Molecular Imprinted Solid-Phase Extraction of Huperzine A from *Huperzia serrata*

Guosong Wang, Qiue Cao, Xiufang Zhu, Xueqiong Yang, Minghui Yang, Zhongtao Ding

Key Laboratory of Medicinal Chemistry for Nature Resource, Ministry of Education; School of Chemical Science and Technology, Yunnan University, Kunming, Yunnan 650091, People's Republic of China

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ABSTRACT: On the basis of the non-covalent interaction between template and monomer, porous molecularly imprinted polymers (MIPs) were synthesized by a thermal-initiated polymerization method using huperzine A as template, acrylamide, or methacrylic acid as function monomer, ethylene glycol dimethacrylate as cross-linking agent. The interaction between template and functional monomers was studied by UV spectrophotometry, which showed a formation of huperzine A-monomer complexes with stoichiometric ratio of 1 : 2 in the pre-polymerized systems. The resultant MIP particles were tested in the equilibrium binding experiment to analyze their adsorption ability to huperzine A, and were characterized by Fourier Transform Infrared (FTIR) study. The recognition properties of MIP were estimated in solid-phase extraction by selecting four compounds (isolated from the Chinese herb *Huperzia serrata*) as substrates, and were compared with and prior to those of the NIP. High affinity and adsorption of MIP1 which was prepared in chloroform with huperzine A as imprinted molecule, and acrylamide (AM) as functional monomer, made an attractive application of MIP1 in separation processes. In final, using MIP1 solid-phase extraction micro-column, huperzine A was enriched and separated from the real extraction sample of *Huperzia serrata*. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 113: 3049–3058, 2009

Key words: Huperzine A; molecular imprinting; molecular recognition; solid phase extraction; *Huperzia serrata*

INTRODUCTION

Molecularly imprinted polymers (MIPs) are macromolecular materials which are artificially prepared by using molecular imprinting technology, and have been widely used for the selective enrichment and pretreatment of target compounds existing in complex matrix.[1–4] MIPs exhibit high affinity and good selectivity to a template molecule, which must be attributed to specific interactions between the template molecule and the functional monomer. Because of their remarkable recognition properties, MIPs have been used in various applications such as drug separations,[5,6] template-assisted synthesis and catalysis,[7,8] bio-mimetic sensors and antibody mimics.[9,10]

MIPs can be used as sorbents with selectivity predetermined for a particular substance, or group of structural analogs, and have been used in solid-phase extraction for separation or clean-up of target compound in low concentrations or in complex matrixes. The improved selectivity of imprinted polymers compared with conventional sorbents may lead to selective enrichment and separation or cleanup of the analytes to levels not achievable with existing methods, and also lead to cleaner chromatographic traces in the subsequent analytical separation. During recent years, molecularly imprinted solid-phase extraction (MISPE) has been used in drug analysis,[11,12] food analysis,[13,14] environmental,[15,16] and biological analysis.[17,18]

Huperzine A, a representative Lycopodium alkaloid isolated from the Chinese herb, *Huperzia serrata* (Thunb) Trev, is a highly specific, potent, and reversible inhibitor of acetylcholinesterase (AChE), which possesses a high efficacy in improving memory in animal model trials and in clinical trials. And it is recognized to be a promising lead structure in the therapy of senile dementia and cognitive impairment in Alzheimer's diseases (AD), which have been attributed to the hypo-function of cholinergic neurotransmission in the brain.[19] In this study, four huperzine A-imprinted polymers and corresponding non-imprinted polymers were prepared. Their adsorptive performances and recognition abilities were studied by means of Ultraviolet (UV), Infrared (IR), and High Performance Liquid Chromatography

Correspondence to: Z. Ding (ztding@ynu.edu.cn).

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(HPLC) analytical methods. The results denoted that, comparing with the non-imprinted polymers (NIPs), the molecularly imprinted polymers (MIPs) had higher adsorption capabilities and better recognition abilities. Moreover, MIP1 prepared in chloroform with huperzine A as imprinted molecule, and acrylamide (AM) as functional monomer, displayed first-rate recognition characteristics. Eventually, the sample of *Huperzia serrata* was analyzed with a self-made off-line MISPE micro-column by liquid chromatography. The results showed that MIP1 could be utilized as a potent sorbent to enrich and separate huperzine A from the complex mixtures.

EXPERIMENTAL

Reagents and materials

Huperzine A, phlegmariurine B, 6β-hydroxylhuperzine A, ferulic acid, and arbutin were isolated from *Huperzia serrata* and their structures were validated by ^{1}H-NMR and ^{13}C-NMR analysis. Acrylamide (AM) was purchased from the Shanghai Chemical Reagent Plant (China). Ethylene glycol dimethacrylate (EDMA, Shanghai Coral Chemical Plant, China) and methacrylic acid (MAA, Suzhou Anli Chemical Plant, China) were purified by distillation before use. 2, 2′-Azobisisobutyronitrile (AIBN, Shanghai Fourth Reagent Plant, China) was recrystallized from ethanol before use. All solvents were analytical-grade or HPLC-grade and used without further purification.

The whole plants of *Huperzia serrata* (Thunb) Trev. (Huperziaceae) were collected in Juhuacun Chinese Herbal Medicine Market, Yunnan Province, People's Republic of China, in December, 2006, and identified by professor Shugang Lu, School of Life Science, Yunnan University, People's Republic of China. A voucher specimen was deposited in the Key Laboratory of Medicinal Chemistry for Natural Resources, Yunnan University, Kunming, People's Republic of China.

