Journal of Chromatography A 1683 (2022) 463529

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Separation of the enantiomers of underivatized amino acids by using serially connected dual column high-performance liquid chromatography-tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 13 June 2022
Revised 19 September 2022
Accepted 21 September 2022
Available online 23 September 2022

Keywords:
Native amino acids
Enantioseparation
Chiral crown ether
Dual column
ZIC-HILIC

ABSTRACT

In this article, a serially connected dual column liquid chromatography-tandem mass spectrometry (LC-MS/MS) method is described for the simultaneous separation and enantioseparation of proteinogenic amino acids. For this purpose, different achiral and chiral stationary phases (CSP) and mobile phase compositions have been tested. As a result of the optimization studies, the best enatioseparation for amino acids were achieved with a combination of zwitterionic and crown ether stationary phases using a gradient of two mobile phases: A (water:TFA 99.5:0.5, % v/v) and B (acetonitrile:ethanol:TFA 85:15:0.5, % v/v/v). The developed method provided simultaneous enantioseparation of all proteinogenic amino acids under this study including isomeric and isobaric ones except for proline. The method was successfully applied to human lung adenocarcinoma cells (A549) and healthy human lung epithelial cells (BEAS-2B) cultivated with D-amino acid containing cocktails in order to evaluate D-amino acids transfer rate in normal and cancer lines. TheD/L amino acid ratios were different in cancer and normal cell lines cultivated as mentioned above for aspartic acid, cysteine, methionine, phenylalanine, and serine.

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1. Introduction

Amino acids are potential biomarkers that have critical role in metabolism, neurotransmission, pain control, gene expression, oxidative stress, detoxification, etc. Twenty proteinogenic amino acids appear in the genetic code and serve as building blocks of the proteins. Except for glycine, proteinogenic amino acids are chiral and exist in two enantiomeric forms. In the living body, predominantlL forms are found [1]. Apart from the L-forms, the trace amounts of D-amino acids have physiological functions, as well as diagnostic value [2–4]. Because of the biological importance of both enantiomers, the chiral separation and the quantification is of utmost importance. Up to date, different analytical techniques are reported for this purpose, including gas chromatography-mass spectrometry, liquid chromatography (LC), supercritical fluid chromatography, and electrodriven methods such as capillary electrophoresis and capillary electrochromatography [5,6]. Among these techniques, liquid chromatography is one of the most preferable due to its high flexibility from the viewpoint of both varieties of the chiral stationary phases (CSPs) and the mobile phase composition. Several CSPs are available for the separation of the enantiomers of the amino acid without derivatization. These CSPs are based on crown ethers [7], macrocyclic antibiotics [8], zwitterionic quinine derivatives [9] and cyclofructan [10] type chiral selectors. Polysaccharide-based materials are also used (but rarely) for the separation of the enantiomers of the underivatized chiral amino acids [11,12]. The alternative approach is converting enantiomers of native amino acids into their derivatives before the separation by using precolumn derivatization reagents [13–15]. The purpose of the derivatization of the samples is the improvement of the detectability of the amino acids by using UV/VIS or fluorescence detectors, their interaction with chiral selectors, as well as by using a single enantiomer of a chiral derivatizing reagent converting enantiomeric pairs into diastereomeric ones. The use of chiral columns is not necessarily required for diastereomeric derivatives. They can be separated on

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https://doi.org/10.1016/j.chroma.2022.463529
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Table 1
List of targeted amino acids with their optimum MRM conditions.

Amino acid	m/z	Collision Energy (eV)	Q1 (V)	Q3 (V)
Alanine	89.80 → 44.10	-13.0	-20.0	-18.0
Arginine	174.90 → 70.10	-23.0	-20.0	-13.0
Asparagine	133.10 → 74.05	-17.0	-10.0	-14.0
Aspartic acid	134.10 → 74.10	-15.0	-14.0	-15.0
Phenylalanine	166.20 → 120.10	-16.0	-12.0	-28.0
Glycine	75.80 → 30.00	-12.0	-15.0	-27.0
Glutamic acid	148.20 → 84.00	-17.0	-10.0	-18.0
Glutamine	147.20 → 83.90	-16.0	-10.0	-17.0
Histidine	156.00 → 110.0	-12.0	-20.0	-25.0
Isoleucine	132.20 → 86.10	-14.0	-14.0	-18.0
Lysine	147.00 → 130.00	-8.0	-14.0	-18.0
Leucine	132.20 → 86.10	-13.0	-14.0	-18.0
Methionine	149.90 → 104.00	-14.0	-16.0	-22.0
Proline	116.00 → 70.00	-16.0	-10.0	-21.0
Serine	106.00 → 60.00	-12.0	-12.0	-18.0
Cysteine	122.05 → 59.00	-24.0	-10.0	-22.0
Tyrosine	181.80 → 136.10	-15.0	-20.0	-15.0
Treonine	119.80 → 74.20	-14.0	-13.0	-16.0
Tryptophan	205.00 → 188.00	-7.0	-11.0	-13.0
Valine	118.00 → 72.00	-15.0	-13.0	-16.0

* Racemic mixture standard. Q1: First quadrupole voltage, Q3: Third quadrupole voltage. The dwell time for each MRM transition was set as 10 ms.

achiral columns. However, indirect chiral separation has some disadvantages such as interferences of impurities from the derivatization reagents, additional costs, and also the extra time required for the sample preparation.

