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Selective separation of active inhibitors of epidermal growth factor receptor from Caragana Jubata by molecularly imprinted solid-phase extraction

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Abstract

A feasibility research was performed to study the possibilities of using a molecularly imprinted polymer as sorbent material in solid-phase extraction for the separation of active inhibitors of epidermal growth factor receptor (EGRF) from Caragana Jubata, a Chinese traditional Tibetan medicine. A molecularly imprinted polymer using quercetin, an active anti-EGFR inhibitor (IC_{50}=15 μ*M*), as the template and acrylamide as the functional monomer was prepared. The polymer was evaluated as a selective sorbent in molecularly imprinted solid-phase extraction. The EtOAc extract of Caragana Jubata was loaded on the polymer, and two novel active anti-EGFR inhibitors were found to be selectively retained after washing the polymer with appropriate solvent to disrupt the non-specific interactions occurring between the sample and the polymer matrix, which were identified as (*E*)-piceatannol (IC_{50}=4.9 μ*M*) and butein (IC_{50}=10 μ*M*). The present work affords us a new potential method for selective separation of bioactive components from herb by using molecularly imprinted polymer as a solid-phase extraction adsorbent.

Keywords: Molecular imprinting; Solid-phase extraction; Epidermal growth factor receptor; Caragana Jubata; Pharmaceutical analysis; Quercetin; Enzyme inhibitors; Flavonoids

1. Introduction

Quercetin, a typical active compound of the flavonoid family, is one of the natural protein tyrosine kinase (PTK) inhibitors that exhibit specificity in the micromolar range [1,2]. The epidermal growth factor receptor (EGFR) belongs to the class of the trans-membrane growth factor receptor PTKs and it can be inhibited by quercetin [3]. EGFR is known to be over-expressed in a large percentage of clinical cancers of various types [4–6] and to be associated with poor prognosis [7,8]. Compounds which can specifically inhibit the tyrosine kinase activity of EGFR are therefore of potential interest as anticancer drugs, and this is an active field of drug development.

As far as we know, direct extraction or separation following by the bioassay guidance of lead compounds from herb has been an important part in the area of drug discovery. However, traditional sepa-

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ration of active components from herb is often tedious and inefficient to some extent resulting from poor affinity and selectivity of conventional materials (e.g., silica-gel, polyamide, ion-exchange types and reversed-phase column). Up to now, much research has been directed toward the variation of the sorption materials in order to achieve the selective interaction with the target molecule [9,10]. One class of such selective sorbent materials is immunosorbents (ISs), which rely upon reversible and highly selective analyte–antibody interactions [11,12]. Nevertheless, the high selectivity and low stability of ISs and the fact that it is both difficult and expensive to obtain biological antibodies, have led researchers to synthesize antibody mimics such as molecularly imprinted polymers (MIPs) in order to selectively extract target substances.

The technique of molecular imprinting, introduced in 1972 by Wulff and Sarhan [13], and much expanded by the work of Arshady and Mosbach in the 1980s [14], has been shown to be capable of producing materials with ''antibody-like'' selectivities [15,16]. Molecularly imprinted polymers are extensively cross-linked polymers containing specific recognition sites with a predetermined selectivity for analytes of interest [17–21]. The chemical and physical robustness of the MIP, in combination with the polymer's selectivity, has proven it to be a good adsorbent for molecularly imprinted solid-phase extraction (MISPE) applications [22–26].

In our previous study [27], a selective and affinitive MIP using quercetin as the template was evaluated by high-performance liquid chromatography (HPLC), and its application for extracting compounds of the flavonoids from gingko leaves was investigated. In the current work, it was elucidated that using the MIP could extract not only the flavonoids but also other types of bioactive compounds from herb, which is not difficult to be understood considering that one potential implementation of MIP is to simulate antibody or enzyme by using the vacant recognition sites complementary to the template in terms of its size, shape and functionality. From the viewpoint of the structure–activity relationship (SAR), most inhibitors against the same enzyme often adopt a similar or even common binding model and therefore are inclined to possess similar structures in terms of size, shape and functional groups. To the binding pocket of one enzyme, only those molecules belonging to a certain size (too large could not enter into the pocket and too small could not occupy the binding sites efficiently) could enter easily and retain stably and thus to be potential inhibitors. Moreover, the possible interactions between the enzyme and the inhibitors such as hydrogen bond, ionic interactions and hydrophobic effects are also utilized in the molecular imprinting. Taking these two important characteristics, steric memory (size and shape), and chemical memory (spatial arrangement of the complementary functionality) of the MIP material into account, using MIPs to extract different types of anti-EGFR inhibitors from herb, as it turned out, has proven to be efficient.

