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Research Article

Extraction of crocin from saffron (*Crocus sativus*) using molecularly imprinted polymer solid-phase extraction

A new molecularly imprinted polymer for extraction of crocin from saffron stigmas was prepared using gentiobiose (a glycoside moiety in crocin structure) as a template. Crocin binding to gentiobiose imprinted polymer (Gent-MIP) was studied in comparison with a blank nonimprinted polymer in aqueous media. Affinity of the Gent-MIP for the crocin was more than the nonimprinted polymer at all concentrations. In Scatchard analysis, the number of binding sites in each gram of polymer (maximum binding sites) and dissociation constant of crocin to binding sites were 18.4 μmol/g polymer and 11.2 μM, respectively. The Gent-MIP was then used as the sorbent in an SPE method for isolation and purification of crocin from methanolic extract of saffron stigmas. The recovery of crocin, safranal and picrocrocin was determined in washing and elution steps. The Gent-MIP had significantly higher affinity for crocin than other compounds and enabled selective extraction of crocin with a high recovery (84%) from a complex mixture. The results demonstrated the possibility of using a part of a big molecule in preparing a molecularly imprinted polymer with a good selectivity for the main structure.

Keywords: Affinity / Crocin / Molecularly imprinted polymer / SPE
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1 Introduction

The preparation of specific recognition sites for molecules has become a major interest and main objective of many researchers in recent years [1–8]. Molecular imprinting is a simple method for synthesis of a specific binding site by radical polymerization of monomers in the presence of a suitable template molecule. Such an MIP could be applied as the sorbent in SPE [4, 6, 9, 10], as the stationary phase in HPLC [11, 12], as the receptor layer in biosensors [13, 14] and as the new drug delivery system [7, 15]. To date, the imprinting of small molecules has been well established and considered almost routine. However, the imprinting of biomacromolecules such as proteins continues to be a significant challenge due to the difficulties with large molecular size, structural complexity, environmental sensitivity of the templates and significantly reduced noncovalent template–monomer interactions in aqueous media [16, 17]. To overcome these limitations, stable short peptide sequences, representative of an accessible fragment of a larger protein of interest can be used. Therefore, if the material can recognize a peptide that represents the exposed part of a protein structure, it will be capable of binding the entire protein as well [18].

Saffron is the dried stigmas of *Crocus sativus* with many different uses as drug, textile dye and culinary adjunct [19]. Saffron and its major active product (crocin), have many therapeutic properties such as antitumoral [20, 21], antioxidant [22], anxiolytic [23, 24], neuronal protective [25], anti-ischemic [22, 26] and protective against DNA damage [27] activities. Crocins are unusual water-soluble carotenoids mono- and di-glycosyl esters of crocetin. Picrocrocin, a colorless glycoside, is the main substance responsible for the bitter taste in saffron. Safranal is responsible for the saffron odor and aroma [19]. Six types of crocins have been detected in saffron extract [19, 28, 29]. All of them are glycoside esters of crocetin. The major component is α-crocin that is a digentiobiosyl ester of crocetin. In other types of crocins, one or two D-glucose with or without gentiobiose occur as carbohydrate residues [19]. Due to its therapeutic properties, introducing a selective extraction method for crocin would be a valuable help in its isolation and purification from saffron stigmas. Therefore, the aim of this study was to prepare a selective MIP for the extraction of total crocin from saffron stigmas.

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Abbreviations: B_{max}, maximum binding sites; **BF,** binding factor; **Gent-MIP,** gentiobiose imprinted polymer; K_D, dissociation constant; **MISPE,** molecularly imprinted SPE; **NIP,** nonimprinted polymer

Crocin is a big and flexible molecule. Its structure is unstable in polymerization condition (50°C or UV). Therefore, it is impossible to use crocin as a template in molecular imprinting procedure. To overcome these limitations, we decided to use a moiety of its structure (gentiobiose) as the template (Fig. 1) in polymerization. The gentiobiose is a disaccharide and insoluble in organic solvents (aprotic solvents are necessary as the porogen in molecular imprinting). Due to having two D-gentiobiose moieties and significantly higher percent in total crocins (60%) [19], α-crocin was considered as the main component in total crocin. The results were promising and demonstrated the possibility of using a part of a molecule in preparing molecular imprinting with a good selectivity for the main molecule.

