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Room-temperature fluorescence spectroscopy of monohydroxy metabolites of polycyclic aromatic hydrocarbons on octadecyl extraction membranes

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ABSTRACT

Urine analysis of monohydroxy metabolites is recognized as an accurate assessment of human exposure to polycyclic aromatic hydrocarbons. Despite the sophisticated arsenal of analytical tools, monitoring of monohydroxy metabolites via simple, cost effective and direct methods of analysis still remains a challenge. This article evaluates the analytical potential of solid-phase extraction room-temperature fluorescence spectroscopy for the problem at hand. Extraction membranes serve the dual purpose of sample pre-concentration and solid substrate for RTF measurements. The potential of our proposition is demonstrated with the analysis of 2-hydroxy-fluorene, 1-hydroxy-pyrene, 3-hydroxy-benzo[*a*]pyrene and 9-hydroxy-phenanthrene in synthetic urine samples. Signal reproducibility is improved with the aid of a sample holder specifically designed for the manual optimization of luminescence signals. Background correction of solid substrates is carried out with the aid of Asymmetric Least Squares. Recovery values for the studied metabolites varied from 99.0 ± 1.2% (3-hydroxy-benzo[*a*]pyrene) to 99.9 ± 0.05% (1-hydroxy-pyrene). With only 10 mL of urine sample, the limits of detection varied from 57 pg mL^{-1} (2-hydroxy-fluorene) to 2 pg mL^{-1} (1-hydroxy-pyrene). Additional figures of merit include a simple experimental procedure for routine screening of numerous samples and compatibility with portable instrumentation for field analysis. Because of the non-destructive nature of fluorescence measurements, membranes can be brought to the lab for subsequent elution and confirmation of compounds via high-resolution techniques.

1. Introduction

Considerable efforts have been made to improve analytical measurements of the fluorescence and phosphorescence of compounds on solid materials [1,2]. As a result, solid-surface luminescence analysis is a widely accepted tool in environmental, pharmaceutical, food and agricultural science [3]. A variety of solid substrates have been used in solid-surface luminescence such as filter paper, silica gel, sodium acetate and polymers. Particularly attractive is the use of solid-phase extraction (SPE) membranes for the analysis of polycyclic aromatic compounds in water samples [4–8]. The analytical merits include simple experimental procedures for routine screening of numerous samples and compatibility with portable instrumentation for field analysis. Because of the non-destructive nature of luminescence measurements, can be brought to the lab for subsequent elution and confirmation of compounds via high-resolution techniques [9–12].

The concept was first applied to the analysis of polycyclic aromatic hydrocarbons (PAHs) in water samples [5,13]. Many PAHs are highly suspect as etiological agents in human cancer [14] and their water monitoring is of great environmental and toxicological importance [15]. Octadecyl extraction membranes were cut in tabs and suspended into aqueous solutions for PAHs extraction. Depending on the PAH, extraction times varied between one and two hours. After drying for 5 min the tabs were examined via front-surface room-temperature fluorescence (RTF). Limits of detection (LOD) were estimated at the parts-per-billion (ng mL^{-1}) level [5,13]. Campiglia and co-workers extended the concept to room-temperature phosphorescence (RTP) analysis. SPE-RTP was applied to the analysis of PAHs [6,16–18], polychlorinated biphenyls [18,19] and polychlorinated dibenzofurans [17,19]. 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. 47 mm octadecyl membranes were cut into 13 mm diameter extraction disks to fit into the substrate holder of the spectrofluorimeter. A rapid air-drying step, which was accomplished by applying positive pressure to the syringe, removed the excess of

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Table 1
Analytical figures of merit of OH-PAH in aqueous solutions and synthetic urine samples.

PAH metabolite	1% Methanol–water					Hydrolyzed synthetic urine				
	$\lambda_{exc}/\lambda_{ems}$ (nm)[a]	LDR[b] (ng mL^{-1})	R^2[c]	LOD[d] (ng mL^{-1})	LOQ[e] (ng mL^{-1})	$\lambda_{ex}/\lambda_{em}$ (nm)[a]	LDR[b] (ng mL^{-1})	R^2[c]	LOD[d] (ng mL^{-1})	LOQ[e] (ng mL^{-1})
2OH-FLU	273/330	1.20–100	0.9999	0.36	1.20	275/328	1.05–100	0.9992	0.32	1.05
1OH-PYR	344/386	0.30–100	0.9999	0.09	0.30	346/385	0.32–100	0.9972	0.10	0.32
3OH-B[a]P	382/434	0.63–100	0.9947	0.19	0.63	384/430	0.81–100	0.9940	0.24	0.81
9OH-PHE	303/384	3.00–100	0.9984	0.90	3.0	307/384	3.82–100	0.9946	1.14	3.82

