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Template Assisted Synthesis of Molecularly Imprinted Polymer for the Extraction of *p*-Coumaric Acid

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In present study, the molecular imprinting polymer (MIP) of *p*-coumaric acid was synthesized by using *p*-coumaric acid as a template, acetonitrile as solvent, 1,4-butanediol dimethacrylate as cross-linker, acrylic acid as monomer and 2,2-azobisisobutironitrile (AIBN) as the initiator. The synthesized polymers were characterized by FTIR and SEM. The results from SEM revealed that the polymer was in spherical shape with size in micro-range. The binding efficiency of polymers was analyzed by adsorption study. The highest rebinding efficiency for MIP was ~ 80% while for non-imprinted polymer (NIP) it was only 24%.

Keywords: *p*-Coumaric acid, Molecularly imprinted polymer, Extraction , Blood serum, Selectivity.

INTRODUCTION

p-Coumaric acid, which is also known as 4-hydrocinnamic acids is a phenolic derivative of cinnamic acid.The phenolic compounds act as antioxidants and their existence in plants plays the role of colouring fruits and flowers and provides protection from environmental stress [1]. *p*-Coumaric acid is one of the most important natural phenolic antioxidants, which is mostly found in edible plants. *p*-Coumaric acid is also absorbed along gastrointestinal tract and reduces the risk of stomach cancer [2]. However, excessive amounts of *p*-coumaric acid will effect on the oxidative damage on DNA and also has an ability to kill tumor cells [1]. *p*-Coumaric acid produces the antioxidant and antimicrobial activities by decreasing low-density lipoprotein peroxidation. Therefore, a method is needed to extract *p*-coumaric selectively from various biological samples.

Molecular imprinting technology (MIT) is used to design the selective sites of polymer matrices with memory of the template molecules [3]. MIT is a green process with potential applications because of its characteristics because of mild conditions, simple operation, low investment, low potential to cause pollution and great selectivity . Molecular imprinting is a very advanced tool in analytical field especially for separating and quantifying various kinds of substances, including drugs and bioactive molecules contained in relatively complex matrices [4]. Molecularly imprinted polymers have been used for the extraction/removal of various compounds from both biological and environmental samples such as dyes [5,6], fungicides [7-9], melamine [10,11], vanillic acid [12], gallic acid [13], cinnamic acid [14] and piperine [15]. Molecular imprinting polymer (MIPs) can be synthesized by different approaches such as non-covalent, covalent and semi-covalent procedures. Since *p*-coumaric acid is an important natural phenolics antioxidant, synthesis of molecular imprinting polymer using *p*-coumaric acid as a template will be a very useful study. In this study, non-covalent approach was used to synthesize molecularly imprinted polymers because of various advantages as compared to covalent. The non-covalent approach is the most widely used method in the preparation of MIPs because of simple preparation procedure. This approach involves multiple non-covalent interactions between the template and the monomers. Interesting examples of non-covalent interactions allowed are hydrogen bonds, ionic interactions, pi-pi, van der Waals forces and hydrophobic interactions [16]. The non-covalent approach seems to be more versatile nowadays [17]. In polymerization process, solvent also known as porogen acts as a medium and

it triggers the formation of a complex between monomer and template, which depends on the strength and the form of interactions [18]. The choice and amount of solvent used in molecular imprinting process will affect the imprinting processes and also physical state such as morphology, toughness, pore size distribution and pore structure [19]. The strength or non-covalent bond is determined by the nature and level of solvents [16]. The important factor that is more affecting the imprinting processes depends on the polarity of solvents. It is important to choose a very low polar solvent in order to prevent the interferences during the formation of template-monomer complex. In this study a non-covalent interaction has been established between *p*-coumaric acid and acrylic acid.

EXPERIMENTAL

p-Coumaric acid, cafeic acid, 1,4-butanedioldimethacrylate and 2,2-azobisisobutyronitrile (AIBN) were purchased from Sigma-Aldrich Co. Ltd. (U.S.A.). Other chemicals such as acrylic acid, acetonitrile (ACN), methanol and acetic acid were obtained other other reputed commerical sources.

