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Application of multivariate analysis to the screening of molecularly imprinted polymers (MIPs) for ametryn

A.R. Koohpaei [a,1], S.J. Shahtaheri [b,*], M.R. Ganjali [c], A. Rahimi Forushani [d], F. Golbabaei [a]

[a] Department of Occupational Health, School of Public Health, Medical Sciences, University of Tehran, Tehran, Iran
[b] Department of Occupational Health, School of Public Health, Center for Environmental Research, Medical Sciences/University of Tehran, Tehran, Iran
[c] Center of Excellence in Electrochemistry, Endocrine & Metabolism Research Center, University of Tehran, Tehran, Iran
[d] Department of Biostatistics, School of Public Health, Medical Sciences, University of Tehran, Tehran, Iran

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Abstract

Among the solid-phase extraction (SPE) techniques, a novel system for a triazine herbicide named ametryn, has been developed based on a molecular imprinted polymer (MIP) phase. Through this method, the synthesis of the complementary to ametryn MIP was accomplished and the factors influencing its efficiency have been optimized. Through the optimization process, the type and the amounts of functional monomer and solvents, template amount, cross-linker, initiator as well as the polymerization temperature were considered to be evaluated. Based on the obtained results, the optimum conditions for the efficient polymerized sorbent, considering the recovery efficiency were solvent: acetonitrile, 6.41 mL; monomer: methacrylic acid, 5.41 mmol; template: 1.204 mmol; cross-linker: 27.070 mmol; initiator: 2.03 mmol; temperature: 40.86 °C. The optimum molar ratio among the template, monomer and cross-linker for ametryn was 1:4.49:22.48. The reversed-phase HPLC-UV was used for the ametryn determination, using an isocratic solvent delivery system (acetonitrile: H_2O, 60:40), flow-rate of 0.8 mL min^{-1} and a UV wavelength of 220 nm. In line with the obtained results, using central composite design (CCD) can increase the precision and accuracy of synthesis and optimization of MIP to ametryn and possibly other similar analogues.

Keywords: Molecular imprinted polymers; Solid-phase extraction; Central composite design; Herbicides; Triazine; Ametryn

1. Introduction

The most widely used group of herbicides, since their discovery in the 1950s, is triazines including ametryn, atrazine, simazine, cyanazine and propazine. Triazinic herbicides were considered as systemic toxicants, causing a variety of acute health effects. On the other hand, the analysis of herbicides, in order to monitor, assess, evaluate and control their effects in the environment, is one of the most important fields in analytical chemistry [1]. In occupational and environmental assessment, a large number of samples are needed. Consequently, inexpensive and rapid analytical techniques are required [2]. In some efforts to reduce the cost, time and use of organic solvents in the organic trace analysis, many researchers have developed miniature methods, requiring fewer sample amounts and thus, less extracting solvents to carry out their analyses. In addition, new approaches such as solid-phase extraction (SPE), supercritical fluid extraction (SFE), accelerated solvent extraction (ASE) and solid-phase microextraction (SPME) using polymer-coated fibers have been investigated, demonstrating more promising approaches in the environmental analysis [3]. Other approaches, which are gaining popularity for the sample clean-up, include the immunoaffinity chromatography [4–6] and the molecular imprinting polymers (MIPs).

In the MIP technique, functional monomers and cross-linkers are polymerized in the presence of a template molecule, which is followed by the template removal from the resultant polymer network to leave a template-fitted cavity. The molecular imprinting technology is less expensive than the antibody production and may offer an alternative when the cost of the antibody production is prohibitive or the antibody performance is a problem.

* Corresponding author. Tel.: +98 91 21779019; fax: +98 21 88951390.
E-mail address: shahtaheri@tums.ac.ir (S.J. Shahtaheri).
[1] Present address: Department of Occupational Health, Qom University of Medical Sciences, Qom, Iran.

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In addition, the molecular imprinted polymers are highly resistant to the organic solvent effects, unlike antibodies (or other biological receptors). Subsequently, the molecular imprints may have applications to the analysis of highly lipophilic compounds (such as PCBs or pesticides) either in a sample clean-up step or in a detection method [7]. The molecular imprinted polymers for triazinic herbicides have been a subject of many investigations in the recent years with interesting application in chemical analysis [8–11]. MIPs are used for SPE and chromatographic separation [1,12–18].

For a general use of the molecular imprinting technology, the class of the imprintable compounds needs to be extended and the existing recognition elements need to be improved in order to meet the requirements in the given application. The large number of compositional (monomers, cross-linkers, porogenic solvents, template to monomer ratios, etc.) and operational (initiation method, polymerization temperature and time) variables, present even in a relatively simple MIP preparation, coupled with the fact that they are dependent with each other, make it an extremely difficult task to optimize an MIP. In this development, a key factor is the identification and the optimization of the main factors, affecting the material structure and the molecular recognition properties.

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 evaluation of the MIPs [19]. 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 [20]. Rather than making every combination in an *n*-dimensional matrix, these methods allow one to vary multiple parameters simultaneously. The utilization of the multivariate analysis of the 'response factor' (e.g. fractional binding of the template) in the polymers, which are synthesized and screened, allows the optimum polymer composition to be predicted. This approach has recently been used in several studies successfully. Navarro-Villoslada et al. [21] used this approach to optimize six key factors in the MIP preparation for the bisphenol A polymers. In that research, the polymers were synthesized as mini-MIPs and screened by HPLC, leading to a rapid and cost-effective identification of the optimized polymers. Kempe and Kempe [22] synthesized beaded polymers by suspension polymerization in mineral oil for their multivariate study and, then, screened for rebinding by radioligand counting methods. In this way, a more extensive screening became feasible and more sophisticated response parameters could be defined, if required using, e.g. competitive binding formats. Davies et al. [23] applied chemometrics to the optimization of highly selective and group selective polymers for sulphonamides. To achieve this aim, they used more sophisticated response factors, based on a multi-analyte competition assay, to estimate the rebinding ability of the synthesized polymers and to select the optimum sulphonamide structures to use as template and for the rebinding studies. The chemometric optimizations of the template/monomer/cross-linker ratios led to the polymers that demonstrated either specificity or group selectivity depending on the experimental design. This methodology can open a new approach for the synthesis of tailor-made phases, called MIPs.

