Iran. J. Environ. Health. Sci. Eng., 2008, Vol. 5, No. 4, pp. 283-296

MOLECULAR IMPRINTED SOLID PHASE EXTRACTION FOR DETERMINATION OF ATRAZINE IN ENVIRONMENTAL SAMPLES

[1]**A. R. Koohpaei,** [*2]**S. J. Shahtaheri,** [3]**M. R. Ganjali,** [4]**A. Rahimi Forushani,** [1]**F. Golbabaei**

[1]Department of Occupational Health, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

[2]Department of Occupational Health, School of Public Health and Center for Environmental Research, Tehran University of Medical Sciences, Tehran, Iran

[3]Center of Excellence in Electrochemistry, Endocrine and Metabolism Research Center, Faculty of Chemistry, University of Tehran, Tehran, Iran

[4]Department of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

Received 23 April 2008; revised 27 June 2008; accepted 29 July 2008

ABSTRACT

Solid phase extraction is one of the major applications of molecularly imprinted polymers fields for clean-up of environmental and biological samples namely molecularly imprinted solid-phase extraction. In this study, solid phase extraction using the imprinted polymer has been optimized with the experimental design approach for a triazine herbicide, named atrazine with regard to the critical factors which influence the molecular imprinted solid phase extraction efficiency such as sample pH, concentration, flow-rate, volume, elution solvent, washing solvent and sorbent mass. Optimization methods that involve changing one factor at a time can be laborious. A novel approach for the optimization of imprinted solid-phase extraction using chemometrics is described. The factors were evaluated statistically and also validated with spiked water samples and showed a good reproducibility over six consecutive days as well as six within-day experiments. Also, in order to the evaluate efficiency of the optimized molecularly imprinted solid-phase extraction protocols, enrichment capacity, reusability and cross-reactivity of cartridges have been also evaluated. Finally, selective molecularly imprinted solid-phase extraction of atrazine was successfully demonstrated with a recovery above 90% for spiked drinking water samples. It was concluded that the chemometrics is frequently employed for analytical method optimization and based on the obtained results, it is believed that the central composite design could prove beneficial for aiding the molecularly imprinted polymer and molecularly imprinted solid-phase extraction development.

Key words: Imprinted solid-phase extraction, chemometrics, herbicides, atrazine

INTRODUCTION

Due to the potential economic benefits of using chemicals such as pesticides, they are now widely used in the modern world for different purposes. Pesticides are a group of chemical compounds that distribute in surface water, soil, air and the food chain; therefore, they are easily found in almost any human environment. Growing concern about the environmental and occupational exposure risk of toxic chemicals to public health has led to an increase in the need for a simple and reliable sample preparation procedure followed by a robust analytical method. However, sample preparation has always been in the shadow of the modern analytical techniques and procedures. Recently, it became apparent that, any mistake occurring in collecting and processing water or biological samples could lead to a substantial error in the final result regardless of the excellent performance of the state of the art of analytical technique applied subsequently. As a result, sample preparation techniques prior to the measurement of trace organic chemicals such as drugs,

*Corresponding author: *shahtaheri@tums.ac.ir*
Telefax: +98 21 8895 1390

pesticides, metabolites and other pollutants in biological and environmental matrices have been a challenging and exciting task in recent years. Hence, these techniques were shifted into the spotlight of the attention of the environmental analysts (Shahtaheri, 1996; 2005a; 2005b; 2007a; 2007b; 2007b; 2008; Liska, 2000) and it is generally accepted that, the most important step in analytical methods is sample preparation (Stevenson, 1999). Historically, liquid–liquid extraction (LLE) has been the preferred technique for clean-up of different samples (Blomgren *et al*., 2002). These extractions resulted in relatively clean extracts with good recoveries; however, they are considered as a time-consuming procedure and the solvents used have often involved environmental and health hazards. In recent years, the classical solid-phase extraction (SPE) has become method of choice in many environmental analytical applications and has overcome many drawbacks of LLE (Masque *et al*., 1998). SPE is a technique which has found wide application in the area of sample preparation, the analyte of interest is being sorbed onto the solid phase; while, the interferents are washed to waste. SPE is cheap, quite fast, gives good recoveries and can be automated; however, despite their attractive features, they do not provide the selectivity needed for very clean extracts, which lead to a partial co-extraction of interfering substances (Pichon, 2007).

A desired grade of selectivity may be obtained using columns packed with materials, which are able to bind the desired analyte with a high grade of selectivity, such as immunoaffinity columns (Baggiani *et al*., 2001); but, this technique is expensive, often time-consuming and has to be performed under very specific conditions to keep the affinity sites intact (Hogendoorn and Zoonen, 2000). However, various formats of immunoassays, based on the use of poly/ monoclonal antibodies, are successfully employed for the clean-up and detection of pesticides such as triazines (Lawrence *et al*., 1996). In the new trends of sample preparation techniques, molecularly imprinted polymers (MIPs) have gained interest as a novel type of sorbent with attractive properties. Such polymers may become complement to antibodies for use in pesticide determination (Siemann *et al*., 1996; Andersson, 2000a).

A MIP is produced by polymerisation of a solution containing a functional monomer, a cross-linker and a template (Sellergren, 2001). Before polymerisation, the functional monomer interacts with the template by, for example, hydrogen, polar, hydrophobic and/or ionic bonds. After polymerisation, the template is removed and final material contains cavities that can selectively bind to compounds very similar in structure, with regard to functional groups and conformation, to the template used. A particularly promising application of MIPs is molecular imprinted solid-phase extraction (MISPE) (Sellergren, 1999) of analytes such as pesticides present in trace concentration or in complex matrices (Chapuis *et al*., 2004; Cacho *et al*., 2006; Caro *et al*., 2006; Carabias-Martinez *et al*., 2006; Sambe *et al*., 2007; Beltran *et al*., 2007). By using MIP phases, very clean extracts can be obtained, allowing quantitation to be performed using more cost-effective instrumentation. MISPE is currently the most advanced application area with respect to the adoption of MIP-based technologies by the wider scientific community. One of the main goals in pesticide water analysis is to reach determination limit of about 0.1 µg/L, which cover all the requirements of the European Union (EU) Drinking Water Directives as well as the US National Pesticide Survey.

For a general use of the MISPE, the existing recognition elements need to be improved to meet the requirements in the given application. The large numbers of variables, coupled with the fact that they are dependent on each other, make it an extremely difficult task to optimize an MISPE. The procedural optimization can be achieved in a traditional trial and error manner or with the assistance of chemometrics. Even using combinatorial methods under the best conditions, a few of the compositional variables can be explored. The complexity of these problems makes the application of chemometric methods an ideal opportunity for the design and the optimization of the MISPE columns (Carro *et al*., 1999). The chemometric approach is based on the use of an

optimum set of experiments (experimental design), which allows the simultaneous variation of all the studied experimental factors (Takeuchi *et al.*, 1999). Rather than making every combination in an n-dimensional matrix, these methods allow one to vary multiple parameters simultaneously.

