**University of Szeged**
**Faculty of Pharmacy**
**Institute of Pharmaceutical Analysis**

# Liquid chromatographic enantiomer separation of amino acid analogs with chiral stationary phases utilizing superficially porous particles

**Ph.D. thesis**

**Dániel Tanács**

**Supervisor:**
**Dr. István Ilisz**

**2023**

**University of Szeged**

Doctoral School of Pharmaceutical Sciences

Head: Prof. Dr. Judit Hohmann D.Sc

Educational program: Pharmaceutical Analysis

Program director: Dr. István Ilisz

Institute of Pharmaceutical Analysis

*Supervisor: Dr. István Ilisz*

**Dániel Tanács**

# Liquid chromatographic enantiomer separation of amino acid analogs with chiral stationary phases utilizing superficially porous particles

**Final Exam committee:**

**Head:** Dr. Loránd Kiss

**Members:** Dr. Krisztián Horváth

Dr. Pál Sipos

**Reviewer committee:**

**Head:** Prof. Dr. István Szatmári

**Reviewers:** Dr. Krisztián Horváth

Prof. Dr. György Szőllősi

**Members:** Dr. Anita Sztojkov-Ivanov

Dr. Andrea Vasas

Szeged

2023

# Introduction

Chirality is a type of molecular asymmetry, what is important in biological systems. Numerous molecules are chiral, e.g., several building blocks of living organisms, such as amino acids (except glycine), peptides, proteins, enzymes, and saccharides. Because of this, a living organism possesses chiral interaction sites, and the enantiomers of chiral molecules may have different biological activities, which can mean different utilization, distribution, metabolism, etc. In most cases, only one of the enantiomers has favorable activity, referred to as eutomers. The other enantiomer, referred to as distomers, can have either no effect, an unwanted side effect, or toxic activity. Consequently, this distinct behavior makes it necessary to study the effect of the enantiomers separately. For example, (*S*)-ofloxacin exhibits high antimicrobial activity, and it is marketed in pure enantiomeric form as levofloxacin, while (*R*)-ofloxacin can show neurotoxic activity. Indacrinone is used as a mixture of both enantiomers. The (*R*)- enantiomer is the eutomer and works as a diuretic, but it has side effects that are countered by the (*S*)- enantiomer. Ibuprofen is used as a racemic mixture because the (*R*)- distomer is inverted by enzymes into the (*S*)- enantiomer, a nonsteroidal anti-inflammatory drug. These are just three examples of different behaviors, but there are several others. While the pharmaceutical industry pays outstanding attention to chiral compounds, it is important to note that they are also present as food additives, agricultural chemicals, or fragrance materials.

The separation of enantiomers is more challenging than the separation of achiral compounds because both enantioselective and non-selective interactions are present, and we have to account for that. For the separation of chiral compounds, chromatographic techniques are the most popular methods. Several decades ago, the separation of the enantiomers of chiral compounds started with indirect methods, in which chiral derivatization reagents were used to produce diastereomers. Nowadays it is a rarely used technique nowadays because of its disadvantages in analytical chemistry. In direct chromatographic methods chiral mobile phase additives or chiral stationary phases are used. The chiral mobile phase additives form complexes with the enantiomers and the formed diastereomers can be separated with achiral columns. These additives can be easily removed.

Chiral stationary phases can effectively separate the enantiomeric molecules. Many different chiral stationary phases are available and a wide range of mobile

phases can be used to achieve the necessary separations. The selectors are chiral molecules that form stronger interactions with one of the enantiomers, which takes a longer time to go through the column, making the separations possible.

## The aim of this work

The aim of this Ph.D. work was to study the enantiomeric separation of amino acid analogs by applying chiral stationary phases immobilized on superficially porous particles. The amino acid analogs investigated have different chemical properties, but structural analogies provide a good basis for interpreting the observed correlations between structure and retention properties. The primary goal of these studies is to explore and evaluate the relationships between the selector's molecular structure the selector and the chromatographic properties of sample compound. The main objectives are:

1. interpretation of the effect of the structure on chiral recognition,
2. evaluation of the effect of the eluent composition and the quality and quantity of various additives on separation,
3. studying the effect of temperature on chromatographic parameters, determining thermodynamic parameters, thermodynamic characterization of separation processes,
4. kinetic characterization of enantioselective separation processes.

