Separation of mAb isoforms using controlled pH gradients and AEX/CEX columns packed with 7 and 10 µm hydrophilic non-porous resin particles

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Introduction

- Biopolymers are highly complex and large molecules.
- Most proteins are expressed in several isoforms.
- Isoforms differ in modifications of individual amino acid side chains, or the N- or C-terminus.
- Typical modifications are deamidation, phosphorylation, acetylation, methylation, oxidation, or glycosylation.
- Isoforms may differ in biological activity and stability.
- Therefore, a thorough characterization and quantification of the isoforms is needed to ensure consistent product quality.
- Fast separation of monoclonal antibody isoforms is important for profiling and mass spectrometric determination.

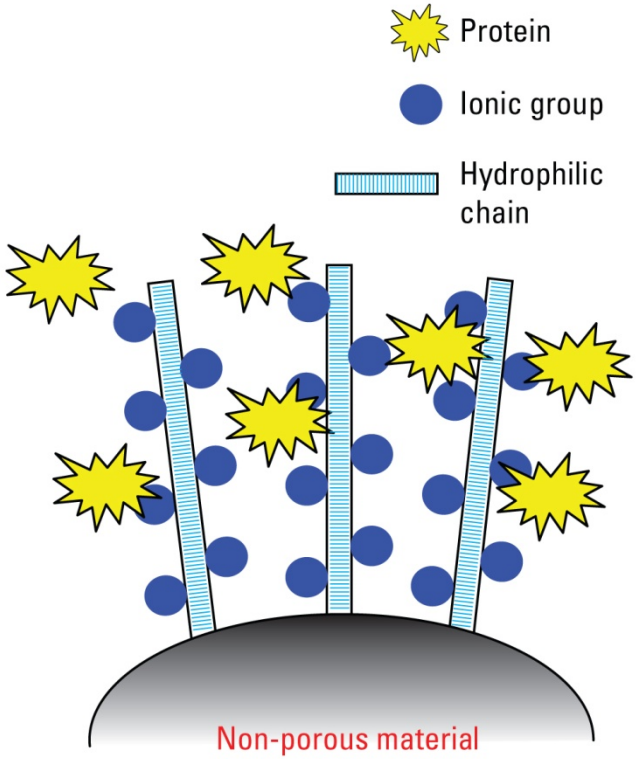
Introduction continued

- 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.

Introduction continued

- Here we report the separation of isoforms of monoclonal antibody using highly controlled pH gradients on TSKgel STAT columns packed with 7 and 10 µm hydrophilic non-porous resin particles.
- This report includes studies using TSKgel SP-STAT (7 µm, 4.6 mm ID × 10 cm) and TSKgel CM-STAT (10 µm, 3.0 mm ID × 3.5 cm) cation exchange columns and TSKgel Q-STAT (7 µm, 4.6 mm ID × 10 cm) anion exchange columns.

TSKgel STAT Columns – a Novel Bonding Chemistry



- The particle surfaces consist of an open access network of multi-layered cation or anion exchange groups.
- Their innovative bonding chemistry, combined with a relatively large particle size, results in a respectable loading capacity and a low operating pressure, making these columns suitable for all HPLC and FPLC systems in biomolecule separations.

Basic Properties of TSKgel SP-STAT and CM-STAT Cation Exchange Columns

Property	TSKgel SP-STAT		TSKgel CM-STAT	
Base material	Cross-linked hydrophilic polymer (mono-disperse particles)			
Pore size	Non-porous			
Functional group	Sulfonate		Carboxymethyl	
Particle size	7 µm	10 µm	7 µm	10 µm
Column size	4.6 mm ID × 10 cm	3 mm ID × 3.5 cm	4.6 mm ID × 10 cm	3 mm ID × 3.5 cm
Application	High resolution (HR) protein separation	High throughput (HT) protein separation	High throughput (HT) protein separation	High throughput (HT) protein separation

Basic Properties of TSKgel Q-STAT and DNA-STAT Anion Exchange Columns

Property	TSKgel Q-STAT		TSKgel DNA-STAT
Base material	Cross-linked hydrophilic polymer (mono-disperse particles)		
Pore size	Non-porous		
Functional group	Quaternary ammonium		
Particle size	7 µm	10 µm	5 µm
Column size	4.6 mm ID × 10 cm	3 mm ID × 3.5 cm	4.6 mm ID × 10 cm
Application	High resolution protein separation	High resolution protein separation	High resolution DNA separation

Tosoh developed novel non-porous anionic exchange (AEX) resins, TSKgel Q-STAT (7 and 10 µm) and TSKgel DNA-STAT (5 µm), with a high loading capacity and a low operating pressure by adopting larger particle sizes (5-10 µm) and by applying a novel bonding chemistry. TSKgel DNA-STAT column is not used in this study.

