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Preparation and Characterization of Molecularly Imprinted Polymer as SPE Sorbent for Melamine Isolation

Nor Azah Yusof [1,2,]*, Siti Khadijah Ab. Rahman [1], Mohd Zobir Hussein [2] and Nor Azowa Ibrahim [1]

[1] Chemistry Department, Faculty of Science, University Putra Malaysia, Serdang 43400, Malaysia;
E-Mails: sitikhadijah.fyuz@gmail.com (S.K.A.R.); norazowa@upm.edu.my (N.A.I.)

[2] Institute of Advanced Technology, Universiti Putra Malaysia, Serdang 43400, Malaysia;
E-Mail: mzobir@upm.edu.my

* Author to whom correspondence should be addressed; E-Mail: azahy@upm.edu.my;
Tel.: +603-8946-6782; Fax: +603-8943-5380.

Abstract: In this paper, a separation procedure combining molecularly imprinted-solid phase extraction (MI-SPE) was developed for the isolation of melamine. The molecularly imprinted polymer (MIP) was prepared using precipitation polymerization method where melamine as template, 9-vinylcarbazole as functional monomer, eththylene glycol dimethacrylate as a cross-linker and benzoyl peroxide as initiator. An off-line MI-SPE method followed by ultra-performance liquid chromatography detection of melamine was established. MIP showed a better affinity toward melamine compared to non imprinted polymer (NIP) with a maximum binding capacity of 53.01 mg/g MIP. Based on the correlation coefficients, the kinetic study indicated that the adsorption of melamine by MIP fit a pseudo-second order model. From isotherm study, adsorption of melamine by MIP increased when the concentration of melamine increased and followed a Freundlich isotherm model, which indicates the sorption can be described by multilayer sorption. The interference study proved that MIP has better binding capacity towards melamine if compared to NIP due to specific sites of melamine occurred in MIP particles.

Keywords: melamine; molecularly imprinted polymer; 9-vinylcarbazole

1. Introduction

Melamine, chemically known as 2,4,6-triamino-1,3,5-triazine, is produced in large amounts (1.2 million tons in 2007) primarily for use in the synthesis of melamine formaldehyde resins for the fabrication of laminates, plastics coatings, commercial filters, glues or adhesives, as well as for dishware and kitchenware [1]. Melamine contamination has been reported in products such as milk, infant formula, frozen yogurt, pet food, biscuits, candy, and coffee drinks. It was previously considered a non-protein nitrogen (NPN) supplement for cattle feed, however, this use has been discontinued [2]. Melamine alone is of low in toxicity; however, it could form insoluble crystals in combination with cyanuric acid, leading to the formation of kidney stones, which may cause kidney failure and ultimately death [3]. In recent years, melamine has been found to be used illicitly as a food additive for a false increase of protein level and due to its high nitrogen content (66.7%). Recent reports demonstrated that this man-made non-nutritive additive was involved in an outbreak of renal injury in infants who ingested regularly the melamine-contained milk products. The melamine-induced acute nephrotoxicity has resulted in several deaths and more than 10,000 of hospitalizations and invoked public anxiety on the food safety in China [4].

The most frequently used methods for the determination of melamine are gas chromatography-mass chromatography (GC-MS) and liquid chromatography-tandem mass chromatography (LC-MS-MS) [5], which always involves in traditional sample pretreatment procedures, such as solid phase extraction and solid phase micro-extraction (SPME). The main problem associated with traditional sorbents of solid phase extraction (SPE) is the low selectivity or low adsorption capacity [6]. A new type of high efficiency adsorbents, molecularly imprinted polymers (MIPs), due to high sample load capacity, high selectivity, low cost and easy preparation, have been widely applied for preconcentration and high efficient separation of trace analytes in diverse matrices, such as natural, agricultural, food products and environmental samples [7,8]. Due to their favorable molecular recognition capability and stability, potential application of MIPs has been investigated in a broad scientific area, such as ligand binding assays, SPE, sensors and catalysis. Among these applications, one of the most widely applied is the MI-SPE, which has been used for the extraction of a broad range of analytes [9]. MIPs are synthetic polymers with highly specific recognition ability for target molecules. In the most common preparation process, monomers form a complex with a template through covalent or non-colvent interactions and are then joined by using a cross-linking agent. After the removal of the template by chemical reaction or extraction, binding sites are exposed which are complementary to the template in size, shape, and position of the functional groups, and consequently allow its selective uptake [10].

The most widely used technique for preparing MIPs is non-covalent imprinting [10]. In this process, the complex of template and functional monomer is formed *in situ* by non-covalent interactions, such as hydrogen bonding, electrostatic forces, van der Waals forces, or hydrophobic interactions. Synthesis of MIP is a relatively straightforward and inexpensive procedure. The MIP is prepared by mixing the functional monomer, template, cross-linker, and initiator in a proper solvent [11]. There are several advantages of this technique including easy preparation of the template monomer complex, easy removal of the templates from the polymers, fast binding of templates to MIPs, and its potential application to a wide range of target molecules [10,12]. However, to maximize the formation of the labile complex of template and monomer, the polymerization conditions must be carefully chosen to

minimize non-specific binding sites. In this research, MIP for melamine was prepared using precipitation polymerization method by dissolving melamine in ethylene glycol and added with 9-vinylcarbazole, benzoyl peroxide and ethylene glycol dimethacrylate (EGDMA) as monomer, initiator and cross linker respectively. The polymers obtained were characterized using Fourier transform infrared spectroscopy (FTIR), thermogravimetric analysis (TGA) and field emission scanning electron microscope (FESEM). The parameters studied include pH, dosage of MIP, kinetics study and adsorption isotherms. The selectivity of the obtained MIP was analyzed by studying the difference in rebinding capabilities towards melamine and structurally related compounds.

