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Gas chromatography–electron-capture detection of urinary methylhippuric acid isomers as biomarkers of environmental exposure to xylene

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Abstract

Methylhippuric acid isomers (MHAs), urinary metabolites of xylenes, were determined, after clean-up by C18-SPE and esterification with hexafluoroisopropanol and diisopropylcarbodiimide, by GC with ECD detection, on an SPB-35 capillary column (30 m, 0.32 mm I.D., 0.25 μm film thickness, $\beta=320$). S-benzyl-mercapturic acid was used for internal standardization. Chromatographic conditions were: oven temperature 162°C, for 14.2 min; ramp by 30°C/min to 190°C, for 3.5 min; ramp by 30°C/min to 250°C, for 4 min; helium flow rate: 1.7 ml/min; detector and injector temperature: 300°C. The sample (1 μl) was injected with a split injection technique (split ratio 5:1). MHA recovery was >95% in the 0.5–20 μmol/l range; the limit of detection was <0.25 μmol/l; day-to-day precision, at 2 μmol/l, was Cv<10%. Urinary MHAs were determined in subjects exposed to different low-level sources of xylenes: (a) tobacco smoking habit and (b) BTX urban air pollution (airborne xylene ranging from 0.1 to 3.7 μmol/m^3). Study (a) showed a significant difference between urinary MHA median excretion values of nonsmokers and smokers (4.6 μmol/l vs. 8.1 μmol/l, $p<0.001$). Study (b) revealed a significant difference between indoor workers and outdoor workers (4.3 μmol/l vs. 6.9 μmol/l, $p<0.001$), and evidenced a relationship between MHAs (y, μmol/mmol creatinine) and airborne xylene (x, μmol/m^3) ($y=0.085+0.34x$; $r=0.82$, $p<0.001$, $n=56$). Proposed biomarkers could represent reliable tools to study very low-level exposure to aromatic hydrocarbons such as those observed in the urban pollution due to vehicular traffic or in indoor air quality evaluation.

Keywords: Xylene; Methylhippuric acid

1. Introduction

Xylene is an aromatic hydrocarbon which exists in three isomeric forms: *ortho-*, *meta-* and *para-*. Technical grade xylene contains a mixture of the three isomers. Approximately 92% of mixed xylenes is blended into petrol, the remainder is used in a variety of solvent applications, particularly in the paint and printing ink industries. Recently xylene became a ubiquitous environmental pollutant, mainly diffused in urban air because of motor vehicle emissions. It is also present in tobacco smoke, being generated by tobacco pyrolysis processes [1]. In humans main metabolic pathway of xylene detoxifi-

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cation proceeds through oxidation of a methyl group, leading to methylbenzoic acid which, after conjugation with glycine yielding methylhippuric acid (MHA), is excreted in urine [2]. Occupational health and safety authorities in most countries recommend a threshold limit value of exposure to airborne xylene of 100 ppm (435 mg/m^3, 5.3 mmol/m^3) in the working environment, and it is included on the Priority Substances List to be assessed under the Canadian Environmental Protection Act [3,4]. The biological monitoring of occupational exposure to xylene has been traditionally performed by means of the measurement of urinary excretion of methylhippuric acid [5]. The improvement of industrial working conditions resulting in lower airborne concentrations, together with the increasing concern for possible health risks posed to the general population by poor quality of urban air, have generated recent interest for the development of sensitive and specific analytical methods for urinary methylhippuric acid determination. These metabolites show the biological specificity required to be successfully applied as a biomarker of xylene exposure, but the available HPLC methods lack the enhanced analytical sensitivity necessary for application in environmental very low-level exposure monitoring (typically, in urban air xylene values range from 0.1 to 3.7 μmol/m^3) [6–10].

In this report, a gas-chromatographic method with ECD detection for the quantification of *o-, m-, p*-methylhippuric acids (derivatized as hexafluoroisopropyl ester) in human urine extracts is described. Validation of the method was obtained by its application to biological monitoring of subjects exposed to different low-level sources of xylene: tobacco smoking habit and urban air pollution.

