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SYNTHESIS OF TETRACYCLINE IMPRINTED POLYMERS WITH METHACRYLIC ACID AS FUNCTIONAL MONOMER IN METHANOL-CHLOROFORM MIXTURE USING BULK AND PRECIPITATION POLYMERIZATION METHOD

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Abstract

The content of tetracycline residues in poultry meat products can cause antibiotic resistance in humans who consume these products, so it is necessary to develop sensitive analytical techniques to determine the levels of tetracycline residues to assess the safety of these products for consumption. The molecular imprinting technique is a method to produce sorbents with molecular recognition capability of target compounds that can be used to increase the selectivity of solid phase extraction to extract tetracycline residues for further analysis. This study aimed to obtain a sorbent synthesized by molecular imprinting technique to analyze tetracycline in poultry meat products. The stages of the research began with the study of the interaction of functional monomers with template molecules, determining the association constants of functional monomers with template molecules, synthesis of imprinted polymer molecules using bulk and precipitation methods, evaluating the ability and adsorption capacity of the synthesized polymers, assess the selectivity of polymers for analog structures and physical characteristics with FTIR. The research showed that methacrylic acid was the best functional monomer with a binding energy value of -27.3776 kcal/mol. The higher adsorption capacity was achieved by Molecularly Imprinted Polymer (MIP) that was synthesized by precipitation method (MIP2) than the other MIP synthesized by bulk polymerization (MIP1) with a value of 0.8748 mg/g and 0.4077 mg/g, respectively. The analogous compounds' imprinting factor values for each MIP were 1.197 and 1.1272. The polymer synthesized by molecular imprinting technique is selective for extracting and analyzing tetracycline from poultry meat matrix.

Keywords: adsorption capacity, molecular imprinting, tetracycline

Introduction

Tetracycline is an antibiotic often used as a growth promoter in the growth of poultry Antibiotic residues in meat can cause resistance effects in humans when consuming these products.[1] Analysis of tetracycline content in poultry meat products requires specific and selective sample preparation because of its low levels and matrix effect.[2] Solid phase extraction has many advantages; it can be an option for extracting and analyzing tetracyclines in poultry meat products.[3] However, solid-phase extraction

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shows a weakness in low selectivity.[4] Molecularly imprinted polymer technology is a solution in sorbent synthesis for more effective solid-phase extraction.[5]

A molecular printed polymer is a synthetic polymer capable of molecularly recognizing target compounds.[6] This ability is obtained from forming functional pockets by removing template molecules from polymers synthesized through the polymerization of functional monomers and crosslinkers in the presence of template molecules.[7] Playful pockets that occur can selectively bind to specific molecules (target compounds) from the mixture and reduce the influence of the matrix in the analysis.[8] Analysis of tetracyclines in poultry meat products using solid phase extraction-molecular imprinted polymers provides accurate information regarding the content of tetracyclines in these products.

The preparation of molecular printed polymers for extraction begins with determining functional monomers with solid interactions with tetracycline molecules as templates.[8] Another component that plays a role in the selectivity of the resulting polymer is the porogen solvent and the polymerization method used.[9] For this reason, this research evaluates the interactions between functional monomers and template molecules, the selection of porogen solvents that support strong interactions between template molecules and functional monomers, and the selection of polymerization methods that produce high selectivity and specificity.

This research is expected to obtain sorbents with molecular recognition capabilities, which are synthesized by molecular imprinting techniques for extracting tetracyclines as residues in poultry meat products.

Methods

Apparatus

The tools used in this study were a 2.0 GHz Intel® Core TM i3-5005U processor, 8 GB DDR3 RAM memory, and a Windows 10 operating system was used for the computational method. Hyperchem 8.0.7 software was used to optimize the geometry of molecules and for binding site prediction and calculation of binding energy. UV-Visible spectrophotometry (Genesis 10), quartz cuvette (Helma), FTIR (Fourier Transform Infrared) (Shimadzu, IR Prestige-21®), oven (Memmert®), digital balance (Amstech®), soxhlet, beaker, 100 mesh, dropping pipette, cuvette, vial, spatula, funnel, volumetric flask, mortar, stamper, and micropipette. The morphological evaluation analysis was carried out by JSM-6610LV JEOL Ltd.

