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Simultaneous determination of 11 monohydroxylated PAHs in human urine by stir bar sorptive extraction and liquid chromatography/tandem mass spectrometry

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ABSTRACT

A simple and sensitive method for simultaneous determination of 11 monohydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) in human urines has been developed based on stir bar sorptive extraction (SBSE) and liquid chromatography/tandem mass spectrometry (HPLC–MS/MS). Factors that may influence the extraction efficiency, such as pH value of matrix, extraction time, desorption solvents and desorption time were optimized. Validation results showed that the method has high sensitivity (quantification limits of 1–3 pg/mL), good reproducibility (RSD between 3.1 and 13.0%) and spiked recoveries (71.9–133.2%). The proposed method was also applied to analysis urines of smokers and nonsmokers, ten trace OH-PAHs were determined and compared between two groups. For the ease operation and satisfactory validiation results, SBSE coupled to HPLC–MS/MS may be an excellent alternative method for trace analysis of OH-PAHs in human urines.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are formed during the incomplete combustion of organic matter under insufficient oxygen conditions [1]. Exposures to PAHs have been identified to associate with increased cancer risks of the skin, bladder and lung [2,3], thus it is of great importance to develop and validate sensitive, specific, noninvasive methods for measuring the exposure to PAHs in humans. Biomonitoring PAHs metabolites especially monohydroxylated PAHs has been proved to be an effective method for evaluating human exposure and body burden. Till now, many works have been carried out to determine OH-PAHs in human urine for exposure assessment [4–13]. However, for the trace levels and complex matrix interference of OH-PAHs in urine, sample process is still a crucial work. To decrease interference and improve sensitivity as far as possible, many efforts have been tried in previous literatures. Typically, OH-PAHs in urine was enzymatic hydrolyzed with β-glucuronidase/sulfatase and subsequently extracted with liquid/liquid extraction [4–6], solid-phase extraction (SPE) [7–12], solid-phase microextraction (SPME) [11] or column switching techniques [13], and then samples were highly concentrated for GC–MS [4,5,7,8,10], (HPLC-FD) [6,9,11] or HPLC–MS/MS analysis [10,12]. Though these methods could meet the analytical requirements, the time-consuming and complicated sample preparation often make the analytical work tedious.

Within the framework of green analytical chemistry, a new clean sample preparation technique, stir bar sorptive extraction (SBSE) has been developed and successfully applied to enrich and selectively determine organic compounds in water, wine, urine and other aqueous matrixes [14–20]. By magnetically stirring the bar in sample solution, target compounds could be simply enriched in the layer of SBSE and then, analytes could be thermally desorbed on-line with GC–MS or liquid desorbed with organic solvents for HPLC analysis. Polydimethylsiloxane (PDMS) is typical coating material in the commercial stir bars. Compared with other sample preparation techniques, SBSE have many advantages, such as ease-of-use, improved sensitivity, high accuracy and low consumption of organic solvents. Desmet [21] and Hiroaki Tao et al. [22] has ever applied SBSE for analyzing OH-PAHs in urine or water with thermal desorption-capillary GC–MS. But in their work, hydroxylated compounds of fluorene and phenanthrene are not included, and in situ-derivation with acetic anhydride are needed before analysis. To simplify sample process and expand application of SBSE in OH-PAHs analysis, we develop a method to simultaneously analyze 11 OH-PAHs in human urines based on SBSE with HPLC–MS/MS detection.

