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Preparation and Characterization of Theophylline-Imprinted Monolithic Column

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Abstract: Monolithic molecularly imprinted column was prepared by an in-situ therm-initiated copolymerization and the effects of essential preparation conditions such as polymerization mixture composition and polymerization condition was investigated. The results showed that the selection of correct porogenic solvents and appropriate polymerization conditions are crucial for the preparation of the monolithic stationary phases. The separation efficiency was only extremely weakly dependent on flow rate and hydrogen-bonding interaction played an important role in the retention and separation. Compared with conventional particle columns and bulk molecular imprinted polymer column, the monolithic column exhibited good stability, ease of regeneration, high separation efficiency, and fast analysis.

Keywords: Monolithic column, molecular imprinted polymer, in-situ polymerization, theophylline

INTRODUCTION

Molecular imprinted polymers (MIP) exhibiting high selectivity and affinity to the predetermined molecule (template) are now seeing a fast growing research. The special binding sites are formed by the self-assembly of the template with functional group and the monomer, followed by a crosslinked

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co-polymerization. After the polymerization, the template was removed from the polymer, leaving recognition sites that, in terms of size, shape, and functionality, are complementary to the template. So, ideally, the resulting MIP can selectively re-bind the template in preference to other closely related structures (1–5). Molecular imprinted polymer has been applied to chiral separation (6, 7), solid extraction (8), biomimic sensor (9, 10), and membrane separation (11, 12).

The conventional approach is to synthesize the MIP in bulk, grind the resulting polymer block into particles, and sieve the particles into the desired size ranges. Such ground and sieved particles have been packed into conventional liquid chromatography columns. Although the process of bulk polymerization is simple, the resulting polymer must be crushed, ground, and sieved to obtain the appropriate particle size, which is tedious and time-consuming. Since only a portion of polymers can be used as packing material, this method suffered high consumption of the template molecules. In addition, the resulting polymer particles are polydispersed both in shape and size, which also has a negative impact on chromatographic performance (13). Uniformly sized and monodispersed particles had been made by suspension polymerization and seed polymerization or multi-step swelling process (14–16). The limits of the above methods are either requiring use of the special dispersing phases/surfactants or too complicated.

The monolithic molecularly imprinted technology represents a novel method for preparation of stationary phases (17, 18). This method combined the advantage of the monolithic column technology and the molecular imprinted technology. These monoliths were prepared by a simple, one-step, in-situ, free-radical polymerization "molding" process directly within the chromatographic column without the tedious procedures of the grinding, sieving, and column packing. The monolithic molecularly imprinted technology has attracted significant interest because of their ease of preparation, high selectivity and sensitivity, high reproducibility, and rapid mass transport (19, 20). These features allow for an efficient and fast separation of especially large biomolecules (21). However, the prepared MIP often suffer from high back pressures and low efficiency, which results in their poor application and practical separation. Moreover, there is still a distinct lack of systematic investigation of fabrication of monolithic MIP column. In addition, newly prepared procedures need to be developed for various materials due to their special structure. In this work, essential preparation conditions and separation characteristics such as the polymerization mixture composition, polymerization condition, template molecule, mobile phase composition, flow rate, and temperature on the retention and separation were investigated. Compared with conventional particle columns, the monolithic column exhibited good stability, ease of regeneration and high-efficiency separation ability. In situ polymerization method is very simple compared with the conventional procedure and its macroporous structure has excellent separation properties.

EXPERIMENTAL

Chemicals

Caffeine and theophylline were obtained from Sigma (ST Louis, MO, USA). The structures of these molecules were shown in Fig. 1. Methacrylic acid (MAA) from Kanto Chemical Co., Inc. (Japan) was distilled prior to use. Ethylene glycol dimethacrylate (EDMA) from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) was extracted with $2\,\mathrm{mol}\cdot\mathrm{L}^{-1}$ sodium hydroxide and water, then dried over anhydroxide magnesium sulfate. α,α'-Azobis (isobutyronitrile) (AIBN) was the product of Junsei Chemical Co., Ltd. (Japan) and was recrystallized prior to use. Toluene was purchased from Orienal Chemical Industries (Japan). Dodecyl alcohol, acetonitrile, chloroform, and methanol are all of HPLC grade and from Duksan Pure Chemical Co., Ltd. (Ansan, Korea). Acetic acid (analytical grade) was purchased from Oriental Chemical Industries (Incheon, Korea). Double distilled water was filtered with 0.45 μm filter membrane before use.