In this article, the use of MIPs as the specific binding matrix for solid phase extraction of a triazine herbicide, named atrazine with regard to sample pH, sample concentration, sample flow rate, sample volume, elution solvent, washing solvent and sorbent mass for environmental matrices was described. The aim of this work was to optimize the main factors affecting the molecular recognition properties of the MISPE by a chemometric approach.

MATERIALS AND METHODS

Reagents

The required reagents for the experimental part of the study were the following: atrazine with purity of greater than 97% and other triazines, including ametryn, cyanazine, simazine and propazine (Riedel-de-Häen, Seelze, Germany), 2,4-dicholorophenoxyacetic acid (2,4-D) (Sigma-Aldrich-Fluka, Milan, Italy), methacrylic acid (MAA, functional monomer, Merck, Germany), ethylene glycol dimethacrylate (EGDMA, co-monomer, Merck, Germany), and 2, 2-azobisisobutyronitrile (AIBN, initiator, Acros, USA). Moreover, the Merck Co. supplied all the used solvents (acetic acid, hydrochloric acid 32%, acetonitrile, and methanol) as well as ammonium acetate and sodium hydroxide pellets, being of analytical reagent grade. Buffer solutions (citrate/ hydrochloric acid, pH=4 and boric acid/potassium chloride-sodium hydroxide, pH=10) were analytical reagent grade (Merck, Darmstadt, Germany). With reference to the 1 mg/L stock solution (by dilution in 1 mL acetonitrile and then, deionized water), the standard solutions were prepared with the dilution of the stock solutions in water. A Purite Purification System provided ultra pure water.

Equipment and chromatographic conditions

A reversed-phase HPLC system (Knaüer Company, Germany) was used for the measurements performance, consisting of a K-1001 series high pressure pump, a K-2006 photo diode-array detector and a VS injection valve, equipped with a 20 µL loop. For the analytes separation on a Chromolith Performance, RR-C_{18}e 100 × 4.6 mm i.d. column (Merch KGa A, Germany) was employed along with column guard (Chromolith Guard Cartridge Kit RP-C_{18}e and 5 cm×4.6 mm i.d., 5 µm) with the following isocratic elution: 50% acetonitrile and 50% mixture of purified water and ammonium acetate (1×10^{-3} M). Atrazine was monitored at 226 nm and quantified with external calibration, using the peak area measurements (R^2 = 0.9993). The chromatogram reproducibility was assured by the triple repetition of each sample. The flow-rate was set at 1.4 mL/min. Optimized chromatographic conditions for other triazines are shown in the Table 1. The system was linked with a LaserJet 1200 series printer for recording the chromatograms, using a 1456-1 Chromogate Data System, Version 2.55. For the polymer synthesis, the employed apparatus comprised soxhlet and a heater unit, a liquid extraction unit (S and S, Germany), a reactor heater system (Memmert, Germany), a nitrogen supply system, an ultrasonic shaker (Tecna-6, Italy), a syringe-filtration unit (FH-0.45 µ, Millipore Corp., USA), PTFE filters (0.2 µ, Sartorius, Germany), an oven (Memmert, Germany), and a shaker (Innova 4000). Also, For the MISPE Procedures, a vacuum manifold (Tajhizteb, Tehran, Iran), a Sibata vacuum pump (Hitachi Ltd. Japan), and a transformer (SE-300, Japan) were used. A digital balance (Sartorius-2024, Germany) was utilized for the weight

Table 1: Chromatographic conditions for triazines analysis

Analyte	Mobile phase (%)		Ammonium acetate (mmol)	Wavelength (nm)	Flow rate (mL/min)	Injection volume(µL)
	Acetonitrile	Water				
Ametryn	60	40	--	220	0.8	20
Cyanazine	85	15	--	256	0.8	20
Simazine	40	60	1	226	1.2	20
Propazine	50	50	1	226	1.2	20

measurement of the reagents (milligram quantities or less). Finally, adjustable-volume pipettors with disposable tips were used to load the sample, washing solvent and eluent into the cartridges (Socorex, Germany).

Polymer synthesis and preparation

For the polymer preparation, the non-covalent bulk polymerization was used as an effective molecular imprinting protocol (Takeuchi *et al*., 1999; Masque *et al*., 2001). In this way, the glassy polymer blocks were attained to be used as powder after being crushed, ground, and sieved. At the beginning, 1 mmol atrazine and 5.83 mmol MAA were added to a 25 mL thick-walled glass tube and afterwards, the mixture was left for 5 min for prearrangement. Subsequently, EDMA (26.28 mmol), AIBN (2.27 mmol), and 5.03 mL acetonitrile were added. The mixture was purged by nitrogen for 5 min and the glass tube was sealed under this atmosphere. Then, it was placed at a thermostated water bath at 55 ^{o}C for starting the polymerization process. After 24 h, the tube was broken and the obtained polymer was ground in a mortar. The particles were thoroughly sedimented three times in methanol to remove fines. The particles with sizes between 50 and 105 μm were collected. Removal of template was performed by soxhlet extraction, using a two-step procedure (methanol: acetic acid (9:1 v: v) as the first step for 16 h and methanol as the second step for 4 h). In the final step, the produced powder was packed in cartridges. Safety precautions were considered during the preparation of the polymerization mixture, grinding, and the extraction of the polymer. These steps were performed in a safety cabinet, as they involved the handling of the toxic compounds: methacrylic acid, ethylene glycol dimethacrylate and 2,2-azobisisobutyronitrile. The optimization of polymer synthesis and template removal procedure took place in our laboratory in order to generate MAA-based binding sites, complementary to triazine herbicides (Koohpaei *et al*., 2008). In parallel, non-imprinted polymers (NIP) were synthesized following the same procedure without the template molecule addition.

Preliminary MISPE procedure

The dry molecularly imprinted polymer/non-imprinted polymer (150 mg) was placed in empty SPE cartridges of 6 mL between two wool-glass frits at the bottom and on the top of the columns. The columns were attached to an SPE vacuum manifold, which was connected, in turn, to a vacuum pump. In the first experiments on extraction, MISPE cartridges were conditioned with 10 mL methanol followed by 10 mL LC-grade water to wet the polymer completely. After drying step (over 2 min), a 10 mL atrazine (100 ng/mL) was passed through the column at approximate flow rate of 3 mL/min. After the sample loading, air was passed through the sorbent for drying the solid phase. In the second step of the extraction, in order to remove the remaining interfering compounds, 1 mL 0.1 M hydrochloric acid, 1 mL LC-grade water and 1 mL acetonitrile was percolated as a basic condition.

