Microchemical Journal 129 (2016) 83–89

Contents lists available at ScienceDirect

Microchemical Journal

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

Room temperature fluorescence spectroscopy of benzo[*a*]pyrene metabolites on octadecyl extraction membranes

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ARTICLE INFO

Article history:
Received 20 April 2016
Received in revised form 6 June 2016
Accepted 6 June 2016
Available online 07 June 2016

Keywords:
Benzo[*a*]pyrene
Benzo[*a*]pyrene metabolites
Solid-phase extraction
Room-temperature fluorescence
Urine analysis
Asymmetric least squares

ABSTRACT

Benzo[*a*]pyrene (B[*a*]P) is a prototypic carcinogenic polycyclic aromatic hydrocarbon (PAH), which requires metabolic activation to produce its detrimental effects. Measurement of B[*a*]P metabolites in human urine could provide a direct way to assess individual differences in susceptibility to PAH-related cancer. This article focuses on the development of screening methodology for the routine analysis of B[*a*]P metabolites in urine samples. It explores the solid-surface room-temperature fluorescence (RTF) properties of 3-hydroxy-benzo[*a*]pyrene, benzo[*a*]pyrene-*trans*-9,10-dihydrodiol, benzo[*a*]pyrene-r-7,t-8,c-9-tetrahydrotriol and benzo[*a*]pyrene-r-7,t-8,c-9,c-10-tetrahydrotetrol previously extracted from urine samples with octadecyl-silica membranes. Relative standard deviations varying from 2.1% (benzo[*a*]pyrene-r-7,t-8,c-9-tetrahydrotriol) to 8.6% (3-hydroxy-benzo[*a*]pyrene) are obtained with the aid of fiber optic probe that eliminates the need for manual optimization of signal intensities. Analytical recoveries from human urine samples varied from 87.5 ± 3.1% (3-hydroxy-benzo[*a*]pyrene) to 99.8 ± 2.5% (benzo[*a*]pyrene-r-7,t-8,c-9,c-10-tetrahydrotetrol). The excellent analytical figures of merit and the simplicity of the experimental procedure demonstrate the potential of this approach for screening biomarkers of PAH exposure in numerous urine samples.

1. Introduction

Considerable efforts have been made to improve luminescence measurements of compounds adsorbed on solid substrates [1,2]. As a result, solid surface luminescence analysis is a widely accepted tool in environmental, pharmaceutical, food and agricultural science [3]. Particularly attractive is the use of solid-phase extraction (SPE) membranes for the on-site analysis of polycyclic aromatic compounds in water samples [4–8]. Attractive features include a simple experimental procedure and compatibility with portable instrumentation. Due to the non-destructive nature of luminescence measurements, extraction membranes can be brought to the lab for subsequent sample elution and fluorophore confirmation via high-resolution techniques [9–12].

Solid surface room temperature fluorescence (RTF) spectroscopy on SPE membranes was first proposed for the analysis of polycyclic aromatic hydrocarbons (PAHs) in water samples [5,13]. Octadecyl silica membranes were cut into tabs and subsequently immersed into aqueous solution for PAHs extraction. Depending on the PAH, the extraction step varied from one to two hours of immersion time. After drying for 5 min, the tabs were examined via front surface room-temperature fluorescence spectroscopy to reach limits of detection (LODs) at the parts-per-billion ($ng \cdot mL^{-1}$) level [5,13]. Later reports extended the use of SPE membranes to the room temperature phosphorimetry (RTP) analysis of PAHs [6,14–17], polychlorinated biphenyls [15,17] and polychlorinated dibenzofurans [17]. Bulky glassware and vacuum pumps, common to classic SPE lab procedures, were replaced with a syringe kit well-suited for manual extraction under field conditions. A rapid air-drying step, which was manually accomplished by applying positive pressure to the syringe, removed the excess of water from the extraction membrane prior to spectroscopic measurements. Total analysis time took less than 10 min per sample and provided LODs at the parts-per-billion ($pg \cdot mL^{-1}$) concentration level.

This article focuses on the urine analysis of PAH metabolites via SPE-RTF. Although chromatographic techniques provide reliable results for the analysis of PAH metabolites [18–28], the development of easy-to-use and cost effective techniques with high sample throughput is relevant for the assessment of PAHs uptake by large populations [22]. Previous articles from our group targeted monohydroxy-PAHs (OH-PAHs) [29,30]. Signal reproducibility from measurements on extraction membranes was improved with the aid of a sample holder specifically

