Journal of Chromatography B, 875 (2008) 531–540

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Development of a gas chromatography/mass spectrometry method to quantify several urinary monohydroxy metabolites of polycyclic aromatic hydrocarbons in occupationally exposed subjects

Laura Campo*, Federica Rossella, Silvia Fustinoni

Department of Occupational and Environmental Health, University of Milan and Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Milan, Italy

ARTICLE INFO

Article history:
Received 13 May 2008
Accepted 8 October 2008
Available online 15 October 2008

Keywords:
Polycyclic aromatic hydrocarbons
Urinary metabolites
GC/MS
Biological monitoring

ABSTRACT

The aim of this study was the development of a method for the determination of 12 urinary monohydroxy metabolites of PAHs, namely 1-hydroxynaphthalene, 2-hydroxynaphthalene, 2-hydroxyfluorene, 9-hydroxyfluorene, 1-hydroxyphenanthrene, 2-hydroxyphenanthrene, 3-hydroxyphenanthrene, 4-hydroxyphenanthrene, 9-hydroxyphenanthrene, 1-hydroxypyrene, 6-hydroxychrysene, and 3-hydroxybenzo[*a*]pyrene. Analytes were determined in the presence of deuterated analogues as internal standards, by GC/MS operating in the electron impact mode. Sample preparation was performed by enzymatic hydrolysis of glucoronate and sulphate conjugates of hydroxy metabolites of PAHs, liquid–liquid extraction with *n*-hexane, and derivatization with a silylating reagent. The method is very specific, limits of quantification are in the range 0.1–1.4 μg/l, and range of linearity is from limit of detection to 208 μg/l. Within- and between-run precision, expressed as coefficient of variation, are <20%; accuracy for most analytes is within 20% of the theoretical value. An application of the proposed method to the analysis of 10 urine samples from coke-oven workers shows that 1-hydroxynaphthalene and 2-hydroxyfluorene were the most abundant compounds (median 61.4 and 69.7 μg/l, respectively), while 6-hydroxychrysene, and 3-hydroxybenzo[*a*]pyrene were always below the quantification limit.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are chemical compounds containing only carbon and hydrogen atoms and made up of two or more condensed aromatic rings. They are formed from combustion of organic matter and for this reason are present, at different concentrations, as common pollutants of domestic and work settings. While PAHs in general may have irritant effects and cause respiratory diseases [1], some of them are classified as known, possible or probable carcinogen to humans by the International Agency for Research on Cancer [2].

Due to the widespread presence of PAHs in the environment and their toxicological relevance, the assessment of exposure to PAHs is important, both for workers (e.g. involved in production of metallurgic coke, aluminium, or carbon electrodes, asphalt workers) and for the general population. To this end, biological monitoring is usually performed. In fact, it permits quantifying the total PAHs intake into the body via respiratory, dermal, and gastrointestinal routes. Following exposure, PAHs are first oxidized in phase I metabolism by P450 enzymes and then reduced or hydrolyzed to hydroxylated metabolites. In phase II metabolism, the hydroxy PAHs are reacted to form glucoronate and sulphate conjugates to facilitate excretion through urine or faeces [3,4].

The choice of a reliable biomarker for PAHs exposure is a crucial point because PAHs are always present as a complex mixture whose composition varies depending on source and temperature of emission. Moreover, each compound, inside the human body, is metabolized to more than one metabolite constituting different positional isomers.

Urinary 1-hydroxypyrene, a metabolite of pyrene, has been proposed as a biomarker of PAHs exposure [5], and it is widely applied in different working environments [6]. However, since multiple exposures to different PAHs always occur, in recent years simultaneous measurement of hydroxylated metabolites coming from different parent compounds has been proposed. Most of the studies report the simultaneous quantification of pyrene, naphthalene, and phenanthrene metabolites [7–10], while very few studies report the quantification of metabolites of other compounds such as fluorene, fluoranthene, chrysene or benzo[*a*]pyrene [11–17]. The analytical assays proposed are usually based on enzymatic deconjugation of the sulphate and glucoronate derivatives of PAHs,

* Corresponding author at: Via S. Barnaba, 8 – 20122 Milan, Italy.
Tel.: +39 02 503 20116; fax: +39 02 503 20111.
E-mail address: laura.campo@unimi.it (L. Campo).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.10.017

extraction of the correspondent hydroxylated compounds from urine via solid-phase or liquid–liquid extraction, high-performance liquid chromatography (HPLC) separation and fluorescence detection (FLD). The use of gas chromatography coupled with mass spectrometry (GC/MS) is less common, although this technique guarantees a high degree of specificity and allows obtaining a better chromatographic resolution of isomeric compounds than HPLC [18]. The GC/MS method reported by Grimmer et al. quantifies ten monohydroxy and five dihydroxy metabolites of different PAHs, but requires a high volume of urine (150 ml) and the use of potentially harmful chemicals such as benzene as solvent and diazomethane as derivatizing agent [11]. Other published methods, requiring less urine volume, limit their application to metabolites of naphthalene and phenanthrene [7,19,20]. An interesting assay involving solid-phase microextraction (SPME) followed by on-fiber derivatization has been proposed by Gmeiner and co-workers in the past and again more recently by other authors [21–24]. This method is quite sensitive and simple, but requires a specific autosampler for the automation; moreover, the brief durability of SPME fiber is a relevant limitation to its routine application [23]. A productive method that permits simultaneous measurement of 24 mono-hydroxy PAHs metabolites has been recently reported by Li et al. [25]. This assay is the result of several improvements that have been introduced over the years to the original assay [26,27] and allows obtaining very high sensitivity (limits of detection in the range of ng/l), thanks to the use of high-resolution mass spectrometry (GC/HRMS). However, this instrumentation is very expensive and seldom available in the laboratory.

In this paper we present a method focused on the measurement of 12 monohydroxy derivatives (OH-PAHs) of some PAHs listed by the U.S. Environmental Protection Agency as priority pollutants: naphthalene, fluorene, phenanthrene, pyrene, chrysene, and benzo[*a*]pyrene. Starting from the above mentioned experiences, we based our assay on enzymatic deconjugation, liquid–liquid extraction, derivatization and analysis by gas chromatography coupled with low-resolution mass spectrometry (GC/MS). Particular attention was dedicated to the optimization of each step in the method in order to obtain a robust and validated assay. An application of the assay to the analysis of urine specimens from coke-oven workers is also shown.

2. Experimental

2.1. Chemicals and standards preparation

1-Hydroxypyrene (1-OHPYR), 1-hydroxyphenanthrene (1-OHPHE), 2-hydroxyphenanthrene (2-OHPHE), 3-hydroxyphenanthrene (3-OHPHE), 4-hydroxyphenanthrene (4-OHPHE), 9-hydroxyphenanthrene (9-OHPHE), 6-hydroxychrysene (6-OHCHR), and 1-hydroxypyrene gluocoronate (1-OHPYRGLU) were purchased as solution in acetonitrile at a concentration of 10 μg/l from Dr. Ehrenstorfer (Labservice Analytica, Anzola Emilia, Italy); 1-hydroxynaphthalene (1-OHNAP) and 2-hydroxynaphthalene (2-OHNAP) were obtained from Merck (Bracco, Milan, Italy), 2-hydroxyfluorene (2-OHFLU), 9-hydroxyfluorene (9-OHFLU), and 3-hydroxybenzo[*a*]pyrene (3-OHBaP) were obtained from Sigma–Aldrich (Milan, Italy). [$^2H_9$]1-hydroxypyrene (1-OHPYRd9; 98%D, Cambridge Isotope Laboratories, Andover, MA, USA) and [$^2H_7$]1-Hydroxynaphthalene (1-OHNAPd7; 97% D, Sigma–Aldrich, Milan, Italy) were used as internal standards.