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2. Material and methods

2.1. Chemicals and supplies

1-naphthol (1-OHNap) and 2-naphthol (2-OHNap) were obtained from Labor Dr. Ehrenstorfer (Augsburg, Germany); 2-hydroxyfluorene (2-OHFlu), 1-hydroxypyrene-d_9 (1-OHPyr-d_9), 3-phenanthrol-d_9 (3-OHPhe-d_9), 2-hydroxyfluorene-d_9 (2-OHFlu-d_9) 1-naphthol-d_8 (1-OHNap-d_8), and 1-phenanthrol (1-OHPhe) was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). 1-hydroxypyrene (1-OHPyr) was purchased from AccuStandard, Inc. (New Haven, USA); 3-hydroxyfluorene (3-OHFlu), 2-phenanthrol (2-OHPhe), 3-phenanthrol (3-OHPhe), 4-phenanthrol (4-OHPhe) and 9-phenanthrol (9-OHPhe) were obtained from Chiron AS (Trondheim, Norway); 9-hydroxybenzo(a) pyrene (9-OHBaP) was purchased from Midwest Research Institute (Kansas City, USA). β-Glucoronidase/aryl sulfatase (85,000 β-glucuronidase units/mL and 7500 sulfatase units/mL) was obtained from Sigma (St. Louis, MO, USA). HPLC-grade methanol and acetonitrile were from J. T. Baker (USA). For sample preparation and HPLC analysis, Milli-Q water (Millipore, Milford, MA, USA) was used. The 20-mm long stir bars (Twister™), coated with 1 mm film thick layer of PDMS were purchased from Gerstel GmbH (Mülheim an der Ruhr, Germany).

2.2. Urine sample collection and preparation

Urine samples were collected in the morning from 8 healthy smokers and 4 nonsmokers. All samples were frozen and stored at −40 °C.

Before use, urine was thawed at room temperature and briefly agitated. For enzymatic hydrolysis of glucuronides and sulfates, 5 ml urine was mixed with 10 mL sodium acetate buffer, and pH value of the solution was then adjusted to 5.0. After 1.5 ng 3-OHPhe-d_9, 3 ng 1-OHPyr-d_9,150 ng 1-OHNap-d_8 and 2-OHFlu-d_9 was added, 30 μL β-Glucuronidase/aryl sulfatase was added for enzymatic hydrolysis, the mixture was incubated at 37 °C overnight.

2.3. Conditioning of SBSE bars and extraction of OH-PAHs

The stir bars were pre-conditioned before use by treating with methanol for cleaning and then heat-treated in a thermal desorption tube at 300 °C under a nitrogen flow (30 mL/min) for 1 h.

After enzymatic hydrolysis, to extract OH-PAHs in urines, a stir bar coated with PDMS was rotated at 1000 rpm for 3 h. Then the stir bar was taken out with tweezers and rinsed slightly with distilled water to remove adsorbed sugars, proteins etc. Then, stir bar was transferred to a 5 mL methanol solution and stirred at 1000 rpm for desorption of OH-PAHs. Finally, the methanol solution was evaporated to dryness and the residue was re-dissolved in 100 μL methanol for analysis.

2.4. LC–MS/MS analysis

Analyses were performed on an Agilent LC system coupled with an API 5500 mass spectrometer (AB Scix, USA). Chromatography separation was performed on an Agilent ZORBAX Eclipse PAH column (1.8 μm, 100 mm × 2.1 mm) with acetonitrile (A) and water (B) applied as mobile phases at the flow rate of 200 μL/min. Gradient elution conditions were as follows: 0–3 min, 40% A; 4–7 min, 55% A; 8–12 min, 75% A; 13–16 min, 90% A; and 17–23 min, 40% A. Negative electrospray ionization at 300 °C was applied with multi-reaction-monitoring (MRM) mode. Mass transitions and retention times for analytes and internal standards are shown in Table 1.

