Preparation and Characteristics of Esculin-Imprinted Polymers

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Four molecularly imprinted polymers (MIPs) were prepared in MeOH with esculin (=6,7-dihydroxycoumarin 6-(β-D-glucopyranoside) = 6-(β-D-glucopyranosyloxy)-7-hydroxy-2*H*-1-benzopyran-2-one) as the imprinted molecule, methacrylic acid (=2-methylprop-2-enoic acid; MAA), acrylamide (= prop-2-enamide; AM), 4-vinylpyridine (=4-ethenylpyridine; 4-VP), or 2-vinylpyridine (=2-ethenylpyridine; 2-VP) as the functional monomer, respectively, as well as ethylene glycol dimethacrylate (=2-methylprop-2-enoic acid ethane-1,2-diyl ester; EGDMA) as the cross-linking agent. The interaction between the template and the functional monomers was investigated by fluorescence and UV spectrophotometry, respectively, which revealed the presence of esculin/monomer complexes in the stoichiometric ratio 1:2 in the pre-polymerization mixture. The resultant polymers were studied in equilibrium binding experiments to evaluate the recognition ability and the binding capacity towards esculin. The results showed that MIP_1, prepared with MAA as the functional monomer, exhibited advantageous characteristics of high binding capacity, optimal imprinting effect, and good selectivity towards esculin. The *Scatchard* analysis indicated that there are two types of binding sites in MIP_1, and its binding parameters including the apparent maximum numbers of binding sites and the dissociation constants were calculated. Finally, by packing an SPE column (SPE = solid-phase extraction) with MIP_1, the esculin was separated and enriched successfully by this sorbent from samples of *Cortex fraxini*, and the average recovery was up to 74.7%.

Introduction. – Molecular imprinting is an experimental technique for preparing polymers possessing the ability of selectively recognizing the imprinted molecule (or the template molecule). The preparation of molecularly imprinted polymers (MIPs) can be described as follows: *i*) the complexation of the imprinted molecule to the polymerizable ligand; *ii*) the polymerization of the complex; *iii*) removal of the imprinted molecule from the polymers after copolymerization. After removal of the imprinted molecule, three-dimensional cavities in the imprinted polymers, which can specifically bind the imprinted molecule, are formed [1].

Because of their outstanding characteristics of selective recognition ability to the imprinted molecule, their ability of resisting machine processes, *i.e.*, high temperature and high pressure, *etc.*, as well as their chemical stability towards acids, alkalis, and many kinds of organic solvents, a number of molecularly imprinted polymers have been prepared for certain target compounds, and used as sorbent to separate and enrich the target compounds [2–8]. Esculin (=6,7-dihydroxycoumarin 6-(β-D-glucopyranoside)), which is one of the main active components of the Chinese traditional medicine *Cortex fraxini*, showed many kinds of biologic activities, such as resisting bacteria, diminishing inflammation, treating arthritis, preventing blood from coagulation, and promoting

blood circulation [9][10]. In this work, as a part of our continuous studies on molecularly imprinted polymers [5–8], four molecularly imprinted polymers, MIP_1–MIP_4, were prepared with esculin as the imprinted molecule. Their adsorptive performances and selective recognition abilities were studied. The results showed that, comparing with the nonimprinted polymers, the imprinted polymers had a high adsorption potential and a good selective recognition ability. Among the four esculin-imprinted polymers, MIP_1, prepared in MeOH with esculin as the imprinted molecule, methacrylic acid (=2-methylprop-2-enoic acid; MAA) as the functional monomer, and ethylene glycol dimethacrylate (=2-methylprop-2-enoic acid ethane-1,2-diyl ester; EGDMA) as the cross-linking agent, exhibited the most advantageous characteristics. Finally, a sample of *Cortex fraxini* was analyzed by HPLC with a MIP_1-SPE column (SPE = solid-phase extraction). The anal. results demonstrated that the prepared MIP_1 might be used as a potent sorbent for the separation and enrichment of esculin from the complex mixture.

Results and Discussion. – 1. *Interaction between Template and Functional Monomer*. 1.1. *Prediction of the Binding by Fluorescence Spectrophotometry.* The solutions containing the same amount of esculin but various amounts of the functional monomer MAA were investigated by fluorescence spectrophotometry by using the excitation and the emission spectra (*Fig. 1*). It could be seen from the spectra that the fluorescence intensity of the analyzed systems markedly decreased on addition of increasing amounts of MAA to the esculin solutions, which indicated that interactions between esculin and MAA exist in these solutions.