2 Materials and methods

2.1 Materials

Crocin was crystallized in our laboratory from ethanol (80%) at −5°C and used as a standard for analysis. About 10 g of saffron stigmas powder was suspended in 25 mL ethanol 80% at 0°C and shaken by vortex for 2 min. After centrifugation at 4000 rpm for 10 min, the supernatant was separated and held at −5°C in darkness. In total, 25 mL of ethanol 80% was added to sediment and the process done again. This extraction stage was repeated several times. The total volume of solvent consumption for 10 g saffron stigmas in extraction process was 200 mL. The resulting solution was kept in a thick-walled glass container at −5°C for 24 days in darkness. The crocin crystals were separated from solution and washed with acetone to remove remaining water. The amount of crystals obtained was 1.7 g. The crystals were dissolved in 120 mL of 80% ethanol kept at −5°C in darkness for extra 20 days. The final amount of crystals obtained at this stage was 1.02 g.

Saffron was purchased from Novin Saffron (Iran). Gentiobiose, methacrylamide, ethylene glycol dimethacrylate, DMSO and safranal were obtained from Sigma-Aldrich (Milwaukee, WI, USA). 2,2′-Azo-*bis*-iso-butyronitrile was purchased from Acros (Geel, Belgium). All solvents used were of HPLC grade. The structures of chemicals used or assayed in this study are shown in Fig. 2.

2.2 Preparation of gentiobiose imprinted polymer

To prepare the gentiobiose imprinted polymer (Gent-MIP), gentiobiose (0.5 mmol) and methacrylamide (4 mmol) were dissolved in DMSO (10 mL) and the solution was incubated at room temperature for 10 min. Then, ethylene glycol

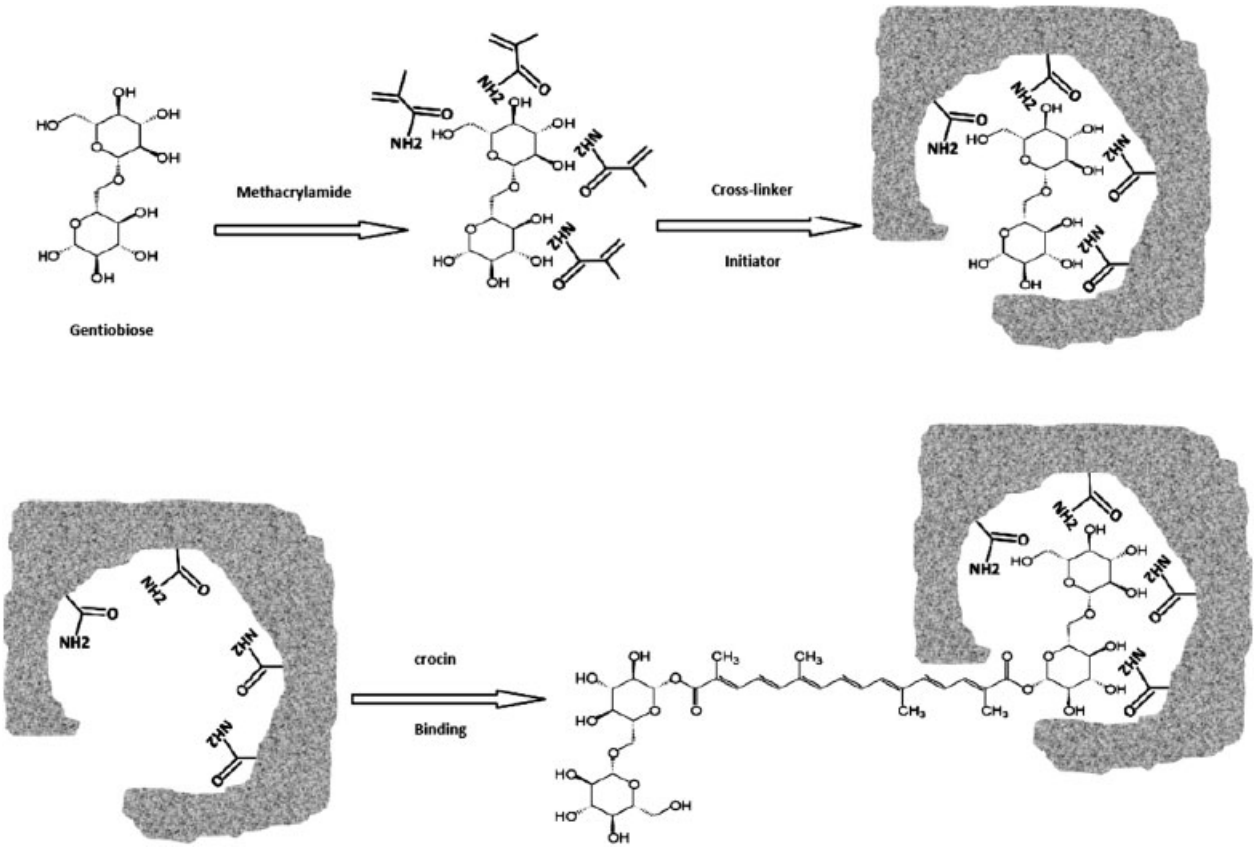

Figure 1. Schematic representation of Gent-MIP preparation and binding of crocin to binding site.

