Preparation and Recognition Properties of Vanillin-Imprinted Polymers

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Some new molecularly imprinted polymers (MIPs) were prepared by different protocols involving vanillin as the imprinted molecule, methacrylic acid (=2-methylprop-2-enoic acid; MAA) as the functional monomer, and ethylene glycol dimethacrylate (EGDMA=2-methylprop-2-enoic acid ethane-1,2-diyl ester) as the cross-linking agent. The adsorption property of the imprinted polymers was studied by UV spectrophotometry and HPLC. The results indicated that the porogen solvent had a certain influence on the adsorption performance of the polymer. The vanillin-imprinted polymer MIP_1 prepared with MeOH as porogen, exhibited advantageous characteristics, *i.e.*, a high binding activity, a good selectivity, and a rapid adsorption equilibrium. The binding parameters studied by *Scatchard* analysis established that there are two types of binding sites in MIP_1. Finally, by packing an SPE column (SPE=solid-phase extraction) with the polymer MIP_1, the vanillin was separated and enriched successfully by this sorbent from the samples of *Vanilla fragrans* and beer.

Introduction. – Molecularly imprinted polymers (MIPs) prepared by the molecular-imprinting technique have attracted considerable attention not only due to their high selectivity and affinity for a predetermined molecule but also due to their strength, cheapness, and longevity of service [1]. These properties allow MIPs to be used in various research fields, such as catalysis [2], sensor technology [3], chemical analysis [4], solid-phase extraction [5], chromatography [6], membrane separation [7], and capillary electrophoresis [8], as well as to separate and enrich a trace analyte from a complex mixture. Up to now, some structurally similar compounds were separated and recognized by using MIPs as sorbents, such as a sugar in a mixture with its optical isomers [9], a specific drug [10][11], or an amino acid in a mixture with its derivatives [12].

Vanillin (=4-hydroxy-3-methoxybenzaldehyde) which has the pleased and fragrant smell of *Vanilla planifolia* can be oxidized slowly in damp air. It is widely used in food industry as food additive for oatmeal, candies, bean milk, and beverages, in tobacco industry, in chemical engineering, and in medicinal chemical engineering [13].

To obtain an imprinted polymer with excellent recognition selectivity and binding activity as sorbent for the separation and enrichment of vanillin, we prepared some molecularly vanillin-imprinted polymers in different porogens by using the molecular-imprinting technique. The results obtained from the study of the adsorption property indicated that one of the imprinted polymers, MIP_1, prepared in MeOH with vanillin as imprinted molecule, methacrylic acid (=2-methylprop-2-enoic acid; MAA) as functional monomer, ethylene glycol dimethacrylate (=2-methylprop-2-enoic acid ethane-1,2-diyl ester; EGDMA) as the cross-linking agent, and MeOH as porogen, might

be a potential sorbent for the separation of vanillin. We also studied the practicability of the prepared polymers by using MIP_1 and the corresponding nonimprinted polymer, $NMIP_1$, as sorbents in the packing of solid-phase extraction (SPE) columns. The results showed that MIP_1 had a higher binding capacity and selectivity to vanillin than to other components in the samples, and could be used as a sorbent to enrich and separate vanillin from the extract of *Vanilla fragrans* and beer.

Results and Discussion. – 1. *Prediction of the Binding by UV Spectrophotometry.* It is crucial that the imprinted molecule and functional monomer form stable host–guest complexes in the pre-polymerization mixture. Thus, we first studied the interaction between the imprinted molecule and the functional monomer at the pre-polymerization stage. The interaction between vanillin and MAA in different solvents including MeOH, $CHCl_3$, MeCN, and toluene was explored by UV spectrophotometry (*Fig. 1*). The results showed that the addition of MAA to vanillin in $CHCl_3$ caused a blue shift of the maximum-absorbance wavelength (from 314.4 to 303.7 nm) and an increase of absorbance (from 0.414 to 0.529) at 314.4 nm. This is typical for H-bonding effects on the n-π^* absorption band of a molecule whose chromophore acts as a proton receptor [14], which suggested that the strong H-bonds existed between vanillin and MAA in $CHCl_3$. The maximum-absorption wavelength of the spectra of vanillin in MeOH, MeCN, or toluene almost did not vary but a slight increase in the absorbance on addition of MAA was observed, which might indicate that a H-bond existed between vanillin and MAA in all four solvents mentioned above (*Fig. 1*). However, the H-bond formed in $CHCl_3$ was stronger than that in the other three solvents.