2. Experimental

2.1. Reagents and solvents

Quercetin, acrylamide, ethylene glycol dimethacrylate (EDMA), azobis(isobutyronitrile) (AIBN) were purchased from Acros Organics (Geel, Belgium). (*E*)-Piceatannol (3,5,3′,4′-tetrahydroxystilbene) and butein (2′,3,4′,4-tetrahydroxychalcone) were isolated by ourselves from Caragana Jubata and identified by the analysis of spectroscopic data. The chemical structures of these two compounds as well as the template, quercetin, are shown in Fig. 1. Before use, the EDMA was distilled under vacuum after being extracted with 10% sodium hydroxide brine and dried over anhydrous magnesium sulfate. AIBN was recrystallized from methanol, and tetrahydrofuran (THF, analytical-reagent grade) was dried by sodium and then distilled. Methanol and acetonitrile were of HPLC grade, glacial acetic acid was of analytical grade. The water was demineralized and purified by a Millipore system.

2.2. Analysis equipment

A HP1100 HPLC system containing a quaternary pump, a variable-wavelength detector, an on-line vacuum degasser and a 20-μl manual injector (Hewlett-Packard, Palo Alto, CA, USA) was used. HPLC analysis of the solutions loaded on the MISPE

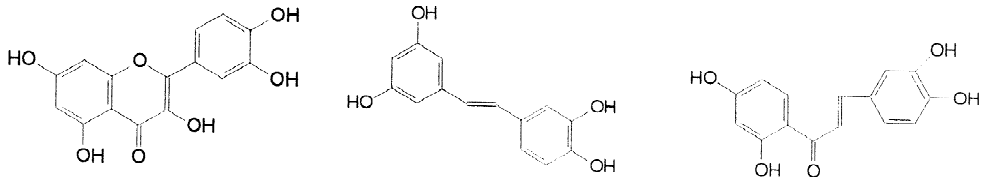

Fig. 1. The chemical structures of the template (quercetin) and two other analytes.

column as well as those eluted from the column was performed using a Zorbax Extend-C_{18} (Agilent, 150×2.1 mm) column. A Mariner time-of-flight (TOF) mass spectrometry (MS) system with an ESI (electrospray ionization) interface was used for the identification of analytes. For ESI, the detailed operated conditions were as follows: the ion polarity was negative; the nebulizer gas and curtain gas were nitrogen with flow-rates of 0.6 and 1.2 ml min^{-1}, respectively; the nozzle and quadrupole were both heated to 140 °C; the spray tip and nozzle potentials were 4800 and 100 V, respectively.

2.3. Preparation of the MISPE column

The preparation of a molecularly imprinted polymer with quercetin as the template and acrylamide as the functional monomer was described in our previous work [27]. The elution of the template was performed by repeated washing on-line in the HPLC system one by one with methanol–acetic acid (9:1, v/v), methanol, methanol–triethylamine (9:1, v/v) and then methanol until a stable baseline was obtained. The MISPE column was packed with 400 mg polymers into 4-ml polyethylene reservoirs, putting two wool-glass frits at the bottom and the top of the column. As a control, non-imprinted polymer without quercetin present during the polymerization was also prepared and washed in the identical manner, and then was packed into a blank SPE column.

2.4. Sample preparation for the extraction test

Air-dried roots of Caragana Jubata were extracted by macerating with 85% ethanol at room temperature. The solvent was evaporated in vacuum and then was partitioned between water, light petroleum, $CHCl_3$, EtOAc and *n*-butanol successively. Bioassay-guided results suggested that the EtOAc extract was efficient in inhibiting the tyrosine kinase activity of EGFR. Therefore, in the following study, only the EtOAc extract of Caragana Jubata was investigated. The EtOAc extract sample (5 mg) was evaporated to dryness in a vacuum centrifuge (RE 52A, Shanghai, China) and then re-dissolved in 5 ml acetonitrile to be qualified as 1 mg ml^{-1}; thereafter it was diluted to 0.1 mg ml^{-1} with acetonitrile before loading onto the MISPE column.