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http://dx.doi.org/10.1016/j.microc.2016.06.010
0026-265X/

designed for the manual optimization of luminescence signals. Background correction of solid substrates was carried out with the aid of Asymmetric Least Squares (ALS), a smoothing algorithm originally devised for baseline correction of chromatographic data [31–33]. 2-hydroxy-fluorene (2OH-FLU), 1-hydroxypyrene (1OH-PYR), 3-hydroxy-benzo[*a*]pyrene (3OH-B[*a*]P) and 9-hydroxy-phenanthrene (9OH-PHE) were determined at the parts-per-trillion concentration level with relative standard deviations (RSDs) varying from 3.5% (2OH-FLU) to 9.5% (9OH-PHE). The application of ALS to SPE-RTF improved the LODs by approximately two orders of magnitude. With only 10 mL of urine sample, the LODs of OH-PAHs varied from 57 pg mL^{-1} (2OH-FLU) to 2 pg mL^{-1}(1OH-PYR). Recovery values from urine samples varied from 99.0 ± 1.2% (3OH-B[*a*]P) to 99.9 ± 0.05% (1OH-PYR) [29].

Herein, the application of SPE-RTF is extended to the urine analysis of benzo[*a*]pyrene-*trans*-9,10-dihydrodiol (B[*a*]P-diol), benzo[*a*]pyrene-r-7,t-8,c-9-tetrahydrotriol (B[*a*]*P*-triol) and benzo[*a*]pyrene-r-7,t-8,c-9,c-10-tetrahydrotetrol (B[*a*]P-tetrol). RSDs varying from 2.1% (B[*a*]P-triol) to 8.6% (3-OH B[*a*]P) were obtained with the aid of a fiber optic probe that eliminates the need for manual optimization of signal intensities. ALS background correction provided LODs at the pg·mL^{-1} range. Analytical recoveries from human urine samples varied from 87.6 ± 3.1% (3OH-B[*a*]P) to 99.8 ± 2.5% (B[*a*]P-tetrol). The excellent analytical figures of merit and the simplicity of the experimental procedure demonstrate the potential of SPE-RTF for screening biomarkers of PAH exposure in numerous urine samples.

2. Experimental

2.1. Chemicals and materials

All solvents were Aldrich HPLC grade. All chemicals were analytical-reagent grade and utilized without further purification. Unless otherwise noted, Nanopure water was used throughout. B[*a*]P-diol, B[*a*]P-triol and B[*a*]P-tetrol were purchased from Sigma–Aldrich. 3OH-B[*a*]P was from Midwest Research Institute. All other chemicals were purchased from Fisher Chemical. The Sep-Pak C-18 membranes were purchased from Varian/Agilent. The synthetic urine solution was manufactured by RICCA Chemical Company (Arlington, TX) and purchased from Fischer Scientific. Human urine samples obtained from an anonymous volunteer group of healthy non-smoking individuals were pooled, frozen and stored at 4 °C until further analysis.

2.2. Preparation of stock solution of B[a]P metabolites

Stock solutions of B[*a*]P metabolites (100 μg/mL) were prepared by dissolving 1.0 mg of standards in 10 mL of methanol. All stock solutions were kept in the dark at 4 °C. Prior to use, stock solutions were monitored via RTF spectroscopy for possible photo-degradation of metabolites. All stock solutions were used within 6 months of preparation. Working solutions of B[*a*]P metabolites were prepared daily by serial dilution of stock solutions.

2.3. Hydrolysis of urine samples

Hydrolysis of urine samples followed the procedure reported previously [29,30]. 8 mL of urine sample were spiked with 1 mL of metabolite stock solution of appropriate concentration and equilibrated for 30 min to allow for the interaction of B[*a*]P metabolites with urine components. Then 500 μL of 0.1 M HCl were added to the sample and the mixture was buffered with 500 μL of 0.05 M potassium biphthalate sodium hydroxide buffer (pH 5.0). The buffered sample was shaken for 30 min at 1400 rpm to allow for urine hydrolysis.

2.4. SPE with octadecyl membranes

A cork borer with an inside diameter of 8 mm was used to dissect 47 mm C-18 membranes into 8 mm extraction disks. 8 mm disks were individually loaded into a stainless steel filter syringe kit (Alltech) and then connected to a 10 mL Hamilton syringe. Manual positive pressure forced all liquid solutions through the disk. The optimization of experimental parameters for best retention of B[*a*]P metabolites on extraction membranes led to the following procedure: standard metabolite solutions and urine samples were processed through extraction membranes previously conditioned with 5 mL methanol and 5 mL water. Following sample extraction, each membrane was sequentially rinsed with 5 mL of water and 5 mL of 1% ammonium hydroxide. Void water was mechanically removed from the membrane by 300 mL of air through the disk with the 100 mL syringe.

