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Optimizing Separation for Charge Variant Analysis (CVA) using bioZen™ 6 µm WCX: Salt versus pH Gradients

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An ion-exchange column, bioZen WCX, was recently introduced and complements the portfolio of bioZen products for biomolecule analysis. bioZen WCX is significantly more efficient than other WCX columns on the market and couples best in class particle technology with inert BioTi™ hardware to give a complete solution. Performing method development for charge variant analysis (CVA) using bioZen WCX columns will be discussed and the benefits of each elution method. The differences in pH and salt gradients for CVA will also be discussed.

Introduction

Charge variants of proteins commonly result from post translational modifications (PTMs) during recombinant production. These PTMs, including C-terminal lysine clipping and glycosylation, result in acidic and basic charged residues relative to the native protein. The most common method to detect and assess these acidic and basic variations is through ion-exchange chromatography (IEX), specifically weak cation-exchange (WCX).

Ion-exchange, unlike most other interactive chromatography mechanisms, is an on / off process and relies on the electrostatic interaction of analyte with stationary phase. The retention of a compound ionically will depend on the number of charges and their location on the particular target molecule. Elution is modulated by the concentration of salt going from a low to high concentration or increasing the mobile phase pH when using base to elute the protein. The elution mechanism selected is important to ensuring a good, reproducible method with consistent results.

With a traditional salt gradient the pH of the mobile phase is kept constant throughout the analysis and, in the case of cation-exchange, the pH will be lower than the pI of the molecule of interest. The ionic strength of the mobile phase will begin at a low salt concentration and throughout the run the concentration of salt is increased to elute the analytes from most acidic to most basic across the gradient. Method parameters such as buffer type, ionic strength, and gradient profile typically will need to be optimized for each compound of interest. Consistent careful buffer preparation made gravimetrically is critical for reproducibility of these methods and the pH must remain constant throughout the method. However, pH adjustment can be used to modulate retention when in salt gradient mode.

With a pH gradient, the initial pH of the mobile phase ensures an opposite charge of the molecule and the stationary phase to facilitate the adsorption. For cation-exchange, the pH will start below the pI of the protein analyte, which provides a highly protonated, cationic species. As the pH of the mobile phases changes and the protein approaches pI this net charge approaches zero and the protein elutes from the column. With a pH gradient salt concentration can be kept low and adjusted for method optimization, if required.

Cetuximab, marketed under the trademark Erbitux®, trastuzumab, marketed under the trademark Herceptin®, infliximab marketed under Remicade® and rituximab marketed as Rituxan® are common monoclonal antibodies in the biopharmaceutical industry. The determination of charge variants in these biotherapeutics is assessed on the bioZen 6 µm WCX column using both a pH and salt gradient.

Materials and Methods

Sample Preparation

Salt Buffer Preparation: (20 mM MES pH 6.0) - 7.81 ± 0.01 g MES hydrate (MW 195.24) was added to a clean 2 L volumetric flask. To this, 1.6 L HPLC grade water was also added. Using a magnetic stir bar the solution was stirred until all solids were dissolved. Using a pH meter the pH of the solution was measured and adjusted to pH 6.0 ± 0.1 with sodium hydroxide (approximately 16 mL). A final volume of 2.0 L was made by adding HPLC grade water. The final mobile phase was filtered using a 0.2 µm membrane filter to remove any particulates.

(20 mM MES + 300 mM NaCl pH 6.0) - 7.81 ± 0.01 g MES hydrate (MW 195.24) and 35.06 ± 0.01 g NaCl (MW 58.44) was added to a clean 2 L volumetric flask. To this, 1.6 L HPLC grade water was also added. Using a magnetic stir bar the solution was stirred until all solids were dissolved. The final mobile phase was filtered using a 0.2 µm membrane filter to remove any particulates.

MES hydrate was purchased from Sigma Aldrich® (St. Louis, MO). Sodium chloride was purchased from VWR (Radnor, PA). Sodium hydroxide was purchased from Fisher Scientific® (Hampton, NH).

pH gradient buffers (CX-1 & CX-2) were purchased from Thermo Fisher® (Hampton, NH).

Cetuximab, rituximab, infliximab, and trastuzumab were purchased from Myoderm® (Norristown, PA) and injected directly onto the column.

For all applications a bioZen 6 µm WCX column was used from Phenomenex (Torrance, CA).