BSTFA [N,O-Bis(trimethylsilyl)trifluoroacetammide with 1% trimethylchlorosilane, GC grade, Fluka, Milan, Italy], Trisil [trimethylchlorosilane and hexamethyldisilazane in pyridine (1:2:10), Pierce, Celbio, Milan, Italy] and Trisil Z [1-(trimethylsilyl)imidazole in pyridine (1:4), Pierce, Celbio, Milan, Italy] were used as derivatizing agents.

β-Glucuronidase/sulphatase (type H-2 from *Helix pomatia*; β-glucuronidase activity 98,000 U/ml and sulphatase activity 2400 U/ml, Sigma–Aldrich, Milan, Italy) and hydrochlorydric acid (HCl, 37%, Carlo Erba, Milan, Italy) were used for enzymatic and acid hydrolysis, respectively.

n-Hexane (GC grade ≥ 99.0%, Fluka, Milan, Italy), *n*-hexane (GC grade, ACS reagent ≥ 99%, Riedel de Haën, Milan, Italy), ethyl acetate (ACS grade, Carlo Erba, Milan, Italy), diethyl ether (ACS grade, Sigma Aldrich, Milan, Italy), dichloromethane (ACS grade, Carlo Erba, Milan, Italy) and methanol (MeOH, plus HPLC, Carlo Erba, Milan, Italy) were used as extraction solvents.

Sodium chloride (NaCl > 99%, Sigma–Aldrich, Milan, Italy), anhydrous magnesium sulphate ($MgSO_4$ > 98% Fluka, Milan, Italy), magnesium sulphate eptahydrate ($MgSO_4$•$7H_2O$, RPE ACS grade, Carlo Erba, Milan, Italy), potassium fluoride (KF, ACS grade, Carlo Erba, Milan, Italy), sodium dihydrogen phosphate (NaH_2PO_4, ACS grade, Carlo Erba, Milan, Italy), potassium iodide (KI, ACS grade, Carlo Erba, Milan, Italy), and sodium carbonate (Na_2CO_3, ACS grade, Carlo Erba, Milan, Italy) were tested as salts to increase ion strength.

Stock solutions of the individual OH-PAHs were prepared in acetonitrile starting from the pure compounds, while working solutions were made by combining aliquots of the stock solutions and then diluting in urine. Working solutions used for the development of the method contained 1-OHNAP and 2-OHNAP at the concentration of 104 μg/l and all the other OH-PAHs at concentration of 10.4 μg/l. Standard solutions for calibration curves were prepared in urine at concentrations of 0.5, 1.0, 2.1, 10.4, 20.8, and 41.6 μg/l for all OH-PAHs except for 1-OHNAP and 2-OHNAP prepared at concentrations of 5, 10.4, 20.8, 104, 208, and 416 μg/l. An unspiked sample of the same urine was kept as a blank. Urine used for the preparation of the calibration and working solutions was a urine pool from non-smoking donors without occupational exposure to PAHs.

From the pure deuterated compounds, an internal standard solution containing 1-OHPYRd9 and 1-OHNAPd7 at a concentration of 5 mg/l and 50 mg/l in acetonitrile (IS) was prepared. 20 μl of IS were added to 2 ml samples to obtain a final concentration of 5 μg/l and 50 μg/l for 1-OHPYRd9 and 1-OHNAPd7, respectively.

Stock solutions in acetonitrile were kept in amber glass vials, while working and standard solutions were kept in polyethylene tubes. All solutions were stored at −20 °C in the dark.

2.2. Equipment

For sample analysis 2 ml amber glass vials sealed with screw open-top closure and silicone-polyperfluoroethylene gaskets were used (Kimble, Superchrom, Milan, Italy).

A 6890 gas chromatograph (Agilent, Cernusco sul Naviglio, Milan), equipped with autosampler for liquid injection and with a 5973 mass spectrometric detector, operating in the electron impact mode (EI) or in the positive chemical ionization mode (PCI), was used to separate and identify OH-PAHs. The split/splitless injection port operated in the splitless mode and was equipped with a 4 mm gooseneck inert liner (CPS Analitica, Milan, Italy). Chromatographic separation was performed on a HP5-MS capillary column (J&W, 30 m length, 0.25 mm internal diameter, 0.25 μm film thickness, CPS Analitica, Milan, Italy).

2.3. GC/MS analysis

The GC analysis was performed in the following conditions: helium carrier gas at a constant flow rate of 1 ml/min; injector temperature 300 °C, gas chromatograph oven temperature programmed from 60 °C (3 min initial hold) to 150 °C at 10 °C/min

(3 min hold), then to 210 °C at 10 °C/min (5 min hold) and then to 320 °C at 10 °C/min (2 min final hold). The approximate retention times obtained under these conditions are reported in Table 1. MS detection was performed in the following conditions: transfer line temperature 280 °C; ion source temperature 300 °C. For mass spectra acquisition, the 50–500 ion mass-to-charge range was scanned, while quantification was performed in the selected ion monitoring mode (SIM). Dwell time was 100 ms. A list of the investigated chemicals and relative internal standards, abbreviations, retention times, chromatographic windows, and molecular quantifying and qualifying ions at mass-to-charge ratio (m/z) acquired for each compound is reported in Table 1.

2.4. MS ionization

To find the best ionization condition for OH-PAHs, EI and CI modes were compared. In the first case, an inert EI source operating at 300 °C at 70 eV was used. In the second case a CI source, not inert, operating at 250 °C in the positive chemical ionization (PCI) mode was used. Methane (Air Liquide, Milan, Italy) and ammonia (100%, Air Liquide, Milan, Italy) were evaluated as reagent gases for PCI.

2.5. Sample preparation optimization

2.5.1. Hydrolysis

To identify the best experimental conditions to obtain hydrolysis of conjugated OH-PAHs, enzymatic and acid hydrolysis were optimized and compared. To this end, the conjugated form of 1-OHPYR, 1-hydroxypyrene glucoronate (1-OHPYRGLU), was used. For each set of experiments, three types of samples were used: a 2 ml urine sample spiked with a known amount of 1-OHPYRGLU (sample 1), a 2 ml urine sample spiked with an equimolar amount of 1-OHPYR (sample 2), and a 2 ml of the unspiked urine sample (blank sample). The reaction yield was calculated from the percentage ratio between the chromatographic area of 1-OHPYR in sample 1 (coming from hydrolysis of the added 1-OHPYRGLU and from endogenous 1-OHPYR) and of 1-OHPYR in sample 2 (coming from the added 1-OHPYR and from endogenous 1-OHPYR), after subtracting from each one the signal of 1-OHPYR present in the blank sample:

$$\frac{[(\text{1-OHPYR}_{\text{sample 1}}) - (\text{1-OHPYR}_{\text{blank sample}})]}{[(\text{1-OHPYR}_{\text{sample 2}}) - (\text{1-OHPYR}_{\text{blank sample}})]} \times 100$$

The need to subtract $\text{1-OHPYR}_{\text{blank samples}}$ arises from the use of matrix containing endogenous 1-OHPYR because of environmental exposure.

In the acid hydrolysis trial, the best reaction time was evaluated by reacting the urine samples with 100 μl of aqueous HCl at 100 °C for a time ranging from 5 to 180 min. The minimum amount of HCl needed to complete hydrolysis was evaluated in the presence of variable acid volumes (100, 200 or 300 μl). Moreover, the influence of the acid pH on IS chromatographic response and on the subsequent step of the assay was evaluated.

In the enzymatic hydrolysis trial, the minimum amount of β-glucuronidase/sulphatase needed to complete hydrolysis was evaluated by performing the reaction in the presence of 10 or 20 μl of enzyme. In these trials, the enzyme along with 20 μl IS was added to 2 ml urine samples buffered with 1 ml of an acetate/acetic acid buffer 0.5 M (pH 5) which was then incubateted overnight at 37 °C. After the appropriate time, samples coming from both acid and enzymatic hydrolysis were extracted with *n*-hexane, derivatized and analyzed via GC/MS in the above reported conditions (Section 2.3). All trials were performed in triplicate.