Material and Methods

HPLC System

- Analyses were carried out using an Agilent-1100 HPLC system running Chemstation (ver B.04.02).

Columns:

The following TSKgel STAT series non-porous resin columns were used for this study:

- Strong cation, polymer
  - TSKgel SP-STAT, 7 μm, 4.6 mm ID × 10 cm (S0004-501N)
- Weak cation, polymer
  - TSKgel CM-STAT, 10 μm, 3.0 mm ID × 3.5 cm (N0018-507N)
- Strong anion, polymer
  - TSKgel Q-STAT, 7 μm, 4.6 mm ID × 10 cm (R0087-501N)

Material and Methods continued

Mobile phase: Controlled pH gradient

- To create controlled pH gradients a pISep kit (CryoBioPhysica, Silver Spring, MD), consisting of a software package and two nearly identical buffer compositions, acidic and basic, composed of small zwitterions with overlapping pKas, was used.
- pISep buffer composition possesses strong, relatively uniform buffering capacity throughout the pH range 2-12.
- The pISep software was used to compute column volume or time-based protocols for the development of single or multistep, linear or nonlinear, pH gradients on ion exchange (IEX) columns over any segment of the pH range 2.4-10.8.

Material and Methods continued

Mobile phase: Controlled pH gradient continued

- Two buffers are mixed together in varying proportions to produce a titration curve smooth enough to allow precise fitting by a high-order polynomial.
- The polynomial equation incorporated into custom software can calculate protocols that are used to drive computer-controlled gradient LC pumps to form pH gradients of any desired shape and slope.
- The scouting process is simple.

Material and Methods continued

- Flow rate: TSKgel SP-STAT (1.0 mL/min or 1.66 min/CV); 1CV = 1.66 mL
  TSKgel CM-STAT (1.0 mL/min or 2.075 min/CV); 1CV = 0.247 mL
  TSKgel Q-STAT (0.8 ml/min or 0.247 min/CV); 1CV = 1.66 mL
- Detection: UV @ 280 nm
- Temperature: ambient
- Injection vol.: 10 µL
- Samples: monoclonal antibody: BI-mAb-2 from Boehringer-Ingelheim (gift from Tosoh Bioscience GmbH)
  concentration: 4.5 g/L in glycine/Na phosphate, pH 6.0

