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Fast and sensitive determination of urinary 1-hydroxypyrene by packed capillary column switching liquid chromatography coupled to micro-electrospray time-of-flight mass spectrometry

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Abstract

The present work reports capillary liquid chromatographic column switching methodology tailored for fast, sensitive and selective determination of 1-hydroxypyrene (1-OHP) in human urine using micro-electrospray ionization time-of-flight mass spectrometric detection. Samples (100 μl) of deconjugated, water diluted and filtered urine samples were loaded onto a 150 μm I.D.×30 mm 10 μm Kromasil C_{18} pre-column, providing on-line sample clean-up and analyte enrichment, prior to back flushed elution onto a 150 μm I.D.×100 mm 3.5 μm Kromasil C_{18} analytical column. Loading flow rates up to 100 μl/min in addition to the use of isocratic elution by a mobile phase composition of acetonitrile/water (70/30, v/v) containing 5 m*M* ammonium acetate provided elution of 1-OHP within 5.5 min and a total analysis time of less than 15 min with manual operation. Ionization was performed in the negative mode and 1-OHP was observed as $[M-H]^-$ at m/z 217.08. The method was validated over the concentration range 0.2–40 ng/ml 1-OHP in pre-treated urine, yielding a coefficient of correlation of 0.997. The within-assay ($n=6$) and between-assay ($n=6$) precisions were in the range 6.4–7.3 and 7.0–8.1%, respectively, and the recoveries were in the range 96.2–97.5 within the investigated concentration range. The method mass limit of detection was 2 pg, corresponding to a 1-OHP concentration limit of detection of 20 pg/ml (0.09 nmol/l) diluted urine or 0.3 ng/ml (1.35 nmol/l) urine.
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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are formed in all kinds of incomplete combustion processes and are widely distributed throughout the environment [1]; elevated PAH exposure may lead to occupational hazards. Examples of such industries are coke plants, aluminium plants, iron and steel foundries, and rubber- and oil manufacturing companies. PAHs are metabolized in the human body by cytochrome P450 monooxygenases, forming dihydrodiol epoxides along the metabolic pathway. These epoxides can react with DNA to covalently bind adducts, which is the initial step of malignant transformation of cells [2–4]. Occupational PAH exposure and elevated cancer risk have been reported

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in several epidemiological studies [5–14]. Thus, monitoring of PAHs in relevant industries is important to industrial hygiene. Although PAH exposure is mainly related to occupational environment, there are several other sources for non-occupational exposure to PAHs such as tobacco smoking, ambient air, food, and water [15–17].

PAH monitoring in workroom atmospheres is traditionally performed by air monitoring, where the compounds are trapped on a filter and/or an adsorbent prior to chromatographic determination, providing a measurement of the PAH workroom air concentration only. Biomonitoring involves analysis of human tissues, blood and excreta for evidence of exposure to chemical substances, and may involve direct measurement of a chemical or a metabolite in a biological matrix or indirect measurement of a biochemical or physiological change that occurs in response to exposure. In order to obtain a measure of the total exposure of an individual to a chemical substance from all routes including inhalation, and dermal and oral pathways, biomonitoring methods are preferred over the more commonly used air monitoring methods. Furthermore, effects of exposure can differ greatly between individuals due to differences in toxicokinetics and toxicodynamics. Thus, individual dose monitoring will probably play an increasingly important role in modern occupational toxicology.

In industries where workers are exposed to PAHs, the level of exposure is related to their working procedures and tasks. Thus, there is a need for selective and sensitive analytical biomonitoring methods covering a wide concentration range in order obtain a measure of the exposure of individuals with different working tasks. Furthermore, non-occupational related exposure to PAHs for non-smokers is only minute, even in urban areas, which adds further to the need for analytical sensitivity if a biomonitoring method for determination of 1-hydroxypyrene (1-OHP) covering a wide exposure range is to be developed.

