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Urinary benzene determination by SPME/GC–MS A study of variables by fractional factorial design and response surface methodology

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Abstract

The urinary excretion of the unmetabolized benzene seems to be a very good index for biomonitoring benzene in occupationally exposed people. The use of solid phase microextraction (SPME) offers important advantages for its determination. Several variables can influence the benzene extraction process. Experimental design methodology was used to estimate the influence of the different variables and to evaluate the simultaneous effect of the more significant variables on the benzene extraction. The results showed that sample temperature, sample volume and their interaction were the more significant factors. A model was found that relates the amount of benzene extracted with the studied variables. The more adequate working conditions were: extraction temperature 15 °C, incubation time 1 min, extraction time 1 min and 2.5 ml of sample volume. The results indicate that this method is capable of providing sensitive and accurate results for the biomonitoring of benzene in urine.

Keywords: Fractional factorial design; Response surface methodology; Benzene

1. Introduction

Benzene is a contaminant with a well established carcinogenic potential [1]. People can be occupationally exposed to benzene, as occurs in service station attendants, for example, where the volume of gasoline sold and the ambient temperature can significantly increase the environmental levels of gasoline vapours [2]. Benzene is also an environmental contaminant to which the general population is exposed. In nonsmokers, the main part of the benzene exposition comes from the emissions of engines and from gasoline vapour. In smokers, it is estimated that 90% of their benzene exposition proceeds from tobacco smoke [3]. The environmental concentrations of benzene can be up to 50 $\mu g/m^3$ in urban zones with high traffic density [4].

The biological control of exposition to benzene is of great importance to prevent its toxic and carcinogenic effects and it is therefore very relevant to develop methods that permit an evaluation of exposition.

Methods based on benzene urinary metabolites, such as phenol and *trans*,*trans*-muconic acid are non specific because these compounds are also generated from sources other than benzene. *S*-Phenyl-mercapturic acid is also a benzene metabolite, which may be employed as a more specific marker of exposure, however the complexity of the analytical method represents a drawback. So, increasing attention has been paid to evaluate the unmetabolized solvent in biological materials as a marker of exposure to benzene. This approach is being used for other solvents too [5,6].

Urinary excretion of unmetabolized benzene would be a useful index for the evaluation of low-level environmental exposure [7–10]. The analytical method usually used involves static or dynamic headspace techniques, coupled with chromatographic analyses. Currently solid phase microextraction (SPME) represents one of the most used techniques for sampling, extraction and concentration of analytes. SPME uses a short length of a coated fibre that can be withdrawn inside a syringe needle, after sampling, for protection and transfer to GC inlet. The fiber is immersed directly into the sample, or exposed to the gas above the sample, and the analytes are retained in the fiber coating.

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The analytes are then thermally desorbed and transferred into the chromatographic column.

Initially SPME was used to extract environmental pollutants from drink, ground and wastewater via direct extraction, but since its introduction there has been a rapid growth in the number of applications of SPME. The first substantive uses of SPME with clinical and toxicological specimens were reported in 1994 and a wide variety of forensic investigations of drugs of abuse and their metabolites from biological matrices have been developed [11–13].

One major challenge in the utilization of SPME for the determination and analysis of unmetabolized organic compounds in urine is the selection of experimental conditions that can provide acceptable peak areas, as the concentration that is expected for these analytes in urine is very small. Several factors can influence the extraction of analytes: temperature of the sample, time of extraction, sample volume, ionic strength, sampling technique (immersion or headspace), pH, etc. There are few studies dealing with the effect of the different variables on the amount of analyte extracted and in these, the traditional method of experimentation is to evaluate only one variable (or factor) at a time, all of the variables are held constant during test runs except the one being studied. This type of experiment reveals the effect of the chosen variable under set conditions assuming that the variables are independent and that the effect will be the same at another level of the remaining variables; it does not show what would happen if the other variables are also changed.

Experimental design allows to estimate the effects of several variables simultaneously. The significant factors affecting the extraction of benzene on the SPME fiber can be deduced, in a screening study, by applying a fractional factorial design, a powerful tool commonly used in exploratory studies characterized by a large number of potentially influential factors [14,15]. Subsequently, response surface methodology (RSM), that consists of a group of mathematical and statistical techniques, are useful in the modelling and analysis of processes in which a response of interests, such as amount of contaminant adsorbed, is simultaneously influenced by several significant variables [14–18].

