Apparent Isoelectric Point (ApI) Separation of Proteins by External pH Gradient IEX Chromatography

pIsep Technology an Overview

CryoBioPhysica, Inc.

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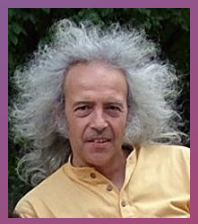

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CryoBioPhysica, Inc.

CryoBioPhysica is a privately owned, new, biotech research company originally engaged in immunological research. The company recently has developed novel and powerful methods for ion exchange chromatography (IEX) based on the controllable formation of external pH gradients on strong anion and cation exchangers, called ***pIsep****.*

These innovative methods have broad applications in the field of chromatographic fractionation and purification of proteins, polysaccharides and other charged biological molecules. They provide better resolution, more flexibility and a less costly substitute for chromatofocusing and ion exchange chromatography with salt elution.

What is pIsep IEX and How It Compares to Polybuffer Chromatofocusing

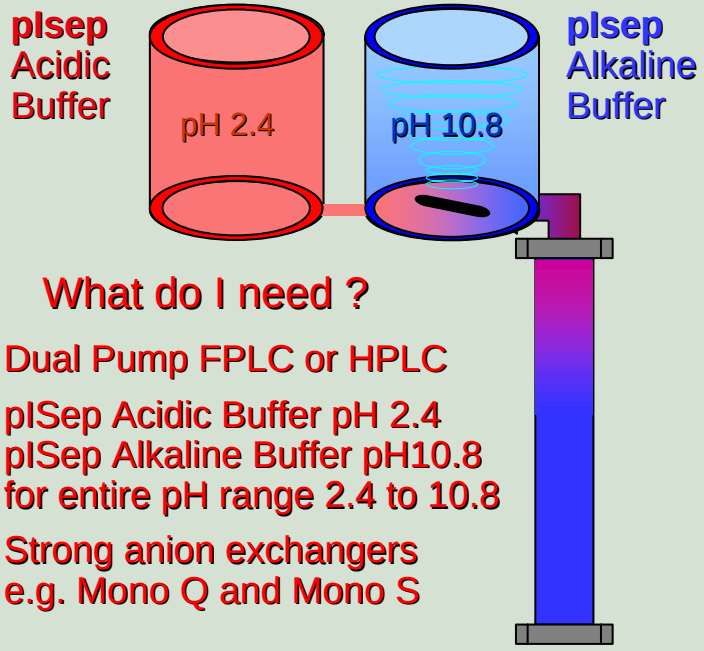
Polybuffer Chromatofocusing	pISep IEX Chromatography
1. Self-generated pH gradients on specially designed weak anion exchangers	1. Externally generated pH gradients on either strong anion or cation exchangers.
2. Separates Proteins in order of their apparent pI from alkaline to acidic pH	2. Separates Proteins in order of their apparent pI from either alkaline to acidic pH or from acidic to alkaline pH
3. Both offer high resolution. Peak width is in the range of 0.04–0.05 pH units or less.	
4. Both provide unique selectivity and support high capacity. Can separate samples containing up to 100 mg per ml of resin per pH unit of gradient.	
5. Focusing in both techniques produces sharp well-separated protein bands.	
6. Three types of Polybuffer cover the pH range 4 to 10.5	6. Single buffer composition covers a broader pH range 2.4 to 10.8
7. Limited control over the formation of the eluting pH gradient depends on the AEX gel	7. Full control over the formation of the pH gradient independent of the AEX or CEX gel
8. Only semi-linear descending alkaline to acidic pH gradients	8. Ascending, descending, stepwise, linear, nonlinear or combined pH gradients
9. Polybuffers need to be removed from the protein fractions	9. pISep buffers no need to be removed from the protein fractions
10. Polybuffers have increased absorptivity below 280nm	10. pISep buffers have low absorptivity below 280 to 254nm safe for flourescence detection and electrophoreses

