



Molecularly imprinted polymer as stationary phase for HPLC separation of phenylalanine enantiomers

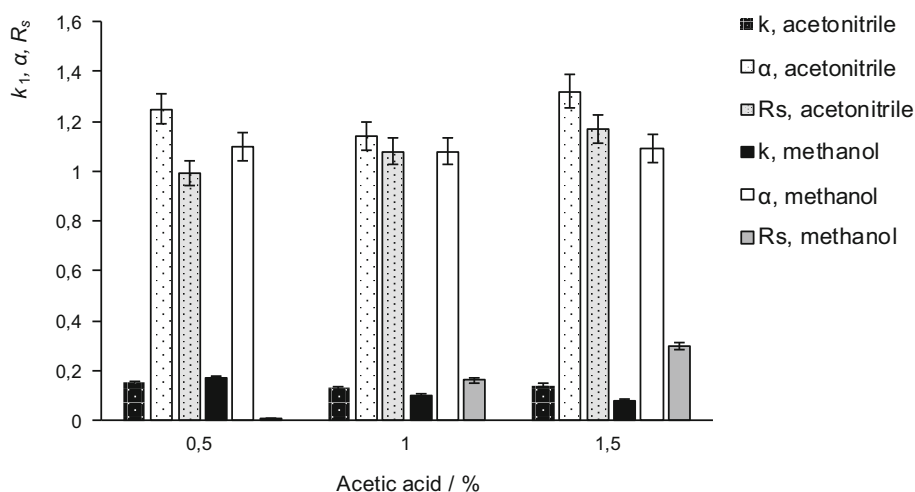
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Abstract

L-Phenylalanine molecularly imprinted polymers were synthesized by bulk polymerization. Methacrylic acid and acrylamide were tested as functional monomers. Ethanol and acetonitrile were used as porogenic solvents. Optimal composition of polymerization mixture was methacrylic acid, template, and ethylene glycol dimethacrylate in molar ratio 1:5:26. MIP was applied as HPLC chiral stationary phase. The influences of the mobile phase composition, flow rate, column temperature, and column length on the efficiency of enantioseparation were investigated. The enantioselective separation of phenylalanine was attained in reversed phase mode at 45 °C with acetonitrile/water containing 1.5% acetic acid (90/10, v/v) as mobile phase (resolution value was 1.49, selectivity factor was 1.38). Applicability of polymeric stationary phase prepared for L-phenylalanine was tested for analysis of dietary supplement sample. The UV detection limits for both enantiomers were 1 mg cm⁻³ (S/N = 3). Good linearity was observed from 1 to 10 mg cm⁻³.

Graphical abstract



Keywords Amino acid · High performance liquid chromatography · Chiral sorbent · Thermodynamics

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Introduction

Since most of biologically important compounds exist in two enantiomeric forms that can possess differences in their physiological activity, the interest for the separation

of optical isomers is still increasing [1]. The determination of the individual enantiomeric forms is essential for quality control and stability studies of drug preparations mainly in pharmaceutical chemistry. The frequently used analytical methods of separation of enantiomers are chromatographic methods [GC and HPLC with chiral stationary phase (CSP), chiral additive in mobile phase or with chiral derivatization] and electromigration methods (CE with chiral additives in electrolyte or with chiral derivatization, CEC with chiral stationary phase). At present, attention is oriented to a chiral polymeric sorbents prepared by molecular imprinting technology [2]. On the base of presence of specific recognition sites, molecularly imprinted polymers (MIP) have ability to recognize and bind target molecule, template. Beside selectivity toward template or structural analogues, they are characterized by high mechanical and chemical stabilities, good adsorption capacity, and reusability [3]. Components of polymerization mixture for MIP synthesis include template, functional monomer, crosslinking monomer, initiator, and porogenic solvent. For the preparation of MIP sorbent with high selectivity, sufficient capacity, and suitable morphology, the optimization of composition of polymerization mixture is needed. When MIP has been utilized as HPLC stationary phase for separation of enantiomers, the imprinting process has been done using pure enantiomeric form as template. The advantage of this approach to chiral separation, being that the order of elution is predictable, is that the imprinted enantiomer will bind to CSP strongly (higher value of elution time) than the non-imprinted enantiomer (lower value of elution time). The disadvantages of MIP CSPs include unsuitability for some types of templates, low sample capacity, low chromatographic peak efficiency arising from slow mass-transfer kinetics. The applications of molecularly imprinted polymer as separation media in HPLC, and also in SFC and CEC, for chiral separation have been extensively reviewed [4–6].