2. Experimental Section

2.1. Materials

Melamine and cyanuric acid were obtained from Sigma Aldrich (St. Louis, MO, USA). 9-vinylcarbazole and ethylene glycol dimethacrylate, were purchased from Fluka (Steinheim, Germany). Ethylene glycol, benzoyl peroxide, acetic acid, citric acid, sodium hydroxide were the product of R&M Chemicals (Essex, UK). Acetonitrile and methanol are all of HPLC grade from Merck. Aqueous solutions were prepared with freshly deionized water obtained with a Sartorius water system (Sartorius, Kuala lumpur, Malaysia). Stock solution of 10 ppm of melamine was prepared by dissolving 10.0 mg of melamine in 10 mL of 5% acetic acid and added with deionized water until 1000 mL using volumetric flask.

2.2. Synthesis of Molecularly Imprinted Polymer (MIP)

MIPs were synthesized using precipitation polymerization method by dissolving 0.5 mmol (0.063 g) of melamine in ethylene glycol (30 mL). After that, 6.0 mmol (1.159 g) of 9-vinylcarbazole as functional monomer, 0.25 mmol (0.061 g) benzoyl peroxide as initiator and 48.0 mmol (9.514 mL) of cross-linker EGDMA were added to the melamine solution. Then the mixture was sonicated until completely dissolved. The resulting mixture was purged with nirogen gas for 5 min and sonicated again for 20 min. Lastly the mixture solution was placed in hot water bath at 60 °C for 20 h. The polymer obtained was collected by centrifugation at 4000 rpm for 5 min and washed with deionized water several times until all unreacted polymer was removed. Polymer obtained was dried overnight in oven at 70 °C. The non-imprinted polymer (NIP) particles were prepared and washed using the same procedure but without the addition of the template melamine.

2.3. Extraction of Melamine from Molecularly Imprinted Polymer (MIP)

100.0 mg of dry MIP was placed in small column and the MIP was washed with water containing 5% acetic acid to remove residual melamine from the MIP. The particles were washed with distilled water, dried in oven and weighted.

2.4. Characterization of the Synthesized MIP

Characterizations of the imprinted polymer were carried out using Fourier transformed infrared (FTIR) spectroscopy Model—100 series by Perkin Elmer (Shelton, CT, USA). About 3 mg of sample powder on diamond holder was irradiated with infrared in the range of 4000–400 cm^{-1}.

TGA analyses were performed using a Metler Toledo TGA/SDTA 851^e model (Metler Toledo, Greifensee, Switzerland) controlled by STARe software version 10.00. TGA was used to characterize the decomposition and thermal stability of studied material. Basically, in this method, a change in thermal stability is examined in terms of percentage of weight loss as a function of temperature. Moisture content and the presence of volatile species can be determined with this technique. About 7–10 mg of Melamine-MIP and NIP samples were placed in alumina crucible and heated from 35 to 1000 °C at a scan rate of 10 °C·min^{-1}. The flow rate of nitrogen used is 20 mL·min^{-1}. The thermogravimetric analyzer measures weight changes in materials with regards to temperature. From this thermal analysis, chemical composition and thermal property of the materials can be predicted.

FESEM analyses were performed using a JEOL-JSM-7600F model (JEOL, Tokyo, Japan). FESEM is used primarily for the study of surface topography of solid materials. It provides a direct image of the topographical nature of the surface from all the emitted secondary electrons. The electron micrograph is virtually a direct image of the real surface structure. The morphology and surface structure of the MIP and NIP were obtained by means of a FESEM, operated at an accelerating voltage of 5 kV. The samples suitable for FESEM were prepared by dispersing the samples in 100% acetone and sonicated for 15 min. The sample solutions were dropped onto metallic sample holder and dried. The micrographs for all samples were recorded at various magnifications.

2.5. Adsorption Studies

Adsorption of melamine from aqueous solutions was investigated in batch experiments. Effects of pH, amount of MIP, adsorption isotherm and kinetics of the fabricated MIP were determined. The adsorption capacity was calculated using the following equation:

$$q = \frac{(C_0 - C_e) \times V}{M}$$ (1)

where q (mg/g) is the amount of total adsorption of melamine, C_0 and C_e are initial and equilibrium concentration of melamine in solution (mg/L), V (L) is the volume of the solution and M (g) is the weight of MIP.

