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Sample Preparation Followed by High Performance Liquid Chromatographic (HPLC) Analysis for Monitoring Muconic Acid as a Biomarker of Occupational Exposure to Benzene

Seyed Jamaleddin Shahtaheri
Farhad Ghamari
Farideh Golbabaei
Abbas Rahimi-Froushani
Mohsen Abdollahi

**School of Public Health & Institute of Public Health Research,
Tehran University of Medical Sciences, Tehran, I.R. Iran.**

Factors affecting solid phase extraction (SPE) of trans,trans-muconic acid (ttMA), as a benzene biomarker, including sample pH, sample concentration, sample volume, sample flow rate, washing solvent, elution solvent, and type of sorbent were evaluated. Extracted samples were determined by HPLC-UV (high performance liquid chromatography-ultraviolet). The analytical column was C18, UV wave length was 259 nm, and the mobile phase was H_2O/methanol/acetic acid run at flow rate of 1 ml/min. A strong anion exchange silica cartridge was found successful in simplifying SPE. There was a significant difference between recoveries of ttMA when different factors were used ($p < .001$*). An optimum recovery was obtained when sample pH was adjusted at 7. There was no significant difference when different sample concentrations were used (*$p > .05$*). The optimized method was then validated with 3 different pools of samples showing good reproducibility over 6 consecutive days and 6 within-day experiments.*

benzene muconic acid sample preparation solid phase extraction
biological monitoring chromatography

1. INTRODUCTION

Due to increasing concern about toxic substances such as benzene and its analogs in the environment and workplaces, it is becoming more important to monitor such chemicals and their metabolites in order to evaluate risk hazards and potential problems caused by exposure to toxic compounds [1, 2, 3]. Benzene is an important industrial compound because of its widespread usage, occurrence in mineral oil, and also in many combustion processes that cause environmental and industrial pollution [2, 4]. Occupational and environmental exposure to benzene occurs mainly via inhalation. Relatively high exposure takes place in manual application techniques such as production, distribution, and handling of gasoline. The predominant sources are emissions from vehicles, petrochemical industries,

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Correspondence and requests for offprints should be sent to Seyed Jamaleddin Shahtaheri, School of Public Health & Institute of Public Health Research, Tehran University of Medical Sciences. P.O. Box 14155-6446, Tehran, I.R. Iran. E-mail: <sjtaheri@sphtums.com>.

and chemical reactions of organic materials. The classification of benzene as a human carcinogen by the International Agency for Research on Cancer (IARC) has resulted in a reduction in occupational exposure levels and, as a consequence, has led to the need for improved biomonitoring techniques [5]. Despite the low metabolic conversion of benzene to trans,trans-muconic acid (ttMA), earlier investigations have revealed that the concentration of ttMA, which is formed and excreted together with phenol, catechol, hyroquinon, S-phenylmercapturic acid, and hyroxyhydroquinone after exposure to benzene, is a suitable urinary metabolite of higher specificity and it is now going to be a more popular biomarker for occupational and environmental exposure to benzene [4, 6, 7, 8]. There are two reasons for selecting ttMA as a biomarker for benzene. First, the exposure limit of benzene is yearly decreased based on new research in which metabolism of benzene to ttMA is well performed at low exposure. Second, ttMA is now considered as a specific metabolite of benzene, showing actual exposure level of individuals to benzene [4, 6, 7, 8]. In biological matrices, either exposed compounds or their metabolites are mostly present at a trace level, making their determination a major problem [9, 10, 11, 12]. Therefore, essential needs for sensitive and selective techniques for the analysis of trace chemicals in environmental and biological matrices have been clearly recognized [9, 10, 13, 14, 15, 16]. Although the use of a detection system has improved the selectivity of the analytical procedures, these sensitive and selective methods require extensive equipment; moreover, they may not be available in most laboratories. Consequently, sample pre-treatment procedures which can be performed in any laboratory have been developed to simplify analytical approaches as these methods reduce expenses, too [9, 10, 13, 14, 15, 16]. Although derivatization reactions, performed either before or after analytical techniques, can enhance the sensitivity of the assay, this extra performance is not often a favorite stage in sample preparation followed by analysis. Although many analytical methods still use liquid-liquid extraction (LLE) to perform sample clean-up [17, 18], in this procedure large volumes of solvents, with an undesirable environmental impact are used. Moreover, because of problems associated with LLE, it is difficult for the method to be automated. In addition, the recovery obtained from LLE is not often suitable and reproducible. In contrast, solid-phase extraction (SPE) methods using silica or bonded silica have proven useful in simplifying sample preparation prior to HPLC-UV (high performance liquid chromatography-ultra violet). Isolation and purification of the compound of interest can be achieved in a short time and only low volumes of` solvents are used during the application of the method. The use of commercially available low-cost vacuum manifolds allows many samples to be processed simultaneously. Furthermore, complete automation of procedures based on SPE is now possible using commercially available instrumentation [19, 20]. A wide range of phases based on silicas are also available from many suppliers, including reversed phase, normal phase, ion exchange, and mixed-mode phases. These phases can be screened and selected, depending on the chemical nature of the analyte [21]. Therefore, the variety of available phases can improve the selectivity of the sample preparation method.

