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SYNTHESIS OF NEW MOLECULAR IMPRINTED POLYMER FOR HIGHLY RECOGNITION OF CHOLIC ACID

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Abstract: In this study, molecular imprinted polymers for highly selective recognition of cholic acid, which is a bile acid, were prepared. Acrylamide, methacrylic acid, methacrylamide were chosen as monomer for the production of molecularly imprinted polymers. Functional monomers were polymerized with various crosslinkers; ethylene glycol dimethacrylate (EGDMA), 1,4- butanediol diacrylate (BUT), trimethylpropane triacrylate (TMT) with target molecule (MIP-EGDMA, MIP-BUT and MIP TMT) and various MIPs were prepared. The cholic acid was removed from MIP with a suitable method. NIP polymers were synthesized without cholic acid (NIP-EGDMA, NIP-BUT and NIP-TMT). For the characterization of synthesized polymers FTIR, DSC, TGA, SEM analyses were used. The parameters that affect the adsorption of target species on polymers such as temperature, pH, and concentration were evaluated. The selectivity and reusability studies were also investigated.

It is concluded that MIPs showed better adsorption capacity than NIPs for all solvents for cholic acid. The adsorption sequencing is MIP-TMT

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> MIP-BUT > MIP EGDMA. The maximum adsorption achieved with ethyl alcohol. The adsorption of cholic acid varies with chancing pH for all produced MIPs and NIPs. It is concluded that the adsorption of cholic acid is not affected by the temperature. The adsorption of cholic acid is followed as L type from Giles adsorption isotherms. The thermodynamic parameters are proved the physical nature of adsorption process. The studies conducted with deoxycholic acid, taurocholic acid that is homolog to cholic acid showed that produced MIPs are highly selective for cholic acid.

***Keywords*:** Acrylamide, Cholic Acid, Methacrylamide, Methacrylic Acid, Molecular Imprinted Polymers (MIP)

Introduction

The liver of an adult human secretes approximately 600-1000 mL bile in a day.[1] The secreted bile has two essential functions. First, the role played in the digestion and absorption of the fat. The bile acids found in the bile are the effective agents of this process. They play a role in both emulsifying big fat particles into multiple smaller particles, which can be broken into pieces by lipase enzymes, and assist to the absorption of fat digestion's final products from the intestinal mucosa. The second function of the bile is the role played on the disposal of the various destruction products from the blood. Bilirubin, which is the product, occurred upon the destruction of cholesterol and hemoglobin is some of them. A very large part (97.5%) of the bile secreted from the liver is formed by water. Lecithin and bile acids form the largest amount of the remaining part.[2,3] Bile acids constitute 60% of the bile. The acids in the bile are cholic acid: chenodeoxycholic acid: deoxycholic acid: lithocholic acid, with the ratio 4:2:1 trace respectively. The most common bile acid is cholic acid.

The solubility of bile acids depends on the number of hydroxyl groups. Cholic acid (in Figure 1) has 3 hydroxyl groups; therefore, its

solubility is higher than chenodeoxycholic acid. Lithocholic acid is one of the least soluble bile acids due to its mono hydroxyl structure. 0.5-1.0 g daily bile acid was synthesized in hepatocyte. This amount may increase up to 3-5 g/day in case of bile loss increases.[4]

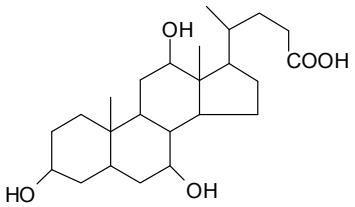


Figure 1. The structure of cholic acid.

Molecularly imprinted polymers are the polymers that are easy to prepare, durable, cheap and have molecular recognition ability.[5,6] Molecular imprinting aims to organize functional monomers around a target (template) molecule with covalent or non-covalent interactions; and then the formation of solid materials having affinity to the template molecule via an adequate operation process.[7-9] At the end of the process, with the removal of the target molecule, complementary cavities, which are specific to the target molecule, are left behind and an ideal material for the processes such as separation, chemical transfer, and catalysis is obtained. Molecularly imprinted polymers (MIP) are polymers with quite high physical and chemical stability against external influences. They are resistant to mechanical forces, high temperature and pressure, acidic and basis metal ions and organic solvents. MIP's storage life is quite long. Also polymers can be reused for more than hundred times without losing "memory effect".[10,11] Molecularly imprinted polymers are used for the separation and purification of the proteins, amino acids, DNA and RNA, peptides, hormones by solid phase extraction of drugs. Another potential application of molecular imprinted polymers is the separation of chiral

compounds, which is important in areas such as some basic researches, drug design, and optic.[12-16]

This study intends the preparation, characterization, and usage of cholic acid imprinted polymers (MIP) in cholic acid adsorption. Acrylamide-methacrylamide-methacrylic acid monomers are selected for the polymer preparation. These monomers are polymerized with the target molecule with the aid of cross-linkers. After the polymerization process, target molecule is removed. MIPs prepared for cholic acid are used in adsorption studies. In addition, MIP samples are compared with Non-Imprinted Polymers (NIP).

Results and Discussion

Molecular imprinted polymers (MIP) are widely used in many fields in recent years, because they carry high binding property by containing binding holes appropriate to the size and functional groups of the target molecule. Bile acids are major metabolites of cholesterol. They play an important role in the expulsion of cholesterol from the body, in the absorption of lipid and fat-soluble vitamins by the formation of micelles. The concentration of bile acids in the body it is associated with many diseases, thus their blood analysis is very important.[17-19]

MIP cross-linked with ethylene glycol dimethacrylate (MIP-EGDMA), MIP cross-linked with 1,4- butanediol diacrylate (MIP-BUT) and MIP cross-linked with trimethylolpropane triacrylate (MIP-TMT) was prepared.

NIP cross-linked with EGDMA (NIP-EGDMA), NIP cross-linked with BUT (NIP-BUT) and NIP cross-linked with TMT (NIP-TMT) was prepared.