Instrumental

Chromatographic evaluation was performed on Waters system (USA) high performance liquid chromatography equipped with Waters-1525 pump, Waters 2996 UV/VIS detector, and Waters-717 automatic injector model. Chromatographic separation was carried out with a Waters C_{18} column (250 × 4.6 mm^2 i.d.; particle size 5 μm). The flow-phase was methanol/0.1 % aqueous ammonium acetate solution (60 : 40, v/v), and detection was carried out at 310 nm. The column was thermostated at 40°C.

Shimadzu-UV-2401 double-beam spectrophotometer (Japan) and Nicolet Magna 630 FTIR spectrophotometer (USA) were used to characterize the polymers. Home-made molecularly imprinted solid-phase extraction (MISPE) micro-column was used to enrich and separate the target compound from the sample solution.

Preparation of MIPs and NIPs

The template molecule huperzine A (0.5 mmol) and functional monomer AM (2.0 mmol) were dissolved with chloroform (10.0 mL) in an ampoule tube. About 3 h later, the cross linker EDMA (20.0 mmol) and the initiator AIBN (20.0 mg) were added into the tube. The solution was purged with nitrogen gas during 10 min. Then, the tube was sealed, and placed into a 60°C water bath. After incubated for 24 h, the bulk rigid polymers were obtained. The bulk rigid polymers were ground into particles in a mortar and sieved to pass through a 0.105-mm sieve (140 mesh per inch). The excessively fine polymer particles were discarded by decantation washing with acetonitrile. The remaining polymer particles were washed with methanol/acetic acid (9 : 1, v/v) until huperzine A could not be detected at 310 nm by UV spectrophotometry in the eluting solution. Finally, MIP1 was washed with methanol again and dried under vacuum.

The same procedure as described above for MIP1 was used to prepare other molecularly imprinted polymers (MIP2-4) by using different functional monomers or different solvents (see Table I). As a control, the non-imprinted polymers (NIP1-4) were similarly obtained as reference polymers except the template was not added.

Binding Experiments of MIPs and NIPs

Huperzine A with known amounts was dissolved with 10.0 mL of solvent (chloroform or methanol) in a 25-mL conical flask attached lid, then added 20.0 mg of the imprinting polymer particles or the non-imprinting polymer particles. The mixture was oscillated for 5 h at room temperature in an oscillator and then transferred into a centrifuge tube. After centrifugation at 4000 rpm for 10 min, the concentration of the free substrate in the supernatant solution was determined by measuring the absorbance at 310 nm by UV spectrophotometry. The binding capacity (Q), which was defined as μmol of the substrate bound per 1 g of polymer particles, was calculated with eq. (1), where C_0 (mol/L) is the initial concentration of substrate, C (mol/L) is the free concentration of substrate in the solution after treated with the polymer particles, V is the volume of adsorption solution, and W (g) is the mass of the polymer particles.

$$Q = (C_0 - C)V \times 10^6/W \quad (1)$$

Adsorption selectivity of MIP1

The column was packed with a MIP1 or NIP1 (100.0 mg, each). The mixture chloroform solution of

TABLE I
Composition and Binding Properties of Polymers[a]

Polymer	Template molecule	In solvent	Monomer	Binding capacity Q (μmol/g)[b]	Imprinted efficiency[c]
MIP1	Huperzine A	Chloroform	AM	132.5	2.06
MIP2		Chloroform	MAA	80.6	1.90
MIP3		Methanol	AM	145.4	1.47
MIP4		Methanol	MAA	110.5	1.50
NIP1	None	Chloroform	AM	64.3	—
NIP2		Chloroform	MAA	42.4	—
NIP3		Methanol	AM	98.9	—
NIP4		Methanol	MAA	73.7	—

[a] The binding properties were determined by adding 0.032 mmol of Huperzine A in 10.0 mL of methanol or chloroform with 20.0 mg of polymer.
[b] Binding capacity (Q) was expressed in μmol of huperzine A bound per 1 g of polymer.
[c] Imprinted efficiency was expressed as the ratio of binding capacity of the imprinted polymer to that of the corresponding non-imprinted one.

huperzine A and other four compounds isolated from *Huperzia serrata* (*i.e.*, phlegmariurine B, 6β-hydroxylhuperzine A, ferulic acid, and arbutin) was passed through the column, and collected the flowing mixture solution from column. The solution was filtered through a 0.45-μm organic micro-filter prior to perform HPLC analysis (C_{18} column 4.6 × 250 mm^2, 5 μm, methanol/0.1% aqueous ammonium acetate solution, 60 : 40, v/v, flow rate 1.0 mL/min. An efficient separation of huperzine A and other components in the mixture was achieved under these conditions).

Establishment of the eluting condition

One hundred micrograms of MIP1 was packed into a column (2.5 × 120 mm^2) to make the MISPE micro-column. The column was rinsed in turn with chloroform, methanol, acetone, and then with chloroform. Huperzine A was dissolved in chloroform to form huperzine A standard solution with concentration of 3.2 mmol/L. The column was loaded with huperzine A standard solution. After column drying, eluting solvent flowed through the column to perform the elution of Huperzine A. The eluting fractions were analyzed by HPLC to detect the content of Huperzine A. On the basis of these data of content of huperzine A in eluate, the optimal eluting condition was selected out.

Sample preparation

The air-dried whole plants of *Huperzia serrata* (500.0 g) were powdered and successively extracted three times at refluxing temperature with ethanol for 4 h every times. The ethanol-extract was filtered through gauze, and the resultant filtrate was distilled to dry. The residue was dissolved with 2% aqueous hydrochloric acid. Then, the in-dissolvable substance was filtered by filter paper. After the pH of the filtrate was adjusted to 9–10 with concentrated ammonia water, the filtrate was extracted with chloroform three times. The chloroform extract was concentrated to give the total alkaloid extract of *Huperzia serrata*, and this extract was used as the sample.

