University of Szeged
Faculty of Pharmacy
Institute of Pharmaceutical Analysis

Enantioselective high-performance liquid chromatographic separations utilizing *Cinchona* alkaloid- and polysaccharide-based chiral stationary phases

Ph.D. thesis

Gábor Németi

Supervisor:
Dr. István Ilisz

2025

University of Szeged

Doctoral School of Pharmaceutical Sciences
Head: Prof. Dr. Judit Hohmann, D.Sc.

Educational program: Pharmaceutical Analysis
Program director: Prof. Dr. István Ilisz, D.Sc.
Institute of Pharmaceutical Analysis
Supervisor: Prof. Dr. István Ilisz, D.Sc.

Gábor Németi

Enantioselective high-performance liquid chromatographic separations utilizing *Cinchona* alkaloid- and polysaccharide-based chiral stationary phases

Final Exam Committee:
Head: Prof. Dr. István Zupkó, D.Sc.
Members: Prof. Dr. Pál Sipos, D.Sc.
Dr. Krisztián Horváth, Ph.D.

Reviewer committee:
Head: Prof. Dr. Dezső Csupor, D.Sc.
Reviewers: Prof. Dr. Attila Hunyadi, D.Sc.
Dr. Krisztián Horváth, Ph.D.
Members: Dr. Anita Sztojkov-Ivanov, Ph.D.
Dr. Andrea Vasas, Ph.D.

Introduction

Enantiomeric pairs, which are non-superimposable mirror images of each other, can exhibit strikingly different biological activities, influencing not only the effectiveness of a drug but also its safety profile. These mirror-image molecules can have varying interactions with biological systems, since various chiral compounds exist in living organisms, such as proteins, sugars, and enzymes. In the world of pharmaceuticals, this discrepancy often leads to differences in therapeutic outcomes because of the different interactions. Furthermore, the desired pharmacological activity is often restricted to one of the enantiomers (eutomer), while the other enantiomer (distomer) may induce unwanted side effects or it can even be toxic during its metabolism. Chirality can be observed not only in pharmaceuticals but also in food additives, chirality can be observed (e.g., amino acids). These different effects of enantiomers emphasize the importance of synthesizing pure enantiomers or separating racemic (or scalemic) mixtures.

Liquid chromatography is the most widely used technique for enantiomeric separations due to its versatility under varying chromatographic conditions. The choice of stationary and mobile phases, which can greatly vary in polarity, plays a crucial role in the separation of analytes. The two members of the enantiomer pairs have the same physical and chemical properties. Consequently, their separation requires a chiral environment, where the enantiomers can form diastereomeric pairs with a selector through secondary interactions of different strengths. The interactions between the selector (SO) and the selectand (SA) can vary depending on the chromatographic environment (e.g., the solvation shell formed by the mobile phase) and the ligand attached to the silica backbone of the HPLC column.

Objectives

The primary objectives of this PhD work were to achieve the liquid chromatographic separation of enantiomeric pairs of pharmacologically important compounds, as well as to investigate the mechanisms of interactions between the chiral SO and the SAs. These potential pharmaceutical agents are typically synthesized as a series of structurally related compounds with diverse functional groups, enabling the examination of differences in retention patterns due to the fine structural dissimilarities of both the SOs and SAs.

In order to assess the relationships between the molecular structure of different families of SOs and the chromatographic characteristics of the enantioseparation of various SAs, the main aims of this study are as follows:

- investigation of the effects of the mobile phase composition, focusing on the quality and ratio of the bulk eluent components, as well as the nature and concentration of acid and base eluent additives, on chromatographic parameters,
- comparative examination of the effects of variations in molecular structures on enantioseparations,
- exploration of the unique characteristics of the higher-order structure of the polysaccharide (PS) chains through the sequential application of diverse eluent compositions,
- thermodynamic characterization of enantioseparations by interpretation of the effect of temperature on chromatographic parameters.

Experimental

Measurements were carried out on two HPLC systems. The first is a Waters Breeze system (Waters Incorporation, Milford, MA, USA) with Empower2 software, and the second system is a Shimadzu Prominence system (Shimadzu Corporation, Kyoto, Japan) with Lab-Solution software. Both chromatographic systems are equipped with the following modules: a binary pump, a photodiode array detector, an autosampler, and a column thermostat, additionally, the Shimadzu system is equipped with a solvent degasser.