Preparation of Monolithic MIP Column

The stationary phase was directly prepared by in-situ polymerization within the confines of a stainless-steel chromatographic column (100 mm × 3.2 mm I.D.). The polymerization mixture composed of template molecule, methacrylic acid, ethylene glycol dimethacrylate (EDMA) and α,α'-Azobis (isobutyronitrile) (AIBN) was dissolved in the porogenic solvents (toluene

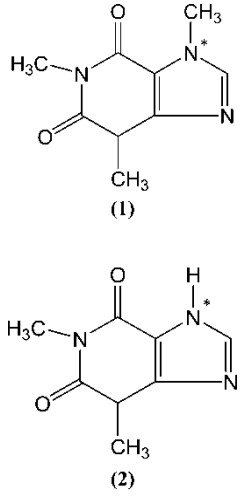


Figure 1. Molecular structure of caffeine (1) and theophylline (2).

and dodecanol) (Table 1). The mixture solution was put into supersonic for 10 min, sparged with helium for 5 min to remove oxygen. The stainless-steel tube sealed at the bottom was filled with the above polymerization mixture and then sealed at the top. The polymerization was performed in a water bath with the temperature maintained at 48°C for 12 h. After the polymerization, the seals were removed; the column was connected to HPLC pump and washed with tetrahydrofuran and methanol/acetic acid (80:20 % v/v) respectively to remove the porogenic solvents and the template molecules. A non-imprinted blank monolithic column (in the absence of template) was prepared and treated in an identical manner, and traditional bulk MIP columns were prepared according to the previous paper (22).

HPLC Analysis

Separation characteristics of the monolithic MIP column were analyzed by a high-performance liquid chromatography system containing Waters 600s Multisolvent Delivery System and a Waters 616 pump (Waters, Milford, MA, USA), Waters 2487 Dual Absorbance UV detector (Waters, Milford, MA, USA) and Rheodyne injection valve (20 μl sample loop). The Millennium 3.2 software (Waters, Milford, MA, USA) was used as data acquisition system. Acetonitrile was used as mobile phase, UV wavelength at 270 nm.

The separation factor (α) was determined by the following equation:

$$\alpha = k_2/k_1 \quad (1)$$

Table 1. Effect of differents porogenic solvents on the separation characteristic of monolithic column

No.	MAA (mmol)	EDMA (mmol)	Toluene (V%)	Cyclohexanol (V%)	Dodecanol (V%)	k_1	k_2	Rs
1	1.38	4.13	—	—	100	<0.78	<0.93	—
3	1.38	4.13	10	—	90	0.88	3.56	2.46
4	1.38	4.13	15	—	85	0.98	5.44	3.42
5	1.38	4.13	—	15	85	0.69	2.30	2.59
6	1.38	4.13	30	—	70	1.17	5.23	3.03
7	1.38	4.13	—	10	90	0.56	2.17	1.98
8	1.38	<3.60	15	—	85	<0.74	<3.42	<2.35
9	1.38	4.13	>40	(>40)	<60	High pressure		

Separated condition: mobile phase: acetonitrile, flow rate: 1.0 mL/min, detection wavelength: 270 nm, k_1: the retention factor of caffeine, k_2: the retention factor of theophylline, *Rs*: resolution of caffeine and theophylline.

where k_2 is the retention factor of the theophylline and k_1 is the retention factor of the caffeine. The retention factor was determined by

$$k = (t_M - t_0)/t_0 \quad (2)$$

where t_M is the retention time of the solute and t_0 is void time of the column. All the procedures were carried out at the room temperature.