[a] Excitation and emission wavelengths.
[b] LDR = linear dynamic range in ng mL^{-1} extending from the limit of quantification (LOQ) to an arbitrarily chosen upper linear concentration.
[c] R = correlation coefficient of calibration curve.
[d] Limit of detection 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.
[e] LOQ defined as $3.3 \times$ LOD.

water from the extraction membrane prior to spectroscopic measurements. The same extraction procedure was later applied to the water analysis of PAHs via SPE-RTF [8]. Total analysis time took less than 10 min per sample and provided LOD at the parts-per-billion (pg mL^{-1}) level.

The main disadvantages of SPE-RTP and SPE-RTF for the quantitative analysis of polycyclic aromatic compounds in water samples are relatively poor precision of measurements and background interference from extraction membranes. The presence of broad, featureless excitation and emission bands deteriorates LOD and often interferes with the determination of weak emitters at the ng mL^{-1} concentration level [6,16,20]. This article presents advances in both fronts. Signal reproducibility is improved with the aid of a sample holder specifically designed for the manual optimization of luminescence signals. Background correction of solid substrates is carried out with the aid of Asymmetric Least Squares (ALS), a smoothing algorithm originally devised for baseline correction of chromatographic data [21] and often applied to matrix interference in chromatographic analysis [22,23]. To the extent of our literature search, the application of ALS to background reduction from extraction membranes and improvement of SPE-RTF precision of measurements has not been reported yet.

The successful application of our proposition is demonstrated with the analysis of 2-hydroxy-fluorene (2OH-FLU), 1-hydroxy-pyrene (1OH-PYR), 3-hydroxy-benzo[*a*]pyrene (3OH-B[a]P) and 9-hydroxy-phenanthrene (9OH-PHE) in synthetic urine samples. Parent PAHs are relatively inert and need metabolic activation to express their carcinogenicity. Because the first step in the metabolic pathway of PAHs forms monohydroxy-PAHs (OH-PAH), urine analysis of OH-PAHs is recognized as an accurate assessment of individual's exposure to PAHs [24,25]. Although chromatographic techniques provide reliable results for the analysis of OH-PAHs [26–31], the development of easy-to-use and cost effective techniques with high sample throughput is relevant to assess PAHs uptake by large populations [30,31].

The research presented here provides screening methodology for urine analysis of OH-PAH at the parts-per-trillion concentration level. Extraction membranes serve the dual purpose of sample pre-concentration and solid substrate for RTF measurements. This approach – which eliminates elution steps and solvent evaporation prior to metabolite determination – provides excellent recoveries via a two-step procedure extremely appealing for routine analysis of numerous samples. Recovery values for the studied OH-PAH varied from 99.0 ± 1.2% (3OH-B[a]P) to 99.9 ± 0.05% (1OH-PYR). The new sample holder improved the precision of measurements for analytical use. Relative standard deviations (RSD) of the studied metabolites varied from 3.5% (2OH-FLU) to 9.5% (9OH-PHE). The application of ALS to SPE-RTF improved the LOD by approximately two orders of magnitude. With only 10 mL of urine sample, the LOD of OH-PAH varied from 57 pg mL^{-1} (2OH-FLU) to 2 pg mL^{-1} (1OH-PYR).

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. 2OH-FLU, 1OH-PYR and 9OH-PHE 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. Its

Table 2
Percentage of OH-PAH retention on C-18 membranes from aqueous solutions and synthetic urine samples.

PAH metabolite[a]	%Retention[b]	
	1% methanol–water	Hydrolysed synthetic urine
2OH-FLU	99.5 ± 0.93	99.2 ± 0.24
1OH-PYR	99.9 ± 0.05	99.4 ± 1.32
3OH-B[a]P	99.0 ± 1.19	99.7 ± 0.49
9OH-PHE	99.4 ± 0.99	96.2 ± 1.35

[a] H-PAH concentration = 50 ppb.
[b] %Retention = $(I_{before} - I_{after})/I_{before} \times 100$, where I_{before} and I_{after} are the fluorescence signals before and after extraction, respectively. All averages are based on three aliquots submitted to the entire extraction procedure.

chemical composition mimicked main components of human urine at the concentrations found in healthy urine samples.