Adsorption of sample solution by MISPE micro-column and HPLC analysis

The SPE glass column (2.5 × 120 mm^2) was packed with a MIP1 or NIP1 (100.0 mg, each). Then, the column was washed in turn with chloroform, acetone, methanol, and then with chloroform. The sample solution (2.0 mL) was filtered through filter papers, and then passed through the column. Then, the column was washed with chloroform, and vacuum was applied trough the column to remove residual chloroform. Eluting solvent (methanol/ammonia water, 98 : 2) flowed through the column to perform the elution of Huperzine A. The eluate were filtered through a 0.45-μm organic micro filter prior to perform HPLC analysis (C_{18} column 4.6 × 250 mm^2, 5 μm, methanol/0.1 % aqueous ammonium acetate solution, 60 : 40, v/v, flow rate 1.0 mL/min). All solutions were measured for three times, and their average values were used to analyze.

RESULTS AND DISCUSSION

Interaction between template and functional monomers

The recognition ability of imprinted polymer towards template molecule depends principally on the preservation of the pre-polymerized host–guest structure in a polymer matrix. Therefore, it is essential to investigate interaction between template

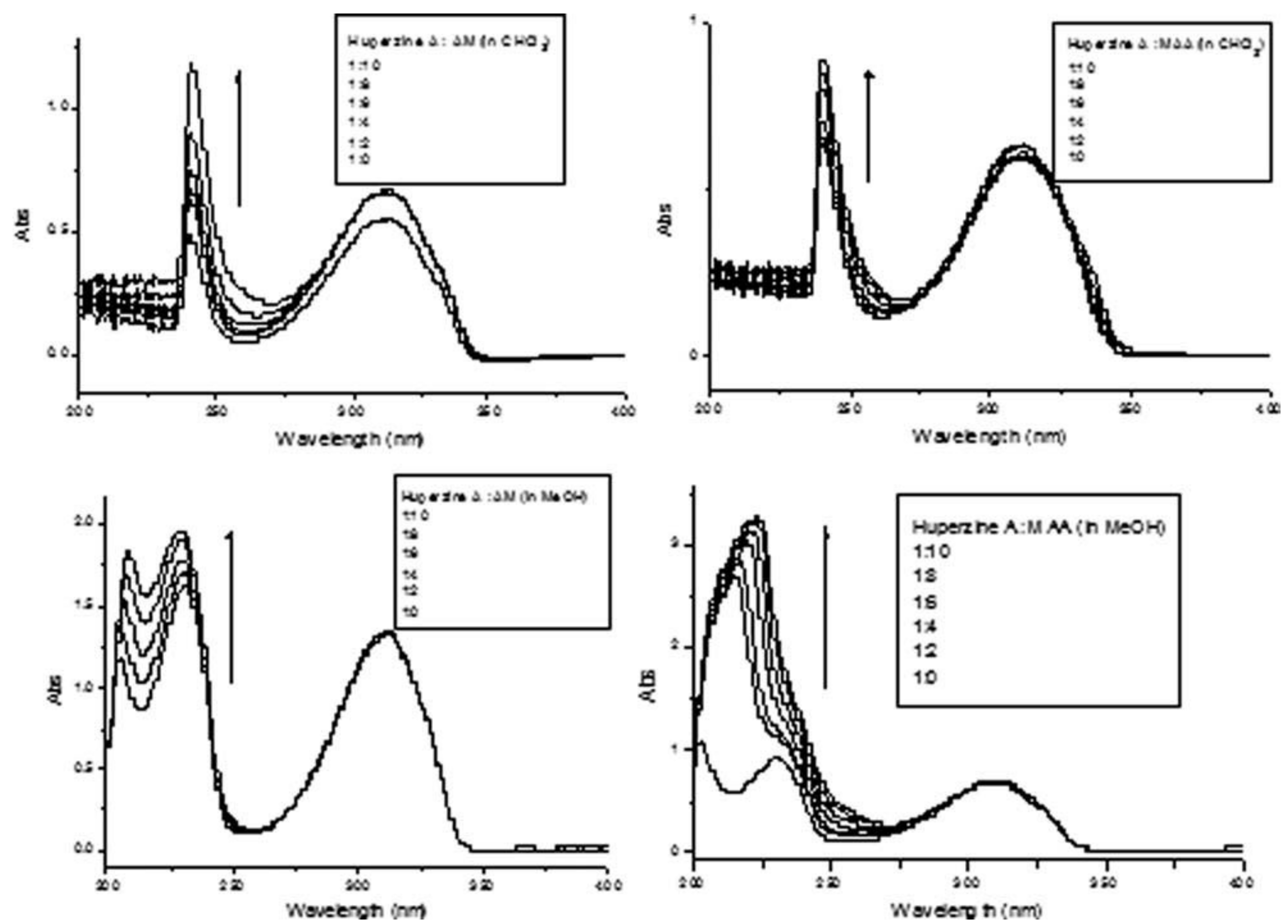

Figure 1 Ultraviolet spectra of huperzine A in the absence and presence of functional monomer. Concentration of huperzine A: 0.05 mmol/L, concentration of monomers: 0, 0.1, 0.2, 0.3, 0.4, 0.5 mmol/L. Corresponding pure solvent (chloroform or methanol) as blanks.

molecule and functional monomer in pre-polymerization stage, which conduces to catch on the reaction mechanism of interaction between template molecule and functional monomer and to predict binding capability and recognition selectivity of imprinted polymer to template molecule. In this work, the interaction of template molecule huperzine A and functional monomer was explored by UV spectrophotometry.