The aim of this work was to estimate the influence of different variables and to evaluate the simultaneous effect of the more significant variables on the benzene extraction by using experimental design methodology. The experimental conditions selected were then applied to developing a method for the determination of benzene in urine to monitor occupational exposure to benzene.

2. Experimental

2.1. Preparation of benzene standards

Standards containing benzene were prepared from pure solvent (99.5% Fluka, Buchs, Switzerland). The first dilution step was performed with methanol (analytical grade, E. Merck, Darmstadt, Germany) whereas further preparation of the standard solutions was carried out with distilled water (Milli-Q quality, Millipore, Bedford, MA, USA). Standard solutions in water were prepared daily. The study of the SPME variables on benzene extraction was performed with benzene solutions of 2 ng/ml. Aliquots were immediately transferred to 5 ml glass vials with a cone-shaped interior containing a triangle-shaped magnetic stirrer. The vials were rapidly sealed with silicone septa and open-top caps.

Calibration standards of benzene were prepared from a working standard solution of 20 ng/ml concentration. In order to minimize evaporation of the benzene during preparation of the standards it was necessary to transfer an aliquot of the working solution into a 2 ml vial, that was sealed with a PTFE/silicone septum, in such a way that there was not headspace in the vial. Subsequently, by using microliter syringes (Hamilton, Bonaduz, Switzerland), the different calibration standards were prepared.

In order to perform accuracy and repeatability measurements, a benzene standard (5000 μg/ml methanol) supplied by Supelco (Bellefonte, PA, USA) was used. Independent samples were prepared by diluting the appropriate amount of the standard in water or in urine to have a concentration of 0.2 ng/ml of benzene. The water- and urine-spiked samples were immediately analysed by SPME sampling method listed below.

2.2. Repeatability in exposed subjects urine samples

A repeatability test was also carried out on urine samples from four service station attendants, analysing at least four aliquots of 2.5 ml from the same urine sample of every exposed worker. Urine samples were collected at the end of the shift. The samples were collected in glass bottles, refrigerated and transferred into 5 ml vials in laboratory. Samples must be plugged tightly as soon as the collection is over. Samples were analysed the same day as they were received.

2.3. Solid phase microextraction method and GC analysis

SPME devices and 100 μm bonded polydimethylsiloxane fiber assemblies were used (Supelco, Bellefonte, PA). The fiber was conditioned in a GC injection port at 250 °C for 1 h to remove fiber contaminants as recommended by the manufacturer. A sample volume was added to the described 5 ml glass vial. Samples were heated or cooled in a thermostatized water bath for a time (incubation time). Then, the SPME fiber was exposed to the headspace of the vial. The solution was stirred with the magnetic stirrer during the incubation time and the headspace extraction. Care was taken to ensure that the same length of the septum-piercing needle passed through the septum. The immersion depth of the fiber into the headspace above the sample during extraction, and also into the injector during desorption, was always maintained the same. After extraction, the fiber was withdrawn into the needle, pulled out from the vial and injected into

the GC. The time lapse between analyte extraction and desorption was as short as possible to minimize evaporation of the analyte from the fiber.

A Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA, USA) equipped with split-splitless injector and coupled to a HP5973 mass selective detector was used for detection of benzene. The column used was a crosslinked methyl silicone capillary column measuring 50 m length, 0.2 mm i.d. with phase thickness of 0.5 μm (Hewlett-Packard, Palo Alto, CA, USA).

The fiber was exposed into the injection port, that uses a Merlin Microseal septum (Merlin, Half Moon Bay, CA) instead a standard septum, in the splitless mode for 2 min at 200 °C. Silanized narrow bore injector liner (0.75 mm i.d., Supelco) were installed. During injection, the column temperature was hold at 40 °C for 2 min and then the temperature was increased at 8 °C/min up to 100 °C. The flow rate of the helium carrier gas was maintained at 1.2 ml/min in constant flow mode.