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HPLC Conditions

Column: bioZen 6 µm WCX
Dimensions: 250 x 4.6 mm
Part No.: 00G-4777-E0
Flow Rate: 1.0 mL/min
Detection: UV @ 228 nm
Temperature: 30 °C
Injection Volume: 15 µL

Mobile Phase Conditions

Method	Mobile Phase A	Mobile Phase B	Gradient Profile
Salt Gradient	20 mM MES (pH 5.6)	20 mM MES + 300 mM NaCl (pH 5.6)	15-45 % B in 30 min
pH Gradient	CX-1 pH gradient buffer A (pH 5.6)	CX-1 pH Gradient Buffer B (pH 10.2)	0-100 % B in 20 min

Results and Discussion

Salt Gradient

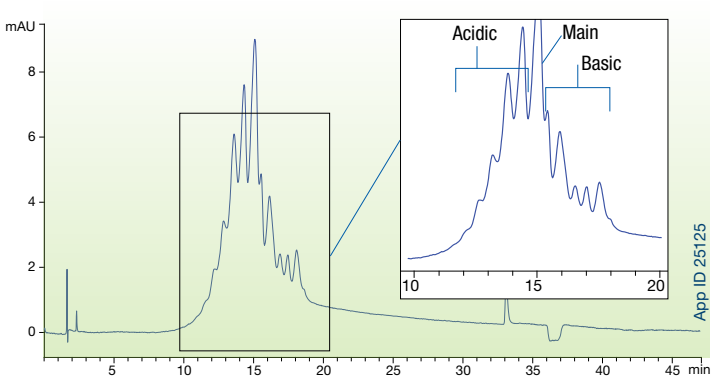
For the salt gradient, morpholino ethane sulfonic acid (MES) in combination with increasing NaCl (300 mM) was utilized (**Figure 1**). For cetuximab, this approach provided 48 % acidic variants and 29 % basic variants. For trastuzumab, this approach provided 27 % acidic variants and 12 % basic variants, for infliximab we saw 14 % acidic and 49 % basic and for rituximab 17 % acidic variants with 14 % basic.

pH Gradient

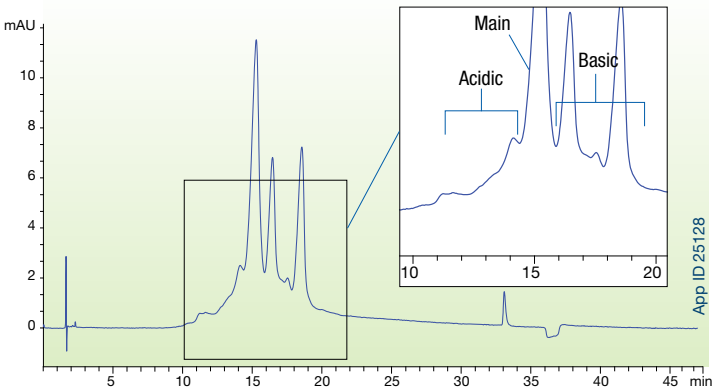
For the pH gradient, CX-1 gradient buffers from Thermo Fisher Scientific® were employed on a 0-100 % linear pH gradient over 20 min (**Figure 2**) For cetuximab, this approach provided 51 % acidic variants and 22 % basic variants. For trastuzumab, this approach provided 23 % acidic variants and 13 % basic variant. For infliximab, 14 % acidic variants were recorded and 47 % basic and for rituximab, we saw 15 % acidic and 18 % basic variants using a pH gradient.

Figure 1.

