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# Chiral separation of aromatic amino acids by capillary electrophoresis using sulphated β-cyclodextrin as a single chiral selector: an experimental and computational study

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Chiral discrimination of aromatic amino acids is crucial in biochemical, pharmaceutical and analytical sciences, yet a clear and mechanistic understanding of cyclodextrin-mediated enantioseparation in capillary electrophoresis (CE) remains incomplete. In this work, the enantioseparation of tryptophan (TRP), tyrosine (TYR), and phenylalanine (PHE) was studied by capillary electrophoresis (CE) using sulphated β-cyclodextrin (S-β-CD) as a single chiral selector under acidic buffer conditions. Separation parameters, including S-β-CD concentration and background electrolyte (BGE) pH, were systematically investigated. Base line separation of the three analytes was achieved using 1.5 mM S-β-CD in 25 mM phosphate buffer at pH 2.5. In addition to analytical performance, the contribution of this study lies in correlating experimental enantiomer migration behavior with qualitative molecular docking analysis to rationalize chiral recognition trends. Docking simulations were performed using sulphated β-cyclodextrin models with substitution degrees representative of the experimental material, allowing comparison of predicted host–guest inclusion tendencies for the individual enantiomers. Molecular docking studies, together with experimental results, indicate that the *R*-enantiomers tend to form more stable inclusion complexes with S-β-CD, which is consistent with their later elution under the applied CE conditions.

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## 1 Introduction

Aromatic amino acids (AAs) like tryptophan (TRP), phenylalanine (PHE), and tyrosine (TYR) serve as common precursors for proteins and phenolic compounds.[1,2] Enantiopure AAs act as crucial components in a wide variety of sectors, such as food, agrochemicals, and pharmaceuticals. Although L-amino acids serve as the essential building blocks of proteins, their *D*-enantiomers have attracted considerable attention because of their various uses in, chemotherapeutics, antibiotics, fluorescent DNA markers, immunosuppressants, deodorants sweeteners, pesticides, and numerous other areas.[2,3] This differentiation between the isomers of AAs underscores the distinct stereochemical tendencies found in nature and stresses the significance of recognizing and employing both enantiomers in different implications.[3,4]

Over the past three decades, CE has demonstrated its effectiveness as a strong alternative to HPLC for enantioselective evaluation.[5–9] In comparison to HPLC, CE provides various unique benefits such as ease of use, reduced analysis durations, high efficiencies, minimal usage of chiral selector (leading to lower operational costs), and alternative separation mechanisms. Cyclodextrins (CDs) and their derivatives are the chiral selectors most commonly utilized in CE.[10–15] They exhibit minimal UV absorbance, dissolve in water, and are widely accessible in various modified versions. The enantioselective recognition arises from a hydrophobic part of the analyte being incorporated into the CD cavity, as well as from hydrogen bonding with chiral hydroxyl groups.[16] Native β-cyclodextrin is limited by its relatively low aqueous solubility and restricted selectivity, which has motivated the extensive use of derivatized cyclodextrins such as methylated, sulfated, carboxymethylated, and sulfobutylated analogues.[17–19] Sulphated β-CDs were reported to separate a greater number of analytes under acidic conditions due to its strong anionic character and enhanced electrostatic interactions.[20–22]

Chiral separation of amino acids by capillary electrophoresis has been reported in the literature, employing chiral selectors such as the crown ether (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid,[23] dual crown ether/cyclodextrin systems,[24] and dual cyclodextrin/dextran combinations.[25] In the realm of structure-driven drug design, molecular docking is an essential

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technique that greatly facilitates the modeling of molecular interactions and the prediction of receptor–ligand binding affinities and modes. In recent years, this approach has gained considerable attention in research focused on chirality in pharmaceutical design. The effectiveness of molecular docking is rooted in its strong capacity to simulate the interactions of pair of enantiomers and the active sites in the chiral selectors. This allows for the anticipation of energy landscapes and the geometric arrangements of selector–selected bindings.[26] These abilities are profoundly beneficial for a comprehensive explanation of the intricate process of chiral recognition.[27,28] Molecular docking significantly helps in establishing elution orders by providing insights into both successful and unsuccessful resolution results.[29] This thorough comprehension is essential for improving enantio-separation methods and enhancing the creation of enantiomerically pure pharmaceuticals.

