Int. J. Environ. Res., 2(2): 169-176, Spring 2008 ISSN: 1735-6865

# Monitoring of Mandelic Acid as a Biomarker of Environmental and Occupational Exposures to Styrene

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Received 26 June 2007; Revi	sed 20 Sep. 2007;	Accepted 7 Oct. 2007
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**ABSTRACT**: Styrene is an important constituent of widely used organic solvents in industries for production of various synthetic materials. The use of solid-phase extraction (SPE) has grown and is a fertile technique of sample preparation as it provides better results than those produced by liquid-liquid extraction. In this study, SPE has been optimized, regarding sample pH, sample concentration, sample flow rate, elution solvent, washing solvent, sample volume, elution volume, and sorbent mass. Through experimental evaluation, a strong anion exchange silica cartridge has been found successful in simplifying sample preparation. The present approach proved that, mandelic acid, as a urinary metabolite of styrene, could be retained on solid phase based on specific interaction. Further study was employed 10% acetic acid to extract the analyte from spiked urine and gave a clean sample. 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 0.85 ml/min, the mobile phase was water/methanol/acetic acid and an ultra violate detector was used, setting at 225 nm. At the developed conditions the extraction recovery was exceeded 98%. The factors were evaluated statically and also validated with three different pools of spiked urine samples and showed a good reproducibility over six consecutive days as well as six within-day experiments.

Key words: Solid phase extraction, Biological monitoring, Optimization, Chromatography

## **INTRODUCTION**

Due to increasing concern about toxic substances such as styrene 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 occupational and environmental exposures to toxic compounds (Keymeulen, et al., 2001; Sperlingova, et al., 2003). Styrene is an organic solvent being commonly used in pharmaceutical processes and considered as an important industrial chemical compound because of its occurrence in mineral oil and its formation in many combustion processes, causing widespread environmental and industrial pollutions (Keymeulen, et al., 2001). Occupational and environmental exposures to styrene occur

mainly via inhalation and relatively high exposure occurs in manual application techniques. Styrene is metabolized to mandelic acid and this urinary compound can be used as a main biomarker for evaluation of human exposure to styrene. Human exposure to styrene causes biological effects, including chronic bronchitis, obstructive pulmonary changes, decreased lung ventilation, asthma, tiredness, nasal secretion and nose irritation (Chung, et al., 2006). Also, its susceptibility of genetic disorders is now under investigation (Teixeira, et al., 2004; Leigh and Speit, 2006). Although mandelic acid (MA) was identified as a urinary metabolite long time ago, it is now going to be a more popular biomarker for different exposures to styrene (Ma, et al., 2005; Ohashi, et al., 2006; Wang, et al., 2006). In biological

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matrices, either exposed compounds or their metabolites mostly are present at a trace level, causing major problems in their determination stages (McDowall, 1989; McDowall, 1989; Shahtaheri, et al., 1995; Shahtaheri, et al., 1998). Therefore, an essential need for sensitive and selective techniques for the analysis of trace chemical compounds in environmental and biological matrices has been clearly recognized (Poole, et al., 1990; Hennion and Scribe, 1993; Maria, 2000; Manini, et al., 2005) The use of detection system has improved the selectivity of the analytical procedures. However, these sensitive and selective methods require extensive equipments; moreover, they may not be available in most laboratories. Consequently, sample pretreatment procedures which can be performed in any laboratory have been developed to simplify analytical approaches as these methods reduce expenses too (Barcelo, 1991; Shahtaheri and Stevenson, 2001; Shahtaheri, et al., 2007). Sometimes, derivatization reactions performed either before or after analytical techniques can enhance the sensitivity of the assay, but, this extra performance is not often a favorite stage in sample preparation followed by analysis. Many analytical methods still use liquid-liquid extraction (LLE) to perform sample clean-up (Ibrahim and Suffet, 1988; Liu and Pleil, 2001) In this procedure, large volume of solvents, having undesirable environmental concerns is used as well as problems associated with the technique to be automated. In addition, the recovery obtained from LLE is not often suitable and reproducible. In contrary, solidphase extraction (SPE) methods using silica or bonded silica have proven useful in simplifying sample preparation prior to HPLC-UV. 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 (Focant, et al., 2004; Pettersson, et al., 2004). A wide range of phases from many suppliers based on silica are also available including reversed phase, normal phase, ion exchange and mixed-mode phases. These phases can be

screened and selected, depending on chemical nature of the analyte (Hennion, 1999). Therefore, the variety of available phases can improve the selectivity of the sample preparation method.

