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Application of Molecularly Imprinted Column for Separation and Purification of Bioactive Compound from *Cirsium segetum Bunge*

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Abstract: Direct application of monolithic column of molecularly imprinted polymers (MIPs) for selective separation and purification of protocatechuic acid from the crude extract of seeds of *Cirsium segetum Bunge* was attempted in present work. An in-situ polymerization technique was used for the preparation of MIPs monolith using protocatechuic acid as the template, acrylamide as functional monomer, 1,1,1-Trimethylolpropane trimethacrylate (TRIM) as cross-linker, and a mixture of tetrahydrofuran-iso-octane (1:2, v/v) as porogenic agents. The preparation procedure was optimized and the retention behavior and selectivity of this monolith evaluated by chromatographic analysis. Then, this monolith was directly connected into the liquid phase chromatographic system to trap target molecule from crude extract. Results indicated its high selectivity for protocatechuic acid, good affinity to its structurally related compounds, and good workability in herb matrix. Sample matrix compounds in a crude extract of this plant could be removed by carefully washing with a solvent, and protocatechuic acid was purified simultaneously by selective desorption with the eluent. The purity and recovery of product obtained can reach 98.7% and 86.1%, respectively.

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Keywords: *Cirsium segetum Bunge*, extraction, in-situ synthesis, molecular imprint, protocatechuic acid

INTRODUCTION

Molecular imprinting is an established approach for preparing polymeric materials possessing highly selective and affinitive properties. In the most common preparation process, monomers form a complex with a template through covalent or non-colvent interactions and are then joined by using a cross-linking agent. After the removal of the template by chemical reaction or extraction, binding sites are exposed which are complementary to the template molecules in size, shape, and position of the functional groups, and consequently allow its selective uptake. Based on such features of the MIPs as the shape size and functionality selectivity, a strong affinity on the target molecule, low cost in the preparation, and the workability in the organic solvent (1,2), it has been used in many areas, including separations (3), immunoassay (4), sensor (5), and artificial enzymes catalysis (6). Especially, MIPs have recently attracted considerable interest in the field of solid phase extraction (SPE) (7,8).

Several polymerization methods can be used to prepare MIPs particles for SPE. Traditional bulk polymerization is relatively simple in the apparatus acquired for synthesis and the reaction conditions can be easily controlled. Unfortunately, the chromatographic performance of these particles is usually unsatisfactory due to their irregular size and shape. Furthermore, the tedious and time-consuming process and low yield of MIPs prevent their industrial production and acceptance in analytical laboratories (9–12). Suspension polymerization can produce high-quality MIPs beads, but the drawback is that water is incompatible with most non-covalent imprinting procedures and liquid fluorocarbons are somewhat expensive (13). Two-step or multi-step swelling polymerization can produce spherical MIPs of uniform size, but their chromatographic performance is still unsatisfactory, despite the highly uniform packing (14). Dispersion and precipitation polymerizations can produce MIPs beads of nanometer size, but the binding sites in the MIP beads are inside their network, causing a slow mass transfer of target molecules (15,16). In addition, special apparatus are needed in order to assure packing homogenization when these MIPs particles prepared by using these methods mentioned as above are packed into a steel column for solid phase extraction or analysis. Compared with those methods, the in-situ polymerization directly prepares molecularly imprinted polymer for HPLC or SPE separation in column, and hence is a very simple and easy method (17,18). Molecularly imprinted polymers monolith (MIPM) synthesized

by this technique were shown to possess higher selective rebinding capability in molecular recognition and solid phase extraction (19–21).

Protocatechuic acid, a pharmaceutically active compound in traditional Chinese medicine *Cirsium segetum Bunge*, has strong inhibition activity on staphylococci, streptococcus, diplococcus lanceolatus, and colibacillus, and the apocrustic and henotic effect on trauma agglutination in the treatment of empyrosis, paediatric pneumonia, bacillary dysentery, etc. Conventional separation techniques for protocatechuic acid were complicated and time- and solvent-consuming and difficult to obtain the products with high purity (>98%) due to the absence of a specific adsorption medium (22). Thin layer chromatography method could only produce a tiny amount of target product and the preparative liquid chromatography separation technique on C_{18} column is a good but expensive approach to purify protocatechuic acid with the need for expensive stationary phase material. Additionally, C_{18} material showed a weak selectivity and separation capability for those compounds with similar polarity (23). The application of MIPs monolith for the separation and purification of protocatechuic acid from the plant crude extract is an interesting attempt. This was not reported in the literatures.

