Exploration of pH-Gradient Ion-Exchange Chromatography for High-Resolution Protein Separations in Biotechnology and Proteomics

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Abstract

Ion-exchange chromatography (IEC) is a versatile separation technique for profiling the charge heterogeneity of biotherapeutic proteins, including monoclonal antibodies. Despite good resolving power and robustness, ionic-strength-based ion-exchange separations are product specific and time consuming to develop. Although salt gradients are more commonly applied, the utilization of pH gradients can provide significant advantages such as: 1) improved separation resolution; 2) lower salt concentration in collected fractions; and 3) the possibility to correlate the protein isoelectric point (pI) data with elution profiles.

Recently, the application of pH-gradient IEC has been described for the separation of standard proteins[1] and monoclonal antibodies.[2,3]

The work shown here describes the application of pH-gradient IEC as compared to salt-gradient IEC for the separation of proteins from various sources. High-resolution separations of a monoclonal antibody and its isoforms were achieved using a new, nonporous, strong cation-exchange resin. Results were compared to those obtained with salt-gradient IEC. Complex protein mixtures typically found in proteomics were separated with pH-gradient IEC. Developed methodology was validated for pH profile shape and precision, retention-time precision, peak capacity, and robustness towards sample solvent composition.

Principles

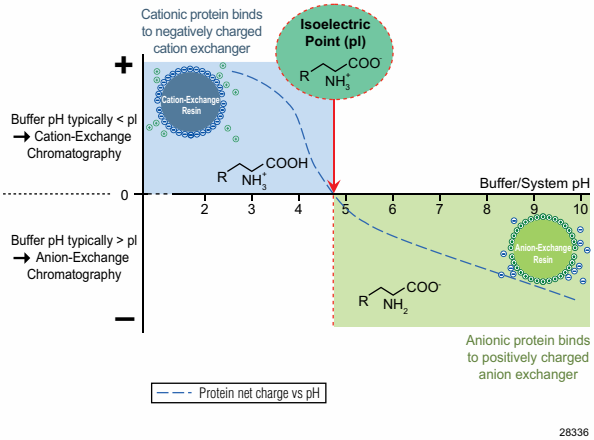
There are two general mechanisms on which proteins are retained and eluted from IEC columns (Figure 1). Use of either a continuous salt (ionic-strength) gradient or a pH gradient result in a high degree of protein fractionation based on protein charge.

In salt-gradient-based IEC, the pH of the buffer system is fixed. In addition to choosing the appropriate pH of the starting buffer, its ionic strength is kept low since the affinity of proteins for IEC resins decreases as ionic strength increases. The proteins are then eluted by increasing the ionic strength (salt concentration) of the buffer to increase the competition between the buffer ions and proteins for charged groups on the IEC resin. As a result, the interaction between the IEC resin and proteins is reduced, causing the proteins to elute.

In pH-gradient-based IEC, the pH of the starting buffer is maintained at a constant level to ensure the proteins obtain the opposite charge of the stationary phase and bind to it. The proteins are eluted by changing the buffer pH so the proteins transition to a net zero charge (ultimately the same charge as the resin) and elute from the column. One of the benefits of pH-gradient-based IEC is that the salt concentration can be kept low, yielding less buffer interferences in, for example, on-line or off-line two-dimensional LC (2D-LC).

High pI proteins are generally separated on cation-exchange columns running a pH-based gradient from low to high pH, and vice versa for low pI proteins.

FIGURE 1. The protein isoelectric point determines the buffer system and column selection. The scheme applies to both salt-gradient-based IEC (one vertical line on the pH axis) as well as pH-gradient-based IEC (along the protein net charge line).



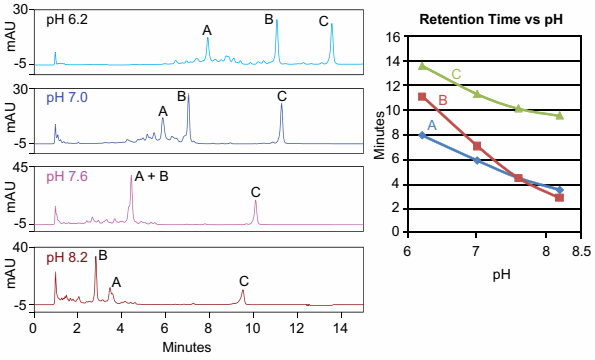
Instrumenta

HPLC experiments were carried out using a Thermo Scientific Dionex UltiMate 3000 Titanium system equipped with:

- SRD-3600 Solvent Rack with low-volume, chemically-inert degasser
- DGP-3600BM × 2 Biocompatible Dual-Gradient Micro Pump
- TCC-3000SD Thermostatted Column Compartment
- WPS-3000TBFC Thermostatted Biocompatible Autosampler with two integrated switching valves
- VWD-3400RS Variable Wavelength Detector with a 2.5 µL flow cell
- PCM-3000 pH and Conductivity Monitor

FIGURE 2. The Thermo Scientific Dionex PCM-3000 is a new inert pH and conductivity monitoring system with low-volume flow cells and quick response time. This unit includes a platform housing the pH and conductivity flow cell and can be mounted on any UltiMate™ 3000 UV-vis detector.

