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An Atrazine Molecularly Imprinted Polymer Synthesized Using a Cooling-Heating Method with Repeated Washing: Its Physico-chemical Characteristics and Enhanced Cavities

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An atrazine molecularly imprinted polymer (MIP) has been successfully produced via a cooling-heating method with repeated washing process. A template (atrazine) was incorporated into a pre-polymerization solution containing a functional monomer (methacrylic acid), a cross-linker (ethylene glycol dimethacrylate), and an initiator (benzoyl peroxide). The pre-polymerization solution was cooled in a refrigerator at -5°C for 60 min. and heated in an oven at 70°C for 150 min. The template removal process was conducted through a repeated washing process to increase the effectiveness of the cavities formation. The FTIR spectrum of the MIP showed that the peaks of amine group decrease significantly, indicating that the concentration of atrazine decreases drastically. HPLC was used to determine accurately the concentration of atrazine and the chromatogram of the MIP confirmed that there is no atrazine left. The repeated washing process was therefore better than the single washing process with 3% of atrazine left. In addition, the MIP obtained by the repeated washing process had the number of cavities of 780, which is higher than that produced by the single washing process as analyzed from SEM images. From the Scatchard plots, it was found that the equilibrium dissociation constant K_D and the maximum number of binding sites B_{max}, which is written as (K_D; B_{max}), of the MIP obtained by the repeated washing process is (7.1 μM; 14.9 mmol/g), respectively. These values are higher than those of the MIP produced by the single washing process.

Keywords: atrazine, binding isotherm, cavity, imprinted polymer, MIP, Scatchard plot, template.

1. INTRODUCTION

For farmers, weeds are the enemy that must be controlled because they compete against the needs of nutrients, water, light, and space to grow crops. Herbicides are considered as effective ways

to control weeds and one of the herbicides is atrazine. Utilizing atrazine, the dose and spray volume used were 1.5 L/ha and 400-600 L/ha, respectively, and the economic benefit is about US$ 15/ha as compared to manual weeding. It has been reported that the use of herbicides increased corn production by 6% [1]. But, as a consequence, if it is used in excess it will be very dangerous for amphibious animals [2], fresh water fish [3], humans, and it can also contaminate the surrounding environment.

In order to determine low concentration atrazine contaminant, previous researchers reported sensors that generally employ molecularly imprinted polymers (MIPs). The MIPs are synthetic polymers with specific binding sites for target molecules such as atrazine, which are prepared by molecular imprinting technique. The virtue of molecular imprinting technique is that sensing properties owned by MIPs make them possible to be applied in many fields, such as chemical and biological elements [4-8], foods [9,10], chromatography [11,12], solid-phase extraction [13,14], and health care as a sensor of atropine [15]. The bond strength of target-binding sites of the MIPs is determined by the dissociation constant [16,17].

In the preparation of MIPs, most of researchers generally use nitrogen flow in pre-polymerization solutions to remove oxygen that disrupts the polymerization process [18-22], before putting the solutions in water bath at 0°C for 4 h. under UV light. The synthesis of an atrazine MIP, in which atrazine serves as analyte molecules or template, using the heating-cooling method rather than nitrogen flow has been reported previously [23]. The results showed that the best way of preparing the polymer is by cooling the pre-polymer solution in a refrigerator at -5°C for 60 min. and then heating it at 70°C for 150 min. Cavities were formed after doing a single washing to the polymer to remove the atrazine template and the atrazine MIP was finally obtained. Moreover, the size distribution of cavities in the atrazine MIP was within the range of 80 to 230 nm and the number of cavities was 499.

The number of cavities essentially affects the ability of the MIP to capture/bind the target molecules because the function of the cavities is to recognize target molecules that have the same shapes of space and physico-chemical properties as the analyte molecules [24,25]. Since the MIP is preceded by the preparation of its polymer and cavities of the MIP exist after a washing process of the polymer, the use of an appropriate process in preparing and washing the polymer may result in more cavities. This paper reports an improvement in obtaining more cavities in an atrazine MIP. A pre-polymer solution was prepared and then cooled at -5°C for 60 min. After heating the cooled pre-polymer solution at 70°C for 150 min., a solid polymer was produced in the solution. The polymer was immersed in acetonitrile for 24 h. and then successive washes in methanol/acetic acid, methanol/aqua bidest, and methanol for 3 times to obtain template-free MIP.

