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Determination of urinary *ortho*- and *meta*-cresol in humans by headspace SPME gas chromatography/mass spectrometry

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Abstract

ortho-Cresol (*o*-C) and *meta*-cresol (*m*-C) are minor urinary metabolites of toluene, a widely used chemical with neurotoxicological properties. A new assay for their determination in human urine is here proposed. Urinary cresol sulphates and glucuronates are submitted to acid hydrolysis, urine is neutralized, added with *o*-cresols-d_8, and analytes are sampled in the headspace of urine by SPME using a polydimethylsiloxane fiber. Analysis is performed by GC/MS using, for separation, either a SupelcoWax10 (for *o*-C) or a chiral CP Cresol (for *o*-C and *m*-C) column. The method is very specific, with a range of linearity 0–5.0 mg/l, within- and between-run precision, as coefficient of variation, <15% and <19%, limit of detection of 0.006 mg/l for *o*-C and 0.007 mg/l for *m*-C. The procedure is applied to the quantification of cresols in urine from workers exposed to toluene and from subjects belonging to the general population.

Keywords: Urinary cresols; Biological monitoring; Occupational exposure to toluene

1. Introduction

Toluene is a very common chemical, which is widely used as a solvent in paints, coatings and many consumer products, as a synthetic intermediate and as an additive in fuel to increase gasoline octane rating [1].

Humans may experience toluene exposure in working settings and in living environments, being toluene a component of cigarette smoke, auto vehicular exhausted fumes, gasoline and various commodities vapours.

Due to its toxicity to the central nervous system, toluene exposure is regulated in many countries. An airborne concentration of 50 ppm (188 mg/m^3) as occupational exposure limit during the work shift is recommended, suggested or established by industrial hygiene associations and/or governmental agencies in different countries [2–5].

Following exposure in humans, toluene is readily transformed into several metabolites, among which the three isomers *ortho*-cresol (*o*-C), *meta*-cresol (*m*-C) and *para*-cresol (*p*-C). These compounds are excreted in urine as glucuronate and sulfate conjugates, accounting for few percent of the inhaled dose [6,7].

For the biological monitoring of occupational exposure to toluene, *o*-C in urine is adopted as determinant both in the biological exposure indices (BEI) list by the American Conference of Governmental Industrial Hygienists (ACGIH) [2] and in the biological tolerance values for occupational exposures (BAT) list by the German Ministry for Employment and Social Affairs (AGS Committee for Hazardous Substances) [3]. A value of 0.5 mg/l for samples collected at the end of the shift is suggested as a BEI, and a BAT of 3.0 mg/l for samples collected at the end of the shift, after a long-term exposure, is listed [2,3]. The "B" notation reported by ACGIH indicates that *o*-C is usually present in a significant amount in biological specimens collected from subjects who have not been occupationally exposed [2].

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The use of urinary *m*-C for the same purpose, has never been investigated. On the contrary, the use of urinary *p*-C is precluded by the large amount of this chemical found in urine of all subjects, regardless the occupational exposure to toluene [7,8].

Several analytical procedures for the determination of urinary *o*-C have been published so far, some of which, by means of a separation performed with either mobile or stationary chiral phase, are suitable also for *m*-C [7–14]. Sample preparation is always required and usually performed, after hydrolysis of conjugates, by extraction of free cresols from urine using organic solvents [7–10,12,15–21], vapour stream distillation [11,22,23] or solid phase using C18 reverse phase silica [24]. Direct headspace sampling of free cresols present in biological fluids following accidental or intentional ingestion is also proposed [14]. While the solvent extraction procedure, due to the high solubility of cresols in water, may be scarcely efficient, the vapour stream distillation needs large sample volume and may be poorly precise and/or affected by loss of analyte due to the use of relatively large distillation apparatus. Techniques such as HPLC and GC are applied for the separation of cresols from each other and/or from other chemicals, and UV, FID detectors are traditionally used for their quantification. The introduction of derivatization with fluorinated or fluorescent chemicals and consequent detection with ECD [7] or fluorimeter [12], as well as the use of MS detector [13,23], have lowered the detection limit of the analytical techniques to few μg/l, allowing the determination of urinary cresols also in non occupationally exposed subjects.