Many investigations have treated chiral selectors as receptors and enantiomers as ligands, though in cases such as cyclodextrins, the interaction is more accurately described as a host–guest inclusion process.[27,28] Docking simulations have been widely applied to study chiral recognition, and several investigations have demonstrated good agreement between simulation results and experimental findings.[27,28,30–36] These studies have provided valuable insights into the interaction energies, binding affinities, and spatial arrangements of enantiomers with chiral selectors. The process of enantioseparation is generally attributed to the formation of transient diastereomeric complexes between the chiral selector and the enantiomers. The difference in the stability of these complexes, reflected in their relative energies, plays a decisive role in determining the efficiency of chiral separation.[37,38]

In this study, sulphated β-cyclodextrin (S-β-CD) was employed as a single chiral selector for the enantioseparation of three structurally related aromatic amino acids, TRP, TYR and PHE by CE under acidic conditions (Fig. 1). The primary contribution of this work is not the introduction of a new selector, but a systematic experimental–computational analysis aimed at rationalizing enantiomer migration order and chiral recognition trends. By integrating experimental migration data with computed binding energies, our approach not only validates the interactions predicted by docking but also identifies the molecular features that govern elution order. Moreover, this integrated approach offers a qualitative, hypothesis generating framework for understanding and improving enantio-separation, providing insights that extend beyond prior studies.

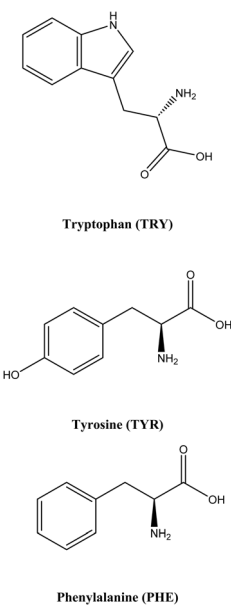


Fig. 1 Chemical structures of amino acids (AAs), tryptophan, tyrosine and phenylalanine.

# 2 Experimental

## 2.1 Instrumentation

The analysis was performed using a CE device from Agilent Technologies (Waldbronn, Germany), specifically model G7100A, linked to a photo diode array detector and utilizing Agilent Chemstation Software. A 50 μm i.d. × 33 cm uncoated fused-silica capillary (with a detection length of 8.5 cm from the capillary's outlet) was utilized, sourced from Agilent Technologies. The pH of the buffer was measured using the Lab pH meter inoLab® pH 7110 (Xylem Analytic Inc., Waldheim, Germany).

## 2.2 Chemicals and reagents

RS-TRP, S-TRP, RS-PHE, S-PHE, RS-TYR, S-TYR, and S-β-CD with extent of labelling 12–15 mole per mole of β-CD, with the supplier catalogue number (389153-5G) were obtained from Sigma-Aldrich (Steinheim, Germany). Sodium phosphate dibasic heptahydrate, sodium hydroxide, and orthophosphoric acid (85%), obtained from Sigma-Aldric (Seelz, Germany). All chemicals were of analytical reagent grade and were used without further purification. Milli-Q water was used for preparation of all solutions (Millipore, Bedford, USA).

## 2.3 CE chiral separation conditions

Enantiomeric separation was conducted under these conditions: a temperature of 25 ± 0.1 °C, an applied voltage of +22 kV, an injection time of 7 s at 0.5 psi hydrostatically, and a detector wavelength of 214 nm. A capillary was prepared by washing with 1 M sodium hydroxide for 20 minutes, then with 0.1 M sodium hydroxide for 8 minutes, and finally with water and background electrolyte (BGE), each for 10 minutes. The BGE consists of 25 mM $H_3PO_4$ modified to the desired pH (2.25, 2.5, 2.75, 3.0 and 3.5) using 25 mM of $NaH_2PO_4$. All standards, samples, and BGE solutions were filtered with a syringe filter having a pore size of 0.20 μm and a diameter of 13 mm (Macherey–Nagel GmBh & Co. KG, Duren, Germany). Before changing each BGE to a different buffer pH, the capillary was flushed with 1 M sodium hydroxide, Milli-Q water, and the suitable BGE for 15, 20, and 30 minutes, respectively. Every day, the capillary was balanced with 0.1 M sodium hydroxide, Milli-Q water, and the working buffer solution for 5, 15, and 20 minutes, respectively. In between runs, the capillaries were rinsed with 0.1 M sodium hydroxide and Milli-Q water for 2 minutes each, followed by a wash with the BGE for 3 minutes.