Underivatized amino acids can be detected using corona charged aerosol, evaporative light scattering, refractive index and mass spectrometry (MS) detectors [16]. Although MS-based hyphenated techniques are useful and versatile tools for the determination of the amino acids, matrix effect and ion suppression need to be carefully controlled to avoid inaccurate results. Accuracy problems can be solved in a certain degree by using isotopically labeled standards [17]. Another challenge comes from the polar nature of amino acids. Due to this reason, polar native amino acids do not retain on conventional nonpolar C18 columns. To overcome this problem, hydrophilic interaction liquid chromatography (HILIC) is used [18]. Additional challenge is a separation of the isomeric and isobaric forms of the amino acids. A Quantification of the amino acids is rather challenging, especially in the case of complex biological matrices, because the L-amino acids can be found in the tissues in high concentrations while the D-forms are generally found on a trace level [17]. In the case of the complex biological media, 2D or 3D separation modes are used for the separation of the enantiomers of the amino acids, which is rather complicated and requires dedicated instrumentation [17,19–21]. Serially connected dual column systems, including achiral and chiral columns in combination, enable efficient enantioseparation and the enantioselective determination of native amino acids. One of the main encountered challenges in this process is to analyze isomeric (leucine/isoleucine) and isobaric (lysine/glutamine) amino acids simultaneously. To solve this problem, both chemo- and enantioselectivity have to be present. Chemoselectivity is of utmost importance since these amino acids also have the same multiple reaction monitoring (MRMs) parameters. In the literature, some successful attempts were published to separate the chiral amino acids [16]. Konya et al. reported the enantioseparation of the chiral amino acids without derivatization by using crown ether based chiral columns and liquid chromatography-time of flight mass spectrometry (LC-TOF-MS) with a total run time of 15 min [22]. However, with this method enantioseparation for proline was not achieved, and also isobaric amino acids lysine and glutamine were coeluted. Serial connections of the chiral anion exchange (Chiralpak QN-AX) and zwitterionic (Zwix(+) and/or Zwix(-)) columns were also used for the enantioresolution of the native amino acids by using LC-MS/MS [23]. Lämmerhofer and co-authors developed a heart-cut two-dimensional ultra-high-performance LC methodology in which a C18 achiral column was used in the first dimension and the tert-butylcarbamoylquinine core-shell chiral column in the second dimension after pre-column derivatization with Sanger's reagent (2,4-dinitrofluorobenzene) yielding chromogenic 2,4-dinitrophenylated amino acids [24]. To achieve higher resolution, a three-dimensional LC with the fluorescence detection system was developed for the native amino acids analysis after the derivatization of the compounds with 4-fluoro-7-nitro-2,1,3-benzoxadiazole [19]. In the first and second dimensions, a separation of amino acids from the matrix components was performed, while in the third-dimension chiral separation was obtained using Pirkle type CSP. As a result, the total run time of three-dimensional analysis is about 3 h per sample [19]. Karongo et al. worked on the shortening of the total run time, and as a result of their attempts, the enantioseparations of the 24 amino acids were achieved in 45 min by using multiple heart cutting online two-dimensional LC-MS [21].

In the present work, our goal was to optimize a combination of achiral and chiral column, as well as a mobile-phase gradient for simultaneous enantioseparation of a mixture of 18 underivatized amino acids with the special emphasis on the separation and enantioseparation of isomeric amino acid pair such as leucine/isoleucine and isobaric pair lysine/glutamine. In order to illustrate the suitability of the method for biological samples; the A549 human lung adenocarcinoma cells and its control BEAS-2B healthy human lung epithelial cells were exposed to the D-amino acids and then the content of the amino acids in the intracellular matrix was analyzed enantioselectively with the present method.

2. Materials and methods

2.1. Chemicals and reagents

D and L forms of alanine, arginine, asparagine, aspartic acid, cysteine, glycine, glutamic acid, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine were purchased from AFG Bioscience (Wood Dale, IL, USA). Methanol, acetonitrile, trifluoroacetic acid (TFA), ethanol, and water were purchased from Merck