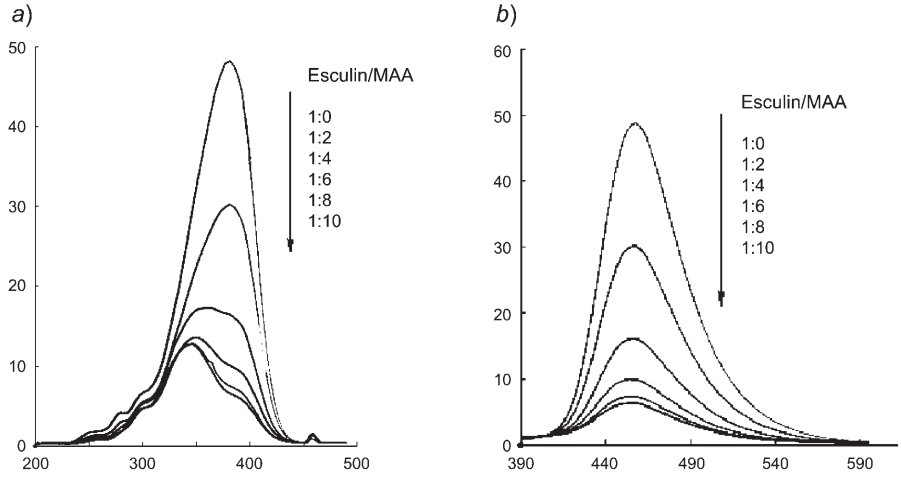

Fig. 1. *Fluorescence spectra of esculin in MeOH in the absence and presence of MAA:* a*) fluorescence excitation* (200–500 nm) *recorded following emission at 455 nm and* b*) fluorescence emission spectra* (390–600 nm) *recorded following excitation at 377 nm*. Concentration of esculin 0.01 mM; concentration of MAA 0, 0.02, 0.04, 0.06, 0.08, 0.10 mM; MeOH as blank reference.

1.2. *Prediction of the Binding by UV Spectrophotometry.* Because of the formation of esculin/monomer complexes, the UV spectra of the mixture of esculin and the

functional monomer were different from that of a pure esculin solution. Under the condition of a surplus amount of the functional monomer in the complex, the composition and the association constant of the coordination compound can be calculated by using difference-UV spectrophotometry [5][11].

The complex (C) formation of the imprinted molecule (A) with the functional monomer (B) can be described by *Eqn. 1*. If the concentration of A (a_0) is much smaller than that of B (b_0), then the equilibrium concentration of B should be approximated as b_0, so the complex concentration (c) can be calculated by *Eqn. 2*, where K refers to the association constant, $n = 1, 2, 3, \ldots$

$$A + nB = C \tag{1}$$

$$c = \frac{a_0 b_0^n K}{1 + b_0^n K} \tag{2}$$

In principle, the absorbance of the mixture can be expressed by *Eqn. 3*. When $b_0 = 0$, the absorbance is given by *Eqn. 4*. When the absorption is determined at a wavelength where B does not show any absorption, the difference of absorbance (ΔA), which expresses the absorption of the mixture with reference to the solution as blank of A, is expressed by *Eqn. 5* where $\Delta e = \varepsilon_C - \varepsilon_A$. Substituting *Eqn. 5* into *Eqn. 2* yields *Eqn. 6*.

$$A = A_A + A_B + A_C = [(a_0 - c)\,\varepsilon_A + (b_0 - nc)\,\varepsilon_B + c\varepsilon_C]\,l \tag{3}$$

$$A_0 = a_0 \varepsilon_A l \tag{4}$$

$$\Delta A = A - A_0 = (a_0 - c)\,\varepsilon_C l - a_0 \varepsilon_A l = \Delta \varepsilon l c \tag{5}$$

$$\frac{\Delta A}{b_0^n} = -K \Delta A + K \Delta \varepsilon_C l a_0 \tag{6}$$

Drawing a plot $\Delta A/b_0^n$ *vs.* ΔA, a good linear relation is found at $n = 2$ (*Table 1*), which demonstrates that esculin and each functional monomer mainly forms a 1:2 complex, held together by cooperative H-bonding. Furthermore, the association constants (K) of the complexes were obtained from the slope and intercept of these lines. The value of K (*Table 1*) showed that all coordination compounds of the imprinted molecule reacting to the functional monomers were stable, and the esculin/MAA complex was the most stable. The results indicated that H-bonding interactions between the imprinted molecule and each functional monomer are generated. Specifically, the interaction between esculin with MAA is strong enough to produce a polymer with good imprinting efficiency.

