Chromatographic Characterization of Molecularly Imprinted Microspheres Synthesized by Aqueous Microsuspension Polymerization: Influences of pH, Kinds and Concentration of Buffer on Capacity Factors

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Molecularly imprinted microspheres (MIMs) were prepared using 4-aminopyridine (4-AP) as template molecule by aqueous microsuspension polymerization. The MIMs were packed into stainless steel column (250 × 4.6 mm I.D.) for selective separation of 4-aminopyridine (4-AP) and 2-aminopyridine (2-AP). The influences of pH, kinds and concentration (c) of buffer on capacity factors were investigated in detail. The relationships of capacity factor (k') with pH and concentration of buffer are quantitatively described firstly. The effects of pH of phosphate and acetate buffer on capacity factors are very different. The relationship between k' and pH can be described by the following equation: $k' = -8.23 + 9.23\ pH - 0.99\ pH^2$ (in phosphate buffer) with $R^2 = 0.9775$ and $k' = 6.79 - 3.76\ pH + 0.68\ pH^2$ (in acetate buffer) with $R^2 = 0.9866$. Furthermore, the capacity factors were also greatly affected by the concentration of acetate buffer in mobile phase while non-imprinted molecule is poorly changed. It increases with decreasing the concentration of buffer—especially in low concentration buffer ($c_{acetate} < 0.02$ mol/L, final concentration in mobile phase). The fit curve of log k' to log c is described by equation: $\log k' = -0.571 - 1.256 \times \log c - 0.186 \times (\log c)^2$ with $R^2 = 0.9979$. The ratio of acetate buffer to methanol was investigated and the optimal ratio for separation of 4-AP and 2-AP is below 1:7.5 (V/V).

Keywords molecularly imprinted microspheres, aqueous microsuspension polymerization, chromatographic stationary phase, influence factors

Introduction

The molecularly imprinted polymers (MIPs) can afford specific recognition of imprint molecules and moderate recognition of the structurally related compounds. They can be used as an attractive alternative or complement to natural antibodies and receptors.[1-5] MIPs have some advantages of physicochemical properties, specifically the ability to tolerate organic solvents, pH value extremes, high pressures and elevated temperatures. They are also used for biosensor,[6-8] membrane separation,[3,9] solid-phase extractions (SPE)[10-15] and chromatographic separation.[16-27] But most MIPs for chromatographic stationary phase are usually synthesized by bulk polymerization. The drawbacks of this method are that the bulk polymer should be crushed, ground and sieved to produce packing particles. The grinding and sieving process is time-consuming and yields only moderate amounts of 'useful' imprinted polymers. The obtained polymer particles are also irregularly shaped, which is unfavorable for chromatographic separation process. In order to avoid the disadvantages of the bulk polymer, another separation medium, continuous rods of MIPs, is prepared by an *in situ* polymerization method,[28] but it often has drawbacks of the high column pressure and the polymerization condition is difficult to control. The available method is micro-

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suspension polymerization technique, which is based on preparation of molecularly imprinted microspheres (MIMs) utilizing a liquid perfluorocarbon as dispersant.[17] The microbeads can be applied directly in chromatographic separation without grinding and sieving. But the liquid perfluorocarbon used is expensive and unfavorable for environment. Another procedure for preparing microspheres is the two- or multi-step swelling polymerization.[18-21] However, the operating steps are complicated and the experimental condition is unable to control. The conventional industrial methods are emulsion or/and micro-suspension polymerization, especially the latter is suited to the formation of cross-linked porous beads. The aqueous micro-suspension polymerization method can produce spherical polymer beads. Furthermore, seldom literatures discuss the influence of pH, kinds and concentration of buffer on capacity factor, which is important to the chromatographic separation utilizing MIMs as stationary phase. In present paper, MIMs were synthesized by aqueous micro-suspension polymerization and characterized by chromatographic model. The influences of pH, kinds and concentration of buffer on capacity factors are investigated in detail.

