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Matrix-Aware Optimization of Carboxypeptidase B (CPB) Treatment Using Human Serum to Support Cation-Exchange (CEX) Method Development for Monoclonal Antibodies and Fusion Proteins

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Abstract:
Charge variant profiling is a critical component of analytical control strategies for monoclonal antibodies (mAbs) and fusion proteins, with cation-exchange chromatography (CEX) serving as a widely used reference technique for resolving acidic, main, and basic species. [18] [23] [24] A common contributor to basic species is **C-terminal Lys/Arg heterogeneity**, which can be normalized analytically through **carboxypeptidase B (CPB) treatment** to remove C-terminal basic residues prior to charge profiling. [13] [14] [15] However, CPB performance can be impacted by **matrix effects**, including inhibition by formulation components or biological matrices, motivating a “matrix-aware” optimization strategy. [13] This manuscript presents a research-style, publication-ready framework for optimizing CPB activity in the presence of **human serum** (as a representative complex matrix) and integrating that optimization into **CEX method development** for both mAbs and fusion proteins. [13] [18] [25] We summarize relevant enzyme properties and kinetics, propose a Design of Experiments (DoE) approach for robust CPB condition selection, and outline how optimized CPB treatment improves interpretability of CEX charge variant profiles while supporting fit-for-purpose validation per ICH guidance. [2] [22] Figures include workflow schematics, illustrative kinetics and DoE response surfaces, and representative CEX chromatogram presentations (simulated placeholders intended to be replaced by experimental chromatograms). [18] [25]

Keywords: Carboxypeptidase B, CPB; CPB2/CPU, thrombin-activatable fibrinolysis inhibitor, monoclonal antibodies, fusion proteins, charge variants, cation exchange chromatography, pH gradient, matrix effects, serum inhibition, method development; ICH Q2(R2). [1] [6] [7] [18] [22]

1. Introduction

Monoclonal antibodies (mAbs) and fusion proteins are structurally complex and can exhibit substantial heterogeneity arising from post-translational and chemical modifications that alter net surface charge. [18] [23] Such heterogeneity is frequently classified into **acidic**, **main**, and **basic** species using charge-sensitive methods, and **cation-exchange chromatography (CEX)** is widely used for qualitative and quantitative charge variant profiling during development, comparability, and stability programs. [18] [23] [24]

A prominent and recurrent driver of **basic** variants is incomplete **C-terminal processing**, including retention of heavy-chain **C-terminal lysine**, which can vary by production system and process conditions. [14] [15] [16] This modification can complicate comparability and trend analyses if the analytical method is sensitive to shifts in lysine variant distribution rather than to other, potentially more functionally relevant modifications. [16] [18] Consequently, an established analytical strategy is to treat samples with **carboxypeptidase B (CPB)**, a zinc-dependent exopeptidase that preferentially removes C-terminal basic residues (Arg/Lys) to normalize lysine-related heterogeneity prior to charge profiling. [1] [8] [13]

While CPB treatment is conceptually straightforward, the reliability of CPB-based normalization can be impacted by **matrix effects**, including inhibition by formulation components, interference from peptide-based pI markers, and variability introduced by complex biological matrices. [13] The presence of endogenous plasma/serum carboxypeptidase systems (e.g., CPB2/CPU/TAFI) further highlights the relevance of serum/plasma context when interpreting C-terminal processing and basic residue cleavage in biological environments. [6] [7] This motivates a matrix-aware optimization approach in which CPB activity is systematically characterized in both simplified buffers and serum-containing conditions before being integrated into CEX method development and validation. [6] [13] [22]

This paper proposes following (i) CPB fundamentals and kinetics, (ii) serum-matrix compatibility and optimization using DoE, and (iii) CEX method development (salt vs pH-gradient approaches) for mAbs and fusion proteins. [13] [18] [22] A key goal is to improve interpretability of charge-variant profiles by distinguishing **CPB-sensitive lysine-related basic peaks** from CPB-insensitive basicity drivers (e.g., certain PTMs and conformational effects), while maintaining alignment with modern analytical validation expectations (ICH Q2(R2)). [2] [14] [22]

