

# A Universal Mobile Phase System for Simple pH Optimization of Mobile Phases for Ion Exchange Chromatography and Hydrophobic Interaction Chromatography

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## ABSTRACT

Separation of biomolecules such as proteins and oligonucleotides using ion exchange chromatography (IEX) often requires optimization of mobile phase pH. However preparing mobile phases with different pHs is time consuming. In this study, a mobile phase system was developed to perform pH scouting without having to prepare multiple sets of mobile phases at different pHs. We demonstrated that this mobile phase system can be used to separate proteins and therapeutic monoclonal antibodies using cation exchange chromatography (CEX), anion exchange chromatography (AEX) and hydrophobic interaction chromatography (HIC). To further simplify the pH scouting process, chromatographic results can be fed into an intelligent software program to assist in selection of the best conditions for the separation.

## INTRODUCTION

High performance liquid chromatography (HPLC) is widely used in protein and oligonucleotide characterization and purification. Size exclusion chromatography (SEC), IEX, and HIC methods are often used to separate impurities from the desired biomolecule of interest. These methods are performed under aqueous conditions, which separates analytes in their native state. The pH of the mobile phase affects the charge state of biomolecules, which in turn can alter the interaction between the biomolecule and the stationary phase. Therefore mobile phase pH optimization is critical to obtain high resolution separations. However, preparing mobile phases with different pHs is time consuming and often a bottleneck for method development.

Here, we present a mobile phase system that allows simple optimization of pH without having to prepare multiple solutions of different pHs. This system can be used for method development of chromatography modes that require pH adjustments in aqueous mobile phases.

## MATERIALS AND METHODS

### Chromatography Columns

Thermo Scientific™ ProPac™ Elite WCX, 5 μm, 4 × 150 mm (P/N 302972)  
Thermo Scientific™ ProPac™ SAX-10, 10 μm, 4 × 250 mm (P/N 054997)  
Thermo Scientific™ MAbPac™ HIC-Butyl, 5 μm, 4 × 150 mm (P/N 088558)

### Sample Preparation

Rituximab, Trastuzumab, Adalimumab and Bevacizumab samples were donated by a local biopharmaceutical company. Ovalbumin was purchased from Sigma-Aldrich. All samples were dissolved in water to a final concentration of 5 mg/mL. For HIC, the sample was diluted two fold with a 2 M NaCl solution.

### HPLC Conditions

#### Instrumentation

Thermo Scientific™ Vanquish™ Flex UHPLC system consisting of the following:  
• Quaternary Pump F (P/N VF-P20-A)  
• Split Sampler FT (P/N VF-A10-A)  
• Column Compartment H (P/N VH-C10-A)  
• Diode Array Detector HL (P/N VH-D10-A)

### Separation Conditions

Mobile phase A: Water  
Mobile phase B: 1 M NaCl for CEX and AEX or 4 M NaCl for HIC  
Mobile phase C: Thermo Scientific™ 10X CX-1 pH Gradient Buffer A, pH 5.6 (P/N 320779)  
Mobile phase D: Thermo Scientific™ 10X CX-1 pH Gradient Buffer B, pH 10.2 (P/N 302780)  
Gradient: shown in Table 1, 2 and 3.  
Flow rate: 1.0 mL/min  
Column temperature: 30°C  
Injection volume: 2 μL for CEX and AEX or 4 μL for HIC  
Detection: UV (280 nm)

**Data Processing:** Thermo Scientific™ Dionex™ Chromeleon™ 7.2.8

Table 1. Gradient method for CEX

Time (min)	%A	%B	%C+%D
0.0	85	5	10
1.0	85	5	10
16.0	70	20	10
17.0	70	20	10
17.1	40	50	10
18.0	40	50	10
18.1	90	5	10
24.0	90	5	10

Table 2. Gradient method for AEX

Time (min)	%A	%B	%C+%D
0.0	88	2	10
1.0	88	2	10
21.0	40	50	10
24.0	40	50	10
24.1	88	2	10
32.0	88	2	10

Table 3. Gradient method for HIC

Time (min)	%A	%B	%C+%D
0.0	0	90	10
1.0	0	90	10
15.0	90	0	10
20.0	90	0	10
20.1	0	90	10
25.0	0	90	10

