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Optimization of Sample Preparation for 1-Hydroxypyrene as a Major Biomarker of Exposure to PAHs Prior to HPLC

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Abstract

Urinary 1-hydroxypuren (1-OHP) is commonly used as a major metabolite and biological indicator of the overall exposure to polycyclic aromatic hydrocarbons (PAHs). For evaluation of human exposure to such compounds, biological monitoring is an essential process, in which, preparation of samples is one of the most time-consuming and error-prone aspects prior to chromatographic techniques. In this study, non classic form of liquid-liquid extraction (LLE) was optimized with regard to solvent type, solvent volume, extraction temperature, mixing type, and mixing duration. Through the extraction process, a mild temperature was used to keep the compound of interest as stable as possible. In this study, a high performance liquid chromatography, using reverse-phase column was used. The isocratic run was done at a constant flow rate of 0.8 ml/min, the mobile phase was methanol/water and a fluorescence detector was used, setting at 242 nm and 388 nm. At the developed conditions, the extraction recovery was exceeded 87.3%, achieving detection limit of 0.2 µg/l. The factors were evaluated statically and the procedure was validated with three different pools of spiked urine samples and showed a good reproducibility over six consecutive days as well as experiments. It was concluded that, this optimized method could simplify sample preparation for trace residue analysis of PAHs metabolites.

Keywords: *Liquid liquid extraction, 1-Hydroxypyrene, PAHs*

Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are generated during the incomplete combustion of organic materials. PAHs as ubiquitous pollutants, present in the environment, are mainly released from motor vehicles and various industrial sources (1, 2). PAHs have gained special attention because some of them are strong mutagens and carcinogens (3, 4). Humans may be exposed to these compounds from a wide variety of sources including occupation (coke-oven, aluminum potrooms, iron foundries, oil refinery and petrochemical industries), environment (air pollutants, drinking water), medical treatment (coal tar) personal habits (smoking) and diet (broiled and smoked foods) (5-11). After absorption in the body, PAHs are largely excreted in urine or feces as hydroxylated metabolites (12, 13). Pyrene is a major component of PAHs in ambient air, making up from 1.1 to 3.8 percent of the total PAHs present in air (14). The cytochrome P_{450} IA family of enzymes oxidizes pyrene at position 1, resulting in 1-Hydroxypyrene (1-OHP), the main metabolite of pyrene, which can undergo further oxidation by another cytochrome P_{450} enzyme to1, 6-dihydrodiol or 1, 8-dihydrodiol pyrene. When cytochrome P_{450} IIB enzymes oxidize pyrene, 4, 5- epoxy, 4, 5- dihydropyrene and 4, 5- dihydrodiolpyrene are formed (15).

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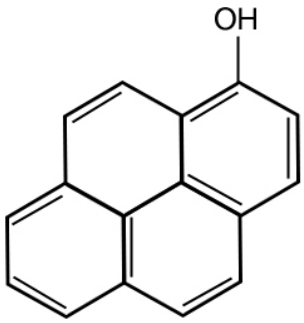

Fig. 1: Chemical *structure of 1-hydroxypyrene*

These metabolites undergo further conjugation by phase II enzymes, such as glucuronosyl transferase/ sulfotrans ferase and glutathione-s-transferase (16). 1-OHP is the major metabolite of pyrene and its concentration in urine has been used as a biomarker of exposure to PAHs since Jongeneelen et al. used high-performance liquid chromatography (HPLC), to develop a method for the measurement of urinary 1-ydronypyrene (17). This method is based on using silica-bonded C18-disposable cartridges named solid phase extraction (SPE) (18). Another method used as an alternative to the method was described by Taguchi (19) in Japan which extracted 1-OHP with a solvent usually methanol with no use of solid sorbent (20). The method by which the interferences are eliminated through sedimentation is addressed as a non-classic form of liquid-liquid extraction (21). The aim of this study was to optimize a method for pretreatment of 1-OHP as a major metabolite of PAHs, using non-classic LLE followed by HPLC-FD. The chemical structure of 1-OHP has been illustrated in Fig. 1

Materials and Methods

Chmicals and reagents 1-Hydroxypyrene standard was obtained from Sigma- Aldrich, USA methanol, ethanol and acetonitril were all HPLC grade and were purchased from Merck Darmstadt, Germany, and water was double distilled and purified using the Purite system.