The aim of this work was the optimization of the main factors, affecting the material structure and the molecular recognition properties of the molecular imprinting polymers by a chemometric approach. Triazinic herbicide ametryn was chosen as the model system of this study. The triazinic herbicides are suitable models for these studies because they are relatively inexpensive and stable. As a consequence, the gram quantities necessary in a typical reaction can be handled without extraordinary precautions.

2. Experimental

2.1. Reagents

Ametryn, with greater than 98.2% purity, was obtained from Riedel-de-Häen (Seelze, Germany), 4-vinylpyridine, methacrylic acid (MAA, functional monomer) [79-41-4] and ethylene glycol dimethacrylate (EGDMA, co-monomer) [97-90-5] were purchased from the Merck Company, Germany. 2,2′-Azobisisobutyronitrile (AIBN, initiator) [78-67-1] was obtained from the Acros Company, USA. All solvents (acetic acid, acetonitrile, dichloromethane and methanol) were of analytical reagent grade (Merck, Germany). Ultra pure water was obtained from a Purite Purification System. Stock standard solutions ($1\,g\,L^{-1}$) were prepared by weighing the solutes, their dissolution in acetonitrile and their storage at $-18\,^{\circ}C$. The standard solutions were obtained with the dilution of the stock solutions in acetonitrile.

2.2. Equipment

All measurements were performed by a reversed-phase HPLC system from the Knauer Company (Germany), 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. The analytes were separated on a Chromolith Performance RR-C_{18}e 100 mm × 4.6 mm i.d. (Merch KGa A, Germany) and column guards (Chromolith Guard Cartridge Kit RP-C_{18}e and 5 cm × 4.6 mm i.d., 5 μm), using isocratic elution as follows: 60% acetonitrile and 40% purified water. Ametryn was monitored at 220 nm and quantified with external calibration using the peak area measurements ($R^2 = 0.9998$). Each sample was repeated three times to assure the chromatogram reproducibility. The flow-rate was set at $0.8\,mL\,min^{-1}$. 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 used apparatus included soxhlets and a heater unit, a liquid extraction unit (S&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 μm, Millipore Corp., USA), PTFE filters (0.2 μm, Sartorius, Germany),

an oven (Memmert, Germany) and a shaker (Innova 4000). The amount of reagents was measured, using a digital balance (Sartorius-2024, Germany) for milligram quantities or less. The quantitative liquid transfers were performed with a pipette (Socorex, Germany).

2.3. Preliminary polymer preparation

In this study, non-covalent bulk polymerization was employed, as one of the successful molecular imprinting protocols [15,24], to obtain glassy polymer blocks to be used as powder after being crushed, ground and sieved. The preliminary molecularly imprinted polymers as center points of the experimental design were prepared as follows: 1-mmol ametryn and 4-mmol MAA were added to a 25-mL glass tube and, afterwards, the mixture was left for 5 min. Subsequently, EDMA (20 mmol), AIBN (2 mmol) and 5-mL acetonitrile were added. The mixture was purged with nitrogen for 5 min and the glass tube was sealed under this atmosphere. It was then placed at a thermostated water bath at 55 °C to start the polymerization process. After 24 h, the tube was broken, the obtained polymer was ground and sieved, and the particles with sizes between 50 and 105 μm were collected. The template was removed by soxhlet extraction using a two-step procedure (methanol:acetic acid washing (9:1, v/v) 18 h as a first step and methanol washing for 6 h as a second step). This procedure was optimized in this study to generate MAA-based binding sites complementary to ametryn. Finally, the produced powder was stored in desiccators. Furthermore, non-imprinted polymers were obtained following the same procedure without the addition of the template molecule. Safety precautions were considered during the preparation of the polymerization mixture, the grinding and the extraction of the polymer. These steps were performed in a safety cabinet, because they involve the handling of the toxic compounds: methacrylic acid, ethylene glycol dimethacrylate and 2,2′-azobisisobutyronitrile.

2.4. Qualitative optimization of the solvents and functional monomers

In order to select the best solvent and functional monomers, a screening plan was performed and through this approach, four experiments were designed (Table 1), namely as ametryn 1 to ametryn 4 (AME_1, AME_2, AME_3 and AME_4). The imprinted polymers were synthesized and examined (except for the solvents and functional monomers), following the same procedure with the preliminary molecularly imprinted polymers.