This study was aimed to achieve the optimum factors necessary for development of an optimum procedure for MA (Fig. 1), leading to a simple protocol of solid phase extraction method.



Fig. 1. Chemical structure of mandelic acid as an urinary metabolite of styrene

#### **MATERIALS & METHODS**

Mandelic acid (99%) (MA) 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 ( $4.00\pm0.02$ ,  $7.00\pm0.02$ , and  $9.00\pm0.02$ ) were also purchased from Merck, Germany. Strong anion exchange (SAX) sorbent (100 mg and 500 mg) was obtained from Macherey-Nagel, Germany and used for solid phase extraction procedure.

A Vac-Elute Vacuum Elution System was used for retention and elution of silica cartridges. A digital pH meter from Hanna, Singapore was used for pH adjustment. The amount of reagents was measured, using a Satorius balance for milligram quantities or less. Quantitative liquid transfers were performed with pipette (Socorex, Germany). The HPLC apparatus used in this study included the following equipments: a K-1001 single piston pump (Knauer, Germany), the analytical column was a C18 (25 cm  $\times$  4.6 mm i.d., 5  $\mu$  m) 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 LaserJet 1200 series printer for recording the chromatograms, using a 1456-1 Chromogate Data System, Version 2.55 (Knauer, Germany). Because the reagents used in this study were HPLC grade, there was no need to filter them. However, the analytical column in HPLC system was equipped with a filter on the top. Solvents and mobile phase used in HPLC analysis were degassed by an on-line degasser attached to the solvent delivery system.

In this study, SPE using bonded silica including SAX (100 mg and 500 mg) has been optimized with regard to sample pH, sample concentration, sample flow rate, elution solvent, washing solvent, sample volume, elution volume, and sorbent mass. The cartridges were conditioned with 3 mL of methanol followed by 3 mL 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, the MA was eluted from the column with 4 mL different solvents. The extracts were then analyzed by HPLC-UV.

The pump was operated at 0.85 ml/min, UV detection wavelength was set at 225 nm, the mobile phase consisted of water/methanol/acetic acid, 69:30:1 (v/v/v), flow rate was 0.85 mL/min, injection volume was 100  $\mu$ L, the analytical column was C18 (25 cm × 4.6 mm i.d., 5  $\mu$ m), and the ambient temperature was used for the chromatographic system. Under these conditions, MA was eluted and detected between 9-10 minutes (Fig. 2). 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 following:

Recovery (%) = peak area (sample)/peak area (standard) ´ 100

## **RESULTS & DISCUSSION**

In order to achieve the optimum chromatographic conditions for analysis of mandelic acid, variables including mobile phase composition, UV wavelength, injection volume, and mobile phase flow-rate were optimized. Analytical column widely used for analysis of such compound is generally reversed phase, in which, C18 was preferred due to its frequent use and efficient results in the trace analysis of organic acids (Tormo and Izco, 2004; Shui. and Leong, 2002). The wavelength of 225 nm was more sensitive for determination of the analyte. In this study, the flow-rate of the mobile phase was also screened, in which, 0.85 mL/min was a suitable flow-rate to get an optimum retention time for MA chromatogram. Using these conditions, the compound of interest was eluted between 9-10 minutes as shown in Fig. 2. The retention time of mandelic acid can be changed by increasing different concentrations of organic modifier 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.

The 500 SAX cartridge was activated and conditioned according to the method explained. 4 ml of sample at different pH values of 2, 4, 6, 7, 8, and 10 were applied. The columns were then washed and retained analyte was eluted using the procedure as explained beforehand. Table 1 shows the influence of sample pH on extraction recovery for MA. The results showed that efficient recovery was obtained from SAX using sample pHs of 6, 7, 8, and 10 (Table 1). From these pH values, sample pH of 7 was selected for further study as this pH seems to be rather mold value. This investigation showed that the pH value of the sample should be adjusted according to the chemistry of the compound of interest. MA is ionizable compound (pKa is 3.85) when its pH is at least 2 units more than pKa. Therefore, it was necessary to adjust the pH of the sample adequately in order to ionize MA 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 the pH bellow pKa, the recovery is poor and a significant difference can be seen when the sample pH is increased more than its pKa (P<0.001) As the Table 1 shows, efficient recovery was achieved at sample pH 6 and above.