### 2.4 Molecular docking methodology

Molecular docking simulations were performed to investigate the possible interactions between the chiral selector sulphated-β-cyclodextrin (S-β-CD) as host and the enantiomers of three amino acids (phenylalanine, tryptophan, and tyrosine) as guest molecules. In this study, S-β-CD with different degrees of substitution (21S-β-CD and 14S-β-CD), along with a seven-substituted host (7S-β-CD) reported in previous studies,[39] were considered. This selection closely reflects the experimental degree of substitution (12–15) and provides a representative set of host molecules for meaningful docking simulations. The energy minimization of sulphated-β-cyclodextrin complexes were performed using the Born implicit solvation model and OPLS-AA force field.[40] The three-dimensional structures of the AA enantiomers (*R* and *S*) were visualized using GaussView 6 software.[41] After that the corresponding PDBQT files were generated, which contain details of the atomic partial charges, atom types, torsional degree of freedom, and polar hydrogens. These files served as input ligand structures docked to the prepared sulphonated-β-CDs utilizing AutoDock tools, and the binding affinity of the complexes was calculated. The binding affinities of the selected AA enantiomers towards the S-β-CD derivatives were calculated using AutoDock Vina.[42] Blind docking was performed with the grid box centered at the host's center of mass (center_$x$ = −0.765 Å, center_$y$ = −0.119 Å, center_$z$ = −0.602 Å) and dimensions of 30 × 30 × 30 Å$^3$, ensuring complete coverage of the host. The S-β-CD host was treated as rigid host, and the exhaustiveness parameter was set to 8, which represents a balance between computational efficiency and search depth. On the other hand, guest amino acids were treated as flexible with all rotatable bonds permitted. The AutoDock Vina parameters were configured as follows: number of modes were set to 9, and energy range was 3 kcal mol$^{-1}$. Each calculation was repeated three times with distinct random seeds to ensure reproducibility and convergence of the predicted binding modes. A maximum of nine binding modes were identified for both *RS*-enantiomers of phenylalanine, tryptophan and tyrosine and their binding affinities ($E_b$) were computed and compared with the experimental results.

**2.4.1 Limitations of the docking methodology.** A rigid-host treatment of S-β-CD, implicit solvation (embedded in the scoring function), and finite conformational sampling based on the exhaustiveness parameter represents a few of the simplifying assumptions used in the docking simulations. In addition, AutoDock Vina scoring functions offer approximate, relative binding tendencies instead of quantitative free energies. Therefore, the docking results are interpreted qualitatively to illustrate potential host-guest interaction patterns and trends rather than to predict binding strengths or elution behaviour.

## 3 Results and discussion

### 3.1 Chiral separation with CE

**3.1.1 Effect of buffer pH.** pH is a crucial factor to optimize since it influences the ionization of the capillary wall's silanol group, consequently impacting the strength of the electroosmotic flow (EOF). The p$K_a$ values for TRP, TYR, and PHE are noted as 2.32 and 9.21,[44] for TYR 2.2 and 9.76,[43] and for PHE 1.83 and 9.13,[44] regarding the acidic and basic groups, respectively. In addition, for pH values below their p$K_a$, all analytes will move as cations within the electrophoretic system. The use of S-β-CD as chiral selector was previously reported in lower pH or acidic buffer.[45] Therefore, in this study the impact of pH on the resolution ($R_s$) was examined within the pH range of 2.25–3.5, utilizing 25 mM phosphate buffer solutions made at varying pH levels that included 1.5 mM S-β-CD. The findings are displayed in Fig. 2. A reduction in resolutions was noted as the pH levels rose from 2.25 to 3.5 for both TRP and TYR.

