



# Simultaneous analysis of PAH urinary mono- and dihydroxylated metabolites by GC-MS-MS following SPE and two-stage derivatization

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Received: 26 June 2021 / Revised: 19 August 2021 / Accepted: 30 August 2021 / Published online: 8 September 2021  
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## Abstract

A new gas chromatography–tandem mass spectrometry method for the determination of mono- and dihydroxylated polycyclic aromatic hydrocarbon metabolites (OH-PAHs and diol-PAHs) in urine was developed and validated. Various sample preparation procedures were compared, namely liquid–liquid extraction (LLE), dispersive solid-phase extraction (dSPE), and SPE, alone or combined. A novel two-stage derivatization approach using 2 silylation reagents was developed, and an experimental procedure design was used to optimize the programmed temperature vaporization–solvent vent injection (PTV-SV) GC parameters. The method focused on 11 target compounds resulting from four- to five-ring suspected carcinogenic PAHs. SPE was identified as an acceptable and more convenient extraction method for all tested metabolites, with extraction rates ranging from 63 to 86% and relative standard deviations lower than 20%. The two-stage derivatization approach successfully allowed first the derivatization of OH-PAHs by MTBSTFA (*N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide) and then diol-PAHs by BSTFA (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide) in a single run. The limits of quantification were in the range of 0.01–0.02  $\mu\text{g l}^{-1}$  for OH-PAHs and 0.02–0.2  $\mu\text{g l}^{-1}$  for diol-PAHs. The intra- and interday precisions were lower than 10%. The method was applied to determine PAH metabolites in urine collected at the beginning and at the end of the working week from 6 workers involved in aluminum production. The mean diol-PAH levels at the end of the week were 10 to 20 times higher (0.86–2.34  $\mu\text{g g}^{-1}$  creatinine) than those of OH-PAHs (0.03–0.30  $\mu\text{g g}^{-1}$ ). These results confirmed the usefulness of this new analytical technique for detecting and characterizing metabolic patterns of PAHs in urine and assessing carcinogenic occupational exposures.

**Keywords** Mono- and dihydroxylated metabolites · Polycyclic aromatic hydrocarbons · Tandem mass spectrometry · Extraction techniques · Derivatization · Urine

## Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants produced by incomplete combustion of organic matter or coal or petroleum distillation. They are emitted in complex and highly variable mixtures in the environment (vehicle exhaust, combustion, cigarette smoke, grilled or smoked food) [1, 2] and in occupational settings (coke oven, aluminum production, road paving, rubber factories). PAHs are made up of

two or more fused aromatic rings, and hundreds of congeners have been identified, including nitro-PAHs and methyl-PAHs that are known for their toxicity [3, 4].

PAH exposure can induce several diseases, of which cancer is the most significant endpoint, and an increased incidence of lung, skin, and bladder cancers were observed in occupational studies [5–7]. Among these various congeners, only a few (4 or more fused rings and including bay regions favoring the production of reactive metabolites) are known or suspected to be carcinogenic and mutagenic. Benzo(a)pyrene (BaP), benzo(e)pyrene (BeP), benzo(a)anthracene (BaA), chrysene (Chr), benzo(b)fluoranthene (BbF), benzo(j)fluoranthene (BjF), benzo(k)fluoranthene (BkF), and dibenzo(a,h)anthracene (DahA) are classified as known carcinogens in Annex VI of European Regulation (EC)1272/2008 and are classified carcinogens (Cat 1B) according to the European Classification Labelling and Packaging (CLP)

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regulation. In addition, exposure to PAHs can lead to other adverse health effects, including diabetes, inflammation, infertility, cardiovascular disease, and poor fetal development [8–13].

PAHs are typical examples of indirect-acting carcinogenic compounds that require metabolic activation in humans. PAHs are metabolized by both phase-I and phase-II cytochrome P450 enzymes to form a series of arene oxides, dihydrodiols, phenols, and quinones, as well as their polar conjugates. The most abundant metabolites are monohydroxylated PAHs (OH-PAHs), but other metabolites, such as dihydrodiols (diol-PAHs), diol epoxides, and tetraols, also result from PAH metabolism. Various OH-PAHs are considered detoxification biomarkers, whereas some diol-PAHs reflect the metabolic activation of parent compounds as precursors of the ultimate reactive metabolites (bay region diol epoxides) inducing DNA adducts [14, 15]. Knowledge of diol-PAH biological level occurrences and intensities compared to those of OH-PAHs is thus critical for a better assessment of human exposure to PAHs and carcinogenic health risks.

Many analytical methods have been developed in recent years to quantitatively determine OH-PAH metabolites in human urine. Usually, after hydrolysis with  $\beta$ -glucuronidase/sulfatase, urine is extracted by liquid–liquid extraction (LLE), solid-phase extraction (SPE), or (more recently) dispersive solid-phase extraction (dSPE). Extracted metabolites are then analyzed by liquid chromatography with fluorescence detection (LC-FLD) or gas chromatography coupled with mass spectrometry-electron ionization mode (GC-EI-MS), high-resolution mass spectrometry (GC-EI-HRMS), or tandem mass spectrometry (GC-EI-MS/MS) following derivatization using BSTFA, N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), or MTBSTFA [16–22]. Most of these methods focused on 2- to 4-ring PAHs (naphthalene, fluorene, phenanthrene) and/or were restricted to OH-PAHs [15, 23]. The rare available analytical methods for diol-PAH quantification were generally developed for 2- and 3-ring specific compounds with sophisticated purification methods [15] or for various metabolites of a single PAH [24, 25]. In addition, some authors used an indirect method involving the conversion of dihydrodiols to phenols by a 16-h acid treatment rather than directly measuring urinary diol-PAHs [23]. Lastly, specificity and sensitivity issues generally preclude the use of a single derivatization reagent, especially in the case of compounds with sterically hindered sites such as diol-PAHs [26]. There is thus a need for more extensive methods allowing the simultaneous determination of OH- and diOH- metabolites of various PAHs in biological samples.

The current study therefore aimed to develop a purification and analytical method based on GC-EI-MS/MS for the analysis of urinary 4- or 5-ring target OH- and diol-PAH metabolites resulting from suspected carcinogenic PAHs (BaA, Chr,

BeP, B<sub>1</sub>JF, DahA, B<sub>1</sub>kF and B<sub>1</sub>bF). The first step consisted of comparing the recovery rates of three purification methods (LLE, SPE, dSPE) performed alone or in combination, and the second step consisted of testing the derivatization rate of a two-stage derivatization compared to a single derivatization. After analytical validation, the most efficient method was applied to determine the levels of OH- and diol-PAHs in urine collected at the beginning and at the end of the working week from 6 workers involved in aluminum production to confirm that the method was sufficiently sensitive to characterize occupational exposure.