3. Results and discussion

3.1. HPLC separation of OH-PAHs

The HPLC separation of the OH-PAHs has been identified as a challenging task in previous published studies [13,23]. To achieve

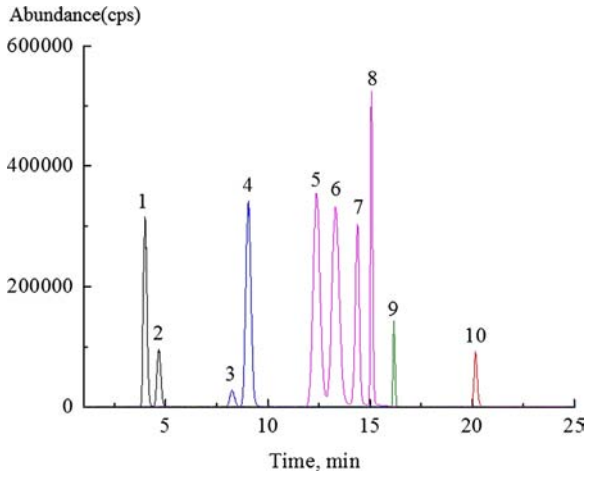

Fig. 1. LC/MS/MS chromatogram of standard OH-PAHs in blank urine. 1: 2-OHNap; 2: 1-OHNap; 3: 3-OHFlu; 4: 2-OHFlu; 5: 3-OHPhe; 6: 2-OHPhe; 7: 9-OHPhe; 8: 1-/4-OHPhe; 9: 1-OHPyr; 10: 6-OHChr.

Table 1
Retention time and MRM parameters used for 11 OH-PAHs.

Analytes	RT (min)	MRM transition		Declustering potential (V)	Collision energy (V)
		Parent ion (m/z)	Daughter ion (m/z)		
2-OHNap	3.95	143.1	115.0	−99	−33
1-OHNap	4.65				
3-OHFlu	8.17	181.0	179.9	−110	−31
2-OHFlu	9.00				
3-OHPhe	12.35				
2-OHPhe	13.32	192.9	164.9	−119	−39
9-OHPhe	14.31				
1-/4-OHPhe	15.03				
1-OHPyr	16.08	216.9	189.1	−114	−47
9-OHBaP	20.34	266.9	239.0	−128	−43
3-OHPhe-d9	11.58	201.9	174.0	−120	−40
1-OHPyr-d9	16.21	226.0	198.0	−155	−47
2-OHFlu-d9	9.00	190.0	188.0	−120	−36
1-OHNap-d8	4.65	151.1	123.0	−115	−37

satisfactory separation, several HPLC columns were evaluated, during which Agilent ZORBAX Eclipse PAH column (1.8 μm, 100 mm × 2.1 mm) proved to be the best analysis column. Fig. 1 showed typical chromatogram of OH-PAHs. Under the optimized HPLC gradients, 9 OH-PAHS could be baseline-separated except for 1- and 4-OHPhe. Consequently, 1-and 4-OHPhe were quantified together by integrating the co-elution peak of 1- and 4-OHPhe, as shown in a LC/MS/MS chromatogram.

3.2. Optimization of SBSE conditions

Sorption extraction is controlled by the partitioning efficiency of solutes between the polymer coating and the sample matrix. According to the literature, parameters such as pH value of matrix, extraction time, desorption solvents and desorption time greatly affect the SBSE efficiency [14]. In this study, these parameters are optimized to develop a novel method for the analysis of OH-PAHs in urine matrix.

In our work, pH value of urine sample was adjusted to 1, 3, 5 and 7 with 1 M HCl. As can be seen in Fig. 2, with the pH value increasing, the response of all the compounds increased, and pH 5 led to the best extraction efficiency for most of the compounds, thus pH 5 was selected for all subsequent experiments.

The extraction-time profiles of the analytes were studied from1 h to 4 h. As can be seen in Fig. 3, with the time increasing, the response of all compounds increased gradually until 3 h, and subsequently decreased slightly, thus the extraction time of 3 h was chosen in the following experiment.

