# Molecularly Imprinted Polymer as SPE Sorbent for Selective Extraction of Diphenylamine in Surface Water

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**ABSTRACT:** This paper describes the development of an analytical methodology to determine diphenylamine (DPA) in surface waters using molecularly imprinted polymers (MIPs) as extraction and pre-concentration material, followed by high performance liquid chromatography (HPLC). A new molecularly imprinted polymer, prepared following a non-covalent approach, was synthesised using DPA as the template molecule, methacrylic acid as functional monomer, ethylene glycol dimethacrylate as cross-linker. The imprinting effect of the polymer was verified by chromatographic evaluation. The polymer exhibited high adsorption capacity and good selective ability. A method for determination of trace DPA was developed using this polymer as enrichment sorbent coupled with high performance liquid chromatography focusing on surface water samples. Under the optimum experimental conditions, the DPA standard is linear within the concentration range studied, that is, from 0.1µg.mL-1 to 1µg.mL-1 ($r^2$= 0.9869). Lower limits of detection (LOD) were 0.02µg.mL-1. Recoveries and relative standard deviation (RSD, n= 4) values for precision in the developed method were 91% and 3.0%. It was demonstrated that the proposed MI-SPE-HPLC method could be applied to direct determination of DPA in surface water.

**Keywords**: Diphenylamine, Molecularly imprinted polymer, Solid phase extraction

## INTRODUCTION

Because of the indiscriminate use of pesticides for different applications, important environmental problems are emerging which are a risk to plant, animal, and human health. Fungicides are one group of these pesticides which are used primarily to control spoilage of crops as a result of fungal attack. They represent approximately 20–25% of pesticides used (Rodriguez et al, 2001). Diphenylamine (DPA, Fig.1) fungicide is systemic pesticide, widely used in agriculture for pre and postharvest treatment for control of a wide range of pathogens (Bramlage et al, 1996). These substances are sprayed over crop fields and hence released to the environment. They readily penetrate plants through the roots and leaves and can directly enter natural waters by drainage from agricultural land. Most of these compounds persist in the environment after their application; some even remain for many years (Johnson and Strickland, 1995). Thus, it is important to develop analytical methods for determining diphenylamine fungicide present in surface water at low levels.

Over the last few years, solid-phase extraction (SPE) has become the most commonly used sample preparation technique, not only for cleaning of the sample matrix but also to enrich the analytes present prior to analysis (Poole, 2003; He et al, 2007). Nevertheless, due to the lack of selectivity of the commercial SPE sorbents, other

materials with high selectivity, such as immunosorbents (IS) and molecularly imprinted polymers (MIPs) (Lanza and Sellergren, 1999), have been developed and applied to extraction procedures. The expensive and time-consuming procedure for the production and isolation of antibodies as well as their lack of availability, have led to MIPs being exploited widely in molecularly imprinted solid phase extraction (MISPE) (Cacho et al, 2004; Haup, 2003).

MIPs are artificial polymers with recognition binding sites able to bind a molecule or its structural analogues from a complex sample (Mayes and Whitcombe, 2005;Tamayo et al, 2007). The polymers are formed by polymerization of functional monomers and a cross-linker in a complementary shape around a template molecule. Template extraction will allow recognition binding sites to remain in the polymer matrix. The application of MIPs as sorbents in solid phase extraction, namely molecularly imprinted solid phase extraction (MISPE), have been successfully reported for many compounds. Some of them are; atrazine (Koohpaei et al, 2008), nitrophenol (Masque et al, 2000), sulfonylureas (Zhu et al, 2002), benzo(a)pyrene (Lai et al, 2004) and carbamazepine (Beltran et al., 2007), etc.