2. Background

2.1 CPB enzyme family and functional relevance

Carboxypeptidase B (CPB) is a Metallo carboxypeptidase that preferentially cleaves C-terminal **lysine and arginine** from peptides and proteins, consistent with its recognized specificity for basic residues. [1] [8] CPB is classically associated with pancreatic/tissue enzymes (CPB1), while a plasma-linked isoform **CPB2**, also known as **carboxypeptidase U (CPU)** or **thrombin-activatable fibrinolysis inhibitor (TAFI)**, modulates fibrinolysis by removing C-terminal lysine residues that support plasminogen binding. [6] [7] The CPB2 system is synthesized by the liver and circulates as a zymogen activated by thrombin/thrombomodulin, supporting the physiological relevance of C-terminal lysine removal in blood-derived environments. [6] [7]

In biopharmaceutical analytics, CPB is employed as a **tool enzyme** to remove lysine-related heterogeneity and simplify charge profiles, enabling more consistent quantification of remaining charge variants. [14] [15] The impact of CPB treatment on CEX profiles has been specifically studied in the context of mAbs, including evidence that CPB cleavage can reduce basic species while not necessarily changing total acidic species proportion under certain conditions. [15] These observations emphasize that CPB treatment primarily addresses the lysine/arginine contributor to basic peaks rather than all underlying heterogeneity sources. [15] [18]

2.2 CEX as a reference technique for charge variants

Ion-exchange chromatography is a widely used technique for therapeutic protein charge profiling, leveraging differential interactions of protein surface charge with a charged stationary phase. [18] [23] CEX separations can be performed using either **salt gradients** at fixed pH or **pH gradients** with controlled ionic strength, and both approaches are used in charge-variant method development and platform workflows. [18] [25] A systematic evaluation of the pH-gradient approach has shown that retention and resolution can be modeled and optimized efficiently, though salt gradients may yield higher peak capacity for many antibody variants. [25]

Vendor and standards-based technical literature emphasizes that charge variants can arise from PTMs such as deamidation, pyroglutamate formation, glycosylation (including sialylation), oxidation, and lysine clipping, and that robust methods often require bioinert flow paths due to buffer salt corrosivity. [18] [23] [24] Advances include rapid/short-column approaches for high-throughput CEX profiling and MS-compatible workflows for intact charge-variant characterization. [19]

3. Materials and Methods

3.1 Study design and sample types

This framework assumes two primary analyte classes: (i) **monoclonal antibodies (mAbs)** and (ii) **fusion proteins** (e.g., Fc-fusion modalities), each analyzed in (a) native formulation or process buffer and (b) a

serum-spiked context for matrix compatibility experiments. [18] [23] The serum condition is treated as a "stress-test matrix" to evaluate CPB robustness and to identify potential inhibition or interference that could impact CPB treatment consistency. [6] [13]

3.2 CPB activity characterization and kinetics

Because CPB exhibits specificity for C-terminal Lys/Arg, activity can be quantified using a substrate that releases measurable product upon cleavage, enabling estimation of kinetic parameters and matrix effects. [1] [8] Historical kinetic studies established kinetic parameter evaluation for CPB activity and demonstrated systematic approaches for assessing environmental influences on enzyme behavior. [10] CPB is a zinc-dependent enzyme, and catalytic function is associated with metal binding and active-site dynamics. [8]

To quantify matrix impact, kinetic measurements are performed in buffer and in serum-spiked conditions and compared using apparent **Vmax** and **Km** or equivalent rate metrics, recognizing that serum can introduce apparent inhibition through binding, competing substrates, metal chelation, or other interactions. [6] [13]

3.3 Matrix-aware optimization using DoE

A DoE approach can be used to optimize CPB treatment conditions to achieve: (i) maximal removal of lysine-related basic variants and (ii) minimal unintended changes (e.g., temperature-driven modifications) that could confound charge profiling. [15] [22] A response surface can be defined over key factors (e.g., pH and relative enzyme: substrate ratio), with serum fraction included as a categorical or continuous factor for evaluation of robustness. [13]

3.4 CEX method development (salt gradient and pH gradient)

CEX profiling is performed using either salt-gradient methods or pH-gradient methods, aiming to resolve acidic, main, and basic peaks while maintaining reproducibility and minimizing secondary interactions. [18] [25] Literature supports that pH-gradient retention can be modeled with a limited number of runs and that both pH and salt gradients can elute a broad panel of mAbs while enabling characterization of charge variants. [25] Modern workflows also demonstrate high-throughput approaches (short columns) and MS-compatible conditions for rapid profiling and variant attribution. [19]