The solvent used for desorption was investigated. To this end, stir bar was immersed in methanol, acetonitrile and methanl/CH_2Cl_2. It was found that recovery obtained by liquid desorption in

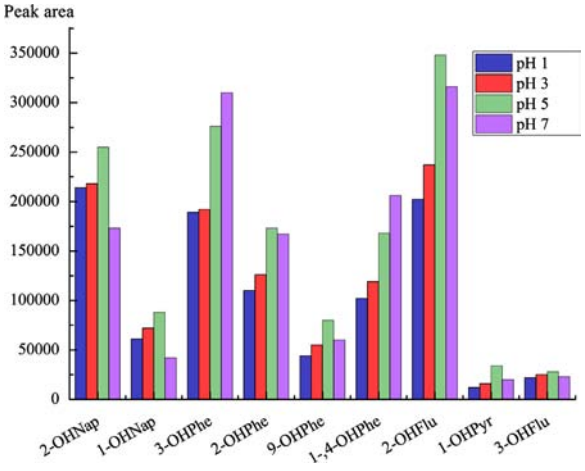

Fig. 2. Influence of pH on the extraction efficiency of SBSE.

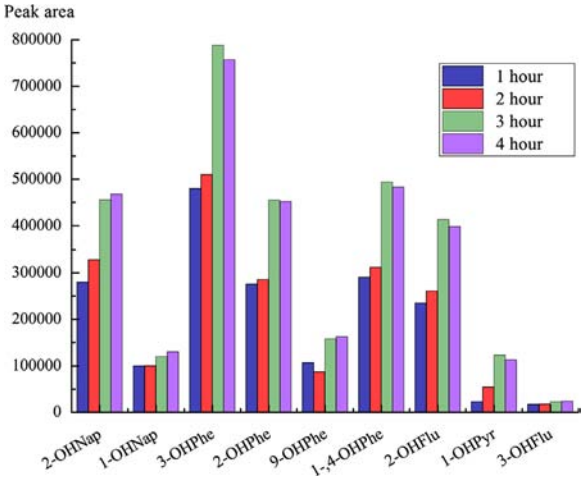

Fig. 3. Influence of extraction time on the extraction efficiency of SBSE.

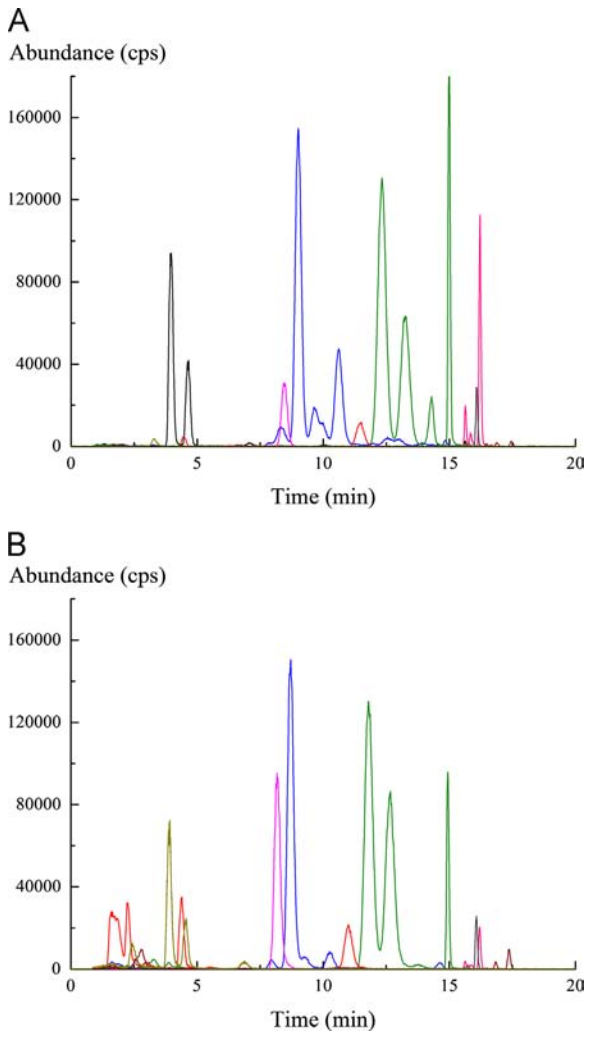

Fig. 4. Urine chromatograms obtained from the (A) SBSE method and (B) C18 SPE method.