Note: Use extreme caution when handling OH-PAH known to be extremely toxic.

2.2. Preparation of stock solutions of PAH metabolites

Stock solutions of PAH metabolites (100 μg/mL) were prepared by dissolving 1.0 mg of standard 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. Spectral profiles and fluorescence intensities of stock solutions remained the same for a period of six months. Working solutions of OH-PAH were prepared daily by serial dilution with the appropriate solvent.

2.3. Hydrolysis of urine samples

Synthetic urine samples (8 mL) were spiked with 1 mL of metabolite stock solution of appropriate concentration and equilibrated for 30 min to allow for the interaction of OH-PAH with urine components such as urea and various salts. Then 500 μL of 0.1 M HCl was 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. Solid-phase extraction

A cork borer with an inside diameter of 10 mm was used to dissect a 47 mm C-18 membrane into 10 mm extraction disks. A 10 mm disk was loaded into a stainless steel filter syringe kit (Alltech) and connected to a 10 mL syringe (Hamilton). Positive pressure was used to force all liquid solutions through the disk. Prior to sample application, the extraction membrane was conditioned with 5 mL of methanol and 5 mL of water. Optimization of experimental parameters concerning the retention of PAH metabolites led to the following procedure: aqueous metabolite solutions or synthetic 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 10 mL water and 10 mL of 20% methanol/water. Void water was mechanically removed with a 100 mL syringe forcing three 100 mL volumes of air through the disk.

2.5. Sample holder for SPE-RTF measurements

The sample holder used for these studies is shown in Fig. 1. Its design allows for the optimization of the fluorescence signal via manual rotation of its cover. The diameter of the cover indention fits into the opening of the sample compartment's lid of the spectrofluorimeter. The extraction disk is mounted on a rectangular platform

Table 3
Reproducibility study of OH-PAH on C-18 membrane.

PAH metabolite	I_{exc}/I_{ems} (nm)[a]	$I_{Background} \pm S_{background}$[c] ($\times 10^4$)			$I_{OH\text{-}PAH} \pm S_{OH\text{-}PAH}$[c] ($\times 10^4$)			Average ± standard deviation[d] ($\times 10^5$)	
		M_1	M_2	M_3	M_1	M_2	M_3	$I_{Background}$	$I_{OH\text{-}PAH}$
2OH-FLU	282/330	3.37 ± 0.07 (2.0)[b]	3.27 ± 0.07 (2.1)[b]	3.49 ± 0.05 (1.4)[b]	7.38 ± 0.03 (0.4)[b]	7.15 ± 0.02 (0.3)[b]	7.63 ± 0.02 (0.3)[b]	0.34 ± 0.01 (2.9)[b]	7.39 ± 0.24 (3.2)[b]
1OH-PYR	348/384	4.82 ± 0.02 (0.4)[b]	5.08 ± 0.05 (1.0)[b]	5.28 ± 0.04 (0.8)[b]	21.2 ± 0.21 (1.0)[b]	22.0 ± 0.20 (0.9)[b]	23.40 ± 0.27 (1.2)[b]	0.51 ± 0.02 (3.9)[b]	22.0 ± 1.10 (5.0)[b]
3OH-B[a]P	383/430	2.95 ± 0.02 (0.7)[b]	3.10 ± 0.04 (1.3)[b]	3.34 ± 0.04 (1.2)[b]	13.9 ± 0.31 (2.2)[b]	12.6 ± 0.31 (2.5)[b]	12.34 ± 0.27 (2.7)[b]	0.31 ± 0.02 (6.4)[b]	12.9 ± 0.83 (6.4)[b]
9OH-PHE	307/382	2.63 ± 0.04 (1.5)[b]	2.85 ± 0.06 (2.1)[b]	2.35 ± 0.01 (0.1)[b]	2.91 ± 0.03 (1.0)[b]	2.39 ± 0.03 (1.2)[b]	2.67 ± 0.04 (1.5)[b]	0.26 ± 0.02 (7.7)[b]	2.60 ± 0.26 (10.0)[b]

[a] Excitation and emission wavelengths.
[b] Relative Standard Deviation (%) = (std dev)/(I_{ave}) × 100.
[c] Average values based on three independent measurements.
[d] Average values calculated as $[M_1 + M_2 + M_3] \times 1/3$; standard deviation $[s_1^2 + s_2^2 + s_3^2]^{1/2}$, where s_1, s_2 and s_3 correspond to their standard deviations of M_1, M_2 and M_3, respectively.