## Experimental

### Standards

Benzo(a)anthracene, trans-8,9-dihydrodiol (> 99%) (8,9-diolBaA), benzo(e)pyrene, trans-4,5-dihydrodiol (> 99%) (4,5-diolBeP), benzo(k)fluoranthene, trans-8,9-dihydrodiol (> 97%) (8,9-diolBkF), 6-hydroxychrysene (> 99%) (6-OHChr), 3-hydroxychrysene (> 99%) (3-OHChr) 3-hydroxybenzo(a)anthracene (>97 %) (3-OHBaA), 8-hydroxybenzo(k)fluoranthene (98%) (8-OHFluo), 9-hydroxybenzo(k)fluoranthene (~ 99%) (9-OHBkF), 10-hydroxybenzo(b)fluoranthene (~ 100%) (10-OHBbF), and 3-hydroxybenzo(e)pyrene (> 91%) (3-OHBeP) were purchased from the National Cancer Institute Chemical Carcinogen Reference Standards Repository (MRI Global, USA). DahA metabolites were not commercially available, whereas several other metabolites ordered contained impurities that prevented them from being identified by GC-MS/MS. In particular, benzo(j)fluoranthene, trans-2,3-dihydrodiol (2,3-diolBjF), benzo(a)anthracene-trans-3,4-dihydrodiol (3,4-diolBaA), benzo(b)fluoranthene-trans-9,10-dihydrodiol (9,10-diolBbF), benzo(b)fluoranthene-trans-11,12-dihydrodiol (11,12-diolBbF), and benzo(e)pyrene-trans-9,10-dihydrodiol (9,10-diolBeP) were thus removed from the study. 6-Hydroxychrysene (13C6, 98%) (6-OHChrC13) at 50  $\mu\text{g/ml}$  in toluene was supplied by Cambridge Isotope Laboratories Inc. (USA).

Individual OH-PAHs and diol-PAHs delivered as solids were dissolved in tetrahydrofuran (THF). Mixed-standard solutions of OH- and diol-PAHs at concentrations of 2, 20, 100, and 1000  $\text{ng ml}^{-1}$  and internal standards at 200  $\text{ng ml}^{-1}$  were prepared in acetonitrile (ACN) and stored at  $-20\text{ }^{\circ}\text{C}$ .

### Chemicals, reagents, and other materials

Ethyl acetate (EA) RPE for analysis was obtained from Carlo Erba (France) and acetonitrile (ACN) HiperSolv Chromanorm ( $\geq 99.9\%$ ), tetrahydrofuran (THF) HiperSolv Chromanorm (> 99%), and acetate buffer AVS titrinorm solution (pH =

4.66) from VWR chemicals (USA). Methanol (MeOH) hypergrade (> 99.9%), pyridine solution (99.8%), and N-tert-butyltrimethylsilyl-N-methyltrifluoroacetamide (> 97%) (MTBSTFA) were delivered by Sigma-Aldrich (USA). *N,O*-Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + TMCS) (99:1) was obtained from Supelco (USA).  $\beta$ -Glucuronidase/arylsulfatase solution was supplied by Roche Diagnostics GmbH (Germany). Sep-Pak Vac 6CC (500 mg) C18 SPE cartridges were supplied by Waters (USA) and sorbent Supel Que Z-Sep and Z-Sep+ by Sigma-Aldrich.

### Extraction and purification method description

Five milliliters of urine was hydrolyzed at 37 °C overnight after the addition of 2.5 ml of acetate buffer and 20  $\mu$ l of  $\beta$ -glucuronidase/arylsulfatase. After adding 50  $\mu$ l of internal standard (200 ng ml<sup>-1</sup>), the purification methods were performed alone (LLE, SPE C18) or in combinations (LLE-SPE C18, LLE-dSPE Z-sep, LLE-dSPE Z-sep+).

- LLE: Hydrolyzed urine was extracted twice with 5 ml of EA in a rotary shaker for 30 min and then centrifuged for 10 min at 4000 rpm. Approximately 9 ml of supernatant was collected and evaporated to dryness under vacuum.
- SPE: Five milliliters of MeOH was added to hydrolyzed urine. The SPE C18 cartridges were conditioned with 5 ml of MeOH followed by 10 ml of purified water using the Milli-Q treatment system (Merck Millipore, Germany). After the sample was loaded on SPE cartridges and purification with 10 ml of MeOH/water (v:v 40%:60%), the compounds were eluted with 7 ml of ACN and evaporated to dryness under vacuum.
- d-SPE: After LLE was performed as indicated above, the upper organic phases were transferred to a new tube containing 500 mg of Z-sep or 200 mg Z-Sep+ sorbent. After shaking for 30 min in a rotary shaker and centrifuging for 10 min at 4000 rpm, approximately 8 ml of purified extracts was evaporated to dryness under vacuum.

In all cases, the robustness of the evaporation step was ensured by using an automated centrifugal vacuum concentrator (coupled to a cold trap) working at stable low temperature (30 °C) and low pressure (50 mbar).

### Derivatization of PAH metabolites

Dry residues resulting from LLE, SPE, or dSPE were dissolved in 60  $\mu$ l of ACN and 20  $\mu$ l of pyridine. Derivatization was first conducted by adding MTBSTFA or BSTFA separately and then conducted by successively adding them to the same sample. This two-stage derivatization was performed by first adding 20  $\mu$ l of MTBSTFA and then 20  $\mu$ l

of BSTFA 15 min later. Furthermore, the influence of temperature on the derivatization rate (%) was studied by conducting the derivatization step at two temperatures (20 °C and 60 °C).

### Instrumentation and analytical conditions

Analyses were performed using an Agilent 7890B gas chromatograph coupled with an Agilent 7000C triple quadrupole mass spectrometer operating in electron impact (EI) ionization mode and equipped with an Agilent CTC PAL autosampler. A DB-5 MS capillary column (30 m, 0.25 mm i.d., 0.25  $\mu$ m film thickness) was used for the separation. The initial oven temperature was set at 70 °C, held for 6.5 min, ramped from 15 °C/min up to 300 °C, and held for 15 min. Helium (purity 99.999%, Air Liquide, France) was employed as the carrier gas with a constant flow of 1.0 ml min<sup>-1</sup>. Programmed temperature vaporization–solvent vent injection (PTV-SV) parameters were optimized by means of an experimental design procedure. Design-Expert software version 12.0.3.0 (State Ease, Inc., Minneapolis, MN, USA) was used to establish an empirical quadratic model that exhibits the relationships between the response (removal of MTBSTFA derivatization reagent in multiple reaction monitoring (MRM) signal) and four PTV parameters (vent flow, vent time, vent pressure, and inlet temperature). BSTFA was not tested due to its lower boiling point that precluded the mass spectrometer ionization source fouling. A statistical procedure was carried out to determine the optimal level of factors minimizing the response.

Under optimum conditions, the temperature of the liner (4 mm internal diameter) with wool (Restek) was held at 84.5 °C, the column head pressure was fixed at 2.8 psi, and the flow rate through the split vent was set at 280 ml min<sup>-1</sup> to eliminate most of the solvent and derivatization reagents. Three microliters of purified and derivatized samples was injected in the solvent vent injection mode into the EI-GC-MS/MS. At 0.72 min of vent time, the analytes were focused to the column in splitless mode for 2 min while the temperature of the PTV injection port was increased at 900 °C min<sup>-1</sup> up to 350 °C and held for 5 min. During this step, 30 psi pulsed pressure was applied for 0.5 min. Finally, the injector was kept at 300 °C with a purge flow of 100 ml min<sup>-1</sup> until the end of the run for cleaning purposes.

The transfer line temperature, ion source, and triple quadrupole were maintained at 350 °C, 325 °C, and 150 °C, respectively. Argon was used as the collision gas, and the flow rate was set at 0.9 ml min<sup>-1</sup>. The quench gas was helium at 2.25 ml min<sup>-1</sup>.

The spectrometer was operated in EI mode with MRM. MRM transitions and retention times ( $t_R$ ) are presented in Table 1. Identification was confirmed by the quantification and confirmation transitions of mass fragments as well as by their ratios for each analyte.