Phenylalanine is an essential amino acid that exists in two isomeric forms. Due to the different biological natures and pharmacological activities of both enantiomers, it is important to develop methods for the effective separation of them. In our previous study [7], HPLC method by utilization of macrocyclic antibiotics, β -cyclodextrin, and cyclofructan 6-based CSPs in reversed-phase, normal-phase, and polar-organic phase separation modes was used for separation of D,L-phenylalanine. The best enantioseparation was obtained on teicoplanin-based CSP in reversed-phase mode. The limited lifetime and cost of commercial CSPs provide the motivation to prepare MIP-based chiral sorbent for separation of phenylalanine enantiomers. The examples of phenylalanine enantiomers imprinting for applicability of resulting polymers such as HPLC CSP, sensors, and electrode for voltammetry [8–15] are

summarized in Table 1. Molecularly imprinted polymers were prepared by utilizing a block or suspension polymerization methods using phenylalanine enantiomer (L- or D-) as the template. Underivatized forms or derivatives (anilides, *tert*-butyloxycarbonyl-, dansyl-) are used [5].

In the present study, a resolution of amino acid racemate was done using MIP CSP prepared by a modified polymerization method with L-phenylalanine as template molecule. The analytical performance of MIP CSP was evaluated. The effects of various operating parameters, such as the selection of a suitable mobile phase composition, flow rate, column length, and column temperature, were investigated for the purpose of achieving efficient separation of phenylalanine enantiomers. The enthalpic and entropic contributions to chromatographic retention of the phenylalanine enantiomers were calculated.

Results and discussion

Synthesis of MIP

MIPs were synthesized by bulk polymerization with non-covalent approach. The properties of resulting sorbent mostly depend on the polymerization mixture composition (type and amount of template, functional monomer, porogenic solvent, crosslinking monomer), synthesis conditions, and polymerization method used. In MIP-L-phenylalanine synthesis, EGDMA was used as crosslinking monomer and initiation was performed thermally at 60 °C with using of AIBN. In the first step of preparation, we selected type of functional monomer and type of porogenic solvent. In the case of MAA as functional monomer, the monomer–template prepolymerization complex was formed through hydrogen bonding between carboxyl group of monomer and amino group of template, and also monomer hydrophilically interacts with the C=O bond in template [16]. Hydrogen bonding interaction takes place between the carbonyl and amino groups of AA and template functional groups. Since the interactions between functional monomers and target molecule are done mainly through hydrogen bonds, thus a less polar solvent with lower dielectric constant, acetonitrile, was chosen as an appropriate porogenic medium. This solvent optimized the formation of monomer–template prepolymerization complex, while in the more polar porogenic solvents the formation of monomer–porogen complexes is preferred. Besides, we optimize the mole ratio of functional monomer, template, and crosslinking monomer for synthesis. As documented in Table 2, in the case of MIP I–III, resulting polymers were soft and not suitable for application as HPLC stationary phases. The formation of blocks of polymers was not obtained with an increasing of

Table 1 Polymerization mixture composition, conditions for MIP-phenylalanine enantiomers preparation, and application of resulting polymer

