i with a 20 μL loop. A 5 μm HICHROM SPHERISORB stainless steel analytical column (150 mm × 4.6 mm I.D.) connected to a guard column (20 mm × 2 mm I.D., packed with the same stationary phase) was used. Both columns were obtained from Varian. Samples were preconcentrated on a 20 mm × 2 mm I.D pre-column packed with nucleosil C18 (10 μm) Phenomenex (CA, USA) stationary phase using an Eldex Duros Series 1000-C pump (Napa, CA, USA). The direction of the mobile phase flow was controlled by a Rheodyne model 7000 six-port switching valve (Berkeley, CA, USA).

2.4. Chromatographic conditions

Analytical separation was performed by the use of two isocratic elution modes at a flow rate of 1 mL min^{-1} as follows: mode 1, mobile phase 65% methanol and 35% water (separation of 4,5-dihydrodiol benzo[a]pyrene and 9,10-dihydrodiol benzo[a]pyrene) and mode 2, mobile phase 85% methanol and 15% water (separation of 3-OH BaP, 9-OH BaP, BaA, BbF, BkF and BaP). The detection wavelengths selected for each compound were as follows: benzo[a]anthracene, λ_{ex} = 284 nm, λ_{em} = 410 nm; for the rest of the compounds, λ_{ex} = 263 nm, λ_{em} = 430 nm.

2.5. PAHs extraction

Due to considerable differences in polarity between the dihydrodiol metabolites and the other compounds, the analytes were recovered in two different SPE and MSPD extracts. Thus, the first extract contained the dihydrodiols-BaP and the second extract contained the hydroxy-BaP together with the PAHs. MSPD and SPE off-line protocols are described below.

2.5.1. Off-line MSPD method

Five milligrams of dried biomass was mixed with 20 μL of 20% ascorbic acid solution and allowed to dry. Next, the entire sample was blended in an agate mortar with 100 mg of C18-PAH silica (preconditioned with 2 mL acetonitrile and dried with vacuum) until a homogeneous mixture was obtained. The mixture was placed into a 1 mL polypropylene cartridge with a polyethylene frit in the bottom, compressed and covered with another polyethylene frit. The following solvent sequence was used to remove interferences from the cartridge: 10 mL of deionized water, 10 mL of a 10:90 (v/v) acetonitrile–water mixture and 5 mL of a 20:80 (v/v) acetonitrile–water mixture. Finally, analytes were eluted from the cartridge in two different extracts identified as D1 (1.5 mL of 40:60 (v/v) acetonitrile–water mixture) and D2 (1 mL of 85:15 (v/v) acetonitrile–water mixture). The entire extracts obtained in the off-line mode were the diluted with water for subsequent preconcentration and analysis by the on-line SPE–HPLC-FD system (see Fig. 1).

2.5.2. Off-line SPE method

Extraction of the analytes from the liquid medium was performed by a simple SPE method, using a cartridge packed with

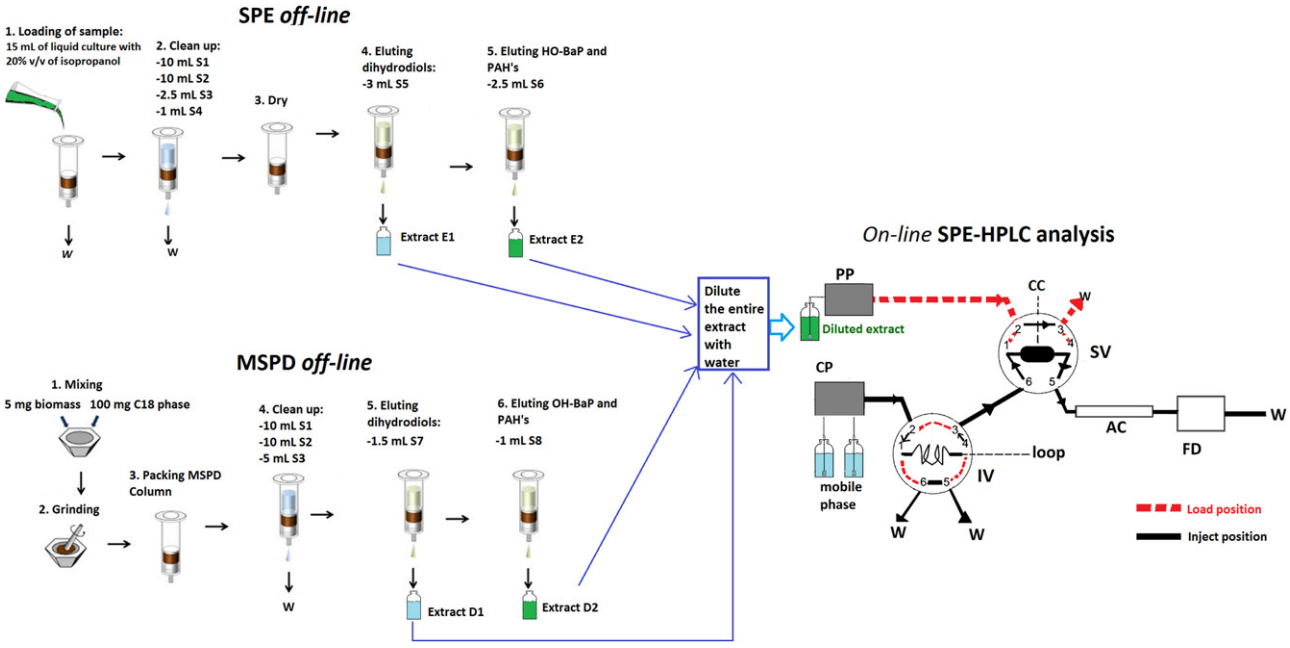

Fig. 1. The methodology scheme including the off-line extraction methods and on-line SPE-HPLC. S1 = water; S2 = ACN/water (10:90, v/v); S3 = ACN/water (20:80, v/v); S4 = ACN/water (30:70, v/v); S5 = ACN/water (55:45, v/v); S6 = ACN 100%; S7 = ACN/water (40:60, v/v); S8 = ACN/water (85:15, v/v); PP = preconcentration pump; CP = HPLC pump; CC = concentration column; IV = injector valve; SV = switching valve; AC = analytical column; FD = fluorescence detector; W = waste. E1 and E2 are extracts from SPE. D1 and D2 are extracts from MSPD.