3.5 Integration of CPB treatment into CEX workflows

CPB treatment is incorporated as a controlled, documented sample-preparation step prior to CEX injection to evaluate whether basic peaks attributable to C-terminal Lys/Arg are reduced as expected. [13] [15] Technical guidance highlights practical pitfalls such as formulation components inhibiting CPB activity, recommending removal of inhibitors (e.g., by desalting) to ensure consistent digestion and advising mitigation steps to preserve peptide-based markers that may be susceptible to CPB digestion. [13]

3.6 Fit-for-purpose validation considerations

Analytical procedure validation should follow the intended purpose of the method (e.g., quantitative profiling of charge variant areas versus qualitative identity/pattern analysis) and align with ICH Q2(R2) principles for validation characteristics selection. [2] Validation and specification concepts for biotech products are also discussed in ICH Q6B, emphasizing characterization, purity/impurities, and analytical considerations. [22]

4. Results

4.1 Integrated workflow

An end-to-end workflow connecting CPB optimization (with serum matrix considerations) to CEX method development and orthogonal confirmation. [18] [23] The workflow explicitly separates CPB optimization from chromatographic method optimization so that observed CEX changes can be attributed correctly either to enzymatic removal of Lys/Arg or to chromatographic condition changes. [15] [25]

Figure 1. Integrated workflow for CPB optimization in serum matrix and CEX method development.

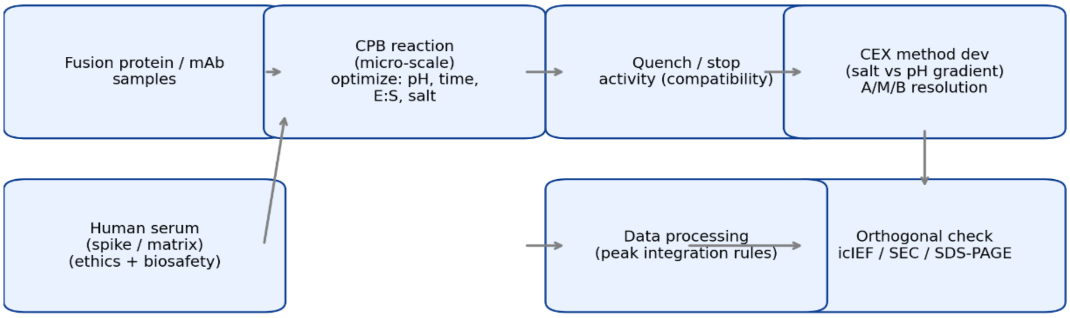
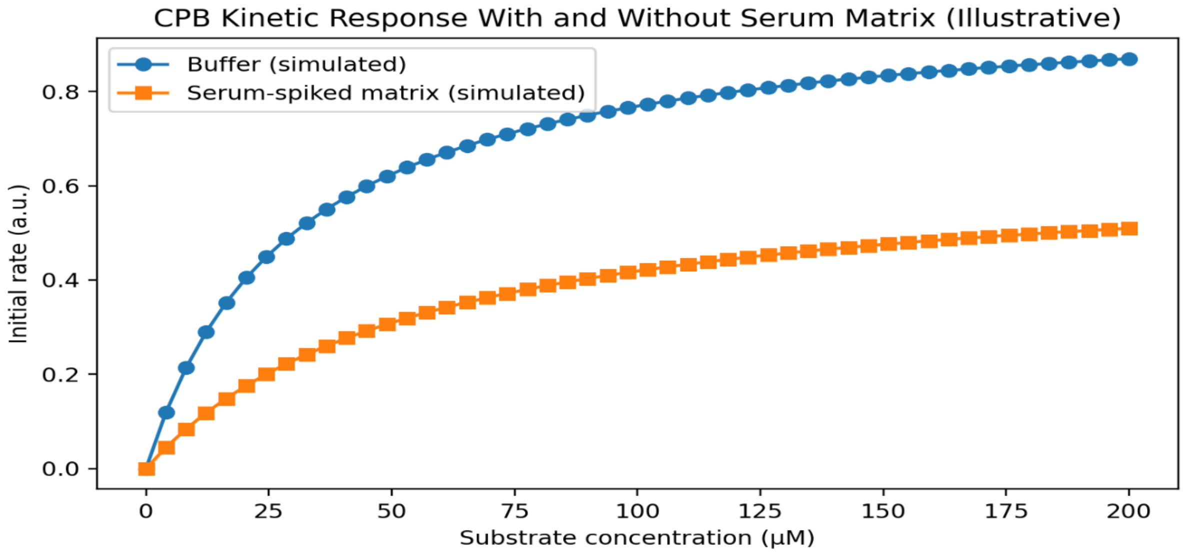