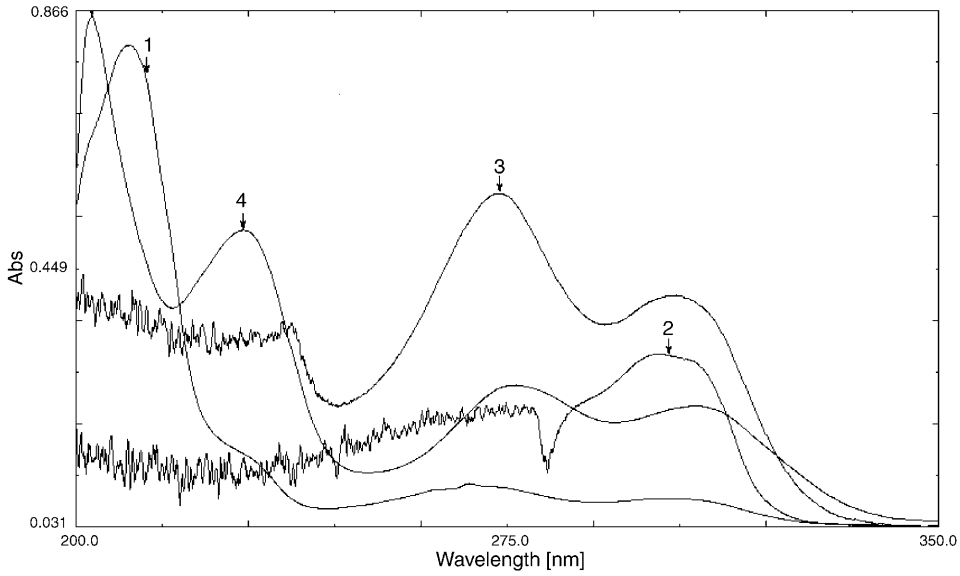

Fig. 1. *UV Spectra of vanillin in the presence of MAA in four different sovents: MeCN* (1), *toluene* (2), *$CHCl_3$* (3), *and MeOH* (4). Concentration of vanillin 0.2 mmol l^{-1}, concentration of MAA 0.8 mmol l^{-1}; corresponding pure MAA solutions as blanks.

2. *Adsorption Performance of the Polymer*. The choice of the solvent is crucial in the preparation of polymers since the solvent as porogen can affect the shape, size, and functionality of suitable three-dimensional cavities in the polymer network. In our work, the adsorption performance of the polymer synthesized in different solvents was studied by UV spectrophotometry. The results listed in *Table 1* pointed out that the type of porogen affected the adsorption performance of the polymer strongly. The optimal imprinted efficiency of the imprinted polymer was obtained by MIP_2 (3.06) which was prepared in $CHCl_3$ as porogen. This result corresponds to the viewpoint that the imprinted efficiency of the imprinted polymer depends on the strength of the H-bonding that existed initially between the imprinted molecule and the functional monomer. The imprinted polymer prepared in MeOH, MIP_1, showed the highest binding activity Q as well as a rather high imprinted efficiency (2.84), although the nonspecific binding activity Q increased remarkably as compared with that of MIP_2, due to the solvent–imprinted molecule interaction. MIP_3, synthesized in MeCN, showed almost no imprinted efficiency (1.29), whereas MIP_4, synthesized in toluene, exhibited a perceptible imprinted efficiency (2.48) but a lower binding activity Q than MIP_1.

