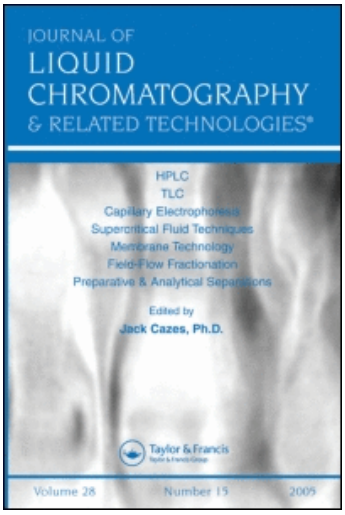

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Special Selectivity of Molecularly Imprinted Monolithic Stationary Phase

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Abstract: A monolithic, molecularly imprinted column was prepared by an *in situ* therm-initiated copolymerization process; the essential conditions of forming a non-covalent molecular imprinting stationary phase are discussed and illustrated with caffeine and theophylline as examples from the angle of molecular construction, and examined by experiments. The results showed that the theophylline-imprinted monolithic column has high selectivity to theophylline; baseline separation of caffeine and theophylline was achieved. However, only weak specific selectivity to the template molecule was observed for the caffeine imprinted monolithic column.

Keywords: Monolithic molecularly imprinted column, Special selectivity, Theophylline, Caffeine

INTRODUCTION

Molecular imprinting is a rapidly developing technique for the preparation of polymers having a high affinity for a target molecule. A molecular imprinted polymer (MIP) can selectively recognize the template molecule even in the presence of compounds with structure and functionality similar to that of the template.[1–5] The special binding sites are formed by the self assembly of the template with a functional group and the monomer, followed by a cross-linked copolymerization. After the polymerization, the template is removed from the polymer, leaving recognition sites that, in term of size, shape, and functionality, are complementary to the template. So, the resulting MIP can

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selectively rebind with the template in preference to other closely related structures. Molecular imprinted polymer has been applied successfully to chiral separation,[6,7] solid extraction,[8] biomimic sensor,[9,10] and membrane separation.[11,12]

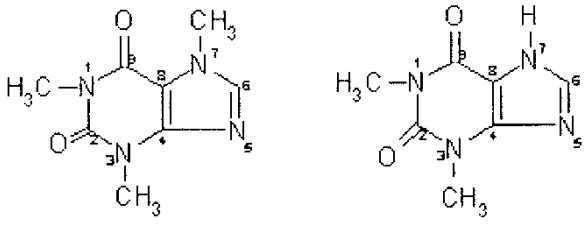
The special binding sites are formed by covalent or, more commonly, non-covalent interaction between the imprinted template with the functional group and the monomer, followed by a crosslinked copolymerization. The molecular recognition effect is a consequence of the presence in the polymerization mixture of template molecules capable of establishing non-covalent interactions with monomers and cross-linkers. The growth of a three-dimensional polymeric structure around the template produces binding sites with proper shape and charge distribution has greatly effected molecular recognition. It has been demonstrated that a significant contribution in the interaction between template molecules and functional groups on the surface of the binding sites is due to the hydrogen bond[13–15] and ion-exchange phenomena,[16] and the environment around the binding site,[17] For example, most MIPs are prepared by non-covalent imprinting and the common systems are based on commodity methacrylic monomers, such as methacrylic acid because its carboxyl group is the most common hydrogen bonding and acidic functional group in molecular imprinting, cross-linked with ethyleneglycol dimethacrylate.[18,19]

In this work, the monolithic MIP column was prepared by a simple, one step, *in-situ*, free-radical polymerization "molding" process directly within the chromatographic column, without the tedious procedures of grinding, sieving, and column packing. The essential conditions of forming non-covalent molecular imprinting stationary phase were discussed and illustrated with caffeine and theophylline as examples, from the angle of molecular construction and examined by the experiments.

EXPERIMENTAL

Materials

Caffeine and theophylline were obtained from Sigma (ST Louis, MO, USA). (The molecule structures were shown in Fig. 1). Methacrylic acid (MAA) was purchased from Kanto Chemical Co., Inc. (Japan). Ethylene glycol dimethacrylate (EDMA) was ordered from Tokyo Kasei Kogyo Co., LTD. (Tokyo, Japan). α,α'-Azobis(isobutyronitrile) (AIBN) was the product of Junsei Chemical Co., Ltd. (Japan). Toluene was from Oriental Chemical Industries (Japan). Dodecyl alcohol, acetonitrile, chloroform, methanol are all of HPLC grade and were purchased 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.



