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Direct analysis of urinary 1-hydroxypyrene using extractive electrospray ionization ion trap tandem mass spectrometry†

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Fast detection (0.5 min) of 1-hydroxypyrene (1-OHP) in urine and hydrolyzed urine without pre-treatment has been successfully achieved by using extractive electrospray ionization ion trap tandem mass spectrometry (EESI-MS/MS) under optimized EESI and MS/MS conditions. Experimental results indicated that for MS/MS analysis the operating parameter of activation Q (AQ) was critical for 1-OHP fragmentation in collision induced dissociation (CID) experiments, and in the EESI process the primary ESI solvent was a key factor for extractive ionization of urinary 1-OHP. The limit of detection (LOD) and limit of quantification (LOQ) were 0.75 and 2.25 μM for both urine and hydrolyzed urine samples. A five-point working curve ranging from 2.29 to 22.91 μM for 1-OHP in urine or hydrolyzed urine was obtained (R^2 = 0.9941 for urine and R^2 = 0.9983 for hydrolyzed urine), and the relative standard deviations (RSD, n = 6) were 2.6–9.7% and 1.5–6.4%, respectively. The developed EESI-MS/MS method was validated by detecting 1-OHP in both urine and hydrolyzed urine samples. Recoveries were determined to >50% and 0.5 min was taken for each measurement, indicating that the proposed method is a promising strategy for high throughput analysis of urinary 1-OHP required for health risk assessment of exposure to polycyclic aromatic hydrocarbons (PAHs).

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of ubiquitous environmental contaminants and of great concern due to their carcinogenic effects. PAHs result from incomplete combustion of organic materials, and humans can be exposed to PAHs from various sources, including occupation (*e.g.*, coke plants, aluminum plants, iron and steel foundries), environment (*e.g.*, air, water and soil pollution), personal habits (*e.g.*, smoking), diet (*e.g.*, broiled and smoked food), and even medical treatment (*e.g.*, coal tar).[1,2] Estimation of exposure to PAHs is necessary for both occupationally and non-occupationally exposed people, and is especially important for occupational exposure regarding greater health risk. Urinary 1-hydroxypyrene (1-OHP) is the most widely used biomarker of internal dose of PAHs, and is capable of evaluating PAH exposure from multiple routes.[3] 1-OHP concentrations detected in urine samples from non-occupationally and occupationally exposed population are 0.5–500 μg L^{-1}.[4,5]

Until now, a number of analytical techniques have been investigated for the analysis of urinary 1-OHP, such as fluorescence spectroscopy (*e.g.*, fixed fluorescence[6,7] and synchronous fluorescence[8]), mass spectrometry (MS)[9,10] (*e.g.*, high resolution MS[11] and tandem MS (MS/MS)[12,13]), electrochemical[14,15] and immunochemical assays.[16,17] Quite recently, methods based on spectroelectrochemical[18,19] and chemiluminescence[20]

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† Electronic supplementary information (ESI) available: (i) Schematic diagram of the homemade EESI source (Fig. S1); (ii) EESI-MS fingerprints of the urine sample containing 2.29 μM of 1-OHP when eleven solvents were applied as primary ESI solvent, respectively (Fig. S2); (iii) effect of NH_4Ac in the primary ESI solvent on 1-OHP ionization efficiency (Fig. S3); (iv) optimization of the ESI voltage, ESI solvent flow rate, sample flow rate and ion-transport capillary temperature (Fig. S4); (v) signal intensity variation with 1-OHP concentration in ultrapure water; (vi) standard addition curves for quantifying 1-OHP in urine and hydrolyzed urine samples (Fig. S6); (vii) preparation of spiked urine samples (Table S1); (viii) preparation of spiked hydrolyzed urine samples (Table S2); (ix) preparation of spiked ultrapure water samples (Table S3); (x) RSDs (n = 6) of each spike concentration in urine, hydrolyzed urine or ultrapure water samples (Table S4); and (xi) spike concentrations and RSDs (n = 6) of the standard addition curves for quantifying 1-OHP in urine and hydrolyzed urine samples (Table S5). See DOI: 10.1039/c3ay40241j

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techniques have also been reported. Among all these methods developed, MS/MS-based methods (*e.g.*, LC-MS/MS,[12] GC-MS/MS[21]) excel in sensitivity and selectivity; however, time-consuming and laborious sample pre-treatments are normally included, which are essentially incompatible with the high throughput analysis of urinary 1-OHP required for health risk assessment of PAHs exposure.

