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Short communication

Analytical method for evaluation of exposure to benzene, toluene, xylene in blood by gas chromatography preceded by solid phase microextraction

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Abstract

Frequency of intentional exposure to organic solvents has been increasing among children and adolescents in Brazil. Analysis of benzene, toluene and xylenes (BTX) in human blood is necessary to diagnose the intentional and accidental exposure to these solvents. A method for BTX determination in blood samples by gas chromatography preceded by solid phase microextration (SPME) from headspace (HS) has been described. SPME has several advantages when compared to other extraction techniques such as simplicity, low cost and solvent-free extraction. The method presents good repeatability (precision was of 2.2–8.0%), accuracy from −4.7 to −9.4%, limit of detection <1.0 ug/mL, linearity from 1.0 to 100 ug/mL for toluene and from 5.0 to 100 ug/mL for the other solvents ($R^2 > 0.99$), which shows to be efficient and adequate for the detection of exposure to BTX in blood samples.

Keywords: Benzene; Toluene; Xylene

1. Introduction

Organic solvents benzene, toluene and xylenes (BTX) are present in a range of commercial products because of their wide industrial use in the production of plastic, paints, glues, solvents, and also as intermediates in the production of other chemical substances [1,2]. Due to its carcinogenicity, the industrial use of benzene is legally limited in Brazil, but monitoring its exposition is still important, because it is a major contaminant of many toluene-based products such as thinners, paints, and adhesives.

When inhaled, these organic compounds are readily absorbed by the lungs. A significant absorption may also occur through the skin, whenever these solvents are in contact with it. Part of the BTX is eliminated unchanged in urine, but most of it is metabolized in the liver and excreted in the form of its metabolites. Toluene and xylenes suffer oxidation followed by conjugation with glycine, producing mainly hippuric and methyl hippuric acid, respectively. Benzene suffers the action of oxidases and hydrolases, besides conjugation with glutation, sulphates and glicuronic acid, producing several metabolites, such as phenol, phenylmercapturic acid, *trans*, *trans*-muconic acid and catechols. Analysis of the concentration of these biotransformed compounds in urine is used for occupational monitoring of workers exposed to these solvents [3]. On the other hand, quantitative analysis of solvents in blood is most likely to correlate with patient's clinical conditions [4].

Inhalant abuse is a significant world wide problem. Availability and low cost of inhalants have contributed for the increase of incidence of intentional inhalation of volatile substances by children and adolescents [5,8–11]. Effects of BTX start rapidly, and may lead to severe poisoning [5]. The resultant toxic effects are similar to those caused by ethanol, causing, at first, euphoria, loss of inhibition, and hallucinations, followed by lethargy, slurred speech, and other depressor effects of the central nervous system (CNS). High doses may lead to convulsions, asphyxia, cardiac dysfunctions, and cessation of breathing, coma and death [1,4,5,8,9]. Levels of toluene in blood can reach concentrations of 500–5000 mg/L, which can induce encephalopathy and severe abnormalities, including brain

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degeneration, cortical atrophy and damage in mental and intellectual performance [6].

Products used for abuse include hair spray, gasoline, paints, diluents, solvents, among others which are easily obtained world wide [5,7,8,10]. Several substances are found in these products, however, toluene is the main constituent [11].

Traditional procedures for BTX analysis make use of sample preparation techniques which use great amounts of solvents, are time consuming, laborious and demand many stages of manipulation. Each one may introduce errors and sample loss especially when volatile compounds are being analyzed [12,13].

Solid phase microextration technique (SPME) presents extraordinary advantages compared to the traditional methods, because it integrates sampling, extraction, concentration and introduction of the sample in a single stage which significantly decreases the use of solvents in the laboratory [2,13–23]. SPME extraction can be performed by direct immersion—when the fiber is introduced and directly exposed in the sample—or by headspace (HS). In headspace extraction, the fiber is exposed to the gaseous phase of the sample, which is obtained by previous heating of the sample in a sealed vial [13,14,21,22,24]. At this point, analytes partition between the matrix and the stationary phase [13] and the adsorption of the analytes in fiber coating takes place [17]. After equilibrium is reached between the involved phases, or after a determined time, the fiber is introduced and exposed in the gas chromatograph (GC) injector so that the thermal desorption of the analytes for the chromatographic column occurs [13,16,18,23,25]. Three phases are involved in the extraction process: fiber coating, headspace and liquid phase of the sample. Consequently, the analyte affinity for these phases will determine extraction efficiency [23].

