Journal of the Serbian Chemical Society
JSCS@tmf.bg.ac.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. *89* (10) 1337–1351 (2024)
JSCS–5791
Original scientific paper
Published 2 November, 2024

Dispersive liquid–liquid microextraction for determining urinary muconic acid as benzene biological indicator

ABOLFAZL MOGHADASI[1,2], SAEED YOUSEFINEJAD[3]*, ESMAEEL SOLEIMANI[2]**, SAJJAD TAGHVAEI[4] and SAEED JAFAI[2]

[1]*Student Research Committee, Shiraz University of Medical Sciences, Shiraz, Iran,* [2]*Department of Occupational Health Engineering, School of Health, Shiraz University of Medical Sciences, Shiraz, Iran,* [3]*Research Center for Health Sciences, Institute of Health, Department of Occupational Health Engineering, School of Health, Shiraz University of Medical Sciences, Shiraz, Iran and* [4]*Department of Mechanical Engineering, Shiraz University, Shiraz, 71936-16548, Iran*

(Received 25 January, revised 5 April, accepted 7 July 2024)

Abstract: The monitoring of occupational exposure to chemicals is essential for assessing the workplace. In the case of hazardous and carcinogenic chemicals, such as benzene, occupational monitoring becomes even more crucial. *Trans*,*trans*-muconic acid (*t*,*t*-MA) is one of the benzene urinary metabolites. Pretreatment methods for *t*,*t*-MA generally include liquid–liquid extraction and solid–phase extraction. Using dispersive liquid–liquid microextraction (DLLME) during sample preparation and extraction can reduce extraction costs and environmental impacts. Furthermore, the process is cost-effective and easy to operate. This study is aimed to develop, optimize, and validate an analytical method for measuring *t*,*t*-MA concentration in urine matrix through DLLME combined with high-performance liquid chromatography. In this method, five variables including pH, the volume of the extractant and the disperser, salt content and the time of centrifugation were optimized using the response surface methodology with a central composite design approach and experimental data. The proposed DLLME was successfully applied to real samples of exposed workers to benzene with extraction efficiencies from 95.8 to 102.4 %. The optimum conditions were pH 8, extractant solvent, 300 µL, disperser solvent, 300 µL, salt, 3.4 % and centrifuge, 3 min. According to the result of this study, the proposed DLLME approach can be effectively applied to the biomonitoring of individuals exposed to benzene.

Keywords: biomonitoring; DLLME; HPLC; central composite design; exposure assessment.

* Corresponding authors. E-mail: (*)yousefinejad.s@gmail.com; (**)esoleimani61@gmail.com
https://doi.org/10.2298/JSC240125066M

INTRODUCTION

As part of the occupational exposure assessment, monitoring occupational exposure to chemicals is necessary.[1] It is important to consider all routes of exposure during this monitoring, including inhalation, ingestion and skin.[2] The occupational monitoring becomes more important when toxic and carcinogenic chemicals are involved, such as benzene.[3] Both the United States Environmental Protection Agency (US-EPA) and the International Agency for Research on Cancer (IARC) have confirmed that benzene is carcinogenic (a class I carcinogen).[4,5] People who have been exposed to benzene acutely may experience central nervous system depression.[6] On the other hand, long-term exposure to benzene may result in anemia, leukemia and immune system alterations.[7] Benzene is present in a wide range of petroleum products, including motor fuel and solvents, in the workplace, in the general environment and at home.[8] Furthermore, both active smokers and second-hand smokers are at risk of exposure to benzene through cigarettes.[9]

Two methods are used to monitor the occupational exposure to benzene: air monitoring and biological monitoring.[10,11] As part of air monitoring, benzene concentrations in breathing air are measured. Comparatively, biological monitoring assesses the amount of benzene entering the body via various routes such as inhalation, skin and digestion.[5,11] Through its metabolites, such as trans, trans muconic acid (*t*,*t*-MA), benzene can be eliminated from the body.[9,12] According to studies, there is a significant correlation between exposure to low levels of benzene (lower than 1 ppm) and urinary *t*,*t*-MA levels.[13] Moreover, some organizations and countries consider urinary *t*,*t*-MA as a benzene biological indicator, including the American Conference of Governmental Industrial Hygienists (ACGIH) and the Occupational Safety and Health Administration (OSHA).[14]

Liquid–liquid extraction (LLE) and solid–phase extraction (SPE) are often used as pretreatments of *t*,*t*-MA.[15,16] These treatments can lead to the separation of *t*,*t*-MA from the urine matrix, which makes its analysis easier.[17] In general, SPE is more effective than LLE.[5] However, SPE is relatively expensive and requires the preparation of columns.[18] By reducing the amount of solvent and maximizing its effectiveness, liquid–liquid microextraction (LLME) can be effectively employed to extract urinary *t*,*t*-MA.[15,19] In addition, recent research has focused on developing more efficient, environmentally friendly and miniaturized methods using microextractions.[16,20] In addition to simplifying the sample preparation and reducing the amount of solvent used, the microextraction techniques can also reduce extraction costs and the impact on the environment.[21]

Due to its high efficiency and rapidity, dispersive liquid–liquid microextraction (DLLME) would be applied for the preconcentration of aqueous samples. DLLME has been employed for the analysis of analytes such as phthalate esters, and bisphenol A.[19] When an extractant is injected and dispersed rapidly in an aqueous solution, tiny droplets of the extract disperse. This increases the contact

surface of the analyte in the sample matrix by extractant solvent. Consequently, the efficiency of extraction will be enhanced, and a large quantity of analytes can be collected rapidly. Additionally, this process is easy to operate and cost-effective.