Huperzine A with known amounts was titrated with the increased amounts of MAA or AM in solvent (methanol or chloroform). After shaken and equilibrated for 3 h, UV spectra (Fig. 1) of the mixtures were scanned by using the corresponding pure solvents as blank references, respectively. It could be seen that UV spectra of the mixtures were different from that of huperzine A, which showed a formation of huperzine A-monomer complexes. To further catch on the interaction mechanics of template and monomers, the theory analysis in pre-polymerization stage was performed as following.[20,21] The difference absorbance was measured by UV spectrophotometry at 220 nm for the huperzine/methanol system, at 240 nm for the huperzine/chloroform system by using the same concentration solution of template molecule as blank references, respectively.

The complex reaction of template (T) with monomer (M) may be described by following reaction equation:

$$T + nM = TM_n \tag{2}$$

The association constant (K) of the reaction can be described as:

$$K = \frac{[TM_n]}{[T][M]^n} \tag{3}$$

In general, the equilibrium concentration of M ($[M]$) could be approximated as b_0 in the condition that initial concentration (b_0) of M is greatly larger than that of T (a_0).

On the basis of *Lambert-Beer* law,[22] drawing a plot with $lg\,[(A_0-A)/(A-A_\infty)]$ vs. $lg\,b_0$, a straight-line was obtained when the stoichiometric ratio n was equal to 2 (Table II), which proved that huperzine A with monomer AM formed a 1 : 2-complex, respectively.

TABLE II
Association Constant (*K*) and Composition (*n*) of Huperzine A-Functional Monomer Complexes[a]

Monomer	Solvent	Regression equation	Correlative coefficient	*K*	*n*
AM	Chloroform	$\lg[(A_0 - A)/(A - A_\infty) = 2.07\lg b_0 + 5.88$	0.9997	7.6×10^5	2
	Methanol	$\lg[(A_0 - A)/(A - A_\infty)] = 2.03\lg b_0 + 5.91$	0.9996	8.1×10^5	2
MAA	Chloroform	$\lg[(A_0 - A)/(A - A_\infty)] = 2.02\lg b_0 + 5.23$	0.9998	1.7×10^5	2
	Methanol	$\lg[(A_0 - A)/(A - A_\infty)] = 2.01\lg b_0 + 5.51$	0.9999	3.2×10^5	2

[a] Concentration of huperzine A: 0.05 mmol/L, concentration of monomers: 0, 0.1, 0.2, 0.3, 0.4, 0.5 mmol/L; Same concentration of monomers in chloroform or in methanol as blanks.

Furthermore, the association constant (*K*), obtained from the intercept of the line showed that the non-cooperative H-bonding interaction between carbonyl group and/or amide group in huperzine A with the functional group of AM are generated, the huperzine A-AM complex might be formed and be quite stable. According to the above methods, the association constants of huperzine A with other monomers in each solvent were also obtained. The results were showed in Table II. The values of *K* showed that all complexes of huperzine A reacting to each functional monomer were quite stable. This indicated that H-bonding interactions between huperzine A and each functional monomer are generated. Further analysis of *K* value, the conclusion could be obtained that the huperzine A complex with AM is more stable than that with MAA in same solvent, which suggested that the polymer prepared with AM as functional monomer would have good binding effect to huperzine A (Fig. 2).

Evaluation of the imprinting effect

The binding capacities of all prepared polymers with AM or MAA as functional monomer to huperzine A were studied with the equilibrium binding experiment by UV spectrophotometry. The results showed that the binding capacities (Q) (Table I) of all imprinted polymers were higher than that of the corresponding non-imprinted polymers. The types of functional monomers affected mainly the adsorption ability of the imprinted polymers to huperzine A. MIP1 prepared with AM as the functional monomer in chloroform, showed the optimal imprinted efficiency (2.06) a higher binding capacity (132.5 μmol/g) in all MIPs. MIP3 prepared also with AM as the functional monomer in methanol, showed the highest binding capacity (145.4 μmol/g) in all imprinted polymers, but the lowest imprinted efficiency (1.47). MIP2 prepared with MAA as the functional monomer in chloroform, showed a higher imprinted efficiency (1.90) and the lowest binding capacity (80.6 μmol/g). And MIP4, prepared with MAA as the functional monomer in methanol, showed a higher binding capacity (110.5 μmol/g) and a lower imprinted efficiency (1.50). The results indicated that AM might be a better functional monomer than MAA in this work, in concordance with the results from the pre-polymerization study.

It is clear that the types of solvents also affected the adsorption performance of the imprinted polymers. Generally, MIPs prepared by non-covalent method in a relatively non-polar organic solvent exhibit better recognition property than those prepared using a polar organic solvent. Methanol is more polar than chloroform, and MIP1 prepared in chloroform showed the highest imprinted efficiency (2.06). Therefore, MIP1 was chosen as the optimal adsorbent for next research.