## RESULTS

### Mobile phase system for simple pH optimization

Four mobile phases were connected to a quaternary pump as shown in Figure 1. Proportioning of line A and line B generated a salt gradient, while line C and line D delivered a certain pH value. Thermo Scientific™ CX-1 pH Gradient Buffers are composed of four zwitterionic buffer species that buffers at different pHs. Buffers connected to lines C and D were made to have a pH values of 5.6 and 10.2 respectively. Since these mobile phases generate a linear pH gradient from 5.6 to 10.2 [Ref 1], it is possible to obtain desired pH values between 5.6 and 10.2 through simple proportioning of eluent C and D. 10X concentrates of these buffers were used for the salt gradient experiments with the percent sums of line C and line D kept at 10%. Table 4 shows the measured pH values at each combination of line C and line D without a column. The calculated and the measured pH values were similar with small deviations.

Figure 1. Mobile phase setup on a quaternary pump

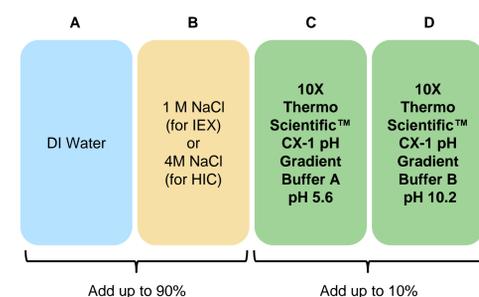


Table 4. Calculated and measured pH values at different percentages of Thermo Scientific™ CX-1 pH Gradient buffers A and B

Program	% C	% D	Calculated pH	Measured pH
I	10	0	5.60	5.53
II	9	1	6.06	6.01
III	8	2	6.52	6.49
IV	7	3	6.98	6.98
V	6	4	7.44	7.50
VI	5	5	7.90	8.05
VII	4	6	8.36	8.53
VIII	3	7	8.82	9.01
IX	2	8	9.28	9.44
X	1	9	9.74	9.85
XI	0	10	10.20	10.22

### Separation of therapeutic mAbs using CEX at different pH conditions

CEX is commonly used to analyze charge variants of therapeutic mAbs. Figure 2 shows the separation of mAb charge variants using a weak cation exchange (WCX) column and the novel mobile phase system. Peak-to-valley calculations were used to determine the separation of peaks not fully baseline resolved. For Rituximab, peak-to-valley ratios of the most adjacent acidic and basic peaks were higher at high pH conditions and the highest at pH 7.9. For both trastuzumab and adalimumab, the best separation was observed at pH 7.9; as neither sample was fully retained on the stationary phase at pH 8.4, higher pH values were not examined for these samples.

### Separation of ovalbumin using AEX at different pH conditions

To demonstrate the use of this mobile phase system for AEX separation, ovalbumin was separated using a strong anion exchange (SAX) column (Figure 3). Lowest pH condition resulted in sharper peaks and higher resolution of both acidic and basic peaks.

Figure 2. Separation of a) Rituximab b) Trastuzumab c) Adalimumab at different pHs using a WCX column

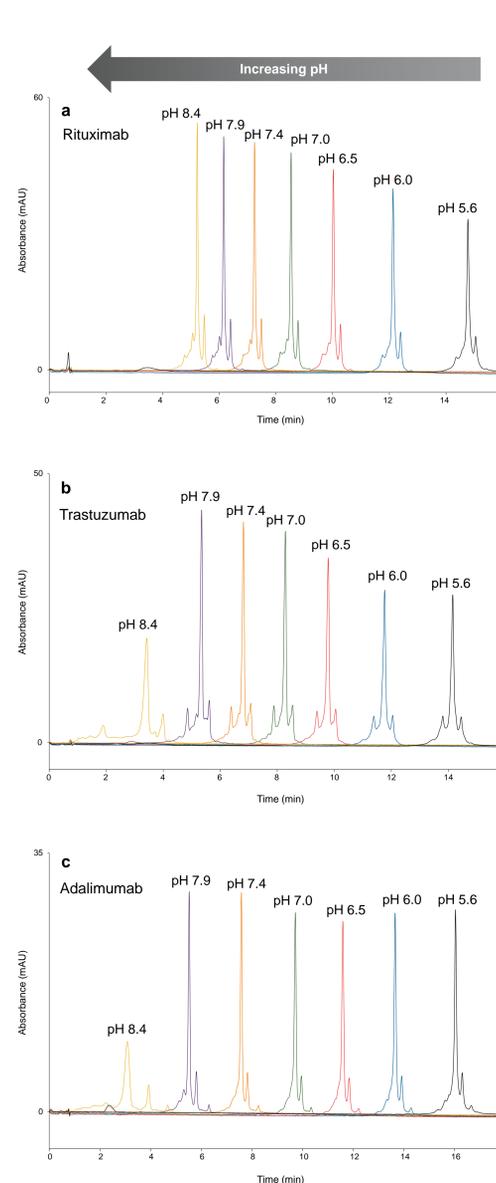
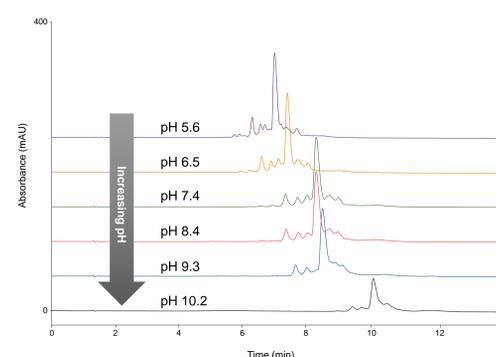