Instrumentation The HPLC system consisted of a k-1001 single piston pump Knauer, Germany, the analytical column was a RP-C18e 5x4.6 mm Mech-KuaA, Germany; the detector was Florescence RF-10AXL Knauer, Germany. to the HPLC.

Method development and optimization

Several parameters were examined to determine the best one that gave the best recoveries of 1-OHP from samples. Florescence wavelengths were obtained from published documents and were set to 242 and 388 for excitation and emission, respectively (17).

The combination of the mobile phase was varied using different combinations of methanol/ water (50/50, 70/30, 88/12, 80/20) and use of 1 mg/l ascorbic acid in a mixture 88% methanol/12% water. Using the best combination of methanol/water determined above, ascorbic acid concentration was further tested between 0.1-2 mg/l. The pH of the mobile phase was kept at 6.8 as the safe pH not to allow the bonded phase to be stripped off, preserving the column life.

Preparation of stock solutions and working standard solutions Stock solutions of 1-OHP (80 mg/l) were prepared freshly every two weeks by dissolving 4 mg 1-OHP in 50 ml methanol and stored at-18 ºC. 1-OHP concentrations in the working standard solutions chosen for the calibration curve were 0, 0.2, 5, 10, 16, 20 mg/l. These working solutions were prepared by further dilution of the stock solutions in methanol. They were prepared freshly daily.

First, for developing the method, water-based samples were used. In order to prepare these kinds of samples, 1 ml of standard solutions mentioned above was placed in a 100 ml volumetric flask and was filled to the mark with HPLC grade water. Then, it was homogenized by ultrasonication bath.

Results

Optimization of chromatographic conditions

Methanol-water compositions of 50: 50, 70: 30, 88: 12, and 80: 20 were used as mobile phase. From these, the 80: 20 methanol/water was the

optimum mobile phase for the HPLC. Average retention time achieved for 1-OHP was 5.05 min. There were no interfering peaks from urine in the spiked urine sample. Fig. 2 shows the chromatogram of a blank injection with just the solvent peak and no other peaks were come out. Fig. 3 presents the chromatogram of 1-OHP raising in 5.05 min.

A calibration curve was constructed in triplicate in the range of 0.2-20 µg/l for 1-OHP.

The linearity of the calibration curve was demonstrated by the good determination coefficient (r^2) obtained for the regression line and was 0.997.

In this study, peak heights were used for analyte determination and the LOQ determined through this study was 0.2 µg/l.

Liquid- liquid extraction Common solvents in the laboratory were tested for the extraction of 1-OHP. They were methanol, ethanol and acetonitril. Recoveries of each solvent were calculated to select the solvent given the best recoveries for the analyte. The best solvent, out of three, was further investigated. The optimum pH was determined for such extraction in a pervious study. So, that pH was used through this study. First, the pH was adjusted to the right amount using a few drops of 0.1 M acetic acid or 2 M NaOH. The solution was thoroughly vortexed and the pH was checked and recorded. These solutions were then treated to the extraction conditions. Briefly, 3×0.5 ml of sample was placed in 3 glass tubes, and different extraction solvents (as listed above) were added. Then, tubes were caped and vortexed followed by placing in ultrasonic bath for 2 min, 20 µl of each was then injected into the HPLC-FD system. Recovery of 1-OHP was plotted against different extraction solvents. As it can be seen, in Table 1 and Fig. 4, the solvent given the highest recovery was methanol and therefore was used for further stages for liquid-liquid extraction. After selecting the optimum solvent, different solvent volumes were evaluated to have the minimum volume of the solvent and also avoiding the usage of large volumes of hazardous organic solvents, besides, it should be enough volume to extract the analyte perfectly. In this stage, 1000, 500, 250 and 125 µl of the selected solvent were used to extract the analyte. Recoveries were all calculated due to the differences in amount of extraction solvent. It was obtained that the best solvent volume for methanol for extracting 1-OHP was 125 µl. Fig. 5 gives the recovery of 1-OHP, using different solvent volumes. Another variable was extraction solvent temperature, in which, after selecting the optimum solvent volume, the solvent temperature was screened at three levels of 4 ºC, 25 ºC, and 40 ºC. Again, in this stage, the recoveries were calculated, and as Table 2 and Fig. 6 show, the best recovery was obtained at 25 ºC (room temperature). Therefore, from this experiment, the best result was used in the following stages of optimization. Another variable was used through this investigation was mixing method of the solvent added to the sample. In this presses, two methods of hand vortex and ultrasonic bath were applied. The method of ultrasonic bath showed 87.50% recovery while, hand- mixing method showed only 58.49% recovery, so, it was well preferred to use ultrasonic bath in mixing the sample with solvent. These results are shown in Table 3. Fig. 7. Mixing duration was also done as another effective parameter at four levels, including 0.5, 1, 2 and 4 min. Recoveries obtained from this experiment have been illustrated in Table 4 and Fig.8. As it can be seen, the best duration of mixing was 2 min and selected for further optimization procedure. The optimized conditions were then used for method validation.