Determination of PAH metabolites in urine from exposed individuals has proven to be accurate and reliable for measurement of PAH exposure [18–20], and the primary metabolite of pyrene, 1-hydroxypyrene (1-OHP), has been widely adapted as a general biomarker for overall PAH exposure [21–23]. Pyrene is a major constituent of most PAH emissions and it is mainly metabolized to 1-OHP, which is readily excreted in urine as more water soluble sulphate- and glucuronide conjugates. Several methods for determination of urinary 1-OHP have been published employing conventional liquid chromatography (LC) [24–28] or gas chromatography (GC) with different derivatization techniques [29,30]. Nevertheless, perhaps the most widely used method for determination of 1-OHP was published by Jongeneelen et al. in 1987 [25]. Their method is based upon enzymatic cleavage of the conjugates, followed by extraction of liberated 1-OHP using disposable SepPak C_{18} solid-phase extraction (SPE) columns and subsequent determination by reversed-phase LC with fluorescence detection. Jongeneelen et al. reported a detection limit of 200 pg 1-OHP/ml using this method. Furthermore, automated column switching LC methodology has been developed for the determination of urinary 1-OHP [31,32].

Attempts have been made to use urinary excretion of 1-OHP as a measure of occupational lung cancer risk, and values ranging from 2.3 to 4.9 μmol 1-OHP/mol creatinine have been suggested as "occupational exposure limits" for different workgroups [33,34].

Packed capillary LC columns are widely recognised for their enhanced mass sensitivity when employed in combination with concentration sensitive detection methods, such as electrospray ionization (ESI) mass spectrometry (MS). Furthermore, the consumption of solvents and sample is greatly reduced, and split-less connection to the ESI source is easily obtained, making miniaturized LC attractive compared to conventional LC for ESI-MS coupling. Thus, by applying sample enrichment and miniaturized column switching techniques, time efficient and highly sensitive methodology is obtainable [35–37]. Miniaturization of the ESI source provides additional improvement of the concentration limit of detection (cLOD) due to the concentration sensitivity of the ionization principle and because the initial droplets emitted from a narrow bore fused-silica capillary are significantly smaller in size compared to droplets emitted from normal stainless steel capillaries, providing more efficient ionization [38].

The aim of this study was to develop a fast, selective and sensitive miniaturized LC–MS method

for determination of urinary 1-OHP covering a wide range of PAH exposure. It was also a goal to avoid the use of time consuming off-line SPE procedures, by implementing high throughput column switching techniques for sample enrichment and on-line sample clean-up, which can easily be automated.

2. Experimental

2.1. Materials and reagents

HPLC grade acetonitrile (ACN) and methanol (MeOH) were obtained from Rathburn Chemicals (Walkerburn, UK) and BDH Laboratory Supplies (Poole, UK), respectively. Water was obtained from a Milli-Q ultrapure water purification system (Millipore, Bedford, MA, USA). Reagent grade ammonium acetate (NH_4Ac), formic acid (FA) and triethylamine (TEA) were provided by Sigma–Aldrich (St. Louis, MO, USA). 1-Hydroxypyrene (1-OHP) and β-glucuronidase/aryl sulphatase were obtained from Acros Organics (Geel, Belgium) and Boehringer (Mannheim, Germany), respectively. All fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). Nitrogen (99.99%) was purchased from AGA (Oslo, Norway).

2.2. Column preparation

The capillary columns were slurry packed using a mixture of ACN and water (70/30, v/v), and the stationary phase material was Kromasil C_{18} (Eka Nobel, Bohus, Sweden). The pre-column (0.150 mm I.D.×30 mm) was packed with 10-μm particles while the analytical column (0.150 mm I.D.×100 mm) was packed with 3.5-μm particles. Valco (Houston, TX, USA) ZU1C unions in combination with Valco FS1.4 polyimide ferrules and Valco 2SR1 steel screens were used as column end fittings.

2.3. Chromatographic system

An Agilent Series 1100 capillary gradient pump (Palo Alto, CA, USA) with an incorporated on-line vacuum degasser was used to deliver the mobile phase (pump 1) providing back-flushed desorption from the pre-column and elution on the analytical column. A Waters 590 LC pump (Milford, MA, USA) was used for sample loading (pump 2). A schematic description of the column switching system used is given in Fig. 1. The mobile phase consisted of ACN/H_2O (70/30, v/v) with added 5 m*M* NH_4Ac, and was delivered at a flow rate of 1 μl/min throughout the whole study, while the ACN/H_2O (5/95, v/v) sample loading solvent mixture was delivered at flow rates of up to 120 μl/min. A Valco Model C4 injection valve or a Rheodyne Model 7725 injection valve (Cotati, CA, USA) (valve 1) was used for manual injections of sample volumes of 0.01–500 μl. An M-435-1 six-port low-dispersion micro injection valve from Upchurch Scientific (Oak Harbour, WA, USA) served as a column-switching valve (valve 2). The column outlet was connected to a SiO/Pt coated fused-silica Picotip provided by New Objective (Woburn, MA,