Sampling for HS is generally used to analyze volatile [13,15,16,22,26–28] and semivolatile [15,23,26,28] compounds and has the advantage of a lower equilibrium time than direct immersion [12,21,26], due to the fact that the diffusion coefficients of gaseous phase present, typically, four orders of magnitude higher than the liquid phase ones [13,17,18]—the time to reach the equilibrium is inversely proportional to the analyte coefficient of diffusion [29]. SPME headspace technique is ideal for biological specimen analyses that contain great amounts of high molecular weight compounds and other interfering ones [2,12,23,30], preventing any contamination in the fiber and in the gas chromatograph column [13,26], and hence allowing a better selectivity for the analyte of interest.

One of the most significant advantages of SPME is the small amount of sample necessary for the preparation process [2,12]. Furthermore, there is the possibility of using the fiber to extract analytes in concentrations to the level of traces (pg/mL) [18,23].

A methodology for volatile organic compounds determination in biological sample is a valuable tool in abuse and emergency toxicology. In this study, the following variables were assayed: heating temperature and sample stirring time in thermoblock, time of sampling adsorption and analytes desorption from the fiber, chromatographic conditions, selectivity of the method, limit of detection, linearity, precision and accuracy of SPME headspace technique for BTX compounds in blood samples.

2. Materials and methods

SPME fused-silica fiber, 10 mm of length, 100 um polydimethylsiloxane-coated (PDMS) was obtained from Supelco (Bellefonte, USA). SPME fiber was pre-conditioned according to manufacturer instructions, inserted in the injector and exposed to a temperature of 200 °C over a 1 h period.

For sample heating a thermoblock was used (Pierce 18.940 model). Polytetrafluoroethylene (PTFE) silicon septum, 20 mm, was used to seal 10 mL vials for headspace. For samples stirring during heating in thermoblock a magnetic bar of 10 mm was used.

All of the reagents used in this study were of analytical degree. Benzene, toluene and methanol were obtained from Merck (Darmstadt, Germany). Sodium chloride, xylene and its isomers were obtained from Carlo Erba (Milan, Italy).

A stock solution of 1000 ug/mL was prepared from each organic compound—benzene, toluene and *o*-, *m*-, *p*-xylenes—diluted in methanol. Working solutions were prepared from the stock solution containing the following concentrations 0.5, 1.0, 5.0, 10, 25, 50 and 100 ug/mL in a pool of whole human blood obtained from individuals not exposed to these solvents. The same procedure was applied to prepare the spicked samples used for precision and accurate validation. This assay is part of a research protocol approved by the Ethics Research Committee of Universidade Federal de São Paulo and is registered under the number 649/01.

Blood samples used for the evaluation of the methodology were collected in vacutainer tube with EDTA and, in case the sample was not promptly analyzed after the collection, they were stored at −20 °C.

2.1. Headspace conditions, adsorption and desorption

Temperatures of 40, 60 and 80 °C for vial heating with sample in thermoblock were tested. The vial was kept for, at least, 20 min to allow the analytes to reach the equilibrium between gas and blood phases. Times tested for adsorption stage of the sample in the fiber were 2, 3 and 5 min and times for desorption of adsorbed components in the fiber in GC injector were 1, 3 and 5 min.

