Extraction of Catechin as a Flavonoid Compound via Molecularly Imprinted Polymers

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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 hydroxyl 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, 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 safy. 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 schema 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 polymerization) 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 summarized 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 QTDS1730 (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](image)

**Table 1.** The used Chemical materials in synthesis of MIPs

| Chemical material | Function | Producer |
|-------------------|----------|----------|
| Quercetin         | Similar structure for template molecule | Sigma-Aldrich |
| Catechin          | Template molecule | Merck |
| Acrylic acid      | Functional monomer | Merck |
| Trimethylolpropano trimethacrylate | Cross-linker | Sigma-Aldrich |
| Azobisobutyronitrile | Initiator | Sigma-Aldrich |
| Acetonitrile      | Porogene | Sigma-Aldrich |
| Methanol/Acetic acid | Elution | Merck |
| Acetone           | Solvent | Sigma-Aldrich |
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 ($Q_e$) of the adsorbent (MIPs) was defined as the difference of the initial ($C_0$) and final ($C_e$) amount (ppm) of catechin in the solution multiple loading value ($V$) over the amount of the used adsorbent (g), based on Equation (1):

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

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

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

$$IF = \frac{Q_{MIPs}}{Q_{NIPs}}$$

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):

$$% \text{Extraction} = \frac{C_e - C_f}{C_0} \times 100$$

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 ($\alpha$)
For measuring selectivity factor ($\alpha$), at first distribution coefficient ($K_d$) of the template should be calculated. Distribution coefficient is introduced by Equation (4):

$$K_d = \frac{C_t}{C_s}$$

where $C_t$ is concentration in the template and $C_s$ is concentration in the solution.
$K_d = \frac{Q_e}{C_e}$ \hspace{1cm} (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 ($\alpha$) of MIPs is an important parameter that establishes the selectivity of the polymers. Selectivity factor is defined as Equation (5):

$$\alpha = \frac{K_d(\text{catechin})}{K_d(\text{quercetin})}$$ \hspace{1cm} (5)

where $K_d(\text{catechin})$ and $K_d(\text{quercetin})$ are distribution coefficients of catechin and quercetin, respectively. Utmost measure of $\alpha$, introduces 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 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 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 spectrometer 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.

| 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 quercetin 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 concentration. 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 IUPAC¹, 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](image1)

![Figure 7. Standard curve for quercetin in distilled water/acetonitrile (1:1 v/v) (Peak surface area Vs concentration (ppm))](image2)

| 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) | Selectivity factor (α) |
|-----------------|-----------------|-----------------|--------------|-----------------|
| 750 | Catechin | 530 | 440 | 0.83 | 7.54 |
| Quercetin | 708 | 84 | 0.11 | 1.1 |

| TABLE 4. BET analysis for MIPs and NIPs |
|-----------------|-----------------|-----------------|-----------------|
| Polymer | Special surface area (m²·g⁻¹) | Volume of the cavities (cm³·g⁻¹) | Mean diameter of the cavities (nm) |
|-----------------|-----------------|-----------------|-----------------|
| MIPs | 45.521 | 0.049 | 4.338 |
| NIPs | 42.206 | 0.044 | 4.203 |

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 Synthesized 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 to 10.767 minutes.

The related peak of the surface area belong to catechin was summarized 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
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.

4. CONCLUSION

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