Preparation and Characterization of Monolithic Poly(methacrylic acid – ethylene glycol dimethacrylate) Columns for High Performance Liquid Chromatography

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Porous polymer monolithic columns were prepared by the direct free radical copolymerization of methacrylic acid and ethylene glycol dimethacrylate within the confines of a chromatographic column in the presence of toluene-dodecanol as a porogenic solvent. The separation characteristics of the monolithic columns were tested by a homologous series of xanthine derivatives, theophylline and caffeine. The effects of the polymerization mixture composition and polymerization condition, mobile phase composition, flow rate and temperature on the retention times and separation efficiencies were 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 temperatures. Hydrogen-bonding interaction played an important role in the retention and separation. Compared with conventional particle columns, the monolithic column exhibited good stability, ease of regeneration, high separation efficiency and fast analysis.

Key Words : Monolithic column, *In-situ* polymerization, Theophylline, Caffeine

Introduction

Monolithic columns, one of the most interesting innovations in column-manufacture technology, are also referred to in the literature as monolithic stationary phase, continuous bed, and rod columns.[1-3] These columns have one common characteristic: they are made of one single piece of an adsorbent material (silica or polymer) that fills the entire length of the column.[4,5] The large through-pores present in this type of stationary phase enable mobile phases to flow through the adsorbent with low flow resistance at high flow rates.[6-10] Because of their ease of preparation, high reproducibility, versatile surface chemistry, and rapid mass transport, monolithic stationary phases have become a rapidly expanding field in chromatographic stationary phase preparation in recent years and some have even been commercialized.[11-15] Their innovative development has led some chromatography researchers to regard them as fourth-generation chromatography adsorbents.[16]

The most innovative feature of the monolithic columns compared to conventional particle beds is their unique porous structure. Conventional particle-based supports consist of a few, micrometer-sized, porous particles. Because the pores within the particles are in close proximity, the liquid inside them remains stagnant. Therefore, the molecules to be separated are transported to the active sites inside the closely packed pores and back to the mobile phase mainly by diffusion. Diffusion alone, however, is a rather slow process, especially in the case of large molecules with a low mobility. Monoliths, on the other hand, consisting of a single piece of porous material, are highly interconnected, and thereby form a network of channels and provide the large surface area needed to achieve sufficient capacity.

Meanwhile, the larger through-pores reduce the flow resistance and thus enable the use of high flow rates at considerably reduced backpressure. Since the flow of the liquid within the channels is driven by the pressure difference, the molecules to be separated are transported to the active sites located on the surface of the channels by convection. The separation efficiency is only extremely weakly dependent on flow rate; therefore, high separation efficiency can be maintained at significantly increased flow rates.[17]

There are presently two main types of monolithic columns: silica[18,19] and porous organic.[20] Monolithic columns were introduced first for organic polymers in the late 1980s and early 1990s. Although the column efficiency provided by polymer monolithic columns is generally lower than that by the silica particles in conventional columns, the polymeric monolithic column exhibits more potential advantages and has a more promising future than its silica-based counterparts because of the simpler preparation process, higher efficiency, easier pore size control, and more adaptability to adjust column selectivity. Organic polymer monolithic materials undergo easy, *in situ* synthesis through thermal or irradiation initiation, during which the pore properties and surface area of the material can be freely controlled by the type and composition of the porogenic solvent, crosslinker and polymerized condition. Given the variety of available monomers and crosslinkers, various separation requirements can be achieved with the polymer monolithic material which enables the production of different polarity and functional groups. Besides, the procedure for synthesizing such kind of material is simple and reproducible, and the polymer monolithic material can be easily situated in the separation support structure, *i.e.* stainless steel column and cartridges,

without any additional packing step, which also makes it an attractive alternative for applications such as separation media.[21-23] However, there is still a distinct lack of systematic investigation into the fabrication of monolithic columns. In addition, new preparation methods for monolithic columns need to be developed for various different materials due to their special structure.