In this work, an improved analytical assay suitable for the determination of a low level of cresols in human urine is presented. This assay is based on the volatility of free cresols that, after hydrolysis of their conjugates, are sampled in the headspace (HS) of urine by SPME and directly injected onto a proper capillary column for separation. The assay uses a small amount of specimen, performs all steps in one vial, introduces an internal standard, avoids the use of solvents and largely reduces the manual work, extracting and injecting the analytes in the chromatographic system automatically. Analysis, performed by GC/MS, ensures good and long lasting performance in separation and high specificity in the identification of the analytes. Two capillary chromatographic columns are proposed: the first to achieve a fast separation of *o*-C, the second, with a chiral stationary phase, to separate both *o*-C and *m*-C. The application of the assay to the determination of *o*-C and *m*-C in rotogravure printing workers and in subjects from the general population is reported.

2. Experimental

2.1. General

2.1.1. Chemicals and standards preparation

o-C (99%+) (Sigma–Aldrich), *m*-C (99%) and *p*-C (99%) (Fluka) were used for standard preparation. *o*-Cresol-d_8 (98 at.% D, Sigma–Aldrich) was used as internal standard. Hydrochloric acid aqueous solution (HCl, 37%, w/w), sodium hydroxide (NaOH), sodium chloride (NaCl), sodium acetate and acetic acid and methanol were purchased at a purification grade of 99% or higher from Carlo Erba Reagenti. Acetic acid and sodium acetate were used to prepare a 1 M buffer solution at pH 4.6 in water. Phenol-β-D-glucuronide (97%) (Sigma–Aldrich) was used to evaluate the efficiency of acid hydrolysis.

The standard solutions of *o*-C, *m*-C for the calibration curve were prepared at the concentration of 5.0, 2.5, 1.2, 0.6, 0.12, 0.06, 0.012 and 0.006 mg/l of each chemical in urine. An unspiked sample of the same urine was kept as blank. The urine used for the preparation of these solutions was a pool of urine from non-smoking donors without occupational exposure to toluene. The solution of *o*-cresol-d_8 to be used as internal standard was prepared at the concentration of 5.63 mg/l in water. The standard solutions of cresols and the internal standard solution were divided into small portions (about 1.2 ml) and stored in the dark at −20 °C.

2.2. Equipment

Gas tight vessels constituted by 2 ml autosampler glass vials sealed with screw open-top closures and silicone-polyperfluoroethylene gaskets (Kimble) were used for sample preparation and analysis.

The SPME devise and fibers with the following coatings: polydimethylsiloxane with 100 μm film thickness (PDMS); polydimethylsiloxane/divinylbenzene with 65 μm film thickness (PDMS/DVB); carbowax/divinylbenzene with 65 μm film thickness (CW/DVB) and polyacrylate with 85 μm film thickness (PA), were used for sampling analytes in the sample headspace (Supelco).

A 5890 II series gas chromatograph system, interfaced with a 5972 mass selective detector operating in the electron impact (EI, 70 eV) mode was obtained from Agilent. Injections were performed using the SPME devise, by a 8200 CX series autosampler (Varian) with agitation system. The split/splitless injector, operating in the splitless mode, was equipped with an inlet liner for SPME (i.d. 0.75 mm, Supelco). The GC was equipped with either a SupelcoWax10 capillary column (30 m length, 0.25 mm i.d. and 0.25 μm film thickness, Supelco) or a CP Cresol capillary column (50 m length, 0.25 mm i.d. and 0.20 μm film thickness, Varian).

2.3. Gas chromatography/mass spectrometry

The mixture containing the cresols and *o*-cresol-d_8 was injected onto the chromatographic column following thermal desorption from the SPME fiber in the inlet kept at 250 °C. Two chromatographic conditions were adopted, according to the column. In both cases carrier gas was He at 1 ml/min, constant flow. For the SupelcoWax10, the oven temperature was kept at 140 °C for 8 min, then the temperature was increased to 225 °C at the rate of 25 °C/min. The oven was kept at

225 °C for 2.5 min and then the temperature increased again to 250 °C at the rate of 25 °C/min, and kept at this temperature for 1 min. The total run time was 15 min. Under these conditions the retention time were: 11.51 min for *o*-C, 12.12 and 12.17 min for *p*-C and *m*-C, 11.47 min for *o*-cresol-d_8. For the CP cresol column, the oven temperature was kept at 50 °C for 8 min, then the temperature was increased to 120 °C at the rate of 25 °C/min. Finally the temperature was kept at 120 °C for 40 min. The total run time was 51 min. Under these conditions the retention times were approximately: 40.33 min for *o*-C, 47.52 min for *p*-C, 49.27 min for *m*-C and 39.69 min for *o*-cresol-d_8.

The MS detector, with the source temperature at 178 °C, acquired signals in the selected ion-monitoring mode. The dwell time was 100 ms. For mass spectra acquisition the ion range *m*/*z* 40–200 was scanned. For analytes quantification the spectrometer was focused at ions *m*/*z* 108 $[CH_3{-}C_6H_4{-}OH]^{\bullet+}$ and 115 $[CD_3{-}C_6D_4{-}OH]^{\bullet+}$ for cresols and *o*-cresol-d_8, respectively.