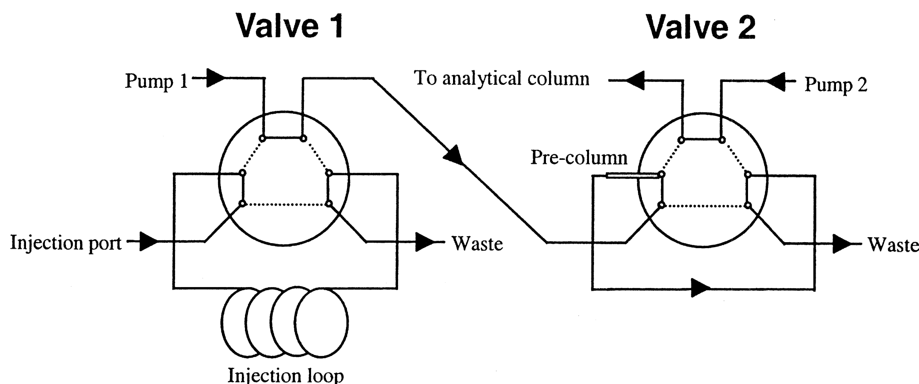

Fig. 1. Schematic description of the column switching system.

USA) with tip O.D. of 50 μm and I.D. of 30 μm. The Picotip was incorporated in a t-coupling from Valco modified to provide nanospray action with the option of applying nebulizer gas. The t-coupling was arranged on an *x*–*y*–*z* stage in order to provide optimization of spray needle position (Micromass, Manchester, UK). Mass analysis of the column effluent was provided by an LCT orthogonal accelerated time-of-flight mass spectrometer (oaTOFMS) equipped with a Z-spray atmospheric pressure ionization ion source prepared for ESI or APCI from Micromass. The chromatographic system was operated at ambient temperature. Electrospray ionization of 1-OHP was performed in the negative mode and the applied voltages were: capillary voltage: −2100 V; sample cone voltage: −50 V; and extraction cone voltage: −3 V. The nebulizer gas pressure was 5 p.s.i. and the source temperature was 100 °C. The TOF-MS instrument was controlled and data were acquired using MassLynx v3.5 software, and mass spectra were acquired in the m/z range 100–500.

2.4. Sample preparation

A stock solution of 1-OHP was prepared in ACN at a concentration of 0.2 mg/ml. Urine from a young, non-smoking athlete served as blank urine, while urine from occupationally PAH exposed individuals were obtained from The National Institute of Occupational Health, Norway. All urine samples were collected in polyethylene containers and immediately frozen at −18 °C. Prior to analysis, the urine and urine blank samples were thawed and mixed to ensure homogeneity, and aliquots of 10 ml urine were diluted with 20 ml 0.1 *M* acetate buffer and adjusted to a final pH of 5.0 using 4 *M* HCl. To diluted urine and urine blank samples 12.5 μl β-glucuronidase/arylsulphatase was added, followed by incubation overnight at 37 °C, in accordance with the procedure of Jongeneelen et al. [25]. Different amounts of the stock solution were added to blank urine aliquots in order to provide calibration and validation solutions of 0.2, 2.0, 10.0, 20.0, and 40.0 ng/ml prepared urine. Prior to injection, all prepared urine samples were passed through 0.22-μm Millex® GV disposable filters (Millipore, Molsheim, France) and diluted 5-fold with water, making the total dilution of the urine sample 15-fold. All solutions were stored at 4 °C.

The urinary creatinine concentration was determined by the use of a Beckman creatinine analyzer 2 (Fullerton, CA, USA).