Table 1
GC/MS parameters for the investigated urinary compounds and relative internal standards.

Analyte	Abbreviation	Retention time (min)	Quantifying ion (*m/z*)	Qualifying ion (*m/z*)	IS[a]	Abbreviation	Retention time (min)	Quantifying ion (*m/z*)	Chromatographic window (min)
1-Hydroxynaphthalene	1-OHNAP	14.52	216	201	1-Hydroxynaphthalene-d7	1-OHNAPd7	14.48	223	12.00–25.00
2-Hydroxynaphthalene	2-OHNAP	14.92	216	201					
2-Hydroxyfluorene	2-OHFLU	21.47	254	239					
9-Hydroxyfluorene	9-OHFLU	19.24	254	239					
1-Hydroxyphenanthrene	1-OHPHE	23.51	266	251					
2-Hydroxyphenanthrene	2-OHPHE	23.93	266	251					
3-Hydroxyphenanthrene	3-OHPHE	23.40	266	251					
4-Hydroxyphenanthrene	4-OHPHE	22.53	266	251					
9-Hydroxyphenanthrene	9-OHPHE	23.05	266	251					
1-Hydroxypyrene	1-OHPYR	29.78	290	275	1-Hydroxypyrene-d9	1-OHPYRd9	29.71	299	25.00–37.00
6-Hydroxychrysene	6-OHCHR	33.13	316	301					
3-Hydroxybenzo(a)pyrene	3-OHBaP	37.36	340	325					

[a] Specific deuterated compound, contained in the IS solution, used for the quantification of each PAH.

2.5.2. Extraction

In the optimization of the extraction, liquid–liquid and solid-phase (SPE) extraction conditions were compared. For liquid–liquid extraction, diethyl ether, ethyl acetate, dichloromethane and *n*-hexane (two brands from different commercial suppliers) were tested as solvents. For SPE extraction, C18 SPE cartridges were used (C18 Bond Elut, 500 mg, Varian, Milan, Italy) and methanol was used as solvent. The extraction trial was performed on 2 ml urine working samples added to 20 μl of the IS solution.

For the liquid–liquid extraction, the following procedure was used: samples were extracted four times with 2 ml aliquots of solvent, the organic extracts were combined, and then evaporated under a gentle stream of nitrogen at room temperature.

For SPE extractions, cartridges were activated with 2 ml MeOH and 2 ml of water, then samples were loaded on cartridges and rinsed with 2 ml aliquots of water/MeOH solutions at different proportions (from 100:0 to 0:100). All elution fractions were collected and evaporated by means of a Speed Vac SC110 evaporator (Thermo Savant, Rodano, Italy). After both the liquid–liquid extraction and SPE step, the residues were derivatized and analyzed by GC/MS. All trials were performed in quadruplicate.

Extraction recovery was evaluated by comparing the chromatographic signal of the extracted urine samples with that of solutions containing OH-PAHs at the same concentration and in the same organic solvent used to perform the extraction.

2.5.3. Ionic strength

Performing liquid–liquid extraction on samples coming from enzymatic hydrolysis, a thick emulsion took shape at the interface between the organic and aqueous phase. This required a centrifugation of the sample to recover the organic phase, and in some cases this was not even effective. To break the emulsion and improve the extraction yield the addition of salt to the aqueous phase was tested. Seven different inorganic salts were tested: NaCl, $MgSO_4$, $MgSO_4$•$7H_2O$, KF, NaH_2PO_4, $MgSO_4$, KI, and Na_2CO_3. In this trial, about 3 ml samples coming from enzymatic hydrolysis (2 ml urine + 1 ml buffer + 20 μl IS + 20 μl enzyme) were saturated with the appropriate salt, shaken, then extracted with *n*-hexane, derivatized, and analyzed by GC/MS. The extraction efficiency obtained with each salt was compared with that obtained in the absence of salt. All trials were performed in triplicate.

2.5.4. Derivatization

Derivatization is a crucial point in analyzing OH-PAHs, since it produces volatile derivatives, suitable for GC/MS analysis. Among different derivatizing reagents, silylating agents are particularly suitable for GC analysis due to their compatibility with the stationary phase of the most common capillary columns. To optimize the derivatization three different silylating agents, producing the same derivative, were compared: BSTFA, Trisil, and Trisil Z. For each reagent, two different temperatures (45 °C and 70 °C) and four different incubation times (15, 30, 60, 90 min), for a total of 24 experimental conditions, were tested. The two reaction temperatures, 45 °C and 70 °C, were chosen as the optimum temperature suggested respectively for BSTFA and Trisil Z by the suppliers, while no specific temperature was suggested for Trisil. Samples were prepared adding 100 μl of each derivatizing agent to the residue coming from the hydrolysis and extraction steps and analyzing them by GC/MS. All trials were performed in triplicate.

2.5.5. Glassware material

The influence of glassware on the recovery of the assay was evaluated by testing the use of different materials, namely polyethylene vials, glass vials and silanized glass vials to perform hydrolysis, extraction, and derivatization steps.

2.6. Optimized procedure for sample preparation

Urine samples were heated at 50° C by means of a dry block until completely thawed. After shaking, 2 ml of urine was transferred to a 7 ml silanized glass vial and added to 20 μl of IS, 1 ml of acetate/acetic acid buffer 0.5 M (pH 5) and 20 μl of β-glucuronidase/sulphatase. The mixture was shaken and incubated at 37 °C overnight. After hydrolysis, the solution was cooled at room temperature for 30 min and then 1 g of $MgSO_4$•$7H_2O$ was added. Samples were extracted twice with 2 ml aliquots of *n*-hexane centrifuging each time for 10 min at about 3000 rpm to facilitate phase separation. The organic extracts were combined in silanized glass vials and then evaporated under a gentle stream of nitrogen at room temperature. The residue was reconstituted with 100 μl BSTFA and incubated for 45 min at 90 °C. After derivatization, samples were cooled at room temperature and rapidly transferred into a 2 ml vial containing a 200 μl conical insert, sealed and analyzed by GC/MS in the conditions reported in Section 2.3.

2.7. Validation of the assay: calibration curves, limits of quantification, within- and between-run precision, accuracy, recovery, and stability of standard solutions

For calibration curves, the standard solutions containing the 12 OH-PAHs in urine and the blank urine, prepared as described in Section 2.1, were analyzed in triplicate using the procedure outlined in Section 2.6. Least squares linear regression analysis was applied to estimate the slope (m) and the intercept (q) of the function $y = mx + q$, where y is the ratio between the chromatographic peak area of each analyte versus IS, and x is the concentration in the sample (μg/l). The limit of quantification (LOQ) of the assay was calculated for each analyte according to the expression $LOQ = (3\,SE_q + q)/m$, where SE_q is the standard error of the intercept [28]. To calculate LOQ, calibration curves at low concentration were used (up to 104 μg/l for naphthols and up to 10.4 μg/l for all the other OH-PAHs).

The within-and between-run precision and accuracy of the assay were determined by analyzing three pools of urine spiked with different and known concentration of each OH-PAH. The same analyst analyzed five replicates of each pool on three different days of the same week [29]. Precision was expressed as percentage coefficient of variation (CV%). Accuracy was estimated as percent ratio between the value calculated from the calibration curve and the theoretical value [29]. Recovery was estimated as the ratio between the signal of each analyte in the working solutions containing 12 OH-PAH in urine and the signal of the same chemicals in acetonitrile.

