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Extraction of Catechin as a Flavonoid Compound via Molecularly Imprinted Polymers

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A B S T R A C T

The aim of this study is synthesis of molecularly imprinted polymers (MIPs) and evaluation for extraction of catechin. Catechin is a bioactive compound which is found abundantly in green tea. In this paper, MIPs was synthesized by precipitation polymerization technique for catechin, acrylic acid and trimethylolpropane trimethacrylate as a template, functional monomer and cross-linker in a molecular ratio of (1:12:12), respectively. Surface morphology in the MIPs by scanning electron microscopy (SEM) demonstrated spheres with nanometric scale. Fourier transform infrared spectroscopy (FTIR) of the polymers showed that catechin molecule was captured in the network copolymers. Porosity of the polymers were analyzed using Brouneur Emmet Teller (BET) technique. Based on BET analysis, specific surface area of the MIPs was 45.5 $m^2.g^{-1}$ while it was 42.2 $m^2.g^{-1}$ for non-imprinted polymers (NIPs). It means that the imprinting process was carried out successfully. Adsorption properties of the polymers were characterized too. The best binding capacity of the MIPs was reported equal to 440 mg. g^{-1} in 750 ppm of the feed concentration whereas it was 84mg.g^{-1} for quercetin (similar structure of catechin). It confirms that the MIPs technology can be introduced as a good candidate for separation process with a satisfactory result in selectivity. The binding capacity of the MIPs was evaluated for natural extract of green tea using a high-performance liquid chromatography (HPLC) device which similar results were obtained. According to above mentioned results, separation and pre-concentration of the bioactive compounds from the extract of medicinal plants can be suggested *via* MIPs technique.

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1. INTRODUCTION

Catechin is a flavonoids compound which is found in a variety of fruits, vegetables and herbal plants. Although catechins are not nutritionally- nutrition for humans, they help improve human health by preventing various diseases [1]. Some Fruits like grapes, apples, strawberries, cherries, and various types of tea, especially green tea are the main sources of catechins [2]. Figure 1 shows the structure of catechin molecule contain five hedroxyl groups.Thanks to their hydroxyl groups, polyphenols such as catechin and quercetin are the most common food antioxidants.

These compounds play an important role in preventing chronic diseases like cancer by inhibiting free radicals. Due to the presence of flavonoid bioactive compounds such as catechins in green tea, Chinese people use green tea as a medicinal beverage. Recently,
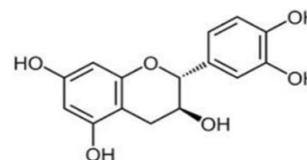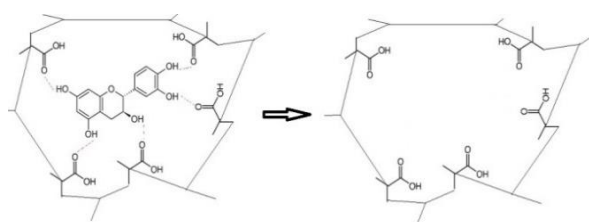

Figure 1. Chemical structure of Catechin molecule

green tea became very popular in many countries including Iran [3]. There are many different methods for separation of the bioactive or special compounds. Using eco-friendly technique is welldwon for human safty. Adsorption of methylene blue by silk cocoon as a natural adsorbent and extraction of the bioactive compound from gringer *via* subcritical water extraction can be mentioned as green methods [4-5]. Intra molecular interaction is a very old concept. Fisher's lock-and-key theory which are analogous to the substrate-enzyme interaction, relates to

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this concept. Today, the molecular imprinting technique is a method for designing and detecting the molecules according to a mimic system, such as antibodies and biological receptors [6-9]. The MIPs are three dimensional network polymers with specific binding sites for template molecules which are obtained by polymerization of functional monomers and cross-linker molecules in the presence of template molecules [10]. Then by elimination of the template molecule, some cavities with a similar structure will be created. Identification and selection of the template molecule is depend on covalent or non-covalent bonding (such as ionic, hydrogen and van der Waals bond) [10-12]. Figure 2 shows a general schem for creating the cavities (especially for Catechin molecule) after tempelate removal from MIPs.

