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Molecularly Imprinted Monolithic Stationary Phases for Liquid Chromatographic Separation of Tryptophan and *N*-CBZ-Phenylalanine Enantiomers

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Abstract Monolithic molecularly imprinted columns were designed and prepared by an *in-situ* thermal-initiated copolymerization technique for rapid separation of tryptophan and *N*-CBZ-phenylalanine enantiomers. The influence of polymerization conditions and separation conditions on the specific molecular recognition ability for enantiomers and diastereomers was investigated. The specious molecular recognition was found to be dependent on the stereo structures and the arrangement of functional groups of the imprinted molecule and the cavities in the molecularly imprinted polymer (MIP). Moreover, hydrogen bonding interactions and hydrophobic interactions played an important role in the retention and separation. Compared to conventional MIP preparation procedures, the present method is very simple, and its macroporous structure has excellent separation properties.

Keywords: molecular imprinted polymer, monolithic column, special molecular recognition, *N*-CBZ-phenylalanine, tryptophan

INTRODUCTION

Molecular imprinted polymers (MIPs) that exhibit high selectivity and affinity to a predetermined molecule (template) are a rapidly growing research focus [1-5]. The special binding sites in MIPs are formed by the self-assembly of a template molecule with specific functional groups and the monomer, followed by a cross-linking copolymerization. The resulting MIP are macroporous matrices that possess microcavities with three-dimensional structures complementary in both shape and chemical functionality to that of the template. The high degree of cross-linking enables the microcavities to maintain their shape after removal of the template, and thus, the functional groups are held in an optimal configuration for rebinding the template, allowing the polymer receptor to 'recognize' the original substrate. MIP has been successfully used to achieve chiral separations [6,7], solid extractions [8], biomimetic sensors [9,10], and membrane separations [11,12].

The conventional approach is to synthesize the MIP in bulk, grind the resulting polymer, and sieve the resulting particles into the desired size ranges. Such ground and sieved particles can then be packed into HPLC columns. Although the process of bulk polymerization is simple, the crushing, grinding and sieving to obtain particles of the appropriate size is tedious and time-consuming and often produces particles that are irregular in size and shape. Furthermore, some interaction sites are destroyed during grinding, which reduces the chromatographic performance and the MIP loading capacity. In order to overcome these problems, uniformly sized and monodispersed particles had been made by other methods, such as suspension polymerization, seed polymerization and the multi-step swelling process [13-15]. Unfortunately, the above techniques also have limits in that they either require use of special dispersing phases/surfactants or are too complicated.

A novel method for the preparation of chromatographic stationary phases, monolithic MIP technology, combines the advantages of molecular imprinting and monolithic column technology [16]. Monolithic MIPs are prepared by a simple, one-step, *in-situ*, free-radical polymerization 'molding' process directly within a column, thus avoiding the tedious procedures of grinding, sieving, and column packing. Compare with other HPLC separation [17-19], monolithic MIP separation has attracted significant interest because of its ease of preparation, high selectivity and sensitivity, and rapid mass transfer. Furthermore, the preparation of this type of MIP is more cost-effective, because it requires much smaller amounts of the template molecules. As a result, the use of monolithic MIP has been rapidly expanding in stationary phase preparation in recent years [20-24].

In this work, monolithic MIP with specific recognition ability for *N*-Carbobenzyloxy(CBZ)-phenylalanine was

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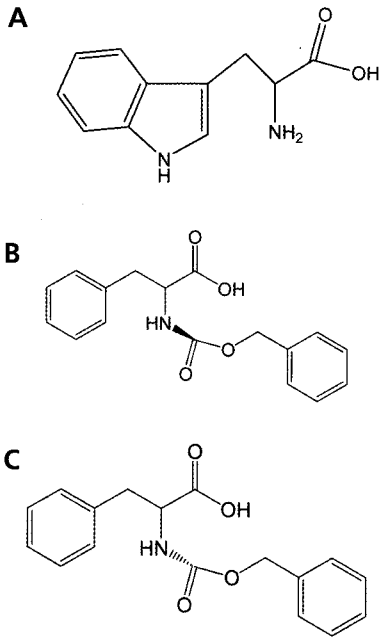

Fig. 1. Molecular structure of (A) tryptophan, (B) *N*-CBZ-L-phenylalanine, and (C) *N*-CBZ-D-phenylalanine.

prepared by *in-situ* thermal-initiated polymerization, using acrylamide as the functional monomer and EDMA as the cross-linker. The chromatographic performance of the monolithic MIP was characterized and demonstrated with a homologous series of amino derivatives, tryptophan and *N*-CBZ-phenylalanine. The effects of the polymerization conditions and separation conditions on specific molecular recognition, as well as possible recognition mechanisms were also discussed.