Recently, the analytical efficiency of MS-based methods has been greatly improved with the introduction of ambient ionization techniques, by which samples can be directly analyzed under ambient conditions without pre-treatment.[22] Direct detection of analytes in different types of samples has been successfully demonstrated by using different ambient ionization techniques such as desorption electrospray ionization (DESI),[23] direct analysis in real time (DART),[24] desorption atmospheric pressure chemical ionization (DAPCI),[25] dielectric barrier discharge ionization (DBDI),[26] low-temperature plasma probe (LTP),[27] atmospheric solids analysis probe (ASAP),[28] laser ablation with electrospray ionization (LAESI),[29] *etc.* Extractive electrospray ionization (EESI) is one of the newly developed ambient ionization techniques and is characterized by facilitating rapid analysis of complex liquid samples.[30–32] In EESI, liquid samples are nebulized to intersect an ESI plume, leading to an on-line microdroplet–microdroplet extraction; then, analytes in liquid samples are extracted into charged microdroplets resulting from the primary ESI solvent and further ionized during the ESI process.[33,34] By applying EESI, ion suppression effects can be effectively reduced due to the extraction process, thus allowing direct and rapid analysis of various complex liquids (*e.g.*, urine, beer, ionic liquid, honey, edible oil).[30,35–37]

In this study, an EESI-MS/MS method for fast detection of urinary 1-OHP has been investigated by using a homemade EESI source coupled to an ion trap tandem mass spectrometer. MS/MS and EESI conditions were systematically optimized, and quantitative analysis of 1-OHP in urine and hydrolyzed urine samples was carried out. Fast detection (0.5 min) of urinary 1-OHP by using EESI-MS/MS was successfully demonstrated, indicating that the proposed method is a promising strategy for high throughput analysis of urinary 1-OHP. Furthermore, the present work also provides helpful information for applying EESI-MS/MS to the analysis of low-polarity organic compounds in complex liquid matrices.

Materials and methods

Materials

1-OHP, β-glucuronidase, ammonium acetate (NH_4Ac, analytical reagent grade) and sodium acetate (NaAc, analytical reagent grade) were purchased from Sigma-Aldrich (St Louis, MO, USA). Methanol (MeOH) (HPLC grade) was provided by Burdick & Jackson (Muskegon, MI, USA). Organic solvents of ethanol (EtOH), 1-propanol (PrOH), 1-butanol (BuOH), 1-pentanol (PeOH), 1-hexanol (HeOH), benzene (BeNE), *n*-pentane (PeNE) and *n*-hexane (HeNE) (GC standards) were obtained from Tianjin Guangfu Fine Chemical Research Institute. Toluene (TeNE) (analytical reagent grade) was brought from Sinopharm Chemical Reagent Co. Ltd. Hydrochloric acid (HCl), ammonia water (NH_4OH, 25–28% by weight as ammonia) and acetic acid (HAc) were supplied by Jiangxi Hongdu Biochemical Co., Ltd., Nanchang Xinguang Fine Chemical Factory and Guangzhou Xilong Chemical Co. Ltd, respectively. Ultrapure water (resistivity 18.2 MΩ cm) was supplied by a Barnstead Nanopure ultrapure water purification system (Thermo Scientific, USA). All chemicals were directly used without further treatment.

