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Determination of heterocyclic aromatic amines in human urine by using hollow-fibre supported liquid membrane extraction and liquid chromatography-ultraviolet detection system

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ABSTRACT

A hollow-fibre supported liquid membrane (HF-SLM) extraction method has been developed for determination of 11 heterocyclic aromatic amines (HCAs) in human urine samples by using high performance liquid chromatography (HPLC) equipped with an ultraviolet (UV) absorbance detector. These compounds were extracted from an alkaline urine sample (donor phase) into the organic solvent residing in the pores of a polypropylene hollow fibre and then back extracted into an acidic solution (acceptor phase) inside the lumen of the hollow fibre. After extraction, HCAs were analyzed by injecting the analyte enriched acceptor phase into the HPLC. The analyte enrichment factors ranged between 241 and 339 obtained in a 90 min extraction time, and method detection limits (MDL) ranged between 0.1 and 0.5 $\mu g\,L^{-1}$ with relative standard deviation (RSD) values between 3.4% and 11%. The extraction technique employed in this work is easy to use and rapid as it involves only a few minutes manipulation of each sample. It is the most economical sample preparation/preconcentration technique to our knowledge as compared to other microextraction techniques.

1. Introduction

In the late 1970s, Japanese scientists reported high mutagenic activity on the surface of grilled meat and fish [1]. This finding led to the discovery of a series of mutagenic and carcinogenic heterocyclic aromatic amines (HCAs) that are formed at ng/g levels during cooking of meat and fish [2]. Today, there are several literature reports where more than 20 HCAs have been isolated and identified from cooked foods [3–5]. Some of these HCAs have also been found in cigarette smoke, soy sauce, wine, vinegar, beer, river water and human urine [6–11].

These compounds (see Table 1) have high mutagenic and carcinogenic properties. It has been observed that the rising cancer incidences in human beings in the developed countries, especially the diseases of colon, large intestine, prostate, liver and kidneys are related to a large extend to the greater consumption of meat as well as preserved meat. The concentration of HCAs in meat depends on cooking temperature and duration of heating. Under normal conditions, the HCAs are formed at very low concentrations but if the cooking temperature is increased above 150 °C the formation of these compounds increases significantly [12].

These amines are formed from free amino acids, creatine/creatinine, and carbohydrates [13,14] during cooking of food, particularly when proteinaceous food is heated at moderate to high temperatures. Their content varies greatly with cooking conditions, so it is difficult to obtain an accurate estimation of the exposure. Thus, methods have been developed to determine these compounds in biological samples (mainly urine samples) [15–19] to reflect recent HCAs exposure. These methods of analysis are usually based on liquid chromatography (LC) [20–24], but gas chromatography (GC) [25,26] and capillary electrophoresis (CE) [27] have also been used. For sample extraction, solid-phase extraction (SPE) is the most commonly used technique [17,20,22–25], but also liquid–liquid extraction (LLE) [16,18,19] and solid-phase microextraction (SPME) [28] have been applied.

There are several emerging alternative extraction techniques based on membrane extraction. Such techniques are typically very simple and cheap and can provide efficient clean-up and high enrichment factors. They are divided into two groups: those based on porous membranes such as Donnan dialysis, microdialysis and electrodialysis, and those based on non-porous membranes, such as supported liquid membrane (SLM) extraction, microporous membrane liquid–liquid extraction (MMLLE), polymeric mem-

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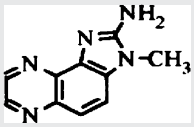
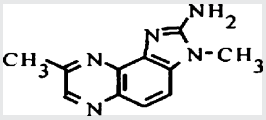
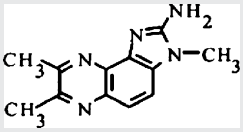
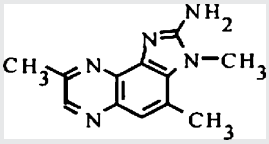
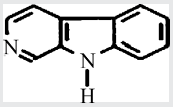
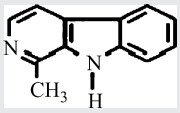
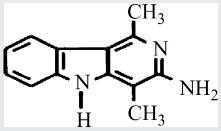
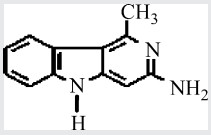
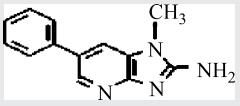
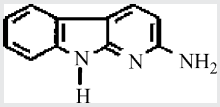
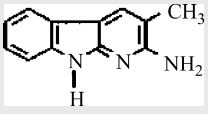
Table 1
Structures of the HCAs studied in this work