**Table 1** MRM transitions for OH and diol-PAHs in urine

Compound	Analytical parameters						Ratio [confirmation]/ [quantification] transitions on spiked urines (%)	
	$t_R$ (min)	Quantification transition	Dwell (ms)	CE (V)	Confirmation transition	Dwell (ms)		CE (V)
8,9-diolBaA	22.16	406→244	10	10	406→303	10	10	44
4,5-diolBeP	22.19	430→340	10	10	430→325	10	10	8
1,2-diolChr	22.45	406→244	10	5	406→303	10	10	45
6-OHChrC13	23.33	364→307	10	10	307→291	10	10	134
6-OHChr	23.33	301→285	25	20	358→301	25	20	77
3-OHChr	24.21	301→245	25	20	358→301	25	20	77
8,9-diolBkF	24.22	430→191	15	10	430→327	10	10	31
3-OHBaA	24.71	301→245	25	20	358→301	25	20	64
8-OHBkF	27.17	382→325	25	20	325→309	25	20	58
10-OHBbF	28.81	325→269	25	20	382→325	25	20	73
9-OHBkF	29.10	382→325	25	20	325→269	25	20	100
3-OHBeP	29.62	382→325	25	20	382→309	25	20	10

### Quality assurance/quality control

The validation of the final method (SPE followed by two-stage derivatization and analysis by EI-GC-MS/MS) was evaluated through the analysis of a pool of 50 blank urine samples (obtained from hospital employees not occupationally exposed to PAHs and that gave their informed consent) artificially contaminated by the mixture of 11 PAH metabolites. Because certified reference materials (CRM) were absent for almost all analytes (except for 6-OHChr), validation tests were performed on blank urine spiked with OH-PAHs and diol-PAHs. The absence of the tested analytes (as well as other potentially coeluting compounds) in the pooled blank urine was previously tested and ensured prior to the validation tests.

The calibration curves were obtained by spiking blank urine samples with the following standard concentrations: 0.08, 0.2, 0.4, 1, 2, and 4  $\mu\text{g l}^{-1}$ . The final method was validated according to the recommendations of the ISO 15189:2012 standards (Medical Laboratories—requirements for quality and competence) for extraction recovery, linearity, accuracy, precision, and limits of quantification (LOQs).

The diol-PAH and OH-PAH extraction recoveries were determined as follows. Briefly, blank urine samples were spiked with two concentrations of each analyte (at 0.4 and 2  $\mu\text{g l}^{-1}$ ) and purified by automated SPE. In a separate experiment, the same blank urine samples were initially processed by automated SPE, and eluates were subsequently spiked with the same analyte concentrations prior to the evaporation step. The extraction recovery (%) was calculated by comparing the chromatographic signal of the extracted spiked urine samples with that of the same urine samples spiked with analytes after the extraction step.

Linearity was determined from three calibration curves prepared on three different days and tested from LOQs up to 20  $\mu\text{g l}^{-1}$  because urinary levels of PAH metabolites > 20  $\mu\text{g l}^{-1}$  were very unlikely to occur in common occupational settings.

Accuracy and precision were calculated from 10 analyses using blank urine samples spiked at a concentration level of 0.4 and 2.0  $\mu\text{g l}^{-1}$  (prepared separately from the calibration standards). Accuracy was assessed by calculating the deviation percentage from the mean of the target value. Precision was determined by calculating relative standard deviations (RSDs, %) intra- and interday. The LOQ was determined according to two different methods. First, LOQ was determined as the lowest concentration at which analytes provided a signal-to-noise ratio higher than or equal to 10 (referred to as LOQ  $S/N > 10$ ). Second, LOQ was determined as the lowest concentration at which 10 successive injections provided both an RSD < 20% and a bias from the target value < 20% (referred to as LOQ Acc-Prec < 20%).

### Application of the analytical method to urine of occupationally exposed workers

The method was applied to urine samples collected from six nonsmoking male workers involved in aluminum plants and occupationally exposed to PAHs. This non-interventional, monocentric, retrospective study involving data and urine samples from human participants was carried out in Grenoble University Hospital according to French current regulation. The investigator (Dr. Persoons, PharmD) signed a commitment to comply with Reference Methodology n°004 issued by French Authorities (CNIL). Subjects were all

informed and did not oppose; written consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Three urine spot samples were collected in 50-ml propylene bottles at the beginning of the shift on the 1st day of the working week (BW-BS), at the end of the shift on the second-to-last workday (EW-ES), and at the beginning of the last shift of the week (EW-ES + 16 h). This strategy was meant to characterize background levels before the working week as well as the post-shift exposure levels. Due to probable skin absorption or elimination kinetics comparable to BaP, the 3rd urine sample aimed to identify delayed urinary excretion peaks. Samples were stored at  $-20\text{ }^{\circ}\text{C}$  until analysis. Simultaneously, urinary creatinine was analyzed using an enzymatic method, and metabolite concentrations were corrected by creatinine levels and further expressed as micrograms per gram creatinine. Statistical analyses were conducted with SPSS Statistics® 26.0 software from IBM. Descriptive statistics were used (geometric mean, GM; geometric standard deviation, GSD) to describe urinary metabolite concentrations. Left-censored data ( $<$  LOQs) were replaced using a multiple imputation method (random value between 0 and the LOQ) following recent recommendations [27]. Mean comparisons for paired samples were conducted using the non-parametric Mann–Whitney test to compare urinary concentrations at ESEW and ESEW + 16 h.

## Results and discussion

### Extraction method development

The results from the different extraction methods tested are presented in Table 2 and Fig. 1. The LLE method tested alone (with EA as the extraction solvent) resulted in signal suppression up to 90% due to the high urinary matrix coextraction, which precluded its use as a unique purification method for urine samples. In addition, LLE is a time-consuming and labor-intensive technique. Studies reporting satisfactory LLE recovery rates in the literature were only interested in OH-PAH metabolites. EA used for 2- and 3-ring OH-PAHs and n-hexane for 4- and 5-ring OH-PAHs or pentane were the most efficient extraction solvents identified [18, 28]. However, there were safety concerns with n-hexane due to its neurotoxic properties. Other authors pointed out the insufficient separation of the 4- to 6-ring OH-PAHs by LLE with dichloromethane, requesting further SPE extraction [19].

The use of LLE with further clean-up using d-SPE Z-Sep (LLE-dSPE Z-Sep) or d-SPE Z-Sep+ (LLE-dSPE Z-Sep+) resulted in a much lower background than LLE alone. The mean recovery rates of the extraction methods were 86% and 70%, with large differences between compounds (73–

107% and 56–84%). LLE-dSPE Z-Sep was efficient in cleaning urinary pigments. As reported, the best removal of matrix interferences seemed to be obtained by the Z-Sep sorbent (compared to C18, PSA, Z-Sep+ and ENVI-Carb sorbents), with increases of 30–80% of analyte responses in the Z-Sep purified extracts and a reduction of 50% of matrix interferences [21]. dSPE sorbents are recommended for reducing sources of interfering compounds, including organic acids, polar pigments, sugars, and fat in biological matrices, with successful use for evaluating urinary OH-PAH metabolites of mothers and newborns [21, 29]. However, LLE-dSPE Z-Sep revealed insufficiently specific clean-up of all compounds that can interfere with diol-PAH metabolites in GC-MS-MS. Indeed, this method did not allow us to quantify 1,2-diol-Chr with reasonable specificity (background noise due to matrix effects of urine samples).

