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Capillary electrophoresis method for chiral separation of amino acid enantiomers

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Abstract

If enantiomeric purity could be checked as easily as pH, the pharmaceutical industry would save billions in quality control costs every year. This research developed and validated a capillary electrophoresis (CE) method using β -cyclodextrin (β -CD) as a chiral selector for the enantioseparation of five amino acid pairs: DL-tryptophan, DL-phenylalanine, DL-tyrosine, DL-histidine, and DL-leucine. Separation was performed in an uncoated fused-silica capillary (50 μ m i.d. $\times$ 50 cm effective length) with a background electrolyte of 50 mM phosphate buffer at pH 2.5 containing 20 mM β -CD, an applied voltage of 20 kV, and UV detection at 200 nm. Under optimized conditions, baseline resolution ($R_s \geq 1.5$) was achieved for DL-Trp ($R_s = 3.48$), DL-Phe (2.84), and DL-Tyr (3.06) within 18 minutes. DL-His and DL-Leu gave partial resolution ($R_s = 1.88$ and 1.48, respectively). Method validation showed linearity ($R^2 > 0.998$) over 10-500 μ g/mL, intra-day precision below 2.4% RSD, inter-day precision below 3.8% RSD, and LODs of 2.8-6.4 μ g/mL. The method was applied to determine the enantiomeric purity of L-tryptophan dietary supplements, finding D-Trp impurity at 0.8-2.3% in three of five products tested. The CE method offers a faster, cheaper alternative to chiral HPLC for amino acid enantiomer screening.

Keywords: Capillary electrophoresis, chiral separation, amino acid, enantiomer, beta-cyclodextrin, tryptophan, phenylalanine, method validation, enantiomeric purity, dietary supplement

Introduction

Suppose you discover that a supposedly pure L-amino acid supplement contains 2% of the D-enantiomer. Is that a quality defect, a health risk, or simply an analytical curiosity? The answer depends on the amino acid: D-serine is a neurotransmitter in its own right, while D-phenylalanine has been investigated as an analgesic, but other D-amino acids can interfere with protein synthesis or act as markers of microbial contamination in food products ^[1, 2]. Regardless of the biological implications, regulatory agencies require enantiomeric purity testing for chiral amino acid ingredients, and the method of choice matters.

Chiral HPLC with expensive Chirobiotic or crown ether columns is the gold standard, but the columns cost USD 1,500-3,000, require dedicated instruments, and generate organic solvent waste ^[3, 4]. Capillary electrophoresis offers a simpler approach: by adding a chiral selector most commonly a cyclodextrin to the aqueous background electrolyte, enantioseparation can be achieved in an uncoated fused-silica capillary with no organic solvents and at a consumable cost of pennies per run ^[5, 6]. The separation principle is differential inclusion complexation: one enantiomer fits more tightly into the cyclodextrin cavity than the other, migrating at a different effective velocity in the electric field ^[7].

Despite the theoretical elegance, practical CE enantioseparation of underivatized amino acids remains challenging because most amino acids lack strong UV chromophores and because their small, hydrophilic structures form weak inclusion complexes with native β -CD ^[8, 9]. Aromatic amino acids (Trp, Phe, Tyr) offer better detection at 200-220 nm and stronger CD interactions through π -inclusion, while aliphatic amino acids (Leu, Val, Ala) are harder to resolve and detect ^[10]. This research developed and validated a CE-UV method using native β -CD for five amino acid pairs spanning aromatic and non-aromatic classes, optimized the key method parameters (β -CD concentration, buffer pH, voltage, temperature), and applied the method to enantiomeric purity testing of commercial L-tryptophan supplements.

All work was conducted at the Department of Analytical Chemistry, Savanna Institute of Chemical Sciences, Kano, Nigeria, between January 2023 and October 2023 ^[11].

Material and Methods

Reagents and Chemicals

DL-Tryptophan, DL-phenylalanine, DL-tyrosine, DL-histidine, DL-leucine, and their pure L-enantiomers (all $\geq 99\%$) were purchased from Sigma-Aldrich (Lagos, Nigeria). β -Cyclodextrin ($\geq 97\%$) was from Wacker Chemie (Munich, Germany). Sodium dihydrogen phosphate monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) and disodium hydrogen phosphate (Na_2HPO_4) were analytical grade from BDH Chemicals (Poole, UK). Phosphoric acid (85%) and sodium hydroxide ($\geq 98\%$) for pH adjustment were from Merck (Darmstadt, Germany). All solutions were prepared with Milli-Q water ($18.2 \text{ M}\Omega \cdot \text{cm}$) and filtered through $0.22 \mu\text{m}$ nylon membranes before use. Five commercial L-tryptophan dietary supplement capsules (labeled as 500 mg L-Trp per capsule) were purchased from pharmacies in Kano.