2.5. Fluorescence background treatment of extraction membranes

Reduction of fluorescence background from extraction membranes was accomplished with a thin layer chromatography (TLC) procedure previously developed in our lab [29]. Each chromatographic run was carried out with 34 mm × 40 mm membranes strips immersed 5 mm deep in methanol for approximately 15 min. This time period was enough for the fluorescence background to reach the maximum migration distance towards the top of the strip. Each membrane strip was submitted to 3 chromatographic runs to achieve the minimum fluorescence background possible. The extraction disks used for SPE-RTF measurements were cut from the strip areas with low fluorescence backgrounds.

2.6. RTF measurements

Fig. 1 shows a schematic diagram of the fiber optic probe (FOP) and the instrumentation used in these studies. The FOP consisted of one delivery and six collection fibers. All the fibers were 3 m long and 500 μm-core diameter silica-clad silica with polyimide buffer coating (Polymicro Technologies, Inc.). The fibers were fed into a 1.2-m-long section of copper tubing that provided mechanical support for lowering the probe into the liquid helium. At the sample end, the fibers were arranged in a conventional six-around-one configuration with the delivery fiber in the center, bundled with vacuum epoxy (Torr-Seal, Varian Vacuum Products), and fed into a metal sleeve for mechanical support. The copper tubing was flared stopping a Swage nut tapped to allow for the threading of a 0.75-mL polypropylene sample vial. At the measurement end, the collection fibers were bundled with vacuum epoxy into a slit configuration, fed into a metal sleeve, and aligned with the entrance slit of the spectrometer.

Fluorescence measurements were carried out with a FluoroMax-3 (Horiba Jobin Yvon, Edison, NJ) equipped with a 450 W xenon arc source. The 1200 grooves/mm gratings in the single excitation and emission monochromators were blazed at 330 and 500 nm, respectively. Their reciprocal linear dispersion was equal to 4.25 nm/mm. The uncooled photomultiplier tube (Hamamatsu, Model R928) detector was operated in the photon- counting mode. Commercial software (DataMax, version 2.20, Horiba Jobin Yvon) was used for automated scanning and fluorescence data acquisition. The excitation fiber and the emission bundle of the fiber optic probe (FOP) were coupled to the sample compartment of the spectrofluorimeter with the aid of a commercial fiber optic mount (F-3000, Horiba Jobin Yvon) that optimized collection efficiency via two concave mirrors. Position alignment of each end of the FOP with the respective focusing mirror was facilitated using commercially available adapters (Horiba Jobin Yvon).

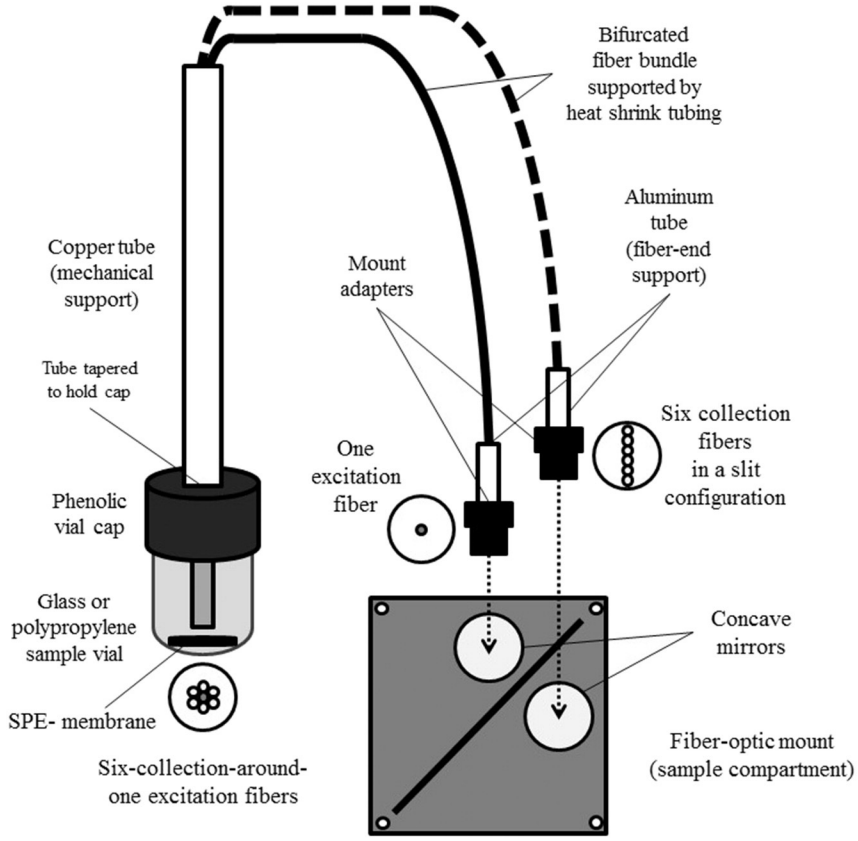

Fig. 1. Fiber optic probe for fluorescence measurements at liquid nitrogen temperature (77K).