MIPs are three-dimensional network copolymers with specific binding sites that are obtained during the polymerization process, in presence of the effective compounds. Fast preparation, easy, cheap, reproducibility and high selectivity are the main reasons that this technique can be suggested for separation of the bioactive compounds. Various polymerization methods (such as bulk and precipitation polymerzation) have been developed for the synthesis of the MIPs. Radical polymerization, as the first method with a great adaptability in selecting functional monomers, in both bulk and precipitation polymerization, has been the most common method [13]. Five original compounds are present in the process of creating MIPs: a template molecule, a functional monomer, a cross-linker, an initiator and a solvent. High selectivity, high mechanical and chemical stability, easy synthesis and cost-effectiveness are the main advantages of the MIPs [14]. These advantages have led to numerous applications of this technique. Applications of the MIPs include sensors, solid phase extraction (SPE), enzymes, biosensors, food safety, detection of micro organisms and especially drug delivery [15-22]. In a study, imprinting of the polymers with herbicides was carried out to produce the adsorbents which could be used to isolate these hazardous compounds from contaminated water [23]. Synthesis of the MIPs was reported to isolate naphthoquinone compound from the extract of the plants [24]. Recently, this technique has also been reported to isolate antifungal compounds from secondary metabolites of *T. virens* [25]. In this study Catechin was used as a target molecule for MIPs synthesis in a molar ratio of (1:12:12) for template, functional monomer and cross-linker respectively via precipitation polymerization technique for the first time. According to the produced adsorbents with great bonding capacities, utilization of this type of intelligent polymers for extraction of the bioactive compounds from medicinal plants can be investigated.

2. EXPERIMENTAL

2. 1. Materials and Methods   The most materials which were used in the polymerization process, the chemicals were HPLC grade. Methanol and acetic acid with high purity were supplied by Merck (Darmstadt, Germany). The list of used chemicals are sumarized in Table 1.

The equipments used in this study include analytical balance manufactured by A&D company, magnetic stirrer model R-50 (Italy), water bath manufacture by Memmert model WB22 (Germany), sonicator model QTD1730 (Korea), Centrifuge manufactured by Hermle company (Germany) and oven. Jenway 6305 UV/Visible spectrometer was used to determine amount of the templates in loading process on the polymers at 278 nm wavelength. The porosity was evaluated by nitrogen gas adsorption/desorption analysis using Brouneur Emmet Teller (BET) analysis (PHS1020-China). The porosity measuring is based on the results of isothermal adsorption at 77 K. Surface morphological information of the MIPs was obtained by scan electron microscope (SEM) model VEGA\\TESCAN (Czech). Amount of Catechin measured by HPLC equipment (Agilent Technologies, Palo Alto, CA, USA) with A G1328B manual injector and C18 Column. Fourier transform infrared spectra (400-4000 cm-1) were recorded for NIPs, Catechin, leached and un-leached MIPs on a

Figure 2. Creating of cavity in MIPs after removing of Catechin

TABLE 1. The used Chemical materials in synthesis of MIPs

Chemical material	Function	Producer
Quercetin	Similal structure for template molecule	Sigma-Aldrich
Catehin	Template molecule	
Acrylic acid	Functional monomer	Merck
Trimethylolpropane trimethacrylate	Cross-linker	Sigma-Aldrich
Azobisisobutyronitrile	Initiator	
Acetonitrile	Porogene	
Methanol/Acetic acid	Elution	Merck
Acetone	Solvent	

Bruker spectrometer (model vector 22; Bruker, Germany).

