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# Box-Behnken Experimental Design for Optimizing HSSPME-GC-MS

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## Abstract

The goal of the present study is to find the optimal conditions of temperature and extraction time, VOC and NaCl amount, and desorb time of extraction reaction that takes place in the vial while performing the HS-SPME procedure for the determination by GC/MS of six analytes (benzene, toluene, ethyl benzene, ortho xylene, metha xylene and para xylene) and the corresponding IS (MIBK) by fitting and analyzing a response surface for each one. The paper shows Box-behnken experimental design (BBD) as a useful technique to improve and optimize an extraction condition and embody the effect that some experimental factors exert on several analytical responses. A five-factor, three-level BBD was used to optimizing. Forty six batches were prepared and evaluated for responses as dependent variables .In the optimal experimental conditions (0.32 gr NaCl,2.67 microliter VOC, 40 ◦C extraction temperature, 15 min extraction time and 60 second desorb time) determined by means of a HS-SPME–GC/MS system is carried out.

**Keywords:** Box–Behnken; optimization; HS-SPME-GC/MS; BTEX

## Introduction:

Response surface methodology (RSM) was used to optimize the extraction parameters such as sample volume, extraction time and temperature, salt addition, time of turbulence of the sample for the HSSPME extraction of urinary BTEX. The optimum extraction conditions were determined using the experimental design called Box-Behnken design (BBD) [1], BBD has been used as a tool for multivariate optimization also are a branch of rotatable or semi rotatable second-order designs that are based on three-level imperfect factorial designs[2]. The strategy developed has been feasible with headspace solid- phase

micro extraction and gas chromatography coupled with mass spectrometry (HS-SPME–GC/MS) technique in urine samples to determine six aromatic compounds namely benzene, ethyl benzene, toluene, ortho xylene, metha xylene and para xylene. Thousands of human-produced toxicants are uninterrupted emitted into the environment and, as a consequence, the basic population is exposed to a large number of contaminants inhaled via air, ingested with food, and adsorbed through skin. The contaminant benzene, toluene, ethylbenzene and xylenes (BTEX) are mainly used as additives in gasolineto enhance octanerating, in displacement of alkyl-lead compounds, and to improve the ignition process. And also extant in main- and side-stream tobacco smoke, which provides one of the largests ources of personal exposure to air pollutants. Meanwhile, toluene, xylene and ethylben-zene are neurotoxic. Benzene is hematotoxic and has been classified as a Famous carcinogen to humans by the International Agency for Research on Cancer (group 1)[3]

Therefore, because of the toxicity of these substances and the fact that legislation is organizing smaller maximum concentrations for these substances the improvement and optimization of methods with low detection limits are very important. The recent studies refers to the use of the gas chromatography with mass spectrometry (GC/MS) for the analysis of these compounds simultaneously with a prior step of preconcentration of the sample like solid-phase microextraction (SPME).SPME as a solvent-free alternative to the older sample preparation and preconcentration techniques was introduced by Pawlyszyn [4],SPME is well adapted to use in conjunction with GC [5–7] different SPME fibers are available commercially for polar, nonpolar and semi polar analytes, but expensive, low mechanical persistence, and low extraction capacity has led to the expansion of new laboratory-made fibers with favorable characteristics [8–10] SPME is a comparatively new method usable for aromatic compounds extraction. It is a very easily and efficient, solvent-free sample preparation method for analysis of organic compounds. A coated silica fiber is directly injected into the sample or into the headspace above the sample (HSSPME). Because there are numerous factors that can influence in the HSSPME and, therefore, in the detection limit of the technique, the optimization step is an essential stage previous to the validation of the method. Among the factors related to HSSPME, The most common are the sample volume, extraction time and temperature, salt addition, time of turbulence of the sample [11,12] .Likewise, It is frequent to compare the result obtained using fibers with different coatings and by direct immersion in the sample or by headspace(HS)[13,14]. In this study we proposed a simple and fast extraction method for the determination of BTEX in URINE. The optimization of effective factors on the HSSPME intensity has been carried out by using Box-Behnken design [15].

## Experimental

## Materials and Methods

Volatile organic compound (VOC), NaOH and MIBK were purchased from Merck(Darmstadt, Germany. The stock standard solution of VOC mixture was prepared at 200 mg/L in MIBK and stored at 4 °C until used.