2. Experimental

2.1. Materials

o-, m-, p-Methylhippuric acid isomers (*o*-MHA, *m*-MHA, *p*-MHA) and S-benzyl-mercapturic acid (internal standard, IS) were obtained from Tokyo Kasei Kogyo (Tokyo, Japan); methanol, chloroform (stabilized with ethanol 1–2%, v/v), acetonitrile, heptane and hydrochloric acid (12 *M*) were purchased from C. Erba (Milano, Italy); hexafluoroisopropanol (HFIP) and diisopropylcarbodiimide (DIC) were from Aldrich (Milano, Italy); all chemicals were of analytical purity grade. Solid-phase octadecyl (Sep-pak C18) cartridges, packed with 360-mg filling, were obtained from Waters (Milano, Italy). Sylanized glass 2-ml vials were purchased from Aldrich (Milano, Italy).

HFIP ester standards were prepared by acid esterification with HFIP and DIC coupling in chloroform; the resulting esters were purified by silica column chromatography. Structures were verified by mass spectrometric fragmentation pattern.

2.1.1. Instrumentation

A 24-port vacuum manifold (Alltech, Milano, Italia) was used for solid-phase extraction (SPE). Gas-chromatographic separation was carried out using a Perkin Elmer 8600 instrument equipped with a ^{63}Ni electron capture detector and a split/splitless injector (Perkin Elmer, Monza, Italy). Analytes were separated with a 30 m×0.32 mm I.D. fused silica column with a 0.25-μm SPB-35 stationary phase [bonded phase, poly(35%-diphenyl–65%-dimethylsiloxane); β=320] (Supelco–Aldrich, Milano, Italy). Initial column temperature was maintained isothermally at 162°C for 14.2 min; the column temperature was then ramped to 190°C by 30°C/min, holding at 190°C for 3.5 min. Finally a clean-up step was applied ramping the temperature by 30°C/min until 250°C and holding it for 4 min. Helium column carrier gas flow rate was 1.7 ml/min and argon–methane (95:5) make-up gas flow rate was 60 ml/min. The injector and detector temperature was set at 300°C. The sample (1 μl) was injected with a split injection technique (split ratio 5:1).

2.1.2. Other methods: xylene environmental air analysis

The diffusion personal samplers were eluted with CS_2 and analysed by gas chromatography/FID after addition of internal standards (fluorobenzene and chlorobenzene). Apparatus: gas-chromatograph/FID 8300 Perkin Elmer; column: DB-5 J&W, 30 m length, 0.25 mm O.D., 1 μm film thickness. Operational conditions: splitless injection at 250°C; oven temperature: 40°C; initial time: 5 min; rate I: 10°C/min; temperature II: 165°C; rate II: 30°C/min; final

temperature 250°C; final time: 2 min. Carrier: He, flow rate 0.9 ml/min [11].

2.1.3. Study group

A group of 113 apparently healthy male workers (83 nonsmokers; 30 smokers, mean number of smoked cigarettes per day=17 cig/day; mean number of smoked cigarettes during the monitored time interval: 5 cig) exposed to BTX air pollution in a large town of Northern Italy was studied. All studied subjects were in the Urban Traffic Service: 51 worked as traffic wardens, while 62 did clerical activities. Information about usual confounding factors (age, alcohol and cigarette consumption, drug assumption, etc.) was recorded by anamnestic questionnaire.

Environmental monitoring of indoor/outdoor xylene pollution was done by means of passive diffusion personal samplers worn in the respiratory zone by each worker from the start of the workshift at 7:30 h to 12:30 h.

Spot urine samples were collected from each subject at 12:30 h (before lunch interval).

2.2. Procedure

2.2.1. Urine sample storage and processing

As soon as possible after spot sample collection, 1-ml aliquots were separated and stored in polyethylene tubes at −18°C until analysis. Before analysis, frozen samples were conditioned at 37°C for 15 min, with frequent stirring.

2.2.2. Standard solutions and calibration curves

Standard solutions of MHA isomers were prepared by individually dissolving the acids in methanol: concentrations were adjusted to 1.0 mmol/l (194 mg/l) for each acid. For calibration purposes, working standard solutions in urine in the range 0.5 to 40.0 μmol/l (92 to 7760 μg/l) of each of the three MHA isomers were prepared. For internal standardization a methanolic solution containing 1.0 mmol/l of S-benzyl-mercapturic acid (IS) was used. Urinary MHA concentrations were calculated by internal standardization, comparing the ratio of metabolite to IS integrated peak height counts obtained from unknown urine sample with that from standard calibration curve.