Hence, in the present work, an in-situ polymerization technique was developed for the preparation of a monolithic column of protocatechuic acid imprinted polymer and then this monolith was directly connected to a liquid phase chromatographic system for on-line SPE or analysis of target molecules. The retention behavior and selectivity of this monolith to target the compound and its structurally related compounds were investigated and the conditions for MIPs solid phase extraction optimized.

EXPERIMENTAL

Chemicals and Materials

Standards of protocatechuic acid (PCA), gallic acid (GLA), 4-hydroxybenzoic acid (4-HBA), chlorogenic acid (CHA), gentisic acid (GA), caffeic acid (CA), 3-hydroxybenzoic acid (3-HBA), and salicylic acid (SA) (their molecular structure are shown in Fig. 1) were provided by the Chemical Research Institute, Hunan Normal University (Changsha, China). Acrylamide, methacrylic acid (MAA), and 1,1,1-Trimethylolpropane trimethacrylate (TRIM) were purchased from Sigma-Aldrich (St. Louis, USA). 2,2′-azobisisobutyronitrile (AIBN), tetrahydrofuran (THF), iso-octane and acetone were from Shanghai Chemical Reagent Co., Ltd (Shanghai, China). Glacial acetic acid (HAc), petroleum ether, ethyl acetate, acetonitrile (MeCN), and methanol (MeOH) were from

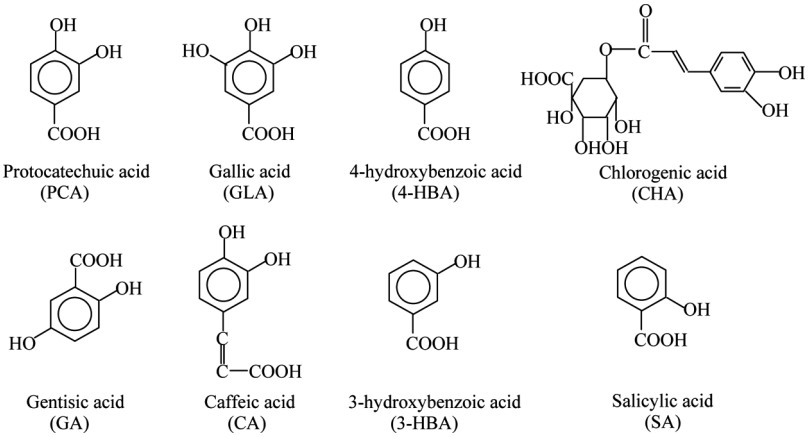

Figure 1. Molecular structure of the compounds.

the Hunan Normal University Reagent Factory (Hunan, China). All the reagents are analytical-grade. Stainless steel column (250 × 4.6 mm I.D.) was kindly donated by the Beijing Jinouya Science & Development Co., Ltd (Beijing, China).

The extract of *Cirsium segetum Bunge* seeds was prepared by the modified method as reference (22). 50.0 g powder of *Cirsium segetum Bunge* seeds was refluxed in petroleum ether for 2 h. After filtration, the solid was extracted with water for 1 h. The solution obtained was filtrated. The liquor was concentrated and then extracted with ethyl acetate. Removal of ethyl acetate from the organic phase produced crude product of protocatechuic acid as the experimental material for MIPs solid phase extraction.

Apparatus

A LC-2010A_{HT} high performance liquid chromatographer from Shimadzu Co., Ltd, (Japan), equipped with a solvent delivery pump, an auto-injector, a system controller, a column oven, an UV detector and a chromatographic workstation, was used for chromatographic analysis.