Name	pK_a	Structure
IQx	–	
MeIQx	5.94	
7,8-MeIQx	6.45	
4,8-MeIQx	6.25	
Norharman	–	
Harman	–	
Trp-P-1	8.55	
Trp-P-2	8.5	
PhIP	5.6	
AαC	4.6	
MeAαC	4.9	

brane extraction (PME) and membrane extraction with a sorbent interface (MESI) [29].

SLM extraction, either using flat membranes or in hollow-fibre configurations, is based on a three-phase (aqueous–organic–aqueous) system, where the organic solvent is held in the pores of a porous membrane supported by capillary forces. It is in contact with two aqueous phases, the donor phase, that is the aqueous sample, and the acceptor phase, usually an aqueous buffer. In the hollow-fibre configuration the latter is placed in the lumen of the fibre [30].

Only a very small amount of organic solvent is used for impregnating the membrane pores. The analytes are extracted from the aqueous donor phase into the organic membrane and then back-extracted to the second aqueous phase, the acceptor. This process is usually driven by differences in pH between the two aqueous phases. For basic analytes such as HCAs, the pH in the aqueous donor needs to be above the pK_a values of the analytes rendering them uncharged. The analytes are then in extractable form and are extracted into the membrane liquid. The analytes diffuse across the membrane and are back-extracted into the acceptor, where the pH is kept below the pK_a value. The basic analytes will thus be charged, non-extractable, and thereby trapped in the acceptor [29].

Recently, hollow-fibre supported liquid membrane extraction has been successfully applied to the determination of 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) [31] in urine and blood plasma. This compound (see below) is the most abundantly found HCA arising from consumption of cooked meat-containing food. In the present work, a similar approach has been developed for extraction/pre-enrichment of 11 HCAs from human urine samples. The main aim of the study was to examine if the developed procedure [31] could be applied to the extraction of other HCAs. In order to detect all the relevant compounds an ultraviolet (UV) detector was used, limiting the detection limits that can be obtained. Using a more sensitive detection system such as LC–MS would help to detect HCAs in unspiked urine samples.

2. Experimental

2.1. Chemicals

The following HCAs (see Table 1 for their structures) were used in this work as model compounds:

2-Amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx), 2-amino-3, 8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3, 4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), 2-amino-3, 7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx), 3-amino-1,4-dimethyl- 5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2- amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP), 2-amino-9*H*-pyrido [2,3-*b*]indole (AαC), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), 2-methyl-β-carboline (Harman), (9Hpyrido[3,4-*b*] indole (Norharman). These HCAs were supplied by Toronto Research chemicals (Toronto, Canada).

High performance liquid chromatography (HPLC)-grade acetonitrile was supplied by Merck (Darmstadt, Germany). Sodium hydroxide was obtained from Scharlan Chemie (Barcelona, Spain). Sulphuric acid and 1-octanol were obtained from Sigma–Aldrich (Steinheim, Germany). All aqueous solutions were prepared by using reagent water purified by a Milli-Q Gradient system (Millipore, Bedford, MA, USA).

The Accurel® PP Q3/2 polypropylene hollow-fibre membranes (200 μm wall thickness, 600 μm inner diameter and 0.2 μm pore size) were obtained from Membrana GmbH (Wuppertal, Germany).

Microsyringes (0.8 mm outer needle diameter and 10–100 μL volume) were purchased from SGE (Australia) and Hamilton Bonaduz AG (Switzerland). The solution pH was measured by a 211 microprocessor pH-meter (Hanna instruments, Rhode Island, USA). A 10-position magnetic stirring device (RO 10 power, IKA-Werke GmbH & Co.KG, Staufen, Germany) was used for sample stirring during extraction.

2.2. Standard solutions and urine samples

Stock standard solutions of HCAs used were prepared in methanol/water (1:1). All these solutions were kept refrigerated at −6 °C. Standard working solutions were prepared by diluting stock solutions with reagent water to 1 mg L^{-1} and stored at 4 °C no longer than a week.