The applied chiral stationary phases (CSPs) can be grouped into two families based on their selectors. *Cinchona* alkaloid-based selectors are chiral ion exchangers, provided by Chiral Technologies Europe (Illkirch, France). The zwitterionic-type ion exchanger **ZWIX(+)** and **ZWIX(−)** (150 × 3.0 mm ID, 3 μm particle size for both columns), and the anion exchanger-type QN–AX and QD–AX columns (150 × 4.6 mm ID, 5 μm particle size for both columns) were applied for the enantioseparation of amino acid derivatives. The PS-based selectors were provided by different manufacturers, they all were applied for the enantioseparation of monoterpene lactone derivatives. The columns of Chiral Technologies Europe, namely Chiralpak IA, IB, IC, ID, IE, IF, and IG, all contain immobilized-type selectors. All Phenomenex columns have the same physical characteristics (250 × 4.6-mm ID, 5 μm particle size). IB and IC are cellulose-based, while the others are amylose-based. Phenomenex columns (Torrance, CA, USA) were of

the coated-type, namely Lux Amylose-1, Lux Cellulose-1, and Lux Cellulose-4, except for Lux i-Amylose-1, which is an immobilized-type column.

The studied analytes can be grouped into three categories: fluorinated β^3-phenyalanines (**FBP**), amino, thio, and oxy analogs of monoterpene lactone derivatives (**ATO**), and azole and benzoazole analogs of monoterpene lactone derivatives (**ABA**). At least one of the two enantiomers ("A" and/or "B") was available in enantiomerically pure form (enantiomeric excess > 99%) for each analyte. The majority of the analytes are examined for the first time in publications related to this work. Although results for a few of the analytes were published in previous studies, they were mostly obtained using different CSPs. The structures of the "A" configurations of the investigated analytes are shown in **Figure 1**.