2.5. SPE procedure

A dry MISPE column was conditioned with 10 ml acetonitrile before 4 ml of the EtOAc extract sample was loaded on. According to the steps as described in Section 2.4, there was 400 μg of matrix components in the 4 ml EtOAc extract sample re-dissolved in acetonitrile. The column was washed with 10×1 ml acetonitrile and then 8×1 ml methanol–acetic acid (9:1, v/v) was used to elute the analytes. Each fraction was evaporated to dryness in a vacuum centrifuge and thereafter re-dissolved in 200 μl of CH_3OH–water (1:9) prior to analysis by reversed-phase (RP) HPLC. Sample analysis was conducted by a HPLC system with a guard column (Dikma) and an analysis column (Zorbax Extend-C_{18}, Agilent, 150×2.1 mm). The mobile phase consisted of solvent A (water) and solvent B (methanol) with the following gradient (B%): 0 min, 10%, 10 min (equilibration), 10%, 50 min, 90%, 51 min (washing), 100%, 61 min (washing), 100%, 62 min (equilibration) 10%. The sample size injected was 20 μl. The flow-rate was 0.5 ml min^{-1}. The detector was set at 254 nm. The column temperature was

ambient. The contents of piceatannol and butein in the sample were determined with the above HPLC gradient using themselves as external references.

2.6. Selectivity study

HPLC evaluation of the MIP column (150×4.6 mm) for the two main analytes was performed at room temperature. The sample size injected was 20 μl and the concentration of sample was 0.1 mmol ml^{-1}. The flow-rate was 1 ml min^{-1}. The detector was set at 254 nm. The capacity factor k' is calculated using the equation, $k'=(t_R-t_0)/t_0$, where t_R is the retention time of a sample and t_0 is the dead time to elute the solvent acetone. The imprinting effect (IF) was defined by the equation IF=k'(MIP)/k'(BP), where k'(MIP) is the capacity factor of the molecularly imprinted polymer and k'(BP) is that of the blank polymer. The relative retention value (α) was calculated by the equation $\alpha=k'$(template)/k'(analyte).

2.7. EGFR Inhibition Assay

The epidermal growth factor receptor was isolated from human A431 carcinoma cell by immunoaffinity chromatography as described in the literature [28]. The epidermal growth factor (EGF), ATP and the antibodies as well as other reagents [e.g., 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium vandate, the substrate peptide and OPD (*o*-phenylenediamine)] used in the enzyme-linked immunosorbent assay (ELISA) procedure were bought from Sigma (USA).

Enzyme reactions were done in a total volume of 100 μl containing 25 *M* HEPES (pH 7.4), 5 m*M* $MgCl_2$, 2 m*M* $MnCl_2$, 50 μ*M* sodium vanadate, 4.0 U EGFR (which also contained enough EGF to make the final concentration 2 μg ml^{-1}), 0.3 m*M* ATP, varying concentration of each compound, and 200 μ*M* of a substrate peptide based on a portion of phospholipase C-γ1 having the sequence Lys–His–Lys–Lys–Leu–Ala–Glu–Gly–Ser–Ala–Tyr^{472}–Glu–Glu–Val. The reaction was initiated by addition of the ATP. After being incubated at room temperature for 30 min, each well was washed with 200 μl of washing buffer [phosphate-buffered saline (PBS)–Tween 20]. Then 100 μl of the appropriate dilution of antibody conjugate was added to each well and the wells incubated for 30 min at room temperature. After washing each well with washing buffer five times, 100 μl of freshly prepared OPD substrate solution was added to each well and incubated for 7 min, in the dark, at room temperature. An orange–yellow color should develop in positive wells. Finally, the reaction was stopped by adding 50 μl of 2 *M* H_2SO_4 to each well.

Assays were conducted in a 96-well microtiter plate, and a Bio-Rad 490 plate reader was used to measure the absorbance at 492 nm. The percent inhibition of EGFR activity was calculated using the equation % inhibition=100[(*A*−*B*)−(*C*−*B*)]/(*A*−*B*), where *A* represents the absorbance after incubation with EGFR and without test sample; *B*, the density after incubation without test sample and without EGFR; *C*, the density after incubation with EGFR and with test sample. In this work, three independent dose–response curves were done for each inhibitor and the IC_{50} value computed. The reported value is a mean, and variation was generally ±10%.

3. Results and discussion

3.1. Extraction of piceatannol and butein from Caragana Jubata

Since the main aim of this work was to study the feasibility of using an MIP prepared with quercetin as the template to directly extract different types of anti-EGFR inhibitors from herb, the selectivity of the polymer for other structurally related flavonoids was not investigated firstly. Also the possible doubts as to whether or not there were flavonoids existing in the herb was not taken into account chiefly. Caragana Jubata, a Chinese traditional Tibetan medicine, was chosen to be studied just because the EtOAc extract of this plant displayed potent inhibitory activity toward EGFR tyrosine kinase. Quercetin was adopted as the template because it is one of the typical natural anti-EGFR inhibitors and is easy to obtain. The application of using the polymer as the sorbent material for SPE was mainly investigated.