SPE extraction with a sep-Pak C18 (SPE C18) showed low background and high recovery rates (the mean ranged from 70 to 86% depending on the analytes) for OH-PAHs and diol-PAHs, with the exception of 8,9-diolBaA and 8,9-diolBkF at low levels (63% and 61%, respectively). These results are comparable to or even better than those obtained using a double SPE in series. Indeed, the recovery rates reported were 50–60% for OH-Chr metabolites with a double extraction by Bond Elut Plexa and Bond Elut C18 SPE [20] or 56% for [13C6]1,2-diolPhe with Strata SDBL and Oasis MAX SPE [15]. An average recovery rate of only 41% for 6-OHChr was found with EnvirElut PAHs due to an absorption of the polar analytes to the SPE bed and the presence of components in the urine matrix interfering with the extraction [16]. Much higher recoveries (69–93% for PAH metabolites including BaA, Chr, and BaP) were obtained with focus cartridges (VARIAN®), which was explained by the addition of a “keeper” (dodecane) to the extracts to minimize losses during the evaporation step following SPE [17].

Finally, the use of LLE followed by SPE gave rise to very low backgrounds, but the mean recovery rates, ranging from 62 to 77%, were lower than those obtained with SPE alone. Nevertheless, RSDs were relatively low (4 to 14%) in comparison to LLE-dSPE methods. The combination of LLE and SPE was probably disadvantaged by the addition of compound losses during the two consecutive extractions.

Because of its highest recovery rates for OH-PAHs and diol-PAHs, the SPE C18 method was retained as the extraction method. Furthermore, this automated method ensured time savings and good reproducibility.

### Two-stage derivatization of OH-PAHs and diol-PAHs

When derivatization reagents were tested separately, diol-PAHs were not derivatized by MTBSTFA due to steric hindrance, while they were derivatized by BSTFA. In addition,

**Table 2** Extraction rates of PAH metabolites following different extraction techniques

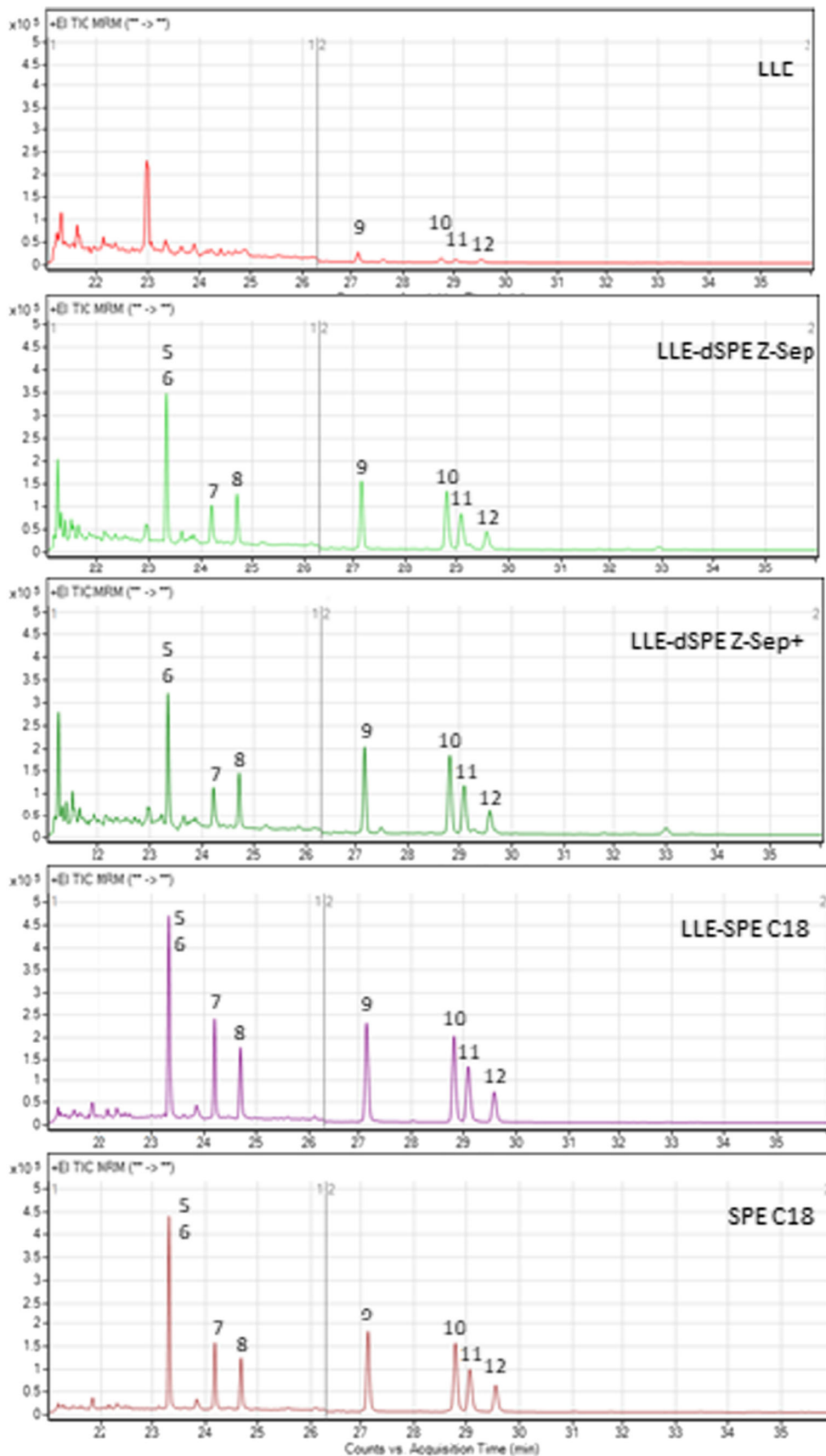
Compound	LLE-dSPE (Z-sep)		LLE-dSPE (Z-sep+)		LLE-SPE (Sep Pak C18)		SPE (Sep Pak C18)		All purification methods
	0.4 $\mu\text{g l}^{-1}$	2 $\mu\text{g l}^{-1}$	0.4 $\mu\text{g l}^{-1}$	2 $\mu\text{g l}^{-1}$	0.4 $\mu\text{g l}^{-1}$	2 $\mu\text{g l}^{-1}$	0.4 $\mu\text{g l}^{-1}$	2 $\mu\text{g l}^{-1}$	
	Mean RSD %	Mean RSD %	Mean RSD %	Mean RSD %	Mean RSD %	Mean RSD %	Mean RSD %	Mean RSD %	
8,9-diolBaA	<b>83</b> 5	<b>75</b> 6	<b>67</b> 19	<b>70</b> 10	<b>66</b> 8	<b>71</b> 6	<b>63</b> 14	<b>72</b> 3	<b>71</b> 9
3-OH BaA	<b>89</b> 5	<b>82</b> 4	<b>78</b> 13	<b>71</b> 21	<b>65</b> 6	<b>69</b> 7	<b>71</b> 2	<b>71</b> 3	<b>75</b> 10
1,2-diolChr	<b>107</b> 22	<b>77</b> 8	<b>84</b> 12	<b>71</b> 11	<b>64</b> 8	<b>77</b> 6	<b>86</b> 17	<b>82</b> 9	<b>81</b> 15
6-OH Chr	<b>96</b> 6	<b>86</b> 3	<b>72</b> 8	<b>68</b> 6	<b>69</b> 5	<b>71</b> 4	<b>71</b> 1	<b>74</b> 2	<b>76</b> 13
3-OH Chr	<b>73</b> 13	<b>85</b> 11	<b>75</b> 6	<b>66</b> 16	<b>66</b> 6	<b>69</b> 7	<b>70</b> 2	<b>71</b> 2	<b>72</b> 8
4,5-diolBeP	<b>85</b> 6	<b>77</b> 4	<b>65</b> 13	<b>66</b> 9	<b>68</b> 8	<b>72</b> 6	<b>68</b> 9	<b>73</b> 3	<b>72</b> 9
3-OH BeP	<b>99</b> 6	<b>89</b> 5	<b>59</b> 11	<b>56</b> 6	<b>70</b> 5	<b>70</b> 4	<b>69</b> 6	<b>72</b> 3	<b>73</b> 19
8,9-diolBkF	<b>80</b> 6	<b>77</b> 8	<b>68</b> 17	<b>65</b> 8	<b>63</b> 14	<b>71</b> 6	<b>61</b> 22	<b>76</b> 4	<b>71</b> 10
8-OH BkF	<b>92</b> 6	<b>86</b> 4	<b>68</b> 10	<b>65</b> 12	<b>62</b> 6	<b>65</b> 7	<b>70</b> 3	<b>70</b> 2	<b>73</b> 15
9-OH BkF	<b>91</b> 6	<b>84</b> 5	<b>81</b> 15	<b>71</b> 19	<b>64</b> 9	<b>67</b> 6	<b>69</b> 5	<b>67</b> 3	<b>75</b> 13
10-OH BbF	<b>91</b> 6	<b>85</b> 6	<b>80</b> 11	<b>71</b> 16	<b>64</b> 8	<b>66</b> 8	<b>69</b> 6	<b>69</b> 3	<b>75</b> 12
All analytes	<b>86</b> 9		<b>70</b> 9		<b>68</b> 5		<b>72</b> 7		