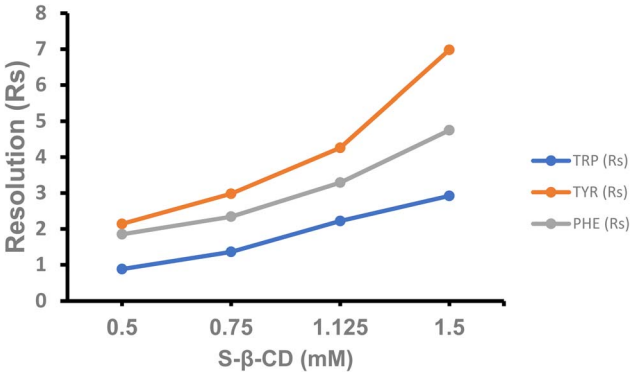


Fig. 2 Effect of S-β-CD concentration on the resolution of AAs (buffer concentration 25 mM, pH 2.5, temperature, 25 °C, and applied voltage, 22 kV).

**3.1.2 Effect of the concentration of S-β-CD.** The concentration of chiral selector is an essential parameter for enantioseparation.[46] To obtain an optimum concentration, S-β-CD at different concentrations was investigated in the range of 0.5 to 1.5 mM. The BGE 25 mM phosphate buffer solution at pH of 2.5. The resolution is increased with the increased of S-β-CD concentration (Fig. 3). Baseline separation of the three analytes was obtained at concentration of 1.125 and 1.5 mM. Electropherograms for the three analytes at optimum conditions were shown in Fig. 4. The migration order was identified by spiking of *S*-enantiomers.

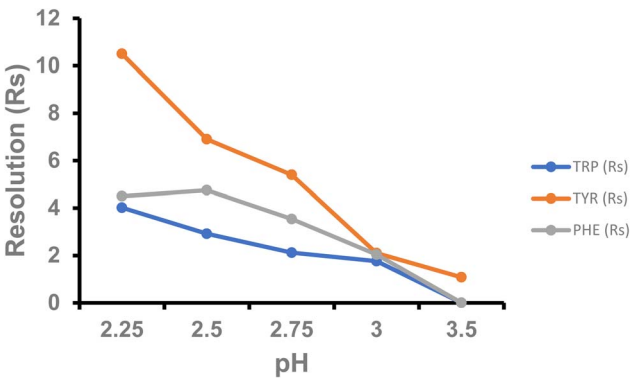


Fig. 3 Effect of pH on the resolution on the resolution of AAs, conditions, BGE 25 mM at different pH mM; S-β-CD concentration, 1.5 mM; temperature, 25 °C, and applied voltage, 22 kV.