2.5. Experimental design approach

The factorial design requires fewer measurements than the classical one-at-a-time experiment to give the same precision. At the same time, it detects and estimates any interaction between the factors, which the classical experiment cannot do. The order of the running experiments was restrictedly randomized to eliminate the possible bias (restricted factor was the polymerization temperature) [25]. The standard approach to the analysis of the experimental design data is to evaluate a list of the main and interaction effects supported by an ANOVA table, indicating which effects are significant [20]. The Minitab, Release 14 for windows statistical software package was used for data manipulation [26]. At the first stage of the experimental design, six factors were selected as potentially affecting the rebinding efficiency. These factors can be the compositional variables, such as the amounts of functional monomer, template, cross-linker, porogen or solvent of polymerization, as well as the operational variables such as the initiator and polymerization temperature. Consequently, a two-level full factorial design of 2^6 was utilized following a linear and quadratic model, containing squared terms. This design involved sixty-four basic experiments, undertaken in random order plus four central points. The values, corresponding to the high (+), low (−) and central (0) points for each factor, are shown in Table 2. At the second stage, a central composite design (CCD) was used with three different α values (1.414, 2, 2.449) in order to assess the α effects on the experimental design result, adding twelve star points to the above 2^6 factorial design. Therefore, 80 runs for each α state were selected or totally 240 runs. For each run in the experiments, non-imprinted polymers (NIPs) were obtained. Table 2 lists the α values given to each factor.

2.6. Equilibrium-rebinding experiments

After the soxhlet extraction and the template removal from the polymer structure, the synthesized polymer was placed at a thermostated oven at 70 °C for 2 h. Afterwards about 1 g of the polymer particles was placed at a beaker, containing a 100 ng mL^{-1} ametryn solution. Finally, the mixture was placed in a digital shaker (100 rpm, 25 °C, for 5 h optimized in this study) and, then, filtered by syringe filter unit (FH-0.45 μm, Millipore Corp., USA). The analyte concentrations in the final solution, representing the analyte amount bound to the polymer, were determined by HPLC-UV as described above. The unbound analyte amount to the polymer was obtained by subtracting the analyte bound amount to the polymer from that of the initial analyte loaded to the polymer.

Table 1
Screening plan for the qualitative optimization of the ametryn molecularly imprinted polymer synthesis

Solvent	Dichloromethane		Acetonitrile	
Functional monomer	4-Vinylpyridine	Methacrylic acid	4-Vinylpyridine	Methacrylic acid
Designed polymer	AME_1	AME_2	AME_3	AME_4

Table 2
Factor levels in the experimental designs

Variable	Low (−)	High (+)	Central (0)	$\alpha = 1.414$		$\alpha = 2$		$\alpha = 2.449$	
				Axial (−α)	Axial (+α)	Axial (−α)	Axial (+α)	Axial (−α)	Axial (+α)
Temperature (°C)	45	65	55	40.86	69.14	35	75	30.51	79.49
Template (mmol)	0.75	1.25	1	0.646	1.353	0.5	1.5	0.387	1.612
Initiator (mmol)	1	3	2	0.586	1.314	0	4	0	4.449
Cross-linker (mmol)	15	25	20	12.93	27.07	10	30	7.775	32.245
Solvent (mL)	4	6	5	3.586	6.414	3	7	2.551	7.449
Monomer (mmol)	3	5	4	2.586	5.414	2	6	1.551	6.449

3. Results

In order to achieve the optimum chromatographic conditions for the ametryn analysis, variables including, the mobile phase composition, UV wavelength, injection volume and mobile phase flow-rate were optimized. Fig. 1 shows the ametryn chromatogram detected at 220 nm (two injections repeatedly).

3.1. Template removing optimization

After the preliminary polymer preparation mentioned earlier, in order to optimize the template removal, the template was removed by soxhlet extraction using a two-step washing procedure. At the first step, the template was washed by 200-mL methanol:acetic acid (100:0, 99:1, 95:5 and 9:1 (v/v)) for 12, 16, 18, 20 and 24 h. For the second step, washing with 200 mL of methanol took place for 2, 4, 6, 8, 10 and 12 h, after each washing at the first step. The ultimate obtained results are illustrated in Fig. 2.

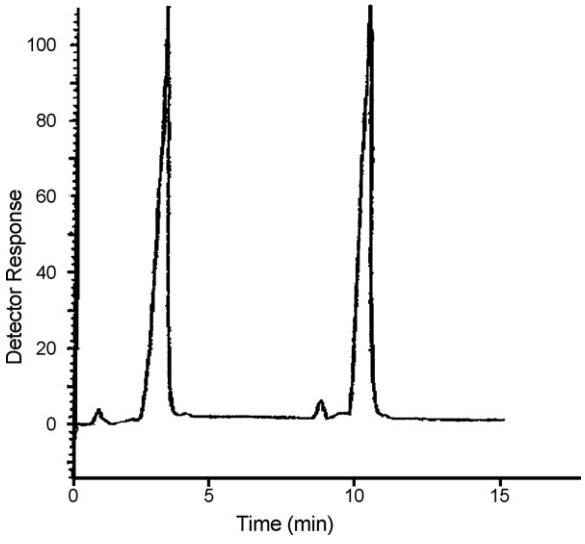

Fig. 1. The HPLC chromatogram of ametryn at the concentration of 100 ng mL^{-1}. Mobile phase, 60% acetonitrile and 40% purified water, flow-rate: 0.8 mL min^{-1}, injection volume: 20 μL, the analytical column: performance RR-C_{18}e 100 mm × 4.6 mm i.d. (Merch KGa A, Germany) and column guards (Chromolith Guard Cartridge Kit RP-C_{18}e and 5 cm × 4.6 mm i.d., 5 μm). UV detection at 220 nm, the ambient temperature was used for the chromatographic system.

