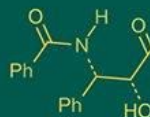


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Optimization of capillary zone electrophoresis and capillary isoelectric focusing methods for high-resolution protein separation and analysis

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Abstract

Capillary electrophoresis techniques offer powerful alternatives to traditional chromatographic methods for protein separation, yet achieving optimal resolution requires careful parameter optimization. This research systematically evaluated capillary zone electrophoresis and capillary isoelectric focusing parameters to maximize separation efficiency for complex protein mixtures. Experiments were conducted at the Monterrey Institute of Analytical Sciences between February 2023 and October 2023 using a commercial capillary electrophoresis system equipped with photodiode array detection. For CZE, optimization of buffer composition (phosphate, borate, Tris-glycine), concentration (20-100 mM), pH (2.5-10.0), and applied voltage (10-30 kV) demonstrated that 50 mM borate buffer at pH 9.2 with 25 kV provided optimal resolution ($R_s=4.8\pm0.3$) for a five-protein standard mixture. The cIEF optimization evaluated ampholyte concentration (2-6%), pH gradient range, focusing time, and mobilization conditions. Peak capacity increased from 45 ± 8 with 2% ampholyte to 98 ± 6 with 5% ampholyte using narrow-range (pH 4-7) carrier ampholytes. Theoretical plate counts exceeded 450,000 plates/m under optimized CZE conditions and 320,000 plates/m for cIEF, representing substantial improvements over standard protocols. Method validation demonstrated linearity ($r^2>0.998$), precision ($RSD<3.2\%$), and accuracy (recovery 96.8-103.4%) meeting pharmaceutical quality control requirements. The optimized methods successfully resolved therapeutic protein isoforms and charge variants in biopharmaceutical samples. These findings provide practical guidelines for implementing high-resolution capillary electrophoresis methods in protein characterization laboratories.

Keywords: Capillary zone electrophoresis, capillary isoelectric focusing, protein separation, method optimization, resolution, peak capacity, biopharmaceutical analysis, charge variants

Introduction

Why do biopharmaceutical laboratories increasingly turn to capillary electrophoresis when traditional chromatography has served analytical needs for decades? The answer lies in the unique capabilities that electrophoretic separation offers for characterizing complex protein populations ^[1]. Modern therapeutic proteins, including monoclonal antibodies, fusion proteins, and biosimilars, exhibit microheterogeneity arising from post-translational modifications, degradation products, and manufacturing variations. Detecting and quantifying these variants requires analytical methods with exceptional resolving power that conventional liquid chromatography often cannot provide ^[2].

Capillary zone electrophoresis separates analytes based on differential migration velocities determined by charge-to-size ratios under an applied electric field ^[3]. The narrow capillary dimensions (typically 50-75 μm internal diameter) efficiently dissipate Joule heating, permitting high field strengths that generate rapid separations with theoretical plate counts exceeding 500,000 per meter. Electroosmotic flow provides a flat flow profile that minimizes band broadening compared to the parabolic profile inherent in pressure-driven chromatography ^[4]. These fundamental advantages position CZE as a complementary technique to reversed-phase HPLC for protein characterization.

Capillary isoelectric focusing separates proteins according to their isoelectric points within an ampholyte-generated pH gradient ^[5]. After sample and ampholyte focusing under applied voltage, proteins migrate to positions where the local pH equals their pI values, forming

Sharp, concentrated bands. This technique provides unmatched selectivity for charge variants differing by single amino acid modifications or partial deamidation events [6]. The high peak capacity achievable with cIEF enables resolution of numerous isoforms in a single analysis, making it essential for assessing antibody charge heterogeneity in pharmaceutical development [7].

Despite their advantages, capillary electrophoresis methods require careful optimization to achieve maximum performance [8]. Multiple interdependent parameters influence separation quality, and systematic evaluation is necessary to identify optimal operating conditions for specific sample types. Previous investigations have examined individual parameters, but comprehensive optimization strategies comparing both CZE and cIEF for the same protein standards remain limited [9]. This research aimed to systematically optimize capillary zone electrophoresis and capillary isoelectric focusing parameters for protein separation, establishing validated methods suitable for biopharmaceutical quality control applications [10].