Preparation of Protocatechuic Acid Imprinted Polymers Monolithic Column

An in-situ polymerization technique was used for the preparation of protocatechuic acid imprinted polymers monolithic column. In a typical process, the reaction mixture containing the template molecules

(protocatechuic acid, 1 mmol), functional monomers (acrylamide, 4 mmol), cross-linking agent (TRIM, 8 mmol), porogenic agents (tetrahydrofuran-iso-octane mixture solvent (1:2, v/v), 3 mL) and initiator (AIBN, 0.1 mmol) are poured into a stainlesssteel column, sealed at one end, and degassed ultrasonically for 15 min. Then the other end is sealed and the polymerization reaction is initiated by heating column in a water bath at 60°C for 12 h. After the removal of template molecules by eluting with MeOH-HAc (9:1, v/v) and MeOH solvents, the column of MIPs monolith was connected directly to the liquid phase chromatographic system (Fig. 2) for on-line SPE or analysis of compounds. As a control, non-imprinted polymers monolith (NIPM) column without a template molecule was also prepared and treated in the same manner.

Chromatographic Evaluation of the Monolith

The MIPM column was directly connected to the liquid phase chromatographic system and the chromatographic evaluation of the monolith

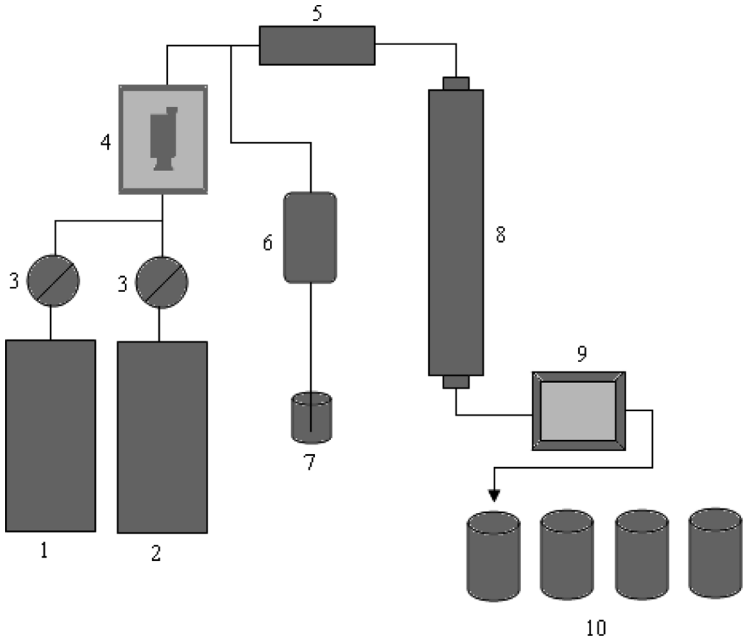

Figure 2. Basic flow chart of molecularly imprinted column solid phase extraction. 1 and 2 Solvent 1 and 2; 3 Valves; 4 Pump; 5 Guard column; 6 Injector; 7 Sample solution; 8 Molecularly imprinted column; 9 UV detector; 10 Fraction collectors.

carried out isocratically at room temperature. The template and its structurally related compounds were prepared into 0.2 mg/mL analytes-MeOH solutions and each sample determined independently. After the MIPM column was equilibrated with acetonitrile, 10 μL of the sample solution was injected into the polymer column and the analyte eluted with acetonitrile as mobile phase at a rate of 0.2 mL/min, recording the retention time of the analyte. Detection was at 280 nm using UV absorption and each determination repeated twice. Then the mobile phase was changed for performing other determination. Acetone was used for the measurement of void time (t_0). The capacity factor (k') was calculated as $(t_R - t_0)/t_0$, where t_R is the retention time of the analyte. The selectivity factor (α) was defined as the ratio of the capacity factor of the template molecule to that of the analogues ($\alpha = k'_{template}/k'_{analogue}$). The imprinting effect (IF) was defined by the equation $IF = k'(MIP)/k'(NIP)$, where $k'(MIP)$ and $k'(NIP)$ was the capacity factors on the MIPM and NIPM, respectively.

Monolith Capacity Test

The MIPM columns were connected into liquid phase chromatographic (LC) system and the column outlet directly into fraction collector. Prior to applying the sample, the column was pre-equilibrated with 10 mL methanol. Then, aliquots (0.5 mL) of 0.2 mmol/L protocatechuic acid in methanol was loaded gradually to the monolith by pump system of LC at a flow rate of 0.2 mL min^{-1}. The loading was stopped when the release of protocatechuic acid could be detected. Then the column was washed with 6 aliquots (1 mL) of methanol, followed by eluting with 5 aliquots (1 mL) of methanol-HAc (9:1, v/v) mixture solution at a flow rate of 0.2 mL min^{-1}. The effluent liquids from each loading, washing, and elution steps were collected and directly analyzed by HPLC on C_{18} column.