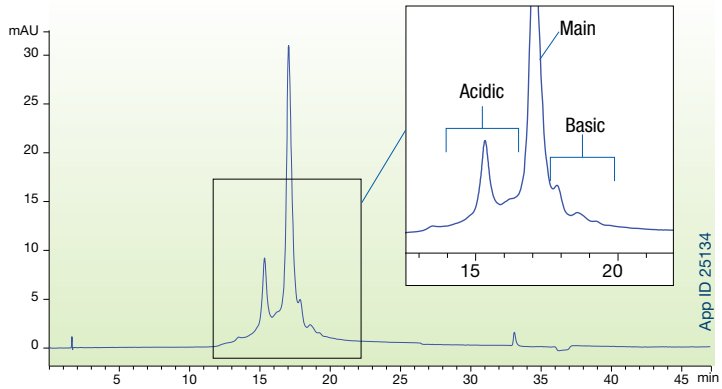
Cetuximab, MES salt gradient



Infliximab, MES salt gradient



Trastuzumab, MES salt gradient



Rituximab, MES salt gradient

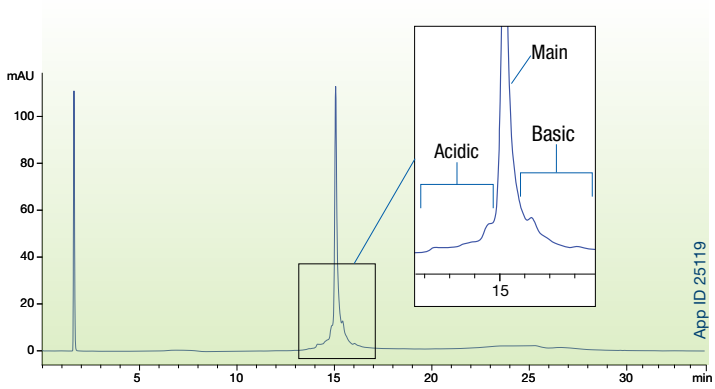


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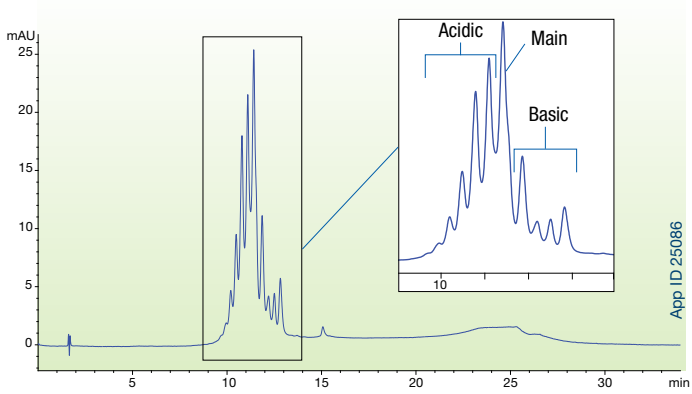
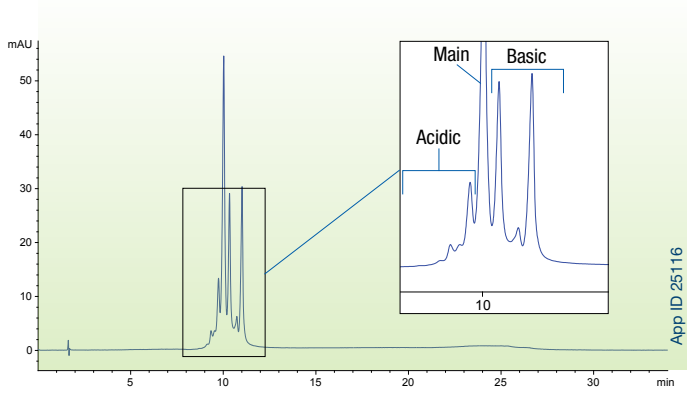
Figure 2.