Materials

The materials used in this study were tetracycline, methacrylic acid (Sigma Aldrich), ethanol (Merck, Germany), acetonitrile (Merck, Germany) 2-2-Azobis-isobutiro-nitrile (AIBN) (Aldrich®), ethylene glycol dimethacrylate (GDMA) (Aldrich®), tetracycline (Sigma Aldrich®), chloroform (Merck®), methanol (Fisher®). Other materials used other than what is said to be pro analysis.

Procedure

Study of the Interaction of Functional Monomer-Template Molecules by Computational Methods

The 3D structures of the template and FMs were created with the freeware application Hyperchem 8.0.7. The molecular structure was optimized using the semi-empirical restricted Hartree-Fock (RHF) approach, which is based on molecular orbital theory. The template-FMs complexes were optimized at the RHF level using the PM3

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approach and a self-consistent field (SCF). All FM selection calculations referred to isolated molecules in the gas phase. The gradient conjugate process (Polak-Ribier) was utilized to optimize the molecule's geometry, with a convergence set at 0.01 Kcal.[10]

Determination of Association Constants

The interaction of functional monomer-template molecules before the polymerization process was investigated using UV titration. As much as 1 ml of 0.001 mmol/liter tetracycline solution in several solvents, namely: ethanol, methanol, mixed solvents methanol-chloroform, and methanol-acetonitrile was put into a 2.5 ml cuvette, then added methacrylic acid solution with a concentration of 0.005 mmol/liter with various volumes, starting from 0 µl to 1000 µl, record the absorbance obtained every time monomer is added. Next, a curve is made between the absorbance delta and monomer concentration to obtain the constant value of the association with the Benesi-Hildebrand equation.

$$\frac{1}{(\text{A-A0})} = \frac{1}{\{\text{K(Amax-A0)[monomer]}\}} + \frac{1}{[\text{Amax-Ao}]}$$

A0 is the absorbance of the template molecule in the absence of monomer; A is the absorbance in the presence of monomer; Amax is the absorbance with the addition of maximum [monomer]; K is the association constant (M-1). The association constant can be determined from the slope/intercept of the straight line from the plot between 1/(A-A0) against 1/[monomer][5.]

Synthesis of Molecularly Imprinted Polymers (MIPs)

Bulk Polymerization Method

444.44 mg of template molecule (tetracycline) with functional monomer (MAA) 340 µl was dissolved in solvent (1:1 methanol-chloroform mixture) in a closed vial and sonicated for 5 minutes. 4 ml of EGDMA as crosslinker and AIBN as initiator were added to the vial. The mixture was sonicated for 40 minutes to remove oxygen. Furthermore, the vial containing this mixture was put into the oven for 24 hours at a temperature of 60 °C. The polymer formed was crushed, sieved with mess 60, and washed using methanol. After washing, the polymer is oven dried. Non-molecular imprinted polymer NIP was also synthesized similarly without adding template molecules/tetracyclines.

Precipitation Polymerization Method

Tetracycline 2.22 g as a template molecule is mixed with 1.7 ml methacrylic acid in a glass bottle and sonicated for 5 minutes. Then 18.9 ml of EGDMA and 5 ml of AIBN were added in 350 ml of mixed methanol-chloroform (1:1). The mixture was sonicated for 30 minutes to remove oxygen, and the glass bottles were tightly closed. Polymerization was carried out on a hot plate magnetic stirrer at 65 °C for 8 hours, and then the polymer formed was washed with a mixture of chloroform-methanol. The composition of the synthesized polymers is shown in Table 1.

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Table 1. Composition of Synthesized MIPs and NIPs

Polymer	The ratio of template molecules: functional monomer: crosslinker	Polymerization Method
MIP1	1:4:20	Bulk
NIP1	0:4:20	Bulk
MIP2	1:4:20	precipitation
NIP2	0:4:20	precipitation

Extraction of Template Molecules

The extraction process begins with preparing the MIP sorbent in filter paper and putting it into the Soxhlet. Extraction was carried out for 24 hours using a solvent mixture of methanol-acetic acid (9:1). After soxhletation; the polymer was washed with chloroform. The extraction process is complete when the resulting washing solution in the MIP sorbent contains no more template molecules when viewed using UV-Vis spectrophotometry.