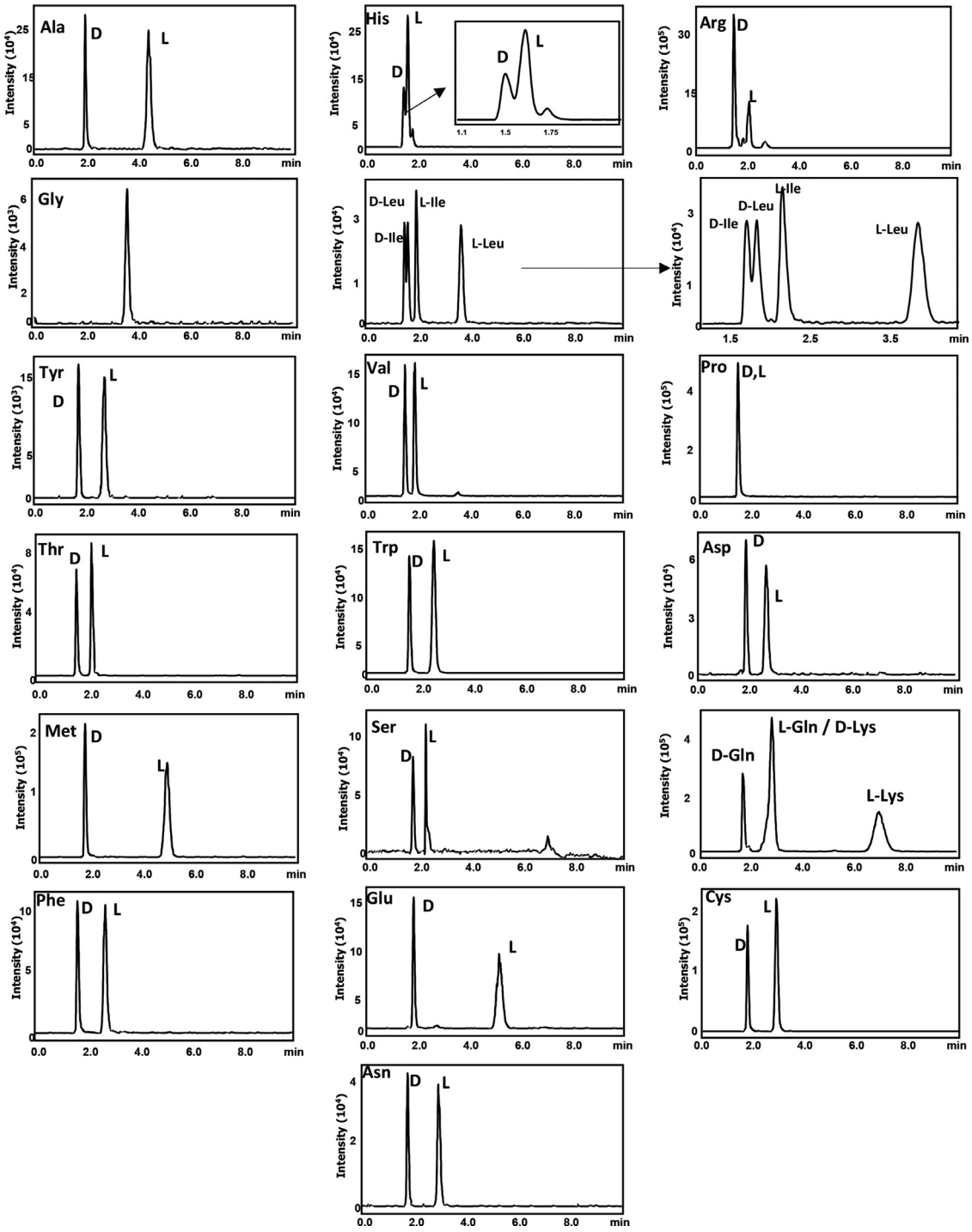


Fig. 1. Chiral separation of amino acids using Crownpak CR-I(+) (150 × 2.1 mm, 5 µm) with mobile phase A and B (5:95 v/v).

(Darmstadt, Germany), while formic acid was obtained from Fluka (St. Gallen, Switzerland). All solvents and chemicals were LC-MS grade.

Chiral columns studied were Lux i-Cellulose-5 (250 × 4.6 mm, 5 µm) (Phenomenex, Torrance, CA, USA), Astec Chirobiotic TAG (250 × 4.6 mm, 5 µm) (Merck, Darmstadt, Germany) and Crownpak CR-I (+) (150 × 3 mm, 5 µm) (Daicel, Tokyo, Japan). Achiral columns were Atlantis dC18 (75 × 4.6 mm, 3 µm) and Cortecs HILIC (50 × 2.1 mm, 1.6 µm) from Waters (Milford, MA, USA), Kinetex HILIC (50 × 2.1 mm, 1.7 µm) and Luna NH_2 (100 × 2 mm, 3 µm) from Phenomenex, SeQuant ZIC-HILIC (100 × 4.6, 5 µm) and SeQuant ZIC-HILIC (50 × 2.5 mm, 3.5 µm) from Merck.

2.2. Standard solutions

The standard stock solutions of D/L-tyrosine, D/L-glutamic acid, and D/L-aspartic acid were individually prepared at a 1000 µg/mL concentration in 1M HCl, while the others were in the water. All stock solutions were aliquoted and stored at -20 °C. A mixed stock

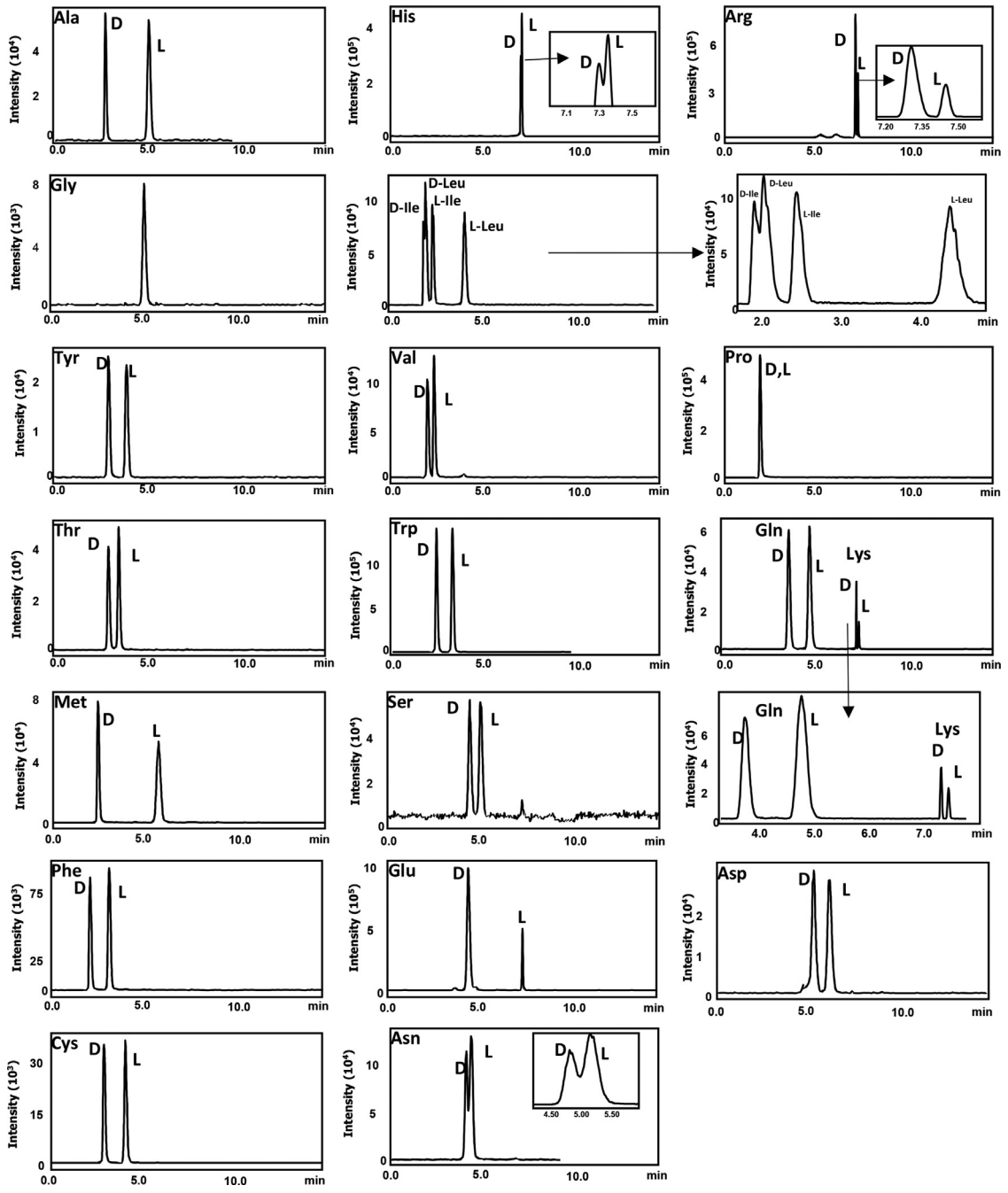


Fig. 2. Enantioseparation of amino acids using serially connected HILIC (50 × 2.1 mm, 3.5 µm) and Crownpak CR-I(+) (150 × 2.1 mm, 5 µm) with mobile phase A and B gradient elution. For further details see Experimental part.

solution containing all amino acids was prepared in the mobile phase at a final concentration of 50 µg/mL for each D/L-amino acid.