In the present study, polymer monolithic columns using methacrylic acid as the acidic monomer and ethylene glycol dimethacrylate as the crosslinker were prepared by a simple, one-step, *in-situ*, free-radical polymerization, "molding" process, directly within the confines of a chromatographic column. The characteristics of the monolithic columns were tested by a homologous series of xanthine derivatives, theophylline and caffeine.[24] The effects of the polymerization mixture composition and polymerization condition, mobile phase composition, flow rate and temperature on the retention times and separation efficiencies were investigated. Compared with conventional particle columns, the monolithic column exhibited good stability, ease of regeneration and high-efficiency separation ability.

Experimental Section

Materials. Caffeine and theophylline were obtained from Sigma (ST Louis, MO, USA). The structures of these molecules were shown in Figure 1. Methacrylic acid (MAA) was purchase from Kanto Chemical Co., Inc. (Japan). Ethylene glycol dimethacrylate (EDMA) was obtained from Tokyo Kasei Kogyo Co., LTD (Tokyo, Japan). α,α'-Azobis (isobutyronitrile) (AIBN) was the product of Junsei Chemical Co., Ltd. (Japan). Toluene was purchased from Oriental 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 0.45mm filter membrane before use.

Preparation of monolithic column. The monolithic columns were prepared by a direct *in situ* polymerization within stainless steel of a 150 mm × 3.9 mm I.D. chromatographic column. The polymerization mixture composed of methacrylic acid, Ethylene glycol dimethacrylate (EDMA)
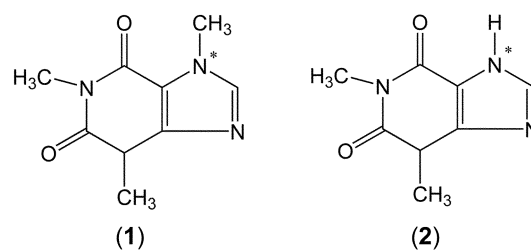

Figure 1. Molecular structures of caffeine (**1**) and theophylline (**2**).

and α,α'-Azobis (isobutyronitrile) (AIBN) was dissolved in the porogenic solvents (toluene and dodecanol) (Table 1). The mixture solution was put into supersonic for 15 min, sparged with helium for 10 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 45 °C for 12 h. After the polymerization, the seals were removed; the column was connected to HPLC pump and washed respectively with tetrahydrofurana for three hour and methanol/acetic acid (80 : 20%v/v) four hour to remove the porogenic solvents and other soluble compounds present in the polymer monolith after the polymerization was completed.

HPLC analysis. Separation characteristics of the monolithic column were analyzed by a 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 32 software (Waters, Milford, MA, USA) was used as data acquisition system. LiChrospher® 100 RP-18 (12 μm) and Lichrospher® Silica (15 μm) was purchase from Merck (Germany). Acetonitrile was used as mobile phase, UV wavelength at 270 nm.

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

$$\alpha = k_2/k_1 \tag{1}$$

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

$$k = (t_M - t_0)/t_0 \tag{2}$$

Table 1. Effect of differents porogenic solvents on the separation characteristic of monolithic column. (Separated condition: mobile phase: acetonitrile, flow rate: 0.5 mL/min, detection wavelength: 270 nm, k_1: the retention factor of caffeine, k_2: the retention factor of theophylline, R_s: resolution of caffeine and theophylline)

No.	MAA (mL)	EDMA (mL)	Toluene (mL)	Cyclohexanol (mL)	Dodecanol (mL)	k_1	k_2	R_s
1	0.085	0.945	–	–	2.000-4.000	< 0.250	< 0.308	–
2	0.085	0.945	2.000-4.000	–	–		High pressure	
3	0.085	0.945	1.250	–	1.250	0.284	1.098	0.892
4	0.085	0.945	1.000	–	3.000	0.326	1.307	1.127
5	0.085	0.945	0.600	–	1.680	0.420	1.334	1.938
6	0.085	0.945	–	0.600	1.680	0.214	1.056	0.842
7	0.085	0.945	–	1.000-2.000	1.000	< 0.210	< 1.124	< 1.328

Where t_M is the retention time of the solute and t_0 is void time of the column, which was determined by acetone as the void marker.[25] All the procedures were carried out at the room temperature.