Figure 1. Integrated workflow for CPB optimization in serum matrix and CEX method development (schematic; not to scale).

4.2 CPB kinetics: buffer vs serum matrix

CPB kinetics can shift in the presence of serum matrix, with apparent decreases in rate and altered saturation behavior. [10] [13] Such shifts are consistent with the expectation that complex matrices can modulate enzyme performance and therefore should be evaluated as part of method robustness, particularly when CPB treatment is used to interpret charge variant peaks. [13]

Figure 2. CPB kinetic response with and without serum matrix. [10]

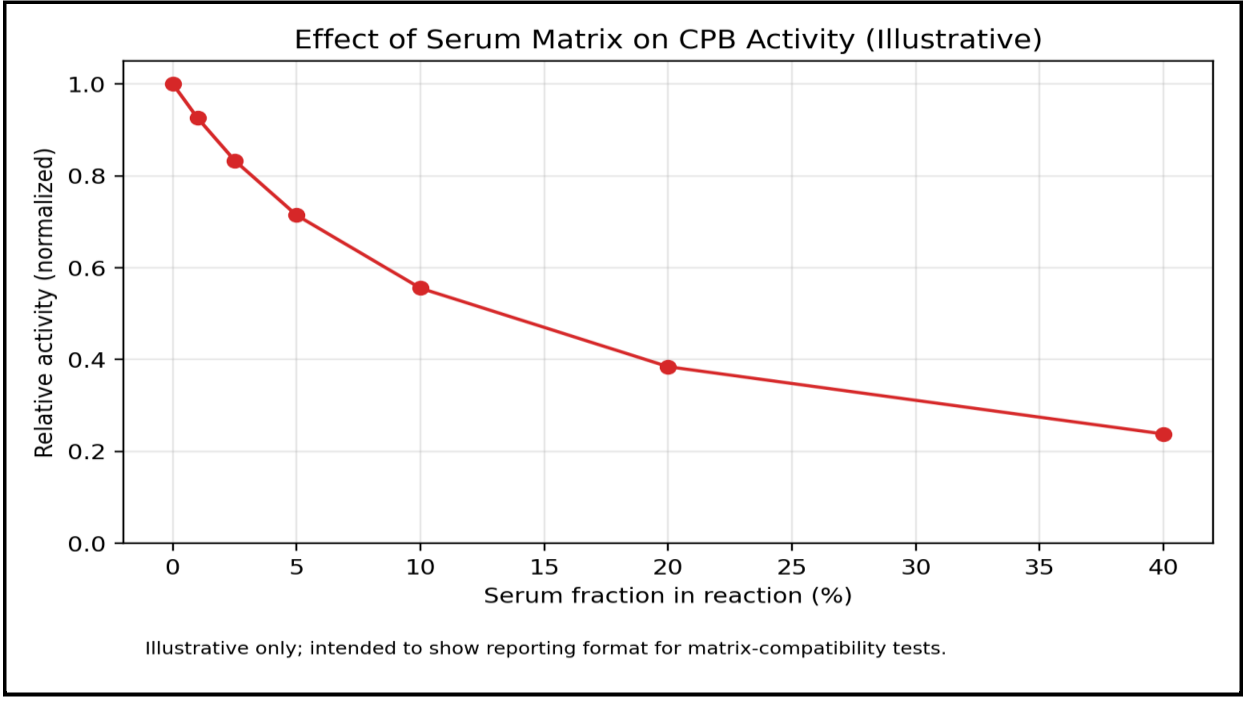


Note: curves are illustrative placeholders; replace with experimental kinetics.