Removal of cholic acid molecule from MIPs

Because of the experiments performed for the removal of cholic acid from cholic acid imprinted polymers, it has been found that the most suitable

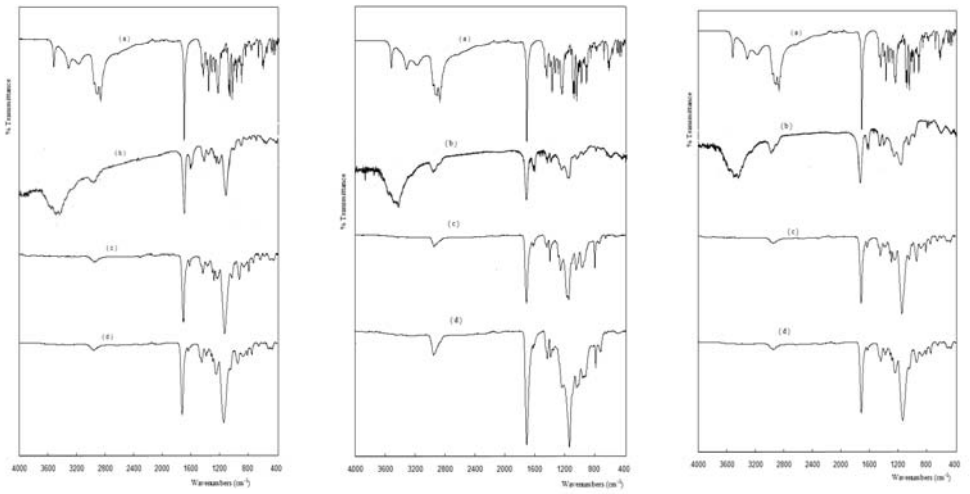
solvent is methanol and methanol was used as the solvent in all cholic acid removal tasks. At the end of this processes MIPs removed cholic acid were prepared (MIP-EGDMA-R, MIP-BUT-R, MIP-TMT-R).

In order to remove the cholic acid from the MIPs; the polymers were washed with methanol. This process has been repeated six times. It was then further washed with distilled water to remove its residues and filtered. This process was done twice. The amount of cholic acid that was removed from the polymer was determined by UV-VIS spectrophotometric method.[20]

Characterization of MIP and NIP

FT-IR Spectroscopic Analysis

FTIR spectrum was taken for the purpose of elucidation of the chemical structure and bonding mechanisms of polymers and the spectrums are given in Figure 2.



(i) (ii) (iii)

Figure 2. FTIR spectra of cross-linked polymer (i: a-Cholic acid; b- MIP-EGDMA; c-NIP-EGDMA; d-MIP-EGDMA-R); (ii: a-Cholic acid; b- MIP-BUT; c-NIP-BUT; d-MIP-BUT-R); (iii: a-Cholic acid; b- MIP-TMT; c-NIP-TMT; d-MIP-TMT-R).

Considering Figure 1, characteristic bands of the monomers that formed MIP were observed (b, c, and d). These bands are OH band observed between 1200-1500 cm^{-1} (1452 cm^{-1}), C-N band observed between 900-1300 cm^{-1} (1248 cm^{-1}) and C=O band observed between 1600-1900 cm^{-1} (1716 cm^{-1}).[22]

From the same figures, it can be seen that the spectrum (a) of the cholic acid carries characteristic bands. Those characteristic peaks can be found in 3521, 3321, and 3192 cm^{-1} number of waves.[23]

The bands that can be observed in MIP spectrum (b) of all three figures cannot be observed in NIP's and washed MIP's spectrums (c and d). These results indicate that cholic acid was included in MIP's and it was removed by washing.

In Figure 2 b-c and d, the presence of vibration peaks belonging to the aliphatic group at about 2800 cm^{-1} was observed, instead of the disappearance of the vibration peaks of the hydroxyl groups. Simplification is observed in the vibrational peaks at 900-1200 cm^{-1} that is C-N bond of the characteristic cholic acid. Less peak vibrations were detected in this region. This also causes a harmonic deviation in the region where the C-N vibration signals in this region are due to the bonds in the hydroxyl portion of the polymer.

When these types of molecules are stimulated with IR light source, the transition of $\Delta v = \pm 2$ or $\Delta v = \pm 3$ is observed as higher quantum numbers go up. Such transitions are defined as overtone bands. They form bands that are approximately two or three times of baseline bands at frequencies. Two different vibrations in one molecule interact with each other to give new absorption peaks.

The frequencies of the new peaks are approximately equal to the

sum or difference of the fundamental frequencies of the two vibrations; these are called "combination" or "difference" bands, and their severity is often low. Overtone, combination and differential bands give a complex look to the vibration spectrum. In our study, this effect was observed in Figure 2(i) a. In Figure 2(i) b-c-d, these effects were lost with the influence of stronger vibration bands, a more simple vibration spectrum being observed.

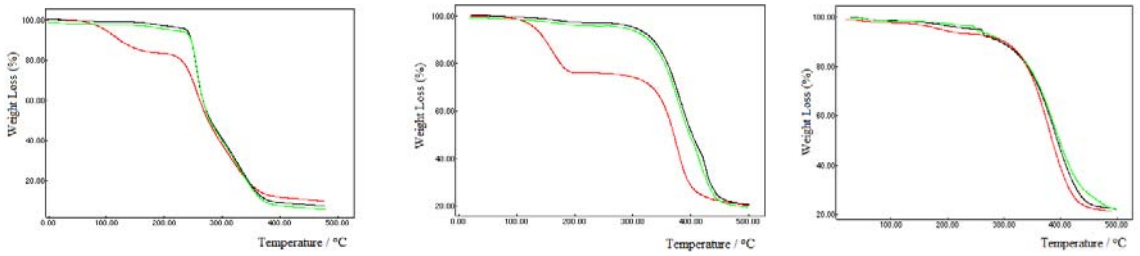
Thermal Analysis

Examination of the thermal behavior of polymeric materials is important in terms of choosing the polymer according to the intended use and purpose. Thermal Analysis of Polymers was performed via theromogravimetric analysis (TGA) and differential scanning calorimetry (DSC).

Theromogravimetric Analysis (TGA)

TG thermograms are given in Figure 3.

Thermal analysis methods are techniques in which some changes in the physical properties of the sample are measured as a function of temperature.



(i) (ii) (iii)

Figure 3. TGA thermograms of the cross-linked polymer (i: ----- MIP-EGDMA; ------NIP-EGDMA; -----MIP-EGDMA-R) (ii: ----- MIP-BUT; -----NIP-BUT; ------MIP-BUT-R) (iii: ----- MIP-TMT; -----NIP-TMT; ------MIP-TMT-R).