Table 1. *Binding Properties of the Molecularly Imprinted Polymers, MIP_1–MIP_4, and of the Nonimprinted Polymers, $NMIP_1$–$NMIP_4$*[a])

	Imprinted molecule	Porogen	Molar ratio of imprinted molecule to functional monomer	Binding activity (Q)[b])	Imprinted efficiency[c])
MIP_1	vanillin	MeOH	1:4	208.9	2.84
MIP_2		$CHCl_3$	1:4	38.5	3.06
MIP_3		MeCN	1:4	71.5	1.29
MIP_4		Toluene	1:4	76.8	2.48
$NMIP_1$	none	MeOH		73.5	
$NMIP_2$		$CHCl_3$		12.6	
$NMIP_3$		MeCN		55.5	
$NMIP_4$		Toluene		31.0	

[a]) The binding properties were determined by adding 7.5 mg of imprinted molecule in 10 ml of porogen solvent in 20.0 mg of polymer. [b]) Binding activity (Q) is expressed in mg of the imprinted molecule bound per 1 g of polymer, which was calculated from the binding assay described in the *Exper. Part.* [c]) The imprinted efficiency is expressed as the ratio of the binding activity of the imprinted polymer with respect to that of the nonimprinted one.

The binding activity Q of MIP_1 is 5 times higher than that of MIP_2, although the imprinted efficiency of MIP_1 is only slightly decreased as compared to that of MIP_2. Therefore, the adsorption dynamics, recognition selectivity, and binding parameters of MIP_1 were studied in detail.

3. *Adsorption-Equilibration Time*. As rapid quantitation is also an important requirement for an assay and separation system, the dynamics of the binding reaction for both the imprinted and nonimprinted polymers with MeOH as porogen was carried out. For this, MIP_1 and $NMIP_1$ were each equilibrated at 25° with a solution of vanillin in MeOH for 0–6 h. The curves of the adsorption dynamics, *i.e.*, the binding activity Q *vs.* time t (*Fig. 2*), pointed out that the adsorption amount of both polymers increased quickly within the first hour, and adsorption equilibrium was reached after about 1 h

for MIP_1 and $NMIP_1$. The adsorption-dynamics properties of an imprinted polymer are determined by its empty caves, which favor the transfer of the imprinted molecule from the liquid to the solid phase. Therefore, the adsorption velocity increased very quickly at the beginning of the adsorption experiment, but once vanillin was adsorbed by the surface of the imprinted polymer, it would obstruct the passage of vanillin to places in the interior of the polymer to a certain extent, causing the decrease of the adsorption velocity. *Fig. 2* also shows that the amount of vanillin bound by $NMIP_1$ was lower than that bound by MIP_1 due to the stronger affinity of the MIP_1 to vanillin as compared to that of $NMIP_1$.

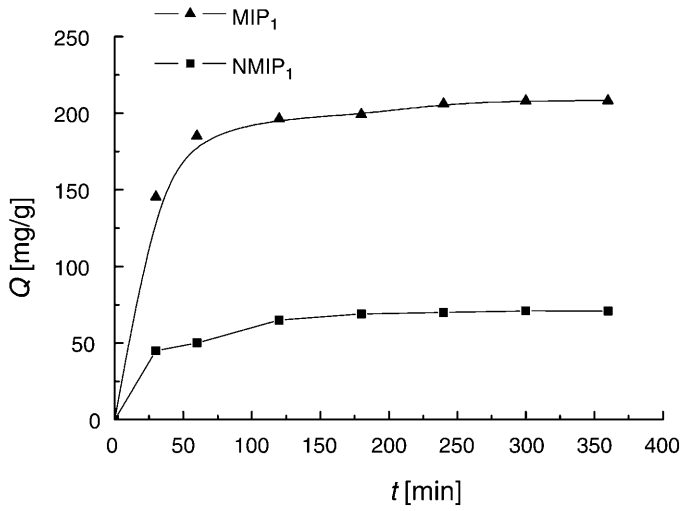

Fig. 2. *Adsorption dynamics of MIP_1 and $NMIP_1$ towards vanillin in MeOH*. Initial concentration of vanillin 7.5 mg/10ml, amount of polymer 20 mg; Q = mg of vanillin bound per 1 g of polymer; volume 10 ml, temp. 25°.