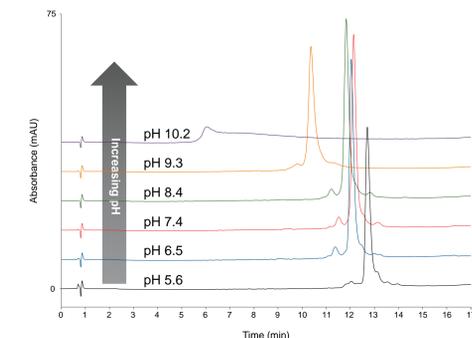
Figure 3. Separation of ovalbumin at different pHs using a SAX column



### Separation of Rituximab using HIC at different pH conditions

HIC is used to separate hydrophilic and hydrophobic variants of proteins. Opposed to IEX, salt gradient for HIC starts from a very high salt concentration and is reduced over time to a low concentration. Proteins generally have higher hydrophobic interaction with the stationary phase at high salt concentrations and elute as the salt concentration decreases. The pH of the mobile phase can affect protein charge, which results in a change in hydrophobicity. Therefore pH conditions could affect the separation of the protein. Rituximab was separated at different pH conditions using a modified mobile phases system. Instead of 1 M NaCl, a 4 M NaCl solution was connected to line B of the quaternary pump. A sodium chloride solution was used instead of ammonium sulfate, since high concentration of ammonium could buffer the mobile phase around pH 9.3. The chromatogram with highest resolution was observed at the lowest pH condition.

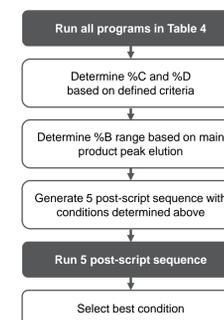
Figure 4. Separation of Rituximab at different pHs using a HIC column



### Workflow automation using chromatogram selection criteria

Different pH conditions can be screened using the programs listed in Table 4. Then the best chromatogram can be selected using several criteria. Here we identified three parameters for the selection of best chromatograms – number of peaks, peak capacity and composite peak-to-valley ratio. These parameters were selected based on calculating these values for several example chromatograms (data not shown). After one or two pH conditions are identified as best conditions, shallower salt gradients can be automatically run with several post-script sequences. The start and the end point of the gradient can be determined by calculating the %B where the main peak eluted in the initial run or the %B where the first and last peaks eluted.

Figure 5. Work flow to obtain optimized chromatograms



## CONCLUSIONS

- A mobile phase system was developed to perform pH scouting without having to prepare multiple sets of mobile phases with different pHs
- This mobile phase system can be applied to CEX, AEX and HIC modes to separate proteins and mAbs.
- Using this mobile phase system, pH scouting process can be automated which can significantly reduce method development time
- A chromatogram selection algorithm can be used to identify optimum buffer conditions.

## REFERENCE

Lin, S. Baek, J. Pohl, C. A Fast and Robust Linear pH Gradient Separation Platform for Monoclonal Antibody (mAb) Charge Variant Analysis, AN 20946.

## TRADEMARKS/LICENSING

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