Table 1
Method optimization (on-line preconcentration). Acetonitrile percentages (% ACN) of the extracts.

Method	Extract	Compounds	Initial % ACN in extract	Extract volume (mL)	Volume of water added (mL)	Final % ACN of diluted extract
MSPD	D1	Dihydrodiols	40	1.5	4.5	10
	D2	OH-BaP, PAHs	85	1	1.1	40
SPE	E1	Dihydrodiols	55	3	13.5	10
	E2	OH-BaP, PAHs	100	2.5	5.8	30

E1 and E2 are extracts from off-line SPE; D1 and D2 are extracts from off-line MSPD.

Table 2
Operation conditions of the on-line SPE-HPLC system.

Operation	Pump active	Valve position		Solvents applied to extract	
		IV	SV	E1 or D1	E2 or D2
Precolumn conditioning	PP	L	L	−5 mL ACN 100%	−5 mL ACN–water (10:90, v/v)
Sample loading	PP	L	L	Flow 2 mL min^{-1}	
On-line clean-up	PP	L	L	−5 mL ACN–water (10:90, v/v)	
Analytes separation	CP	I	I	Mobile phase MeOH–water (65:35, v/v) Flow 1 mL min^{-1}	Mobile phase MeOH–water (85:15, v/v) Flow 1 mL min^{-1}
Precolumn reconditioning	PP	L	L	−5 mL ACN 100%	−5 mL ACN–water (10:90, v/v)
Direct injection of standard	CP	I	L	Mobile phase MeOH–water (65:35, v/v) Flow 1 mL min^{-1}	Mobile phase MeOH–water (85:15, v/v) Flow 1 mL min^{-1}

PP = preconcentration pump; CP = pump of the HPLC system; IV = injector valve; SV = switching valve. I = inject; L = load. E = extract obtained from SPE step and diluted; D = extract obtained from MSPD step and diluted.

300 mg of Chromabond C18-PAH and previously conditioned with 4 mL of acetonitrile and 8 mL of water. First, 15 mL of sample mixed with 3.75 mL of isopropanol, to obtain a 20% solution, was passed through the cartridge. Afterwards, a sequence of solvents was passed through the cartridge to clean the sample: 10 mL of water, 10 mL of 10:90 (v/v) acetonitrile–water mixture, 2.5 mL of 20:80 (v/v) acetonitrile–water mixture and 1 mL of 30:70 (v/v) acetonitrile–water mixture. Finally, as in the MSPD method, the analytes were eluted from the cartridge in two different extracts designated as E1 (3 mL of 55:45 (v/v) acetonitrile–water mixture) and E2 (2.5 mL of acetonitrile). The entire extracts obtained in the off-line mode, were diluted with water, preconcentrated on-line and analyzed as in the MSPD method (see Fig. 1).

2.6. On-line SPE preconcentration and HPLC-FD analysis of MSPD and SPE extracts

A schematic diagram of the entire off-line/on-line methodology for determination of the analytes with SPE or MSPD and HPLC-FD is shown in Fig. 1. The on-line system consisted of a concentration precolumn coupled to HPLC-FD. The entire extracts obtained from off-line SPE or MSPD were diluted to the acetonitrile percentages indicated in Table 1. Then, the diluted extracts were loaded onto the concentration column (CC) which was packed with a C18 phase and connected to the switching valve (SV). This operation was performed using the preconcentration pump (PP). Table 2 describes the operating conditions used for the on-line system.

2.7. Method validation

2.7.1. Off line/on-line method (MSPD/SPE–HPLC-FD)

The linearity and precision of the MSPD/SPE–HPLC-FD method were evaluated by analyzing dry biomass samples spiked at five concentration levels (20, 60, 80, 120, 200 pg mg^{-1}). For each concentration, three replicates were made and the RSD was calculated. Precision was evaluated in terms of reproducibility by analyzing five extracts, on different days, prepared from samples spiked at two different concentration levels (20 and 120 pg mg^{-1}). The accuracy was evaluated by plotting the recovered amount of each analyte vs. the amount initially added to the spiked samples; the

Table 3
Optimization of the off-line MSPD elution sequence.

Compound	Recovery in fraction%									Global recovery % (±RSD)
	Clean-up		Analyte elution							n = 3
	S1	S3	S4				S5			
	S2	1–5	1	2	3	4	1	2	3	
9,10-Dihydrodiol BaP	NI	–	32	44	–	–	–	–	–	76 (±3)
4,5-Dihydrodiol BaP	NI	–	29	36	6	–	–	–	–	71 (±1)
9-OH BaP	NI	–	–	–	–	–	16	–	–	16 (±8)
3-OH BaP	NI	–	–	–	–	–	7	–	–	7 (±10)
BaA	NI	–	–	–	–	–	91	–	–	91 (±3)
BbF	NI	–	–	–	–	–	89	–	–	89 (±2)
BkF	NI	–	–	–	–	–	87	–	–	87 (±4)
BaP	NI	–	–	–	–	–	70	10	–	80 (±3)

S1 = 10 mL water HPLC; S2 = 10 mL ACN/water (10:90, v/v); S3 = 5 × 1 mL ACN/water (20:80, v/v); S4 = 4 × 0.5 mL ACN/water (40:60, v/v); S5 = 3 × 0.5 mL ACN/water (85:15, v/v). NI = not injected. – = not detected. BaA = benzo[a]anthracene; BbF = benzo[b]fluoranthene; BkF = benzo[k]fluoranthene; BaP = benzo[a]pyrene.

slope of the curve indicated the overall recovery. The experimental limits of detection (LODs) were determined at a signal-to-noise ratio (S/N) of 3, and the limits of quantification (LOQs) were determined at S/N of 10 at the optimum λ_{em} and λ_{ex} of each compound.