2. MATERIALS AND METHODS

2.1. Chemicals

Chemical used in this work were atrazine pestanal (molar mass of 215.68 g/mol), methacrylic acid (MAA), ethylene glycol dimethacrylate (EDMA), benzoyl peroxide (BPO), methanol, acetonitrile, chloroform, acetic acid, and aqua bidest. All the chemicals were purchased from Sigma Aldrich.

2.2. Methods

Figure 1 represents the overall process of synthesis and sensing test of atrazine MIP. The synthesis was initiated by inserting atrazine as a template into a tube containing chloroform. Later, MAA as a functional monomer, EDMA as a cross-linker, and BPO as an initiator were added sequentially to make a pre-polymer solution. The pre-polymer solution was then stirred for 15 min. before it was put into a refrigerator at -5°C for 60 min. Next, the cooled solution was heated at 70°C for 150 min. Finally, the solid polymer product was formed in the solution. It was then filtered and crushed to obtain polymer particles. The resulting polymer particles were washed to remove the atrazine template from them. As a result, cavities are left in the polymer particles and the polymer particles with cavities are known as atrazine MIP particles. They can be used to identify a target that has physico-chemical properties similar to the template. When the target is exposed to the MIP, then the MIP will recognize and be able to bind the target. The sensing test of MIP is aimed to see the effectiveness of the MIP in recognizing the target.

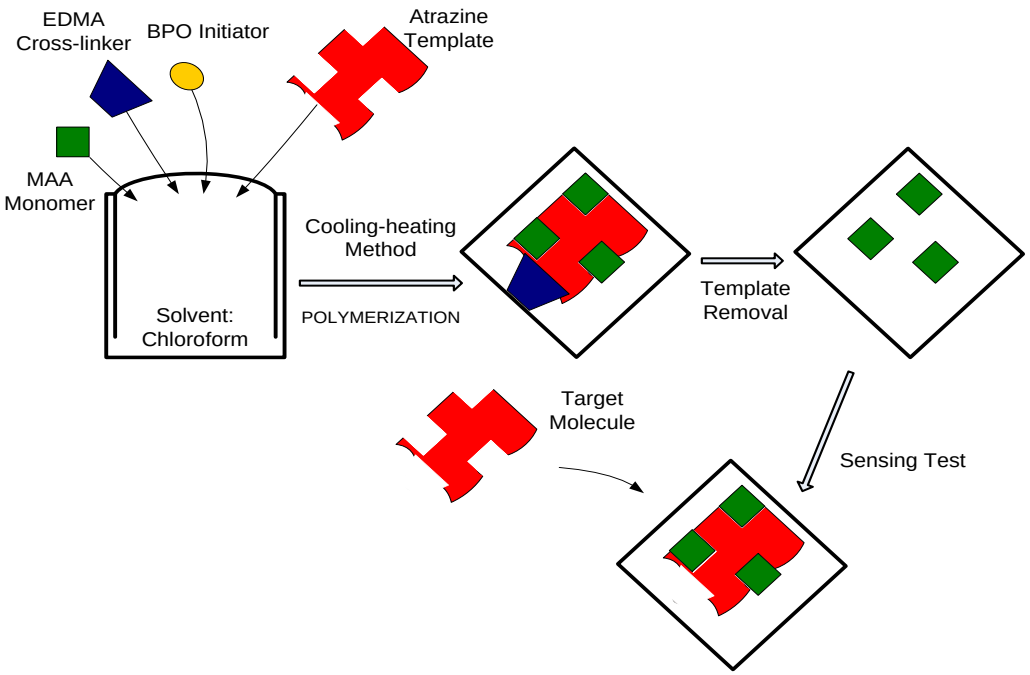


Figure 1. The process of preparing an atrazine molecularly imprinted polymer (MIP) and testing its sensing characteristics [23].