Materials and methods

Chemicals and materials

4-Aminopyridine (4-AP) and 2-aminopyridine (2-AP) were purchased from Sigma Company. Pyridine (PD), phenylamine (PA) and 4-Methylpyridine (4-MP) were purchased from Shanghai Chemical Reagent Factory No. 1 (Shanghai, China). Methacrylic acid (MAA) was purchased from Beijing Donghuan Chemical Reagent Factory (Beijing, China). Ethylene glycol dimethacrylate (EGDMA) was purchased from Suzhou Anli Chemical & Engineering Co. Lt. (Suzhou, China). 2,2′-Azobis (2-isobutyronitrile) (AIBN) was supplied by Special Chemical Reagent Factory of Nankai University (Tianjin, China). Polyvinyl alcohol 400 (d.p.: 400, saponification value: 87—89 mol%) was purchased from Beijing Organic Chemical Reagent Factory No. 2 (Beijing, China). Other analytical reagents were from Tianjin Chemical Reagent Co. Lt. (Tianjin, China). Acetonitrile and methanol are of HPLC grade. All the acetic acid, acetate, phosphoric acid, phosphate and other chemicals are of analytical grade. Doubly distilled water was used throughout.

Preparation of MIMs

Polyvinyl alcohol 400 (5.5 g) was dissolved in 120 mL of doubly distilled water at 90 ℃ by stirring in the beaker. Then the aqueous solution was transferred into a reactor flask fitted with a mechanical stirrer which adhered to a counter, reflux condenser, nitrogen inlet and dropping funnel. The template molecule 4-AP (2 mmol) and functional monomer MAA (8 mmol) were dissolved in 15 mL of chloroform (as porogen) in a glass beaker. The cross-linker EGDMA (50 mmol) and the initiator AIBN (180 mg) were added to this mixture and sonicated to dissolve. The total organic mixture was admitted into the aqueous solution in flask at 400 rpm under a gentle stream (0.8 L/min) of nitrogen (99.99%). Then the temperature was raised to 60 ℃ for polymerization. The process was allowed to proceed for 24 h. The microspheres were washed with doubly distilled water, methanol and 5% acetic acid of methanol subsequently. Non-imprinted microspheres were prepared in the same way without the addition of template molecule.

High performance liquid chromatography (HPLC) *and methods*

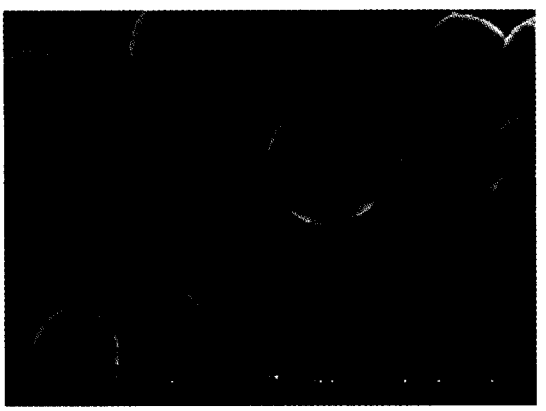
The microspheres were packed into a stainless steel column (250 mm × 4.6 mm I.D.) by slurry method using a Haskel DSTV-150 pump at 600 kg/cm^2 pressure and using a mixture of methanol and 2-propanol as slurry medium. The column was rinsed on-line with 5% (V) acetic acid of methanol until a stable baseline was obtained. HPLC was performed using a Shimadzu-LC-4A pump equipped with SIL-1A injector, a Shimadzu SPD-2AS UV detector and a CTO-2AS column oven. The chromatographic data were collected by ANASTR chromatographic work system (Version 5.2). Column oven temperature was 25 ℃. The flow-rate was 1.0 mL/min. The volume of injection was 20 μL. Detection wavelength was 246 nm. The mobile phase was the acetate buffer and methanol (1:10, V/V) or phosphate buffer and methanol (1:10, V/V). The capacity factor was calculated according to standard chromatographic procedure as $k' = (t - t_0)/t_0$, where t and t_0 are retention time and void time, respectively. The t_0 was determined by the elution time of a small negative peak observed upon injection of the sample solvent (acetonitrile). All pH and concentration were calibrated on a PXD-12 digital ionic meter

(Jiangsu, China). Except for the chromatograms, the other figures were drawn with Origin Version 6.1.