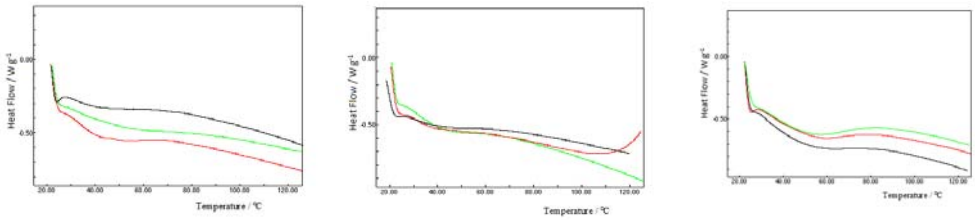
Some of these physical properties are melting point, boiling point, dehydration point and isomer passing point. Thermal analysis methods are widely used in the structure analysis of materials, in the control of their purity, in the examination of especially clay, ceramics, glass and other fillers.

The TGA is called the thermal distortion curve. The TGA shows weight loss of samples at a given temperature. TGA thermograms provide the composition of the compound. It also shows the temperature range (s) without weight loss. This means that the material is stable.

From TGA thermograms, it is seen that prepared polymers show multi-step thermal decomposition reaction. Thermograms of NIPs and washed MIPs were similar, whereas thermograms of MIP are different. Therefore, it can be said that target molecule stripped well. In all polymers, the first mass loss occurred at temperatures up to about 100° C is due to the evaporation of the solvent bonded to the structure of the polymers. Structural degradation of the polymer is not observed at this temperature. No structural decay was observed on NIP and washed MIP polymers due to heat effect up to 200 °C. Decomposition process occurs in the range of 200-400 ^{O}C, via intra and intermolecular reactions.[24] This temperature varies for different cross-linkers (200 ^{o}C for EGDMA, 300 ^{o}C for BUT, and 350 ^{o}C for TMT). These results demonstrated that TMT provide thermal stability to the prepared polymer. In temperatures above 400° C, mass degradation of the remaining polymer takes place due to the main chain scission.

Differential Scanning Calorimetry (DSC)

The DSC technique is based on a system of measuring the heat flow between the sample and the reference sample. whether is endothermic or exothermic of the DSC reaction shows the amount of heat transferred that is measured.



(i) **(ii)** **(iii)**

Figure 4. The DSC curve of the crosslinked polymer (i: ----- MIP-EGDMA; -----NIP-EGDMA; -----MIP-EGDMA-R); (ii: ----- MIP-BUT; -----NIP-BUT; -----MIP-BUT-R); (iii: ----- MIP-TMT; -----NIP-TMT; -----MIP-TMT-R).

Glass transition temperatures of MIPs, washed MIPs and NIPs were determined by differential scanning calorimetry (DSC). The DSC curve of the polymers is presented in Figure 4. Tg values that were driven from the thermograms are given in Table 1.

Table 1. The glass transition temperatures of polymers made with different cross-linkers.

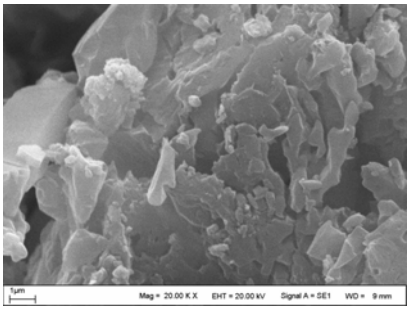
Polymer type	**MIP/ ^{o}C**	**NIP/ ^{o}C**	**MIP –R / ^{o}C**
EGDMA	40,81	42,04	39,89
BUT	47,56	49,93	44,50
TMT	55,53	61,70	54,84

One of the important factors that affect glass transition temperature is crosslinking. Glass transition temperature is higher for the polymers, that crosslinking is provided by trifunctional TMT compared to the ones produced with bifunctional EGDMA and BUT. As can be seen from the table, T_g values of MIPs is lower than NIPs, due to the plasticizing effects of the template molecule found in their structure. Polymers retain their hard and glassy structures up to about 55 °C.

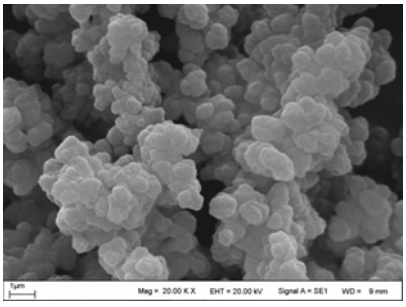
Scanning Electron Microscopy (SEM)

SEM images of the polymers prepared with three different cross-linkers are shown in Figure 5.

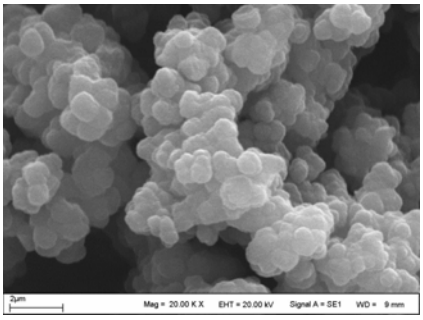
Surface morphology and cross-sectional structures of cholic acid imprinted polymers was investigated via scanning electron microscopy (SEM). After the examination of the SEM images of the polymer, it is seen that there is an image change in the polymer matrix of cholic acid imprinted polymers. It shows a significant difference between the NIPs and MIPs. After the molecular imprinting process, it is observed that gaps in MIPs are in standard size and homogeneously distributed compared to NIPs. Changing of cross-linkers also caused images differences between MIP images.