2. *Binding Studies of the Polymers.* The adsorption performance of the polymers prepared with different functional monomers was studied by UV spectrophotometry. The results in *Table 2* point out that the adsorption capacity (Q) of the imprinted polymers is better than that of the nonimprinted polymers, and the type of the functional monomer affects intensively the adsorption performance of the polymers. The optimal imprinted efficiency (3.39) and the highest adsorption capacity (105.8 μmol/g) for the imprinted polymers were found for MIP_1, prepared with

Table 1. *Association Constant* (K) *and Composition* (n) *of the Coordinated Complexes Esculin/Monomer*[a])

Monomers[b])	Regression equation	Correlative coefficient r	K	n
MAA	$(\Delta A/b_0^2)\cdot 10^6 = 13.28 - 10.7\Delta A$	0.9988	$1.07\cdot 10^7$	2
AM	$(\Delta A/b_0^2)\cdot 10^6 = 25.41 - 8.13\Delta A$	0.9990	$8.13\cdot 10^6$	2
4-VP	$(\Delta A/b_0^2)\cdot 10^6 = 22.80 - 6.88\Delta A$	0.9988	$6.88\cdot 10^6$	2
2-VP	$(\Delta A/b_0^2)\cdot 10^6 = 14.11 - 3.25\Delta A$	0.9985	$3.25\cdot 10^6$	2

[a]) Concentration of esculin: 0.01 mM, concentrations of the functional monomers: 0, 0.02, 0.04, 0.06, 0.08, and 0.10 mM. The same concentration of esculin in MeOH as blanks. [b]) MAA = Methacrylic acid = 2-methylprop-2-enoic acid; AM = acrylamide = prop-2-eramide; 4-VP = 4-vinylpyridine = 4-ethenylpyridine; 2-VP = 2-vinylpyridine = 2-ethenylpyridine.

Table 2. *Binding Properties of Polymers*[a])

	Imprinted molecule	Functional monomer[b])	Esculin/monomer (molar ratio)	Binding capacity Q [μmol/g][c])	Imprinted efficiency[d])
MIP_1	esculin	MAA	1 : 4	105.8	3.39
MIP_2		AM	1 : 4	88.6	2.73
MIP_3		4-VP	1 : 4	47.8	1.94
MIP_4		2-VP	1 : 4	57.9	2.02
$NMIP_1$	none	MAA		31.2	
$NMIP_2$		AM		32.5	
$NMIP_3$		4-VP		24.7	
$NMIP_4$		2-VP		28.6	

[a]) The binding properties were determined by adding to 2.0 mmol of esculin in MeOH (10 ml) 20.0 mg of polymer. [b]) MAA = Methacrylic acid = 2-methylprop-2-enoic acid; AM = acrylamide = prop-2-enamide; 4-VP = 4-vinylpyridine = 4-ethenylpyridine; 2-VP = 2-vinylpyridine = 2-ethenylpyridine. [c]) Binding capacity (Q) is expressed in μmol of the imprinted molecule bound per 1.0 g of polymer. [d]) The imprinted efficiency is expressed as the ratio of binding capacity of the imprinted polymer with respect to that of the nonimprinted one.

MAA as the functional monomer in MeOH, which corresponds to the viewpoint that the extent of the imprinted efficiency for the imprinted polymers depends on the strength of the H-bonding that exists initially between the imprinted molecule and the functional monomer. MIP_2, prepared with AM as the functional monomer, showed a high adsorption capacity (88.6 μmol/g) as well as a perceptible imprinted efficiency (2.73). But MIP_3 (47.8 μmol/g, 1.94), prepared with 4-VP, and MIP_4 (57.9 μmol/g, 2.02), prepared with 2-VP, showed a lower adsorption capacity and imprinted efficiency. These results were in agreement with the findings of the binding by fluorescence and UV spectrophotometry.