2.2.3. Analytical procedure

Organic acids were isolated from urine using solid-phase extraction (SPE). The C18 cartridges were preactivated with 3 ml of methanol and 4 ml of water and conditioned with 2 ml of 0.06 *M* HCl. Aliquots (1.0 ml) of urine, acidified with 50 μl of 12 M HCl and added with 5 μl of methanolic IS solution, were loaded under negative pressure onto the cartridge. The cartridge was washed in sequence with 2 ml of 0.06 *M* HCl and 2 ml of heptane. MHAs were then eluted with 2 ml of a mixture of chloroform–methanol (92:8, v/v). The aqueous upper layer was aspirated by means of a Pasteur glass pipette and discarded; the chloroform layer was collected and dried by addition of 50–80 mg of anhydrous magnesium sulphate. For derivatization, a 100-μl aliquot of eluant was then tranferred to a silanized 2-ml vial and evaporated at 35°C under N_2 stream, the residue was dissolved with 100 μl of chloroform and added with 15 μl of DIC and 10 μl of HFIP. The esterification reaction was driven for 10 min at room temperature, in a rotating shaker. The excess reagent was removed by evaporation at 35°C under N_2 stream and the derivatized residue reconstituted in 100 μl of acetonitrile for subsequent GC–ECD analysis.

3. Results

3.1. Chromatographic separation

Chromatographic profiles obtained from blank and spiked urines are shown in Fig. 1: Fig. 1A and B refer respectively to a blank urine and to the same urine spiked with 2.0 μmol/l (388 μg/l) each of *o-, m-, p*-methylhippuric acids; all samples contain 2.0 μmol/l of IS. All hexafluoroisopropyl esters of interest are baseline-resolved and elute as sharp, symmetrical peaks with half-height widths of 2–5 s depending on their retention times. The day-to-day precision of retention times, determined on 12 analytical series in 1 month, gave a relative standard deviation (RSD)<2%.