The aim of this work was to synthesize diphenylamine imprinted polymers for use as a sorbent in the SPE cartridge. Then, the MISPE procedure for extraction of the trace amount of diphenylamine in surface water was developed and its subsequent its HPLC analysis was optimized.
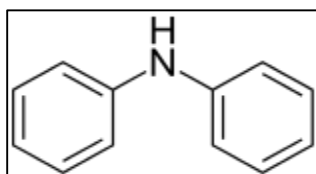


**Fig. 1. The structures of DPA**

## 2. Experimental

### 2.1. Materials and reagents

Analytical grade diphenylamine was purchased from Sigma-Aldrich. Acetonitrile, methanol, chloroform and acetic acid were purchased from Merck and were analytical or HPLC grade. Methacrylic acid (MAA) as the functional monomer, ethylene glycol dimethacrylate (EDMA) as the cross-linker and α,α′-Azoisobutyronitrile (AIBN) were purchased from Merck (Hohenbrunn, Germany). MAA was distilled under vacuum to remove the inhibitor prior to polymerization. Ultra pure water used for HPLC analysis and MISPE process were obtained from a Direct.Q3 Water Purification System (Millipore Corporation, USA). Diphenylamine standard solutions were prepared by dilution of the stock solution (5 $\mu g.mL^{-1}$ in methanol) with LC- grade water.

### 2.2. Instrumentation

For analyzing diphenylamine, high performance liquid chromatography (Agilent technology 1200 series HPLC) was used. The instrument was equipped with CO-2060 column oven, Bin pump sl De 63060570, and Tcc sl de 64156237 Dad detector. The detector was set at 285 nm. The chromatographic column was $C_{18}$ (250×4.6 mm i.d.; Supelco, USA). The mobile phase was a mixture of methanol:water (70:30) containing 5 μL $H_3PO_4$ at a flow rate of 1.5 $mL.min^{-1}$. The column temperature was fixed at 40C°. The injection volume was 10 μL.

### 2.3. Preparation of MIP

MIP was prepared using bulk polymerization method by dissolving 0.5 mmol of DPA, 2 mmol of functional monomer MAA, 10 mmol of cross-linker EGDMA in 10 mL of chloroform in a 20 mL borosilicate glass bottle, equipped with a rubber cap. This mixture was stirred over night at 50 ◦C for the formation of a complex of imprint molecule and monomers. After added 0.25 mmol of AIBN, the solution was saturated with dry nitrogen for 5min and the bottle was placed in a thermostated oil bath at 60 ◦C for 24 h. After polymerization, the polymer was ground with a mortar and pestle, and sieved to give particles with size dimensions between 25 μm and 50 μm and then repeatedly suspended in acetone to remove the small particles. The product was extracted with methanol containing 10% acetic acid using a Soxhlet apparatus for 48 h. Then the product was washed with methanol for three times and dried under vacuum at room temperature. The non-imprinted polymer (NIP) particles were prepared and washed using the same recipe but without the addition of the template DPA.

### 2.4. Procedure of SPE

In order to face the sensitive and quantitative determination of DPA at the required levels, a SPE procedure was carried out using this imprinted polymer as enrichment sorbent. SPE procedure was performed using a

VisiprepTM-DL SPE vacuum manifold from Supelco (Bellefonte, PA, USA). Empty SPE tubes were packed with 100 mg of imprinted or non-imprinted polymer using two PTFE frits at each end. SPE cartridges were preconditioned with 2 mL of ultra pure water and 2 mL of methanol to activate the sorbent before the enrichment procedure. 50 mL of DPA solution were uploaded onto the preconditioned cartridge. After loading, the vacuum was still applied to the cartridges for 5 min in order to remove the residual solvent. 1 mL of acetonitrile were added to the cartridges to eliminate molecules retained by physical adsorption by the polymer. Eluting step was performed using 1 mL of methanol/acetic acid (9/1, v/v) mixture solution. The elution fractions were dried under a gentle nitrogen stream. The residue was dissolved with 1 mL of methanol and filtered through a 0.45 μm nylon filter for subsequent HPLC analysis.

## 2.5. MISPE procedure of spiked surface water

The performance of the optimized MISPE procedure was evaluated for trace analysis of DPA in surface water. Surface water was collected from a farmland and its pH was adjusted to 6 (optimized pH obtained in this study). The surface water was filtered to remove particulate and spiked with DPA at: 0.2, 0.5, 0.7, and 1.0 μg.mL$^{-1}$. Then, MISPE of 100 mL of .0.2 μg.mL$^{-1}$ (sample loading flow rate: 1 mL.min$^{-1}$) and as well as 20 mL of 0.5, 0.7, and 1 μg.mL$^{-1}$ (sample loading flow rate: 1 mL.min$^{-1}$) were carried out.