The *Scatchard* analysis is the common method used for polymer studies, which can be described by *Eqn. 7* [12][13], where Q is the amount of the substrate bound to the polymer, Q_{max} is the apparent maximum number of the binding sites, K_d is the equilibrium dissociation constant, and [substrate] is the concentration of the free substrate in solution after adsorption.

$$Q/[\text{substrate}] = Q_{max}/K_d - Q/K_d. \quad (7)$$

The equilibrium-adsorption experiments were carried out by varying the initial concentration of esculin from 0.02 mmol/ml to 0.3 mmol/ml in MeOH in the presence of 20.0 mg of MIP_1, and the obtained data were plotted according to *Eqn. 7* (*Fig. 2*). The *Scatchard* plot for MIP_1 was not linear, which indicated that the binding sites in MIP_1 were heterogeneous with respect to the affinity to esculin. But there are two distinct linear sections within the plot, which implied that two types of binding sites were generated in MIP_1. Based on the slope and intercept of the two linear sections, the apparent maximum dissociation constants K_{d1} and K_{d2} were calculated to be $1.99 \cdot 10^{-3}$ M and $3.73 \cdot 10^{-3}$ M, as well as the apparent maximum numbers Q_{max1} and Q_{max2} to be 105.2 μmol · g^{-1} and 318.5 μmol · g^{-1}, respectively.

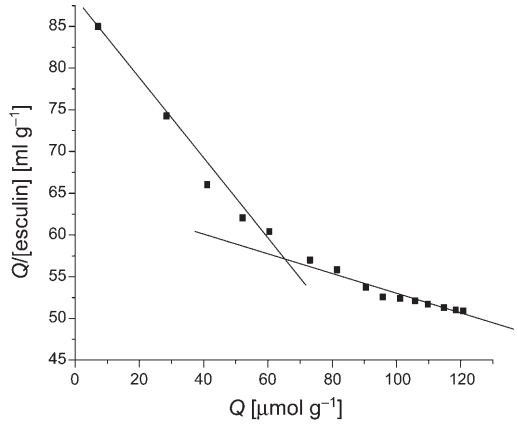

Fig. 2. Scatchard *plot of MIP*$_1$. Amount of MIP_1 20.0 mg; Q = μmol of esculin bound per 1.0 g of MIP_1; range of initial concentration of esculin 0.02 – 0.3 mmol/ml; [esculin] = concentration of free esculin in the solution after adsorption; volume 10 ml; temp. 25°.

3. *Dynamics of Binding Reaction*. It is important for a separation and determination system to produce rapid analytical results. Therefore, the dynamics of the binding reaction for the imprinted polymer MIP_1 to esculin was carried out by mixing esculin (2.0 mmol) with MIP_1 (20.0 mg) in MeOH (10 ml) at 25° for 0 – 6 h. The curve of the adsorption dynamics, *i.e.*, the binding capacity Q *vs.* time t (*Fig. 3*), pointed out that the adsorption amount of MIP_1 to esculin increased quickly within the first 1.5 h, and the adsorption equilibrium was reached after *ca.* 2.5 h. MIP_1 performed this kind of adsorption-dynamics properties on account of its empty caves, which favor the transfer of esculin from the liquid to the solid phase. Therefore, the adsorption velocity increased quickly at the initial stage of the adsorption experiment, but once the surface of MIP_1 adsorbed enough esculin, it would hinder to a certain extent the passage of esculin to places in the interior of MIP_1, thus causing the decrease of the adsorption velocity.

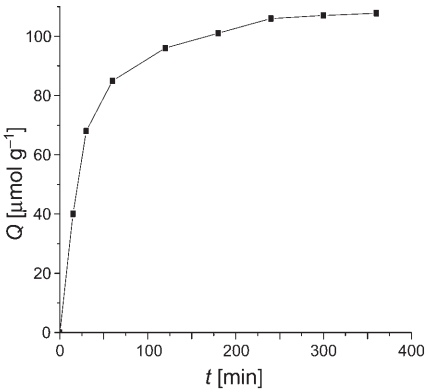

Fig. 3. *Adsorption dynamics of MIP$_1$ towards esculin in MeOH.* Initial concentration of esculin 0.2 mmol/ml; amount of MIP$_1$ 20.0 mg; Q = μmol of esculin bound per 1.0 g of MIP$_1$; volume 10 ml; temp. 25°.