Table 2
Calibration curves and detection limits of 11 OH-PAHs.

Compound	Range (ng/mL)	Linear regression equation	Correlation (r)	LOQ (ng/mL)
1-OHNap	0.01–100	$Y=0.0114X+0.00222$	1	0.002
2-OHNap	0.01–100	$Y=0.0289X+0.00757$	1	0.003
2-OHFlu	0.01–100	$Y=0.0238X+0.0378$	1	0.002
3-OHFlu	0.01–100	$Y=0.00223X+0.00069$	0.9999	0.022
1-OHPhe	0.01–40	$Y=0.0682X+0.0157$	0.9999	0.003
2-OHPhe	0.01–40	$Y=0.169X+0.0318$	0.9999	0.003
3-OHPhe	0.01–40	$Y=0.174X+0.0443$	0.9999	0.003
4-OHPhe	0.01–40	$Y=0.0682X+0.0157$	0.9999	0.003
9-OHPhe	0.01–40	$Y=0.094X+0.00369$	1	0.002
1-OHPyr	0.01–40	$Y=0.0523X+0.0103$	0.9999	0.003
9-OHBaP	0.01–40	$Y=0.132X+0.0493$	0.9990	0.001

Note: 1-/2-OHNap were quantified using 1-OHNap-d8; 2-/3-OHFlu were quantified using 2-OHFlu-d9; 1-/2-/3-/4-/9-OHPhe was quantified using 3-OHPhe-d9, 1-OHPhe and 4-OHPhe could not be separated chromatographically and were quantified together; 1-OHPyr, 9-OHBaP were quantified using 1-OHPyr-d9.

methanol was slightly higher than that in acetonitrile and methanl/CH_2Cl_2. Thus, methanol is chosen as the desorption solvent, and 1 h desorption time was found to be enough and suitable in the SBSE procedure.

Table 3
The precision and recovery of OH-PAHs.

Compound	Intra-day precision (%)	Inter-day precision (%)	Recovery (%)	
1-OHNap	3.08	6.89	Low	116.7
			Middle	99.76
			High	104.5
2-OHNap	9.28	6.29	Low	95.72
			Middle	97.00
			High	94.68
2-OHFlu	5.68	8.76	Low	89.32
			Middle	92.37
			High	97.76
3-OHFlu	4.65	7.66	Low	90.95
			Middle	98.31
			High	93.89
9-OHBap	13.0	10.9	Low	104.0
			Middle	82.05
			High	71.86
1,4-OHPhe	5.47	11.3	Low	115.8
			Middle	122.7
			High	118.7
2-OHPhe	6.88	10.8	Low	100.4
			Middle	78.16
			High	74.44
3-OHPhe	5.42	6.40	Low	111.2
			Middle	103.4
			High	99.23
9-OHPhe	7.40	3.26	Low	113.3
			Middle	73.88
			High	80.58
1-OHPyr	12.4	4.72	Low	133.2
			Middle	103.0
			High	126.7

3.3. Comparison of clean up effect of SBSE and solid extraction

In previous reference, OH-PAHs in urine were typically cleaned up by C18 solid phase extraction after enzymatic hydrolysis. In this work, the C18 SPE method was also applied for sample cleanup as reported before [24]. In detail, urine sample was transferred to a C18 cartridge, the loaded cartridge was washed with 10 mL water and 10 mL CH_3OH/H_2O (v/v 1:4) subsequently, finally the cartridge was thoroughly dried with nitrogen and eluted with 7.5 ml *n*-hexane/methanol. The eluate was evaporated to dryness under reduced pressure at room temperature. The residue was re-dissolved in 0.1 mL methanol.