pISep Software – a Representative Plot

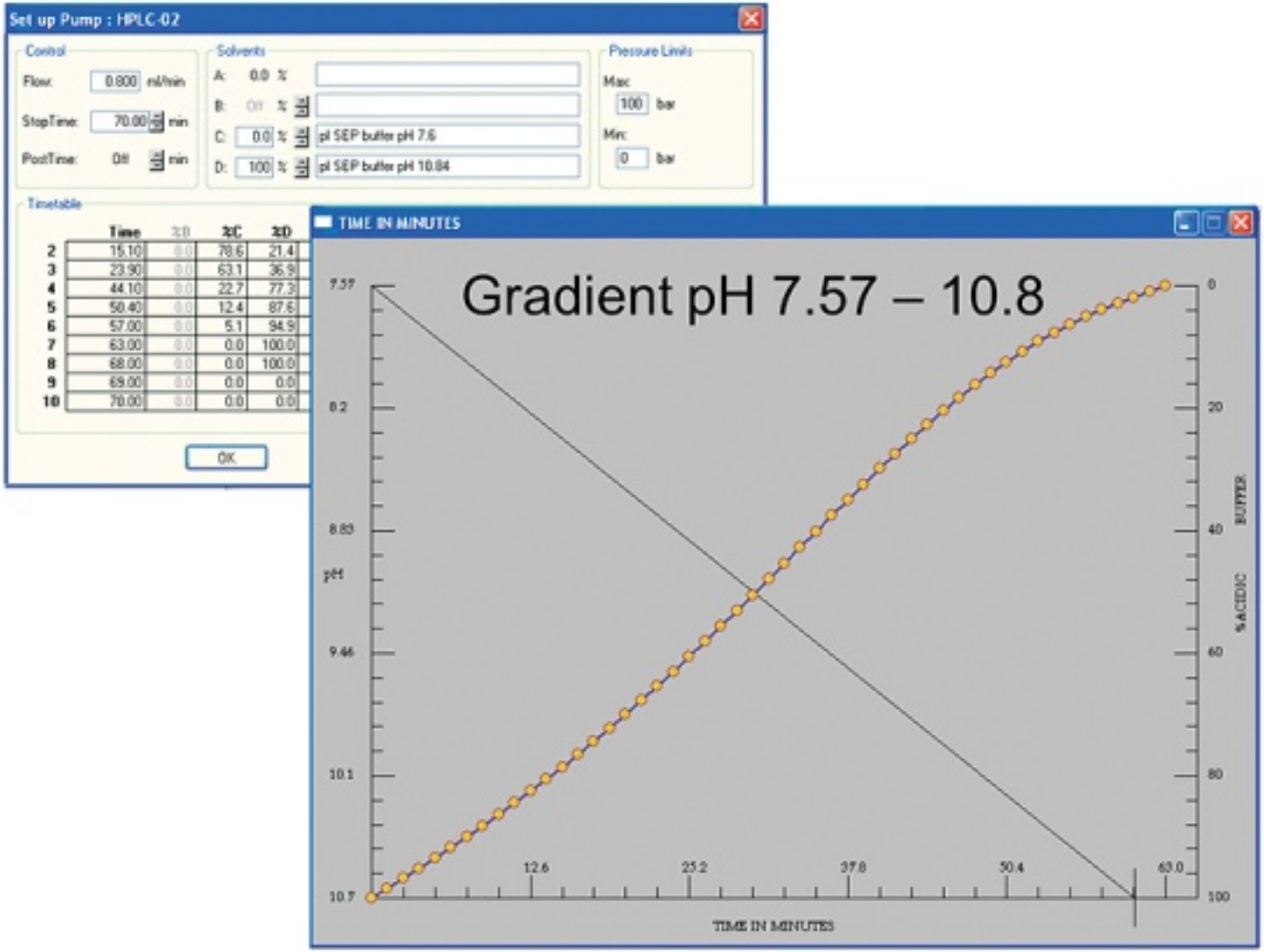
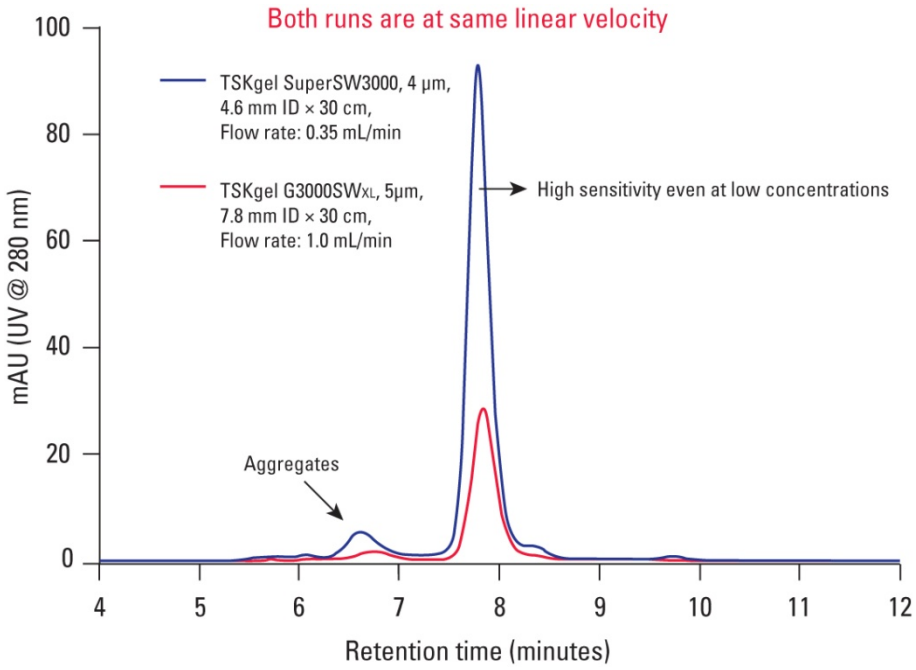