## 3. Results and discussion

### 3.1. Preparation conditions of molecularly imprinted polymer

The preparation process of MIP is quite simple, but a number of factors which affect the polymerization and imprinted effect have to be taken to account. For the synthesis of the MIP, MAA was chosen as the functional monomer because, from the previous experience, this is the best functional monomer in order to establish selective hydrogen bonding interactions with the functional groups of DPA. Another important parameter that affected the capability of MIP synthesized in the synthesis process is molar ratio of template/monomer/cross-linker. Only appropriate molar ratio of template/monomer can afford high selectivity with MIP. And the amount of cross-linker should be high enough to maintain the stability of the recognition sites (Andersson et al, 1999). Through complex optimization experiments, the MIP synthesized at the molar ratio of 1:4:20 (template/monomer/cross-linker) in 10 mL of chloroform showed better affinity and selectivity and were chosen as synthetical conditions of DPA-MIP.

## 3.2. Effect of variables on MIP application

### 3.2.1. Effect of pH

The effect of pH on the sorption of DPA was investigated by varying the solution pH from 2.0 to 10.0. Several experiments were performed by equilibrating 100 mg of the imprinted particles with 50 mL of solutions containing 0.5 μg.mL$^{-1}$ of DPA under the desired range of pH. The pH dependence of extracted percentage of DPA is shown in Fig. 2. The results show that, the DPA recovery was increased to pH 6.0. The optimal pH should be applied to better embed of analyte in the polymer cavities. Because of make convenient configuration of DPA, the pH of sample loading should be adjusted. In these circumstances suitable hydrogen bonding with polymer was attained. Therefore, pH 6.0 was chosen as the suitable pH for further studies.
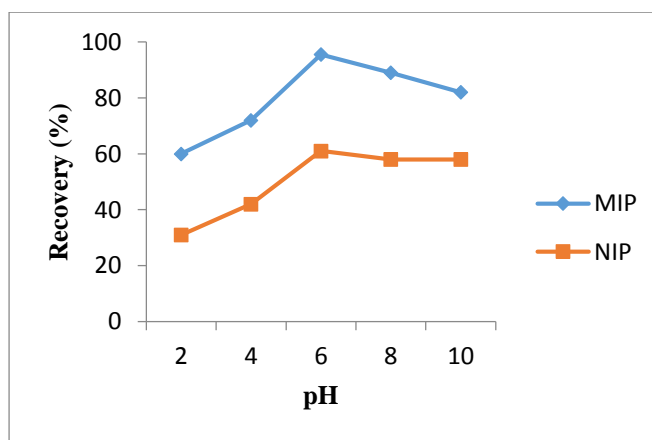


**Fig. 2. Effect of analyte pH on the percentage recovery.**

### 3.2.2. Effect of eluent volume

The eluent volume is also a crucial parameter to be optimized in SPE. The chosen volume of eluent must be just sufficient to elute the analyte from the sorbent. Thus, recoveries of DPA were studied in applying different eluent volumes of 0.25–2.00 mL. The results indicated that good recoveries were achieved at 1 mL of methanol/acetic acid (9:1), and more volume provided similar recovery values, which showed that 1 mL of methanol/acetic acid (9:1) was enough to provide a quantitative elution of the analyte from the sorbent (Fig. 3).