## Experimental

Experiments were carried out on a Waters® ACQUITY UPLC® H-Class PLUS System (Waters Incorporation, Milford, MA, USA). The system contained the following modules: a quaternary solvent manager, a sample manager, a column manager, a PDA detector, and a QDa mass spectrometry detector. The system was managed by Empower 3 software (Waters).

Chiral columns were available in two different internal diameters (i.d.), 3.0 mm and 2.1 mm; all columns were 100 mm long. Chiral selectors are based on teicoplanin (TeicoShell), modified teicoplanin (NicoShell), teicoplanin aglycone (TagShell), vancomycin (VancoShell), isopropyl carbamate functionalized cyclofructan-6 (LarihcShell-P), and *Cinchona* alkaloid-based *tert*-butyl carbamate quinine (Q-Shell). All columns were provided by AZYP (LLC, Arlington, TX, USA). Their courtesy is highly appreciated.

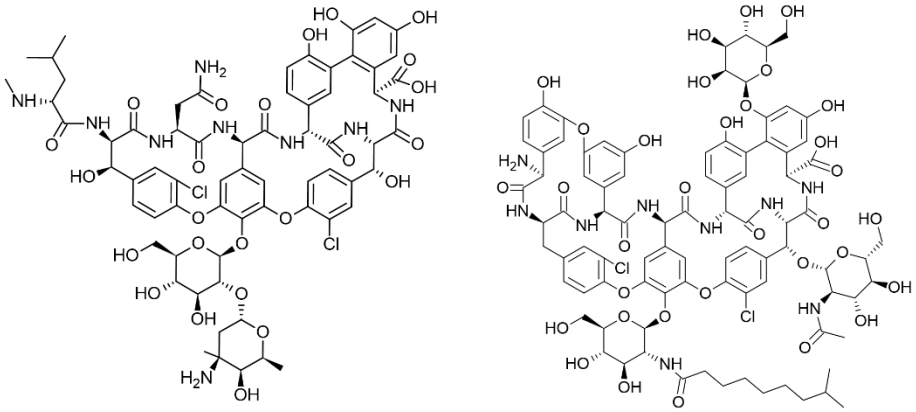

**Figure 1.** The structure of vancomycin (left) and teicoplanin (right)

Vancomycin was one of the first macrocyclic glycopeptides used as chiral selector and it is still widely used nowadays due its good separation capabilities. Teicoplanin is also an often-used selector. These selectors, shown in **Figure 1.**, were also heavily utilized during my studies.

The studied analytes can be grouped into three categories: $\beta^2$-amino acids, fluorinated $\beta$-phenylalanines, and $\alpha$-substituted proline analogs. The structures of the analytes are shown in **Figure 2**.