4. *Adsorption Selectivity of the Polymers.* The adsorption selectivity of MIP$_1$ and the corresponding nonimprinted analogue NMIP$_1$ was investigated by HPLC by determining the amount of esculin and its four analogues, *i.e.*, esculetin (=6,7-dihydroxycoumarin), 4-methylesculetin (=4-methyl-6,7-dihydroxycoumarin), coumarin (=2*H*-1-benzopyran-2-one), and 7-hydroxycoumarin. The equations of working curves with good linear relation of five substrates (*Table 3*) were obtained by using the peak area as independent variable, and the concentration of the substrate as dependent variable [14].

Table 3. *Equations of Working Curves of Substrates* ($n=5$)

Substrates	Regression equation[a])	Correlation coefficient r
Esculin	$C=5\cdot10^{-7}A+0.0851$	0.9999
Esculetin	$C=1\cdot10^{-6}A+0.1524$	0.9998
4-Methylesculetin	$C=1\cdot10^{-6}A+0.2819$	0.9998
Coumarin	$C=1\cdot10^{-5}A+0.1116$	0.9996
7-Hydroxycoumarin	$C=1\cdot10^{-6}A+0.1686$	0.9998

[a]) C and A in the regression equation represent the concentration of substrate in μmol/ml and the area of peak, respectively.

The binding selectivity of MIP$_1$ and NMIP$_1$ (200.0 mg, each) to the five substrates was investigated in the standard-mixture solution (10 ml) which contained the same amounts (2.0 mmol) of the substrates in MeOH. The chromatogram of this standard-mixture solution is shown in *Fig. 4*. The ratio of adsorption in *Table 4* was obtained by dividing the content of the substrates in the initial mixture solution by the difference of content of the substrates in the residual solutions and in the initial mixture solution, respectively. It can be seen that comparing with the nonimprinted analogue NMIP$_1$, MIP$_1$ provided a larger binding capacity and a better recognition ability to each substrate, and MIP$_1$ ensured a larger binding capacity and a better recognition ability to

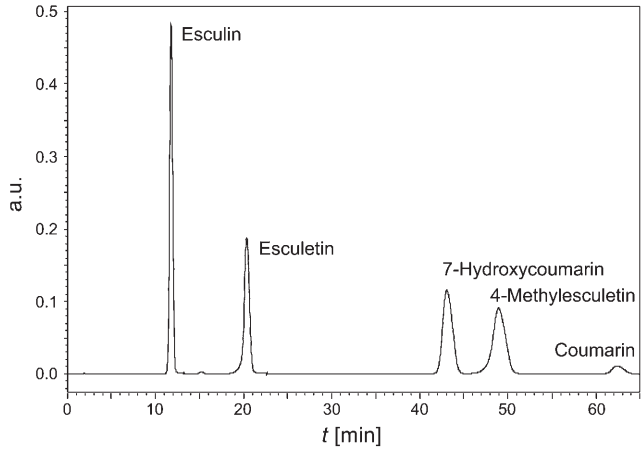

Fig. 4. *Chromatogram of the standard mixture.* Concentration of each substrate 0.2 mmol/ml.

Table 4. *Adsorption of MIP_1 and $NMIP_1$ to Substrates in Standard-Mixture Solutions*[a])

Substrates	Substrate content in residue [mmol/ml]		Ratio of adsorption [%][b])	
	MIP_1	$NMIP_1$	MIP_1	$NMIP_1$
Esculin	0.071	0.158	64.5 (1.72)	21.0 (1.83)
Esculetin	0.170	0.166	15.0 (1.71)	17.0 (1.80)
4-Methylesculetin	0.171	0.165	14.5 (1.73)	17.5 (1.87)
Coumarin	0.186	0.172	7.0 (1.99)	14.0 (2.01)
7-Hydroxycoumarin	0.160	0.157	20.0 (1.72)	21.5 (1.84)

[a]) Data in parentheses are r.s.d. [%]. [b]) See text.

esculin than to the other four substrates (all at the same concentration), although MIP_1 also bound partially the other four substrates.