For the SPME a polydimethylsiloxane/divinylbenzene fiber (PDMS/DVB, 65 micro meter film thickness) was used. It was supplied by Supelco (Bellefonte, PA, USA).

***Instrumentation and software***

The analysis of the samples was carried out using a gas chromatograph Agilent 6890N equipped with a splitless injector, coupled to an Agilent 5975 quadrupole mass selective detector (Agilent Technologies, PaloAlto, CA, USA). Chromatographic separation was achieved with the J&W DB-5MS capillary column with dimensions of 25 m × 0.25 mm I.D. × 0.25 mictrometer film thickness (J&WScientific,Folsom,CA,USA).

## Experimental procedure

## Sample Prepration

All samples were prepared by adding 3ml drug-free Urine to 4 microliter internal standard (MIBK, 100 mg/L) and specified volumes of VOC (200 ppm) with NaCl values assigned according to BBD table (Table 2). The total volume was transferred to a 5 mL vial to perform the HS-SPME process and was encapsulated. Then, vials were sealed with a silicone/PTFE septum and placed in the shaker bath. Afterwards, the samples are extracted with the HS-SPME procedure according to the type of sample, as explained in follow.

## Parameters of SPME and GC–MS

The PDMS/DVB fiber was conditioned according to the specifications of the manufacturer before its first use (250 ◦ C for 30 min). The sampling depth was fixed at 20 mm. The depth of penetration of the needle in the injector port was 35 mm and the fiber was desorbed at 280 ◦ C. During the extraction process the vial was placed in a shaker bath and the sample was agitated during 500 s followed by 5 s in rest. Injections were performed in splitless mode. The injector was kept at a temperature of 280 ◦ C. The oven temperature was maintained at 40 ◦ C and was increased until 190 ◦ C with a heating rate of 20 ◦ C/min.

Head space solidphase micro extraction

Different parameters that effected the HSSPME extraction efficiency were studied and optimized, incloding VOC volume (2-6 microliter), NaClamount (0-0.5 gr), extraction time (10-20 min), extraction temperate(40-60 C) and desorbe time(30-90 sec).

Experimental Design

Based on the results of single-factor experiments a 3-level, 5- factor Box-Behnken design requiring 46 experiments was performed for optimization of HSSPME to acquire the maximum extraction. Table1 represents the levels of each factor. The experimental data (area in GC) are recorded in Table2. Experimental design and statistical analysis of the results were performed by Minitab statistical Software. All experiments were repeated three times.

**Table1. The chosen levels of variables for Box- Behnken design.**

| nomber | VARIABLE | Low | Middle | High |
|---|---|---|---|---|
| 1 | VOC( microliter) | 2 | 4 | 6 |
| 2 | NaCl(gr) | 0 | 0.25 | 0.5 |
| 3 | Extraction time | 10 | 15 | 20 |
| 4 | Extraction temperate | 40 | 50 | 60 |

| | | | | |
|---|---|---|---|---|
| 5 | Desortion time | 30 | 60 | 90 |

**Table2. The required quantities of the VOC, NaCl amount, temperature and time of extraction and desorption time based on BBD.**