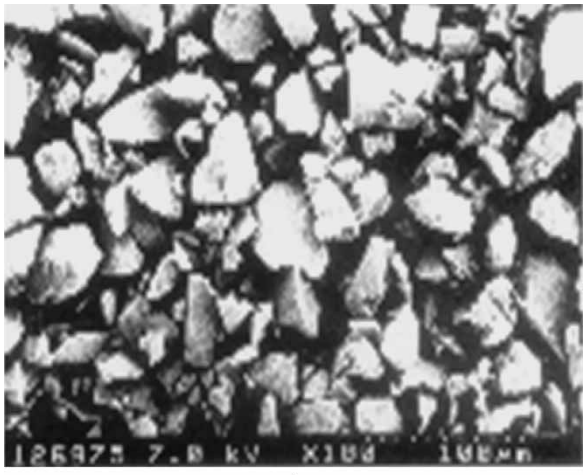
Characterization of Monolithic Stationary Phases

After the chromatographic experiments had been completed, the column was washed with methanol/acetic acid (4:1 v/v) for 30 min. The bottom column fitting was removed and the monolith inside the column was pushed out of the tube using the pressure of the methanol mobile phase at a flow-rate of 4 ml/min. The cylindrical monolith was dried under 50°C for 12 h and cut into pieces with a razor blade. The pore properties and microscopic analysis of the monolith was carried out in an S-4200 Scanning Electron Microscopy (Hitachi, Japan) at 3.0 kV.

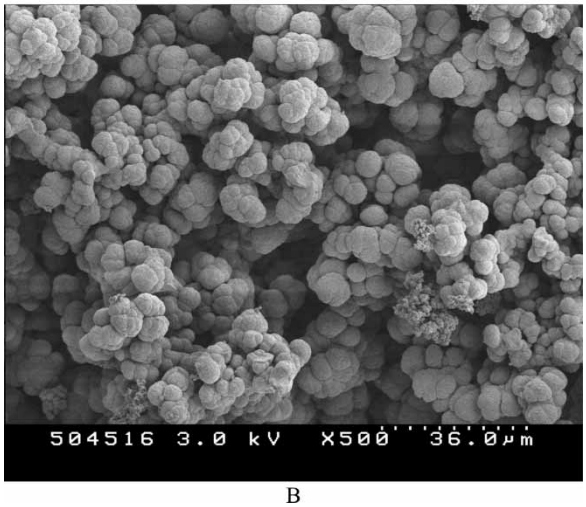
RESULTS AND DISCUSSION

Comparisons with Traditional Particle Column and Bulk MIP Column

The obvious advantages of monolithic Column are their porous, highly interconnected, network structure, which support the formation of a network of channels and provide the large surface area needed to achieve sufficient capacity. Compared to a packed column, in which high efficiency and high speed are mutually exclusive and enable the mobile phases to flow through the adsorbent with low flow resistance at high flow rates. Figure 2 showed that the monolithic MIP column consists of a single piece of porous material that fills the entire length of the column. The mesopores located on the column skeleton are highly interconnected, forming a network of channels and providing the large surface area needed to achieve sufficient capacity. Meanwhile, the large through-pores present in this type of stationary phase. It could reduce flow resistance, allowing the use of high flow rates at considerably reduced backpressure. From the Fig. 3 we could see that theophylline and caffeine could not be separated on conventional Lichrospher 100 RP-18 particle column and blank monolithic column, at the same time good separation was obtained on the monolithic MIP column. From Fig. 4, we could see that in comparison to the conventional bulk packed column, low backpressures were obtained on the monolithic MIP column at different



A



B

Figure 2. Scanning electron microscope (SEM) of the monolithic MIP columns and bulk MIP column. (A: bulk MIP column, B: monolithic MIP column)

flow rates. When flow rate was 4.0 mL/min, the column pressure was observed only 7.68 MPa on the monolithic column.

Effect of the Preparation of the Monolithic Column

The monolithic MIP columns were prepared by a simple, in-situ, free-radical polymerization without the tedious procedures of the grinding, sieving, and