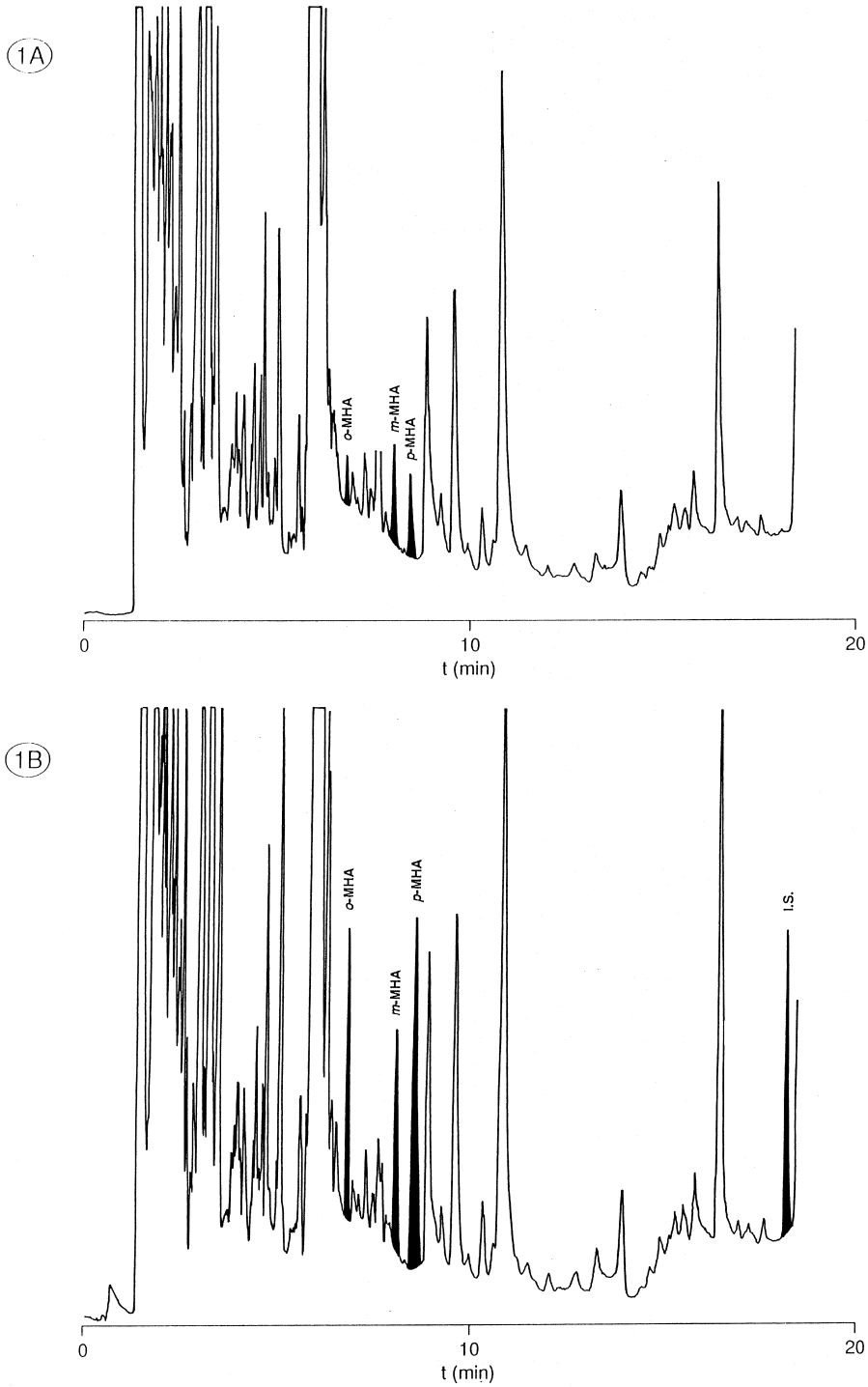

Fig. 1. Representative chromatograms of methylhippuric acids in urine: (A) unspiked urine from a subject exposed to xylene pollution in the urban environment (*o*-methylhippuric acid: 0.41 μmol/l; *m*-methylhippuric acid: 1.62 μmol/l; *p*-methylhippuric acid: 0.59 μmol/l); (B) the same urine enriched with 2 μmol/l of each isomer and IS.

Table 1
Calibration curve parameters for MHA isomers in urine

Metabolite	Concentration range (nmol/ml)	Slope (ml/nmol)	Intercept	Correlation (r)	Limit of detection (nmol/ml)
o-MHA	0.5÷5.0	7.54	14.55	0.983	0.24
m-MHA	0.5÷5.0	9.95	14.05	0.987	0.22
p -MHA	0.5÷5.0	12.01	7.97	0.991	0.25

3.2. Calibration, recovery, and reproducibility

Calibration curves were linear in the interval 0.5–20.0 μmol/l for all the studied metabolites; the limits of detection (LOD) were obtained from spiked urine calibration curves (four different added MHA isomer concentrations in the range 0.5–5.0 μmol/l, 5 determinations for each point) by use of the intercept (a) and standard error of its estimate SE(a) of the regression line for MHA concentration versus signal [12]. The limit of detection, calculated from $y=a+3SE(a)$, resulted in LOD$<$0.25 μmol/l for all the MHA isomers (Table 1). The recovery of the overall method was investigated by working up ten urine samples spiked with a mixture of the MHA isomers. To estimate the recovery, a standard mixture of all MHA isomers plus the internal standard was added to chloroform or urine. The standards were measured in biological samples with and without added MHAs (added amounts of each acid: 0, 1 and 10 μmol/l urine). The recovery was calculated from the ratio of standard in chloroform vs. the concentration in biological samples (after subtraction of blank values) based on five repeated experiments: observed recoveries were in all cases higher than 95%. Matrix-matched calibration curves were eventually preferred, because more variable results and lower percentage recoveries from SPE cartridges were observed with aqueous solutions.

Derivatization yield, calculated by comparison of the signal obtained by a known molar amount of MHAs made to react with DIC and HFIP in chloroform with that given by direct injection of the same absolute molar quantity of MHA-esters, also gave results higher than 90%. The repeatability (precision within a run of ca. 8 h) of the method was determined by analysis of 6 aliquots of a urine containing metabolism-derived MHA isomers (2.01±0.13 μmol/l of *o*-MHA, 1.13±0.08 μmol/l *m*-MHA and 2.04±0.11 μmol/l *p*-MHA) and 6 aliquots of the same urine enriched with 2 μmol/l of each MHA isomer. In addition, the reproducibility (between-day precision) between different assays during a period of 30 days was determined ($n=5$). The results, expressed as coefficients of variation (Cv%), are shown in Table 2.