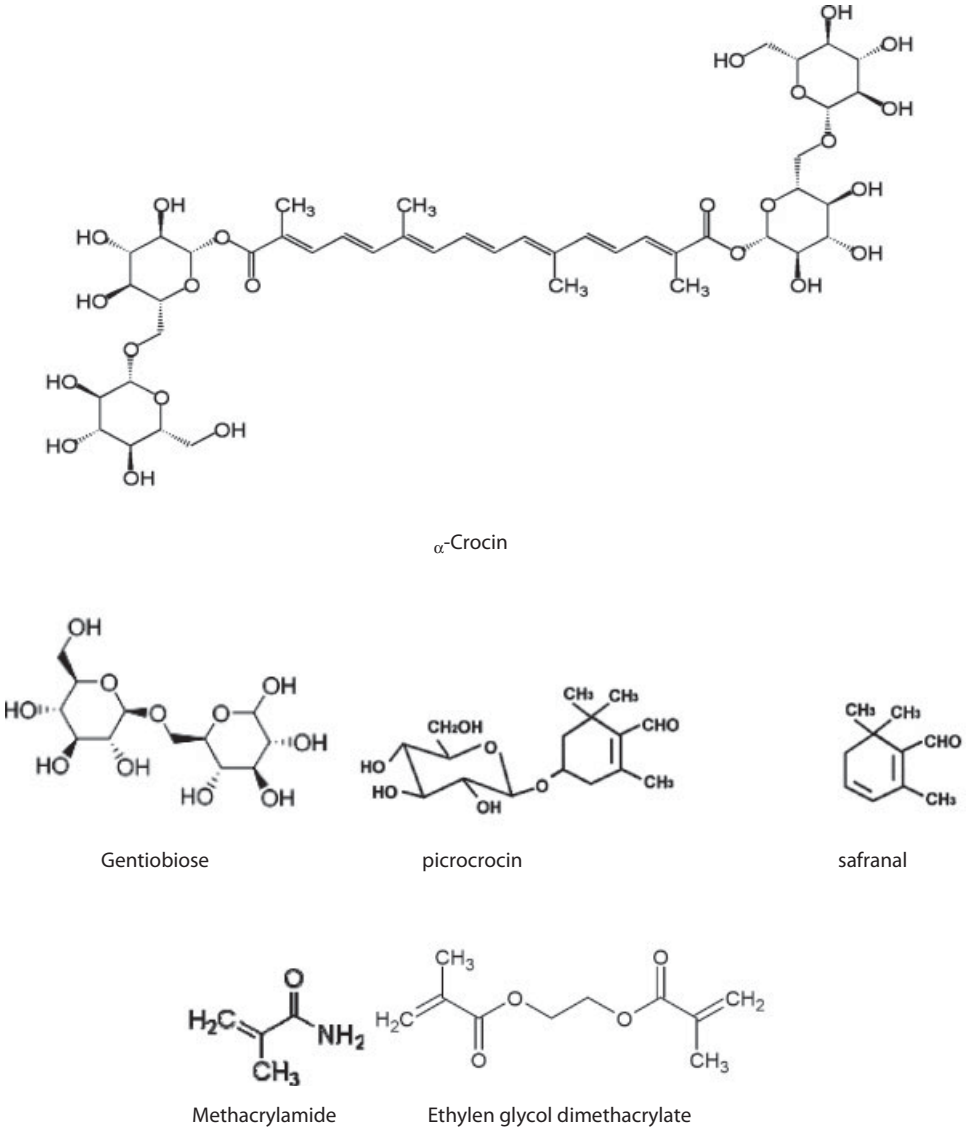

Figure 2. Structure of compounds used in this study.

dimethacrylate (20 mmol) and 2,2′-azo-*bis*-iso-butyronitrile (10 mg) were added. This solution was sparged with oxygen-free nitrogen for 10 min. The tube was sealed under nitrogen stream and heated at 50°C for 24 h to complete radical polymerization. The polymer obtained was ground and passed through a 200-mesh sieve (particles less than 75 μm). A blank polymer (nonimprinted polymer, NIP) was synthesized, in the absence of gentiobiose, following the same procedure as described above. The Gent-MIP and NIP particles were washed several times with methanol/acetic acid (80:20, v/v) mixture, followed by methanol. The polymer particles were dried at 45°C for 24 h.