Spiked sample preparation

Spiked samples were prepared by diluting 1-OHP stock solution with individual solutions (*e.g.*, urine, hydrolyzed urine, ultrapure water) (see Tables S1–4 in the ESI† for more details), and always freshly prepared on each experimental day. The stock solution of 1-OHP (0.46 mM) was obtained by dissolving 10.0 mg of 1-OHP in 100 mL of MeOH, and stored in 20 mL brown vials in the dark at 4 °C. Urine samples were collected from non-PAH-exposed healthy volunteers (24–26 years old) and kept at −20 °C before use. Hydrolyzed urine samples were prepared according to the procedure reported by Fan *et al.*[38] In brief, 4 mL of urine was mixed with 0.5 mL of 0.1 M HCl, 1.5 mL of 0.5 M NaAc–HAc buffer solution (pH = 5) and 10 μL of β-glucuronidase; then, the mixture was incubated at 37 °C in a water bath overnight.

EESI-MS/MS conditions

A homemade EESI source (Fig. S1†) was set up and coupled to a commercial LTQ-XL mass spectrometer (Finnigan, San Jose, USA) as described previously,[39,40] *i.e.*, the distance a between the end-tips of two sprays and the distance b between the spray tips and the MS inlet were about 1 and 10 mm, respectively. The angle α between the two sprays and the angle β between individual sprays and the MS inlet were around 60° and 150°, respectively. The flow rates of the primary ESI solvent and sample solution were set to 3 μL min^{-1} and 5 μL min^{-1}, respectively. High purity nitrogen gas (N_2, purity ≥ 99.999%) supplied by a gas cylinder (pressure 1.2 MPa) was used for nebulizing the primary ESI solvent and sample solution.

The LTQ-XL mass spectrometer was operated in the negative ion detection mode. The ESI voltage was set to −4 kV. The temperature of the ion-transport capillary was 400 °C. The maximum ion injection time was 200 ms and the automatic gain control was enabled to regulate the number of ions injected into the cell. For MS/MS analysis, collision induced dissociation (CID) experiments were carried out. The ion at m/z 217 was selected as the precursor ion; the isolation width and activation time were set to 2.0 Da and 30 ms, respectively. Helium gas (He) was used as the collision gas and the pressure in the collision cell ranged from 0.82–0.83 × 10^{-5} Torr. It is noteworthy that EESI and MS/MS conditions mentioned above were applied as initial conditions, which were further optimized in the study.

Before experiments, the homemade EESI source was checked by analyzing a 1-OHP MeOH solution (0.092 μM) to ensure that the homemade source worked properly. Besides, a MeOH solution containing 0.23 μM of 1-OHP and a urine sample containing 2.3 μM of 1-OHP were used for optimizing MS/MS

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and EESI conditions, respectively. For each sample, at least six independent measurements were carried out.

Results and discussion

Optimization of MS/MS conditions

In CID experiments, two operating parameters were optimized, *i.e.*, activation Q (AQ) and normalized collision energy (NCE) (Fig. 1). AQ is the RF frequency used to fragment ions;[41,42] NCE scales the amplitude of the voltage applied to the ions, and this scaled voltage is related to the translational kinetic energy of ions during their interaction with the neutral atoms (He) in the ion trap.[43]

When AQ was in the range of 0.10–0.30, the deprotonated ion of 1-OHP, *i.e.*, $[M-H]^-$ at *m*/*z* 217, was ineffectually fragmented at NCE 0–100%. Then, as the value of AQ was continually increased from 0.35 to 0.70, the fragment ion of 1-OHP ($[M-H-CO]^-$ at *m*/*z* 189) was produced due to the neutral loss of CO (28 Da) at NCE ≥ 30% (Fig. 1b and 2), and the highest intensity of the signal at *m*/*z* 189 was achieved at NCE 35–55% at each AQ value. By comparing the highest intensity at individual AQ ranging from 0.35 to 0.70, the highest intensity was identified when AQ was 0.40, 0.45 and 0.50. Thus, for MS/MS analysis, AQ and NCE were set to 0.40 and 40% in the following experiments, respectively. Additionally, the intensity of the peak at *m*/*z* 189 in MS/MS spectra was used in the following studies of EESI conditions optimization, matrix effect and quantitative analysis.