Evaluation of Adsorption Capacity

20 mg of MIPs and NIPs sorbents were added to 5 ml of tetracycline solution with concentrations of 4 ppm, 6 ppm, 8 ppm, 10 ppm, and 12 ppm. The mixture was then shaken for 10 minutes at 120 rpm, allowed to stand for 18 hours at room temperature, and filtered. Tetracycline levels in the filtrate were measured using a UV-Vis spectrophotometer. The amount of adsorbed tetracycline was calculated from the difference between the filtrate's initial and free tetracycline concentrations. The same test was also performed on the NIP. The final results were plotted on the Freundlich isotherm curve to see the adsorption capacity of the sorbent.

Evaluation of MIPs Selectivity

20 mg of MIP sorbent was mixed into 5 ml of tetracycline and oxytetracycline with a concentration of 8 ppm, shaken at 120 ppm for 10 minutes, allowed to stand for 18 hours at room temperature, and filtered. The filtrate was measured with a UV-Vis spectrophotometer. The sorbent selectivity parameter can be seen from the value of the imprinting factor.

IF = Kd (MIP)/Kd(NIP)

IF: imprinting factor
Kd : Coeffisien Distribution
MIP: Molecularly imprinted polymers
NIP: Non-molecularly imprinted polymers [9]

Physical Characterization with Fourier Transform Infra-Red and Scanning Electron Microscope

In this final stage, the MIPs sorbent is crushed with KBr and then templated into a pellet-like state. MIPs pellets that have been formed using the FTIR instrument were observed. Transmission measurement starts from the wave number 4000-400cm^{-1}. Determination of functional groups was performed on MIPs before and after extraction by the soxhletation method of MIPs sorbent and NIPs sorbent. The surface morphologies of the polymers were observed using SEM by placing MIP and NIP on silicon and then putting them in the SEM instrument.

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Result

Table 2. Binding Energy Various Monomers Functional with Tetracycline

Complex TET-Functional Monomers	ΔE (kcal/mol)	Number of Hydrogen Bonds
Methacrylic acid (MAA)	-27,3776493	6
4-vinyl benzoic acid (4-VBA)	-25,095754	6
4-vinyl pyridine (4-VP)	-22,0871442	6
Acrylamide (AM)	-21,444608	4
2-Hydroxyethylmethacrylate (HEMA)	-21,2811154	6
2-Fluoromethyl acrylic acid (TFMAA)	-21,095754	5
Methacrylamide (MMA)	-17,2635128	6
Acrylic Acid (AA)	-14,3676338	5