4. *Recognition Selectivity of Imprinted Polymer.* The recognition selectivity of MIP_1 was studied by HPLC by measuring the amount of isomer bound by the polymer exposed to the mixture of vanillin and *o*-vanillin (=2-hydroxy-3-methoxybenzaldehyde), a positional isomer of vanillin, in MeOH. The results (*Table 2*) established that MIP_1 exhibited high selectivity for vanillin in the presence of *o*-vanillin, and there was almost no cross-reactivity for *o*-vanillin, while the bound amount of vanillin was similar in *Exper. 3–5* to that in *Exper. 1* when *o*-vanillin was not present.

5. *Binding Properties of the Imprinted Polymers.* The equilibrium dissociation constant and the apparent maximum number of binding sites of the imprinted polymers were mainly estimated by a *Scatchard* analysis as described in *Eqn. 1* [15], where Q is the amount of substrate bound to the polymer, Q_{max} is the apparent maximum number of binding sites, K_d is the equilibrium dissociation constant, and [substrate] is the concentration of free substrate in the solution after adsorption.

$$Q/[\text{substrate}] = Q_{max}/K_d - Q/K_d \quad (1)$$

Table 2. *Recognition Selectivity of MIP$_1$*

Exper.[a])	Bound amount [%]	
	vanillin	*o*-vanillin
1	50%	
2		17.7%
3	46.7%	10.0%
4	48.0%	13.3%
5	53.3%	16.7%

[a]) The initial concentrations were as follows: *Exper. 1*, 0.3 mg/ml of vanillin; *Exper. 2*, 0.3 mg/ml of *o*-vanillin; *Exper. 3*, 0.3 mg/ml of vanillin + 0.3 mg/ml of *o*-vanillin; *Exper. 4*, 0.3 mg/ml of vanillin + 0.15 mg/ml of *o*-vanillin; *Exper. 5*, 0.15 mg/ml of vanillin + 0.3 mg/ml of *o*-vanillin.

The equilibrium binding experiments were carried out by varying the initial concentration of vanillin from 0.3 to 3.6 mg/10 ml in MeOH in the presence of 20 mg of MIP_1, and the obtained data were plotted according to *Eqn. 1* (*Fig. 3*). The *Scatchard* plot for MIP_1 was not linear, which indicated that the binding sites in MIP_1 are heterogeneous with respect to the affinity for vanillin. But there are two distinct sections within the plot which can be regarded as straight lines, suggesting that two types of binding sites were produced in MIP_1. This result is consistent with many binding studies of imprinted polymers [15]. The linear regression equations of the two linear sections of the *Scatchard* plot are given in *Table 3*, besides Q_{max} and K_d of the higher-affinity and the lower-affinity binding sites, calculated according to the regression equations.