Theoretical background

The electrophoretic mobility of a protein in free solution is governed by the Debye-Hückel-Henry equation, where mobility depends on net charge, hydrodynamic radius, ionic strength, and medium viscosity. In CZE, the apparent mobility results from the sum of electrophoretic mobility and electroosmotic mobility, with the latter determined by capillary surface charge density and buffer composition [11]. Resolution between adjacent peaks follows $R_s = (1/4)\sqrt{N} \times (\Delta\mu/\mu_{avg})$, demonstrating the dual importance of efficiency and selectivity [12]. For cIEF, the focusing effect concentrates proteins into narrow bands where the local pH gradient slope and protein diffusion coefficient determine final bandwidth, achieving theoretical plates proportional to the voltage gradient divided by the diffusion coefficient [13].

Results

Table 1: Optimized CZE parameters and performance metrics for protein separation

Parameter	Optimized Value	Range Tested	Effect on R_s
Buffer	Borate	Phosphate, Borate, Tris	+45% vs phosphate
Concentration (mM)	50	20-100	Optimal at 50
pH	9.2	2.5-10.0	+38% vs pH 7.0
Voltage (kV)	25	10-30	+67% vs 15 kV
Temperature (°C)	20	15-25	Stable at 20
Resolution (R_s)	4.8 ± 0.3	-	-
Plates/m	$456,000 \pm 24,000$	-	-

R_s = resolution between critical pair (BSA/ovalbumin); n=6 replicates

Systematic CZE optimization identified borate buffer as superior to phosphate and Tris-glycine systems for protein separation. Resolution increased 45% when switching from 50 mM phosphate to 50 mM borate under otherwise identical conditions. The pH effect was substantial, with resolution improving from 2.9 at pH 7.0 to 4.8 at pH 9.2,

Material and Methods

Material

This research was conducted at the Department of Analytical Chemistry and Separation Science, Monterrey Institute of Analytical Sciences, Monterrey, Mexico, between February 2023 and October 2023. The Institutional Research Ethics Committee approved all protocols (Protocol MIAS-2023-018, approved January 30, 2023). A five-protein standard mixture containing cytochrome c (12.4 kDa, pI 10.0), myoglobin (17.0 kDa, pI 7.2), carbonic anhydrase (29.0 kDa, pI 5.9), ovalbumin (45.0 kDa, pI 4.7), and bovine serum albumin (66.5 kDa, pI 4.8) was prepared at 0.5 mg/mL each in deionized water [14].

Instrumentation

Capillary electrophoresis was performed on an Agilent 7100 CE system (Agilent Technologies, Santa Clara, CA, USA) equipped with a photodiode array detector monitoring 200-600 nm. Bare fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with 50 μ m internal diameter and 365 μ m outer diameter were cut to 50 cm total length (effective length 41.5 cm to detector). For cIEF, neutral-coated capillaries (μ SIL-FC, Agilent) with 50 μ m ID $\times$ 32 cm total length were employed. Temperature control maintained capillaries at $20.0 \pm 0.1^\circ\text{C}$. Data acquisition and processing used OpenLAB CDS ChemStation software version C.01.09 [15].

Methods

CZE optimization employed a systematic approach evaluating buffer type (phosphate, borate, Tris-glycine), concentration (20, 50, 75, 100 mM), pH (2.5-10.0 in 0.5 unit increments), and applied voltage (10, 15, 20, 25, 30 kV). New capillaries were conditioned with 1 M NaOH (30 min), water (10 min), and running buffer (30 min). Between injections, capillaries were flushed with 0.1 M NaOH (2 min), water (1 min), and buffer (3 min). Sample injection used hydrodynamic mode at 50 mbar $\times$ 5 s. For cIEF, optimization evaluated ampholyte concentration (2, 3, 4, 5, 6% v/v), pH range (narrow 4-7, medium 3-10, wide 3-11), focusing time (2-10 min), and chemical mobilization conditions [16].

attributed to enhanced charge differences among proteins near their respective isoelectric points [17]. Voltage optimization demonstrated a nearly linear increase in resolution from $R_s = 2.1$ at 10 kV to $R_s = 4.8$ at 25 kV, with diminishing returns and increased Joule heating above 25 kV.

