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Article

Headspace Solid-Phase Microextraction Procedure for Gas-Chromatography Analysis of Toluene in Urine

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O tolueno é amplamente usado em aplicações industriais e de laboratório, e está relacionado a problemas de abuso social. O tolueno inalterado em urina é considerado um bioindicador mais específico da exposição ocupacional ao solvente. Entretanto, sua análise apresenta algumas dificuldades de ordem analítica, relacionadas aos baixos valores detectados em urina. Nesta pesquisa foi desenvolvido um método cromatográfico em fase gasosa com detecção por ionização em chama e usando-se a técnica de headspace-microextração em fase sólida em dois diferentes tipos de fibra: polidimetilsiloxana (PDMS) e carboxen-PDMS. Após a otimização das variáveis dos processos de extração, o uso da fibra carboxen-PDMS demonstrou menor limite de quantificação (12,5 ng mL^{-1}), melhor eficiência de extração (até 28,1%) e repetibilidade (CV < 4,9%) com relação à de PDMS. O método foi aplicado na análise de tolueno em urina de trabalhadores expostos a baixos teores do solvente em oficinas de reparo de veículos.

Toluene is widely used in industrial and laboratory applications and in many countries is related with social problems of abuse. Unaltered urinary toluene was introduced as a bioindicator of occupational exposure to the solvent, but its analysis presents difficulties due to the low levels of the compound excreted in urine. A gas-chromatography/flame ionization method for toluene in urine is described using headspace solid-phase microextraction and establishing the better conditions for two different extracting phases: polydimethylsiloxane (PDMS) and carboxen-PDMS. The carboxen-PDMS fiber showed lower quantifying limit (12.5 ng mL^{-1}), better extraction efficiency (up to 28.1%) and repeatability (CV < 4.9%) than PDMS coating. The method was applied to analysis of toluene in urine of workers from car repair shops exposed to low solvent levels.

Keywords: urinary toluene, HS-SPME, sample preparation, gas-chromatography

Introduction

Toluene is one of the most widely used solvents in industry and human exposure to its vapors can result in acute or chronic intoxication. In automotive repair and repainting shops, workers use solvent-containing paints and diluents. The composition of the solvent varies according to the purpose of the product; however, toluene continues to be the most prevalent and is mixed with other compounds such as ethers, ketones and hexane.[1] Both acute and chronic occupational exposure to toluene and other solvents represents a health risk, with the central nervous system being the most affected site.[2] The threshold limit value-time weighted average (TLV-TWA) of 50 ppm of toluene is recommended to prevent its toxic effects to the occupationally exposed workers.[3] Therefore, actual exposure, including inhalant and dermal absorption, can only be ascertained through indicators evaluated in biological specimens.

Some toluene is eliminated in exhaled air and in urine as metabolite by-products, mainly hippuric acid and *o*-cresol. Biological monitoring of workers is currently undertaken by measuring urinary hippuric acid, despite its non-specificity to toluene or occupational exposure.[4,5] Since metabolism of benzoic acid and proteins may yield hippuric acid, its urinary levels in non-exposed subjects is in the range of g *per* g creatinine.

Urinary toluene itself is a more specific biomarker and could be recommended as a biomarker of choice for solvent exposure,[6] but its analysis may be problematic because the very low levels excreted in urine - estimated at about 0.005% of the total amount absorbed.[7,8] The development

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of an analytical method with adequate sensitivity and reliability to apply in routine biomonitoring of the exposed workers is thus a relevant undertaking.

Solid-phase microextraction (SPME) integrates sampling, extraction, concentration and sample introduction into a single step and offers a simple, solvent-free alternative to traditional methods of sample preparation. Headspace-SPME (HS-SPME) is a modification of SPME in which fused-silica fibers coated with a thin layer of selective coating is used to trap and concentrate volatile analytes directly from the headspace.

The HS-SPME technique is promising for analysis of volatile organic compounds (VOCs) in complex matrices.[9-12] This technique has been used for simultaneous analysis of benzene, toluene, ethylbenzene and xylenes (BTEX) in urine, as biomarker in occupational and environmental exposure. In the reported methods polydimethysiloxane (PDMS) fiber coating is used following the detection by gas-chromatography coupled to mass spectrometry (GC-MS).[13-16] Other techniques are described aiming sample preparation for urinary organic solvents analysis as dynamic headspace (purge-and-trap) gas chromatographic with photoionization detection[17] and static headspace followed by GC-MS.[18]

The high sensitivity of HS-SPME technique relative to conventional headspace may be advantageous in labs where exposure is routinely evaluated by an economically more accessible system, such as gas chromatography with flame ionization detector (GC/FID), instead the GC-MS apparatus recommended when headspace is used in sample preparation.