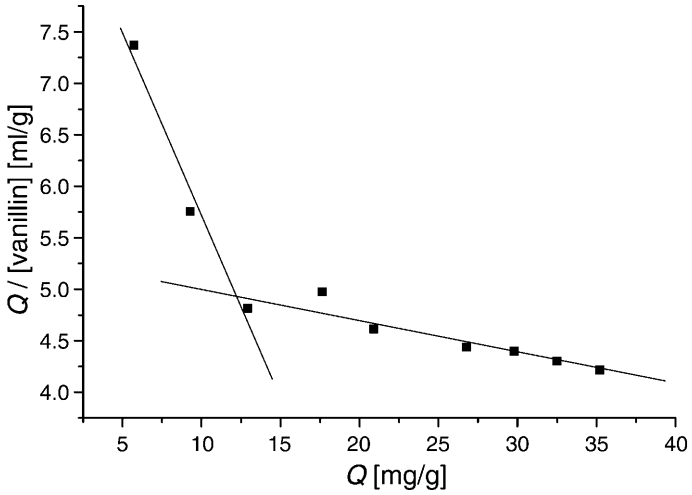

Fig. 3. Scatchard *plot of MIP$_1$*. Amount of MIP_1 20 mg; Q = mg of vanillin bound per 1 g of MIP_1; initial concentration of vanillin 0.3–3.6 mg/10 ml; [vanillin] = concentration of free vanillin in the solution after adsorption; volume 10 ml, temp. 25°.

Table 3. *Binding Properties of MIP$_1$* (see *Eqn. 1*)

	Imprinted-molecule concentration [mg/10 ml]	Regression equation[a])	K_d [mg/ml]	Q_{max} [mg/g]
MIP_1	0.3–0.9	$y = -0.3549x + 9.2827$	2.82	26.2
	0.9–3.6	$y = -0.0316x + 5.3312$	31.6	168.7

[a]) y and x in the regression equation represent Q/[imprinted molecule] in ml/g and Q in mg/g, resp.

6. *Samples Analysis.* The content of vanillin in the untreated samples of *Vanilla fragrans* and of beer, in the residual solutions obtained after treatment of the samples with a polymer; and in the eluates (obtained after treatment with a polymer from the latter by elution with MeOH/AcOH 9:1) were determined by using HPLC under the established chromatographic conditions [16]. The chromatograms of these sample solutions, residue solutions, and eluates are shown in *Figs. 4* and *5* (for data, see *Table 4*). The ratio of adsorption was obtained by dividing the content of the initial sample solution (= amount in sample) by the difference of content of the residue solutions (= amount in residue) and the initial sample solution. The adsorption recovery was calculated as the ratio of the amount released from the polymer (= amount in elute) to that in the sample solution. The results listed in *Table 4* reveal that MIP_1 provided a satisfactory selective adsorption recovery, and comparing with $NMIP_1$, MIP_1 showed a higher selectivity towards vanillin, thus allowing for the enrichment and separation of vanillin from *Vanilla fragrans* and from beer.

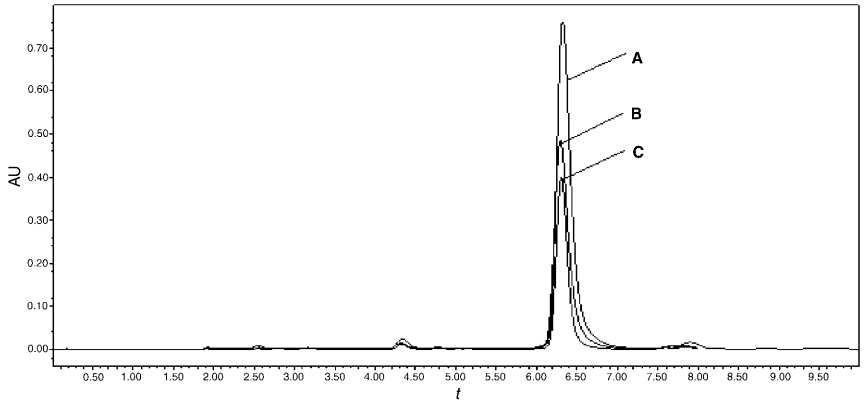

Fig. 4. *Chromatograms of the MeOH extract of* Vanilla fragrans. **A** = vanillin in the initial sample solution of *Vanilla fragrans*; **B** = vanillin in the eluate from MIP_1 (elution with MeOH/AcOH 9:1) after treatment of **A** with MIP_1; **C** = vanillin in the residue solution after treatment of **A** with MIP_1.