FIGURE 3. Salt-gradient-based IEC at different pH levels reveals the importance of buffer pH selection for selectivity of the chromatographic method.



Column: Thermo Scientific ProPac SCX-10, 4 mm i.d. × 250 mm
Mobile Phase: A: 25 mM Phosphate
B: Same as A + 0.5 M NaCl
Gradient: 0–50% B in 15 min
Flow Rate: 1.00 mL/min
Detection: UV at 280 nm
Peaks: A: α-Chymotrypsinogen (pI = 8.5)
B: Ribonuclease A (pI = 9.45)
C: Cytochrome C (pI = 10.2)

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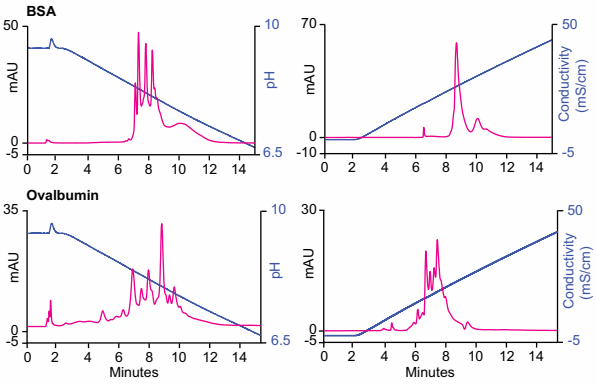
FIGURE 4. Comparison of pH-gradient-based AEC (left) and salt-gradient-based AEC (right).



pH Gradient

Column:	ProPac SAX-10, 4 mm i.d. × 250 mm
Mobile Phase:	**A:** 20 mM Piperazine + 20 mM triethanolamine + 20 mM bis-tris propane + 20 mM N-methylpiperazine, pH = 3.7 (titrated with HCl) **B:** 20 mM Piperazine + 20 mM triethanolamine + 20 mM bis-tris propane + 20 mM N-methylpiperazine, pH = 9.7 (titrated with HCl)
Gradient:	0–100% B in 15 min
Flow Rate:	1.00 mL/min
Detection:	UV at 280 nm

Salt Gradient

Column:	ProPac SAX-10, 4 mm i.d. × 250 mm
Mobile Phase:	**A:** 20 mM TRIS, pH 8.5 **B:** Same as A + 0.5 M NaCl
Gradient:	0–100% B in 15 min
Flow Rate:	1.00 mL/min
Detection:	UV at 280 nm

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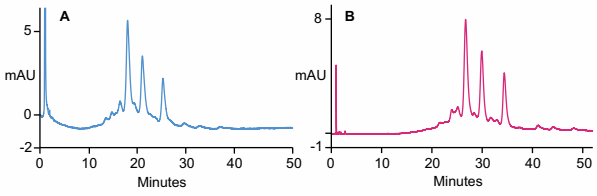
IEC for Monoclonal Antibody Analysis

Salt-based cation-exchange chromatography is the gold standard for charge variant analysis of monoclonal antibodies (MAbs). The Thermo Scientific ProPac WCX-10 and Thermo Scientific MAbPac SCX-10 are two high-performance, industry-leading, charge variant analysis columns, featuring unique selectivity and high resolving power. The MAbPac™ SCX-10 column is complimentary to the ProPac™ WCX-10 column for monoclonal antibody variant analysis. The MAbPac SCX-10 column offers alternative selectivity and provides higher resolution and efficiency for variant analysis of most monoclonal antibody samples than the ProPac WCX-10 column (see Figure 5). Figure 6 shows an analytical method utilizing a pH gradient.

FIGURE 5. Typical high-resolution, salt-gradient-based IEC chromatograms for separations using A) ProPac WCX-10, 4 mm i.d. × 250 mm (left) and B) MAbPac SCX-10, 4 mm i.d. × 250 mm (right) columns.



Column:	ProPac WCX-10, 4 mm i.d. × 250 mm	Column:	MAbPac SCX-10, 4 mm i.d. × 250 mm
Mobile Phase:	**A:** 20 mM MES pH 5.6 + 60 mM NaCl **B:** 20 mM MES pH 5.6 + 300 mM NaCl	Mobile Phase:	**A:** 20 mM MES pH 5.6 + 60 mM NaCl **B:** 20 mM MES pH 5.6 + 300 mM NaCl
Gradient:	0–100% B in 15 min	Gradient:	0–100% B in 15 min
Flow Rate:	1.00 mL/min	Flow Rate:	1.00 mL/min
Detection:	UV at 280 nm	Detection:	UV at 280 nm

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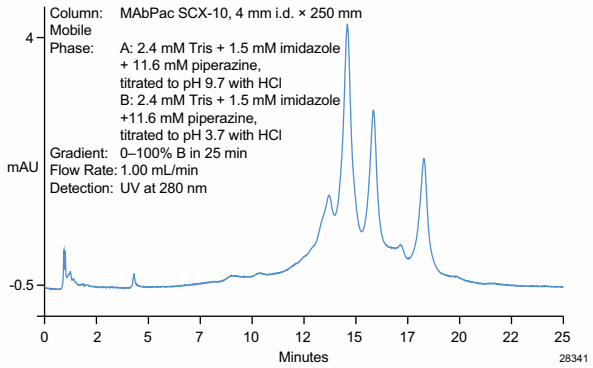
FIGURE 6. pH-gradient-based IEC of a monoclonal antibody separation using a MAbPac SCX-10, 4 mm i.d. × 250 mm column.

