

Evaluation of New Selective Molecularly Imprinted Polymers for the Extraction of Resveratrol from *Polygonum Cuspidatum*

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Abstract: Four different molecularly imprinted polymers (MIPs) were prepared using resveratrol as the template, methacrylic acid (MAA) or acrylamide (AA) as functional monomers, 2, 2'-azobisisobutyronitrile (AIBN) as the initiator, and thermo- or photo-induced polymerization. The ability of the different polymers to rebind selectively not only the template but also other phenols was evaluated. In parallel, the influence of the different templates and functional monomers used during polymer syntheses on the performance of the obtained MIPs was also studied through different rebinding experiments. The binding ability and selectivity of the polymer were studied by static balance method and Scatchard analysis. It was concluded that AA-based polymer by photo-induced polymerization presents the best properties to be used as a selective absorbent for the extraction of resveratrol.

Keywords: molecularly imprinted polymers, resveratrol, isotherms, binding sites.

Introduction

Molecular imprinting is a technique used for preparing polymers with synthetic recognition sites having a predetermined selectivity for analyte of interest. The imprint is obtained by arranging polymerization functional monomers around a template (the analyte). Complexes are then formed through molecular interactions between the analyte and the monomer precursors. The complexes are assembled in the liquid phase and fixed by cross-linking polymerization. Removal of the template from the resulting polymer matrix creates vacant recognition sites that exhibit affinity for the analyte.

It has been shown previously, that molecularly imprinted polymers (MIPs) possess high selectivity and sensitivity for templates.¹⁻⁴ Synthesis of MIPs is a relatively straightforward and inexpensive procedure. Moreover, these polymers demonstrate very good thermal and mechanical stability and can be used in aggressive media.⁵ MIPs have been widely studied for chromatographic separation⁵⁻⁹ and as selective elements

of chemical sensors.¹⁰⁻¹² In particular, the application of MIPs for solid-phase extraction (SPE) is a field of intense development.^{13,14}

Around the template molecule, a three-dimensional polymeric structure produces bi proper shape and charge distribution.^{15,16} The diversity of the natural compound components leads to the complication of the active ingredients extraction. The molecular imprinted polymer's specificity and affinity can be applied to extract some ingredients of natural compound directly.¹⁷

Resveratrol, trans-3,5,4-trihydroxy stilbene, is one of the major stilbene phytoalexins found in various families of plants, but grapes, peanuts, and their products are considered the most important dietary sources of the resveratrol. Resveratrol had diverse biological properties.¹⁸⁻²² Especially their use as drug for heart-vein disease and cancer disease is very attractive. In this paper, the resveratrol-imprinted polymer was prepared successfully using the technique of molecular imprinting with acrylamide as the functional monomer and ethylene glycol dimethacrylate as the cross-linker. The combination ability and selective performance of the polymer were studied by static balance combination and Scatchard

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analyses. The further study could be used in the direct extraction, enrichment and determination of the resveratrol in herbal medicine.

Experimental

Instrumentation. The chromatographic separation was performed on a Shimadzu 2010A liquid chromatograph (Shimadzu, Japan) with a guard column equipped with a UV-Vis diode array detector. TGL-16G freeze centrifuge was purchased from Shanghai Science Instrument Factory (Shanghai, China). ZD-2 speed-governor-oscillator was obtained from Jiangsu Wanhua Instrument Factory (Jiangsu, China). CQ-6 ultrasonic cleaner was obtained from Shanghai Huchao Ultrasonic Instrument Ltd (Shanghai, China).

Materials. Acetonitrile, methanol, acrylamide (AA), and tetrahydrofuran (THF) were purchased from Hunan Normal University (Hunan, China). Resveratrol, polydatin and Biphenol A were purchased from Sigma (USA). Methyl acrylic acid (MAA), ethylene glycol dimethylacrylate (EGDMA) and 2,2-azobisisobutyronitrile (AIBN) were obtained from Tianjin Kemi Reagent Development Center (Tianjin, China). The water was distilled twice.