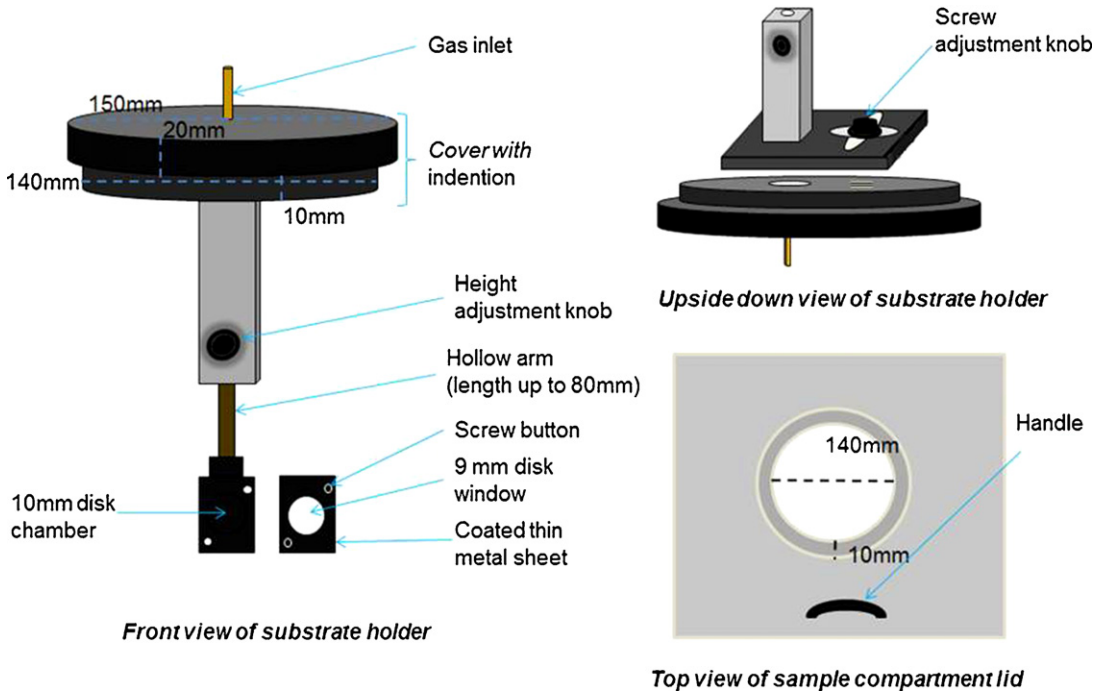

Fig. 1. Solid-substrate holder for solid-surface room-temperature fluorescence measurements.

and held in place by a plate with a circular window for sample excitation. The substrate platform is connected to the cover with the aid of a cylindrical rod made hollow for gas purging and sample degassing. The height of the platform inside the sample compartment is adjustable by means of a knob placed on the rectangular rod of the cover. The cover rod allows for *x*–*y* adjustment of the substrate position with respect to the excitation beam. Once the excitation beam had been aligned with the circular window of the platform, no further optimization was needed. Maximum fluorescence intensities were observed rotating the platform to around 45° in relation to the excitation beam.

2.6. RTF measurements

Steady state excitation and fluorescence spectra and signal intensities were recorded with a commercial spectrofluorimeter (Photon Technology international). The excitation source was a continuous-wave 75 W pulsed xenon lamp with broadband illumination from 200 to 2000 nm. The excitation and emission monochromators had the same reciprocal linear dispersion (4 nm mm^{-1}) and accuracy (±1 nm with 0.25 nm resolution). The gratings were blazed at 300 and 400 nm, respectively. Detection was made with a photomultiplier tube with spectral response from 185 to 650 nm. The instrument was computer controlled using commercial software (Felix32) specifically designed for the system. Appropriate cut off filters were used to reject straight-light radiation and second-order emission.