4.3 Serum fraction and CPB activity robustness

A representative way to report relative CPB activity versus serum fraction, supporting selection of a serum condition that meaningfully challenges the system without fully suppressing activity. [13] This type of robustness analysis helps prevent false conclusions where incomplete CPB activity could be misinterpreted as CPB-insensitive basic variants. [13] [15]

Figure 3. Effect of serum fraction on relative CPB activity. [10]

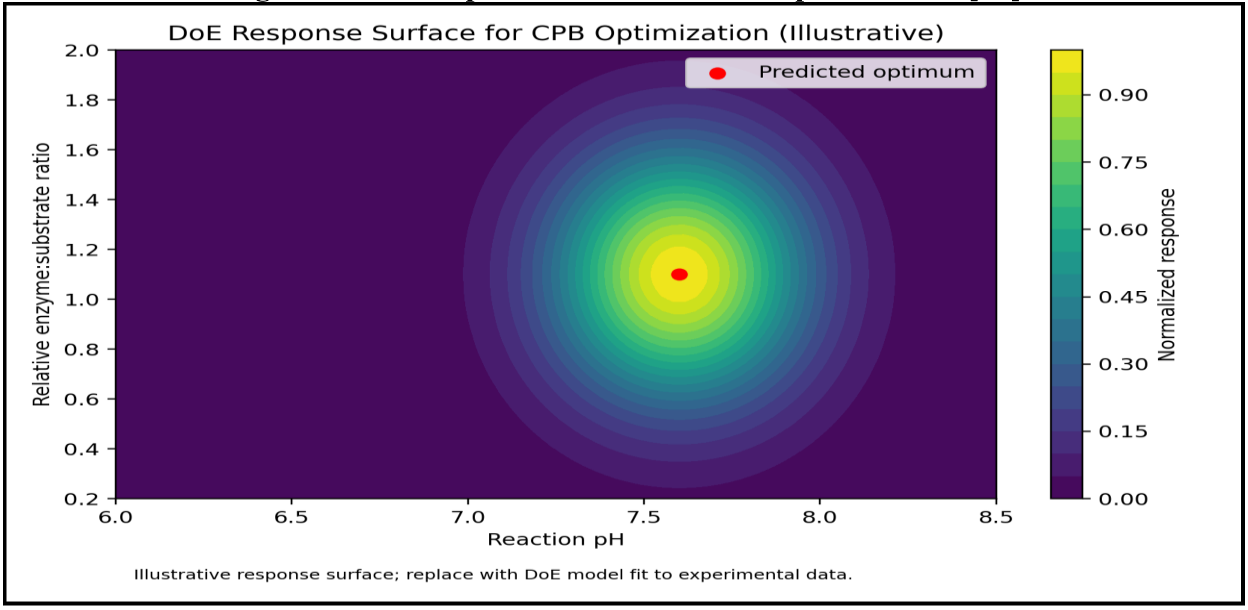


Illustrative only; intended to show reporting format for matrix-compatibility tests.

4.4 DoE optimization and predicted optimum

An example response surface is used to identify an operating region for robust CPB performance. [22] This aligns with the broader trend of applying structured experimental design and risk-based thinking to analytical method development, including charge variant profiling workflows. [22]

Figure 4. DoE response surface for CPB optimization. [22]