2.7.2. Off-line/on-line method (SPE/SPE–HPLC-FD)

The linearity and precision of the SPE/SPE–HPLC-FD method were evaluated by analyzing samples of liquid culture medium (free from microorganisms) spiked at five concentration levels. For the compounds 4,5-dihydrodiol BaP and 9,10-dihydrodiol BaP, the concentrations used were 5, 20, 40, 80 and 120 pg mL^{-1}. For the remaining analytes, the concentrations used were 10, 20, 40, 80 and 120 pg mL^{-1}. For each concentration level, three replicates were made and the RSD was calculated. Precision was evaluated in terms of reproducibility by preparing and analyzing five extracts, each one in different days, from samples spiked at two different concentration levels (10 and 80 pg mL^{-1}). The accuracy, experimental LODs and LOQs were determined as for the MSPD method.

2.8. BaP exposure tests

Several 50 mL Erlenmeyer flasks, each one containing 15 mL of liquid culture of *S. capricornutum* were divided into three groups as follows: (i) Exposed Microalgae Culture (EMC) – a liquid culture with a cell density between 2 and 5×10^6 cells mL^{-1} exposed to 5 ng mL^{-1} of BaP; (ii) Non Inoculated Control (NIC) – medium containing the aforementioned BaP concentration but without inoculation of algal cells; this group was introduced for the monitoring of abiotic losses of BaP; (iii) Blank Microalgae Culture (BMC) – a liquid culture with the same cell density, but without BaP.

The flasks were shaken on a rotary shaker (100 rpm) at room temperature (20 ± 2 °C), with the same photoperiod (16/8-h light/dark) used for the stock algal culture but illuminated with yellow light provided by a 40-watt incandescent lamp. Warshawsky et al. [8] demonstrated that the use of yellow light avoid the formation of toxic compounds for the microalgae, for example, quinones. Thus, triplicate flasks from each group were analyzed with the developed methodology at 0.75, 1.5, 3, 6, 24 and 48 h of exposure. The residual concentrations of BaP and the metabolites produced in the medium and algal biomass were determined.

3. Results and discussion

3.1. Miniaturized off-line MSPD

To optimize the miniaturized MSPD method, using a very small amount of sample, a sample/adsorbent ratio of 1:20 (5 mg/100 mg) was used. This ratio was found to be satisfactory even thought it differed from the typical 1:1 or 1:4 ratio reported in the literature for other MSPD applications [22,32–34]. Thus, various 5 mg samples of dry biomass was spiked with 20 μL of solution of PAHs and metabolites at 500 ng mL^{-1} to obtain a sample fortified at 2 ng mg^{-1}. After dispersion and packing in a cartridge, the sequence of cleaning and elution solvents applied to the MSPD cartridge was optimized by testing different volumes of water, acetonitrile or mixtures of acetonitrile/water (identified as S1–S5). The effluent was collected in 1 mL or 0.5 mL fractions in order to check the possible breakthrough of analytes. Each fraction was analyzed by injecting 20 μL into the HPLC-FD system, and the recoveries were calculated in comparison to the area obtained by the injection of 20 μL of a standard solution of all compounds at 20 ng mL^{-1}. The results of these experiments to follow the elution profile are shown in Table 3, where it can be observed that there was no breakthrough of any analytes in the S1–S3 fractions. Analytes 9,10-dihydrodiolbenzo[a]pyrene (1) and 4,5-dihydrodiol benzo[a]pyrene (2) were eluted by the S4 solvent mixture (D1 extract) and the remaining analytes (9-hydroxy benzo[a]pyrene (3)); 3-hydroxy benzo[a]pyrene (4); benzo[a]anthracene (5); benzo[b]fluoranthene (6); benzo[k]fluoranthene (7) and benzo[a]pyrene (8) were eluted by the S5 solvent mixture (D2 extract). Analytes could be recovered in small extract volumes (<1.5 mL). Dihydrodiols and PAHs showed good recoveries (>71%), but the hydroxy-BAP metabolites showed very low recoveries (<17%). In this respect, the literature reports significant instability of these compounds under different conditions of light, pH, temperature, air, etc. [16]. Therefore, efforts were made to increase the recoveries of these metabolites from different types of samples with the use of different compounds such as acids, surfactants and antioxidants [26,35–37]. Substances such as HNO_3, H_2SO_4, sodium lauryl-sulfate, sodium heptan-sulfate and ascorbic acid were added to the sample prior to extraction. The effects of these chemical modifiers on the recoveries of the MSPD procedure can be found in Table 4, where the data show that 20% (w/v) ascorbic acid in water gave the best results. Under this condition, recoveries were increased from 16 and 7% to 54 and 55% for compounds 3 and 4, respectively. It is important to note that the ascorbic acid markedly improved the recoveries of the hydroxy-BaP compounds (3 and 4) but the recoveries of the dihydrodiols compounds and benzo[a]anthracene (5) decreased. This effect can be attributed to the modification of the pH of the samples because dihydrodiol recoveries also decreased when solutions of HNO_3 were used. Therefore, 20% ascorbic acid was established as optimal for the MSPD, allowing recoveries of greater than 50% for all analytes.

