EFFICIENT USE OF PH GRADIENTS IN THE ION EXCHANGE ANALYSIS OF PROTEINS

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INTRODUCTION

The analysis of proteins is complicated by their large size and by the heterogeneity of chemical properties. Because these factors affect the selectivity of the analysis, it is necessary to combine multiple analytical techniques that are sensitive to different physical and chemical properties to ensure complete characterization of a protein sample. Within the set of chromatographic techniques included in this approach, ion exchange can be the most discriminating. Realization of the full power of ion exchange requires optimization of both the column and the mobile phase. Among the parameters requiring optimization, the pH of the separation buffer has the largest effect on the selectivity of the method. Efficient techniques have been developed for screening separations with a selection of columns over a series of different pH buffers. It is, in addition, recognized that pH gradients can provide particularly useful protein separations. Such gradients could complement the use of iterative experiments over small increments of pH. In practice, pH gradients have not been widely applied because they are difficult to control. Because pH is a logarithmic function, simple buffer mixing does not give the desired pH profile. As the target pH deviates further from the pK, pH changes become very large with small changes in proportions. It is, therefore, also complicated to control the pH profile when changing column dimensions or flow rate. We have developed and evaluated algorithms and a software user interface to simplify this process. The technology is implemented on a four solvent pump and the gradient table is programmed directly in units of pH and salt concentration. The system uses a "buffer system" that is freely defined by the user to calculate the required proportions of the four mobile phase reservoirs and to recalculate those proportions at each pump stroke. We will show the use of this system for both anionic and cationic buffers. Several examples of protein separations, including monoclonal antibodies, will also be shown.

METHODS

Instrumentation

Waters ACQUITY H-Class Bio System
Waters ACQUITY TUV Detector
Waters WFCIII Fraction Collector
Waters Empower 2 Chromatography Data System
GE Healthcare Monitor pH/C-900

Reagents

Water, Optima® LC/MS (Fisher Scientific)
Sodium Chloride (Sigma S7653)
Multi-Buffer
- Mobile Phase A: Acidic Components
  - 48 mM Trizma® Hydrochloride (Sigma T-6666)
  - 30 mM Imidazole-Cl (Sigma 56752)
  - 58 mM Piperazine-Cl (Sigma 284904)
- Mobile Phase B: Basic Components
  - 48 mM Trizma® Base (Sigma T-1503)
  - 30 mM Imidazole (Sigma I202)
  - 58 mM Piperazine (Sigma 80621)

All solutions filtered with Millicup-LH Vacuum-driven bottletop filter unit (Millipore Cat No SJLHM4710)

Samples

Protein mix: all at 1.25 mg/mL in water (All from Sigma)
- Chicken Lysozyme
- Horse cytochrome c
- Bovine ribonuclease
- Bovine α-lactalbumin
- Bovine carbonic anhyrdrase
- Soybean trypsin inhibitor
- Horse myoglobin
- Bovine α-chymotrypsinogen

Monclonal antibodies:
- Chimeric and Humanized formulated drug products at approximately 20 mg/mL in phosphate-buffered saline

Columns

ProteinPak Hi Res CM, 7 µm, 4.6 x 100 mm
ProteinPak Hi Res SP, 7 µm, 4.6 x 100 mm
ProteinPak Hi Res Q, 7 µm, 4.6 x 100 mm
Brand A Q, 8 µm, 5.0 x 100mm
Brand B DEAE, 8 µm, 8.0 x 75mm

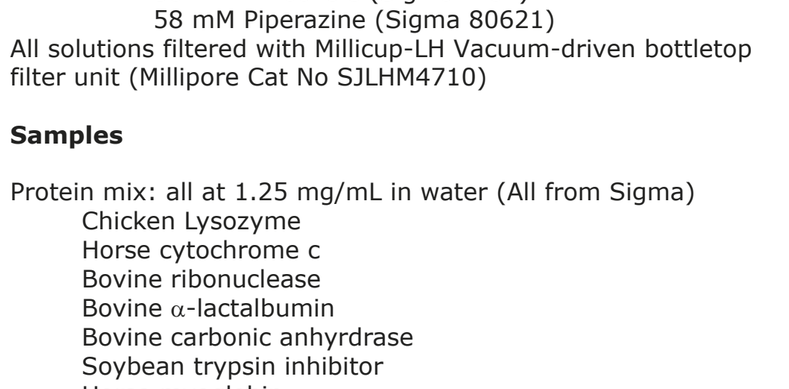

Figure 1. Principle of Auto•Blend Plus™ Technology . The four mobile phase reservoirs of the ACQUITY UPLC® H-Class System are filled with concentrated stocks and the Auto•Blend Plus™ Technology algorithm is used to calculate the proportions required from each reservoir for a specified pH and salt concentration.