This study aimed at achieving optimum factors necessary for the development of an optimized procedure for ttMA (Figure 1), leading to a simple protocol of the SPE method [22].

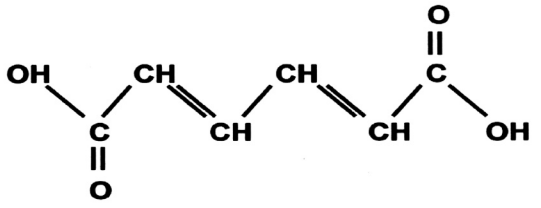


Figure 1. Structure of trans,trans-muconic acid (ttMA).

2. MATERIAL AND METHODS

2.1. Reagents and Chemicals

Trans,trans-muconic acid (99%) (ttMA) as standard was obtained from Merck, Germany. Methanol, ethanol, acetonitril, and acetic acid (all HPLC grade), deionized water, and standard

buffered solution at three pH values (4.00 ± 0.02, 7.00 ± 0.02, and 9.00 ± 0.02) were also purchased from Merck, Germany. Octadecyl (C18) and strong anion exchange (SAX) sorbents (100 mg and 500 mg) were obtained from Macherey-Nagel, Germany, and used for a solid phase extraction procedure.

2.2. Apparatus

A Vac-Elute Vacuum Elution System (Negin Co., Iran) was used to retain and elute silica cartridges. A digital pH meter from Hanna, Singapore, was used for pH adjustment. The amount of reagents was measured, using a CP 225D Satorius balance (Sartorius, Germany) for milligram, or lower, quantities. Quantitative liquid transfers were performed with a pipette (Socorex, Germany). The HPLC apparatus used in this study included the following equipment: a K-1001 single piston pump (Knauer, Germany), the analytical column was C18 (25 cm × 4.6 mm i.d., 5 µm) purchased from Hichrom Limited, Reading, UK. The detector was a K-2600 LC-UV spectrometer obtained from Wellchrom, Germany. The system was linked with a Hewlett Packard (USA) LaserJet 1200 series printer for recording chromatograms, using a 1456-1 Chromogate Data System, Version 2.55 (Knauer, Germany). Solvents and mobile phase used in HPLC analysis were degassed with an on-line degasser attached to the solvent delivery system.

2.3. Optimized Sample Preparation

In this study, SPE using bonded silica including SAX and C18 (100 and 500 mg) was optimized with regard to sample pH, sample concentration, sample flow rate, elution solvent, washing solvent, sample volume, elution volume, sorbent type, and sorbent mass. The cartridges were conditioned with 3 ml of methanol followed by 3 ml of HPLC water. Care was taken to prevent the cartridges from drying. The samples were then passed through the columns at a flow-rate of 1–2 ml/min. The cartridges were then washed with 3 ml of different solvents. Finally, ttMA was eluted from the column with 4 ml of different solvents. The extracts were then analyzed with HPLC-UV.

2.4. Chromatographic conditions

The pump was operated at 1 ml/min, UV detection wavelength was set at 259 nm, the mobile phase consisted of water/methanol/acetic acid, 69:30:1 (v/v/v), flow rate was 1 ml/min, injection volume was 100 µl, the analytical column was C18 (25 cm × 4.6 mm i.d., 5 µm), and ambient temperature was used for the chromatographic system. Under these conditions, ttMA was eluted and detected in 7–8 min. In this study, peak area was used as detector response, and extraction recoveries were calculated by comparison of the peak area in the chromatogram of extracts with those in the chromatogram of standard solutions prepared in the same solvent as follows:

$$\text{Recovery (\%)} = \frac{\text{peak area (sample)}}{\text{peak area (standard)}} \times 100.$$

More experiments were performed on spiked urine to validate the present method. Spiked urine can be a suitable model as it may contain interfering constituents similar to a real sample [23]. The spiked samples of 50 ml of ttMA were used for extraction followed by HPLC-UV determination. Linear standard curve (for extracted samples) over the range of 0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1 µg/ml were obtained every day (n = 6) with the correlation coefficient of .999 or greater. The extraction procedure was reliable and reproducible from day-to-day and within-day.

3. RESULTS

3.1. Optimization of Chromatographic Analysis

In order to achieve optimum chromatographic conditions for an analysis of ttMA, variables including mobile phase composition, UV wavelength, injection volume, and mobile phase flow-rate were optimized. Figure 2 shows a chromatogram of ttMA at concentration of 5 µg/ml detected at around 8 min.

