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High-Performance Liquid Chromatographic Enantioseparation of Unusual Cyclic *β*-Amino Acids

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1. INTRODUCTION

Chirality and the separation of chiral compounds have become increasingly important in recent decades in the pharmaceutical industry and modern analytical chemistry regarding both scientific and economic perspective.

Chiral molecules in living organisms have paramount importance because amino acids in proteins, sugars, enzymes, *etc.* are chiral compounds. Often only one of the enantiomers is responsible for the therapeutic effect (eutomer), while the other isomer (distomer) should ideally be harmless. The distomer, however, may trigger some undesirable side effects or even may be toxic. In the pharmaceutical and drug industries, the existence of chirality became particularly important after the thalidomide tragedy in the 1960s. Thalidomide, in its racemic form, was put on the market as a sedative. The (+)-isomer was even therapeutic but the harmful (–)-form was shown to be responsible for the catastrophic malformations of embryos when thalidomide was administrated to women during pregnancy. It is understandable, therefore, that chirality has become a very important issue in terms of the safety of drug production. Stereoisomeric mixtures as drug substances have been allowed to be marketed only in reasonable cases for a long time.

Varied procedures, among others, crystallization, chiral derivatization, enzymatic resolution, *etc*. are available for the preparation of pure enantiomers. To check the chiral purity of the end product is always indispensable regardless the preparation method used. Pharmaceutical drug development and impurity control require highly reproducible techniques with high sensitivity and stereoselectivity. For this purpose, the most suitable and most frequently used method is high-performance liquid chromatography (HPLC) with the application of chiral columns.

β-Amino acids have attracted considerable research interest during the past few decades due to their biological relevance. They play a central role in modern chemical research because of their important implication in synthetic and medicinal chemistry. They have unique biological, neurological, and pharmaceutical activities. The incorporation of conformationally constrained *β*-amino acids may provide peptides with a more rigid structure. This allows the investigation of receptor binding processes and preparation of peptide-based drug molecules with high biological potential. Many of the investigated compounds have various biological (anti-influenza, antifungal) activity.

2. AIMS

The primary aim of this work was to develop simple, readily available chiral HPLC methods for the resolution of stereoisomers of *β*-amino acids, endowed both biological and pharmaceutical interest on newly developed chiral stationary phases (CSPs), such as chiral ligand-exchange-based (CLEC) and zwitterion-exchange-based (ZWIX) CSPs.

- The enantioseparation of isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogues on chiral ligand-exchange column has been studied. The influence of mobile phase composition and Cu(II) salts with different anions, the effect of $Cu(ClO_4)_2$ concentration, the structure of analytes, and temperature on the chiral recognition have been examined.
- The enantioseparation of isoxazoline-fused 2-aminocyclopentanecarboxylic acid stereoisomers was investigated on new types of zwitterionic chiral stationary phases based on *Cinchona* alkaloids, with the aim of optimizing chromatographic conditions (mobile phase composition, bulk solvent composition, temperature, *etc*.).
- For the enantioseparation of monoterpene-based *β*-amino acids, zwitterionic chiral stationary phases based on *Cinchona* alkaloids were used, with the aim of developing new chromatographic methods for their chiral separation.
- The separation efficiencies of the two types of CSPs (CLEC and ZWIX) were compared on the basis of chromatographic data obtained for isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogues.

3. EXPERIMENTAL

3.1. Apparatus

Measurements were carried out on alternative HPLC systems.

System I: a Waters Breeze system consisting of a 1525 binary pump, a 2487 dual-channel absorbance detector, a 717 plus auto sampler, and Empower 2 data manager software (Waters Chromatography, Milford, MA, USA).

System II: a 1100 Series HPLC system from Agilent Technologies (Waldbronn, Germany) consisting of a solvent degasser, a pump, an autosampler, a column thermostat, a multiwavelength UV-Vis detector, and a corona charged aerosol detector from ESA Biosciences, Inc. (Chelmsford, MA, USA). Data acquisition and analysis were carried out with ChemStation chromatographic data software from Agilent Technologies.

3.2. Columns applied

Chiral ligand-exchange CSP [Column IV: sodium *N*-((*R*)-2-hydroxy-1-phenylethyl)-*N*-undecylaminoacetate-based CSP, 150×4.6 mm I.D., 5 μm particle size] was prepared from (*R*)-phenylglycinol by covalently bonding (*R*)-*N,N*-carboxymethyl undecyl phenylglycinol monosodium salt, its derivative, to Kromasil silica gel.