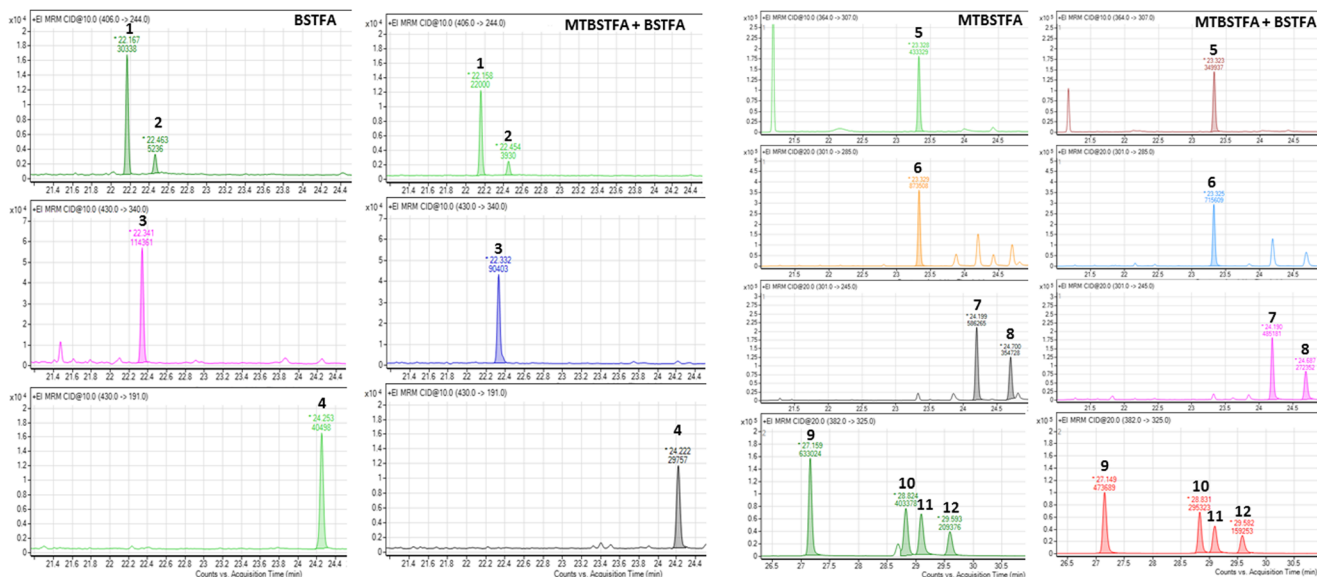
OH-PAHs were derivatized by both reagents. When derivatization was performed by adding MTBSTFA in the first step and BSTFA in the second step, the peak areas of diol-PAHs and OH-PAHs were equivalent to those derivatized only by BSTFA or MTBSTFA, respectively (Fig. 2). In fact, all OH-PAHs were completely derivatized at the first step (MTBSTFA), while diol-PAHs were derivatized only at the second step (BSTFA). These results were in agreement with previous studies. While derivatization of OH-PAHs with  $\leq 3$  rings can be performed with BSTFA or MTBSTFA, MTBSTFA should (whenever possible) be used for metabolites with  $> 3$  rings due to more specific fragmentation patterns for the analyzed compounds [26]. However, for diol-PAHs with sterically hindered sites, BSTFA should be preferred because steric hindrance lowers the analytical responses of MTBSTFA-derivatized compounds or inhibits derivatization. BSTFA was successfully used to derivatize diol-phenanthrene (1,2-diol-Phe and 3,4-diol-Phe) in the urine of smokers and nonsmokers [15]. Furthermore, we did not find any significant influence of temperature on derivatization efficiency, with an average of only a 20% increase for diol-PAHs and no difference at all for OH-

PAHs at 60 °C when compared to that of room temperature. The same results were already reported for OH-PAHs. Indeed, derivatizations with the MSTFA of OH-Chr, OH-BaA, and OH-BaP were quite insensitive to slight variations in derivatization conditions, such as varying temperature, amount of reagent, and derivatization time [30].

Consequently, the two-step derivatization method was set at room temperature and included initial derivatization of OH-PAHs by MTBSTFA followed by derivatization of diol-PAHs by BSTFA. This strategy was justified as it saved time and resulted in much more specific mass fragments for OH-PAHs following MTBSTFA derivatization. To the best of our knowledge, this is the first time that a two-stage derivatization was used in a single run to analyze various urinary 4- and 5-ring PAH metabolites, including both OH-PAHs and

**Fig. 1** TIC chromatograms of extracted urine samples depending on the extraction technique (LLE, LLE-dSPE, LLE-SPE, SPE). 5: 6-OH-ChrC13, 6: 6-OH-Chr, 7: 3-OH-Chr, 8: 3-OH-BaA, 9: 8-OH-BkF, 10: 10-OH-BbF, 11: 9-OH-BkF, 11: 3-OH-BeP. Diol-PAH compounds (1: 8,9-diolBaA, 2: 1,2-diolChr, 3: 4,5-diolBeP, 4: 8,9-diolBkF) are not represented on these graphs due to very low responses in TIC acquisition





**Fig. 2** MRM chromatograms of extracted urine samples spiked with Diol-PAH/OH-PAH derivatized with BSTFA, MTBSTFA, or a two-stage derivatization (at room temperature). 1: 8,9-diolBaA, 2: 1,2-diolChr, 3: 4,5-diolBeP, 4: 8,9-diolBkF, 5: 6-OHChrC13, 6: 6-OHChr, 7: 3-OHChr, 8: 3-OHBaA, 9: 8-OHBkF, 10: 10-OHBBF, 11: 9-OHBkF,

11: 3-OHBeP. Comparison of derivatization rates must take into account different sample volumes, as dry residues spiked with a single reagent (BSTFA or MTBSTFA) were dissolved in 100  $\mu$ l whereas residues spiked by the combination of both reagents (BSTFA + MTBSTFA) were dissolved in 120  $\mu$ l

diol-PAHs. This is partly explained by the fact that published studies did not consider both OH-PAHs and diol-PAHs in their analytical techniques [16–18, 31, 32]. However, a similar sequential one-pot silylation method was used for the analysis of fatty acids and sterol lipids in human plasma by Kloos et al. [33]. This strategy was also used with MSTFA and MBTFA for the determination of plasma dibasic amino acids [34].