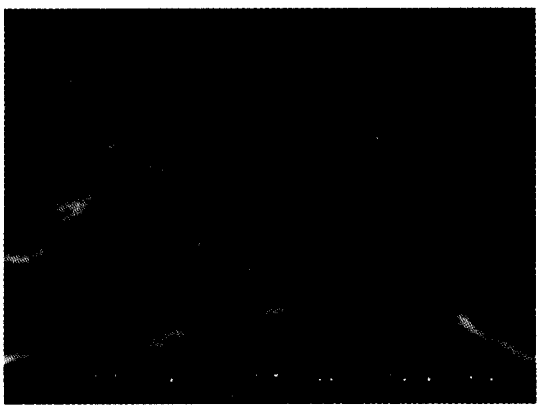
Results and discussion

Aqueous micro-suspension polymerization and selectivity

Aqueous microsuspension polymerization has some advantages as compared with other polymerization procedures. First, the polymers obtained are microspheres without grinding. Second, the shape of microspheres is favorable of chromatographic separation. Third, it is very cheap and favorable of environment utilizing water as dispersing phase. Last, the steps of manipulation are simple. The typical scanning electronic microgram (SEM) of MIMs prepared by this method is shown in Fig. 1. Fig. 1a is the size distribution of microsphere with 1200 amplified factors. The distribution of particle size is about 6—20 μm. Fig. 1b is the surface state of the microsphere with 20000 amplified factors. The porous surface, which was favorable for chromatographic separation, was observed on the surface of microsphere.



(a)



(b)

Fig. 1 Scanning electron micrograph (SEM) of MIMs by micro-aqueous suspension polymerization (performed by a Hitachi S3500 N Scanning Electron Microscope; a × 1200; b × 20000).

MIMs obtained were packed into stainless steel column as chromatographic stationary phase. In order to investigate the selectivity of MIMs column, a non-MIMs column was prepared at the same condition used for MIMs column. Fig. 2 shows that the capacity factor of MIMs column (4.17) was far higher than that of non-MIMs column (1.01). It indicates that MIMs column exhibits good selectivity for 4-AP as compared with non-MIMs column. This is because that the imprinting cavities play an important role against template molecule (4-AP) as previous discussed.

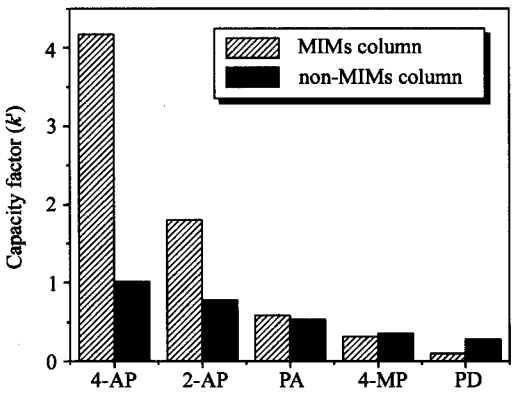


Fig. 2 Comparison of capacity factors for analytes on imprinted column and non-imprinted column.

Influences of pH *and kinds of buffers on capacity factor*

In order to control the influence of concentration of buffer, the total concentration of acetate or phosphate was controlled at 0.02 mol/L, then the pH values were adjusted from 2.0 to 7.0 with acetic acid or phosphoric acid, respectively. The results showed that the pH, kinds and concentration of buffer greatly affected on capacity factors of 4-AP and 2-AP (Fig. 3). Fig. 3a indicates the influences of pH values on capacity factors in acetate buffer of mobile phase and Fig. 3b does that in phosphate buffer. It shows that the influences of pH on the capacity factors for 4-MP in both buffers are very similar. In other word, the capacity factors are less affected by the pH values and kinds of buffer, and also are under 1.0. This is because 4-MP possesses only one binding site under the

nitrogen and is unable to strongly interact with the binding sites of MIMs stationary phase. Although 2-AP is a non-imprinted molecule, its capacity factors (k' is 3.01—4.78 in phosphate buffer and 2.45—3.83 in acetate buffer) seem higher than that of 4-MP (k' is 0.556—0.661 and 0.214—0.403 in respective buffer). It is attributed to the numbers of interaction functionalities in the substrate.[30]