2.7. Software

Routines for data pre-treatment and processing were written in MATLAB [32]. Baseline routines for emission background correction were adapted from routines previously described in the literature for baseline correction of chromatographic data [33]. Implementation of the ALS algorithm included a smoothing parameter equal to 1×10^7, 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

The four metabolites chosen for this study were selected as a tentative means of comparing our analytical figures of merit (AFOM) with previously reported data. Early methods focused on the analysis of 1OH-PYR [27]. Considering that humans are usually exposed to mixtures of PAHs, subsequent methods expanded their scope to a larger number of OH-PAH. Major attention has been paid to screening metabolites of EPA-PAHs, i.e. OH-PAH resulting from human exposure to PAHs included in the Environmental Protection Agency (EPA) priority pollutants list. The general approach follows the sequence of urine hydrolysis, sample cleanup and preconcentration and chromatographic analysis. The purpose of the hydrolysis step (either enzymatic or acidic) is to dissociate OH-PAH from their glucuronide and/or sulfate conjugates. Metabolites are typically extracted from urine samples via SPE using commercial cartridges containing silica particles derivatized with C-18 alkyl chains. OH-PAH are eluted from SPE cartridges with milliliter volumes of methanol and pre-concentrated to micro-litters for chromatographic analysis.

One of the main sources of metabolite loss in chromatographic procedures is the evaporation of the eluting solvent. Recoveries as low as 45–48% have been reported for 3OH-B[a]P [29,34], one of the metabolites studied here. Benzo[*a*]pyrene is the most toxic EPA-PAH and its concentration alone is often used as a measure of risk [12]. Reported recoveries for the other three metabolites have varied from 62 to 82% (2OH-FLU) [34,35], 80 to 99% (1OH-PYR) [29,34], and 93 to 96% (9OH-PHE) [29,34].

3.1. RTF analytical figures of merit (AFOM) of OH-PAH in aqueous solutions and urine samples

The initial survey of room-temperature excitation and fluorescence spectra was carried out in methanol/water (1% v/v) solutions and hydrolyzed urine samples. 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 2 nm excitation and emission band-pass. No attempts were

made to adjust slit widths for optimum spectral resolution, nor were the spectra corrected for instrumental response. The 2 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 due to the presence of urine.

Table 1 summarizes the 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 five OH-PAH 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 [36] with satisfactory results. The limits of detection (LOD) 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 [37]. The strong fluorescence intensity resulting from the rigid and delocalized π-electron system of OH-PAH provides LOD and LOQ values at the ng mL^{-1} concentration level.

3.2. Extraction efficiency of SPE membranes

The percentages of extraction (%*E*) were calculated with the formula $\%E = (I_{BE} - I_{AE}/I_{BE}) \times 100$, where I_{BE} and I_{AE} refer to the fluorescence signals before and after extraction, respectively. In all cases, the volume of extracted sample was 10 mL. The mass of extracted metabolite did not surpass the nominal breakthrough mass (30 mg) of extraction membranes [38]. Table 2 compares %*E* values obtained from standard solutions to those from hydrolyzed urine samples. All values are the averages of three aliquots submitted to the entire extraction procedure. Within a confidence interval of 95%, all %*E* are equivalent to 100% [39]. This fact excludes the possibility of matrix interference on the retention of OH-PAH. Similar results were obtained with other OH-PAH concentrations within the LDR of Table 1. Keeping in mind that metabolite elution is not required for the determination of OH-PAH via SPE-RTF, the %*E* values in Table 2 correspond to the analytical recovery of the method.

3.3. Reproducibility of RTF measurements on extraction membranes

Previous work in our lab studied the deposition pattern of PAHs on extraction membrane surfaces [40]. We monitored the fluorescence images of membranes used to extract trace concentration levels (ng mL^{-1}) of PAHs dissolved in 1% methanol/water (v/v) solutions. Random deposition patterns of PAHs were observed at all concentration levels. The heterogeneous deposition of PAHs on the surfaces of extraction membranes leads to poor reproducibility of measurements. One way to improve precision of measurements is to irradiate the entire area of the solid substrate via laser excitation with a fiber optic probe [40]. With commercial spectrofluorimeters equipped with broad band irradiation sources and excitation monochromators, one possibility is to opening the excitation slits for complete PAH excitation on the extraction membrane. Unfortunately, opening excitation slits compromises spectral resolution and deteriorates the selectivity of the technique. A practical way to overcome this limitation is to using the sample holder in Fig. 1. This device optimizes the position of the extraction membrane for maximum fluorescence signal. Maximum intensity results from the irradiation of the membrane area with the highest mass of deposited PAH.