The binding distribution coefficient (K_D) of the imprinted polymer MIP_1 and $NMIP_1$ to esculin and its four analogues was also calculated (*Fig. 5*). It was found that K_D of MIP_1 to esculin is larger than to the other four substrates. Comparing with $NMIP_1$, K_D of MIP_1 to each substrate is also larger than that of $NMIP_1$ to each one.

Esculin is the glucoside of esculetin, and has similar functionalities for H-bond formation as the analogues mentioned above. But the MIP_1 prepared with esculin as the imprinted molecule provides specific cavities with regard to the shape of the esculin molecule, as shown by the high selectivity for esculin binding. The results established that the template effect plays an important part in the process of preparing specific polymers.

5. *Sample Analysis.* 5.1. *Sample Analysis.* The adsorption-separation ability of MIP_1 and $NMIP_1$ (200.0 mg, each) to esculin in a sample solution (10 ml) of the chinese traditional medicine *Cortex fraxini* was studied by HPLC. The anal. results are shown in *Table 5* and *Fig. 6*. The ratio of adsorption in *Table 5* was obtained by dividing the content of esculin in the initial sample solution by the difference of content of esculin in

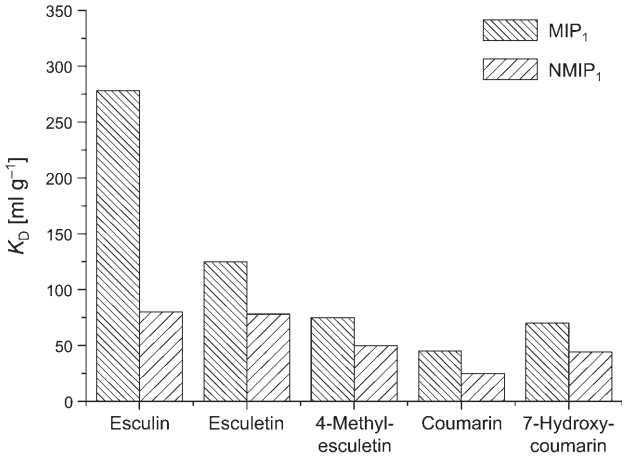

Fig. 5. *Selectivity of MIP_1 and $NMIP_1$ recognizing substrates.* Amount of polymer 20.0 mg; initial concentration of each substrate 0.2 mmol/ml; volume 10 ml; temp. 25°; adsorption time 6 h; $K_D = c_p/c_s$; c_p [μmol/g] = amount of substrate bound to MIP_1 or $NMIP_1$; c_s [μmol/ml] = equilibrium concentration of substrate in solution after adsorption.

Table 5. *Adsorption of MIP_1 and $NMIP_1$ to Esculin in a Sample Solution of* Cortex fraxini ($n = 3$)[a])

	Content of esculin [mmol/ml]		Ratio of adsorption [%][b])
	initial	residual	
MIP_1	0.154	0.021	86.4 (2.1)
$NMIP_1$	0.154	0.101	34.4 (2.0)

[a]) Data in parentheses are r.s.d. [%]. [b]) See text.

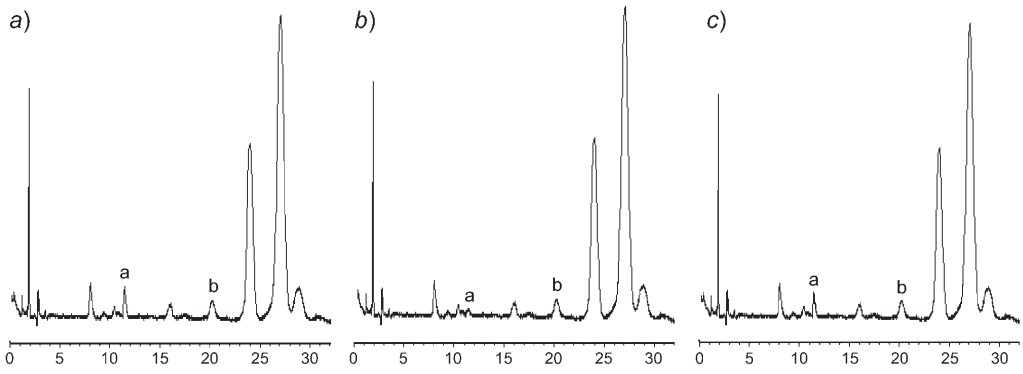

Fig. 6. *Chromatograms of a sample of* Cortex fraxini: a*) initial sample*, b*) sample after treatment of the initial sample with MIP_1, and* c*) sample after treatment of the initial sample with $NMIP_1$*; the peaks a and b in each plot represent esculin and esculetin, respectively.

the residual solution and in the initial sample solution. It can be seen from *Table 5* and *Fig. 6* that MIP_1 could adsorb mainly esculin from the sample, and performed a larger binding capacity and a better recognition ability to esculin than to other components of the sample.