A gentle vacuum was applied between each step. After polymer drying, atrazine was quantitatively extracted three times with 3 mL methanol and analyzed using an HPLC-UV system. In order to optimize MISPE elution step, different methanol values of 1, 2, 2×1, 3, 3×1, 4, 4×1, 5, and 5×1 mL were applied. Also, the columns were washed with 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mL acetonitrile and its influence on extraction recovery was examined. Another experiment performed during this section of study was evaluation of the different methanol and water values (9, 8, 7, 6, 5, 4 and 3 mL) on atrazine recovery. Finally, the effect of first vacuum time in washing step (5, 10, 15, 20, 25 and 30 min), on the atrazine recovery was investigated. Table 2 gives guideline for experiments.

Table 2: Guideline for experiments in the central status of the MISPE

Experiment	1	2	3	4	5	6	7	8	9	10
Elution Methanol (mL)	1	2	2×1	3	3×1	4	4×1	5	5×1	--
Washing Acetonitrile (mL)	1	2	3	4	5	6	7	8	9	10
Conditioning (mL) Methanol and water	9	8	7	6	5	4	3	--	--	--
Vacuum time (min)	5	10	15	20	25	30	--	--	--	--

Qualitative optimization of MISPE procedure

In order to select the best solvents in washing and elution steps and remove non-specific interactions and interferences absorbed to the columns, the cartridges were washed with different acetonitrile values of 7 and 6 mL and different acetic acid volume percent (0 and 0.1 with 7 mL acetonitrile and 0.1, 0.2, 0.3, 0.4 and 0.5 with 6 mL acetonitrile) in the 7 experiments (n=3). However, care should be taken that, no analyte-sorbent bonding is broken during washing stage. Another experiment performed was evaluation of the elution solvent composition on atrazine recovery. Nine solvents composition (methanol and acetic acid) were screened for their ability to produce optimum elution of the retained atrazine from the MIP columns. They were 2×1 mL methanol (followed by 1%, 2%, 3%, 4% and 5% acetic acid) and 3×1 mL methanol (followed by 0, 1%, 2% and 3% acetic acid). The MISPE procedure was used and examined as described above (except for the solvents composition that was considered in the screening stage). Analyte was eluted under the same condition as those described in the previous section.

Experimental design approach for SPE procedures on MIP

For the experimental part, the approach of the factorial design was preferred to the classical one at a time experiment, because the first approach requires fewer measurements than the second one to give the same precision. Another advantage of the factorial design is the fact that, it is able to detect and estimate any interaction between the factors.

For the elimination of possible bias, the order of the running experiments was restrictedly randomized (restricted factor was the flow-rate of the sample). The standard approach to the analysis of the experimental design data is to evaluate a list of the main and interaction effects, indicating that which effects are significant (wu and Hamada, 2000). The data were analyzed with the aid of the statistical software package, Minitab, Release 14, for windows (Jamshidian and Nourizad, 2004). The primary stage of the experimental design involved the selection of five factors which could influence the recovery efficiency. These factors could be the operational variables such as sample pH, sample concentration, sample flow rate, sample volume, and sorbent mass. Accordingly, a two-level full factorial design of 2^5 was utilized, following a linear and quadratic model, containing squared terms. This led to 32 basic experiments, undertaken in random order plus four central points. As the second stage of the experimental part, a central composite design was used with α values equals to 2 for the assessment of the α effects on the resulting data, adding ten star points to the above 2^5 factorial design. For these reason, 46 runs were selected. Table 3 depicts the values, corresponding to the high (+), low (–) and central (0) points and α values for each factor. For each run in the experiments, NIP columns were obtained and examined.

Table 3: Factor levels in the experimental designs

Variable	Low (–)	High (+)	Central (0)	α=2	
				Axial(–α)	Axial(+α)
Sample flow rate (mL/min)	2	4	3	1	5
Sample concentration (ng/mL)	65	145	100	10	190
Sample volume (mL)	7.5	12.5	10	5	15
Sorbent mass (mg)	100	200	150	50	250
Sample pH	4	10	7	1	13

In order to evaluate the capacity of MISPE cartridges, different volumes of 10 µg/L atrazine (25, 50, 100, 200, 300, 400, 500, and 1000 mL) were added to the MIP columns. Also, in order to evaluate the volume break-through, one mL sample of 0.1 µg/mL atrazine was diluted into different volumes, 25, 50, 100, 200, 300, 400, 500, and 1000 mL and added to the MISPE cartridges. The columns were washed and eluted according to the optimized method.

Method validation and identification of atrazine in drinking water

Drinking water samples were spiked with five different amounts of atrazine to reach a final

concentration of 10, 65, 100, 145 and 190 ng/mL. The calibration graphs were constructed by plotting the peak area of the analytes versus its concentration. The intra- and inter-day precision and accuracy data were obtained with the assay of spiked drinking water samples. Other triazines samples, ametryn, cyanazine, simazine and propazine were also examined to evaluate the cross-reactivity.

RESULTS

In order to define the optimum chromatographic conditions for the atrazine analysis, specific parameters were optimized including the mobile phase composition, the UV wavelength, the injection volume and the mobile phase flow rate. The atrazine chromatogram was detected at 226 nm (Fig. 1). The results obtained from central status of the MISPE section are illustrated in Fig. 2. Also, the results of qualitative optimization of the MISPE are shown in Fig. 3.

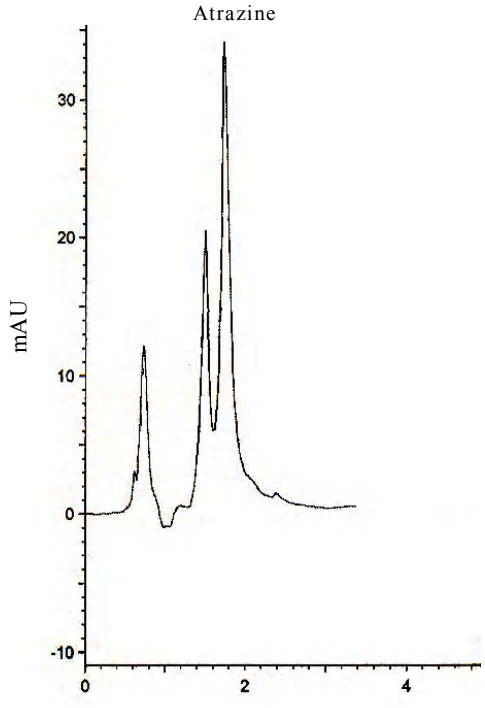

Fig. 1: The HPLC chromatogram of drinking water spiked of atrazine at the concentration of 1 µg/mL