The analyses of melamine (C_e) performed using Waters Acquity UPLC system controlled by Empower software (Milford, MA, USA). It is equipped with UV as detector and Zic-Hilic PEEK HPLC column (150 mm × 2.1 mm, 5 μm, 200 Å) was used as the column imported by SeQuant, MERK (Darmstadt, Germany). The mobile phase is 10.0 mM sodium heptane sulfonate (pH 3.0) and acetonitrile (87:17, *v/v*), and its flow rate was set at 1.2 mL/min with wavelength of 235 nm for melamine and 215 nm for cyanuric acid.

MIP and NIP particles were placed in SPE column and conditioned with 2 mL of deionized water and methanol, successively. After that, the sample was passed through the columns at a flow rate of

0.25 mL/min, and the sample was collected. The final concentration of melamine and cyanuric acid (C_e) were determined using UPLC analysis.

For pH study, pH was adjusted to 3, 4, 5, 6, 8 and 9 with acetic acid or sodium hydroxide (NaOH). The adsorption isotherms were studied using MIP (10.0 mg) in melamine solution (10 mL) at different concentration (1, 20, 60, 100, 200 and 400 ppm). MIP dosage was studied using 100 ppm of melamine (10 mL) for different amount of MIP (5.0, 10.0, 20.0, 40.0 and 100.0 mg). Selectivity of the fabricated melamine-molecularly imprinted polymer-9-vinylcarbazole (Mel-MIP-9VC) and non imprinted polymer-9-vinylcarbazole (NIP-9VC) towards melamine was studied by studying the adsorption of the MIP towards cyanuric acid, analogue of melamine. A solution (10 mL) containing 10.0 ppm of each compound was mixed together and treated with Mel-MIP-9VC and NIP-9VC (10.0 mg) at room temperature.

3. Results and Discussion

3.1. Characterization of MIP

3.1.1. Fourier Transform Infrared Spectroscopy

FTIR spectroscopy is a suitable method to determine the functional groups and types of bonds presence in MIP. Melamine, monomer and NIP were used as reference to confirm the polymerization and interaction with template that has occurred. FTIR spectra for melamine, pure monomer 9-vinylcarbazole, Mel-MIP-9VC and NIP-9VC are shown in Figure 1. Weak N–H amines can be observed for melamine and Mel-MIP-9VC spectra, at 3413 and 3643 cm^{-1} but this N–H stretch peak is absence in monomer and NIP-9VC spectra. This peak occurs when melamine is exists. All of these three spectra show a weak band of C–H stretching at 3046 cm^{-1} for monomer of 9-vinylcarbazole, 2953 cm^{-1} for Mel-MIP-9VC and 2954 cm^{-1} for NIP-9VC spectra. The presence of two significant peaks around 1727 (C=O stretching) and 1145 (C–O stretching) supports the existence of poly(EDGMA) as cross-linker in the obtained Mel-MIP-9VC and NIP-9VC [13]. This result suggested that the polymerization process had occurred successfully. The C=N stretch vibrations shows medium band for melamine compound at 1632 cm^{-1}, while intensity of this vibration band for Mel-MIP-9VC disappear due to overlapping with C=O absorptions. The C=C stretch vibrations shows very strong band for monomer-9VC at 1636 cm^{-1}, while intensity of this vibration band for Mel-MIP-9VC and NIP-9VC are weak due to overlapping with C=O absorptions. The C–N stretching absorption only appeared in monomer-9VC at 1157 cm^{-1} while, C–N vibrations in Mel-MIP-9VC and NIP-9VC compounds often overlap with the C–O absorptions, which also appeared in this region. C–H bending bands and C–H out of plane, in CH$_2$ from 9-vinylcarbazole gave medium vibration bands in the range of 1448–1454 cm^{-1} and 745–752 cm^{-1} respectively. The proposed interaction between monomer (9-vinylcarbazole) and the template (melamine) is shown in Figure 2.