2.4. HS SPME

To define the most convenient HS SPME experimental conditions the following parameters were evaluated: different fiber coatings, addition of saturating amount of salt to the solution of analytes, agitation during sampling, sampling time, desorption time. All the trials were performed sampling cresols from 0.5 ml of urine spiked with cresols at the concentration of about 1.4 mg/l each, inserted in a gas tight vial and kept at room temperature (∼25 °C). Finally, once the overall conditions for HS SPME were identified, the recovery of the extraction procedure was evaluated.

2.4.1. Comparative evaluation of different SPME fiber coatings

The following fiber coatings with relative GC inlet desorbtion temperatures were compared: PDMS, 250 °C; PDMS/DVB, 250 °C; CW/DVB, 240 °C; PA, 250 °C. For this experiment sampling time of 5 min and desorption time of 3 min were chosen. SPME sampling may be performed either at the equilibrium or for shorter time. The second mode is preferable when the coating is sampling by adsorption (PDMS/DVB and CV/DVB fibers), instead that by absorption (PDMS and PA fibers). In fact, for a fiber sampling by adsorption, at the equilibrium a competitive displacement of the analytes caused by other, more abundant, chemicals in the biological fluid, may take place. For this reason a short sampling time was chosen for a first comparison among fibers [25].

2.4.2. Effect of salt addition and agitation

The effect of addition of saturating amount of NaCl and agitation during sampling were separately and additionally evaluated. Saturation was obtained adding 300 mg of NaCl to each vial. Agitation was performed automatically, using the agitation accessory furnished with the autosampler that works by vibrating the SPME fiber, with consequent transmission of the movements to the vial and sample. For this experiment the following conditions were used: PDMS fiber, sampling time of 20 min, desorption time of 8 min, desorption temperature of 250 °C.

2.4.3. Sampling and desorption time

Using the PDMS fiber, the kinetics of HS SPME absorption of cresols from urine and thermal desorbtion of analytes from fiber were evaluated. For absorption times ranging from 3 to 40 min were investigated. For desorption, performed at 250 °C, times ranging from 3 to 15 min, were investigated.

2.4.4. Recovery

The recovery of *o*-C and *m*-C in the optimized HS SMPE conditions (see Section 2.6) was evaluated. For this trial the chromatographic signal obtained from 0.3 ml standard solution at 0.600 mg/l (180 ng of each cresol), was compared with the chromatographic signal obtained by direct injection of the same amount of analytes in 0.5 μl of methanol. For the liquid injection the chromatographic conditions were as described above, except that an inlet liner with a 4.0 mm i.d. (Supelco) was used [26].

2.5. Hydrolysis of cresol conjugates

To define the most convenient conditions to perform hydrolysis of cresol esters using aqueous HCl at 37% (w/w) the following parameters were evaluated: reaction time, reactant amount, influence of saturating amount of NaCl in the sample during hydrolysis. A further experiment was performed to define the conditions for neutralizing and buffering the reaction medium after the hydrolysis. To optimize the reaction time and reactant amount, due to the lack of commercial standards of conjugated cresols, phenol-β-D-glucuronide was used as a surrogated. In the other tests a pool of urine from occupationally exposed subjects was used.

The best reaction time, i.e. the minimum time necessary to complete hydrolysis, was evaluated reacting 0.5 ml of phenol-β-D-glucuronide 0.5 mM in water with 50 μl of aqueous HCl at 100 °C for time ranging from 5 to 90 min. The chromatographic signal of the hydrolyzed phenol was compared with that of an aqueous solution of free phenol at the same concentration. The minimum amount of HCl needed to complete hydrolysis was evaluated performing the reaction in the presence of variable volume of acid (from 40 to 80 μl).

2.6. Sample preparation

The urine samples were left at room temperature until completely thawed. After shaking and waiting for a few minutes, 0.3 ml of urine surnatant were transferred in a 2 ml glass vial containing 300 mg of NaCl, added with 50 μl aqueous HCl 37% and readily sealed. The mixture was vigorously shaken and cresols conjugates hydrolyzed at 100 °C for 60 min. The solution was cooled at room temperature for

15 min and then chilled at −20 °C for 90 min. To the chilled sample were added, in the following order, 50 μl of NaOH 10 M, 300 μl of acetate/acetic acid buffer 1 M and 50 μl of internal standard solution. The sample was placed in the autosampler tray at room temperature for analysis. *o*-C and *m*-C were sampled in urine headspace using the SPME devise equipped with the PDMS fiber. Sampling was operated at room temperature for 20 min. Analytes injection was performed by thermal desorption of SPME fiber in the GC inlet operating in the splittless mode. Desorption was performed at 250 °C for 8 min. The analytical separation was done by GC/MS in the conditions reported above (see Section 2.3).