Synthesis of Polymers. 0.57 g of the template molecule (resveratrol) and 1.065 g of the functional monomer (AA) were dissolved in 25 mL acetonitrile at 0–5 °C. Then, 15 g of cross-linker (EGDMA) and 0.057 g of initiator (AIBN) were added slowly under stirring. During the addition (10–15 min) the temperature was held constant at 0 °C. The mixture was purged with nitrogen for 10 min and sealed in vacuum. The sealed bottle was polymerized under ultraviolet ray at 4 °C for 24 h. The resultant stick polymer was crushed in a mortar and passed through a griddle of 75 μ m. It was placed in a Soxhlet apparatus with 5% acetic acid and methanol solution for 24 h to remove the template molecule and unreacted compounds. Then acetic acid and methanol were removed by acetonitrile. The polymer was repeatedly sedimentated with acetone to get rid of the tiny particles and dried in vacuum desiccator. The obtained polymer was polymer-A (PA). A blank polymer polymer-B (PB) was also synthesized with the same procedure, but lacked the template molecule. Polymer-C (PC) was synthesized using polymer-A synthesis method but not under ultraviolet ray at 4 °C for 24 h. Poly-

merization was carried out in a water bath regulated 60 °C for 24 h. The synthetic procedure of polymer-D (PD) was the same as polymer-C but the functional monomer was MAA. The synthetic procedure of polymer-E (PE) was the same as polymer-A but the solvent was THF. The synthetic conditions of the polymers were shown in Table I.

Determination of Binding Capacity for MIPs. Binding capacities of MIPs for resveratrol were determined spectrophotometrically at 303 nm. The standard solutions of resveratrol were prepared by acetonitrile and its absorbances were measured against a reagent blank prepared with the same amount of acetonitrile, but no resveratrol. A calibration graph was found between the absorbance and concentration of resveratrol. An accurately weighed 0.1000 g portion of the polymer was transferred into a 250 mL conical flask, 10.0 mL of 4.5 mmol/L resveratrol–acetonitrile standard solution was added and the flask shaken for 5 h. This solution was centrifuged at 4,500 rpm for 5 min. The centrifugate (1.00 mL) was transferred into 5.0 mL volumetric flask and diluted to the mark with acetonitrile. The absorbance of the solution was measured at 303 nm with spectrophotometer. The concentration of resveratrol was estimated based on calibration graph of resveratrol.

Measurement of Adsorption Isotherms. 400 mg of the resultant polymer was added to 1, 2, 3, 4, 5, 7, 10, 13, 17 mL of acetonitrile which contained 1 mg/mL of resveratrol. They were diluted to 40 mL with acetonitrile and surged in ZD-2 oscillator for 12 h at room temperature. The surged samples were extracted by centrifugal effect. The upper solution was diluted with acetonitrile and detected by HPLC-UV at 303 nm. Then the concentration of resveratrol was determined. The adsorption capability of molecularly imprinted polymer was estimated according to the concentration change of resveratrol.

HPLC Analysis. A reversed-phase high performance liquid chromatography (RP-HPLC) method was developed to determine resveratrol. The separation was performed on an ODS C₁₈ column (250×4.6 mm, 5 μ m), with the mobile phase of CH₃OH–H₂O (45:55, v/v). The flow rate was 0.8 mL/min and the detection wavelength was 303 nm. The result showed that there was a good linear relationship between the area and the concentration of resveratrol. The corresponding regression equation is $Y=15879.28X+12350.2$, $r=0.9996$.

Table I. Conditions of Polymer Synthesis

Polymer	PA	PB	PC	PD	PE
Template Molecule	Resveratrol	-	Resveratrol	Resveratrol	Resveratrol
Functional Monomer	AA	AA	AA	MAA	AA
Porous Solvent	acetonitrile	acetonitrile	acetonitrile	acetonitrile	THF
Polymerization Method ^a	Photo-p	Photo-p	Heat-p	Heat-p	Photo-p

^aPhoto-p i.e. light initiated polymerization was polymerized under UV radiation for 24 h at 4 °C. Heat-p i.e. heat initiated polymerization was polymerized for 24 h at 60 °C (water).