Figure 1. IR spectra for monomer of 9-vinylcarbazole, Mel-MIP-9VC and NIP-9VC.
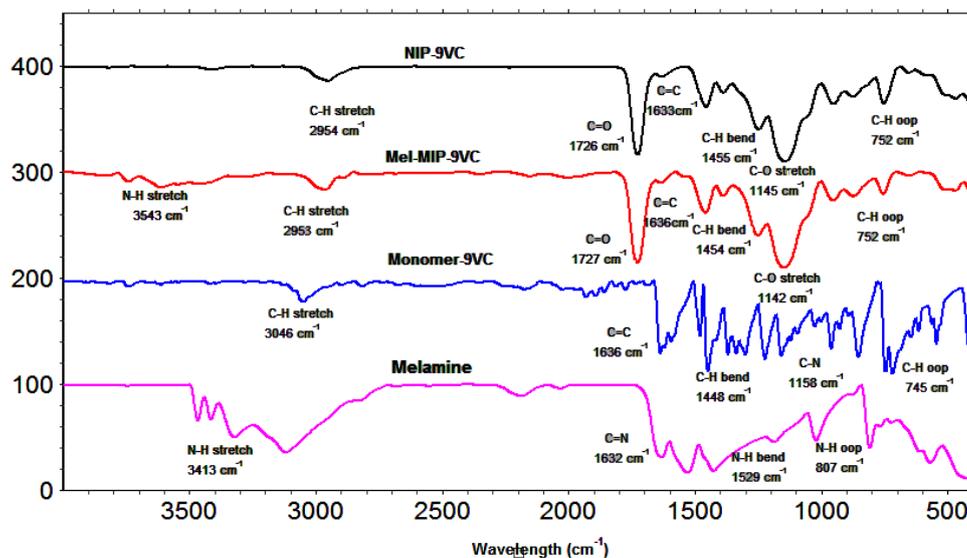
Figure 2. Proposed interaction between monomer (9-vinylcarbazole) and the template (melamine).
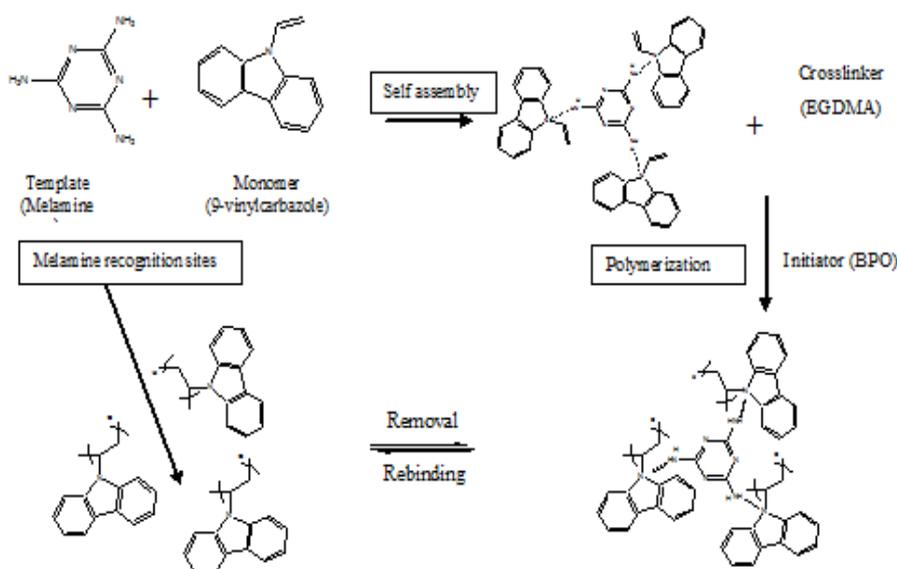
3.1.2. Thermal Stability for MIP

The TGA thermogram was used in order to investigate the difference of decomposition stage of monomer, MIP and NIP particles and to study the stability of the polymer at high temperature condition. Figure 3, shows thermogram data for monomer-9VC, Mel-MIP-9VC and NIP-9VC particle, respectively. The thermogram of Mel-MIP-9VC and NIP-9VC have shown a similar kind of degradation pattern. Monomer-9VC starts to decompose T_i at 110 °C and displays final decomposition temperature T_f at 287 °C. The initial decomposition T_i of Mel-MIP-9VC and NIP-9VC is at 398 and 220 °C and final decomposition T_f at 456 and 451 °C, respectively. It indicates monomer-9VC has lower thermal stability compared to Mel-MIP-9VC and NIP-9VC. This could be due to monomer has lower

molecular weight compared to polymer matrix. This result also showed that Mel-MIP-9VC has higher thermal stability compared to NIP-9VC. This indicates that the MIP is quite stable at high temperature.

Figure 3. TGA curve for monomer-9VC, Mel-MIP-9VC and NIP-9VC.
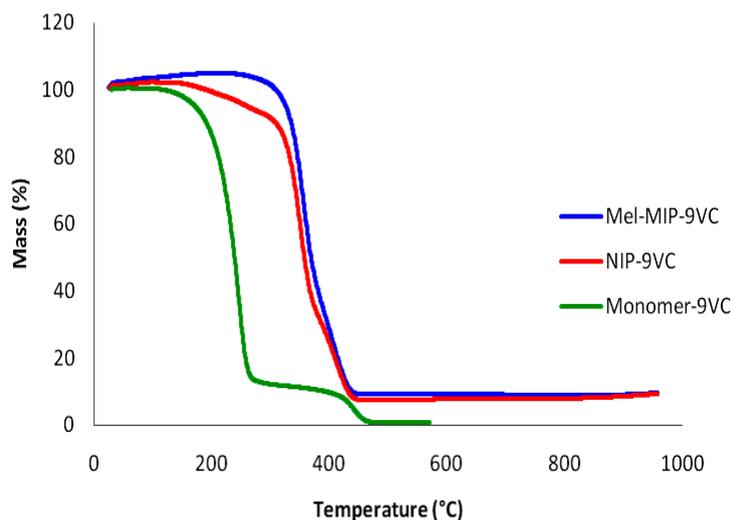
3.1.3. Field Emission Scanning Electron Microscope (FESEM)

The surface morphology FESEM images of the Mel-MIP-9VC and NIP-9VC under the magnifications 25,000 are shown in Figure 4. The Mel-MIP-9VC particles are more globular while NIP-9VC particles are denser, smooth and have low porosity. NIP morphology appears to be smoother due to the fact that no specific binding sites have been created by melamine compound in the NIP polymer particles. The globular and porous MIP have higher adsorption capacity towards target (in this case melamine) compared to a dense and flaky NIP, possibly due to the porosity and higher surface area. The specific binding sites created also contribute to the higher adsorption of MIP towards melamine.