RESULTS

Principles of Auto•Blend Plus™ Technology

Auto•Blend Plus™ Technology is the use of specific algorithms to program a quaternary solvent manager to deliver buffers of a specified pH and salt concentration. The function is derived from the practice of preparing buffer solutions by blending acidic and basic stock solutions. In the quaternary solvent manager, the percentage of flow drawn from each of the four reservoirs provides the same blending as manual buffer preparation. A typical system configuration is shown in Figure 1. The software calculates the proportions of reservoirs A, B, C, and D based on the definitions of the buffer system. Proportions are calculated from the known pKa of the buffer or from an empirical calibration table. The required proportions from each reservoir are calculated at each pump stroke to deliver the pH and salt concentration specified by the separation method. The system and software have been validated for accurate pH delivery, both at constant pH and with simple pH gradients.

In the present study, a mixture of buffers proposed by Rea, *et al. (Biopharm International, November, 2010, pp. 44-52)* is used to cover a wider range of pH in a single system. These cationic buffers are most appropriate for anion exchange, but they have been used in cation exchange. Both modes are tested here.

Accuracy of pH Gradient Delivery

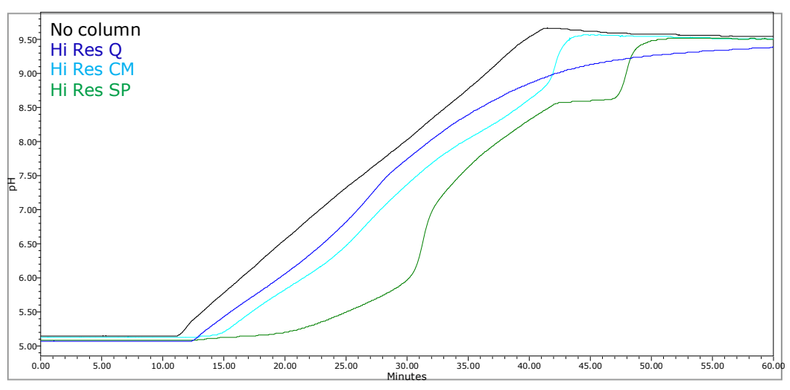

Figure 2. Alteration of Cationic Buffer pH Gradient by Ion exchange columns. The gradient of increasing pH is used for elution in cation exchange. The distortion with cation exchange columns is expected since the buffering species binds to the column. The large discontinuity on the anion exchange column must reflect secondary binding of the non-protonated buffer species to base packing material.

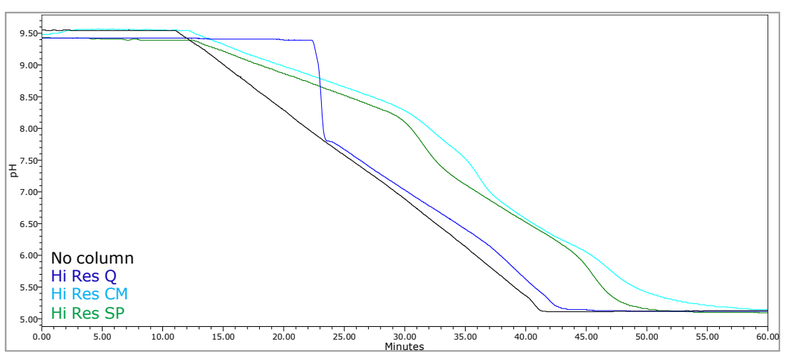

Figure 3. Alteration of Cationic Buffer pH Gradient by Ion exchange columns. The gradient of decreasing pH is used for elution in anion exchange. The distortion with cation exchange columns is expected since the buffering species binds to the column. The large discontinuity on the anion exchange column must reflect secondary binding of the non-protonated buffer species to base packing material.