From the schematic description given in Fig. 1, the detailed stages to prepare an atrazine MIP and to test its sensing characteristics are as follows.

2.3. Polymerization process

Atrazine as template (0.025 g) was put into chloroform as solvent (2.01 mL), and then the solution was stirred for 15 min. MAA as functional monomer (0.3 mL), EDMA as cross-linker (0.525

mL), benzoyl peroxide (BPO) as an initiator (0.07 g) were added sequentially to prepare a pre-polymer solution. The pre-polymer solution was poured into vials, which then were sealed and stored in a refrigerator at -5°C for 60 min. Next, the vials were then placed in an oven at 70°C for 150 min. After the heating process, the solid polymer was obtained in the solution.

In the same way, a polymer without atrazine (non-imprinted polymer/NIP) was prepared to make comparison to the MIP.

2.4. Template removal process

The obtained solid polymer was crushed to produce fine polymer particles. The fine polymer particles were immersed in acetonitrile for 24 h. and then filtered to remove atrazine template molecules. The template removal was further achieved by successive washes the polymer in methanol/acetic acid (0.625 mL/12.5 mL), methanol/aqua bidest (12.5 mL/6.375 mL), and methanol (1 h., 20 h., and 1 h.), respectively. The template-free polymer (MIP) particles were collected and dried in vacuum.

Structural/physical characteristics of the MIP particles were examined by using a scanning electron microscope (SEM) JEOL-JSM-6510LV. In addition, their chemical characteristics were studied by employing an energy dispersive x-ray spectroscopy (EDS) and a Fourier transform infrared (FTIR) spectrometer FT/IR-4200 type A.

2.5. Sensing test

The steps to prepare test solutions are as follows. Initially, the stock solution (5000 ppm of atrazine solution) was prepared by dissolving 50 mg of atrazine in 10 mL of methanol. The atrazine standard solutions were obtained by diluting 0.5, 1.0, 1.5, 2.0, 2.3, and 2.4 mL of the stock solution with 5 mL of methanol/acetic acid in a 1:1 ratio. The obtained standard solutions were 500, 1000, 1500, 2000, 2300, and 2400 ppm, respectively. One mL of each standard solution was inserted into a test tube. Further, 5 mL of chloroform, 5 mL of acetic acid, and 10 mL of methanol was respectively added into the test tube to get a test solution that will be used to test sensing properties of the MIP particles. Therefore, the test solutions were 0.11, 0.221, 0.331, 0.442, 0.508 and 0.530 mM, in which the each concentration is called as initial concentration of atrazine solution (C).

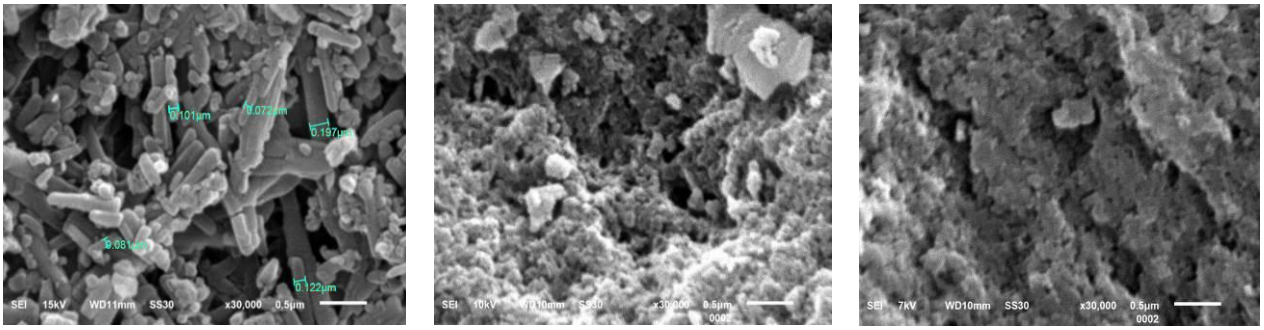
For testing sensing properties of the MIP, 10 mg of the MIP particles was put into a test solution. The mixture was then stirred for 12 h to allow the MIP particles bind atrazine molecules (targets) of the test solution. As a result, the current concentration of atrazine solution becomes lower because of a number of atrazine molecules bound by the MIP particles. The stirred mixture was then centrifuged at 2500 rpm for 15 min. to accumulate the atrazine-binding MIP particles. Finally, a 4.5 micron filter paper was used to separate the atrazine-binding MIP particles from the mixture and the remaining concentration of atrazine solution, which is labeled as F, was determined using a reversed-phase high performance liquid chromatography (HPLC) HP-1100 series with Waters 2487 detector. The number of atrazine molecules (targets) bound by the MIP particles per unit-weight, which is