2.7. ALS software

Routines for data pre-treatment and processing were written in MATLAB [34]. Baseline routines for emission background correction were adapted from routines previously described for baseline correction of chromatographic data [35]. Implementation of the ALS algorithm included a smoothing parameter equal to 1 × 107, an asymmetry parameter equal to 0.001, an order of differences in penalty equal to 3 and a single regularization parameter, whose value was 1.

3. Results and discussion

Considerable efforts have been made to develop bio-analytical assays for the determination of PAH metabolites in physiological fluids.

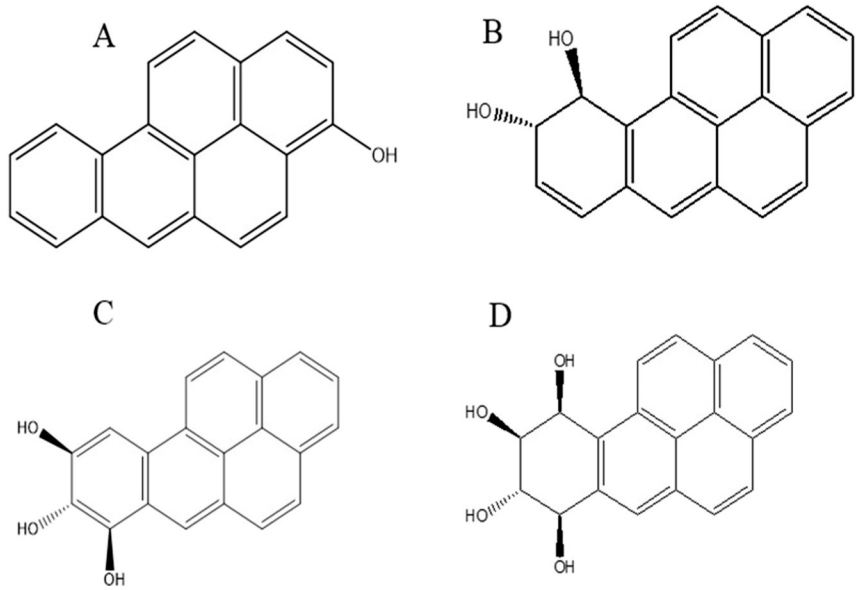

Fig. 2. Molecular structures of (A) 3-hydroxyl-benzo[*a*]pyrene; (B) Benzo[*a*]pyrene-*trans*-9,10-dihydrodiol; (C) Benzo[*a*]pyrene-*r*-7,*t*-8,*c*-9- tetrahydrotriol ; (D) Benzo[*a*]pyrene-*r*-7,*t*-8,*c*-9,*c*-10- tetrahydrotetrol.

Table 1
Analytical Figures of Merit reported for B[*a*]P metabolites analysis in urine samples by different analytical methods.

Metabolites	LOD (ng L^{-1})	Recovery %	Sample preparation	Instrumental method	Analysis time (min)[a]	Reference
3-OH B[*a*]P	1615	40	SPE, evaporation	HPLC	40	[18]
3-OH B[*a*]P	40	48	SPE, evaporation	HPLC	45	[19]
3-OH B[*a*]P	44	68	SPE, evaporation	HPLC	41	[20]
3-OH B[*a*]P		80	SPE, evaporation	LC-MS/MS	7	[21]
B[*a*]P-diol		79	SPE, evaporation	HPLC	70	[22][b]
B[*a*]P-diol		90	SPE, evaporation	Spectrofluorometric detection		[23]
B[*a*]P-diol	70		LLE[a], evaporation, HPLC fraction, derivatization	GC–MS	45	[24][c]
B[*a*]P-tetrol	10	34	SPE, evaporation, HPLC fraction	Fluorescence spectroscopy		[25]
B[*a*]P-tetrol	50		SPE, evaporation	HPLC	14	[26]
B[*a*]P-tetrol		44	SPE, evaporation	GC–MS/MS	14	[27]
B[*a*]P-tetrol			SPE, evaporation, HPLC fraction, derivatization	GC–MS/MS	15	[28]

[a] Instrumental analysis only; does not include sample preparation procedures.
[b] In rat urine.
[c] LLE = liquid − liquid extraction.

A combination of preparation techniques is often necessary to reach the limits of detection of the instrumental method of analysis [36–39]. Analysis of metabolites has been accomplished via high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) and gas chromatography (GC). Ultraviolet–visible absorption and RTF detection have been widely used with both HPLC and CE [40–45]. The ultimate specificity belongs to high-resolution MS either coupled to gas chromatography (GC–MS) or HPLC (HPLC-MS) [46–51].