2.3 Batch adsorption assay

Dry Gent-MIP or NIP (10 mg) was incubated in 2 mL aqueous solution of crocin and was shaken at room temperature for 1 h. After centrifugation (14000 rpm for 5 min), 1 mL of supernatant was diluted with deionized water and assayed with UV–Vis spectrophotometer at 440 nm (λ_{max} of crocin). The amount of crocin bound to the polymers was calculated by subtracting the free crocin after equilibration from the initial amount. Each test was carried out four times and mean ± SD was reported.

The binding factor (BF) was calculated in each solvent according to the following equation:

$$BF = \frac{K_{MIP}}{K_{NIP}} \quad (1)$$

where K was the partition coefficient for each polymer and calculated in Eq. (2):

$$K = \frac{\text{bound crocin/gpolymer}}{[\text{Free}]} \quad (2)$$

where [Free] was the crocin concentration in solution after equilibrium and bound crocin was the amount of crocin bound *per* gram dry polymer. When the crocin concentration was varied in solution, a Scatchard plot was constructed according to Eq. (3):

$$\frac{\text{Bound}}{[\text{Free}]} = -\frac{\text{Bound}}{K_D} + \frac{B_{max}}{K_D} \quad (3)$$

where Bound was the amount of crocin bound to Gent-MIP at equilibrium; [Free] was the crocin concentration at equilibrium; B_{max} was the maximum binding sites and K_D was the dissociation constant. The values of K_D and B_{max} could be calculated from the slope and intercept of the straight line derived from a plot of Bound/Free *versus* Bound.

2.4 Optimization of molecularly imprinted SPE procedure

Briefly, 60 mg of Gent-MIP in 3 mL methanol was slurry packed into an empty polypropylene cartridge. The column was washed several times with methanol and finally conditioned with 2 mL ACN. Crocin (50 μg) in 50 μL methanol was loaded onto the column. For safranal, 50 μL of its methanolic standard (75 ng/mL) was loaded onto the column and for picrocrocin, 50 μL of methanolic extract of saffron stigmas was loaded onto the Gent-MIP column. After each loading, the column was washed with different volumes of ACN and finally eluted with 10 mL methanol. To prepare a methanolic solution of saffron extract, 200 mg of saffron stigmas powder was macerated in 10 mL methanol and was shaken, continuously, in darkness for 48 h. After centrifugation, the supernatant was used for the analysis of compounds and extraction of crocins.

2.5 Analysis of crocin and other compounds

Total crocin determination was carried out by UV–Vis spectrophotometer (UV-1700 Pharmaspec model) from Shimadzu (Japan). Chromatographic determination of components was carried out on a Younglin (South Korea) Acme 9000 system, consisting of SP930D solvent delivery module, SDV50A Solvent Mixing Vacuum Degasser, Column Oven CTS30, UV730 Dual Wavelength UV/VIS detector and ODSA C18 (4.6 × 250 mm, 5 μm) column. The data analysis was performed by Autochro-3000 software. The injection volume was 20 μL, the flow rate was 0.5 mL/min and the column temperature was fixed at 30°C. A gradient method was used for chromatographic determination of crocins and picrocrocin. The mobile-phase composition was changed linearly from 20 to 80% ACN in water at 20 min. For the analysis of safranal, an isocratic method was used and mobile-phase composition was 76% ACN in water [30]. For the determination of crocins, safranal and picrocrocin, the UV detector was set to 440, 308 and 250 nm, respectively.

3 Results and discussion

3.1 Gentiobiose as a template

Crocin is a big, flexible and unstable molecule in polymerization condition. Therefore, it could not be used as a suitable template in molecular imprinting. As described before, some researchers have used epitope approach to synthesize a selective polymeric binding site for large peptides [18]. Gentiobiose is a disaccharide in crocin structure and is stable during polymerization process. But, insolubility of this carbohydrate in organic solvents was another problem. An effective noncovalent molecular imprinting is usually carried out in organic solvents with minimum solvent–monomers and solvent–template interactions. In aqueous media, noncovalent interactions between monomers and template are weak. In a study by Okutucu *et al.*, molecular imprinting was done in DMSO to prepare a binding site for galactose as a carbohydrate [31]. Thus it seemed that DMSO could be a good medium for noncovalent molecular imprinting for carbohydrates. Due to its small size and stability in polymerization procedure, gentiobiose was used as the template and because of good solubility in DMSO it was selected as the solvent in polymerization procedure.