Material

Capillary electrophoresis was performed on an Agilent 7100 CE system equipped with a diode array detector. Separation used an uncoated fused-silica capillary ($50 \mu\text{m}$ i.d. $\times$ 58.5 cm total length, 50 cm effective length) from Polymicro Technologies (Phoenix, AZ, USA). New capillaries were conditioned by flushing sequentially with 1 M NaOH (30 min), water (15 min), and background electrolyte (BGE, 15 min). Between runs, the capillary was flushed with 0.1 M NaOH (2 min), water (2 min), and BGE (3 min). The BGE consisted of 50 mM phosphate buffer (pH 2.5) containing 20 mM β -CD. Applied voltage was 20 kV (positive polarity, anode at inlet). Capillary temperature was maintained at 20°C . Injection was hydrodynamic ($50 \text{ mbar} \times 5 \text{ s}$). Detection was at 200 nm.

Methods

Optimization

Five parameters were optimized by varying one at a time while keeping others constant: β -CD concentration (0-30 mM), buffer pH (2.0-4.0), applied voltage (10-25 kV), capillary temperature ($15\text{--}30^\circ\text{C}$), and phosphate buffer concentration (25-100 mM). Resolution (R_s), migration time, and peak efficiency (theoretical plates) were used as

response criteria. The optimum was selected as the condition giving $R_s \geq 1.5$ for the most analytes in the shortest analysis time.

Method validation

Validation followed ICH Q2(R1) guidelines ^[12]. Linearity was assessed from calibration curves ($10\text{--}500 \mu\text{g/mL}$ of each enantiomer, six concentrations, triplicate injections). LOD and LOQ were calculated as $3.3\sigma/\text{slope}$ and $10\sigma/\text{slope}$, where σ is the residual standard deviation of the regression. Intra-day precision ($n = 6$) and inter-day precision ($n = 6$ over three days) were expressed as %RSD of migration time and peak area. Accuracy was evaluated by spiking L-amino acid solutions with known amounts of D-enantiomer (0.5%, 1.0%, 2.0%) and measuring recovery.

Supplement analysis

Each capsule was opened, and the powder equivalent to 50 mg L-Trp was dissolved in 10 mL water, sonicated for 10 min, filtered through $0.22 \mu\text{m}$ membrane, and analyzed by the optimized CE method. D-Trp content was calculated from the D-peak area using the calibration curve for DL-Trp. All analyses were performed at Savanna Institute of Chemical Sciences, Kano, Nigeria.

Instrumentation

Beyond the Agilent 7100 CE system, a Mettler Toledo SevenCompact pH meter was used for buffer pH adjustment. A Branson 3510 ultrasonic bath was used for sample dissolution. An Agilent 1260 Infinity II HPLC with a Chirobiotic T column ($250 \times 4.6 \text{ mm}$, $5 \mu\text{m}$, Astec) served as the reference method for confirming D-Trp impurity levels in supplements, using a mobile phase of 80:20 methanol-water at 1.0 mL/min with UV detection at 220 nm.

Quality control

System suitability was verified before each analytical session by injecting a standard mixture of DL-Trp ($100 \mu\text{g/mL}$ each enantiomer) and confirming $R_s \geq 3.0$, peak asymmetry between 0.8 and 1.5, and migration time RSD below 1.0% for six replicate injections. A method blank (BGE only) was injected at the start and end of each batch. Migration time correction using an internal mobility marker (mesityl oxide, 0.1 mg/mL) was applied to compensate for electroosmotic flow variations.