The proposed method is simple and rapid with good precision. The recoveries were 95.81–103.65% and RSD was 1.79%.

MISPE of Sample Extracts. Fifty millilitres of methanol solution including 0.25 $\mu\text{mol/L}$ resveratrol extracted from *Polygonum cuspidatum* was filtered to remove insoluble substance. Twenty-five millilitres of filtrate was separately loaded onto PA column; the column was washed with 10 mL water, 10 mL ethanol. Finally, it was eluted with 5 mL methanol including 5% acetic acid. This fraction was evaporated to dryness and dissolved in 2 mL of acetonitrile for final analysis by HPLC–UV.

Results and Discussion

Imprinting Mechanism. Many imprinted polymers were synthesized using MAA as functional monomer and ethylene dimethacrylate (EDMA) or ethylene glycol dimethacrylate (EGDMA) as cross-linker by heat initiated or photo initiated polymerization. In principle, MIPs are synthesized by cross-linking complexes of template molecules and functional monomers. After removing the template molecules from the polymers, binding sites are formed by functional monomer derived residues complementary for the template molecules. According to the principle, the stability of monomer–template complexes present in the solution prior to polymerization as well as the polymerization reaction itself undoubtedly play a dominant role in determining the recognition performance of the polymers. An understanding of the physical rules governing the quality and quantity of MIPs recognition sites will contribute to design polymerization systems for producing polymers with good recognition property.

The template molecule, resveratrol, is a stilbene compound. The resveratrol molecule has three phenolic hydroxyls and a double bond which link with two benzenes. This structure ensures to form a three-dimensional molecular imprinting

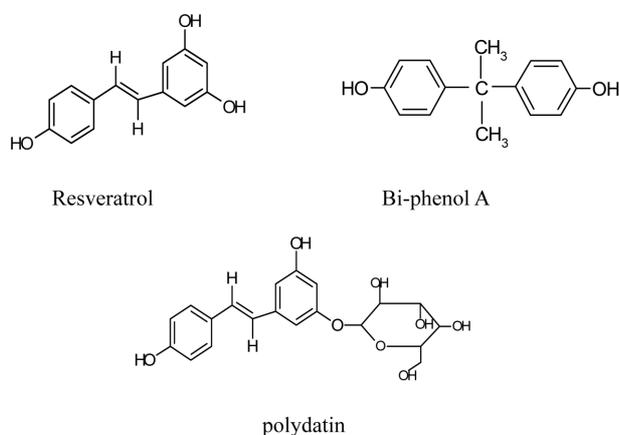


Figure 1. Structures of substrates resveratrol, bi-phenol A and polydatin.

with functional monomer (AA) and cross-linker (EGDMA). The oxygen atom functional groups of resveratrol molecule can form hydrogen bond with functional monomer (AA). According to the Scatchard model the processing of data showed that there were three bonding sites in the MIPs (Section 3.4). Three binding sites are located at the point that the OH group which link with phenolic hydroxyls of resveratrol molecule bond with carboxyl of MAA through hydrogen bond. In order to remove the imprint molecule from MIPs after cross-linking, it is necessary to break the non-covalent bond between resveratrol molecule and carboxyl group of the polymer. This was performed by continuous extraction with methanol containing 5% acetic acid in a Soxhlet extractor. Figure 2 is a schematic representation of the synthesis of molecularly imprinted polymer for resveratrol.

Adsorption Capacity for All MIPs. According to 2.4, the binding capacity of all MIPs was measured and calculated according to following equation:

$$Q = [(C_i - C_f) V_s \times 1000] / M_{MIPs}$$

Where Q is adsorption capacity of MIPs ($\mu\text{mol/g}$), C_i the initial resveratrol concentration ($\mu\text{mol/mL}$), C_f the final resveratrol concentration ($\mu\text{mol/mL}$), V_s the volume of solution tested (mL), M_{MIPs} the mass of dried polymer (mg). The adsorption capacity is an important parameter to evaluate MIP for extract bioactive compounds from Chinese herbs. The adsorption capacity for PA is 132.18 $\mu\text{mol/g}$. It means that 1 g PA can absorb 30.16 mg resveratrol. But 1 g PE, PC, and PD can only absorb 22.39, 17.74, and 11.56 mg resveratrol, respectively. PB almost doesn't absorb any resveratrol. So PA is evaluated by Scatchard analysis and chosen to extract resveratrol from *Polygonum cuspidatum*.