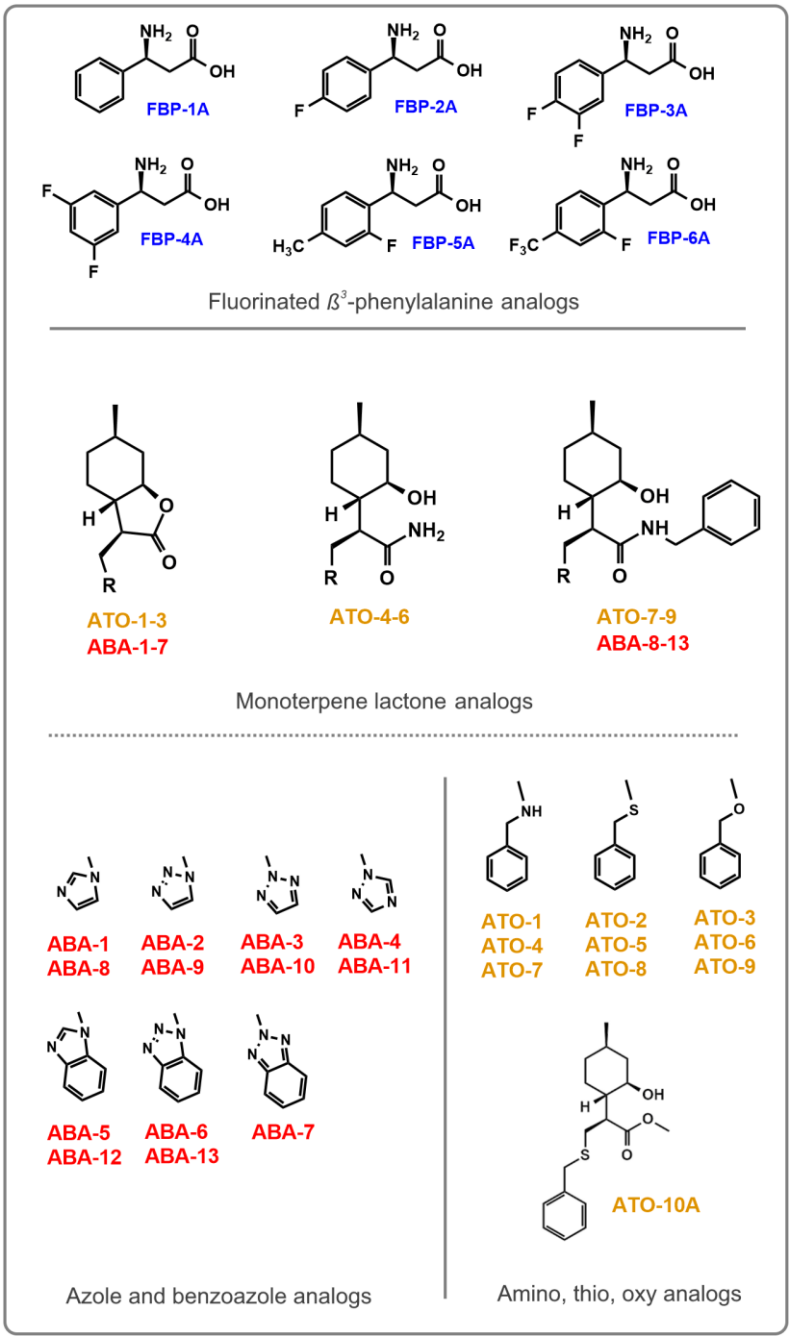

Figure 1. Structures of the analytes ("A" configurations)

Results

I) Results obtained on column selection and mobile phase composition

The enantiomers of FBP analytes were efficiently separated with good selectivity using ZIE CSPs at a moderate or even low MeCN ratio, while the anion exchangers lacked significant enantiorecognition ability. The potential effectiveness of polar organic mode (POM) to normal phase mode (NPM) with PS-based CSPs has been demonstrated with ATO and ABA analytes. The differences between coated and immobilized PS-type columns were found to be more pronounced in POM, indicating diverse effects on the retention mechanisms due to the different solvation shells.

In the case of zwitterionic *Cinchona* alkaloid-based CSPs, retention and selectivity increased with an increasing amount of aprotic MeCN relative to the protic MeOH in the bulk eluent composition. However, selectivity and resolution usually changed according to a maximum curve, reaching the maximum at a composition of MeOH/MeCN 50/50 or 25/75 (*v/v*). All **FBP** analytes were separated with good selectivity at a moderate or low ratio of MeCN in the bulk mobile phase applying the ZIE-type columns. In contrast, the applied anion exchanger columns were found to be unsuitable for the enantioseparation of the studied **FBP** derivatives.

With PS-based CSPs, retention showed typical NP behavior when separating the enantiomers of **ATO** analytes as the retention factor decreased with increasing alcohol concentrations present in the *n*-hexane bulk eluent. Enantiomer elution order (EEO) reversals were observed in several cases when an alcohol of a different nature or concentration was present in the mobile phase. The retention factors of the first peaks were lower, while the enantioselectivities and resolutions were higher on the coated-type column in NPM. In most cases, retention factors, applying only neat alcohols in PO mode when separating the enantiomers of **ABA** analytes, decreased in the order EtOH > MeOH > 1-PrOH > 2-PrOH. Regarding the types of the PS columns, higher differences can be seen, depending on the nature of the PO solvents in POM, than in NPM. Differences in retentions between the coated- and immobilized-type CSPs were dependent on the mobile phase, suggesting a more complex influence on the retention mechanism due to the different solvation shells associated with each column type. The eluent compositions used in the further studies were selected based on these results.

II) Results obtained on the effects of the mobile phase additives

Neither the nature of the base nor the concentration of additives influenced significantly the enantioselectivity and resolution on either the ZIE- or PS-based CSPs. However, retentions of FBP analytes were notably affected in the case of ZIE CSPs. In contrast, retentions of ATO and ABA analytes on PS-based CSPs were found to be only slightly affected by the additives, more likely depending on the acid-base characteristics of the studied analytes.

Acid and base additives function as counterions in the case of ZIE CSPs. The nature of the base affected the retention when separating the enantiomers of **FBP** analytes; the elution strength was TEA < DEA < EA in all cases. Retention decreased with increasing counterion concentration, and it could be described with the simple stoichiometric displacement model. Neither the nature of the base nor the concentration of additives had a marked effect on selectivity and resolution. This effect underscores the importance of counterion selection in optimizing retention without compromising selectivity or resolution.

In the case of PS-based CSPs, when separating either the **ATO** or **ABA** analytes, the additives had slight effects on the chromatographic parameters. An important exception was observed when the enantioseparation of two of the most basic **ABA** monoterpene derivatives deteriorated severely when only formic acid was added into the mobile phase both in NP and polar ionic (PI) modes, where dissociation or ion-pair formation was a plausible cause of the altered interaction mechanism. This effect suggests a dependence on the acid-base characteristics of the analytes. For other analytes, the impact of the additives was found to be minimal in the case of PS-type CSPs.