Rismanchian *et al.* developed a partitioned dispersive liquid–liquid microextraction (PDLLME) method based on chloroform extraction for the extraction of urinary *t*,*t*-MA.[22] It consists of a two-stage procedure in which tetrahydrofuran is mixed with chloroform followed by centrifuging and drying using nitrogen flow. The metabolite is then prepared for injection into HPLC by resolving it in methanol. Despite all its advantages, this method has some limitations, such as a long extraction time, the use of solvents in relatively high amounts, and the use of tetrahydrofuran, which is highly volatile. Therefore, this study was designed to develop a dispersive liquid–liquid microextraction method that is simpler, requires fewer solvents, and uses a solvent with lower volatility.

The purpose of this study was to develop a valid method using DLLME and high-performance liquid chromatography (HPLC) for the extraction of *t*,*t*-MA from urine matrix. The biological monitoring of individuals exposed to benzene was successfully performed using the proposed method.

EXPERIMENTAL

Reagents and solutions

Hydrochloric acid (HCl, 37 %, Merck), chloroform (CHCl, Merck), *t*,*t*-MA (Sigma–Aldrich) analytical grades were used. Acetonitrile (CH_3CN) in liquid chromatography grade (Merck), methanol (CH_3OH) in gas chromatography grade (Merck), and NaCl (purity > 99 %, Sigma Aldrich) were used in the suggested microextraction/chromatographic method as well.

Purified deionized water was produced by a Direct-Q 3UV Millipore system (Molsheim, France).

Apparatus

The chromatographic analysis was carried out using an HPLC system (HPLC, Knauer, Smartline system 1000, Berlin, Germany) coupled with a UV detector (Knauer, 2000) at 274 nm. A C18 analytical column was used to separate the analyte (Knauer, Eurospher 100-5, 150 mm×4.6 mm). The utilized mobile phase was a mixed solvent containing acetic acid (1 %) and methanol with a volume ratio of 70:30 with a flow rate of 1.0 ml/min for elution. A 100 µl Hamilton syringe was used for injecting the sample into a 20 µL stainless steel injection loop. For pH measurement, a Metrohm 827 pH-meter (Metrohm, Switzerland) was used. Organic solvents were separated from sample solutions using a Hettich EBA 20 centrifuge.

Experimental design

The DLLME efficiency could be affected by a variety of factors, including solution pH, percentage of salt, the quantity of both dispersers and extractants and the centrifuge time. Analytes solubility, sample matrix surface area, and interactions between the sample matrix and the extraction could be affected by these parameters. By optimizing each parameter, the efficiency of the extraction can be maximized. Thus, determining the optimal conditions for experiments is an essential step in the extraction process. The optimisation of the extraction process parameters can be achieved through experimental design. Using an experimental design method, time is saved, efficiency is improved, parameter interactions are investigated, and errors are

reduced with fewer runs. In addition, experimental design methods can be used to optimize process parameters systematically and cost-effectively.

Central composite design (CCD) has been used to link polynomial models with experimental data utilizing response surface methodology (RSM). This approach enables researchers to identify the optimal combination of input parameters and to understand the interactions between them. RSM is an approach to modelling the relationships between a response variable and a set of predictor variables. RSM is used when there is interest in understanding how the response variable responds to changes in the predictor variables and for the response optimization. CCD is an efficient and cost-effective way to gain deeper insight into the system behaviour and to optimize the design.

A CCD with five variables and five levels was used in this study. The variables included pH (*A*), the quantity of the extractant (*B*, µL), the volume of the disperser (*C*, µL), the amount of salt (*D*, %), and the centrifugation time (*E*, min). The utilized factors and their levels are summarized in Table I.

TABLE I. The central composite design matrix and responses

Variable	Level			Star points (α = 2.0)	
	Low (–1)	Central (0)	High (+1)	$-\alpha$	$+\alpha$
Extractant solvent volume, µl	100	150	200	50	250
Disperser solvent volume, µl	200	300	400	100	500
Salt amount ,%	2	4	6	0	8
Centrifuge time, min	2	3	4	1	5
pH	4	6	8	2	10

Standard solutions and calibration curve

A stock solution of 100 ppm of *t*,*t*-MA was prepared by dissolving *t*,*t*-MA in a mixture of deionized water and methanol (1:4 volume ratio). The stock was diluted five times, then the standard solutions were prepared from the 20 ppm solution (used for spiked urine samples and calibration curve). Urine samples of non-smokers and healthy volunteers who were not occupationally exposed to benzene were used for the calibration curve. To reduce and remove coarse suspended particles and molecules, the samples were centrifuged (5000 rpm; 10 min), then filtered through a membrane (pore size = 0.45 µm) and were diluted 1:2. After finding the optimal conditions using CCD, seven urine samples were prepared and analysed in the optimum conditions for calibration curves, including non-spiked urine and six spiked samples (2, 1, 0.5, 0.1, 0.05 and 0.01 ppm).

DLLME procedure

According to the proposed extraction method, Fig. 1 illustrates the DLLME procedure schematically. To minimize the matrix effect, urine was centrifuged (10 min, 5000 rpm). 2 mL of urine was diluted 1:2 with deionized water. A stepwise addition of HCl and NaOH solution was applied to adjust the sample pH (pH 8). The urine was then injected with 300 µL of chloroform. Next, the salt concentration was adjusted by the required percentage of NaCl (3.4 %), and gently shaking the solution. Afterward, 300 µL of dispersive (acetonitrile) was added, which resulted in a cloudy solution. The cloudy solution remained stable for approximately 10 min. Finally, the cloudy solution was centrifuged at 4000 rpm for 4 min. The extracted phase was separated from the bottom of the solution using the syringe and then injected into the HPLC for further analysis.