2. 2. Synthesis of the MIPs And NIPs For synthesis of MIPs, the target molecule (catechin), acrylic acid monomer (AA), and trimethylolpropane trimethacrylate crosslinker (TRIM) with molecular ratio of 1:12:12 were used [26]. Precipitation polymerization reaction was carried out in a flat-botton flask. Initiator of the reaction was 2,2-azobisisobutyronitrile and acetonitrile was used as a porogene. At first catechin was dissolved in acetonitrile solvent and then other reactants were added at 0°C. Ultrasonic process was performed for 5 min after adding each compound. The mixture was purged for 5 min using nitrogen gas to remove oxygen molecules from the flask. The flask took place in a water bath at 60°C on the stirrer for polymerization reaction for duration of 24 h. Finally the synthesized polymer was centrifuged and kept in oven at 50°C over night. The synthesis of non-imprinted polymer (NIPs) was performed according to the same method but without the presence of any template.

2. 3. Removal of the Catechin From The MIPs For separation of the catechin molecules from the synthesized polymer, the MIPs were elluted on the stirrer with methanol/acetic acid as a ellutant (9:1v/v) for several times in room temperature. The process was continued until the absorbance of the extracted solution at the wavelength of 278 nm, reached to zero. The leached MIPs was washed with pure methanol and distilled water respectively and put in oven at 60°C for furthure use.

2. 4. Evaluation of the MIPs

2. 4. 1. Catechin Standard Absorption Curve At first the standard curve for different concentration of catechin in acetonitrile/water solvent(1:1v/v) vs absorbance in wavelength at 278 nm was drown to measure the binding capacity of the MIPs (see Figure 3).
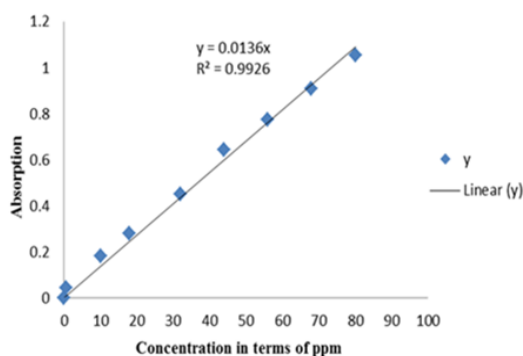

Figure 3. The standard curve for catechin in acetonitrile/distilled water (1:1 v/v)

According to the chart with the equation of $y = 0.0136x$, the correlation coefficient was 0.9926, which is very desirable.

2. 4. 2. Measuring Binding Capacity of the MIPs In order to evaluate the data obtained from the adsorption analysis, a parameter was defined as the adsorption capacity, which determines the adsorbent performance and is an index to compare the adsorbent's performance. The binding capacity (Qe) of the adsorbent (MIPs) was defined as the difference of the initial (C0) and final (Ce) amount (ppm) of catechin in the solution multiple loading value (V) over the amount of the used adsorbent (g), based on Equation (1):

$$Q_e = \frac{(C_0 - C_e)}{m}V \qquad (1)$$

where Qe (mg/g) is known as binding capacity.

2. 4. 3. Imprinting Factor The imprinting factor (IF) is defined according to Equation (2):

$$IF = \frac{Q_{MIPs}}{Q_{NIPs}} \qquad (2)$$

in which, Q_{MIPs} and Q_{NIPs} are binding capacity of the MIPs and NIPs, respectively.

2. 4. 4. Yield of the Extraction The yield of extraction or percent of the MIPs adsorption can be calculated as Equation (3):

$$\%Extraction = \frac{C_0 - C_e}{C_0} * 100 \qquad (3)$$

where C_0 and C_e are initial and final concentration of the feed in loading process.

2. 4. 5. Selectivity of the MIPs In general, the MIPs were evaluated for their diagnostic properties relative to the template molecule. Chromatographic and equilibrium adsorption analyses on the discontinuous system are commonly used to study the selectivity of the imprinted materials. In such experiments, a certain mass of the chemical compound which is similar to the template molecule (base on its structure) is added to the solution containing the MIPs. After loading procedure, by measuring amount of the remained molecule in solution, the quantity of the adsorbed by MIPs can be calculated [27]. Figure 4 shows the structure of Quercetin molecule (a flavonoid compound) which is similar to Catechin molecule structurally. This compound was used for selectivity test of the MIPs.