The aim of this study was to develop a HS-SPME method using GC/FID to identify urinary toluene and to evaluate the performance of two different phases, polydimethylsiloxane (PDMS) and crosslinked carboxen-PDMS. The method was applied to analysis of the solvent in urine of workers occupationally exposed to toluene in car repair shops.

Experimental

Chemicals and standard solutions

Analytical grade methanol (Mallinckrodt Baker Inc., Phillipsburg, USA), toluene (AccuStandard Inc., New Haven, USA), pyridine (Vetec Química Fina Ltda., Rio de Janeiro, Brazil), carbon disulfide (E. Merck, Darmstadt, Germany), and sodium chloride (Neon Comercial Ltda., Ipiranga, Brazil) were used. Stock standard solutions (1 mg mL^{-1} and 1 µg mL^{-1}) of toluene and pyridine were prepared in methanol. Spiked urine samples (5-3000 ng mL^{-1}) were prepared fresh from stock solutions prior to the analysis.

Equipment and optimization of gas-chromatography conditions

A GC 1000 gas-chromatograph with a FID detector (Ciola Gregori Ltda., São Paulo, Brazil) equipped with splitless injector inlet liner interfaced to a PC with DANI DS 1000 integrator (Dani Strumentazione Analitica, Monza, Italy) and IQ3 software for data acquisition was used; vortex shaker (Marconi, Piracicaba, Brazil); magnetic stirrers (Supelco, Bellefonte, USA); water bath with digital temperature control interface and circulation mechanism for heating (B. Braun Biotech International GmbH, Melsungen, Germany); PTFE/silicone septum and 15 mL headspace vials (Supelco); fiber holder for manual use; and fibers of 100 µm polydimethysiloxane (PDMS) and 75 µm cross-linked carboxen-PDMS (Supelco Bellefonte, USA). Optimal conditions for chromatographic resolution and efficiency were established. The parameters evaluated were: column; temperatures of injector, oven and detector; and carrier gas flow-rate (nitrogen). Desorption of analytes from the fibers - time and temperature of the injector port - was also established.

Sample treatment

Eight milliliter of urine, previous homogenized, were transferred to the headspace/SPME vial (15 mL capacity) containing an adequate amount of sodium chloride and 1 cm magnetic bar. The vial was sealed with a silicone septum covered internally with polytetrafluoroethylene (PTFE) and heated in a water bath with a digital temperature control interface under magnetic stirring. After reaching the equilibrium, septa was pierced with SPME needle and the SPME fiber was exposed to the headspace for sorption of the volatile compounds.

HS-SPME variables

The variables evaluated for each extracting phase (100 µm PDMS and 75 µm carboxen-PDMS fibers) were: temperature and time of heating, time of exposure of the fiber in the headspace and mass of sodium chloride added to the matrix (Table 1).

Table 1. Variables of the HS-SPME toluene extraction from urine using fibers of 100 µm PDMS and 75 µm carboxen-PDMS

Variable	values
heating temperature / °C	26, 30, 35, 40, 45 and 50
heating time / min	10, 20 and 30
exposure time / min	1, 3, 5, 7 and 10
sodium chloride mass / g	1.0 , 2.0 and 3.0

Selectivity, calibration, limit of detection, limit of quantification, repeatability and extraction efficiency

In the establishment of the figures of merit, urine was sampled from non-smoking subjects unexposed to solvents. The method selectivity for the two fibers was studied by adding the following volatile solvents to the urine: *n*-hexane, *n*-heptane, isooctane, methanol, ethanol, diethyl ether, acetone, ethyl acetate, benzene, chloroform, methyl-isobutyl-ketone and xylenes. These samples were extracted and analyzed under optimized chromatographic conditions. To evaluate the linear range of the analytical method, calibration curves were obtained by analyzing urine samples spiked with toluene (n = 5 for each concentration) over the concentration range of 100-3000 ng mL^{-1} (6 points) when using PDMS fiber and between 12.5-200 ng mL^{-1} (6 points) for the carboxen-PDMS fiber. Plots of toluene/internal standard peak area ratios *versus* analyte concentration were constructed and the relationships were determined by linear least-squares regression analysis. The lower and higher values of the linear range were defined taking in account the LOQ, the coating film thickness and the sensitivity of each fiber. The limit of detection (LOD) of assays was the lowest value distinguished from zero with confidence, i.e. that resulting in a signal-to-noise ratio of 3 (S/N = 3). Quantification limit (LOQ) corresponded to the lowest concentration measured with precision and resulting a signal-to-noise ratio of 10 (S/N = 10). The repeatability of the assay was estimated by analysis of urine samples spiked at low, medium and high toluene concentrations within the linear range of each fiber (six for each concentration level). To establish extraction efficiency, toluene was diluted in carbon disulfide in concentrations of 12.5, 50.0 and 200.0 ng mL^{-1} for carboxen-PDMS and 100, 1000 and 3000 ng mL^{-1} for PDMS fibers. These solutions were injected directly into the gas chromatograph and the results were compared with those obtained from analysis of urine spiked with identical concentrations of toluene.