Various storage conditions were examined to minimize the loss of urinary metabolites before instrumental analysis. Untreated urine 1ml-aliquots were stored at −18°C for 6 months, without significant modifications of metabolite concentrations; SPE eluate could be stored at 4°C for 2 weeks, or, when

Table 2
Precision in the GC–ECD analysis of 6 urine samples unspiked or spiked with 2 μmol/l of three MHA isomers

Metabolite	Concentration		Repeatability[a] (Cv%)	Reproducibility[a] (Cv%)
	(nmol/ml)	(ng/ml)		
o-MHA	2.00	388	6.5	7.2
o-MHA	4.08	791	5.1	5.9
m-MHA	1.13	219	6.9	7.6
m-MHA	3.14	609	4.9	6.5
p-MHA	2.04	395	5.0	6.4
p-MHA	4.42	857	4.5	5.9

[a] Given as relative standard deviation Cv%.

taken to dryness, it could be stored at 4°C for at least 60 days; the reaction mixture could be stored for up to 5 days at 4°C. Once the sample was ready for GC injection, analysis was performed as soon as possible, because a decrease of about 50% in signal intensity was observed after 24 h.

3.3. Field validation of the analytical method

Validation of the analytical procedure was obtained by its application to biological monitoring of subjects exposed to different environmental low-level sources of xylenes, such as (a) tobacco smoke and (b) BTX urban air pollution.

In study (a) the influence of tobacco smoking habit on MHA urinary levels of 62 indoor white-collar workers was considered. Detectable amounts of MHA isomers were found in all analyzed samples: *m*-MHA was the major metabolite, representing 50% of all excreted MHAs; *o*-MHA and *p*-MHA accounted respectively for about 30% and 24%. Results are summarized in Table 3. A statistically highly significant relationship was evidenced among excretion values of the three isomers (multiple regression analysis, $r^2=0.86$, $p<0.001$, $n=62$). When comparison was made between smoker and nonsmoker results, it appeared that smoker MHA median excretion was significantly increased (nearly doubled) over that of nonsmokers ($p<0.001$) (Fig. 2).

Study (b) was intended to evaluate the possibility of using urinary MHAs to assess exposure to xylene derived from urban air pollution: MHA excretion was investigated in nonsmoker subjects ($n=83$) grouped according to their indoor ($n=45$) or outdoor

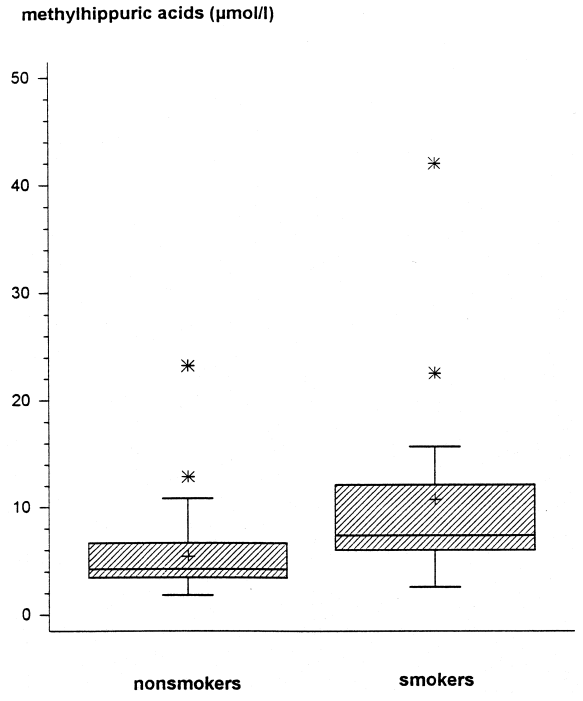

Fig. 2. Concentrations of total methylhippuric acids (calculated as the sum of *o*-, *m*- and *p*-isomers, µmol/l), determined in urine samples collected at 12:30 h, in subjects classified according to cigarette smoking (nonsmokers $n=45$, smokers $n=17$). The plotted data are divided into four areas of frequency. The box encloses the middle 50%. The horizontal line inside the box represents the median. The lower vertical line extends from the first quartile to the smallest data point within 1.5 interquartile ranges from the first quartile, the other whisker extends from the third quartile to the largest data point within 1.5 interquartile ranges from the third quartile. Data values that fall beyond the whiskers but within 3 interquartile ranges (suspected outliers, *) are plotted as individual points. Mean values are indicated by the + symbol.