The MS detector, with the source at 230 °C, was used in the selected ion monitoring mode and focused at *m/z* 78 molecular ion. Standard autotunes with perfluorotributylamine were carried out on a daily basis. The benzene retention time was 7.8 min.

2.4. Variable screening

The influence of different variables and their interactions on SPME procedure was checked by using experimental design. As benzene is a ubiquitous environmental contaminant, there is a variable background level of benzene in urine in the general population [10]. So this study was developed with water as the matrix, since urine is mainly aqueous.

To ascertain the individual effects of temperature of the sample, incubation time, extraction time, sample volume and ionic strength on the retention of benzene, a half-fractional factorial design, for five variables at two levels, was applied. Two experiments were performed at the centre of the experimental design. A replicate was carried out in different days and using different benzene solutions, so a total of 36 runs were performed. The experimental variables considered and the design matrix are shown in Table 1. The design matrix was generated and the results evaluated by using Statgraphics V4. 1 software (Manugistics, 1998).

2.5. Simultaneous effect of the selected variables on the SPME extraction of benzene

The extraction process is controlled by different variables that can interact. One experimental approach is to apply the RSM for modelling the amount extracted as a function of the selected variables. We used a second-order, response surface design, with three levels factorial for each factor in three blocks, to be carried out in three different days, and a central point per block was applied to the variables that had a significant influence; in all blocks of experiments the runs were randomized. The factors and their low, central and high levels were: sample temperature (10, 37.5, and 60 °C), incubation time (1, 8, and 15 min) and sample volume (0.5, 1.5, and 2.5 ml). The matrix involved 30 runs. The experiment at the centre of the experimental field was performed four times. Statgraphics software was used to obtain the combination of values that draw the surface response. Table 2 presents the values of the variables for the response surface experiment.

Table 1
Fractional factorial design matrix of the screening experiment

Run	T (°C)[a]	t_{inc} (min)[b]	t_{ext} (min)[c]	V (ml)[d]	S[e]
1	37.5	15.5	8	1.5	No
2	15.0	30.0	1	0.5	No
3	15.0	30.0	1	2.5	Yes
4	60.0	30.0	15	2.5	Yes
5	60.0	30.0	1	2.5	No
6	15.0	1.0	15	0.5	No
7	60.0	30.0	15	0.5	No
8	60.0	1.0	15	0.5	Yes
9	60.0	1.0	1	0.5	No
10	60.0	30.0	1	0.5	Yes
11	15.0	30.0	15	0.5	Yes
12	15.0	1.0	15	2.5	Yes
13	15.0	1.0	1	0.5	Yes
14	60.0	1.0	1	2.5	Yes
15	15.0	1.0	1	2.5	No
16	15.0	30.0	15	2.5	No
17	60.0	1.0	15	2.5	No
18	37.5	15.5	8	1.5	Yes

[a] Temperature of the sample.
[b] Incubation time.
[c] Extraction time.
[d] Sample volume.
[e] Ionic strength.

3. Results and discussion

3.1. Factor screening

Some parameters were preliminary considered in order to define the experimental field. Variables such as sampling procedure and stirring were selected according to the literature. Headspace extraction was selected because adsorption of biomacromolecules could decrease the affinity for the analyte of interest with biological matrixes [12,19]. Besides HS is cleaner than immersion sampling so that longer lifetime of SPME fiber can be expected.

Conventional magnetic stirring was also selected in this study. A rapid equilibrium between aqueous and vapour phases can be achieved by constantly stirring the aqueous sample to generate a continuously fresh surface [20]. A well agitated aqueous phase simply means that the mass transport in the aqueous phase is much faster than that in the other two phases and is not a limiting step for whole diffusion process. A 5 ml vial was selected to allow small volumes of the bio-