Cinchona alkaloid-based zwitterionic stationary phases Chiralpak ZWIX(+)™ and ZWIX(-)™ columns were from Chiral Technologies Europe (Illkirch, France) (150 mm×3 mm I.D., 3 μm particle size for each column).

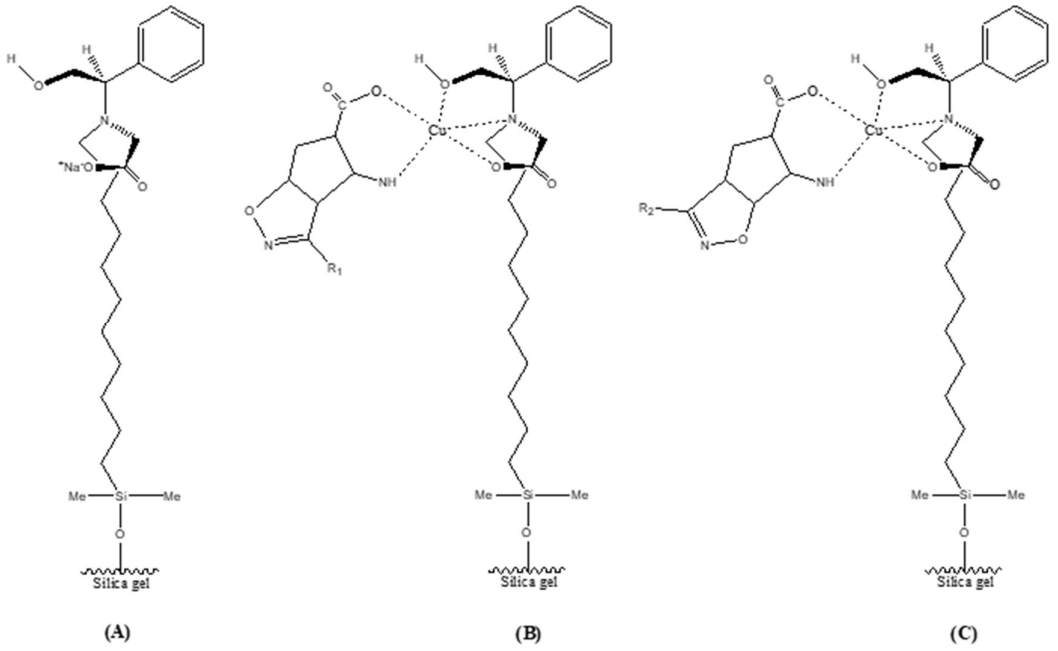

Figure 1. *Structure of the selector of CLEC (A) and its terner complex with isoxazoline-fused 2-aminocyclopentanecarboxylic acids* ***1,2*** *(B) and* ***3,4*** *(C)*

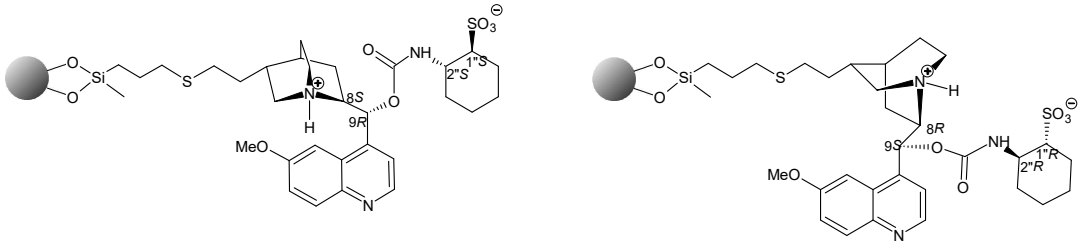

Figure 5. *Structure of QN-based [ZWIX(+)™] and QD-based [ZWIX(-)™] columns*

4. RESULTS

During the work of enantioseparation of two types of unusual β-amino acid analogues, isoxazoline-fused 2-aminocyclopentanecarboxylic acids and monoterpene-based β-amino acids were investigated. The chromatographic measurements were carried out on a chiral ligand-exchange and two *Cinchona* alkaloid-based stationary phases.