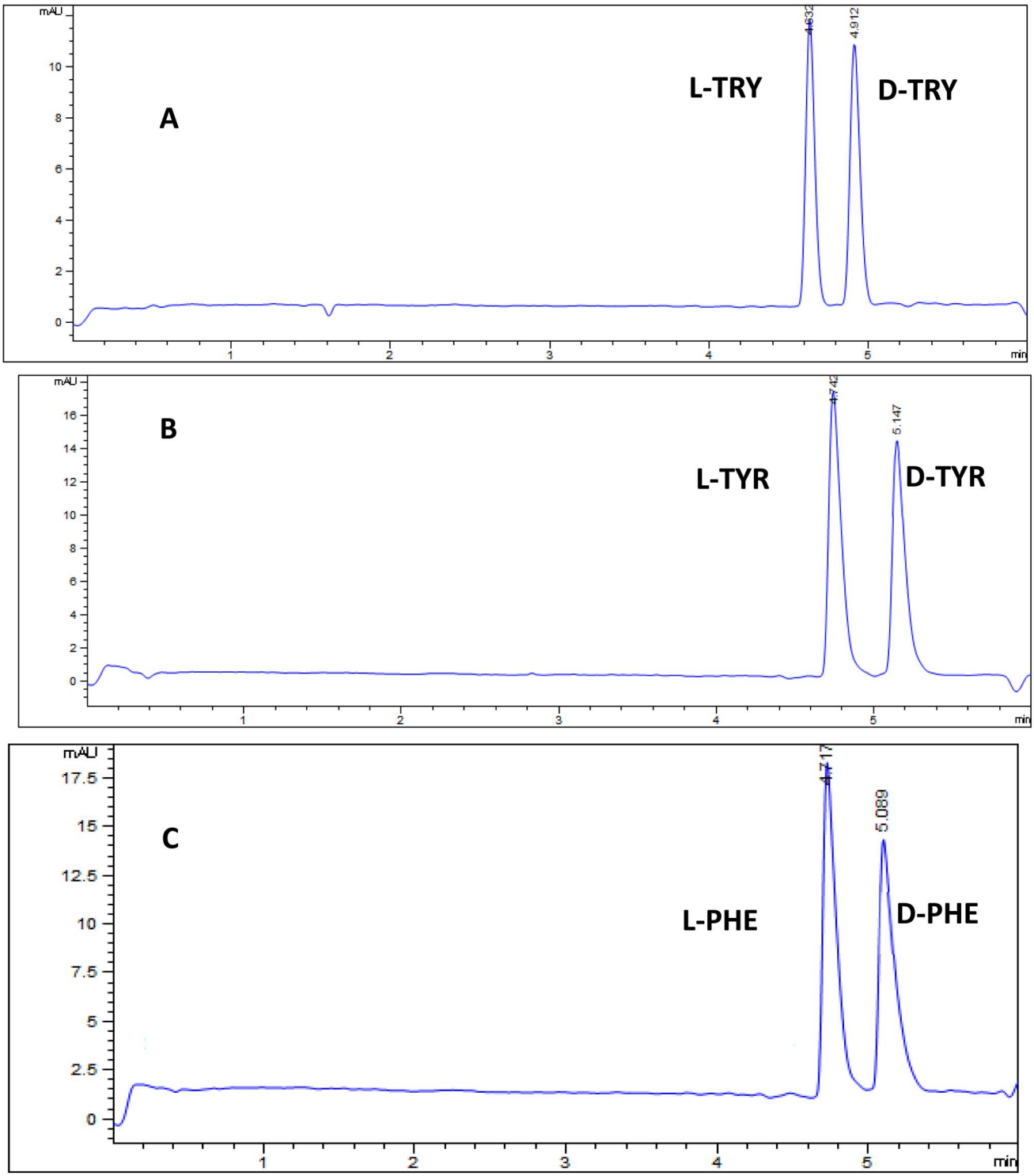


Fig. 4 Electropherograms for (A) TRY, (B) TYR (C) PHE obtained at BGE consisted of 25 mM phosphate buffer at pH 2.5, temperature 25 °C, applied voltage of 22 kV.

**3.1.3 Effect of instrumental parameters.** The effect of instrumental parameters such as applied voltage, capillary temperature, and injection time was also investigated. In this study applied voltage was examined between 16 and 24 kV. An optimal peak shape and satisfactory migration time were obtained at 22 kV. The impact of temperature (19–28 °C) on peak efficiency was also investigated. Ideal peaks were achieved when separated at 25 °C. To further lower the detection limits, the injection time was adjusted between 3 to 9 seconds. Through hydrostatic injection, an injection time of 7 seconds was determined to be optimal.

From the above experiments the optimum conditions were BGE 25 mM phosphate buffer at pH 2.5, S-β-CD concentration

Table 1 Resolution, separation factor and theoretical plate numbers obtained as the optimum conditions

| Parameters/analyte | Resolution ($R_s$) | Selectivity factor ($\alpha$) | Theoretical plate numbers ($N$) first enantiomer |
|---|---|---|---|
| D/L-TRP | 4.02 | 1.02 | 35 268.84 |
| D/L-TYR | 10.5 | 1.10 | 18 235.8016 |
| D/L-PHE | 4.75 | 1.08 | 16 723.6624 |

Table 2 Within days reproducibility for the repeated injection of different concentration of DL-TRP DL-TYR, and PHE standard

| | RSD % | | RSD % | | RSD % | |
|---|---|---|---|---|---|---|
| | Migration time | | Migration time | | Migration time | |
| Factor conc. (μg mL$^{-1}$) | L-TRP | D-TRP | L-TYR | D-TYR | L-PHE | D-PHE |
| Intraday precision ($n = 6$) | | | | | | |
| 50 | 2.98 | 1.70 | 3.62 | 2.98 | 3.07 | 3.19 |
| 100 | 2.92 | 2.87 | 3.01 | 2.10 | 2.92 | 2.87 |
| 150 | 1.83 | 2.42 | 3.25 | 3.84 | 2.79 | 3.10 |