(1) (2)

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

Preparation of Monolithic MIP Column

The stationary phase was directly prepared by in-situ polymerization within the confines of a stainless-steel chromatographic column tube of 150 × 3.9 mm I.D. Schematic illustrations of the imprint formation and molecular recognition was shown in Figure 2. The polymerization mixture composed of 0.20 mmol template molecule, 0.060 g free radical initiator (AIBN), 0.085 mL monomer and 0.945 mL cross-linker (EDMA) was dissolved in the porogenic solvents (1.00 mL toluene and 3.00 mL

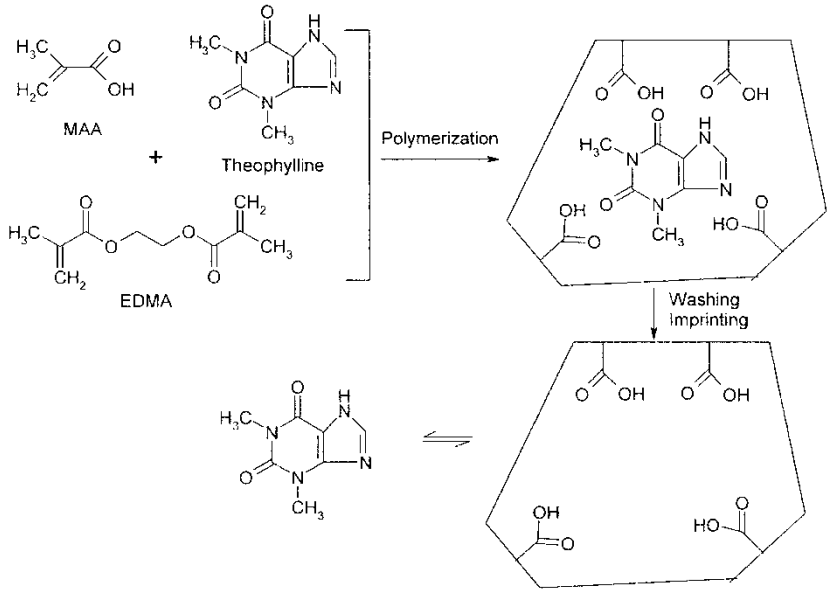


Figure 2. Schematic illustrations of the imprint formation and molecular recognition.

dodecanol). The mixture was put into a supersonic bath for 15 min and 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 55°C for 24 h. After the polymerization, the seals were removed; the column was connected to an 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 blank monolithic column was prepared and treated in an identical manner.

HPLC Analysis

Separation characteristics of the monolithic MIP 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. Acetonitrile was used as mobile phase and 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 the theophylline and k_1 is the retention factor of the caffeine. The retention factor was determined by

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

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. All the procedures were carried out at room temperature.

RESULTS AND DISCUSSION

Effect of Molecular Structure of Template on the Selectivity

According to the elementary theory of MIP, the template and monomer could form a stable complex, which is the elementary condition for MIP. In our experiment, methacrylic acid was used as the monomer because its carboxyl group is the most common hydrogen bonding and acidic functional group in molecular imprinting cross-linked with ethyleneglycol dimethacrylate. From the molecular structure of theophylline and caffeine we could see that the only differences between the two templates lie in N^7. For the caffeine

molecule, the hydrogen on N^7 was placed by methyl, whereas active hydrogen still existed on N^7 in theophylline. So, the amino group in theophylline could form a hydrogen bond by the active hydrogen on N^7 with the monomer, and produce specific sites in the polymer. Caffeine, on the other hand, since no amino group exists in the molecular structure and the amide group could only form a very weak hydrogen bond with the monomer, it is hard to produce effective specific sites.

On the other hand, because the molecular volume of hydrogen is very small, oxygen and nitrogen could form a hydrogen bond with the carboxyl in the monomer. So, even if the oxygen, which is between two methyls in theophylline, it is not capable of forming a hydrogen bond because of its steric hindrance; three special binding sites could form a stable template-monomer complex with methacrylic acid. For the caffeine molecule, no active hydrogen is offered for forming hydrogen bonds. Moreover, since the volume of the methyl group is larger than hydrogen; it could block the hydrogen bond forming by steric hindrance. From the molecular structure, theophylline shows higher rigidity than caffeine. As is known, a template with a high rigidity tends to fasten the binding site, and the binding site formed by a template of lower rigidity shows higher flexibility and is harmful to the special selectivity. So theophylline imprinted polymers have shown better molecular recognization ability than caffeine-imprinted polymer.

Effect of the Preparation Procedure on the Selectivity

The proportion of mixture composition and polymerization temperature defines the monolithic structure and separation characteristic without further processing. In this work, cyclohexanol, dodecanol, and toluene were tested for their compatibility as porogenic solvents. The investigations revealed that the high selectivity molecular imprinted monolithic stationary phases could be obtained using the low polar porogenic solvents of toluene and dodecanol as a porogenic mixture. The porogenic solvents should be of relatively low polarity, in order to reduce the interferences during complex formation between the imprinted molecule and the monomer. It is very important to obtain high selectivity MIP. The ratio of toluene and dodecanol also affects the separation performance through the change in pore structure of the monolithic stationary phases. With increasing the proportion of the good toluene solvent, the mean pore size decreased and the specific area and resolution factor increased. However, when there is too much toluene in the porogenic mixture, the resulting stationary phase had too small a pore diameter to allow the mobile phase to flow through. Thus, a balance had to be found between the requirements of low flow resistance and large surface area of the polymer. The amount of cross linker should be high enough to maintain the stability of the recognition sites. When the

percentage of cross-linker was lower than 60%, the MIP showed a weak recognition ability or not at all. 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, thus, the functional groups are held in an optimal configuration for rebinding the template, allowing the receptor to 'recognize' the original substrate.