3.2 Binding study

3.2.1 Best solvent composition for binding study

Due to stronger hydrogen interactions between template and binding sites, the binding study is usually carried out in organic solvents [4, 32]. But the solubility of crocin in nonaqueous solvents is poor. Thus, binding tests were performed in aqueous solvents. The values of bound crocin to polymers were dependent on binding properties of polymers and indicated their affinity for binding crocin. The adsorption measurements in water (Table 1) showed that binding of crocin to Gent-MIP was more than to NIP ($p = 0.01 < 0.05$). This difference suggested that there were binding sites in Gent-MIP for gentiobiose moiety of crocin. Acidifying the water with acetic acid increased the difference between Gent-MIP and NIP in binding crocin,

Table 1. Binding of crocin (100 μg/mL) to Gent-MIP and NIP in different solvents ($n = 4$)[a)]

solvent	BF $K_{Gent\text{-}MIP}/K_{NIP}$	Bound crocin (mg/g NIP)	Bound crocin (mg/g Gent-MIP)
Water	1.3	14.5 ± 0.7	15.5 ± 0.3
Acetic acid 2%	1.6	11.5 ± 0.6	13.0 ± 1.0
Acetic acid 5%	1.3	8.6 ± 0.4	9.9 ± 1.1
Acetic acid 10%	1.0	7.3 ± 0.9	7.4 ± 1.4

a) Each data represent mean ± SD.

but this increase depended on the amount of acetic acid in solution. The most difference was seen at 2% ($p = 0.004 < 0.05$) and the least was at 5% acetic acid in water ($p = 0.04 < 0.05$). At 10% there was not any difference between Gent-MIP and NIP ($p = 0.47 > 0.05$).

These data and values of BF (Table 1) indicated that adding acetic acid, in little amounts (2%), increased the crocin solubility and breaking of the crocin–polymer bonds. But the chance of breaking of crocin–NIP was more than that of crocin–Gent-MIP bonds. Increasing the acetic acid from 2 to 5 and 10% caused a decrease in BF. Therefore, acidifying the water beyond 2% acetic acid increased the chance of breaking of bonds in both polymers. Hence, 2% acetic acid in water was selected as the solvent for binding study and Scatchard analysis.

3.2.2 Scatchard analysis

The conventional batch adsorption method involves the incubation of ligand solution with polymer at different concentrations and measurement of ligand bound to polymer after a fixed time [4, 33, 34]. In this study, binding of crocin to Gent-MIP was more than NIP at all concentrations. From the Scatchard plot (Fig. 3) and equation ($Y = -0.0891X + 1.6396$, $R^2 = 0.9634$), K_D and maximum binding capacity (B_{max}) were 11.2 μM and 18.4 μmol/g Gent-MIP, respectively. The K_D-value obtained in other studies ranged from low micromolar to molar (lamotrigine K_D = 16.6 μM [4], sulfamethoxazole K_D = 18.8 μM [11], theophylline K_D = 1.5 M [35]). The small K_D in this study (11.2 μM) was in the lower end of this range and indicated a high affinity of Gent-MIP for binding crocin.

3.3 Optimization of MISPE

The aim of this study was the extraction of crocin from its methanolic solution. Thus, methanol was selected as the loading solvent. In washing step, a solvent should be applied which yields the maximum selectivity and recovery of crocin. Several solvents (methanol, THF, water and ACN) were tested for this purpose. When methanol and THF were used as washing solvents, a high percentage of crocin was removed from Gent-MIP column. It meant that these two solvents were not suitable for washing step in extraction process. Also, many compounds of saffron, *e.g.* safranal, are insoluble in water. Thus, methanol, THF and water were not suitable washing solvents. However, after washing the cartridge with 3, 4 and 5 mL ACN only 9, 16 and 26% of total crocin were removed from Gent-MIP. The data for safranal were 97, 100 and 100% and for picrocrocin were 86, 88 and 93.5%. Therefore, ACN washed maximum amount of safranal and picrocrocin, whereas a small amount of crocin was removed. Thus, optimized molecularly imprinted SPE (MISPE) condition was as follows: washing condition, 4 mL ACN; elution condition, 10 mL methanol.