Conclusions. – Imprinted polymers were prepared in different porogen solvents by using vanillin as imprinted molecule, and the adsorption performances including the binding parameters of the imprinted polymers were studied. The results revealed that the porogen had a certain influence on adsorption performance of the polymer, and the cooperative H-bonding interaction between the imprinted molecule and the monomer enhanced the selectivity of the imprinted polymer. The highest binding activ-

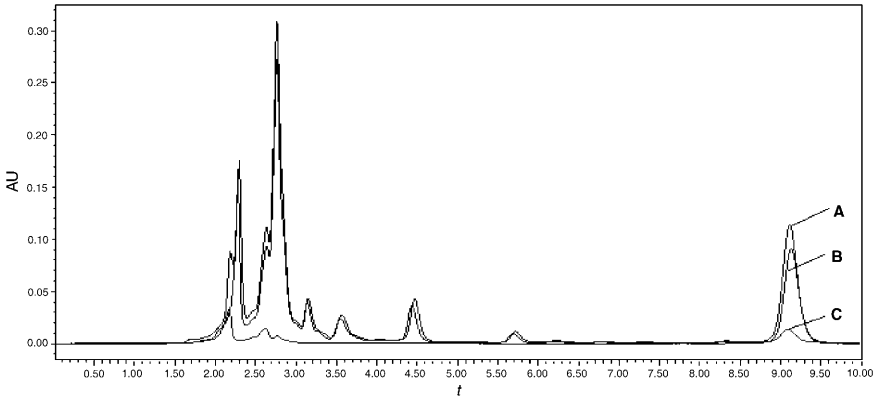

Fig. 5. *Chromatograms of beer samples.* **A** = vanillin in the initial sample solution of beer; **B** = vanillin in the eluate from MIP_1 (elution with MeOH/AcOH 9:1) after treatment of **A** with MIP_1; **C** = vanillin in the residue solution after treatment of **A** with MIP_1.

Table 4. *HPLC Results for the Adsorption of Vanillin from* Vanilla fragrans *and from Beer by MIP_1 or $NMIP_1$* ($n=3$)[a])

	Amount in sample [mg/ml]	Amount in residue [mg/ml]		Ratio of adsorption [%][b])		Amount in eluate [mg/ml]		Recovery [%][b])	
		MIP_1	$NMIP_1$	MIP_1	$NMIP_1$	MIP_1	$NMIP_1$	MIP_1	$NMIP_1$
Vanilla fragrans	0.61(1.2)	0.24(1.4)	0.49(1.5)	60.7	19.6	0.29(1.4)	0.09(1.5)	78.4	75.0
Ber	0.075(1.6)	0.003(1.3)	0.055(1.3)	96.0	26.7	0.058(1.2)	0.016(1.5)	80.6	80.0

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

ity as well as a perceptible imprinted effect was obtained with MIP_1 prepared by using MeOH as porogen. Because of its advantages, such as a high binding activity, good selectivity, and rapid adsorption equilibrium, MIP_1 might be a suitable sorbent material for the separation and enrichment of vanillin. The vanillin from the samples of *Vanilla fragrans* and of beer were successfully enriched and separated, respectively, by using a MIP_1-packed SPE column. This method could be particularly useful when vanillin has to be removed from a mixture or enriched in a mixture containing structurally very similar compounds.

The project was financially supported by the *Natural Science Foundation of China* (No. 30260014 and No. 30560178) as well as by the *Natural Science Foundation of Yunnan Province* (No. 2003B0004M).

Experimental Part

Materials and Instruments. Vanillin and *o*-vanillin were purchased from the *Shanghai Xinhua Spice Factory* (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. Azobisisobutyronitrile (=2,2′-azobis[2-methylpropanenitrile]; AIBN; *Shanghai Fourth Reagent Plant*) 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-580* pump, *Waters-486* UV/VIS detector, and *Waters-U6k* injector model. UV Spectra: *Shimadzu-UV-2401* double-beam spectrophotometer (Japan); *HZ* constant-temp.-bath oscillator (China); λ_{max} in nm.