In order to evaluate the effect of sample concentration on SPE performance, different concentrations of MA ranged from 50 to 1600  $\mu$ g/mL were prepared using deionized water, using sample pH of 7. 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 1 gives the recoveries obtained after passing 4 mL sample at different sample concentrations followed by elution with 4 ml acetic acid 10%. As can be seen, the recoveries are independent of sample

Sample pH	Mean(%)±SD (N=6)	Sample concentration(µg/ml)	Mean(%)±SD (N=6)
2	61.33±3.98	50	99.41±1.55
4	$62.45 \pm 4.86$	100	$99.84{\pm}1.11$
6	$110.85 \pm 4.90$	200	$99.48 \pm 2.06$
7	$104.88 \pm 6.39$	400	$107.45 \pm 1.20$
8	$102.08 \pm 2.76$	800	$109.96 \pm 1.75$
10	$105.76 \pm 4.07$	1600	$113.76 \pm 4.07$

Table 1. Effects of sample pH and sample concentration on recovery of mandelic acid from strong anion
exchange (SAX) sorbent

4 mL of samples at concentration of 50 mg/mL passed through 500 mg sorbent conditioned with 3 mL methanol followed by 3 mL deionized water, eluted by 4 mL acetic acid 10%

concentrations over the concentrations range studied. Although some differences can be seen between obtained recoveries, all recovery values are satisfactory scientifically as the results show range recoveries of 99.41-113.76. During this experiment, the breakthrough fraction was also analyzed and no breakthrough of the compound was detected. The minimum sample concentration of 50  $\mu$ g/mL used in this experiment was half of the Permissible Exposure Level (PEL) which is 100  $\mu$ g/ml, (recommended by OSHA for evaluation of occupational exposure to styrene). Therefore, preferably, this concentration was used for experimental tests.

Another experiment performed during this study was evaluation of the eluent strength on MA recovery. Six solvents were screened for their ability to produce optimum elution of the retained MA from the SAX sorbent. They were ethanol, acetonitril, acetic acid 5% (HOAc), acetic acid 7%, acetic acid 10% and methanol. The same sequence of conditioning, washing, and elution were used as in previous section. The results of this process are shown in Table 2. 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 strongly retained analyte making elution difficult and subsequent given poor recovery from ionic sorbents. Although three different percentages of acetic acid can extract MA efficiently without no significant difference (p>0.5), acetic acid 10% seems to be an optimum eluent compare to ethanol, acetonitril, and methanol (p<0.05) to break completely hydrophilic interaction between sorbent and analyte of interest. According to the chemistry and solvent composition, it can increase the solubility of the analyte as well as minimizing the physical losses on sample handling. However, in case, other percentages (5%, 7%) of acetic acid can also be of preference considerations.

 Table 2. Effects of eluent type and sample volume on recovery of mandelic acid from strong anion exchange (SAX) sorbent

Eluent type	Mean(%)± SD (N=6)	Sample Volume(mL)	Mean(%) ±SD (N=6)
Ethanol	0	1	103.15±2.64
Acetonitril	0	10	96.22±1.42
HOAc 5%	106.81±2.67	20	96.75±2.17
HOAc 7%	$105.21 \pm 2.28$	50	91.06±1.80
HOAc10%	102.23±2.21	100	93.90±3.02
Methanol	0	200	86.16±1.98

Sample pH of 7 at concentration of 50 µg/mL Sorbents (500 mg) were conditioned with 3 mL methanol followed by 3 mL deionized water, eluted by 4 ml acetic acid 10%

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 eluent volume must be just sufficient to elute the compound of interest from the sorbent. The result obtained from an evaluation of 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 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. Solvent with grater 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 require an increased elution volume to be applied.

In order to evaluate the volume breakthrough of the silica cartridges, one mL sample of 400  $\mu$ g/mL MA was diluted into different volumes, 1 mL, 10 mL, 20 mL, 50 mL, 100 mL, and 200 mL and added to the SAX column. The column was washed and eluted according to the optimized method. The results have been shown in Table 2 demonstrating that up to 200 mL of sample could be applied without significant loss of recovery (at least 86.16±1.98 for 200 ml sample volume). This allowed accurate measurement as low as 8  $\mu$ g/mL MA (less than one tenth of PEL) when a large sample volume is applied on the column, therefore, resulting in a possible trace enrichment of the analyte.