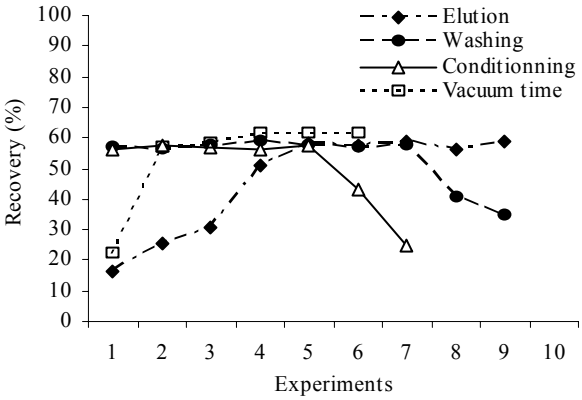

Fig. 2: Mean recovery of atrazine in different status of elution, washing, conditioning and first vacuum time in washing step (n=3)

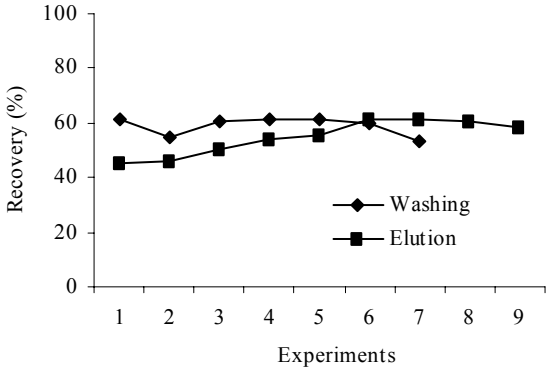

Fig. 3: Mean recovery of atrazine in different composition of elution and washing solvent, (n=3)

Quantitative optimization of the MISPE

Table 4 exhibits the type of optimization design chosen in this work and the so-called response surface model: the central composite design (CCD), where the axial points are located on the sphere surrounding the two-level factorial design. The obtained results have been summarized in Tables 5 and 6. Main effects plots (Fig. 4) depict the response surface plots for atrazine. Based on the quantitative optimization results, the effect of sample flow-rate decreasing over the range of central composite design (1, 0.9, 0.8, 0.7, 0.6, and 0.5 mL/min) on the atrazine recovery was investigated (Table 7). The results of the capacity of MISPE cartridges and volume break-through are shown in Fig. 5. The effect of amount of 0.1 M hydrochloric acid on the atrazine recovery has been shown in Table 8. The specificity of the atrazine MIP was determined via the cross-reactivity of similar and different pesticides (Table 9).

Table 4: The experimental designs for MISPE

Factor[a] / Run	A	B	C	D	E	Factor / Run	A	B	C	D	E	Factor / Run	A	B	C	D	E	Factor / Run	A	B	C	D	E
1	0	0	0	0	0	13	+	+	–	+	+	25	–	–	+	–	+	37	+α	0	0	0	0
2	0	0	0	0	0	14	+	+	–	–	–	26	–	–	–	+	+	38	-α	0	0	0	0
3	+	+	+	+	+	15	+	–	+	–	+	27	–	–	–	–	–	39	0	+α	0	0	0
4	+	+	+	+	–	16	+	–	–	+	+	28	–	+	+	–	–	40	0	-α	0	0	0
5	+	–	+	+	+	17	+	–	–	+	–	29	–	+	–	–	+	41	0	0	+α	0	0
6	+	+	+	–	+	18	+	–	+	–	–	30	–	–	+	–	–	42	0	0	-α	0	0
7	+	+	+	–	–	19	–	+	+	–	+	31	–	–	+	+	+	43	0	0	0	+α	0
8	+	–	+	+	–	20	–	–	–	–	+	32	–	+	+	+	–	44	0	0	0	-α	0
9	+	+	–	+	–	21	–	–	+	+	–	33	–	+	–	–	–	45	0	0	0	0	+α
10	+	–	–	–	–	22	–	+	+	+	+	34	–	+	–	+	+	46	0	0	0	0	-α
11	+	+	–	–	+	23	–	–	–	+	–	35	0	0	0	0	0						
12	+	–	–	–	+	24	–	+	–	+	–	36	0	0	0	0	0						

[a] (A: Flow-rate of sample, B: Concentration, C: Sample volume, D: Mass of sorbent, E: pH of sample)

Table 5: Mean recovery on the molecular imprinted and the non-imprinted polymers in the response surface methodology model

Run	Recovery (Mean, N=3) MIP SD	NIP SD	Run	Recovery (Mean, N=3) MIP SD	NIP SD	Run	Recovery (Mean, N=3) MIP SD	NIP SD	Run	Recovery (Mean, N=3) MIP SD	NIP SD
1	61.93 0.26	19.48 0.36	13	47.03 0.73	21.43 0.42	25	73.36 0.26	14.69 0.39	37	37.11 0.26	10.59 0.33
2	61.75 0.14	19.51 0.35	14	52.23 0.61	18.56 0.4	26	72.48 0.2	24.42 0.35	38	86.41 0.24	19.41 0.42
3	41.84 0.24	21.25 0.45	15	43.56 0.28	14.62 0.35	27	77.67 0.3	15.89 0.22	39	71.48 0.28	24.27 0.55
4	40.55 0.4	24.03 0.22	16	42.30 0.2	19.11 0.45	28	77.08 0.26	18.55 0.28	40	61.08 0.9	19.50 0.4
5	39.17 0.59	19.45 0.39	17	40.77 0.28	21.52 0.42	29	84.53 0.26	18.58 0.2	41	55.35 0.22	19.14 0.35
6	42.64 0.26	16.44 0.4	18	41.11 0.33	16.42 0.22	30	71.76 0.17	16.57 0.33	42	67.37 0.6	19.38 0.49
7	40.23 0.42	19.08 0.57	19	78.14 0.48	16.62 0.37	31	66.57 0.3	24.52 0.44	43	47.47 0.28	29.41 0.22
8	38.43 0.3	21.49 0.39	20	79.46 0.35	14.21 0.39	32	77.56 0.35	29.21 0.32	44	62.58 0.32	10.97 0.3
9	45.69 0.17	23.38 0.36	21	64.45 0.41	26.38 0.3	33	82.91 0.2	18.32 0.3	45	0	0
10	48.83 0.58	10.61 0.37	22	66.85 0.22	26.71 0.22	34	77.40 0.26	26.56 0.32	46	0	0
11	54.46 0.59	16.34 0.37	23	79.61 0.44	26.25 0.37	35	61.44 0.17	19.61 0.2			
12	49.98 0.35	14.75 0.47	24	75.23 0.41	28.54 0.28	36	61.39 0.57	19.76 0.22			

Table 6: The estimated response surface regression coefficients for the mean recovery on the MISPE

Term	Coefficient	P_{Value}	Term	Coefficient	P_{Value}
Constant	58.004	0.001	A×B	-2.29	0.692
Flow-rate (A)	-29.64	0.001	A×C	-0.03	0.995
Concentration (B)	3.38	0.198	A×D	0.94	0.856
Volume (C)	-6.55	0.09	A×E	2.58	0.618
Mass (D)	-5.63	0.022	B×C	-0.31	0.958
pH (E)	0.299	0.897	B×D	0.399	0.945
A×A	13.33	0.024	B×E	-0.38	0.948
B×B	15.52	0.012	C×D	2.14	0.679
C×C	12.93	0.028	C×E	-0.47	0.928
D×D	6.59	0.244	D×E	-2.87	0.579
E×E	-48.42	0.001			

