

Research Paper

Determination of Hippuric Acid and Methylhippuric Acid Isomers in the Urine of Gas Stations Workers



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ABSTRACT

Background: The purpose of this study was to determine the concentration levels of hippuric acid and 2, 3, 4- methyl hippuric acid isomers as biological indicators of exposure to toluene and xylene isomers in the urine of the gas stations workers of Ahvaz, Iran.

Methods: The urine sample was taken the first time in January and the second time in June 2018. Eight gas stations were selected in Ahvaz, Khuzestan Province, Iran. The study sample was 24 males; three workers participated in the study from each gas station. Two workers in each gas station served as the exposed group, and one secretary from each gas station was the control group. The participants' ages ranged from 21 to 49 years. Each participant was requested to fill out a consent form to participate in the study and a questionnaire containing personal information such as age, smoking, weight and height, and work experience.

Results: The mean concentration of hippuric acid, 2, 3 and 4- methyl hippuric acid in the investigated urine of the workers were 0.245, 0.017, 0.012, and 0.011 g/g creatinine, respectively. There was no relationship between variable of season and levels of hippuric acid, 2, 3 and 4- methyl hippuric acid in the urine of exposed and control groups.

Conclusion: There was no significant difference in the analyzed results of urine samples between control and exposure groups. There was at least one methyl hippuric acid isomer, indicating that all subjects were exposed to xylene, which was lower than the threshold limit value. In conclusion, the variable of season cannot cause a significant change in the metabolites of toluene and xylene isomers. Exposure of workers to toluene and xylene at gas stations is within acceptable limits.

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1. Introduction

Volatile Organic Compounds (VOCs) are among the most important air pollutants in the workplace. Toluene and xylene isomers are classified as VOCs. Also, gasoline contains volatile organic compounds, especially toluene and xylene isomers (ortho, meta, and para-xylene). Gas station workers can have respiratory exposure to toluene and xylene isomers due to their volatility [1]. The recommended permissible exposure limit to toluene and xylene isomers by National Institute for Occupational Safety and Health (NIOSH) is 100 ppm. Toluene and xylene isomers can be absorbed through the skin and respiratory tract [2]. Toluene and xylene are classified by International Agency for Research on Cancer (IARC) in group 3 carcinogens. Although there is not enough evidence for their carcinogenic effects in humans, according to some reports, several types of cancers are associated with these substances. Therefore, performing further study on these substances is important. Toluene can cause neurobehavioral disturbances and ototoxic effects in humans [3, 4]. Xylene can cause central nervous system depression and respiratory system problems [5].

Biomonitoring the urinary metabolites of toluene and xylene isomers is an accepted way for assessing the exposure level [6]. Urinary ortho-cresol and Hippuric Acid (HA) are the most common measurable factors for biomonitoring the toluene metabolites. In some studies, ortho-cresol and in others, HA has been proposed as the major toluene metabolites. However, in some studies, none has been suggested, and some other compounds have been proposed for biomonitoring [7, 8]. The level of urinary 2-, 3-, and 4-Methylhippuric Acid (MHA) is proportional to the removal level of ortho, meta, and para-xylene from blood, and most of the absorbed xylene isomers are converted into MHA isomers. They are excreted in the urine after metabolism [9]. The recommended threshold limit value for workers' urine samples by NIOSH is 1.6 g/g creatinine for HA and 1.5 g/g creatinine for MHA isomers. In the provided method by NIOSH, the metabolites are extracted from urine samples by solid-phase extraction technique.

Since the purpose of this study was to determine the HA and MHA isomers levels as the biological indicators of toluene and xylene isomers in the urine of Ahvaz gas stations workers (not to develop a method), we decided to choose an approved method (NIOSH 8301) to determine the levels of the urinary metabolites. However, for the precise determination of the metabolites and separa-

tion of 3-MHA and 4-MHA peaks in the provided method by NIOSH, we made some changes and improved it.