3. Results and discussion

Enrichment procedures are necessary to perform LC trace determination of urinary 1-OHP, and by employing column switching techniques, improved concentration sensitivity as well as time-efficient on-line sample clean-up is achieved. Off-line sample enrichment and clean-up methods are often tedious and time consuming, and hence do not meet the increasing demands for speed and cost efficiency. Utilization of MS detection also often increases time efficiency, providing mass selectivity leading to reduced demand for fully optimized separations and baseline resolved components. To the authors' knowledge, this is the first published study on biomonitoring of 1-OHP using miniaturized LC–MS.

3.1. Mobile and stationary phase considerations

Mobile phase optimization of LC–MS separations is often a more challenging task than when using more conventional UV or fluorescence detectors, due to the fact that the MS signal is strongly dependent on the ionization of the solute which often is highly related to the mobile phase properties. In order to determine the mobile phase composition providing the best signal to noise (S/N) ratio for 1-OHP, different mobile phase additives were evaluated in combination with MeOH/water (50/50, v/v) or ACN/water (50/50, v/v). The tested additives were TEA, NH_4Ac, acetic acid and formic acid at concentrations of 0, 5, 10, 20 and 50 m*M*. Solutions containing 4 μg/ml 1-OHP were analysed by direct infusion into the ESI-TOF-MS at a flow rate of 1 μl/min, and mass spectra were acquired for 1 min. The mass spectra were combined and 1-OHP was observed at m/z 217.08 $[M–H]^-$, whereas the monoisotopic mass of 1-OHP is 218.0732 Da, and the corresponding $[M–H]^-$ is 217.0654 Da. All the different evaluated additives displayed a signal intensity maximum at a concentration of 5 m*M* both in combination with ACN or MeOH as organic modifier. In accordance with ESI theory, the basic TEA additive provided the best S/N ratio when operating

in negative mode, while FA provided by far the lowest. However, when basic additives at high pH are used in combination with most silica based column packing materials, the silica particles can potentially dissolve, giving rise to high background signals and deteriorated column performance, even when used in combination with highly end-capped stationary phase materials designed for alkaline mobile phases. Fortunately, by using NH_4Ac as mobile phase additive, the signal intensity was 50% of that obtained using TEA. Hence, NH_4Ac was used as mobile phase additive as a compromise between signal intensity and column performance. As the organic component of the mobile phase, ACN was chosen over MeOH due to the fact that it provided three times higher S/N ratio in combination with NH_4Ac.

Based on the high bonding density and experience gained from previous work, Kromasil C_{18} with 3.5-μm particles was used as the stationary phase material. By using an isocratic mobile phase of ACN/water (70/30, v/v) containing 5 m*M* NH_4Ac and a column length of 10 cm (0.15 mm I.D.), elution of 1-OHP was obtained in less than 6 min using a flow rate of 1 μl/min.

The advantages of TOF-MS include high mass resolution and extremely fast acquisition of mass spectra as compared to quadrupole and ion trap mass analysers. These features provide very selective and sensitive detection of compounds of interest due to the option of extracting single ion chromatograms (SIC) with relatively high mass accuracy. However, in order to avoid suppression effects during ionization, 1-OHP was sought to be eluted well separated from major interfering species originating from the urine sample matrix. Most methods published for urinary 1-OHP analysis make use of a solvent gradient in separation optimization. However, an isocratic elution mode is simpler, more practical and potentially faster as no column re-equilibration is required.

3.2. Micro-ESI optimization

In micro-ESI/nanoelectrospray the needle orifice diameter is much smaller than in conventional ESI, resulting in the emission of much smaller primary droplets that undergo subsequent droplet fissions and evaporation, finally resulting in ion emission. These miniaturized ESI sources have several advantages compared to conventional ESI sources, such as enhanced ionization efficiency and higher tolerance toward salt concentration, which can be import when working with biological matrices.

The TOF-MS employed in this study was equipped with a micro-ESI/nanoelectrospray interface consisting of a stainless steel t-coupling from Valco, modified to accommodate coated fused-silica spraying tips and to provide optional nebulizer gas. SiO/Pt coated fused-silica tips with I.D. 30 μm and O.D. 50 μm were used, which provided stable spray performance in combination with the solvent mixtures used. However, when applying capillary voltages below −2.2 kV the coating gradually started to deteriorate as a result of electrical discharges and electrochemical stress [39–41], and at a capillary voltage of −2.5 kV the lifetime of a tip was reduced to only half an hour of operation. However, when using a capillary voltage of −2.1 kV no deterioration of the tip was observed during more than 100 h of operation, and only a 5% reduction in signal intensity was observed as compared to operation at −2.5 kV. The sample cone and the extraction cone were held at −50 and −3 V, respectively, and no fragmentation of 1-OHP was observed.