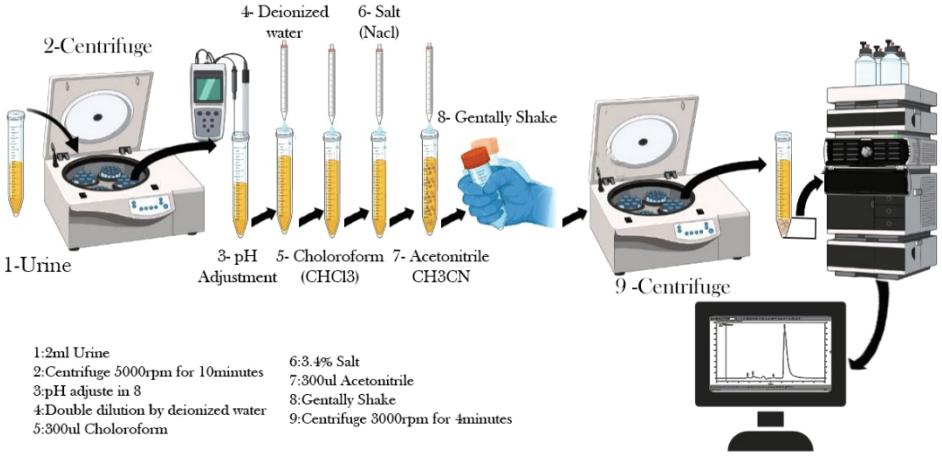

Fig. 1. Schematic procedure for the utilized DLLME technique for determination of *t*,*t*-MA.

Collection of real samples

Urine samples were collected from four occupationally exposed workers from Petrochemical company at end-of-shift. All samples were analysed within a week. The samples were stored at –20 °C before analysis. All procedures associated with the collection of urine samples and human participation conformed to the relevant regulations and the Helsinki Declaration's ethical principles. Shiraz University of Medical Sciences Ethics Committee approved this study.

RESULTS AND DISCUSSION

Selection of extraction and dispersive solvents

Two extractants including carbon tetrachloride and chloroform, and three dispersants including acetonitrile, methanol and acetone were used as potential solvents for extraction of urinary *t*,*t*-MA. Both extractants were tested with all dispersers. In addition, all possible experiments were carried out under three pH conditions: pH 2, 7 and 10. For each experiment, 2 ml of centrifuged urine was diluted 1:2 with deionized water. The urine was treated with 100 µL of extractant and a dispersive solvent. It was observed that the extractant phase was separated from the urine phase without centrifugation. Ultimately, the extractant/dispersive couple that produced the most effective extraction efficiency and the separation of the organic phase within the shortest possible time was chosen as the extraction and dispersive solvent.

In different conditions, carbon tetrachloride made two phases between 15–50 min with three dispersants. The result showed that the organic phase separation using carbon tetrachloride and acetonitrile at pH 10 was obtained at 14 min, while using carbon tetrachloride and methanol at pH 7separation time was 75 min. It is emphasized that the separation without centrifugation is possible for application in automated separation in future studies. In addition, in various conditions, the separation of organic phase from urine were observed using chloroform with three dis-

persants in times longer than 60 min. Finally, chloroform was chosen as the extractant and acetonitrile as the dispersant.

Optimization variables; RSM-CCD

As shown in Table S-I (Supplementary material to this paper), the experimental runs were ordered randomly in the CCD matrix to prevent uncontrolled variables. The CCD matrix was used to ensure that all experimental runs in the suggested DLLME were conducted in an unbiased manner. This ensured that the results of the experiment were not influenced by external factors which can be a guarantee for the application of the method. The results of the experiment were then analysed to determine the variable's effect on the peak area of the extracted *t*,*t*-MA as the outcome of model. Following the collection of responses associated with each run (Table S-I), the quadratic polynomial model was fitted based on the ANOVA analysis results. A backward elimination variable method was used to establish a reined model and to eliminate factors or interaction variables with non--significant *p*-values (> 0.1) and the final results are as follows:

$$\text{Peak Area} = 192.5428 + (1.554873A) + (0.544503B) + (-5.74414C) + \\ + (-73.9784\times\text{D}) + (-232.203E) + (-0.00577AB) + (-0.08685-) + \\ (0.532968\times\text{B}\times\text{E}) + (2.733038CD) + (10.83993CE) + (20.52228DE) \quad (1)$$

To confirm the capability of the obtained linear regression model (MLR), Fisher's statistical test (F-test) was employed. In the model (Eq. (1)), the *F*-value (41.17) was higher than the critical *F*-value in the required degree of freedom, which shows its significance. The results of the Fisher's statistical test indicated that the multiple linear regression model was statistically significant. Therefore, the model can be used to predict the outcome of the experiment.

To confirm the validity of MLR models obtained for this suggested DLLME, a non-significant lack of fit (*LOF*) is another critical criterion to consider. According to the current model, the *F*-value of *LOF* was 0.4875, indicating that it was not significant. Moreover, it indicates that the proposed MLR is free of pure errors. The squared regression coefficients of MLR model were calculated for the evaluation of overall fitness and predictive ability, such as the calibration R^2 (R^2_{cal}), adjusted R^2 (R^2_{adj}) and prediction R^2 (R^2_{pred}). This showed that the model was able to accurately predict the peak area of *t*,*t*-MA after applying the microextraction based on the given inputs.