A comparison of sample total ion chromatograms between the two methods is shown in Fig. 4. Obviously, the sample obtained with SBSE extraction had a cleaner chromatograms in comparison with that obtained by C18 solid phase extraction. Especially for 2-OH-Nap, interferences are greatly decreased, and responses for 3-OH-Flu and 9-OH-phe improved greatly, indicating that SBSE has certain selectivity for OHPAHs.

3.4. Validation of the SBSE method

The blank urine sample spiked with 11 OH-PAHs was taken for analysis to evaluate the developed method. The data of linear range, correlation coefficients, LODs, LOQs, average recoveries and reproducibility for the OH-PAHs under the optimized experimental conditions were listed in Tables 2 and 3. It can be seen from the data that the developed SBSE–HPLC-MS/MS method presents a good performance. The linear range was between 0.01 and 40/100 ng/ml with good regression coefficients ($R^2 > 0.999$). The detection limit for each OH-PAHs was calculated as three times the standard derivation at the lowest spiked standard concentration of blank urine (Table 2), and those were in the range of 1–3 pg/mL, lower than the reported literature [7,10,23]. The recoveries of all the OH-PAHs ranged from 71.9% (9-OHBap) to 133.2% (9-OHPhe).

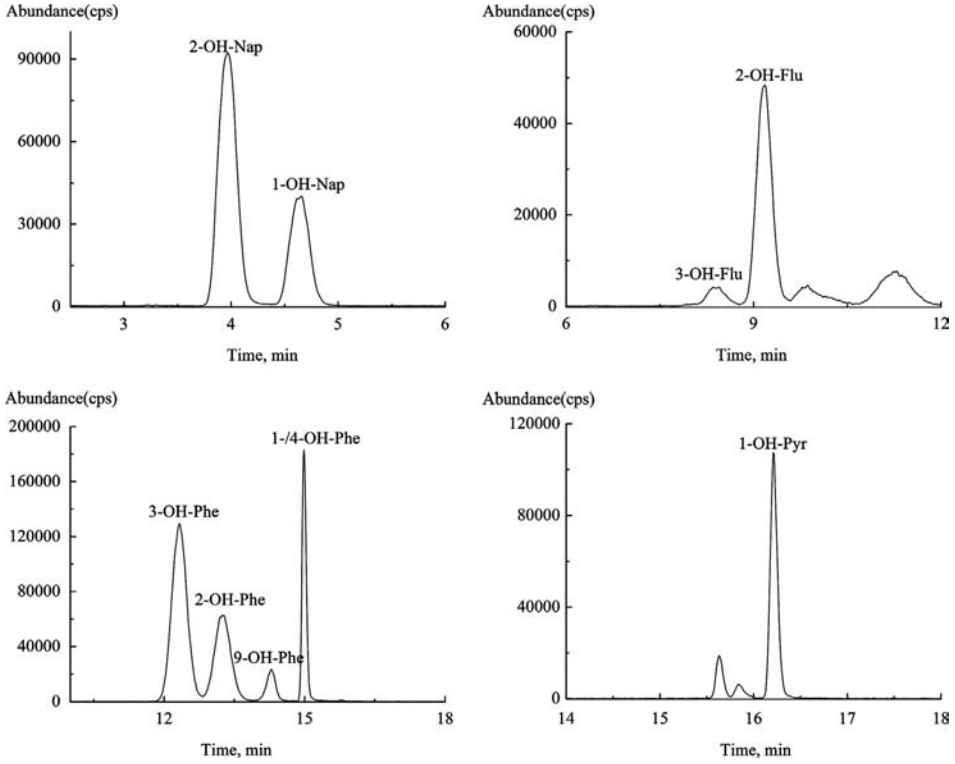

Fig. 5. Chromatogram of smoker's urine. 1:2-OHNap; 2:1-OHNap; 3:3-OHFlu; 4:2-OHFlu; 5:3-OHPhe; 6:2-OHPhe; 7:9-OHPhe; 8:1-/4-OHPhe; 9:1-OHPyr.