3.3. Large volume injection

Kromasil C_{18} 10-μm particles were used in the pre-column, providing a compromise between sample capacity and column back pressure during sample loading at elevated flow rates. Despite the fact that similar alkyl ligands were used on both the analytical column and the pre-column, the use of 10-μm pre-column particles has the potential of providing refocusing (reconcentration) of the solutes on the analytical column containing 3.5-μm particles. A composition of ACN/water (5/95, v/v) with 5 m*M* NH_4Ac was used as sample loading solution, providing efficient sample clean-up and 1-OHP enrichment. Thus, the column switching method eliminated the use of costly and time consuming off-line SPE procedures. The 5% ACN content in the sample loading solution did not influence the enrichment procedure, and provided efficient solvatization of the alkyl ligands between injections of the aqueous samples. Furthermore, the pre-column was washed

with ACN with 5 mM NH_4Ac for 5 min between every third injection.

Urine contains numerous compounds that can be retained on C_{18} particles and compete with analytes of interest, potentially resulting in column overloading. Thus, the urine samples were diluted 15-fold with water prior to injection. In order to determine the maximum sample volume of diluted urine that could be injected on the pre-column without column overloading and subsequent analyte break-through, water-diluted and spiked blank urine was injected onto the pre-column using different injection loop volumes of 10, 50, 75, 100, 125, 200 and 500 μl. The samples were spiked with different amounts of 1-OHP, always giving an absolute injected amount of 1 ng 1-OHP independent of the volume. The loading flow rate was 100 μl/min in accordance with an optimized procedure (see below). The peak areas were compared in order to reveal any analyte break-through, and the results presented in Fig. 2 clearly indicate that the capacity limit of the pre-column is reached at ~125 μl injected diluted urine. Thus, an injection volume of 100 μl was used for further studies in order to avoid band broadening, break-through and potential reproducibility problems associated with operating close to the pre-column capacity limit. The injection valve was held in the inject position for 3 min to completely consume the sample

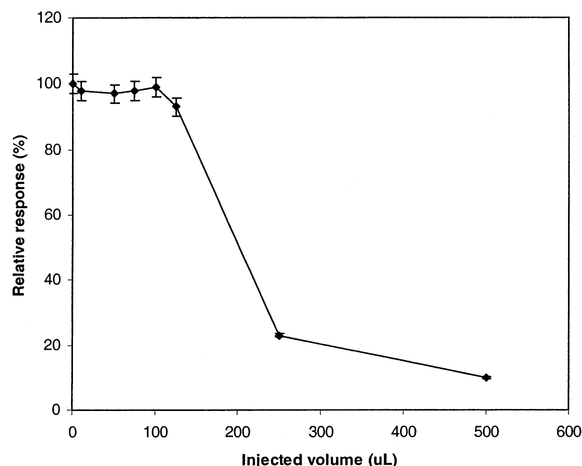

Fig. 2. Evaluation of sample capacity of large volume urine injections as a function of injection volume. An absolute amount of 1 ng 1-OHP was injected using different loop volumes. Error bars represent standard deviation ($n=3$).

loop volume, to flush connecting capillaries and provide on-line sample clean-up.

Large volume injections in capillary LC can potentially be a very time consuming task considering that the optimal flow rate with regard to efficiency on 0.150 mm I.D. columns is ~1 μl/min. However, by loading and focusing the sample onto a short pre-column providing low back pressure, the sample loading time is reduced substantially. Unfortunately, high loading flow rates can potentially affect the sample enrichment process and contribute to band broadening and sample break-through, in addition to problems related to high pressure operation, e.g. valve leakage and pump limitations. In order to evaluate the loading flow rates, 1 ng 1-OHP was loaded onto the column switching system using a sample loop volume of 100 μl prior to back-flushed elution onto the analytical column. The peak areas remained constant over the investigated range of flow rates, clearly indicating that 1-OHP was completely retained on the pre-column regardless of the sample loading flow rates. The highest applicable flow rate was confined by the 300-bar upper pressure limit of the six-port switching valve from Upchurch. Loading flow rates of up to 120 μl/min were possible within the pressure limit of the pump and no valve leakage was observed. However, a flow rate of 100 μl/min was employed in further studies, in order to reduce the risk of valve leakage.