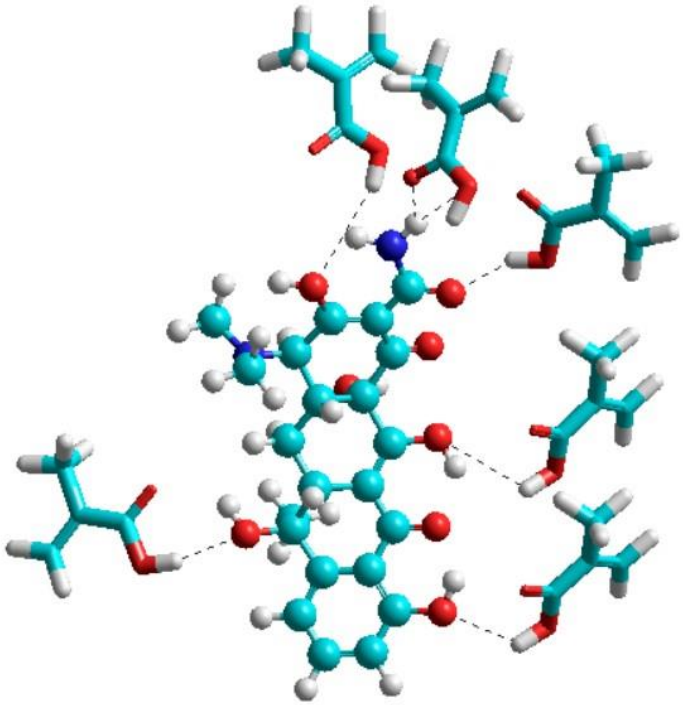

Figure 1. The interaction between tetracycline molecules and methacrylic acid

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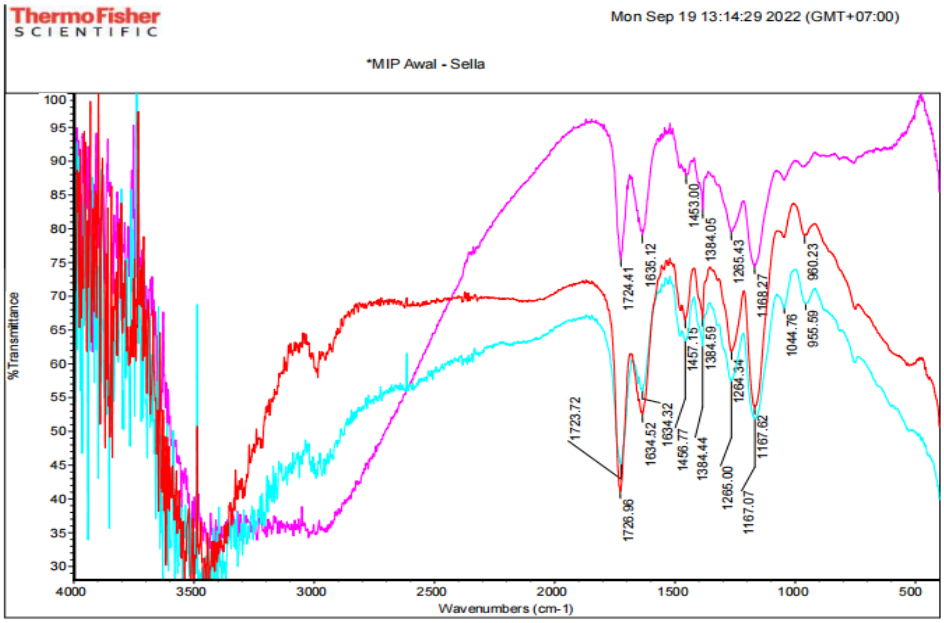

Figure 2. FTIR spectrum of MIP sorbent before extraction (purple), MIP after extraction (red), and NIP (blue)