labeled as B_{bound}, is therefore given by $(C\text{-}F)V_s/W$, where V_s is the total volume of solvents and W is the weight of MIP particles. For all measurements, the injection volume was 50 µL, the mobile phase was 0.01 M of acetic acid in acetonitrile (50:50), the flow rate was 1 mL/min, and the detection employed UV light at 260 nm.

3. RESULTS AND DISCUSSION

3.1. Physico-chemical characteristics

Figures 2.(b) and 2.(c) show SEM images of the polymer before removing the atrazine template and its atrazine MIP after the template removal, respectively. The SEM image of standard atrazine given in Fig. 2.(a) is for comparison purposes. It is seen that the surface of the atrazine MIP after the template removal is cleaner than that of polymer before the template removal. This finding is a coarse indication that the template removal process is effective.



(a) (b) (c)

Figure 2. SEM images of (a) standard atrazine, (b) polymer before removing the atrazine template, and (c) the atrazine MIP after template removal.

Rough chemical compositions are given by the elemental analyses of the polymer and its atrazine MIP as shown in Table 1. It is found that the amount of C, N, and O atoms do not change significantly with the template removal while that of Cl atoms does with an order of magnitude reduction. Since atrazine has Cl atoms in its chemical composition, this result implies that atrazine was significantly reduced after the washing or template removal process.

Table 1. Chemical compositions of the MIP, analyzed by EDS.

Element	Before template removal (%)		After template removal (%)	
	Mass	Atom	Mass	Atom
C	47.14	53.10	46.12	51.46
N	26.69	25.77	28.99	27.74
O	24.02	20.31	24.77	20.75
Cl	2.15	0.82	0.12	0.05

The number of cavities of a MIP surface was determined by following the method given in Ref. [26] using SEM images with sizes of 254×337 mm^2. Figure 3 compares the cavity size distribution of an atrazine MIP produced by the previous single washing process [23] to that obtained by the present repeated washing. It is shown that the size of cavities in the range of 80 to 230 nm is dominant while those with larger sizes are insignificant. Moreover, the number of cavities with the size in the range of 80 to 230 nm obtained under the repeated washing process is 780, which is higher than that produced under the single washing process. The repeated washing process is therefore better than the single washing process in synthesizing the atrazine MIP.