Based on previous experiences, possible adsorption of 1-OHP on the filters was not investigated, and the recoveries of the method support this assessment. Due to the fact that the developed methodology needs little sample preparation, the use of expensive internal standards was avoided.

3.4. Method validation

The column switching method was validated in the concentration range 0.2–40 ng/ml 1-OHP in prepared urine samples, corresponding to deconjugated 1-OHP urine concentrations of 3–600 ng/ml. A sample loading flow rate of 100 μl/min and an injection volume of 100 μl was employed.

The extracted SIC for m/z 217.08 was used for establishing the limit of detection (LOD). The mass LOD (mLOD) of the method was 2 pg 1-OHP ($S/N=3$) in pre-treated urine, corresponding to a

concentration LOD (cLOD) of 20 pg/ml (0.09 nmol/l) diluted urine or 0.3 ng/ml (1.35 nmol/l) urine.

The linearity of the method was established by spiking deconjugated blank urine samples with 1-OHP in the range 0.2–40 ng/ml ($n=5$). The on-line SPE column switching LC method was linear within the investigated concentration range with a coefficient of correlation of 0.997.

The within- and between-assay precision were established by injecting six sets of samples at three spiking concentration levels (0.2, 10 and 40 ng/ml) within 1 and 6 days, respectively, by the same analyst. The within-assay ($n=6$) and between-assay ($n=6$) precision were in the range 6.4–7.3 and 7.0–8.1%, respectively, as summarized in Table 1. The relative standard deviations of the within- and between-assay precision remained almost constant throughout the validated concentration range.

The recoveries of 1-OHP were established by comparing the resulting peak areas from different spiked urine concentration levels with peak areas from corresponding absolute amounts of 1-OHP urine samples injected directly onto the analytical column using 10-nl sample volume loop ($n=3$). The recoveries at the different concentration levels were in the range 96.2–97.5%, as summarized in Table 1. The recoveries remained almost constant throughout the validated concentration range.

The within- and between-assay precision of retention times was below 2%, and the column efficiency remained constant throughout the study. The same pre-column and analytical column were used throughout the study, and identical chromatographic performance was observed when the pre-column and the analytical column were replaced with columns prepared identically. All calibration solutions and real sample solutions were stable for at least 2 months when stored at 4 °C.

3.5. Determination of 1-OHP in urine from exposed workers

Fig. 3 shows the chromatographic profile of 1-OHP in urine from an occupationally exposed worker, illustrated by the resulting TIC and the extracted SIC for m/z 217.08. 1-OHP is well separated from any interfering compounds in both TIC and SIC mode under isocratic conditions with a retention time of 5.5 min. The overall analysis time was less than 15 min, including pre-column loading, sample clean-up, switching, elution and resetting procedures. The background subtracted mass spectrum of the corresponding 1-OHP peak profile is presented in Fig. 4, and shows no sign of co-eluting compounds or adduct formation. The urinary content of creatinine in the urine sample was 15.6 m*M*, and the measured 1-OHP concentration was 6.17 μmol/mol creatinine, corresponding to 21 ng/ml deconjugated 1-OHP in the untreated urine sample.

4. Conclusions

This paper describes a fast, simple and reliable method for sensitive and selective determination of 1-OHP in human urine. The methodology offers on-line sample clean-up and analyte preconcentration through direct injection of urine after deconjugation, dilution and filtration. In addition, the column switching method can easily be automated for high throughput analysis of large sample series and subsequent assessment of PAH exposure in groups of individuals of particular interest. The developed column switching system might also be modified to include the determination of other PAH metabolites in urine in order to obtain a more accurate measurement of PAH exposure. The method is to be used for