Applications of pIsep Technology

- **Applicable to analytical and large scale preparative purifications of proteins:**
  - ✓ Enzymes
  - ✓ Antibodies -- separates isoforms of immunoglobulins (even monoclonal antibodies possessing heterogeneously charged sugar chains)
  - ✓ Vaccines
  - ✓ Biological pesticides
  - ✓ Blood proteins
  - ✓ Milk whey proteins
- **Can replace salt-elution Ion Exchange Chromatography. Reduces separation steps and eliminates all of the desalting steps associated with IEX**
- **Can be used as a polishing step following:**
  - ✓ Affinity Chromatography
  - ✓ Hydrophobic interaction chromatography
  - ✓ Reversed phase chromatography
  - ✓ Ion-exchange chromatography
  - ✓ Gel filtration chromatography
- **pIsep can separate protein isoforms differing by a single amino acid or species with variations in glycosylation**
- **Analytical Quality Control/Quality Analysis – pIsep can detect degradation or folding variants of proteins**

pH Gradient formation - How It Works
pIsep IEX vs. Polybuffer Chromatofocusing

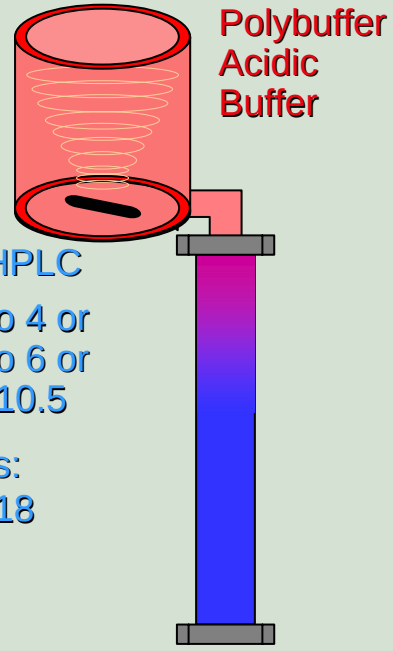
pISep IEX Chromatography
External pH Gradient



What do I need ?

1. Dual Pump FPLC or HPLC
2. pISep Acidic Buffer pH 2.4
   pISep Alkaline Buffer pH10.8
   for entire pH range 2.4 to 10.8
3. Strong anion exchangers
   e.g. Mono Q and Mono S