Sample collection

Urine samples were obtained from 15 non-smoking volunteers who worked in vehicle repair shops. Subjects often handled paint and solvents containing toluene as the principal aromatic solvent. Spot urine specimens were collected directly from the donor in 50 mL polyethylene vials. Aliquots of 8 mL each were immediately transferred to headspace vials containing the internal standard and salt and then sealed. Samples were analyzed up to 3 h following collection.

Results and Discussion

Toluene sampled in urine may be an indicator of its general bioavailability. Gas-chromatography-flame ionization detection is a simple, low cost and reliable system but usually it is not sensitive to unaltered solvents in biological materials when using headspace extraction alone. Thus, coupling HS with SPME as extracting technique can permit GC-FID detection of unaltered solvents in urine.

The optimal conditions established for chromatographic analyses were: capillary column with 100% crosslinked polyethylene glycol phase HP-Innowax (30 m × 0.53 mm i.d. × 0.5 µm film thickness) (Agilent Technologies, Wilmington, USA); detector temperature of 250 °C; injector temperature of 260 °C; carrier gas flow-rate (N_2, 99.999%) of 3.0 mL min^{-1}; and injector operation in splitless mode. Column temperature programming for urine analysis was: 60 °C (initial temperature) held for 3 min, 8 °C min^{-1} to 150 °C, held for 1 min, and 40 °C min^{-1} to 220 °C (final temperature) with a hold time for 3 min (total run time: 21 min). Complete desorption of the analytes was reached after holding the fiber for 3 min in the GC injector port set at 260 °C. Chromatograms yielded adequate separation of toluene and internal standard and a practically noiseless baseline (Figure 1).

Since sources of toluene exposure may contain other solvents, chromatographic conditions were based on resolving analyte and internal standard from substances that might evaporate and adsorb/absorb onto the fiber. There was no co-elution phenomenon between the peaks of target analytes and possible interfering compounds studied. Methanol used as diluent for preparation of the solutions did not interfere with either analyte or the internal standard peaks in the chromatogram after extraction from urine.

Four variables were identified as potentially affecting efficiency: extraction time and temperature of heating, exposure time of the fiber in headspace, and quantity of salt added. Table 2 summarizes results of the optimal HS-SPME extraction conditions established for each coating phase.

Table 2. Optimized values of the variables influencing toluene extraction from urine samples with HS-SPME using phases 100 µm PDMS and 75 µm carboxen-PDMS

Variable	extracting phase	
	PDMS	carboxen-PDMS
heating temperature / °C	26	30
heating time / min	20	20
exposure time / min	3	10
sodium chloride mass / g	3.0	3.0