3.2. Off-line SPE

According to some publications, the solubility of PAHs in water decreases with an increase in the number of aromatic rings. To improve the solubility of these compounds in the liquid media, isopropanol has been added at different concentrations [20,38]. Here, different SPE cartridges were loaded with 15 mL samples of liquid culture spiked with metabolites and PAHs at 8 ng mL^{-1} and containing different percentages of isopropanol. After application of the elution sequence to the cartridge, all the analytes were recovered by using 4 mL of ACN 100% to assure a complete elution of analytes; this volume was optimized in preliminary experiments. The extracts were analyzed by injecting a 20 μL volume into the HPLC-FD system, and the recoveries were calculated in comparison to the area obtained by injection of 20 μL of a standard solution of all compounds at 30 ng mL^{-1}. The results are presented in Table 5, where it can be observed that the best recoveries for PAHs (5–8), were obtained with the addition of 20% isopropanol. For metabolites, 15% isopropanol was the best choice, but 20% was selected in order to improve the SPE extraction of PAHs and to maintain the recoveries of all compounds above 62%.

Subsequently, the clean-up and elution sequence was optimized using water, acetonitrile and mixtures of water/acetonitrile (S1–S6). Desorption of analytes was observed in eluents S5 and S6, which were therefore collected in 1 mL fractions. The results are shown in Table 6, where it can be noted that only the cleaning solvent S4 has sufficient polarity to desorb a small percent (<3%) of the compounds 9 and 10 BaP dihydrodiol from the cartridge. Good simplification of the matrix (i.e., clean-up) without significant losses of this compound was obtained through the use of the cleaning solvents S1–S4. Although the solution S6 (a total of 3 mL of 100% ACN) allowed recovery of the hydroxy-BaP and PAHs, this volume was reduced to 2.5 mL without significantly decreasing the recoveries of each compound. With the SPE conditions optimized, the analytes were recovered selectively in the two extracts E1 and E2: 3 mL of S5 (PAH dihydrodiols) and 2.5 mL of S6 (hydroxy-PAHs and PAHs). In addition, good reproducibility (RSD 1–5%) in these trials

Table 4
Effects of chemical modifiers on recoveries applied to the off-line MSPD procedure.

Compound	Recovery % (±RSD) $n = 3$ Modifier[a]								
	WM	HSNa	LSNa	HNO_3		H_2SO_4	Ascorbic acid		
		1 M	1 M	0.1 M	1 M	0.1 M	15%	20%	40%
9,10-Dihydrodiol BaP	77 (±4)	71 (±3)	73 (±3)	52 (±1)	ND	50 (±4)	56 (±3)	52 (±4)	42 (±2)
4,5-Dihydrodiol BaP	79 (±2)	76 (±4)	79 (±2)	95 (±4)	90 (±2)	67 (±5)	66 (±3)	61 (±3)	57 (±2)
9-OH BaP	16 (±1)	16 (±4)	14 (±3)	33 (±3)	42 (±1)	18 (±4)	53 (±6)	54 (±5)	61 (±4)
3-OH BaP	7 (±11)	7 (±9)	6 (±10)	26 (±9)	54 (±10)	15 (±8)	61 (±9)	55 (±13)	53 (±11)
BaA	91 (±2)	90 (±1)	92 (±4)	85 (±2)	83 (±5)	80 (±2)	88 (±3)	76 (±4)	77 (±4)
BbF	89 (±5)	91 (±2)	93 (±4)	83 (±3)	81 (±2)	80 (±3)	87 (±2)	82 (±4)	81 (±3)
BkF	87 (±4)	88 (±4)	89 (±2)	85 (±4)	81 (±2)	83 (±4)	87 (±2)	87 (±4)	85 (±6)
BaP	90 (±4)	91 (±2)	93 (±3)	86 (±4)	83 (±5)	81 (±4)	88 (±4)	86 (±4)	83 (±6)

[a] 20 μL added to sample.
WM = without modifier; HSNa = sodium heptanesulfate; LSNa = sodium lauryl-sulfate. BaA = benzo[a]anthracene; BbF = benzo[b]fluoranthene; BkF = benzo[k]fluoranthene; BaP = benzo[a]pyrene.

Table 5
Method optimization (off-line SPE step). Recoveries (+RSD) at three isopropanol percentages added to the sample.

Compound	% isopropanol		
	0%	15%	20%
9,10-Dihydrodiol BaP	77 (±5)	96 (±4)	89 (±4)
4,5-Dihydrodiol BaP	67 (±4)	87 (±3)	84 (±3)
9-OH BaP	59 (±7)	68 (±5)	69 (±4)
3-OH BaP	47 (±9)	64 (±10)	62 (±9)
BaA	49 (±5)	66 (±4)	75 (±3)
BbF	37 (±5)	64 (±5)	68 (±3)
BkF	38 (±6)	63 (±7)	72 (±4)
BaP	36 (±7)	63 (±6)	68 (±5)

BaA = benzo[a]anthracene; BbF = benzo[b]fluoranthene; BkF = benzo[k]fluoranthene; BaP = benzo[a]pyrene.

was obtained for all compounds in these trials, except for 3-OH BaP (RSD 9%).

3.3. Optimization of the on-line preconcentration

On-line preconcentration was included in the methodology because it allows very low limits of detection. However, in this work, the extracts recovered by the SPE and MSPD methods could not be preconcentrated directly on the C18 precolumn because they were dissolved in a high percent of acetonitrile, which caused the breakthrough of the analytes. To address this issue, several standard solutions were used to simulate the extracts obtained from each method. Each solution was diluted with water in order to optimize the percentage of ACN and improve recoveries, while keeping the final volume of the diluted solution constant. Fig. 2 shows the results obtained for each preconcentrated solution: simulated E1 and E2 extracts (from SPE) or simulated D1 and D2 extracts (from MSPD).