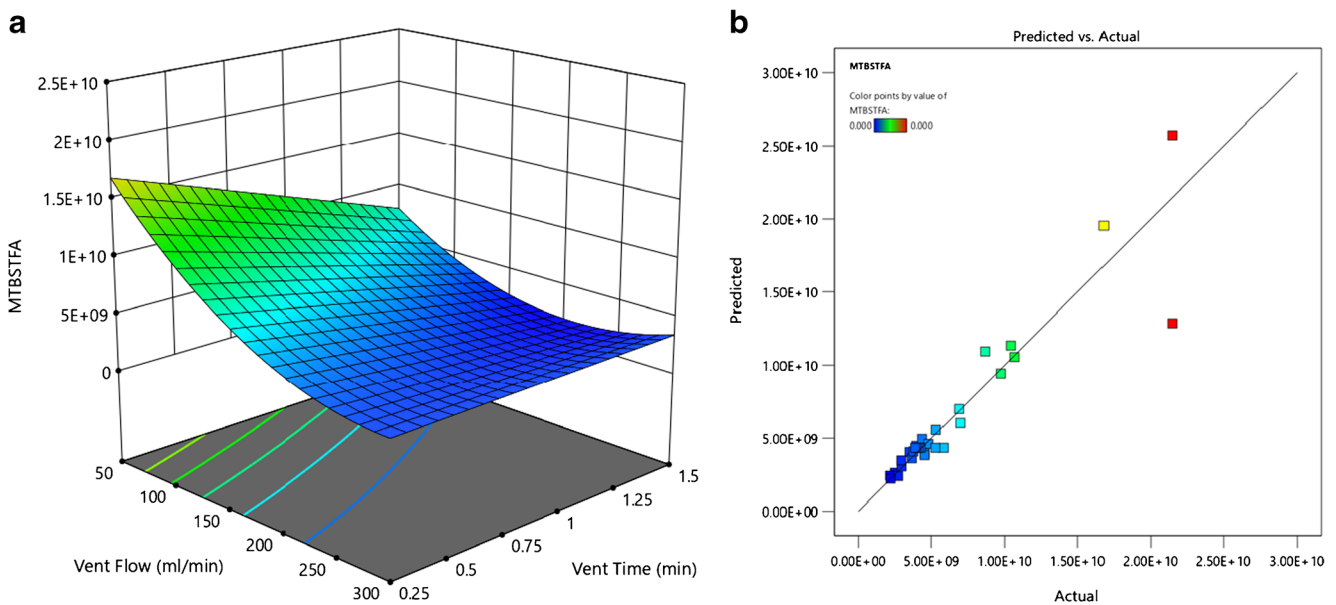
### GC-MS-MS method optimization

The optimization protocol aimed to remove excess solvents and derivatization reagents without losing the compounds of interest to prevent premature fouling of the EI source, causing a decrease in signal and therefore a decrease in sensitivity. A particular focus was given to MTBSTFA, which is the most difficult to eliminate due to its high boiling point (175  $^{\circ}$ C) compared to BSTFA (50  $^{\circ}$ C). Computer-based optimization techniques based on response surface methodology (RSM) were applied to discover the optimum operating conditions for solvent vaporization in a short time period with marginal trials. According to analytical knowledge, vent flow, vent time, vent pressure, and inlet temperature were identified as key factors. The range of tested values for the 4 optimized parameters were [50–175–300  $\text{ml min}^{-1}$ ] for the vent flow, [0.25–0.875–1.5 min] for the vent time, [2–6–10 psi] for the vent pressure, and [70–77.5–85  $^{\circ}$ C] for the liner inlet temperature. A central composite design (reported in the [supplementary material](#)) was used to optimize these PTV

parameters, which consisted of determining the smallest MTBSTFA MRM signal (184 $\rightarrow$ 77) [35]. Finally, the combined effects of vent flow and vent time factors on the MTBSTFA MRM signal were illustrated with the help of a response surface (Fig. 3a). Figure 3b shows the observed response values in comparison with the predicted values, which depicts a good fit. An optimum setting of the four factors was generated by the numerical optimization hill climbing technique. The optimal conditions in the actual scaling were vent flow = 280  $\text{ml min}^{-1}$ , vent time = 0.72 min, vent pressure = 2.8 psi, and temperature = 84.5  $^{\circ}$ C. Similar optimization techniques of PTV injection in solvent vent mode were used by other authors for the analysis of the 16 EU-priority PAHs in food. Vent time, vent flow, and vent temperature were previously identified as critical parameters, and vent time showed the highest effect on the analytical response (signal intensity) of the target PAHs [36].

Regardless of the conditions selected during optimization, speed injection was adjusted at 1  $\mu\text{l s}^{-1}$ , the final temperature of the injector was maintained at 350  $^{\circ}$ C, and the temperature ramp was 900  $^{\circ}\text{C min}^{-1}$ . The splitless mode was maintained for 2 min with an increase in inlet pressure of 30 psi for 0.5 min to reduce the residence time of the low volatile analytes in the liner. Finally, 5 min after the injection, the inlet liner temperature was set at 300  $^{\circ}$ C until the end of the run, with a split flow at 100  $\text{ml min}^{-1}$  to remove the high-boiling compounds through the split vent. These GC conditions were close to those described by Grova et al. [19] for the analysis of OH-PAHs in rat hair.





**Fig. 3** **a** Response surface plots showing the influence of vent flow and vent time on the MTBSTFA MRM signal (vent pressure and liner inlet temperature respectively adjusted to 6 psi and 77.5 °C). **b** Plot between observed and predicted value of MTBSTFA MRM signal

## Method validation

The method validation data for the 11 compounds are reported in Table 3. LOQs ranged from 0.005 to 0.02  $\mu\text{g l}^{-1}$  for OH-PAHs and from 0.02 to 0.20  $\mu\text{g l}^{-1}$  for diol-PAHs, regardless

of the calculation method used ( $S/N > 10$  or Acc-Prec  $< 20\%$ ). Intraday precision was in the range [1–7%] and interday precision was [2–13%], except for 1,2-diolChr at 0.4  $\mu\text{g l}^{-1}$  (14% and 25%, respectively). A wide linear range was obtained from the LOQ to 20  $\mu\text{g l}^{-1}$  for all metabolites.