The *Scatchard* analysis[23,24] described by eq. (4) is commonly used to study mechanism of the polymers rebinding template,

$$Q/[substrate] = Q_{\max}/K_d - Q/K_d \quad (4)$$

where, Q is the binding amount of substrate bound to the polymer, $Q_{\max}$ is the apparent maximum number of the binding sites, K_d is the equilibrium

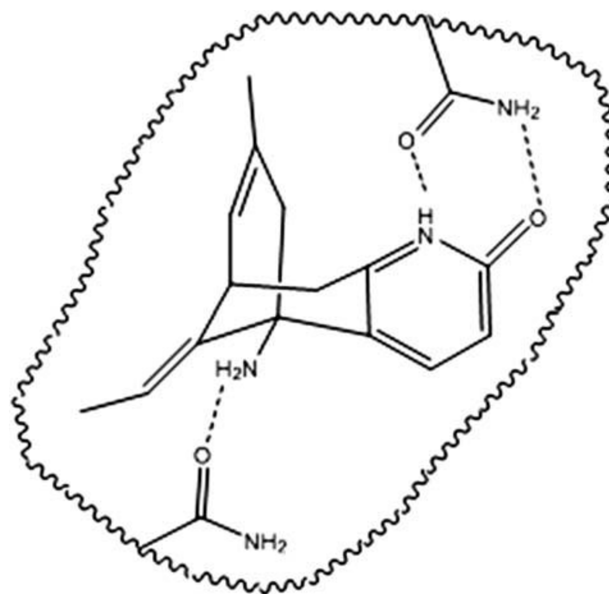

Figure 2 Schematic imprinting effects of huperzine A and AM.

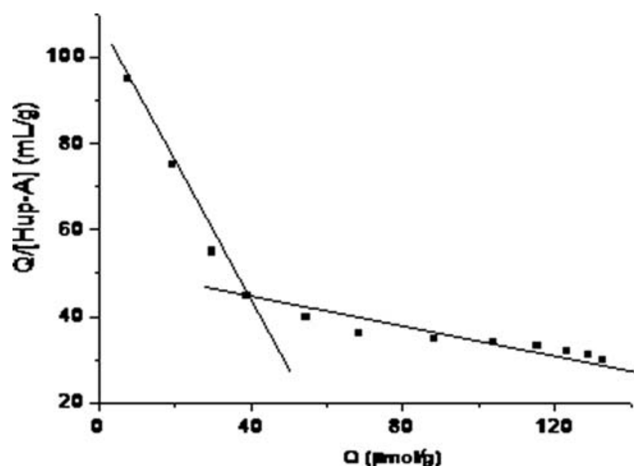

Figure 3 Scatchard plot of MIP1. Amount of MIP1 20.0 mg; Q = μmol of huperzine A bound per 1 g of MIP1; the range of initial concentration of huperzine A 0.02–3.2 mmol/L. [*Hup-A*] = concentration of free huperzine A in solution after adsorption; volume 10.0 mL.

dissociation constant, and [*substrate*] is the equilibrium concentration of the free substrate in solution after adsorbed by the polymers.

The adsorption experiments were carried out by varying the initial concentration of substrate (huperzine A) from 0.5 mmol/L to 3.2 mmol/L in chloroform in the presence of 20.0 mg of MIP1, and the obtained data were plotted according to eq. (4). The *Scatchard* plot for MIP1 (Fig. 3) was not rectilinear, but two crossed straight lines, which implied that the binding sites in MIP1 to huperzine A were heterogeneous and that two types of binding sites were in MIP1. From the intercept and slope of the two linear sections, the apparent maximum dissociation constant (K_{d1}) and the apparent maximum number (Q_{max1}) of the higher affinity binding sites were found to be 0.94 mmol/L and 69.8 μmol/g, as well as the apparent maximum dissociation constant (K_{d2}) and the apparent maximum number (Q_{max2}) of the lower affinity binding sites to be 5.23 mmol/L and 273.3 μmol/g, respectively.

Dynamics of binding reaction

To obtain quickly analytical results, the reactive dynamics for MIP1 rebinding huperzine A was studied by mixing huperzine A (0.05 mmol) with MIP1 (20.0 mg) in chloroform (10.0 mL) at 25°C for 0–6 h. The dynamic curves, i.e., the binding capacity Q versus time t (Fig. 4), pointed out that the binding amount of MIP1 to huperzine A increased quickly within the first 1.5 h. After approximately 5–6 h, the rebinding experiment reached the equilibrium. This kind of adsorption-dynamics properties might be on account of the empty caves existed in MIP1, which favor to transfer huperzine A from the liquid phase to the solid phase. At the initial stage of the rebinding experiment, the adsorptive velocity increased quickly. When the surface layer and lower layer of MIP1 had adsorbed lots of huperzine A, the passage of more huperzine A into the higher layer of MIP1 must be hindered, and causing the decrease of the adsorption velocity.

FTIR spectra of the polymers

MIP1 and NIP1 rebinding with template molecule huperzine A were studied by the FTIR spectra (Fig. 5). It could be seen that the wave numbers of carbonyl group stretching vibrations adsorption in MIP1 before and after rebinding with template molecule varied from about 1634 cm^{-1} to about 1613 cm^{-1}. This indicated that hydrogen bonding interaction existed between carbonyl group in MIP1 with NH_2 or NH group in huperzine A. The adsorption intensity of associated NH_2 or NH group with hydrogen bonding in high wave numbers region (3100–3300 cm^{-1}) increased evidently, which implied the associated NH_2 or NH group with hydrogen bonding increased in the imprinted sorbent MIP1 after rebinding huperzine A, because of the interaction between MA and huperzine A. In addition, IR spectrum of the polymer appeared a stronger-adsorption peak in about 1647 cm^{-1}, corresponded to the stretching vibration adsorption of carbonyl group in huperzne A, and many weak-adsorption peaks of huperzne A in the fingerprint region when MIP1 rebound with template molecule, which proved that template molecule huperzine A had been adsorbed successfully onto the imprinted polymer MIP1. On the contrary, the FTIR spectra of the non-imprinted polymer NIP1 before and after rebinding with template molecule had few evident changes. Above all