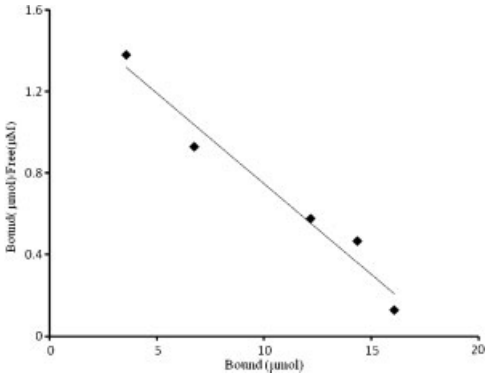

Figure 3. Scatchard plot of binding of crocin to the Gent-MIP. Gent-MIP (10 mg) was incubated with different concentrations of crocin in 2% acetic acid at room temperature for 1 h.

3.4 Validation of MISPE procedure

The extraction procedure was validated using MISPE process as described in Section 2.4 for the extraction of crocin from saffron. After washing with 4 mL ACN and elution with 10 mL methanol, the recovery of total crocin was 84%. In this condition, the amount of safranal and picrocrocin in final solution was 0 and about 10%.

Figures 4–6 show typical chromatograms of solutions before and after extraction by MISPE. These data showed that the optimized MISPE was practically efficient in the

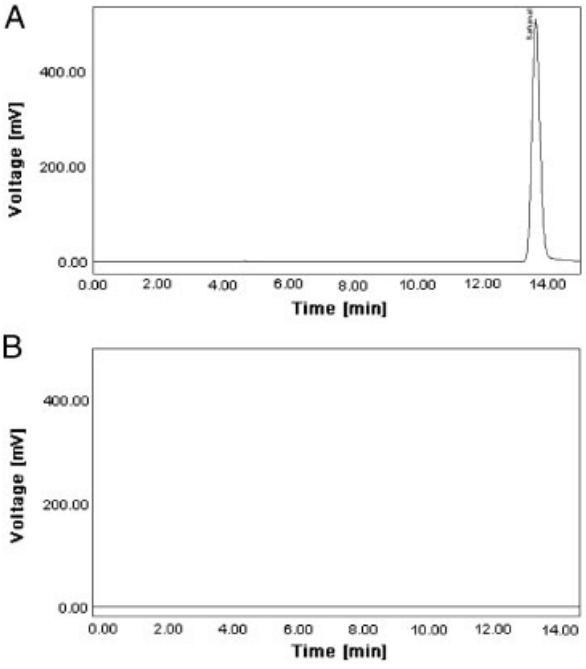

Figure 4. Chromatogram of a methanolic solution of saffranal, before (A) and after (B) MISPE procedure at 308 nm (λ_{max} of safranal). Washing solvent: 4 mL ACN and elution solvent: 10 mL methanol. Mobile phase for an isocratic method: 76% ACN in water.