Table 1
Within- and between-assay precision and recovery of the method

Analyte	Concentration level (ng/ml)	Within-assay precision (% RSD, $n=6$)	Between-assay precision (% RSD, $n=6$)	Recovery
1-OHP	0.2	7.3	8.1	97.3
	2			96.2
	10	6.4	7.0	97.1
	20			96.9
	40	7.1	7.8	97.5

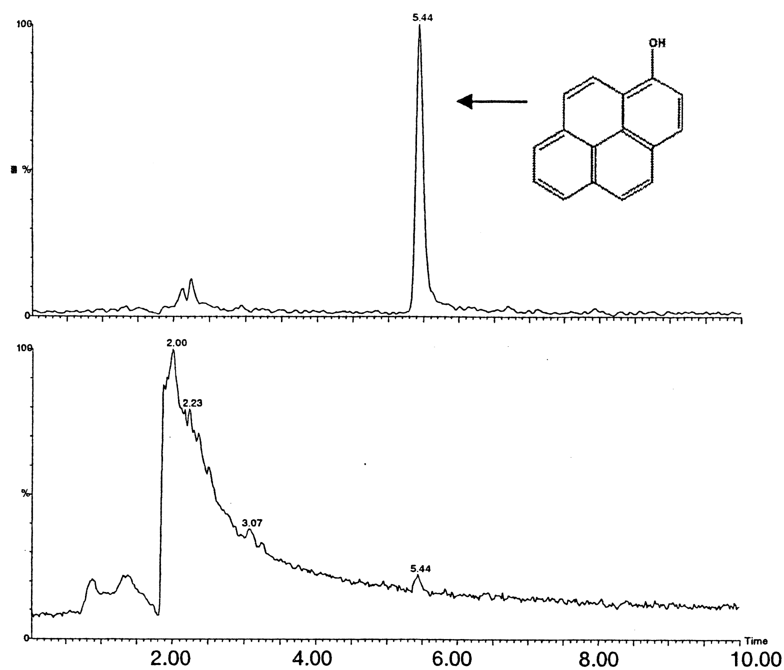

Fig. 3. Determination of 1-OHP in urine obtained from an occupationally exposed individual. A 100-μl aliquot of pre-treated urine was injected and the determined urinary 1-OHP content was 6.17 μmol/mol creatinine. The total ion chromatogram (TIC) (bottom) and the extracted SIC (top) for the mass 217.08 are displayed.

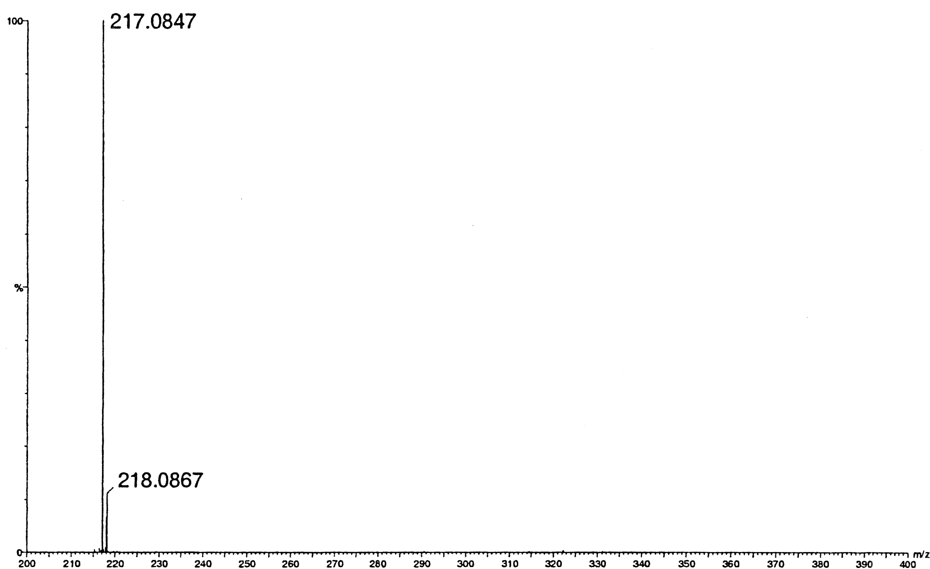

Fig. 4. Mass spectrum of the 1-OHP peak profile in Fig. 3.

measurement of low-dose PAH exposure of workers from relevant industries.

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