Table 3
Urinary concentration of *o*-methylhippuric, *m*-methylhippuric and *p*-methylhippuric, and total methylhippuric acids (calculated as the sum of the 3 isomers), expressed in µmol/l, observed in urine samples obtained at 12:30 h, before lunch, from indoor subjects, classified as nonsmokers and smokers

Metabolite (µmol/l)	Nonsmokers						Smokers					
	mean	SD	median	min	max	*n*	mean	SD	median	min	max	*n*
o-Methylhippuric acid	1.7	0.9	1.4	0.5	5.3	45	2.4	1.6	2.0	1.0	6.8	17
m-Methylhippuric acid	2.5	2.0	2.0	0.5	12.3	45	6.0	5.3	4.1	0.7	26.0	17
p-Methylhippuric acid	1.2	0.8	1.0	0.5	5.0	45	2.3	2.0	1.7	0.8	9.1	17
Total methylhippuric acids	4.7	3.7	4.3	1.8	23.2	45	10.7	9.5	8.1	2.5	42.0	17

SD=standard deviation; min=minimum value; max=maximum value; *n*=number of subjects.

(n=38) working environment. The comparison between MHA median values of office vs. street workers showed statistically significant differences for all three isomers ($p<0.001$), with outdoor workers excreting about a twofold amount of MHA more than indoor ones (Table 4). Moreover a statistically significant correlation was evidenced between urinary excretion of MHAs (y, μmol/mmol creatinine) and xylene environmental concentrations (x, μmol/m^3) ($y=0.085+0.34x$; $r=0.82$, $p<0.001$, $n=56$) (Fig. 3).

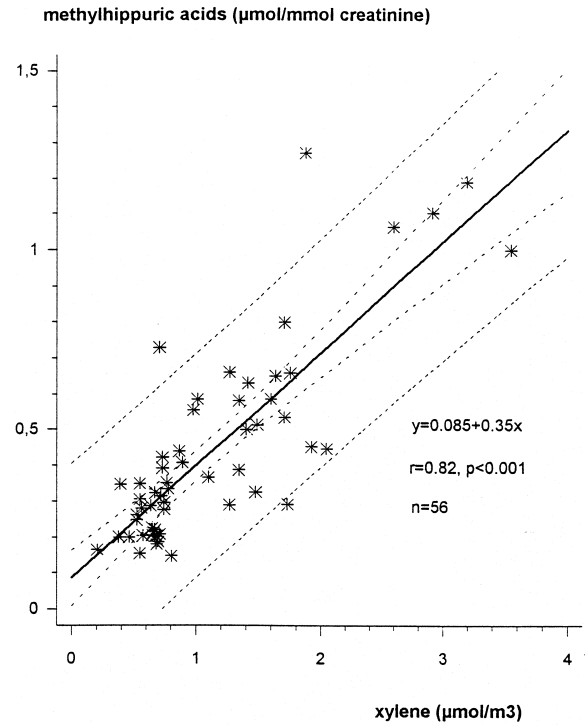

Fig. 3. Relationship observed between airborne xylene concentration (μmol/m^3) and total methylhippuric acids (μmol/mmol creatinine) in nonsmoker subjects.

4. Discussion

4.1. Work-up procedure

Numerous papers have been published on work-up methods for carboxylic acids in biological samples. In the past liquid–liquid extraction techniques have been frequently used; however, they often give insufficient clean-up and are generally laborious when many samples need to be treated. More recently the use of SPE columns has proved to be successful for isolation and recovery of trace organic compounds in complicated matrices and SPE is currently being used as an effective alternative to other extractive techniques for biological samples. The SPE protocol here proposed is a very efficient procedure that gave quantitative recovery of the organic acids at the low concentrations of interest, allowing shorter work-up times and decreased organic solvent consumption relative to liquid–liquid extraction; besides, SPE overcame the potential for emulsion that is a problem in any solvent extraction procedure requiring agitation of samples. The SPE cartridges could be repeatedly recycled without any noticeable change in performance (at least 3 times, after being regenerated by eluting through 3 ml of methanol and 4 ml of water) thus contributing to lower the analysis cost.