2.7. Linearity, limit of detection, within- and between-run precision, accuracy

For calibration curve, the calibration solutions of *o*-C and *m*-C in urine (each at a concentration ranging from 0.006 to 5 mg/l) and the urine blank, prepared as described in Section 2.1.1, were analyzed in triplicate using the procedure outlined above. 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 the *o*-C or *m*-C versus *o*-cresol-d_8, and x is the *o*-C or *m*-C concentration in the sample (mg/l). The limit of detection (LOD) of the assay, for each component, was calculated according to the expression:

$$\mathrm{LOD} = \frac{3\mathrm{SE}_q + q}{m}$$

where SE_q is the standard error of the intercept [27].

The within-and between-run precision and accuracy of the assay using the SupelcoWax10 and the CP Cresol columns were determined by analyzing three pools of urine spiked with known concentration of both *o*-C and *m*-C (theoretically 0.012, 0.12, 1.2 mg/l each). The same analyst analyzed five replicates of each pool on three different days. For the determination of accuracy the deviation of the mean value of the 3 days from the theoretically value was used. In particular this value should be within 15% of the theoretically value except for concentrations close to the LOD, where it should not deviate by more than 20% [28,29].

2.8. Stability of standard solutions

The stability of the standard solutions (free *o*-C and *m*-C in urine), and of the internal standard solution (*o*-cresol-d_8 5.63 mg/l in water), stored in the dark at −20 °C, was evaluated at various time points over 6 months.

2.9. Application of the assay to the biological monitoring

The urine samples were obtained from 20 healthy male workers (age 20–55 years) employed in the rotogravure printing industry, exposed to toluene during printing or maintenance of machineries (median personal airborne exposure, as time weighted average, 38 mg/m^3). As controls, 10 subjects belonging to the general population, without occupationally exposure to toluene, matched with exposed subjects for gender, health status and age, were investigated. At the beginning of the study, each subject received information about the aim of the research and a written informed consent was obtained. A spot urine sample was obtained from each subject, for workers at the end of the exposure, for controls, in the morning, as second urine of the day. For urine collection, 10 ml polyethylene tubes were used. Samples were cooled at −20 °C and delivered to the laboratory, where they were kept at the same temperature in the dark until analysis.

3. Results

3.1. Chromatographic separation

In Fig. 1a and b the single ion mass-chromatograms of a standard solution containing 0.6 mg/l of *o*-C and *m*-C, obtained registering the ions *m*/*z* 108 (*o*-C and *m*-C, above) and 115 (*o*-cresol-d_8, below) using a SupelcoWax10 column and a CP Cresol column, respectively, are reported. With the first column, the chromatographic peak of *o*-C was well resolved and quantified, while the peak of *m*-C was partially co-eluted with that of *p*-C and can be barely identified as a shoulder

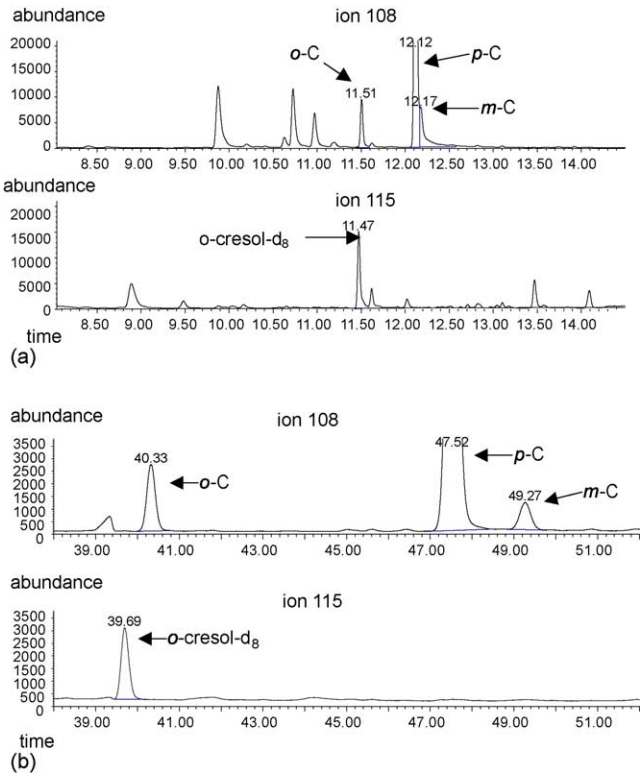

Fig. 1. Single ion mass-chromatograms obtained registering the ions *m*/*z* 108 and 115 for cresols and internal standard *o*-cresol-d_8, in a standard solution containing *o*-C and *m*-C each at the concentration of 0.6 mg/l, using the SupelcoWax10 column (a) and the CP Cresol column (b).