Adsorption Isotherm Models. Figure 3 shows the binding isotherm of all MIPs for resveratrol via static balance combination method. PC and PA were synthesized by heat initiation and photo initiation respectively in the same polymerization solution; the later was a little better than the former (Figures 3(a) and 3(b)). PA and PD were synthesized using different functional monomers. Molecular imprinted polymer (PD) using methyl acrylic acid as functional monomer has lower adsorption capability (Figures 3(a) and 3(d)). It was in accordance with the theoretical conclusion. Compared PC with PE synthesized by different porogenic agents, the affinity between the functional monomer and the template molecule had no obvious difference (Figures 3(b) and 3(c)). It indicated that the template molecule also could form hydrogen bond with acrylamide in THF and the synthesized molecular imprinted polymer had high adsorption capability to the template.

Characteristic of the Recognition of the MIPs for Resveratrol. MIPs for resveratrol were prepared based on the synthetic condition. The binding capacities of the MIPs to

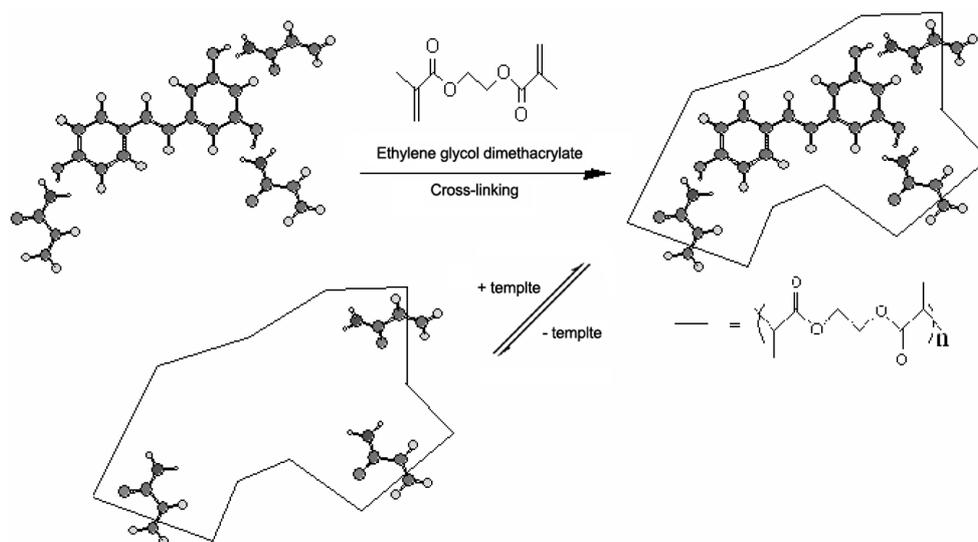


Figure 2. Schematic representation of molecular imprinting for creating a template-selective binding site utilizing a pre-polymer: (a) Resveratrol as a template is mixed with the pre-polymer to give complex species and (b) cross-linking of the complex with ethylene glycol dimethacrylate results in formation of a binding site complementary to resveratrol.

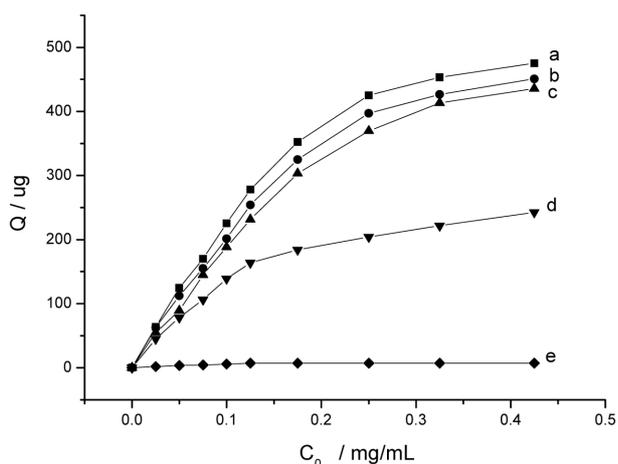


Figure 3. Binding isotherm of imprinted polymers for resveratrol. (a) PA; (b) PC; (c) PE; (d) PD; (e) PB (Blank polymers).