Method validation In order to determine the method applicability, it was necessary to be validated. So, 6 working solutions with 0, 0.2, 2, 10, 15, and 20 µg/l concentrations were prepared and a calibration curve was prepared daily.

To validate the method, day to day and within day reproducibility were determined.

Day to day reproducibility Solutions with concentrations of 2, 10, and 20µg/l were prepared and for 6 consecutive days, they were extracted using the optimized method. Standard deviation, mean values and coefficient of variation were calculated. Table 5 shows the results of this stage.

Within day reproducibility In order to determine within day reproducibility, solutions with concentrations of 2, 10, and 20 µg/l were prepared and 1-OHP concentration was calculated using each day's calibration curve and it was done for 3 consecutive days and each day for one of the selected concentrations. Each experiment was done 6 times. Table 6 gives the results of these experiments.

Table 1: Recovery of 1-OHP by LLE method using different solvents

Solvent type	Recovery Mean±SD N=5
Ethanol	42.50±2.12
Methanol	87.00±3.95
Acetonitril	79.80±7.49

Table 2: Recovery of 1-OHP by LLE method using different extraction solvent temperatures

Temperature (ºC)	Recovery Mean±SD N=5
4	32.11±4.19
25	86.80±1.83
40	39.30±5.68

Table 3: Recovery of 1-OHP by LLE method using different mixing types

Mixing method	Recovery Mean±SD N=5
Hand vortex	58.49±6.97
Ultrasonic	83.50±4.76

Table 4: Recovery of 1-OHP by LLE method using different mixing duration

Mixing duration (min)	Recovery Mean±SD N=5
0.5	69.81±2.02
1	79.33±3.54
2	87.3±1.29
4	87.30±2.41

Table 5: Day to day reproducibility

Day	2 µg/l	10 µg/l	20 µg/l
1	1.81	9.21	18.97
2	1.73	8.72	17.91
3	1.80	8.71	18.59
4	1.69	9.09	19.05
5	1.65	8.91	19.22
6	1.82	8.98	18.02
Mean	1.75	8.94	18.58
SD	0.07	0.2	0.52
CV	4.04	2.23	2.8

Table 6: Within day reproducibility

Experiment	2 µg/l	10 µg/l	20 µg/l
1	1.79	9.02	16.83
2	1.71	8.91	18.31
3	1.69	8.72	17.31
4	1.88	7.99	19.02
5	1.82	8.27	17.69
6	1.90	9.06	18.88
Mean	1.80	8.66	18.01
SD	0.09	0.44	0.88
CV	4.79	5.05	4.87