Additionally, since the fragment ion of 1-OHP was obtained only when AQ ≥ 0.35, AQ was more critical for effective fragmentation of 1-OHP than NCE. Similar phenomena have also been observed for chlorophenols[42] and creatinine.[44] The possible explanation is that at lower AQ (*e.g.*, AQ ≤ 0.30 in this study), precursor ions are ejected from the cell or quench in the ion trap with the increase of NCE, and thus the internal energy of precursor ions could not be accumulated for fragmentation. In contrast, when AQ is greater than the threshold, precursor ions would be trapped in the cell at increased NCE; meanwhile, the internal energy of the ions can be accumulated rapidly, resulting in effective fragmentation.[45]

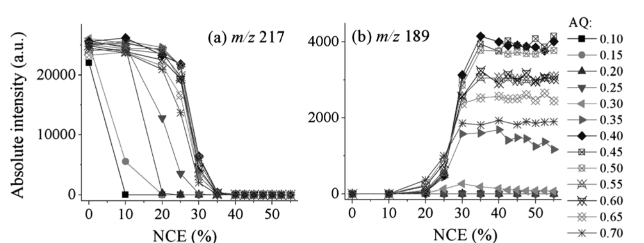

Fig. 1 Intensity variation of (a) deprotonated 1-OHP ($[M-H]^-$ at *m*/*z* 217) and (b) its fragment ion ($[M-H–CO]^-$ at *m*/*z* 189) with activation Q (AQ) and normalized collision energy (NCE).

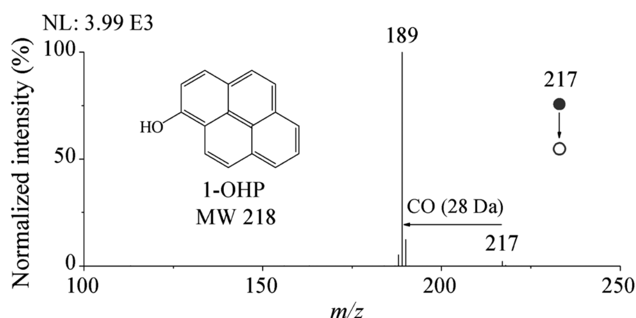

Fig. 2 EESI-MS/MS spectrum of the 1-OHP MeOH solution (0.092 μM).

Optimization of EESI conditions

EESI conditions of primary ESI solvent composition, ESI voltage, primary ESI solvent flow rate, sample flow rate and ion-transport capillary temperature were optimized to improve extraction and ionization efficiencies.

The primary ESI solvent was optimized by using eleven solvents of different polarities, including water, MeOH, EtOH, PrOH, BuOH, PeOH, HeOH, BeNe, ToNE, PeNE and HeNE (Fig. 3a). The most polar solvent was water ($E_T^N = 1.000$) while the least polar solvents were PeNE and HeNE ($E_T^N = 0.009$) (Fig. 3b). E_T^N is the normalized solvent polarity parameter, which is derived from the empirical solvent polarity parameter $E_T(30)$ (kcal mol^{-1}).[46]

Among the eleven solvents investigated, the highest intensity was obtained when MeOH and PeOH were applied (Fig. 3a). This is probably because when MeOH is used as the primary ESI solvent, more primary ions are produced (Fig. S2†), leading to higher ionization efficiency. In the case of PeOH, the solubility of 1-OHP in PeOH is increased, and better extraction efficiency is achieved. This result also indicates that in the EESI process the selection of the primary ESI solvent is a compromise between solvent extraction and ionization abilities, *e.g.*, MeOH excels in the ionization process, although it extracts less urinary 1-OHP than other less polar alcohols; PeOH performed better in the extraction process, compensating for its deficiency in ionization efficiency. Besides, EESI-MS mass spectra (over the mass range of *m*/*z* 50–1000) of the urine sample were different from each other (Fig. S2†), when eleven solvents were used as primary ESI solvent, respectively, implying the different extraction and/or ionization abilities of the solvents. However, since our work