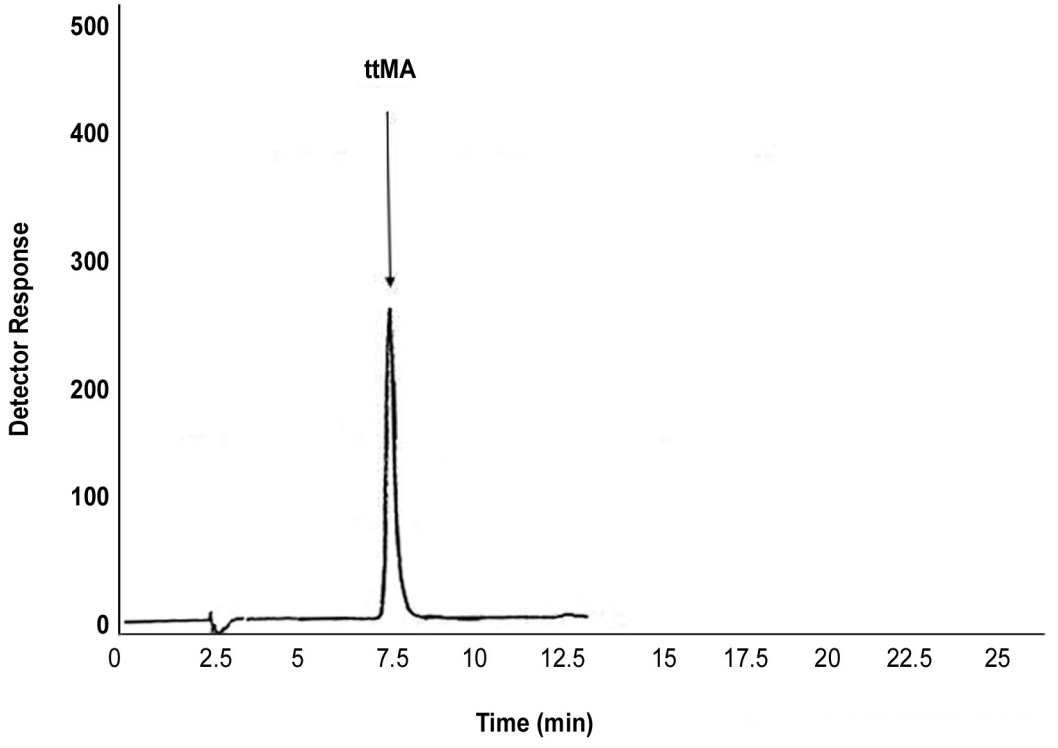


Figure 2. HPLC chromatogram of trans,trans-muconic acid (ttMA) at concentration of 5 µg/ml.

3.2. Optimization of Solid Phase Extraction

3.2.1. Sorbent selection

In order to optimize SPE, there were several factors with which retention and elution could be altered. First, sorbents, including C8, C18, and SAX, containing 100 and 500 mg of bonded silica were evaluated for extraction recovery of ttMA. After conditioning, columns with 3 ml of methanol followed by the same volume of deionized water, 4 ml of ttMA standard at different concentrations were applied. The retained analyte was washed with 3 ml of acetic acid 1% followed by elution with 4 ml of acetic acid 10%. The results are illustrated in Table 1.

TABLE 1. Recovery of ttMA Obtained From Sorbents

Sorbent Type	Recovery (*M* ± *SD*)
SAX	99.80 ± 2.00
C18	40.80 ± 0.75
C8	47.80 ± 2.00

Notes. ttMA—trans,trans-muconic acid, SAX—strong anion exchange, C18—octadecyl; *N* = 6. Four ml of sample (50 µg/ml) were used, conditioning with 3 ml of methanol followed by 3 ml of deionized water, eluted in 4 ml of acetic acid 10%.

3.2.2. Sample pH

The 500-mg C8, C18, and SAX cartridges were activated and conditioned according to the method explained. Four ml of sample at different pH values of 3, 5, 7, 9, and 11 were applied. The columns were then washed and the retained analyte was eluted using the same procedure as explained beforehand. Figure 3 shows the influence of sample pH on extraction recovery for ttMA.

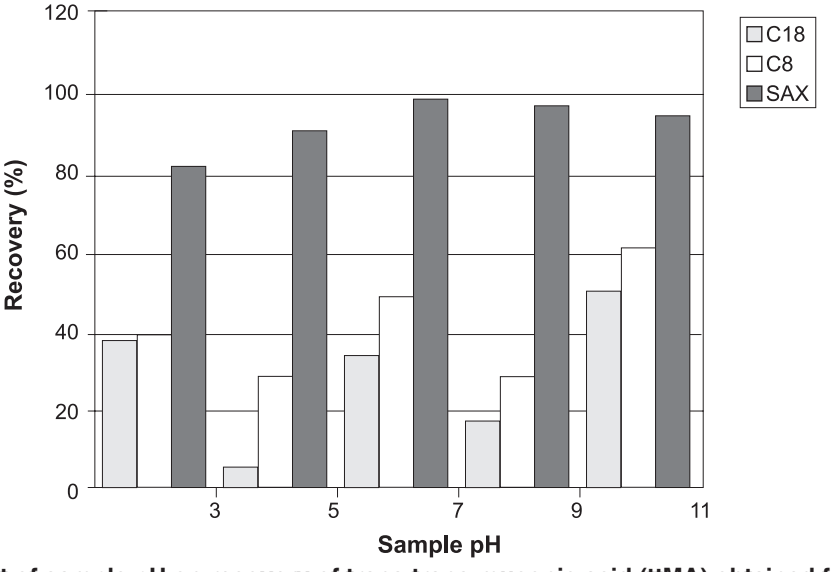


Figure 3. Effect of sample pH on recovery of trans,trans-muconic acid (ttMA) obtained from sorbents (500 mg). *Notes*. SAX—strong anion exchange.