Following demonstration of the feasibility of using large sample volumes, the effect of sample flow rate on the MA recovery was investigated. Flow rate ranges of 1 mL/min, 2, 4, 8, and 10 mL/ min. were used in these experiment 100 ml of sample was utilized on the column and the same extraction sequence was employed. No significant reduction of recovery was found for sample flow rate up to 2 mL/min (Table 3). However, 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 100 mL sample volume. It may be concluded that the interaction between the MA and sorbents SAX is not as strong as enough to run sample at flow rate more than 2 ml/min. However, strong interaction between the analyte and the sorbent can cause

the elution process to be performed more difficult, in which, more elution solvent should be applied, providing less analyte concentration taken place.

In order to remove unbound material and interferences adsorbed to either the silica support or the phases bonded to the silica, the column was washed with 3 ml of different solvents immediately after the retention stage. The volume of 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 washing stage. The results obtained from this experiment have been illustrated in Table 3. As it can be seen, washing the SAX column, using all solvents is significantly efficient (recovery ranges: 92.87-102.80). However, considering the efficiency, the use of acetic acid 1% may have more advantageous as this solvent is more similar to the eluent (acetic acid 10%), by which, the most closely related interference compounds can be removed at this step. To increase the percentage of acetic acid may resulted in the compound of interest to be co-washed followed by a decrease in MA recovery as in can be seen in the Table 3 when acetic acid 2% was used. However, this recovery 92±1.82) is still scientifically acceptable and may be used if removal of more closely related analogues is needed.

The quantity of the sorbent was also evaluated, indicating that, the greater quantity of the sorbent, the greater the sample breakthrough volume, and greater the elution solvent volume. However, through this study, cartridges containing 500 mg sorbent was more efficient (Table 3).

Sample flow	Mean(%)±SD	Washing	Mean(%)±SD	Sorbent	Mean(%)±SD
rate(ml/min)	(N=6)	solvent	(N=6)	mass (mg)	(N=6)
1	100.76±1.18	H <sub>2</sub> O	97.48±0.90		
2	90.01±1.63	Ethanol	99.16±1.31	100	74.46±5.83
4	52.75±2.92	Acetonitril	99.66±1.13		
8	28.81±3.17	OHAc 1%	$102.80{\pm}1.88$		
10	$21.18 \pm 2.02$	Methanol	98.51±1.77	500	102.23±1.79
		OHAc 2%	92.87±1.82		

 Table 3. Effects of sample flow rate, washing solvent, and sorbent mass on recovery of mandelic acid from strong anion exchange (SAX)

Sample pH was adjusted at 7 with concentration of 50 µg/ml. Sorbents were conditioned with 3 mL methanol followed by 3 mL deionized water, eluted by 4 mL acetic acid 10%



Fig. 2. HPLC chromatograms of *a*) standard sample at 400 μg/mL, *b*) blank urine sample, spiked urine samples of mandelic acid at concentrations of *c*) 50 μg/mL, *d*) 400 μg/ml, *e*) 1600 μg/mL. Mobile phase, water/ methanol/acetic acid, 69:30:1 (v/v/v), flow rate, 0.85 mL/min injection volume: 100 μl, the analytical column: C18 (25 cm × 4.6 mm i.d., 5 μm), UV detection at 225 nm, the ambient temperature was used for the chromatographic system

# CONCLUSION

The procedure developed during this study, has shown that solid phase extraction using silicabonded is more advantageous and efficient than liquid-liquid extraction. Depending on chemical and physical properties of the analyte, manipulating of the factors, including sample pH, sample volume, sample concentration, sample flow rate, 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 treatment of different classes of pollutants such as pesticides and different hydrocarbons can make the technique to be popular when a selective and sensitive trace residue analysis is required. The authors 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 more developed in the near future.

#### ACKNOWLEDGEMENT

This research has been supported by Tehran University of Medical Sciences and Health Services grant. Hereby, sincere cooperation is highly appreciated. The authors also thank Mr. Mahdi Bahjati for his kind assistance.

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