MATERIALS AND METHODS

Chemicals

Tryptophan and *N*-CBZ-phenylalanine were obtained from Sigma (ST Louis, MO, USA). The molecular structures are shown in Fig. 1. Ethylene glycol dimethacrylate (EDMA) from Tokyo Kasei Kogyo Co., LTD (Tokyo, Japan) was extracted with 2.0 M NaOH solution and water and dried over anhydrous magnesium sulfate. α, α′-Azobis (isobutyronitrile) (AIBN) was the product of Junsei Chemical Co., Ltd. (Japan) and was recrystallized prior to use. Toluene (Extra Pure) was purchased from Oriental Chemical Industries (Japan). Ac-rylamide (AM), acetic acid (AA), dodecanol, acetonitrile (ACN), chloroform, and methanol were all Extra Pure or HPLC grade and purchased from Duksan Pure Chemical Co., LTD (Ansan, Korea). Double distilled water was filtered with a 0.45-μm filter membrane before use.

Preparation of Monolithic MIP Column

The monolithic MIP was directly prepared by in-situ polymerization within the confines of a stainless-steel chromatographic column tube (100 × 3.2 mm I.D.). The schematic preparation process of the monolithic MIP column is shown in Fig. 2. 0.0086 g template molecule (*N*-CBZ-L-phenylalanine), 0.009 g AIBN, 0.0610 g acrylamide, and 0.795 mL EDMA were dissolved in the appropriate porogenic solvents (toluene and dodecanol). The solution was ultasonicated for 10 min, sparged with helium for 5 min to remove oxygen. The polymerization mixture was then packed into the HPLC column. Subsequently, the polymerization reaction took place in the column in a water bath at 48°C for 16 h. After the polymerization, 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 other soluble compounds. A non-imprinted blank monolithic column was prepared in the absence of template and treated in an identical manner.

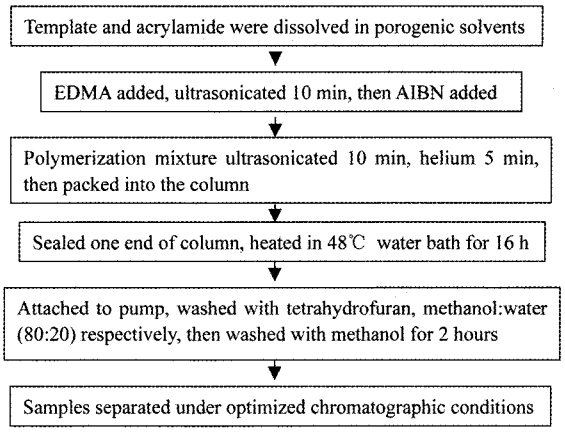

Fig. 2. Schematic preparation process of the monolithic MIP column.

HPLC Analysis

The separation characteristics of the monolithic MIP column were analyzed by a liquid chromatography system containing a Waters 600s Multisolvent Delivery System, a Waters 616 pump (Waters, Milford, MA, USA), a Waters 2487 Dual Absorbance UV detector (Waters) and Rheodyne injection valve (20 μL sample loop). The Millennium 32 software (Waters) was used for data acquisition. The UV detection wavelength was set at 270 nm.

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

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

where k_2 is the retention factor of *N*-CBZ-phenylalanine, and k_1 is the retention factor of tryptophan. The retention factors were determined by:

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

where t_M is the retention time of the analyte, and t_0 is the

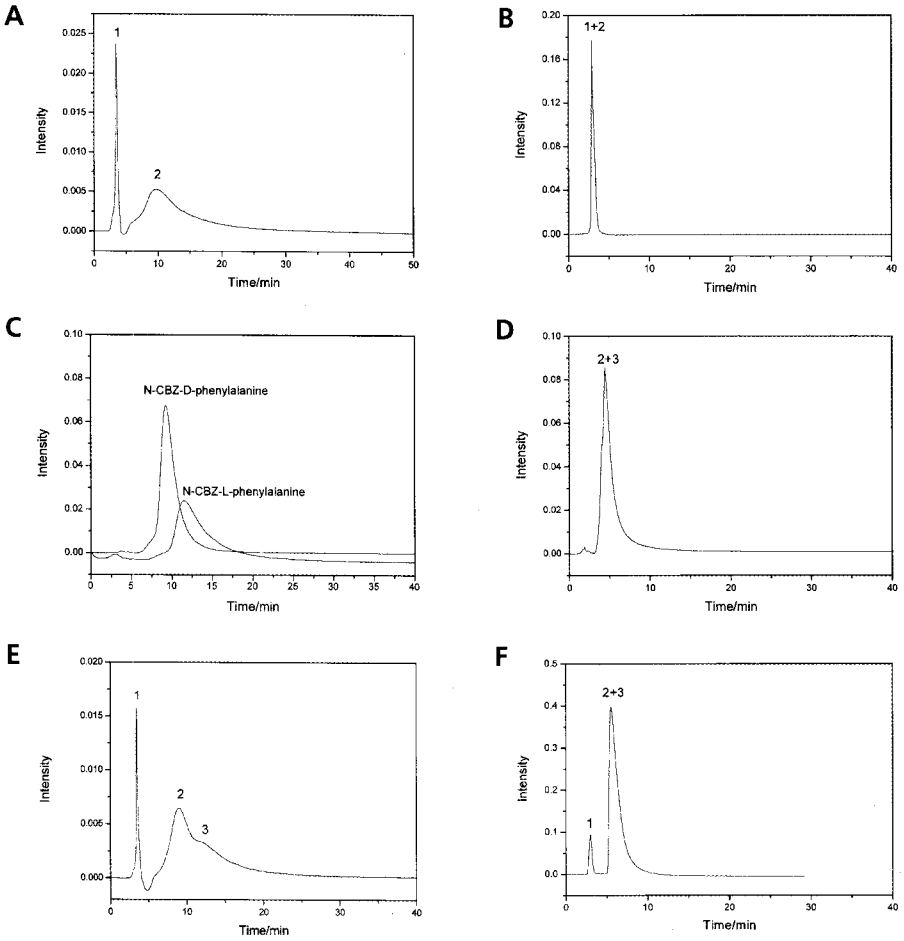

Fig. 3. Chromatograms of tryptophan and *N*-CBZ-phenylalanine on different columns. (A, C, E: monolithic MIP column; B: C_{18} particle column; D: blank MIP column; F: silica particle column. Mobile phase: 1% AA + 99% ACN; flow rate: 0.5 mL/min. Peak 1: tryptophan; Peak 2: *N*-CBZ-D-phenylalanine; Peak 3: *N*-CBZ-L-phenylalanine).

void time of the column. All procedures were carried out at room temperature, unless otherwise noted.

Characterization of Monolithic Molecular Imprinting Stationary Phases

After all 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 5 mL/min. The cylindrical monolith was then dried under vacuum at 40°C for 24 h and cut into pieces with a razor blade. Microscopic analysis of the monolith was carried out in an S-4200 Scanning Electron Microscopy (Hitachi, Japan) at 3.0 kV.

RESULTS AND DISCUSSION

Separation on the Monolithic MIP Column

To evaluate the recognition properties of the monolithic MIP column, two homologous series of amino acids derivatives, *N*-CBZ-phenylalanine and tryptophan, were applied to the monolithic MIP column, the blank MIP column, a C_{18} column, and a silica particle column. As shown in Fig. 3, higher apparent separation factors were obtained on the monolithic MIP column than on the blank MIP, traditional C_{18} or silica particle column. Baseline separation of tryptophan and *N*-CBZ-D-phenylalanine on the monolithic MIP column is due to the macroporous structures and the strength and stability of the non-covalent interactions, *i.e.* hydrogen bonds, between the template and monomers.