Template	Functional monomer	Polymerization method, crosslinker, initiator, porogen, conditions	MIP application, sample	References
L-Phenylalanine	β -CD + MAA	Suspension, EGDMA, AIBN, acetonitrile, 23 °C	HPLC CSP (MP: acetonitrile containing 1.0% HAc/water), D,L-phenylalanine ($R_s = 1.46$, $\alpha = 1.41$)	[8]
D-Phenylalanine	MAA	Block, EGDMA–trifluoroacetic acid, AIBN, toluene, 24 h, 60 °C	HPLC CSP (MP: ethanol/30 mmol dm ⁻³ acetate buffer), D,L-phenylalanine ($R_s = 1.38$, $\alpha = 2.56$)	[9]
D-Phenylalanine	MAA	Block, EGDMA, 4,4'-azo-4-cyanovaleric acid, methanol/water (80:20), 48 h, 40 °C	HPLC CSP (MP: 1.5 mmol dm ⁻³ glycine), D,L-phenylalanine ($\alpha = 1.41$)	[10]
L-Phenylalanine, D-phenylalanine	MAA	Block, EGDMA, <i>N,N</i> -methylenebisacrylamide, 3-aminopropyltrimethoxysilane, toluene, ethanol, DMSO, 24 h, 15 °C	MIP electrode for voltammetry	[11]
L-Phenylalanine	MAA	Block, EGDMA, AIBN, acetonitrile, toluene, tetrahydrofuran, chloroform, 24 h	Adsorbent, human phenylketonuria serum	[12]
L-Phenylalanine	β -CD + maleic acid anhydride	Block, <i>N,N</i> -methylenebisacrylamide, ammonium persulfate, water/acetic acid/methanol, 55 °C	MIP sensor, injection sample	[13]
L-Phenylalanine	AA	Suspension, EGDMA, AIBN, acetonitrile, 24 h, 60 °C	MIP sensor, milk	[14]
Dansyl L-phenylalanine	MAA + 4-VP	Precipitation, EGDMA, AIBN, 12 h, 50 °C	MIP sensor	[15]

AA acrylamide, AIBN 2,2-azo-bis-isobutyronitrile, β -CD β -cyclodextrin, CSP chiral stationary phase, DMSO dimethyl sulfoxide, EGDMA ethylene glycol dimethacrylate, HAc acetic acid, HPLC high performance liquid chromatography, MAA methacrylic acid, MP mobile phase, 4-VP 4-vinylpyridine, R_s resolution value for HPLC separation of phenylalanine enantiomers, α selectivity factor for HPLC separation of phenylalanine enantiomers

Table 2 Synthesized molecularly imprinted polymers for template L-phenylalanine

MIP	I	II	III	IV	V
Functional monomer	AA	AA	AA	AA	MAA
Crosslinker	EGDMA	EGDMA	EGDMA	EGDMA	EGDMA
M:T:S ^a	1:4:20	1:4:20	1:4:20	1:5:26	1:5:26
Porogen	Ethanol	Acetonitrile	Acetonitrile	Acetonitrile	Acetonitrile
Initiator	AIBN	AIBN	AIBN	AIBN	AIBN
Polymer type	Soft	Soft	Soft	Hard block	Hard block

AA acrylamide, EGDMA ethylene glycol dimethacrylate, AIBN 2,2-azo-bis-isobutyronitrile, MAA methacrylic acid

^aTemplate: monomer: crosslinking monomer

polymerization time to 30 h (time 24 h was used for MIP preparation). From this reason, the higher amounts of template and crosslinker in the polymerization mixture were tested.

The suitable composition of polymerization mixture for L-phenylalanine imprinting being MAA or AA as functional monomers with acetonitrile as porogen (molar ratio template: functional monomer: crosslinking monomer 1:5:26). In second step, polymers MIP IV and V were tested from the aspect of enantioselectivity. Analyte solvent affected solvation of its molecules, swelling of the polymer, and finally sorption process of the analyte on

MIP, and from these reasons the selectivities of polymers for enantiomer recognition were evaluated in different solvents and mixtures (water, methanol/water 1/1, methanol, acetonitrile/water 1/1) by testing the response of MIPs to L and D enantiomeric forms. Results presented in Table 3 show that higher values of efficiency factor (EF) were obtained for template, L-phenylalanine, which indicate the ability of MIP IV and MIP V to recognize enantiomeric forms of phenylalanine. The highest difference of EF values of L-form and D-form was observed for the MIP V when water/methanol mixture was used as analyte solvent (EF-L-phe = 1.28, EF-D-phe = 0.93; statistically

Table 3 Effect of analyte solvent on values of efficiency factor for MIP-L-phenylalanine and NIP

	EF ^a							
	Water		Methanol–water 1/1		Methanol		Acetonitrile–water 1/1	
	L-Phe	D-Phe	L-Phe	D-Phe	L-Phe	D-Phe	L-Phe	D-Phe
MIP IV	1.11	1.07	1.06	0.90	1.04	0.92	1.09	0.95
NIP IV	1.10	1.09	0.91	0.91	0.95	0.94	0.98	0.98
MIP V	1.14	1.08	1.28	0.93	1.07	0.95	1.11	0.93
NIP V	1.19	1.20	1.08	1.05	0.95	0.94	1.00	1.02

L-phe, L-phenylalanine, *D-Phe* D-phenylalanine *EF* efficiency factor

^aConditions of sorption: 0.25 g of MIP or NIP, 60 min, 23 °C; RSD < 3.1% ($n = 3$)

significant difference, *t* test, 95%). These results suggest that MIP V is enantioselective for L-phenylalanine. The EF values of both enantiomeric forms obtained for blank polymers (NIP) are similar (statistically not significant differences, *t* test, 95%), indicating that the NIPs are not able to recognize the enantiomers of phenylalanine due to the absence of enantiospecific cavities.