Stability of the standard solutions (free OH-PAHs in urine) was evaluated as long-term, short-term and freeze-and-thaw stability [29]. To this end separate aliquots at low and high concentrations (20.8 and 416 μg/l for naphthols and 2.08 and 41.6 μg/l for all the other OH-PAHs) were prepared and stored in polyethylene tubes at −20 °C. For long-term stability, samples were analyzed after 30, 50, and 80 days. For short-term stability, solutions were thawed and kept at room temperature for 6 h before analysis. This time was chosen on the basis of the expected time that samples could be kept at room temperature in the laboratory's daily practice. For freeze-and-thaw stability, solutions were submitted to three 12-h cycles of freezing at −20 °C and thawing at room temperature before analysis. In each trial, three aliquots at each of the low and high concentrations were analyzed. Stability was evalu-

ated comparing the calculated concentrations to their theoretical values.

2.8. Application of the assay to the biological monitoring

The assay was applied to urine samples obtained from 10 male coke-oven workers, all smokers, exposed to PAHs during their daily work. The subjects were informed about the aims of the research and signed an informed consent. Urine samples were collected in 100 ml polyethylene bottles at the end of the work-shift, after at least 3 consecutive days of work. Samples were stored in the dark at −20 °C until analysis.

Samples were prepared and analyzed according to the procedure described in Sections 2.6 and 2.3 and quantified using calibration curves prepared as described in Section 2.7.

3. Results and discussion

3.1. Molecule ionization and chromatographic separation

In EI spectra the molecular ion $[M]^{+\bullet}$ corresponding to the trimethylsilyl ion $[Si(CH_3)_3OPAH]^{+\bullet}$ was obtained for each compound with an abundance of at least 82%. The ions corresponding to $[M^{+\bullet} - CH_3{\bullet}]^+$ (abundance range 10%–100%), $[M^{+\bullet} - Si(CH_3)_3{\bullet}]^+$ (13%–48%), and $[M^{+\bullet} - OSi(CH_3)_3{\bullet}]^+$ (<10%–100%), were also obtained for each compound.

In PCI with methane as reagent gas, the proton transfer ionization process was observed for all OH-PAHs. In fact the protonated molecular ion $[M+H]^+$, corresponding to the trimethylsilyl ion $[Si(CH_3)_3OPAH+H]^+$, was obtained for each compound with an abundance of 100%. For hydroxynaphthalenes, hydroxyfluorenes,

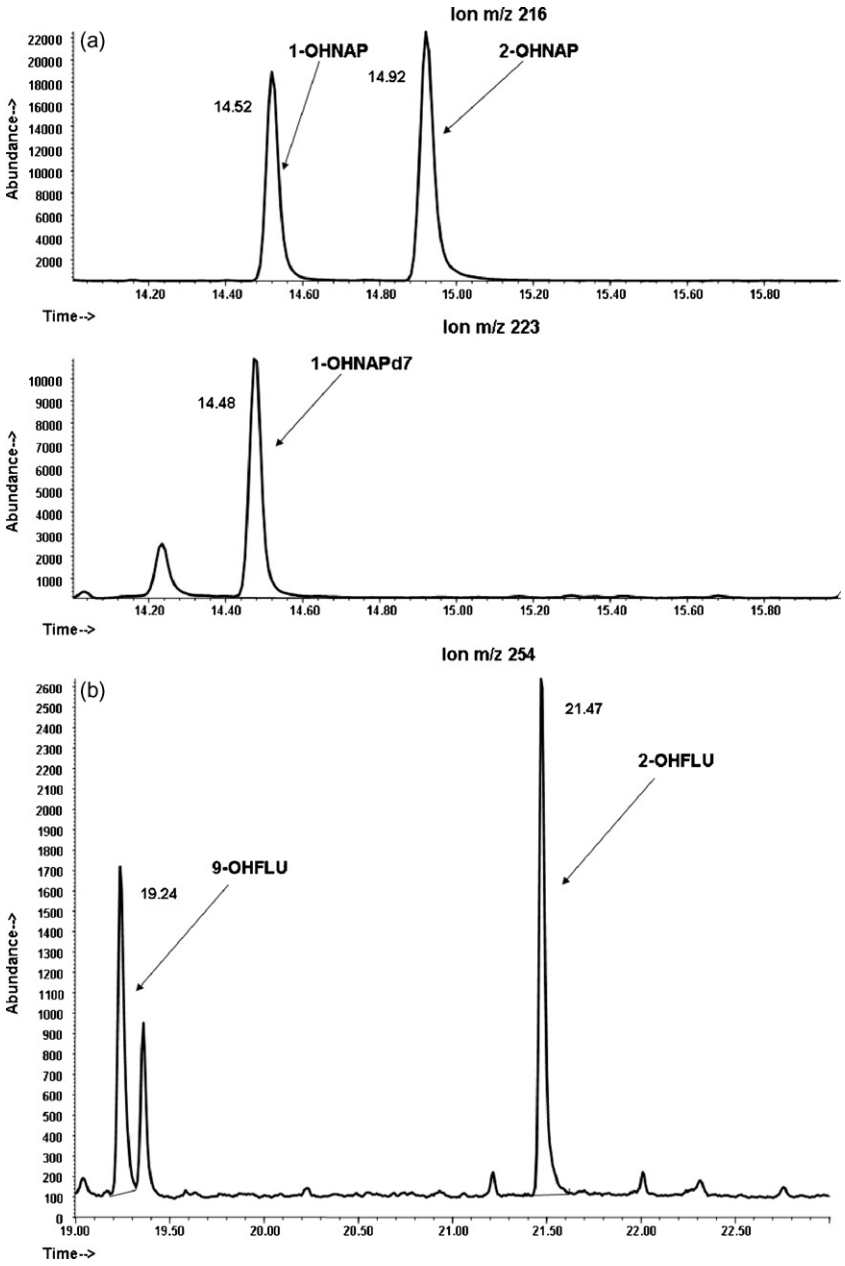

Fig. 1. Single ion chromatogram of a urine sample from an unexposed subject, spiked with OH-PAHs and IS. (1a), 1-OHNAP, 2-OHNAP 1-OHNAPd7; (1b), 2-OHFLU and 9-OHFLU; (1c) 1-OHPHE, 2-OHPHE, 3-OHPHE, 4-OHPHE, and 9-OHPHE; (1d) 1-OHPYR and 1-OHPYRd9; (1e) 6-OHCHR; (1f) 3-OHBaP.