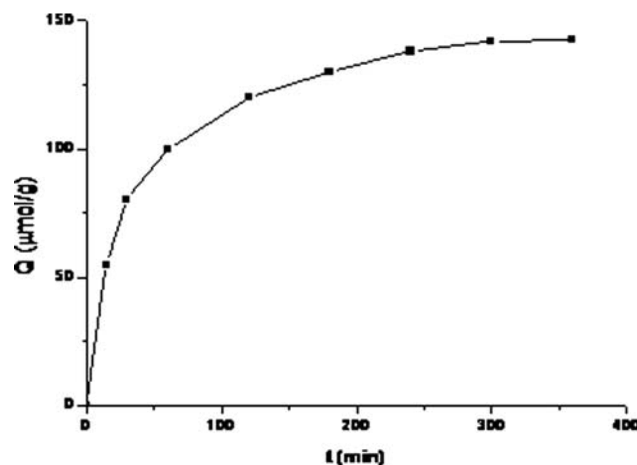

Figure 4 Adsorption dynamics of MIP1 towards huperzine A in chloroform. Initial concentration of huperzine A 3.2 mmol/L; Amount of MIP1 20.0 mg; Q = μmol of huperzine A bound per 1 g of MIP1; volume 10.0 mL.

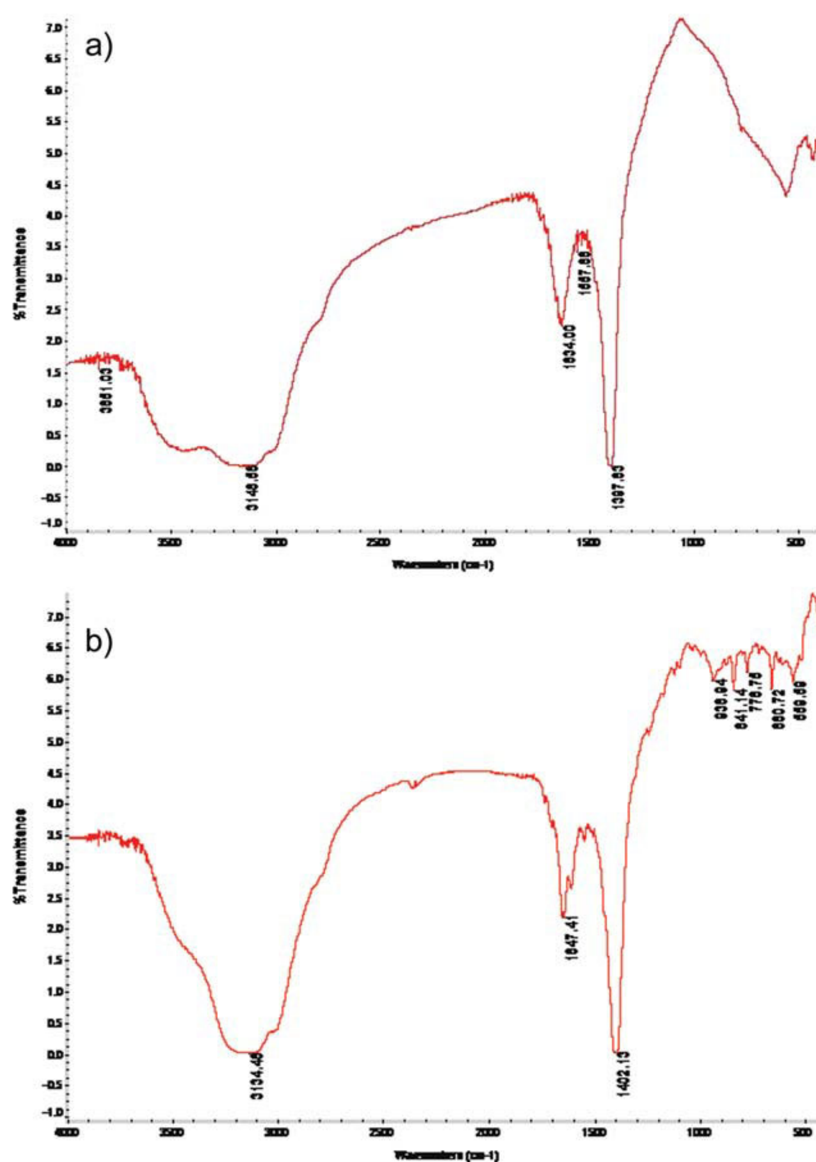

Figure 5 FTIR spectra of MIP1. (a) MIP1 before rebinding with template and (b) MIP1 after rebinding with template. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

results indicated that the template molecule played a well-defined role in the process of preparing MIP1.