Separation of enantiomers on MIP-L-phenylalanine chiral stationary phase

MIP-L-phenylalanine (MIP V) was used as stationary phase for HPLC separation of enantiomers of phenylalanine. The effect of the composition of the mobile phase, amount of ionic modifier in mobile phase, the column temperature, and the length of the column on chromatographic characteristics (retention factor, resolution of enantiomers, selectivity factor) of enantiomers of phenylalanine and other amino acids were investigated.

Effect of mobile phase composition

A HPLC column (42 mm × 5 mm I.D.) containing 300 mg of the stationary phase MIP-L-phenylalanine was used for separation of phenylalanine enantiomers. Separation of enantiomers was performed in a reverse phase mode with mobile phases consisted of a mixture of methanol or acetonitrile and water, as solvents proved to be the most appropriate in assessing the MIP properties. Decrease of amount of organic modifiers in mobile phases from 100 to 0% caused decrease of retention factor values from 0.8 to 0.2; however, the separation of the enantiomers was not achieved. Decreasing the flow rate of the mobile phase from 0.5 to 0.3 cm³ min^{−1} did not significantly affect the efficiency of enantioseparation. To achieve an efficient separation of the individual enantiomers of phenylalanine, the effect of concentration of ionic modifiers, acetic acid and triethylamine, in mobile phase on the chromatographic characteristics were tested. Partial separation of enantiomers was reached in mobile phases with the addition of

acetic acid as ionic modifier (Fig. 1). Using the mobile phase with methanol as the organic modifier (methanol/water 90/10 with 0.5–1.5% of acetic acid), the retention factors of the enantiomers ranged from 0.08 to 0.017 and the enantiomers were not separated ($R_s < 0.3$). The change of the organic modifier for acetonitrile results to separation of the enantiomers of D,L-phenylalanine. By increasing the amount of acetic acid in the mobile phase from 0.5 to 1.5%, the values of enantiomers resolution increased (Fig. 1) from 1 to 1.2. Suitable composition of the mobile phase for HPLC separation of the D,L-phenylalanine enantiomers on the MIP-L-phenylalanine stationary phase was acetonitrile/water (90/10, v/v) containing 1.5% acetic acid.

Effect of temperature

The column temperature affects the interaction between the separated substances, the stationary phase, and the mobile phase, and also influenced the chromatographic characteristics. The effect of column temperature on retention and resolution of enantiomers was investigated in the range from 5 to 45 °C with the aim to increase the efficiency of phenylalanine enantioseparation. The dependence of the resolution values on the column temperature is shown in Fig. 2. Increase in column temperature caused the increase in values of resolution of enantiomers and the values reached its maximum at 45 °C. The retention factor values were also markedly influenced by the column temperature. The effect of the column temperature on the retention of phenylalanine enantiomers showed the same trend as for resolution, the increase in column temperature in the monitored temperature range results in an increase in values of retention factor of the enantiomers.

Thermodynamic studies can provide information on possible enantiorecognition mechanisms for selected CSP. The thermodynamic parameters for the two enantiomers could be obtained from the Van't Hoff's plots, which showed linear behaviour in studied temperature range (R^2 higher than 0.995, Table 4). Values of the partial molar enthalpy (ΔH_i) and partial molar entropy (ΔS_i) for the

Fig. 1 Effect of acetic acid concentration in mobile phase on chromatographic characteristics, retention factor (k), selectivity (α), and resolution (R_s), of L- and D-phenylalanine. Chromatographic conditions: stationary phase: L-phenylalanine (42 mm \times 5 mm, 0.3 g of MIP V), mobile phase: acetonitrile or methanol/water (90/10 v/v) containing acetic acid; column temperature: 25 °C; flow rate: 0.3 cm³ min⁻¹; detection: UV 210 nm