In the optimization of the procedure, different SPE cartridge fillings [trimethylaminopropyl (SAX), phenyl, octyl, C18] were tested, with best results

Table 4
Urinary concentration of *o*-methylhippuric, *m*-methylhippuric, *p*-methylhippuric, and total methylhippuric acids (calculated as the sum of the 3 isomers), expressed in μmol/l, observed in urine samples obtained at 12:30 h, after 5-h workshift, from nonsmoker subjects, classified according to their working environment: indoor=white-collar office workers, outdoor=street workers

Metabolites (μmol/l)	Indoor						Outdoor					
	mean	SD	median	min	max	*n*	mean	SD	median	min	max	*n*
o-Methylhippuric acid	1.7	0.9	1.4	0.5	5.3	45	2.5	1.4	2.1	0.8	7.1	37
m-Methylhippuric acid	2.5	3.9	2.0	0.5	12.3	45	3.7	2.3	2.9	0.6	9.8	38
p-Methylhippuric acid	1.2	0.8	1.0	0.5	5.0	45	1.8	0.9	1.5	0.6	4.1	38
Total methylhippuric acids	4.7	3.7	4.3	1.8	23.2	45	8.1	4.4	6.9	3.0	19.5	37
Airborne xylene (μmol/m^3)	0.6	0.2	0.5	0.2	1.2	45	1.4	0.7	1.3	0.4	3.1	37

SD=standard deviation; GM=geometric mean; min=minimum value; max=maximum value; n=number of subjects.

obtained by use of C18. The main losses were found during the washing step and the method was therefore optimized by testing various washing solutions. HCl solutions (0.06 *M*) with concentrations of methanol in the range 0–30% were tried, but an increasing loss was found for all concentrations of methanol added compared to pure 0.06 *M* HCl. Several buffer solutions were also tried such as phosphate buffer and Tris buffer in a pH range 3–7 and 0–10% additions of methanol. However, all buffers gave losses and it was concluded that best results were those obtainable with 0.06 *M* HCl without any addition of methanol. Great consideration was also payed to the choice of solvents for SPE elution with the aim of minimizing elution of interferents: chloroform–methanol mixture was eventually preferred, because it gave quantitative recovery of the metabolites of interest together with reduced background.

The use of coupled SAX– and C18–SPE was also investigated, but this technique did not result in improved purification of samples; it was more laborious and decreased recoveries of MHAs were obtained compared with the C18 cartridges alone.

4.2. Derivatization

A general esterification procedure suitable for simultaneous derivatization and measurement of metabolites of aromatic hydrocarbons, all of which are carboxylic acids, was sought. GC–ECD is known to be a very sensitive technique and thus a derivatization procedure that allows the formation of volatile esters of MHAs employing a derivatization agent which itself contains the ECD detectable function seemed very interesting. The carbodiimide-coupled esterification of carboxylic acids is a well known reaction that has been done under a variety of conditions; but although HFIP and DIC have been used to esterify amino acids for in vitro peptide synthesis, there appears to be only few references to the use of these two reagents for the purpose of trace analysis, and no reference is available for application to biological samples [13]. HFIP has here demonstrated to be an excellent derivatization reagent for carboxylic acids: its volatility (b.p. 59°C) facilitates removal, it is commercially available at reasonable cost and in high purity grade. DIC has the advantage, in respect to other carbodiimides, of being a liquid, easily handled by syringe and soluble in all the solvents used. Besides, the DIC-coupled esterification of MHAs occurs very rapidly at room temperature, The HFIP esters are stable to column chromatography and the six fluorine atoms of the alcohol moiety confer to the derivative good ECD sensitivity. In the considered instance, chloroform was shown to give optimized reaction conditions in comparison with other experimented solvents such as heptane, isooctane, cyclohexane, toluene, dichloromethane and ethylacetate. Lastly, the absence of clean-up procedures after the esterification step further on reduced the probability of analyte losses. Acetonitrile was shown to be the most efficient solvent for residue dissolution after the esterification step. An intermediate polarity column with a thin film thickness was used for the analysis because of the lower elution temperature and relatively shorter analysis time achieved. Thousands of injections have been made without any observed column aberrations.