For the HPLC analysis, mobile phase used was a mixture of methanol-water-acetic acid (19:80:1.0, v/v/v) at a rate of 1.0 mL/min. Injection volume was 10 μL and UV detection wavelength was 280 nm. Each determination was repeated twice. A standard curve method was used for the quantification of analytes.

Extraction Test

Column connection, fraction collection and HPLC analysis were the same as described in the previous section. For the extraction test, 2 mL

methanol solution of crude extract was loaded onto the monolith column, followed by washing with 6 aliquots (1 mL) of methanol and eluting with 4 aliquots (1 mL) of methanol-HAc (9:1, v/v) mixture solution at a flow rate of 0.2 mL min^{-1}.

RESULTS AND DISCUSSIONS

Preparation of Molecularly Imprinted Polymer Monolith

The preparation process for the MIPM by the in-situ polymerization method is quite simple and a column filled with molecularly imprinted polymers can be directly obtained after the polymerization reaction was finished. However, a number of factors need to be taken into account to assure good character of the MIPs monolithic column. First the mole ratio of Template-Functional monomer-Cross linker was tested by preliminary experiments, indicating that the polymer prepared under this ratio of 1:4:8 had good absorption capacity. The influence of functional monomers types on the performance of the MIPM was considered. For this purpose, two functional monomers (MAA and acrylamide) were respectively used for the preparation of the molecularly imprinted polymers under fixed mole ratio of the template to the functional monomer (1:4). Results showed that the monolith obtained by using acrylamide as functional monomer had a higher capacity factor (43.0) and a better imprint efficiency (5.18) than that obtained by using MAA (10.2 for the capacity factor and 3.19 for the imprint efficiency) under the same conditions. Hence, acrylamide was selected for the following preparation procedure for protocatechuic imprinted polymeric monolith. Then, the type of cross-linker was optimized. The influence of two cross-linkers (TRIM and EDMA) on the retention behavior of the MIPM was compared under the same conditions. Result showed that while the capacity factor was for the monolith was 43.0 when TRIM was used as the cross-linker, it was only 19.1 when using EDMA. Therefore, TRIM was chosen. In addition, a significant problem involved in the in-situ preparation technique was the choice of appropriate porogen in order to assure good penetrability for the MIPs monolithic column. By the preliminary test, it was found that using a mixture of THF-iso-octane could produce good effect. This was also proved in previous work (24,25). It was found by our investigation that when the ratio of THF to iso-octane in the porogenic mixture was 1:2 (v/v), the MIPM column with low backpressure could be obtained.

Chromatographic Performance of the Polymer Monolith

Retention Behavior

The chromatographic retention behavior of MIPM and NIPM columns to the template molecule was evaluated by LC using different mobile phases. First, acetonitrile was used as the mobile phase for this test. Then acetonitrile solution containing different amount of water was for this test. Table 1 lists the values of capacity factors obtained using different eluents. It was found that when acetonitrile was used as the mobile phase, the MIPM column took on the strongest retention to protocatechuic acid molecule and the capacity factor ($k' = 40.3$) was the highest. In fact, the template could not be eluted out of the MIPs column in 60 min under this condition. With the increase of water content in acetonitrile, the capacity factor of the monolith decreased dramatically. As water content in acetonitrile was 5% (v/v), the monolith of MIPs almost lost its imprinting effect. This showed that hydrogen-bonding might be the dominant interaction in the rebinding of this monolith. The same situation happened when methanol was used as the mobile phase. Especially, when methanol-acetic acid (9:1, v/v), a more polar and protonic solvent, was used, the imprinting effect disappeared and a symmetrical chromatographic peak shape was appeared. This was due to that these protonic solvents (water, methanol or acetic acid) could seriously disturb hydrogen bonding interaction between protocatechuic acid and amino groups on recognition sites in the polymer matrix (26).