4.1. Enantioseparation of isoxazoline-fused 2-aminocyclopentanecarboxylic acids on a chiral ligand-exchange stationary phase

The effects of the mobile phase composition [H_2O/MeOH and H_2O/EtOH containing 0.2 mM total concentration of $Cu(ClO_4)_2$] showed that, in most cases, U-shaped retention curves were observed. Both higher and lower water contents afforded the largest k_1 values. This was probably due to enhanced hydrophobic interactions between the analyte and the CSP. However, when the alcohol content of the mobile phase increased from 5 to 15%, k_1 first decreased, but further increases in the MeOH or EtOH content induced increases again. This suggests that the separation may be controlled by a HILIC mechanism at high alcohol contents.

The nature of the alcohol modifier (MeOH, EtOH, PrOH, and 2-PrOH) exerted major effects on the retention. Namely, k_1 increased with increasing carbon number of the alcohol. This phenomenon allowed the conclusion that increasing carbon number was disadvantageous for polar interactions between the mobile phase and the analytes.

The effects of the concentration of $Cu(ClO_4)_2$ were also investigated. On increasing concentration, Cu(II) gave a U-shaped k_1 curve. The increase in $Cu(ClO_4)_2$ concentration in the mobile phase promoted the formation of several SA complexes in the eluent thus shortening k_1. At higher Cu(II) concentrations more intricate complexation events occurring on the column may affect separation.

A comparison of the chromatographic data acquired with the use of $Cu(ClO_4)_2$, $Cu(NO_3)_2$ or $CuSO_4$ demonstrated that larger k_1 values were obtained by the application of nitrate or sulphate ions; perchlorate ions, in turn, resulted in lower k_1. The apolar character of the amino acid–Cu(II)-anion complex is suggested to induce an increase in retention when nitrate or sulphate ions are used.

The structure of the SAs also influenced chiral recognition. As concerns steric interactions, the position of the methyl or ethyl group affected k_1, α, and R_S values. In most cases, *trans*-isomers (**C,D**) exhibited larger enantioseparation than *cis*-isomers (**A,B**).

The effects of the temperature were investigated between 5–45 °C for all SAs. According to thermodynamic parameters determined in all cases, enantioseparations, in most cases, are enthalpically driven (except **2A,2B**, **3A3B**, and **4A,4B**).

4.2. Enantioseparation of isoxazoline-fused 2-aminocyclopentanecarboxylic acids on Cinchona alkaloid-based CSPs

The *Cinchona* alkaloid-based zwitterionic CSPs afforded the best results in PIM when a mixture of MeOH and MeCN was applied. Retention, selectivity, and resolution of zwitterionic amino acids in most cases increased with increasing MeCN content. This was probably due to the decreased solvation effect of MeCN and the stronger electrostatic interaction of the SAs with the SO.

For comparison, the concentration of the acid and base components as mobile phase additives was investigated by varying the ratio of TEA and AcOH in polar ionic mobile phase. The results indicated that an acid excess (acid to base ratio 2:1) was favourable, while under basic conditions (1:4), a decrease in retention parameters was observed. The explanation for this phenomenon was that the ion-exchange effect and the stochiometric displacement model dominate the retention mechanism.

Seven different base modifiers and two acid modifiers were applied for the separation of analytes **1A,1B**, and **4C,4D**. As experimental results showed, k_1, α, and R_S values differ slightly when the base or acid component was varied. In most cases, k_1 increased as the degree of alkyl substitution or apolar character and bulkiness of aliphatic moiety on the *N* atom of base increased (EA<DEA<TEA; PA<TPA; BA<TBA). The nature of the applied acid also influenced the resolution. For the same analytes the presence of FA instead of AcOH, in most cases, resulted in slightly higher k_1 values when the same base additive was applied.

The differences of the structure of SAs influenced the chromatographic behaviour. CSPs based on *Cinchona* alkaloids interact in different ways with analytes. Higher k_1 values were generally observed on ZWIX(-)^{TM}; however, QN-based CSP distinguished the two enantiomers more efficiently in several cases.

For the differentiation of the four diastereomers of isoxazoline-fused 2-aminocyclopentanecarboxylic acid in a single chromatographic run, the chromatographic conditions were optimized. For the separation of **1A**–**1D,** however, two different columns and eluent systems had to be applied.

The effect of temperature was investigated between 5–50 °C on both CSPs. Chromatographic parameters decreased with increasing temperature, but an increase in α was

registered in some cases. The thermodynamic parameters were determined in all cases, and enthalpy-controlled enantioselective discrimination was found in most cases. Entropy-controlled separations could also be observed with increasing temperature. On ZWIX(+)^{TM} for **2A,2B** with eluent c, T_{iso} was 14 °C; the change in elution sequence with changing temperature for **2A,2B**.