2.2. Chromatographic parameters

Chromatographic separation was performed in Varian 3300 Gas Chromatograph equipped with flame-ionization

detector (FID) and 4290 Integrator. Nitrogen was used as the carrier gas at a constant flow of 6.0 mL/min, injector and detector temperature were kept at 200 and 250 °C, respectively. The GC was equipped with splitless injector inlet liner (1060/1061 Varian) for analytes thermal desorption from the SPME fiber. Analyses were performed with DB-624 megabore column (30 m × 0.53 mm i.d. × 3 um film thickness). Oven temperature was kept at 40 °C for 2 min, increasing it at a 6 °C/min rate until it reached 105 °C, keeping this temperature for 2 min. Other chromatographic slopes were tested to evaluate the best selectivity and separation of chromatographic peaks.

2.3. Headspace extraction procedure

In a headspace vial of 10 mL containing a magnetic bar of 10 mm and 1.0 sodium chloride, 2.0 mL of previously homogenized blood sample was added. The vial was sealed with a septum and closed up to prevent evaporation of volatile compounds. The vials were kept heated at 40 °C under magnetic agitation in thermoblock during 20 min.

After the equilibrium, septa were pierced with SPME needle and then, the SPME-PDMS fiber was exposed to headspace during 2 min to effect the adsorption of volatile components present in the sample. After that, the SPME fiber was collected and then inserted directly in the GC injector at 200 °C, to allow thermal desorption of substances adsorbed for GC column. The fiber was kept in the injector for 3 min, then collected inside the needle and withdrawn from the chromatograph. Total time of the chromatographic analysis was 15 min.

In order to check the existence of any carry-over phenomena and/or external contamination between analyses, blank analyses were carried out by exposing the SPME fiber in the injector for 2 min and then analyzed as a regular sample.

2.4. Analytical validation

The study of chromatographic interference for detection of the co-elution phenomenon between substances was performed from tests of addition of solvents with chromatographic retention times close to BTX, according to data obtained in literature [4,24,27]. Individually, methanol, ethanol, diethyl ether, acetone, *n*-hexane, methyl ethyl ketone, ethyl acetate, chloroform and trichloroethylene were added and analyzed.

Calibration curves were constructed by analyzing working solutions containing blood spiked with 0.5–100 ug/mL of BTX. No internal standard was used. For attainment of dynamic interval and the linearity, analyses with these working solutions were carried out in a similar way. Coefficient of linear correlation (R^2) was used to predict the slope of the curve (a) and the intersection (b) of the calibration curve $y = ax + b$, where "y" corresponds to the analyte chromatographic area and "x" to its blood concentration (ug/mL). Quantification of the results was obtained via an external calibration, using the calibration curve.

Limit of detection (LOD) of the assay for each aromatical hydrocarbon was considered as the lowest detected value different from zero, due to the fact that the sample blank analysis did not show any chromatographic signal.

The precision of the method was expressed as variation coefficient, CV%. The repeatability was estimated by replicate analyses of samples spiked at concentrations of 10, 50 and 100 ug/mL of BTX ($n = 6$).

The accuracy of the method was obtained via the replicate analyses of samples spiked at concentrations of 100 ug/mL ($n = 6$). Accuracy was expressed as the percentage of deviation between the true and the measured value.

The data were plotted in the software Windows Excel spreadsheet and the statistical analyses of the study were performed according to the ANOVA method.

After validation, the method was used to quantify organic solvents in blood sample collected from an individual suspected of being an abusive inhalant user.

3. Results

The results of linearity, coefficient of linear correlation (R^2), limits of detection, intraassay precision and accuracy are summarized in Table 1.

The method studied showed a linear relationship at dynamic ranges for benzene, toluene and xylenes. The correlation coefficients of the calibration curves were all $R^2 > 0.99$. Precision for replicate analysis was lower than 8.0% for all BTX in the three levels evaluated. The accuracy of the method ranged from −4.7 to −9.4%.