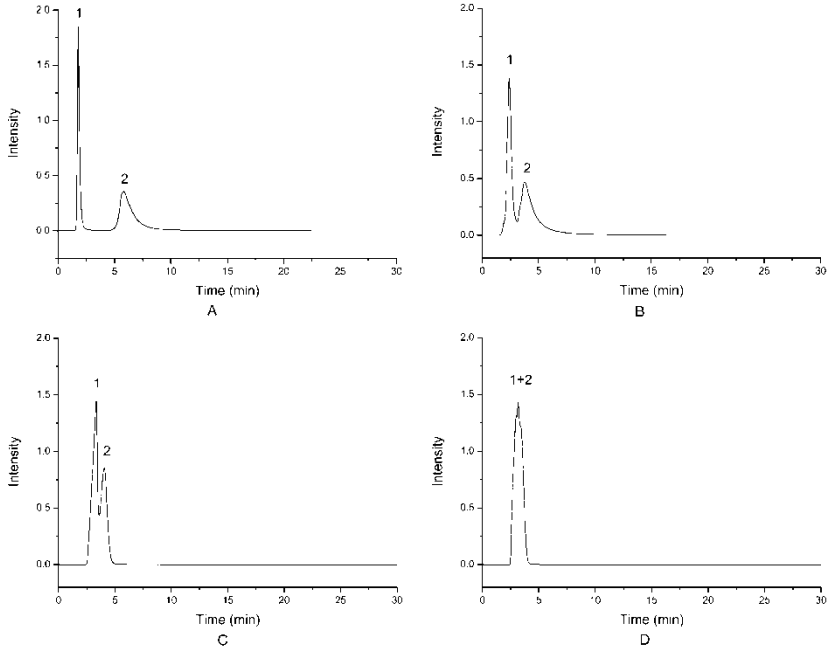


Figure 3. Chromatograms of caffeine and theophylline on different columns. (A), bulk-imprinted column (B), blank monolithic column (C) and Lichrospher 100 RP-18 particle column (D) (Mobile phase: acetonitrile, Flow rate: 1.0 mL/min, detection wavelength: 270 nm, Peak 1: caffeine; Peak 2: theophylline).

column packing. The proportion of mixture composition and polymerization temperature defines the monolithic structure and separation characteristic without further processing. Although the preparation process of the molecularly imprinted monolithic stationary phases is quite simple, however, some numbers of factors have to be taken into account. Among these factors, the selection of the porogenic solvents is crucial for the preparation of monolithic MIP stationary phases. Firstly, template molecule, initiator, monomer, and cross-linker have to be soluble in the porogenic solvents. Secondly, the porogenic solvents should produce large pores, in order to assure good flow-through properties of the resulting polymer. Thirdly, the porogenic solvents should be of relatively low polarity, in order to reduce the interferences during complex formation between the imprint molecule and the monomer, as the latter is very important to obtain high selectivity MIP. In this work, several porogenic solvents, n-heptane, cyclohexanol, dodecanol, and toluene were tested for their compatibility. The investigations revealed that the monolithic stationary phases using n-heptane, cyclohexanol, and dodecanol as porogenic solvents showed low selectivity for theophylline and caffeine. Meanwhile, monolithic stationary phases with high selectivity

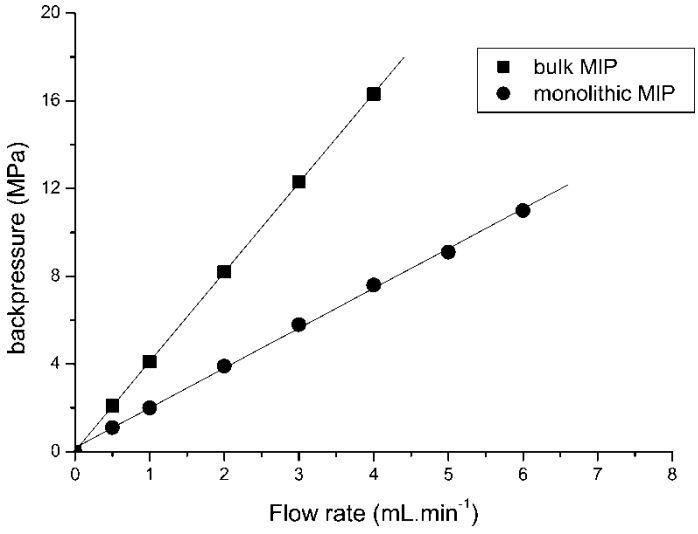
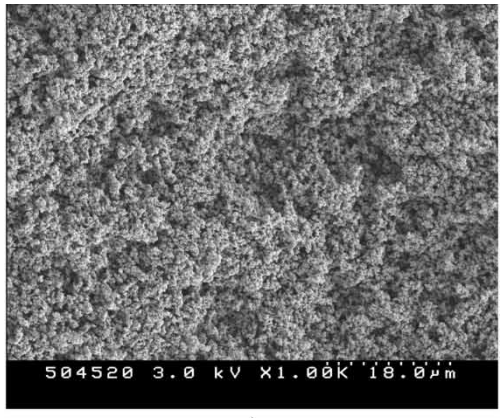


Figure 4. The relationship of backpressure and flow rates on monolithic MIP column and bulk MIP column. (column: 100 mm 3.2 mm I.D., mobile phase: acetonitrile)

and low backpressure could be obtained using the low polar porogenic solvents of toluene and dodecanol as porogenic mixture.