Table 2
Response surface design with three level factorial in three blocks

Run	Block	T (°C)[a]	t_{inc} (min)[b]	V (ml)[c]
1	1	37.5	1	2.5
2		37.5	8	0.5
3		60.0	8	2.5
4		60.0	1	1.5
5		37.5	8	1.5
6		10.0	8	1.5
7		10.0	15	2.5
8		10.0	1	0.5
9		60.0	15	0.5
10		37.5	15	1.5
11	2	60.0	15	2.5
12		37.5	8	2.5
13		10.0	1	2.5
14		60.0	8	1.5
15		37.5	8	1.5
16		10.0	15	1.5
17		37.5	15	0.5
18		10.0	8	0.5
19		37.5	1	1.5
20		60.0	1	0.5
21	3	60.0	1	2.5
22		60.0	8	0.5
23		10.0	15	0.5
24		10.0	8	2.5
25		37.5	8	1.5
26		37.5	15	2.5
27		10.0	1	1.5
28		37.5	1	0.5
29		37.5	8	1.5
30		60.0	15	1.5

[a] Temperature of the sample.
[b] Incubation time.
[c] Sample volume.

logical fluids to be collected. The ratio of sample volume to headspace volume for headspace analysis in 5 ml-vials was determined by varying the sample volume between 0.5 and 2.5 ml.

In the dynamic or static headspace analysis unmetabolized compounds can be separated from biological specimens by increasing the temperature, usually to above 60 °C, in sealed vials. In SPME analysis of drugs and volatile organic compounds in body fluids temperatures between 35 and 80 °C are very common [11,12,21]. Sample temperature must be increased to improve analytes transfer from the liquid to the headspace but to increase analytes concentration in the fiber temperature must be kept low. Therefore, the temperature of the sample was studied between 15 and 60 °C in the experimental design.

The addition of a salt to an aqueous matrix increases the ionic strength of the solution. For many organic analytes, aqueous solubility decreases with increasing ionic strength, and thus the partitioning of the analytes from the aqueous solution to the headspace is improved. DeBruin et al. investigated the effect of NaCl concentration over the range of 0–4.0 M on the extraction of aromatic amines, and they found the amount of analyte extracted onto the fiber increased with salt concentration [19]. So we have considered the salt variable as absence or saturated NaCl solution.

Table 3
Effects of the factors and interactions obtained by fractional factorial design

Factor/interaction	Effect
Average	16499 ± 792
A: Sample temperature	−17306 ± 1680
B: Incubation time	−8313 ± 1680
C: Extraction time	−4656 ± 1680
D: Sample volume	17739 ± 1680
E: Ionic strength	2687 ± 1584
AB	6719 ± 1680
AC	825 ± 1680
AD	−6636 ± 1680
AE	−1741 ± 1680
BC	1058 ± 1680
BD	−557 ± 1680
BE	−1699 ± 1680
CD	−50 ± 1680
CE	161 ± 1680
DE	−278 ± 1679
Block	−369 ± 1584

Once the variables and their experimental field were selected, the influence of the variables and their interactions on the extraction of benzene was checked. The estimated effects of the factors and their interactions can be seen in Table 3, which also shows the standard error of each of the effects. Only four factors appeared to be significant: sample temperature and sample volume; in minor extension, incubation and extraction times. The volume of sample has a positive influence on the response contrary to the temperature of the sample and incubation and extraction times. The study also shows that there were significant interactions between these factors, the effect of incubation time and sample volume is greater at low temperatures.

The results from this first step led to the elimination of two variables: extraction time and the presence of salt. Therefore no salts were added to the sample and extraction time was fixed to 1 min. The results indicate that 1 min is enough for the headspace extraction of benzene. Zhang and Pawliszing studying the extraction time of BTEX from water found that the time for benzene was very short, approximately 40 s [20].

3.2. Simultaneous effect of the temperature of the sample, incubation time and sample volume on the SPME extraction of benzene

Due to the previous results only the most significant variables were used to define the experimental matrix of the design (see Table 2), and a low temperature, 10 °C, was checked. Analysis of variance (ANOVA) was performed on the design to assess the significance of the model. The results obtained can be seen in Table 4. Sample temperature, sample volume, and their interaction had the largest effect