Selectivity of the Monolith

Selectivity test was carried out by using the template molecule and its structurally related compounds, which were comparable to the compounds co-existing in the crude extract of *Cirsium segetum Bunge* seeds. Methanol, the common solvent in the extraction of this plant, was used as the mobile phase for this test. Capacity factor, relative retention values (α), and imprinting efficiency value (*IF*) are listed in Table 2. It could be seen that the MIPs monolith exhibited the strongest affinity to the template and most of the structurally related compounds tested were inclined to retain on the MIPM column rather than on the NIPM column. The highest selectivity of the monolith of MIPM for protocatechuic acid was induced during the imprinting process, resulting from the high complementarity of the template molecule to the recognition sites in molecular shape, size and functional groups.

Table 1. Chromatographic retention performance of protocatechuic acid on the MIPM using different mobile phases*

Parameters	Polymers	Acetonitrile	1% Water in acetonitrile	3% Water in acetonitrile	5% Water in acetonitrile	Methanol	10% HAc in methanol
k'	B4	8.3	4.5	2.8	2.3	1.7	1.2
	P4	40.3	16.7	8.9	2.5	5.2	1.2
IF		5.18	3.7	3.2	1.1	3.1	1

*k': Capacity factors; *IF*: Imprinting effect. P4 was the molecularly imprinted column prepared under optimized conditions and B4 was the non-imprinted column prepared under the same conditions but without the template.

Table 2. Chromatographic parameters for protocatechuic acid and other relative compounds on the MIPM and NIPM columns*

Factors	Polymers	PCA	GLA	4-HBA	GA	3-HBA	CA	SA	CHA
k'	P4	5.2	3.6	3.3	2.9	2.8	1.6	1.0	0.6
	B4	1.7	1.6	1.5	1.3	1.1	1.5	1.2	1.1
α	P4	1	1.4	1.6	1.8	1.9	3.3	5.5	8.4
	B4	1	1.1	1.1	1.3	1.6	1.2	1.4	1.6
IF		3.1	2.3	2.0	2.2	2.6	1.1	0.8	0.6

*Each sample was injected independently. The sample volume and concentration injected was 10 μL and 0.2 mmol L^{-1}, respectively. Chromatographic conditions: mobile phase: methanol; flow rate: 0.2 mL min^{-1}; UV detection wavelength: 280 nm. P4 was the molecularly imprinted column prepared under optimized conditions and B4 was the non-imprinted column prepared under the same conditions but without the template.

The selectivity test results may also give aspects of the molecular recognition mechanism. This is reflected in the relative retention values (α). The higher α values for CHA, SA, and CA showed relatively weak retention capacity and low selectivity of the MIPs monolith to them. For CHA and CA, steric hindrance during the molecular size and the shape recognition by the polymer network possibly accounts for their poor retention. However for SA, in spite of the similarity in molecular structure to the template, intramolecular hydrogen bonding might be formed under the test conditions, decreasing its retention on the monolith. The difference of molecular structure of the GLA, 4-HBA, GA and 3-HBA with that of the template in the position and amount of functional groups brings them slightly poor matching with the microcave structure induced by protocatechuic acid. Owing to the good selectivity and affinity of the MIPs monolith to protocatechuic acid, this MIPs monolith column might be attempted for selective adsorption of the target compound from complicated herbal crude extracts.

Application Extraction

Monolith Capacity Test

This test was for the purpose of using the MIPM for the following extract study. Herein, the solvent used in the protocatechuic acid solution was methanol. In general, this polar and protic solvent can disturb the specific non-covalent interaction between the analytes and recognition sites, decreasing the rebinding capacity and weakening the affinity of the

Table 3. Loaded and released amount of protocatechuic acid in the MIPM column capacity test by loading, washing and elution steps

	Amount of protocatechuic acid (μg)	
Procedures	NIPM	MIPM
Loaded	46.2	77.0
Sorbed	37.3	63.8
Washing	33.5	6.0
Elution	0.2	48.9
Total	33.7	54.9
Recovery (%)	90.3	86.1

MIPM. However, methanol was selected to evaluate the adsorption capacity of this monolith because methanol could sufficiently dissolve the various components in the extracts of this plant seeds.