Table 3 summarizes the precision of SPE-RTF measurements made with the sample holder in Fig. 1. All extraction membranes were dissected from 47 mm-diameter disks belonging to the same commercial lot. Three extraction membranes per metabolite (M_1, M_2 and M_3) were used to calculate the average background signals. The same is true for the average intensities of extracted metabolites. Standard deviations from single membrane measurements were based on signal intensities recorded after three repetitive optimizations of the substrate holder position. The relative standard deviations of single membrane measurements were equal or lower than 2%, which demonstrates the reproducibility of the signal optimization procedure. Fluorescence background was observed from all the examined membranes. It consisted of a broad, featureless emission band between 350 and 500 nm. Comparison of average background values shows considerable intensity variations with excitation and emission wavelengths. The same is true with their respective standard deviations. As expected, relatively large background variations deteriorate the reproducibility of measurements from extracted metabolites.

3.4. Fluorescence background treatment of extraction membranes

Several attempts were made to decrease the fluorescence background of commercial membranes. All attempts assumed the presence of at least one inherent impurity as the source of fluorescence background. These included: (a) "pre-flushing" individual membrane disks with the syringe kit using increasing volumes (10, 20 and 30 mL) of high purity methanol; (b) ultraviolet irradiation of membrane disks for eight hours in a photochemical reactor equipped with a total of twelve lamps irradiating at 254 nm, 300 nm and 320 nm, and thin-layer chromatography (TLC) of membrane strips using high purity methanol as the mobile phase. All these attempts were chosen based on their previous success for background reduction of paper substrates [1,2,7,41–43].

The best background reductions with extraction membranes were obtained via TLC. Each chromatographic run was carried out with 34 mm × 40 mm membrane strips immersed 5 mm deep in methanol. Visual inspection of membrane strips under an ULTRALUM UV–VIS irradiation box equipped with 254 and 365 nm revealed the maximum migration distance of the fluorescence background towards the top of the strip after 10–15 min of chromatographic run. The membrane disks used for further measurements were cut from the strip area with low fluorescence background. Fig. 2 correlates the background intensity to the number of TLC runs. Intensities plotted in the graph correspond to average values calculated from 3 individual measurements of 6 membrane disks. The fluorescence background reaches a minimum after 3 chromatographic runs. Based on this fact, all further experiments were than carried out with extraction disks previously treated with 3 TLC runs.

3.5. AFOM of OH-PAH via SPE-RTF

The SPE-RTF AFOM of the four studied metabolites are summarized in Table 4. SPE was carried out with 10 mL of standards prepared in 1% 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. The LDR were obtained with a minimum of five OH-PAH concentrations. No efforts were made to experimentally obtain the upper concentration limits of the calibration curve. 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 [8]. The correlation coefficients of the calibration curves and the slopes