The molecular structures of the metabolites chosen for this study are shown in Fig. 2. These four compounds provide an example of the rich and heterogeneous distribution of the metabolic products B[*a*]P produce. B[*a*]P is the most toxic PAH in the EPA priority pollutants list and it is often used as a measure of risk. A summary of the analytical approaches developed for the determination of B[*a*]P metabolites in urine samples is presented in Table 1. The extent of our literature search revealed no reports on the analysis of B[*a*]P-triol in urine samples.

3.1. RTF Analytical Figures of Merit (AFOM) of B[a]P Metabolites in Aqueous Solutions

Fig. 3 shows the room-temperature excitation and fluorescence spectra of B[*a*]P metabolites in methanol/water (10% v/v) solutions. All measurements were made from standard (1 × 1 cm) quartz cuvettes filled with undegassed solutions. All spectra were collected at 90° from the excitation beam using 3 nm excitation and emission band-pass. No attempts were made to adjust slit widths for optimum spectral

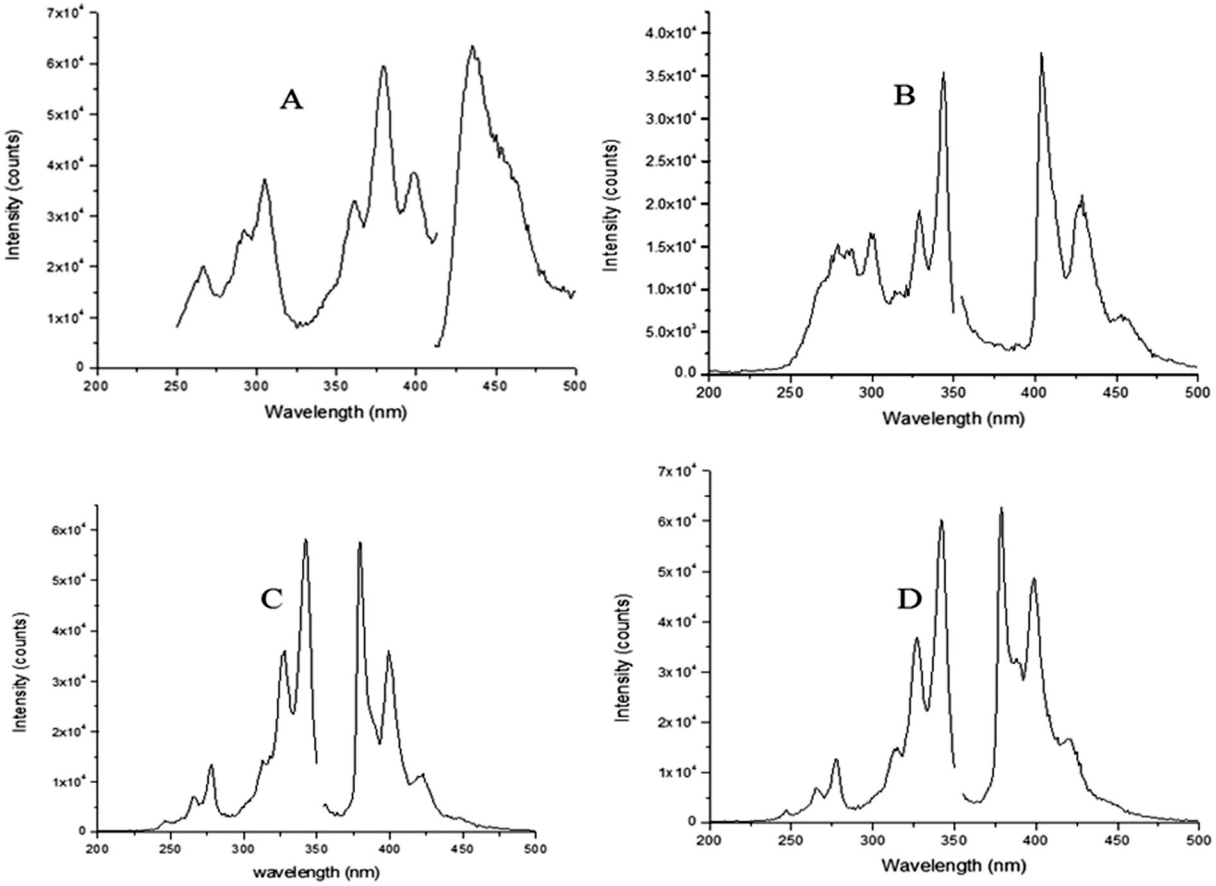

Fig. 3. Room-temperature excitation and fluorescence spectra of (A) 20 ng·mL^{-1} 3-hydroxy Benzo[a]pyrene, (B) 20 ng·mL^{-1} Benzo[*a*]pyrene-*trans*-9,10-dihydrodiol, (C) 20ng·mL^{-1} benzo[a]pyrene-r-7,t-8,c-9-tetrahydrotriol and (D) 20ng·mL^{-1} Benzo[*a*]pyrene-*r*-7,*t*-8,*c*-9,*c*-10-tetrahydrotetrol recorded from pure standard solutions prepared in 10% methanol/water (v/v).