3.2.3. Sample concentration

In order to evaluate the effect of sample concentration on SPE performance, different concentrations of ttMA, including 0.01, 0.1, 1, 10, and 100 μg/ml, as has been mentioned in Table 2, were prepared using deionized water.

3.2.4. Eluent type

Evaluation of eluent strength on ttMA recovery was another experiment performed during this study. Seven solvents were screened for their ability to produce optimum elution of the retained ttMA from the SAX and C8 sorbents. They were acetonitril, ethanol, methanol, acetic acid 5%, acetic acid 7%, acetic acid 9%, and acetic acid 10%. The same sequence of conditioning, washing, and elution were used as in the previous section. The results of this process are shown in Figure 4.

TABLE 2. The Recovery of ttMA From SAX and C8 at Different Sample Concentrations (μg/ml)

	Recovery (*M* ± *SD*)				
Sorbent	**0.01**	**0.10**	**1**	**10**	**100**
SAX	72.00 ± 6.70	97.00 ± 7.80	100.00 ± 3.00	101.00 ± 2.40	92.00 ± 6.20
C8	35.80 ± 0.66	43.20 ± 1.70	63.00 ± 3.46	38.00 ± 1.70	28.4 ± 4.90

Notes. ttMA—trans,trans-muconic acid, SAX—strong anion exchange; $N = 6$.

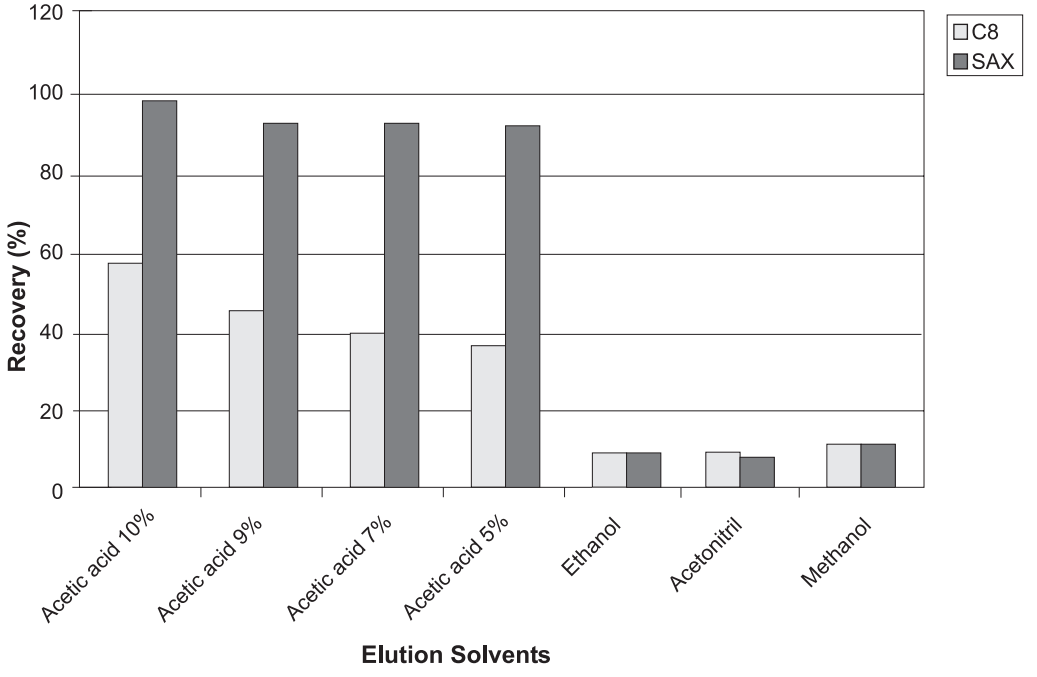


Figure 4. The recovery of trans,trans-muconic acid (ttMA) from sorbents SAX and C8, using different elution solvents at concentration of 50 µg/ml. *Notes*. SAX—strong anion exchange.

3.2.5. Eluent volume

Enrichment of the analyte in SPE is achieved by applying large volumes of sample and eluting the analyte in a minimum volume of eluent. The volume of the eluent must be just sufficient to elute the compound of interest from the sorbent. The result obtained from an evaluation of the volume of elution showed that the smallest satisfactory volume for acetic acid from 500 mg of sorbent, was 4 ml.