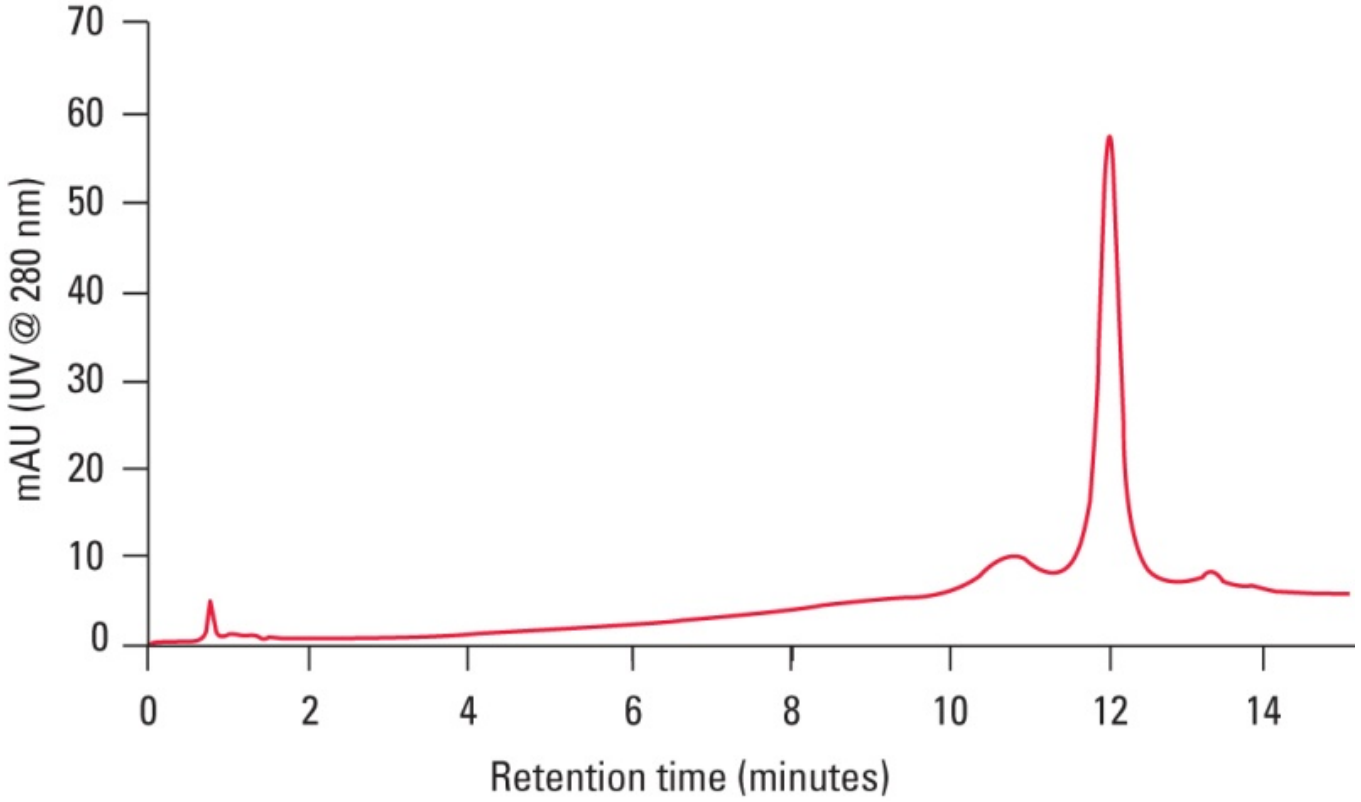
Figure 1: Analysis of Monoclonal Antibody using Size Exclusion Chromatography



Mobile phase:	0.1 mol/L phosphate buffer (mono/dibasic), 0.1 mol/L NaCl, 0.05% NaN_3, pH 6.8
Flow rate:	1 mL/min
Detection:	UV @ 280 nm (micro flow cell)
Injection vol.:	5 µL

Size exclusion chromatographic separation is used for the quality control study in the purification of monoclonal antibodies.

Figure 2: Salt Gradient-based Separation of a Monoclonal Antibody using a Strong Cation Exchange TSKgel SP-STAT Column

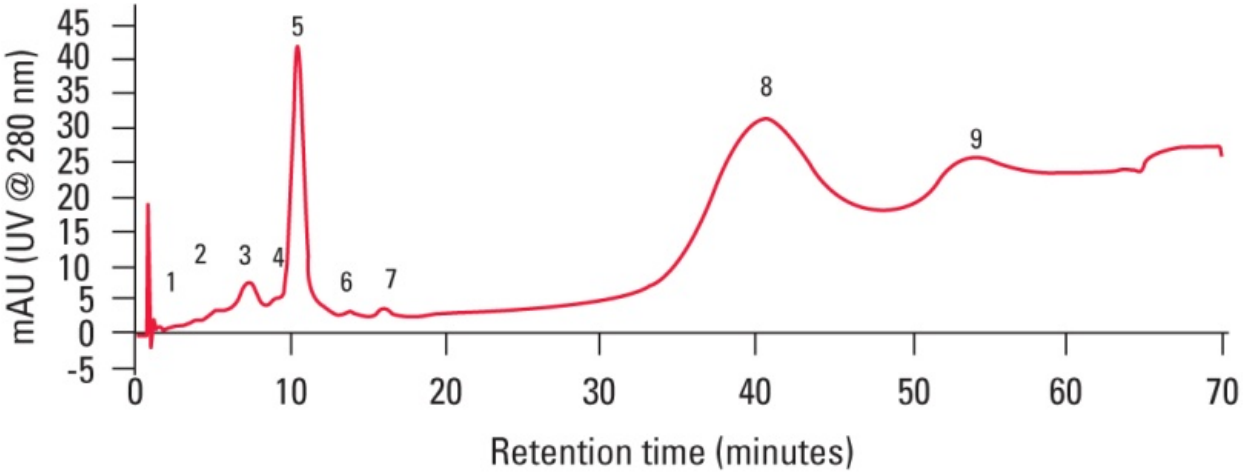


Linear salt gradient

A: 10 mmol/L phosphate buffer containing 10 mmol/L Na_2SO_4
B: 10 mmol/L phosphate buffer containing 10 mmol/L Na_2SO_4 with 1 mol/L NaCl