4.3. Enantioseparation of monoterpene-based 2-aminocyclopentanecarboxylic acids on Cinchona alkaloid-based CSPs

Non-aqueous polar ionic experimental conditions with MeOH and MeCN as bulk solvent were applied in this project. Similar to previous results, the retention of *β*-amino acids, in all cases, increased substantially with increasing MeCN content. Characteristically, a certain amount of MeCN on both columns enhanced selectivity and resolution with a few exceptions.

For a more detailed investigation of the effects of the nature of the acid and base components in the mobile phase, five different bases (NH_3, EA, DEA, TEA, and PA) and two acids (FA and AcOH) as mobile phase additives were selected. The nature of the applied acid and base modifiers exhibited only a slight effect on the chromatographic parameters. According to the experimental results, k_1 values increased on both columns with both acid modifiers as the degree of ethyl substitution on the nitrogen atom of the base increased. With a few exceptions, the application of TEA or NH_3 resulted in the largest and the smallest k_1 values, respectively. The effects of the nature of the acid additives on the retention are complex; however, it seems that there is a slight difference in the eluent strength when AcOH or FA was applied.

The effects of temperature were investigated on both CSPs between 10–50 °C and the thermodynamic parameters were determined in all cases. Usually, enantioselective discriminations were enthalpy controlled; however, for SAs **6**, **8**, and **9** on ZWIX(-)^{TM} CSP with mobile phase MeOH/MeCN (50/50 *v/v*) containing 25 mM TEA and 50 mM FA, separation was entropically driven.

The structures of *β*-amino acid analogues also influenced the chiral recognition. On both CSPs, at a given mobile phase composition, k_1 values for the *trans* analogues (SAs **8** and **9**) were larger than for the *cis* ones (SAs **5**–**7**) in most cases. For comparison of the diastereomers (SAs **5** and **8**), k_1 values were larger for SA **8** on both columns, while the difference of the structure of analytes **5** and **8** exhibited only a slight effect on enantioselectivity. The extra methyl group at position 2 on SA **6** sterically hindered the main ionic interactions, resulting in lower k_1, α, and R_S values.

Elution sequences were determined in all cases. ZWIX(+)^{TM} and ZWIX(-)^{TM} CSPs behave like pseudo enantiomeric stationary phases, and the sequence of elution of SAs, in principle, should be reversed. On ZWIX(+)^{TM}, the elution sequence of SAs **5**, **6**, **7**, and **9** was found to be $A < B$, while it was $B < A$ for SA **8**. In contrast, the opposite trend was registered on the ZWIX(-)^{TM} CSP.

4.4. Comparison of chiral ligand-exchange and Cinchona alkaloid-based stationary phases

The basic principle of CLEC is reversible coordination between the immobilized selector and analyte within the transition metal cation coordination sphere. Zwitterionic chiral ion-exchangers may be regarded as Pirkle-type CSPs but the ionizable groups participate in the retention process. It is essential for both columns that SA contains free amino and carboxyl groups. In the case of isoxazoline-fused cyclopentane analogues, the separation ability of these two types of CSPs can be compared.

A comparison of k_1 values indicates that isoxazoline-fused 2-aminocyclopentane carboxylic acids exhibit stronger interactions with CLEC CSP. Retention factors were generally lower on ZWIX(+)™ and ZWIX(-)™ CSPs than on the CLEC column.

Regarding the chromatographic efficiency of the two types of SOs, in most cases, α and R_S were higher on CLEC when k_1 values were lower on both ZWIX(+)™ and ZWIX(-)™ CSPs.

The effect of temperature on chiral recognition was investigated on both types of CSPs. A variable-temperature study was carried out on CLEC over the temperature range 5–45 °C and on ZWIX(+)™ and ZWIX(-)™ columns over the temperature range 5–50 °C. The increase in temperature influenced the chromatographic parameters. In the studied temperature range, both enthalpically- and entropically-driven enantioseparations were observed on both types of CSPs.

A comparison of chromatographic data allows the conclusion that both types of columns are capable for the separation of these types of SAs.