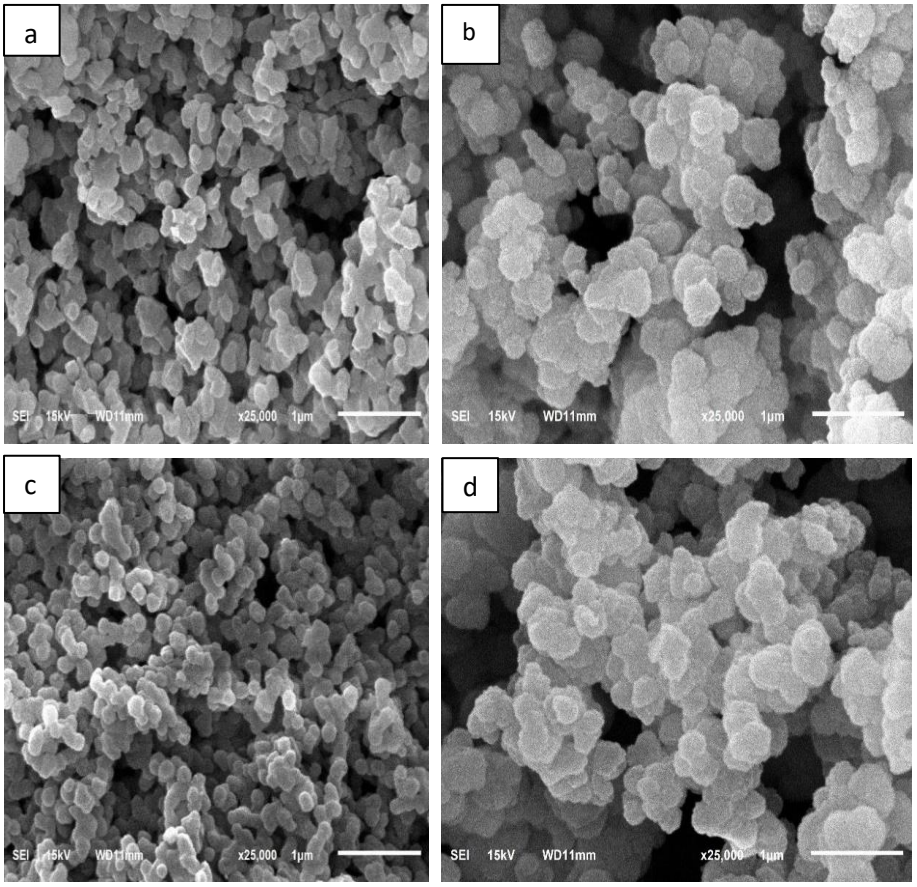

Figure 3. Scanning electron microscope (SEM) of molecular imprinted polymer (MIP) 1 (a), non-imprinted polymer (NIP) 1 (b), MIP 2 (C), and NIP 2 (D)

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Table 3. Weights and Colors of Synthesized Polymers

Polymer	Color	Mass (gr)
MIP1	Pale yellow	4.9630
NIP1	white	2.5764
MIP2	yellow	78.766
NIP2	white	83.313

Table 4. Tetracycline Extraction Cycle by Soxhletation

24-hour cycle	Absorbent
1x	0.161
2x	0.092
3x	0.035
4x	0.001

Table 5. The adsorption capacity of the Freundlich Isotherm Equation

Polymer	R^2	M	a (mg/gr)
MIP1	0.9756	0.4670	0.4077
NIP1	0.9416	0.5689	0.2543
MIP2	0.9559	0.8846	0.8748
NIP2	0.9588	0.9531	0.2879

Table 6. Calculation results of Kd and IF

Substance	Kd		IF	Kd		IF
	MIP1	NIP1		MIP2	NIP2	
Tetracycline	8.09131	6.75482	1.197	4.524285	4.013748	1.1272
Oxytetracycline	6.61960	6.43426	1.0298	2.943459	2.778954	1.05918

Discussion

A study of the interaction between template molecules and functional monomers can predict the stability of the complex formed at the pre-polymerization stage.[11] The selectivity and specificity of the synthesized polymer are determined by the computational strength of the template molecule complex and the functional monomer. This interaction can be seen from the value of the bond energy. The lower the bond energy, the stronger the interaction.[12] The tetracycline and methacrylic acid molecules show a bond energy of -27,3776493 kcal/mol, as shown in Table 2. In addition, the simulation results obtained information on the interaction of hydrogen bonds between tetracycline and methacrylic acid. This bond is the strongest non-covalent bond.[13] Figure 1 shows the interaction between tetracycline molecules and methacrylic acid.

The solvent affects the interaction between the template molecule and the functional monomer. The choice of solvent in synthesizing molecularly imprinted polymers is essential in determining the resulting polymer's analytical performance.[13] The solvent used must not interfere with the interaction of the template molecule with the functional monomer.[14] The association constant indicates the stability of a template molecule complex and a functional monomer in a particular solvent. Solvents with weak hydrogen bond capacities are better than those with strong ones.[15] From the research results, the association constants of the complex between tetracycline and methacrylic acid were 2,922.571 M^{-1} in the methanol-chloroform solvent, 2,737.636 M^{-1} in the methanol-acetonitrile solvent, 1,967.285M^{-1} in ethanol solvent and 647.087 M^{-1} in methanol.

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The bulk polymerization method was the first widely used technique because it was fast, did not require special expertise, and the process was simple.[16] The weakness of this method is that the shape of the particles is not uniform because the process of making it through grinding and sieving processes can damage some of the bond sites that are formed.[17] Precipitation polymerization is a method of synthesis of molecularly printed polymers that produces homogeneous polymer particles. This method requires stirring during the polymerization process and large amounts of template molecules and solvents.[18] The weights and colors of the synthesized polymers can be seen in Table 3.

In the synthesis of printed polymer molecules using a template molecule: functional monomer: crosslinker ratio of 1:4:20. The non-covalent approach used begins with the formation of a template molecule complex: functional monomer spontaneously through the interaction of hydrogen bonds, electrostatic interactions, van der Waals forces and dipole-dipole bonds, then this complex is locked by crosslinking.[19] Polymerization is carried out using an oven at 60°C using an AIBN initiator, which decomposes at this temperature to initiate polymerization.[20] Methacrylic acid was chosen as a functional monomer because it has functional groups (C=O and OH) that can form hydrogen bonds with tetracycline molecules.