Gradient: 0-5% B over 15 minutes

Figure 3: pH Gradient-based Ion Exchange Separation of a Monoclonal Antibody Separation using a Strong Cation Exchange TSKgel SP-STAT Column

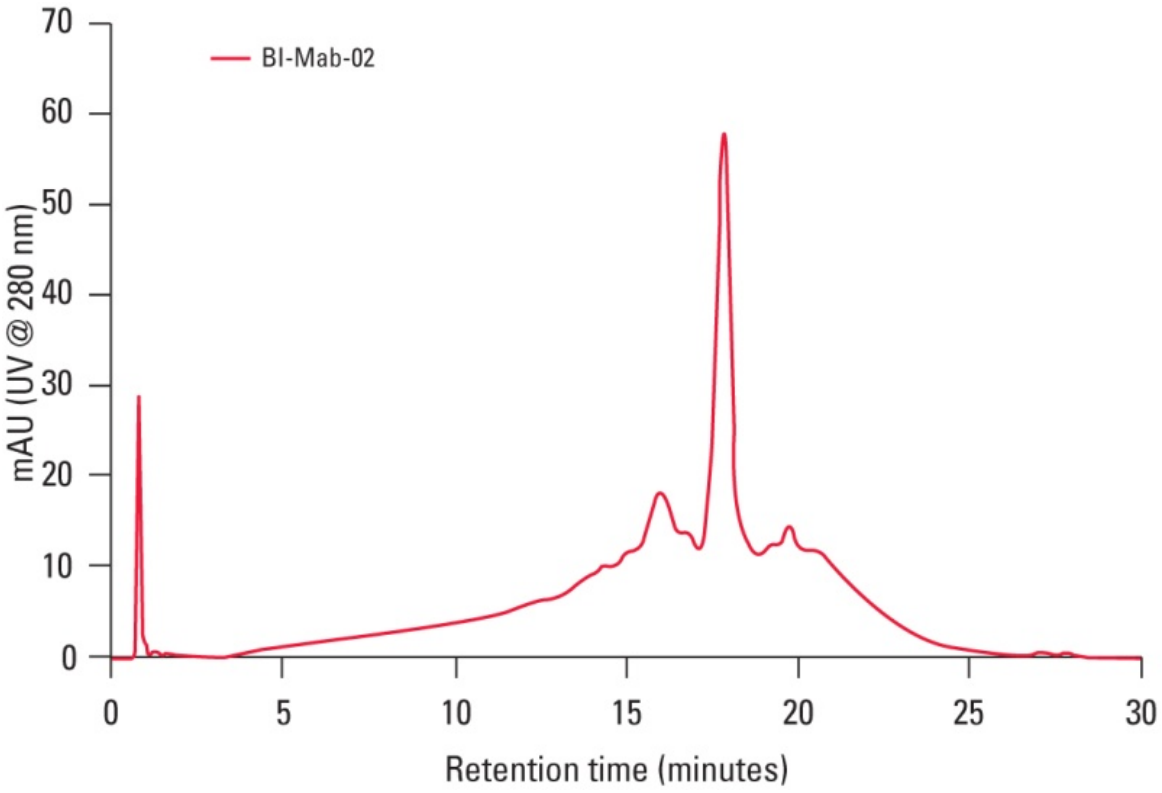


Time (min)	%B	pH
0	0	7.6
15.1	21.4	8.6
23.9	36.9	8.7
44.1	77.3	9.7
50.4	87.6	10.1
57.0	94.9	10.4
63.0	100.0	10.8
70.0	100.0	10.8
70.1	0	7.6

Gradient: pH 7.57-10.8
Buffer A (pH 7.57) is made by mixing pISep buffer (pH 2.4) and pISep buffer (pH 10.83) as calculated by pISep software

Nine isoforms are visible in this separation.
More shallow profile shown in figure 4.