5. PUBLICATIONS

5.1. Papers related to the thesis

I. **Zsanett Gecse**, István Ilisz, Melinda Nonn, Nóra Grecsó, Ferenc Fülöp, Rajalingam Agneeswari, Myung Ho Hyun and Antal Péter
High-performance liquid chromatographic enantioseparation of isoxazoline-fused 2-aminocyclopentanecarboxylic acids on chiral ligand-exchange stationary phase.
Journal of Separation Science 36 (**2013**) 1335-1342. **i.f.: 2.594**

II. Zoltán Pataj, István Ilisz, **Zsanett Gecse**, Zsolt Szakonyi, Ferenc Fülöp, Wolfgang Lindner, Antal Péter
Effect of mobile phase composition on the liquid chromatographic enantioseparation of bulky monoterpene-based β-amino acids applying chiral stationary phases based on Cinchona alkaloid.
Journal of Separation Science 37 (**2014**) 1075-1082. **i.f.: 2.737**

III. István Ilisz, Zoltán Pataj, **Zsanett Gecse**, Zsolt Szakonyi, Ferenc Fülöp, Wolfgang Lindner, Antal Péter
Unusual temperature-induced retention behavior of constrained ß-amio acid enantiomers on the zwitterionic chiral stationary phases ZWIX(+)^{TM} and ZWIX(-)^{TM}.
Chirality 26 (**2014**) 385-393. **i.f.: 1.886**

IV. István Ilisz, **Zsanett Gecse**, Gyula Lajkó, Melinda Nonn, Ferenc Fülöp, Wolfgang Lindner, Antal Péter
Investigation of the structure-selectivityrelationships and van't Hoff analysis of chromatographic separations of unusual isoxazoline-fused 2-aminocyclopentanecarboxylic acids on Cinchona alkaloid-based chiral stationary phases.
Journal of Chromatography A 1384 (**2015**) 67-75. **i.f.: 3.926**

Total impact factor: 11.143

5.2. Other publications

V. László Sipos, István Ilisz, Anita Aranyi, **Zsanett Gecse**, Melinda Nonn, Ferenc Fülöp, Myung Ho Hyun and Antal Péter
High-performance liquid chromatographic enantioseparation of unusual isoxazoline-fused 2-aminocyclopentane-carboxylic acids on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based chiral stationary phases.
Chirality 24 (**2012**) 817-824. **i.f.: 1.894**

VI. István Ilisz, **Zsanett Gecse**, István Szatmári, Ferenc Fülöp, Antal Péter
High-performance liquid chromatographic enntioseparation of naphtol substituted tetrahydroisoquinolines on polisaccharide-based chiral stationary phases.
Biomedical Chromatography 28 (**2014**) 142-151. **i.f.: 1.723**

VII. István Ilisz, **Zsanett Gecse**, Zoltán Pataj, Ferenc Fülöp, Géza Tóth, Wolfgang Lindner, Antal Péter
Direct high-performance liquid chromatographic enantioseparation of secondary amino acids on Cinchona alkaloid-based chiral zwitterionic stationary phases. Unusual temperature behavior.
Journal of Chromatography A 1363 (**2014**) 169-177. **i.f.: 4.169**

VIII. Nóra Grecsó, István Ilisz, **Zsanett Gecse**, László Schönstein, Ferenc Fülöp, Antal Péter
High-performance liquid chromatographic enantioseparation of amino alcohol analogues possessing 1,2,3,4-tetrahydroisoquinoline skeleton on polisaccharide-based chiral stationary phases.
Biomedical Chromatography 29 (**2015**) 788-796. **i.f.: 1.729**

IX. István Ilisz, **Zsanett Gecse**, Gyula Lajkó, Enikő Forró, Ferenc Fülöp, Wolfgang Lindner, Antal Péter
High-performance liquid chromatographic enntioseparation of cyclic β-amino acids on zwitterionic chiral stationary phases on Cinchona alkaloids.
Chirality, 27 (**2015**) 563-570. **i.f.: 2.025**

Commulative impact factor: **22.683**

5.3. Conference lectures

I. **Gecse Zsanett**, Nonn Melinda, Fülöp Ferenc, Péter Antal
Izoxazolin gyűrűvel kondenzált ciszpentacin származékok folyadékkromatográfiás vizsgálata koronaéter alapú királis állófázisokon
XXXV. Kémiai Előadói Napok
Szeged, Hungary, 29-31, Oktober, 2012, Abstr.: p. 61, oral presentation