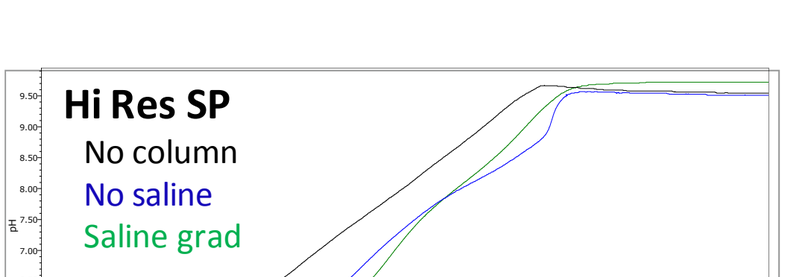

Figure 4. Alteration of Cationic Buffer pH Gradient by Sulfopropyl Cation Exchanger. The chromatographic system under Auto•Blend Plus control delivers the programmed gradient to the head of the column. A substantially different pH profile emerges from the column. The changes induced by the column are different in the presence of a simultaneous gradient of increasing sodium chloride. The distortion is not so large as shown below for the carboxymethyl exchanger.

Figure 5. Alteration of Cationic Buffer pH Gradient by Carboxymethyl Cation Exchanger. The chromatographic system under Auto•Blend Plus control delivers the programmed gradient to the head of the column. A substantially different pH profile emerges from the column. The changes induced by the column are different in the presence of a simultaneous gradient of increasing sodium chloride.

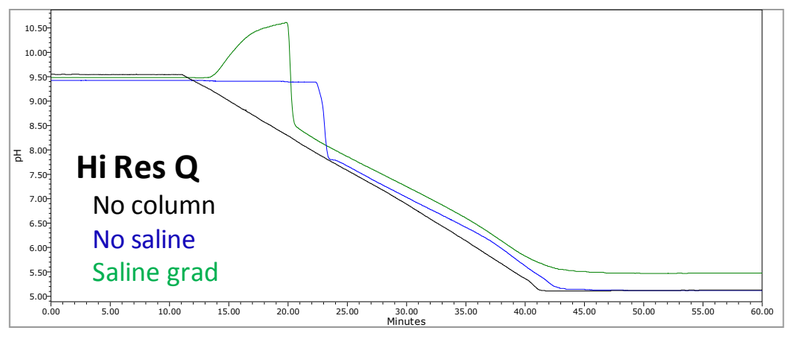

Figure 6. Effect of Salt Gradient on pH Gradient in Anion Exchange. Substantial buffer interaction with packing material is apparent

Effects of pH Gradients in Protein Separations

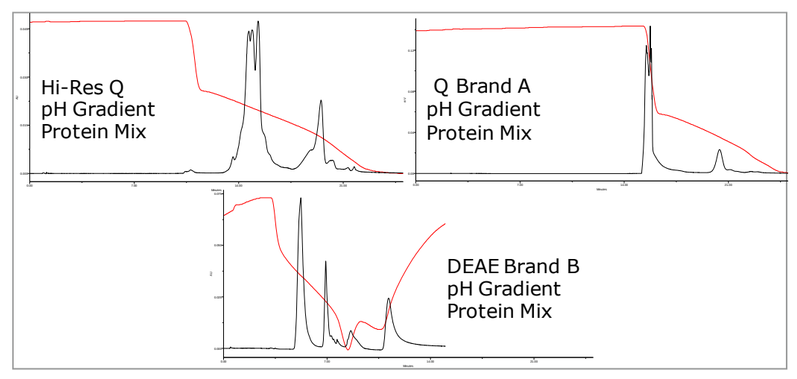

Figure 7. Separation of Protein Standard Mixture with Anion Exchange Chromatography. The mixture of proteins gives different selectivity patterns on the different columns and with the alternate separation gradients. The timing of the separation is different on the DEAE column that has significantly different dimensions. The gradient is, however, scaled to occur over the same number of column volumes. As shown below, cation exchange is more useful for this sample mixture.