The ratio of toluene and dodecanol also affected the separation performance through variation in pore structure of the monolithic stationary phases. From Table 1 and Fig. 5 we could see that with increasing toluene proportion, the mean pore size decreased and the specific area and resolution factor increased. However, when the proportion of toluene in the porogenic mixture rose above 40%, the pore diameter of the resulting stationary phase was too small to allow the mobile phase to flow through. Thus, in finding a balance between the requirements of low flow resistance and large surface area, a ratio of 15% toluene and 85% dodecanol was used in this experiment as the optimal porogenic mixture.

This work also shows that the amount of cross-linker should be high enough to maintain the stability of the recognition sites. The results showed that the percentage of crosslinking agent in monomer mixture (V%) should be even higher than 60% of monomer mixture. When the percentage was lower than 60%, the MIP showed no or weakly recognition ability. These may be because the high degree of cross-linking enables the microcavities to maintain three-dimensional structure complementary in both shape and chemical functionality to that of the template after removal of the template, and thus, the functional groups are held in an optimal configuration for rebinding the template, allowing the receptor to "recognize" the original substrate. The optimized cross-linker percentage of 86% was using in this work.



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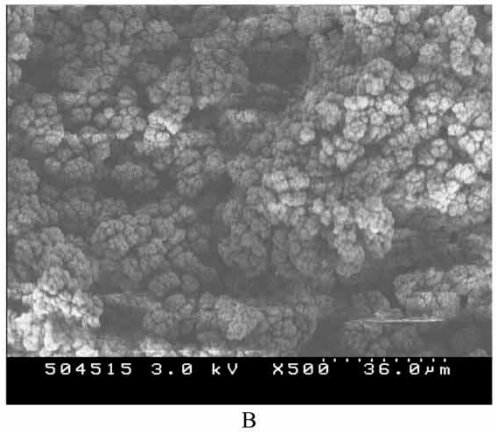

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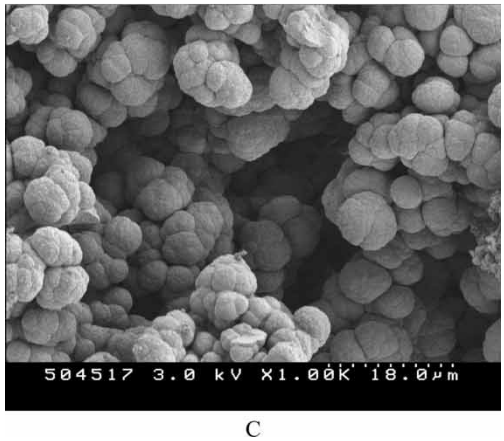

C

Figure 5. SEM of the monolithic MIP columns prepared by different porogenic solvents. (Proportion of toluene and dodecanol (V%): A: 40/60; B: 30/70; C: 15/85)

Finally, the polymerization conditions, such as polymerization temperature and polymerization time affect the efficiency and selectivity of the resultant polymeric stationary phases. Usually, most people using 60°C as the polymerization temperature. But the initiation of the polymerization reaction was very fast and therefore hard to control at this temperature. This resulted in low reproducibility of molecular imprinted monolithic stationary phases. Furthermore, the relatively high temperatures have a negative impact on the complex stability, which reduced the reproducibility of the monolithic stationary phases and produced high column pressure drops. Thus, the relatively low temperature of 48°C with a prolonged reaction time of 12 h was selected in order to yield a more reproducible polymerization. The reaction time of 12 h was chosen because the polymerization remained incomplete at less than 9 h, whereas the column pressure increased at more than 12 h.