As can be seen in Tables II and S-II of the Supplementary material, R^2_{cal} demonstrates the proposed CCD model successfully models 92.8 % of the data. Its goodness of fit was confirmed by an R^2_{adj} that was greater than 0.8. The R^2_{pred} (0.807) and the R^2_{adj} (0.894) were consistent. An indication that the prediction ability is very good is the closeness between R^2_{pred} and R^2_{adj} with a difference of less than 0.2.[23] The degree of precision is another statistic used to measure signal-

-to-noise (*S*/*N*). A precision greater than 4 is considered acceptable and based on Tables II and S-II, a signal-to-noise ratio of 18.54 is considered appropriate.[19]

TABLE II. Analysis of variance (ANOVA), summary statistics of the quadratic model – statistical parameters of the MLR model, in the current microextraction study

Std. Dev.	6.723	R^2	0.970
Mean	45.068	R^2_{adi}	0.946
C.V., %	14.917	R^2_{pred}	0.831
PRESS	3570.196	Adeq. Precision	22.585

It is evident from all the above metrics that the factors and interactions included in the suggested MLR model are sufficiently correlated. The peak area is used as the response value of the *t*,*t*-MA recovery derived from DLLME. Based on the plot in Fig. 2a, it can be seen that the predicted peak area is in good agreement with the experimental peak area values, which indicates the ability of the proposed model to make accurate predictions. Using the residual value (the difference between actual and predicted response) is a criterion for determining the applicability domain of an MLR. A narrow range for the studentized residual of the outcome model is shown in Fig. 2b, demonstrating its reliability.[24] Furthermore, all residual values are scattered randomly on either side of the zero line, indicating that there has been no systematic error.[25]

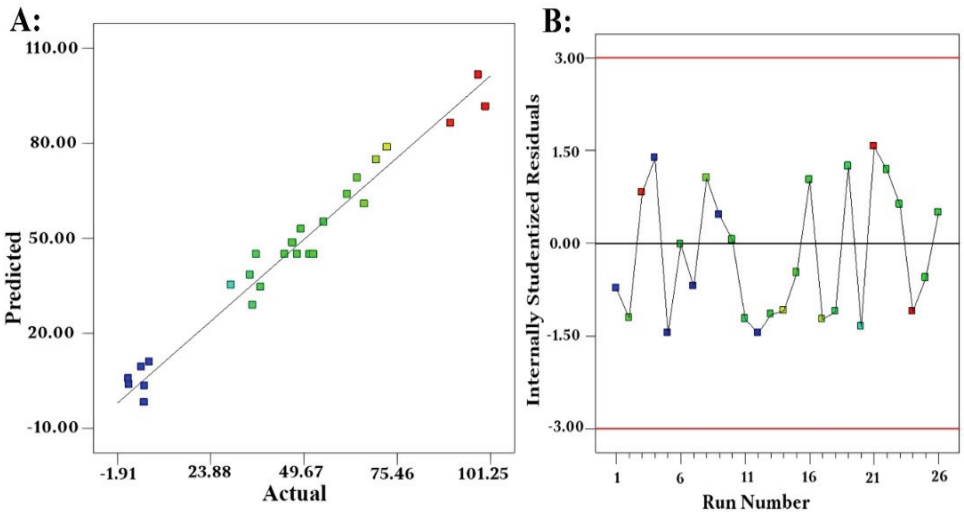

Fig. 2. The internally studentized residuals *vs*. the performed runs (A); and the predicted value vs. actual response (B).

MLR model includes some interaction terms, such as the interaction between *AC*, *AE*, *CD* and *CE*, as shown in Eq. (1). Three-dimensional (3D) response surface plots have been used to describe the mixed effects of factors in between-factors-

-interaction terms and to determine optimal values for the independent parameters in the suggested DLLME. Additionally, these curves are useful in identifying possible interactions between two independent variables to provide information about the maximum responses.

In Fig. 3A, it is demonstrated that the matrix pH and the disperser volume simultaneously affect the response (peak area of *t*,*t*-MA). pH plays an important role in all extraction techniques involving basic or acidic analytes. Several experiments were conducted to investigate the effect of pH on *t*,*t*-MA extraction from the sample solutions (Table III). As can be seen in Fig. 3a, the maximum response was observed by increasing the pH and volume of the disperser solvent. It is therefore necessary to increase the pH of the sample to achieve the highest extraction rate. The maximum response was observed at pH 6.

Fig. 3B illustrates the effect of different amounts of dispersive and extraction on the extraction of *t*,*t*-MA. To determine whether the quantity of disperser affects the response level, a variety of examinations were designed, using the acetonitrile volumes ranging from 100 to 500 μL(Table II). The maximum response (peak area) was achieved when acetonitrile value was increased. Various tests have been conducted with various quantities of chloroform ranging from 50 to 250 μL to determine the impact of the volume of extractant solvent (Table II). As a result of these tests, which can be seen in Fig. 3B, increasing both dispersing and extractant solvents provided increasing the *t*,*t*-MA peak area.

Based on the variations in salt versus pH (Fig. 3C), it appears that increasing pH led to increase in the efficiency of the suggested DLLME and consequently increased *t*,*t*-MA peak area. However, the increasing salt percentage has no significant effect on the analyte signal when compared with pH.

Fig. 3D shows the interaction between centrifuge time and extractant solvent volume. Assuming that the metabolite concentration, the salt amount, and the volume of dispersive solvent were constant, increasing the extractant solvent needs a decrease in the centrifuge time to reach an enhancement in the peak area of metabolite. In other word, by the addition of extractant solvent, increasing the centrifuge time showed negative effect on the extraction efficiency (Fig. 3D).

As shown in Fig. 3E, increasing the centrifugation time can decrease the microextraction efficiency in higher amount of salt. Thus, it can be suggested that simultaneously increasing the salt amount and centrifugation time has a negative effect on the peak area.

The change in the peak area in different amounts of pH and centrifugation time is represented in Fig. 3F. In lower pH values, increasing the centrifuge time can decrease the peak areas of the metabolite and microextraction efficiency. On the other hand, at higher values of pH, increasing centrifuge time can lead to higher peak areas.