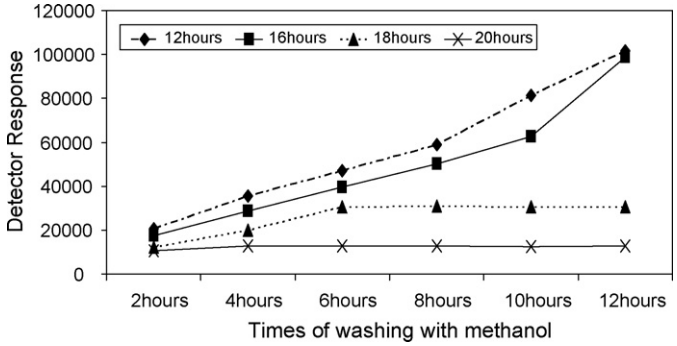

Fig. 2. Template removal with methanol:acetic acid (9:1, v/v).

Table 3
The rebinding mean percentages of ametryn on the MIP *vs.* the NIP in the rebinding optimization procedure ($N = 3$)

Time (h)	Recovery on the MIP (mean ± S.D.)	Recovery on the NIP (mean ± S.D.)
1	41.494 ± 0.29	1.25 ± 0.07
2	47.45 ± 0.35	3.36 ± 0.3
3	71.18 ± 0.21	5.346 ± 0.12
4	79.19 ± 0.23	7.413 ± 0.09
5	87.50 ± 0.27	8.48 ± 0.25
6	87.12 ± 0.14	8.38 ± 0.31
7	87.29 ± 0.19	8.375 ± 0.47

3.2. Equilibrium-rebinding experiments optimization

After the template removal optimization, a procedure for optimizing the rebinding experiments was selected. In this procedure, 1 g of the polymer particles was placed in a beaker containing a solution of 100 ng mL^{-1} of ametryn in a digital

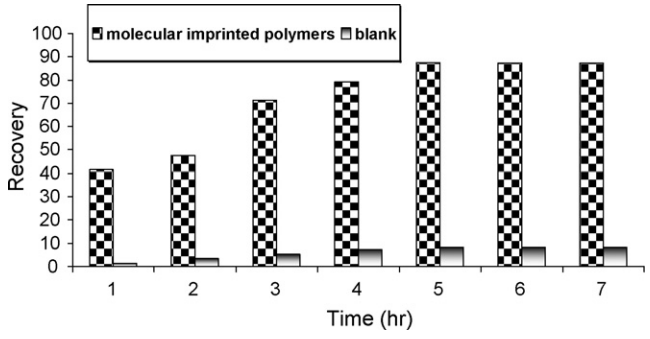

Fig. 3. Effect of the selected times on the rebinding of MIP *vs.* NIP in the rebinding optimization procedure ($N = 3$).

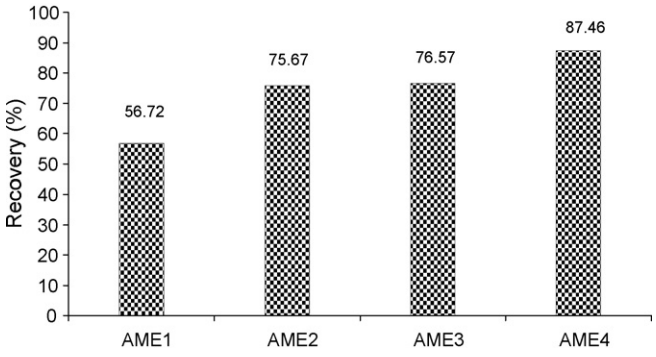

Fig. 4. Rebinding of ametryn to the MIP with different functional monomers and solvents ($N = 5$).

shaker (100 rpm, 25 °C) for 1–7 h. Each time the analyte concentrations in the final solution were determined by HPLC-UV as mentioned earlier. The results of this equilibrium optimization have been shown in Table 3 and Fig. 3.

3.3. Qualitative optimization of the molecularly imprinted polymer

As mentioned in the previous section, ametryn was the template molecule; MAA and 4-vinylpyridine were used as functional monomers, while acetonitrile and dichloromethane were utilized as porogens. The results of this library have been shown in Fig. 4.

3.4. Quantitative optimization of the molecularly imprinted polymer

When the number of independent variables is small, then overlying the response surfaces and choosing the optimum conditions constitute a simple and usually highly effective method.

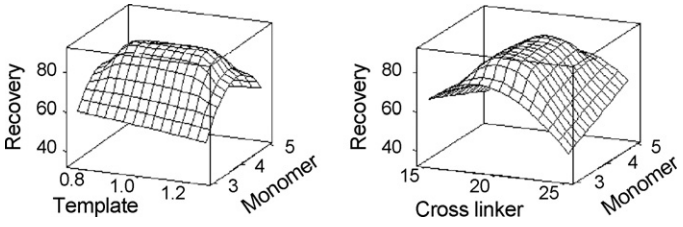

Fig. 5. Response surfaces estimated (recovery%) for atrazine using the central composite design obtained by plotting (a) template (mmol) *vs.* functional monomer (mmol) and (b) cross-linker (mmol) *vs.* functional monomer (mmol).

Table 4 exhibits the type of optimization design chosen in this work and the so-called response surface model: the central composite design, where the axial points are located on the sphere surrounding the two-level factorial design. The obtained results are summarized in Tables 5 and 6.

The data in Tables 5 and 6 were evaluated by ANOVA at the 5% significance level. The data indicate that, in most cases, interactions took place between principal factors (Table 7). This means that the respective response hypersurfaces in the multi-dimensional factorial space are considerably curved in the domain of the experimental design, *i.e.* the influence of one factor on the optimization parameter depends on the values of all the other factors. For instance, Fig. 5 depicts the response surface plots for ametryn, where the response surface curvature and factor interactions are evident. These data can also be presented by main effects plot (Fig. 6) and interaction effects plot (Fig. 7).