Results and Discussion

Comparisons with traditional particle column. The obvious advantage of monolithic column is their porous,
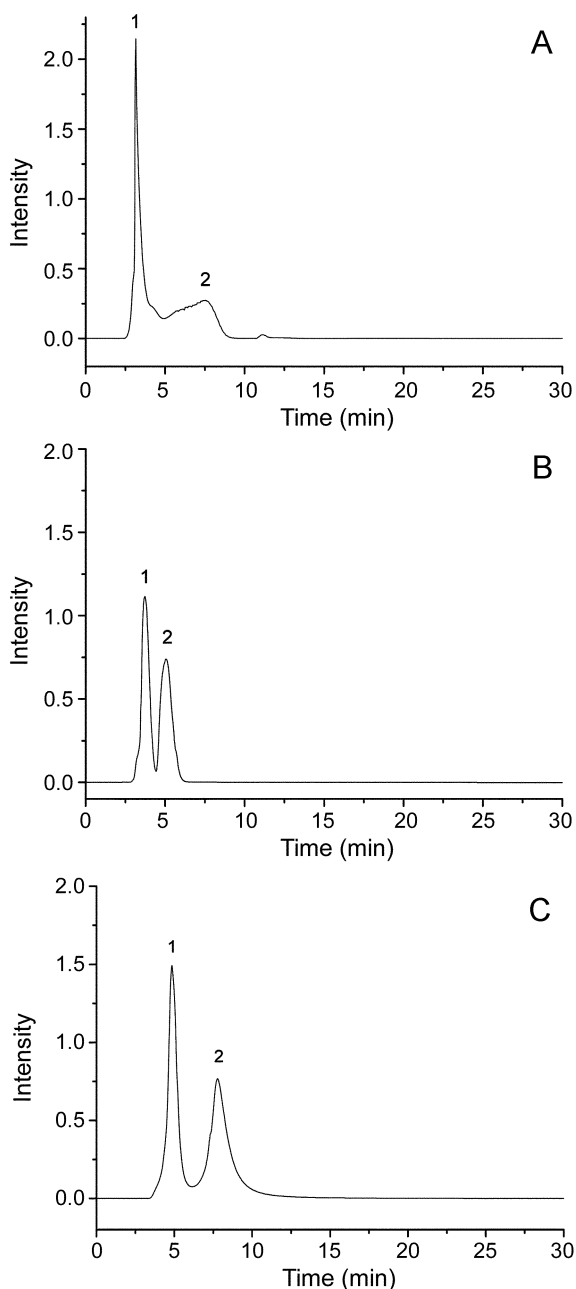

Figure 2. Chromatograms of caffeine and theophylline on different columns. (A: Chromatograms on Lichrospher® 100 RP-18 particle column, B: Chromatograms on Lichrospher® Silica column, C: Chromatograms on polymer monolithic column, mobile phase: A: 20% acetonitrile +80%water; B: methanol; C: acetonitrile, flow rate: 0.5 mL/min, detection wavelength: 270 nm, peak 1: caffeine; peak 2: theophylline)

highly interconnected network structure, which supports the formation of a network of channels and provides the large surface area needed to achieve sufficient capacity. Compared to the conventions packed column, in which high efficiency and high speed are mutually exclusive, the monolithic column allows a high flow rate which enables faster separation and lower consumption of mobile phase. In this experiment, Lithospher® 100 RP-18 particle column and Lichrospher® Silica column were used as conventional particle columns and compared with the monolithic column at different separation condition. Water, methanol, acetonitrile, 2-propanal and hexane were used as mobile phase respectively. At the optimized condition, theophylline and caffeine can't be completely separated on conventional Lithospher® 100 RP-18 particle column and better separation was obtained at the Lichrospher® Silica column (Figure 2). The retention time of the samples on Silica column increased with the polar decreasing of mobile phase, but the separation factor not increased obviously because the peaks broaden. Theophylline and caffeine can't be washed out within 60 min if using more than 60% of hexane in 2-propanol as mobile phase. Moreover, the separation efficiency decreased and column pressure increased obviously with the increasing of mobile phase flow rate on the traditional particle column. Theophylline and caffeine can be absolute separation on the monolithic column using acetonitrile as mobile phase. The influence of flow rate variation on the separation efficiency is shown in Table 2. The chromatographic efficiency decreased obviously with the increasing of flow rate on the conventions packed column because the pores within the particles are close to each other and the liquid inside them is stagnant. Therefore, the molecules to be separated are transported inside the close pores and back to the mobile phase mainly by diffusion. Compared to the conventions packed column, the monolithic column shown an extremely small dependency of separation efficiency on flow rate is due to the flow of the liquid within the macropore network channels is driven by