2. 4. 6. Selectivity Factor (α) For measuring selectivity factor (α), at first distribution coefficient (K_d) of the template should be calculated. Distribution coefficient is introduced by Equation (4):
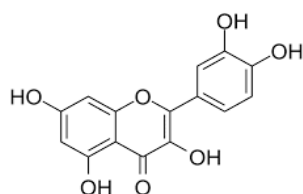
Figure 4. Chemical structure of Quercetin molecule

$$K_d = \frac{Q_e}{C_e} \tag{4}$$

where Q_e and C_e are the binding capacity (mg.g-1) and final concentration (mg/l) of the feed in loading process, respectively. Selectivity factor (α) of MIPs is a important parameter that establish the selectivity of the polymers. Selectivity factor is defined as Equation (5):

$$\alpha = \frac{K_d(\text{catechin})}{K_d(\text{quercetin})} \tag{5}$$

where $K_d(\text{catechin})$ and $K_d(\text{quercetin})$ are distribution coefficients of catechin and quercetin, respectively. Utmost measure of α, introduce high selectivity of the MIPs.

2. 4. 7. Applicability Test for MIPs with Natural Product

2. 4. 7. 1. Preparation of the Green Tea Extract
The extract of green tea was carried out in methanol solvent at 70ºC for 90 min [28]. It contains several flavonoids compounds which most of them are catechin and its derivatives. Amount of catechin before and after loading procedure on MIPs can be measured by HPLC equipment at retention time of 10.567 minutes of chromatogram according to standard curve (Figure 5).

2. 4. 7. 2. Loading Natural Product Extract on the MIPs
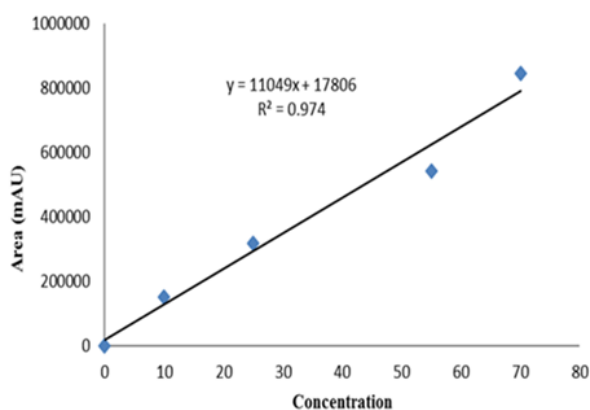The extracted solution from green tea was

Figure 5. Standard HPLC curve for Catechin (Peak surface area *vs* concentration (ppm))

loaded on the MIPs. At first 20 µl of the extract was diluted with 10 ml methanol and then 10 ml of this solution with 10 mg of the leached MIPs was put in a conical flask. Loading process took place on the magnetic stirrer for 2h. Before and after loading process, the amount of Catechin in the solution was measured by HPLC equipment. Catechin was detected at retention time 10.567 minutes after injection in C18 column. Standard HPLC curve in different concentration of pure Catechin was prepared (Figure 5) and the binding capacities were calculated.

As illustrated in Figure 5, the regression of the curve is 0.974, which shows an unexpected deviation from the straight line [28].

3. RESULTS AND DISCUSSION

3. 1. Measurement of the Binding Capacity for MIPs
The amount of the polymer and the volume of solution in each loading was 10 mg and 20 ml, respectively, and loading was continued for two hours at the ambient temperature and in a batch system. The loading solvent at all loadings was distilled water-acetonitrile (1:1 volume ratio). Both the adsorption of the filtered solution after loading process and adsorption of catechin solution in feed (before loading) was measured by UV spetrometer at a wavelength of 278 nm. The calculations and final results are summarized in Table 2. For evaluating of the MIPs, the same steps was performed using NIPs particles [29]. Measurement of the binding capacity was performed three times, and the results were relatively identical in all replicates.