**Table 3** Validation data for the analysis of OH-PAHs and diol-PAHs in urine by GC-MS/MS

Analyte	Concentration ( $\mu\text{g l}^{-1}$ )	Recovery % ( $\pm$ RSD)	Accuracy (%)	Intra-day precision (% RSD)	Inter-day precision (% RSD)	Linearity ( $R^2$ , regression coefficient)	Limit of quantitation ( $S/N \geq 10$ ) ( $\mu\text{g l}^{-1}$ )	Limit of quantitation (Acc-Prec $< 20\%$ ) ( $\mu\text{g l}^{-1}$ )
8,9-diolBaA	0.4	63 ( $\pm 14$ )	112.6	3	8	0.9999	0.06	0.02
	2.0	72 ( $\pm 3$ )	104.7	1	5			
3-OHBaA	0.4	71 ( $\pm 2$ )	106.1	3	6	0.9999	0.02	0.02
	2.0	71 ( $\pm 3$ )	100.1	2	4			
1,2-diolChr	0.4	86 ( $\pm 17$ )	109.3	14	25	0.9999	0.18	0.20
	2.0	82 ( $\pm 9$ )	105.4	4	9			
6-OHChr	0.4	71 ( $\pm 1$ )	94.7	2	8	0.9997	0.03	0.02
	2.0	74 ( $\pm 2$ )	99.7	1	4			
3-OHChr	0.4	70 ( $\pm 2$ )	103.9	2	5	0.9998	0.03	0.02
	2.0	71 ( $\pm 2$ )	98.4	2	4			
4,5-diolBeP	0.4	68 ( $\pm 9$ )	111.7	3	13	0.9998	0.18	0.20
	2.0	73 ( $\pm 3$ )	102.3	2	5			
3-OHBeP	0.4	69 ( $\pm 6$ )	93.4	4	9	0.9999	0.01	0.02
	2.0	72 ( $\pm 3$ )	100.3	1	4			
8,9-diolBkF	0.4	61 ( $\pm 22$ )	108.3	7	10	0.9998	0.06	0.08
	2.0	76 ( $\pm 5$ )	103.9	3	7			
8-OHBkF	0.4	70 ( $\pm 3$ )	102.9	3	6	0.9995	0.005	0.01
	2.0	70 ( $\pm 2$ )	99.3	1	2			
9-OHBkF	0.4	69 ( $\pm 5$ )	97.7	3	6	0.9989	0.01	0.01
	2.0	67 ( $\pm 3$ )	97.4	3	6			
10-OHBbF	0.4	69 ( $\pm 6$ )	102.3	2	4	0.9990	0.01	0.02
	2.0	69 ( $\pm 3$ )	99.4	2	6			

The analyte recoveries by SPE were between 61 and 86%, with the majority of analytes exhibiting recoveries  $\geq 70\%$  at both low and high levels.

The current LOQs were comparable with those previously reported for multi-analyte determination of OH-PAHs by GC coupled with low-resolution mass spectrometry [18, 32]. However, the worse results for metabolites of high-molecular-mass PAHs (6-OHChr, 3-OHBaP) were explained by their high boiling points requesting higher temperatures and resulting in longer retention times and broader chromatographic peaks [18]. Such conditions probably resulted in column bleeding and contributed to the lowering of the signal to noise ratio, with an obvious decrease of analytical performance. Analytical techniques using high-resolution mass spectrometry (HR-MS) logically reported lower LOQs, ranging from 0.001 to 0.01  $\mu\text{g l}^{-1}$  [17, 28]. To the best of our knowledge, there are no studies reporting LOQs for urinary 4-/5-ring diol-PAHs, but the sensitivity of the present method was similar to that for OH-PAHs. The worse intra-/interday precision obtained for 1,2-diolChr at 0.4  $\mu\text{g l}^{-1}$  could be explained by the fact that this concentration was close to the LOQ of this compound (0.2  $\mu\text{g l}^{-1}$ ).

Urinary BaP metabolites were not included in the current analytical method due to the other existing methods already developed in our laboratory with much higher sensitivities: HPLC fluorescence for 3-OHBaP (LOQ, 0.05  $\text{ng l}^{-1}$ ) [37] and GC-NCI-MS-MS for tetraol-BaP (LOQ, 0.02  $\text{ng l}^{-1}$ ) [38].

One of the limits of our study lies in the potential coelution of other PAH metabolites sharing the same retention times and mass fragments in GC-MS-MS. Indeed, other authors showed coelution and common quantification/confirmation MS transitions for 3-OHBaA/9-OHBaA, 6-OHChr/11-OHBaA, 8-OHBkF/12-OHBkF, and 9-OHBkF/7-OHBaP. In our study, 8-OHBkF/9-OHBkF and 10-OHBbF were never detected, whereas the levels of the two remaining compounds (3-OHBaA and 6-OHChr) may potentially be overestimated and reflect [3-OHBaA+9-OHBaA] and [6-OHChr+11-OHBaA] concentrations, respectively. The compounds in the first set (3 + 9-OHBaA) both conveniently refer to the same parent compound (BaA), whereas the compounds of the second set are more of concern when referring to different parent compounds. Grova et al. [39] found that rats orally exposed to BaA did not eliminate 11-OHBaA at detectable levels, ensuring that 6-OHChr levels were not interfered with by 11-OHBaA in their study. This lack of specificity of our method for some metabolites has to be kept in mind when using it for risk assessment purposes.

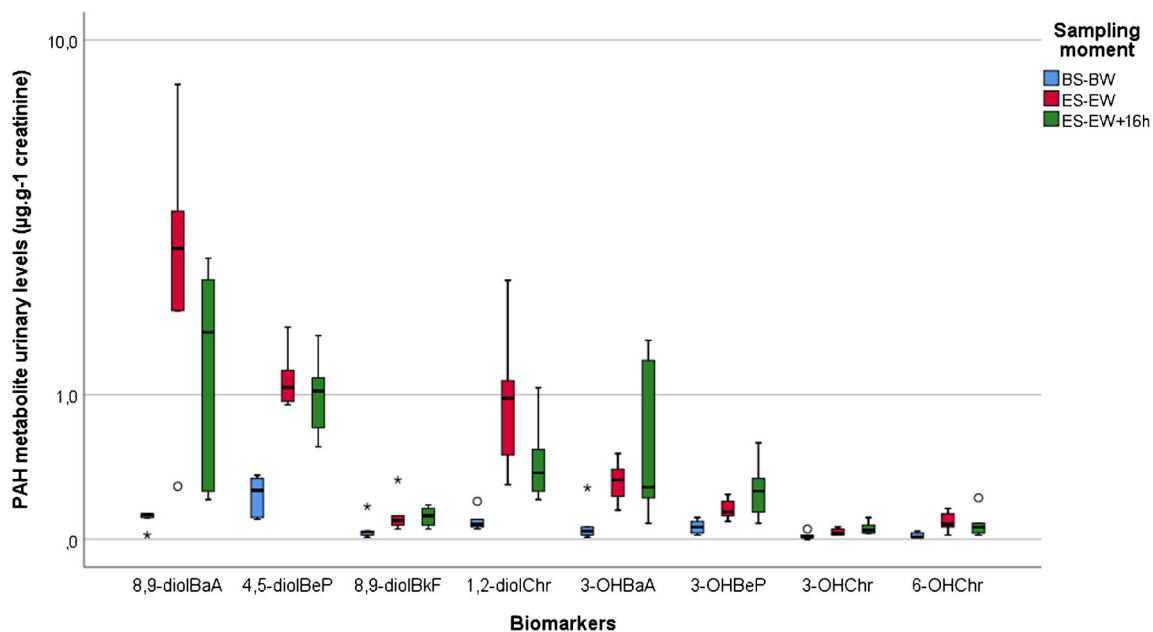
### Application in urine of workers

The urinary levels of OH-PAHs and diol-PAHs of workers involved in aluminum production are presented in Fig. 4.