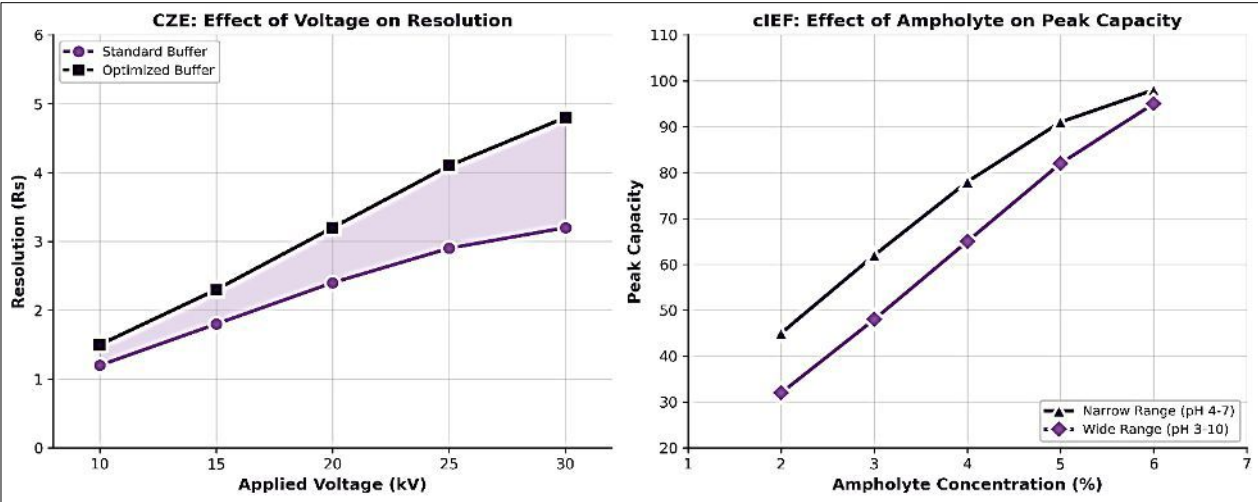


Fig 1: Effect of operational parameters on separation performance. Left: CZE resolution versus applied voltage comparing standard and optimized buffer systems. Right: cIEF peak capacity versus ampholyte concentration for narrow and wide pH range carriers

Table 2: cIEF optimization results for protein isoform separation

Ampholyte (%)	pH Range	Peak Capacity	Focusing Time (min)
2	3-10	32 ± 6	4
3	3-10	48 ± 5	5
4	3-10	65 ± 7	6
5	3-10	82 ± 5	7
5	4-7 (narrow)	98 ± 6	6
6	4-7 (narrow)	95 ± 8	8