Unspiked urine samples were obtained from volunteers after intake of 250 g fried chicken together with 200 g boiled potatoes and 25 g green peas, collecting the urine twelve hours after eating, as it was reported in a previous study [32].

2.3. HPLC analysis

HPLC analyses of HCAs were performed using an 1100 Series HPLC instrument, (Agilent Technologies, Heilbronn, Germany), consisting of a quaternary pump, a vacuum degasser, thermostated autosampler, column compartments and ultraviolet detector. The HPLC data obtained were processed by Agilent Chemstation revision B.01.03 software. All data were evaluated using Microsoft Excel.

The chromatographic separation was carried out by a reverse phase HPLC system, using a C_{18} column RP-Ace 5 (250 mm × 4.6 mm × 5 μm particle size) (ACT, Scotland). The column was kept at 22 °C during the whole sequence. Optimum separation in short time was achieved with a binary mobile phase at gradient conditions and a flow rate of 1 mL min^{-1}. Solvent A was acetonitrile and solvent B was aqueous acetic acid–ammonium acetate buffer 30 mM (pH 4.5). The gradient elution program was: 0–1 min 5% A; 1–15 min, 5–30% A; 15–18 min 30% as isocratic, 18–30 min, 30–55% A and then 30–35 min, 55–5% A to return to initial conditions. UV detection was carried out at three wavelengths of 265 nm (IQx, MeIQx, 7,8-DiMeIQx, 4,8-DiMeIQx, Trp-P-1 and Trp-P-2), 287 nm (Harman and Norharman) and 315 nm (PhIP, AαC and MeAαC).

2.4. HF-SLM extraction procedure

2.4.1. Preparation of the HF extraction device

The hollow fibre was cut into 4.2 cm long pieces and then one end of each piece was heat-sealed by squeezing with a pair of forceps and touching a hot soldering iron. The heat-sealed fibres were cleaned with acetone, air-dried and stored in a closed glass tube. For extraction, the unsealed end of one fibre was connected to a needle of a 100-μL HPLC syringe that was filled with acidic acceptor solution, which was injected into the lumen of hollow fibre. Kept attached to the needle of the syringe, the hollow fibre was dipped into an organic solvent (1-octanol) for a few seconds in order to fill the hollow-fibre pores with organic solvent. The hollow fibre was shaken in clean water for 30 s. Shaking the fibre in clean water eliminated the possible surplus organic solvent on the hollow-fibre surface. The hollow fibre was then ready for sample extraction.

2.4.2. Sample extraction

The sample (3 mL urine) was transferred into a small 4-mL glass vial and diluted to 4 mL with 0.5 M NaOH. Then a magnetic stir bar (8 mm × 3 mm) was added to the vial. After that, the already prepared hollow-fibre extraction device was immersed into the sample so that the hollow fibre was completely covered by the sample. Each sample vial was put on one spot of a multi-spot magnetic stirring device.

After the selected extraction time, the hollow fibre attached to the syringe was taken out from the sample, the sealed end was cut and the acceptor phase inside the hollow-fibre lumen was transferred to an HPLC glass insert inside an HPLC vial by attaching the hollow fibre to another needle of an air-filled syringe and depressing the syringe plunger. The recovered acceptor volume (9–11 μL) was then measured by weighing the HPLC vial before and after filling the acceptor solution. This corresponds to approximately 80–95% of the nominal volume of the lumen of the fibre, which is 11.5 μL. Then, 5 μL of the extract was injected into the HPLC using the autosampler.

3. Result and discussion

In order to obtain high enrichment and extraction efficiency of the HCAs using a hollow-fibre supported liquid membrane (HF-SLM) technique, previously optimized parameters [31] such as sample and acceptor pH, stirring speed and organic solvent were used. In this study, optimization of extraction time was carried out in urine samples containing 11 HCAs.