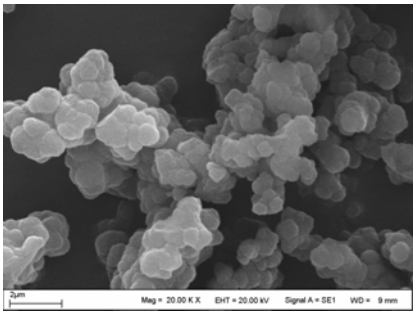
Cholic acid



NIP

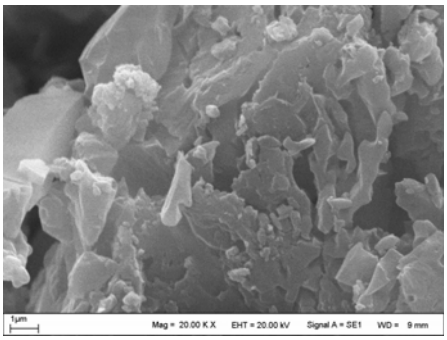


MIP-EGDMA

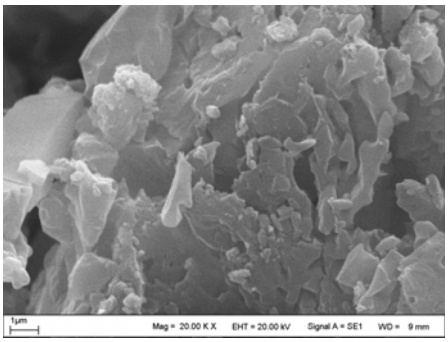


MIP-EGDMA-R

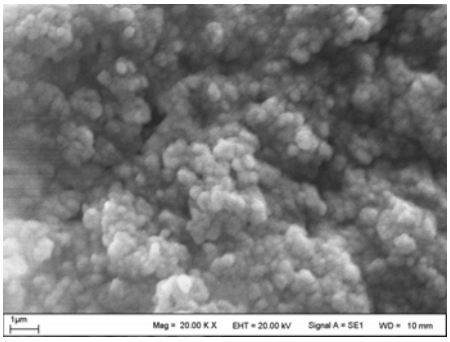
a.



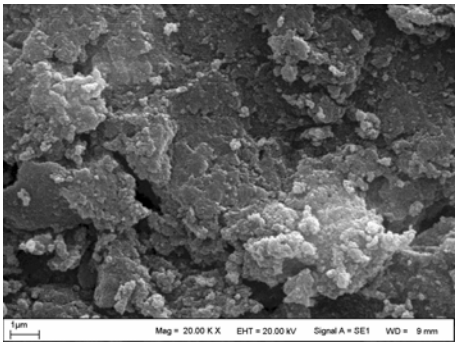
Cholic acid



NIP

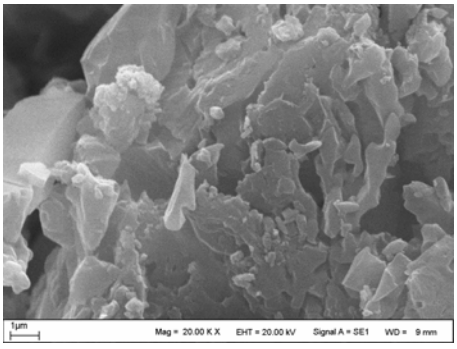


MIP-BUT

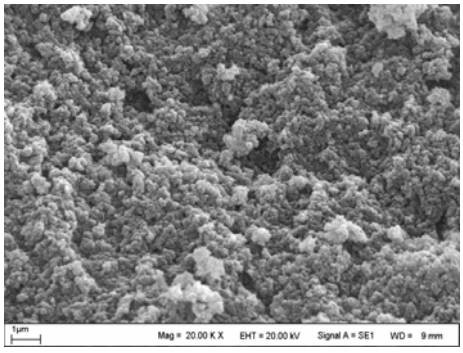


MIP-BUT-R

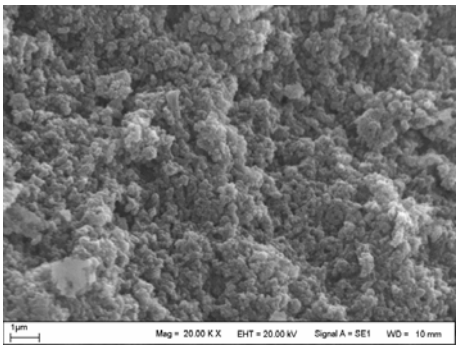
b.



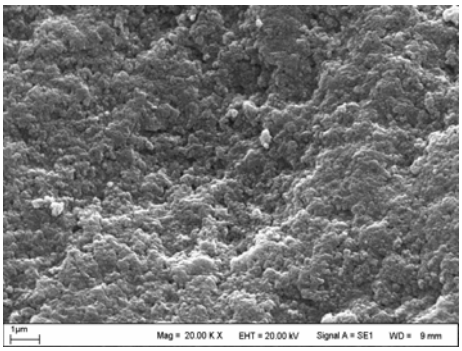
Cholic acid



NIP



MIP- TMT



MIP- TMT-R

c.

Figure 5.a. SEM images of the cross-linked polymer with the EGDMA; **5.b.** SEM images of the cross-linked polymer with the BUT; **5.c.** SEM images of the cross-linked polymer with the TMT

Because of MIP characterization studies, an example for the proposed mechanism for cholic acid imprinted polymer synthesis was chosen as MIP-EGDMA and it is presented in Figure 4a. Accordingly, carboxyl and amino groups of AAm, MAA, MAN monomers, which were cross-linked to EGDMA, were able to make hydrogen bond with the hydroxyl group of cholic acid molecule. Methanol mixture that was used for removing cholic acid from MIP has disrupted this interaction, which was resulted with the detachment of the molecule and the molecule template is formed on MIP.

Mechanism of MIP

Multi-monomer system was applied in preparing cholic acid imprinted MIP. These monomers are neutral acrylamide and methacrylamide, and acidic methacrylic acid. Therefore, it was possible to obtain a solid polymer, as well as to prepare a suitable mold, which is potentially able to make hydrogen bonding for cholic acid. Methacrylic acid, can act as both donor and acceptor while creating hydrogen bond. This feature is advantageous in binding experiments for holding back many molecules. Acrylamide can create strong ties with the mold in polar solvents.

While preparing cholic acid imprinted MIP, in order to investigate the effect of cross-linker three different cross-linkers were tested. Thus, it was intended to form the most adequate polymer mesh, which is stable and hold the molecule in its memory after the removal of the template molecule.