Table 2. Separation characteristic of theophylline and caffeine at different flow rates. (No. 1-6: polymer monolithic column, No. 7-8: Lichrospher® Silica column, k_1: the retention factor of caffeine, k_2: the retention factor of theophylline, a: separation factor of caffeine and theophylline, N_1: the number of plates for caffeine, N_2: the number of plates for theophylline, R_s: resolution of caffeine and theophylline)

No.	Flow rate	k_1	k_2	α	N_1	N_2	R_s
1	0.2 mL/min	0.443	1.386	3.126	490	207	2.039
2	0.3 mL/min	0.431	1.331	3.086	459	222	2.006
3	0.5 mL/min	0.420	1.334	3.175	476	195	1.938
4	1.0 mL/min	0.428	1.251	2.923	461	191	1.827
5	2.0 mL/min	0.420	1.219	2.902	443	182	1.791
6	3.0 mL/min	0.416	1.203	2.892	438	180	1.785
7	0.5 mL/min	0.292	0.598	2.048	340	196	1.126
8	1.0 mL/min	0.266	0.501	1.883	308	162	0.887

the pressure difference and the molecules to be separated are transported by convection. From Table 2 we could see the monolithic column allowing the separation efficiency to be maintained at significantly increased flow.

Effect of the preparation condition of the monolithic column. The proportion of mixture composition and polymerization temperature defines the monolithic structure and separation characteristic without further processing. Although the preparation process of the monolithic stationary phases is quite simple, a few factors have to be taken into account, of which the selection of the porogenic solvents is crucial for the preparation of the monolithic stationary phases. Firstly, the 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. In this study, several porogenic solvents, including cyclohexanol, dodecanol, and toluene, were tested for their compatibility. The monolithic stationary phases using cyclo-hexanol and dodecanol as porogenic solvents showed low selectivity for theophylline and caffeine. Meanwhile, mono-lithic stationary phases with high selectivity and low back-pressure 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. With increasing toluene pro-portion, the mean pore size decreased and the specific area and resolution factor increased. However, when the pro-portion of toluene in the porogenic mixture rose above 30%, 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 26% toluene and 74% dodecanol was used in this experiment as the optimal porogenic mixture.

The polymerization conditions of temperature and time affect the efficiency and selectivity of the resultant poly-meric stationary phases. The commonly used polymeri-zation temperature is 60 °C. Although complete polymeri-zation was achieved in 4 h at 60 °C in the present study, the initiation of the polymerization reaction was very fast and therefore hard to control at this temperature. This reduced the reproducibility of the monolithic stationary phases. Furthermore, the relatively high temperature had 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 45 °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 study, the effect of mobile phase composition on the separation was investigated using methanol, water and acetonitrile, with the
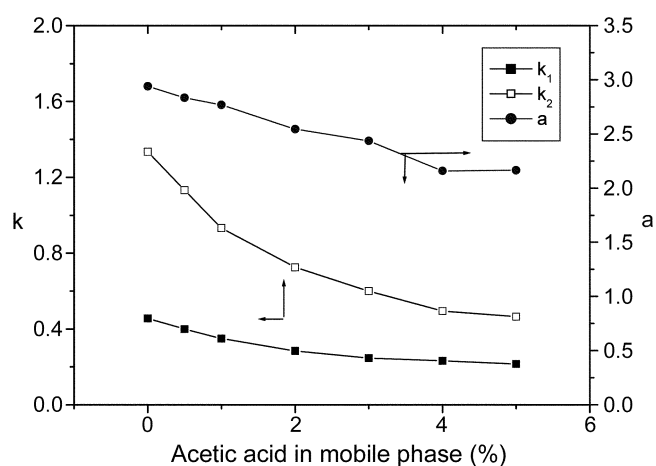