A typical SPE procedure consists of four steps: conditioning of the column material, loading of the

sample, washing to remove non-specifically bound molecules and finally elution of the analytes. In order to select the loading, washing and elution conditions, a large variety of solvents including chloroform, acetonitrile, methanol, ethanol, water, toluene, dichloromethane and THF were compared. Suitable solvents were found for loading (acetonitrile), washing (acetonitrile) and elution (methanol–acetic acid, 9:1, v/v). A 4-ml volume of herb extract containing 400 μg matrix components was loaded directly onto the MISPE column after conditioning. Then the column was washed with 10×1 ml acetonitrile to eliminate those components non-specifically bound to the polymer. Finally the analytes, piceatannol and butein, were eluted with 8×1 ml methanol–acetic acid (9:1, v/v). The same procedure was used for the blank SPE column. The contents of piceatannol and butein were measured in washing and each individual elute fraction and the results are shown in Table 1.

From the data shown in Table 1, it could be obviously observed and therefore concluded that: (1) using 10×1 ml acetonitrile as washing solvent, the two analytes (piceatannol and butein) were washed out almost completely in the blank SPE column. On the contrary, these two components were retained on the MISPE column until eluted by methanol–acetic acid (9:1, v/v), which demonstrated that they could be specifically adsorbed onto the MIP material. In fact, the existence of these two anti-EGFR inhibitors in this plant was unknown at first. The fractions washed from the MISPE column as well as those eluted from it were collected and evaporated to dryness in a vacuum centrifuge, and then these two parts were evaluated for their anti-EGFR bioactivities by ELISA. The results showed that the former fraction (washed) possessed rather low activity while the latter (eluted) displayed higher anti-EGFR activity than the crude extract loaded on the column. The conclusion of the blank SPE column was the opposite: the active anti-EGFR components were washed out firstly with little reservation on the BSPE column, which testified that the active components in the crude extract of this herb could be selectively separated and concentrated through the MISPE procedure. It was confirmed afterward that the eluted fractions from the MISPE column mainly contained two inhibitors of EGFR, piceatannol and butein, with IC_{50} values of 4.9 and 10 μ*M*, respectively. These two compounds as well as the template were identified by high-resolution ESI-TOF-MS: quercetin ($[M–H]^-$ ion at 301.0343), butein ([M–

Table 1
Evaluation of the MISPE procedure for extraction of piceatannol and butein as anti-EGFR inhibitors from herb compared to the blank SPE column

SPE step	Amount of the inhibitors (piceatannol and butein)			
	MISPE		BSPE (blank SPE)	
	Butein (μg)	Piceatannol (μg)	Butein (μg)	Piceatannol (μg)
Loading (total)	400 μg matrix components			
Washing				
1–3 ml	–[a]	–	–	23
4–7 ml	–	0.3	16.7	1.8
8–10 ml	–	0.2	1.6	0.9
Elution				
1 ml	–	1.5	0.3	–
2 ml	–	3.4	–	–
3 ml	0.6	17.5	–	–
4 ml	0.4	2.0	–	–
5 ml	11		–	–
6 ml	2.0		–	–
7 ml	1.9		–	–
8 ml	0.3		–	–
Elution (total)	16.2	24.4	18.6	25.7

[a] Not possible to detect the analytes due to the matrix interferences.

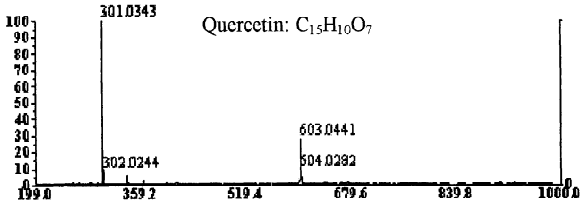

Fig. 2. The mass spectra of the template compound.