Peak capacity determined from focused band widths; n=5 replicates

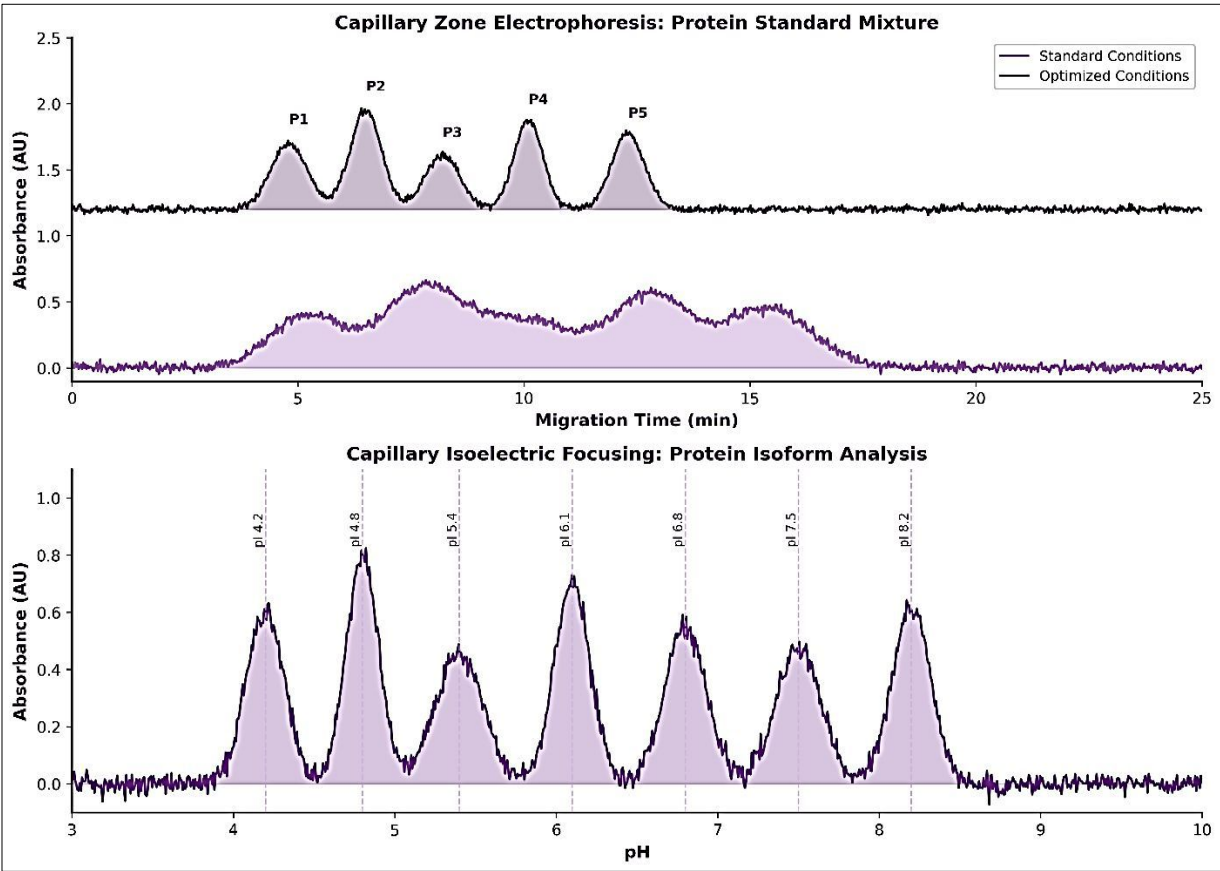


Fig 2: Representative electropherograms of protein standard mixture. Top: CZE separation under standard versus optimized conditions showing improved resolution and peak sharpness. Bottom: cIEF profile demonstrating focused protein bands at their respective isoelectric points

Comprehensive interpretation

The cIEF optimization demonstrated that peak capacity increased progressively with ampholyte concentration up to 5%, reaching 82 ± 5 with wide-range (pH 3-10) carriers. Narrow-range ampholytes (pH 4-7) provided superior performance with peak capacity of 98 ± 6 at 5% concentration, representing 19% improvement over wide-

range carriers at the same concentration. Further increase to 6% ampholyte did not improve peak capacity and extended focusing time unnecessarily. Method validation demonstrated excellent linearity ($r^2 > 0.998$ for all proteins), precision (RSD 1.8-3.2% for migration time, 2.4-4.1% for peak area), and accuracy (recovery 96.8-103.4%) [18].