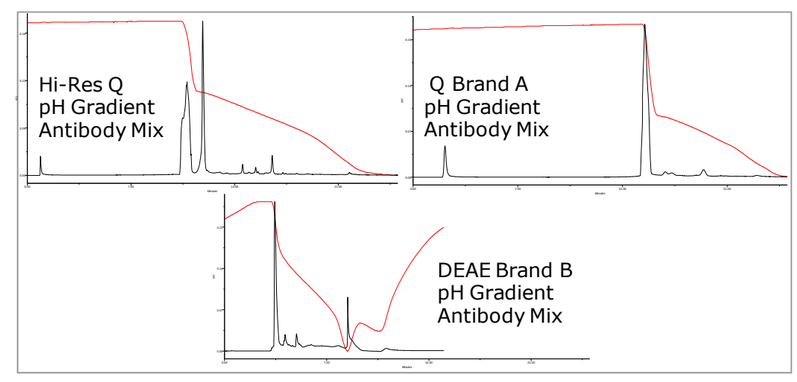

Figure 8. Separation of Monoclonal Antibodies with Anion Exchange Chromatography. The mixture of monoclonal antibodies, including Lysine-truncation variants gives different selectivity patterns on the different columns and with the alternate separation gradients. The timing of the separation is different on the DEAE column that has significantly different dimensions. The gradient is, however, scaled to occur over the same number of column volumes. As shown below, cation exchange is more useful for this sample mixture.

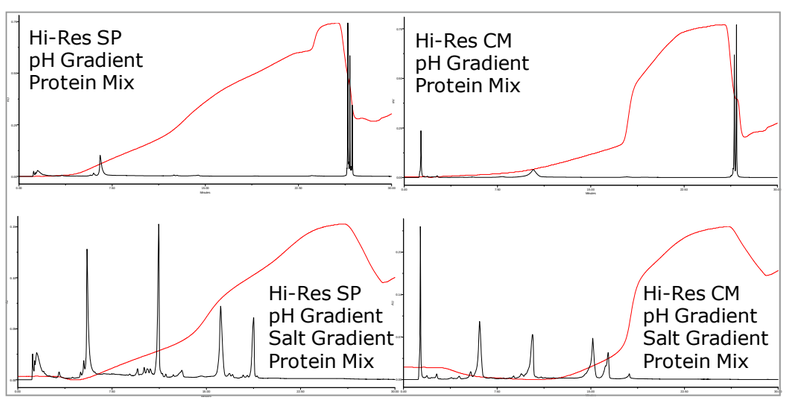

Figure 9. Separation of Protein Standard Mixturewith Cation Exchange Chromatography. The mixture of proteins representing a wide range of isoelectric points and sizes, gives different selectivity patterns on the different columns and with the alternate separation gradients. These molecules are distinguished by a range of chemical and physical differences. For such samples, the combination of pH and ionic strength gradients offers a useful option for controlling the separation.

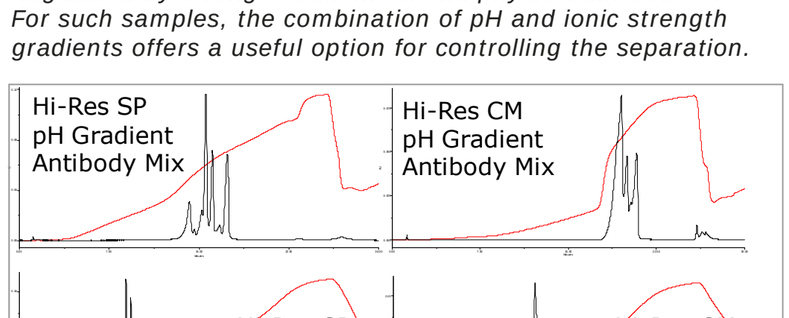

Figure 10. Separation of Monoclonal Antibodies with Cation Exchange Chromatography. The mixture of monoclonal antibodies, including Lysine-truncation variants gives different selectivity patterns on the different columns and with the alternate separation gradients. These are large molecules distinguished by small chemical differences. For such samples, pH gradients offer useful options in controlling the separation.

CONCLUSION

- **Proteins can be separated in ion exchange with gradients of pH**
- **Auto•Blend Plus Technology generates accuratepH gradients from multi-component buffer systems**
- **Ion exchange columns alter the applied pH gradient in ways that are specific to the particular column packing material**
- **Cationic buffers can be successfully used with cation exchange columns with allowance for significant deviations from the programmed gradient upon passage through the column**
- **Some protein samples separate best in pH gradients while other separate best with ionic strength gradients**

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