Table 2
RTF Analytical Figures of Merit in 10% methanol/water solutions.

B[*a*]P metabolite	λexc/λem[a] (nm)	LDR[b] (ng mL^{-1})	R^{2}[c]	RSD[d]	LOD[e] (ng mL^{-1})
3-OH B[*a*]P	380/435	3.7–40	0.9902	4.5	1.2
B[*a*]P-diol	344/404	2.4–40	0.9918	1.0	0.7
B[*a*]P-triol	343.5/379	1.1–40	0.9975	1.7	0.3
B[*a*]P-tetrol	344/379	0.97–40	0.9979	2.0	0.3

[a] Excitation and emission wavelengths.
[b] LDR = linear dynamic range extending from the limit of quantification (LOQ) to an arbitrarily chosen upper linear concentration. LOQ defined as 3.3 × LOD.
[c] Correlation coefficient of calibration curve.
[d] RSD = relative standard deviation in %. Calculated as RSD = $[S_{av}/X_{av}] * 100$; where S_{av} is the standard deviation and X_{av} is the average intensity of three independent measurements from three aliquots.
[e] Limit of detection in calculated as $3 \times S_B/m$, where S_B is the standard deviation of 16 blank measurements and m is the slope of the calibration curve.

resolution, nor were the spectra corrected for instrumental response. The 3 nm band-pass provided signal-to-blank ratios higher than 3 for all the studied metabolites at the trace concentration level. No significant changes were observed from spectra of B[*a*]P metabolites in hydrolyzed urine samples or adsorbed on extraction membranes.

Table 2 summarizes the RTF AFOM of the studied metabolites. All measurements were made at the maximum excitation and fluorescence wavelengths of each compound. Each calibration curve was built with a minimum of four B[*a*]P metabolites concentrations. For each concentration plotted in the calibration graph, the RTF intensity was the average of three determinations taken from three sample aliquots (N = 3). No efforts were made to experimentally obtain the upper concentration limit of the calibration curve. The correlation coefficients of the calibration curves were close to unity, indicating a linear relationship between metabolite concentration and signal intensity. The linearity was also evaluated by an ANOVA test as suggested by IUPAC [52] with satisfactory results. The LODs were calculated using the equation LOD = $3 \times S_B/m$, were S_B is the standard deviation of 16 blank determinations and m is the slope of the calibration curve. The limits of quantitation (LOQ) were calculated with the formula LOQ = $10 \times S_B/m$. The slopes of the calibration curves were obtained with the least squares method. The strong fluorescence intensity resulting from the rigid and delocalized **π**-electron system of B[*a*]P metabolites provides LOD and LOQ values at the ng·mL^{-1} concentration level.

3.2. Extraction efficiency of SPE membranes

Examination of previously reported SPE procedures shows a wide range of recoveries for the studied metabolites (see Table 1). The general trend is to elute B[*a*]P metabolites from SPE cartridges with milliliter volumes of methanol, which are then evaporated to micro-litters (or even dryness) for sample pre-concentration. Table 3 summarizes the extraction efficiencies (%E) of the studied metabolites obtained via SPE-RTF. Since metabolite elution is not required for the determination of metabolites on the surface of the extraction membrane, the %E values in Table 3 correspond to the analytical recovery of the method. The %E values were calculated with the formula %E = (IBE − IAE/IBE) × 100, where IBE and IAE refer to the fluorescence signals before and after extraction, respectively. In all cases, the volume of extracted sample was

Table 3
Percentage of metabolite retention on C-18 membranes from aqueous solutions, synthetic urine and human urine samples.

B[*a*]P metabolite	Aqueous solution[a]	Synthetic urine	Human urine
3-OH B[*a*]P	87.6 ± 3.1	90.3 ± 2.8	82.1 ± 4.9
B[*a*]P-diol	99.2 ± 3.1	97.8 ± 2.8	93.9 ± 3.5
B[*a*]P-triol	98.6 ± 4.3	98.1 ± 2.2	92.4 ± 3.8
B[*a*]P-tetrol	99.8 ± 3.0	98.9 ± 3.0	91.9 ± 4.1

[a] 10% methanol/water solutions.

Table 4
SPE-RTF AFOM of B[*a*]P metabolites.