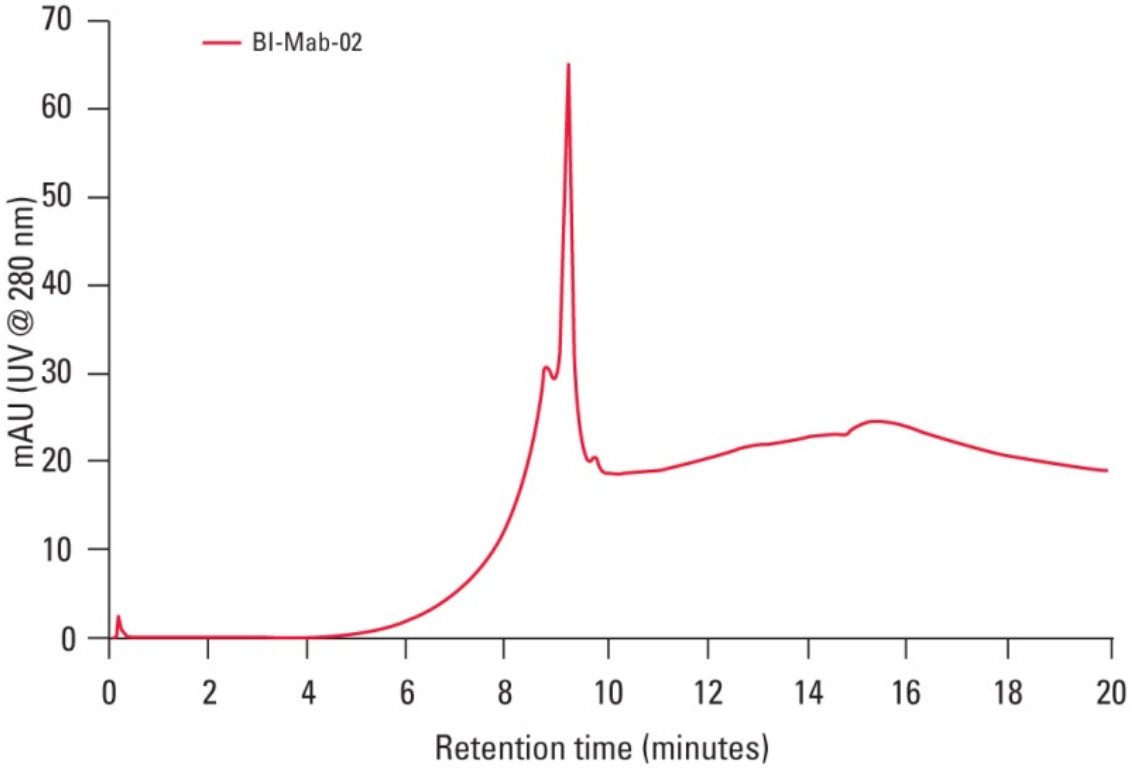
Figure 4: pH Gradient-based Separation of a Monoclonal Antibody Separation using a Strong Cation Exchange TSKgel SP-STAT Column



Time (min)	%B	pH
0	0	6.8
15.7	59.2	6.8
29.8	67.4	7.2
18.6	100.0	7.5
20.0	100.0	7.5
20.1	0	6.8

Gradient pH 6.8-7.46
Buffers A (pH 6.8) and B (pH 7.46) are made by mixing pISep buffer (pH 2.4) and pISep buffer (pH 10.83) as calculated by pISep software

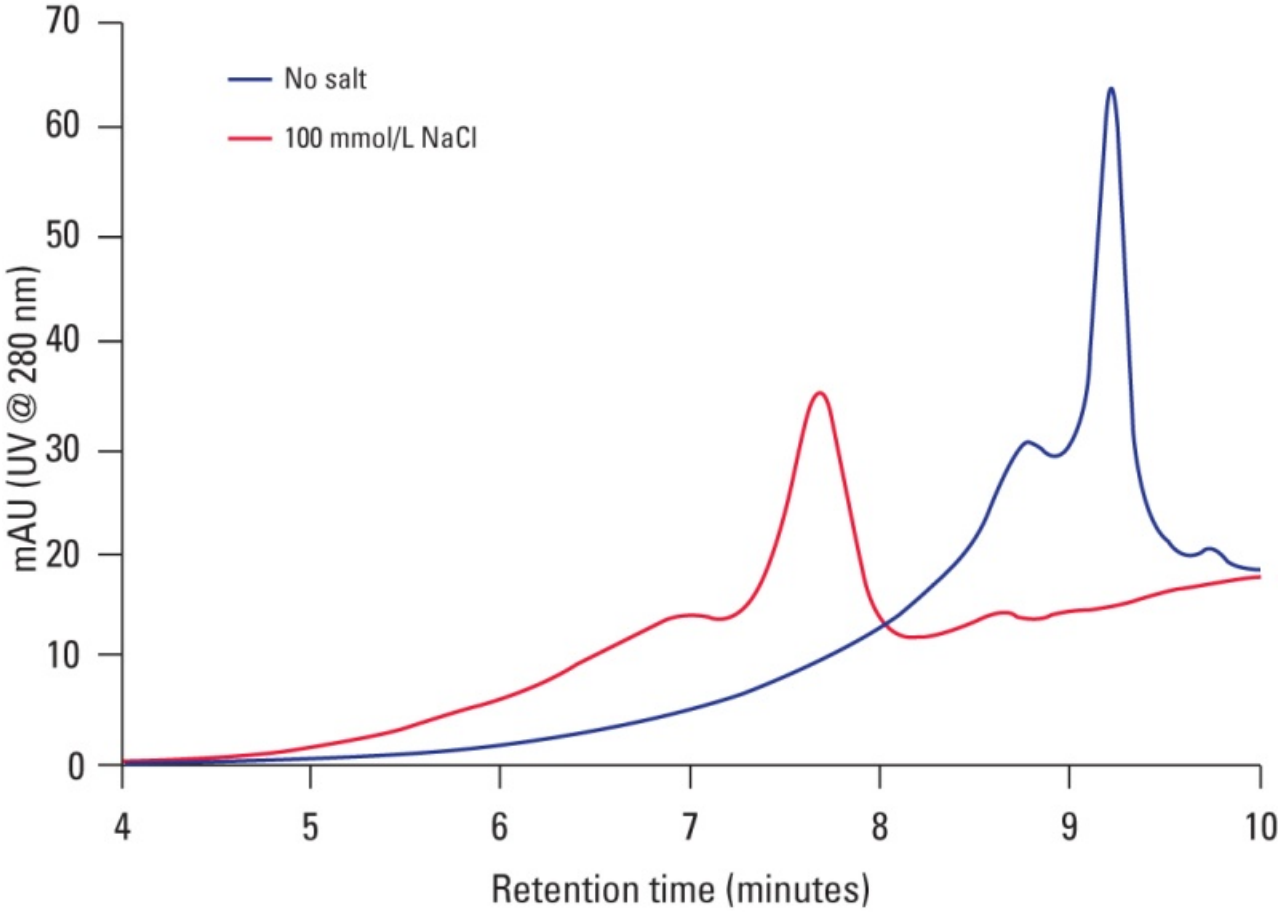
Figure 5: pH Gradient-based Separation of a Monoclonal Antibody using a Weak Cation Exchange TSKgel CM-STAT Column