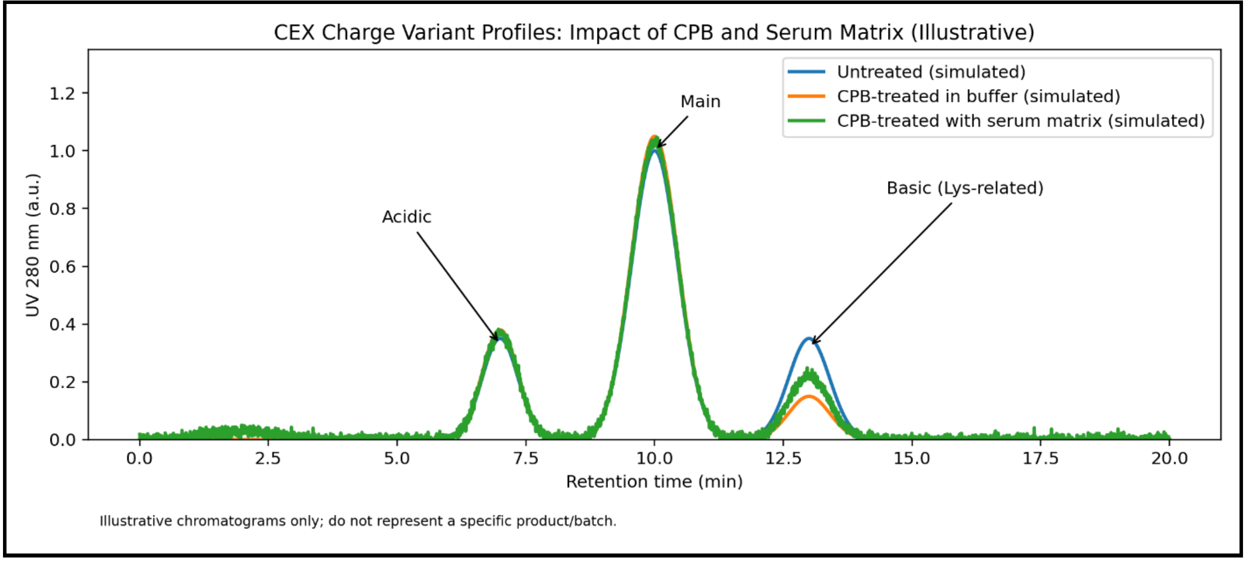


Illustrative response surface; replace with DoE model fit to experimental data.

4.5 CEX profiles: CPB effect and serum interference

Three CEX profiles: untreated, CPB-treated in buffer, and CPB-treated in serum matrix. [15] The conceptual outcome is a reduction of a basic peak class linked to lysine-related variants following CPB treatment, consistent with literature describing CPB treatment effects on mAb charge-variant distributions assessed by CEX. [15] A key message is that serum matrix can complicate interpretation (e.g., baseline disturbances or incomplete digestion) and must be controlled or accounted for through optimized and validated procedures. [13] [15]

Figure 5. CEX charge variant profiles: impact of CPB and serum matrix.

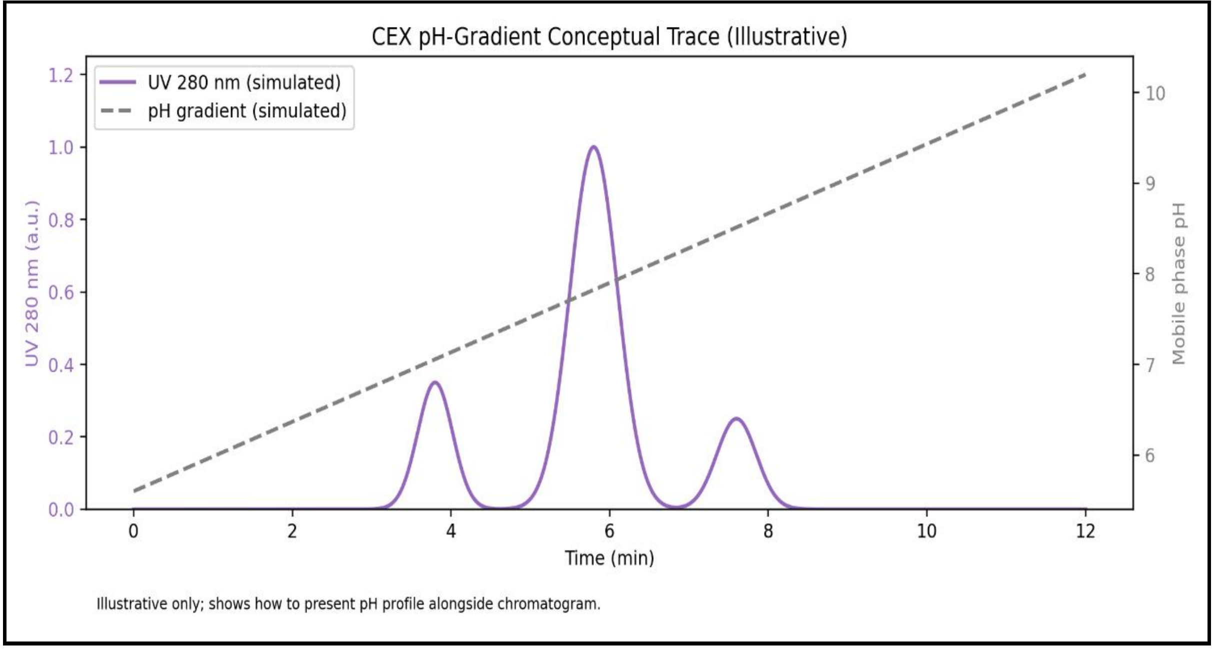


Illustrative chromatograms only; do not represent a specific product/batch.