4. Discussion

The phenomenon of selective recognition resulting from polymer imprinting and the associated polymer recognition properties has been observed and reported in great detail. Despite the popularity in the literature published within the past decades,

Table 4
The experimental designs for ametryn

Run	A	B	C	D	E	F	Run	A	B	C	D	E	F	Run	A	B	C	D	E	F	Run	A	B	C	D	E	F
1	0	0	0	0	0	0	21	−	+	+	−	+	+	41	+	−	−	−	+	+	61	+	+	+	−	+	−
2	0	0	0	0	0	0	22	−	+	−	+	+	+	42	+	+	−	−	+	−	62	+	−	+	+	−	+
3	−	−	+	−	+	+	23	−	−	−	−	−	−	43	+	−	+	+	−	−	63	+	−	−	+	+	−
4	−	+	+	−	−	+	24	−	+	−	+	−	+	44	+	+	−	+	+	+	64	+	−	−	−	−	−
5	−	+	−	−	−	+	25	−	−	−	+	+	+	45	+	+	−	−	+	+	65	+	−	+	−	−	+
6	−	−	+	−	−	+	26	−	−	+	+	+	−	46	+	−	+	−	+	−	66	+	−	+	−	−	−
7	−	−	+	+	+	+	27	−	+	−	+	+	−	47	+	+	−	+	+	−	67	0	0	0	0	0	0
8	−	+	−	−	+	+	28	−	−	−	−	−	+	48	+	+	+	−	−	−	68	0	0	0	0	0	0
9	−	+	+	−	−	−	29	−	−	+	−	+	−	49	+	−	−	−	+	−	69	+α	0	0	0	0	0
10	−	+	+	+	+	−	30	−	−	−	−	+	−	50	+	+	+	−	−	+	70	−α	0	0	0	0	0
11	−	+	−	+	−	−	31	−	−	+	−	−	−	51	+	−	+	+	+	−	71	0	+α	0	0	0	0
12	−	+	−	−	−	−	32	−	+	+	+	−	−	52	+	−	−	+	−	−	72	0	−α	0	0	0	0
13	−	+	+	−	+	−	33	−	−	−	+	+	−	53	+	+	+	−	+	+	73	0	0	+α	0	0	0
14	−	+	+	+	+	+	34	−	−	−	−	+	+	54	+	−	−	+	−	+	74	0	0	−α	0	0	0
15	−	−	−	+	−	+	35	+	+	−	−	−	+	55	+	+	+	+	+	−	75	0	0	0	+α	0	0
16	−	−	+	+	−	−	36	+	+	+	+	−	−	56	+	−	+	+	+	+	76	0	0	0	−α	0	0
17	−	−	−	+	−	−	37	+	+	−	+	−	−	57	+	+	−	+	−	+	77	0	0	0	0	+α	0
18	−	−	+	+	−	+	38	+	−	−	+	+	+	58	+	+	+	+	+	+	78	0	0	0	0	−α	0
19	−	+	−	−	+	−	39	+	+	+	+	−	+	59	+	+	−	−	−	−	79	0	0	0	0	0	+α
20	−	+	+	+	−	+	40	+	−	+	−	+	+	60	+	−	−	−	−	+	80	0	0	0	0	0	−α

Factor A: temperature; factor B: template; factor C: initiator; factor D: cross-linker; factor E: solvent; factor F: functional monomer.

Table 5
Mean equilibrium rebinding on the molecular imprinted and the non-imprinted polymers in the full factorial design model

Run	Recovery (mean, $N=5$)		Run	Recovery (mean, $N=5$)		Run	Recovery (mean, $N=5$)		Run	Recovery (mean, $N=5$)		Run	Recovery (mean, $N=5$)	
	MIP	NIP		MIP	NIP		MIP	NIP		MIP	NIP		MIP	NIP
1	87.34	8.41	13	59.78	10.59	25	54.40	10.29	37	43.40	15.49	49	76.37	17.45	61	51.29	12.55
2	87.47	8.28	14	89.64	10.63	26	47.57	35.56	38	46.45	12.31	50	41.38	14.52	62	46.37	11.59
3	64.44	12.48	15	52.59	9.47	27	55.42	11.30	39	57.27	16.44	51	41.71	37.33	63	38.37	35.38
4	48.52	12.41	16	45.43	34.67	28	57.24	11.86	40	56.52	14.33	52	35.35	36.49	64	72.36	17.56
5	47.52	12.58	17	43.28	34.78	29	81.68	15.52	41	53.32	16.29	53	41.49	13.41	65	52.29	14.47
6	60.53	12.60	18	54.28	9.28	30	82.67	15.81	42	51.58	12.44	54	44.58	11.37	66	73.59	17.44
7	61.34	8.52	19	59.67	10.43	31	81.25	15.48	43	37.52	36.49	55	50.36	13.51	67	87.45	8.41
8	47.75	17.56	20	65.57	14.63	32	53.38	13.61	44	80.28	13.52	56	53.36	10.55	68	87.44	8.56
9	57.44	8.33	21	49.70	11.33	33	46.31	33.29	45	39.23	19.27	57	73.51	17.55			
10	58.52	11.34	22	88.48	11.64	34	61.45	14.28	46	73.46	17.46	58	81.34	12.52			
11	51.75	13.60	23	80.58	15.62	35	39.60	14.34	47	47.49	13.55	59	47.46	10.55			
12	55.62	8.92	24	80.44	15.37	36	45.49	15.66	48	49.46	10.31	60	49.34	13.37			