S=7.217, R-Sq=92.4%, R-Sq (adj) =86.3% .The analysis was done using coded units

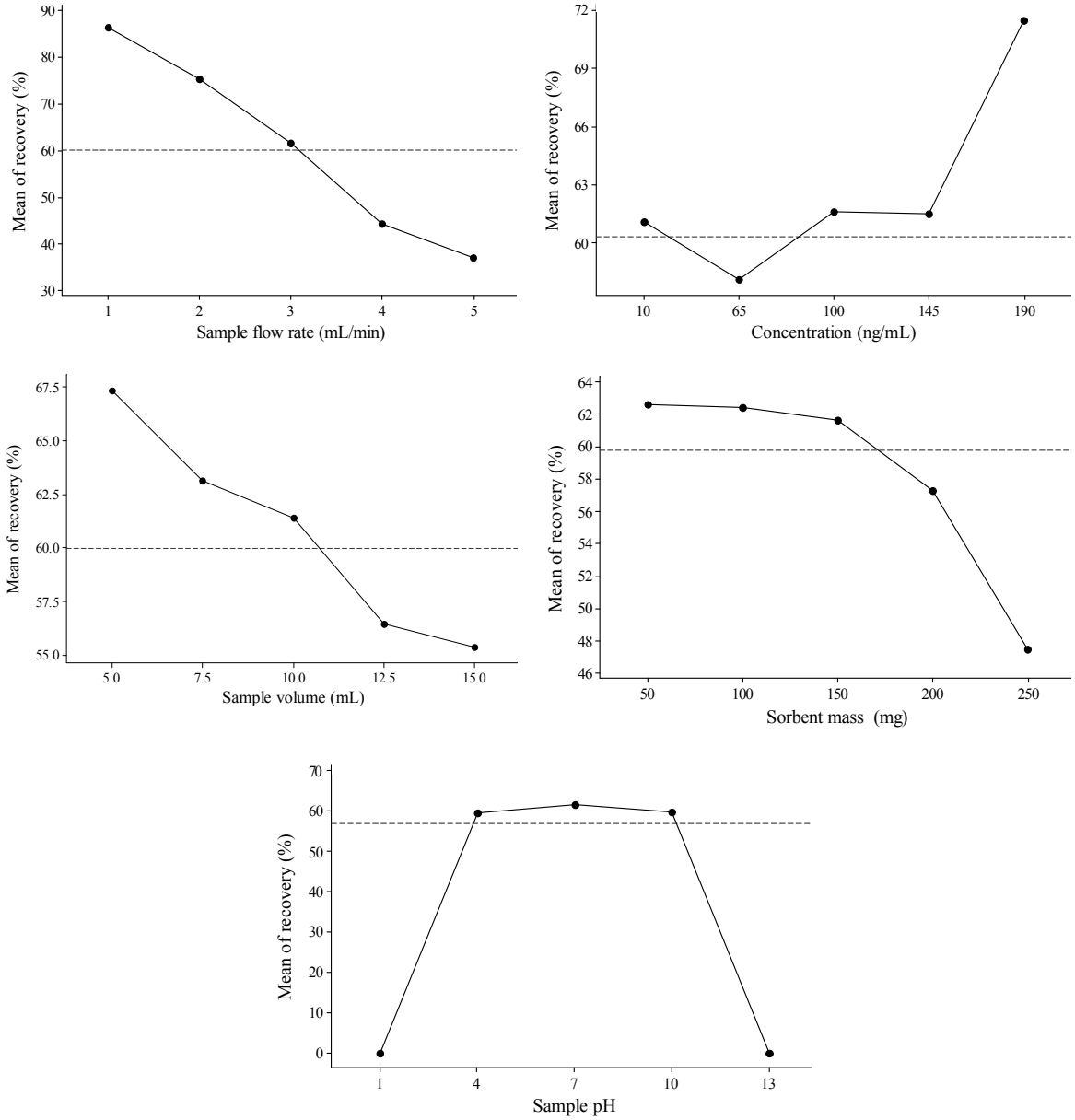

Fig. 4: Main effect plots of atrazine MISPE variables in the central composite design

Table7 : The effect of flow-rate of sample on recovery of atrazine in MISPE

Flow-rate (mL/min)	1	0.9	0.8	0.7	0.6	0.5
Recovery Mean±SD (N=3)	86.06±0.51	87.15±0.19	90.69±0.78	93.29±0.32	93.64±0.26	93.16±0.39

Table8: The effect of amount of 0.1M hydrochloric acid on the atrazine recovery

Amount (mL)	1	1.5	2	2.5	3	3.5	4	4.5	5
Recovery Mean (%) (N=3)	81.37	84.58	87.38	91.52	92.76	92.52	88.48	80.37	70.67
SD	0.39	0.61	0.28	0.35	0.35	0.13	0.31	0.18	0.92

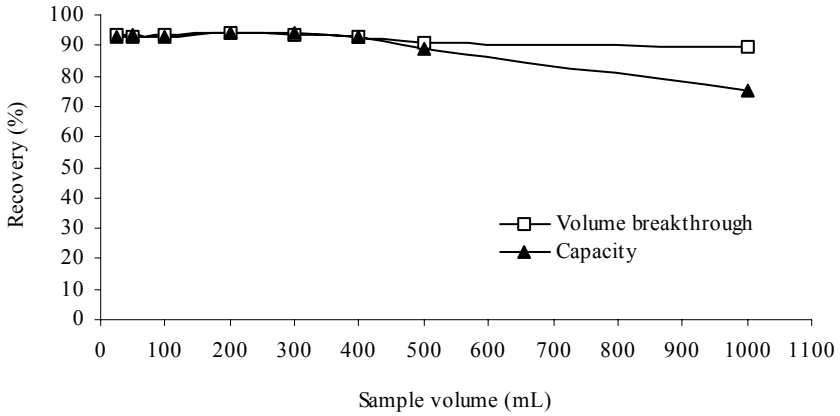

Fig. 5: Effect of different volume on the capacity and breakthrough of the MISPE columns

Table 9: Recoveries of some triazines and non-related herbicide

Analyte	Mean recovery (%) ± S.D.[a]	
	MIP	NIP
Atrazine	93.42±0.28	-
Simazine	96.37±0.39	1.66±0.57
Cyanazine	94.35±0.29	2.66±0.57
Propazine	95.24±0.14	-
Ametryn	29.95±0.33	-
2,4-D	-	-

[a]S.D., standard deviation for n=3. (-) Not detected.

Method validation

More experiments were performed on spiked drinking water to validate the present method (Table 10). Spiked water sample can be a suitable model as it may contain interfering constituents similar to the real sample (Laurens *et al*., 2002).