2.3. Cell culture

A549 (CCL-185^{TM}, ATCC, Washington, DC, USA) human lung adenocarcinoma cells and BEAS-2B (CRL-9609^{TM}, ATCC) human lung epithelial cells were cultured with Dulbecco's Modified Eagle Medium (DMEM) supplemented with penicillin-streptomycin (10,000 U/mL) and 10 % fetal bovine serum. The D-amino acid application for both cell lines was performed in the same medium spiked with a D-amino acids cocktail (Table S2). The cells were grown under standard culture conditions for 48 h (37 °C and 5% CO_2) in a humidified incubator. All cultures that reached 100% confluency were harvested.

2.4. Sample preparation

Petri dishes with cell lines that reached 100% confluency were taken out from the incubator and quickly washed with phosphate buffer saline solution. Afterwards, 1 mL of methanol:water (9:1, v/v) was added, and the cells were quickly frozen in liquid nitrogen to quench cells' metabolism. The frozen cells were scraped off with a cell scraper into Eppendorf tubes. The Petri dishes were washed by adding 1 mL of a mixture of methanol:water (9:1, v/v), and the extracts were combined. After the extracts were centrifugated at

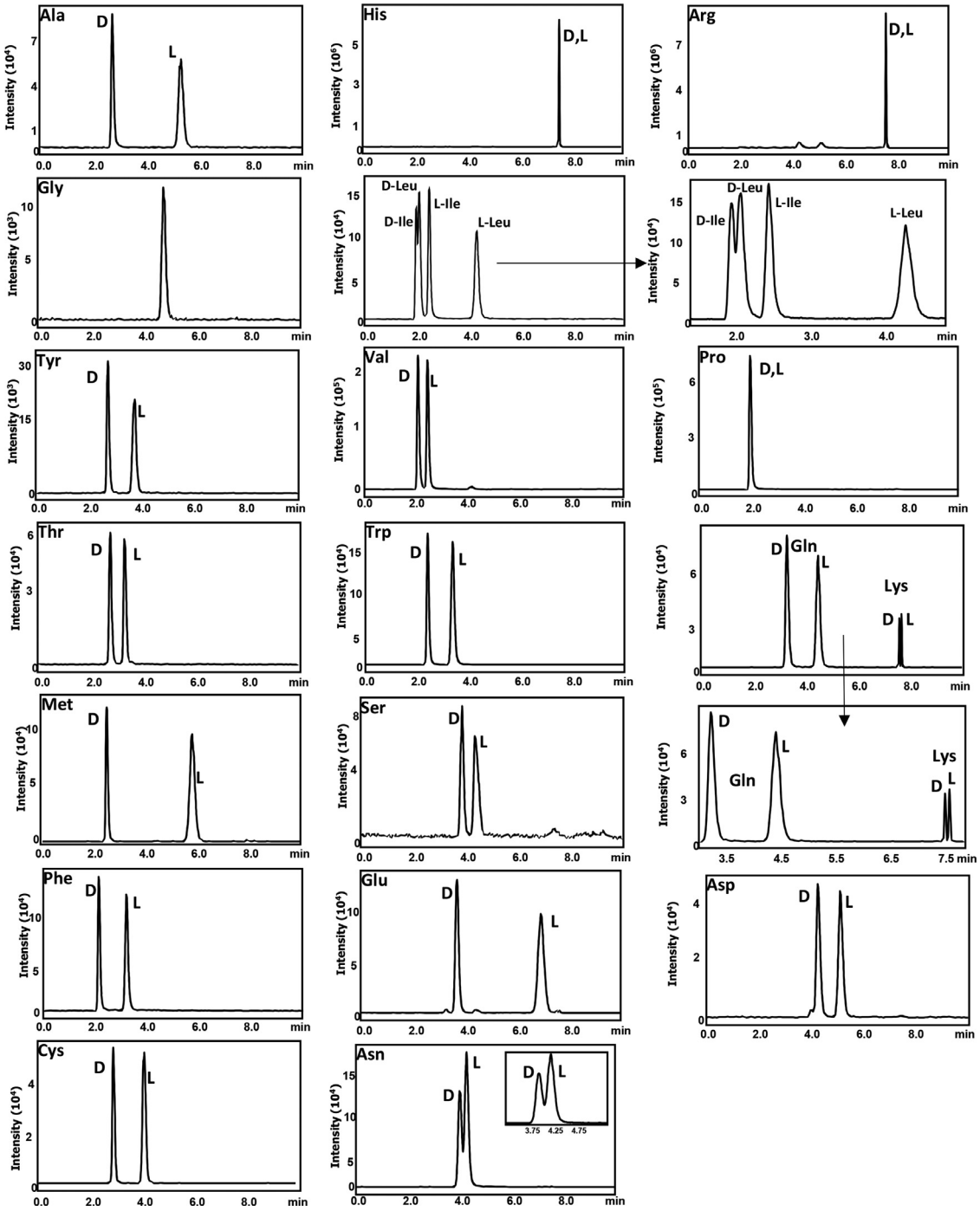


Fig. 3. Enantioseparation of amino acids using serially connected in reversed order (Crownpak CR-I(+) (150 × 2.1 mm, 5 µm) and HILIC (50 × 2.1 mm, 3.5 µm)) with mobile phase A and B in gradient elution. For further details see Experimental part.

18,188 g for 10 min and the supernatant was transferred to a 2 mL Eppendorf tube and stored at -80 °C until analysis.

A 200 µL aliquot of the cell culture sample was evaporated to dryness. Then the samples were reconstituted with 200 µL mobile phase (acetonitrile/ethanol/water/ 0.5% TFA, 85:10:5:0.5, % v/v/v). After vortexing for 1 min and centrifugation at 18,188 g for 3 min, a 150 µL of supernatant was transferred into a vial and analyzed by LC-MS/MS.