the selectivity of MIP mechanisms and their rational control has not entirely been recognized and still is under question. Therefore, there is the need to optimize the extraction procedure more detailed. This becomes apparent in contributions as published by Martin-Esteban in 2001. It was found that, after imprinting a polymer against the drug tamoxifen for solid-phase extraction purposes, a control polymer imprinted against propanol exhibited greater affinity for tamoxifen than the tamoxifen imprinted polymer despite the obvious differences in the molecular structure of the template molecules. Such irregularities necessitate detailed study and analysis of the MIP systems prior to a more widespread acceptance as a facile analytical technique [27]. As already stated, the first step in the MIP preparation involves the prearrangement of the template and the used monomer. The applied temperature as well as the employed template, monomer, cross-linker and solvent (porogen) are obviously the key parameters for the achievement of a successful selective MIP [28]. Since all the mentioned parameters have a strong influence on the overall MIP performance in terms of affinity, selectivity, loading capacity, etc., their proper selection (qualitative and quantitative) will ensure that polymers with appropriate properties are obtained for a particular application. As it has been mentioned in Section 2.3, in order to start the optimization process by an experimental design approach, a preliminary polymer synthesis was designed. In general, a template–monomer molar ratio of 1:4 provides enough stability for the formed complex, assuring the achievement of the desired imprint effect. As a consequence, this ratio was selected as a start point for optimization process. From the results given in Fig. 3, it was deduced that NIP was not capable of binding a relatively high degree of ametryn in comparison with the ametryn MIPs. This indicated that the MIPs might have not rebound a large ametryn amount through non-specific binding interactions.

It should be noted that generally there are some problems associated with the use of MIPs in analytical separations, such as leakage of the imprinted molecules. The leakage of the imprinted molecules during the elution stage was evident on several occasions, which led to an uncertainty in the concentration determination. For that reasons, each method development at one stage must confirm the absence of interference from the bleeding template species. One approach to circumvent this problem is the polymer heat-treatment, accompanied with excessive washes under strong washing conditions [29]. This treatment is claimed to greatly reduce or even eliminate the template bleeding from the MIP phase. Therefore, a two-step washing procedure (methanol:acetic acid and methanol), based on soxhlet extraction, was optimized in this study to generate MAA-based binding sites complementary to ametryn. Soxhlet extraction is easy to handle, however, involves large volumes of solvent and takes 6–24 h [30,31]. In accordance with many authors, the results showed that by using methanol:acetic acid (9:1, v/v) 18 h and methanol 6 h as the template washing regime, the template would be removed completely from the polymer structure (Fig. 2) [32].

In order to select properly the functional monomer and solvent, as it is displayed in Table 1, a screening library was obtained. From Fig. 4, it could be concluded that AME_4 pre-

Table 6
Mean equilibrium rebinding on the molecular imprinted and the non-imprinted polymers in the star points of central composite design model ($N = 5$)

Run	$\alpha = 1.414$		Run	$\alpha = 2$		Run	$\alpha = 2.449$	
	Recovery (mean ± S.D.)			Recovery (mean ± S.D.)			Recovery (mean ± S.D.)	
	MIP	NIP		MIP	NIP		MIP	NIP
69	77.60 ± 0.06	9.67 ± 0.08	69	45.53 ± 0.07	15.22 ± 0.01	69	35.40 ± 0.05	25.41 ± 0.07
70	89.46 ± 0.09	8.42 ± 0.03	70	–	–	70	–	–
71	86.54 ± 0.05	8.33 ± 0.02	71	85.37 ± 0.04	8.48 ± 0.07	71	84.31 ± 0.03	8.58 ± 0.05
72	59.56 ± 0.06	8.36 ± 0.02	72	43.46 ± 0.10	8.24 ± 0.07	72	32.35 ± 0.06	8.40 ± 0.05
73	85.33 ± 0.03	12.55 ± 0.05	73	84.42 ± 0.05	14.42 ± 0.07	73	84.40 ± 0.02	15.27 ± 0.06
74	75.47 ± 0.09	9.16 ± 0.01	74	–	–	74	–	–
75	85.27 ± 0.02	17.84 ± 0.01	75	79.30 ± 0.02	25.70 ± 0.01	75	70.28 ± 0.01	30.52 ± 0.06
76	61.57 ± 0.10	9.33 ± 0.05	76	52.53 ± 0.06	9.43 ± 0.02	76	45.51 ± 0.04	9.49 ± 0.05
77	88.54 ± 0.05	8.51 ± 0.06	77	81.70 ± 0.05	10.27 ± 0.03	77	75.53 ± 0.07	11.40 ± 0.03
78	71.47 ± 0.05	11.54 ± 0.05	78	65.32 ± 0.05	12.59 ± 0.09	78	54.35 ± 0.08	13.51 ± 0.05
79	80.38 ± 0.05	13.45 ± 0.04	79	79.28 ± 0.06	14.22 ± 0.01	79	61.59 ± 0.03	25.40 ± 0.08
80	70.37 ± 0.02	14.19 ± 0.00	80	54.29 ± 0.01	17.32 ± 0.05	80	38.28 ± 0.04	24.48 ± 0.07

sented the highest specificity by binding nearly 11%, as much of its closest member in the library (AME_3). The right selection of functional monomers is important in molecular imprinting, because the interaction with functional groups affects the affinity of MIPs. MIPs have mainly been synthesized by the non-covalent approach, using methacrylic acid as the functional monomer and ethylene glycol dimethacrylate as the cross-linker monomer. In general, acidic functional monomers (such as MAA) are normally used for the basic template, whereas 4-vinylpyridine (4-VP) and methacrylamide are used for the acidic templates [24].