TABLE 2. Evaluation of the binding capacity on MIPs and NIPs

Feed (ppm) (before loading)	Polymer	Feed (ppm) after loading	Binding capacity (mg/g)	Extraction percentage	IF
1000	MIP	897	206	10.3	2.28
	NIP	955	90	2.2	
750	MIP	492	**516**	34.4	2.41
	NIP	595	310	20.6	
600	MIP	493	310	25.8	2.50
	NIP	538	124	10.3	
500	MIP	401	198	19.8	2.47
	NIP	460	80	8	
400	MIP	356	88	4.4	2.44
	NIP	382	36	1.8	
250	MIP	221	58	2.9	1.98
	NIP	235	30	1.5	

The absorption curves of MIPs and NIPs showed that the utmost binding capacity was 516 (mg.g^{-1}) for MIPs which occurred at a concentration of about 750 ppm (Figure 6). This difference in adsorption implies the presence of specific binding sites in the MIPs for catechin, that indicates the nanoporous MIPs was well synthesized.

3. 2. Selectivity Analysis of the MIPs With Quercetin
At first, standard curve was obtained for different concentration of quecetin in acetonitrile-water solvent (1:1v/v). Measurement was carried out by UV spectrometer at 370 nm (Figure 7).

Selectivity analysis of quercetin was performed using a 750 ppm quercetin solution, because the synthesized MIPs showed the best binding capacity in this feed conentration. Quercetin had a good absorbance at the wavelength of 370nm, so the quantity of quercetin was measured in this wavelength while acetonitrile and
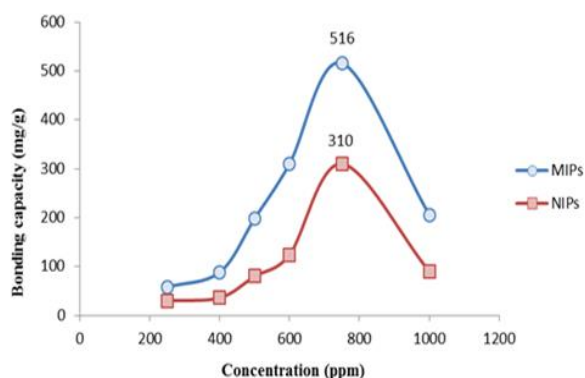
distilled water has no absorbance. The related results were summarized in Table 3.

At a concentration of 750 ppm, the imprinted polymer with catechin had a binding capacity of 440 mg.g^{-1} and 84 mg.g^{-1} for catechin and quercetin, respectively. This indicates a high selectivity of the synthesized polymer imprinted the specificity of nanopores created within the AA-based molecular imprinted polymer network.

3. 3. Evaluation of the Polymers Based on Adsorption-Desorption Analysis
Based on the adsorption-desorption analysis by nitrogen gas, the specific surface area in MIPs was 45.5, while the specific surface area in NIPs was 42.2. These values indicated that the imprinting of the polymers was desirable. The data in Table 4 presents the formation of nanopore molecular imprinted polymers. We found that MIPs had both a larger cavity volume and diameter than NIPs which indicates that MIPs had a higher specific absorption to catechin compared to NIPs. Also, based on the hole classification of the IUPAC[1], the mesopores are compounds with diameters between 2 - 50 nm. Therefore, according to the obtained average diameter of the cavities, the synthesized MIPs can be classified in the mesopores group.

3. 4. Morphology Study
Imaging by scanning electron microscopy (SEM) proved the spherical and almost uniform shape of the particles in nanometric size. According to Figure 8, the particle size of the MIPs with diameter about 142 nm was observed.