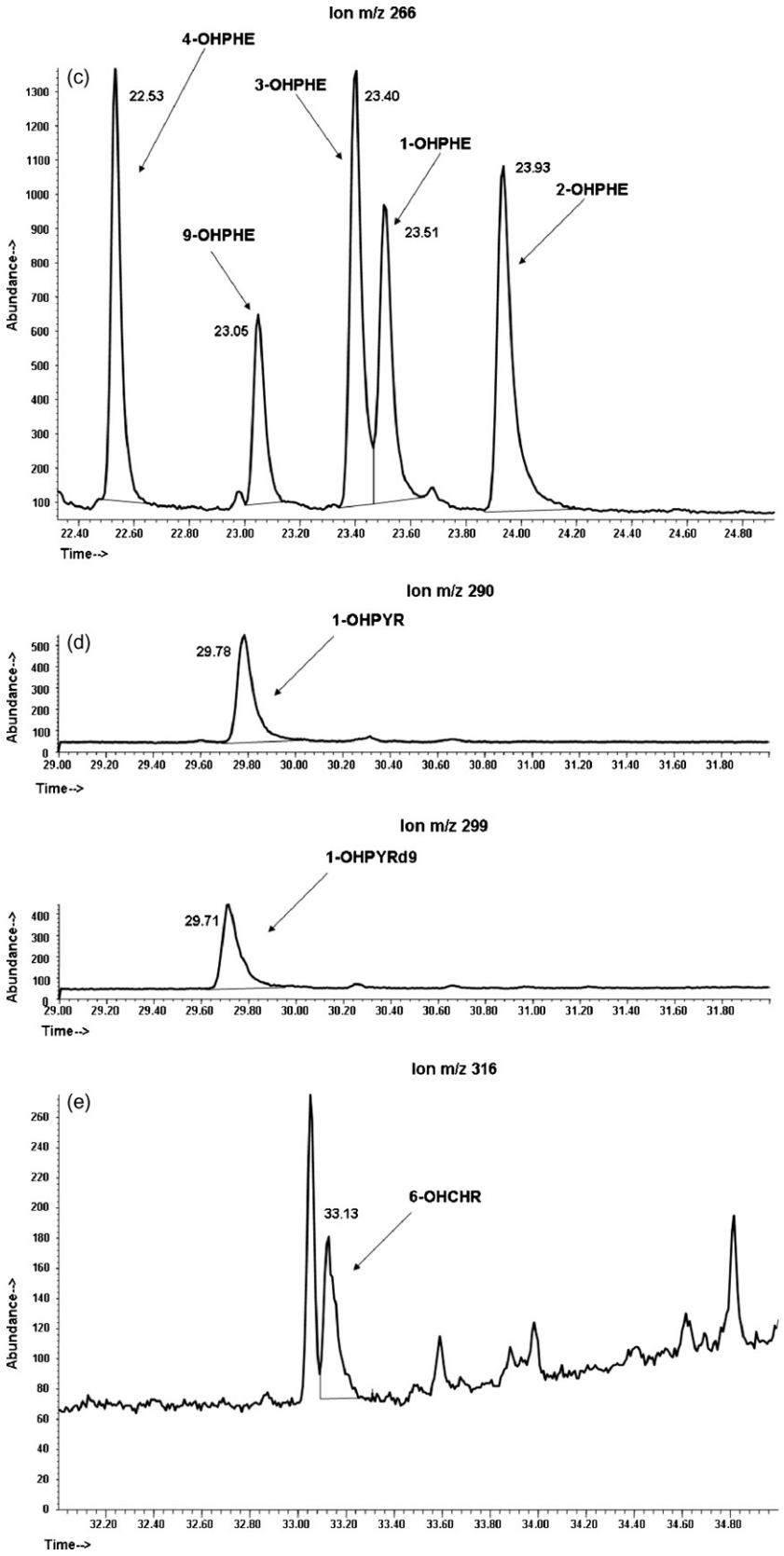

Fig. 1. (*Continued*)

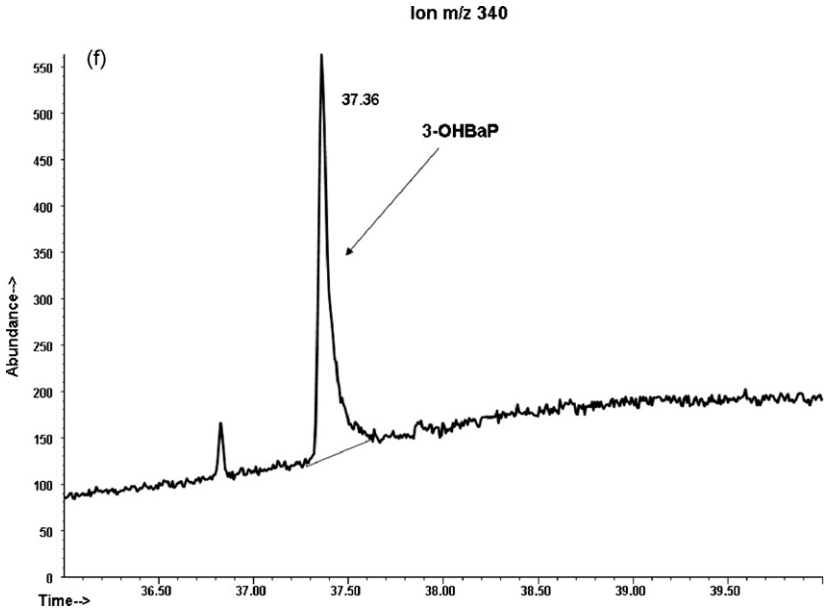

Fig. 1. (*Continued*).

and hydroxyphenanthrenes the adduct ion $[M+C_2H_5]^+$ was also observed (<5–24%).

In PCI with ammonia as reagent gas, only the proton transfer ionization process was observed, with the formation of the protonated molecular ion $[M+H]^+$ (abundance 100%). However, in the mass spectra of 9-OHFLU the $[M+H]^+$ ion was completely absent, while the $[M+H-Si(CH_3)_3]^+$ ion (m/z 182, abundance 100%) and the adduct ion $[M+NH_4]^+$ were observed (16%).

Quantification was performed in the selected ion monitoring mode acquiring, for each compound, the molecular ions $[M]^{+\bullet}$ or $[M+H]^+$ ($[M+NH_4]^+$ for 9-OHFLU), respectively in the EI and CI mode. Comparing EI and PCI, we found that single ion chromatogram areas were lower in PCI, both with methane and ammonia, than in EI. Moreover, chromatographic peaks of 1-OHPYR, 1-OHPYRd9, and high-molecular weight OH-PAHs were characterized by a low signal to noise ratio, thus leading to scarce sensitivity. For this reason, the use of EI as ionization mode was preferred and used in the further optimization of the assay.

The deuterated compounds 1-OHNAPd7 and 1-OHPYRd9 were used as internal standards. No other deuterated analogues were commercially available, and the use of existing C^{13}-labeled compounds was considered cost-prohibitive. Therefore, 1-OHNAPd7 was used as internal standard for 2- and 3-rings analytes, while 1-OHPYRd9 was used as internal standard for 4- and 5-rings analytes (see Table 1).

Fig. 1 shows the EI single ion chromatogram of a urine sample from an unexposed subject: the sample was spiked with 1-OHNAP and 2-OHNAP at a concentration of 104 μg/l, the other OH-PAHs at a concentration of 10.4 μg/l, 1-OHNAPd7 at a concentration of 50 μg/l and 1-OHPYRd9 at 5 μg/l. The analytes were univocally assigned based on their retention times and mass-to-charge ratio. A good chromatographic separation of all considered compounds was obtained using isothermal conditions for the elution of positional isomers.

3.2. Optimization of the sample preparation

3.2.1. Hydrolysis

In the acid conditions, a complete hydrolysis of conjugated 1-OHPYRGLU was attained upon reacting the analyte for 90 min at 100 °C with 300 μl of 37% aqueous HCl. We noted that the strong acid conditions led to depletion of the deuterated IS due to exchange between deuterium and hydrogen atoms. For this reason IS was added to samples only after the acid hydrolysis reaction. This procedure is justified also by the observation that the deuterated compounds here used as IS are in the deconjugated form and thus not useful to control hydrolysis. Obviously, the problem of deuterium-hydrogen exchange would not be observed using C^{13}-labeled analogues as internal standard [25].

As regards enzymatic hydrolysis, recently published data underlined the importance of using an enzyme with both β-glucoronidase and arylsulphatase activity since OH-PAHs are excreted as a mixture of glucoronide and sulphate conjugates [25,27]. We observed that the use of 10 μl of enzyme was not sufficient to complete the reaction (yield 67%), while the use of 20 μl of enzyme led to obtaining a yield higher than 97%.

Although similar yields were obtained for enzymatic and acid hydrolysis of 1-OHPYRGLU, the following extraction step was negatively influenced by the very low pH of the acid hydrolysis, in particular for 6-OHCHR and 3-OHBaP. Not even buffering the sample let us obtain satisfactory results, so enzymatic hydrolysis was finally chosen for the subsequent optimization of the assay.