Polybuffer Chromatofocusing
Internal pH Gradient

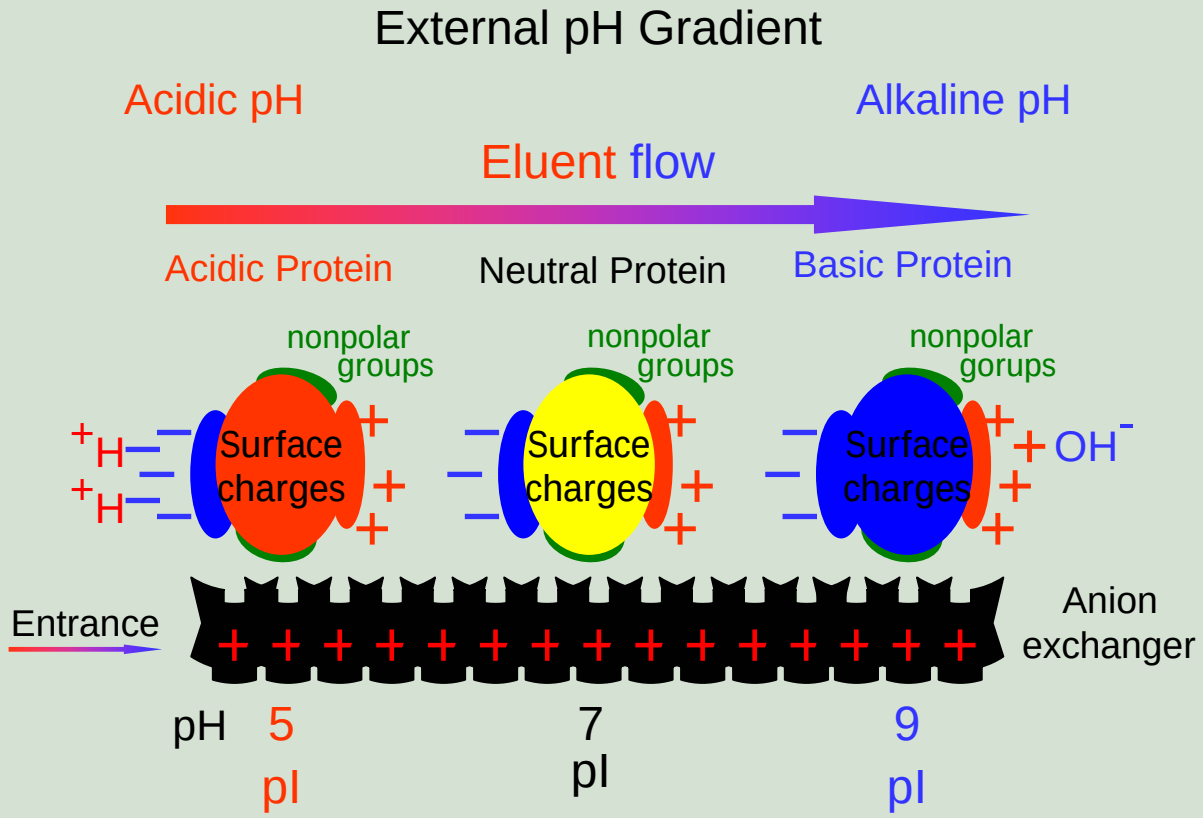


What do I need ?

1. Single Pump FPLC or HPLC
2. Polybuffer 74 for pH 7 to 4 or
   Polybuffer 96 for pH 9 to 6 or
   Pharmalyte for pH 8 to 10.5
3. Weak anion exchangers:
   Mono P, PBE94, PBE118
   Specially designed
   for chromatofocusing

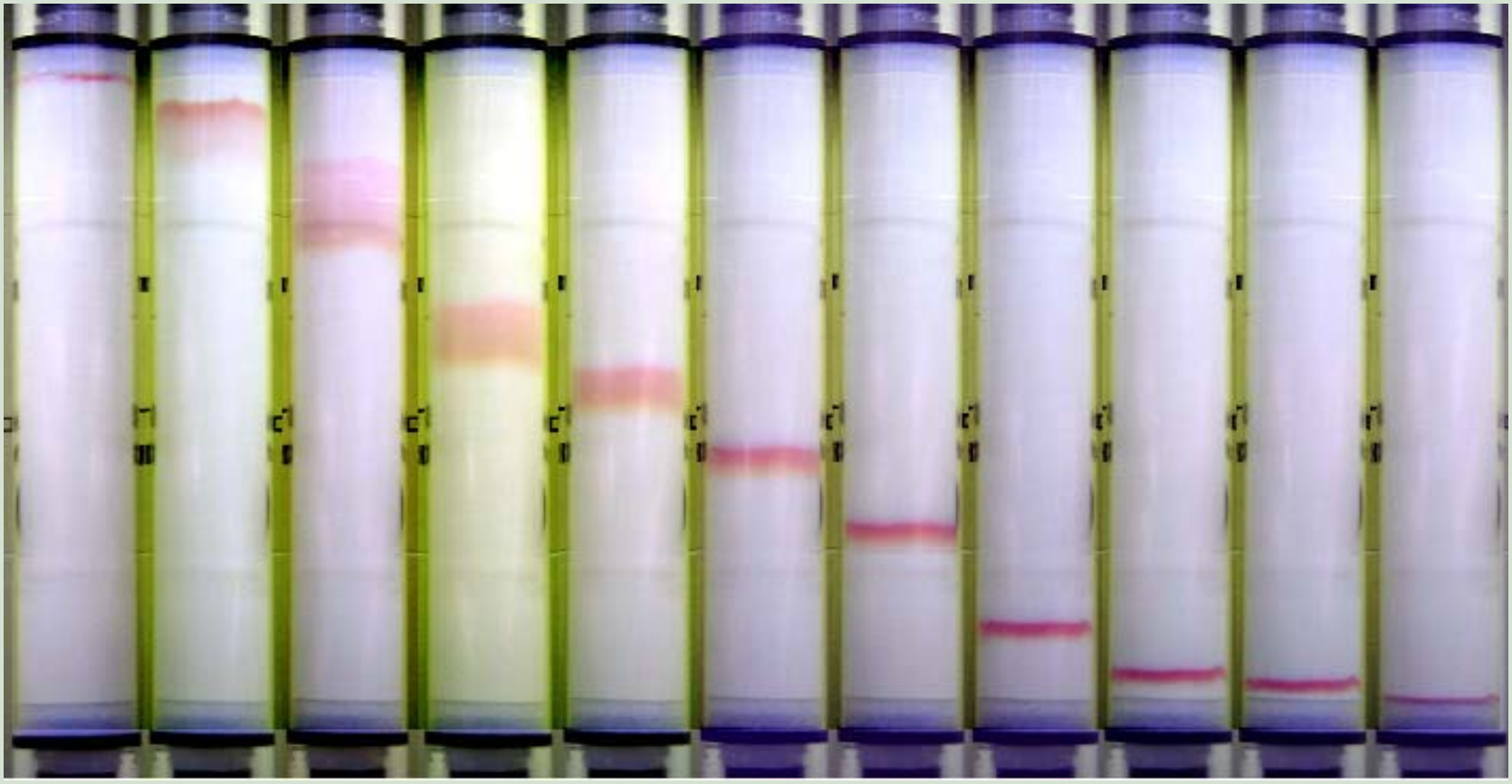
This cartoon shows the pH gradient formation during pIsep (on the left) compared with the pH gradient formation during Polybuffer chromatofocusing (on the right). In pIsep, an external pH gradient is continuously formed as the pH of the eluting buffer entering the column is changed by diluting the alkaline pIsep buffer with the acidic pIsep buffer. Both the slope and the shape of the gradient are entirely controlled by the chromatographer and do not depend on the properties of the strong anion exchanger. The Polybuffer retained pH gradient is generated automatically inside a weak ion exchange column by interaction between the multibuffer species of the Polybuffer and the exchanger. The chromatographers' control over the formation of this pH gradient is limited. The slope and shape of the gradient are dependent on at least three independent variables: the length of the column, the Polybuffer concentration and the binding properties of the ion exchanger.