$H]^-$ ion at 271.0601) and piceatannol ($[M–H]^-$ ion at 243.0652). The mass spectra are shown in Figs. 2–4. It was the first time that these two anti-EGFR

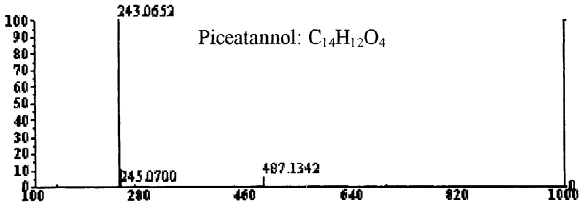

Fig. 3. The mass spectra of the first three fractions eluted from the MISPE column. (The first three eluted fractions were evaporated to dryness and then re-dissolved in 200 μl methanol for MS detection).

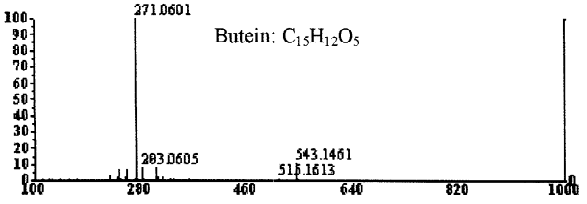

Fig. 4. The mass spectra of the last three fractions eluted from the MISPE column. (The last three eluted fractions were treated with the same procedure as described in Fig. 3.)

inhibitors were reported to be isolated from this plant. (2) In the MISPE procedure, about 16.2 μg of butein and 24.4 μg of piceatannol were eluted from 400 μg EtOAc extract of Caragana Jubata, which meant that the contents of these two compounds are 4.1 and 6.1%, respectively. There was totally about 21.6 μg of butein and 30.5 μg of piceatannol in 400 μg EtOAc extract of Caragana Jubata by the validation of HPLC, revealing the actual contents of these two compounds to be 5.4 and 7.6%, respectively. Therefore the recovery of these two analytes in the elution step could be calculated to be 76% for butein and 80% for piceatannol. The loss of the compounds may arise from two causes: one is the loss on the polymer, and the other is the insufficient adsorption of the samples. But this outcome was still satisfactory. (3) From the elution step it could be found that piceatannol was the main component eluted in the first four fractions while butein was mostly eluted in the last four ones. In fact, we found that if methanol was used as the elution solvent, mainly piceatannol was eluted, while butein could not be eluted efficiently by methanol until methanol–acetic acid (9:1, v/v) was used, which suggested that these two analytes could be differentiated furthermore by different elution solvents resulting from the little difference in the adsorption capabilities of these two compounds on the the MIP column.

Chromatographic traces with and without prior MISPE column treatment are shown in Figs. 5–7. From Fig. 5 it could be found that there were plenty of components in the EtOAc extract prior to the MISPE column, while after washing the column to eliminate the interferences non-specifically binding to the polymer, only two main compounds, piceatan-

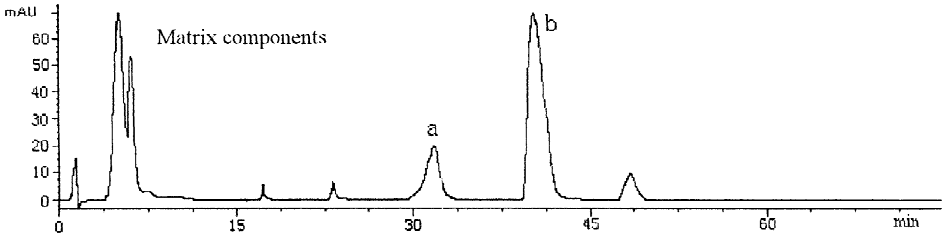

Fig. 5. Chromatographic trace of the herb extract prior to the MISPE column (a: piceatannol, b: butein). Sample analysis was conducted by HPLC with a guard column (Dikma) and an analysis column (Zorbax Extend-C_{18}, Agilent, 150×2.1 mm). The mobile phase consisted of solvent A (water) and solvent B (methanol) with the following gradient (B%): 0 min, 10%, 10 min (equilibration), 10%, 50 min, 90%, 51 min (washing), 100%, 61 min (washing), 100%, 62 min (equilibration) 10%.

