Analyzing Charge Heterogeneity in Monoclonal Antibodies via SCX-HPLC using BioPro SF 5 µm

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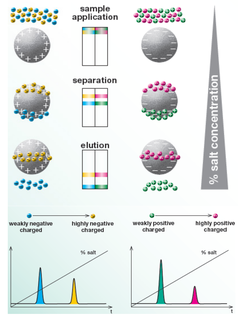
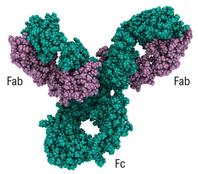
Introduction

Strong cation exchange chromatography (SCX) coupled with high-performance liquid chromatography (HPLC) is a robust analytical technique for separating and characterizing charge variants in biopharmaceuticals. This study explores SCX-HPLC for analyzing charge heterogeneity in three widely used therapeutics: Amatuximab, Trastuzumab, and NISTmab. Utilizing the YMC BioPro SF column (5 µm), known for its exceptional resolving power for acidic variants at lower pressure, we optimized critical parameters such as pH, salt gradient, and flow rate to achieve high-resolution separation of acidic, basic, and main peak isoforms. The findings reveal distinct charge variant profiles for each therapeutic, providing insights into their post-translational modifications and stability. These results underscore the utility of SCX-HPLC and advanced column technologies like YMC BioPro SF in ensuring the quality and consistency of biopharmaceutical product.

IEX Mechanism Overview





Mechanism: separation according to charge state electrostatic interactions

Elution order: weakly charged molecules → highly charged molecules

Benefits: preservation of native state versatile method development

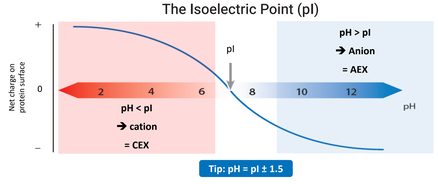
Applications: peptides/proteins, antibodies (mAbs), antibody-drug-conjugates (ADCs)

→ **Requirements:**
- → consideration of analytes isoelectric points (pI)

→ Mobile phase: gradient elution
- → aqueous salt-/buffer solutions
- → change of salt concentration and/or pH

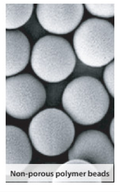
→ Stationary phase: hydrophilic, positively or negatively charged, porous or non-porous
- → AEX: quaternary ammonium, diethylamino ethyl
- → CEX: sulfopropyl, carboxymethyl

The Isoelectric Point (pI)



Tip: pH = pI ± 1.5

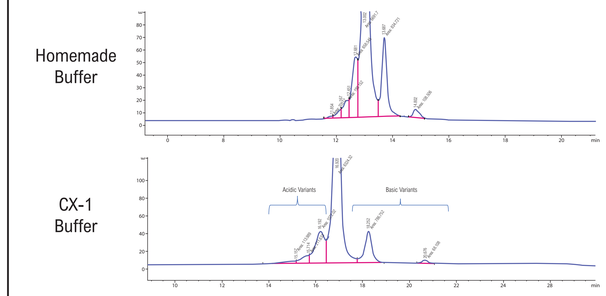
	BioPro IEX QF	BioPro IEX SF
Matrix	hydrophilic polymer (polymethacrylate)	hydrophilic polymer (polymethacrylate)
Particle size / µm	3, 5	3, 5
Pore size / nm	non-porous	non-porous
Charged group	$-CH_2N^+(CH_3)_3$	$-(CH_2)_3SO_3^-$
Counter ion	Cl^-	Na^+
Available pH range	2.0–12.0	2.0–12.0
Temperature range	4–60 °C	
Pressure limit	3 µm: 18–25 MPa (2,610–3,625 psi) 5 µm: 6–12 MPa (870–1,740 psi)	
Column hardware	PEEK	



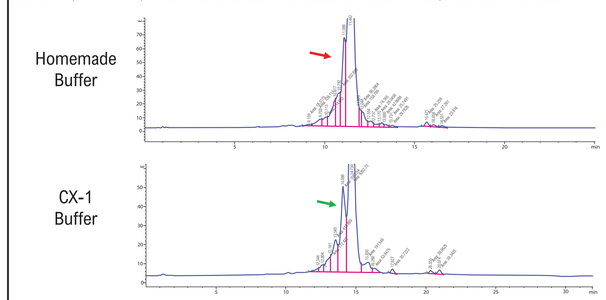
Mobile Phase Comparison BioPro SF 5 µm SCX 100 x 4.6 mm