Ametryn contains functional groups, being able to form cyclic hydrogen bonds with MAA. These bonds are one of the strongest non-covalent interactions and have proved the basis of some of the most successful MIPs in the literature. Nevertheless, some authors chose other functional monomers such as 4-VP and methacrylamide [24]. It has been demonstrated that MIPs offer the highest selectivity, if for the MIP preparation; the samples were dissolved in the used porogen [33]. Based on analysis considerations, in the qualitative optimization of the solvents and functional monomers, equilibrium-rebinding experiments were conducted with the aid of acetonitrile. Therefore, the highest rebinding was observed in AME_4 (where acetonitrile and MAA were used). Acetonitrile has been found to be a suitable mobile phase solvent for low to medium-polar templates, such as triazines with $pK_a = 1$–4 and $\log P_{ow} = 1.7$–3.4, presumably due to its weak hydrogen-bonding capacity and, subsequently, its limited ability to compete for the hydrogen-bonding sites. Furthermore, it solvates the methacrylate polymer backbone well and it is polar enough to dissolve a large number of compounds [34]. On the other hand, the morphology was influenced by the properties of the porogen, since the swelling of the polymers is dependent on the surrounding medium [35]. Thus, the swelling is most pronounced in chlorinated solvents, such as dichloromethane, as compared with solvents like acetonitrile. This swelling behavior may lead to changes in the three-dimensional configuration of the functional groups, taking part in the recognition in the sites resulting in poorer binding capacity, as it was observed in AME_2 as in comparison with AME_4.

Generally, α or the star points indicator in the experimental design should be chosen between 1 and $\sqrt{K}$ (K is the input

Table 7
The estimated response surface regression coefficients for the mean equilibrium rebinding on the molecular imprinted polymer[a]

Term	Coefficient	S.E. coefficient	T	P-value	Term	Coefficient	S.E. Coefficient	T	P_{value}
Constant	87.71	1.654	53.008	0.001	A × C	0.010	1.24	0.008	0.994
Temperature (A)	−5.60	0.86	−6.45	0.001	A × D	0.05	1.24	0.038	0.969
Template (B)	0.44	0.86	0.52	0.605	A × E	0.008	1.24	0.006	0.995
Initiator (C)	0.87	0.86	1.01	0.316	A × F	−0.077	1.24	−0.061	0.951
Cross-linker (D)	−1.24	0.86	−1.44	0.156	B × C	−1.39	1.24	−1.12	0.266
Solvent (E)	3.55	0.86	4.12	0.001	B × D	17.41	1.24	13.97	0.001
Monomer (F)	1.21	0.86	1.41	0.163	B × E	1.28	1.24	1.027	0.309
A × A	−4.32	3.31	−1.3	0.198	B × F	6.87	1.24	5.52	0.001
B × B	−14.80	3.29	−4.49	0.001	C × D	−0.44	1.25	−0.34	0.729
C × C	−7.45	3.30	−2.25	0.321	C × E	1.17	1.25	0.93	0.352
D × D	−14.43	3.31	−4.35	0.001	C × F	−0.38	1.25	−0.308	0.760
E × E	−7.84	3.31	−2.37	0.021	D × E	2.32	1.25	1.85	0.070
F × F	−12.47	3.31	−3.77	0.001	D × F	16.59	1.25	13.22	0.001
A × B	−0.14	1.24	−0.11	0.912	E × F	1.53	1.25	1.22	0.227

[a] $S = 5.035$, $R^2 = 93.6\%$, R^2 (adj) = 90.3%. The analysis was done using coded units.

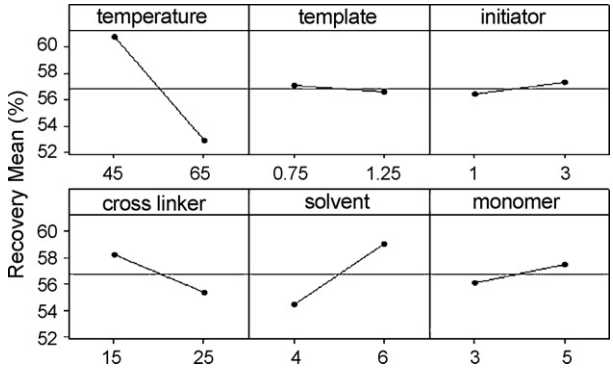

Fig. 6. Main effects plot in the factorial 2^6 design.

factors in coded units) and rarely outside this range [25]. As a result, in order to evaluate the effects of the α values on the model, three α values, *i.e.*, $\sqrt{2}$, $\sqrt{4}$ and $\sqrt{6}$ (K equals to 6) were chosen. According to the one-way ANOVA test, it was revealed that the differences between the α values were not significant ($P = 0.756$). In line with the resulting data in Table 7, the linear effects of the temperature and the solvent along with the quadratic effects of the template, cross-linker, solvent and the functional monomer on the mean equilibrium rebinding were significant. Also, the R^2 (adj) value was 90.3%, meaning that the six studied factors could explain 90.3% of the variation in the equilibrium-rebinding percentage. The results of the central composite design were validated using ANOVA. The P-value for the model (0.001) was lower than the critical value of the significance set, below 0.05. The R^2 value for a valid model is 0.6 or greater. For this model, the R^2 was 0.806, indicating that the made predictions would be reasonably accurate. The variation domain for each factor defines the parameter space within which the variable can change. For this reason, the selection of the variation domain was based on prior experiments. The molar values for the monomer were selected between 1.551 and 6.449. It was thought that using values lower than 2; it would create binding sites of insufficient opportunity for the template interaction. Conversely, the use of high values for the template would produce binding sites with too many points for the template interactions, resulting in the lack of specificity. Also, the molar values for the cross-linker were selected between 7.775 and 32.245.