Fig. 2A and B shows that extracts E1 and D1, containing BaP dihydrodiols, had the best recoveries when they were diluted to 10% ACN. An increase in the percentage of ACN in the extracts caused breakthrough of the analytes. Fig. 2A also shows that for the extract E2, the OH-BaP compounds had the best recoveries with 30% ACN; the PAHs in this extract had the best recoveries when the extract was diluted to 40% or 45% ACN. Thus, in the final off-line/on-line protocol, the E2 extracts were diluted to 30% ACN to preferentially achieve the highest recoveries of metabolites which are produced in very small quantities in degradation bioassays. For the D2 extracts, Fig. 2B shows that the highest recovery of the OH-BaP was obtained with 40% ACN whereas for PAHs, it was obtained with 50% ACN. For both, E2 and D2 extracts, increasing the percentage of acetonitrile to 55%, decreased the recoveries due to the breakthrough of the analytes from the precolumn. Decreasing the percentage of acetonitrile also decreased the recoveries, this time due to the adsorption of PAHs and metabolites in the vessels at a higher proportion of water. Therefore, for the final method protocol, D2 extracts were diluted to 40% ACN. In general, good recoveries were obtained for the dihydrodiols (86–104%) and lower recoveries were obtained for the other compounds (65–84%), but these values were sufficient to achieve excellent detection limits. Due to the instability of the compound 3-OH BaP during the on-line analysis, it was not possible to determine that metabolite with adequate accuracy and reproducibility in both biomass samples and liquid cultures. Therefore, the following sections discuss only the results obtained with the remaining metabolites and PAHs.

Table 6
Optimization of the off-line SPE elution sequence.

Compound	Clean-up		Analytes elution						Total recovery % (±RSD)
	S1, S2, S3	S4	S5			S6			
			1	2	3	1	2	3	
9,10-Dihydrodiol BaP	NI	3	39	31	6	–	–	–	79 (±4)
4,5-Dihydrodiol BaP	NI	–	32	41	9	–	–	–	82 (±3)
9-OH BaP	NI	–	–	–	–	69	–	–	69 (±4)
3-OH BaP	NI	–	–	–	–	62	–	–	62 (±9)
BaA	NI	–	–	–	–	54	14	5	73 (±3)
BbF	NI	–	–	–	–	48	16	6	70 (±3)
BkF	NI	–	–	–	–	46	19	6	71 (±4)
BaP	NI	–	–	–	–	41	22	4	67 (±5)

S1 = 10 mL water; S2 = 10 mL ACN/water (10:90); S3 = 2.5 mL ACN/water (20:80); S4 = 1 mL ACN/water (30:70); S5 = 3 × 1 mL ACN/water (55:45); S6 = 3 × 1 mL ACN 100%. NI = not injected. – = not detected. BaA = benzo[a]anthracene; BbF = benzo[b]fluoranthene; BkF = benzo[k]fluoranthene; BaP = benzo[a]pyrene.