The enantioselectivity of the monolithic MIP was evaluated by comparing the molecular recognition of *N*-CBZ-phenylalanine enantiomers on the monolithic MIP column. As shown in the chromatogram (Fig. 3C), *N*-CBZ-L-phenylalanine and its D-enantiomer were partially separated on the monolithic MIP column. When 99% acetonitrile and 1% acetic acid were used as the eluent, the retention factor of *N*-CBZ-L-phenylalanine on the imprinted monolith was 5.83, but only 4.42 for its D-enantiomer. As far as these two analytes are concerned, since their chemical composition and functional groups

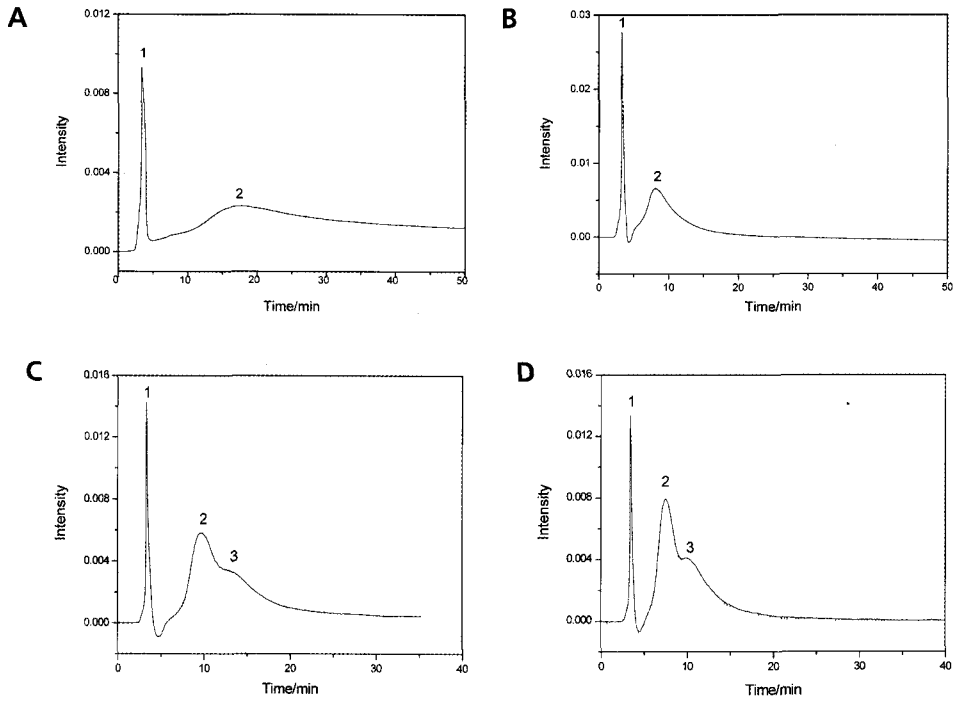

Fig. 4. Chromatograms of tryptophan and *N*-CBZ-phenylalanine on monolithic MIP column with different separation conditions. (A: 100% ACN; B: 2% AA + 98% ACN; C: 0.5% AA + 99.5% ACN (20°C); D: 0.5% AA + 99.5% ACN (50°C). Flow rate: 0.5 mL/min. Peak 1: tryptophan; Peak 2: *N*-CBZ-D-phenylalanine; Peak 3: *N*-CBZ-L-phenylalanine).

are the same, they can only be distinguished by their steric structures; *N*-CBZ-L-phenylalanine contains L-phenylalanine unit, whereas its D-enantiomer contains D-phenylalanine. Enantioseparation on the blank MIP column was also evaluated, and no enantiorecognition ability was observed (Fig. 3D). Based on the results of the monolithic MIP, it appears that L-phenylalanine in the template played a crucial role in both the formation of the template-monomer complex and enantiorecognition. Although the monolithic MIP column appears to have some enantioseparation ability, two conjoining peaks were obtained for the enantiomers, and further improvements are needed to enhance the enantioseparation.

Effect of Separation Conditions on Molecular Recognition

The influence of mobile phase composition on the molecular recognition properties was investigated using methanol, acetonitrile, chloroform, water, and phosphate as the mobile phase, however only acetonitrile produced satisfactory separation. The effects of polar additives in the mobile phase were also evaluated (Fig. 4). Changes in the ratio of acetic acid to acetonitrile from 0:100 to 5:95 (v/v) resulted in decreases in the retention factor as the proportion of acetic acid in the mobile phase increased (Fig. 5). Furthermore, the addition of acetic acid in the mobile phase also resulted in low enantioselectivities and low resolutions. The apparent enantioselectivity factor (α) also decreased as the proportion of water or acetic acid in mobile phase increased, suggesting that polar additives can interfere with the hydrogen-bonding interactions between the MIP matrix and the functional groups of the analytes.