Three compounds (10-OHBbF, 8-OHBkF, and 9-OHBkF) were systematically undetectable ( $< 0.01 \mu\text{g l}^{-1}$ ). Eight others were detected in the majority of urine samples, including 4 diol-PAHs (8,9-diolBaA, 1,2-diolChr, 4,5-diolBeP, 8,9-diolBkF) and 4 OH-PAHs (3-OHBaA, 3-OHBaP, 3-OHChr, 6-OHChr). While only 59% of metabolite levels were below the LOQ for urine samples collected before the working week (BS-BW), they were almost all detectable at the end of the week (90% and 86% for urine collected at EW-ES and EW-ES + 16 h, respectively). 3-OHBaA and 3-OHBaP were always detected in the urine of workers regardless of the sampling time. At the end of the working week, the levels of diol-PAH metabolites were more abundant than those of OH-PAHs, regardless of the PAH compound. The mean (GM) ES-EW levels of diol-PAHs ranged from 0.86 to 2.34  $\mu\text{g g}^{-1}$  creatinine (except for 8,9-diolBkF, 0.1  $\mu\text{g g}^{-1}$ ), whereas those of OH-PAHs ranged from 0.03 to 0.30  $\mu\text{g g}^{-1}$  creatinine. Sixteen hours after the end of the shift, 8,9-diolBaA and 1,2-diolChr concentrations were found to be significantly lower than those at ES-EW ( $p = 0.03$ ), whereas similar levels were observed for other metabolites, revealing different elimination patterns depending on the considered biomarker. No correlations were found between the levels of diol-PAHs and OH-PAHs for the considered biomarkers. However, high interindividual variabilities were observed, with average relative standard deviations (RSDs) of 67% (range, 30–132%) and 89% (range, 38–234%) at ES-EW and ES-EW + 16 h, respectively. This high between-worker variability of metabolic pathways involving carcinogenic PAHs is toxicologically relevant as it may reflect different susceptibilities for developing cancer.

Urinary levels of > 4-ring PAH metabolites are 1000 to 10,000 times lower than those of < 4-ring PAH metabolites due to a major elimination in feces of high-molecular-weight metabolites [40]. These very low levels require highly sensitive analytical methods, explaining undetectable levels of 6-OHChr/3-OHBaP observed in the urine of coke oven workers by others (LOQ = 1–1.4  $\mu\text{g l}^{-1}$ ) [18]. Despite much lower LODs (2–10  $\text{ng l}^{-1}$ ), less than 5% of urine samples had detectable levels of OH-Chr and OH-BaA in the NHANES cohort (2748 participants from the general US population) [41]. More recently, Lankova measured detectable levels ( $> 0.01 \mu\text{g l}^{-1}$ ) of 2–4-ring PAH (Naph, Fluo, Phe, Pyr) metabolites in > 70% of urine samples from women living in a highly polluted industrial agglomeration [21].

For diol-PAHs, very few data are available, and only 2-/3-ring compounds have been studied. Among 97 highly PAH-exposed workers, urinary diol metabolites of phenanthrene were quantified [23]. The average levels of 1,2-diol-Phe, 3,4-diol-Phe, and 9,10-diol-Phe were 7.3, 0.3, and 4.8  $\mu\text{g g}^{-1}$  creatinine, respectively. More recently, Luo et al. determined that urinary levels of 1,2-diol-Phe and 3,4-diol-Phe were detected in controls in the range of 0.2–2  $\text{nmol/6 h}$



**Fig. 4** PAH metabolites ( $\mu\text{g/g}$  creatinine) measured in BSBW (blue), ESEW (red), and BSEW16 (green) urine samples from 6 male aluminum plant workers occupationally exposed to PAH

urine, with the influence of cigarette smoking on the regioselective metabolism of Phe metabolites [15].

To our knowledge, this is the first time that the diol-PAH metabolites of BaA, BeP, BkF, and Chr were measured in the urine of workers and compared with the levels of OH-PAH metabolites. At the end of the working week, the highest levels were found for 8,9-diol-BaA, with concentrations twice as high as those of 1,2-diol-Chr and 4,5-diol-BeP and five times higher than those of 3-OH-BaA and 3-OH-BeP. The lowest levels were found for 3-OH-Chr and 6-OH-Chr, while those of 10-OH-BbF/8-OH-BkF and 9-OH-BkF remained undetectable.

In a study on rats orally exposed to a mixture of PAHs, Grova et al. [39] found strong linear dose versus urinary concentration relationships for several OH-PAHs, with correlations ranging from  $R^2 = 0.56$  to  $R^2 = 0.82$ . 3-OHChr and 6-OHChr were identified as the most suitable metabolites to assess chrysene exposure, and 8-OHBkF/11-OHBbF were also assumed to be relevant biomarkers of exposure to BkF and BbF. Among the tested metabolites, some were detected at the lowest levels of exposure (3-OHChr, 8-OHBkF, 11-OH-BbF), whereas others remained undetectable at the highest exposure dose (10-OHBbF). Our results are in accordance with the results of Grova et al. for OH-PAHs, with 3-OHChr/6-OHChr and 3-OHBaA being systematically detected in the urine of recruited workers. In addition, we also detected four diol-PAHs that were not studied elsewhere. In our study, the selection of the tested metabolites resulted from a literature review on the toxicity and detoxification pathways of each PAH, aiming at selecting at least one metabolite of the

toxicity pathway and one of the detoxification pathways for each compound.

Even if both results cannot be compared due to interspecies variabilities in metabolic pathways, they provided evidence that these metabolites are of interest to reflect exposure to their parent compounds. All the metabolites tested in the Grova study required more than 12 h to be entirely excreted, and the majority of them presented excretion peaks at 6–8 h following the last exposure. This is in accordance with our results, where no significant differences were observed between ES-EW and ES-EW + 16 h for a majority of metabolites, indicating elimination half-lives of several hours.

## Conclusions

This analytical method allowed the simultaneous determination of the monohydroxylated and dihydroxylated metabolites of 4- and 5-ring PAHs in urine. Different extraction techniques (LLE, dSPE, SPE) were compared, and SPE C18 was selected to achieve satisfactory recovery rates and low backgrounds. A two-stage derivatization approach including the initial derivatization of OH-PAHs by MTBSTFA followed by derivatization of diol-PAHs by BSTFA was developed and validated in a single run. After optimizing the GC injection parameters through an experimental design procedure, the final method allowed eleven compounds to be quantified at very low levels (LOQs ranging from 0.01–0.02  $\mu\text{g l}^{-1}$  for OH-PAHs to 0.02–0.2  $\mu\text{g l}^{-1}$  for diol-PAHs). In aluminum workers, the urinary levels of diol-PAHs were higher than

those of OH-PAHs at the end of the working week. This analytical technique will be helpful to further characterize the patterns of metabolic pathways resulting from occupational exposures to carcinogenic PAHs and to reflect the bioactivation and detoxification pathways of high-molecular-weight PAHs, including a “bay region.” The next step will be to add the determination of the hydroxylated metabolites of 2- and 3-ring PAHs to the current analytical method.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00216-021-03638-4>.

**Author contribution** Marie Marques and Renaud Persoons contributed to the analysis and interpretation of the results. All authors listed have made substantial, direct, and intellectual contributions to the work and approved it for publication.

**Funding** This study was funded by the French sanitary agency ANSES (Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail), Project n° 2014-1-176.

**Data availability** The raw data supporting the conclusions of this article will be made available by the authors within respect of General Data Protection Regulation.

## Declarations

**Conflict of interest** The authors declare no competing interests.

**Informed consent** Informed consent was obtained from all participants for inclusion in the study.

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