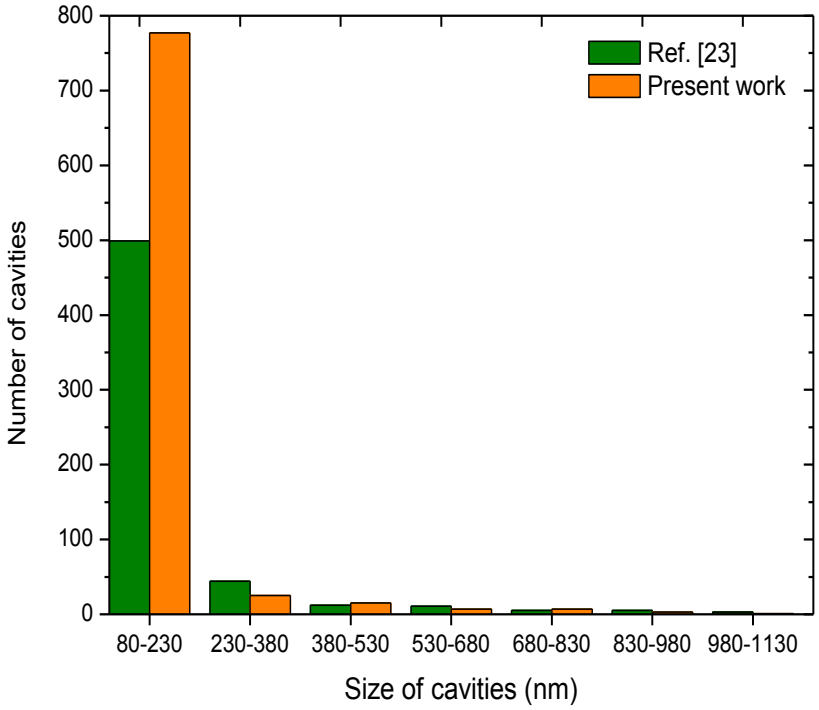


Figure 3. Cavity size distributions of atrazine MIP as reported in Ref. [23] due to the single washing process and in the present work that was produced with the repeated washing process.

The FTIR spectra of the polymer and its atrazine MIP are depicted in Fig. 4. Their infrared vibrational peak characteristics are assigned by labels from A to N and given in Table 2. The FTIR spectrum of the polymer before the template removal has the following peaks. The weak peaks at 3402 cm^{-1} (label A) and 3332 cm^{-1} (B) are attributed to the N–H stretching of primary and secondary amines, respectively. The peaks at 2924 cm^{-1} (C) and 2854 cm^{-1} (D) are assigned to the asymmetrical and symmetrical C–H stretching of methylene group, respectively. The peaks at 1724 cm^{-1} (E) and 1388 cm^{-1} (H) are due to the C=O stretching and O-H bending of carboxylic acid group, respectively. The peak at 1624 cm^{-1} (F) is designated to the N-H bending of primary amine while that at 1457 cm^{-1} (G) is appointed to the C-H bending of amine and carboxylic acid groups. The peaks at 1265 cm^{-1} (I) and 1157 cm^{-1} (J) belong to the C-N stretching of primary and secondary aromatic amines, respectively. The weak peaks in the region 909-660 cm^{-1} (K, L, and M) are originated from the N-H

wagging of amine. All of the peaks therefore establish that the polymer was formed from the MAA monomer with carboxylic acid group, the EDMA cross-linker with carboxylic acid group and the atrazine template with amine group.

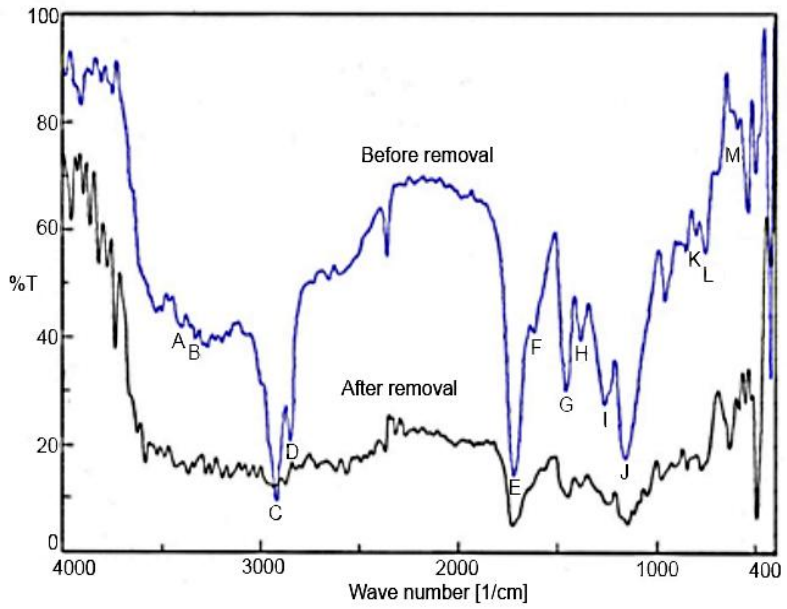


Figure 4. FTIR spectra of the polymer before removing the atrazine template and its atrazine MIP obtained after the template removal.