| run | Des,Time (sec) | Ex,Time (min) | Ext.Temp | VOC (microL) | NaCl (gr) | run | Des,Time (sec) | Ex,Time (min) | Ext.Temp | VOC (microL) | NaCl (gr) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 30 | 15 | 50 | 2 | 0.25 | 24 | 60 | 15 | 50 | 2 | 0 |
| 2 | 90 | 15 | 50 | 2 | 0.25 | 25 | 60 | 20 | 50 | 4 | 0 |
| 3 | 60 | 10 | 40 | 4 | 0.25 | 26 | 90 | 15 | 50 | 6 | 0.25 |
| 4 | 60 | 15 | 40 | 2 | 0.25 | 27 | 60 | 15 | 60 | 4 | 0.5 |
| 5 | 30 | 10 | 50 | 4 | 0.25 | 28 | 60 | 15 | 60 | 6 | 0.25 |
| 6 | 60 | 20 | 60 | 4 | 0.25 | 29 | 60 | 15 | 50 | 4 | 0.25 |
| 7 | 60 | 15 | 50 | 2 | 0.5 | 30 | 60 | 15 | 50 | 4 | 0.25 |
| 8 | 60 | 20 | 50 | 6 | 0.25 | 31 | 60 | 10 | 60 | 4 | 0.25 |
| 9 | 60 | 20 | 50 | 2 | 0.25 | 32 | 30 | 20 | 50 | 4 | 0.25 |
| 10 | 60 | 20 | 40 | 4 | 0.25 | 33 | 60 | 10 | 50 | 4 | 0.5 |
| 11 | 60 | 15 | 50 | 4 | 0.25 | 34 | 60 | 15 | 50 | 6 | 0 |
| 12 | 90 | 15 | 40 | 4 | 0.25 | 35 | 60 | 20 | 50 | 4 | 0.5 |
| 13 | 60 | 15 | 40 | 6 | 0.25 | 36 | 30 | 15 | 50 | 4 | 0.5 |
| 14 | 60 | 15 | 50 | 6 | 0.5 | 37 | 90 | 10 | 50 | 4 | 0.25 |
| 15 | 60 | 15 | 50 | 4 | 0.25 | 38 | 90 | 15 | 60 | 4 | 0.25 |
| 16 | 30 | 15 | 60 | 4 | 0.25 | 39 | 60 | 15 | 40 | 4 | 0.5 |
| 17 | 60 | 15 | 60 | 4 | 0 | 40 | 60 | 15 | 40 | 4 | 0 |
| 18 | 60 | 10 | 50 | 2 | 0.25 | 41 | 90 | 20 | 50 | 4 | 0.25 |
| 19 | 30 | 15 | 50 | 6 | 0.25 | 42 | 30 | 15 | 40 | 4 | 0.25 |
| 20 | 90 | 15 | 50 | 4 | 0 | 43 | 60 | 10 | 50 | 4 | 0 |
| 21 | 60 | 15 | 60 | 2 | 0.25 | 44 | 90 | 15 | 50 | 4 | 0.5 |
| 22 | 60 | 10 | 50 | 6 | 0.25 | 45 | 30 | 15 | 50 | 4 | 0 |
| 23 | 60 | 15 | 50 | 4 | 0.25 | 46 | 60 | 15 | 50 | 4 | 0.25 |

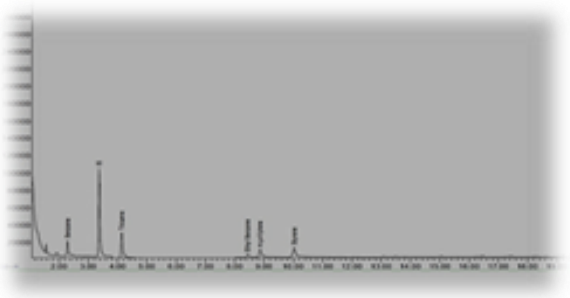


**Fig. 1.Chromatographic profiles of urinary BTEX**

## Result and discussion

The chromatographic profil of a urine sample were presented in Fig. 1. Each chemical was univocally identified by its retention time and mass to charge ratio, and this was confirmed by the use of the proper deuterate internal standard. In this study, in order to achieve a better understanding of the effects of the independent variables and their interactions on HSSPME, 3D response surface plots for the extent responses were generated based on the model equation. As the regression model has five independent variables, one variable was kept at constant at the center level for each plot, thus, a total ten response 3D plots were formed for responses. Figure2 shows the 3D response surfaces as the functions of two variables at the center level of other variables. By analyzing three-dimensional response surface, the following optimum values of these significant factors were presented in Table 3. In the optimum condition the highest HSSPME was achieved and corresponding to Fig.2, all of the independent variables have the most positive effect on the HSSPME at their highest levels.

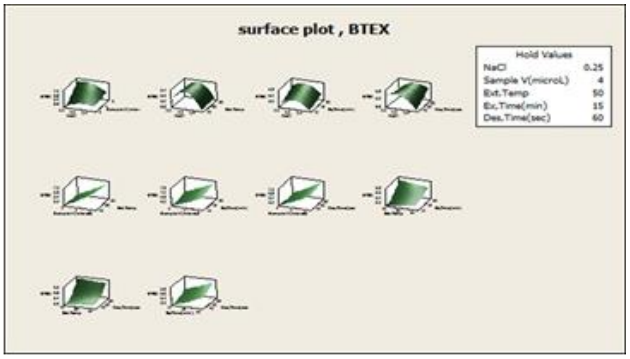



**Fig2. 3D response surfaces as the functions of two variables at the center level of other variables for urinary BTEX with HSSPME.**

In optimizing condition of HSSPME (Table 3), calibration regession graph was shown in Fig.3 and was presented in Table. 4. In order to examine the applicability of the proposed method, we measured the analytes concentration in the real samples. Urine samples from different persons were supplied by the diagnostic laboratories (Tehran, Iran). To examine the reliability of the method certain amounts of standard solution (VOC) was added to the sample solutions and analyzed according to the proposed method. The results show a good and acceptable linear relationship ( Table. 5).