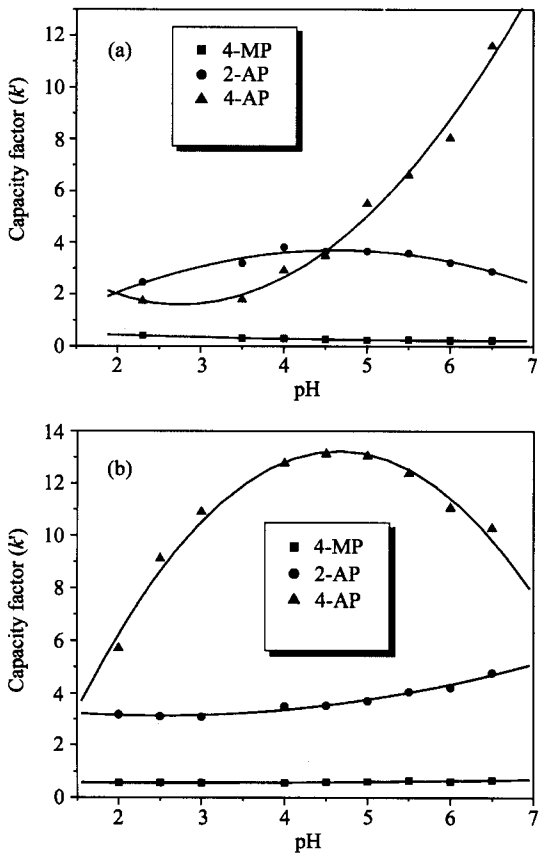


Fig. 3 Influences of pH and kinds of buffer on capacity factors (k') (a: acetate buffer, b: phosphate buffer, c = 0.02 mol/L, buffer: methanol = 1:10, V/V).

Fig. 3 also shows that the influences of pH values on capacity factors for template molecule (4-AP) are very different in phosphate and acetate buffers of mobile phase. In acetate buffer, the capacity factors for 4-AP increase with the increasing of pH values (Fig. 3a). This is because the retention time depends on both mobile phase and stationary phase. In low pH of acetate buffer, the carboxyl in MIMs stationary phase is neutral and difficult to interact with the 4-AP by ion-exchange interaction. The solubility of mobile phase plays a main role in chromatographic retention and the capacity factor of 4-AP is lower. In high pH of acetate buffer, reversibly, the carboxylate ion with negative charge can interact with the 4-AP in the mobile phase by ion-exchange interaction. The recognition sites in MIMs plays a principal role in the chromatographic separation due to the specific recognition sites. As a result, the retention time increases with increasing pH value in mobile phase. The influence of pH of acetate buffer on capacity factors is described by the second fit curve with equation: $k' = 6.793 - 3.764\ \mathrm{pH} + 0.682\ \mathrm{pH}^2$ with $R^2 = 0.9866$.

In phosphate buffer, as can be seen from Fig. 3b, the influences of pH values on capacity factors are increased firstly and reduced subsequently with increasing pH values. The fitting curve of capacity factor to pH value presents parabola shape. In pH < 2.0 or pH > 7.0 mobile phase, the capacity factor for 4-AP is gradually close to that of 2-AP. The template molecule and non-imprinted molecule are slightly separated by MIMs stationary phase under this condition. As a result, the recognition properties of MIMs to 4-AP disappeared. It is contributed that, in low pH of buffer, the retention process is similar to that in acetate buffer. But with the increasing of pH values, the phosphate ion will be changed from low charged group ($H_2PO_4^-$) to highly charged group (HPO_4^{2-}). As well as known, the electrostatic force is proportional to charges. Therefore, the electrostatic interaction between 4-AP and HPO_4^{2-} in mobile phase is probably strong enough to overcome the interaction of carboxylate ion in MIMs to 4-AP and the retention time is decreased reversibly with increasing pH values of phosphate buffer in mobile phase. The influence of pH values of phosphate buffer on capacity factors is described by the second fit curve of $k' = -8.235 + 9.232\ \mathrm{pH} - 0.992\ \mathrm{pH}^2$ with $R^2 = 0.9775$. This equation was differentiated and the maximum pH value was obtained at pH_{max} 4.65.