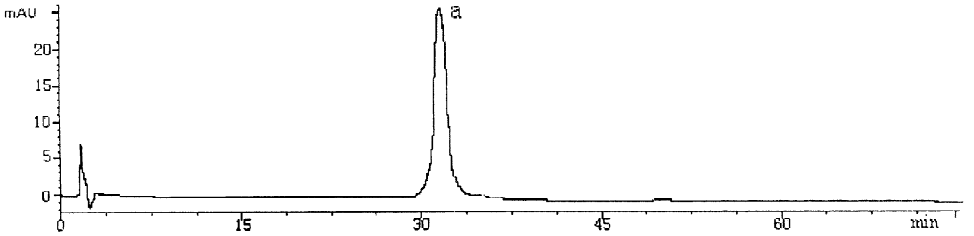

Fig. 6. Chromatographic trace of the first three eluted fractions from the MISPE column (a: piceatannol). (The first three eluted fractions were evaporated to dryness and then re-dissolved in 200 μl methanol for analysis. Sample analysis was conducted by the same HPLC system as in Fig. 5).

nol and butein were selectively trapped (Figs. 6 and 7). The analytes were affirmed by MS. As described above, the content of butein in the herb extract was lower than that of piceatannol, but the peak area of butein was larger than that of the piceatannol (Fig. 5), which may be due to the higher response of butein during UV detection at 254 nm.

3.2. Selectivity study of the quercetin-imprinted polymer

The selectivity test was carried out for the template molecule itself and the two main anti-EGFR inhibitors, piceatannol and butein. Methanol was used as the mobile phase. In the present study, the main subject was to directly extract efficient inhibitors from herb, therefore only those grasped components (piceatannol and butein) were tested for the selectivity of the polymer. The relative retention value (α) and imprinting effect value (IF) are listed in Table 2.

From the results listed in Table 2 it could be observed that the quercetin-imprinted polymer exhibited the strongest affinity to the template molecule itself. Butein seemed to be retained longer on the

Table 2
Chromatographic parameters for quercetin and other two compounds

	Quercetin	Butein	(*E*)-Piceatannol
k'(MIP)	6.98	2.07	1.96
k'(BP)	2.00	1.28	1.43
α(MIP)	1	3.37	3.56
α(BP)	1	1.56	1.40
IF	3.49	1.62	1.37

MIP column than piceatannol although it displayed lower anti-EGFR activity than the latter, which may be explained by the similarity of its chemical structure to that of the template. It could be expected that using piceatannol with the higher activity (IC_{50}=4.9 μM) instead of quercetin (IC_{50}=15 μM) as the template molecule to synthesize the corresponding polymer, the ability of which to mimic the binding pocket of the enzyme will be improved furthermore. This is another research task underway in our group.

4. Conclusion

Our study indicates that by SPE, a molecularly

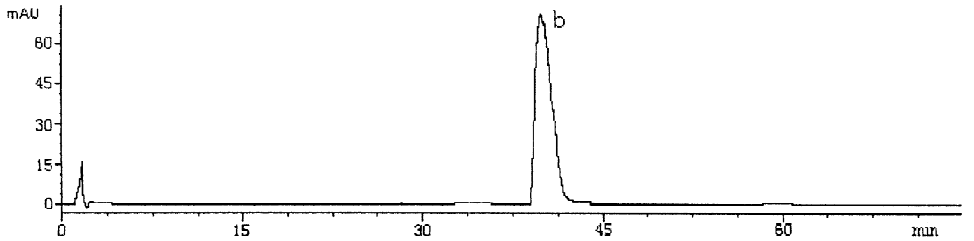

Fig. 7. Chromatographic trace of the last three eluted fractions after the MISPE column (b: butein). (Sample analysis was performed with the same procedure as described in Fig. 6).

imprinted polymer prepared with quercetin, a known natural anti-EGFR inhibitor, as the template could grasp other different anti-EGFR inhibitors from herb directly and efficiently. As far as we know, most previous literature about the application of MISPE [29–33] mainly focus on the extraction of the derivatives or isomers of the template or only the template molecule itself from various types of sample matrices, such as biological fluids in undiluted form or diluted with buffer or water [34–36], heptane extracts of human plasma [37], and organic solvent extracts of beef liver [38] or human serum [39], while using MISPE to extract different types of compounds with similar biological functions (e.g., bioactivities) to the template from herb has rarely been reported. Although the concept is not new: previous work [40,41] had indicated that the molecularly imprinted polymers could effectively mimic the recognition patterns exhibited by the natural receptors and thus could recognize a variety of small molecules, the screening of plant extracts in this study seems particularly suited for such application. The sites imprinted with a known inhibitor (in this case quercetin) could trap analogues that exhibit stronger inhibiting properties. In the present research work, using an MIP as the sorbent material in solid-phase extraction for separation of active anti-EGFR components from Caragana Jubata was confirmed to be feasible and effective, which afforded us an efficient approach for the discovery of anti-cancer lead compounds avoiding the tediousness and inefficacy of traditional isolation. This is promising for further drug development.

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