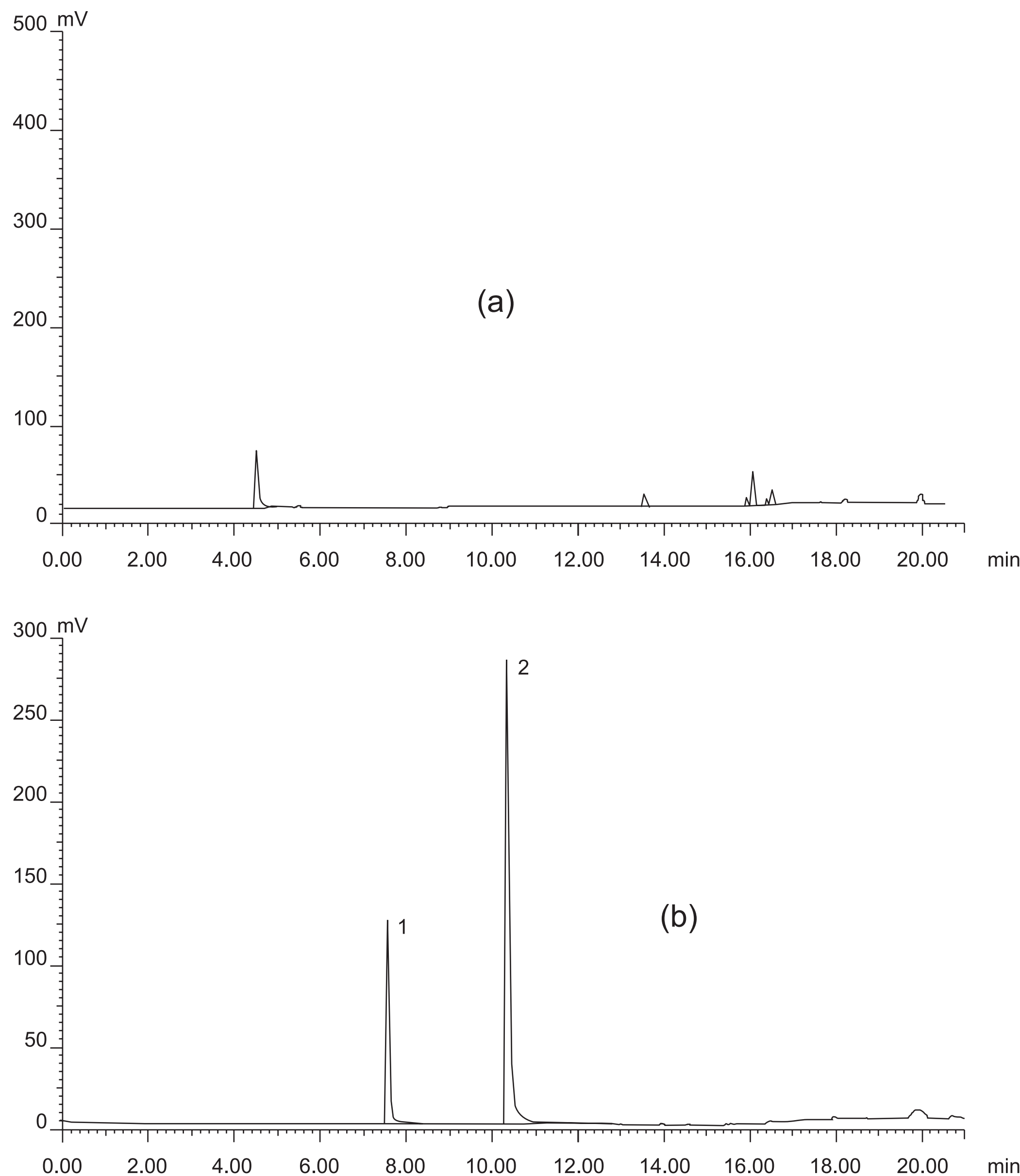

Figure 1. (a) Chromatogram of blank of urine and (b) Chromatogram of urine containing 80 ng mL^{-1} of toluene (1) and 500 ng mL^{-1} of pyridine (internal standard, 2) extracted by carboxen-PDMS fiber.

HS-SPME extraction of volatile substances is particularly affected by fiber chemistry and type of matrix modification used. Although HS-SPME is equilibrium rather than an exhaustive extraction technique, by careful adjustment of the extraction conditions significant enhancement in sensitivity can be achieved to enable the detection of volatile analytes.[11]

Moreover, SPME from aqueous matrices requires stirring to allow rapid extraction by transporting analytes from the bulk of the solution to the vicinity of the fiber and to reduce the "depletion zone" effect.[19] The spiking of urine samples was performed immediately before analysis, as recommended by Ikeda,[20] to prevent loss by volatilization of the solvents.

Heating temperature and time of exposure of the fiber in headspace were the parameters that most influenced the results. Table 3 shows results of the validation parameters established for the two fibers studied.