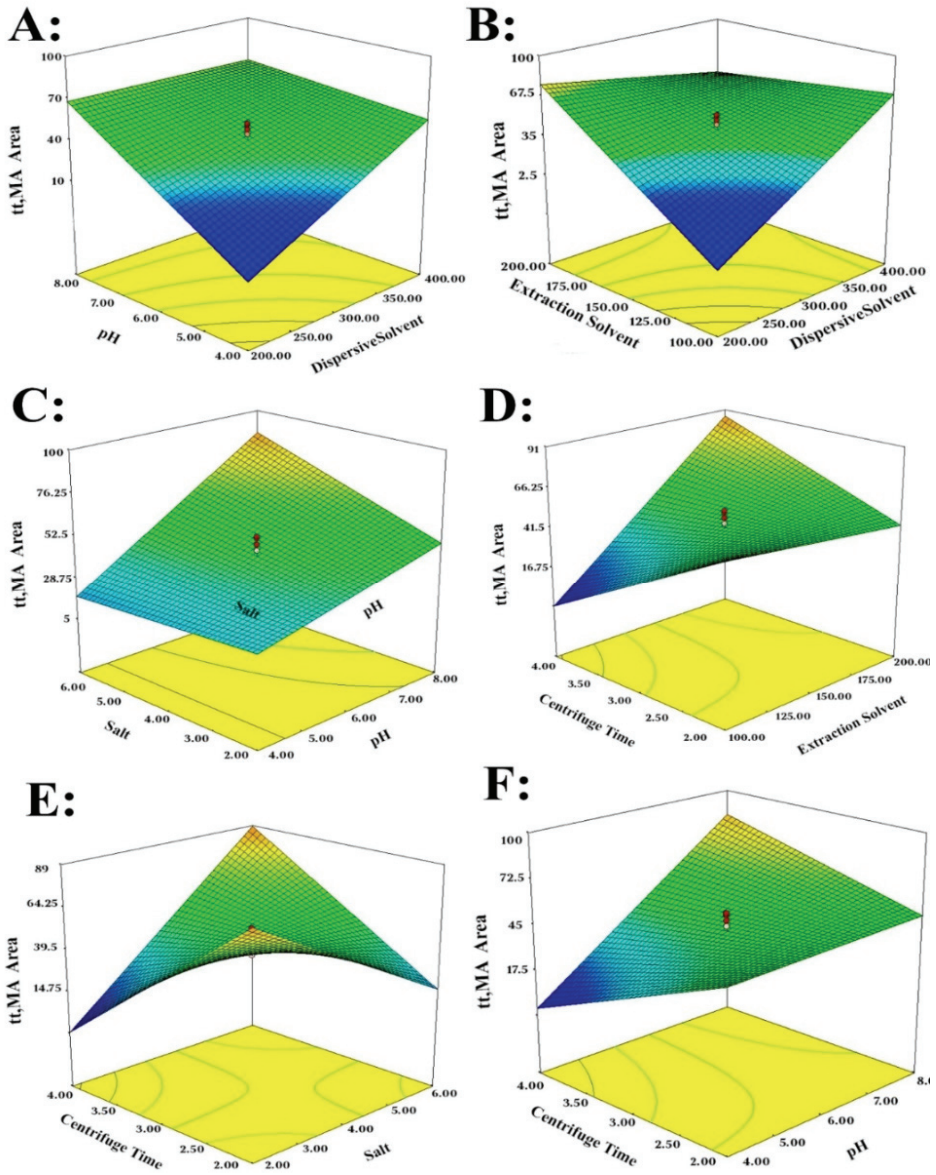

Fig. 3. Three-dimensional response surface plots of between-factor interaction terms.

TABLE III. Analytical characteristics of the method

Correlation coefficient (r^2)	0.9996
LDR, µL/ml	0.008–5.0
LOD, µg/ml	0.0024
LOQ, µg/ml	0.008
Calibration equation	$y = 2\times10^6x + 474423$

Optimum conditions

As given above, the statistical analysis of the suggested MLR model showed the validity of the proposed DLLME and thus can be applied to find the optimum conditions. For the determination of the optimal experimental conditions for the extraction of *t*,*t*-MA, the simplex optimization was applied. As shown in Table S--III (Supplementary material), the optimum conditions were as follows: pH 8, extractant solvent volume, 300 μL, volume of disperser solvent, 300 μL, applied salt content, 3.4 % and centrifuge time, 4 min.

To determine the *t*,*t*-MA concentration, a calibration curve was drawn using spiked samples of *trans*-muconic acid under the optimum conditions. The calibration graph (Fig. 4a and b) was drawn by spiked values of 0.01, 0.1, 0.5, 1 and 2 ppm. A calibration graph with $R = 0.9996$ was derived (Table III). Fig. 4a illustrates the obtained peaks.

Matrix effect

The chromatograms of two spiked urine samples with two *t*,*t*-MA concentrations extracted by the proposed DLLME method were compared with similar spiked in distilled water, each with three replicates. The results showed a significant change of peaks in urine and water because of the matrix effect (change of more than 17–20 % in *RSD*).

Thus, it was decided to perform the calibration curve in the urine sample. On the other hand, to show the presence or absence of matrix effect in different urine samples, two concentrations of *t*,*t*-MA were spiked into three urine samples obtained from persons without exposure to benzene (0.5 and 1.0 μg mL^{-1}). The change in urine samples was estimated by following the *RSD* between samples.