Table 2. The FTIR vibrational frequencies and their associated functional groups [27,28].

Label	Frequency (cm^{-1})	Vibrations
A	3402	N–H stretching of primary amine
B	3332	N–H stretching of secondary amine
C	2924	Asymmetrical C–H stretching of methylene group
D	2854	Symmetrical C–H stretching of methylene group
E	1724	C=O stretching of carboxylic acid
F	1624	N-H bending of primary amine
G	1457	C-H bending of amine and carboxylic acid groups
H	1388	O-H bending of carboxylic acid
I	1265	C-N stretching of primary aromatic amine
J	1157	C-N stretching of secondary aromatic amine
K	806	N-H wagging of amine. The position of this band depends on the degree of H-bonding.
L	756	
M	699	

Having applied repeated washing to the polymer, the atrazine template was removed from the polymer and the atrazine MIP was then obtained. It is shown in Fig. 4 that all of the peaks observed in the FTIR spectrum of the polymer become weaker drastically after the atrazine template was removed. This finding emphasizes that the concentration of atrazine decreases significantly due to the

application of the present repeated washing process. It implies that the formation of cavities is effective and the number of cavities is therefore notably high as determined by the SEM image analyses in Fig. 3.

Accurate quantities of the atrazine template in the polymer and its atrazine MIP were determined by employing the reversed-phase HPLC. Figure 5 presents HPLC chromatograms of the atrazine template in the polymer and atrazine MIP. It is found that the atrazine template in the polymer has a peak at 5.805 min. When the repeated washing process has been applied to the polymer to obtain the atrazine MIP, its HPLC chromatogram shows no peak meaning that atrazine becomes undetectable in the atrazine MIP. This indicates that the present repeated washing process is very effective to eliminate atrazine in the polymer. Noting that the single washing process left 3% of atrazine in the atrazine MIP [29], the present repeated washing process therefore offers a useful way to obtain better atrazine MIPs.

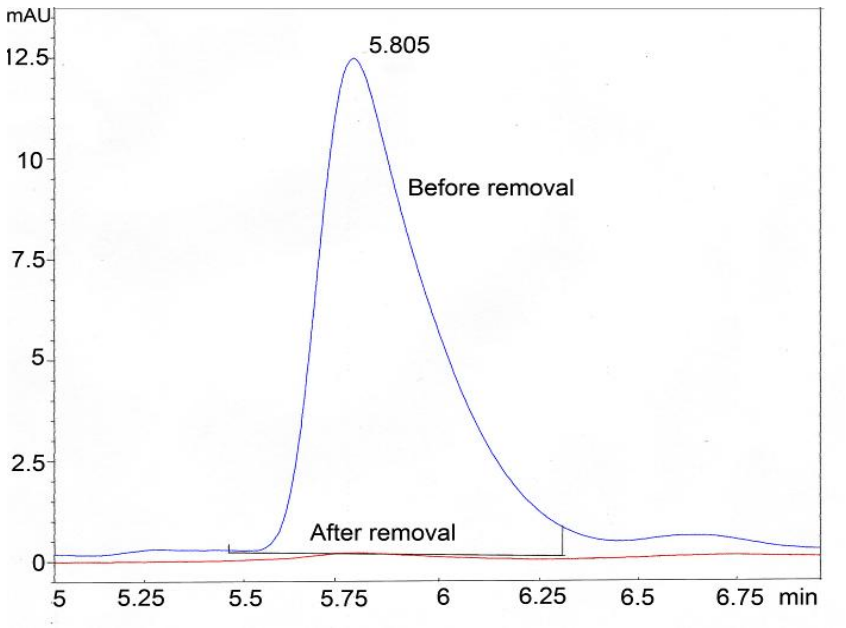


Figure 5. HPLC chromatograms of atrazine in the polymer before removing the atrazine template and in its atrazine MIP obtained after the template removal.