Selectivity of the polymers binding substrates

The selectivity of the imprinted polymer MIP1 and the corresponding non-imprinted polymer NIP1 to huperzine A and four other substrates isolated from *Huperzia serrata* (Fig. 6), i.e., phlegmariurine B, ferulic acid, 6β-hydroxylhuperzine A, and arbutin, were investigated by MIP-SPE micro-column. The binding capacities of MIP1 or NIP1 (100.0 mg, each) to five substrates in the mixture consisting of five substrates (5.0 mg, each) were determined in chloroform. Each peak in the chromatogram was identified by adding the standard substance one by one into the mixture. The ratio of adsorption in Table III was obtained by dividing the amount of the substrates in the initial mixture by the difference of amount of the substrates in the residual and in the initial mixture, respectively. It can be seen that MIP1 could mainly adsorb huperzine A in the mixture substrate solution with a larger ratio of adsorption to huperzine A (86.6 %) than that of NIP1 (15.8 %). Moreover, MIP1 performed a larger ratio of adsorption to huperzine A than to other four substrates (all at the same concentration). Although both MIP1 and NIP1 bound partially other four substrates, the ratio of adsorption of MIP1 and NIP1 to four substrates only had a little disparity for each other, which showed that MIP1 had selective recognition ability to huperzine A in mixture substrates chloroform solution. This

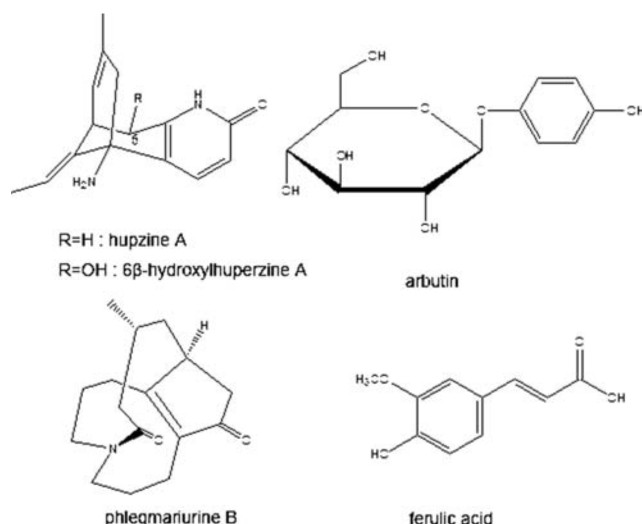

Figure 6 Structure of huperzine A and other substrates.

indicated that MIP1 prepared in this work could be used as a practical material to enrich and separate huperzine A from the complex matrixes.

It is different for huperzine A in structure from phlegmariurine B, ferulic acid, 6β-hydroxylhuperzine A, and arbutin. Although the functional groups in phlegmariurine B, ferulic acid, 6β-hydroxylhuperzine A, and arbutin could also form hydrogen bond with AM, MIP1 could rebind huperzine A selectively for theirs structural distinction compared with NIP1. It is because MIP1 possessed the specific cavities created by template printing with functionality of functional monomer in a complementary fashion.

Optimization of MISPE conditions

MISPE micro-columns were packed with the optimal polymer materials MIP1 and the corresponding NIP1 (100.0 mg, each), respectively. Their performances as sorbents for extracting huperzine A were compared, and the washing, eluting conditions were optimized.

Chloroform and other different solvents were employed in eluting step and the HPLC data were listed in Table IV. It can be seen that methanol containing 2–5% of ammonia water had the best eluting effect (almost 100%) to huperzine A adsorbed by both MIP1 and NIP1 micro-column, which showed that methanol containing 2–5% of ammonia water was effective to be used as the eluting solvent for performing the elution of huperzine A adsorbed by MIP1 or NIP1 micro-column. Therefore, the methanol containing 2% of ammonia water was used as the eluting solvent to elute huperzine A adsorbed by MIP1.

It can be also seen that the bad eluting effect were obtained by using chloroform as the eluting solvent. In this case, only 13% of huperzine A was eluted from the MIP1 micro-columns, whereas in the NIP ones, this percentage was 69%, which showed that our materials MIP1 are very useful as sorbents for SPE procedure. On the basis of these results, chloroform was chose to be used as the loading and washing solvent.

Sample analysis

The ability of MIP1 and NIP1 (100.0 mg, each) extracting target compound, huperzine A, from the sample of the Chinese traditional herb, *Huperzia serrata*, was studied through an off-line MISPE micro-column. The existence of huperzine A in the herb extract was confirmed by adding the standard substance of huperzine A, into the extract solution, and the content of huperzine A in the herb extract was determined by HPLC. After loading the MISPE micro-column with the sample of herb extract

TABLE III
Adsorption of MIP1 and NIP1 to Five Substrates of the Mixture[a]

Substrates	Substrate content in residual (mg)		Ratio of adsorption (%)[b]	
	MIP1	NIP1	MIP1	NIP1
Huperzine A	0.67	4.21	86.6	15.8
Phlegmariurine B	3.11	4.13	37.8	17.4
Ferulic acid	3.53	4.05	29.4	19.0
6β-Hydroxylhuperzine A	3.98	4.20	20.4	16.0
Arbutin	3.72	4.38	25.6	12.4

[a] Intial amount of each substrate was 5.0 mg.
[b] Ratio of adsorption was calculated as the ratio of the difference of amount of substrate in residual and in initial mixture to initial amount of substrate.

TABLE IV
Percentage of Collected Huperzine A in Eluting Fractions[a]

Eluting solvent	MIP1	NIP1
Ethanol	61 ± 3.2[b]	70 ± 3.0
Methanol	78 ± 2.1	90 ± 2.1
Acetone	23 ± 1.5	55 ± 1.4
Acetonitrile	38 ± 1.8	62 ± 1.9
Chloroform	13 ± 1.5	69 ± 1.5
Methanol/ammonia water, 95 : 5 (v/v)	100 ± 2.2	100 ± 2.2
Methanol/ammonia water, 98 : 2 (v/v)	100 ± 2.0	100 ± 2.0

[a] Loading step: 2.0 mL of chloroform solution, flow rate about 0.1 mL/min. Eluting step: 5.0 mL of the eluting solvent, flow rate about 0.1 mL/min.
[b] The values following " ± " are R.S.D.