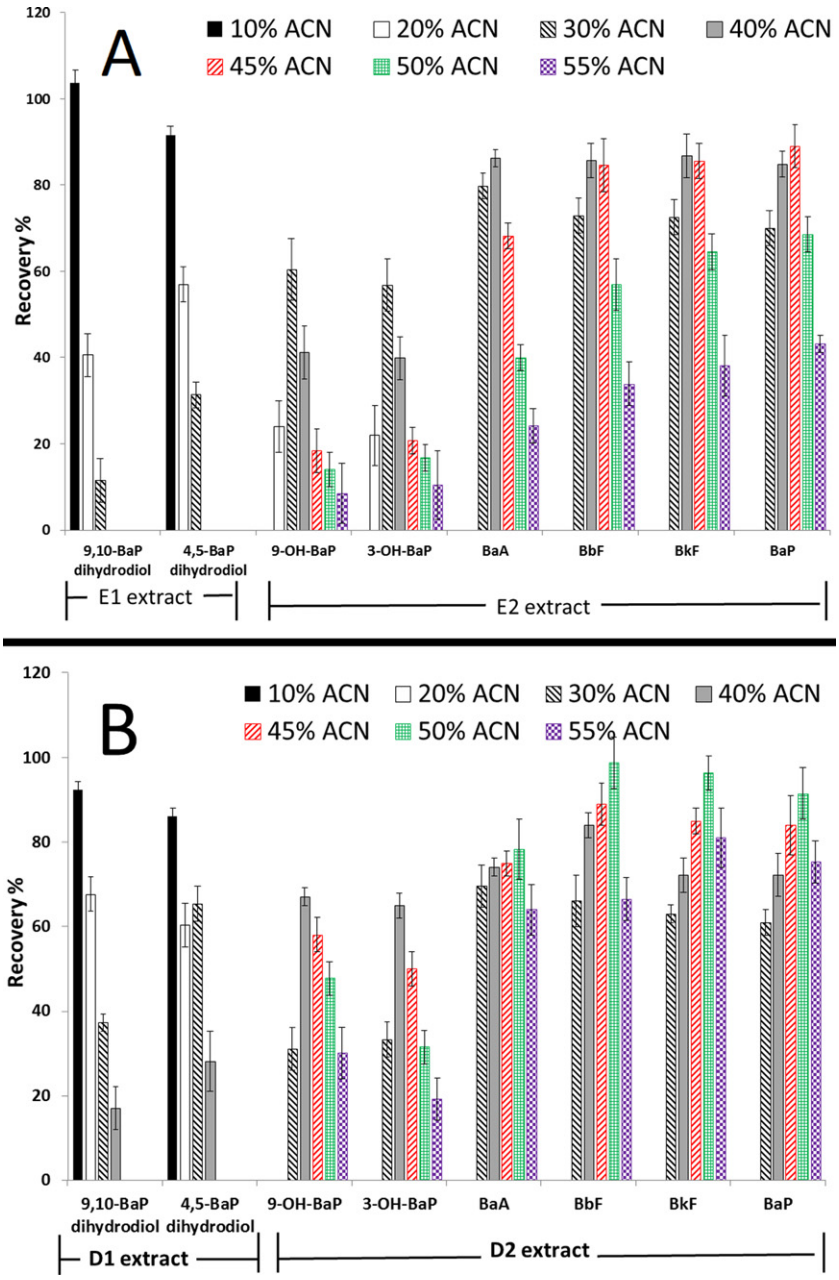

Fig. 2. Optimization of the dilution of simulated extracts for on-line SPE-HPLC preconcentration. (A) Effect of the percentage of acetonitrile on analyte recoveries loading 20 mL (E1) or 8 mL (E2) of hidro-organic standard solutions at 8 pg mL^{-1} and 27 pg mL^{-1}, respectively. (B) Effect of the percentage of acetonitrile on analyte recoveries loading 6 mL (D1) or 2 mL (D2) of hidro-organic standard solutions at 45 pg mL^{-1} and 160 pg mL^{-1}, respectively.

3.4. Validation

3.4.1. Off line/on-line method (MSPD/SPE–HPLC-FD)

3.4.1.1. Linearity and accuracy. The accuracy of the MSPD/SPE–HPLC-FD method developed here was evaluated by the determination of the recoveries of analytes from spiked dry biomass samples. For each compound, curves were constructed for the added vs. recovered amounts and the slope and correlation coefficients (r^2) were calculated. Good correlation coefficients (>0.9912) were obtained for all compounds. The global recoveries were obtained from the slope of this curve; they ranged from 40% to 66% for 9-OH BaP and BkF, respectively. These results are shown in Table 7. These recovery values were in agreement with EURACHEM and the AOAC International (Association of Official Analytical Chemists), which considers a method to be accurate if the recoveries are between 40 and 120% for analytes at ppb levels (pg mg^{-1}) [39].

3.4.1.2. Precision and lower limits. Table 7 also shows the precision of the MSPD/SPE–HPLC/FD method, which was evaluated with the relative standard deviation parameter RSD in terms of reproducibility (inter-day) at two levels of fortification (8 and 120 pg mg^{-1}). Adequate precision was found for all compounds; RDS values were ≤4.73%. These values were in agreement with AOAC which considers a method to be precise when RSD% is less than 15%. The LODs and LOQs were calculated at the optimum wavelengths. The LODs ranged from 2.0 and 6.5 pg mg^{-1} and the LOQs ranged from 6.1 and 18.7 pg mg^{-1}.

Table 7
Parameters of the added vs. recovered amount curves, precision and lower limits.

Compound[c]	9,10-Dihydrodiol BaP	4,5-Dihydrodiol BaP	9-OH BaP	BaA	BbF	BkF	BaP
Off-line/on-line MSPD/SPE-HPLC method							
Recovery (%) (slope × 100)	66	55	40	57	53	62	51
Linearity (r^2)[a]	0.9985	0.992	0.9982	0.9985	0.9941	0.9912	0.995
Reproducibility (RSD)							
8 pg mg^{-1}	4.13	4.73	2.9	2.06	3.22	2.8	3.31
120 pg mg^{-1}	2.77	3.09	2.35	1.87	2.65	2.06	2.97
LOD (S/N = 3) (pg mg^{-1})	3.3	2	2.4	3.2	6.5	5.7	2
LOQ (S/N = 10) (pg mg^{-1})	9.9	6.2	7.2	9.6	18.7	17.3	6.1
Off-line/on-line SPE/SPE-HPLC method							
Recovery (%) (slope × 100)	74	65	42	49	41	45	38
Linearity (r^2)[b]	0.9913	0.9955	0.998	0.9967	0.9965	0.9978	0.9985
Reproducibility (RSD)							
20 pg mL^{-1}	5.59	5.93	4.32	4.14	7.36	6.68	5.47
80 pg mL^{-1}	3.75	3.99	2.89	2.04	5.54	4.03	3.08
LOD (S/N = 3) (pg mL^{-1})	1.3	0.8	0.9	1	2.3	2.1	1
LOQ (S/N = 10) (pg mL^{-1})	4.4	2.5	2.8	3.2	7.5	6.9	3.4

r^2 = correlation coefficient. Precision expressed as relative standard deviation (RSD), limits of detection (LOD); limits of quantification (LOQ).
Compounds: BaA = benzo[a]anthracene; BbF = benzo[b]fluoranthene; BkF = benzo[k]fluoranthene; BaP = benzo[a]pyrene.
[a] Linearity range for all compounds was 20–200 pg mg^{-1}.
[b] Linearity range for compounds 9,10 and 4,5-dihydrodiol BaP was 5–120 pg mL^{-1}; for the rest of compounds the range was 10–120 pg mL^{-1}.
[c] Compound 3-OH BaP did not show good parameters of validation when on-line method was used.

3.4.2. Off-line/on-line method (SPE/SPE–HPLC-FD)

3.4.2.1. Linearity and accuracy. The accuracy of the SPE/SPE–HPLC-FD method was evaluated by determination of recovery efficiencies of spiked liquid culture samples. Curves for the added vs. recovered amounts were constructed and gave excellent correlation coefficients ($r^2 > 0.9913$). Table 7 shows these values and the global recoveries obtained from the slope of this curve, which ranged from 38% to 74% for BaP and 9,10-dihydrodiol BaP, respectively. These results were considered acceptable, similar to those of the MSPD method.