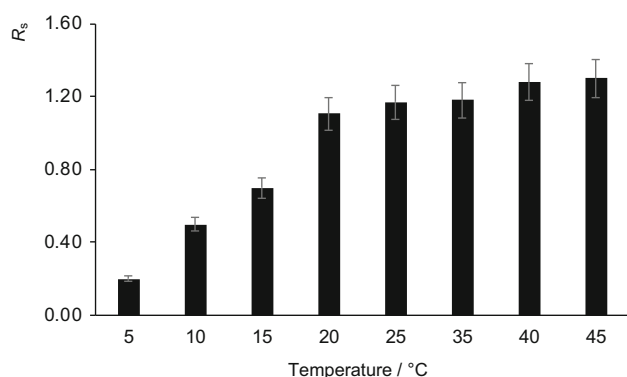
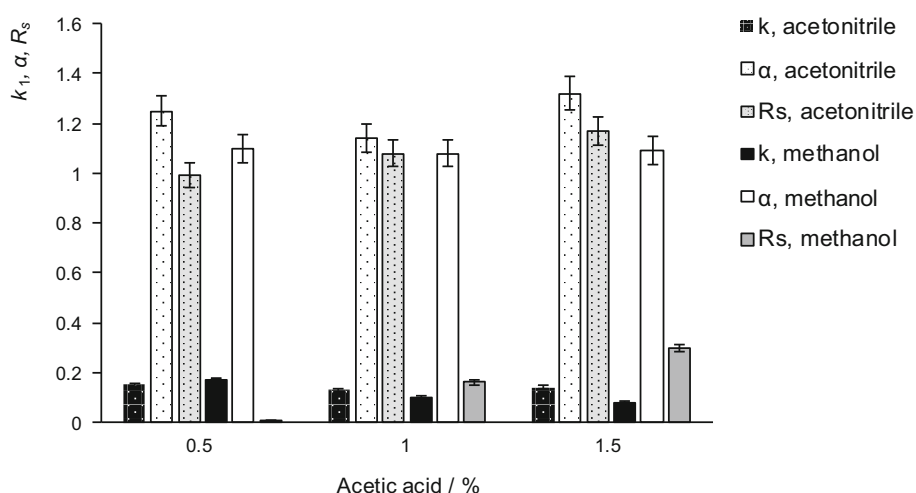


Fig. 2 Effect of column temperature on chromatographic resolution of L- and D-phenylalanine. Chromatographic conditions: see Fig. 1

phenylalanine enantiomers were calculated from the slope and intercept of the straight lines [Eq. (2)]. Since the value of phase ratio (Φ) was not known, the approach based on examination of the energetic terms of the Van't Hoff equation individually was elected. The first term ($-\Delta H/$

RT) gives the enthalpic contribution to the retention ($\ln k$) at given temperature and the second term ($\Delta S/R + \ln \Phi$) provides the entropic contribution [17, 18]. Values of enthalpic and entropic contributions to retention of the phenylalanine enantiomers and other thermodynamic data (ΔH_i , $\Delta(\Delta H)$, $\Delta(\Delta S)$, $\Delta(\Delta G)$) are summarized in Table 4. The enthalpic and entropic contributions are comparable, around 50%. The differences in contributions between the individual enantiomers are statistically insignificant (49.5/50.1 and 50.5/49.9% for enthalpic and entropic contributions, respectively). These results indicate that similar interaction mechanisms were responsible for enantio-recognition of enantiomeric forms on CSP. The value of enthalpy change for transfer from the mobile phase to the stationary phase is lower for the second eluted enantiomer, which indicates that interactions between the stationary phase and the second eluted enantiomer (L) are more enthalpy-favourable than for first eluted enantiomer (D).