3.2.2. Extraction

In liquid–liquid extraction maximum recovery was obtained for every solvent with the first extraction, only low percentages of metabolites were recovered with the second extraction, and nothing with the following extractions. Therefore, the number of extractions was set at two. Every solvent was effective for PAHs metabolite extraction, with the best recovery obtained by using ethyl acetate for 1-OHNAP, 2-OHNAP, 9-OHFLU, 2-OHFLU, 4-OHPHE and 9-OHPHE and *n*-hexane for 3-OHPHE, 1-OHPHE, 2-OHPHE, 1-OHPYR, 6-OHCHR and 3-OHBaP. *n*-Hexane was finally preferred, because it gave much cleaner chromatographic profiles, satisfying recovery for all the OH-PAHs of interest, and was easily removed by evaporation. In particular comparing the two brands of *n*-hexane, better chromatograms with less interfering peaks were obtained using *n*-hexane from Fluka.

In SPE, a 50:50 MeOH/water solution was sufficient to begin the elution of 1-OHNAP and 2-OHNAP, while solutions with higher proportions of organic solvent were necessary to elute the other analytes, with 3-OHBaP demanding at least a 80:20 MeOH/water solution. From these results, a 40:60 MeOH/water solution was chosen as washing condition, while the use of pure MeOH was used as extraction condition. The extraction was repeated four times with 2 ml aliquots of MeOH and, for all analytes, maximum recovery was obtained with the first aliquot.

Comparing liquid–liquid extraction with *n*-hexane and SPE, slightly higher recovery was observed with the second; in any case, the use of methanol was discontinued because it led to several chromatographic interferences; due to this liquid–liquid extraction with *n*-hexane was finally chosen.

3.2.3. Ionic strength

We noted that every tested salt was effective in breaking the emulsion, but only $MgSO_4 \bullet 7H_2O$ improved the yield of the extraction, while the addition of other salts had no influence (NaCl, KI, NaH_2PO_4) or even a negative influence ($MgSO_4$, Na_2CO_3, KF) on the extraction. From these results, the addition of a saturating amount of $MgSO_4 \bullet 7H_2O$ was positively evaluated.

3.2.4. Derivatization

All the tested silylating agents were effective in forming the trimethyl silyl derivatives. For almost all analytes, the best results were obtained with BSTFA for an incubation time of 90 min at 45 °C, and this condition was finally chosen for derivatization. Slightly higher chromatographic areas were obtained only for naphthols, with an incubation time of 60 min at 45 °C.

3.2.5. Glassware material, urine sediment dissolution and GC/MS cleaning

In the final optimization of the assay, the possibility of using disposable polyethylene vials, as alternative to glass vials, was evaluated. It was noted that interfering peaks, possibly due to release of plasticizers from polyethylene plastic, appeared in correspondence of the chromatographic peaks of 1-OHNAP and of 9-OHFLU, causing difficulties for their quantification. For this reason, the use of polyethylene vials was discontinued.

The possibility of loss of OH-PAHs as a consequence of their absorption on the wall of glass materials was evaluated by comparing the chromatographic areas obtained performing the extraction and derivatization step of the assay in glass or silanized glassware. As shown in Fig. 2, higher chromatographic areas for all analytes were obtained using silanized glassware.

It has been reported that OH-PAHs in their conjugated form may be adsorbed on sediment particles of urine. Thus, the OH-PAHs recovery and the effective concentration is less than estimated if a proper procedure for sediment dissolution is not followed [30,31]. Regarding this issue, we noted that liquid–liquid extraction was easier and recovery was higher if samples were heated at 50 °C until completely thawed, before sampling the required volume. This procedure permitted dissolving the urine sediment completely and thus homogenizing the sample.

In daily practice, we noted a rather rapid worsening of GC/MS performance due to the progressive soiling of the GC/MS system (inlet liner, inlet seal, and ion source) that led to the loss of linearity in calibration curves, in particular for high-molecular mass OH-PAHs. To overcome this problem, we strongly recommend cleaning/replacing the GC/MS components about every 70 analytical runs.

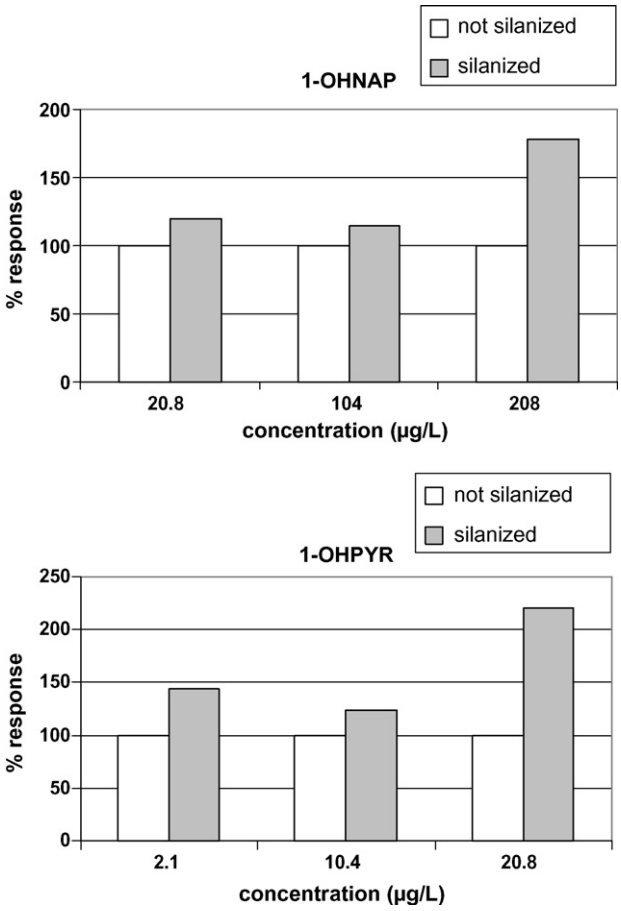

Fig. 2. Chromatographic response for 1-OHNAP and 1-OHPYR performing the assay in glass or silanized glass vials. Chromatographic response is reported as percent of the response obtained using not silanized glassware.

3.3. Validation of the assay: calibration curves, limits of quantification, within- and between-run precision, accuracy, recovery, stability of standard solutions

Calibration curves obtained for the investigated analytes were linear all through the respective concentration ranges investigated. The determination coefficients r^2 were higher than 0.996 for all analytes. Limits of quantification (LOQ) were in the 0.1–1.4 μg/l range (Table 2). These levels are lower than levels previously reported for gas chromatography coupled with low-resolution mass spectrometry assay [7,32], while they are of course higher than those obtained with high-resolution mass spectrometry [25].

Good analytical features, in line with the US-FDA requirements for validation of bio-analytical methods, were in general obtained for low-molecular weight analytes (CV below 20%, accuracy within ±20%, Table 2). Worse results, especially regarding accuracy, were obtained for metabolites of high-molecular mass PAHs, 6-OHCHR and 3-OHBaP. The mean overall recovery of the assay was 84% for all analytes, with the exception of 6-OHCHR and 3-OHBaP, for which a recovery below 40% was obtained.

The low performance obtained for 6-OHCHR and 3-OHBaP is due to chromatographic difficulties caused by the high boiling points of these compounds. Their chromatographic elution is characterized by long retention times and requires high temperatures. In these conditions, broad chromatographic peaks and column bleeding are observed. Both factors contribute to lowering signal to noise ratio, with obvious decrease of analytical performance. Also the relevant difference between these compounds and 1-OHPYRd9 used

Table 2
Recovery, limit of quantification, within- and between-run precision and accuracy for the determination of OH-PAHs.