Table 4
ANOVA on the data obtained using the response surface methodology

Effect	Sum of squares	d.f.	Mean square	*F*-ratio	*P*-value
A: Incubation time	1.8837E8	1	1.8837E8	16.47	0.0007
B: Sample volume	1.9171E9	1	1.9171E9	167.67	0.0000
C: Sample temperature	2.7737E9	1	2.7737E9	242.60	0.0000
AA	6.4141E7	1	6.4141E7	5.61	0.0293
AB	1.0264E7	1	1.0264E7	0.90	0.3560
AC	5.1813E7	1	5.1813E7	4.53	0.0473
BB	1.1299E8	1	1.1299E8	9.88	0.0056
BC	6.1655E8	1	6.1655E8	53.92	0.0000
CC	1.3002E8	1	1.3002E8	11.37	0.0034
Blocks	4.3756E7	2	2.1878E7	1.91	0.1764
Total error	2.0581E8	18	1.1434E7		
Total (correlation)	6.0897E9	29			

$R^2 = 0.9662$; standard error of estimation = 3381.39; mean absolute error = 2217.88; *F*-ratio: MS factor/MS error.

on the response. The ANOVA table partitions the variability in the response into separate pieces for each of the effects. It then tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. In this case, eight effects have *P*-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level.

For the experiment, regression technique was used to fit the parameters to a response surface. The equation that describes the benzene peak area in terms of the significant variables is as follows:

$$\begin{aligned}\text{Peak (area)} = {} & 9254 - 1678t_{\text{inc}} + 33590V - 649T \\ & + 62.4t_{\text{inc}}^2 - 132t_{\text{inc}}V + 11.9t_{\text{inc}}T \\ & - 4059V^2 - 287VT + 7.0T^2 \end{aligned} \quad (1)$$

Because it was not possible to simultaneously plot the experimental response as a function of the significant variables that control the adsorption process, we will consider separately the effects of two variables on the response. These graphs offer a visual means of understanding how the factors influence the measurement system.

Fig. 1 shows the response surface developed by the model for incubation time and sample volume at temperature values of 60 °C (Fig. 1a) and 10 °C (Fig. 1b). For 60 °C a maximum is found for a sample volume of 2 ml. Probably, as temperature increases higher relation headspace/sample volume is needed. The situation is different at 10 °C where the benzene peak increases almost linearly with sample volume. Nevertheless, for a T value of 10 °C the peak counts was 53,000 whereas at 60 °C the maximum was only 12,000. For the 5 ml vial used, 2.5 ml is the maximum amount of sample volume for the fiber stayed above the liquid phase without contacting the sample; therefore it is the smaller headspace/sample ratio. This fact allows the equilibrium between the headspace and the fiber coating is reached quickly [19,20]. Fig. 1b also shows that the peak counts have a little decrease when incubation

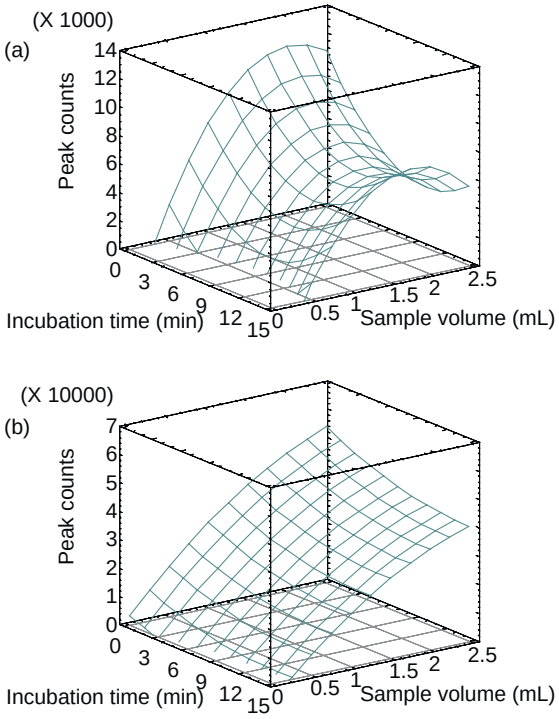

Fig. 1. Benzene area peak as a function of sample volume and incubation time. (a) Sample temperature of 60 °C. (b) Sample temperature of 10 °C.

time increased; therefore incubation time appears scarcely influential.