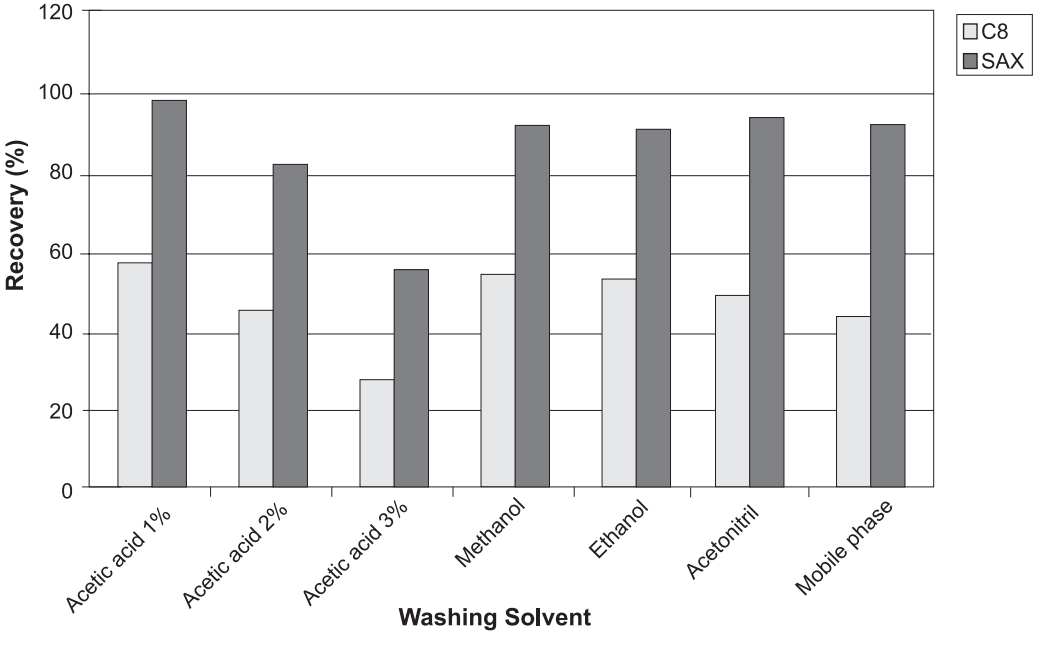


Figure 5. Effect of washing solvent on recovery of trans,trans-muconic acid (ttMA) obtained from sorbents (500 mg). *Notes*. SAX—strong anion exchange.

3.2.6. Washing solvent

In order to remove unbound material and interferences adsorbed to either the silica support or the phases bonded to silica, the column was washed with 3 ml of different solvents immediately after the retention stage. The volume of the solvent can be increased until the unwanted materials have been clearly removed. However, care should be taken that no analyte-sorbent bonding is broken during the washing stage. The results obtained in this experiment have been illustrated in Figure 5.

3.2.7. Sample volume

In order to evaluate the volume breakthrough of the silica cartridges, a 1-ml sample of 1 µg/ml of ttMA was diluted into different volumes of 10, 50, 100, 500, and 1000 ml loaded to columns C8 and SAX. The columns were washed and eluted according to the optimized method. The results are shown in Table 3 demonstrating that up to 500 ml of sample could be applied without significant loss of recovery (at least 90.40 ± 2.30 for 500 ml of sample volume).

3.2.8. Sample flow

Following a demonstration of the feasibility of using large sample volumes, the effect of sample flow rate on trans,trans-muconic acid recovery was investigated. Flow rate ranges of 1, 2, 4, 8, 10, and 20 ml/min were used in these experiment. One hundred ml of sample was utilized on the column and the same extraction sequence was employed. No significant reduction in recovery was found for sample flow rate up to 2 ml/min when SAX was used (Figure 6).

TABLE 3. Effect of Sample Volume (ml) on Recovery of ttMA Obtained From Sorbents

	Recovery (*M* ± *SD*)					
Sorbent	**1**	**10**	**50**	**100**	**500**	**1000**
SAX	100.40 ± 1.30	94.40 ± 3.00	89.60 ± 1.30	91.00 ± 1.9	90.40 ± 2.30	40.00 ± 1.40
C8	63.00 ± 3.50	60.402.00 ±	41.40 ± 2.70	39.60 ± 1.1	28.20 ± 1.90	15.00 ± 3.60

Notes. ttMA—trans,trans-muconic acid, SAX—strong anion exchange; sample volume: 500 mg, *N* = 6.