Protein Elution in Anionic pIsep



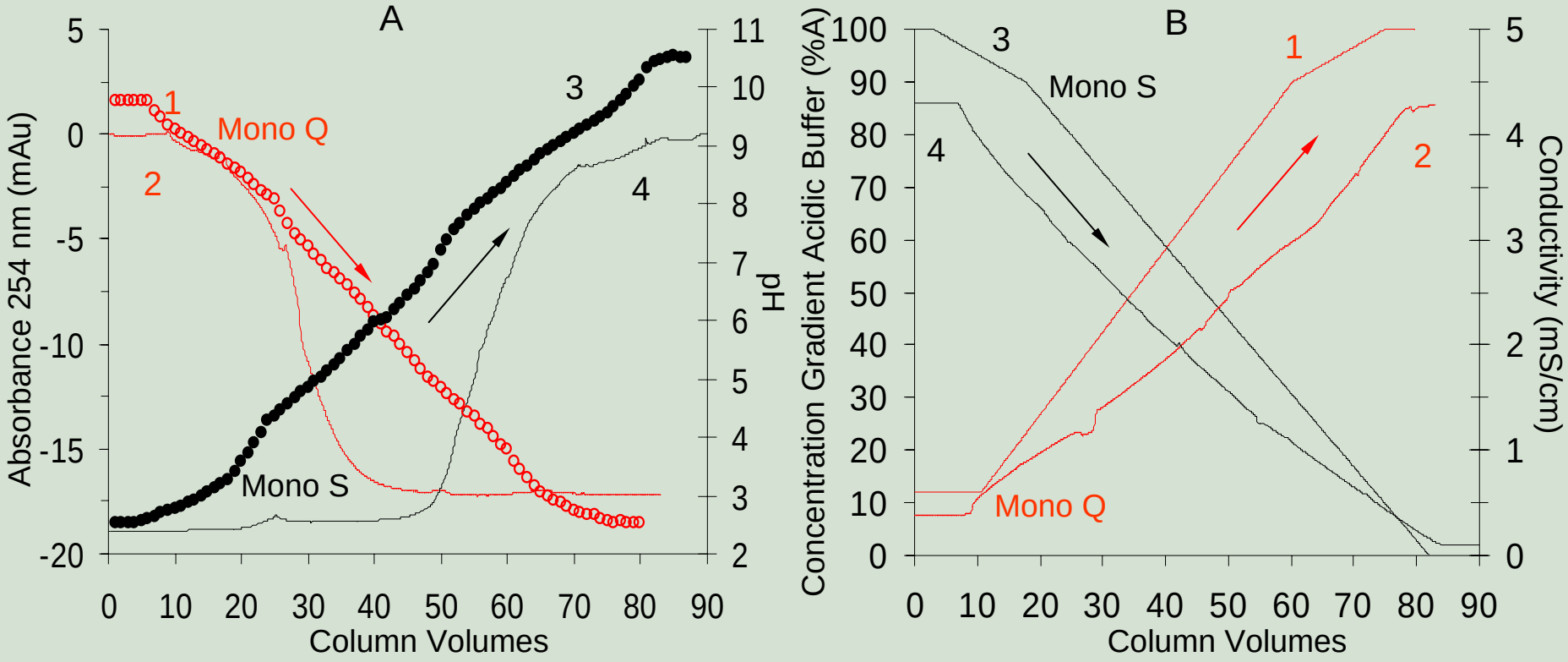
This cartoon shows the elution of proteins by a descending pH gradient that is being developed from the entrance towards the exit of a strong anion exchange column by a continuous flow of an increasingly acidic elution buffer. A protein at its **pI** does not interact with the anion exchanger and is thus neither bound nor repelled from it. Because of that when the pH of the eluent reaches the **pI** of a bound protein, the protein is released from the column and starts moving with the front of the pH gradient.