IR spectra of polymer particles were recorded with Thermo Scientific Nicolet-S10. Scanning electron microscope (Jeol JSM-6390LA) was used to study the morphology of polymer particles. Reversed-phase high performance liquid chromatography (RP-HPLC) was conducted on Shimadzu LC-20A and used to evaluate the batch binding of polymer particles.

Preparation of molecularly imprinted polymer *via* precipitation polymerization: Molecularly imprinted polymer (MIP) was prepared by non-covalent approach of precipitation polymerization. Initially, 0.5 mmol template (*p*-coumaric acid), 4 mmol functional monomers (acrylic acid), 16.0 mmol of cross-linker 1,4-butanedioldimethacrylate and 30 mg 2,2′-azobisisobutyronitrile (AIBN) were dissolved in a flask containing 80 mL of acetonitrile. The reaction mixture was then sonicated for 15 min followed by the deoxygenation under nitrogen for 15 min. After nitrogen purging, polymerization was carried out at 60 ºC for first three hours and then temperature was increased to 80 ºC for next 3 h. Non-imprinted polymer was also prepared in the same way but without template (*p*-coumaric acid). The synthesized beads were centrifuged and collected after washing with methanol. The removal of template from imprinted polymer beads was carried out by washing with mixture of methanol and acetic acid (7:3, v/v). The removal of template was monitored by using HPLC. This process was repeated until template was not detected by HPLC.

Batch binding assay: In order to evaluate the rebinding capacity of MIP and NIP, batch binding assay was conducted. In this test, a set of 20 conical flasks were prepared, 10 conical flasks were added with 0.1 g of MIP and another 10 flasks were added with 0.1 g of NIP each. After that, 10 mL of 20 ppm standard solution of *p*-coumaric acid was added to every conical flask. Then, all conical flasks were kept on shaker and agitated at 150 rpm. The marked flasks were collected at different time intervals (30, 60, 90, 120, 150, 180, 210, 240, 270 and 300 min). The concentration of free *p*-coumaric acid in the filtrate at every time interval was recorded by using HPLC.

The degree of extraction of *p*-coumaric acid was calculated by using the following equation:

$$\text{Extraction } (\%) = \frac{C_i - C_f}{C_i} \times 100 \quad (1)$$

where C_i and C_f represent the concentration of *p*-coumaric acid in solution from the interval between initial concentration (C_i) and final concentration (C_f).

The reversed-phase high performance liquid chromatography (RP-HPLC) was carried out by using acetonitrile, water, acetic acid (60:39.5:0.5, v/v/v) as a solvent and C18 column as a stationary phase. The flow rate of sample was 1 mL/min and wavelength for analysis was 268 nm.

Effect of dosage of MIP on rebinding efficiency: The binding capacity of MIP was tested by using different amount of MIP (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 g). A series of seven conical flasks were used in which different amounts of MIP was taken and followed by the addition of 20 mL of 20 ppm of template solution. The conical flasks were then kept on shaker and were agitated at 150 rpm for 60 min. The concen-tration of free *p*-coumaric acid in the filtrate was recorded by using RP-HPLC. The binding capacity of *p*-coumaric acid was calculated using eqn 1.

Effect of initial concentration on rebinding capacity: In this study, amount of dosage (0.5 g) and agitation time (150 rpm for 60 min) were kept constant. Firstly, different concentrations (10, 15, 20, 25 and 30 ppm) of *p*-coumaric acid were prepared. Then, 0.5 g of MIP was added in a series of 5 conical flasks separately, followed by the addition of 20 mL of different concentrations (10, 15, 20, 25 and 30 ppm) of *p*-coumaric acid. The concentration of free *p*-coumaric acid in the filtrate was recorded using RP-HPLC. The binding capacity of *p*-coumaric acid were calculated using eqn 1.

Effect of pH on rebinding efficiency: The amount of dosage (0.5 g), template concentration (20 ppm) and agitation time (150 rpm for 60 min) were kept constant. This study was conducted at three different pH′s (3, 7 and 9). A set of three conical flasks were added with 0.5 g of MIP. After that 20 mL of 20 ppm of template solution having three different pH were added into three different flasks. The concentration of free *p*-coumaric acid in the filtrate by different pH standard solutions were recor-ded using RP-HPLC.