3.1. Extraction time profile

Extraction efficiency and enrichment factor are dependent on the period of extraction. Hence, the extraction time was studied in order to obtain the highest extraction efficiency and enrichment. Figs. 1 and 2 describe the influence of the extraction time on the extraction efficiency and enrichment factor of HCAs, respectively. The optimized conditions of 100 mM H_2SO_4 as acceptor phase, sample pH 10, 1-octanol as an extraction solvent, 660 rpm as stirring speed and 4.2 cm length of HF were used to optimize the extraction time. Spiked HCAs in urine were extracted for different extraction times ranging from 10 to 150 min. The enrichment factor

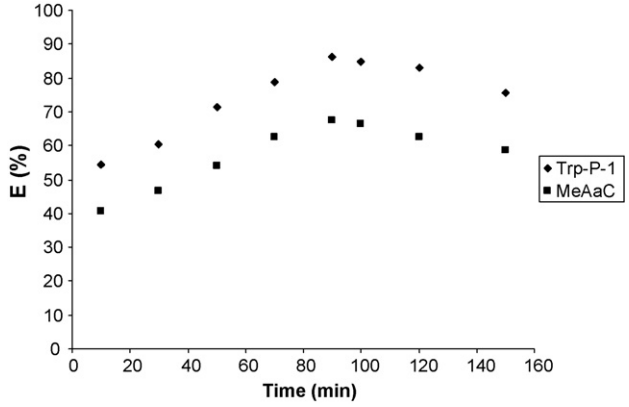


Fig. 1. Effect of extraction time on extraction efficiency of HCAs. Only those compounds with highest and lowest extraction efficiencies are shown.

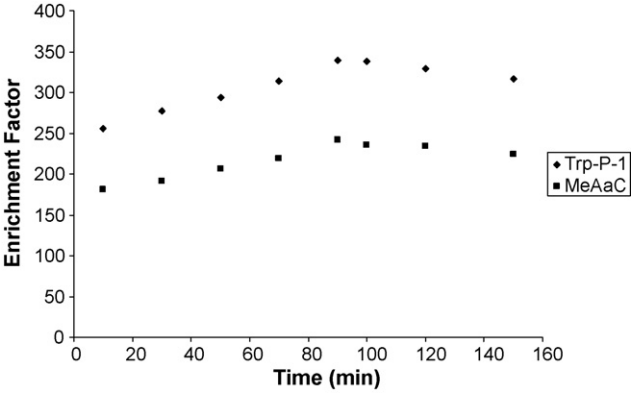


Fig. 2. Effect of extraction time on enrichment factor of HCAs. Only those compounds with highest and lowest enrichment factors are shown.

Table 2
Analytical performance of the HF-SLM system in urine samples

Analyte	Linearity (R^2) range (1–50 μg L^{-1})	Limits (μg L^{-1})		Extraction efficiency (%) (%RSD)	Enrichment (%RSD)	Precision (%RSD)	
		MDL	MQL			Repeatability ($n=3$)	Reproducibility ($n=9$)
IQx	0.9995	0.25	1.0	81.4 (0.61)	293 (2.92)	4.7	7.5
MeIQx	0.9991	0.1	0.5	81.3 (2.83)	292 (1.67)	3.4	5.5
7,8-DiMeIQx	0.9988	0.25	0.5	82.3 (0.52)	296 (2.37)	7.6	6.8
4,8-DiMeIQx	0.9980	0.25	0.5	84.5 (1.43)	304 (1.66)	6.1	7.0
Norharman	0.9918	0.5	1.0	72.0 (7.85)	259 (5.52)	7.4	9.3
Harman	0.9889	0.5	1.0	84.9 (5.88)	305 (6.75)	7.2	6.0
Trp-P-2	0.9955	0.1	0.25	89.2 (3.47)	321 (5.54)	3.8	6.6
Trp-P-1	0.9970	0.1	0.25	94.3 (0.38)	339 (3.08)	5.0	8.9
PhIP	0.9819	0.5	1.0	79.5 (7.62)	286 (8.27)	6.8	6.7
AαC	0.9643	0.5	1.0	73.5 (4.28)	264 (6.03)	9.3	8.5
MeAαC	0.9999	0.5	1.0	67.0 (7.99)	241 (7.70)	11.1	11.2

increased with time up to 90 min and then remained constant for some time period, indicating that equilibrium is obtained for all compounds. At longer than 90 min extraction time, the enrichment factors and extraction efficiencies were slightly decreased, probably due to the instability of the organic solvent at longer time. On the basis of these findings and also considering the high enrichment factor for all HCAs after 90 min, this extraction time was selected as optimal for the rest of the experiments. It should