It can be observed from Table 3 that the initial amount loaded on the NIPM column was lower than that on the MIPM column, but part of loaded molecules were still released from the NIPM column at the loading step because of non-specific adsorption of protocatechuic acid molecules on the NIPM. Moreover, almost all of protocatechuic acid sorbed was removed from the NIPM at washing steps and only a little can be released from the MIPM. Most of protocatechuic acid was desorbed from the MIPM at the elution steps. This resulted from the specific adsorption capacity in the MIPM matrix, beneficial for the selective separation of target compound by washing with solvent.

The final amount of protocatechuic acid recovered from the MIPM was 54.9 μg and the recovery was 86.1%. Further, the final specific amount on the MIPM was 21.2 μg, which was calculated by the deduction of non-specific adsorption amount (33.7 μg) from 54.9 μg amount on the MIPM. Hence, it could be deduced that a higher capacity could be achieved by imprinting method. The adsorption capacity of the produced MIPM column was acceptable for trapping certain compounds from herb crude extract.

Extraction of Real Sample

First a real sample solution was prepared by dissolving 1 g *Cirsium segetum Bunge* seeds extract in 10 mL methanol, followed by the dilution to 500 mL with methanol. The content of protocatechuic acid in this real sample solution was determined by HPLC and it was 26.2 μg mL^{-1}.

The HPLC chromatogram of this sample solution shown in Fig. 3 indicated that there mainly exist three compounds, i.e., protocatechuic acid, chlorogenic acid and caffeic acid with retention times of 9.6, 20.7 and 26.9 min, respectively, and some matrix impurity compounds in this sample solution.

At the beginning of the extraction, aliquots (0.5 mL) of the real sample solution were loaded gradually on the MIPM by using pump system of LC. The loading rate was 0.2 mL min^{-1}. When the fourth loading was finished, protocatechuic acid was detected in the effluent liquid collected. As a result, the total adsorbed amount of target compound protocatechuic acid on the MIPM column was 52.3 μg. Then, washing and elution steps were carried out in the section titled "Extraction Test." The effluent liquids from the MIPM column at every washing and elution step were analyzed by HPLC and the analysis result are shown in Fig. 4 and Table 4. Minor peaks of sample matrix compounds were not listed. It could be observed from chromatograms in Fig. 4 that at wash steps, most of the impurity compounds including CHA and CA were desorbed out of the MIPM columns, however, only a small amount of template molecules was washed out during washing steps. When the elution step was carried out, chromatographic analysis indicated that the release of protocatechuic acid was mainly in Elution 1–3 steps and there was only a tiny of impurity compounds in the effluent liquids in this stage. As the elution step 4 was finished, no protocatechuic acid was found in the effluent by the HPLC analysis.

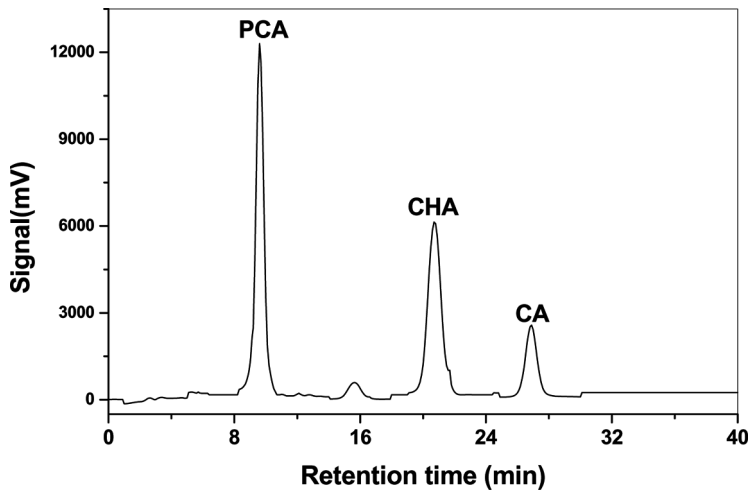

Figure 3. HPLC chromatogram of seeds extract of *Cirsium segetum Bunge* on C_{18} column.