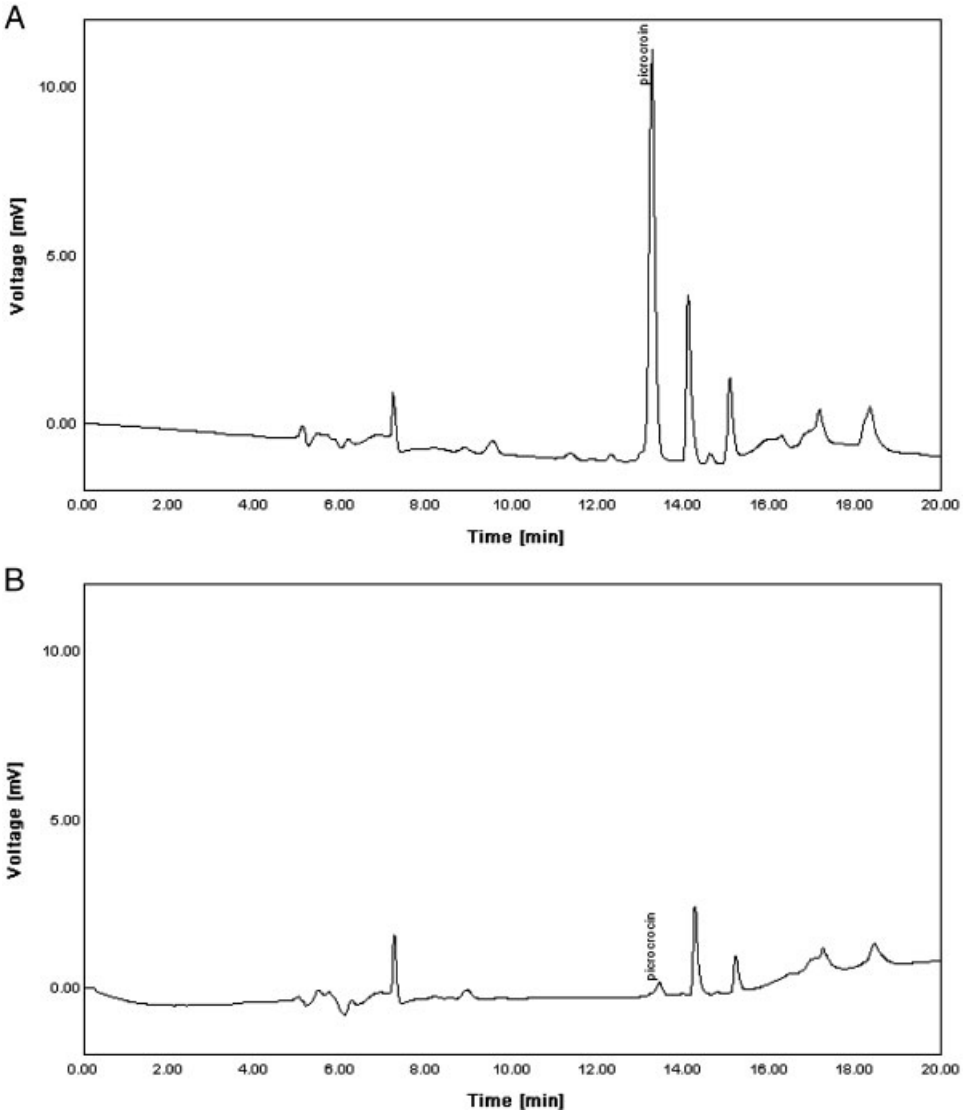

Figure 5. Chromatogram of a methanolic solution of saffron extract before (A) and after (B) MISPE procedure at 250 nm (λ_{max} of picrocrocin). Washing solvent: 4 mL ACN and elution solvent: 10 mL methanol. Mobile phase for a gradient method: The mobile phase composition for the gradient method was changed linearly from 20 to 80% ACN in water at 20 min.

extraction of total crocin from saffron. The HPLC analysis of methanolic solutions before (Figs. 4A, 5A, 6A) and after (Figs. 4B, 5B, 6B) MISPE procedure indicated that the recoveries of safranal and picrocrocin were 0 and 10%, respectively. But the data for total crocin were about 84%. It meant that, in washing step, safranal (100%) and picrocrocin (about 90%) were removed from polymer, whereas only 16% of crocin was washed with 3 mL ACN. Therefore, the Gent-MIP could selectively bind and extract crocin from saffron stigmas.

Some workers have used MISPE as a technique for the extraction of chemicals from biological sources. In a research, Claude *et al*. reached to 70% recovery in the extraction of betulin and betulinic acid from plane bark [36]. In a study, mean recovery of bisphenol A in MISPE procedure from milk was 81% [37] and the data for extraction of α-tocopherol from bay leaves using molecularly imprinted polymer as a sorbent were 60% [9]. In comparison with above-mentioned studies, the MISPE process in this study had a high recovery (84%) and could be used as an optimized method for the extraction of crocin from saffron.

The recovery of α-crocin in 3, 4 and 5 mL of ACN (in washing step) was less than other types of crocin (Table 2). Consequently, its recovery in elution step (in 10 mL methanol) was the most between all crocin types. Because of having two gentiobiose groups, α-crocin could bind stronger to polymer than other types and this is the reason why its waste in ACN is the least. This finding indicated that Gent-MIP synthesized in this study was not a simple resin and the existence of gentiobiose in structure of crocin was important in binding of this molecule to the polymer.

4 Concluding remarks

A molecularly imprinted polymer (Gent-MIP) was prepared using gentiobiose as the template. Crocin binding to polymer was characterized in comparison with a blank