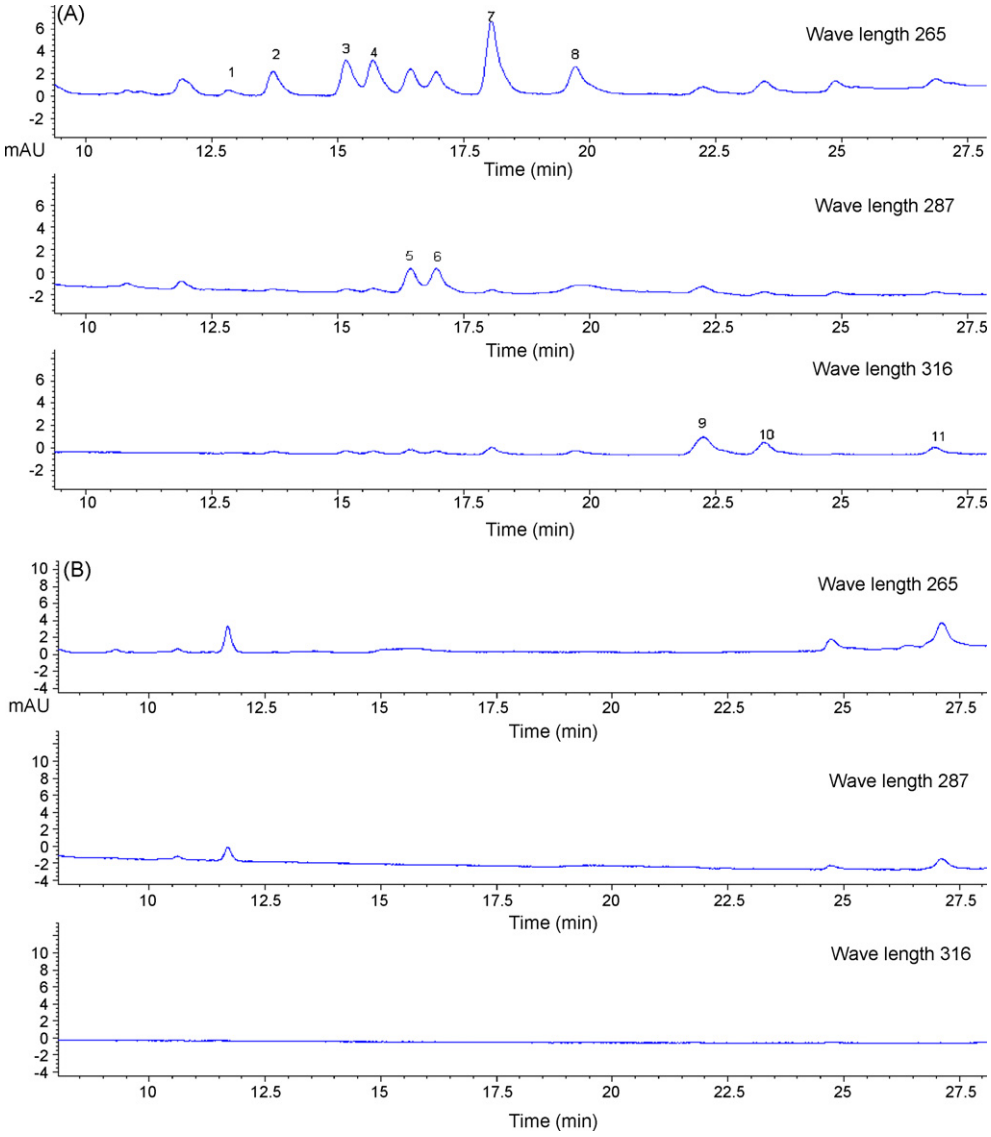


Fig. 3. HPLC-UV chromatograms of (A) spiked urine sample and (B) blank urine sample. Peaks: (1) IQx, (2) MeIQx, (3) 7,8-DiMeIQx, (4) 4,8-DiMeIQx, (5) Norharman, (6) Harman, (7) Trp-P-2, (8) Trp-P-1, (9) PhIP, (10) AαC, (11) MeAαC.

be observed that the actual handling time for each sample is only a few minutes, so several extractions can easily be performed in parallel adding virtually no time to the time needed for the HPLC run.