3. 5. Infrared (IR) Spectroscopy
Infrared spectroscopy for pure catechin, NIPs, leached (after elution) and un-leached (before elution) of the MIPs was carried out by Fourier transform infrared (FTIR)

Figure 6. The variation of the binding capacity of the polymers *Vs* concentrations of Catechin

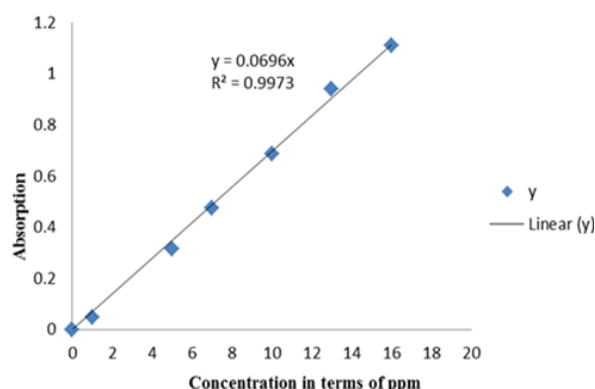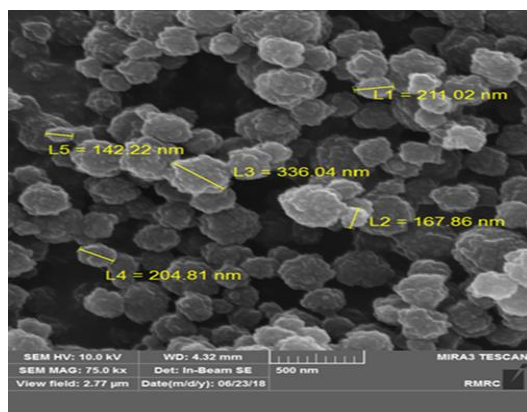**Figure 7.** Standard curve for quercetin in distilled water/acetonitrile (1:1 v/v) (Peak surface area *vs* concentration (ppm))

TABLE 3. Selectivity results with quercetin molecule for the synthesized MIPs base on Catechin template

Before loading (ppm)	Loading solution	After loading (ppm)	Binding capacity (mg.g^{-1})	(K_d)	Selectivity factor (α)
750	Catechin	530	440	0.83	7.54
	Quercetin	708	84	0.11	

TABLE 4. BET analysis for MIPs and NIPs

Polymer	Special surface area (m^2.g^{-1})	Volume of the cavities (cm^2.g^{-1})	Mean diameter of the cavities (nm)
MIPs	45.521	0.049	4.338
NIPs	42.206	0.044	4.203

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[1] International Union of Pure and Applied Chemistry

Figure 8. Imaging by scanning electron microscopy (SEM)

Figure 9. FTIR spectrum of the MIPs (after and before elution), NIPs and pure catechin molecule

spectroscopy at the frequency of 500-4000 cm^{-1} by potassium bromide (KBr) salt, and recorded infrared (IR) spectra are shown in Figure 9. Although particles exhibited similar peaks due to having the same functional groups (such as CO, -OH, and carbonyl C=O), which indicates the similarity in the solid structure of the polymers, the difference between the IR spectra of the compounds was expected. A band related to the OH group of carboxylic acids (AA) was recorded at the frequency of 3000 cm^{-1}, while this band for pure catechin (related to phenol groups of catechin) was more elongated and broad in the range of 3200-3550 cm^{-1}. Due to the presence of common functional groups in MIPs before elution, on top of the functional groups related to the polymer structure resulting from the catechin, some peaks like the peak at 3000 cm^{-1} were wider and stronger. The peak related to C=C in the aromatic ring of catechin appeared at a frequency of about 1650 cm-1, and the ester C=O peak was recorded at the frequency range of 1735-1750 cm^{-1}. This peak exists in catechin and un-leached MIPs spectrum while it was omitted in the leached MIPs. Also the related peak is absent in NIPs spectrum. It means that, after removal of catechin from the MIPs, most of catechin will be removed, so this peak will be disappeared in IR-Spectrum. Absorbance in 1750cm-1 indicates steric carbonyl group which should not be found in catechin spectrum whereas the other spectrum involve this peak. However, the carboxylic acid C=O peak was expected to appear at the 1780-1710 cm^{-1} range, but due to its proximity to OH groups and intermolecular hydrogen bonds, this peak appeared at a lower frequency of about 1670 cm^{-1} [30].