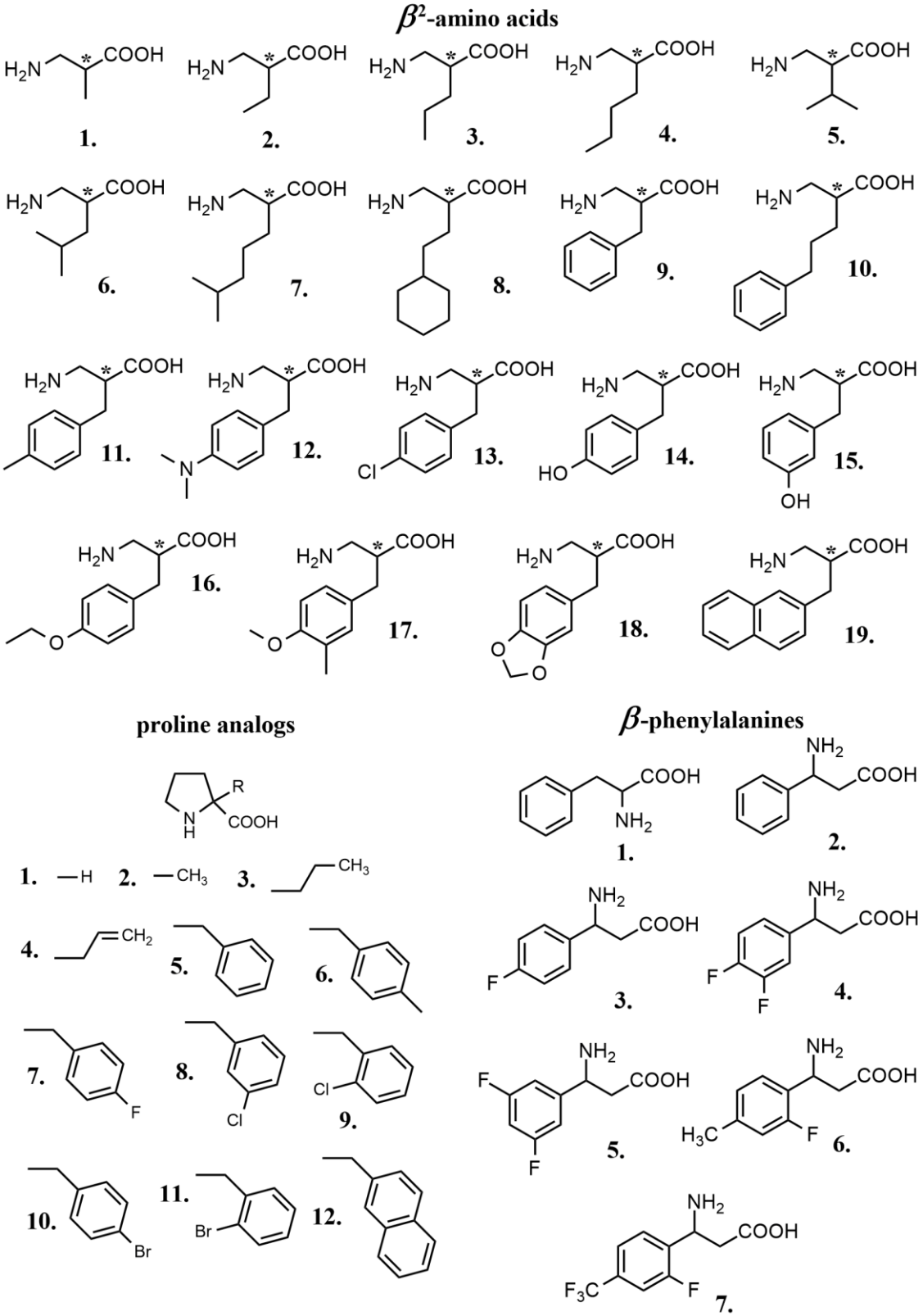


**Figure 2.** The structure of the analytes

# Results

**i) Studying the effects of mobile phase composition**

**The eluent composition studies showed that the most effective mobile phase for the macrocyclic glycopeptide selectors was $H_2O$/MeOH as eluent containing polar modifier. This eluent had the shortest retention times with selectivities and resolutions comparable with the $H_2O$/MeOH eluent without a modifier and MeOH/MeCN eluent containing polar modifier.**

The effects of the mobile phase composition were studied first. Based on these results, selected compositions were used in further studies. The retention times were lowest in the $H_2O$/MeOH eluent system containing polar modifiers, probably due to the good solvation in the eluent, and the addition of polar modifiers resulted in further improvements. The retention times were significantly higher with eluent systems utilizing MeCN in $H_2O$/MeCN containing triethylammonium acetate (TEAA) or MeOH/MeCN containing TEAA. Increasing MeCN content afforded highly increased retention times. As concerns selectivities, they increased (or reached a maximum) with increasing MeOH content in $H_2O$/MeOH based eluents. In mobile phases containing MeCN, separations decreased with increasing MeCN content, especially in polar ionic mode. Based on these results, the $H_2O$/MeOH eluent system containing TEAA was the most effective and it was used in most of our later studies. Note, however, that other eluents were also used in several cases.

**ii) Studying the effect of the nature of mobile phase additives**

**Overall, TEAA was the best additive; however, it is not compatible with MS detection. Acetic acid and ammonium acetate provided good chiral separations in several cases, making them MS compatible alternatives.**

Chiral separations can be sensitive to the nature of the mobile phase additives, which was also observed here. TEAA was an effective additive and often used in chiral chromatography, applying macrocyclic glycopeptide selectors and spectrophotometric detection. However, it is incompatible with MS detection, which can be a significant disadvantage. MS compatibility is an increasing requirement nowadays and it was our aim to search for an alternative to TEAA for these separations. We studied the efficiency of several polar modifiers such as acetic acid,

ammonium acetate and ammonium formate. They were found to show results comparable to those of TEAA.