Chromatographic Separation of Caffeine and Theophylline

Molecularly imprinted monolithic stationary phases have been successfully prepared and applied to the chromatographic separation of caffeine and theophylline. Different mobile phases, such as acetonitrile, methanol-water, acetonitrile-acetic acid was tested for effects of selectivity. The experiment showed that the polarity of the mobile phases have significant effects on the selectivity. With increasing the polarity of the mobile phase, the selectivity of caffeine and theophylline decreased. This implies that the hydrogen-bonding interaction and hydrophobic interaction can play an important role in the retention and separation. The best retention factor was attained when only acetonitrile was used as the mobile phase. The obtained chromatograms are shown in Fig. 3. We could see theophylline exhibited excessively long retention times and peak tailing on the theophylline-MIP column. The retention factors (k), and separation factors (α), of these compounds are listed in Table 1. It can be seen that even though rather small differences existed in their structure, different imprinted polymers showed different selectivity for their templates. When caffeine was used as the template, the template's retention factor increased when compared with the blank polymer, but was lower than those of theophylline imprinted compounds. The theophylline imprinted polymer, on the other hand, showed strong retention for theophylline. This difference can be discussed in term of the different basic characteristics of the template. It is known that when N-heterocycles were used as templates, it was thought that they could combine with the carboxylic acid in MAA with the nitrogen ring through the H-bond. The strength of the monomer template interaction is therefore expected to be influenced by the basicity of the template; the more basic template will interact more strongly with MAA producing a large population of sites, and results in stronger selectivity for the template. Among them, the basicity of theophylline is stronger (pKb 8.8) than caffeine (pKb 10.4). Because of its weak basicity, caffeine can only form weak complexes with MAA through the H-bond. Hence, only a small amount of selective sites was produced and most surface of the polymer remains nonselective. The results of caffeine and theophylline imprinted polymers are reasonable according to the above discussions.

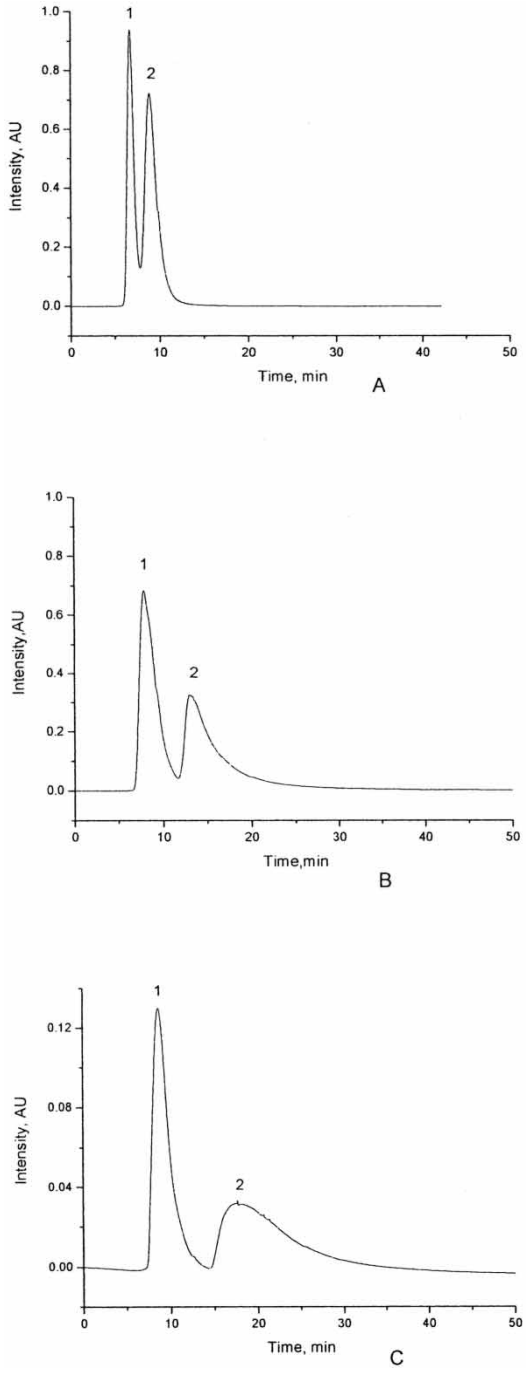


Figure 3. Chromatograms of caffeine and theophylline on different columns.

Table 1. Retention factors and separation factors of caffeine and theophylline at different columns

Column	k_1 (Caffeine)	k_2 (Theophylline)	α
Theophylline MIP	0.446	2.142	4.802
Caffeine MIP	0.407	1.335	3.281
Blank column	0.238	0.587	2.471

CONCLUSIONS

From the experimental results, different template MIPs had different molecular recognitions to the templates and the structural analogues, according to the molecular structures of these compounds. Theophylline imprinted polymers showed better selectivity on the close analogues than caffeine imprinted polymers. The molecular recognition mechanism acting on the columns was mainly dependent on the hydrogen bonding interactions.

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