4.3. Analytical precision

Internal standardization had a relevant role in determining the sufficient precision of the proposed analytical procedure. However, because S-benzyl-mercapturic acid is known to be a minor metabolite of toluene recently identified in urine of occupationally exposed workers, its use as internal standard could perhaps appear questionable. In fact, before resolving to adopt this chemical as IS, many different organic acids were tried, but, due to the complexity of urine GC elution profile, S-benzyl-mercapturic acid was the only one running in a free zone of the chromatogram. Its choice was also supported by two observations: first, during the search for an IS, more than 200 specimens from environmentally exposed subjects were analyzed and only in a few cases (less than 10%) trace concentrations of possibly S-benzyl-mercapturic acid were observed, equivalent to concentrations lower than 0.15 μmol/l; secondly, after S-benzyl-mercapturic acid was finally adopted as IS, we took controlling peak heights of IS in samples

analyzed over a 6 month-period of time, and a low dispersion of values was observed (Cv<15%, n=158), pointing out reduced interference from ''physiological'' S-benzyl-mercapturic acid on results. Anyway, when occupational exposure to toluene can not be ruled out and the presence of S-benzyl-mercapturic acid in urine is suspected, the choice of a different IS should be considered.

4.4. Analytical specificity

To check proper identification of urinary metabolites, more than a hundred urine samples were analyzed either by the proposed procedure and on a nonpolar SPB-1 capillary column: comparable results were in general obtained ($y=0.06+0.92x$, $r=0.89$, $p<0.01$), with some notable exceptions that on SPB-1 gave *o*-MHA results up to 10 times higher than on SPB-35. Visual inspection of *o*-MHA peak shapes from SPB-1 showed enlargement and asymmetry of the peak, suggesting the presence of coeluting interferents; this consideration, while not fully exhaustive, seems to indicate that adopted analytical conditions confer improved specificity to the procedure. Independent confirmations of adequate analytical specificity could also be derived from results of our field study: it appears quite doubtful that, being metabolites not correctly identified, significant correlations among the three isomers or between environmental xylene concentration and urinary excretion of MHAs could be casually found; these observations should thus verify the good quality of metabolite identification.

Available analytical methods were generally devised for the measure of exposure to xylene in occupational environment and, due to their elevated values of detection limit, have limited applications for the biological monitoring of urban environmental pollution. The lowest published LOD for urine MHA is 1 μmol/l [16]: in the present study 50% of nonsmoker results are below such a limit. The proposed method, showing a detection limit of 0.25 μmol/l (about 50 mcg/l) for each methylhippuric acid isomer in urine samples, allowed quantification of MHAs in all studied samples and proved to be reproducible and robust in its application.

4.5. Study group results

A bibliographic search in the literature from 1972 to 1998 revealed only few reports on urinary MHA excretion in non occupationally exposed subjects. MHA values here observed for subjects exposed to urban airborne pollution are in agreement with those found by other authors, who reported mean excretion values from 0.25μmol/l for *p*-MHA up to 100 μmol/l for *o*-MHA [14–16]. The reliability of the proposed analytical method for the biological monitoring of low-level xylene exposure is confirmed by the results obtained from the studied groups: the biological sensitivity of the biomarker allowed evidencing of a statistically significant difference in MHA excretion between smokers and nonsmokers and an increased excretion in outdoor with respect to indoor workers. Moreover, it revealed the presence of a significant relationship between low-level airborne xylene concentrations (ranging from 0.1 to 3.7 $\mu mol/m^3$) and urinary excretion of MHAs. Also the specificity of the biomarker appears satisfying: in fact the narrow distribution of urinary values observed in nonsmokers witnesses limited interferences from physiological, alimentary or voluptuary variables on urinary excretion of xylene metabolites.

The proposed method could thus represent a reliable tool that allows the study of low-level exposure to aromatic hydrocarbon such as that observed in the urban pollution due to vehicular traffic or indoor air quality evaluation.

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