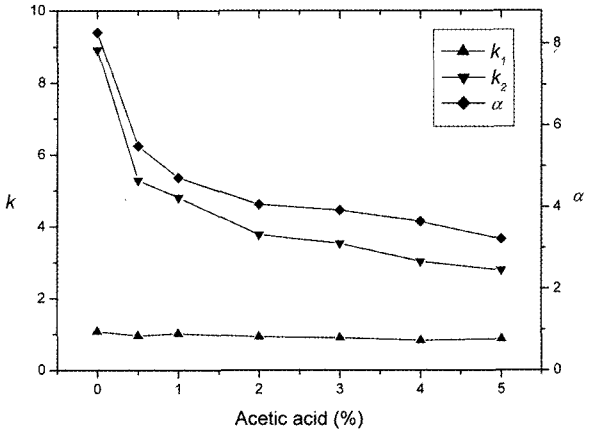

Fig. 5. Effect of mobile phase composition on retention and separation. (Mobile phase: ACN + AA; flow rate: 0.5 mL/min; k_1 and k_2: retention factors for tryptophan and *N*-CBZ-D-phenylalanine, respectively; α: separation factor of tryptophan and *N*-CBZ-D-phenylalanine).

To investigate the effect of temperature on the diastereoseparation and enantioseparation, the temperature was adjusted from 20 to 50°C, and the molecular recognition of the column was analyzed. Three replicate injections were made for each analyte, and the column was equilibrated with the mobile phase for about 30 min fol-

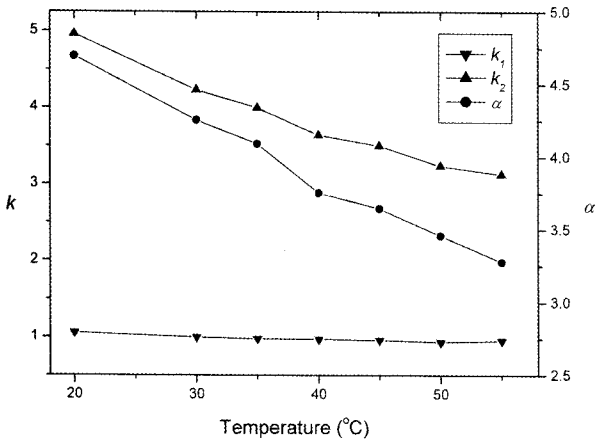

Fig. 6. Effect of temperature on retention and separation. (Mobile phase: 99.5% ACN + 0.5% AA; flow rate: 0.5 mL/min; k_1 and k_2: retention factors for tryptophan and *N*-CBZ-D-phenylalanine, respectively; α: separation factor of tryptophan and *N*-CBZ-D-phenylalanine)

lowing each temperature change. The results showed that the retention factor of tryptophan and *N*-CBZ-phenylalanine all decreased with increasing temperature (Fig. 6). This indicates that the analytes adsorb more weakly to the MIP as the temperature increases, and therefore migrate more rapidly through the MIP stationary phase. Furthermore, the apparent diastereoselectivity factors decreased as the elution temperature increased, because the higher temperature decreased the interaction between the imprinted molecule and the polymers more than the interaction between non-imprinted molecule and the polymers. Therefore, a lower temperature leads to a higher diastereoselectivity. However, the enantioselectivity of *N*-CBZ-L-phenylalanine and *N*-CBZ-D-phenylalanine did not decrease with the increase in temperature. In fact, when the temperature was increased from 20 to 50°C, the separation factor of *N*-CBZ-L-phenylalanine and *N*-CBZ-D-phenylalanine increased slightly from 1.32 to 1.43. Therefore, although the retention factor for *N*-CBZ-phenylalanine enantiomers decreased, the special recognition sites in the monolithic MIP have greater ability to discriminate between the different steric structures of D- or L-phenylalanine as part of the template molecule at the higher temperature.