resveratrol were measured according to 2.3. The binding isotherm, displayed in Figure 3, shows that the change of Q value tends to flat with a rise in the concentration of resveratrol. This indicated that the cavities of the MIPs were gradually saturated by resveratrol molecule. In the molecularly imprinted polymer technique, Scatchard model was often used to evaluate the characteristic of the recognition of the MIPs. Scatchard equation is follows: $Q/C = (Q_{max} - Q)/K_d$. In the equation: Q is binding capacity; C concentration of resveratrol (mg/L); Q_{max} maximum apparent binding capacity; K_d disassociation constant at binding site. Test found a relationship between Q/C and Q , namely, Scatchard plot showed in Figure 4. The Scatchard plot obtained for the being of

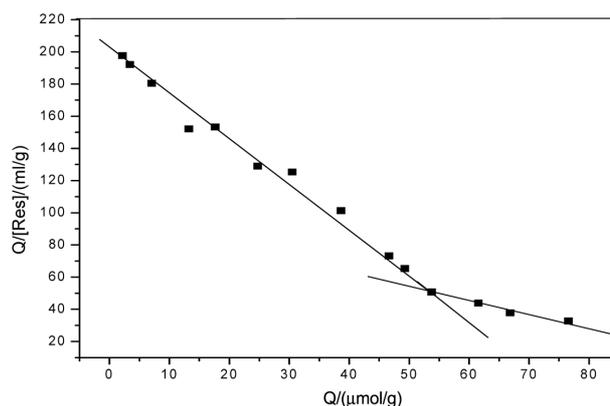


Figure 4. Scatchard plot for the binding nature of PA.

resveratrol to its polymer imprint consists of two distinct straight lines. By plotted Q/C versus Q (Figure 4), the dissociation constant of the binding sites K_d and the apparent maximum number of the binding sites (Q_{max}) can be obtained. Scatchard curve is divided into two segments (Figure 4). Both parts give a nice linear line with different slopes, suggesting two kinds of binding sites be formed in the scope of studied template concentration. According to the slope and intercept in Scatchard curve, the binding parameter to resveratrol could be obtained. K_{d1} and Q_{max1} with high affinity of binding sites are 2.665×10^{-3} mol/L and $74.71 \mu\text{mol/g}$ respectively. K_{d2} and Q_{max2} with low affinity of binding sites are 7.998×10^{-4} mol/L and $Q_{max2} = 116.27 \mu\text{mol/g}$ respectively. The Scatchard plot showed two lines with different slopes corresponding to high and low affinity populations of binding sites. i.e., K_{d1} and K_{d2} were disassociation constant

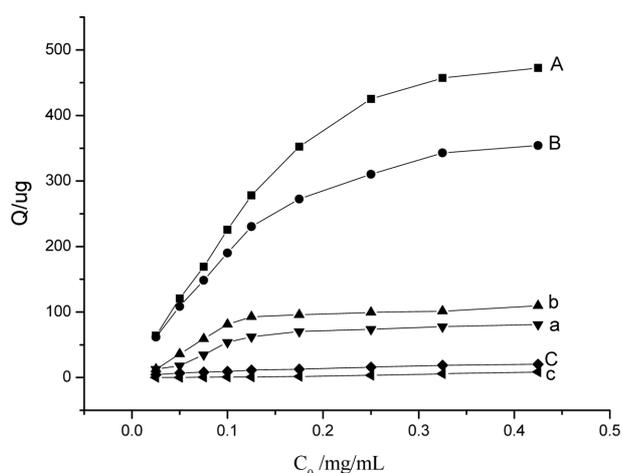


Figure 5. Binding properties of PA and PB for substrates: (A), (B), and (C): Amount of substrates bound to PA substrates vs. initial concentrations; (a), (b), and (c): Amount of substrates bound to PB substrates vs. initial concentrations: (A) and (a) for resveratrol; (B) and (b) for polydatin; (C) and (c) for bi-phenol A.