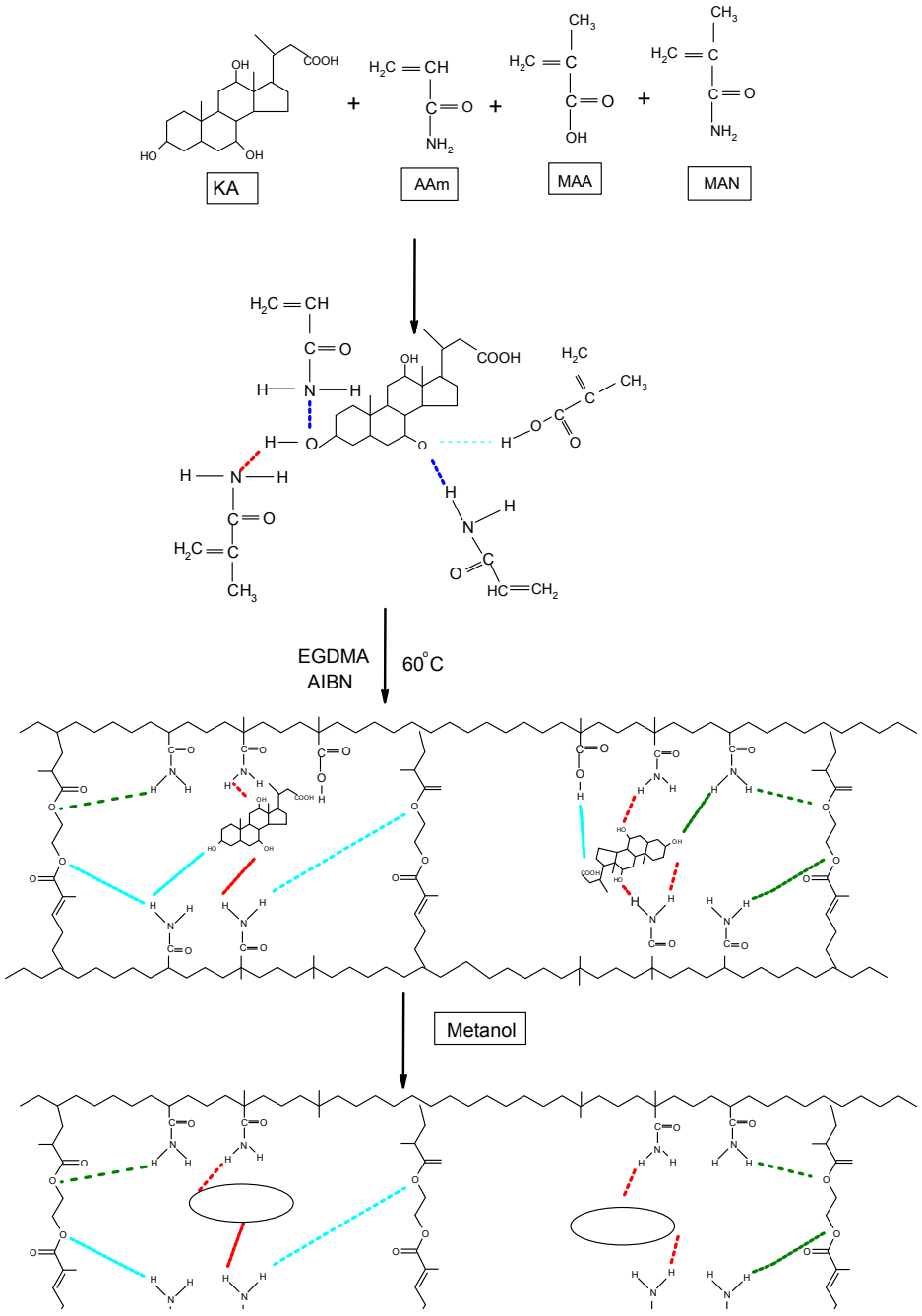


Figure 6. Proposed mechanism for the formation of MIP-EGDMA. (*This is an illustration. Only the location of the H bonds is shown and the bond length must not have considered).

Cholic Acid Adsorption

Cholic acid adsorption was performed on NIP and cholic acid removed MIP samples. After kinetic examination of cholic acid adsorption, adsorption time was determined and the effects of different solvents, pH, temperature, and concentration on adsorption were investigated. Selectivity and repeatability studies were conducted with these polymers.

Adsorption Kinetics

MIP-EGDMA with cholic acid adsorption was examined for 25 ^{o}C at 500 min. Adsorption reached saturation at the end of 350 min. Therefore, adsorption time was selected at 6 h.

Comparison of MIP's and NIP's Cholic Acid Adsorption

MIPs have adsorbed more cholic acid molecule compared to NIPs. When two solvents were compared, EtOH seems to be the best solvent. Bonding in the polymers occurs via both electrostatic and hydrophobic interactions. The equilibrium between the electrostatic and hydrophobic interactions shifts as the dielectric constant increases. When imprinted polymers are used to recognize cholic acid, its carboxyl group can make hydrogen bond with the hydroxyl group of the cholic acid. This interaction is subject to deterioration with the increase of the polarity of the solvent. Since the polarity of MeOH is higher than EtOH, it affected the bonding and more adsorption took place in EtOH.[25]

The Effect of pH, Temperature, Concentration on Cholic Acid Adsorption

In this part of the study, the effect of pH, temperature, and concentration on imprinted polymers, which affects cholic acid adsorption equilibrium, has been investigated.

pH Effect

It is well-known that the amount of substance adsorbed by the polymers, which contains ionizable acidic or basic groups in their structure, varies with pH. In order to investigate this effect, the adsorption capacity of the solutions with different pH was examined.

The curves showing the change of the amount of adsorbed substance with pH were drawn and presented in Figure 7.

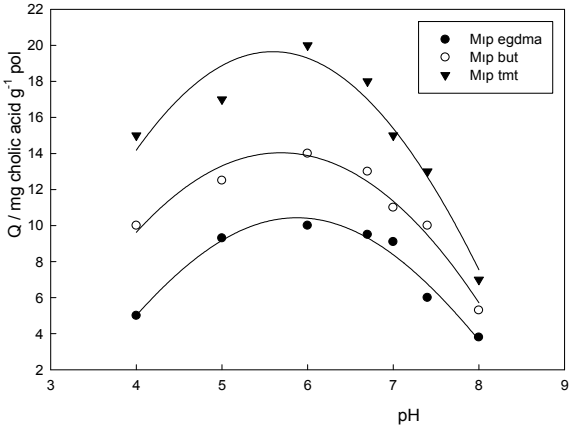


Figure 7. The effect of pH.