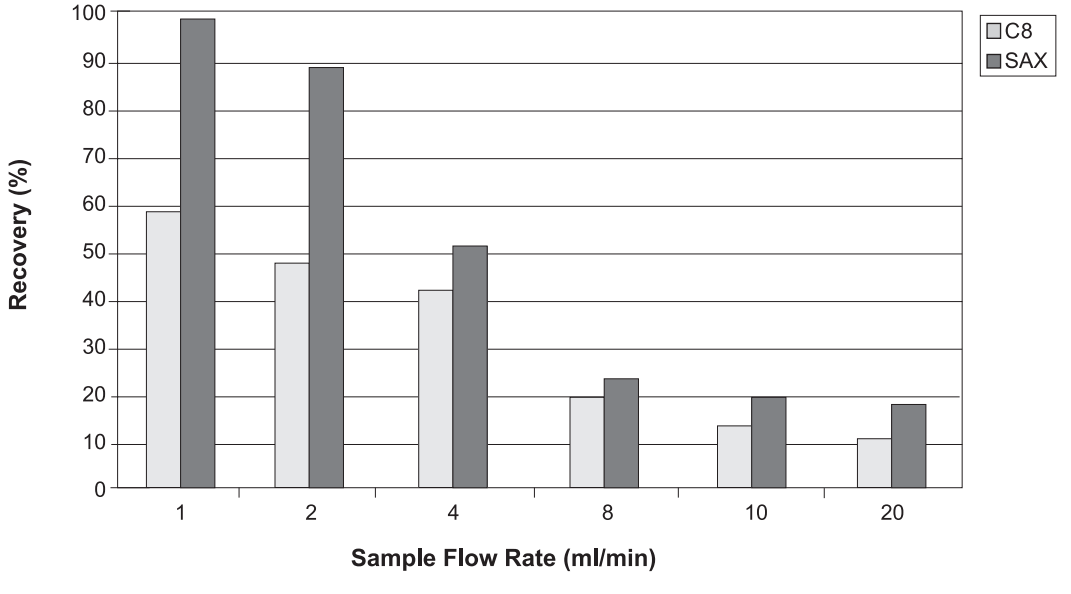
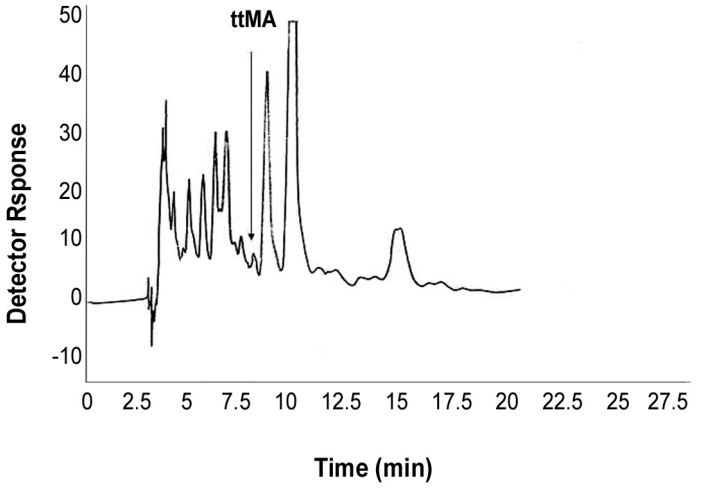
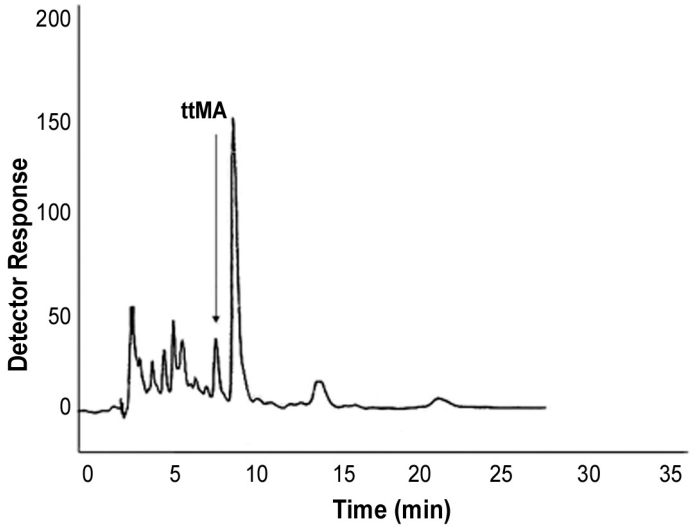


Figure 6. Effect of sample flow rate on recovery of trans,trans-muconic acid (ttMA) obtained from sorbents (500 mg). *Notes*. SAX—strong anion exchange.