2. Materials and Methods

Sampling

In this study, eight gas stations were selected in Ahvaz, Khuzestan Province, Iran, and we conducted our study from January to June 2018. The study sample was 24 males, i.e., three workers participated in the study from each gas station. Two workers in each gas station served as the exposed group, and one secretary from each gas station as the control group. The participants' ages ranged from 21 to 49 years. Each participant was requested to fill out a consent form to participate in the study and a questionnaire containing personal information such as age, smoking, weight and height, and work experience. The criteria for entering the study were the lack of kidney problems and being a non-smoker. Temperature change in two seasons may affect the volatility of HA and MHA isomers. As the volatility increases, the level of exposure may increase. Therefore, the urine sample was taken two times from each person as follows. The first time in the cold season (January) and the second in the warm season (June). So, 48 urine samples were collected. Also, all urine samples were collected at the end of the shifts, after 8 hours of work. Thymol crystals were used as a preservative for the urine samples.

Ethical clearance

Written informed consent was obtained from the participants before collecting the samples. Also, the study protocol was approved by the Ethics Committee on Medical Research, Vice-Chancellor for Research and Technology, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (Code: IR.AJUMS.REC.1396.549).

Study materials

2-Methylhippuric acid and 4-methylhippuric acid were purchased from Sigma-Aldrich (Switzerland). 3-Methylhippuric acid was purchased from Sigma-Aldrich (Japan). Also, HA, ethyl acetate, tetrahydrofuran, phosphoric acid, hydrochloric acid, calcium chloride, magnesium chloride, methanol, sodium chloride, sodium sulfate, trisodium citrate, sodium oxalate, potassium dihydrogen phosphate, potassium chloride, ammonium chloride, urea, and creatinine were purchased from Merck (Germany).

Preparation of standard solutions

Synthetic urine was made before preparing the standard solutions (Table 1) [10]. Synthetic urine is used for making and diluting the standard solutions. Five mixtures were made containing HA, 2-MHA, 3-MHA, and 4-MHA at different concentrations, as shown in Table 2. To extract standards from each of the five mixtures, 1 mL of each mixture was poured into a borosilicate glass tube. Then, 80 μ L of 6N hydrochloric acid, 0.3 g of sodium chloride, and 4 mL of ethyl acetate were sequentially added and stirred by rotation for 2 min. Then, the mixture was centrifuged at 4000 rpm for 6 min. After centrifugation, 200 μ L of the upper layer was transferred to a High-Performance Liquid Chromatography (HPLC) vial and dried in a water bath with mild nitrogen flow for 30 min at 37 °C. The residue was dissolved in 200 μ L of mobile phase, and 30 μ L of this solution was injected into the HPLC [11-14].

Creatinine determination

The creatinine level of each sample was determined using Cell Biolabs' Company Urinary Creatinine Assay Kit, which is based on the Jaffe method used to determine the creatinine levels in blood and urine [15, 16].

Instrumentation

In this study, the Shimadzu HPLC system and Shimadzu LC-Solution software were used to determine the amount of urinary metabolites. The HPLC was equipped with a KNAUER C18 column with a dimension of 4.6 \times 250 mm and particle size of 5 μ m. The HPLC mobile phase consisted of 910 mL phosphate buffer (12 mM, pH= 2), 45 mL tetrahydrofuran, and 45 mL methanol at a flow rate of 1.5 mL/min. Also, the detection wavelength of the UV detector was 216 nm for all analyses, and the column temperature was set at 48 °C [14].

Preparation and analysis of urine samples

To extract metabolites from urine samples, 1 mL of each sample was poured into a borosilicate glass tube, and 80 μ L of 6N hydrochloric acid, 0.3 g of sodium chloride, and 4 mL of ethyl acetate were sequentially added and then stirred by rotation for 2 min and centrifuged at 4000 rpm for 6 min. After centrifugation, 200 μ L of the upper layer was transferred to an HPLC vial and dried in a water bath with mild nitrogen flow for 30 min at 30 °C. The residue was dissolved in 200 μ L of mobile phase, and 30 μ L of this solution was injected into the HPLC [11, 14]. The pH of urine samples was between 4.3 to 7.6. The Limit of Detection (LOD) Limit of Quantification (LOQ) are shown in Table 3.