Focusing in pIsep



These photographs show the focusing effect observed during anionic **pIsep** of horse myoglobin on Mono Q HR 5/5. Myoglobin molecules at the rear of the chromatographic band are at a pH lower than their pI and, as a consequence, are repelled from the anion exchanger and move faster than molecules at the front of the band where the pH is more alkaline than the pI of the protein. The result is progressive narrowing of the band and elution of myoglobin as a very sharp peak.

Very Wide pH-Range Buffer System for External Gradient and External Gradient Reverse pIsep

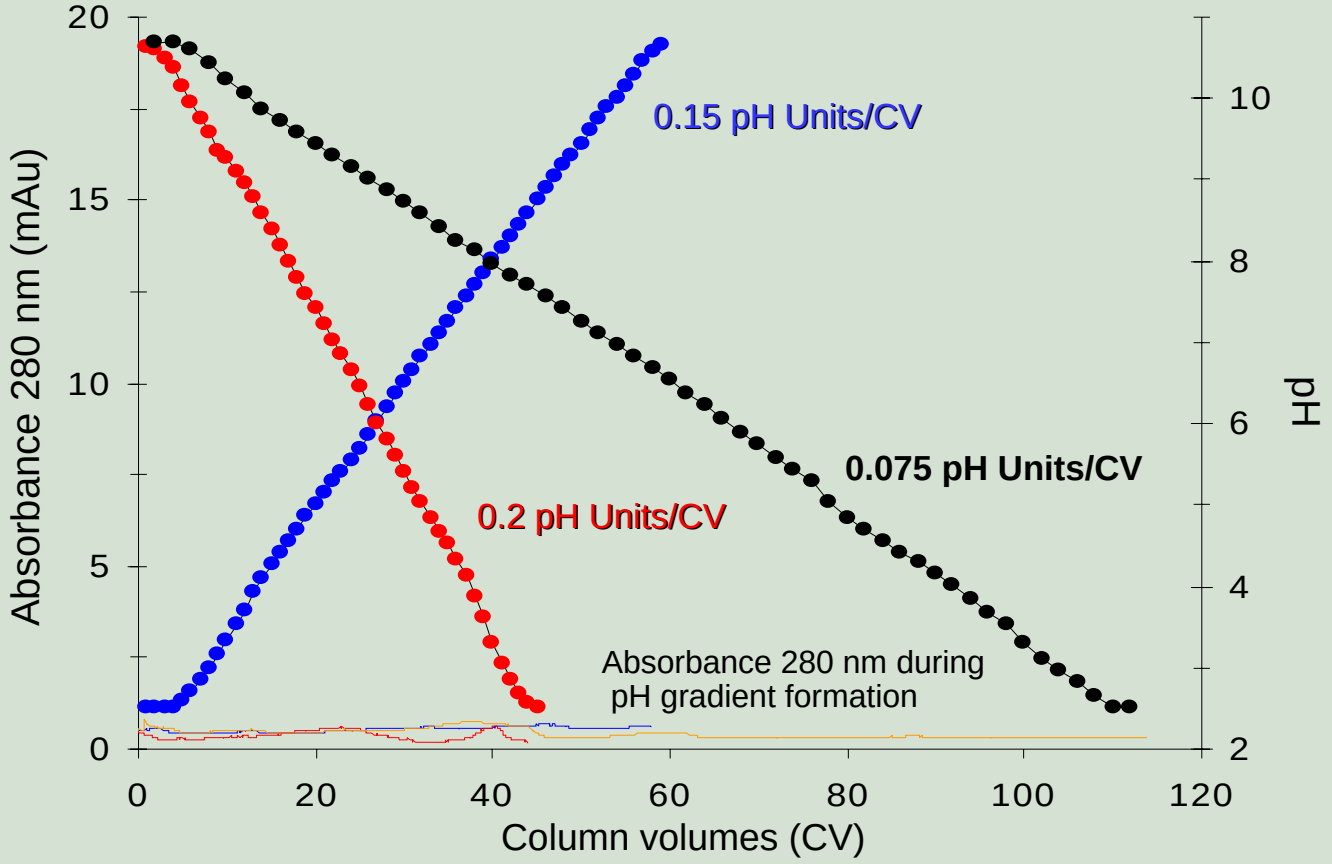


These graphics present pH profiles and effluent absorbance profiles observed during external pH gradient anionic **pIsep** (curves 1, 2 left panel) and reverse, cationic **pIsep** (curves 3, 4 left panel). This powerful buffering system allows separation of charged molecules over 7.3 pH units (anionic **pIsep**) and 8.3 pH units (reverse, cationic **pIsep**). Programmed gradient formation and effluent conductivity observed during external gradient anionic and reverse, cationic **pIsep** are presented in the right panel (curves 1, 2 and 3, 4). pH gradients were developed with ÄKTA FPLC on Mono Q and Mono S HR 5/5 columns connected in the eluent flow. Flow 1 ml/min.

pIsep Kit: A Window to Protein Purification Success

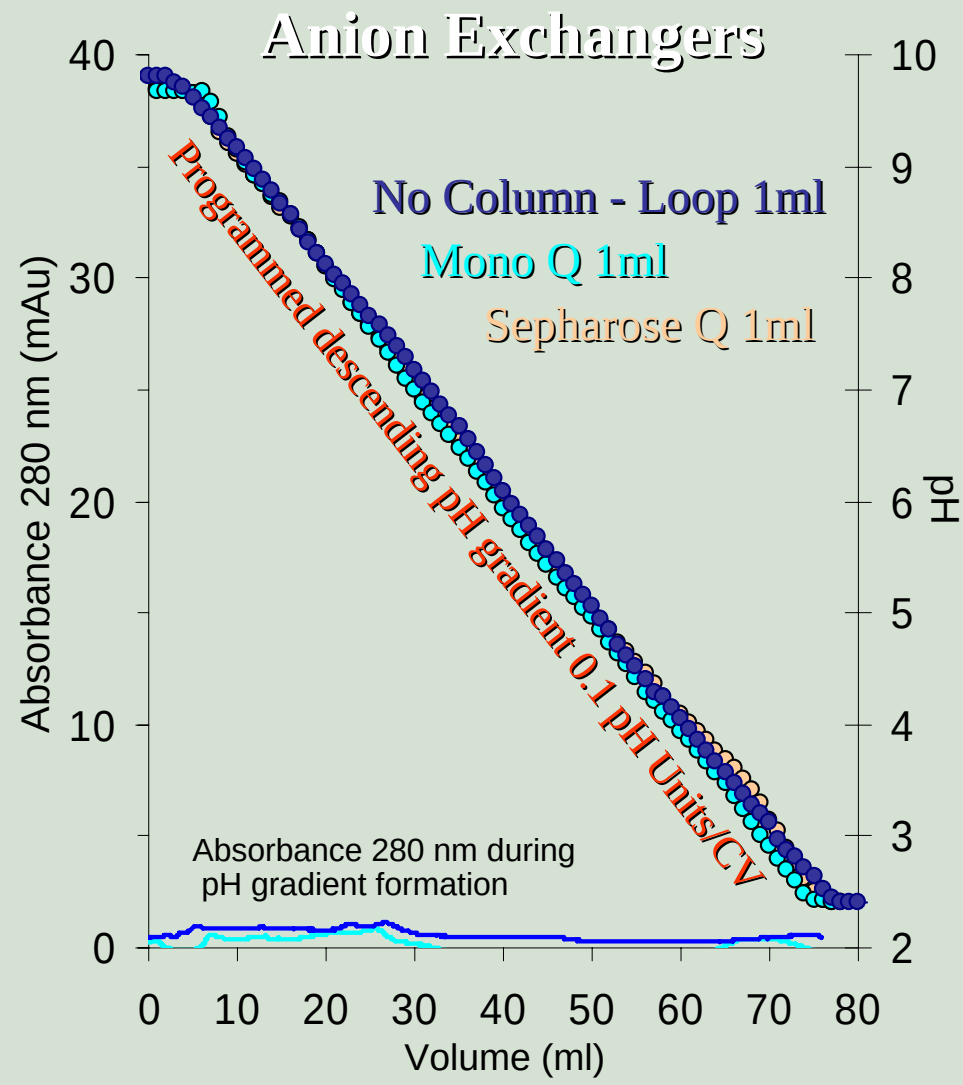
CryoBioPhysica offers a kit that includes **pIsep** buffer concentrates and a software package for optimization of **pIsep**. The software will calculate the chromatographic parameters necessary to develop complex pH gradients containing multiple linear segments of user-defined length and slope, as well as nonlinear concave and convex pH gradients. Together the **pIsep** buffers and the software package provide the necessary flexibility to achieve complete control over optimization of the protein separation process.