5.2. *Method Validation*. To validate the reliability of the method by using an MIP_1-packed solid-phase-extraction (SPE) column and MeOH/AcOH 9 : 1 as the eluant, and to check the lifetime of effectiveness of the MIP_1-SPE column, three different amounts of esculin were added to the known esculin concentration of solutions of the medicinal material *Cortex fraxini*. These solutions were then passed in turn through the same MIP_1-SPE column. After washing the column with MeOH/AcOH, esculin in the eluates was determined under the above-described HPLC conditions, and the degrees of recovery were calculated (*Table 6*). The average recovery degrees were 70.0–78.0%, and the relative standard difference of the method was less than 3.2% although the repeated use can cause a perceptible decrease of the efficiency of the MIP_1-SPE column. The results showed that MIP_1, prepared in this work, could be used to separate and enrich esculin from *Cortex fraxini*, and the self-established method to separate and enrich esculin from complex mixtures is reliable, as well as the MIP_1-SPE column can be used repeatedly within limits of times.

Table 6. *Analytical Results of the Addition of the Standard Substance Esculin to Samples of* Cortex fraxini ($n = 3$)[a])

Exper.	Added [mmol]	Found [mmol][a])	Recovery [%][b])	r.s.d. [%]
1	0.50	1.16	78.0	2.7
2	1.00	1.53	76.0	3.1
3	1.50	1.82	70.0	3.2

[a]) The concentration of esculin in the initial sample solutions is 0.077 mmol/ml; volume 10 ml. [b]) The adsorption recovery was calculated as the ratio of the difference of the found amount and the amount of esculin in the initial sample to the added amount.

Conclusions. – In this work, four new molecularly imprinted polymers and four nonimprinted polymers were prepared by polymerization in MeOH. The adsorption capacity and selectivity of the imprinted polymers were studied and compared with those of the corresponding nonimprinted polymers. The results demonstrated that MIP_1, by involving MAA as the functional monomer, has a larger adsorption capacity and a higher selectivity to esculin than MIP_2–MIP_4, and the specific cavity spheres as template enhance the selectivity of the imprinted polymer sterically. Based on these results, a new method was established to separate and enrich esculin from samples of *Cortex fraxini*, by using a MIP_1-packed SPE column. This method, which is simple, convenient, and reliable, could be particularly useful to separate, enrich, and determine esculin in a mixture containing structurally very similar compounds.

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Experimental Part

General. Esculin was purchased from *Tokyo Kasei Kogyo Co. Ltd.* (Japan). Esculetin, 4-methylesculetin, 4-vinylpyridine (4-VP), and 2-vinylpyridine (2-VP) were purchased from *Acros Organics Co.* (USA). The 7-hydroxycoumarin was purchased from *Sigma Co.* (Germany). Coumarin and acrylamide (AM) were purchased from the *Shanghai Chemical Reagents Plant* (China). Ethylene glycol dimethacrylate (EGDMA; *Shanghai Coral Chemical Plant*, China) and methacrylic acid (MAA; *Suzhou Anli Chemical Plant*, China) were purified by distillation before use. Azobis[isobutyronitrile] (=2,2′-azobis[2-methylpropanenitrile]; AIBN; *Shanghai Fourth Reagent Plant*, China) was recrystallized from EtOH before use. All other reagents were used without further purification. Solvents were of anal. grade. HPLC: *Waters* system (USA) consisting of a *Waters 1525* pump, *Waters 2996* UV/VIS detector, and *Waters 717* automatic injector model. UV Spectra: *Shimadzu UV-2401* double-beam spectrophotometer (Japan); *HZ* constant-temp.-bath oscillator (China); *Hitachi 850* fluorometry (Japan); home-made molecularly imprinted polymer solid-phase extraction (MIP-SPE) glass column; λ_{max} in nm.