As can be seen from the graph, cholic acid molecule adsorbed by polymers varies with pH. Up to pH 6, adsorption increases with the increase of pH. It reaches the maximum value at pH 6. Over pH 6, adsorption decreases with the increase of pH. In low pH, the adsorption occurs between carboxyl groups of cholic acid and amine groups of the polymer via hydrogen bonds. pKa is about 6 for carboxyl groups. When pH<6, carboxyl groups fail to ionize, and the adsorption between the polymer and cholic acid molecule occurs only via hydrogen bonds. When pH is high, the adsorption is reduced because the electrostatic repulsion between ionized carboxyl groups in the polymer and cholic acid is increased. Therefore, the amount of adsorbed substance decreased after pH> 6.[26,27]

Temperature Effect

No significant change occurred on the adsorbed amount of cholic acid in the tested temperature range. Thus, it seems that the temperature has no significant effect on adsorption.

Concentration Effect

The equilibrium concentration of the solutions was determined by working charts prepared before. For each concentration, equilibrium concentration of the solute (C_B) in the adsorbent was calculated using initial (C_T) and balance (C_D) concentrations and from this value the amount of the adsorbed substance was found by the equation $q = \frac{C_B . V}{m}$. Isotherm curves obtained by the plot of adsorbed substance amount (Q) versus the equilibrium concentration are presented in Figures 8, 9 and 10.

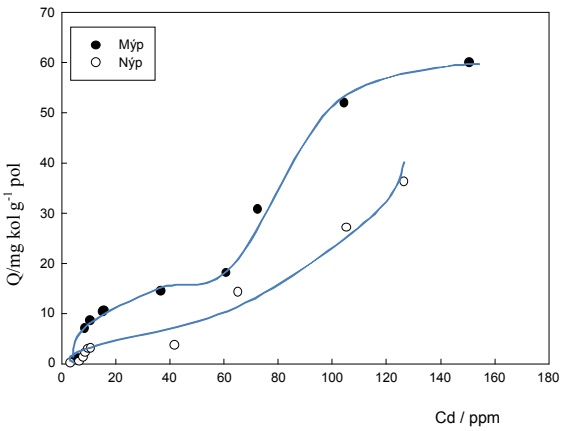


Figure 8. Temperature curves of cholic acid adsorption.

Regarding the cholic acid molecules adsorption isotherms of the polymers cross-linked to EGDMA, it can be said that according to the classification of Giles' adsorption isotherms MIP-EGDMA is similar to L4 type and NIP-EGDMA is similar to L3 type curves.[28]

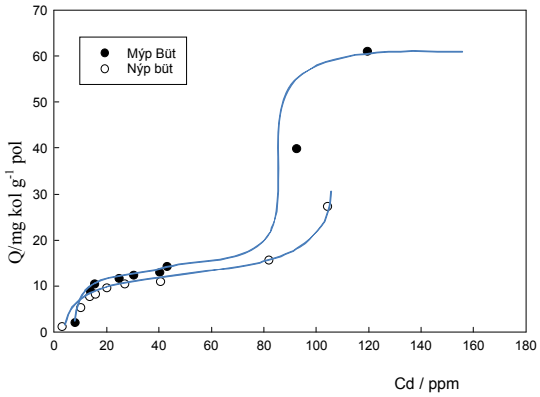


Figure 9. Temperature curves of cholic acid adsorption.

Regarding the cholic acid molecules adsorption isotherms of the polymers cross-linked to BUT, it can be said that according to the classification of Giles' adsorption isotherms, MIP-BUT is similar to L4 type and NIP-BUT is similar to L3 type curves.[28] Q/mg kol g^{-1} pol

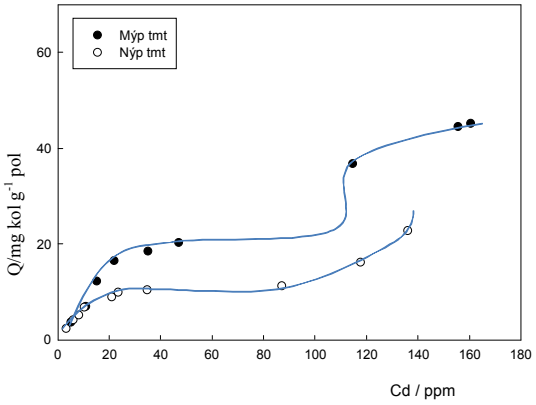


Figure 10. Temperature curves of colic acid adsorption.

According to the classification of Giles' adsorption isotherms, the most characteristics feature of L type curves is; adsorbed molecules have strong inter-molecular interaction with the adsorptive molecules. By creating memory effect via imprinting processes, a strong interaction

between the adsorptive type and the adsorbed is established. It was observed that adsorption type does not change with the difference of cross-linker. From the adsorption isotherms, Ki= initial binding constant, K= equilibrium binding constant, n= monolayer coating and Θ= maximum occupancy rate was calculated and they are given in Table 2.

Table 2. The binding constants of polymers.

	Ki x 10^3/ L mol^{-1}	**K/ L mol^{-1}**	**n x10^3**	**Θ**
MIP-EGDMA	10,4	2,5	4,1	0,6
NIP-EGDMA	10,0	1,0	10,0	0,3
MIP-BUT	41,4	13,9	3,0	0,7
NIP-BUT	4,3	1,0	4,3	0,5
MIP-TMT	67,4	29,8	2,3	0,9
NIP-TMT	6,3	1,3	5,0	0,8

The amount of adsorption was changed with the change cross-linker type. Adsorption sequence according to cross-linker is as TMT > BUT > EGDMA. As can be seen from the table, K and Ki values are higher for MIPs. MIPs made more adsorption compared to NIPs. Single-layer coating has been realized, but the layers are not full.

Table 3. Thermodynamic Parameters of Polymers.