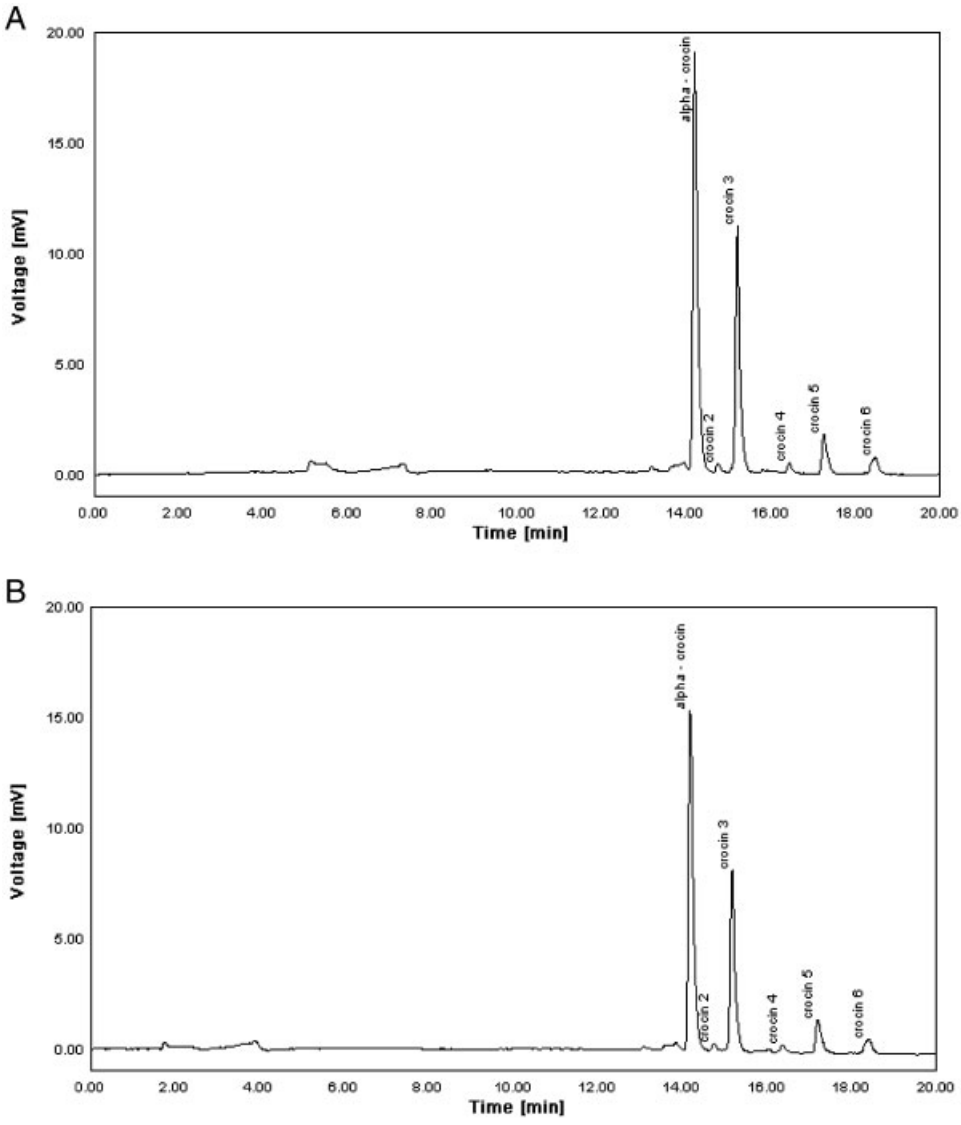

Figure 6. Chromatogram of a methanolic solution of saffron extract before (A) and after (B) MISPE procedure at 440 nm (λ_{max} of crocin). Washing solvent: 4 mL ACN and elution solvent: 10 mL methanol. The mobile-phase composition for the gradient method was changed linearly from 20 to 80% ACN in water at 20 min.

Table 2. Recovery of crocin types after percolation of ACN through a Gent-MIP column[a)]

ACN (mL) (washing solvent)	Recovery %	Recovery %	Recovery %	Recovery %	Recovery %	Recovery %
	α-Crocin (RT = 14.1 min)	Crocin 2 (RT = 14.7 min)	Crocin 3 (RT = 15.2 min)	Crocin 4 (RT = 16.4 min)	Crocin 5 (RT = 17.2 min)	Crocin 6 (RT = 18.4 min)
3	5.5	9	13	53	16	13
4	11	15	35	73	18	21
5	18	20	42	100	19	25

a) RT is the retention time of the peak in chromatograph. The mobile-phase composition for the gradient method was changed linearly from 20 to 80% ACN in water at 20 min.

nonimprinted polymer. Affinity of the Gent-MIP for crocin was studied using Scatchard analysis. The Gent-MIP was used as the sorbent in MISPE procedure for the extraction of crocin from saffron. The optimized process was applied for the extraction of crocin from methanolic extract of saffron stigmas. The recovery of safranal, picrocrocin and crocin was determined in washing and elution steps. The results indicated that the Gent-MIP prepared in this study was selective for crocin and had a high affinity for binding this compound. This study showed that the Gent-MIP was

suitable for use in MISPE of crocin with a high recovery (84%) and selectivity from saffron stigmas. The results demonstrated the possibility of using a part of a big unstable molecule (gentiobiose) to prepare a molecularly imprinted polymer with a good selectivity for the main molecule.

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The authors have declared no conflict of interest.

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