Effect of the Mobile Phase Composition

In this paper, the effect of composition of the mobile phase on the separation was investigated using methanol, water, and acetonitrile as mobile phase. The best separation was obtained by using acetonitrile as mobile phase. The effects of polar additives in the mobile phase were also evaluated with the mixtures of acetonitrile-acetic acid as the mobile phase. The experiment showed that the polarity of the organic solvents had a significant effect on the retention behavior of the template (see Fig. 6). The retention factors of caffeine and theophylline all decreased with the increasing of mobile phase polarity. Such difference can be explained by the presence of a hydrogen-bonding interaction between the polymer with imprinting molecules and the hydrophobic cavity generated by these molecules in the polymeric matrix. With the increasing of mobile phase polarity, the k_1 value of caffeine change slightly and the k_2 value of theophylline changes quickly. When only acetonitrile was used as the mobile phase, the best retention factor was attained. The hydrogen-bonding interactions were destroyed with the increasing of polarity modification and retention factors decreased when the mixture of acetic acid and acetonitrile were used as the mobile phase. The results imply that the hydrogen-bonding interaction and hydrophobic interaction can play an important role in the retention and separation.

Effect of the Flow Rate on the Separation

Monolithic columns have been studied as materials, having advantages inherent to their network-type one-piece structures. The microscopic structure of monolithic columns is porous and composed of interconnected networks of pores. Through-pores provide flow paths along the column, and

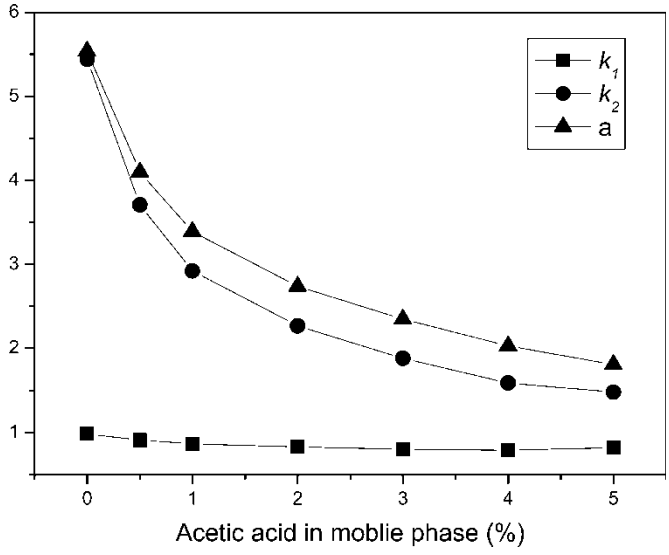

Figure 6. Effect of mobile phase composition on retention factor and separation factor. (Determination condition: mobile phase: acetonitrile, flow rate: 1.0 mL/min, detection wavelength: 270 nm, k_1: the retention factor of caffeine, k_2: the retention factor of theophylline, a: separation factor of caffeine and theophylline)

the size and density of the macropore network causes the monolithic columns to have a high external porosity, consequently, having a large permeability and a low column hydraulic resistance. At the same time, the network of mesopores is responsible for the large specific surface area of the monolith, hence for the retention volumes observed for most analytes. For these reasons, the monolithic columns are efficient at high flow-rates and allow the achievement of very high efficiencies. In this experiment, the flow rate of the mobile phase was investigated in the range of $0.5 \sim 6.0$ mL/min. Although the migration times of the caffeine and theophylline decreased with the increasing of the flow rate, but just a slight decrease of the separation efficiency was found when increasing the flow rate (Fig. 7). The results shown that the dependency of separation efficiency on flow rate is extremely small, therefore, separation efficiency can be maintained at significantly increased flow rates. This is the typical characteristic of a monolithic column.

Effect of Temperature on the Separation

The effects of different temperatures changing from 24°C to 60°C on the separation were also investigated. The results of Figure 8 showed that under higher temperature, the retention time of caffeine only extremely weakly