2.5. Instruments

The LC-MS/MS used in this study consisted of a Shimadzu (Kyoto, Japan) LC-20AXR system coupled to a Shimadzu 8030 MS/MS triple quadrupole tandem mass spectrometer. The mass spectrometer was operated in positive electrospray ionization and MRM mode. Data acquisition and processing were carried out using the Lab Solution Software version 5.72.

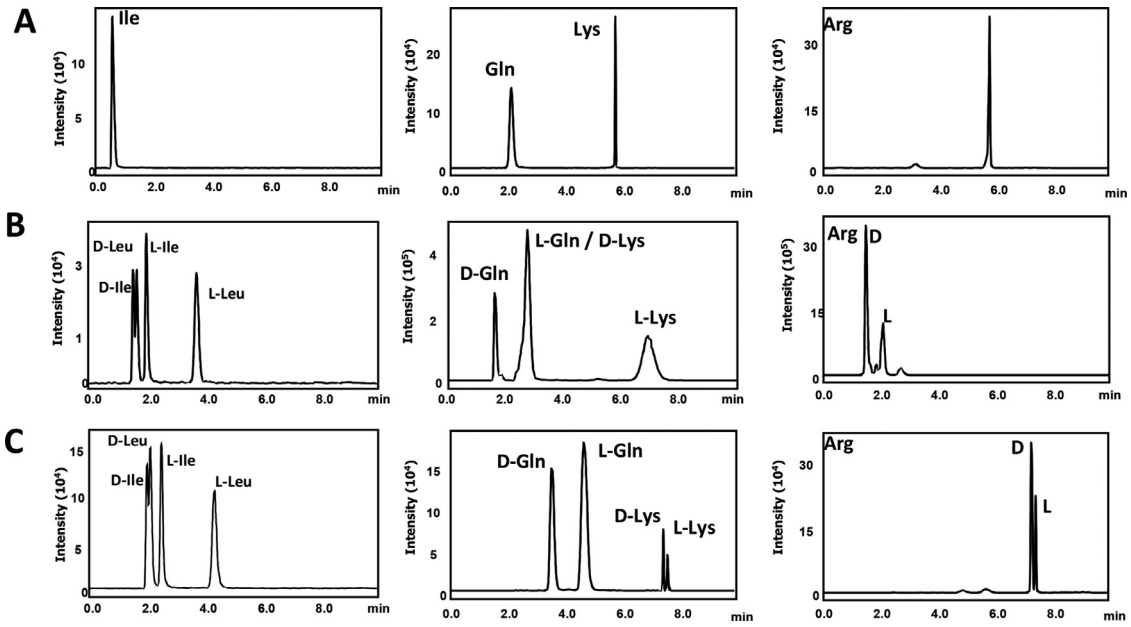


Fig. 4. Enantioseparation of isoleucine, leucine, arginine, glutamine and lysine at optimal chromatographic conditions (A) HILIC (50 × 2.1 mm, 3.5 µm) (B) Crownpak CR-I(+) (150 × 2.1 mm, 5 µm) and (C) serially connected HILIC (50 × 2.1 mm, 3.5 µm) and Crownpak CR-I(+) (150 × 2.1 mm, 5 µm). For further details see Experimental part.

2.6. Optimized Liquid chromatography and mass spectrometry conditions

All experiments were carried out using a serially connected column in the order of achiral SeQuant ZIC-HILIC, 50 × 4.6 mm, 3.5 µm) column and chiral column Crownpak CR-I (+), 150 × 3 mm, 5 µm). The flow rate was set to 0.4 mL/min and the column temperature was set to 30 °C. A gradient elution program with mobile phases A (water:TFA 99.5:0.5, % v/v) and B (acetonitrile:ethanol:TFA 85:15:0.5, % v/v/v) was run. The gradient elution was applied to the mobile phase as follows: from 0 to 3 min the mobile phase contained 95% B; from 3 to 6 min the percentage of B decreased to 25%; from 6 to 7 min the B percentage increased to 95%, and kept at this ratio for 3 min. The sample injection volume was 10 µL, while the run time was 12 min. In all experiments, collision activated dissociation (CAD) gas (argon) served as medium (arbitrary units) and nebulizer at a gas flow rate of 3 mL/min, drying gas flow rate of 15 L/min, desolvation line temperature of 250 °C and heat block temperature of 400 °C were set for the analysis.

2.7. Optimization of MS/MS parameters

MRM conditions of each amino acid were determined in order to perform simultaneous analysis of the D and L forms of proteinogenic amino acids. For this purpose, standard stock solutions of each amino acid were prepared at 1 µg/mL concentrations by diluting the standard solutions with acetonitrile: water (1:1, % v/v, both containing 0.1% FA). Afterward, the injections of each amino acid were made directly into the mass spectrometer using a mobile phase of acetonitrile: water (1:1, % v/v, both containing 0.1% FA) at a flow rate of 0.3 mL/min and MRM conditions (fragmentation ions, collision energies, and quadrupole voltages) were determined (Table 1).

3. Results and discussion

The optimization of the method for simultaneous analysis of proteinogenic amino acids without derivatization was performed in three steps; (i) column selection, (ii) mobile phase optimization, and (iii) selection of column combination.

3.1. Chiral column selection

The CSP of the chiral column is the critical part to achieve a chiral separation. Therefore, we firstly tested three different CSPs (polysaccharide-based, teicoplanin, and crown ether). The organic modifier selection for the mobile phase is based on the analyte and CSPs. Among many other organic modifiers, tetrahydrofuran, acetonitrile, ethanol and methanol were frequently and successfully used for the analysis of amino acids [22]. Therefore, acetonitrile/ethanol/water/TFA (80:15:5:0.5, % v/v/v/v) was selected for initial mobile phase. Among these chiral columns with the given mobile phase, the polysaccharide-based chiral column did not provide an enantioseparation for any of the native chiral amino acids studied (Fig. S1). The teicoplanin-based chiral column under given conditions separated the enantiomers of proline and histidine (Fig. S2). Finally, the chiral separation of amino acids was tested with the crown ether-based chiral column, and as shown in Fig. S4 enantioseparations were obtained for almost all of the studied chiral amino acids except for proline. These results confirm previously reported data [22].