Tetracycline extraction from the polymer was carried out using the soxhlet method. This extraction aims to form a three-dimensional cavity capable of explicitly rebinding template molecules or structural analogs.[21] Extraction using the soxhletation method was chosen because of several advantages, namely repeated contact between the MIP polymer and a new solvent, less solvent required, and simpler use.[22] Soxhletation was performed until tetracycline was no longer found in the filtrate. The solvent used was methanol with the addition of 10% acetic acid. The time cycle required for tetracycline extraction can be seen in Table 4.

The adsorption capacity was evaluated to understand the distribution of the functional pockets and the affinity of the functional pockets in the polymer for the target analytes.[23] Isotherm adsorption curves describe the phenomenon of binding analytes to functional pockets in polymers. The amount of analyte bound to the polymer compared to the analyte free in solution after equilibrium is measured as a parameter to calculate how many active pockets are formed. The Freundlich isotherm is an adsorption isotherm model that is most often used in determining MIP affinity, assuming that absorption occurs on a heterogeneous surface and adsorption occurs in a multilayer manner.[24] Table 5 shows that the MIP affinity resulting from the precipitation method is higher than the bulk method. The m value represents the homogeneity index; when the value is close to 1, the sorbent is homogeneous, and if the m value is close to 0, then the sorbent is heterogeneous. The value of a represents the affinity of the sorbent; the greater the value of a, the greater the capacity of the analyte bound to the sorbent.[25]

Analog molecule template molecules are used to test the selectivity of the synthesized polymer. Oxytetracycline has a core structure similar to tetracycline, so it can be used to test sorbent selectivity. The distribution coefficient is used to see the ratio of the absorbed analyte to the total amount of analyte. Imprinting Factor (IF) can also be calculated as the ratio of analyte distribution in MIPs and NIPs.[26] The IF was estimated to see whether the MIPs interactions synthesized using template molecules could be more specific for tetracyclines than NIPs.[26] A good IF value is more than 1. The greater the IF value, the more precise the resulting MIPs is against tetracycline compared to NIPs.[27] The IF value of MIPs is expected to be greater for tetracycline analytes than other analytes because tetracycline is a template molecule used during the MIPs synthesis process.

The selectivity test is shown in Table 6. The MIPs IF values for the two different analytes showed a result of more than 1. These results indicated that PTM's affinity for tetracycline was more significant than that for oxytetracycline. These results suggest that

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the resulting MIP is good enough for the two substances tested. KD value on tetracycline has a big difference between MIP and NIP. This shows that the MIPs formed is more selective towards tetracycline due to molecular interactions.[28]

The MIPs and NIPs' physical characteristic tests use the FTIR instrument, which aims to see the template and monomer interaction during the formation of the pre-polymerization complex and mixing the template in the polymer during rebinding.[19] In Figure 2, there is a shift in vibrational absorption in the MIPs before extraction and after extraction in the C=O group on the MIPs sorbent, namely before 1726.96 cm^{-1} and after 1724.41 cm^{-1} where there is a decrease in electron density due to lower and broader absorption. Methacrylic acid has a vinyl group and is at a wave number of 1000-900 cm^{1}.[29] The success of polymerization can be determined from the presence or absence of twin peaks, proving that the reaction is taking place ideally because there are no vinyl groups.[30] SEM was used to characterize the morphology of MIPs. In comparison to sorbent NIPs, the sorbents had smaller particle sizes and higher porosity, as seen in Figure 3. The higher porosity level of MIP compared to NIP indicates that the MIP has developed a cavity or recognition side to the target molecule, with a high porosity profile providing a larger adsorption area, allowing it to deliver strong tetracycline adsorption capabilities.

Conclusion

MIP sorbent for tetracycline extraction can be synthesized using methacrylic acid as a monomer, chloroform: methanol as a porogen, and EGDMA as a crosslinker in a ratio of 1:4:20. MIP sorbent can be synthesized by using both bulk method and precipitation polymerization method. The sorbent from precipitation polymerization had a higher adsorption capacity (0.8748 mg/g) than the bulk method (0.4077 mg/g). Both methods are selective for tetracycline, but the precipitation method is more demanding than the bulk method.

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