Table 4 The coefficients of Van't Hoff's plots and thermodynamic data for the phenylalanine enantioseparation on MIP-L-phenylalanine CSP

Parameter	D-Phenylalanine	L-Phenylalanine
Van't Hoff's plot	$\ln k = -7856.9/T + 24.2$ $R^2 = 0.995$	$\ln k = -6781.1/T + 21.2$ $R^2 = 0.996$
Enthalpic term ^a ($-\Delta H_i/RT$) (%)	24.7 ± 0.5 49.5	21.3 ± 0.6 50.1
ΔH_i	65.3×10^3	56.4×10^3
Entropic term ($\Delta S_i/R + \ln \Phi$) (%)	24.2 ± 0.6 50.5	21.2 ± 0.5 49.9
$\Delta(\Delta H)$	-8.9×10^3	
$\Delta(\Delta S)$	-24.9	
$\Delta(\Delta G)^a$	3.1	

^aCalculated at a temperature of 318 K, ΔH_i and $\Delta(\Delta H)/J \text{ mol}^{-1}$, ΔS_i and $\Delta(\Delta S)/\text{mol}^{-1} \text{ K}^{-1}$, $\Delta(\Delta G)/J \text{ mol}^{-1} \text{ K}^{-1}$, $R/J \text{ mol}^{-1} \text{ K}^{-1}$

Effect of column length

To increase the efficiency of separation of enantiomers of phenylalanine, an increase of column length from 42 to 85 mm was tested (I.D. was 5 mm, amount of stationary phase was increased from 0.3 to 0.6 g). Increasing the column length had a positive effect on the separation of the enantiomers (higher values of resolution R_s 1.49 and selectivity factor $\alpha = 1.38$), resulting from the increase in the number of binding sites in stationary phase. Chromatogram of separation of phenylalanine enantiomers on MIP-L-phenylalanine stationary phase at optimal HPLC conditions is shown in Fig. 3a.

MIP enantioselectivity

Prepared MIP-L-phenylalanine, due to the presence of chemical functionalities in recognition sites which are responsible for selective binding, has ability to separate the enantiomeric forms. The shape complementarity of imprinted cavity resulted in higher absorbability and a longer retention time for L-phenylalanine than for D-phenylalanine. Separation of enantiomers was not observed when the column was packed with the NIP (Fig. 3b). The retention times of enantiomers were shorter (5.4 min) in comparison with MIP CSP (5.8 min for D-phenylalanine, 7.8 min for L-phenylalanine). The enantioselectivity of CSP was also tested for other structurally related compounds from the amino acid group (methionine, alanine, asparagine). Separation of enantiomers was not observed and retention times were short (4.6 min) which indicated that compounds were not retained on the CSP. This indicates that prepared MIP is enantioselective for phenylalanine enantiomers.

Chiral stationary phase reusability, stability, and applicability

The reusability of MIP-L-phenylalanine as CSP was evaluated by the repeatable separation phenylalanine racemate under optimal chromatographic conditions. More than forty injections of racemate solution were realized without changing retention times and resolution values (differences less than 7%).

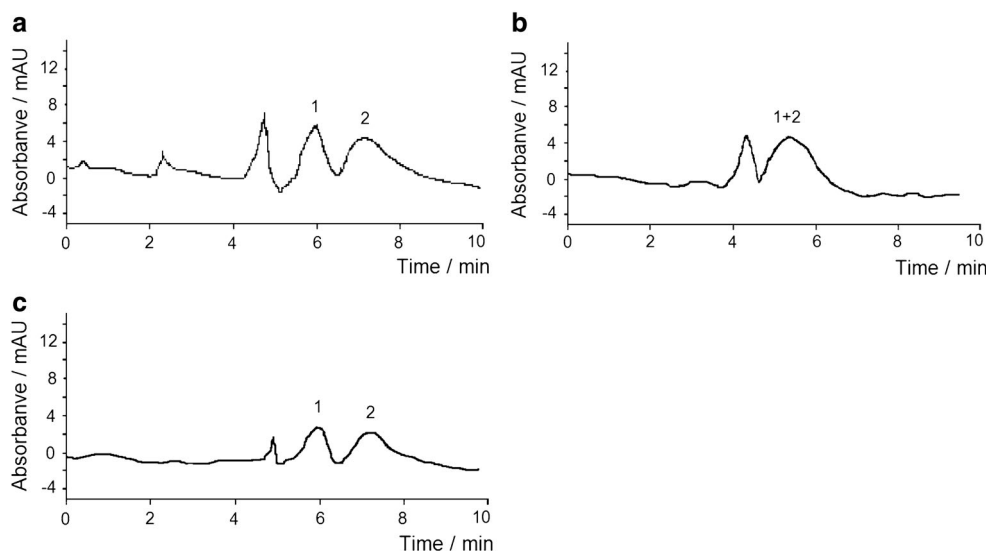
In our previous work focused on HPLC separations of D,L-phenylalanine, the resolution values 1.6, 1.1, and 0.7/1.2 on teicoplanin in reversed phase mode, β -cyclodextrin in normal phase mode, and isopropylcarbamate cyclofructan 6 CSPs in polar organic or normal phase modes, respectively, were obtained [7]. Comparable resolution value (1.5), good separation factor, and shorter analysis time (about 10 min in comparison with 18 min for teicoplanin CSP) for enantiomeric separation on MIP-based CSP using a reversed phase system were reported in this study.