Table 4
The concentration of OH-PAHs in human urine.

Concentration (ng/mL)		2-OH Nap	3-OH Phe	3-OH Flu	1-OH Nap	2-OH Phe	1-OH Pyr	2-OH Flu	9-OH Phe	1,4-OH Phe
Smokers	1	12.1	1.4	3.63	12.0	0.7	1.5	3.6	0.3	3.1
	2	78.2	3.7	17.7	27.8	1.8	8.6	12.6	1.8	6.4
	3	1.7	6.0	3.5	1.9	3.3	4.0	15.9	0.4	9.6
	4	4.1	1.7	1.4	1.5	0.6	1.3	3.9	0.4	1.8
	5	33.2	2.0	9.2	10.3	0.9	2.8	7.7	0.9	4.3
	6	4.9	1.3	1.3	1.0	0.7	3.1	2.6	0.1	2.0
	7	20.6	1.9	4.1	12.9	0.8	2.2	4.2	0.7	3.3
	8	12.2	2.4	6.1	4.6	1.2	3.8	7.7	0.6	3.0
	Average	20.8	2.6	5.9	9.0	1.3	3.4	7.3	0.7	4.2
Non-smokers	1	2.8	1.1	0.43	2.2	0.69	1.9	1.9	0.04	1.2
	2	4.2	0.9	0.52	1.9	0.65	1.7	1.6	0.05	0.9
	3	2.0	1.1	0.60	1.7	0.73	1.5	2.7	0.02	1.1
	4	5.1	1.9	0.72	3.5	1.42	3.9	5.1	0.16	4.2
	Average	3.5	1.2	0.57	2.3	0.87	2.2	2.8	0.06	1.8

The precision of the proposed method was evaluated using within and between day repeatability calculated as R.S.D on three replicates, and the variations were between 3.1% (1-OHNap) and 13.0% (9-OHBap). The good reproducibility, high sensitivity and recovery well indicated that the developed method may be a good method for analyzing OH-PAHs in urine matrix.

3.5. Analysis of OH-PAHs in human urine

The developed method was also applied to urines obtained from 8 smokers and 4 nonsmokers. Fig. 5 shows a chromatogram obtained from a smoker′s urine. All the compounds are well separated with good shape. Except for 9-OHBaP, all other OH-PAHs could be detected in all samples. This may be because that 9-OHBaP has 5 rings structure, it is excreted mainly from feces, and the concentration in urine is fairly low.

The analytical results of urine samples were shown in Table 4. Since PAHs are widely present in food, environment, cigarette smoke and automobile exhaust, it is not difficult to understand that OH-PAHs could also be detectable in urines of nonsmokers. From the data, it can be seen that 2-OH-Nap, 1-OH-Nap and 2-OH-Flu are mostly abundant, 2-OH-Phe and 9-OH-phe are in trace level. In smokers' urines, concentrations of OH-PAHs also differed greatly between individuals, which could be ascribed to different smoking behaviors and metabolism between individuals. Compared the data between the two groups, it is found that the concentrations of OH-PAHs are obviously higher in urines of smokers than that of nonsmokers, showing that smoking can greatly increase the intake of the harmful compounds. Moreover, ten OH-PAHs are varied in reflecting the difference between smokers and nonsmokers, 2-OH-Nap, 3-OH-Flu and 9-OH-Phe are more sensitive to smoking, showing their potential applications to assess PAHs exposures in future.

4. Conclusion

A method for simultaneous determination of 11 OH-PAHs in human urine based on the SBSE and HPLC–MS/MS method has been developed and validated. For the high sensitivity and good reproducibility, the method was successfully applied to the analysis of trace OH-PAHs in smokers and nonsmokers' urine. Compared to the previous method, the developed method has many other advantages, such as ease operation, decreased interference and less organic solvent consumption. Thus, this method may be a potentially alternative in assessing PAHs exposure to environmental in human urine.

Acknowledgements

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