Homemade Buffer	MPA: 20 mM MES, pH 5.6 MPB: 20 mM HEPES + 150 mM NaCl, pH 8.0
CX-1 Buffer	MPA: CS-1A MPB: CX-1B

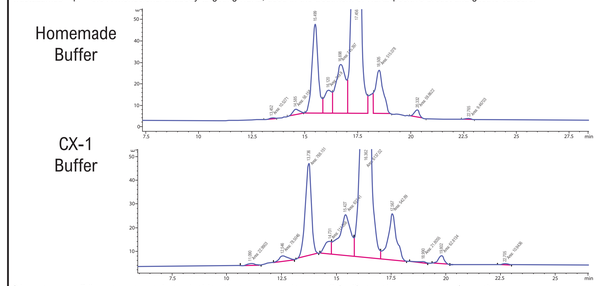
NISTmab – pI = 9.2 A monoclonal antibody reference material developed by NIST for biopharmaceutical research and analytical method validation.



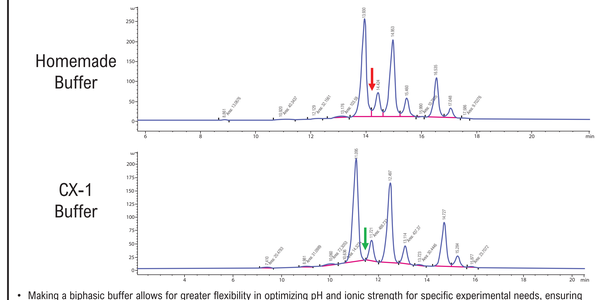
Adalimumab – pI = 8.4 A TNF-alpha inhibitor used to treat autoimmune diseases like rheumatoid arthritis, psoriasis, and Crohn's disease.



Trastuzumab – pI = 8.8 A monoclonal antibody targeting HER2, used in the treatment of HER2-positive breast and gastric cancers.



Situximab – pI = 7.8 a monoclonal antibody that targets and blocks interleukin-6 (IL-6), a protein involved in inflammation

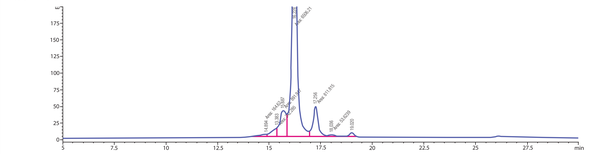


- Making a biphasic buffer allows for greater flexibility in optimizing pH and ionic strength for specific experimental needs, ensuring better compatibility with various analytes and separation conditions.
- In contrast, Thermo CX-1 premade buffer offers convenience but may not provide the same level of customization required for specialized applications.
- After testing the initial screening gradient from 0 to 100% B, we refined the gradient to maximize resolution for individual charged variants. Overall, the CX-1 buffer produced the best peak shape.

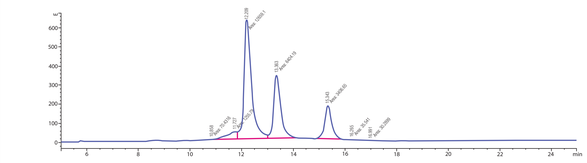
Salt Gradient

Column:	BioPro IEX SF 5 µm	**Gradient:**	Time (min) % MPA %MPB 0 90 10 20 70 30
Dimension:	100 x 4.6 mm		
Mobile Phase A:	20 mM HEPES pH 7.4	**Flow Rate:**	0.5 mL/min
Mobile Phase B:	20 mM HEPES pH 7.4	**Detection:**	FLD @Ex = 280nm Em= 360nm
		Temperature:	Ambient