Influence of concentration of buffer on capacity factor

Our studies show that the influence of concentration of buffer on capacity factor for imprinted molecule is very great. Present paper will discuss the influence of concentration of acetate buffer on capacity factor. Due to the too high concentration of phosphate ($c_{\mathrm{phosphate}} > 0.05$ mol/L, final concentration in mobile phase), it was poorly dissolved in methanol and precipitated out. Therefore, 0.002, 0.005, 0.01, 0.02, 0.05 and 0.1 mol/L ac-

etate buffers (final concentration in mobile phase, pH 6.0) were investigated. As can be seen from Fig. 4, the capacity factors of non-imprinted molecules (2-AP and 4-MP) are subtly affected by the concentration of acetate buffer while the imprinted molecule (4-AP) is greatly affected by it. The result is identical with the influence of pH values. The capacity factor for 4-AP, especially in low concentration of buffer (< 0.02 mol/L), increased abruptly with the decreasing of concentration of buffer. It can be suggested that, in high concentration of buffer, the binding sites of carboxylate ion in MIMs are likely to be occupied by large quantity of ions. As a result, the imprinted molecule (4-AP) is difficult to interact with the recognition sites by ion-exchange interaction. In low concentration of buffer, the recognition sites in MIMs are seldom occupied by the ion in mobile phase and the template action may play a main role in chromatographic retention. As a result, the capacity factor increases greatly. It further testifies the fact that the ionic interactions of MIMs stationary phase to template molecule (4-AP) play a main role. Data in Fig. 4 are taken in logarithm. Then curves fitting for log k' to log c are obtained in Fig. 5. The parameters of the fitting curve are presented in Table 1. They showed that the R^2 of curve fitting for imprinted molecule (4-AP) is 0.9979 (close to 1.0000) and is higher than that of non-imprinted molecule with R^2 below 0.99. Therefore, the curve fitting of log k' to log c is the most suitable for imprinted molecule (4-AP). The equation is $\log k' = -0.5710 - 1.2562 \times \log c - 0.1857 \times (\log c)^2$ with $R^2 = 0.9979$. It indicated that the k' value decreases in hyperbola with increasing the concentration of buffer. The retention behavior of 4-AP on the MIMs column could be explained by ion-exchange model.[31,32]

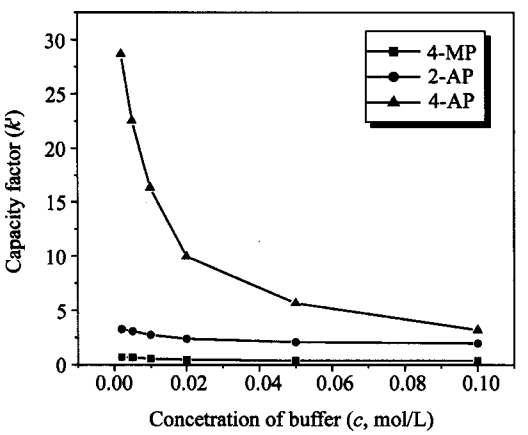


Fig. 4 Influence of concentration of acetate buffer on capacity factor (pH 6.0 buffer).

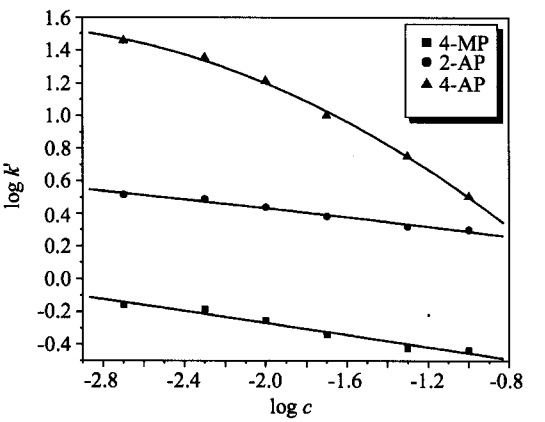


Fig. 5 Curve fitting of log k' to log c for analytes in acetate buffer (pH 6.0 buffer).