Figure 3. Effect of mobile phase composition on retention factor and separation factor. (mobile phase: acetonitrile, flow rate: 0.5 mL/min, detection wavelength: 270 nm, k_1: the retention factor of caffeine, k_2: the retention factor of theophylline, a: the separation factor of caffeine and theophylline)

latter showing the best separation. The effects of polar additives in the mobile phase were also evaluated with mixtures of acetonitrile-acetic acid as the mobile phase (Figures 3 and 4). The experiment showed that with the increasing of the solvent polarity in the mobile phase, the retention factors of caffeine and theophylline all decreased. The best retention factor was attained with 100% acetonitrile as the mobile phase. These results can be explained by the presence of ionic pair and hydrogen-bonding interactions between the stationary phase and the sample. In our experiment, methacrylic acid was used as the monomer and its carboxyl group is the most common hydrogen-bonding and acidic functional group. From the molecular structure of theophylline and caffeine, the only evident differences between the two templates lay in N*. For the caffeine molecule, the hydrogen on N* was placed by methyl, whereas active hydrogen still existed on N* in theophylline. So the amino group in theophylline could form a hydrogen bond by the active hydrogen on N* with the carboxyl in methacrylic acid. In addition, the near oxygen and nitrogen also could form a hydrogen bond with the carboxyl in the monomer. For the caffeine molecule, no active hydrogen was offered for forming the hydrogen bond on N*. More-over, the volume of the methyl group was much large than that of hydrogen; it could, therefore, block the hydrogen bond forming by steric hindrance. Hence, theophylline exhibited excessively long retention times and peak tailing on the monolithic column. As the polar additives can inter-fere with the hydrogen-bonding interactions between the carboxylic acid of methacrylic acid in monolithic columns and the functional group of the analytes, the retention factors of caffeine and theophylline decreased with increasing content of the polarity solvent in the mobile phase.

Effect of the flow rate on the separation efficiency. Monolithic columns have been studied as materials, with the inherent advantages of their network-type, one-piece struc-
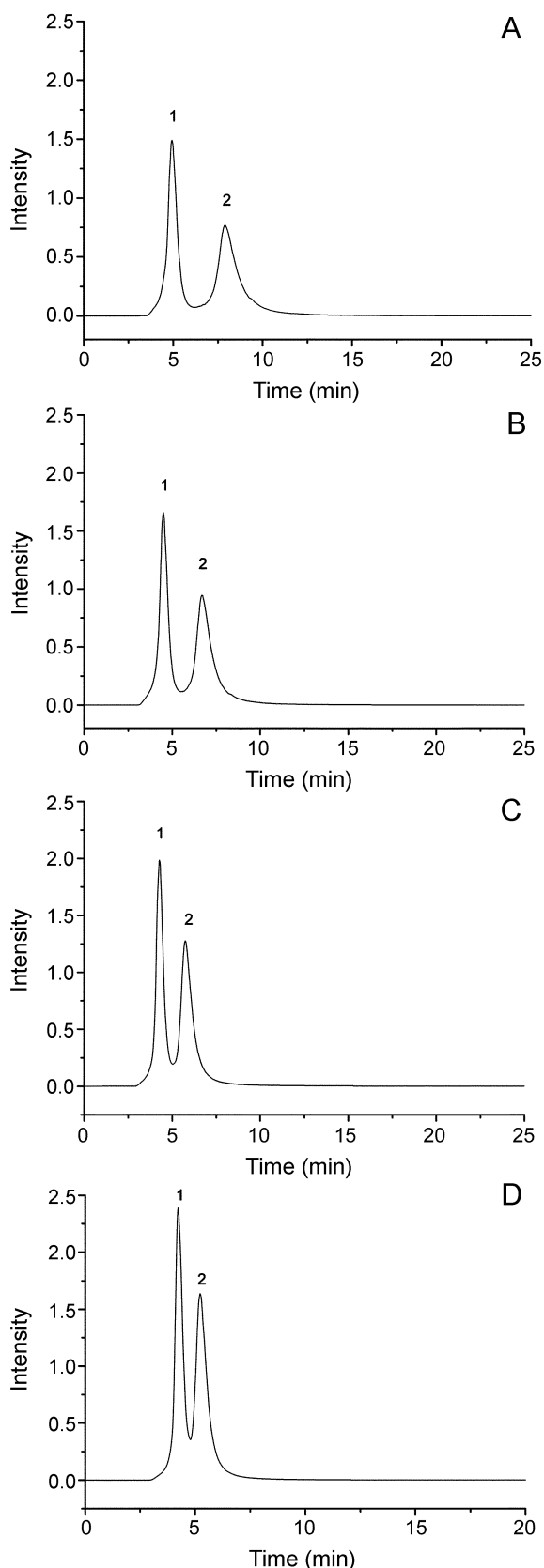
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Figure 5. Effect of different temperatures on the separation factor and retention factor. (mobile phase: acetonitrile, flow rate: 0.5 mL/min, detection wavelength: 270 nm, k_1: the retention factor of caffeine, k_2: the retention factor of theophylline, a: separation factor of caffeine and theophylline.)