Table 10: Day-to-day (D-day) and within-day (W-day) reproducibility of atrazine spiked in drinking water; sample volume: 10 mL (N=6)

Statistic data	Concentration added (ng/mL)									
	10		65		100		145		190	
	D-day	W-day	D-day	W-day	D-day	W-day	D-day	W-day	D-day	W-day
Mean	9.3	9.23	60.27	60.32	92.02	93.15	135.9	135.31	178.66	178.03
SD	0.09	0.12	0.43	0.39	0.55	0.36	0.41	0.61	0.42	0.57

DISCUSSION

Despite the popularity in the literature published within the past decades, the selectivity of MISPE mechanisms and their rational control has not entirely been recognized and is still under question. Therefore, there is a need to optimize the MISPE extraction procedure in more details. Since all the conditioning, loading, washing, and elution step parameters (both type and amounts) have a strong influence on the overall MISPE performance in terms of affinity, selectivity, loading capacity, etc., their proper selection (qualitative and quantitative) will ensure that polymers with appropriate properties have been obtained successfully.

In this study, the use of MISPE of a triazine herbicide, named atrazine with regard to qualitative and quantitative parameters for drinking water samples was described. Since the main aim of this work was the optimization of the main factors affecting the molecular recognition properties of the MISPE by a chemometric approach, the atrazine MIP was packed into cartridges as described in the experimental section. In the first step and in agreement with the other studies (Zander *et al*., 1998; Baggiani *et al*., 2001) the bleeding of residual template from the polymer was checked by washing the MISPE cartridges with successive methanol fractions (3×1 mL each). The chromatograms of all fractions were found to be free of atrazine at the sensivity of the UV detector. For atrazine, retention on the blank column was lower than for the MIP column, which suggested that the polymer had been successfully imprinted (Table 5).

As it has been mentioned above, in order to start the optimization process by an experimental design approach, a preliminary MISPE procedure was designed. From the results given in Fig. 2, it was deduced that 3×1 mL methanol as eluent could be applied for efficient elution. However, the eluent volume must be just sufficient to elute the compound of interest from the sorbent. Also, based on the obtained results (Fig. 2), the best acetonitrile volume for washing step of MISPE was 7 mL and methanol and water volume for conditioning step was 5 mL. It should be noted that, water volume in the washing step, was selected in accordance with the water volume in the conditioning step (5 mL). The retention of the analytes on a sorbent from an aqueous medium may be strongly affected by the presence of water embedded in the sorbent after passage of the sample (Carabias-Martinez *et al*., 2006). Accordingly, it was examined that, how the drying time can affect the extraction recoveries. As the drying time increased up to 20 min, so did the extraction recovery of atrazine. Therefore, 20 min for first vacuum in washing step was selected.

In order to the optimize of the MISPE qualitatively, different composition of acetonitrile and acetic acid for washing step and different composition of methanol and acetic acid for elution step were considered to be studied. The results have been shown in Fig. 3, demonstrating that 6 mL acetonitrile plus 0.3% v/v acetic acid instead of the 7 mL acetonitrile can be used. Also, 3×1 mL methanol as eluent could be applied for efficient elution without acetic acid. However, it should be noted that, the enrichment of the analyte in MISPE is achieved by applying large volumes of sample and eluting the analyte in a minimum volume of eluent ideally.

As it has been mentioned above, in the experimental design, the evaluation of five factors was considered. The flow-rate of sample was the first variable. The amounts for the flow-rate were selected between 1 and 5 mL/min. The higher flow-rates were obtained using reduced pressure at the MIP-column outlet. Significant reduction of recovery was found for sample flow-rate from 1 to 5 mL/min (Fig. 4). By combining the response surfaces and based on the response optimizer data, it was finally possible to suggest the optimum conditions for the flow-rate i.e. 1 mL/min. It seems that using lower sample flow-rates would significantly increase the extraction recovery. From the result given in Table 7, it was deduced that, 0.7 mL/min could be applied for sample flow-rate.

The next parameter studied was the concentration. The amounts for the sample concentration were selected between 10 and 190 ng/mL. 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. However, it was revealed that unusually selectivity and template affinity were better at higher concentration (Fig. 4). This phenomenon has been previously shown (Lavignac *et al*., 2006) where it was proposed that, at higher concentrations, the ability of atrazine to generate atrazine-atrazine complexes, both in solution and on the polymer surface, results in increased atrazine selectivity.

In order to evaluate the effect of sample volume on the MISPE performance, different volume of sample ranged from 5 to 15 mL as mentioned in Table 2 were prepared using deionized water. Significant reduction of recovery was found for sample volumes from 5 to 15 mL/min (Fig. 4). This phenomenon can

be explained by the heterogeneous surface of the polymer involving the presence of binding sites or cavities of different energy levels. Application of a small volume of sample, allows to the analytes interacting with a larger number of binding sites than when higher sample volumes are applied. It seems that, in case of application of a higher sample volume, i.e. 15 mL, the partial breakthrough volume for some binding site was attained. However, it should be noted that, the heterogeneity of the binding sites is not a limiting factor for using MISPE because the retention remains always selective since the compound of interest is not retained on the non-imprinted polymer.
Another parameter studied was the pH of the sample. The amounts for the sample pH were selected between 1 and 13. In this experiment, the effect of sample pH on retention of atrazine on the columns was assessed. Fig. 4 illustrates the effect of the sample pH on atrazine retention, showing the recovery obtained using different sample pH. From the obtained results, the recoveries were found to be similar at sample pH of 4-10. Atrazine is relatively stable in neutral, weakly acidic, and weakly alkaline media, and rapidly hydrolyzed to the hydroxyl derivatives in strong acids and alkalis. However, atrazine is not protonated at neutral pH (D' Agostino *et al*., 2006). Therefore, a pH of about 7.0 proved to be optimum for application of samples to the SPE cartridge containing the MIP to atrazine.
Finally, In order to evaluate the effect of sorbent mass on MISPE performance, different mass ranged from 50 to 250 mg were selected as mentioned in Table 2. To check the influence of the sorbent mass in the recovery values, a series of empty SPE cartridges were filled with different amounts of polymer. Based on the obtained results, the extraction recoveries were not significantly improved when amounts of sorbent above 150 mg were used. It seems that, the difficulty in passing the sample through the system increases with the increase in polymer mass. In the other hand, problems with non-specific adsorption to the polymer can be reduced by the use of small amounts of MIP, thereby; the polymer surface area available for non-specific adsorption is reduced (Andersson, 2000b). Accordingly, by combining the response surfaces, it was finally possible to suggest the optimum conditions for the sorbent amounts of 125 mg.
The data in Table 5 was evaluated by ANOVA at the 5% significance level (Table 6). Regarding the results presented in Table 6, among the linear effects, the most crucial variables were the flow-rate and mass of the sorbent. Among quadratic effects; the effects of the flow-rate, sample concentration, volume of sample, and pH of sample were significant ($P \leqslant 0.05$). Also, it can be derived that, the interaction between the studied variables (from A×B to D×E) were not significant ($P < 0.05$). Moreover, the R-Sq (adj) value was 86.3%, showing that, the five studied factors could explain 86.3% of the variation in the recovery percentage. The results of the central composite design (CCD) were validated using ANOVA. The *P*-value for the model (0.001) was lower than the critical value of the significance (below 0.05). The R^2 value for a validated model is 0.6 or greater. For this model, the R^2 was 0.827, indicating that the model would be reasonably accurate.
The extraction recoveries were lower than 20% for the spiked water samples indicating a matrix effect. This loss of extraction recovery can be explained by the presence of cations in the water samples and an ion-exchange mechanism (Chapuis *et al*., 2003). In order to eliminate matrix effect, regenerating the interaction site and achieve the best results for extraction of atrazine from spiked drinking water, the volume effect of amount of HCl 0.1 M (1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 mL after sample loading) on the recovery of atrazine was investigated. The same sequence of conditioning, loading and elution were used as explained beforehand. The obtained results have been illustrated in Table 8 and showed that the smallest satisfactory volume for hydrochloric acid was 3 mL.
The results have been shown in Fig. 5, demonstrating that for column capacity up to 400 mL of sample could be applied without significant loss of recovery (at least 92.81±0.56 for 400 mL sample volume) and for volume break-through, no significant reduction of recovery was found in the total of the study range (25-1000 mL). Also, it should be noted that, based on a separate experiment, the atrazine MIP exhibited good stability and selectivity even after the 45 enrichment and desorption studies. Usually the specificity of the atrazine MIP is determined via the cross-reactivity of several triazine herbicides (high and intermediate cross-reactivity) and