**iii) Studying the effect of counter ion concentration, application of the stoichiometric displacement model**

**In all cases, retentions decreased with increasing counter ion concentration, but selectivities did not change or just slight improvements were observed. These results showed that only weak ionic interactions are present and they have only a minor effect on chiral separations.**

The stoichiometric displacement model predicts a linear relationship between the logarithm of the retention factor and the logarithm of the counter ion concentration, if ion exchange mechanisms are present. Its slope will be proportional to the effective charge and shows the contribution of the ionic interactions in retentions and separations. We also applied this model in our studies.

The increase in counter ion concentration decreased the retentions for all three groups of analytes independently of the applied column. The absolute value of the slopes was relatively small in all cases, indicating that only weak ionic interactions are present. The retention factors of the second eluting enantiomer practically possess the same slopes with increasing counter ion content as the first eluting enantiomer. This means that selectivity does not change with the increasing amount of counter ion. That is, the counter ion concentration has only a limited effect on the enantioselectivity.

**iv) Evaluating structure–retention relationships**

**Differences could be seen based on the side groups of the amino acids. The side groups bearing have an aromatic group showed both higher retentions and selectivities in numerous cases. Retentions, selectivities, and resolutions also depended not just on the nature of the substitutent groups on the aromatic ring, but also on their position. The *meta* position on the aromatic ring was generally the most advantageous.**

$\beta^2$-Amino acids were separated into two groups – aliphatic and aromatic analytes –based on their side chain. They were studied on the TeicoShell and TagShell columns. The results showed a strong relationship between the size of the aliphatic side chain and both retention and selectivity. Retentions decreased with the increase

of the size of the chain, which suggests steric hindrance effects. In addition, the nature and position of substituent on the aromatic side chain of $\beta^2$-amino acids greatly affected chiral discrimination. It revealed that the *para* position for substitution was favored regarding retention. However, in several cases, it was not true for selectivity and resolution.

VancoShell and LarihcShell-P columns were used for studies with fluorinated $\beta$-phenylalanines. Data showed that the number and position of F atoms with electron-withdrawing character possess a strong effect on chiral recognition. The $H_2O$/MeOH mixture containing acid and base additives was the best choice for the mobile phase. The increasing number of F atoms generally has a negative effect on selectivity, while the influence of their *ortho* and *para* position depends on the chiral selector applied. The introduction of a F atom in the *para* position decreased the selectivity on the LarihcShell-P column, but increases were found on the VancoShell column.

The best eluent for the $\alpha$-substituted proline analogs with aliphatic side chain was $H_2O$/MeOH containing acetic acid as mobile phase with MS detection. For the analogs with aromatic side chains, TEAA as additive was also successfully applied. It was also registered that substituents on the aromatic ring with electron-donating properties were favorable, whereas electron-withdrawing groups decreased the selectivity. The *para* or *meta* position was the most advantageous for the modifier groups, while the *ortho* position caused significant steric hindrance in enantiorecognition. The introduction of a naphthyl moiety caused increased selectivity and resolution but also increased the retention, indicating strong $\pi$–$\pi$ interactions.

The $\alpha$-substituted proline analogs were studied on both TeicoShell and TagShell columns. The teicoplanin aglycone is a modified teicoplanin made by removing the three sugar units of the teicoplanin from the aglycone unit. This can cause different enantiorecognition in several cases. The differences in the enantioselective free energies calculated for the TeicoShell and TagShell columns shed light on the role of sugar units in the enantiorecognition. In most cases, the TagShell column was more effective for the separation of proline analogs.