Time (min)	%B	pH
0	0	5.1
2.2	15.4	6.1
4.6	30.0	7.0
7.0	45.5	8.0
9.4	66.5	9.0
11.5	88.1	9.9
13.7	100.0	10.8

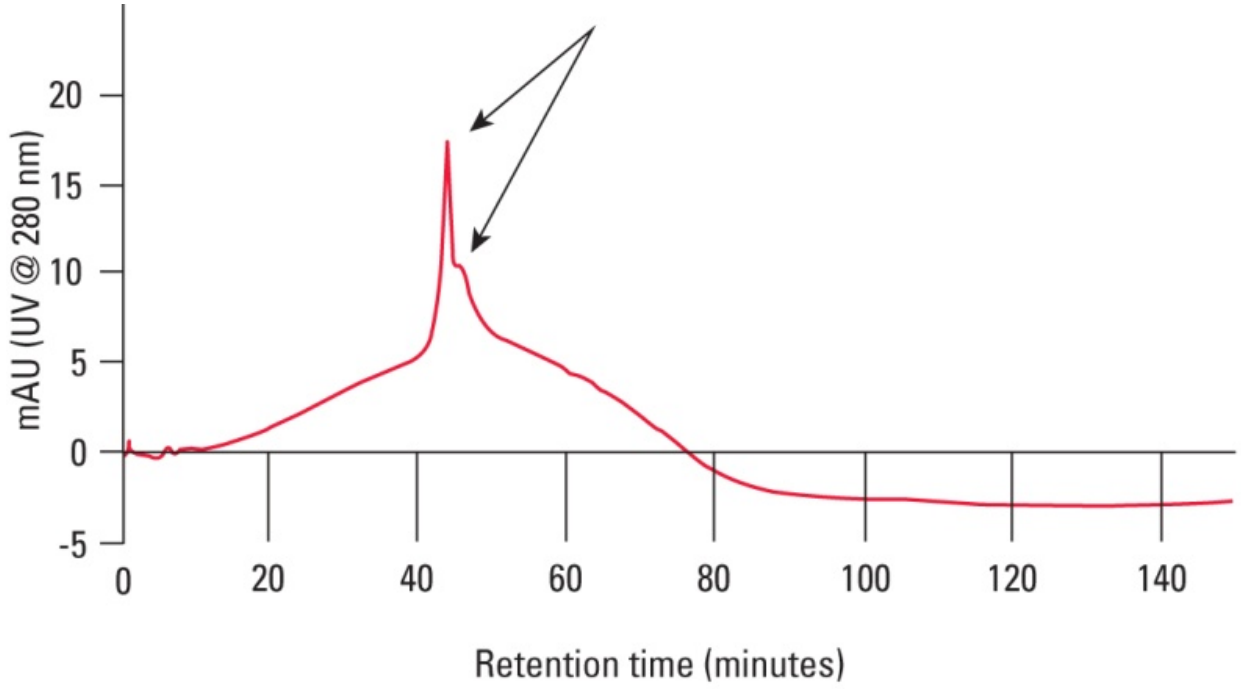
Gradient pH 5.1-10.83
Buffer A (pH 5.1) is made by mixing pISep buffer (pH 2.4) and pISep buffer (pH 10.83) as calculated by pISep software

Figure 6: Effect of Salt in pH Gradient-based Weak Cation Exchange Separation of a Monoclonal Antibody using a TSKgel CM-STAT Column



The resolution between the isoform peaks could be improved by the addition of 100 mmol/L NaCl.