3.2. Extraction efficiency and enrichment

The characteristic of extraction depends on the extraction efficiency, i.e. the fraction of analyte extracted from the sample. The extraction efficiencies were determined by using Eq. (1).

$$E = \frac{(C_A \times V_A)}{(C_S \times V_S)} \quad (1)$$

where C_A and V_A are the concentration of analytes in the acceptor and the aqueous volume of the acceptor phase, respectively. C_S and V_S are the initial concentration of the analyte in the sample and the sample volume, respectively. It was observed that for all these 11 compounds, the extraction efficiency was in a small range due to the similar nature of the compounds. This extraction efficiency was sufficient for determination of these compounds in urine samples with good precision. Table 2 shows the extraction efficiencies (E) and enrichment factors (E_e) for all studied HCAs under the selected conditions. The enrichment factor represents the number of times of the analyte concentration increased by the extraction process. It is defined as in Eq. (2).

$$E_e = \frac{C_A}{C_S} \quad (2)$$

3.3. Method validation

3.3.1. Analytical performance in urine samples

The performance parameters of the HF-SLM technique, such as linearity, detection and quantification limits, relative standard deviations (RSD), extraction efficiencies and enrichment factors are listed in Table 2. The urine samples were spiked at 1 $\mu g L^{-1}$ for the validation of this analytical method. The enrichment factors ranged from 241 for MeAαC to 339 for Trp-P-1, and are suitable for the determination of these HCAs in urine samples. The linearity was checked for HCAs spiked urine samples at concentration ranging from 1 to 50 $\mu g L^{-1}$ by analyzing each concentration in triplicate. All the analytes showed good linearity with squared regression coefficient (R^2) ranging from 0.9643 to 0.9999. The method detection limits (MDL) ranged from 0.1 $\mu g L^{-1}$ for MeIQX, Trp-P-1 and Trp-P-2 to 0.5 $\mu g L^{-1}$ for PhIP, AαC, MeAαC, Harman and Norharman. The repeatability (three samples in one day) and reproducibility (nine samples in three consecutive days in triplicate analysis each day) were tested in urine samples spiked at two concentrations (1 $\mu g L^{-1}$ and 10 $\mu g L^{-1}$). It was observed that the RSD was lower at 10 $\mu g L^{-1}$ than at 1 $\mu g L^{-1}$. The RSD of the experimental procedure ranged from 3.4% to 11.1% for repeatability and from 5.5% to 11.2% for reproducibility. Thus the method showed good precision for all the HCAs studied.

3.3.2. Method application

The developed HF-SLM technique was finally applied to the analysis of urine samples. Three urine samples collected from healthy male adults (aged 22–26 years) were subjected to extraction and analysis. The optimized parameters were followed and extraction was done as described above. However, none of the target compounds were found in any of the urine samples tested. This might be due to the fact that HCAs are rapidly absorbed from the intestinal tract and only a few percent are present as parent compound in the urine [17,18]. The main aim of the study was to examine if the hollow-fibre supported membrane extraction method that has been developed for PhIP [31] could be applied for the extraction of other HCAs. In the previous study, a fluorescence detector was used, permitting low detection limits for PhIP, but this detector is not applicable to all the compounds studied here. A more universal detector, such as a mass spectrometer would permit detection of HCAs in unspiked urine samples at low concentrations. Promising studies with this approach are in progress. One of the urine samples was spiked at a concentration of 10 $\mu g L^{-1}$ of HCAs and extracted in a usual manner. From the spiked urine sample all the 11 HCAs were successfully extracted. Fig. 3 shows the HPLC-UV chromatograms of spiked and blank urine samples.

4. Conclusion

A fast and simple procedure based on the exhaustive SLM equilibrium sampling in a hollow fibre combined with HPLC has been developed to extract HCAs from urine samples. The experimental setup is very simple and highly affordable. Among all microextraction techniques reported, this technique is the most economical sample preparation/preconcentration technique. The hollow fibre is disposable, so single use of the hollow fibre reduces the risk of cross-contamination and carry-over problems. The proposed method allows the effective recuperation of 11 HCAs into one single extract. Moreover, the method was applied to the analysis of spiked human urine giving good qualitative and quantitative results. This procedure can be successfully used for the analysis of urine samples in the study of human exposure to HCAs.

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