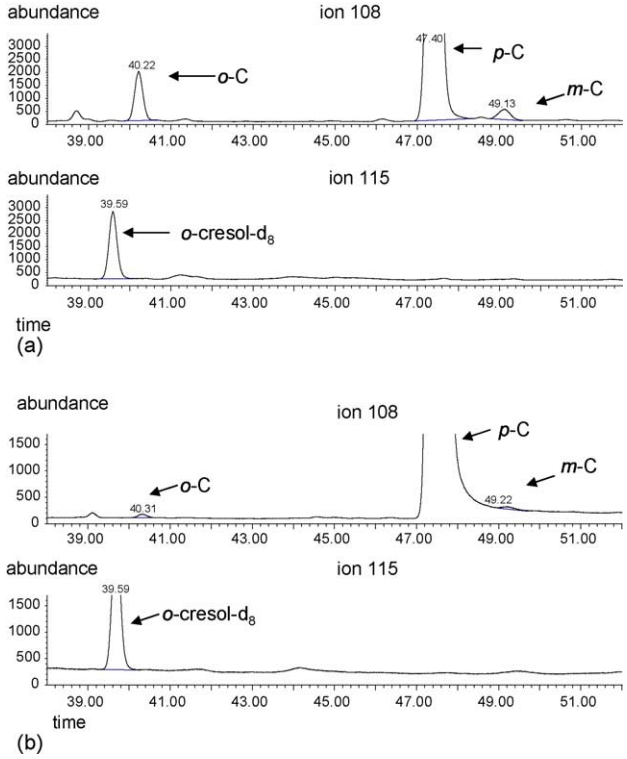

Fig. 2. Single ion mass-chromatograms obtained registering the ions *m/z* 108 and 115 for cresols and internal standard *o*-cresol-d_8, in urine samples from a rotogravure printing worker (a) and a control (b) using the CP Cresol column. The concentrations of *o*-C and *m*-C in the sample of worker were 0.403 and 0.233 mg/l and in the sample of control were 0.014 and 0.024 mg/l.

on the right side of it (Fig. 1a). Using the second column the peaks of the three cresols were well separated, although much longer chromatographic time was needed. The analytes were univocally assigned based on their retention times and mass to charge ratio. In Fig. 2a and b the single ion mass-chromatograms obtained with a CP Cresol column analyzing specimens collected from a printing workers and a control, are shown. The concentrations of *o*-C and *m*-C in the sample of worker were 0.403 and 0.233 mg/l and in the sample of control were 0.014 and 0.024 mg/l.

3.2. HS SPME

3.2.1. Comparative evaluation of different SPME fiber coatings

The comparison among the different fiber coatings showed that the CV/DVB fiber has the greatest affinity for cresols (100%), followed by the PA fiber (54–62%), the PDMS fiber (38%) and by PDMS/DVB (35–36%). Based on these results the CV/DVB fiber was initially selected for further experiments. Lately on the CV/DVB fiber was abandoned due to its scarce durability. PA fiber was not further investigated, and PDMS fiber was instead adopted based on the good sensitivity achieved even with this fiber and on its long lasting performance.

3.2.2. Effect of salt addition and agitation

The effect of salt addition and agitation during HS SPME sampling are reported in Table 1. Following the addition of saturating amount of NaCl a great improvement of extraction efficiency was obtained. On the contrary agitation, while represented an additional source of stress for the fiber, only slightly increased the signal of cresols. Based on these evidences sampling was performed in the presence of NaCl without agitation.

3.2.3. Sampling and desorption time

In Fig. 3 the kinetics of HS SPME absorption of cresols from urine (Fig. 3a) and of thermal desorption (Fig. 3b) are shown. Maximum absorption and desorption were obtained starting from times 20 and 8 min, respectively.

3.2.4. Recovery

The recovery of *o*-C and *m*-C in the standard solutions at 0.6 mg/l was 7.2 and 5.4%, respectively, as a mean value of five replicates.