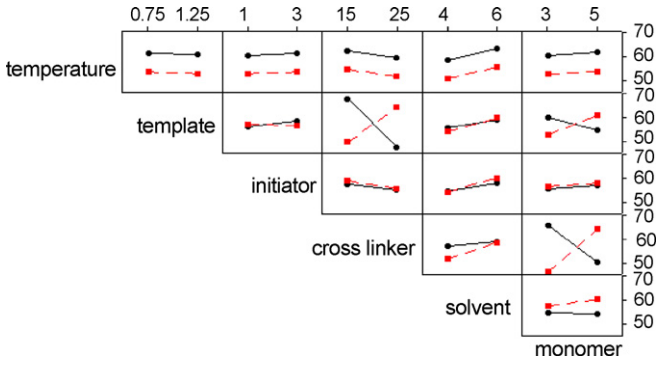

Fig. 7. Interaction effects plot in the factorial 2^6 design.

The designed 80 MIPs in the CCD had differing physical characteristics, ranging from extremely hard to much softer MIPs that contained lowest cross-linking ratios. Even the polymer in the run numbers 70 (without initiator) and run numbers 74 (very low temperature) were not successfully synthesized (Table 6). In agreement with Fig. 6, in the studied ranges, except temperature and solvent, other factors did not exhibit any statistically significant trend under the linear effect mode, when considered as main isolated factors ($P \leq 0.05$). The temperature values were selected between 30.51 and 79.49 °C. Temperature produced a negative influence. The present results indicated that the polymerization temperature played a crucial role in the performance of the synthesized material. It is believed from the earlier reported studies that the polymer affinity and specificity were significantly improved by optimizing the polymerization temperature [36]. Usually, lower temperature values will stabilize the template–functional monomers complexes [37]. It must be noted that the polymerization temperature also affects the polymerization process and the polymer structure, influencing the quality and the quantity of the MIPs recognition sites. It should be noted that 40.86 °C was the lowest level that has been predicted by central composite design results as theoretical optimum. It means that, the lowest possible level temperature must be selected. Therefore, it was decided to set the temperature at the lowest possible level of 55 °C to avoid the binding sites degradation.

As it has been reported, the optimization of the amount porogen is important for the recognition ability and the other MIP properties, such as surface area, swelling capacity and pore volume. Normally, the hydrogen-bonding capacity of the porogens played a significant role in the recognition properties of the materials [38]. The porogen values in the model were selected between 2.551 and 7.449 mL. Regarding the results of Table 7, among the linear effects, the most crucial variables were the temperature and the solvent used in the polymerization as well as among quadratic effects, the effects of the template, cross-linker, solvent and functional monomer were significant ($P \leq 0.05$) (solvent is also identified as a crucial variable when it is treated as quadratic term). Also, in agreement with Fig. 7, it can be derived that the interaction between the template, cross-linker and monomer (B × D, B × F and D × F) was significant ($P \leq 0.05$). According to the obtained results, it was revealed that the molar relationship between the functional monomer and the template was found to be important with respect to the number and quality of the MIP recognition sites. The low monomer:template (M/T) ratios led to less than optimal complexation on account of insufficient functional monomer. On the contrary, when the M/T ratios were too high, non-selective binds were yielded [39]. Furthermore, it was considered that, the application of high molar ratio for the cross-linker would produce an MIP, lacking the flexibility to allow the rapid mass transfer of the rebind analyte to and from the binding site. On the contrary, an MIP, formed with low molar ratios for the cross-linker, would not possess the mechanical strength to retain the binding site memory. Based on the result of ANOVA (Table 7), it can be concluded that, central composite design approach is useful in predicting an optimum

template:monomer:cross-linker (T:M:C) ratio for the production of an efficient ametryn MIP. By combining the response surfaces and based on the response optimizer data (weight of one and importance of one), it was finally possible to suggest the optimum conditions to be adopted in the MIP synthesis for ametryn, *viz.*, theoretical optimum temperature 40.86 °C, porogen 6.410 mL, cross-linker 27.070 mmol, initiator 2.03 mmol, monomer 5.41 mmol and template 1.204 mmol. The response surface model displayed the optimum template:monomer:cross-linker ratio, presented in Fig. 5. The predicted optimal T:M:C molar ratio for an ametryn MIP was 1:4.49: 22.48.

5. Conclusion

MIP applications in the industrial and environmental fields are still rare, although they have become more common in the recent years. The new MIP studies are mainly based on developing imprinted polymers for new target analytes of biological and environmental interest. MIPs may be limited by their low yield of specific binding sites, so in some cases these polymers have low sample load capacity and high non-specific binding. For this reason, studies currently being developed involve the optimization of the MIP synthesis in order to improve the capacity and/or the selectivity of the MISPE sorbents. Chemometrics is frequently employed for analytical method optimization and it is believed that the central composite design could prove beneficial for aiding the MIP development, such as the prediction of an optimum template:monomer:cross-linker ratio together with the optimization of the temperature and the amount of solvent and initiator for the production of an ametryn MIP, capable of rebinding the highest ametryn capacity.

Future works may also consider using different parameters. For example, different types of functional monomer, cross-linker and solvent could be compared for their respective rebind performances. Other factors like type of initiator, polymerization techniques and type of cross-linker could also be incorporated into the factorial and central composite design. However, limitation on the number of factors is essential, as the number of experiments required for modeling will increase proportionally. Finally, in reference with other works [23], this study has shown that chemometrics can be used as a rational approach to the MIP optimization.

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