Frequency peaks at 2370 cm^{-1} are related to the asymmetric tensile frequency of CO2 molecules present in the air and combine with the sample during the formation of KBr tablets [31]. The most obvious difference between the IR spectra of the catechin molecule and other polymeric compounds originated from the C=C bond in the catechin. In the catechin spectrum, this absorption was observed at a frequency of about 1660 cm^{-1} and was expected to appear in the MIPs spectrum before elution. However, it was hidden due to the presence of a peak at about 1670 cm^{-1} that belonged to the carboxylic group (C=O). This group, due to the presence of hydrogen bonds, had resonance [32].
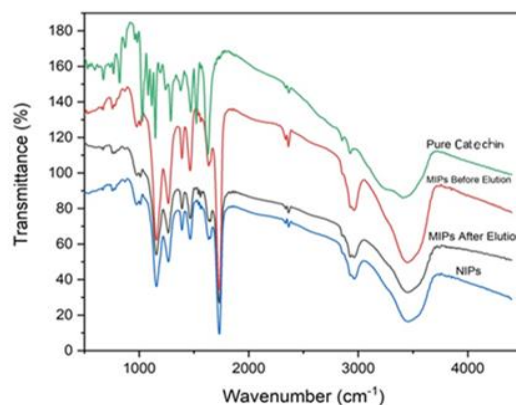
**3. 7. Evaluation of the Synthesied MIPs for Separation of Catechin from Natural Extract of Green Tea**   The amount of the absorbed catechin by MIPs was measured base on comparing of the chromatogram in before and after loading process [33-34]. Figure 10 shows the related chromatogram of green tea extract before loading on the MIPs. In this figure, catechin was appeared in retention time close 10.567 minutes. Figure 11 shows the related chromatogram of green tea extract after loading on the MIPs. In this figure, catechin was appeared at retention time of close to10.767 minutes.

The related peak of the surface area belong to catechin was sumarized in Table 5.

According to the standard curve in Figure 5, the binding capacity of the MIPs was calculated about 14.07 mg.g-1. It means that the adsorption of catechin by nanoporous MIPs was carried out succesfully.
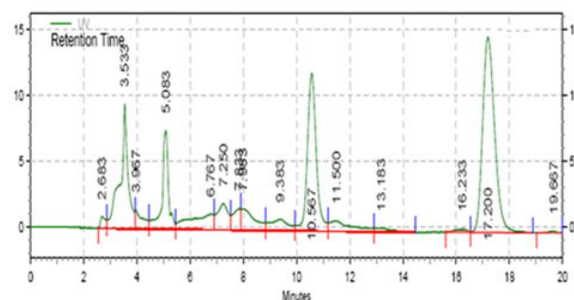
Figure 10. HPLC Chromatogram of green tea extract before loading
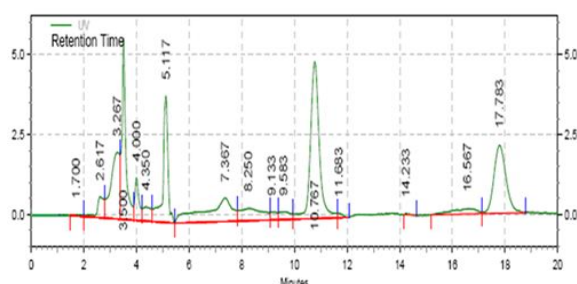
Figure 11. HPLC Chromatogram of green tea extract after loading

TABLE 5. HPLC results for natural extract of Green tea in loading process on MIPs

Green Tea Extract	Catechin (*ppm*)	Peak surface area (*mAU*)	Binding capacity (Q_e) (mg.g^{-1})
Before loading	23.57	278244	-
After loading	8.81	115212	14.07