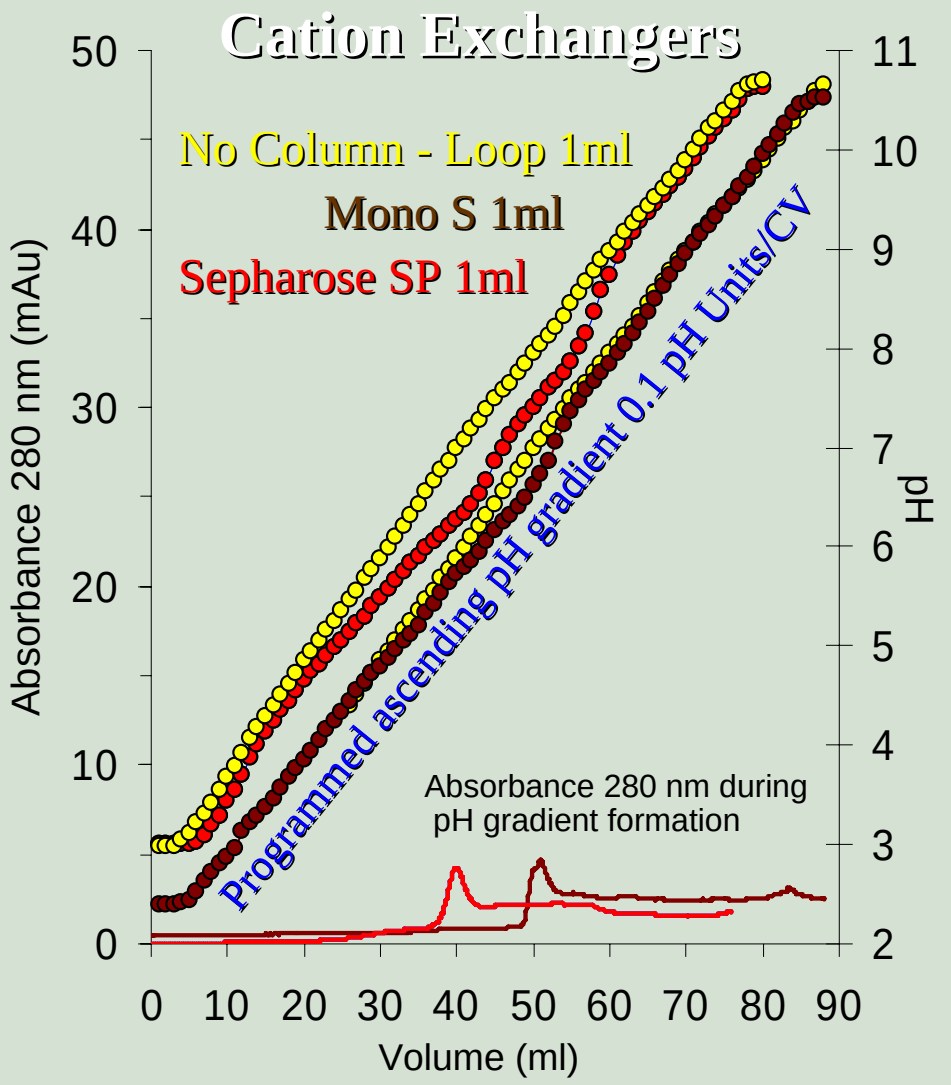
Together pIsep Buffers and pIsep Software Allow Controlled Formation of Linear pH Gradients