Figure 4. The Morphology of (**a**) Mel-MIP-9VC and (**b**) NIP-9VC using field emission scanning electron microscopy (FESEM).
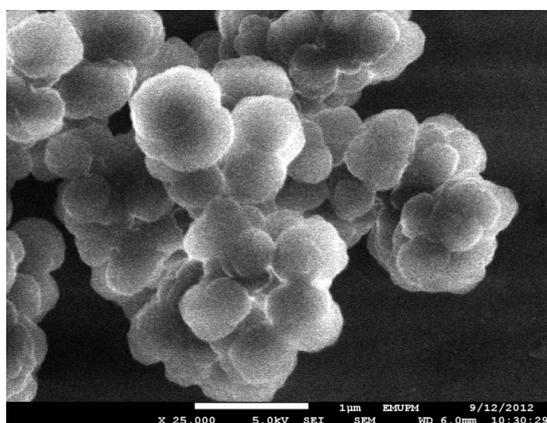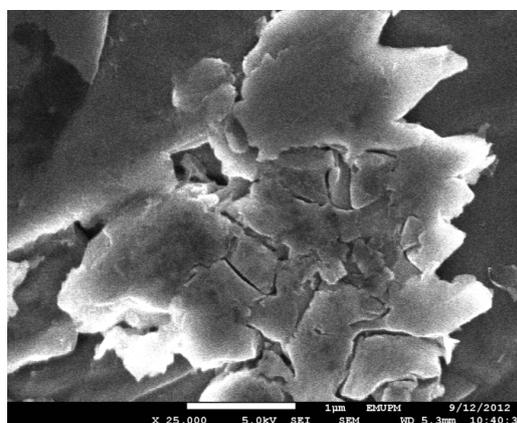
(a)   (b)

3.2. Adsorption Studies of MIP

3.2.1. pH Study

The optimum pH was determined by studying the effect of different pH towards adsorption capacity of melamine. Figure 5 shows the effect of different pH for adsorption of melamine by Mel-MIP-9VC and NIP-9VC. From the graph, it can be seen that the adsorption of melamine increases in the pH range of 3.0–5.0, but decreases beyond this range. This is due to higher formation of hydrogen bond between hydrogen in protonated melamine and the amine group of 9-vinylcarbazole compound. When pH is more than 6.0, melamine mainly exists as a neutral compound, its hydrogen bonding interaction with the polymer is weakened (likely to be due to deprotonation of the amino groups in melamine). From the Figure 5, it is observed that the adsorption capacity of melamine by MIP is higher than NIP. It is due to the fact that MIP exhibits a higher adsorption capacity of melamine compared to NIP. The weak adsorption of melamine template on NIP is due to non-specific interaction with the polymer matrix [10].

Figure 5. Effect of pH on adsorption of melamine by Mel-MIP-9VC and NIP-9VC particles. Experiment was conducted by using 10.0 mg of molecularly imprinted polymer (MIP) with 10.0 ppm of melamine solution.
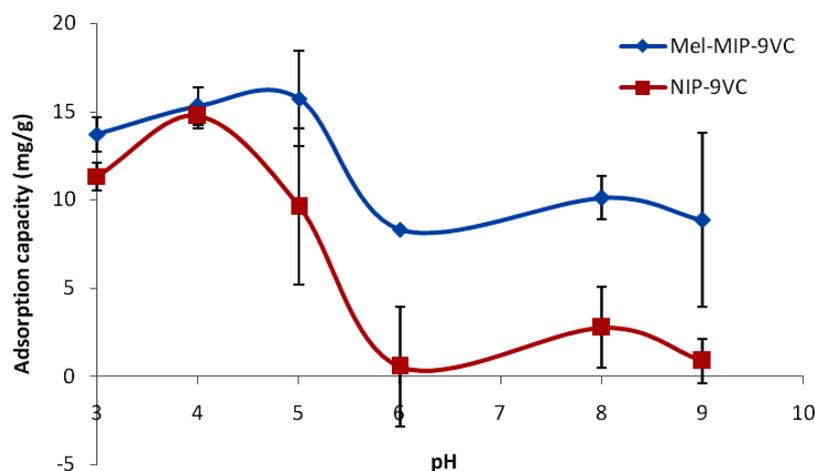
3.2.2. Effect of Dosage of MIP

The dependence of Mel-MIP-9VC sorption on sorbent dosage was studied by varying the amount of absorbents from 5.0 to 100.0 mg, while keeping other parameters (pH, concentration, and contact time) constant. From Figure 6, the percentage removal of melamine by the MIP has increased by increasing the MIP dosage. This is expected due to the fact that the increasing MIP dosage has more surface area available for adsorption of melamine due to increase in imprinting sites in the MIP [14].