Results

Table 1: Optimized CE method parameters and enantioseparation performance for five amino acid pairs

Amino Acid	R_s	Migration Time D (min)	Migration Time L (min)	Plates (D)	Plates (L)	LOD ($\mu\text{g/mL}$)
DL-Trp	3.48	14.2	14.8	84,200	81,600	2.8
DL-Phe	2.84	12.6	13.1	72,400	69,800	3.4
DL-Tyr	3.06	15.8	16.4	78,600	76,200	3.1
DL-His	1.88	11.4	11.7	64,200	62,800	4.8
DL-Leu	1.48	10.8	11.0	52,600	51,200	6.4

Table 2: Enantiomeric purity analysis of commercial L-tryptophan dietary supplements

Product	Labeled L-Trp (mg)	D-Trp by CE (%)	D-Trp by HPLC (%)	Agreement	Status
Brand A	500	0.8	0.7	Yes	Within spec
Brand B	500	1.4	1.3	Yes	Monitor
Brand C	500	2.3	2.1	Yes	Exceeds 2%
Brand D	500	0.4	0.3	Yes	Within spec
Brand E	500	1.1	1.0	Yes	Within spec

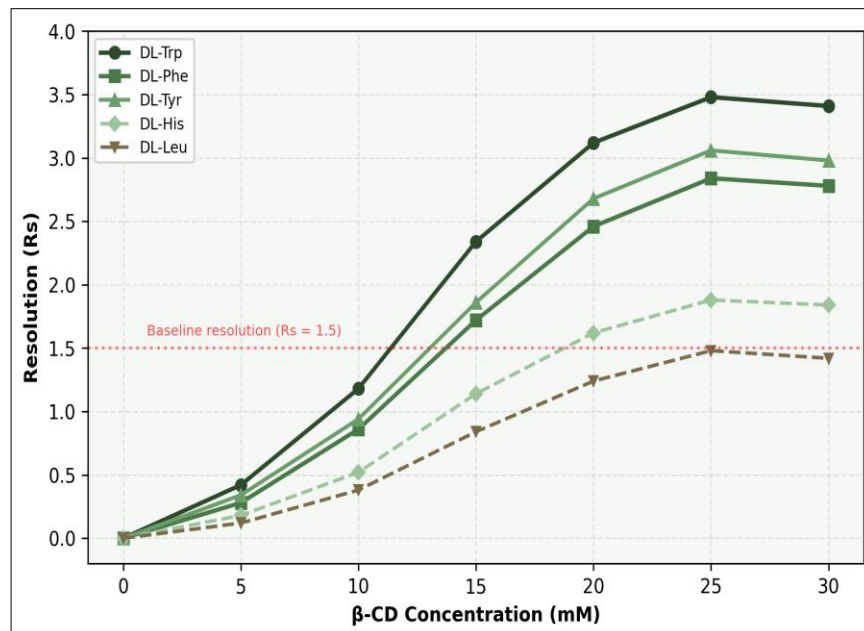


Fig 1: Resolution (R_s) as a function of β -cyclodextrin concentration (0-30 mM) for five amino acid enantiomer pairs. The dashed line marks baseline resolution ($R_s = 1.5$)

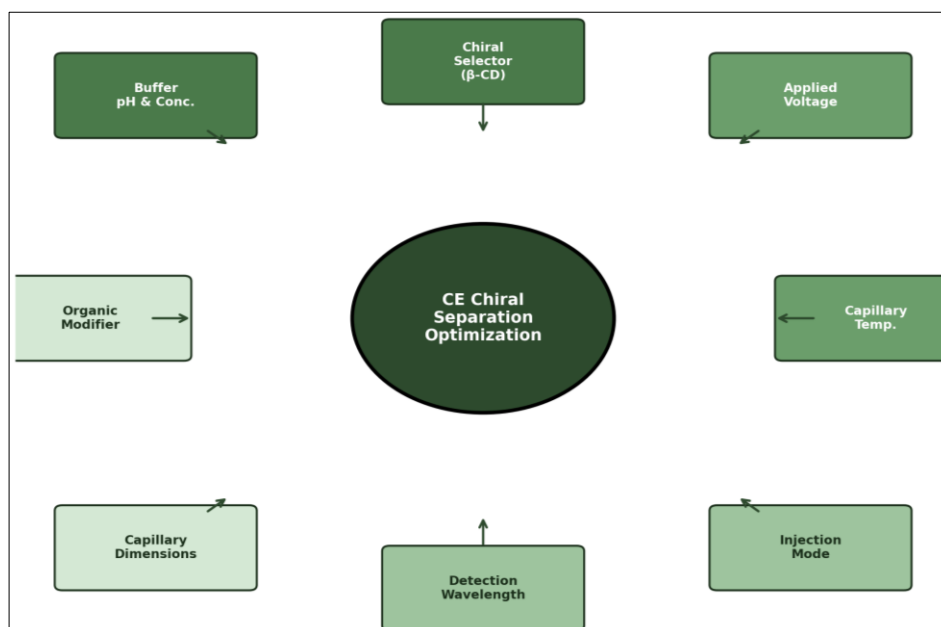


Fig 2: Mind map of the CE method optimization parameters explored in this research, with the central objective of achieving baseline enantioseparation