Statistical analysis

All statistical analyses were conducted using the SPSS version 20. The data were expressed as Mean \pm Standard Deviation (SD). To compare the groups after analyzing the normal distribution of the findings and the homogeneity of variances, we investigated the data using the Independent t-test, the Chi-square test, and Analysis of Variance (ANOVA). The differences were considered statistically significant at $P < 0.05$.

3. Results and Discussion

In this study, the concentration levels of HA and 2-, 3-, and 4-MHA were evaluated in the urine samples of workers of Ahvaz gas stations. According to the results obtained from the analysis of the urine samples and the statistical method of ANOVA, the mean concentrations of HA, 2-, 3-, and 4-MHA in the exposed group were 0.163, 0.02, 0.017, and 0.015 g/g of creatinine, respectively. The mean concentrations of HA, 2-, 3-, and 4-MHA in the control group urine samples were 0.407, 0.011, 0.002, and 0.005 g/g creatinine, respectively (Table 4). No significant difference was found between the exposed and control groups for HA ($P=0.114$), 2-MHA ($P=0.253$), 3-MHA ($P=0.062$), and 4-MHA ($P=0.413$).

The mean concentrations of HA in the urine samples of workers in gas stations numbers 1 and 2 were higher compared to other gas stations (Figure 1). The mean concentrations of 2-MHA in workers' urine samples in gas stations numbers 6 and 2 were higher than those of other gas stations (Figure 2). The mean concentrations of 3-MHA in the urine samples of workers in gas stations numbers 3 and 1 were higher compared to other gas stations (Figure 3). The highest mean concentration of 4-MHA was seen in workers of gas station number 3 (Figure 4). The duration of the analysis program was 18 minutes that is shown in Figure 5.

The work experiences in the control and exposure groups were 4.37 and 4.06 years, respectively. The participants' age and BMI in the groups of this research were almost the same (Table 5).

Figure 5 displays the chromatograms of HA and 2-, 3-, and 4-MHA. Complete separation of all four chromatogram peaks was observed in 18 minutes. Accordingly, peaks appeared after 7.38 minutes for HA, after 10.01 minutes for 2-MHA, after 15.62 minutes for 4-MHA, and after 16.33 minutes for 3-MHA.

Table 1. Composition of the synthetic urine

Salt and Compounds	g/L	mM
Calcium chloride (CaCl ₂ ·2H ₂ O)	0.65	4.4
Magnesium chloride (MgCl ₂ ·6H ₂ O)	0.65	3.2
Sodium chloride (NaCl)	4.60	78.7
Sodium sulphate (Na ₂ SO ₄)	2.30	16.2
Trisodium citrate (Na ₃ C ₆ H ₅ O ₇ ·2H ₂ O)	0.65	2.6
Sodium oxalate [Na ₂ (COO) ₂]	0.02	0.15
Potassium dihydrogen phosphate (KH ₂ PO ₄)	4.2	30.9
Potassium chloride (KCl)	1.60	21.5
Ammonium chloride (NH ₄ Cl)	1.00	18.7
Urea [(NH ₂) ₂ CO]	25.0	417
Creatinine (C ₄ H ₇ N ₃ O)	1.10	9.7

Biological monitoring data of toluene and xylenes isomers metabolites showed that the urinary levels of HA and 2-, 3-, and 4-MHA were very low in the exposed workers. Also, the urinary levels of HA and 2-, 3-, and 4-MHA were very low in the nonexposed workers (Table 4). Therefore, the determined metabolites in exposed and nonexposed workers were lower than the Biological Exposure Indices (BEI) suggested by The American Conference of Governmental Industrial Hygienists (ACGIH). Duangduan Yimrungruang investigated the health risk assessment of volatile organic compounds on gas service station workers in 9 gas stations in Chonburi Province, Thailand, from October to December 2007. They collected urine and personal air samples of workers (a total of 27 samples). According to their results, the concentrations of toluene and xylenes were significantly higher in the air of the workplaces compared to office places ($P < 0.05$). Urine samples were analyzed for VOCs metabolites, including t,t-muconic acid, HA,