Fig. 2 shows the effect of sample volume and sample temperature at 1 min of incubation time. Increasing the sample temperature decreases the amount extracted. Low temperature has a positive effect on the adsorption. Therefore, the global process is exothermic. Fustinoni et al. found that the average intensity of the chromatographic signals, calculated with the same extraction time, was significantly reduced when temperature increases from 40 to 60 or 80 °C. Based on these results, 40 °C were selected by the authors but, probably, a lower temperature would improve extraction of BTX in urine [20]. As there is not a significant difference between the results obtained from the model at 10 and 15 °C, this latter was selected.

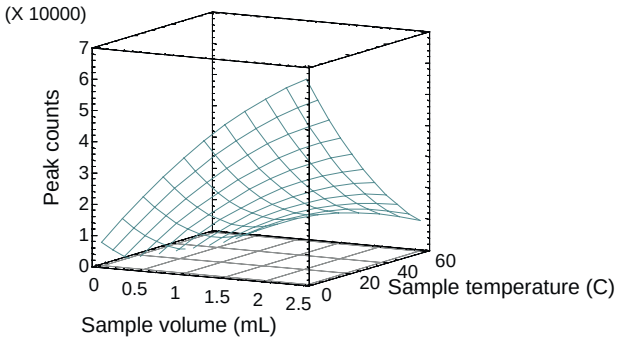

Fig. 2. Benzene area peak as a function of sample volume and sample temperature (incubation time of 1 min).

In conclusion, the more adequate working conditions were: extraction temperature 15 °C, incubation time 1 min, extraction time 1 min and 2.5 ml of sample volume. The total time of the analyte extraction was just 2 min. These experimental conditions are an advantage as compared to reported extraction times of 45 min [19,22–24] allowing more samples to be analysed in a day.

3.3. Analytical performance

Calibration samples of different concentrations between 40 and 2000 ng/l, including blanks, were measured according to the operating conditions described above. The obtained eight-point calibration curve shows good linearity over the studied range. Linear correlation was as follows: $y = 964.3 + 20128x$ (y: ion peak area; x: concentration of benzene (ng/ml); $r^2 = 0.999$). The detection limit of the proposed method, based on the residual standard deviation of the regression line, was 0.043 ng/ml [25]. It should be taken into account that calibration curves and blank anal-

Table 5
Precision and accuracy of the method in standard benzene samples

	Benzene concentration in spiked samples (ng/ml)	
	Water matrix	Urine matrix
	0.201	0.232
	0.217	0.213
	0.184	0.194
	0.219	0.199
	0.202	0.210
	0.192	0.192
Mean	0.203	0.207
R.S.D. (%)	6.8	7.3
Overall uncertainty[a]	15.2	18.3

[a] $[(|\bar{x} - x_{true}| + 2s)/x_{true}] \times 100$, where $\bar{x}$ is the mean value of the repeated measurements; x_{true} the true concentration value and s is the standard deviation of measurements.