(a) 0.1 μg/ml



(b) 1 μg/ml



(c) 10 μg/ml

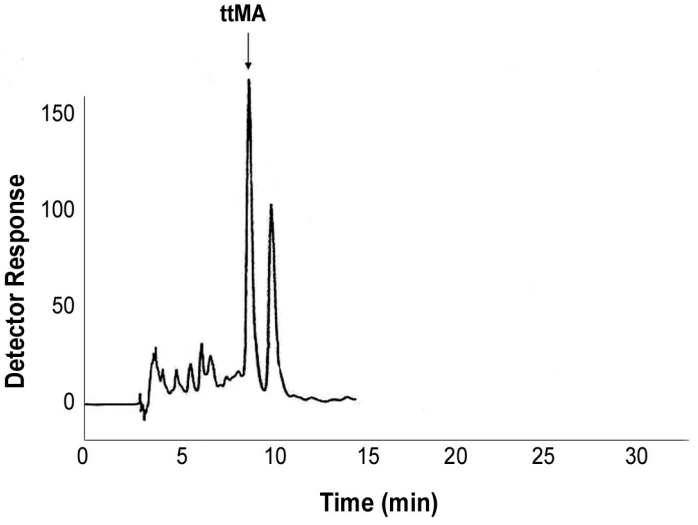


Figure 7. HPLC chromatograms of spiked urine sample of trans,trans-muconic acid (ttMA) at concentrations of (a) 0.1 μg/ml, (b) 1 μg/ml, (c) 10 μg/ml. Mobile phase, water/methanol/acetic acid, 69:30:1 (v/v/v), flow rate, 1 ml/min injection volume: 100 μl, analytical column: C18 (25 cm × 4.6 mm i.d., 5 μm), UV detection at 259 nm, the ambient temperature was used for the chromatographic system.

TABLE 4. Day-to-Day (D-day) and Within-Day (W-day) Reproducibility of ttMA Spiked in Urine

	Concentration Added (μg/ml)					
	0.1		1		10	
Statistical Data	D-day	W-day	D-day	W-day	D-day	W-day
M (SD)	0.097 (0.007)	0.1 (0.01)	0.99 (0.025)	0.98 (0.03)	9.90 (0.3)	9.87 (0.27)
RSD	7.20	10.00	2.50	3.06	3.00	2.74

Notes. ttMA—trans,trans-muconic acid; sample volume: 50 ml, $N = 6$.

3.2.9. Validation of optimized method

As spiked urine may contain some interference similar to a real sample [23], it can be considered as an appropriate sample for validation of the optimized method. Therefore, samples containing 50 ml of ttMA were used for extraction followed by HPLC-UV determination (see Table 4). Figure 7 also shows HPLC chromatograms of spiked urine samples of ttMA at concentrations of 0.1, 1, and 10 μg/ml.

4. DISCUSSION

Analytical columns widely used for analysing such compounds are generally reversed phase, in which C18 is preferred due to its frequent use and efficient results in trace analysis of organic acids [24, 25]. The wavelength of 259 nm was more sensitive for determining the analyte. In this study, the flow-rate of the mobile phase was also screened; 1 ml/min was a suitable flow-rate to get an optimum retention time for a ttMA chromatogram. Using these conditions, the compound of interest was eluted in 7–8 min as shown in Figure 2. The retention time of ttMA can be changed by increasing different concentrations of organic modifiers in the mobile phase. Therefore, retention time (k' value) can be varied by changing the composition of the mobile phase in order to isolate the analyte from interferences contained in the sample solution.

From the result given in Table 1, it was deduced that a SAX cartridge was more satisfactory for efficient recovery of ttMA. It seems that the polarity of the sorbents as well as the hydrophlilicity of the compound can be major factors for the mechanisms that took place. As can be seen, a few similar interactions took place with both C8 and C18. The quantity of the sorbents was then also screened. It was indicated that the greater the quantity of the sorbent, the greater the sample breakthrough volume, and the greater the elution solvent volume. Due to the type of interaction, providing an efficient recovery, it seems that a SAX cartridge can be a more advantageous phase for further optimization steps.

The results showed that efficient recovery was obtained from SAX using sample pH of 7. For the compound, however, the amount of the analyte recovered from SAX at sample pH values of 3, 5, 9, and 11 was also efficient. From these pH values, sample pH of 7 was selected for further study as this pH seems to be a rather moderate value. This investigation showed that the pH value of the sample should be adjusted according to the chemistry of the compound of interest. ttMA is an ionizable compound (pKa is 3.85) when pH is at least 2 units higher than pKa. Therefore, it was necessary to adjust the pH of the sample adequately in order to ionize ttMA completely and ensure that the compound was in appropriate ionic or weakly associated form to achieve efficient retention by the solid phase sorbent using ionic interaction mechanism. At pH below pKa, the recovery can be broken down because of non-stability of silica-bonded cartridges at these low pH values. As Figure 3 shows, a more efficient recovery was achieved at sample pH value of 7. The extraction recoveries obtained from C8 and C18 at different values of pH were still low. It seems that when sample pH is above pKa, ttMA is in an ionized form, in which a suitable mechanism interaction cannot take place as the solid phase is a non-polar sorbent and retention cannot take place on non-polar cartridges like C8 and C18. When pH is equal to pKa, ttMA is in 50:50 ionized forms, so the retention and the recovery are low. Although at sample pH below pKa, ttMA can be in a molecular form to make a non-polar interaction, C18 is not

stable; therefore, the compound of interest cannot be retained adequately on both C8 and C18.