Selectivity test: A competitive template (caffeic acid) was used to check the selectivity of MIP towards *p*-coumaric acid. A mixture of solution containing both *p*-coumaric acid and caffeic acid with equal concentration was prepared. Then, 20 mL of resulting solution was added into two different conical flasks containing 0.5 g of MIP and NIP. Both the conical flasks were then agitated on a shaker at 150 rpm for 60 min.

The concentrations of *p*-coumaric acid and caffeic acid after adsorption were recorded by using RP-HPLC. The distribution ratios of *p*-coumaric acid and cafeic acid between the MIP or NIP with acetonitrile were determined using eqn. 2:

$$\text{Distribution ratio } (K^D) = \frac{(C_i - C_f)V}{C_i m} \quad (2)$$

where C_i = initial concentration in the solution; C_f = final concentration in the solution; V = volume of acetonitrile used; m = mass of MIP/NIP used.

The selectivity coefficients for *p*-coumaric relative to binding competitor (caffeic acid) for MIP and NIP were calculated by using eqn. 3:

$$\text{Selectivity coefficient}\left(K^{sel}_{p\text{-Coumaric/caffeic}}\right)=\frac{K^{D}_{p\text{-Coumaric acid}}}{K^{D}_{\text{Caffeic acid}}} \quad (3)$$

where, $K^{D}_{p\text{-coumaric acid}}$ = distribution ratio for *p*-coumaric acid; K^{D}_{Caffeic} = distribution ratio for caffeic acid.

The relative selectivity coefficient (K′) was determined using eqn. 4:

$$K' = \frac{k(MIP)}{k(NIP)} \quad (4)$$

Preparation of spiked blood serum: Human blood (10 mL) was collected from a healthy person by standard phlebotomy technique. Informed consent was obtained from the donor prior to blood draw. In order to extract the blood serum, the blood sample collected was left to clot for 20 min. The clotted blood was then centrifuged at 2500 rpm for 15 min. After that blood serum was collected and diluted with pure water in the ratio of 10:1 and then 20 ppm of *p*-coumaric was spiked with diluted serum solution. Now, 10 mL of spiked serum was added into a flask containing 0.5 g of MIP and then followed with the same procedure of batch binding assay.

RESULTS AND DISCUSSION

FT-IR analysis: The FTIR spectra as shown in Fig. 1 provides information about the functional groups present in both MIP and NIP. Broad peaks at 3462.43 and 3461.20 cm^{-1} in both MIP and NIP spectra indicates the presence of intermolecular bonded OH group, respectively. A sharp, strong and significant peaks were observed at 1726.28 cm^{-1} in both MIP and NIP, these peaks were assigned to the C=O bond stretching. The broad band observed in the range of 2954.87-2954.04 cm^{-1} showed the vibration mode of C-H stretching of aliphatic compound together with asymmetric and symmetric CH_2 stretching in both MIP and NIP. The small peaks at 1385.70, 1385.83 and 1384.96 cm^{-1} may be observed because of the C-H bending of alkane group. The peaks in the region 1300-1000 cm^{-1} correspond to the C-O-C stretch of ester group present in 1,4-butanediol methacrylate. The =C-H bending of alkene group can be identified in the range of 973.20-956.73 cm^{-1} for both MIP and NIP . The activity of C=C stretching of aromatic rings give peaks around 1600-1400 cm^{-1}, a peaks at 1470.09 cm^{-1} was observed in MIP, which indicated incorporation of *p*-coumaric acid in the polymer matrix. The presence of peaks around 1263-1262 cm^{-1} were mostly contributed by the ester group of functional monomer (acrylic acid) and cross-linker (1,4-butanediol dimethacrylate).

SEM analysis: The surface morphology of MIP was observed by using scanning electron microscope under ×5,000 magnification. Every polymer vary in their shapes and sizes depends on the type of polymerization, functional monomers, porogenic solvents, and cross-linker used in polymer synthesis. From the image as shown in Fig. 2, it is obvious that the method clearly produces reasonably spherical beads with uniform shape and size.The spherical beads are usually produced by heterogeneous polymerization techniques [20] such as emulsion polymerization [11], precipitation polymerization [8]. The choice of porogenic solvents for polymerization will also influence the surface morphology of a polymer [21]. By using high volume of cosolvent content spherical particles with a rather smooth surface can be obtained. Previous studies showed that molecularly imprinted polymers (MIPs) prepared by acetonitrile exhibited a regular spherical shape [22].