mandelic acid, and MHA. Duangduan Yimrungruang reported that the mean concentrations of HA and MHA in the urine samples of gas station workers were 0.28 and 0.06, and in the control group were 0.23 and 0.02 g/g creatinine, respectively. These metabolites in gas station workers and control workers were close. In the present study, the mean concentrations of HA and MHA in the urine samples of the gas stations workers were 0.02 and 0.05 and in the control workers 0.2 and 0.01 g/creatinine, respectively [17].

The study showed no high concentrations of HA and 2-, 3-, and 4-MHA in Ahvaz gas stations workers compared to the control group ($P = 0.114$). These results are similar to the results of studies that confirmed low concentrations of urinary HA and 2-, 3-, and 4-MHA among the gas stations workers compared to the BEI, as recommended by ACGIH. However, these similar studies showed a significant relationship between respiratory

Table 2. Concentrations of different mixtures of standards (mg/L)

Mixtures of Standards	HA	2-MHA	3-MHA	4-MHA
Mixture 1	500	500	500	750
Mixture 2	250	250	250	375
Mixture 3	125	125	125	187.5
Mixture 4	62.5	62.5	62.5	93.75
Mixture 5	31.25	31.25	31.25	46.875

Table 3. LOD and LOQ studied biological indicators

Biological Indicators	LOD (mg/L)	LOQ (mg/L)
HA	9.61	31.25
2-MHA	9.61	31.25
3-MHA	9.61	31.25
4-MHA	14.423	46.875

HA: hippuric acid; MHA: methylhippuric acid.

exposure to toluene and xylene isomers with their metabolites [18, 19]. The main metabolite of toluene is benzoic acid, and benzoic acid can be rapidly conjugated to glycine and converted to HA, and excreted by the urine. Also, there is a direct relationship between toluene and HA levels in occupational exposure [20].

Some studies recommend un-metabolized toluene in low exposures with toluene [21]. According to some other studies, o-cresol is a more specific metabolite in toluene occupational exposure, and HA is the metabolite of other substances in the urine, and there is significantly urinary HA in background levels [22-25]. However, HA is still measured as one of the metabolites of toluene. In a study conducted on the paint workers at metal furniture manufacturers in Thailand, only HA was measured as a

toluene metabolite. However, toluene concentrations in the workplace ranged from low to high levels. The concentration of toluene in the workplace for exposed workers was between 12 and 198 ppm. There was a significant relationship between exposure to toluene and urinary HA level. Considering the possibility of low to high concentrations of toluene in different workplaces, only one toluene metabolite (AH) should not be considered for each workplace [26]. However, in some studies, such as Edoardo De Rosa et al. study, to investigate the exposure to low concentrations of toluene (11.4 to 67.4 ppm or 42 to 253 mg/m³), HA is recommended as a better biomarker compared to o-cresol [27].

It seems that the use of clinical symptoms and pathological manifestations simultaneous with biological

Table 4. Mean±SD, MSE, HA, 2-, 3-, and 4-MHA in the urine of the exposed and control groups obtained by ANOVA test

Biological Indicators	Mean (g/g Creatinine)	Std. Deviation	Std. Error of Mean	BEIs (Recommended by ACGIH)	
Hippuric acid	Unexposed	0.407	0.834172	0.208	
	Exposed	0.163	0.154141	0.027	1.6 g/g creatinine
	Total	0.245	0.501164	0.072	
2-Methyl hippuric acid	Unexposed	0.0111	0.006114	0.001	
	Exposed	0.0202	0.031179	0.005	1.5 g/g creatinine
	Total	0.0172	0.025925	0.003	
3-Methyl hippuric acid	Unexposed	0.0025	0.004624	0.001	
	Exposed	0.0171	0.030232	0.005	1.5 g/g creatinine
	Total	0.0122	0.025655	0.003	
4-Methyl hippuric acid	Unexposed	0.005	0.008537	0.002	
	Exposed	0.0151	0.048143	0.008	1.5 g/g creatinine
	Total	0.0118	0.039686	0.005	

BEI: Biological Exposure Indices; ACGIH: The American Conference of Governmental Industrial Hygienists.