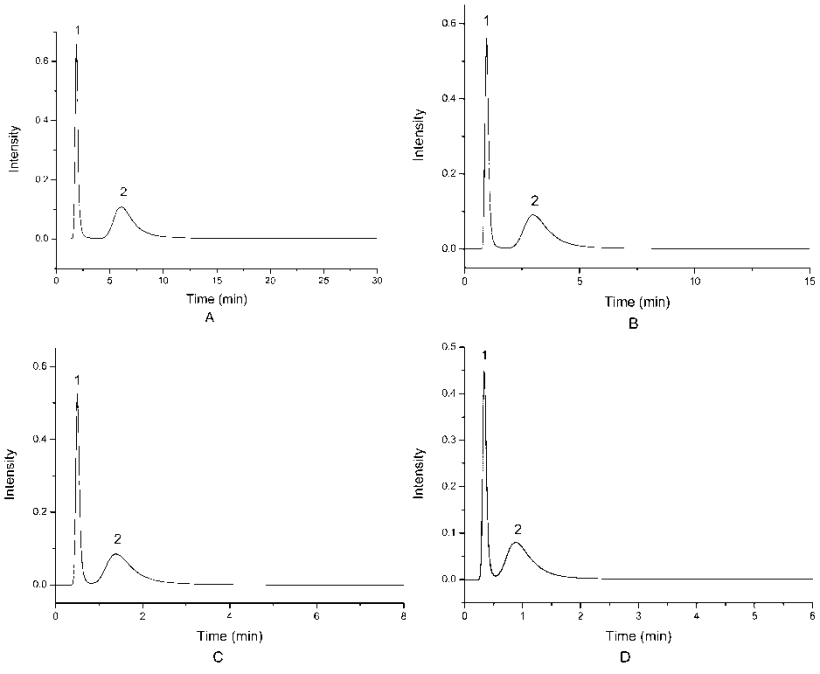

Figure 7. Chromatograms of caffeine and theophylline at different flow rates. (Determination phase: acetonitrile, flow rate: A: 1.0 mL/min, B: 2.0 mL/min; C: 4.0 mL/min; D: 6.0 mL/min; detection wavelength: 270 nm, Peak 1: caffeine; Peak 2: theophylline)

changed, but the retention time of theophylline changes much faster than the one in caffeine. The retention factor of theophylline decreasing with increasing temperature is because the adsorptions of the analytes to the substrate weaken with increasing temperature, allowing the analytes to migrate faster through the monolithic column. That means that the hydrogen-bonding interaction and hydrophobic interaction between the template and polymer weakened with increasing temperature. Furthermore, the separation factors decreased with increasing elution temperature, due to higher temperature decreasing the interaction between the theophylline and the polymers more than the interaction between the caffeine molecule and the polymers. Therefore, a lower temperature will lead to a higher separation.

CONCLUSION

The results showed that the proportion of pre-mixture composition and the polymerization condition combine to define the monolithic structure without further processing. Therefore, the proper selection of porogenic solvents

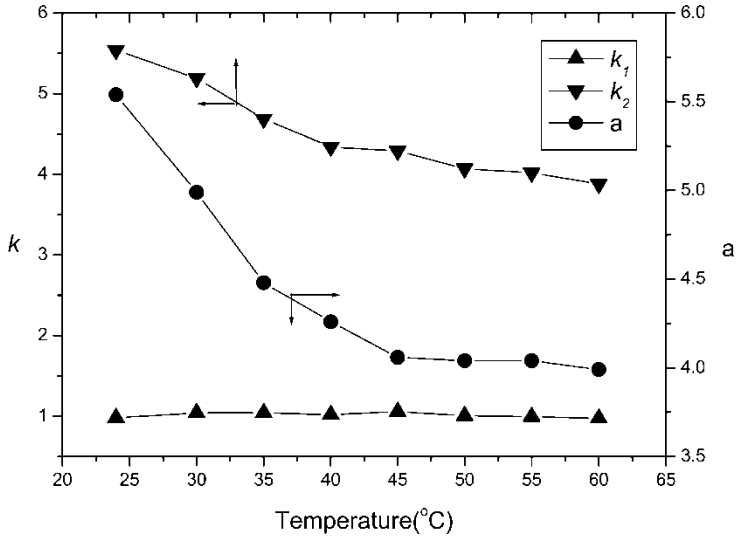

Figure 8. Effect of different temperatures on the separation factor and retention factor. (Determination condition: mobile phase: acetonitrile, flow rate: 1.0 mL/min, detection wavelength: 270 nm, k_1: the retention factor of caffeine, k_2: the retention factor of theophylline, a: separation factor of caffeine and theophylline)

and polymerization temperature is crucial for the preparation of the monolithic stationary phases. The method of in situ polymerization is simple and rapid and the consumption of chemicals is low. Moreover, the dependency of separation efficiency on flow rate is extremely small and hydrogen-bonding interaction play an important role in the retention and separation. The study results presented here have substantiated the significant research interest in monolithic MIP columns compared with conventional particle columns and bulk MIP columns due to their ease of preparation, high separation efficiency, and rapid mass transport.

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