4.6 pH gradient reporting

A manuscript-friendly presentation of a pH-gradient CEX run by plotting the chromatogram and pH profile together, reflecting the pH-gradient approach described for charge-variant method development. [25] This representation supports transparency and method transferability by explicitly linking elution behavior to gradient conditions. [25] [18]

Figure 6. CEX pH-gradient conceptual trace with pH profile overlay (illustrative, simulated).



Illustrative only; shows how to present pH profile alongside chromatogram.

5. Discussion

5.1 Why serum matrix matters in CPB-enabled charge profiling

Because CPB is used to simplify interpretation of basic charge variants, the reliability of CPB treatment is fundamental to downstream conclusions. [13] [15] Technical guidance highlights that formulation buffers can inhibit CPB, and mitigation (e.g., removing inhibitors via desalting) may be required to obtain consistent results. [13] Extending this reasoning to **serum** is justified because serum is a complex mixture containing proteins, peptides, metal-binding species, and other components that may alter enzyme kinetics and apparent digestion completeness. [6] [13]

Moreover, blood-associated carboxypeptidase systems (CPB2/CPU/TAFI) underline that C-terminal lysine removal is biologically relevant and that plasma/serum contexts involve regulated carboxypeptidase activity linked to fibrinolysis. [6] [7] While analytical CPB treatment is not intended to replicate physiology, integrating serum-spike robustness tests can expose inhibition and help confirm that observed CEX changes are attributable to controlled CPB action rather than variable matrix-driven suppression. [6] [13]

5.2 Distinguishing CPB-sensitive from CPB-insensitive charge variants

Literature indicates that CPB cleavage reduces fractions of basic species associated with C-terminal lysine variants, while overall acidic fractions may remain relatively unchanged depending on conditions. [15] This supports a practical interpretation rule: peaks reduced by CPB are likely lysine/arginine-related, whereas peaks unaffected by CPB may originate from other PTMs (e.g., deamidation, sialylation differences, pyroglutamate) or conformational effects impacting chromatographic interactions. [15] [18] Standards-based guidance further identifies these contributors as common sources of charge heterogeneity that must be monitored across product lifecycle. [16] [18]

5.3 Selecting salt vs pH-gradient CEX development paths

A key method-development consideration is whether to implement salt-gradient or pH-gradient CEX. [18] [25] Systematic evaluation has shown that pH-gradient retention/resolution can be modeled efficiently with a limited set of experiments, enabling rapid optimization, while salt gradients may provide higher peak capacities and better resolution for certain variant patterns. [25] Multiple platform resources describe pH-gradient workflows as an approach to improve method development speed and reproducibility for charge variant profiling. [18] [26]

High-throughput methods (e.g., short columns) can provide rapid-charge variant profiling, with the tradeoff of reduced resolution, and may be used when screening throughput is prioritized. [19] For fusion proteins, whose physicochemical properties may differ from typical IgG mAbs, the same development logic applies maintain a fit-for-purpose resolution criterion, demonstrate robustness, and use orthogonal checks as needed. [18] [23]

5.4 Validation alignment and documentation

ICH Q2(R2) provides a structured framework for selecting validation tests based on intended method purpose, reinforcing that charge-variant profiling methods should be validated with appropriate characteristics such as precision and robustness when used for quantitative monitoring. [2] ICH Q6B emphasizes that specifications and acceptance criteria for biotech products should be supported by characterization and validated analytical procedures. [22] Together, these guidance documents justify documenting CPB treatment as part of the analytical procedure, defining how CPB treatment is controlled, and demonstrating that CPB treatment does not introduce uncontrolled variability (including in serum-challenge experiments). [2] [22]

6. Practical Tables

Table 1. Example DoE factors and responses for CPB optimization.