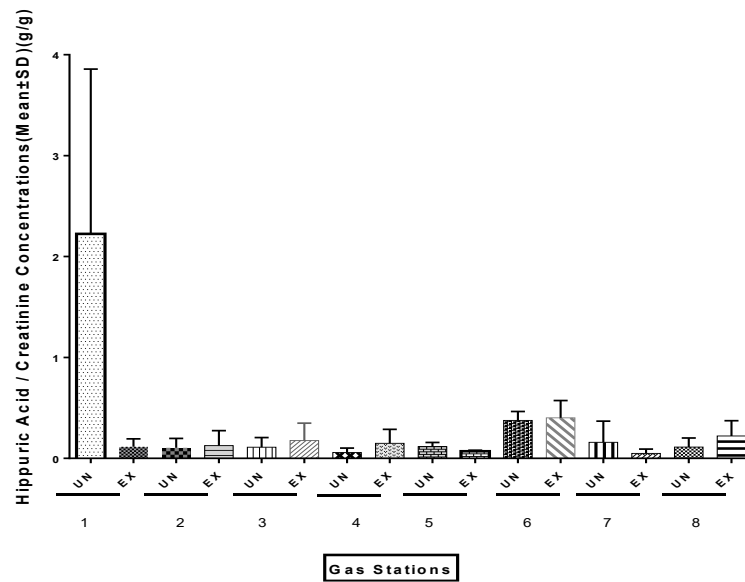


Figure 1. Mean concentrations of HA (g/g creatinine) at each gas station in the control and exposed groups regardless of the season variable

UN: Control group; EX: Exposure group.

monitoring for toluene exposed workers can obtain better results to determine the range of the exposure and the effects of toluene.

HA level is up to 1 g/g creatinine in unexposed workers. Also, 2-, 3-, and 4-MHA were not found in unexposed workers [11]. Therefore, in this study, the presence of MHA isomers in the urine samples of the control and

exposure groups indicated that both groups were exposed to xylene isomers.

HA levels were higher in the control group compared to the exposure group. Because HA values were below 1 g/g creatinine in both groups, the reason cannot be justified because of more exposure to toluene. One of the reasons for the presence of hippuric acid in nonexposed

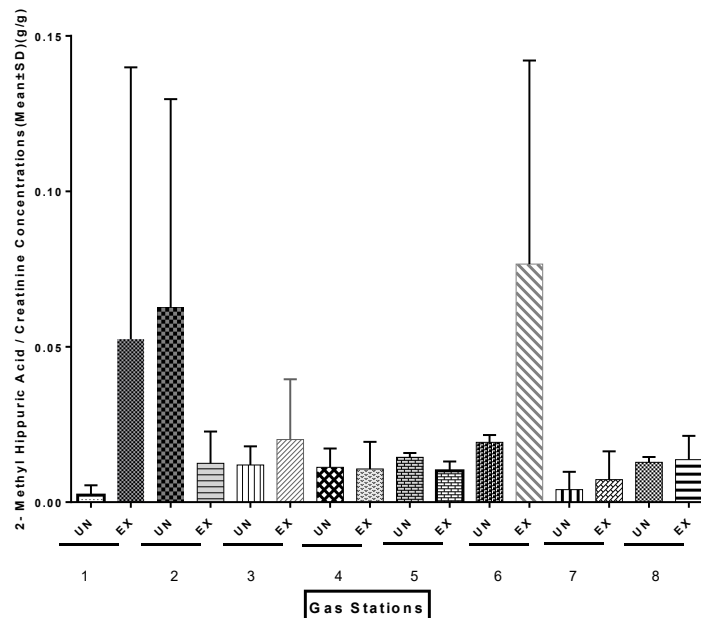


Figure 2. Mean concentrations of 2-MHA (g/g creatinine) at each gas station in the control and exposed groups regardless of the season variable

UN: Control group; EX: Exposure group.