3.2.3. Isotherm Study

Equilibrium isotherm plays an important role in the predictive modeling for analysis and design of adsorption systems. The adsorption isotherm is also an invaluable tool for the theoretical evaluation and interpretation of thermodynamic parameters. Langmuir and Freundlich adsorption isotherms are

considered in this study for predictive modeling of adsorption of melamine by MIP. In this adsorption study, the experiment was carried out at various concentration of melamine (1, 20, 60, 100, 200 and 400 ppm). Figure 7 shows that the sorption capacity for melamine by MIP is significantly increased as the equilibrium concentrations increased. The maximum sorption capacity for melamine by Mel-MIP-9VC is 6.57 mg/g.

Figure 6. Removal of melamine at different dosage of Mel-MIP-9VC, the concentration of melamine solution is 10.0 ppm.
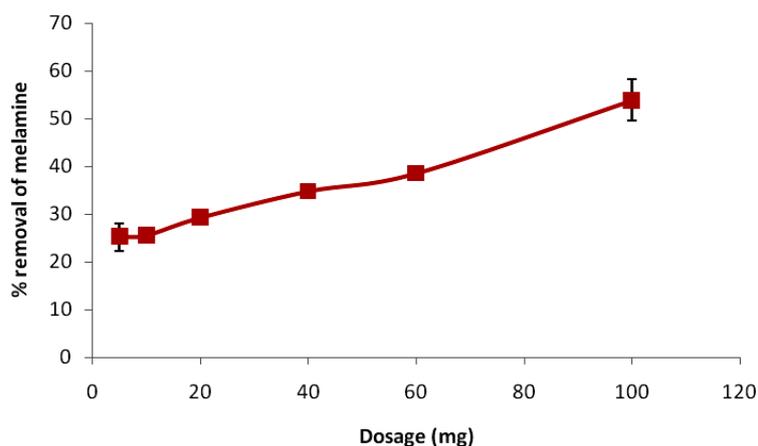
Figure 7. Adsorption capacity of various concentration of melamine by Mel-MIP-9VC. Dosage of MIP is 10.0 mg.
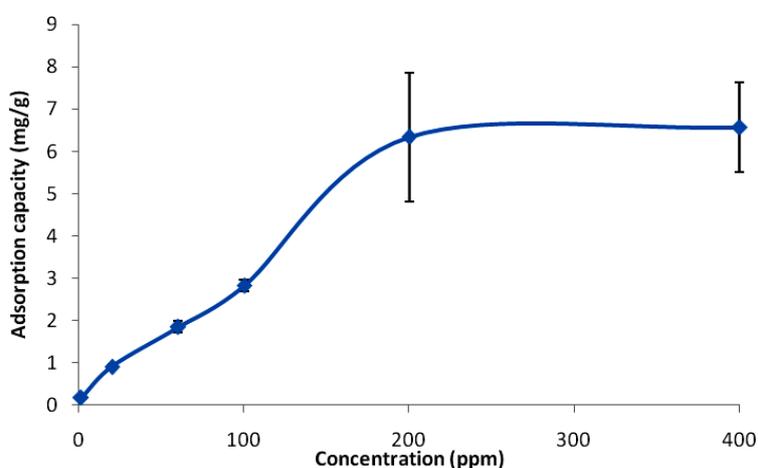
Both Langmuir and Freundlich plots for the sorption for melamine are shown in Figure 8 and the related constants are given respectively in Table 1. The Freundlich model has higher correlation coefficient, R^2 compared to Langmuir model. The value of calculated K_F for freundlich model is comparable to the experimental value q_e. Based on this, it can be concluded that the sorption of melamine by MIP follows the Freundlich adsorption isotherm model. The Freundlich isotherm gives the relationship of equilibrium between liquid and solid phase based on the multilayer adsorption (heterogenous surface). The Freundlich isotherm explains better probably due to multiple interaction and adsorption that occur during the uptake of melamine and there is possibility involving multiple

layers of adsorbates. These two characteristics of adsorption are well described by Freundlich model rather than Langmuir model [15].

Figure 8. (**a**) Langmuir plot for adsorption of melamine by Mel-MIP-9VC; and (**b**) Freundlich plot for the adsorption of melamine by Mel-MIP-9VC.

(a)

(b)
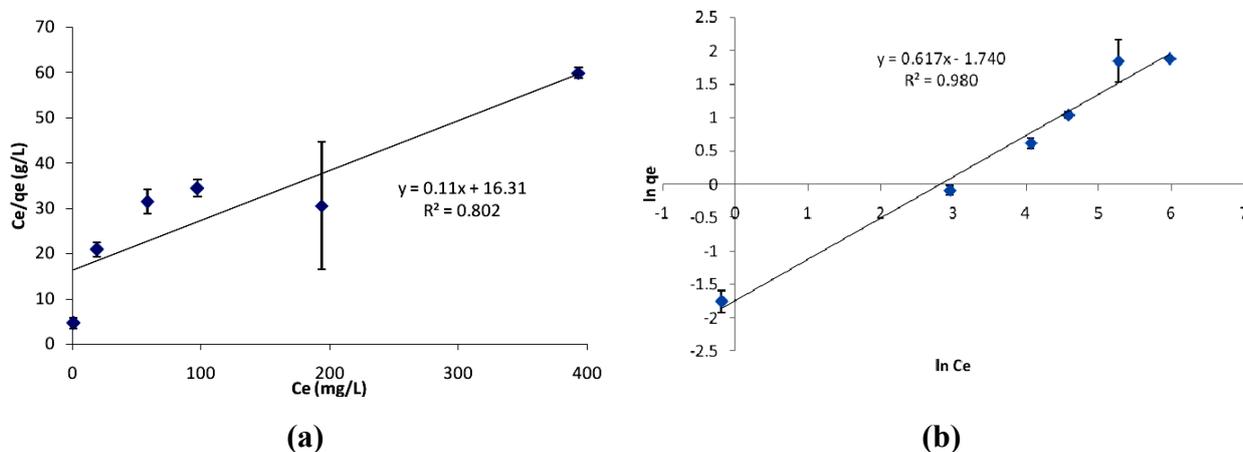
Table 1. Langmuir and Freundlich isotherm for sorption of melamine.