**Table3. Variables and Optimized Responses in Box–Behnken Design for BTEX HSSPME**

| Var. | NaCl (gr) | VOC (uL) | Ext. time(min) | Ext.temp(C ) | Des. Time (sec.) |
|---|---|---|---|---|---|
| Opt. | 0.32 | 2.76 | 15.1 | 40.6 C | 60.4 |

**Table4. Summary of results of regression analysis for responses.**

| Peak | Benzene | Toluene | Ethyl benzene | m,p-xylene | o-xylene |
|---|---|---|---|---|---|
| RT | 2.113 | 4.182 | 8.509 | 8.935 | 10.097 |
| 0-1 | 16084 | 80390 | 59615 | 180246 | 167532 |
| 0-2 | 17821 | 77321 | 51201 | 174302 | 152301 |
| 0-3 | 15022 | 78541 | 50210 | 197320 | 149321 |
| SD | 1413 | 1545.21 | 5167.71 | 11949.07 | 9768.18 |
| LOD | 0.045 | 0.036 | 0.053 | 0.085 | 0.087 |
| LOQ | 0.149 | 0.119 | 0.176 | 0.281 | 0.287 |
| Slope | 93685 | 128375 | 290638 | 421062 | 336605 |

**Table5. Analyze of BTEX in real sample.**

| Peak | Benzene | Toluene | Ethyl benzene | m,p-xylene | o-xylene |
|---|---|---|---|---|---|
| RT | 2.113 | 4.182 | 8.509 | 8.935 | 10.097 |
| Area Rep.1th | 684542 | 842228 | 1883082 | 4442709 | 3942943 |
| Area Rep.2th | 658221 | 834969 | 2016157 | 4841336 | 4249021 |
| Area Rep.3th | 675573 | 887418 | 1986302 | 4832310 | 4151533 |
| SD | 13381 | 28419 | 69827 | 227587 | 156366 |
| Mean | 672779 | 858472 | 1961847 | 4705452 | 4114501 |
| RSD | 1.99 | 3.32 | 3.56 | 4.84 | 3.8 |

Optimization of HSSPME/GCMSS is a complex process that requires one to consider a large number of variables and their interactions with each other. The present study certainly demonstrates the use of a Box–Behnken statistical design is valid for optimizing BTEX concentration with HSSPME/GCMSS . The derived polynomial equations and contour plots aid in predicting the values of selected independent variables for preparation of optimum extraction with desired properties.

## Conclusions

HSSPME-GC-MASS appeared to be a simple and reliable method for determination of BETX in Urine. With the HSSPME technique, the low concentration samples were conveniently analyzed and with BBD

technique, Optimum extraction conditions were achieved. This simple, low-cost and reliable method is suitable for quantitative analysis and quality control for Urinary BTEX. Also, the proposed method provided a good limit of detection and a relatively wide linear working range.

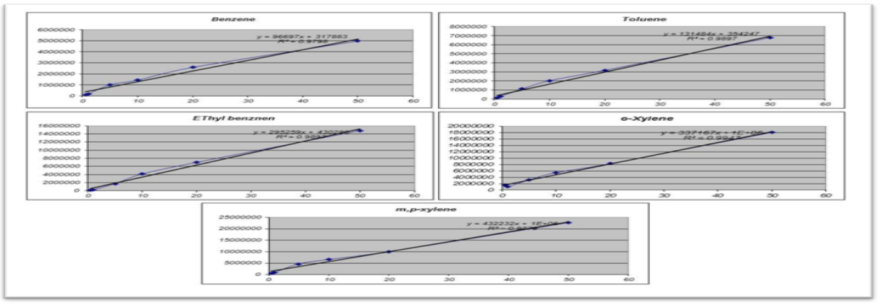



**Fig3. Calibration regession graph of Benzene, Toluene, Ethyl Benene,Ortho Xylene,Metha and Para Xylene.**

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