3.4.2.2. Precision and lower limits. The results in Table 7 show the adequate method precision that was obtained for all compounds; RSD values were ≤4.13% in terms of reproducibility (inter-day) at two levels of fortification (20 and 80 pg mL^{-1}). The LOD range was 0.8–2.3 pg mL^{-1} and the LOQ range was 2.5–7.5 pg mL^{-1}. All LOQ values were below the maximum contaminant level (MCL = 200 pg mL^{-1}) established by the EPA for BaP in water samples [40].

3.5. Application of the methodology to BaP exposure assays

The validated methodology was used to measure the residual amounts of BaP and the amounts of certain metabolites in both components of the exposed cultures after different exposure times. The results of the exposure assays showed that BaP was metabolized by microalgae as demonstrated by the decreasing BaP concentrations over time, presented in Fig. 3. Interestingly, the amount of this compound recovered from the non-inoculated cultures (NIC) was less than the expected value. Assuming an analyte recovery of 100%, with 15 mL of sample at 5 ng mL^{-1}, the expected amount of recovered BaP is 75 ng. However, in NIC, the observed levels were only approximately 31 ng at the first time point measured (45 min) and this result was in accord with the global recoveries obtained for BaP (approximately 40%). It was also observed that the amount of BaP in these samples decreased as the time of exposure increased; this result was unexpected because the cultures contained no microalgae. Therefore, this result may be explained in terms of the low polarity of heavy molecular weight PAHs, like BaP. It has been reported that PAHs trend to be adsorbed to the container when they are in aqueous solutions [20]. There was no evidence of photodegradation because no signals, different from those in the blank samples, were found in the non-inoculated cultures (NIC) that were spiked with BaP. Furthermore, Warshawsky et al. [8] studied the effect of different types of light on the photodegradation of BaP by *S. capricornutum* and they demonstrated that with the use of yellow light the process decreases significantly.

On the other hand, in the exposed microalgae cultures (EMC), the major amount of BaP was found in the biomass. It could be proposed that there was rapid adsorption of BaP to the cells, in contrast to the small residual quantity released to the liquid culture. At 45 min of exposure, averages of 23 and 5 ng were found in the biomass and liquid samples, respectively. These values show that almost 82% of recovered BaP was present in the microalgae samples. This effect has been reported for other compounds and it may be explained by the lipophilic composition of the cell membranes which have a higher affinity for nonpolar compounds than such compounds have to water [8,12,41].

From an analytical perspective, the very low detection limits achieved by the methodology developed here allowed a new biological study to be performed. This study demonstrated that with a combination of biosorption and biodegradation capacities, the microalgae *S. capricornutum* was able to reduce the BaP concentration in the liquid medium to values below the EPA limit for water (0.2 ng mL^{-1}). After 3 h of exposure, only 1.7 ng of BaP were present in aqueous liquid culture; the concentration in the 15 mL sample was 0.11 ng mL^{-1}.

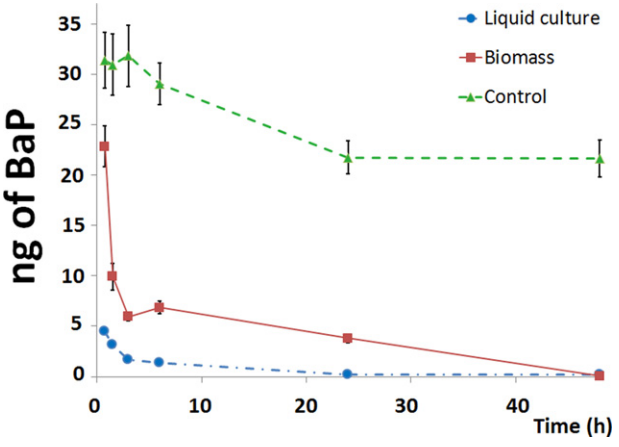

Fig. 3. Recovered amounts of BaP vs. time curves (A).

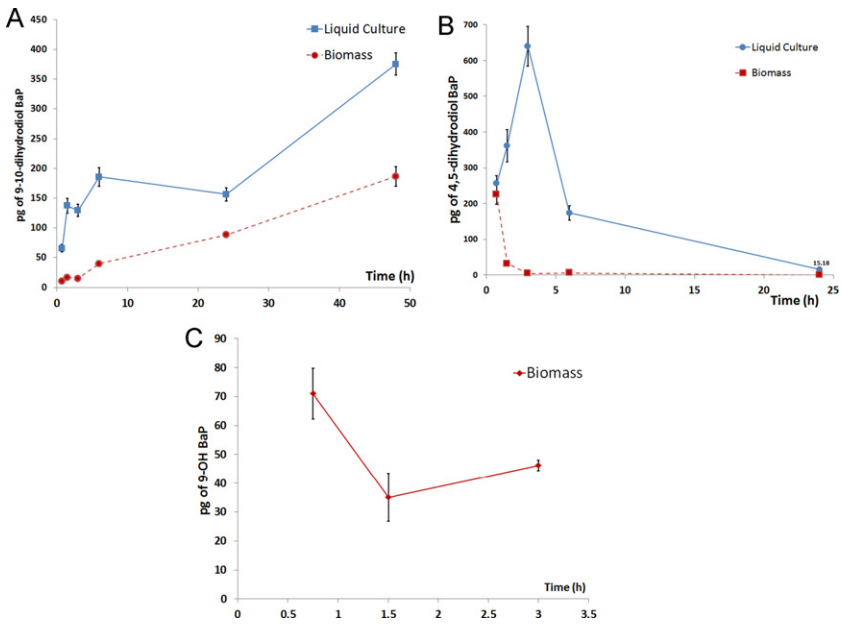

Fig. 4. Recovered amounts of (A) 9,10-dihydrodiol BaP; (B) 4,5-dihydrodiol BaP and (C) 9-OH BaP vs. time curves from the exposure to 5 ng mL^{-1} of BaP assay.