Analyte	% Recovery[a]	LOQ (μg/l)	Theoretical concentrations (μg/l)	Day 1		Day 2		Day 3		Overall	
				CV%[b]	% Theoretical[d]	CV%[b]	% Theoretical[d]	CV%[b]	% Theoretical[d]	CV%[c]	% Theoretical[d]
1-OHNAP	53.1	0.9	20.8	12.2	111	1.4	100	1.3	97	7.2	103
			104	5.2	88	3.3	99	10.9	112	12.0	100
			208	4.3	95	4.8	102	10.3	93	5.2	97
2-OHNAP	71.5	0.9	20.8	11.7	113	1.0	98	1.6	102	7.2	105
			104	6.8	89	2.8	97	12.3	114	12.8	100
			208	3.8	97	4.2	102	9.8	96	3.6	98
2-OHFLU	94.1	0.1	2.1	10.5	134	1.4	134	1.7	134	0.0	134
			10.4	14.8	100	19.4	100	13.7	127	14.1	109
			20.8	13.9	100	9.0	124	13.3	103	11.6	109
9-OHFLU	85.2	0.2	2.1	9.1	101	3.0	101	18.9	101	0.0	101
			10.4	14.3	97	5.9	113	8.2	121	11.2	110
			20.8	8.3	99	6.9	111	13.8	109	6.1	106
1-OHPHE	78.1	0.3	2.1	10.1	77	5.5	77	4.7	77	0.0	77
			10.4	17.2	92	4.6	100	5.0	115	14.5	103
			20.8	14.7	100	7.9	108	13.5	115	7.7	108
2-OHPHE	109.0	0.1	2.1	6.7	89	1.6	89	8.7	89	0.0	89
			10.4	23.0	94	2.4	100	4.1	118	4.0	104
			20.8	10.6	97	5.0	109	14.0	112	7.9	106
3-OHPHE	126.9	0.2	2.1	13.1	85	2.4	85	9.0	85	0.0	85
			10.4	25.1	85	2.7	97	6.2	114	14.5	99
			20.8	11.9	97	5.4	111	14.4	111	7.7	106
4-OHPHE	83.2	0.1	2.1	8.3	105	1.6	70	15.0	105	21.7	94
			10.4	16.5	91	22.7	119	27.0	140	21.1	117
			20.8	9.6	98	8.2	109	14.0	112	6.9	106
9-OHPHE	50.1	0.2	2.1	7.7	67	1.9	67	11.4	67	0.0	67
			10.4	23.8	80	13.7	94	3.0	107	10.1	94
			20.8	10.5	94	2.5	107	13.5	107	9.2	102
1-OHPYR	88.7	0.5	2.1	4.9	103	4.2	92	2.9	92	6.7	96
			10.4	6.9	79	3.8	90	4.9	96	9.7	89
			20.8	5.6	79	4.2	90	10.5	103	13.1	91
6-OHCHR	23.4	1.4	2.1	33.7	97	18.0	77	22.0	77	13.3	84
			10.4	12.3	58	6.8	69	6.2	93	24.1	73
			20.8	7.4	70	3.7	81	3.4	104	20.8	85
3-OHBaP	36.3	1.0	2.1	18.9	106	20.0	87	8.3	73	19.2	89
			10.4	8.2	76	9.3	75	20.8	94	13.4	81
			20.8	11.8	75	5.9	63	5.7	112	30.7	83

[a] Values are expressed as percent of the chromatographic signal obtained from OH-PAHs solutions in acetonitrile.
[b] Within-run precision.
[c] Between-run precision.
[d] Accuracy.

as internal standard plays a role, and this underlines the importance of the use of specific internal standards [26].

A decreasing trend in accuracy of urinary standard solutions was evidenced in the long-term stability trial for all analytes. Actually, in the first 30 days the decrease of accuracy was less than 20%, while major decreases (up to 30%) were observed after 50 or 80 days of storage, especially for high-molecular weight OH-PAHs. The tendency for PAHs to be absorbed on surfaces with which they come in contact has been described in the past [33,34]. The same behaviour could be ascribed also to OH-PAHs, considering that the presence of the hydroxyl group only partially modifies the apolar nature of the PAH molecules, especially for those with higher molecular mass [35].

As regards short-term and freeze-and-thaw stability, accuracy was within ±20% of the theoretical values, so no indications about instability arise from these conditions. However, it is worth remembering that these stability experiments were carried out on urinary solutions spiked with OH-PAHs in their unconjugated form, while in real samples OH-PAHs are mostly present as conjugated derivatives, and other authors have reported no instability for conjugated OH-PAHs in biological samples [18,31].

3.4. Application of the assay to biological monitoring

Table 3 shows the results of the application of the developed assay to samples from coke-oven workers. All OH-PAHs were found at concentrations above the LOQ, with the exception of 6-OHCHR and 3-OHBaP that were always below the LOQ.

The OH-PAHs levels found in these subjects are indicative of a high environmental exposure to PAHs. In particular, the median level of 1-OHPYR is more than 6 times higher than the benchmark level of 6.3 μg/l proposed for this working activity [36] and is also higher than that reported in some recent European studies [37,38]. The absence of quantifiable amounts of 6-OHCHR and 3-OHBaP is not surprising, considering that metabolites of high-boiling PAHs are mainly excreted in faeces [4], and very low excretion levels (in

Table 3
Levels of urinary OH-PAHs in ten coke-oven workers. Results are expressed in μg/l.

Analytes	Median (μg/l)	Min (μg/l)	Max (μg/l)
1-OHNAP	61.4	48.6	143.3
2-OHNAP	56.3	37.9	119.6
2-OHFLU	69.7	9.7	260.3
9-OHFLU	20.3	5.6	42.7
1-OHPHE	25.1	3.3	71.7
2-OHPHE	11.9	1.3	24.6
3-OHPHE	26.1	3.0	59.4
4-OHPHE	1.9	0.4	5.3
9-OHPHE	3.2	1.0	8.6
1-OHPYR	38.7	4.5	147.1
6-OHCHR	<1.4	<1.4	<1.4
3-OHBaP	<1.0	<1.0	<1.0

the range of the low ng/l) are expected even for highly exposed subjects [39]. Results of this application highlight that the major contribution to exposure comes from naphthalene (43.4% to the total amount of excreted metabolites) and fluorene (28.5%). This result is in line with previous studies on coke-oven environment for which these chemicals were the most abundant airborne pollutants [40–42]. As far as we know, this is the first time that the simultaneous detection of 12 metabolites coming from different parent compounds is reported in occupationally exposed subjects. The investigation of several OH-PAHs opens the possibility of better understanding total exposure to PAHs [43].

3.5. Conclusions

In this manuscript a specific and sensitive method based on low-resolution GC/MS for the quantification of 12 hydroxylated metabolites of PAHs in human urine is described. The use of GC permits overcoming the problem of chromatography resolution often reported when using liquid chromatography [14,18]. Every step of the analytical assay has been developed testing different operative conditions in order to optimize it, and this allowed obtaining quantification limits lower than those declared by other authors using similar assays. The validation of the assay shows that good features in terms of precision, accuracy, and sensitivity for the analysis of metabolites of low-molecular weight compounds have been obtained, while more difficulties, especially in terms of accuracy and recovery, are encountered in quantification of metabolites of high-molecular mass PAHs. The stability of OH-PAHs in urinary solutions was also assessed. The application of the developed method to the quantification of OH-PAHs in samples from coke-oven workers shows that this assay is useful for biological monitoring of occupational exposure to PAHs, while more sensitive methods are necessary to quantify OH-PAHs, especially the high-molecular weight ones, in subjects environmentally exposed, for which much lower exposure is reported [17,43].