4. CONCLUSION

Recently, the researches for separation of the bioactive compounds from natural extracts of medicinal plants or removal of the hazardous compounds from water has been increased. MIPs is one of the most suitable methods (adsorbent) with high selectivity in this regards. In this study, a highly stable and selective adsorbent was successfully synthesized according to precipitation polymerization reaction, in a molecular ratio of 1:12:12 for the first time. The results confirmed a good binding capacity of the synthesized MIPs with high selectivity for catechin molecule. Due to its low cost, relatively easy synthesis, high stability and selectivity, this technique has being developed in the production of enzymes, hormones, sensors, development of isolation and diagnostic methods, drug delivery, water purification, environmental chemistry, etc. Since, herbal plants contain a lot of the bioactive compounds which are very effective in treatment of the human diseases like cancer, it is nessesary to find a suitable method for separation of these compounds and pre-concentration of them. Results of this research indicated that, MIPs technique can be suggested for extraction and pre-concentration of the bioactive compounds from medicinal plants.

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Persian Abstract

چکیده

هدف از این مطالعه سنتز پلیمرهای قالب مولکولی و ارزیابی آن برای استخراج مولکول کاتچین می باشد. کاتچین یک ترکیب زیست فعال است که به وفور در چای سبز یافت می‌شود. در این مقاله پلیمرهای قالب گیری شده مولکولی (MIPs) به روش واکنش پلیمری شدن رسوبی در حضور کاتچین، آکریلیک اسید، تری متیلول پروپان تری متاکریلات به ترتیب به عنوان مولکول هدف، منومر عاملی، اتصال دهنده عرضی به نسبت (۱:۱۲:۱۲)، سنتز گردید. ریخت شناسی سطحی پلیمر با استفاده از میکروسکوپ الکترونی روبشی (SEM) ذرات کروی پلیمر با ابعاد نانومتری را نشان داد. مطالعه پیوندهای شیمیایی موجود در ذرات پلیمر با استفاده از طیف سنجی تبدیل فوریه مادون قرمز(FTIR) از تسخیر شدن مولکول کاتچین در داخل پلیمر قالب گیری شده، دلالت دارند. تخلخل سنجی پلیمر، با استفاده از روش برونر، امت، تلر (BET) مورد تجزیه و تحلیل قرار گرفت. براساس این آنالیز، سطح ویژه در پلیمرهای قالب گیری شده برابر ۴۵.۵ m².g⁻¹ بوده در حالی که این مقدار در پلیمرهای قالب گیری نشده (NIPs) برابر ۴۲.۲ m².g⁻¹ بوده است. این بدان معناست که قالب گیری در پلیمر به خوبی انجام شده است. همچنین ویژگی‌های جذب سطحی پلیمر نیز انجام شد. پلیمر قالب گیری شده در محلول بارگذاری با غلظت ۷۵۰ppm، دارای بیشترین ظرفیت اتصال برای کاتچین به مقدار ۴۴۰mg.g⁻¹ بوده است درحالیکه ظرفیت اتصال برای مولکول کوئرستین (مولکولی با ساختار مشابه کاتچین) برابر ۸۴mg.g⁻¹ بوده است. این موضوع بیانگر آنست که فناوری قالب گیری مولکولی می‌تواند به عنوان یک گزینه خوب با گزینش پذیری بالای پلیمر در فرآیند جداسازی معرفی شود. همچنین اندازه گیری ظرفیت اتصال پلیمرهای قالب‌گیری شده بر روی عصاره طبیعی چای سبز با استفاده از دستگاه کروماتوگرافی مایع با عملکرد بالا(HPLC) انجام پذیرفت که نتایج مشابه حاصل شد. بر اساس نتایج اشاره شده، جداسازی و تغلیظ ترکیبات زیست فعال از عصاره گیاهان دارویی با استفاده از فناوری قالب گیری مولکولی، پیشنهاد می گردد.