III) Results obtained on the structure–retention relationships

The presence of the trifluoromethyl group in the FBP analytes led to weaker enantioselective interactions when higher MeCN ratios were utilized in the mobile phase on ZIE CSPs. A direct correlation between retention and size of the ATO-analytes was revealed on PS-based CSPs. Amylose-based CSPs demonstrated superior separation performance overall. Coated-type PS-based CSPs require special attention in the case of using binary mobile phases.

Interestingly, one **FBP** derivative showed lower selectivities at higher MeCN ratios than the other **FBP** analogs, which suggests that the trifluorination of the methyl group on the phenyl ring has a pronounced effect on the enantioselectivity. EEOs were determined in all cases with *R* < *S* for all **FBP** analytes on the **ZWIX(–)** column, and *S*

< *R* on the **ZWIX(+)** column without any exception. When comparing the columns, the chromatographic parameters were larger on the **ZWIX(–)** CSP than on the **ZWIX(+)**, and the EEOs were reversed.

The amylose-based CSPs overall offered a better fit for the studied **ATO** and **ABA** analytes than the cellulose-based CSPs, independent of the applied chromatographic mode. The EEOs of monoterpene lactone derivatives were varied, seemingly in an unsystematic way. Retention of **ATO** analytes on PS-based CSPs showed a correlation with the size of the analytes in NPM. On the contrary, the nature of the polar solvent in POM affects retention and enantiorecognition in several ways, resulting in pronounced differences, even in the case of structurally related compounds. The effects of binary mobile phases required further investigations due to peculiar retention anomalies (a.k.a. hysteresis) that are plausibly explained by the changes in the higher-order structures of PS chains, which mainly affect the coated-type CSPs.

IV) Results obtained on the hysteresis phenomena

Hysteresis phenomena were observed in various instances when applying PS-based CSPs for the enantioseparation of ABA analytes. The PrOH-containing binary eluent systems showed the highest deviations in the chromatographic parameters depending on the direction from which the given composition is approached. The logarithmic value of hysteresis of selectivity (based on the configuration), analogous to the selectivity, was first introduced in order to comparably present the hysteretic values.

The hysteretic behavior of PS-based CSPs was documented in several cases together with EEO reversals in the case of the enantioseparations of **ABA** analytes. The hystereticity factor (v) describes the hysteresis of the retention factor and it is defined based on the direction from which the given composition is approached. To decide whether hysteresis is present or not, simplified confidence intervals of hystereticity were calculated, and hysteresis loops and tables with data of hystereticity and hysteresis of selectivity (based on the configuration) were constructed. This hysteresis of selectivity can be accurately and comparably presented by the (v_A/v_B) values. Additionally, information can be extracted about the percentage contribution of each enantiomer to the hysteresis. Our research group was the first to propose this novel evaluation methodology for hysteresis data, including the calculation of confidence intervals, hysteresis of selectivity, percentage contribution of an enantiomer to the overall hysteresis, and the logarithmic approach, ultimately allowing for the discovery of hysteresis–configuration

relationships. The highest hysteresis was observed in POM in binary eluent systems containing 1- and 2-PrOH, while in the presence of MeOH, EtOH, and MeCN, typically modest or low hysteresis was found. In general, only low hystereticity can be expected in NPM, in the case of cellulose-based CSPs or immobilized CSPs. The undesirable effects of hysteresis can be prevented with proper column handling.

V) Results obtained on the effect of temperature

In the case of all FBP and ATO analytes, increasing the temperature of the separations resulted in lower retention factors and decreased selectivities in the majority of cases, indicating that these separations were enthalpically driven. The calculated apparent thermodynamic parameters supported this premise.