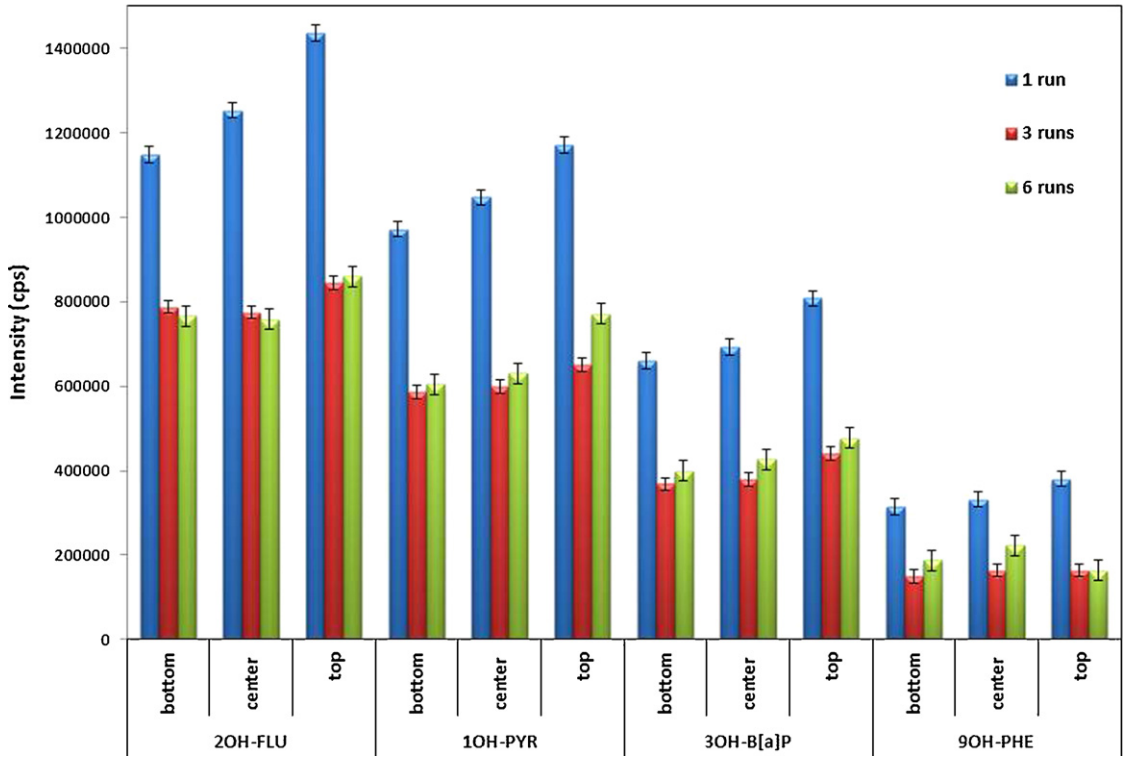

Fig. 2. Fluorescence background of extraction membranes as a function of chromatographic runs with methanol as the carrier solvent. Wavelength measurements were the following: $\lambda_{exc}/\lambda_{em}$ = 282/330 nm 2OH-FLU, $\lambda_{exc}/\lambda_{em}$ = 348/384 nm 1OH-PYR, $\lambda_{exc}/\lambda_{em}$ = 383/430 nm 3OH-B[a]P, and $\lambda_{exc}/\lambda_{em}$ = 307/382 nm 9OH-PHE.

Table 4
Room-temperature fluorescence analytical figures of merit of OH-PAH on C-18 membranes.

PAH metabolite	l_{exc}/l_{em}(nm)[a]	LDR[b]	R^2[c]	LOD[d]
2OH-FLU	282/330	4.33–100	0.9987	1.30
1OH-PYR	348/384	0.85–100	0.9944	0.26
3OH-B[a]P	383/430	2.35–100	0.9984	0.71
9OH-PHE	307/382	12.83–100	0.9969	3.85

[a] Excitation and emission wavelengths.
[b] LDR = linear dynamic range in ng mL^{-1} 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 16 blank measurements and m is the slope of the calibration curve.

of the log–log plots (data not shown) were close to unity, indicating a linear relationship between OH-PAH concentration and fluorescence intensity. Satisfactory results were also obtained by the ANOVA test suggested by IUPAC [36]. The RSD at medium linear concentrations were lower than 10%. The LOD were estimated at the parts-per-billion to sub-parts-per-billion concentration levels. Their comparison to the LOD values in Table 1 shows no advantage of pre-concentrating the sample prior to RTF.

Table 5 compares the slopes of the calibration curves, blank intensities and their standard deviations from aqueous solutions to those obtained on extraction membranes. As expected, sample pre-concentration 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. Better SPE-RTF LOD could have been obtained by extracting 100 mL volumes of standard solutions, i.e. an alternative with little appealing in the screening context of human urine samples.

3.6. Background correction via ALS

The ALS algorithm uses the Whittaker smoother for discrete (time) series, which minimizes the function [21]:

$$Q = \sum_i v_i (y_i - f_i)^2 + p \quad (1)$$

where y is the data (experimental signal), f a smooth trend (or baseline estimation), v prior weights, and p the asymmetry parameter. The successful implementation of ALS to the background correction of extraction membranes was achieved with a smoothing parameter equal to 1×10^7, an asymmetry parameter equal

Table 5
Slope and blank signals of OH-PAH in aqueous solution and on extraction membranes.