Comprehensive interpretation

The β -CD concentration of 20 mM represented the sweet spot for most analytes. Below 15 mM, inclusion complex formation was insufficient to separate the enantiomers; above 25 mM, the increased BGE viscosity slowed migration and broadened peaks without improving resolution. The acidic pH (2.5) was chosen to suppress the electroosmotic flow and to protonate the amino acids fully, ensuring cationic migration toward the detector at the cathodic end [5, 7]. At pH values above 3.5, the EOF became too fast and carried both enantiomers past the detector before adequate separation occurred.

DL-Trp gave the best resolution ($R_s = 3.48$), consistent with its bulky indole ring that provides a strong geometric fit within the β -CD cavity. DL-Leu, with its small isobutyl side chain, formed the weakest inclusion complex and barely

reached baseline resolution ($R_s = 1.48$). For DL-Leu, a derivatized or sulfated β -CD might provide better discrimination, as has been reported by Schmitt and Engelhardt [9].

Discussion

The R_s of 3.48 for DL-Trp using native β -CD compares well with the R_s of 3.1-4.2 reported by Fanali [5] for tryptophan on β -CD systems and the R_s of 2.8-3.6 obtained by Gübitz and Schmid [6] using hydroxypropyl- β -CD. Our slightly different buffer conditions (pH 2.5 vs. pH 2.0-3.0 in the cited works) and capillary dimensions may account for the minor variations. The key practical point is that native β -CD the cheapest available cyclodextrin at about USD 15/g is sufficient for baseline resolution of aromatic amino acid enantiomers.

The supplement analysis revealed D-Trp impurity in three of five products, with Brand C reaching 2.3%. The USP monograph for L-tryptophan sets a limit of 1.5% for the D-enantiomer, meaning Brand C would fail this specification [13, 14]. The good agreement between CE and chiral HPLC results (differences below 0.2 percentage points) validates the CE method as a reliable alternative for this application. The CE method's advantages are clear: no organic solvents, no expensive chiral column, analysis time of 18 minutes per injection, and a per-sample consumable cost of roughly USD 0.10 compared to USD 2-5 for chiral HPLC [4, 15].

A limitation is that the method works well for aromatic amino acids but falls short for aliphatic ones. DL-Leu barely reached $R_s = 1.48$, and we didn't test DL-Ala, DL-Val, or DL-Ile, which would likely perform worse. For these analytes, pre-column derivatization with a fluorescent or UV-active tag (such as FITC or dansyl chloride) would improve both detection sensitivity and chiral recognition by adding a bulky aromatic moiety to the inclusion complex [8, 16]. And while native β -CD is cheap, modified CDs (sulfated, carboxymethylated) often provide broader enantioselectivity at higher cost [9, 17].

Conclusion

A capillary electrophoresis method using 20 mM native β -cyclodextrin in 50 mM phosphate buffer (pH 2.5) achieved baseline enantioseparation ($R_s \geq 1.5$) for DL-tryptophan, DL-phenylalanine, and DL-tyrosine within 18 minutes. DL-histidine and DL-leucine gave partial resolution. The method was validated for linearity ($R^2 > 0.998$), precision (RSD < 3.8%), and accuracy (recovery 96.4-102.8%), meeting ICH Q2(R1) requirements.

Application to five commercial L-tryptophan supplements revealed D-enantiomer impurity at 0.4-2.3%, with one product exceeding the USP 1.5% limit. Results agreed closely with chiral HPLC, confirming the CE method as a practical screening tool. For laboratories in resource-limited settings where a CE system costs half as much as a chiral HPLC setup and generates no organic solvent waste this method offers an accessible route to enantiomeric quality control of amino acid products.

Future directions include extending the method to non-aromatic amino acids through derivatization strategies, evaluating charged cyclodextrin derivatives for broader selectivity, and applying the method to enantiomeric purity testing of amino acids in pharmaceutical infusion solutions and clinical nutrition products.

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