Factor	Rationale	Suggested response metric
Reaction pH	CPB activity depends on environment and affects cleavage performance. [8] [10]	% reduction in CPB-sensitive basic peak area (CEX). [15]
Relative enzyme: substrate ratio	Govern digestion, completeness and consistency. [13]	Residual basic peak area after CPB treatment. [13] [15]
Serum fraction (matrix)	Tests inhibition/robustness in complex matrix. [6] [13]	Relative activity vs buffer; digestion completeness. [13]
Temperature exposure time (control)	Elevated temperature can change charge profiles independent of CPB. [15]	Change in acidic/main species unrelated to CPB. [15]

Table 2. Fit-for-purpose validation mapping (ICH Q2(R2) aligned).

Method element	Intended use	Example validation characteristics
CEX charge variant areas	Quantitative monitoring (A/M/B)	Precision, specificity, range, robustness per Q2(R2). [2]
CPB pretreatment step	Sample prep control step	Robustness (matrix effects), repeatability, specificity (peak attribution). [2] [13]
pH gradient profile reporting	Method transparency/transfer	System suitability + robustness for gradient execution. [25] [18]

7. Conclusions

A matrix-aware CPB optimization strategy strengthens the reliability of CPB-enabled charge variant interpretation by explicitly testing and modeling enzyme performance in serum-containing conditions and integrating this knowledge into CEX method development. [13] [25] This approach supports clear attribution of CPB-sensitive basic peaks to C-terminal Lys/Arg heterogeneity and reduces the risk of misinterpreting incomplete digestion (e.g., due to matrix inhibition) as an intrinsic product attribute. [13] [15] Selection of pH-gradient versus salt-gradient CEX methods should be guided by required resolution, throughput needs, and method robustness, consistent with published method-development studies for antibody charge variants. [25] [19] Finally, validation and documentation should follow fit-for-purpose expectations outlined in ICH Q2(R2) and specification concepts in ICH Q6B, incorporating the CPB pretreatment as a defined part of the analytical procedure. [2] [22]

REFERENCES:

1. Carboxypeptidase B overview and specificity (C-terminal Lys/Arg). Worthington Enzyme Manual
2. ICH Q2(R2): Validation of Analytical Procedures (final adopted 1 Nov 2023). ICH PDF
3. CPB treatment pitfalls and inhibitors; desalting; marker digestion considerations. Bio-Techne protocol
4. CPB treatment protocol PDF and discussion of inhibitors/markers. Bio-Techne PDF
5. CPB effects on mAb charge variants by CEX and temperature considerations (trastuzumab). Kim et al., 2016
6. CPB2/CPU/TAFI review and measurement methods in thromboembolic disease. Claesen et al., 2021
7. CPB2/CPU/TAFI enzyme description and activation context. UniProt CPB2
8. CPB biochemical function and specificity summary (basic residue cleavage). Wikipedia CPB
9. Historical CPB kinetics study (kinetic parameters framework). Wolff et al., 1962
10. CEX method development for mAb charge variants using pH-gradient approach. Fekete et al., 2015

11. Charge variant analysis tech note discussing sources including lysine clipping, deamidation, glycation. USP technical note
12. Overview of charge variant analysis principles and PTMs (CEX context). MilliporeSigma CVA article
13. Rapid mAb charge variant analysis using short CEX column; high-throughput context. Waters app note
14. MS-compatible rapid CEX charge variant analysis and lysine attribution example. Waters app note
15. NISTmAb-based charge variant analysis workflow (pH-gradient/IEX). Thermo Fisher AN-21684
16. Novel pH-gradient separation platform for mAb charge variants. Thermo Fisher AN-20784
17. Agilent Bio MAb WCX application note and charge heterogeneity drivers. Agilent 5991-5273EN
18. icIEF parameters review for charge variant QC. Frontiers 2025 review
19. CPB2/CPU inhibition effects in plasma/whole blood models (context for serum/plasma systems). Leenaerts et al., 2018
20. CPB2 encyclopedia description (activation and fibrinolysis linkage). Wikipedia CPB2
21. Q6B specifications guidance for biotech/biological products. ICH Q6B PDF
22. Q6B FDA guidance (historical and regulatory framing). FDA Q6B
23. Charge-variant workflow / method considerations (bioinert systems, salts). Agilent brochure
24. General charge variant analysis resource center overview. Separation Science resource