some other pesticides (low cross-reactivity) (Siemann *et al*., 1996). Based on the obtained results from Table 9 and similar to the other works (Lavignac *et al*., 2006), atrazine, simazine, cyanazine and propazine are structurally very similar and it was therefore unsurprising that their recoveries on the atrazine imprinted column were similar. The minor observed variation in recoveries can be accounted for small variations in the interaction energies between the molecules and the recognition sites of the polymer (Chapuis *et al*., 2003), size, and the additional methyl substitution. In contrast, the recoveries for the thiomethyl analogue, ametryn was very lower than the values obtained for the chlorine substituted analogues of atrazine, simazine, cyanazine, and propazine. Thiotriazines possess a thiomethyl group that is larger than the chlorine atom of the atrazine template. It seems that a steric hindrance phenomenon limits the access to the designed cavities (Chapuis *et al*., 2004; Carabias-Martinez *et al*., 2006). Also, the compound with low cross-reactivity, 2,4-dicholorophenoxy acetic acid (2,4-D) was not retained on the atrazine MIP. This result confirms the high selectivity of the extraction on MIP. For atrazine, recovery on the blank column was very lower than for the MIP column, which suggested that the polymer had been successfully imprinted (Table 5). In addition, similar to other study (Chapuis *et al*., 2004) in order to decrease the non-specific interactions and obtain maximal selectivity, 1% methanol was added to the samples. Methanol was selected for its high eluting strength. However, this amount of methanol should be as low as possible because it should decrease the retention of compounds retained on the surface of the polymer without affecting the overall retention in the imprints. The addition of 1% methanol causes a significant drop in extraction recoveries on the non-imprinted polymers (Table 9).

For the validation of the present method, the drinking water spiked samples of 10 mL of atrazine were used for extraction followed by HPLC-UV determination. Linear standard curve (for extracted samples) over the range 10-190 ng/mL were obtained each day (n=6) with correlation coefficient of 0.998 or greater. The extraction procedure was reliable and reproducible from day-to-day and within-day (Table 10). The relative standard deviation (RSD) of 0.97, 0.71, 0.59, 0.3 and 0.23 were obtained for 10, 65, 100, 145 and 190 ng/mL respectively for day-to-day and 1.3, 0.65, 0.39, 0.45 and 0.32, at the same concentrations, respectively for within-day, showing suitable accuracy and precision. The detection limit of the method (signal/noise: 3:1) using drinking water spiked sample volume of 10 mL was 0.01 µg/mL as well as reproducible and quantitative recoveries, ranging from 93% to 96% for triazine herbicides were possible.

ACKNOWLEDGEMENTS

This research has been supported by Tehran University of Medical Sciences grant (project No.132-56-73). Hereby, the cooperation of the University and also the Center for Environmental Research (CER) is highly appreciated. The authors also thank Ms. Fereshteh Eshaghzadeh, Mrs. Razieh Divani and Mr. Mirghani Seyedsomeh for their kind assistance.

REFERENCES

Andersson, L.I., (2000a). Molecular imprinting: Developments and application in the analytical chemistry field. J. Chromatogr. B., **745**: 3-13.

Andersson, L. I., (2000b). Molecular imprinting for drug bioanalysis: A review on the application of imprinted polymers to solid-phase extraction and binding assay. J. Chromatogr. B, **739**: 163-173.

Baggiani, C., Giovannoli, C., Anfossi, L., Tozzi, C., (2001). Molecularly imprinted solid-phase extraction sorbent for the clean-up of chlorinated phenoxyacids from aqueous samples. J. Chromatogr. A., **938**: 35-44.

Beltran, A., Caro, E., Marce, R. M., Cormack, P. A. G., Sherrington, D. C., Borrull, F., (2007). Synthesis and application of a carbamazepine-imprinted polymer for solid-phase extraction from urine and wastewater. Anal. Shim. Acta, **597**: 6-11.

Blomgren, A., Berggren, C., Holmberg, A., Larsson, F., Sellergren, B., Ensing, K., (2002). Extraction of clenbuterol from calf urine using a molecularly imprinted polymer followed by quantitation by high-performance liquid chromatography with UV detection. J. Chromatogr. A., **975**: 157-164.

Cacho, C., Turiel, E., Martin-Steban, A., Ayala, D., Perez-Conde, C., (2006). Semi-covalent imprinted polymer using propazine methacrylate as template molecule for the clean-up of triazines in soil and vegetable samples. J. Chromatogr. A., **1114**: 255-262.

Carabias-Martinez, R., Rodriguez-Gonzalo, E., Herrero-Hernandez, E., (2006). Behavior of triazine herbicides and their hydroxylated and dealkylated metabolites on a

propazine-imprinted polymer comparative study in organic and aqueous media. Anal. Shim. Acta., **559**: 186-194.

Caro, E., Marce, R. M., Cormack, P. A. G., Sherrington, D. C., Borrull, F., (2006). Novel enrofloxacin imprinted polymer applied to the solid-phase extraction of fluorinated quinolones from urine and tissue samples. Anal. Shim. Acta., **562**: 145-151.