3.3. Hydrolysis of cresol conjugates

3.3.1. Optimization of reaction conditions

The kinetic of acid deconjugation of phenol-β-D-glucuronide using 50 μl of HCl at 100 °C is shown in Fig. 4. A reaction time of 60 min and a minimum amount of HCl of 50 μl were selected as the best condition to perform the hydrolysis. In this condition the reaction yield was higher then 97%.

No decrease in the yield of the acid hydrolysis was observed when the reaction was performed in presence of NaCl. This condition was tested because the maximum recovery of cresols by HS SPME sampling was obtained in the presence of saturating amount of this salt. However, the addition of salt just before HS SPME sampling, would mean a possible loss of analytes, due to the presence of free cresols in

Table 1
Effect of the addition of saturating amount of NaCl to urine and/or of sample agitation during SPME on the chromatographic response of cresols

Analyte	Sampling conditions			
	No NaCl; no agitation	No NaCl; agitation	NaCl; no agitation	NaCl; agitation
o-C	1.0	1.0	10.2	11.4
m-C	1.0	1.0	10.1	11.1
o-Cresol-d_8	1.0	1.0	10.5	11.8

Data are expressed as relative response assuming that the chromatographic response obtained in the absence of both NaCl and agitation is equal to 1.

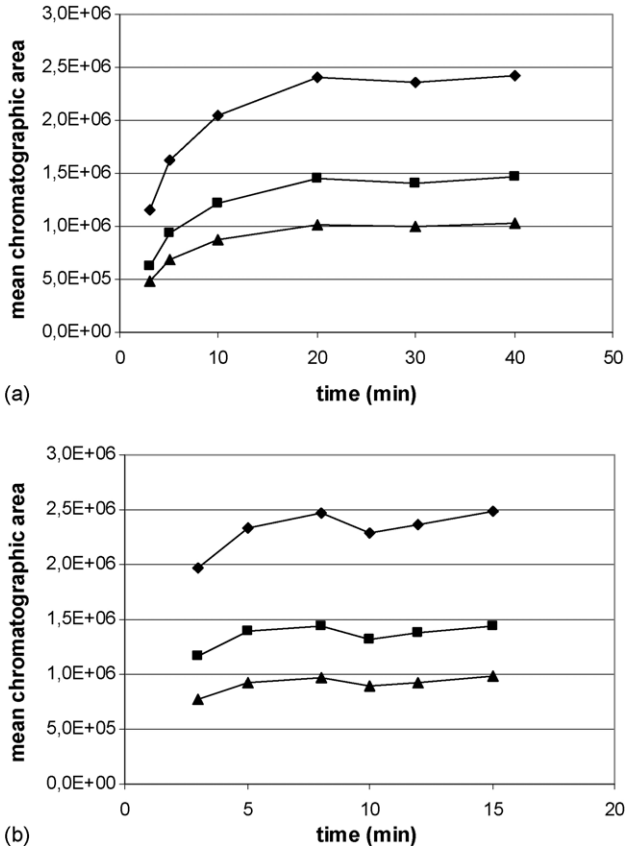

Fig. 3. Kinetic of adsorption of cresols from urine by HS SPME using a PDMS fiber at room temperature, in the presence of saturating amount of NaCl (a); and kinetic of thermal desorption of cresol from PDMS fiber, in the injection liner of GC at 250 °C (b). (♦) *o*-C; (■) *m*-C; (▲) *o*-cresol-d_8.

the sample that, being volatile, may leave the reaction vessel while opening during addition. The result of this experiment allowed the introduction of saturating amount of NaCl in the sample since the beginning of the assay.

Following acid hydrolysis the reaction medium was neutralized and buffered to achieve a pH ranging from 5 to 6 by addition of aqueous NaOH 10 M (50 μl) and 1 M acetate/acetic acid buffer (300 μl). This pH is low enough to maintain cresols unsalted, i.e. available for partitioning between aqueous solution and headspace, but close to neutrality, to avoid damage of SPME fiber and GC column. However this operation, performed opening the reaction vial in the presence of volatile analytes, could lead to their loss. To avoid such inconvenient it was lowered the analytes volatility by freeze-cooling the sample at −20 °C for 90 min before opening vial. The introduction of the cooling step resulted in no observable decrease in the chromatographic signal of the analytes.