3.2. Optimization of chromatographic conditions

The problem in this analysis on the crown ether-based column was the coelution of isobaric amino acids lysine and glutamine. Therefore, the mobile phase composition was first optimized to solve this problem. For this reason, isocratic mobile phase conditions were evaluated. The effect of water content in the mobile phase has been tested at different percentages (2, 5, 10, 15, 20 and 25%) (Supplementary Figs. S3–S8), and it was observed that the enantioseparation decreased with increasing water content in the mobile phase (Fig. S8). At the same time, the decreasing water content caused the peak distortion apparently due to the low solubility of amino acids in the mobile phase (Fig. S4). To overcome these problems, ethanol was replaced with methanol to ensure the required polarity and thus, increase the solubility of the amino acids. The enantioseparation of amino acids

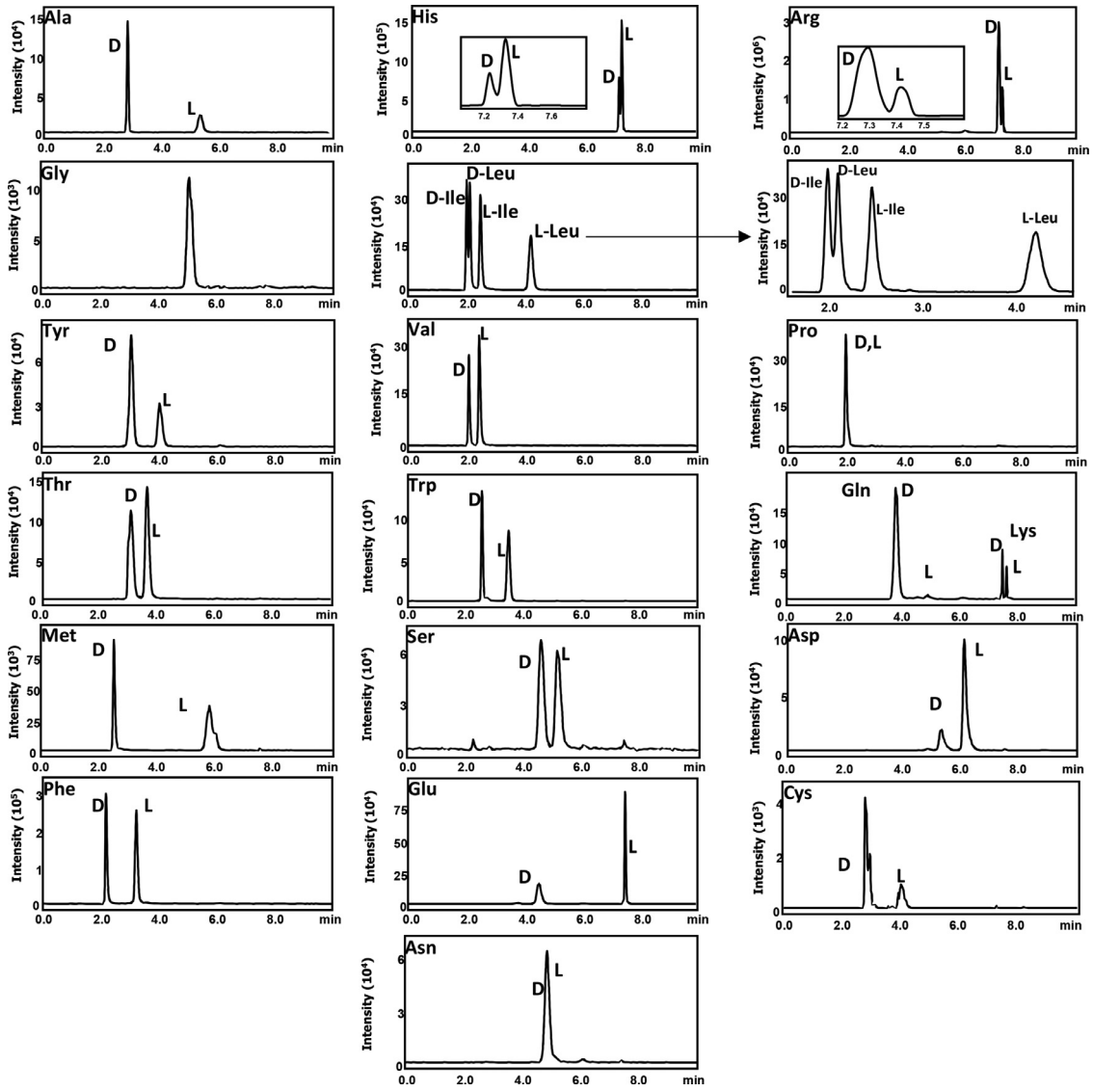


Fig. 5. Representative chromatograms for enantioseparation of amino acids from the intracellular matrix of A549 cells incubated with D-amino acids cocktail enriched cell culture medium. Chromatographic conditions: Column HILIC (50 × 2.1 mm, 3.5 µm)/Crownpak CR-I(+) (150 × 2.1 mm, 5 µm)) with mobile phase A and B in gradient elution. For further details see Experimental part.

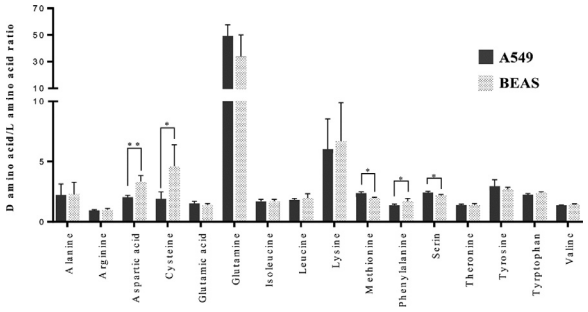


Fig. 6. D and L amino acid ratios in the intracellular matrix in cancer (A549) and normal/healthy (BEAS) cell lines ($n = 3$) cells incubated with D-amino acids cocktail enriched cell culture medium for 48 h.

was tested with an anhydrous mobile phase containing acetonitrile:methanol:TFA (85:15:0.5, % v/v/v). However, sufficient enantioseparation was not observed for all chiral amino acids and some of the amino acid peaks were distorted. Then the water content in the mobile phase of acetonitrile:methanol:TFA (85:15:0.5, % v/v/v) was increased. The overall peak shapes and resolution of D and L enantiomers of amino acids, as well as separation of isobaric amino acids (lysine and glutamine), were not improved compared to the ethanol-containing mobile phase. Therefore, ethanol was selected as a mobile phase modifier because it is less toxic than methanol. Then different gradient elution programs for ethanol-containing mobile phases were tested with increasing water in acetonitrile:ethanol:TFA (85:15:0.5, % v/v/v). It was observed that the initial amount of water (5% v/v) was critical for obtaining acceptable enantioseparations and peak shape (Fig. 1).