Carro, A. M., Cobas, J. C., Rodriguez, J. B., Lorenzo, R. A., Cela, R., (1999). Application of chemometric techniques to the optimization of the solid-phase extraction of 27 pesticides before GC-MIP-AES analysis. J. Anal. At. Spect., **14**: 1867-1873.

Chapuis, F., Pichon, V., Lanza, F., Sellergren, B., Hennion, M.C., (2003). Optimization of the class-selective extraction of triazines from aqueous samples using a molecularly imprinted polymer by a comprehensive approach of the retention mechanism. J. Chromatogr. A, **999**: 23-33.

Chauis, F., Pichon, V., Lanza, F., Sellergren, B., Hennion, M.C., (2004). Retention mechanism of analytes in the solid-phase extraction process using molecularly imprinted polymers application to the extraction of triazines from complex matrices. J. Chromatogr. B., **804**: 93-101.

D´ Agostino, G., Alberti, G., Biesuz, R., Pesavento, M., (2006). Potentiometric sensor for atrazine based on a molecular imprinted membrane. Biosensors and Bioelectronics, **22**: 145-152.

Hogendoorn, E., Zoonen, P. V., (2000). Recent and future developments of liquid chromatography in pesticide trace analysis. J. Chromatogr. A., **892**: 435-453.

Jamshidian, A.R. and Nourizad, M., (2004). Design and analysis of experiments with Minitab 14. 1st edition, Arkan publications, Tehran, Iran.

Koohpaei, A. R., Shahtaheri, S. J., Ganjali, M. R., Rahimi Forushani, A., Golbabaei, F., (2008). Application of multivariate analysis to the screening of molecularly imprinted polymers (MIPs) for ametryn. Talanta, **75**: 978-986.

Laurens, J. B., Mbianda, X. Y., Spies, J. H., Ubbink, J. B., Vermaak, W. J. H., (2002). Validated method for quantitation of biomarkers for benzene and its alkylated analogues in urine. J. Chromatogr. B., **774**: 173-185.

Lavignac, N., Brain, K. R., Allender, C. J., (2006). Concentration dependent atrazine–atrazine complex formation promotes selectivity in atrazine imprinted polymers. Biosensors and Bioelectronics, **22**: 138-144.

Lawrence, J. F., Menard, C., Hennion, M. C., Oichon, V., Le Goffic, F., Durand, N., (1996). Evaluation of immunoaffinity chromatography as a replacement for organic solvent clean-up of plant extracts for the determination of triazine herbicides by liquid chromatography. J. Chromatogr. A., **752**: 147-154.

Liska, I., (2000). Fifty years of solid-phase extraction in water analysis-historical development and overview. J. Chromatogr. A., **885**: 3-16.

Masque, N., Marce, F., Borrull, F., (2001). Molecularly imprinted polymers: New taylor-made materials for selective solid-phase extraction. Trends Anal. Chem., **20**(9): 477-486.

Masque, N., Marce, R. M., Borrull, F., (1998). New polymeric and other types of sorbents for solid-phase extraction of polar organic micropollutants from environmental water. Trends Anal. Chem., **17** (6): 384-394.

Pichon, V., (2007). Selective sample treatment using molecularly imprinted polymers. J. Chromatogr. A., **1152**: 41-53.

Sambe, H., Hoshina, k., Haginaka, J., (2007). Molecularly imprinted polymers for triazine herbicides prepared by multi-step swelling and polymerization method their application to the determination of methylthiotriazine herbicides in river water. J. Chromatogr. A., **1152**: 130-137.

Sellergren, B., (2001). Molecularly imprinted polymers: Man-mimics of antibodies and their applications in analytical chemistry, techniques and instrumentation in analytical chemistry. Vol. 23, Elsevier, Amsterdam.

Sellergren, B., (1999). Polymer and template-related factors influencing the efficiency in molecularly imprinted solid-phase extractions. Trends Anal. Chem., **18** (3):164-174.

Shahtaheri, S. J., (1996). PhD Thesis. Trace pesticide analysis using immuno-based solid-phase extraction. Robens Institute of Health and Safety, University of Surrey, Guildford, England.

Shahtaheri, S. J., Mesdaghinia, A., Stevenson, D., (2005a). Evaluation of factors influencing recovery of herbicide 2,4-D from drinking water. Iranian Journal of Chemistry and Chemical Engineering (IJCCE), **24**: 33-40.

Shahtaheri, S. J., Ghamari, F., Golbabaei, F., Rahimi-Foroushani, A., Abdollahi, M., (2005b). Sample preparation followed by high performance liquid chromatographic analysis for monitoring of muconic acid as a biomarker of occupational exposure to benzene. International Journal of Occupational Safety & Ergonomics (JOSE), **11** 337-388.

Shahtaheri, S. J., Khadem, M., Golbabaei, F., Rahimi-Foroushani, A., (2007a). Solid phase extraction for evaluation of occupational exposure to Pb (II) using XAD-4 sorbent prior to atomic absorption spectroscopy. International Journal of Occupational Safety and Ergonomics (JOSE), **13**: 137-145.

Shahtaheri, S. J., Khadem, M., Golbabaei, F., Rahimi-Foroushani, A., (2007b). Optimization of sample preparation procedure for evaluation of occupational and environmental exposure to nickel. Iranian Journal of Public Health, **36**: 73-81.

Shahtaheri, S. J., Ibrahimi, L., Golbabaei, F., Hosseini, M., (2007c). Optimization of solid phase extraction for 1-hydroxypyrene as a major biomarker of exposure to PAHs prior to high performance liquid chromatography. Iranian Journal of Chemistry and Chemical Engineering (IJCCE), **26**: 75-81.

Shahtaheri, S. J., Abdollahi, M., Golbabaei, F., Rahimi-Foroushani, A., (2008). Sample preparation followed by HPLC for monitoring of mandelic acid as a biomarker of

environmental and occupational exposure to styrene. International Journal of Environmental Research, **2**: 169-176.

Siemann, M., Andersson, L. I., Mosbach, K., (1996). Selective recognition of the herbicide atrazine by noncovalent molecularly imprinted polymers. J. Agric. Food Chem., **44**: 141-145.

Stevenson, D., (1999). Molecular imprinted polymer for solid phase extraction. Trends Anal. Chem., **18** (3): 54-158.

Takeuchi, T., Fukuma, D., Matsui, J., (1999). Combinatorial molecular imprint-ing: An approach to synthetic polymer receptors. Anal. Chem., **71**: 285-290.

Wu, C. F. J. and Hamada, M., (2000). Experiments: planning, analysis, and parameter design optimization. 1st edition, John Wiley and Sons INC., New York, NY.

Zander, A., Findlay, P., Renner, T., Sellergren, B., Swietlow, A., (1998). Analysis of nicotine and its oxidation products in nicotine chewing gum by a molecularly imprinted solid-phase extraction. Anal. Chem., **70**: 3304-3314.