	ΔG/ j mol^{-1}	**ΔH/kj mol^{-1}**	**ΔS/ j mol^{-1} K^{-1}**
MIP-EGDMA	-6268	-5,1	21,0
NIP-EGDMA	-2486	-6,1	8,3
MIP-BUT	-34537	-2,1	115,9
NIP-BUT	-2527	-3,0	8,5
MIP-TMT	-73757	-15,0	247,5
NIP-TMT	-3121	-14,6	10,4

The values in Table 3 show that the adsorption is physisorption

(physical adsorption). Physical adsorption is an exothermic process. Thus, ΔG is negative. Negative ΔH implied that the adsorption is physical. On the other hand, positive ΔS value indicates the presence of hydrophobic interactions.[29]

Selectivity

In order to examine the selectivity of the target type used to imprint MIPs, deoxycholic acid and taurocholic acid, which are similar to cholic acid among the bile acids, were tested. Prepared MIPs were used in the adsorption test with the solution containing the mixture of cholic acid, deoxycholic acid and taurocholic acid and the amounts of adsorbed substance was presented. The selectivity of the target molecule of the prepared MIP is given in Table 4.

Table 4. The selectivity of the target molecule of the prepared MIP.

	% Cholic acid / Qmg target molecules g^{-1}	**% Deoxycholic acid/ Q mg target molecules g^{-1} pol**	**% Taurocholic acid / Q mg target molecules**
MIP-	62	54	12
MIP-BUT	69	28	3
MIP-TMT	100	59	27

The sequencing of cholic acid imprinted MIPs adsorption with different molecules is cholic acid > deoxycholic acid > taurocholic acid for each of the three cross-linker. The selectivity of the prepared MIP is high. As a result of removing template molecule from the polymer, chemical and topological (size, shape and three-dimensional structure) memory effect towards the cholic acid molecule, which was imprinted into the polymer, has obtained and the cholic acid molecule in a mixture could be selectively connected again with the polymer. The obtained results showed that, cholic acid is better recognized by cholic acid stamped polymers compared to similar compounds forming the solution.

Repeatability

After adsorption, in order to remove cholic acids molecule bound to the polymers, it was washed six times with MeOH and twice with 250 mL distilled water. Adsorption-desorption processes were repeated five times. Repeatability (% RE) was calculated from Equation 2 and displayed in Figure 11.

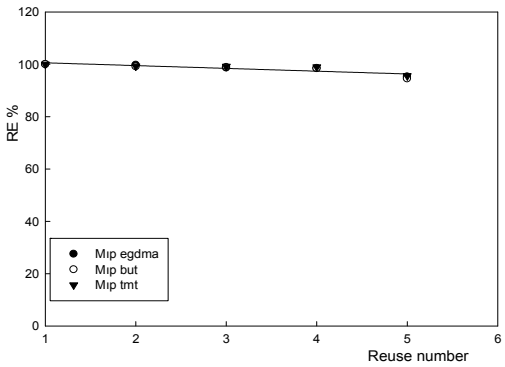


Figure 11. Repeatability for colic acid adsorption of MIPs.

As the result of adsorption-desorption processes, repeated five times, it was observed that memory effect and structural integrity of the polymers were maintained.

Experimental

Materials

Cholic acid (Ch) was obtained from Alfa Aesar (Karlsruhe, Germany). Acrylamide (AAm), methacrylamide (MAN), methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), methanol (MeOH), acetic acid (AcA), sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, 1,4 butanediol diacrylate (But) were obtained from Merck (Darmstadt, Germany). α-α'-azobisisobutyronitrile (AIBN) was obtained from Fluka (Chemie AG CH-9470 Buchs). Dimethyl sulfoxide (DMSO) was obtained from Carlo Erba (Rohano, Italy). Tween 20, Triton X 100 were purchased from Ambresco (Solon, Ohio).

Preparation of MIPs and NIPs

To synthesize cholic acid imprinted polymers (MIP); 1 mmol of cholic acid was dissolved in 4 mL of methanol and 0.5 mmol of acrylamide, 1 mmol methacrylamide and 2.5 mmol of methacrylic acid were added to this solution to dissolve the monomers and it was stirred for thirty min to ensure that monomers interact with the cholic acid. Twenty mmol of cross-linker (EGDMA, TMA or BUT) dissolved in 5 mL methanol, was added to the mixture and stirred for thirty minutes to complete the crosslinking. After adding 10 mg AIBN as the initiator, it was allowed to dissolve for five minutes, and then the solution was put to 100 mL flask; N_2 gas was bubbled for 10 min to remove molecular oxygen from the environment and it was polymerized in the water bath at 60^o C, for approximately four hours. The prepared polymers were washed with a little methanol. It dried in petri dishes at room temperature. Non-imprinted polymers (NIPs) were prepared using same process without addition of cholic acid. MIP and NIP polymers were obtained as solid powders.

The contents of the prepared cholic acid imprinted polymers (MIP) and cholic acid non- imprinted polymers (NIP) are given in Table 5.

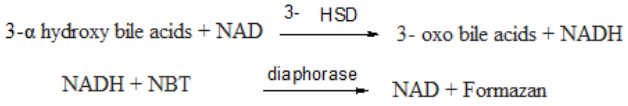
Table 5. The amount of chemicals used in the preparation of molecular imprinted polymers

Polymer	The amount of chemicals							
	Cholic mmol	AAm mmol	MANmmol	MAA mmol	AIBN mg	BUT mmol	TMT mmol	EGDMA mmol
MIP-1	1	0,5	1	2,5	10	----	---	20
NIP-1	----	0,5	1	2,5	10	----	---	20
MIP-2	1	0,5	1	2,5	10	20	----	----
NIP-2	----	0,5	1	2,5	10	20	----	----
MIP-3	1	0,5	1	2,5	10	----	20	----
NIP-3	----	0,5	1	2,5	10	----	20	----

(AAm, Acrylamide; MAN, methacrylamide; MAA, methacrylic acid; EGDMA, ethylene glycol dimethacrylate; BUT, 1,4-butanediol diacrylate; TMT, Trimethyl propane triacrylate; AIBN, α-α'-azobisisobutyronitrile).

Removal of Cholic acid from MIPs

To remove cholic acid from MIPs; the polymers were washed with methanol by stirring in a magnetic stirrer at 150 rpm for 24 h and filtered after washing. This process was repeated six times. Then, in order to get rid of the residue, it was washed again with distilled water by stirring in a magnetic stirrer at 150 rpm for 24 h. Polymers filtered after washing. This operation was also performed twice. The amount of cholic acid removed from the polymer was determined by UV-VIS spectrophotometric method.[20] In this method, bile acids are oxidized to 3-oxo bile acids by dehydrogenase enzyme. The resulting NADH diaphorase enzyme is employed in the reaction where nitro blue tetrazolium is transformed to formazan. Since the amount of formazan is equivalent to the amount of bile acid, absorption measurement performed at 530 nm wavelengths is used to find the amount of bile acids.[21]



(Method 1)

The same washing process was applied to NIPs as well. After washing, the polymers were dried in petri dishes at room temperature.