The applicability of MIP-L-phenylalanine CSP for separation of phenylalanine enantiomers in samples of nutrition supplement was tested. HPLC analysis shows presence of both enantiomeric forms in sample of nutrition supplement (Fig. 3c) above LOD of proposed method (LOD (UV 210 nm) for D- and L-phenylalanine was 1.0 mg cm^{-3}). The other components of the nutritional supplement did not interfere with the enantiomeric forms of phenylalanine (under the HPLC conditions used).

Conclusion

In this work, an L-phenylalanine imprinted polymer was synthesized by thermal bulk polymerization with non-covalent approach using methacrylic acid as functional

Fig. 3 Chromatograms of D,L-phenylalanine separation on MIP-L-phenylalanine (a), NIP (b), and sample of dietary supplement on MIP-L-phenylalanine stationary phases. Chromatographic conditions: stationary phase: MIP-L-phenylalanine or NIP (85 mm \times 5 mm, 0.6 g of sorbent), mobile phase: acetonitrile/water (90/10 v/v) containing 1.5% acetic acid; column temperature: 45 °C; flow rate: $0.3 \text{ cm}^3 \text{ min}^{-1}$; detection: UV 210 nm; (1) D-phenylalanine, (2) L-phenylalanine



monomer and acetonitrile as porogen. Chiral stationary phase based on MIP exhibited a good potential for separation of enantiomers of D,L-phenylalanine under optimal conditions. The shape complementarity of imprinted cavity resulted in a longer retention time for L-phenylalanine than for D-phenylalanine. The resolution value and selectivity factor were 1.49 and 1.38, respectively. On the basis of thermodynamic study, it was observed that the enthalpic and entropic contributions to the retention ($\ln k$) are comparable, around 50% for both enantiomers, and indicate their similar interaction mechanisms of enantioseparation on prepared CSP. The MIP-L-phenylalanine CSP exhibited a good stability and reusability for enantioseparation of phenylalanine racemate. The applicability of chromatographic column packed with lab-made MIP CSP for separation of phenylalanine enantiomers in the sample of nutrition supplement was shown. The advantage of MIP enantioselective sorbent is the ability to control the elution order of enantiomers by changing the enantiomeric form of template used for imprinting.

Experimental

Materials

D,L-Phenylalanine (> 98%) and L-phenylalanine (> 98%) was purchased from Sigma-Aldrich. Acetonitrile, methanol (VWR International, Slovakia; gradient-grade purity), ethanol (Centralchem, Slovakia; for UV), acetone, acetic acid, (Centralchem, Slovakia; analytical grade), trifluoroacetic acid, triethylamine, 2,2'-azobisisobutyronitrile (AIBN), methacrylic acid (MAA), acrylamide (AA), ethylene glycol dimethacrylate (EGDMA) (Merck, Germany; reagents were for synthesis grade) were used for synthesis and testing of polymers. Sample of dietary supplement was obtained from a local pharmacy.

HPLC instrumentation and conditions

The liquid chromatograph Agilent 1200 series, consisting of a binary high-pressure pump, injection valve Rheodyne (20-mm³ injection loop), column thermostat, and diode array detector, was used. The mixtures of acetonitrile or methanol with water and acetic acid at different ratios were used as mobile phase. Separations of the enantiomers were performed on lab-made MIP-L-phenylalanine chromatographic columns of different length (42 mm × 5 mm I.D.) or (85 mm × 5 mm I.D.). The tested flow rates were 0.2, 0.3, and 0.5 cm³ min⁻¹ and the column temperatures were maintained at 5–45 °C. The chromatograms were recorded at detection wavelength 210 nm. All solutions were injected in three replicates. Dead retention time was

determined as retention time of methanol or acetonitrile (depend on mobile phase type).