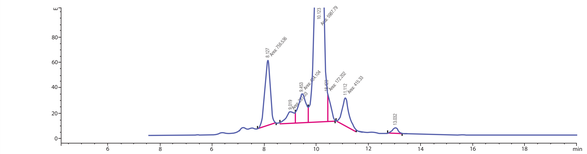
NISTmab



Amatuximab

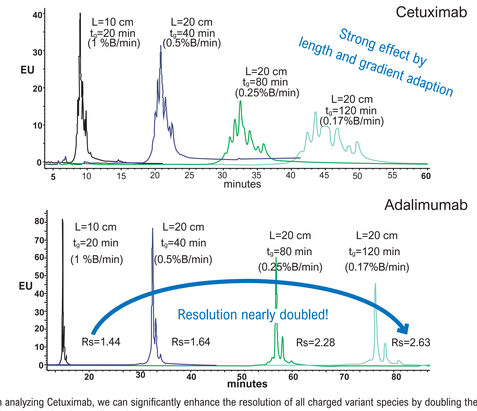


Trastuzumab



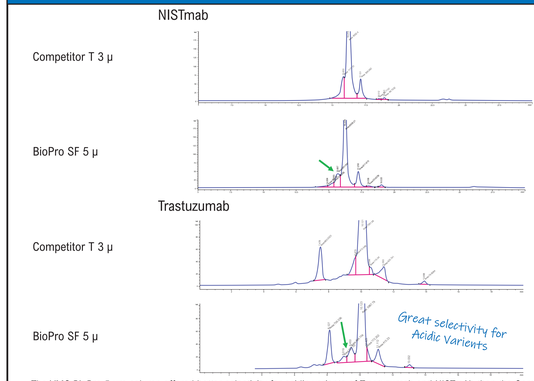
- The YMC BioPro SF column is ideal for salt and pH gradient analysis of charged variants due to its highly uniform, non-porous hydrophilic resin, which provides exceptional resolution and minimal secondary interactions.
- Its high efficiency and low backpressure enable precise control over gradient conditions, ensuring reproducible separation of closely related charge variants with superior peak shape and robustness.

Impact of Column Length



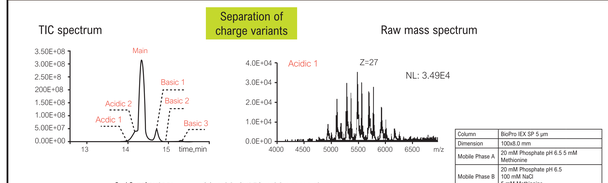
- When analyzing Cetuximab, we can significantly enhance the resolution of all charged variant species by doubling the column length and adjusting the gradient slope.
- The same approach can be applied to Adalimumab, where we have nearly doubled the resolution value for one of the basic variants.
- This setup also improves peak capacity and detection sensitivity, leading to more accurate characterization of charge heterogeneity

Competitor T 3 µm SCX Mono-Dispersed 100 x 4.0 mm Comparison



- The YMC BioPro 5 µm column offered better selectivity for acidic variants of Trastuzumab and NISTmAb than the Competitor T 3 µm, due to its larger particle size, which enhances mass transfer and analyte interactions. This increased surface area improved resolution of closely eluting charge variants.

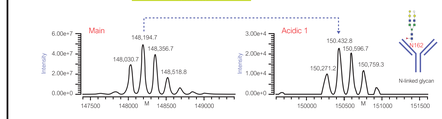
LC/MS



2-18 minutes: 20 mM → 150 mM ammonium acetate pH 5.6 → pH 7.4

+

Identification of charge variants



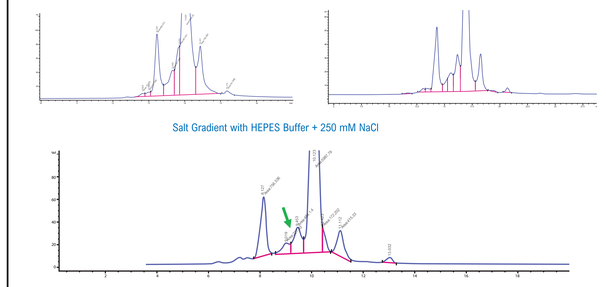
Column	BioPro IEX SP 5 µm		
Dimension	100x8.0 mm		
Mobile Phase A	20 mM Phosphate pH 6.5 5 mM Methionine		
Mobile Phase B	20 mM Phosphate pH 6.5 100 mM NaCl 5 mM Methionine		
Gradient	Time (min)	% MPA	% MPB
	0	70	30
	18	25	75
Flow Rate	4 mL/min		
Detection	UV @ 280 nm and LC/MS		
Temperature	Ambient		

LC/MS, when paired with the YMC BioPro SF column, is critical for charged variant analysis as it offers precise molecular weight determination and improves the detection of low-abundance species. This combination enhances resolution, quantification, and structural characterization of charge variants.

Conclusion Trastuzumab Charged Variant Analysis via pH and Salt Gradients

Initial Screen: Using CX-1 Buffer 0→100 % B

Optimized: using CX-1 Buffer under focused



- YMC BioPro SF column provides excellent resolution for charged variant analysis of monoclonal antibodies using biphasic buffers, CX-1 premade buffer, and salt gradients.
- The initial screening gradient for Trastuzumab, followed by the focused CX-1 gradient, enhances separation efficiency.
- Optimized salt gradient with HEPES and sodium chloride further refines resolution, improving charge variant differentiation.