References

[1] International Programme on Chemical Safety (IPCS), Selected non-heterocyclic polycyclic aromatic hydrocarbons, in: Environmental Health Criteria 202, World Health Organization, Geneva, 1998.
[2] International Agency for Research on Cancer (IARC), IARC monographs on the evaluation of carcinogenic risks to humans, Air Pollution, Part 1, Some Non-heterocyclic Polycyclic Aromatic Hydrocarbons and Some Related Industrial Exposures. Available on line at http://monographs.iarc.fr/ENG/Meetings/92-pahs.pdf (Last accessed February 4th, 2008).
[3] J. Angerer, C. Mannschreck, J. Gündel, Int. Arch. Occup. Environ. Health 70 (1997) 365.
[4] A. Ramesh, S.A. Walker, D.B. Hood, M.D. Guillén, K. Shneider, E.H. Weyland, Int. J. Toxicol. 23 (2004) 301.
[5] F.J. Jongeneelen, R.B.M. Anzion, P.T.J. Scheepers, R.P. Bos, P.T. Henderson, E.H. Nijenhuis, S.J. Veenstra, R.M. Brouns, A. Winkes, Ann. Occup. Hyg. 32 (1988) 35.
[6] Á.M. Hansen, L. Mathiesen, M. Pedersen, L.E. Knudsen, Int. J. Hyg. Environ. Health 211 (2008) 447.
[7] B. Serdar, S. Waidyanatha, Y. Zheng, S.M. Rappaport, Biomarkers 8 (2003) 93.
[8] H. Kim, S.-H. Cho, J.-W. Kang, Y.D. Kim, H.-M. Nan, C.-H. Lee, H. Lee, T. Kawamoto, Int. Arch. Occup. Environ. Health 74 (2001) 59.
[9] V. Väänänen, M. Hämeilä, H. Kontsas, K. Peltonen, P. Heikkilä, J. Environ. Monit. 5 (2003) 739.
[10] R. Preuss, H. Drexler, M. Bottcher, M. Wilhelm, T. Brüning, J. Angerer, Int. Arch. Occup. Environ. Health 78 (2005) 355.
[11] G. Grimmer, J. Jacob, G. Dettbarn, K.-W. Naujack, Int. Arch. Occup. Environ. Health 69 (1997) 231.
[12] A. Toriba, T. Chetiyanukornkul, R. Kizu, K. Hayakawa, Analyst 128 (2003) 605.
[13] X. Xu, J. Zhang, L. Zhang, W. Liu, C.P. Weisel, Rapid Commun. Mass Spectrom. 18 (2004) 2299.
[14] T. Chetiyanukornkul, A. Toriba, T. Kameda, N. Tang, K. Hayakawa, Anal. Bioanal. Chem. 386 (2006) 712.
[15] M. Lafontaine, C. Champmartin, P. Simon, P. Delsaut, C. Funck-Brentano, Toxicol. Lett. 162 (2006) 181.
[16] R. Fan, Y. Dong, W. Zhang, Y. Wang, Z. Yu, G. Sheng, J. Fu, J. Chromatogr. B 836 (2006) 92.
[17] Z. Li, C.D. Sandau, L.C. Romanoff, S.P. Caudill, A. Sjodin, L.L. Needham, D.G. Patterson Jr., Environ. Res 107 (2008) 320.
[18] P. Jacob III, M. Wilson, N.L. Benowitz, Anal. Chem. 79 (2007) 587.
[19] M. Yang, M. Koga, T. Katoh, T. Kawamoto, Arch. Environ. Contam. Toxicol. 36 (1999) 99.
[20] S.G. Carmella, M. Chen, H. Yagi, D.M. Jerina, S.S. Hecht, Cancer Epidemiol. Biomarkers Prev. 13 (2004) 2167.
[21] G. Gmeiner, C. Krassnig, E. Schmid, H. Tausch, J. Chromatogr. B 705 (1998) 132.
[22] G. Gmeiner, P. Gärtner, C. Krassnig, H. Tausch, J. Chromatogr. B 766 (2002) 209.
[23] C.J. Smith, C.J. Walcott, W. Huang, V. Maggio, J. Grainger, D.G. Patterson Jr., J. Chromatogr. B 778 (2002) 157.
[24] T. Luan, S. Fang, Y. Zhong, L. Lin, S.M.N. Chan, C. Lan, N.F.Y. Tam, J. Chromatogr. A 1173 (2007) 37.
[25] Z. Li, L.C. Romanoff, D.A. Trinidad, N. Hussain, R.S. Jones, E.N. Porter, D.G. Patterson Jr., A. Sjödin, Anal. Chem. 78 (2006) 5744.
[26] C.J. Smith, W. Huang, C.J. Walcott, W. Turner, J. Grainger, D.G. Patterson Jr., Anal. Bioanal. Chem. 372 (2002) 216.
[27] L.C. Romanoff, Z. Li, K.J. Young, N.C. Blakely III, D.G. Patterson Jr., C.D. Sandau, J. Chromatogr. B 835 (2006) 47.
[28] C. Miller, J.N. Miller, in: R.A. Chalmers, M. Masson (Eds.), Statistics for Analytical Chemistry, Chapter 4, 96, Ellis Horwood Limited, Chichester, 1984, p. 96.
[29] U.S. Food and Drug Administration, Guidance for Industry, Bioanalytical Method Validation, U.S. Food and Drug Administration. Available on line at http://www.fda.gov/cder/guidance/4252fnl.pdf, 2001 (Last accessed February 4th, 2008).
[30] P. Simon, M. Lafontaine, P. Delsaut, Y. Morele, T. Nicot, J. Chromatogr. B 748 (2000) 337.
[31] Y. Li, A.C. Li, H. Shi, S. Zhou, W.Z. Shou, X. Jiang, W. Naidong, J.H. Lauterbach, Rapid Commun. Mass Spectrom. 19 (2005) 3331.
[32] N. Grova, F. Monteau, B. Le Bizec, C. Feidt, F. Andre, G. Rychen, J. Anal. Toxicol. 29 (2005) 175.
[33] A. López Garcia, E. Blanco Gonzalez, J.L. García Alonso, A. Sanz-Medel, Anal. Chim. Acta 264 (1992) 241.
[34] M.S. Garcia-Falcon, M. Pérez-Lamela, J. Simal-Gándara, J. Agric. Food Chem. 52 (2004) 6897.
[35] L. Wolska, M. Rawa-Adkonis, J. Namieśnik, Anal. Bioanal. Chem. 382 (2005) 1389.
[36] F.J. Jongeneelen, Ann. Occup. Hyg. 45 (2001) 3.
[37] B. Pesch, M. Kappler, K. Straif, B. Marczynski, R. Preuss, B. Rossbach, H.-P. Rihs, T. Weiss, S. Rabstein, C. Pierl, M. Sherenberg, A. Adams, H.U. Käfferlein, J. Angerer, M. Wilhelm, A. Seidel, T. Brüning, Cancer Epidemiol. Biomarkers Prev. 16 (2007) 1863.
[38] J.H.M. Van Delft, M-J.S. Steenwinkel, J.G. Van Asten, N. De Vogel, T.C.D.M. Bruijntjes-Rozier, T. Schouten, P. Cramers, L. Maas, M.H. Van Herwijnen, F.J. Van Schooten, P.M. Hopmans, Ann. Occup. Hyg. 45 (2001) 395.
[39] K. Förster, R. Preuss, B. Rossbach, T. Brüning, J. Angerer, P. Simon, Occup. Environ. Med. 65 (2008) 224.
[40] B. Rossbach, R. Preuss, S. Letzel, J. Drexler, J. Angerer, Int. Arch. Occup. Environ. Health 81 (2007) 221.
[41] P. Strunk, K. Ortlepp, H. Heinz, B. Rossbach, J. Angerer, Int. Arch. Occup. Environ. Health 75 (2002) 354.
[42] J. Unwin, J. Cocker, E. Scobbie, H. Chambers, Ann. Occup. Hyg. 50 (2006) 395.
[43] M. Wilhelm, J. Hardt, C. Schulz, J. Angerer, Int. J. Hyg. Environ. Health 211 (2008) 447.