**v) Exploring the influence of temperature**

**Increasing the column temperature decreased the retention times in all cases and decreased the selectivities in most cases, meaning that these separations were enthalpically driven. The calculated thermodynamic values also confirmed this interpretation.**

Thermodynamic studies were carried out by changing the column temperature (5–50 °C) for the measurements. In nearly all cases, retentions and selectivities decreased with increasing temperature, which reveals enthalpically driven separations. This was further confirmed by the calculated $-\Delta(\Delta H^0)$, $-\Delta(\Delta S^0)$, and $Q$ values. The propylene-substituted proline analyte on the TagShell column was the only exception, since it worked in an entropy-driven separation mechanism. The $-\Delta(\Delta H^0)$ and $-\Delta(\Delta S^0)$ values for $\beta^2$-amino acids on the TeicoShell and TagShell columns and for fluorinated $\beta$-phenylalanines on the VancoShell and LarihcShell-P columns, with a few exceptions, changed in a relatively narrow range (generally between 1.0–7.5 for the $-\Delta(\Delta H^0)$ and 2.0–10.0 for the $-\Delta(\Delta S^0)$), while for proline analogs this range was larger (between 2.0–11.0 for the $-\Delta(\Delta H^0)$ and 4.0–28.0 for the $-\Delta(\Delta S^0)$). The *ln α vs. 1/T* curve for 2F,4Me-substituted $\beta$-phenylalanine on the VancoShell column was a non-linear curve, indicating a change in the retention mechanisms in the investigated temperature range, which is a relatively rare case.

**vi) Kinetic studies**

**Typical and unusual van Deemter curves were observed. The results showed that plate heights and curve shapes depended not just on the structure of the analyte and selector, but also on the composition of the eluent, too.**

The van Deemter analysis describes the kinetics of selector–selectand interactions by changing the linear flow rate. All columns were available in two internal diameters (3.0 mm, and 2.1 mm). Using the linear flow rate, the different column sizes were easy to compare, because the result is independent of the internal volume of the column.

In terms of best selectivity and performance, not the most optimal eluents were selected to decrease the backpressures, which can become considerably high at higher flow rates. The shape of the curves and their minimum position depend both on the geometrical size (i.d.) of the column and on the structure of the analytes and even on

the nature of the mobile phase. Unusual van Deemter curves were observed in the case of proline analogs and, a few cases, of the $\beta^2$-amino acids and fluorinated $\beta$-phenylalanines. According to Felletti et al., this effect is caused by the strong retention of the analytes on the selector, which results negligible diffusion of the analytes in the stationary phase [1].

It is important to note that the internal volume of the UHPLC instrument was not optimized, and making these optimizations could lower the plate heights.

# Biblography

[1] S. Felletti *et al.*, *J. Chromatogr. A.* **1630**, 461532 (2020).

# List of publications, presentations, and posters

## Publications related to the thesis

i. **D. Tanács**, R. Berkecz, A. Misicka, D. Tymecka, F. Fülöp, D.W. Armstrong, I. Ilisz, A. Péter: Enantioseparation of *β*-amino acids by liquid chromatography using core-shell chiral stationary phases based on teicoplanin and teicoplanin aglycone
Journal of Chromatography A. 1653 (2021) 462383.
https://doi.org/10.1016/j.chroma.2021.462383.
**if.: 4.601**

ii. **D. Tanács**, R. Berkecz, S. Shahmohammadi, E. Forró, D.W. Armstrong, A. Péter, I. Ilisz: Macrocyclic glycopeptides- and derivatized cyclofructan-based chiral stationary phases for the enantioseparation of fluorinated *β*-phenylalanine analogs
Journal of Pharmaceutical and Biomedical Analysis. 219 (2022) 114912.
https://doi.org/10.1016/j.jpba.2022.114912.
**if.: 3.571**

iii. **D. Tanács**, R. Berkecz, D.W. Armstrong, A. Péter, I. Ilisz: Enantioseparation of
a-substituted proline analogs with macrocyclic glycopeptide-based chiral stationary phases immobilized on superficially porous particles of silica applying liquid chromatography with ultraviolet and mass spectrometric detection
Journal of Chromatography A. 1697 (2023) 463997.
https://doi.org/10.1016/j.chroma.2023.463997.
**if.: 4.601**