Table 1
Results of technical validation for BTX analysis in blood samples

Compound	Dynamic range (ug/mL)	Correlation coefficient (R^2)	Limits of detection (ug/mL)	Accuracy (%)	Intraassay precision (%)		
					10 ug/mL	50 ug/mL	100 ug/mL
Benzene	5.0–100	0.9971	1.0	−9.4	5.2	4.3	5.8
Toluene	1.0–100	0.9989	0.5	−4.7	4.5	4.5	3.7
p-Xylene	5.0–100	0.9989	1.0	−2.7	6.2	5.6	2.8
m-Xylene	5.0–100	0.9991	1.0	−2.7	6.2	5.8	2.8
o-Xylene	5.0–100	0.9988	1.0	−2.3	8.0	4.6	2.2

Table 2
Retention time of possible interfering compounds evaluated

Compound	Retention time (min)
Methanol	1.76
Ethanol	1.84
Diethyl ether	1.93
Acetone	2.17
n-Hexane	3.21
Methyl ethyl ketone	4.04
Ethyl acetate	4.41
Chloroform	4.93
Benzene	6.3
Trichloroethylene	7.33
Toluene	10.11
p-Xylene	13.29
m-Xylene	13.5
o-Xylene	14.38

There was no co-elution phenomenon between the peaks of target-analytes (BTX) and possible interfering compounds in selectivity evaluation (Table 2). The chromatogram presented a satisfactory separation of benzene, toluene and xylene isomers peaks and a baseline which was practically noiseless (Fig. 1). Methanol was used to prepare the working solutions.

The best headspace conditions were obtained in heating temperature of 40 °C in thermoblock, for there was no formation of clots in the blood sample which resulted in the best chromatographic signal. The different tested times of adsorption did not show significant difference, therefore, the lowest time evaluated was established (2 min); there was no significant difference in desorption times 3 and 5 min, although the time 1 min presented residues in the fiber when it was injected again in the chromatograph, which is an evidence of insufficient time to get through desorption. Due to this fact, the lowest time was chosen (3 min).

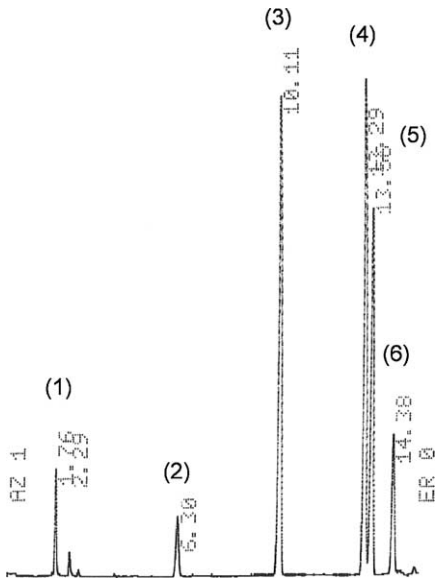

Fig. 1. Chromatographic analysis of working solution of BTX 10 ug/mL in blood sample by headspace SPME/GC/FID. Key: (1) methanol, (2) benzene, (3) toluene, (4) *p*-xylene, (5) *m*-xylene, (6) *o*-xylene.

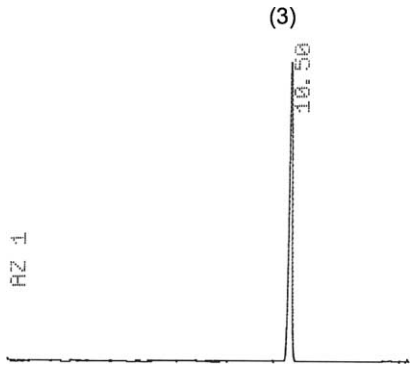

Fig. 2. Chromatographic analysis of a real blood sample from an inhalants user by headspace SPME/GC/FID. The toluene concentration detected was 16.1 ug/mL. Key: (3) toluene.

Crossed contaminations can also occur in the septum, in the vials and with solvent vapors present in the laboratory atmosphere. Blank analyses performed between the run samples confirmed that external contamination and carry-over between the samples was not evident.

The Chromatogram of the analysis of a sample collected from one inhalant user is shown in Fig. 2. This sample presented only toluene at concentration 16.1 ug/mL which was obtained from an interpolation of the calibration curve.