Ultraviolet and Fluorescence Spectrum Characteristics. A series of solns. containing various amounts of MAA and a fixed amount of esculin in MeOH were prepared at first, and their fluorescence absorption spectra were determined by using pure MeOH as blank reference.

The same amounts of esculin were added into a series of 10-ml tubes for comparison. The different amounts of MAA, AM, 2-VP, or 4-VP were gently increased and added to the tubes. These solns. were diluted to graduation with MeOH, shaken, and settled for *ca.* 15 min. Then, the difference-UV absorption spectra of these solns. were scanned by using the same concentration of esculin in MeOH as blank references, and the absorbance was measured at 232 nm for the esculin/MAA system, at 260 nm for the esculin/AM system, at 248 nm for the esculin/2-VP system, and at 236 nm for the esculin/4-VP system.

Preparation of Polymers. The mixture of esculin (0.5 mmol) and MAA (2.0 mmol) in MeOH (10 ml) was stirred heavily at r.t. for *ca.* 3 h in a 25-ml conical flask. Then, EGDMA (2.7 ml) and AIBN (15.0 mg) were added. The soln. was purged with N_2 for 5 min and then transferred into an ampoule. The ampoule was sealed under vacuum and placed into a shaker bath at 60° for 24 h. The bulk rigid polymer obtained was ground into particles in a mortar and passed through a 75-μm sieve. The polymer particles were washed with MeOH/AcOH 9:1 (*v*/*v*) until esculin could no longer be detected at 340 nm by spectrophotometry in the eluate. Finally, after being washed with MeOH to remove residual AcOH and dried to constant weight *in vacuo*, the polymer MIP_1 was obtained.

The same procedure as described for MIP_1 was used to prepare the other molecularly imprinted polymers (MIP_{2-4}) by using different functional monomers (see *Table 2*). As a control, the nonimprinted polymers ($NMIP_{1-4}$) were obtained in the same way, except that the template was not added.

Binding Studies by UV Spectrophotometry. Polymer particles (20.0 mg) in a conical flask were mixed with different amounts of esculin in MeOH (10 ml). The mixture was oscillated in a constant-temp.-bath oscillator at 25° for *ca.* 6 h and then transferred into a centrifuge tube. After centrifugation at 4000 r.p.m. for 10 min, the concentration of the free substrate in the supernatant soln. was determined by measuring the absorbance at 340 nm by UV spectrophotometry. The binding/adsorption capacity (Q), which was defined as μmol of the substrate bound per 1.0 g of polymer, was calculated with *Eqn. 8*, where C_0 [mmol/ml] is the initial substrate concentration, C [mmol/ml] the concentration of the free substrate in the soln. after adsorption, V [ml] the volume of the soln., and W [g] the mass of the polymer.

$$Q = (C_0 - C)\,V \times 10^3/W. \tag{8}$$

Preparation of Sample Solution. The chinese medicine *Cortex fraxini* was purchased from Fulintang in Kunming. The slivers of *Cortex fraxini* (2.164 g) in MeOH (2 × 25 ml) were extracted twice at reflux temp. for 2 h. The extract was filtered through filter paper and the resultant filtrate was used as the sample soln.

Adsorption by MIP-SPE Column and HPLC Analysis. The SPE glass column (12 mm × 110 mm) was packed with a MIP_1 or $NMIP_1$ (200.0 mg, each). Then, the column was washed with H_2O/MeOH/AcOH 40:6:0.1 (2 × 5 ml) and MeOH, respectively. The sample soln. (10 ml) was filtered through filter papers, and then passed through the column. If the volume of the eluate solns. was less than 10 ml, the

volume of the solns. was completed to 10 ml with pure MeOH. The eluate solns. were filtered through a 0.45-μm organic micro filter prior to HPLC analysis (*C18* column (4.6 mm × 250 mm, 5 μm), H_2O/MeOH/AcOH 40 : 6 : 0.1, flow rate 1.5 ml/min). An efficient separation of esculin and other components in the standard-mixture solns. and in the real-sample solns. was achieved under these conditions. The peak identification of each substrate was performed by adding the corresponding reference substance to the standard-mixture or sample solns. All solns. were determined three times, and their average values were used.

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