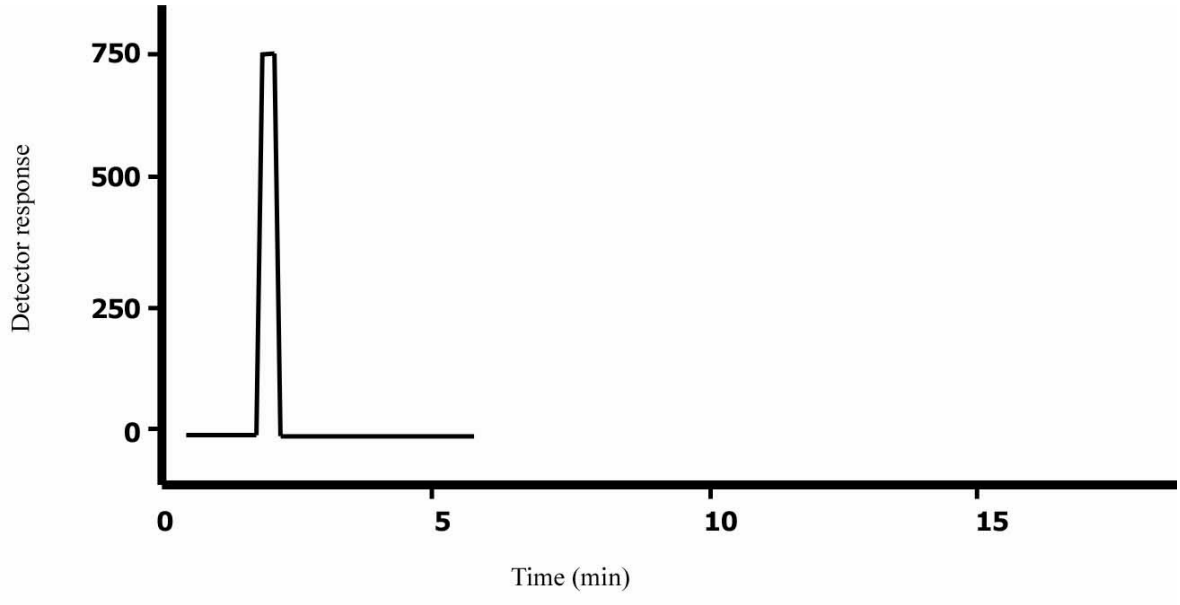

Fig. 2: Chromatogram of a blank sample

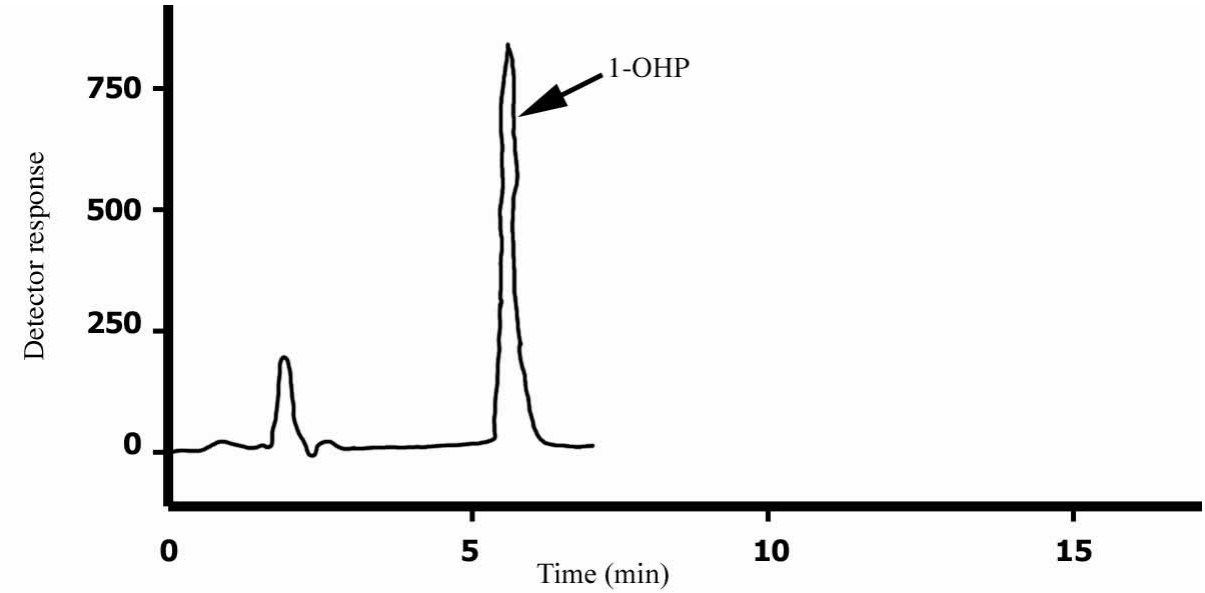

Fig. 3: Chromatogram of 1-OHP using optimized conditions

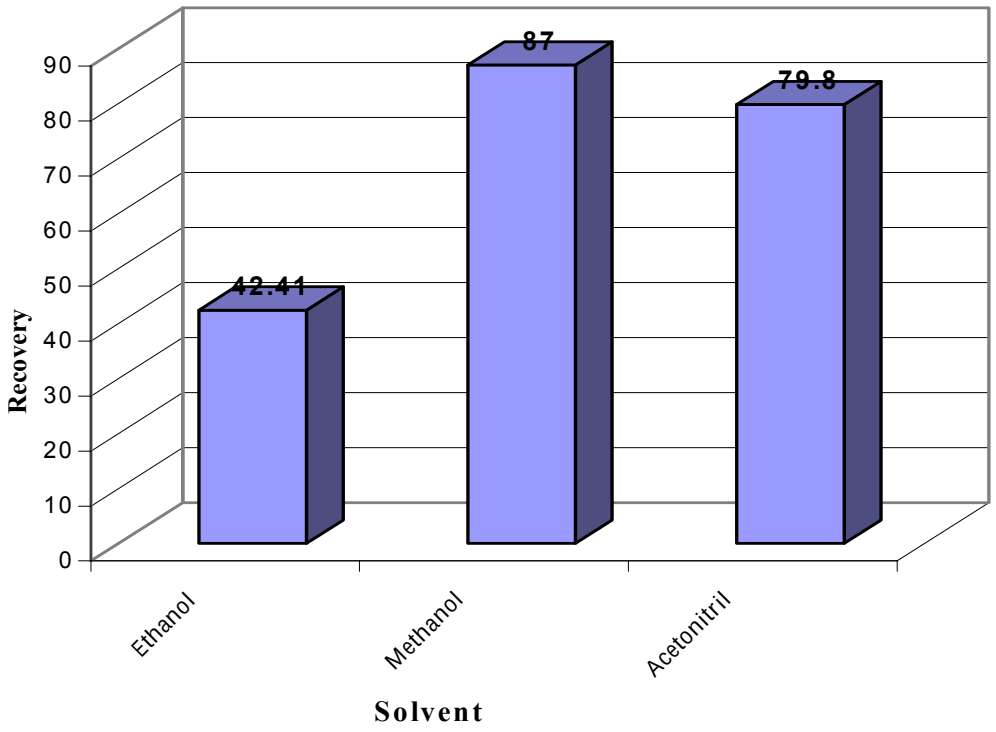

Fig. 4: Recovery of 1-OHP using different solvents

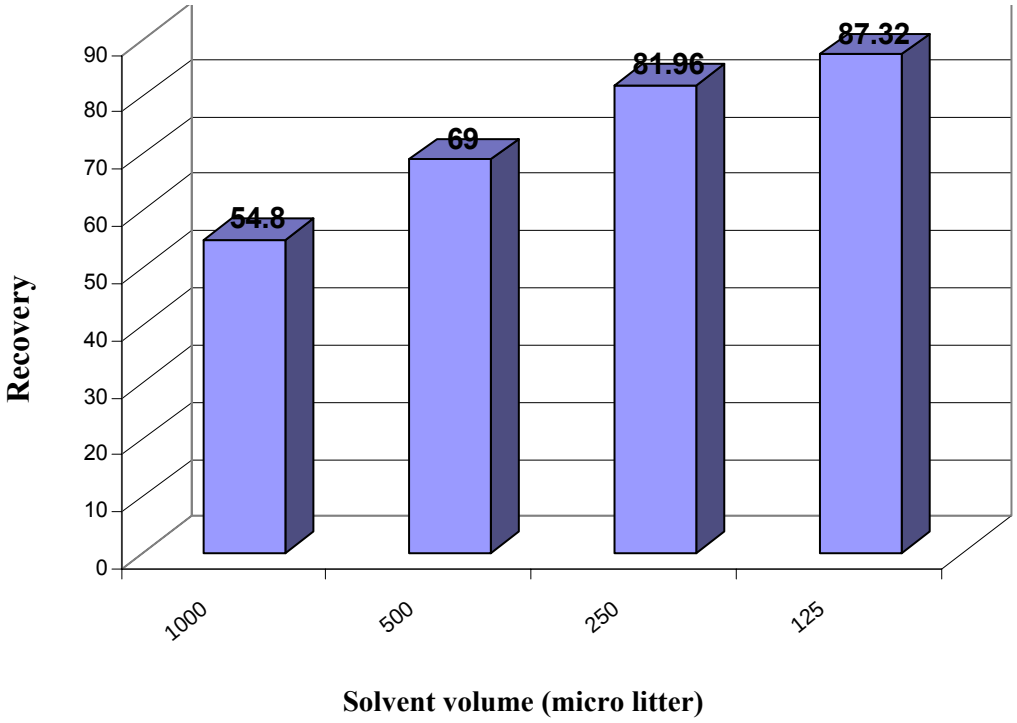

Fig. 5: Recovery of 1-OHP using different solvent volumes

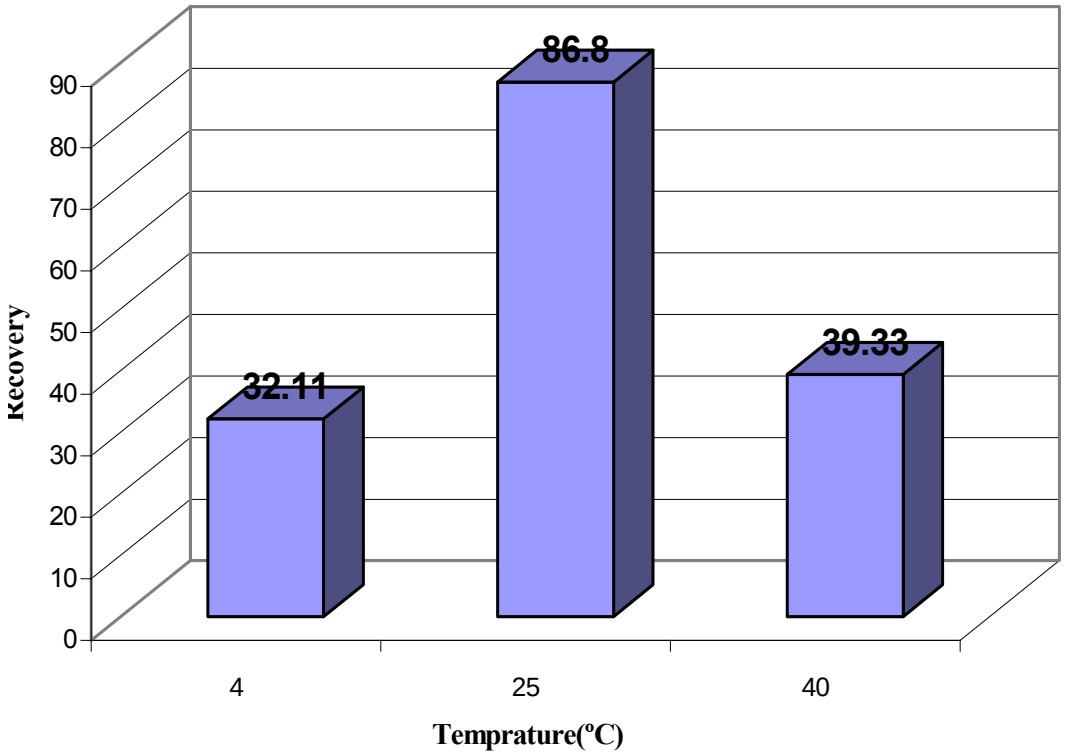

Fig. 6: Recovery of 1-OHP using different solvent temperatures

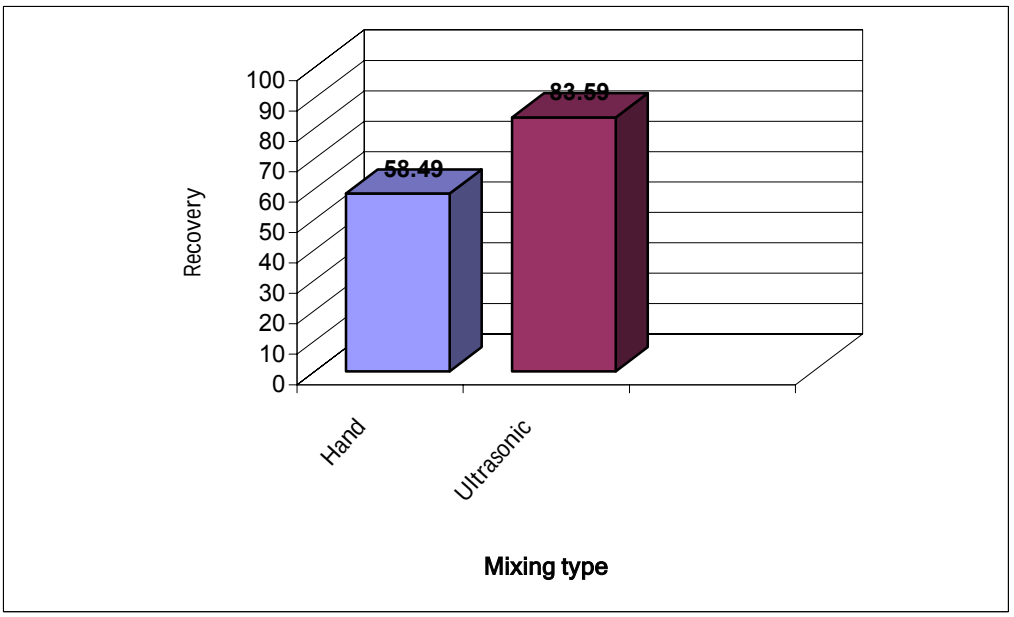

Fig. 7: Recovery of 1-OHP using different mixing types

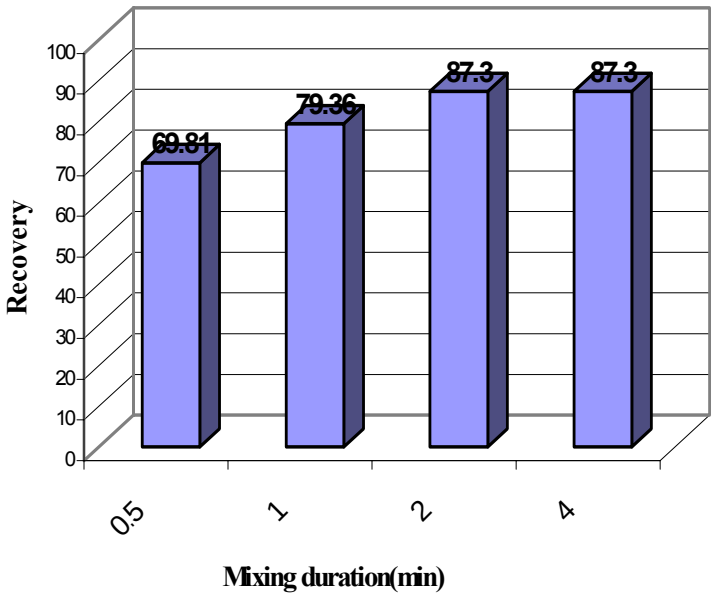

Fig. 8: Recovery of 1-OHP using different mixing durations

Discussion