4. Discussion

The most significant disadvantages perceived in traditional methods for solvent extraction in biological samples are usually time-consuming and demand multiple stages. Furthermore, excessive amounts of organic solvents are needed, and laboratory staff may also be exposed to hazardous substances.

SPME is a useful technique for the analysis of volatile compounds because it is simple, fast, sensitive, and solvent-free. Headspace sampling is important to protect the fiber from any damages caused by high molecular weight substances and other non-volatile contaminants present in the sample matrix [12,21]. Moreover, the chromatogram is represented by a practically noiseless baseline and the fiber can be reused about 100–200 times, thus decreasing analysis cost.

Analyte transfer from the first phase to the polymeric phase is promptly initiated when the coated fiber comes in contact with the sample or its headspace [12]. For most cases, SPME extraction is considered completed when analyte concentration reaches a balance of distribution between these involved phases [12,14,19,20]. In practical terms, this means that, once the equilibrium is reached, the extracted amount remains constant, within experimental error limits, and is non-dependent on whether extraction time increases [5,12].

The necessary time to reach the extraction equilibrium is influenced by several factors, such as the chemical compounds involved, thickness and composition of stationary phase of the SPME fiber, volume of the sample and headspace, time and temperature of extraction, sample stirring, addition and amount of sodium chloride [13,16,19–23,28,29]. These factors must therefore be carefully analyzed for the extraction optimization of each compound over validation.

Stirring technique, with magnetic bars with pre-defined speed, is a procedure applied to decrease "zone of depletion" which occurs in the neighborhoods of the fiber as a result of the fluid barrier formed [12]. These mechanisms make mass transference from condensed phase to gaseous phase easy [20,22], increase analytes recovery and speed up the process of chemical equilibrium [12,21,23]. Addition of sodium chloride in the sample improves the efficiency of volatile compounds extraction in the matrix, for it increases the ionic force of the solution, and consequently it decreases their solubility and increases the partition coefficient [13].

The temperature of 40 °C for the thermoblock was established, since for sensitivity represented by obtained peak areas was satisfactory, and indeed at this temperature there was no formation of clots in the blood sample, which would affect stirring rate and component release to the headspace, yielding non-reproducible results. Heating temperature of sample has a significant effect to the process kinetics, due to analyte vapor pressures [12].

To keep precision and reproducibility, these conditions and other ones such as incubation temperature, sample agitation and volume, times of adsorption and desorption must be kept constant [5]. Time and temperature used in desorption influence recovery and these need to be optimized. The conditions of the GC were adjusted to perform an efficient separation at a reasonable run time. Final oven temperature of 105 °C was established to allow the elution of all extracted compounds of the column. As there is no use of extraction solvents, injections were performed in the splitless mode to assure complete analyte transference and sensitivity.

The method showed to be of easy performance, requiring a small volume of blood (2 mL) showed satisfactory repeatability, limit of detection, linearity, and accuracy, which therefore makes the use of internal standards not necessary. The applicability of the method was confirmed by the analysis of a real sample collected from an individual suspected of inhalant abuse.

Sensibility of 0.5 ug/mL may seem insufficient when compared with literature data for analysis of BTX by other methods [2,20,23,30]. But LOD can reach 0.02 ug/mL by altering GC range, and since the objective of this method was to diagnose abuse of BTX, it is necessary to maintain the range tested to guarantee linearity in high concentrations, which are frequent between inhalant abusers.

Chromatographic separation of xylene isomers is hard to be achieved, but usually they do not need to be quantified separately, because its concentration can be obtained from by the sum of isomers peaks.

5. Conclusions

The validated analytical method for BTX determination in this study presented efficiency in detection, identification and quantification of benzene, toluene and xylenes compounds in blood samples. The results of the validation were satisfactory in terms of linearity, specificity, limits of detection, precision, and accuracy within the range studied. This method has been readily and routinely applied in our laboratory, showing evidence to be adequate for the sample analysis of individuals that are suspected of extreme exposure to these organic solvents.

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