Preparation of solutions and sample

Stock standard solutions of D,L-phenylalanine and L-phenylalanine (concentration 10 mg cm⁻³) were prepared in mobile phase used. The working standard solutions were prepared daily by appropriate dilution of the stock solution with mobile phase. The solutions were filtered through a syringe nylon filtre of 0.45 µm pore size prior to HPLC separation. Solutions were found to be stable when stored at 4 °C.

Accurately weighed amount of 0.3 g of powdered dietary supplement sample was mixed with 100 cm³ of mobile phase and stirred on a mechanical shaker at 23 °C for 10 min. Finally, the mixture was centrifuged at 4000 rpm for 10 min at 23 °C. The supernatant was removed and used for chromatographic analysis.

Synthesis of molecularly imprinted polymers

The mixture of template, functional monomer, and porogen (3 cm³) (molar ratios of template, functional monomer, and crosslinking monomer are shown in Table 2) was stirred in ultrasound bath for 10 min. Afterwards, crosslinking monomer (EGDMA), initiator (0.191 g AIBN, 1.16 mmol), 0.6 cm³ acetic acid, 2 cm³ acetonitrile, and 0.125 cm³ trifluoroacetic acid were added and the final mixture was thermally polymerized (60 °C, 24 h). The resulting polymer was crushed, sieved through the 80-µm sieve, and washed with acetone to remove fine particles. The imprinted template molecules were removed from the polymer by the Soxhlet extraction with mixture methanol/acetic acid (9/1 v/v, 200 cm³, 24 h). Blank, non-imprinted polymers (NIPs) were prepared by the same procedure and reagents but without addition of a template into the polymerization mixture.

Evaluation of MIP performance

Binding capacity was determined by static adsorption experiment. Amount of 0.15 g of dried polymer (MIP or NIP) was suspended in 10 cm³ of D,L-phenylalanine solution, concentration 0.02 mg cm⁻³. Solutions of analyte were prepared in water, methanol, methanol/water (1/1 v/v), and acetonitrile/water (1/1 v/v). The suspension was incubated at temperature 23 °C for 60 min under shaking (180 rpm), and finally centrifuged at 4000 rpm. The residual enantiomer concentrations were determined by HPLC method [7].

The specific binding capacity was calculated as difference of amount of analyte bound to MIP and NIP. The

efficiency factor (EF) was used to evaluate the recognition property of MIPs. The EF values were calculated according to the following formula:

$$EF = \frac{\text{Concentration of analyte before application on MIP or NIP}}{\text{Concentration of analyte after adsorption on MIP or NIP}} \quad (1)$$

Chromatographic evaluation

The separation ability of CSP towards D- and L-phenylalanine was evaluated by chromatographic characteristics. The retention factor (k) values of first and second eluted enantiomer were calculated as the ratio of differences between retention time of enantiomer (t_R) and dead time (t_M) to dead time as follows: $k = (t_R - t_M)/t_M$. The selectivity factor was calculated as the ratio of retention factors of second and first eluted enantiomer ($\alpha = k_2/k_1$). The resolution values (R_S) was calculated by ratio of difference between retention times of enantiomers (t_{R1} , t_{R2}) to sum of peak widths at half peak height ($w_{0.5,1}$, $w_{0.5,2}$) as follows: $R_S = 1.18 \times (t_{R2} - t_{R1}) / (w_{0.5,1} + w_{0.5,2})$.

Temperature study

For evaluation of temperature influence on enantiomeric retention, the Van't Hoff's expression was used:

$$\ln k_i = -\Delta H_i / RT + \Delta S_i / R + \ln \Phi, \quad (2)$$

where k is retention factor, R is the gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), T/K is the column temperature, Φ is the phase ratio [defined as ratio of volume of the stationary (V_S) and mobile phase (V_M)], ΔH is enthalpy of transfer of the solute from the mobile phase to the CSP, ΔS is the entropy of transfer of the solute from the mobile phase to the CSP, and α is selectivity coefficient. The change in free

energy $\Delta(\Delta G)$ accompanying the separation of enantiomeric forms is given by the equation:

$$\Delta(\Delta G) = \Delta(\Delta H) - T\Delta(\Delta S). \quad (3)$$

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