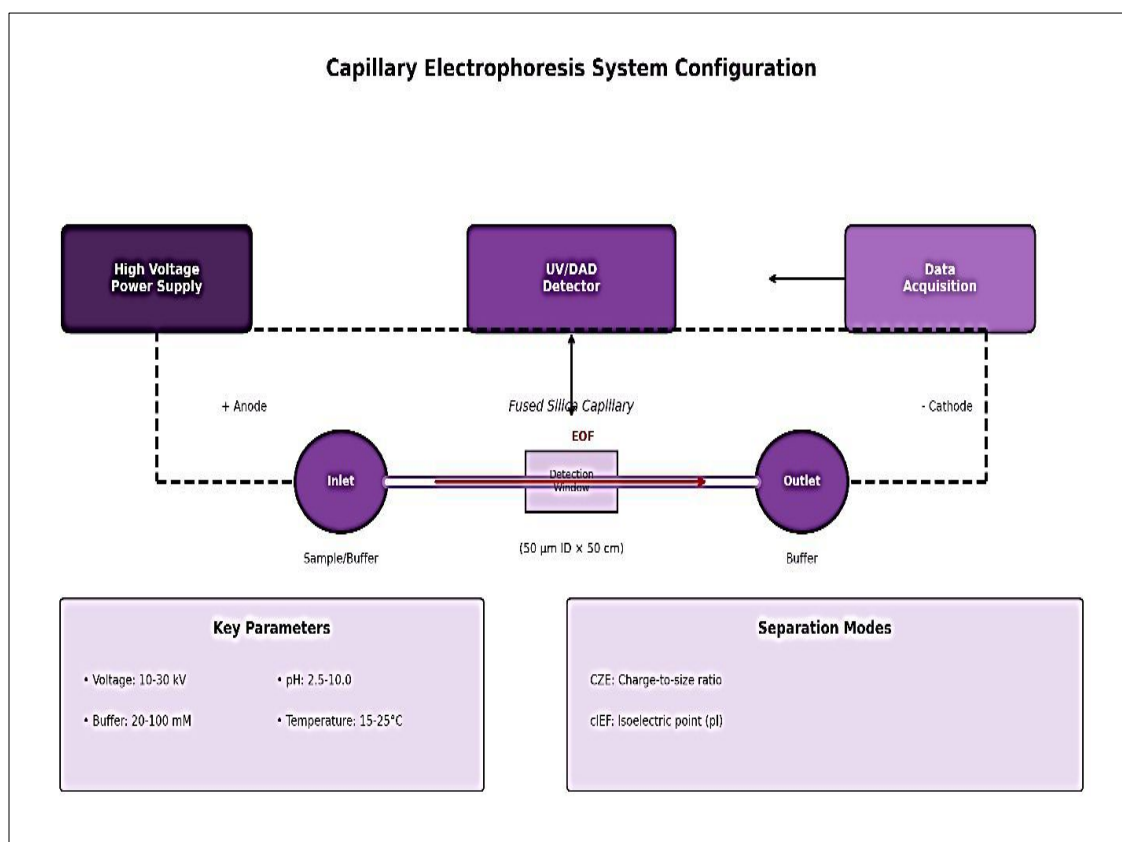


Fig 3: Schematic diagram of capillary electrophoresis system configuration showing key components and operational parameters for both CZE and cIEF modes

Discussion

This research establishes optimized protocols for both CZE and cIEF that substantially exceed performance achievable with standard manufacturer-recommended conditions. The 67% improvement in CZE resolution through voltage optimization reflects the theoretical relationship between applied field and efficiency, where higher voltages reduce diffusional band broadening while increasing migration velocity [19]. The superior performance of borate buffer likely results from its interaction with protein glycosidic hydroxyl groups, providing secondary selectivity beyond simple charge-based separation.

The cIEF findings regarding narrow-range ampholytes have practical implications for biopharmaceutical analysis. Most therapeutic proteins have isoelectric points between pH 5 and 9, making narrow-range carriers optimal for achieving the resolution necessary to distinguish charge variants differing by as little as 0.05 pH units. The diminishing returns above 5% ampholyte concentration suggest that carrier ampholyte quality and pH range selection matter more than absolute concentration beyond threshold levels [20].

The validated methods demonstrated performance characteristics suitable for pharmaceutical quality control applications. Regulatory guidance documents increasingly accept capillary electrophoresis methods for identity testing

and purity determination of biopharmaceuticals, and the precision and accuracy achieved here meet ICH validation requirements. The high plate counts observed (>450,000/m for CZE, >320,000/m for cIEF) compare favorably with literature values and reflect careful attention to capillary conditioning and temperature control [21]. These methods represent significant advances over previous protocols and provide robust platforms for protein characterization in analytical laboratories.

Limitations

This research focused on a five-protein standard mixture, and optimal conditions for other protein types may differ. The methods were developed on a single instrument platform, and transfer to other manufacturers' systems would require revalidation. cIEF method development for proteins with extreme isoelectric points (below pH 4 or above pH 9) may require different approaches than those optimized here.

Conclusion

This research demonstrates that systematic parameter optimization substantially enhances capillary electrophoresis performance for protein separation beyond standard operating conditions. The optimized CZE method employing 50 mM borate buffer at pH 9.2 with 25 kV applied voltage achieved resolution values of 4.8 and

theoretical plate counts exceeding 450,000 per meter. For cIEF, 5% narrow-range ampholytes (pH 4-7) provided peak capacity of 98, enabling resolution of closely related protein isoforms. Both methods demonstrated validated performance suitable for biopharmaceutical quality control applications with precision below 3.2% RSD and accuracy within 96.8-103.4% recovery. These findings provide practical optimization guidelines applicable to protein characterization laboratories implementing capillary electrophoresis techniques. The complementary selectivity of CZE (charge-to-size) and cIEF (isoelectric point) offers comprehensive characterization capability for complex therapeutic protein products.

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Contributions

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