As mentioned above, TFA was used as a mobile phase modifier. However, it is not the first choice as a mobile phase modifier in LC-MS applications due to its causing contamination of ion source and diminishing of ionization efficiency, especially in negative ionization mode. Therefore, alternatives such as formic acid, ammonium formate, and ammonium acetate, more compatible with mass spectrometry systems, were tested instead of TFA. However, we did not obtain enantioseparations while TFA, as the additive, provided

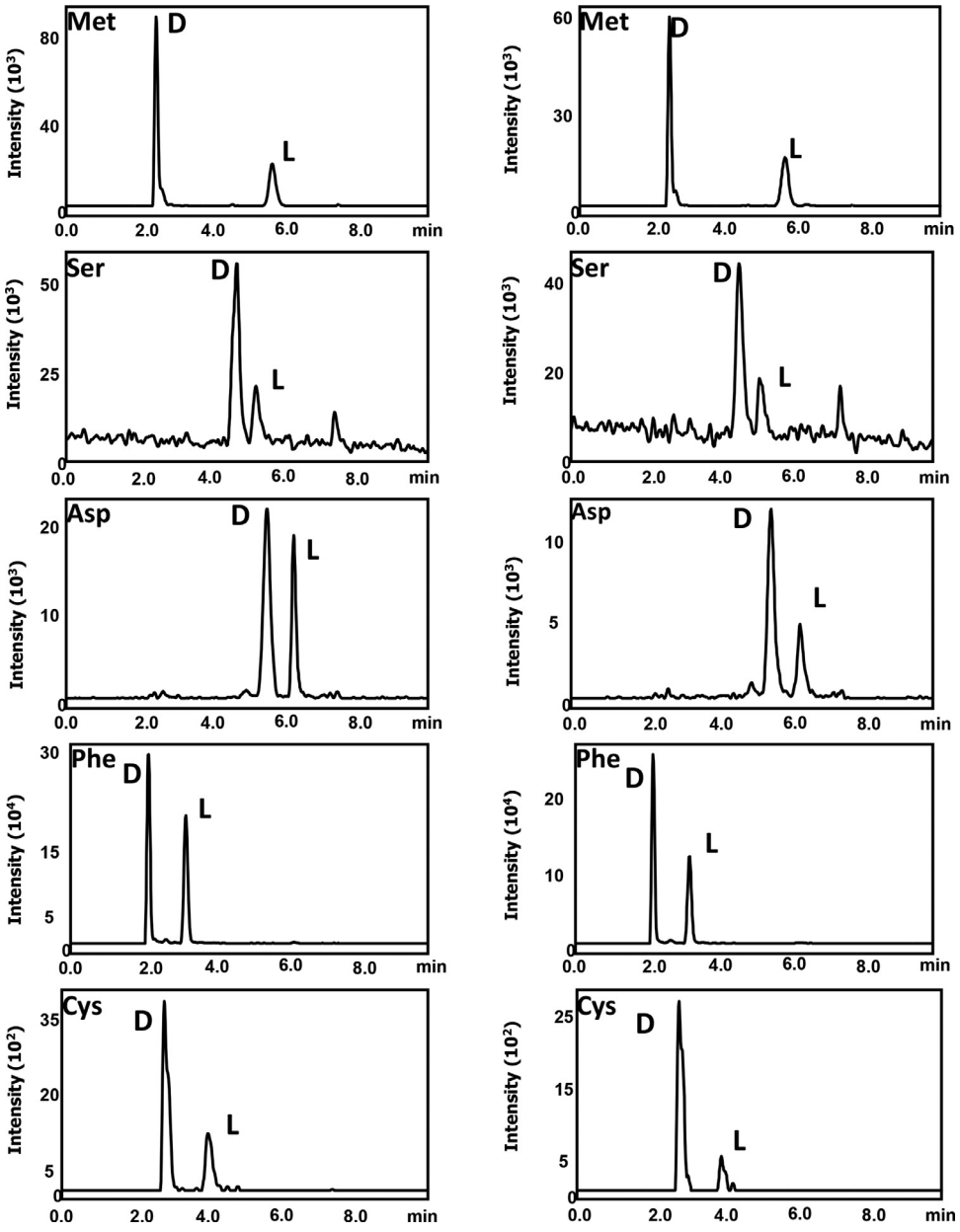


Fig. 7. Representative chromatograms for significantly altered metabolites compared to A549 (Left panel) and BEAS (Right panel) cell lines incubated with D-amino acids cocktail enriched cell culture medium for 48 h.

it. Therefore, TFA has been selected as a mobile phase modifier for chiral separations. TFA, an ion-pairing agent, is known to have a dominant effect on chiral separations, however, the mechanism has not been completely understood. It might partially be due to the fact that TFA provided an increased binding of the ammonium group of the amino acids to the crown ether functionality of the stationary phase and electrostatic repulsion counteracting the hydrophilic partition mechanism [22] or just the strength of other acids is not sufficient for effective suppression of deprotonation of carboxylic function in amino acids.

3.3. Selection of achiral-chiral column combination

Enantioseparation of most of the amino acids was achieved with the crown ether-based chiral column. However, isobaric amino acids D/L-lysine and D/L-glutamine coeluted on this column. These amino acids cannot be analyzed without prior chromatographic separation, since they have the same MRM values. Similarly, isomeric D/L-leucine and D/L-isoleucine are also needed to be separated chromatographically. Crown ether column allows partial separation of D/L-leucine and D/L-isoleucine, but not D/L-lysine and D/L-glutamine. Therefore, secondary nonchiral reversed- and HILIC-type systems were tested for achiral separation before a chiral separation.