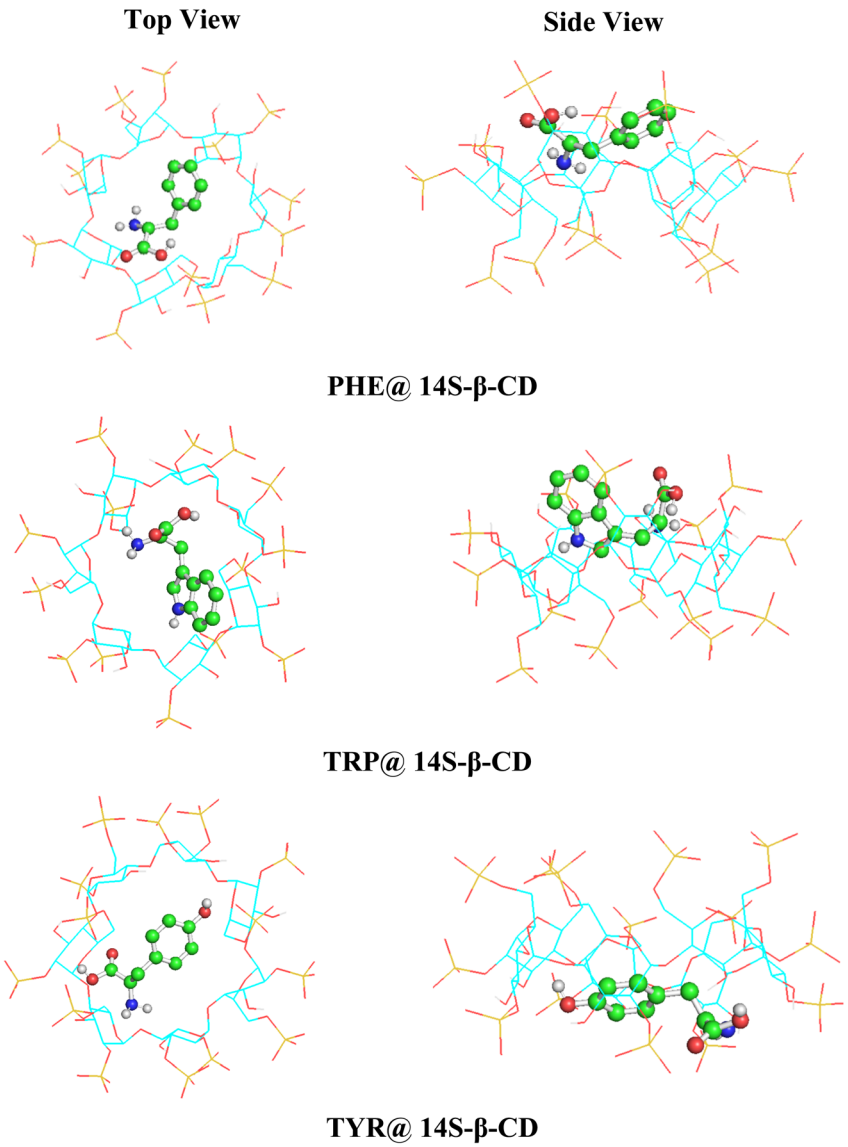


Fig. 5 3D structures of diastereomeric complexes of AA enantiomers and 14S-β-CD.

1.5 mM, applied voltage 22 kV, capillary temperature 25 °C, and injection time is 7 S. The resolution ($R_s$), selectivity factor, ($\alpha$) and theoretical plate number ($N$) obtained at these optimum conditions are shown in Table 1.

The intra-day precision and migration-time repeatability were evaluated by repeated CE analyses of standard racemic solutions of TRP, TYR, and PHE at three concentration levels (50, 100, and 150 μg mL$^{-1}$). The results are presented in Table 2. The relative standard deviation (RSD) values for migration times of all enantiomers were consistently below 4%, demonstrating good repeatability and short-term precision of the method.

The reported CE methods are typical based on higher cyclodextrin concentrations and/or dual-additive systems (*e.g.*, S-β-CD (0–20 mM)) with dextran sulfate[25] or cyclodextrin-crown ether combinations[24] to achieve acceptable enantioresolution, often with longer analysis times and increased method complexity. In contrast, the proposed method achieves baseline separation of TRP, TYR, and PHE using a single chiral selector (S-β-CD) at a markedly lower concentration (1.5 mM) and with a shorter runtime, while maintaining good separation efficiency. This comparison clearly demonstrates that the present strategy offers improved simplicity and efficiency without compromising resolution, and further distinguishes it by coupling analytical performance with mechanistic insight from molecular docking, which is absent in most earlier CE studies.