Figure 4. Chromatograms of caffeine and theophylline at different mobile phase composition. (mobile phase: 1: acetonitrile; 2: 99.5% acetonitrile+0.5% acetic acid; 3: 98% acetonitrile+2% acetic acid; 4: 96% acetonitrile+4% acetic acid, flow rate: 0.5 mL/min, detection wavelength: 270 nm, peak 1: caffeine; peak 2: theophylline)
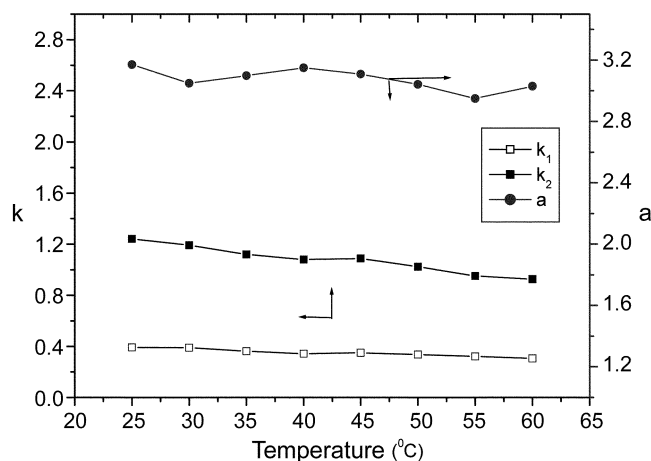
tures. The microscopic structure of monolithic columns is porous and composed of two interconnected networks of pores. Through-pores provide flow paths through the column, and the size and density of the macropore network gives the monolithic columns a high external porosity and, consequently, a large permeability and low column hydraulic resistance. 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 can also be used in long, connected series, thereby giving very high efficiencies. In this study, the flow rate of the mobile phase was investigated over the range of 0.2-3.0 mL/min (Table 2). Although the migration times of the caffeine and theophylline decreased with increasing flow rate, only a slight decrease of the separation efficiency was found with increasing flow rate. The results showed an extremely small dependency of separation efficiency on flow rate, thereby allowing the separation efficiency to be maintained at significantly increased flow rates. This is the typical characteristic of monolithic columns.

Effect of temperature on the separation efficiency. The effects of temperature, over the range from 25 °C to 60 °C, on the separation were also investigated (Figure 5). The results showed that at higher temperature, both theophylline and caffeine migrated fast, and that theophylline changed faster than caffeine. Both k_1 and k_2 decreased with increasing temperature because the adsorption of the analytes to the substrate weakened with increasing temperature, allowing the analytes to migrate faster through the monolithic column. Furthermore, the separation factors decreased with increasing elution temperature, because the higher temperature decreased the interaction between the theophylline and the polymers more than that between the caffeine molecule and the polymers. Therefore, a lower temperature will lead to a higher separation. However, with increasing column temperature, the separation and retention factors were only slightly

changed. The results demonstrated that the dependency of separation efficiency on temperature is small, and that high separation efficiency can be maintained under higher temperature.

Conclusion

The results showed that the mixture composition proportion and the polymerization condition combine to define the monolithic structure without further processing. Therefore, the proper selection of porogenic solvents and polymerization temperature is crucial for the preparation of the monolithic stationary phases. The method of *in situ* polymerization is simple and rapid, with a low consumption of chemicals. Moreover, the dependency of separation efficiency on flow rate and temperatures is extremely small and hydrogen-bonding interaction plays an important role in the retention and separation. The study results presented here have substantiated the significant research interest in monolithic columns compared with conventional particle columns due to their ease of preparation, high separation efficiency, and rapid mass transport.

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