II. Grecsó Nóra, **Gecse Zsanett**, Nonn Melinda, Fülöp Ferenc, Péter Antal
Izoxazolin gyűrűvel kondenzált 2-aminociklopentán karbonsav analógok sztereoizomerjeinek elválasztása ligandumcserés királis állófázison
XXXV. Kémiai Előadói Napok
Szeged, Hungary, 29-31, Oktober, 2012, Abstr.: p. 62, oral presentation

III. **Gecse Zsanett**, Grecsó Nóra, Nonn Melinda, Fülöp Ferenc, Péter Antal
Izoxazolin gyűrűvel kondenzált ciszpentacin származékok sztereoizomerjeinek elválasztása ligandumcsrés királis állófázison
Elválasztástudományi Vándorgyűlés 2012
Hajdúszoboszló, Hungary, 7-9, November, 2012, Abstr.: P-41, poster presentation

IV. **Gecse, Zsanett**, Grecsó Nóra, Nonn Melinda, Fülöp Ferenc, Péter Antal
Izoxazolin gyűrűvel kondenzált 2-aminociklopentán karbonsav származékok sztereoizomerjeinek elválasztása ligandumcserés királis állófázison
Elválasztástudományi Ankét 2013
Budapest, Hungary, 13. May, 2013, Hotel, Mercure Budapest Buda, oral presentation

V. Péter Antal, **Gecse Zsanett**, Ilisz, István, Szatmári István, Fülöp Ferenc
Tetrahidroizokinolin-vázas analógok királis kromatográfiája
Vegyészkonferencia 2013
Hajdúszoboszló, Hungary, 26-28, June, 2013, poster presentation

VI. **Zsanett Gecse**, István Ilisz, István Szatmári, Ferenc Fülöp, Antal Péter
HPLC Enantioseparation of Naphthol-Substituted Tetrahydroisoquinolines on Polysaccharide-Based Chiral Stationary Phases
9th Balaton Symposium on High-Performance Separation Methods
Siófok, Hungary, 4-6, September, 2013, Abstr.: P-44, poster presentation

VII. Ilisz I., Aranyi A, **Gecse Zs.,** Wernisch S., Lindner W., Péter A.
Enantioseparation of Imino-Acids on Newly Developed Zwitterionic Chiral Stationary Phases
9th Balaton Symposium on High-Performance Separation Methods
Siófok, Hungary, 4-6, September, 2013, Abstr.: P-45, poster presentation

VIII. **Gecse Zsanett,** Ilisz, István, Fülöp Ferenc, Péter Antal
Naftol szubsztituált tetrahidroizokinolin analógok HPLC elválasztása polizacharid alapú királis állófázisokon
XXXVI. Kémiai Előadói Napok
Szeged, Hungary, 28-30, Oktober, 2013, Abstr.: p. 37, oral presentation

IX. A. Péter, I. Ilisz, **Z. Gecse**, D. W. Armstrong, M.-H. Hyun, W. Lindner
High-performance liquid chromatographic enantioseparation of unusual amino acids
Advances in Chromatography and Electrophoresis & Chiranal 2014
Olomouc, Czech Republic, 10-14, February, 2014, oral presentation

X. Antal Péter, István Ilisz, **Zsanett Gecse,** Wolfgang Lindner
High-performance liquid chromatographic enantioseparation of unusual amino acids
30th International Symposium on MicroScale Bioseparations
Pécs, Hungary, 27 April - 1 May, 2014, oral presentation

XI. **Gecse Zsanett,** Ilisz, István, Nonn Melinda, Fülöp Ferenc, Péter Antal
Ikerionos típusú állófázisok alkalmazása izoxazolin gyűrűvel kondenzált 2-aminociklopentán karbonsav származékok sztereoizomerjeinek királis megkülönböztetésére
Elválasztástudományi Vándorgyűlés 2014
Egerszalók, Hungary, 12-14, November, 2014, Abstr.: P-15, poster presentation

XII. Grecsó Nóra**,** Ilisz, István, **Gecse Zsanett,** Schönstein László, Fülöp Ferenc, Péter Antal
1,2,3,4-tetrahidroizokinolin vázú aminoalkoholok és származékakik enantiomerjeinek folyadékkromatográfiás elválasztása poliszacharid alapú állófázisokon
Elválasztástudományi Vándorgyűlés 2014
Egerszalók, Hungary, 12-14, November, 2014, Abstr.: P-18, poster presentation