There was no change in *t*,*t* retention time and the *RSD* between different urine samples was lower than 7.2 (for 0.5 μg mL^{-1}) and 6.8 % (for 1.0 μg mL^{-1}). However, for better performance it could be suggested to do the calibration curve in a pool of not-exposed urine samples. Fig. 4 shows the chromatograms of spiked urine samples with concentrations of 0.01, 0.1, 0.5, 1 and 2 μg mL^{-1}.

Method validation

A number of merit measures have been evaluated to assess the effectiveness of the optimized method. These measures include quantification limit (*LOQ*), precision, linear dynamic ranges (*LDR*s), detection limit (*LOD*), correlation coefficients of the calibration curve (R^2), and relative recovery (*RR*), Table III.

To assess the precision of the proposed method, three spiked urine samples in different levels were used. Three replicates were done on three days and the relative standard deviation (*RSD*) was calculated (Table III). The *RSD*s were in the range of 5.1–6.8 %. To evaluate the method's accuracy, the average of the extraction recovery (*ER*) in the spiked samples was calculated (Table IV). The average

ER in the spiked samples was between 95.8–102 % and confirmed the ability of the extraction method for the analyte.

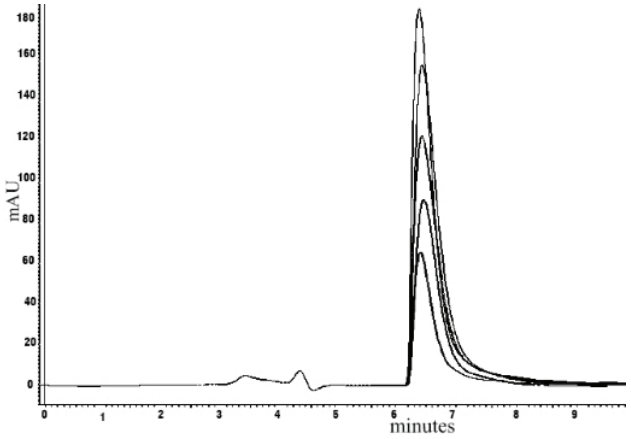

Fig. 4. Chromatogram of spiked urine samples after extraction in optimum conditions with concentrations of 0.01, 0.1, 0.5, 1 and 2 μg mL^{-1}.

TABLE IV. Results of the validation of accuracy and precision of suggested DLLME; *ER* – extraction recovery; *RSD* – relative standard deviation

Spiked level, μg/ml	Intra-day *RSD*, % (n = 3)	Average *ER*, %
0.5	<6.1	102.4
1	<6.8	98.6.5
2.5	<5.1	95.8

Application of the optimized DLLME; biomonitoring of benzene-exposed workers

The established DLLME method was applied to the four urine samples of petrochemical workers exposed to benzene. The suggested method and the conventional standard laboratory method strong anion exchange in SPE (SAX-SPE), were used to determine the quantity of *t*,*t*-MA (Table V). A good agreement was observed between the proposed and standard methods.

TABLE V. The result of real sample analysis by the suggested DLLME and standard SPE

Sample ID	Predicted conc., μg/mL		Residual μg/mL	Creatinine mg dL^{-1}	Predicted adjusted conc. (μg/g creatinine)	
	DLLME	SPE			DLLME	SPE
Worker 1	0.85	0.89	–0.04	92	923.9	967.4
Worker 2	0.745	0.692	0.053	81	919.8	854.3
Worker 3	0.38	0.43	–0.06	95	400.0	452.6
Worker 4	1.03	1.16	–0.13	88	1170.5	1318.2

Comparison with other methods

In Table S-IV (Supplementary material), a comparison of the developed DLLME with other methods is presented with previous similar methods using HPLC-UV. Based on the comparison, it can be concluded that the proposed DLLME resulted in acceptable analytical figures of merit. Further, compared with most previous methods, a lower volume of samples and solution was consumed, and the extraction times were decreased.

The proposed DLLME technique is an efficient and simple extraction method for target metabolite (*t*,*t*-MA) from the urine matrix. Additionally, the *LOD* and *LDR* values were comparable to other developed extraction methods for the urinary *t*,*t*-MA, and the analysis by HPLC–UV. The suggested DLLME method with low *LOD* (2.4 μg/L) and *LOQ* (8 μg/L) showed sufficient sensitivities for benzene biomonitoring, especially at low levels of benzene exposure. On the other hand, as can be seen in Table S-IV (Supplementary material), the extraction time is lower than in most of the previous reports, which make it suitable for the automatic extraction systems as well. Accordingly, the proposed DLLME analysis can be used to determine *t*,*t*-MA in urine samples in a sensitive, user-friendly, time- and cost-effective manner.

The method developed by Rismanchian *et al.* was the most similar to the method suggested in this study.[22] Nevertheless, there are some differences between the developed DLLME method and the PDLLME method introduced by Rismanchian *et al*. In the presented DLLME method in this study, solvents are employed in smaller amounts than the PDLLME method. According to Rismanchian *et al.* study, 5 ml of sample, 200 μL of chloroform, 2000 μL of tetrahydrofuran, 20 μL of methanol, and nitrogen result in a total of 7220 μL of sample and solvent was required.