3.2. Concentrations of atrazine bound to the polymers

Figure 6 compares the saturation binding of atrazine MIP to that of NIP. The horizontal axis represents the initial concentration of atrazine and vertical axis is atrazine concentration that is bound in the cavity of the MIP. It is seen that the amount of atrazine molecules bound to the cavities of the MIP (B_{bound}) increases linearly with increasing the initial concentration of atrazine (C). The same trend is also given by the NIP but with a lower slope. This indicates that the ability of atrazine MIP to bind targets is better than that of NIP. In other words, the number of cavities of the atrazine MIP will

therefore determine its sensing properties. The atrazine sensing properties get improved when the number of cavities of the atrazine MIP enhances.

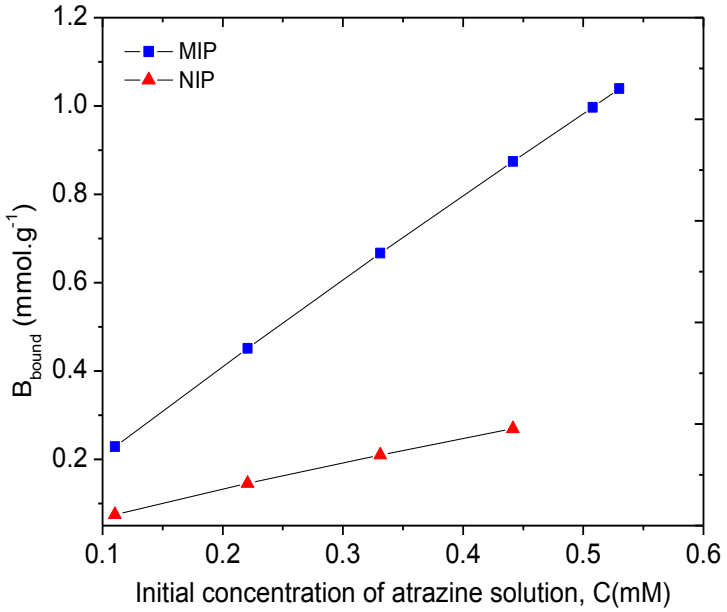


Figure 6. The relation between the number of bound atrazine molecules (targets) and initial concentration of atrazine solution for MIP and NIP.

To determine the apparent maximum number of binding sites and the equilibrium binding constant, the curves in Fig. 6 is re-plotted as a Scatchard plot according to Eq.(1).

$$\frac{B_{bound}}{C} = -\frac{1}{K_D} B_{bound} + \frac{B_{\max}}{K_D} \qquad (1)$$

where B_{bound} is the concentration of atrazine bound to the MIP, C is the guest concentration of atrazine, B_{max} is the apparent maximum number of binding sites, and K_D is the equilibrium dissociation constant.

The Scatchard plots for 1 mL of atrazine solution are shown in Fig. 7. It is found that the values of (K_D; B_{max}) of the MIP and NIP are (7.1 μM; 14.9 mmol/g) and (2.8 μM; 1.99 mmol/g), respectively. The obtained values of K_D and B_{max} indicate that the ability of the MIP to recognize the target is better than that of the NIP. This is because the MIP has cavities that are intentionally prepared to bind targets having the same physico-chemical properties as the template. On the other hand, the cavities do not exist in the NIP so that these particles cannot bind to the target. Moreover, the values of K_D and B_{max} of the MIP prepared by the present repeated washing process are higher than those of obtained the MIP prepared by the previous single washing process [23], which were 4.69 μM and 9.87 mmol/g. This suggests that the increased number of cavities in the MIP makes it better in binding the targets.