Influence of ratio of buffer to methanol

In order to testify above result, present paper further researched the influence of ratio of the buffer to methanol (V/V) on capacity factor. The ratios of 1:20, 1:15, 1:10, 1:7.5 and 1:5 were investigated. As can be seen from Fig. 6, the influence of the ratio of buffer to methanol is similar to that of the concentration of buffer. It is easy to understand that the carboxylate ion in MIMs stationary phase is combined by the cation in highly concentration of buffer. The higher the ratio of buffer to methanol

Table 1 Parameters for curve fitting of log k' to log c

Analyte	$\log k' = a + b_1 \log c + b_2(\log c)^2$	R^2
4-MP	$\log k' = -0.6457 - 0.1943 \log c - 0.0028 (\log c)^2$	0.9619
2-AP	$\log k' = 0.1340 - 0.1612 \log c - 0.0062 (\log c)^2$	0.9801
4-AP	$\log k' = -0.5710 - 1.2562 \log c - 0.1857 (\log c)^2$	0.9979

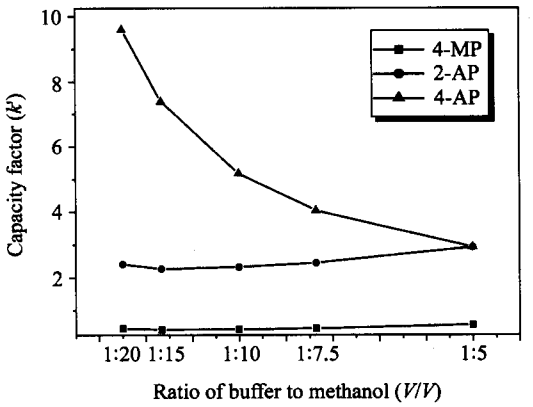


Fig. 6 Influence of the ratio of acetate buffer to methanol on capacity factor (pH 6.0, $c = 0.05$ mol/L).

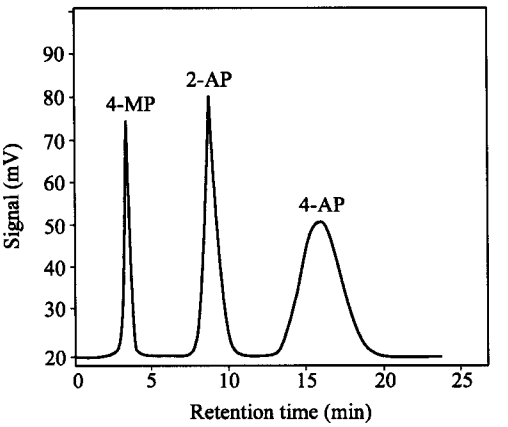


Fig. 7 Representative chromatogram of MIMs stationary phase (acetate buffer 0.02 mol/L, pH 6.0, buffer: methanol = 1:10, $V:V$).

is, the more the ions are present in mobile phase, the more the carboxylate ion in MIMs is occupied. As a result, the interaction of carboxyl in MIMs to 4-AP is weakened, and even completely eliminated by the high concentration of ion. And the capacity factor for 4-AP is reduced with the increasing of the ratio of buffer to methanol. In addition, the higher the ratio of buffer to methanol is, the more water is present in mobile phase. Therefore, the hydrogen bond between MIMs and imprinted molecule is also weakened with increasing the water in mobile phase. It is also shown that the capacity factors for imprinted molecule approach to that for non-imprinted molecule when the ratio is above 1:7.5 (V/V). In other words, the imprinted molecule and non-imprinted molecule are poorly separated by MIMs under this condition. Considering long-tailed peak in low ratio of buffer to methanol, the ratio of 1:10 is chosen in this paper. According to above discussion and results, the optimal conditions for separation 4-AP and 2-AP are listed in Table 2. A representative chromatogram is shown in Fig. 7.

Table 2 Optimal conditions for separation and determination of 4-AP

Buffer	Acetate	Phosphate
pH value	6.0	4.6
Concentration of buffer (c)	0.02	0.02
Ratio of buffer to methanol (V/V)	1:10	1:10

Conclusion

Molecularly imprinted microspheres (MIMs) for HPLC were prepared by aqueous micro-suspension polymerization. The influences of pH, kinds and concentration of buffer on the retention behaviors of MIMs were investigated in detail. The relationships of capacity factor (k') to pH and concentration (c) of buffer were quantitatively described firstly. The MIMs prepared were successfully used to separate 4-AP and 2-AP by HPLC model. Furthermore, the MIMs obtained could be used as solid-phase extraction (SPE) materials for extracting target molecules according to the aim.

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