at binding site of low affinity and at binding site of high affinity, respectively. Based on the existence of three binding sites in the MIPs (PA) we infer that three binding sites are located at the point that the OH group which link with phenolic hydroxyls of resveratrol molecule bond with carboxyl of AA through hydrogen bond. Certainly, there is still much work to be done in efforts to verify which phenolic hydroxyls of resveratrol molecule bond with carboxyl of AA.

Selectivity of Molecularly Imprinted Polymers. Figure 5 shows the binding properties of PA and PB for substrates by equilibrium adsorption. The low adsorption capability of non-imprinted polymers to resveratrol (Figure 5(A) and 5(a)), polydatin (Figure 5(B) and 5(b)) and Bi-phenol A (Figure 5(C) and 5(c)) confirmed that the affinity between molecularly imprinted polymer and the template molecule was enhanced by specific binding sites. The combination of binding sites is more co-operative than that of two hydrogen bonds, which have strong action. Further more, the binding sites and the template structure made the polymer selective so as to recognize the template molecule. The adsorption of non-molecular imprinted polymer came from the non-single combination such as monomer functional group arranged at random. It doesn't involve cooperation of hydrogen bond in

specific position. Non-imprinted polymers had no strong adsorption to the template molecule.

The selectivity of molecular imprinted polymer can also be expressed by static adsorption distribute coefficient K_D and separation factor α with the formula $K_D = C_p/C_s$, where C_p (mmol/g) is the concentration of the substrate on molecular imprinted polymer, C_s (mmol/mL) is the concentration of the substrate in the solution.

Separation factor is the proportion of K_D of two different substrates. $\alpha = K_{Di}/K_{Dj}$, where i and j refer to the template and the substrate respectively. When i is equaled to j , $\alpha = 1.00$. Table II showed K_D and α of different polymers to three substrates with similar structure.

It's found that the K_D and α of non-imprinted PB to three substrates were of little discrepancy. There were no selective binding sites in PB. It depended on non-selective combination on the surface to adsorb the template molecule.

The K_D of PA to the substrates was much higher than PB. It suggested that PA using resveratrol as the template molecular produced obvious effect. PA had cavities with the appropriate shape and function to specifically binding resveratrol, thus the K_D and α to three substrates are discrepantly great. The high selectivity and recognition to resveratrol were much stronger than the other two substrates. Resveratrol-imprinted polymer had high selectivity and recognition to resveratrol while definite selectivity and recognition to polydatin. Because polydatin had only dextrose moiety in structure while the molecular size is larger than resveratrol. Resveratrol-imprinted polymer adsorbed large amount of resveratrol, but only few of polydatin. It had the lowest selectivity and recognition to Bi-phenol A. Resveratrol had a single C=C while Bi-phenol A is one carbon atom linking two benzenes. Changes in space structure lead to lower recognition. The results further confirmed that there were binding cavities in specific position whose magnitude depended on the bulk of the template and position lies on the template position. The cavities were complementary to resveratrol, so the polymer had memory ability to the template molecule. The distinction of PD and PA indicated that the functional monomer had significant effect on binding capacity of MIP. The K_D and α of molecular imprinted polymer using methyl acrylic acid as functional monomer to the template molecular and the other two substrates hadn't apparent distinction, which further showed acrylamide was easier to form hydrogen bond than methyl acrylic acid in polar solution. The comparison of