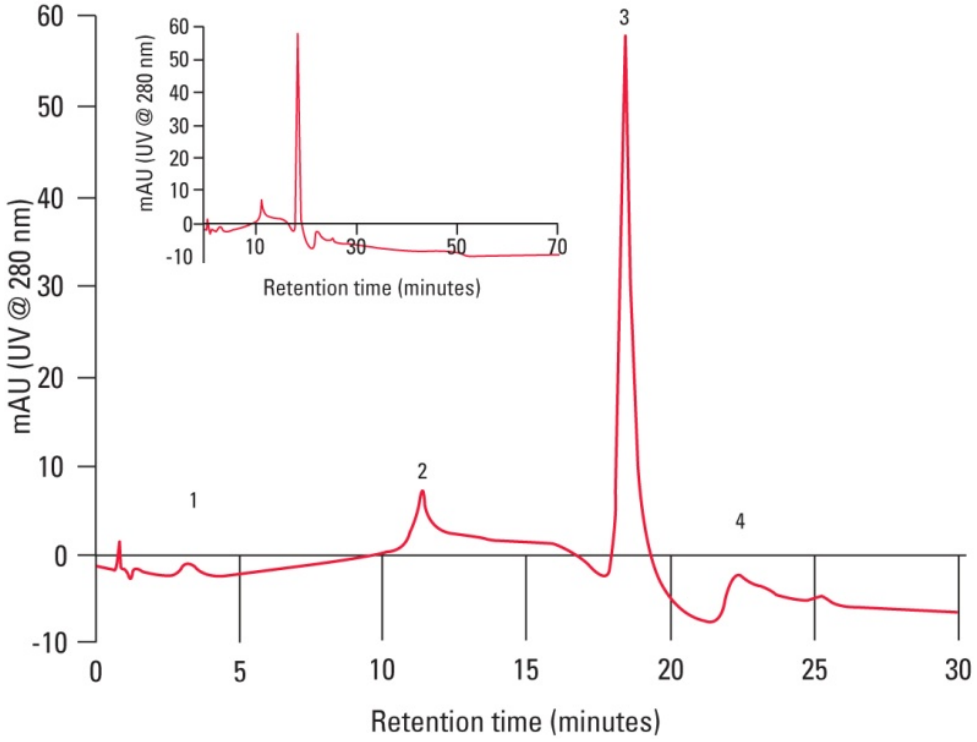
Figure 7: pH Gradient-based Anion Exchange Separation of a Monoclonal Antibody using a TSKgel Q-STAT, 7 μm, 4.6 mm ID × 10 cm Column



Time (min)	%B	pH
0	100.0	10.8
3.1	98.6	10.6
15.6	92.4	10.0
49.9	64.4	8.3
62.4	56.5	7.7
106.1	32.4	5.5
152.9	12.2	3.2
156.0	10.8	3.0
160.0	10.8	3.0
160.1	100.0	10.8

- Analysis shows 2-3 major isoforms.
- The peaks shown by the arrows need further resolution.

Figure 8: pH Gradient-based Separation of a Monoclonal Antibody using a Anion Exchange TSKgel Q-STAT Column



Time (min)	%B	pH
0	100.0	9.0
8.4	81.2	8.6
14.4	69.3	8.2
24.0	52.9	7.8
60.0	0	6.0
70.0	0	6.0
70.1	100.0	9.0

Gradient pH 9-6; Slope 0.1; 50 points per linear step
Buffers A (pH 6) and B (pH 9) are made by mixing pISep buffer (pH 2.4) and pISep buffer (pH 10.83) as calculated by pISep software

- The peaks are better resolved by the precise modification of the pH gradient.
- A number of isoforms are separated.
- Large changes in resolution can also sometimes be achieved in controlled pH gradient elution simply by changing the range of elution.

Conclusions

- Controlled pH gradient-based ion exchange chromatography can be a very good method for the separation of protein isoforms.
- Good resolution was found for pH gradient-based separations using a broad range universal buffer system like pISep.
- TSKgel STAT columns can be used to separate monoclonal antibody isoforms using controlled pH gradient.