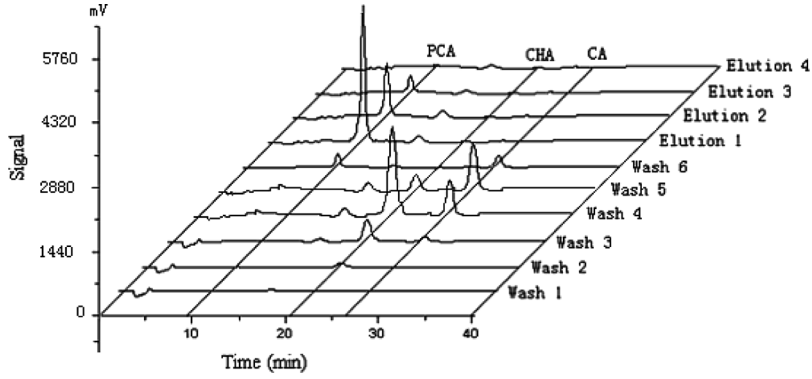

Figure 4. HPLC chromatograms on C_{18} column of effluents liquids collected at every washing and elution steps.

Also, chromatographic peak areas and the contents (listed in Table 4) of the protocatechuic acid and main impurity compounds (CHA and CA) for each effluent liquid collected at every wash and elution step also indicated that the release of the major impurity compounds was at the wash steps while that of the protocatechuic acid was mainly at the elution steps. For the effective separation and purification of protocatechuic acid, the effluent liquids from the elution 1 to 3 steps were collected and evaporated to dryness under vacuum to obtain product in which the content of protocatechuic acid reached 98.7%. The chromatogram of the product obtained

Table 4. Peak areas and contents of the template and part of impurity compounds in each effluent determined by high performance liquid chromatography on C_{18} column

	Peak area ($\times 10^2$)			Content (μg/mL)		
Procedures	PCA	CHA	CA	PCA	CHA	CA
Wash 1	0	0	0	0	0	0
Wash 2	0	8.8	0	0	0.9	0
Wash 3	0	185.5	10.6	0	18.9	2.7
Wash 4	0	47.1	44.7	0	4.8	11.4
Wash 5	0	21.5	70.9	0	2.2	18.1
Wash 6	28.6	0	19.2	3.1	0	4.9
Elution 1	306.1	0	0	33.1	0	0
Elution 2	66.7	0	0	7.2	0	0
Elution 3	19.4	0	0	2.1	0	0
Elution 4	0	0	0	0	0	0

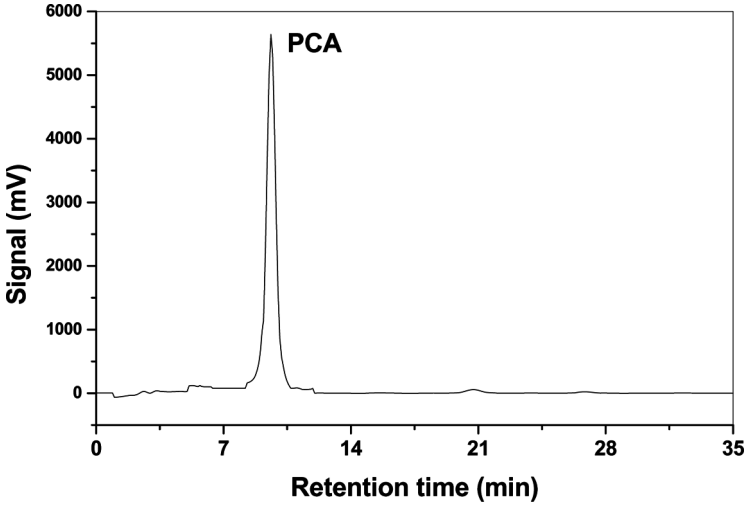

Figure 5. HPLC chromatogram of protocatechuic acid product.

was shown in Fig. 5. This MIPM solid phase extraction took on considerable application foreground and can be developed into a novel technique for the selective separation and purification of protocatechuic acid from the crude extract of the seeds of *Cirsium segetum Bunge*.

CONCLUSION

This study has shown that the MIPM column can perform efficient extraction of certain active compounds from herb extract. Protocatechuic acid could be directly trapped by MIPM from the crude extract of *Cirsium segetum Bunge* seeds. Additionally, this MIPM exhibited the highest affinity to the template molecules, and showed good workability in the herb matrix. It could also retain those structurally related compound to the template molecule. However, such matrix compounds could be removed by carefully washing with the solvent, and the template was purified simultaneously in this way. Direct application of the protocatechuic acid imprinted polymer monolith for the separation and purification of the target compound from this plant was shown to be feasible in the present work.

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