Based on previous findings, the crosslinkers have three main important roles in molecular imprinting such as it provides control on the morphologies of polymer matrices, it helps the imprinted binding site to be always stable and also takes part in mechanical stability of the polymer matrix [23]. High

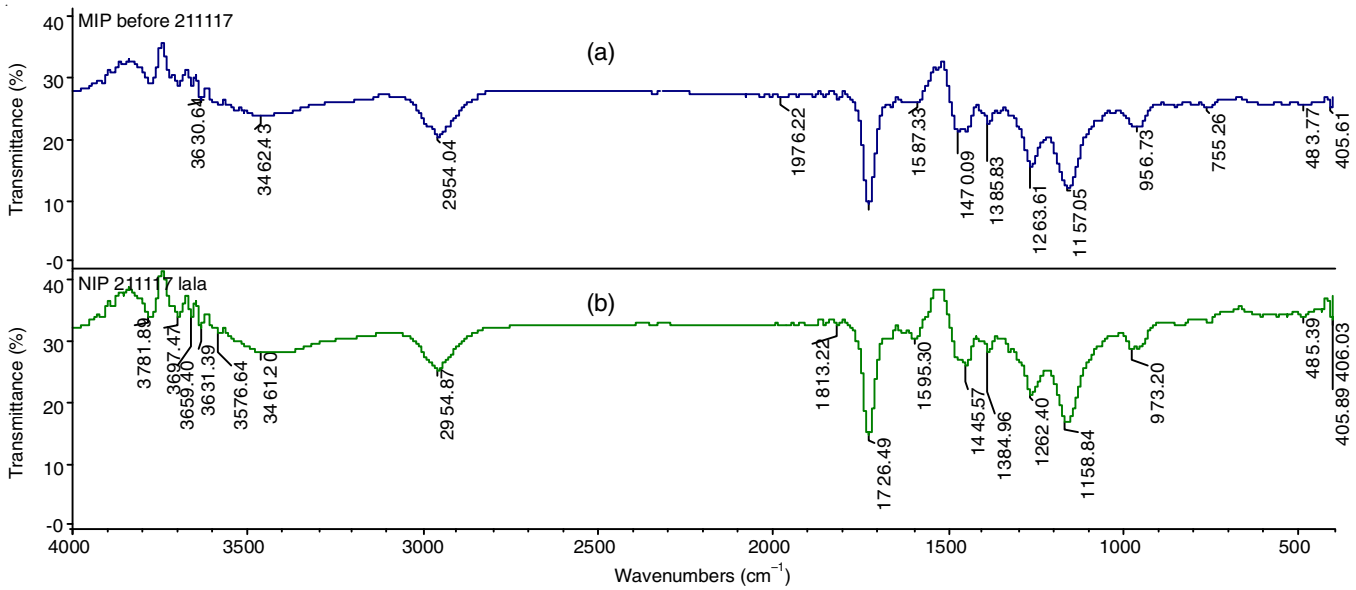

Fig. 1. FTIR spectra of (a) MIP and (b) NIP

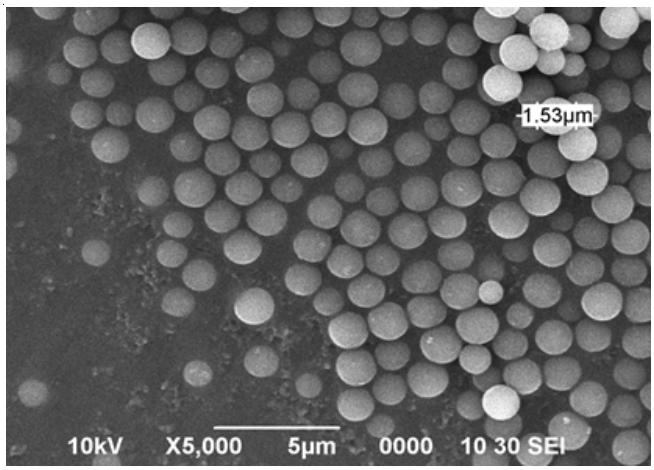

Fig. 2. SEM micrograph of MIP under x5,000 magnification

amounts of cross-linkers are preferred to access macroporous materials so that materials with great mechanical stability can be generated and to keep the stability of the recognition sites [16].