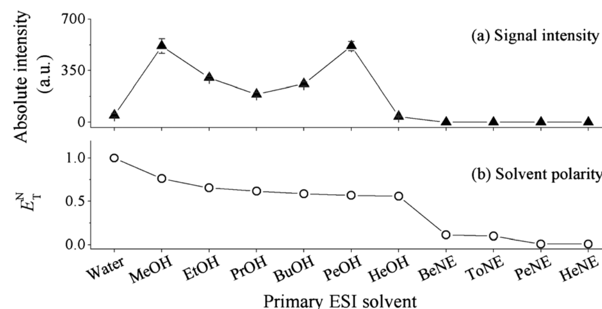

Fig. 3 (a) Intensity variation of the signal at *m*/*z* 189 when eleven solvents were used as primary ESI solvent, respectively and (b) polarities of the solvents. E_T^N is the normalized solvent polarity parameter and is derived from the empirical solvent polarity parameter $E_T(30)$ (kcal mol^{-1}).[46]

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has been focused on urinary 1-OHP detection, these phenomena were not further investigated.

Effects of supporting electrolytes in primary ESI solvent on ionization efficiency were also studied. Five solvents of different polarities containing 1.0×10^{-4} M of NH_4Ac were used as primary ESI solvent, respectively, and the most significant improvement was observed for MeOH added with NH_4Ac (Fig. S3†). Furthermore, when MeOH was added with 1.0×10^{-7} to 1.0×10^{-2} M of NH_4Ac, the signal intensity was increased with the increase of NH_4Ac concentration, and the highest intensity was achieved when 1.0×10^{-2} M of NH_4Ac was applied (Fig. 4). Similar results were obtained when 1.4×10^{-3} to 1.4×10^{-1} M of NH_4OH was added into MeOH (Fig. 4). Finally, the primary ESI solvent composition was optimized to MeOH containing 1.0×10^{-2} M of NH_4Ac.

Other optimal EESI conditions were acquired as follows: the ESI voltage was set to −3 kV (Fig. S4a†) and the ESI solvent flow rate was 1 μL min^{-1} (Fig. S4b†); for sample flow rate, despite the fact that higher signal intensity was achieved at higher sample flow rate, the mass spectrometer might be contaminated, and thus the appropriate sample flow rate was determined to 4 μL min^{-1} (Fig. S4c†); as for the ion-transport capillary temperature, 400 °C was adopted (Fig. S4d†) regarding the maximum temperature permitted on the instrument is 450 °C.

Matrix effect

The matrix effect has been tentatively studied by analyzing four types of mixtures of hydrolyzed urine and ultrapure water. The mixtures included 100% hydrolyzed urine, hydrolyzed urine : water (75 : 25, v/v), hydrolyzed urine : water (50 : 50, v/v) and 100% water (Fig. 5). Spiking concentrations of 1-OHP in each type of mixture were 2.29 μM and 11.45 μM. For the solutions containing 2.29 μM of 1-OHP, signal intensities of 1-OHP were comparable to each other, and similar phenomena were also observed for the solutions spiked with 11.45 μM of 1-OHP. These results indicate that 1-OHP signal intensity was not affected by matrix compounds even when 100% hydrolyzed urine sample was directly used, suggesting that the matrix effect can be effectively reduced by using EESI.

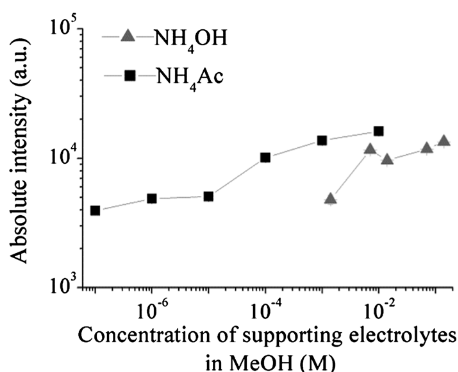

Fig. 4 Signal intensity variation with concentration of NH_4OH or NH_4Ac added to MeOH. Note that logarithmic axes are used in this figure.