Occupational and non- occupational exposure to PAHs can be monitored by measuring urinary 1- OHP. In order to determine the amount of this metabolite in urine, there are several methods (11, 17-21) which could be used for this purpose. Two main methods are non- classic liquid-liquid extraction and solid- phase extraction or SPE. Despite the need for a higher level of technology, SPE is now a days more in use. But there are some limitations in using such technology in every country. So, in this study, we challenged the proposed non- classic LLE optimized by Taguchi (1993), whether was practical to be used in occupational monitoring laboratories in Iran, or not.

Thus, based on reported methods (19, 20), several parameters critical for recovery from an extraction method were selected and examined as follows: using different organic solvent, solvent volumes, different solvent temperatures, different mixing methods and different mixing durations. The results showed that the best recoveries could be obtained when using 125 µl of methanol in room temperature (25°C) and 2-4 min of ultra soniction. There was a similarity to the SPE method developed by Jongeneelen (17). It showed that the metabolite (1-OHP) could be extracted successfully. While, extracting a 0.5 ml sample, 125 micro liters of the organic solvent (methanol) was able to do both extraction and concentration, so that, the recovery obtained was 87.32%. This recovery was higher than that of reported earlier (19, 20) and equal to the SPE method (16-18), it could be easily used in the laboratories testing the urinary metabolite of PAHs as well, no need to bonded-silica sorbents (C18). In comparison to Taguchi method (19), the solvent volume used was one half and mixing duration for the best recoveries was reduced to 2 min in comparison to the other methods (15-20).

In conclusion, the assay for 1- OHP using non-classic liquid-liquid extraction could be easily applied to monitoring occupational or non-occupational exposure to PAHs and other similar compounds released in the environment as well as work places.

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