**Sum if.: 12.773**

## Other publications

iv. T. Orosz, A. Bajtai, T. Minh Le, **D. Tanács**, Z. Szakonyi, F. Fülöp, A. Péter, I. Ilisz: Chiral high-performance liquid and supercritical fluid chromatographic enantioseparations of limonene-based bicyclic aminoalcohols and aminodiols on polysaccharide-based chiral stationary phases
Biomedical Chromatography. 33 (2019) e4517.
https://doi.org/10.1002/bmc.4517.
**if.: 1.911**

v. **D. Tanács**, T. Orosz, Z. Szakonyi, T.M. Le, F. Fülöp, W. Lindner, I. Ilisz, A. Péter: High-performance liquid chromatographic enantioseparation of isopulegol-based *β*-amino lactone and *β*-amino amide analogs on polysaccharide-based chiral stationary phases focusing on the change of the enantiomer elution order
Journal of Chromatography A. 1621 (2020) 461054.
https://doi.org/10.1016/j.chroma.2020.461054.
**if.: 4.601**

vi. A. Bajtai, **D. Tanács**, R. Berkecz, E. Forró, F. Fülöp, W. Lindner, A. Péter, I. Ilisz: High-performance liquid chromatographic evaluation of strong cation exchanger-based chiral stationary phases focusing on stationary phase characteristics and mobile phase effects employing enantiomers of tetrahydro-*β*-carboline and 1,2,3,4-tetrahydroisoquinoline analogs
Journal of Chromatography A. 1644 (2021) 462121.
https://doi.org/10.1016/j.chroma.2021.462121.
**if.: 4.601**

vii. **D. Tanács**, T. Orosz, I. Ilisz, A. Péter, W. Lindner: Unexpected effects of mobile phase solvents and additives on retention and resolution of N-acyl-D,L-leucine applying *Cinchonane*-based chiral ion exchangers
Journal of Chromatography A. 1648 (2021) 462212.
https://doi.org/10.1016/j.chroma.2021.462212.
**if.: 4.601**

viii. **D. Tanács**, A. Bajtai, R. Berkecz, E. Forró, F. Fülöp, W. Lindner, A. Péter, I. Ilisz: *Cinchona*-alkaloid-based zwitterionic chiral stationary phases as potential tools for high-performance liquid chromatographic enantioseparation of cationic compounds of pharmaceutical relevance
Journal of Separation Science. 44 (2021) 2735–2743.
https://doi.org/10.1002/jssc.202100264.
**if.: 3.614**

ix. R. Berkecz, **D. Tanács**, A. Péter, I. Ilisz: Enantioselective liquid chromatographic separations using macrocyclic glycopeptide-based chiral selectors
Molecules. 26 (2021) 3380.
https://doi.org/10.3390/molecules26113380.
**if.: 4.927**

**Total if.: 37.028**

## Posters and presentations

x. **Dániel Tanács**, Ferenc Fülöp, Antal Péter, István Ilisz: Ultrahigh-performance liquid chromatographic enantioseparation of some $\beta^2$-amino acids
26$^{th}$ International Symposium on Analytical and Environmental Problems, 23-24. November 2020., Szeged; Presentation

xi. **Tanács Dániel**, Berkecz Róbert, Aleksandra Misicka, Dagmara Tymecka, Fülöp Ferenc, Daniel W. Armstrong, Péter Antal, Ilisz István: $\beta^2$-Aminosavak enantioszelektív elválasztása teikoplanin és teikoplanin-aglikon szelektorral rendelkező héjszerkezetű királis állófázisok segítségével
METT 25, a Magyar Elválasztástudományi Társaság 25 éves jubileumi konferenciája, 18-21. October 2021., Egerszalók; Poster

xii. **Dániel Tanács**, Róbert Berkecz, Antal Péter, István Ilisz: Enantioselective separations with high- and ultrahigh-performance chiral liquid chromatography stationary phases
27$^{th}$ International Symposium on Analytical and Environmental Problems, 22-23. November 2021., Szeged; Presentation

xiii. **Dániel Tanács**, Róbert Berkecz, Sayeh Shahmohammadi, Enikő Forró, Daniel W. Armstrong, Antal Péter, István Ilisz: Liquid chromatographic enantioseparation of fluorinated $\beta$-phenylalanine analogs utilizing superficially porous particles

33rd International Symposium on Chromatography, 18-22. September 2022., Budapest; Poster

xiv. **Dániel Tanács**, Róbert Berkecz, Antal Péter, István Ilisz: Enantioselective separations of proline analogs with macrocyclic glycopeptide-based chiral stationary phases

28th International Symposium on Analytical and Environmental Problems, 14-15. November 2022., Szeged; Presentation