Prediction of the Binding by UV Spectrophotometry. A series of solns. containing various amounts of MAA and a fixed concentration of vanillin in MeOH, $CHCl_3$, MeCN, or toluene were prepared, and their UV absorption spectra determined by using the corresponding MAA solns. as blanks.

Preparation of Polymers. Imprinted polymers were prepared with vanillin as the imprinted molecule, MAA as the functional monomer, EGDMA as the cross-linker, and AIBN as the initiator. The procedure for the synthesis of MIP_1 is as follows: The mixture of vanillin (2 mmol) and MAA (8 mmol) in MeOH (10 ml) in a glass ampoule was shaken at r.t. for *ca.* 3 h. Then EGDMA (60 mmol) and AIBN (60 mg) were added. The polymerization mixture was sparged by N_2 for 5 min, then the ampoule was sealed under vacuum and the polymerization carried out in a shaker bath at 60° for 24 h. The bulk rigid polymer obtained was ground in a mortar and passed through a 75-μm sieve. The polymer was washed (*Soxhlet* extraction) with 10% AcOH/MeOH until vanillin could no longer be detected at 270 nm by spectrophotometry in the eluent. The particles were then washed with MeOH to remove residual AcOH and dried to constant weight under vacuum.

The other imprinted polymers were synthesized by the same procedure as described for MIP_1 but with different porogens (see *Table 1*). Similarly, the non imprinted blank polymers (NMIP) were obtained in the absence of an imprinted molecule.

Binding Studies by Spectrophotometry. The polymer particles (20.0 mg) in a conical flask were mixed with different amounts of vanillin in MeOH (10 ml). The mixture was oscillated in a constant-temp.-bath oscillator at 25° for 6 h and then transferred into a centrifuge tube. After centrifugation at 4000 rpm for 10 min, the concentration of the free substrate in the supernatant soln. was determined by measuring the absorbance at 270 nm by spectrophotometry. The binding activity (Q), which was defined as mg of vanillin bound per 1 g of polymer, was calculated by *Eqn. 2*, where C_0 (mg/ml) is the initial substrate concentration, C (mg/ml) the concentration of free vanillin in the soln. after adsorption, V (ml) the volume of the soln., and W [g] the mass of the polymer.

$$Q = (C_0 - C)V/W \tag{2}$$

Recognition of MIP by HPLC. To MIP_1 (20 mg) in a 20-ml conical flask, the mixture of different amounts of vanillin and *o*-vanillin in MeOH (10 ml) was added. After oscillation of the mixture in a constant-temp.-bath oscillator at 25° for 6 h, the soln. was filtered over an organic micro filter, and the filtrate (10 μl) was subjected to HPLC (*C18* column, H_2O/MeOH/AcOH 55 : 42 : 3, flow rate 1.0 ml/min) to quantitatively determine the concentration of vanillin and *o*-vanillin remaining in the soln. at 268 nm and r.t. An efficient separation of vanillin and *o*-vanillin was achieved.

Samples Preparation and Analysis by HPLC. The samples of *Vanilla fragrans* and of beer were analyzed by HPLC. *Vanilla fragrans* (2.051 g) in MeOH (50 ml) was extracted by refluxing for 2 h. The CO_2 contained in the beer was removed by treatment in an ultrasonic bath for 30 min. The sample solns. thus prepared were then filtered through filter papers, and the filtrate (10 ml) was loaded into the home-made SPE glass column (110 mm × 12 mm), which was packed with an MIP or NMIP (200 mg). Then the column was washed with MeOH/AcOH (2 × 5 ml). If the volume of residue and eluate solns. was less than 5 ml, the volume of the solns. was completed to 5 ml. The sample solns. were filtered through a 0.45-μm organic micro filter prior to HPLC analysis.The peak identification of vanillin was performed by adding a standard substance into the sample solns. All the sample solns. were tested three times, and their average values were used.

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Received June 22, 2006