PAH metabolite	Slope[a]		Average blank intensity ± standard deviation[b]	
	H_2O	Membrane	H_2O (×10^3)	Membrane (×10^4)
2OH-FLU	8.6×10^2	7.2×10^3	1.90 ± 0.10	3.5 ± 0.31
1OH-PYR	2.4×10^3	3.2×10^4	0.62 ± 0.07	3.9 ± 0.29
3OH-B[a]P	1.6×10^2	1.3×10^4	0.70 ± 0.09	3.4 ± 0.31
9OH-PHE	5.8×10^2	2.4×10^3	9.2 ± 0.17	2.5 ± 0.31

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

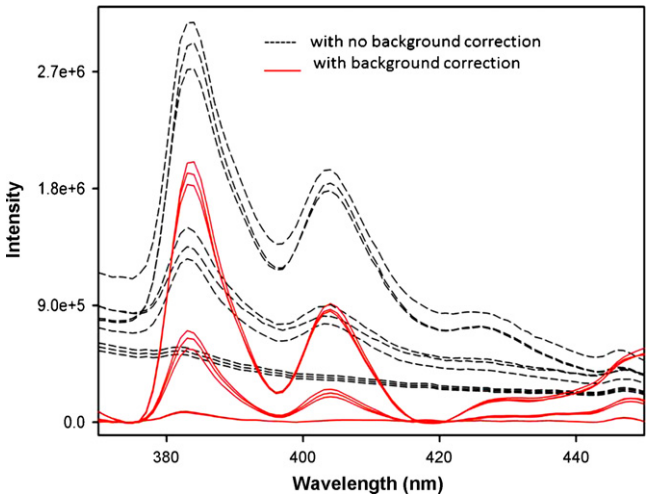

Fig. 3. Fluorescence spectra of 2.5 and 5 ng mL^{-1} of 1OH-PYR recorded from extraction membranes with and without background correction.

Table 6
RTF analytical figures of merit of OH-PAH on C-18 membrane with background correction.

PAH metabolite	l_{exc}/l_{em}(nm)[a]	LDR[b]	R^2[c]	LOD[d]
2OH-FLU	282/330	0.17–5.0	0.9939	0.057
1OH-PYR	348/384	0.006–5.0	0.9944	0.0017
3OH-B[a]P	383/430	0.25–5.0	0.9949	0.0075
9OH-PHE	307/382	0.036–5.0	0.9951	0.011

[a] Excitation and emission wavelengths.
[b] LDR = linear dynamic range in ng mL^{-1}.
[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.

to 0.001, an order of differences in penalty equal to 3 and a single regularization parameter, whose value was 1. Fig. 3 provides a visual demonstration of the clear improvement upon ALS implementation. The background intensity (I_B) of extraction membranes (see Fig. 3A) was considerably reduced and so it was the standard deviation (S_B) of the average background signal. At the maximum fluorescence wavelength of 1OH-PYR (380 nm), the I_B and S_B values before and after ALS treatment were $4.11 \times 10^5 \pm 0.44 \times 10^5$ counts per second (cps) and $5.4 \times 10^2 \pm 2.09 \times 10^2$ cps, respectively. Table 6 summarizes the AFOM obtained via SPE-RTF-ALS. Comparison to Table 5 reveals LOD improvements ranging from 79.8 × (2OH-FLU) to 158.8 × (1OH-PYR). The LOD in Table 6 meet the expectations for the analysis of OH-PAH in urine samples.

4. Conclusion

Several features make SPE-RTF spectroscopy a well-suited approach for screening OH-PAH in urine samples. Its straightforward experimental procedure is attractive for routine analysis of numerous samples. The direct determination of OH-PAH on the surface of the extraction membrane eliminates the need for subsequent elution steps and provides excellent (~100%) metabolite recoveries (see Table 2). Acceptable precision for analytical work is possible with the aid of a substrate holder specifically designed for manual optimization of maximum fluorescence signals. Background correction of extraction membranes via ALS provides LOD at the parts-per-trillion concentration level. With 10 mL of urine samples, the LOD varied from 60 (2OH-FLU) to 2 pg mL^{-1} (1OH-PYR). These values compare favorably to LOD previously reported via other methods of analysis, including HPLC [26–28,44,45] and RTF-excitation emission matrix spectroscopy of SPE extracts [20]. "Chemometric work in our group is searching for a well-suited algorithm capable to handle the simultaneous determination of co-extracted metabolites as well as the pharmacological interference that might occur in urine samples of unhealthy individuals."

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