### 3.2.3. Effect of flow rate

The influence of analyte retention time was estimated by transmitting 50 mL of DPA solution (0.5 µg.mL$^{-1}$) at the pH 6.0 in different flow rates. The selective binding of DPA to the imprinted polymer had to be optimized in order to evade of reduced sensitivity of the MIP sorbent. In this system, the selectivity can be improved by ease of access to the binding sites within the porous polymer particles. As can be seen (Fig. 4) the best flow rate was attained to be 1.0 mL.min$^{-1}$. In higher flow rates, the analyte does not have sufficient residence time for effective interactions with sorbent and in lower flow rates, the template molecule reaches to equilibrium with MIP and have enough time to diffusion from MIP cavities to solution.
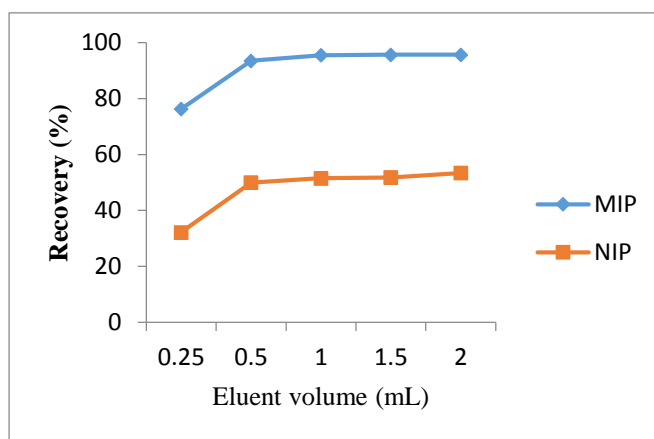


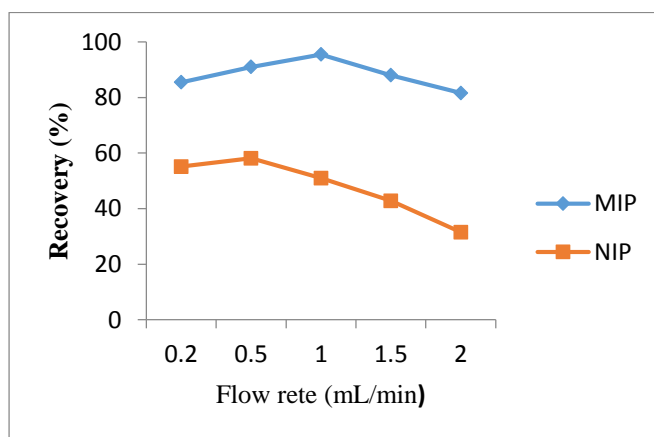**Fig. 3. Effect of Eluent volume on the percentage recovery.**



**Fig. 4. Effect of flow rate on the percentage recovery.**

### 3.2.4. Retention capacity and enrichment factor of MIP

A significant step in any MIP-based procedure is the determination of retention capacity because it permits to achieve high extraction efficiency. Various concentration of DPA in the range 0.1–2.0 µg.mL$^{-1}$ at the same volume (50 mL) was passed through the column for measuring retention capacity. The retention capacity (mg adsorbed DPA/g of sorbent) was obtained to be 9.9 mg.g$^{-1}$. The enrichment factor as an important parameter on preconcentration step was determined by passing 100.0mL of DPA solution with the concentration of 0.5 µg.mL$^{-1}$

through the MIP column. After analyte elution with 2.0mL methanol/acetic acid (9/1, v/v), HPLC analysis was carried out and the results show that the enrichment factor is 50.

### 3.2.5. Reproducibility of the MI-SPE column

Regeneration property is one of the important advantages of MIPs. MIPs can be used again to adsorb the interested analyte after regeneration. In order to reuse the polymers after each extraction, a short regeneration step was performed by washing with 3 mL of methanol/acetic acid (9/1, v/v). The results (see Fig. 5) showed that the MIP could be used many times and maintained their adsorption capacity at an almost constant value. This confirmed the reliability and efficacy of the proposed method for the analysis of DPA residues in real samples.
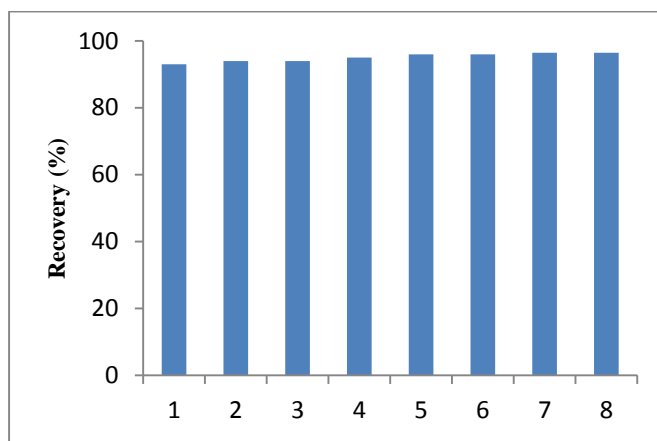