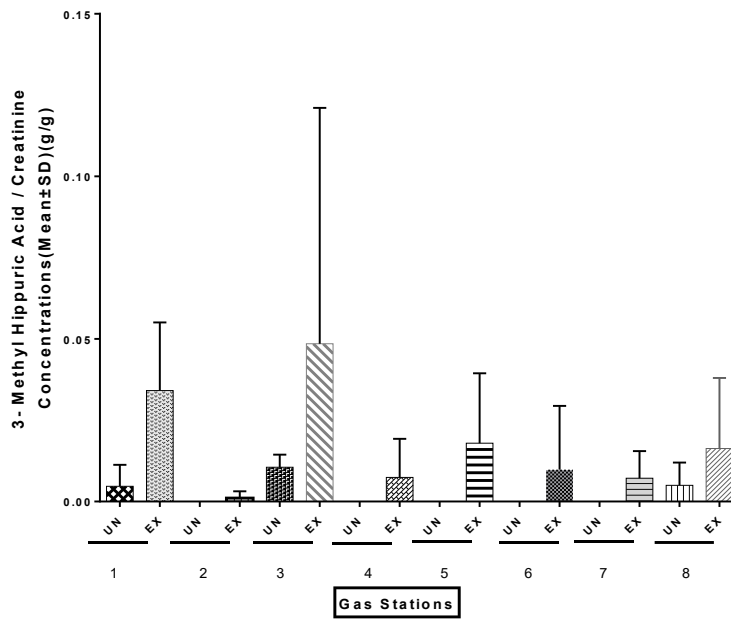


Figure 3. Mean concentrations of 3-MHA (g/g creatinine) at each gas station in the control and exposed groups regardless of the season variable

UN: Control group; EX: Exposure group.

workers to toluene is related to their diet [28, 29]. However, MHA isomer levels were lower in the control group compared to the exposure group.

There was no relationship between seasons (winter and summer) and levels of HA, as well as 2-, 3-, and 4-MHA

in the urine of exposed and control groups. After the injection of standards based on NIOSH 8301, the peaks of 3-MHA and 4-MHA completely overlapped. The mobile phase in NIOSH 8301 method contained water, acetonitrile, and glacial acetic acid with ratios of 84, 16, and 0.025%, respectively [11].

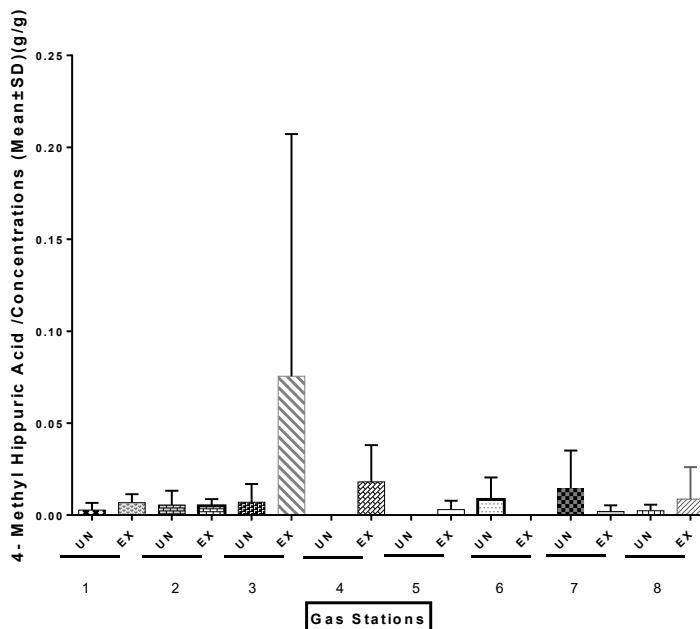


Figure 4. Mean concentrations of 4-MHA (g/g creatinine) at each gas station in the control and exposed groups regardless of the season variable

UN: Control group; EX: Exposure group.