B[*a*]P metabolite	$\lambda_{exc}/\lambda_{em}$[a]	LDR[b] (ng mL^{-1})	R^{2}[c]	LOD[d] (ng mL^{-1})
3-OH-B[*a*]P	381/425	9.9–40	0.9746	2.9
B[*a*]P-diol	345/405	2.8–40	0.9935	0.8
B[*a*]P-triol	345/380	4.2–40	0.9981	1.3
B[*a*]P-tetrol	345/375	2.9–40	0.9967	0.9

[a] Excitation and emission wavelengths.
[b] LDR = linear dynamic range extending from the limit of quantification (LOQ) to an arbitrarily chosen upper linear concentration. LOQ defined as 3.3 × LOD.
[c] Correlation coefficient of calibration curve.
[d] Limit of detection calculated as $3 \times S_B/m$; where S_B is the standard deviation of 6 blank measurements and m is the slope of the calibration curve.

10 mL. The mass of extracted metabolite did not surpass the nominal breakthrough mass (30 mg) of extraction membranes. Within a confidence interval of 95% (N = 3), all %E were equivalent to 100%. The same is true for all the concentrations within the LDRs in Table 2. The %E values obtained from standard solutions in 10% methanol/water are statistically the same (P = 95%; $N_1 = N_2 = 3$; $t_{crit} = 2.78$) as those obtained from urine samples. This is an indication that the chemical compositions of synthetic and human urine samples do not interfere with the retention of B[*a*]P metabolites on extraction membranes.

3.3. AFOM of B[a]p metabolites via SPE-RTF

The SPE-RTF AFOMs of the four studied metabolites are summarized in Table 4. SPE was carried out with 10 mL of standards prepared in 10% methanol/water (v/v). For each concentration plotted in the calibration graph, the RTF intensity was the average of at least three determinations taken from three extraction disks (N = 9). The LDRs were obtained with a minimum of five B[*a*]P metabolites concentrations. No efforts were made to experimentally obtain the upper concentration limits of the calibration curves. It is important to note, however, that the highest concentration plotted in each calibration curve did not surpass the breakdown volume of the SPE device [16]. The correlation coefficients of the calibration curves and the slopes of the log-log plots (data not shown) were close to unity, indicating a linear relationship between B[*a*]P metabolite concentration and fluorescence intensity. Satisfactory results were also obtained by the ANOVA test suggested by IUPAC [52]. The RSDs at medium linear concentrations were lower than 10%. The LODs were estimated at the parts-per-billion to sub-parts-per-billion concentration levels. Their comparison to the LOD values in Table 2 shows no advantage of pre-concentrating the sample prior to RTF.

Table 5 summarizes the slopes of the calibration curves, blank intensities and standard deviations obtained from aqueous solutions and extraction membranes. Sample pre- concentration via SPE provides 10-fold improvements on the slopes of the calibration curves. The main reason for the lack of LOD improvements is the higher intensities of blank signals and their respective standard deviations on extraction membranes.

Table 5
Slope and blank signals obtained from calibration curves in Table 5.

B[*a*]P metabolites	Slope[a]		Average blank intensity ± standard deviation[b] (cps)	
	H_2O (×10^3)	Membrane (×10^4)	H_2O (×10^3)	Membrane (×10^4)
3-OH B[*a*]P	3.9	2.3	8.5 ± 0.6	6.2 ± 0.5
B[*a*]P-diol	2.0	8.5	1.3 ± 0.2	5.5 ± 0.2
B[*a*]P-triol	2.6	8.1	2.2 ± 0.3	7.4 ± 0.2
B[*a*]P-tetrol	2.8	8.2	2.3 ± 0.3	7.3 ± 0.2

[a] Slope of linear dynamic range obtained via the least squares method.
[b] Average values based on three individual measurements recorded from the extraction membrane submitted to the entire procedure.

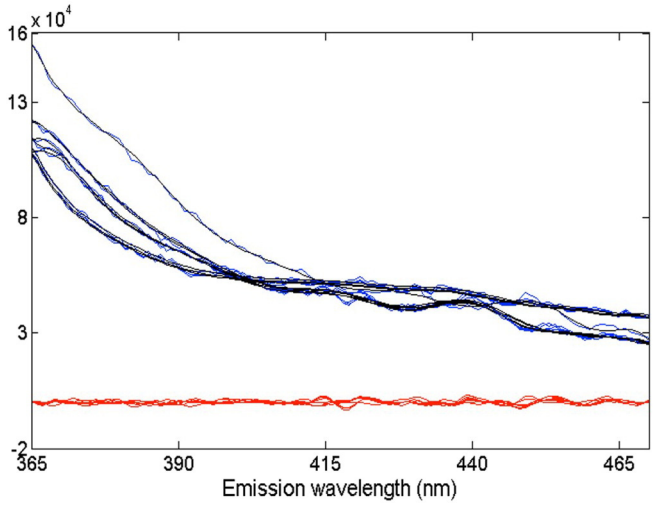

Fig. 4. Blank signals before (top; blue) and after (bottom; red) ALS background correction.