The present DLLME method uses 2 ml of sample, 300 ml of chloroform, and 300 ml of acetonitrile (in total 4600 μL of solvent and sample). Secondly, Rismanchian *et al.*'s protocol is longer than the current protocol (more than 15 min, and the suggested method is approximately 8 min). As well as this, the linear range of the present DLLME method (0.0008–5 μg/mL) is much lower than that in Rismanchian *et al.*'s study (0.1–10 μg/mL), which is more suitable for the evaluation of metabolites at low concentrations. Additionally, Rismanchian *et al.*'s study used tetrahydrofuran (2000 μL), a volatile substance with a boiling point of 66 °C and a vapour pressure of 162 mm Hg* at 25 °C, whereas in the present study, acetonitrile (300 μL) was used, which has lower volatility (boiling point 82 °C, vapor pressure 73 mm Hg at 25 °C). Additionally, acetonitrile has a saturation concentration of 9.6 % at 20 °C and tetrahydrofuran has a saturation concentration of 19.1 % at 20 °C. Due to its higher volatility and tendency to vaporize,

* 1 mm Hg = 133.3 Pa

tetrahydrofuran will have a higher concentration in the air at the same temperature and pressure. The same working conditions result in a higher risk of the operator being exposed to tetrahydrofuran. In Rismanchian *et al.*'s protocol, three solvents and nitrogen gas were used, while in the present protocol, only two solvents were used. Therefore, the presented DLLME method has overcome the limitations of the PDLLME method by having less complexity, less extraction time, less solvent consumption, less linear range, and fewer exposures to the operator than the PDLLME method developed by Rismanchian *et al*.

CONCLUSIONS

The present study proposes a new and efficient DLLME for *t*,*t*-MA, a well--known benzene metabolite. The developed DLLME was coupled with HPLC–UV and showed significant efficiency for benzene biomonitoring. Moreover, the proposed DLLME method is highly efficient, requires a short extraction time, and exhibits high selectivity and accuracy. The proposed approach was also successfully applied to real urine samples of benzene-exposed workers with the extraction efficiency ranging from 95.8 to 102.4 %.

In this study, a multivariate approach was applied to optimize variables that could affect the preconcentration of the *t*,*t*-MA to identify the optimum conditions. It has been demonstrated that the suggested DLLME approach can be effectively applied for biomonitoring of individuals who have been occupationally exposed to benzene in industrial settings. However, the use of organic solvent is a limitation of this approach. However, the main goal of this work was to suggest a simple approach to be applicable in an automatic microextraction, which is in progress in our research group. On the other hand, very small quantities of solvent, in the microliter range, are sufficient for the suggested DLLME method.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/12789, or from the corresponding author on request.

Acknowledgements. The present paper was a part of PhD Thesis conducted by Abolfazl Moghadasi at Shiraz University of Medical Sciences (Shiraz, Iran). The authors would like to appreciate the financial support provided by the Shiraz University of Medical Sciences (Grant No. 24565).

ИЗВОД

ДИСПЕРЗИВНА ТЕЧНО-ТЕЧНА МИКРОЕКСТРАКЦИЈА ЗА ОДРЕЂИВАЊЕ МУКОНСКЕ КИСЕЛИНЕ КАО БИОЛОШКОГ ИНДИКАТОРА БЕНЗЕНА У УРИНУ

ABOLFAZL MOGHADASI[1,2], SAEED YOUSEFINEJAD[3], ESMAEEL SOLEIMANI[2], SAJJAD TAGHVAEI[4] и SAEED JAFAI[2]

[1]Student Research Committee, Shiraz University of Medical Sciences, Shiraz, Iran, [2]Department of Occupational Health Engineering, School of Health, Shiraz University of Medical Sciences, Shiraz, Iran, [3]Research Center for Health Sciences, Institute of Health, Department of Occupational Health Engineering, School of Health, Shiraz University of Medical Sciences, Shiraz, Iran и [4]Department of Mechanical Engineering, Shiraz University, Shiraz, 71936-16548, Iran

Праћење професионалне изложености хемикалијама је од суштинског значаја за процену радног места. У случају опасних и канцерогених хемикалија, као што је бензол, надзор на раду постаје још важнији. *Trans*,*trans*-муконска киселина (*t*,*t*-MA) је један од метаболита бензена у урину. Методе претходног третмана за *t*,*t*-MA генерално укључују течно–течну и екстракцију на чврстој фази. Употреба дисперзивне течно–течно микроекстракције (DTTME) током припреме узорка и екстракције може смањити трошкове екстракције и утицаје на животну средину. Штавише, процес је исплатив и једноставан за руковање. Ова студија је имала за циљ да развије, оптимизује и валидира аналитичку методу за мерење концентрације *t*,*t*-MA у матриксу урина применом DTTME у комбинацији са течном хроматографијом високих перформанси. Пет варијабли укључујући pH, количину екстрактанта и супстанце за дисперговање, количину соли и време центрифугирања оптимизовано је применом централног композитног дизајна са методологијом површине одговора и експерименталних података. Предложени DTTME приступ је успешно примењен на реалне узорке, добијене од радника изложених бензену, са ефикасношћу екстракције од 95,8 до 102,4 %. Оптимални услови су pH 8, растварач за екстракцију, 300 μL, растварач за дисперговање, 300 μL, 3,4 % раствор соли и време центрифугирања, 3 min. Према резултатима ове студије, предложени DTTME приступ се може ефикасно применити на биомониторинг појединаца изложених бензену.