Table 5. Characteristics of the study participants

Characteristics	Control	Exposure	Total
Age (y)	32.5	35.5	34.29
Work experience (y)	4.375	4.062	4.16
Weight (kg)	71.25	69.687	70.16
Height (cm)	172.25	168.5	169.75
Body Max Index (kg/m ²)	23.99	24.46	24.3

Instead of the provided mobile phase in NIOSH 8301, the other mobile phase provided by Inoue et al., including methanol, acetic acid, and water, with the ratios of 20, 0.8, and 79.2%, was replaced for the detection of HA and MHA isomers, respectively. Although 3-MHA and 4-MHA were separated, there was still a great deal of overlapping [30].

To complete the separation of 3-MHA and 4-MHA-peaks in the chromatogram, used from a mobile phase, which was in a developed method. Tardif et al. to detect the HA and MHA isomers. The mobile phase is including potassium phosphate buffer (pH= 2), methanol and tetrahydrofuran were used with the ratios of 91, 4.5, and 4.5%, respectively (Figure 5) [14].

4. Conclusion

In the absence of exposure to toluene, the concentration of urinary HA was less than 1 g/g creatinine except for the urinary concentration of HA in the control group at one of the gas stations. There was no significant differ-

ence in the analyzed results of urine samples between the control and exposure groups. None of the other subjects had been exposed to toluene. MHA isomers in the urine were not observed in the absence of exposure to xylene isomers. In all samples, there was at least one MHA isomer, indicating that all subjects were exposed to xylene, which was lower than the threshold limit value. At the gas stations, urinary HA and MHA isomers levels were less than the threshold limit value. The analyzed results of urine samples show no significant difference between the control and exposure groups. HA levels were higher in the control group than in the exposure group. There was no relationship between the variable of season and levels of HA, and 2-, 3- and 4-MHA in the urine of the exposed and control groups. In the analysis method, 3-MHA and 4-MHA peaks were significantly separated. However, they were not completely separated. The season's variable cannot cause a significant change in the metabolites of toluene and xylene isomers in Ahvaz. Exposure of workers to toluene and xylene isomers at gas stations is within an acceptable range. The modified method of this study can be used in other studies.

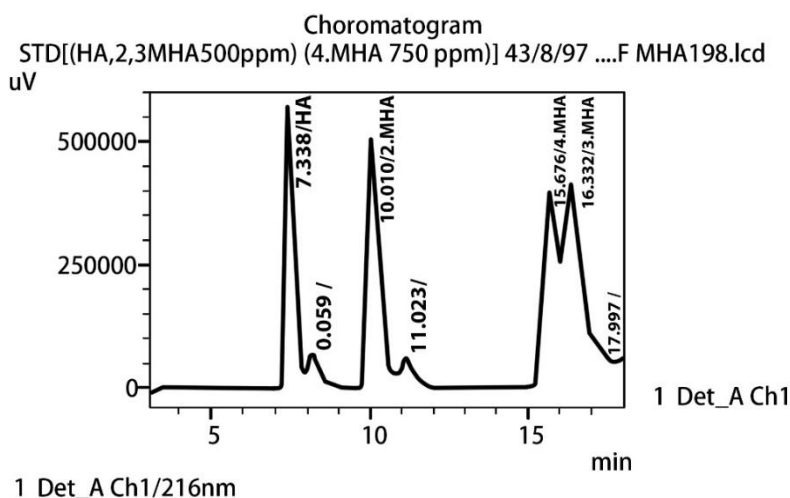


Figure 5. Chromatograms of Hippuric Acid (500 mg/L), 2-Methylhippuric Acid (500 mg/L), 3-Methylhippuric Acid (500 mg/L), and 4-Methylhippuric Acid (750 mg/L)

Ethical Considerations

Compliance with ethical guidelines

The study protocol was approved by the Ethics Committee on Medical Research of Ahvaz Jundishapur University of Medical Sciences in Ahvaz, Iran (Code: IR.AJUMS.REC.1396.549).

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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