**Fig. 5. Regeneration property experiment of the MI-SPE column (repeat 8 times).**

### 3.3. Calibration curve and analytical parameters

In this work, a series of standards in the range 0.1–1.0 µg.mL$^{-1}$ of DPA was prepared in methanol and used to determine the analytical parameters. The response of HPLC was schemed against the concentration of this compound (Fig. 6) and calibration curve equation was build up by least-squares method. The linear dynamic range for the DPA determination was 0.1–1.0 µg.mL$^{-1}$ . The detection limit was 0.02 µg.mL$^{-1}$, and the relative standard deviation (RSD %) for four replicate measurements of 0.5 µg.mL$^{-1}$ of DPA was 3.0%.



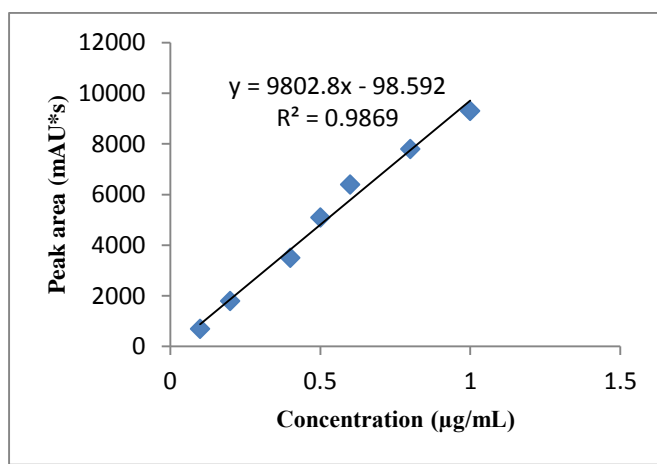**Fig. 6. Scheme of HPLC response against the concentration of DPA, the scheme confirms the linear dynamic range of 0.1–1.0 µg.mL$^{-1}$.**

### 3.4. Real sample analysis

The recommended method has been applied successfully for the extraction and determination of DPA in the surface water samples. The analysis was performed by using the spiked samples. The results are summarized in Table 1 (each analysis was done three times) and good recoveries in all samples were achieved. In order to

remove the interference of real sample constituents, the column was washed with washing solution (methanol 10%) after loading real samples on it and then it was eluted by elution solvent to measure DPA content (the same as described procedure). It was therefore possible to measure trace amounts of a target analyte in surface water samples by MISPE using HPLC.

**Table 1. Results of DPA determination in real sample analysis (n = 3).**

| Sample | DPA added (μg.mL$^{-1}$) | Average of DPA found (μg.mL$^{-1}$) | Recovery (%) |
|---|---|---|---|
| Surface water | - | Not detected | - |
| | 0.2 | 0.19±0.06 | 95.0 |
| | 0.5 | 0.46±0.05 | 92.0 |
| | 0.7 | 0.64±0.05 | 91.4 |
| | 1.0 | 0.92±0.04 | 92.0 |

## 4. Conclusion

The DPA imprinted polymer was prepared by bulk polymerization using MAA and EGDMA as the functional monomer and cross-linker, respectively. The obtained MIP showed good selectivity and affinity for DPA. Accordingly, a method was successfully developed for analysis of DPA in surface water by using the obtained MIP as the SPE sorbent coupled with HPLC. The limit of quantitation of the proposed method was 0.02μg.mL$^{-1}$. Moreover, the average recovery was higher than 91% (RSD: 3.0%). It should focus on the DPA determination in other samples in further research. This paper also offered a new method to determine other analytes in different samples.

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