Polydimethylsiloxane is more resistant, indeed the film is the thickest among the fibers commercially available, but its efficiency is poorer than carboxen-PDMS (Table 3). Better sensitivity, although shorter dynamic range, of carboxen-PDMS phase relative to pure PDMS in extraction of toluene, benzene, ethylbenzene

Table 3. Parameters of merit for urinary toluene by HS-SPME/GC-FID using 100 μm PDMS and 75 μm carboxen-PDMS fibers

Validation Parameter	PDMS	carboxen-PDMS
linearity range/(ng mL^{-1})	100-3000	12.5-200
Regression	y = 0.4861x – 0.044	y = 0.0143x + 0.021
correlation coefficient (r)	0.9971	0.9997
LOD/(ng mL^{-1})	50	5.0
LOQ/(ng mL^{-1})	100	12.5
intra-assay precision/CV%	7.0-11.9	3.9-4.9
extraction efficiency/%	2.9-3.2	26.7-28.1

and xylenes (BTEX) from soils was reported by Ezquerro *et al.*[21]

The pores in carboxen-PDMS fibers gradually taper so that minor gaseous molecules diffuse more deeply into them and remain adsorbed and rigorous desorption conditions are needed to ensure no carryover analytes.[11] This is not observed in pure PDMS phase, where retention is by partition. Peak broadening may occur with SPME because the analyte can delay desorption from the fiber, particularly when using carboxen-PDMS coating. The temperature slope was increased to 220 °C to avoid potential interference of extracted compounds strongly retained in the column, which may appears as ghost peaks. This is relevant because the composition of urine is variable.

The LOQ for urinary toluene is lower (1.60 ng mL^{-1}) when using HS-SPME followed by gas-chromatography - mass spectrometry detection.[16] For blood toluene analysis using HS-SPME and GC/FID, the LOD reported by Alegretti *et al.*[9] was 500 ng mL^{-1}. Both of these studies used 100 μm PDMS fibers.

Levels of blood toluene may reach concentrations ranging from 500 to 5,000 μg mL^{-1} under extreme exposure.[9,12] Although the quantity of solvent is about ten times lower in urine than in blood,[7] PDMS phase is more suitable than crosslinked carboxen-PDMS fiber for quantifying high toluene levels in urine when considering both the linearity range achieved and polarity.

Urine offers advantages in relation to blood as sample to be used in biomonitoring exposure due to its non-invasive sampling and higher volume available. The addition of salt eliminates the ionic strength variations common in biological matrices as urine.

Table 4 presents the results from urine analysis of samples collected from exposed workers and extracted by the two SPME fibers. To evaluate the level of toluene absorption by the workers, hippuric acid was also analyzed in the samples according to the Kira (1997) method.[22] All samples were analyzed in duplicate.

Table 4. Toluene in urine (ng mL^{-1}) from the exposed volunteers, extracted by PDMS and carboxen-PDMS fibers

samples	PDMS	Carboxen-PDMS
1	nq	15.2
2	nq	17.8
3	nq	19.5
4	nq	29.7
5	nq	39.5
6	nq	39.9
7	nq	42.7
8	nq	42.8
9	nq	52.8
10	nq	55.6
11	nq	69.5
12	nq	70.1
13	112.2	109.4
14	121.18	118.2
15	124.3	122.9

nq = not quantified (LOQ-PDMS fiber = 100 ng mL^{-1}).

The toluene in the end-of-shift spot urine sample appears to be the marker of choice for biological monitoring of occupational exposure at low air solvent levels.[23] The correlation between toluene concentration in the air and the end-of-shift urine sample was excellent as reported by Ducos *et al.*[6] In exposure of workers to 50 ppm of toluene (TLV-TWA), the concentration of the solvent excreted in urine is approximately 75.6 ng mL^{-1}.[6] Therefore, the carboxen-PDMS fiber is more appropriate for monitoring subjects exposed to low concentrations (LOQ = 12.5 ng mL^{-1}).

Since exposure of the extracting phase is not made by direct immersion the fiber it may be used for extraction several times (about 150), particularly when using a matrix rich in non-volatile compounds, such as urine. Another advantage of headspace is that there is frequently an important decrease in extraction time relative to direct immersion of the fiber.[24]

In exposed workers the use of a carboxen-PDMS fiber for toluene extraction allowed for the detection of the unaltered solvent using GC-FID in all samples; PDMS extraction permitted detection in only three of these. The absorption of toluene by the workers was probably low, as supported by urinary hippuric acid levels found. These values varied between 0.18 and 1.52 g *per* g creatinine, indicating exposure below the biological exposure index (BEI) for this metabolite adopted by ACGIH, 2006 (1.60 g *per* g creatinine).[3]

Conclusions

The use of polydimethylsiloxane fibers resulted in better chromatographic efficiency and a broader linear range, although carboxen-PDMS coating showed lower LOQ and better precision and extraction efficiency when applied to analysis of urinary toluene by GC/FID. Routine biomonitoring of exposed workers could be more economically accessible using the method described.

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