### 3.2 Molecular docking study

Molecular docking was performed to gain insight into the possible binding affinities of amino acid enantiomers for chiral recognition and enantioseparation on the Chiral CD-Ph column. The interactions between the *R*/*S* enantiomers of three amino acids (phenylalanine, tryptophan, and tyrosine) and sulphated-β-cyclodextrin (21S-β-CD, 14S-β-CD, and 7S-β-CD) were investigated and their 3D structures are shown in Fig. 5, S1 and S2 (see SI). The AA enantiomers interact with the cavity of sulphated-β-cyclodextrin, forming inclusion complexes with the host.

It is crucial to emphasize that the molecular docking results provide qualitative insights that are consistent with the experimental elution order of enantiomers.[47] The computed binding energies for the enantiomeric complexes ranged from −4.4 to −6.7 kcal mol$^{-1}$ indicating that host–guest complex formation is energetically favourable as values listed in Table 3.

Across all amino acids studied, the docking results suggest a trend where *R*-enantiomers may form slightly more favourable interactions through hydrophobic and hydrogen-bonding interactions with S-β-CD compared to their S-counterparts, which is qualitatively consistent with the experimental observation that *S*-enantiomers elute earlier. In the case of phenylalanine, the computed $\Delta E_b$ difference between enantiomers is very small (~0.1 kcal mol$^{-1}$), falling within the typical uncertainty of docking scoring functions. Therefore, these results should be interpreted as hypothesis generating evidence that rationalizes potential interaction patterns and trends rather than providing precise predictions of elution order. Overall, docking complements experimental observations by revealing potential interaction patterns without making definitive mechanistic claims.

Table 3 Binding affinities ($E_b$, kcal mol$^{-1}$) of the *R*/*S* enantiomers of the three amino acids towards sulphated-β-CDs

| | 21S-β-CD | | 14S-β-CD | | 7S-β-CD | |
|---|---|---|---|---|---|---|
| | $E_b$ (kcal mol$^{-1}$) | | $E_b$ (kcal mol$^{-1}$) | | $E_b$ (kcal mol$^{-1}$) | |
| Amino acids | *R* | *S* | *R* | *S* | *R* | *S* |
| PHE | −5.8 | −5.5 | −4.5 | −4.4 | −5.8 | −5.7 |
| TRP | −6.7 | −6.5 | −5.5 | −5.3 | −6.5 | −6.3 |
| TYR | −5.9 | −5.6 | −4.7 | −4.6 | −5.9 | −5.8 |

## 4 Conclusion

It can be concluded that, to the best of our knowledge, this is the first systematic report combining S-β-CD (substitution degree 12–15) with molecular docking to study the enantioseparation of this particular set of amino acids under the reported buffer conditions. The key parameters influencing enantioseparation, including the concentration of chiral selector and the pH of BGE, were optimized. The migration sequence of the enantiomers was determined through spiking experiments, revealing that the *R*-enantiomers possess more negative binding energies and migrate later, thereby confirming that the *S*-enantiomer elutes first in the electropherogram. Additionally, the mechanism of enantiomeric recognition by S-β-CD was explored by integrating experimental observations with molecular docking analysis. The docking results suggest similar binding modes for the enantiomers and highlight qualitative trends in host–guest inclusion and interaction patterns with S-β-CD. While the calculated binding energies are approximate and not quantitatively predictive, the observed qualitative trends are consistent with the experimental migration behaviour. Overall, the combined experimental and computational approach provides molecular level insight into possible enantiomer S-β-CD interactions and supports the use of docking as a qualitative, hypothesis generating tool for understanding chiral recognition and guiding the design of chiral separation systems. Furthermore, this study not only offers a practical approach for the enantioseparation of chiral amino acids but also provides a fresh understanding of the chiral recognition mechanism of S-β-CD. It also demonstrates the viability of employing S-β-CD for the chiral analysis of these substances in pharmaceutical formulations.

## Conflicts of interest

The authors declare no competing interests.

## Data availability

The data supporting this article have been included as part of the figures, tables, and supplementary information (SI). Supplementary information is available. See DOI: https://doi.org/10.1039/d5ra07410j.

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