Rebinding assay of MIP: The highest rebinding efficiency for both MIP and NIP was achieved at 60 min of time interval (Fig. 3). It is important for MIP to have higher rebinding efficiency because the presence of template in MIP during synthesis process would lead to a greater capacity of high-affinity binding sites. The removal of template in MIP from the polymer matrix leaves cavities of complementary shape, size and chemical functionality to the template [24]. NIP does not exhibit a specific binding site of template leads to poor selectivity towards template molecules can also explain the factor that gives low efficiency in NIP. The other reason may be the random distribution of functional monomers in the NIP also gave a lower binding affinity than MIP. The highest rebinding time interval (60 min) was selected for other adsorption studies.

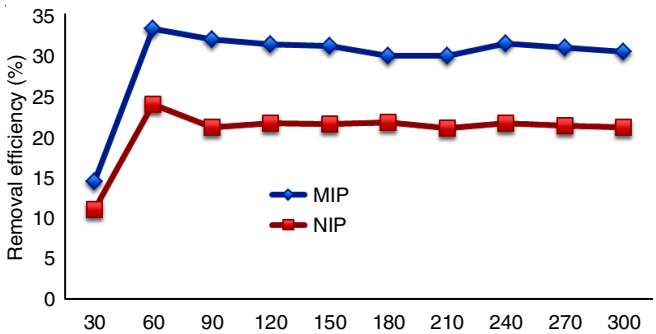

Fig. 3. Removal efficiency of MIP and NIP against time

Effect of polymer dosage on rebinding efficiency: One of the factors that affects the rebinding capacity of MIP is the amount of polymer dosage used in the batch binding process. Fig. 4 shows the rebinding capacity of MIP against the amount of polymer dosage. The rebinding capacity increased with the increase in the dosage of polymer upto 0.5 g and then after that it decreases. This indicated that at higher amount of polymer there exists an interparticle interaction, which results in the agglomeration of particles. This agglomeration could hide the

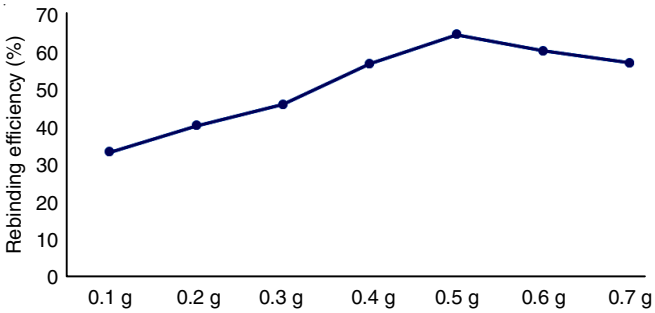

Fig. 4. Rebinding efficiency at different dosage of MIP

available binding sites in the polymer particles and results in the less uptake of template molecule.

Effect of concentration of template on rebinding efficiency of MIP: Fig. 5 shows the rebinding capacity of MIP at different concentrations of template analayte (*p*-coumaric acid). The rebinding capacity of MIP increases upto a certain optimum level (20 ppm) after that further increase in concentration of template have decreased the rebinding capacity of MIP. This observation can be explained in the way that there is an optimum number of binding sites available in the polymer particles. This optimum rebinding capacity was achieved at a concentration level of 20 ppm of template. This indicated that all the binding sites have already saturated with the template molecule and further increase in concentration have reduced the possibility of interacting with the binding sites. This reduced the rebinding capacity of MIP at very higher concentration of template molecule.