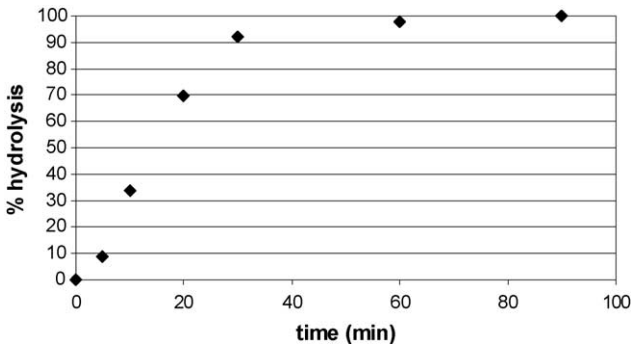

Fig. 4. Kinetic of hydrolysis of phenol-β-D-glucuronide to give phenol, using 50 μl of HCl 37% (w/w) at 100 °C.

3.4. Linearity, limit of detection, within- and between-run precision, accuracy

The calibration curves obtained for *o*-C using the SupelcoWax10 column and for *o*-C and *m*-C using the CP Cresols column were linear all over the investigated range (0–5 mg/l), with correlation coefficient typically higher then 0.999. The analytical limit of detection was 0.006 mg/l for *o*-C, with both columns, and 0.007 mg/l for *m*-C. Within- and between-run precision and accuracy of the assay using the two columns are summarized in Tables 2 and 3.

3.5. Stability of standard solutions

Following this experiment it was found that the standard solutions and the internal standard solution kept in the dark at −20 °C, were stable for, at least, 6 months.

3.6. Application of the assay to the biological monitoring

The statistics of the excretion of urinary *o*-C and *m*-C in rotogravure printing workers and in controls are summarized in Table 4.

4. Discussion

The present work describes an original analytical procedure based on HS SPME GC/MS, useful to perform routine analysis of urinary cresols among subjects occupationally and non-occupationally exposed to toluene.

The major feature introduced by this assay, in comparison with the past procedures, is the use of SPME to extract the analytes from the headspace of specimen and directly inject them onto the chromatographic system. The advantages of using SPME are to skip tedious, time consuming and unsafe solvent extractions or vapour distillation and to give the opportunity of performing the whole operation automatically, enhancing precision, saving time and, consequently, lowering costs.

In our study several SPME fibers were compared for their capability of extracting cresols from urine: although the CW/DVB fiber was found to be the most efficient, it was also poor durable: the major problem was the loss of adhesion of coating from fused silica substrate. The PDMS fiber

Table 2
Within-run and between-run precision and accuracy for the determination of urinary *o*-C with the SupelcoWax10 column

		Theoretical concentration *o*-C (mg/l)		
		0.012	0.12	1.2
Day 1	Mean ($N = 5$)	0.0124	0.117	1.20
	CV%*	6.5	2.1	2.3
	% Theoretical	103.6	97.6	99.8
Day 2	Mean ($N = 5$)	0.0112	0.119	1.26
	CV%*	14.1	1.5	2.1
	% Theoretical	93.2	99.3	104.7
Day 3	Mean ($N = 5$)	0.0119	0.120	1.23
	CV%*	6.3	1.6	2.5
	% Theoretical	99.0	100.3	102.2
Overall	Mean ($N = 15$)	0.0118	0.119	1.23
	CV%**	9.7	2.0	2.9
	% Theoretical***	98.6	99.1	102.3

* Within-run precision.
** Between-run precision.
*** Accuracy.

adopted instead was, at least, 1/2 less efficient, but due to the satisfactory sensitivity reached also with this fiber, it was chosen for routine use. In fact, our previous experience in the determination of urinary BTEX by PDMS fiber proved its long lasting performance [30,31]; moreover the recent experience with analysis of cresols confirms that the PDMS fiber allowed hundreds of samples to be analyzed without major loss of performance. The PA fiber, initially investigated in this study, and suggested for sampling cresols from blood in forensic toxicology [32], showed higher extraction efficiency in comparison with the PDMS fiber and therefore a further improvement in the sensitivity of the assay is expected from its adoption.

The choice of sampling analytes in headspace of urine, instead that by immersion of the fiber in the specimen, was based on various considerations: the high vapour pressure of cresols insures a significant presence of analytes in the headspace of specimen also at room temperature, moreover sampling for immersion in a complex matrix as urine represents a major source of stress for the fiber and may shorten its life, finally the kinetic of partitioning of analytes between urine headspace and fiber is much faster then that between urine solution and fiber, due to the greater activity of molecules in the vapour phase, so that longer extraction time would be necessary if the immersion procedure was adopted.