The effect of temperature was investigated for both types of CSPs in the temperature range of 10–50 °C. The apparent thermodynamic parameters were calculated by the *van't Hoff* equation. Confidence intervals, rarely applied in the evaluation of *van't Hoff* plots, helped the evaluation of data obtained on both the ZIE- and PS-type columns. Retention factors decreased with increasing temperature in all cases, while selectivities mostly decreased as well. When the ZIE columns were utilized, both $\Delta(\Delta H^0)$ and $\Delta(\Delta S^0)$ values changed in a narrow range and they were influenced more significantly by the structural peculiarities of the **FBP** analytes rather than changes in the flow rate, even if the analytes are structurally closely related. A clear tendency can be seen between the MeCN content and the thermodynamic parameters when changing the MeCN ratio in the bulk eluent. Relatively small differences in thermodynamic parameters between analytes indicate similar characteristics in the separation process, except for the trifluoromethylated phenylalanine derivative **FBP–6**, which showed no significant change in selectivity as a function of temperature on **ZWIX(+)** in 100% MeOH mobile phase, while all other separations were enthalpically driven.

The calculated apparent thermodynamic parameters of the **ATO** analytes were more diverse, as well as their structure, compared to the **FBP** analytes. Most of the enantioseparations were enthalpically driven. Conversely, a few of the enantioseparations were entropically driven, while the driving force of some other enantioseparations could not be stated at a 95% confidence level due to the relatively wide confidence intervals in their *Q* values. Amylose-based CSPs showed more negative $\Delta(\Delta H^0)$ and $\Delta(\Delta S^0)$ values than cellulose-based CSPs, while only slight differences were found when SOs with different phenylcarbamate groups were compared.

List of publications, presentations, and posters

Publications related to the thesis

I. **G. Németi**, R. Berkecz, S. Shahmohammadi, E. Forró, W. Lindner, A. Péter, I. Ilisz: Enantioselective high-performance liquid chromatographic separation of fluorinated *ß*-phenylalanine derivatives utilizing Cinchona alkaloid-based ion-exchanger chiral stationary phases: Enantioselective separation of fluorinated *ß*-phenylalanine derivatives
Journal of Chromatography A, 1670 (2022) 462974
https://doi.org/10.1016/j.chroma.2022.462974
if.: 4.1 (Analytical Chemistry: Q1)

II. A. Bajtai, **G. Németi,** T. M. Le, Zs. Szakonyi, A. Péter, I. Ilisz: Enantiomeric separation of newly synthesized amino, thio, and oxy derivatives of monoterpene lactones, amides, and ester applying polysaccharide-based chiral stationary phases in normal-phase mode
Journal of Chromatography A, 1672 (2022) 463050
https://doi.org/10.1016/j.chroma.2022.463050
if.: 4.1 (Analytical Chemistry: Q1)

III. **G. Németi**, R. Berkecz, T. M. Le, Zs. Szakonyi, A. Péter, I. Ilisz
High-performance liquid chromatographic enantioseparation of azole analogs of monoterpene lactones and amides focusing on the separation characteristics of polysaccharide-based chiral stationary phases
Journal of Chromatography A, 1717 (2024) 464660
https://doi.org/10.1016/j.chroma.2024.464660
if.: 3.8 (2023, Q2)
Sum if.: 12.0

Other publications

IV. A. Bajtai, Gy. Lajkó, **G. Németi**, I. Szatmári, F. Fülöp, A. Péter, I. Ilisz: High-performance liquid chromatographic and subcritical fluid chromatographic separation of α-arylated β-carboline, *N*-alkylated tetrahydroisoquinolines and their bioisosteres on polysaccharide-based chiral stationary phases
Journal of Separation Science, 42 (2019) 2779–2787
https://doi.org/10.1002/jssc.201900228
if.: 2.9 (2.878, Q2)

V. R. Berkecz, **G. Németi**, A. Péter, I. Ilisz: Liquid chromatographic enantioseparations utilizing chiral stationary phases based on crown ethers and cyclofructans
Molecules, 26 (2021) 4648–4866
https://doi.org/10.3390/molecules26154648
if.: 4.9 (4.927, Q2)

VI. J. P. Mészáros, **G. Németi**, J. M. Poljarevic, T. Holczbauer, N. V. May, É. A. Enyedy: Effect of the additional carboxyl group in half-sandwich organometallic 2,4-dipicolinate complexes on solution speciation and structure
European Journal of Inorganic Chemistry, 19 (2021) 1858–1868
https://doi.org/10.1002/ejic.202100122
if.: 2.6 (2.551, Q2)

VII. J. P. Mészáros, V. F. S. Pape, G. Szakács, **G. Németi**, M. Dénes, T. Holczbauer, N. V. May, É. A. Enyedy: Half-sandwich organometallic Ru and Rh complexes of (*N*,*N*) donor compounds: effect of ligand methylation on solution speciation and anticancer activity
Dalton Transactions, 50 (2021) 8218–8231
https://doi.org/10.1039/D1DT00808K
if.: 4.6 (4.569, Q1)