Characterization of MIP and NIP

FTIR analysis, Thermal analysis, surface morphology analysis for characterization of MIP and NIP was performed.

FTIR Studies

Infrared spectrum of MIP and NIP's were obtained by using a FTIR spectrophotometer [Perkin Elmer Spectrum 100 FT-IR Pike Gladii brand / EC]. FTIR-ATR spectra were taken in the wavenumber range of 4000-400 cm^{-1}.

Thermal Analysis

Examination of the thermal behavior of the MIP and NIP is important for purpose to use of the polymers. Therefore, MIP and NIP theromogravimetric analysis (TGA) and Differential Scanning Calorimetry (DSC), theromogravimetric analysis were applied.

Theromogravimetric Analysis (TGA)

MIP and NIP theromogravimetric analysis (TGA) was performed with Shimadzu TGA-50 brand model instrument. TG thermograms of polymers were collected at a heating rate of 10 ^{o}C dk^{-1} and under a nitrogen atmosphere.

DSC

DSC analysis of the MIP and the NIP are made by a computer linked to Differential scanning calorimeter that is the brand Shimadzu DSC-50 models. Approximately 10 mg of dry sample was heated at a heating rate of 10 ^{o} C/min in nitrogenous (N_2) atmosphere, thermograms were taken.

Surface Morphology

The surface morphology of MIP and NIP was examined using scanning electron microscopy (SEM). The surface morphology of MIP and NIP was investigated by scanning electron microscopy (Leo EVO 40XVP).

Cholic Acid Adsorption on Molecular Imprinted Polymers

Cholic Acid Adsorption, on NIP and cholic acid removed MIP samples have been investigated. For this purpose, first cholic acid adsorption was investigated kinetically and adsorption time was set. The effects of various solvents, pH, temperature, and concentration on adsorption have been investigated. Selectivity and repeatability studies were conducted with these polymers.

During the adsorption study, cholic acid concentration was determined by UV-VIS spectrophotometer. The amount cholic acid adsorbed by the polymer was calculated using Equation 1.

$$Q = \frac{(c_i - c_s) \cdot V}{m} \cdot 100 \quad (1)$$

Q = Adsorbed amount of cholic acid / mg cholic acid (g polymer)$^{-1}$

C_i = initial concentration of the solution / ppm

C_s = final concentration of the solution / ppm

V = Volume of the solution / mL

m = Mass of polymer / g

Adsorption Kinetics

In order examine the adsorption kinetics, cholic acid adsorption by MIP-EGDMA was examined for 300 min, at 25 ^{o}C and adsorbed amount of cholic acid was plotted against time. Equilibrium time was determined and used in the study.

Cholic acid adsorption of MIP and NIP with different solvents

After the removal of cholic acid from the MIPs prepared using various cross-linkers, cholic acid adsorption has been performed on these polymers and on NIP samples, which had not contained cholic acid.

Methanol and ethanol were used as solvent on the binding of cholic acid because the kit used in cholic acid analysis only works on methanol and ethanol. 20 mL of 100-ppm cholic acid solution was added to 0.02 g of polymers. It waited in an incubator at 25 °C for 6 h. The amounts of cholic acid were determined using UV-VIS spectrophotometry

The Effect of pH, Temperature, Concentration on Cholic acid adsorption

In this part of the study, the effect of pH, temperature, concentration, which may affect cholic acid adsorption equilibrium, has been investigated.

pH Effect

In order to examine the effect of pH on the adsorption of cholic acid molecule, 20 mg of dry powder polymer was added into cholic acid solution of 50 ppm concentration, adjusted to seven different pH levels with phosphate buffer (pH; 4, 5, 6, 6.5, 7, 7.4, 8) and waited in an incubator at 25 °C for 6 h. After this period, cholic acid concentrations of the solutions were found by the method described in method 1, the adsorbed amount was calculated from Equation 1.

Temperature Effect

In order to examine the effect of temperature on the adsorption of cholic acid molecule, 20 mg of dry powder polymer was added into cholic acid solution of 50 ppm concentration adjusted to pH: 6 with phosphate buffer and waited in an incubator for 6 h at six different temperatures (20, 25, 30, 35, 40 °C). After this period, cholic acid concentrations of the solutions were calculated from equation 1.

Concentration Effect

In order to examine the effect of cholic acid concentration on the adsorption, 20 mg of dry powder polymer was added into cholic acid solutions of 5-300 ppm concentrations adjusted to pH: 6 with phosphate buffer and waited in an incubator for 6 hours at 25 °C. After this period, cholic acid concentrations of the solutions were found by the method described in method 1, the adsorbed amount was calculated from equation 1.

Selectivity

In order to investigate the selectivity of imprinted polymers towards the target type used in the imprint, 20 mg of dry polymer was added into the mixture of 50 ppm cholic acid, 50 ppm deoxycholic acid and 50 ppm taurocholic acid solution, adjusted to pH: 6 and waited in an incubator for 6 h at 25 °C. The adsorption equilibrium concentrations of cholic acid have been identified and the adsorbed amount of substance was calculated.

Repeatability

In order to perform adsorption-desorption analysis of the imprinted polymers, 1 g dry polymer was put into 50 ppm cholic acid solution, adjusted to pH 6 and waited in an incubator for 6 h at 25 °C. After the adsorption, equilibrium concentration of cholic acid was determined and the amount of adsorbed substance was calculated. After adsorption, cholic acids bounded to the polymer were removed by washing with methanol. Adsorption-desorption process was repeated five times. Repeatability (RE %) was calculated from equation 2.

Repeatability; $$RE\ \% = \frac{Qs}{Q1}\,100 \qquad (2)$$

Qs: the amount of cholic acid in the end of the adsorption measurements;

Q_1: the amount of cholic acid in the initial of the adsorption measurements

Conclusions

Consequently, MIP polymers synthesized in this study, are the materials with improved adsorption capacity, effectiveness and selectivity, which can be reused repeatedly. The most distinctive feature of these polymeric materials from the other polymers is their selectivity towards a specific type.

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