Among the major advantages of the proposed assay is the possibility of performing the whole procedure using an autoinjector vial as the only vessel. In this vial the steps of hydrolysis, extraction and injection are performed consecutively, without sample transfer, therefore avoiding loss of analytes and/or introduction of handling errors. To achieve this result the volume of urine, salt, acid and neutralizing solutions

Table 3
Within-run and between-run precision and accuracy for the determination of urinary *o*-C and *m*-C with the CP cresol column

		Theoretical concentration					
		o-C (mg/l)			*m*-C (mg/l)		
		0.012	0.12	1.2	0.012	0.12	1.2
Day 1	Mean ($N = 5$)	0.0111	0.113	1.12	0.0103	0.110	1.13
	CV%*	1.5	9.7	9.1	2.6	6.7	9.6
	% Theoretical	92.3	94.5	93.6	85.6	91.8	94.0
Day 2	Mean ($N = 5$)	0.0142	0.117	1.18	0.0151	0.131	1.19
	CV%*	6.0	9.9	3.0	7.5	5.9	3.2
	% Theoretical	118.1	97.6	98.0	125.5	109.3	98.8
Day 3	Mean ($N = 5$)	0.0110	0.111	1.24	0.0100	0.109	1.24
	CV%*	9.6	4.3	0.2	8.5	3.8	2.5
	% Theoretical	91.7	92.7	103.2	83.6	90.8	103.3
Overall	Mean ($N = 15$)	0.0121	0.114	1.18	0.0118	0.116	1.19
	CV%**	18.9	10.9	4.4	8.5	11.9	4.8
	% Theoretical***	100.8	94.7	98.3	98.3	96.7	98.9

* Within-run precision.
** Between-run precision.
*** Accuracy.

Table 4
Summary of statistics for urinary *o*-C and *m*-C in 20 rotogravure printing workers and in 10 controls belonging to the general population

Subjects (*N*)	Statistics	*o*-C (mg/l)	*m*-C (mg/l)
Controls (10)	Mean ± S.D.	0.042 ± 0.057	0.156 ± 0.151
	Median	0.017	0.089
	Min–max	0.006–0.194	0.024–0.423
Rotogravure printing workers (20)	Mean ± S.D.	0.286 ± 0.128	0.357 ± 0.272
	Median	0.253	0.290
	Min–max	0.062–0.556	0.091–1.056

introduced in the vial were accurately calibrated and minimized to reach a final total volume of about 0.7 ml. In this way at the end of sample preparation steps, the headspace volume needed for SPME sampling was still available in the vial.

The analysis performed by gas chromatography allows achieving good performance in separation using capillary columns. The SupelcoWax10 column, due to its polarity, gives good resolution of polar compounds such as *o*-C. However, under this condition, *m*-C and *p*-C are co-eluted. A complete separation of the three cresols was possible using a specific chiral column CP Cresol, although with much longer retention times.

To preserve both columns and SPME fiber from acid vapours it was introduced a careful neutralization step after hydrolysis: under these conditions the SupelcoWax10 column demonstrated its robustness allowing a large number of samples to be analyzed without loss of performance and a great stability of retention times. On the contrary the chiral CP Cresol column showed to be more delicate and sensitive to humidity, with retention times that tend to shorten with time.

Although with the CP Cresol column also *p*-C was completely resolved and could be easily identified, the attempts to extend the assay also to its quantification failed. Due to the fact that *p*-C is physiologically excreted in concentration that is, at least, one order of magnitude larger then *o*-C and *m*-C, also in the absence of occupational exposure to toluene, a much wider calibration range is required for its quantification.

The use of a MS detector improves specificity and sensibility of the assay: the identification of the cresols is based on both the acquisition of the single ion *m/z* 108, and on the retention time of the peak. The single ion chosen is the most representative for abundance and is highly specific, being the molecular ion of cresols. Moreover, the single ion-monitoring mode allowed elimination of the peaks deriving from the interfering compounds present in the biological matrix.

Finally, the use of MS detector allowed the introduction of *o*-cresol-d_8 as internal standard. This molecule, commercially available, is the best choice for correction of casual errors that may occur during both HS SPME sampling and GC/MS analysis. On the contrary the lack of a conjugated internal standard does not allow checking for the efficiency of the hydrolysis.

Using the outlined conditions, the procedure was accelerated to prepare as many as 40 samples/day, using the SupelcoWax10 column or 20 sample/day with the CP Cresol column.

So far the assay was successfully applied to routine analysis of hundreds of urine samples. As example, typical levels found in rotogravure printing workers exposed to toluene during printing or maintenance of printing machines and in a suitable group of controls, are shown in Table 4. The urinary level of *o*-C found in the investigated subjects is in line with those reported in previous studies [7,8,33–35].

Based on the features of the above described analytical procedure, in terms of urine volume required, time necessary for performing the assay, sensibility, specificity and precision, we conclude that the present approach represents a useful tool for the biological monitoring of exposure to toluene through the determination of urinary *o*-C and *m*-C.

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