VIII. **G. Németi**, R. Berkecz, A. Péter, W. Lindner, I. Ilisz: Cinchona alkaloid-based zwitterionic chiral stationary phases applied for liquid chromatographic enantiomer separations: an updated overview
Book chapter of G.K.E. Scriba, ed., Chiral Separations, Springer New York, New York, NY, expected release in 2025

Total if.: 26.92

Presentations

I. **G. Németi**, S. Shahmohammadi, E. Forró, A. Péter, I. Ilisz: Investigation of enantioselective HPLC separations of fluorinated β^3-phenylalanine derivatives
27th International Symposium on Analytical and Environmental Problems; Szeged; Hungary; November 22–23; 2021

II. **G. Németi**, T. M. Le, Zs. Szakonyi, A. Péter, I. Ilisz: HPLC separations of *N*-azole compounds in polar organic and normal phase mode utilizing amylose-based chiral stationary phases
28th International Symposium on Analytical and Environmental Problems; Szeged; Hungary; November 14–15; 2022

III. **G. Németi**, D. Ozsvár, R. Berkecz, A. Péter, W. Lindner, I. Ilisz: Enantioselective separation of substituted amino acids utilizing *Cinchona* alkaloid-based chiral stationary phases
29th International Symposium on Analytical and Environmental Problems; Szeged; Hungary; November 13–14; 2023

Posters

I. **Németi G.**, Forró E., Fülöp F., Péter A., Ilisz I.: Fluorozott β^3-fenilalaninszármazákok folyadékkromatográfiás kölcsönhatásainak vizsgálata ioncserélő típusú királis állófázisokon
METT25; Egerszalók, 2021 Október 18–20

II. **G. Németi**, T. L. Minh, Zs. Szakonyi A. Péter, I. Ilisz: High-performance liquid chromatographic separations in polar organic and normal phase mode utilizing polysaccharide-based chiral stationary phases
33rd International Symposium on Chromatography; Budapest, Hungary, September 18–22; 2022

III. **G. Németi**, D. Ozsvár, R. Berkecz, P. Antal, W. Lindner, I. Ilisz: HPLC study of the enantioselective separation of β-methyl-substituted amino acids applying ionexchanger-based chiral stationary phases
13th Balaton Symposium on High-Performance Separation Methods; Siófok, 2023 Szeptember 4–6

IV. **Németi G.**, T.M. Le, Szakonyi Zsolt, Péter Antal, Ilisz István
Pirimidinszármazékok folyadékkromatográfiás kölcsönhatásainak vizsgálata poliszacharid típusú királis állófázisokon
Elválasztástudományi Vándorgyűlés 2024; Visegrád–Lepence, 2024. November 7–9.

Acknowledgments

I would like to express my gratitude to my supervisor, Professor István Ilisz, Head of the Institute of Pharmaceutical Analysis, for his invaluable guidance and for providing me with the opportunity to make my M.Sc. and Ph.D. work. I am grateful to Professor Antal Péter for his mentorship, who also guided my work. I also would like to thank Dr. Tímea Orosz, a former Ph.D. student, who guided me in my initial years in the research group. Their mentorship and support were essential to the advancement of my knowledge and research in this field.

I would like to thank my colleagues, Dr. Attila Bajtai, Dr. Dániel Tanács, Dr. Tímea Körmöczi, Dr. Róbert Berkecz, and Gabriella Vasas for their advice in diverse fields, Melinda Guba and Dr. Dániel Ozsvár for their help with my measurements, as well as to all other members of the Institute of Pharmaceutical Analysis.

I also want to thank Professor Árpád Molnár for proofreading my thesis.

I would like to express my sincerest gratitude to my parents and sister for their support and encouragement.

This work was supported by UNKP–22–3–SZTE–156 New National Excellence Program of the Ministry for Culture and Innovation from the source of the National Research, Development and Innovation Fund, and by the National Research, Development and Innovation Office–NKFIA through project K137607. Project no. TKP2021–EGA–32 has been implemented with the support provided by the Ministry of Culture and Innovation of Hungary from the National Research, Development and Innovation Fund, financed under the TKP2021–EGA funding scheme.