3.4. Background correction via ALS

Several experimental attempts have been made in our lab to reduce the fluorescence background of extraction membranes [29]. All attempts have assumed the presence of at least one fluorescence impurity as the source of observed background. In addition to the TLC procedure previously described in Section 2.5, background reduction attempts included pre-flushing individual disks with increasing volumes (10, 20 and 30 mL) of pure methanol and ultraviolet irradiation in a photochemical reactor equipped with a total of twelve lamps with emission maxima at 254 nm, 300 nm and 320 nm. The best background reductions were obtained with the TLC procedure.

A more efficient approach to improve LODs was found to be the treatment of background data with the ALS algorithm [29]. This algorithm uses the Whittaker smoother for discrete (time) series, which minimizes the function [51]:

$$Q = \sum_i vi(yi - fi)^2 + p$$

where y is the data (experimental signal), f a smooth trend (or baseline estimation), υ prior weights, and p the asymmetry parameter. The implementation of ALS to the background correction of extraction membranes was best achieved with a smoothing parameter equal to 1×107, an asymmetry parameter equal to 0.001, an order of differences in penalty equal to 3 and a single regularization parameter. Fig. 4 shows the improvement that was obtained with the implementation of ALS. The background intensity (I_B) of extraction membrane was considerably reduced and so it was the standard deviation (S_B) of the average background signal. Table 6 summarizes the effect of ALS on the SPE-RTF AFOMs of the studied metabolites. Comparison to Table 2 reveals LOD improvements ranging from 25× (B[*a*]P-triol) to 42× (B[*a*]P-diol). Upon background correction with ALS, the LODs via SPE-RTF meet the expectations for the analysis of B[*a*]P metabolites in urine samples [36–45].

Table 6
SPE-RTF-ALS AFOMs of B[*a*]P metabolites.

PAH metabolite	$\lambda_{exc}/\lambda_{em}$[a]	LDR[b] (ng mL^{-1})	R^2[c]	LOD[d] (ng mL^{-1})
3-OH– B[*a*]P	381/425	0.3–40	0.9924	0.1
B[*a*]P-diol	345/405	0.1–40	0.9995	0.02
B[*a*]P-triol	345/380	0.2–40	0.9987	0.05
B[*a*]P-tetrol	345/375	0.1–40	0.9966	0.03

[a] Excitation and emission wavelengths.
[b] LDR = linear dynamic range extending from the limit of quantification (LOQ) to an arbitrarily chosen upper linear concentration. LOQ defined as 3.3 × LOD.
[c] Correlation coefficient of calibration curve.
[d] Limit of detection calculated as 3 × SB/m; where SB is the standard deviation of 6 blank measurements and m is the slope of the calibration curve.

4. Conclusion

Several features make SPE-RTF spectroscopy an attractive approach for screening B[*a*]P metabolites in urine samples. The use of the FOP allows for a straightforward procedure with potential application to the routine analysis of numerous samples. The direct determination of B[*a*]P metabolites on the surface of the extraction membrane eliminates the need for subsequent elution steps and provides excellent metabolite recoveries (~100%). Background correction of extraction membranes via ALS provides LODs at the parts-per-trillion concentration level. With 10 mL of urine samples, the LODs varied from 20 pg·mL^{-1} (B[*a*]P-tetrol) to 110 pg·mL^{-1} 3-OH-Pyr). These values compare favorably to LODs previously reported via chromatographic methods [18–28].

The statistical equivalence of the overall recoveries in Table 3 demonstrates the lack of interference from human urine samples of unknown composition. The accurate determination of mono-hydroxy PAH metabolites with highly overlapped excitation and fluorescence spectra has been demonstrated in our lab via excitation-emission matrix [53] and/or synchronous excitation [54] spectroscopy. Spectral overlapping was resolved with the aid of multi-way partial least-squares and residual bi-linearization [53,54]. Accurate determination of 3-OH-B[*a*]P, B[*a*]P-diol, B[*a*]P-triol and B[*a*]P-tetrol on octadecyl membranes without the need of previous separation has been recently accomplished in our lab with the aid of time-resolved excitation–emission matrix spectroscopy. The application of parallel factor analysis (PARAFAC) to resolved spectral overlapping in time-resolved excitation emission matrices will be the subject of a near future publication.

Acknowledgements

A. V. Schenone acknowledges Fulbright and Fundación Bunge y Born for her fellowship. H. C. Goicoechea acknowledges UNL (project # CAID II) and CONICET (project # PIP 0345) for financial support. F. Barbosa Jr. and A. D. Campiglia are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial support (project # 2014/00986-6).

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