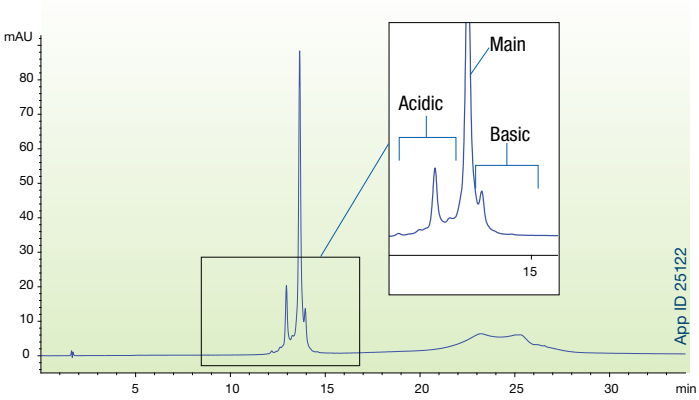
Cetuximab, pH gradient buffer



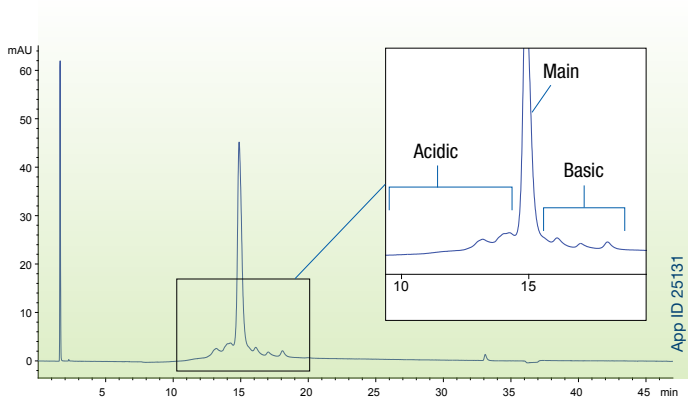
Infliximab, pH gradient buffer



Trastuzumab, pH gradient buffer



Rituximab, pH gradient buffer



Conclusion

Both gradients effectively separate the acidic and basic variants from 4 neutral mAbs (cetuximab, trastuzumab, infliximab and rituximab). In all cases there were differences in the reported values of acidic and basic variants and these variations were specific to each antibody. With trastuzumab there was a superior separation observed for the acidic variants when using a salt gradient however, for cetuximab the opposite was found. Infliximab and rituximab showed the best correlation between the two elution methods. When working with a salt gradient the complexity of reproducible buffer preparation can impact the uniformity of the results, which is negated with pH gradients when commercially available proprietary buffers are used. Each elution method will give different retention times and which can be seen in the examples above with retention much longer when running a salt gradient compared to the pH gradient. It is important to be aware that when running a salt gradient the pI of the protein will not determine the separation which is dependent on the number of accessible charges. Ultimately, the surface charge density between the phase and the mAb will determine separation and is antibody specific. In contrast, a pH gradient will typically elute proteins according to what their isoelectric point would predict.

bioZen™ Ordering Information

bioZen Columns (mm)						Biocompatible Guard Cartridges		
	50 x 2.1	100 x 2.1	150 x 2.1	50 x 4.6	150 x 4.6	for 2.1 mm	for 4.6 mm	Holder
				—	—	/3pk	—	ea
bioZen 2.6 µm Glycan	00B-4773-AN	00D-4773-AN	00F-4773-AN	—	—	AJ0-9800	—	AJ0-9000
				—	—	/3pk	—	ea
bioZen 1.6 µm Peptide PS-C18	00B-4770-AN	00D-4770-AN	00F-4770-AN	—	—	AJ0-9803	—	AJ0-9000
		—				/10pk	/10pk	ea
bioZen 3 µm Peptide PS-C18	00B-4771-AN	—	00F-4771-AN	00B-4771-E0	00F-4771-E0	AJ0-7605	AJ0-7606	KJ0-4282
				—	—	/3pk	—	ea
bioZen 1.7 µm Peptide XB-C18	00B-4774-AN	00D-4774-AN	00F-4774-AN	—	—	AJ0-9806	—	AJ0-9000
						/3pk	/3pk	ea
bioZen 2.6 µm Peptide XB-C18	00B-4768-AN	00D-4768-AN	00F-4768-AN	00B-4768-E0	00F-4768-E0	AJ0-9806	AJ0-9808	AJ0-9000
						/3pk	/3pk	ea
bioZen 3.6 µm Intact C4	00B-4767-AN	00D-4767-AN	00F-4767-AN	00B-4767-E0	00F-4767-E0	AJ0-9809	AJ0-9811	AJ0-9000
bioZen 3.6 µm Intact XB-C8	00B-4766-AN	00D-4766-AN	00F-4766-AN	00B-4766-E0	00F-4766-E0	AJ0-9812	AJ0-9814	AJ0-9000
	50 x 4.6	**100 x 4.6**	**150 x 4.6**	**250 x 4.6**	**300 x 4.6**		**for 4.6 mm**	**Holder**
	—			—		—	/3pk	ea
bioZen 1.8 µm SEC-2	—	—	00F-4769-E0	—	00H-4769-E0	—	AJ0-9850	AJ0-9000
bioZen 1.8 µm SEC-3	—	00D-4772-E0	00F-4772-E0	—	00H-4772-E0	—	AJ0-9851	AJ0-9000
					—	—	/10pk	ea
bioZen 6 µm WCX	00B-4777-E0	00D-4777-E0	00F-4777-E0	00G-4777-E0	—	—	AJ0-9400	KJ0-4282

Sample Preparation Ordering Information

Format	bioZen Solid Phase Extraction	Sorbent Mass	Part Number	Unit
Microelution 96-Well Plate				
	bioZen N-Glycan Clean-Up	5 mg/well	8M-S009-NGA	1/box

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