Firstly, a C18 column was tested, and as expected, no retention of amino acids was observed on this column due to the high organic solvent content in the mobile phase (Fig. S9). Secondly, polar columns such as Luna NH_2 and HILIC columns (Kinetex, Cortecs and SeQuant ZIC-HILIC) were tested. Among these columns, a better separation was obtained with SeQuant ZIC-HILIC columns compared to the others on which all amino acids were eluted almost at dead volume (Figs. S10–S12). Both (long and short) ZIC-HILIC columns retained amino acids selectively. ZIC-HILIC was selected due to the shorter analysis time without losing chromatographic

resolution and providing preferable peak shapes (symmetrical and sharp). In addition, the particle size and length of the selected short HILIC column were more compatible with the MS system, where a lower flow rate is required for robust and reproducible ionization. Moreover, this shorter analysis time might reduce peak broadening compared to the long column, especially in dual systems. Therefore, experiments were carried out with a shorter and smaller particle size ZIC-HILIC column (50 × 2.5 mm, 3.5 µm) (Fig. S13). Due to the strong interactions of histidine with ZIC-HILIC column, it could not be eluted under isocratic conditions within 10 min.

The dual-column system for simultaneous enantioseparation of amino acids was made by serially connecting ZIC-HILIC (50 × 4.6 mm, 3.5 µm) and Crownpak CR-I (+) (150 × 3 mm, 5 µm) columns. The dual-column system was tested with a mobile phase acetonitrile:ethanol:water:TFA (80:15:5:0.5, %v/v/v/v) under isocratic conditions. As mentioned above, D/L -histidine did not elute under these conditions due to its strong interaction with the stationary phase. Therefore, HILIC-type gradient elution was applied (Fig. 2). The dual column provided a better chiral separation for isobaric D/L-lysine and D/L-glutamine compared to the single chiral column, but it did not improve the separation between isomeric D/L-leucine and D/L-isoleucine which are the other pair to be separated (Fig. 2). In addition, the dual column diminished the chiral separation of D/L histidine due to peak broadening on the HILIC column. On the other hand, good separation for D/L arginine was obtained while the L-arginine peak in the mixture of amino acids was distorted when only the chiral column was used. We also tested the reverse sequence of the columns using the same gradient elution program. In this case, first, a chiral separation was performed with the crown ether column and then achiral separation on the HILIC column. We observed the same separation ability for most amino acids except for lately eluted D/L-arginine and D/L-histidine, for which enantioseparations were lost (Fig. 3). This was the result of the gradient elution mode. Although, the same gradient elution was used in the reverted column sequence. Because the water percentage of the mobile phase in the chiral column was not the same at the time of the initial column sequence. Thus, the higher water content in the chiral column led to narrowing peaks but at the same time loss of the enantioseparation of lately eluted amino acids. Therefore, the serial dual-column in the order HILIC first followed by chiral crown ether column has been found optimal for simultaneous enantioseparation of amino acids (Fig. 4).

3.4. System suitability of the method

In this study, the ratio of D/L-amino acids in intracellular matrix was aimed to determine. The study does not include an absolute quantification for D and L amino acids. Therefore, a partial method validation studies including system suitability, and selectivity were applied to all proteogenic amino acids.

The system suitability was assessed by six replicate analyzes of D/L-amino acids at a concentration of 1 µg/mL. The retention times, capacity factors, resolutions, theoretical plate numbers and peak asymmetries were within acceptable criteria (Table S2). To examine the effect of the sample matrix on the ionization efficiency of D/L amino acids, standard solutions were spiked into both water and pooled cell intracellular matrix at four different (1, 2, 3 and 5 µg/mL) concentrations. The peak areas of intracellular and water spiked were compared (Table S3) and the matrix effects for amino acids were found in the acceptable range except for D-isoleucine and L-alanine. The results show that the sample matrix has no negative effect on the ionization and fragmentation of amino acids.

3.5. Application to cell culture samples

The developed method has been applied to A549 human lung adenocarcinoma cells and its control BEAS-2B healthy human lung epithelial cells (Fig. 5). The cells were exposed to D amino acids that was added to the cultivation medium. The D/L-amino acids were detected in the intracellular matrix and their D to L-enantiomer peak area ratios for each amino acids in cancer and normal cell lines are shown in Fig. 6. The results indicated that the ratio of enantiomers for most amino acids is similar in cancer and normal cell lines except for aspartic acid, cysteine, methionine, phenylalanine, and serine (Fig. 7). In recent studies, the potential functional role of D-amino acids in cancer cell proliferation was suggested [4,25].

4. Conclusion

Here we describe the simultaneous enantioselective determination of amino acids except for D/L-proline without preliminary derivatization on a serially connected HILIC and chiral crown ether-based columns. The direct analysis has the advantages over method with derivatization such as eliminating interferences of impurities from the derivatization reagents, additional costs, and also the extra time required for the sample preparation/derivatization. The developed serial column system seems suitable to determine the content of amino acids in intracellular matrix enantioselectively and can be used to understand the role of D-amino acids in cancer and normal cell lines or for mechanistic and biomarker research studies for the early diagnosis of various diseases.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Tuğçe Öztepe: Investigation, Methodology, Formal analysis, Writing – original draft. **Nermin Büşra Kale:** Methodology, Formal analysis. **Tuba Reçber:** Methodology, Formal analysis. **İpek Baysal:** Methodology, Writing – review & editing. **Samiye Yabanoğlu-Çiftçi:** Methodology, Writing – review & editing. **Mehmet Gumustas:** Investigation, Conceptualization, Methodology, Writing – original draft. **Sedef Kır:** Investigation, Conceptualization, Methodology, Writing – original draft. **Bezhan Chankvetadze:** Investigation, Conceptualization, Methodology, Supervision, Writing – review & editing. **Emirhan Nemutlu:** Investigation, Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Data Availability

Data will be made available on request.

Acknowledgments

This project (ID: 120Z600) was funded by The Scientific and Technological Research Council of Turkey (TÜBİTAK).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2022.463529.

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