Adsorption model	q_e (exp.) (mg/g)	K_L (mg/g)	K_F (mg/g)	b_L (L/mg)	b_F (L/mg)	R^2
Langmuir constants	6.5725	9.0909	–	0.035	–	0.802
Freundlich constants		–	5.697	–	0.617	0.980

3.2.4. Kinetic Study

Figure 9 shows the adsorption of melamine by Mel-MIP-9VC at different adsorption time from 1 to 20 min while keeping other parameters constant (pH, concentration and dosage). Based on Figure 9, the maximum adsorption of melamine by Mel-MIP-9VC is 53.01 mg/g. The graph shows that the adsorption capacity of melamine increased by increasing of adsorption time up to 5 min and decrease slightly beyond that.

Figure 9. Adsorption capacity of melamine by Mel-MIP-9VC. Experiment was conducted by using 50.0 mg of MIP with 100 ppm of melamine solution.
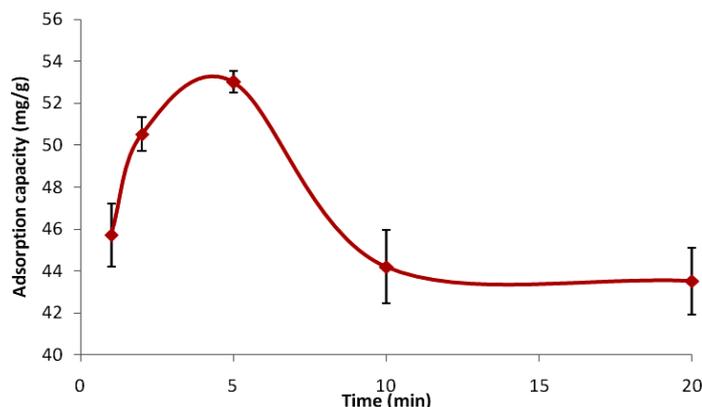
The sorption kinetics data for melamine were also analyzed using pseudo-second order equation based on the adsorption equilibrium capacity as shown in Equation (2):

$$\frac{dq}{dt} = k_2(q_e - q)^2 \tag{2}$$

Integrating the equation above and applying the initial conditions, the equation becomes:

$$\frac{t}{q} = \frac{1}{k_2}q_e^2 + \frac{t}{q_e} \tag{3}$$

where, q_e is sorption capacity of melamine at equilibrium (mg/g), k_2 is second order rate constant (min^{-1}), and q is sorption capacity of melamine at any time (mg/g).

Pseudo-second order model (Figure 10b) provides better correlation of the sorption data compared to the pseudo-first order model (Figure 10a). The pseudo-second order kinetic model gives the best fitting of kinetic data for melamine adsorption with higher correlation coefficient, R^2. The values of rate constant are summarized in Table 2. The q_e value calculated for second order model is closed to experimental value compared to the first order model. It can be concluded that the pseudo-second order is preferred as the sorption kinetic model for sorption of melamine rather than pseudo-first order model. The pseudo second order model indicates that chemisorption is involved in adsorption of melamine. The rate limiting step may be chemical sorption involving valency forces through sharing or exchange of electron between sorbent and sorbate [16].

Figure 10. (**a**) Pseudo first order for sorption of melamine by MIP; and (**b**) Pseudo second order for sorption of melamine by MIP.

(a)   (b)
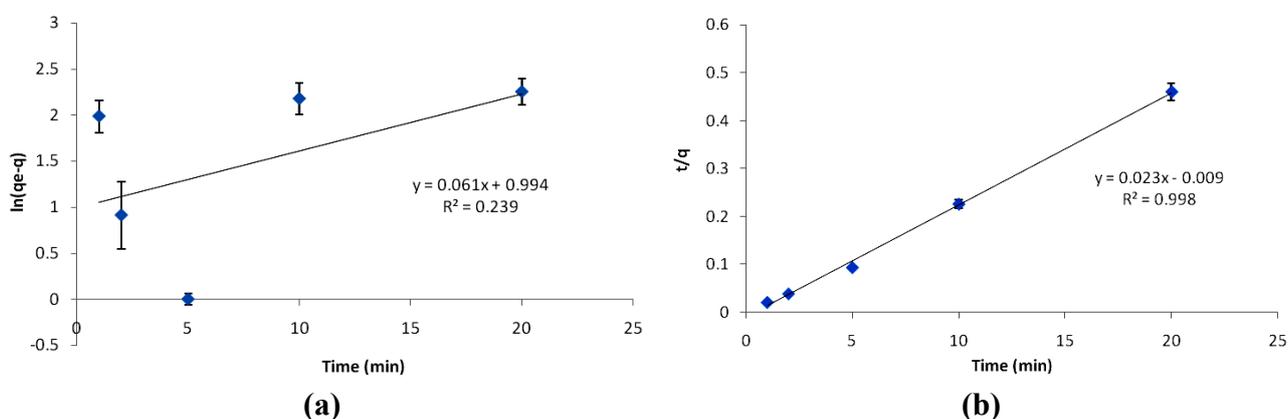
Table 2. Pseudo first order and pseudo second order sorption rate constant for melamine sorption.