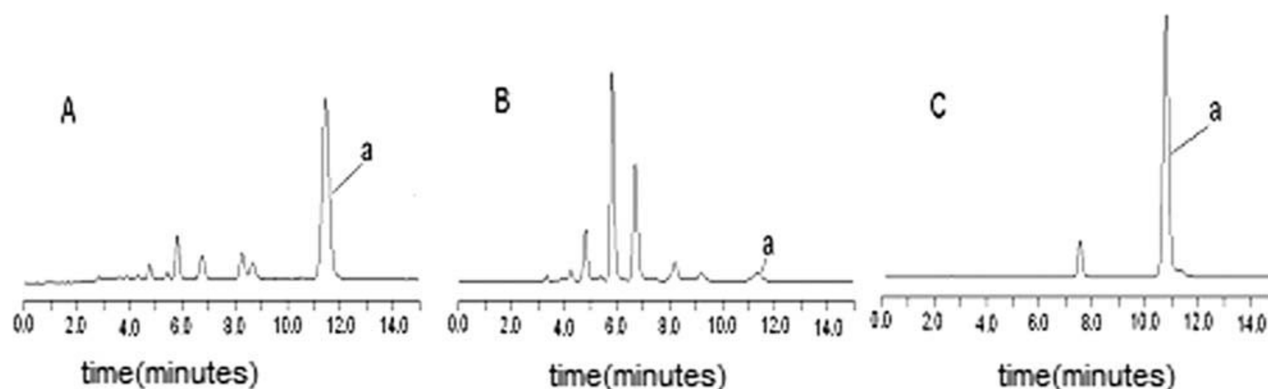

Figure 7 Chromatograms of the sample solution of *huperzia serrata*. **A** is the initial sample solution, **B** is the sample solution after treated by MIP1, **C** is the eluting solution from MIP1 (elution with methanol/ammonia water, v/v, 98 : 2); the peak of **a** in each plot is huperzine A.

solution, the column was washed with chloroform (5 mL) and then eluted with methanol/ammonia water (98 : 2, v/v; 5 mL). The content of huperzine A in the washing solution and in the eluate was analyzed, respectively. The Chromatograms of the sample are showed in Figure 7, and the analytical results of the sample are listed in Table V. Ratio of adsorption in Table V was calculated as the ratio of the amount of huperzine A in eluate to initial amount of huperzine A. Ratio of adsorption (89.4 %) of MIP1 was very higher than that (32.6 %) of NIP1. It could be concluded that MISPE column could enrich principally and separate huperzine A from the sample of herb extract, and presented a higher binding capacity and better recognition ability to target compound, huperzine A, than to other components in the sample of *Huperzia serrata*.

Method validation and reusability of MIPSPE micro-column

To verify the recovery and the lifespan of effectiveness about the MISPE micro-column, three different amounts of huperzine A were added into the sample of herb extract that contains a known amount of huperzine A, respectively. Each of above sample solutions (2.0 mL) was loading into the selfsame MISPE micro-column in turn, subsequently washing the column with chloroform. After eluting the column with methanol/ammonia water, the eluates were assembled together, respectively. The amount of huperzine A in the eluate was determined and the degrees of recovery were calculated (Table VI). The results showed MIP particles showed acceptable loss in the adsorption capacity after sorption/desorption cycle three times with the same sorbent. Above 90 percent of the average degrees might be a wonderful result, though the reiteration could result in a downward tendency of the efficiency of the MISPE micro-column. These results manifested that the self-established methods is dependable to enrich and separate huperzine A from the sample of herb, *Huperzia serrata*, and as well as the MISPE micro-column could be used repeatedly to enrich and separate huperzine A from complex mixtures within limits of times.

CONCLUSIONS

In this article, four new huperzine A-imprinted polymers and four corresponding non-imprinted polymers, were synthesized by thermal-initiated co-

TABLE V
Adsorption of MIP1 and NIP1 to Huperzine A in Sample Solution of *Huperzia serrata*

	Amount of huperzine A (μmol)		Ratio of adsorption (%)[a]
	In initial	In eluate	
MIP1	62.0	55.4	89.4 ± 2.2
NIP1	62.0	20.2	32.6 ± 2.7

[a] Ratio of adsorption was calculated as the ratio of the amount of huperzine A in eluate to initial amount of huperzine A. The values following " ± " are R.S.D.

TABLE VI
Analytical Results of the Addition of Huperzine A to Sample Solution of *Huperzia serrata*[a]

Experiment	Added (μmol)	Found (μmol)	Recovery (%)[b]
1	25.0	85.2	92.8 ± 2.0
2	50.0	107.4	90.8 ± 2.3
3	150.0	191.7	86.5 ± 2.9

[a] Amount of huperzine A in initial sample solution was 62.0 μmol; Loading volume of the sample solution was 2.0 mL.
[b] The recovery was calculated as the ratio of the difference of found amount and amount of huperzine A in initial sample to added amount.

polymerization using acrylamide (or methacrylic acid) as the functional monomer in chloroform (or methanol). Binding capacity and adsorption selectivity of the imprinted polymers were studied, and compared with each other and with those of the corresponding non-imprinted polymers. The results demonstrated that MIP1 by involving AM as functional monomer in chloroform, having effective artificial recognition sites for huperzine A, showed a larger binding capacity and higher recognition ability to huperzine A. Finally, by using a MIP1-packed SPE micro-column, a new method for extracting and separating huperzine A from the sample of *Huperzia serrata* was established. The method, which is convenient and valid, could be used to extract, separate and determine huperzine A from an intricate mixture.

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