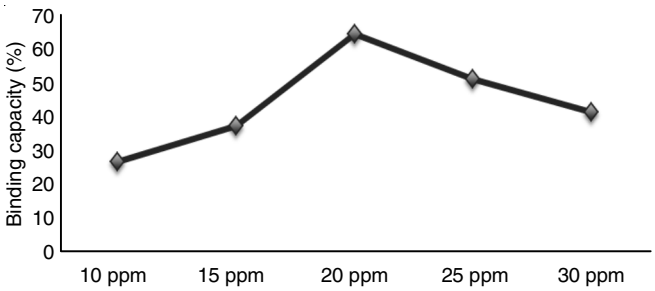

Fig. 5. Binding capacity of MIP against concentration of template (*p*-coumaric acid)

Effect of pH on rebinding efficiency: The rebinding efficiency of imprinted polymer was evaluated at different pH conditions. This test was conducted to study behaviour of imprinted polymer weather it is a pH-sensitive polymer or not, which exhibits a conformational change when treated with different pH values which leads to swelling or shrinking behaviour of the polymer [25].

Table-1 shows that the rebinding efficiency of MIP with varying pH of template solution, which clearly indicates that the highest rebinding capacity was achieved at pH 7 a neutral condition as compared to acidic or basic conditions. The results concluded that neutral (pH 7) condition is the optimum condition for the interaction of template and the imprinting site. The specific binding sites on template may have altered due to the alteration of pH. The presence of H^+ ions or OH^- ions in the acidic or basic solutions may have changed functionality

TABLE-1
REBINDING EFFICIENCY OF MIP AT DIFFERENT pH

pH	Removal efficiency (%)
3	40.0
7	65.9
9	40.9

of template molecule due to the hydrogen bonding or with some other interactions. This change in functionality could have decreased the rebinding efficiency with the MIP. It has been reported that the binding energy of the selectivity sites on MIP decreased in the non-optimum pH range [25].

Selectivity of MIP: Selectivity test could be a possible way to evaluate the properties of MIP of *p*-coumaric acid as a sensing material. In this regard, a competitive template (caffeic acid) which exhibited most of the chemical and physical properties of *p*-coumaric acid was chosen.

Table-2 shows the distribution ratio, selectivity coefficients and relative selectivity coefficient of MIP and NIP for both *p*-coumaric acid and caffeic acid. It shows that the distribution ratio and selectivity coefficients of *p*-coumaric acid both in MIP and NIP were higher than the competitive template (caffeic acid). Moreover, the MIP results higher distribution ratio for both *p*-coumaric acid and caffeic acid than NIP. This situation is due to the lack of binding sites in non-imprinted polymer.

TABLE-2
DISTRIBUTION RATIO, SELECTIVITY COEFFICIENTS AND RELATIVE SELECTIVITY COEFFICIENT OF MIP AND NIP

Template	K_D (MIP)	K_D (NIP)	K^{sel}	K'
p-Coumaric acid	26.04	8.83	4.27	–
Caffeic acid	6.10	3.75	2.25	1.9

The higher distribution ratio of MIP towards *p*-coumaric acid was due to the specific recognition sites of template molecule (*p*-coumaric acid) available on MIP for binding interactions. On the other hand, a distribution ratio of MIP towards caffeic acid was low because of non-specific binding interactions [26].

Extraction of *p*-coumaric from spiked blood serum: It is well known fact that *p*-coumaric acid has good antioxidant properties and mostly found in human daily diet. So there is a possibility that *p*-coumaric acid can be found abundantly in human blood. Therefore, a test has been performed for the rebinding efficiency of MIP in the blood serum spiked with *p*-coumaric acid. A good amount of *p*-coumaric acid has been extracted from the blood serum. The rebinding efficiency of MIP was about 80% as compared to NIP which was only 25.6%. This is because MIP has specific binding sites towards *p*-coumaric acid as compared to NIP.

Conclusion

A molecular imprinted polymer of *p*-coumaric acid was synthesized by precipitation polymerization with non-covalent approach.The surface morphology of MIP revealed that the polymer particles were dispersed with same size and shape. The selectivity test proved that MIP showed more sensing properties towards *p*-coumaric acid in the presence of competitive template (caffeic acid).The synthesized MIP was successfully used to extract *p*-coumaric acid from blood serum. The maximum removal efficiency of MIP was 80% of *p*-coumaric acid. This can be concluded that MIP could be used in future for the solid phase extraction or in column packing material.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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