The results concerning the appearance of the metabolites *cis*-4,5-dihydrodiol BaP, cis-9-10-dihydrodiol BaP and 9-OH BaP showed that these compounds could be quantified in extracts from both liquid media and biomass samples. Fig. 4 shows the quantities recovered for each compound at different exposure times, and Fig. 5 shows representative chromatograms obtained from the extracts in these experiments. Fig. 4A shows that the compound 9,10-dihydrodiol BaP appeared in liquid media in less than 45 min and its amount in both biomass and liquid media increased with the time of exposure. The production of this metabolite was slow in contrast to 4,5-dihydrodiol BaP, which unlike the other metabolites was produced in maximal amounts at 3 h of exposure (see Fig. 4B). After 3 h, the amount of 4,5-dihydrodiol BaP decreased and finally, at 24 h of exposure, this metabolite could not be detected. Because, there are no reports about a posterior metabolism of this compound by the microalgae *S. capricornutum* and because the cultures were maintained in axenic conditions, the disappearance of this compound from the medium was attributed to its greater instability in aqueous solution than the other dihydrodiol. These results obtained for the dihydrodiols show the importance of monitoring PAHs metabolites because metabolism could increase the bioavailability of these contaminants in the nature. The behavior of these metabolites during the exposure tests is shown in Fig. 5.

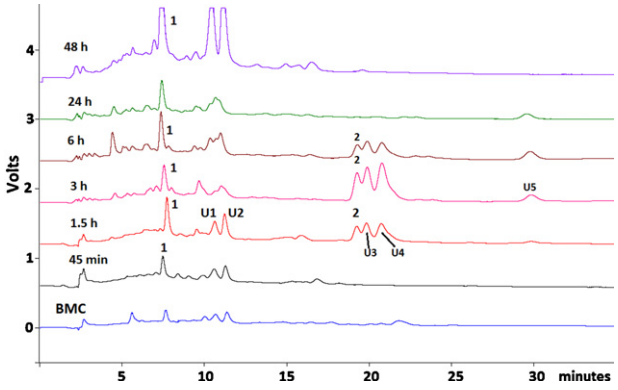

Fig. 5. Appearance of analytes in extract E1 from the exposure to 5 ng mL^{-1} of BaP assay. BMC = Blank Microalgae Culture. 1 = 9,10-dihydrodiol BaP; 2 = 4,5-dihydrodiol BaP. U1–U5 = unidentified compounds.

With respect to 9-OH BaP, the results showed that it was produced in very low amounts during the first 3 h of exposure, and after this time, this metabolite could not be determinate in cells. In liquid media, it could not be detected at any time. Thus, in contrast to the dihydrodiols BaP, the 9-OH BaP was only found in the biomass and it was produced in minor quantities (<70 pg). This result is in agreement with reports that the microalgae group metabolizes PAHs preferentially by the dioxygenase route instead of the monoxygenase route, so that production of dihydrodiols is favored [30].

The chromatograms from Fig. 5 show the appearance of other unidentified compounds, which were produced by metabolic activity of the microalgae because they were not found in the control cultures NIC or BMC. The compounds are labeled in Fig. 5 as U1–U5. The behavior of microalgae in an exposed culture at 50 ng mL^{-1} of BaP was similar to the behavior from the experiments at 5 ng mL^{-1}. Warshawsky et al. [8] reports the formation of several compounds, such as epoxides or quinones, in addition to other dihydrodiols and hydroxyl BaP that can be produced by *S. capricornutum*. However, further research must be performed to determine the concentration and structure of each compound.

4. Conclusions

A very sensitive and reproducible methodology was developed to determine the amounts of BaP metabolites and PAHs present in cultures of *S. capricornutum*. With the use of the off-line SPE or MSPD extraction and the on-line SPE/HPLC-FD system, the final analytical protocol produce a rapid process, with a simplified sample treatment compared to more common methods used based on traditional liquid–liquid extraction. The developed and validated methodology in this work provides very low LOQs at levels of pg mL^{-1} and pg mg^{-1}. Thus, it can be used to determine the small amounts of BaP metabolites that are produced by microalgae cultures in exposure test, using a very small size of sample of biomass and liquid media and without requiring the use of a very high concentration of BaP in an exposure bioassay. The microalgae cultures exposed to 5 ng mL^{-1} of BaP were able to reduce the levels of contaminant in the liquid medium to less than the EPA limit (<0.2 ng mL^{-1}). The results obtained are in agreement with

information reported in previous works by other authors in that the metabolites favored by the microalgae are the dihydrodiols. Though, there are some reports that monitored PAHs degradation using this type of samples, the current work presents a validated methodology that allows determination of the recovered quantities of different PAHs. Most previous reports are focused on determining the degradation only of less polar PAHs and BaP, but the current methodology could be a useful tool to provide more information about the degradation of heavy PAHs with low water solubility.

Acknowledgements

This work was supported by the *Dirección General de Asuntos de Personal Académico* from the Universidad Nacional Autónoma de México (DGAPA-UNAM) Projects PAPIIT IN223111 and PAPIME PE202310. Also, the authors thank the Consejo Nacional de Ciencia y Tecnología de México – CONACyT for the project 166389 and the doctoral scholarship awarded to J.J. Olmos Espejel (No. 204859).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2012.09.015.

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