Table II. K_D and α Values of Different Polymers

Substrates	PA		PB		PC		PD		PE	
	K_D	α	K_D	α	K_D	α	K_D	α	K_D	α
Resveratrol	1.158	1.00	0.132	1.00	1.067	1.00	0.549	1.00	1.140	1.00
Polydatin	0.978	1.18	0.245	0.54	0.925	1.15	0.697	0.79	0.846	1.35
Bi-phenol A	0.0008	1447.5	0.015	8.8	0.0195	54.72	0.0149	36.85	0.001	1140

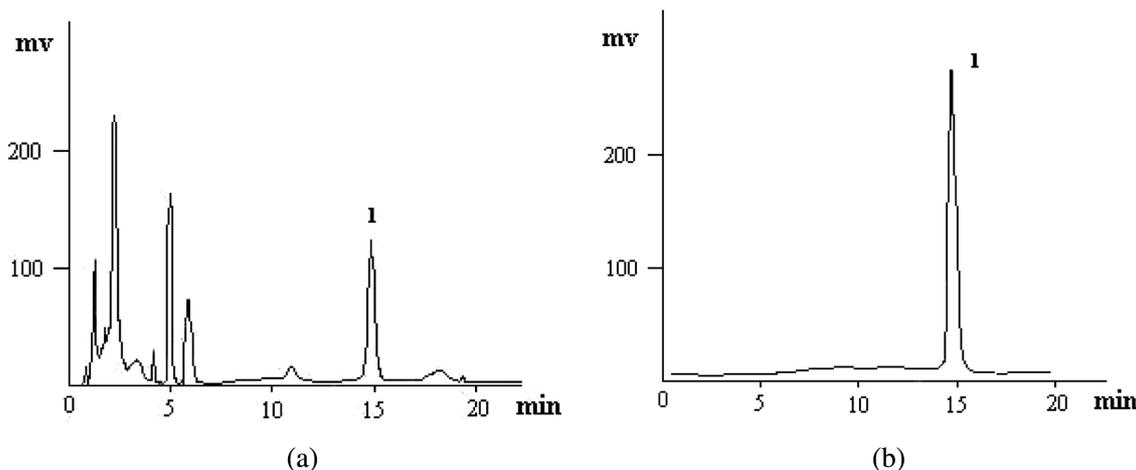


Figure 6. Chromatograms of methanol extract from *Polygonum cuspidatum* before extracting (a) and after enriching and separating by PA (b).

PE and PA showed that molecular imprinted polymer using THF as porogenic agents had much higher selective recognition to the template molecule than the other two substrates. The template molecule and the functional monomer both could form hydrogen bond in THF solution; therefore, molecular imprinted polymer with selective adsorption was obtained.

MISPE of Resveratrol from *Polygonum Cuspidatum* Extracts. Apart from these theoretical considerations, and according to the obtained results, PA seems to be able to retain selectively all the tested analytes with high enough capacity and affinity. Thus, a preliminary evaluation on the use of this polymer in MISPE of resveratrol in a real sample (*Polygonum cuspidatum*) was carried out. Figure 6(a) shows the chromatogram of methanol extract from *Polygonum cuspidatum* without extraction by PA and obviously it is complex. Figure 6(b) shows the chromatogram after enriching and separating by PA. In Figure 6(b) most interferences were removed and high purity of resveratrol (>99.21%) was attained from *Polygonum cuspidatum*.

This preliminary evaluation is very promising and thus both PC and PE polymers are under further evaluation in our laboratory for the development of molecularly imprinted solid-phase extraction methods for the determination of phenyls from environmental and food samples.

Conclusions

A molecular imprinted polymer (MIP) was synthesized using the technique of molecular imprinting with acrylamide as the functional monomer, resveratrol as the template in polar solvent, namely acetonitrile. The selectivity and affinity in acetonitrile were studied. The results show that resveratrol-imprinted polymer has higher selective recognition to resveratrol compared with non-imprinted polymer in aceto-

nitrile. At the same time, resveratrol-imprinted polymer also has high adsorption to polydatin, which has similar structure with resveratrol, suggesting cross-reactivity. It has low adsorption to Bi-phenol A, which has apparent distinction with resveratrol in structure. Study in this paper is promising to offer definite experiment and theory foundation in the application of molecular imprinted polymer in herbal medicine.

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