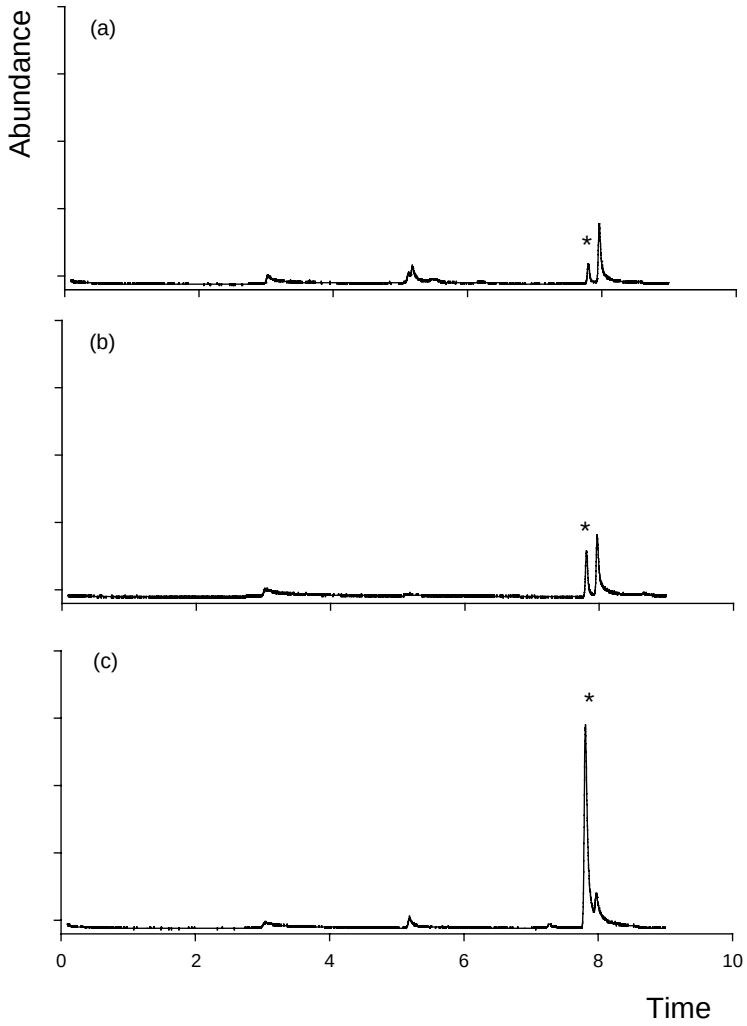

Fig. 3. Mass chromatograms of m/z 78 obtained from (a) a calibration blank, (b) urine of occupationally exposed subject No. 1 in Table 6, and (c) urine from subject No. 2 in Table 6. (*) Benzene peak.

Table 6
Repeatability of the determination of benzene in urine samples from occupationally exposed workers

Subject	Benzene concentration (ng/ml)	R.S.D. (%)
1	0.131, 0.103, 0.118, 0.099, 0.119, 0.099	11.8
2	0.506, 0.492, 0.495, 0.555	5.7
3	0.181, 0.169, 0.183, 0.211, 0.192	8.4
4	0.057, 0.061, 0.068, 0.054	10.1

ysis should be carried out each day as well as autotunes. Blank samples were always considered, as it is impossible to completely eliminate benzene from the analytical system. The values obtained for this samples are very constant. This behaviour has been yet observed for other trace-level contaminants [24].

The repeatability and accuracy of the method was determined by conducting six replicate extractions of independent benzene standard of 0.2 ng/ml that were prepared from a commercial standard both in water and urine matrix. The results are shown in Table 5. The relative standard deviation (R.S.D.) was 6.8% for water samples and 7.3% for urine standard. These R.S.D. values were in agreed with reported values. For example, precision values were found in the range from 2.7 to 10.9% R.S.D. for aromatic amines in different matrixes [19]. In replicate measurements using internal standard, a coefficient of variation of 6% for urine samples spiked with benzene at the concentration of 1250 ng/l were reported [22]. On headspace methyl *tert*-butyl ether extraction from water and SIM detection, R.S.D. values of 10% were observed [24].

The accuracy of the method has also been checked; the mean value of the six replicate analyses can be seen in Table 5. A good correspondence was obtained between the standard concentration values, 0.2 ng/ml, and the SPME results. Results of overall uncertainty of the measuring procedure are also showed in Table 5. Overall uncertainty is expressed, on a relative basis, by a combination of bias and precision [26]. The results for uncertainty of the proposed method were satisfactory and similar, or lower, to NIOSH methods for unmetabolized contaminants in biological fluids (phenol, *p*-cresol and pentaclorophenol in urine, ethanol, pentaclorophenol and toluene in blood) [27]. Results are also similar to another method that applies SPME to the determination of VOCs in exhaled breath samples [28].

Table 6 shows the results for the urine samples from four subjects occupationally exposed to benzene in different service stations. The R.S.D. for the aliquots analysed ranged from 5.7 to 11.8%. A representative chromatogram of the urine samples corresponding to subject Nos. 1 and 2 is shown in Fig. 3. A mass chromatogram corresponding to a blank sample has also been included. As expected, R.S.D. was higher for urine samples than for standards. It should be kept in mind that the precision of urine results represents the entire method, including the sampling stage.

The results indicate that this method is capable of providing sensitive and accurate results for the biomonitoring of benzene in urine in the assessment of occupational exposure to this contaminant. It can also be applied to the determination of benzene in polluted water in environmental analysis.

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