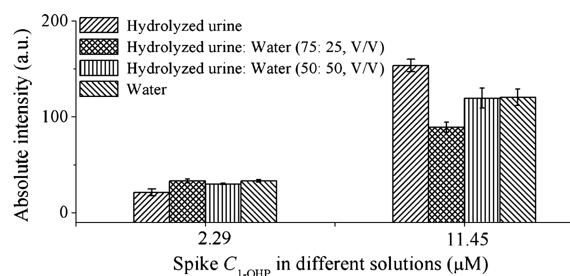

Fig. 5 Signal intensities obtained by EESI-MS/MS when four types of mixtures of hydrolyzed urine and ultrapure water were analyzed.

Quantitative analysis of urinary 1-OHP

With optimized EESI and MS/MS conditions, the limit of detection (LOD) ($S/N = 3$) for 1-OHP in ultrapure water samples (urine is about 95% water) is 0.35 μM (Fig. S5 and Table S5†); for urine and hydrolyzed urine samples, LOD ($S/N = 3$) was 0.75 μM and limit of quantification (LOQ) ($S/N = 10$) was 2.25 μM (Fig. 6). The signal intensity was linearly related to the 1-OHP concentration in the range of 2.29–22.91 μM ($R^2 = 0.9941$ for urine and $R^2 = 0.9983$ for hydrolyzed urine) (see insets in Fig. 6a and b), and relative standard deviations (RSDs) were 2.6–9.7% for urine ($n = 6$) and 1.5–6.4% for hydrolyzed urine ($n = 6$) (Table S5†). 0.5 min was taken for each measurement and 8 min was required for six independent measurements of one sample. Intra-day variations were in the range of 5.8–19.9% ($n = 6$) for urine samples and 4.4–9.2% ($n = 6$) for hydrolyzed urine samples.

Furthermore, the developed method was tentatively validated by analyzing urine and hydrolyzed urine samples containing 2.29 μM of 1-OHP. It is noteworthy that a standard addition method was adopted for the quantification of 1-OHP in real samples instead of an external calibration method. This is because influences on quantification of urinary 1-OHP caused by different matrix compositions of different samples can be avoided. Based on the standard addition curves (Fig. S6 and Table S6†), 1-OHP concentrations in urine and hydrolyzed urine were determined to 1.19 and 1.16 μM, respectively, and recoveries were around 50%.

Finally, it should be noted that the proposed EESI-MS/MS method is applicable to the detection of urinary 1-OHP even without chromatographic separation (*e.g.*, LC). This is because 1-OHP is the dominant metabolite of pyrene[48] and no chromatographic separation is required.

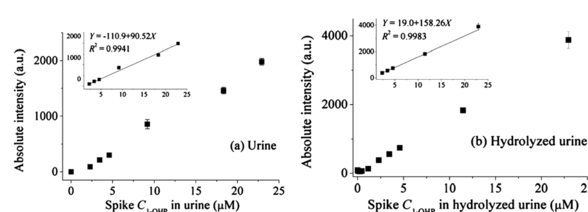

Fig. 6 Signal intensity variation with 1-OHP concentration ($C_{1\text{-OHP}}$) in (a) urine and (b) hydrolyzed urine; insets in (a) and (b): linear relationship between signal intensity and $C_{1\text{-OHP}}$ ranging from 2.29–22.91 μM. The error bars are the standard deviation (SD) of the mean value obtained from six independent measurements.

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Conclusions

In this study, an ambient MS method for fast quantification of urinary 1-OHP has been successfully demonstrated by using a homemade EESI source coupled to an ion-trap tandem mass spectrometer. Instead of spending hours on sample pre-treatment required in conventional methods (*e.g.*, HPLC, HPLC-MS), 0.5 min is taken for each measurement with the EESI-MS/MS method, which suggests that the method would be a promising strategy for high throughput analysis of urinary 1-OHP required for PAH risk assessment. Besides, the method sensitivity could be further improved by applying an ion funnel between the EESI source and MS interface;[47] the method accuracy and reproducibility can also be improved with the improvement of the homemade EESI source.

Acknowledgements

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