Effect of Monolithic MIP Preparation Conditions on Molecular Recognition

Although the preparation process for the monolithic MIP is quite simple, a number of factors must be taken into account. Among these factors, the selection of the proper porogenic solvent is crucial for the preparation of monolithic MIP stationary phases. An appropriate amount of porogenic solvent is used to obtain suitable permeability for the monolithic column. Insufficient addition of the porogenic solvent results in uniform monolithic structure, and the sample solution would have to pass through the column under large backpressure. Several

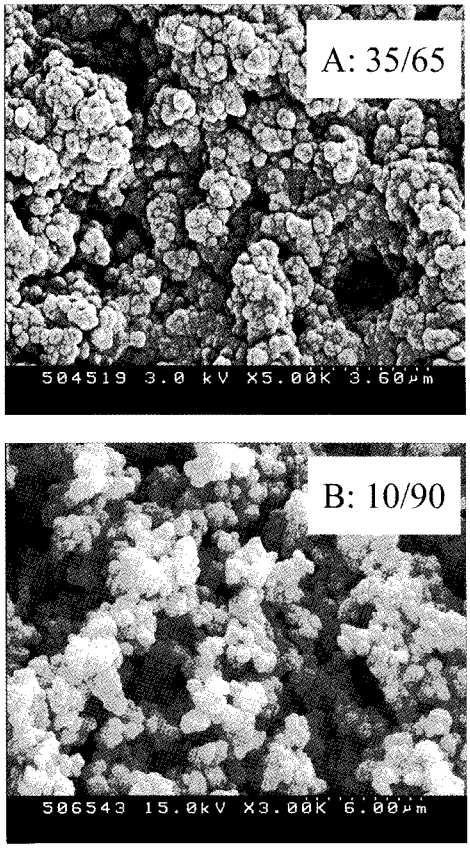

Fig. 7. Scanning electron microscope (SEM) of the monolithic MIP column. (porogenic solvents: toluene and dodecanol (v/v, % as indicated)).

porogenic solvent mixtures, including cyclohexanol and dodecanol, toluene and dimethyl sulfoxide, toluene and dodecanol, and isooctane, were tested for their compatibility. Toluene and dodecanol, porogenic solvents with low polarity, were found to generate MIP with higher selectivity and good flow-though properties. The mixture of 0.160 mL toluene and 1.450 mL dodecanol served best as the porogenic solvent, and the structure of the optimized monolithic column is shown in Fig. 7. From the SEM image, numerous macropores and flow-through channels can be seen inlaid in the network skeleton of the imprinted monolith. These macropores and channels allow the mobile phase to flow through the monolith with low flow resistance, thus enabling fast mass transfer of the solutes.

Variations in the monomer/crosslinker ratio not only produce different porous structures, but also lead to imprinted polymers with different compositions. Higher crosslinker content results in more highly crosslinked MIP, which increases the rigidity to allow preservation of the cavity structure after the template is split off. In other words, a high degree of cross-linking enables the microcavities to maintain a three-dimensional structure that is complementary in both shape and chemical functionality to that of the template, even after removal of the template. Thus, the functional groups in the microcavities are held

in an optimal configuration for rebinding the template, allowing the polymer receptor to 'recognize' the original substrate. The cross-linker percentage was optimized at 93% for this study.

Polymerization temperature is regarded as the most convenient variable that can adjust the pore size distribution of the macroporous media, because it does not result in changes in the composition of the reaction mixture. Due to complexity of the phase separation, it was believed that temperature could affect the polymer morphology in different ways, *e.g.* by generating free radicals and forming cross-linked nuclei. Typically, 60°C is used as the polymerization temperature. However, the initiation of the polymerization reaction was very fast and, therefore, hard to control at this temperature, resulting in low reproducibility of the molecular imprinted monolithic stationary phase. Thus, the relatively low temperature of 48°C was utilized with a prolonged reaction time of 16 h in order to yield a more reproducible polymerization.

CONCLUSION

Monolithic MIP stationary phase was successfully prepared by *in-situ* thermal polymerization, and its specific molecular recognition abilities for enantiomers and diastereomers were discussed. Specious recognition was dependent on the stereo structures and the arrangement of functional groups in the MIP cavities. Moreover, hydrogen-bonding interactions played an important role in both retention and separation. In contrast to traditional particle columns, the presented monolithic MIP methods have substantiated the significant research interest in monolithic MIP columns due to the ease of preparation, good stability, ease of regeneration and high-efficiency separation capability.

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