Kinetic model	q_e (exp.) (mg/g)	q_e (calc.) (mg/g)	k_1 (min^{-1})	k_2 (g·mg^{-1}·min^{-1})	R^2
First order	53.01	2.702	0.061	−	0.239
Second order		43.478	−	2.10×10^5	0.998

3.2.5. Selectivity Study

Selectivity of MIP with regards to NIP was studied and result is shown in Figure 11. Cyanuric acid was used to evaluate the cross selectivity of the synthesized MIP. Cyanuric acid is an analogue of melamine compound. From Figure 11, the adsorption capacity of Mel-MIP-9VC towards melamine is higher than NIP-9VC, indicating specific binding of MIP. The result also indicated that MIP particles have higher adsorption capacities towards melamine compared to cyanuric acid, while the adsorption capacities of melamine and cyanuric acid by NIP are almost similar to each other. The imprinting sites formed in the MIP have the capability to distinguish target molecules through their size, shape and functional group distribution [17]. However, NIP only adsorb melamine and cyanuric acid on surface due to non-existence of the imprinting sites in polymer network.

Figure 11. Selectivity study of melamine by MEL-MIP-9VC and NIP-9VC in the presence of cyanuric acid. The solution (10 mL) containing cyanuric acid (10.0 ppm) and melamine (10.0 ppm) was treated with 10.0 mg of MIP and NIP particles.
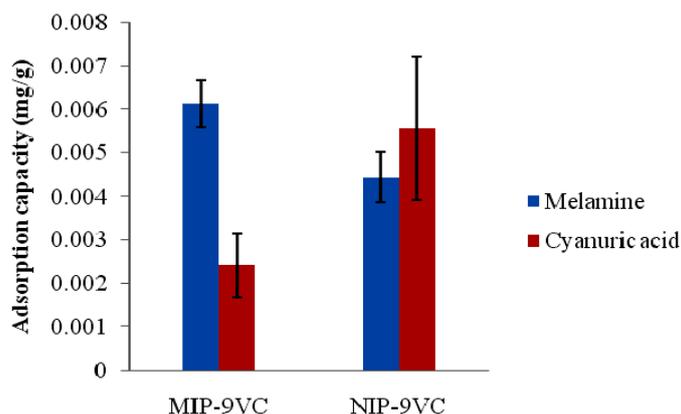
Distribution and selectivity coefficient of cyanuric acid with respect to melamine were calculated using the following equation:

$$K_d = \frac{[C_i - C_f]}{M} \times V \tag{4}$$

where K_d is distribution coefficient, C_i and C_f the initial and final solution concentration, respectively. V (mL) is the volume of the solution and M (g) is the amount of MIP. The selectivity coefficient for the binding of melamine compound in the presence of competitor species can be obtained from equilibrium binding data according to:

$$k = \frac{K_d(\text{Malamine})}{K_d(\text{Cyanuric acid})} \tag{5}$$

where k is the selectivity coefficient, k values of the imprinted polymers with cyanuric acid compound allow an estimate of the effect of imprinting on selectivity. The relative selectivity coefficient k' can be defined as:

$$k' = \frac{k_{\text{imprinted}}}{k_{\text{control}}} \tag{6}$$

Table 3 summarized K_d, k and k' values of melamine in the presence of cyanuric acid. The k' value for Mel-MIP-9VC of Melamine/cyanuric acid is 3.1651 indicating a good selectivity towards melamine [18,19].

Table 3. Selectivity study of the MIP and NIP towards melamine and cyanuric acid.

Compound	K_d MIP (mg/g)	K_d NIP (mg/g)	k_{MIP}	k_{NIP}	k'
Melamine	0.0061	0.0045	2.5337	0.8005	3.1651
Cyanuric acid	0.0024	0.0057			

4. Conclusions

MIP has been successfully used as sorbents for selective removal of melamine. MIP for Mel-MIP-9VC was synthesized using precipitation polymerization method. MIP has been characterized by Fourier transform infrared spectroscopy (FTIR), thermo-gravimetric analysis (TGA) and field emission scanning electron microscopy (FESEM). The binding capacity of melamine by MIP is observed to be higher than NIP. The obtained MIP also showed good selectivity and affinity towards melamine over cyanuric acid, the analogue of melamine.

Acknowledgments

Conflicts of Interest

The authors declare no conflict of interest.

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