Ideally, the extraction recovery should not be sample concentration dependent. In other words, for the method to be useful there should be no significant difference in recovery over the expected concentrations range of the compound to be analyzed. Table 2 gives the recoveries obtained after passing a 4-ml sample at different sample concentrations followed by elution with 4 ml of acetic acid 10%. As can be seen, the recoveries are independent of sample concentrations over the concentrations range studied. Although some differences can be seen between the obtained recoveries, all recovery values are satisfactory scientifically as the results show range recoveries of (72.00 ± 6.70)–(101.00 ± 2.40). However, sample pH of 7 was used for this experiment; therefore, recoveries obtained for C8 at all concentrations were poor. During this experiment, the breakthrough fraction was also analyzed and no breakthrough of the compound was detected.

Understanding the chemistry of the compound under analysis such as its hydrophilicity or ionizability can be useful in designing appropriate conditions to obtain efficient extraction recovery. Highly hydrophilic compounds result in a strongly retained analyte making elution difficult and leading to subsequent poor recovery from ionic sorbents. Although four different percentages of acetic acid can extract ttMA efficiently without no significant difference ($p > .5$), acetic acid 10% seems to be an optimum eluent compared to other solvents ($p < .05$) to break completely the hydrophilic interaction between the sorbent and the analyte of interest. According to the chemistry and the composition of the solvent, it can increase the solubility of the analyte as well as minimize physical losses on sample handling. However, other percentages (5, 9, and 7%) of acetic acid can also be preferred.

The result obtained from an evaluation of the elution volume showed that the smallest satisfactory volume for acetic acid, from 500 mg of sorbent, was 4 ml. As a consequence, the volume required to elute the analyte from the sorbent, depends on two important parameters. First, the capacity factor (k') of the compound of interest, showing the strength of its retention. A solvent with greater elution strength can be used to elute an analyte in less volume but may incorporate undesirable contaminants into the eluted fraction; secondly, the sorbent mass used in SPE, in which using a larger sorbent mass cartridges requires an increased elution volume to be applied.

As it can be seen in Figure 5, C8 is still performing as a non-efficient sorbent. In contrast, washing the SAX column, using all solvents, is significantly efficient (recovery ranges: 90–100) except for acetic acid 3%, in which this washing solvent is much closer to the eluent. However, considering the efficiency, the use of acetic acid 1% may have more advantages as this solvent is safer and also relatively similar to the eluent (acetic acid 10%), by which the most closely related interference compounds can be removed at this step. An increase in the percentage of acetic acid may result in the compound of interest being co-washed followed by a decrease in ttMA recovery as it can be seen in Figure 5 when acetic acids 2 and 3% were used.

The sample volume experiment allowed an accurate measurement as low as 0.002 µg/ml of ttMA when a large sample volume (500 ml) is applied on the column, resulting in a possible trace enrichment of the analyte. This experiment has shown that the column could retain 1 µg of ttMA, which is compatible with current chromatography detection systems. However, the efficiency of ttMA when using C8 at all sample volumes is considerably low.

However, with the experiment of evaluating the flow rate effect on extraction, the recovery was reduced at flow rates of 4–10 ml/min and most likely the compound was eluted in breakthrough, which is not detectable in a 100-ml sample volume. It may be concluded that the interaction between ttMA and sorbents SAX and C8 is not strong enough to run sample at a flow rate of more than 2 ml/min. However, a strong interaction between the analyte and the sorbent can cause the elution process to be more difficult, in which more elution solvent should be applied, providing less analyte concentration has taken place.

Finally, in order to validate the method, reproducibility of the optimized method was

performed for day-to-day and within-day experiments. A linear standard curve (for extracted samples) over the range concentrations of 0.1, 1, and 10 µg/ml was obtained ever day for 6 consecutive days (n = 6) with the correlation coefficient of .999 or greater. In within-day evaluation, six experiments were performed per day for 3 consecutive days. The extraction procedure was reliable and reproducible from day-to-day and within-day. Coefficients of variation (CV%) of 7.20, 2.50, and 3.00 were obtained for 0.1, 1, and 10 µg/ml respectively for day-to-day and 10.00, 3.06, and 2.74, at the same concentrations respectively for within-day, showing suitable accuracy and precision (see Table 4). Through these experiments reproducible and quantitative recoveries, ranging from 97 to 100%, were also possible. Figure 7 shows HPLC chromatograms of spiked urine samples of ttMA at concentrations of 0.1, 1, and 10 µg/ml.

5. CONCLUSION

Through this study factors influencing SPE were optimized, showing an efficient sample preparation procedure for muconic acid as a biomarker of benzene, as a solid phase extraction method using bonded silica has more advantages than LLE. Depending on the chemical and physical properties of the analyte, manipulating factors including sorbent type, sample pH, sample volume, sample concentration, sample flow rate, and type and volume of eluent can play essential roles in optimizing the method, providing reliable, easy to use, and cost-effective procedure to overcome difficulties associated with other sample preparation techniques. Applicability of the method for treating different classes of pollutants such as pesticides and different hydrocarbons can make the technique popular when a selective and sensitive trace residue analysis is required. The authors are sure that the SPE is a highly fertile area for sample preparation methods and, based on the needs and facilities, these method protocols can be further developed in the near future.

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