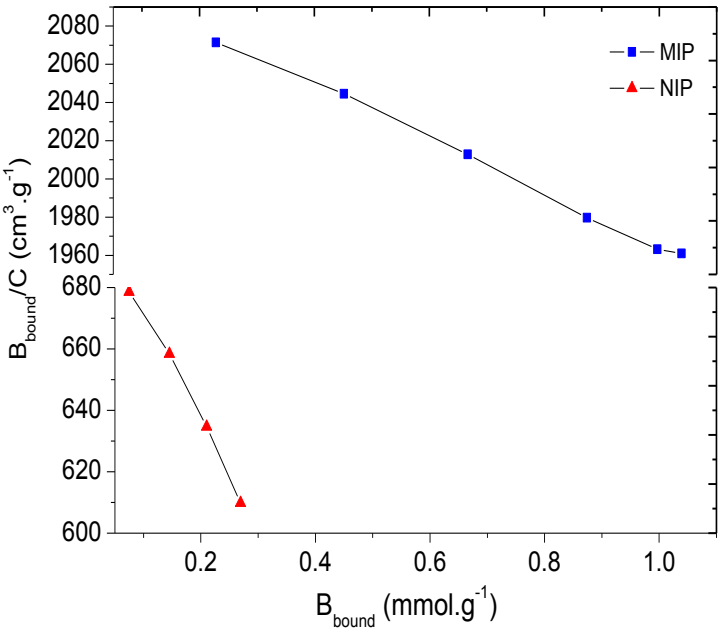


Figure 7. Scatchard plots of atrazine MIP and NIP for 1 mL of atrazine solution.

In several studies, binding isotherm is usually plotted as the concentration of analyte bound to the solid phase (B_{bound}) against the concentration of free analyte in remaining solution (F) [24]. Figure 8 demonstrates the binding isotherm curves of the MIP and NIP. It is seen that concentrations of atrazine molecules bound by the MIP and NIP increases with increasing the free atrazine in the remaining concentration of atrazine solution. However, for the same concentration of atrazine molecules bound by the MIP and NIP, the concentration of free atrazine for the NIP is higher than that for the MIP. This shows that the NIP tends to not bind the atrazine molecules (targets) because no cavity is available.

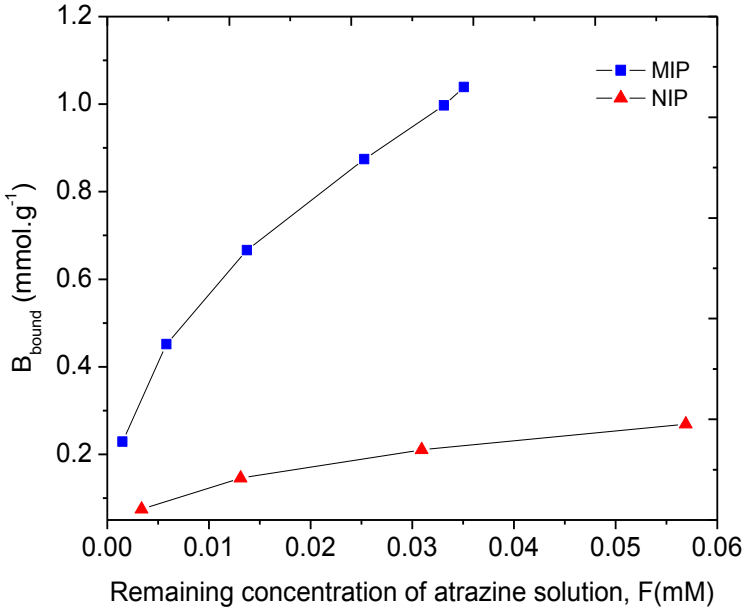


Figure 8. The relation between the number of bound atrazine molecules (targets) and the remaining concentration of atrazine solution for MIP and NIP.

4. CONCLUSION

The cooling-heating method with repeated washing process has been successfully applied to produce an atrazine molecularly imprinted polymer (MIP). It has been found that concentration of atrazine decreases considerably as given by the FTIR spectrum of the MIP. The HPLC chromatogram of the MIP has verified that there is no atrazine left. As the single washing process has left 3% of atrazine, the repeated washing process is therefore better. Moreover, the number of cavities in the MIP obtained by the repeated washing process is 780, which is far higher than that is produced by the single washing process as analyzed from SEM images. From the Scatchard plots, it has been obtained that the equilibrium dissociation constant K_D and the maximum number of binding sites B_{max}, which is written as (K_D; B_{max}), of the MIP obtained by the repeated washing process is (7.1 μM; 14.9 mmol/g). These values are higher than those of the MIP produced by the single washing process.

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