(Примљено 25. јануара, ревидирано 5. априла, прихваћено 7. јула 2024)

REFERENCES

1. C. Carvalhais, M. Querido, C. C. Pereira, J. Santos, *Work* **69** (2021) 3 (https://doi.org/10.3233/WOR-205302)
2. L. De Maria, C. Ledda, A. Caputi, F. Mansi, E. S. S. Cannone, S. Sponselli, D. Cavone, F. Birtolo, E. Cannizzaro, G. M. Ferri, V. Rapisarda, L. Vimercati, *Front. Public Heal.* **8** (2020) 271 (https://doi.org/10.3389/fpubh.2020.00271)
3. Y. R. Li, C. J. Xie, C. X. Qiu, Q. Y. Lin, Y. M. Liu, *Chin. J. Ind. Hygiene Occupat. Dis.* **37** (2019) 119 (https://doi.org/10.3760/CMA.J.ISSN.1001-9391.2019.02.007)
4. S. B. Wilbur, S. M. S. Keith, O. Faroon, D. Wohlers, *ATSDR's Toxicol. Profiles* (2002) (https://doi.org/10.1201/9781420061888_ch38)
5. N. Mirzaei, K. Naddafi, Raminnabizadeh, K. Yaghmaeian, M. S. Assanvand, S. Maroufizadeh, M. Hoseini, S. Adabi, M. Yunesian, *Acta Medica Mediterr.* **32** (2016) 1471 (https://www.actamedicamediterranea.com/archive/2016/special-issue-4/urinary-benzene-as-a-biomarker-of-environmental-exposure-to-benzene-in-males-in-the-general-population/document)

6. T. Tunsaringkarn, J. Suwansaksri, S. Soogarun, W. Siriwong, A. Rungsiyothin, K. Zapuang, M. Robson, *Asian Pacific J. Cancer Prev.* **12** (2011) 223 (https://journal.waocp.org/article_25501_cd29634a0ebf3dad97742bb9edc1e432.pdf)
7. E. A. Natelson, *Am. J. Hematol.* **82** (2007) 826 (https://doi.org/10.1002/ajh.20934)
8. M. Borgie, A. Garat, F. Cazier, A. Delbende, D. Allorge, F. Ledoux, D. Courcot, P. Shirali, Z. Dagher, *Chemosphere* **96** (2014) 122 (https://doi.org/10.1016/j.chemosphere.2013.09.034)
9. S. M. Rappaport, S. Kim, Q. Lan, R. Vermeulen, S. Waidyanatha, L. Zhang, G. Li, S. Yin, R. B. Hayes, N. Rothman, M. T. Smith, *Environ. Health Perspect.* **117** (2009) 946 (https://doi.org/10.1289/ehp.0800510)
10. E. Soleimani, *Rev. Anal. Chem.* **39** (2020) 168 (https://doi.org/10.1515/revac-2020-0116)
11. S. Viegas, M. Z. Jeddi, N. B. Hopf, J. Bessems, N. Palmen, K. S. Galea, K. Jones, P. Kujath, R. C. Duca, H. Verhagen, T. Santonen, R. Pasanen-Kase, *Int. J. Environ. Res. Public Health* **17** (2020) 5884 (https://doi.org/10.3390/ijerph17165884)
12. I. Pilia, M. Campagna, G. Marcias, D. Fabbri, F. Meloni, G. Spatari, D. Cottica, C. Cocheo, E. Grignani, F. De-Giorgio, P. Cocco, E. D'aloja, *Int. J. Environ. Res. Public Health* **18** (2021) 4644 (https://doi.org/10.3390/ijerph18094644)
13. F. Ghamari, A. Bahrami, Y. Yamini, F. G. Shahna, A. Moghimbeigi, *Anal. Chem. Insights* **2016** (2016) 65 (https://doi.org/10.4137/Aci.s40177)
14. Threshold Limit Values (TLVs®) and Biological Exposure Indices (BEIs®), 2022, https://www.techstreet.com/standards/2022-threshold-limit-values-tlvs-and-biological-exposure-indices-beis?product_id=2242171#jumps (accessed 1 January, 2022)
15. F. Dehghani, F. Omidi, O. Heravizadeh, S. Yousefinejad, *Sci. Rep.* **11** (2021) 15751 (https://doi.org/10.1038/s41598-021-95174-5)
16. A. Damokhi, S. Yousefinejad, S. Jafari, E. Soleimani, F. Dehghani, *J. Mol. Liq.* **386** (2023) 122506 (https://doi.org/10.1016/j.molliq.2023.122506)
17. D. Basu, S. Bag, S. Mukherjee, G. Goel, *J. Acad. Clin. Microbiol.* **21** (2019) 66 (https://doi.org/10.4103/jacm.jacm_20_18)
18. A. Barbieri, L. Sabatini, A. Accorsi, A. Roda, F. S. Violante, *Rapid Commun. Mass Spectrom.* **18** (2004) 1983 (https://doi.org/10.1002/rcm.1580)
19. S. Abbaszadeh, S. Yousefinejad, S. Jafari, E. Soleimani, *J. Sep. Sci.* **44** (2021) 3126 (https://doi.org/10.1002/jssc.202100044)
20. M. Tehranirokh, M. Van den Bronk, P. Smith, Z. Dai, K. Ragunathan, A. Muscalu, S. Mills, M. C. Breadmore, R. A. Shellie, *J. Chromatogr., A* **1642** (2021) 462032 (https://doi.org/10.1016/j.chroma.2021.462032)
21. M. Rezaee, Y. Yamini, S. Shariati, A. Esrafili, M. Shamsipur, *J. Chromatogr., A* **1216** (2009) 1511 (https://doi.org/10.1016/j.chroma.2008.12.091)
22. M. Rismanchian, K. Ebrahim, Z. Ordudari, *Chem. Pap.* **73** (2019) 2485 (https://doi.org/10.1007/s11696-019-00800-2)
23. S. Yousefinejad, F. Honarasa, N. Saeed, *J. Sep. Sci.* **38** (2015) 1771 (https://doi.org/10.1002/jssc.201401427)
24. M. Nekoeinia, S. Yousefinejad, A. Abdollahi-Dezaki, *Ind. Eng. Chem. Res.* **54** (2015) 12682 (https://doi.org/10.1021/acs.iecr.5b02982)
25. S. Yousefinejad, F. Honarasa, H. Montaseri, *RSC Adv.* **5** (2015) 42266 (https://doi.org/10.1039/c5ra05930e).