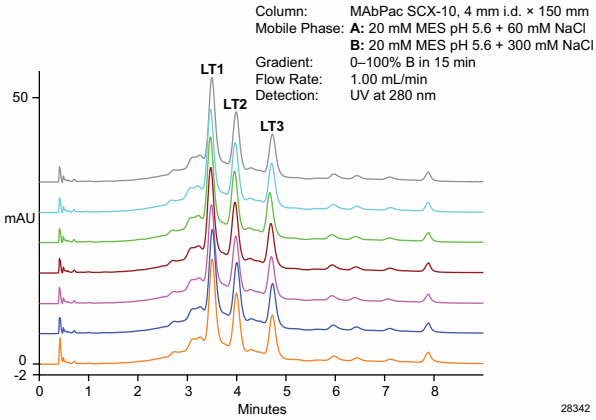


Enhancing Sample Throughput in Charge Variant Analysis

Depending on the requirements set for the charge-variant analysis, the gain in analysis time may become more important than the loss of an acceptable level of separation power. In this case, there are several options which do not seriously affect the resolution.

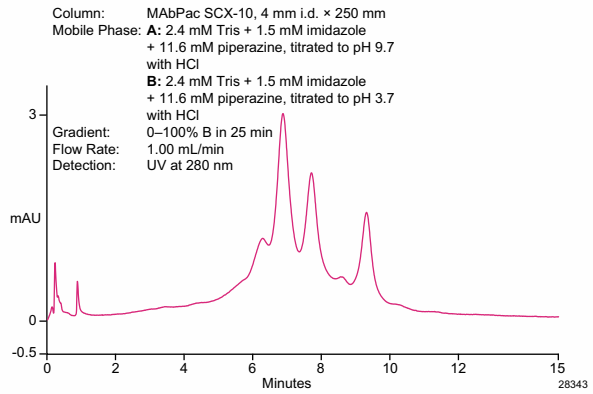
One can accelerate the current method by increasing the gradient slope, or maintain the same gradient while utilizing a high-throughput (shorter) column. The example shown in Figure 7 illustrates a relatively small loss in resolution compared with trace B in Figure 5, even though the total analysis time was reduced more than fourfold. Method robustness was also unaffected by the reduction in analysis time. The lysine truncations are depicted as: LT1, no lysine; LT2, one lysine; LT3, two lysines.

FIGURE 7. Example of an accelerated salt-gradient-based IEC.



The speed of pH-gradient-based IEC can also be increased considerably, as shown in Figure 8. A run with a total analysis time of 60 min was reduced to 30 min by using a shorter (50 mm) MAbPac SCX-10 column, while maintaining a similar gradient.

FIGURE 8. Example of an accelerated pH-gradient-based IEC.



Conclusions

- pH-gradient-based IEC can be a very good alternative to salt-gradient-based IEC.
- Good resolution was found for pH-gradient-based separations with both long and short SCX columns.
- One of the benefits of pH-gradient-based IEC is that the salt concentration can be kept low, yielding less buffer interferences (e.g., on-line or off-line two-dimensional LC [2D-LC]).
- pH-gradient IEC is promising for high throughput and fast screening of proteins and antibodies.

References

1. Ahamed, T. et al., Selection of pH-Related Parameters in Ion-Exchange Chromatography Using pH-Gradient Operations. *J. Chromatogr., A* **2008**, *1194* (1), 22–29.
2. Farnan, D.; Moreno, G. T. Multi-Product High-Resolution Monoclonal Antibody Charge Variant Separations by pH Gradient Ion-Exchange Chromatography. *Anal. Chem.* **2009**, *81* (21), 8846–8857.
3. Rea, J. C.; Moreno, G. T.; Lou, Y.; Farnan, D. Validation of a pH Gradient-Based Ion-Exchange Chromatography Method for High-Resolution Monoclonal Antibody Charge Variant Separations, *J. Pharm. Biomed. Anal.* **2011**, *54* (2), 317–323.
4. Kaliszan, R.; Wiczling, P.; Markuszewski, M. J. pH Gradient High-Performance Liquid Chromatography: Theory and Applications. *J. Chromatogr., A* **2004**,*1060*, 165–175.
5. Ahamed, T. et al., pH-Gradient Ion-Exchange Chromatography: An Analytical Tool for Design and Optimization of Protein Separations. *J. Chromatogr., A* **2007**, *1164*, 181–188.
6. Tsonev, L. I.; Hirsh, A. G. Theory and Applications of a Novel Ion Exchange Chromatographic Technology Using Controlled pH Gradients for Separating Proteins on Anionic and Cationic Stationary Phases, *J. Chromatogr., A* **2008**, *1200*, 166–182.

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