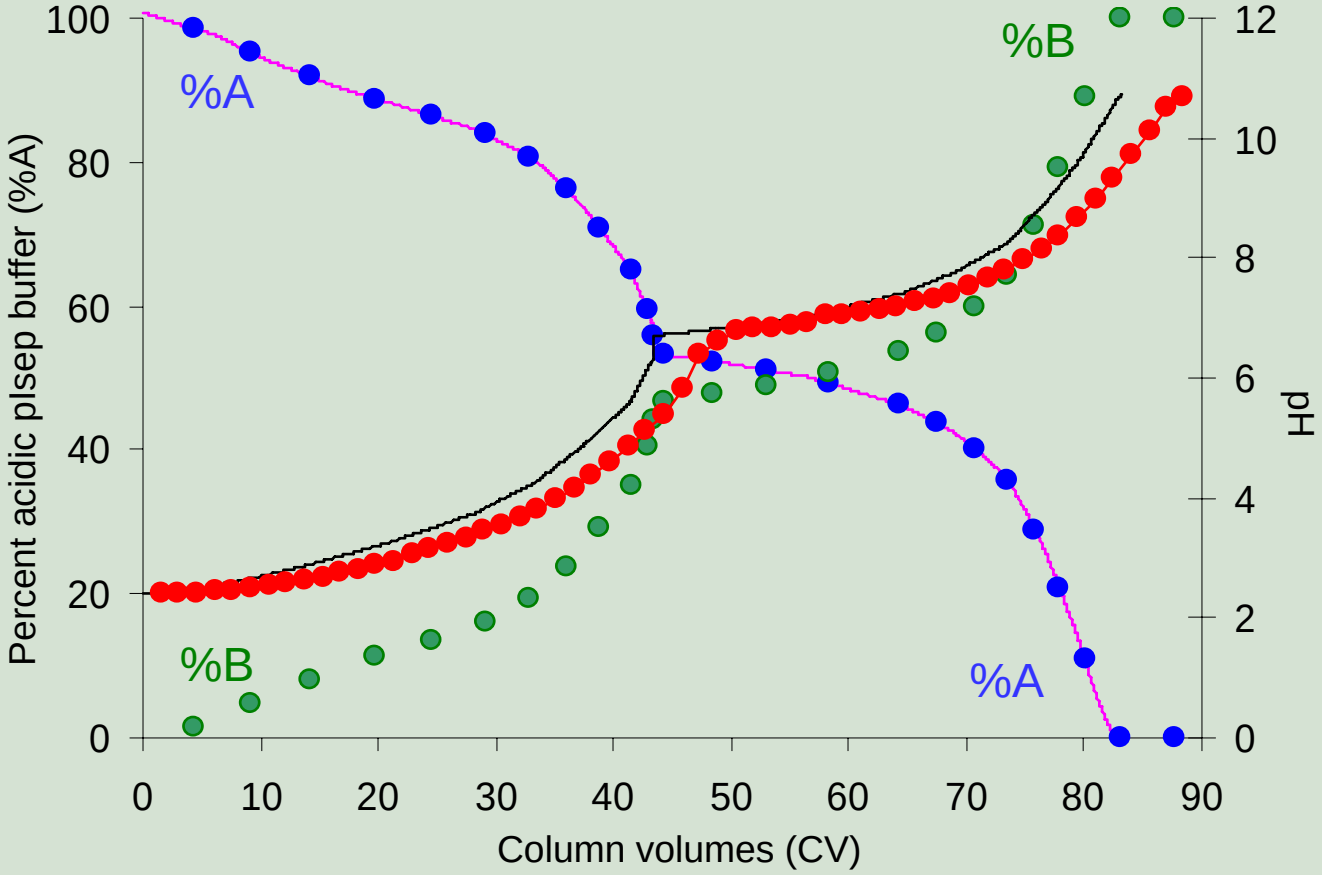
Examples of linear pH gradients created in the pIsep software and reproduced using pIsep buffers on an ÄKTA FPLC. The red dotted line demonstrates a descending, experimentally generated linear pH gradient with a slope of 0.2 pH units per column volume; The blue dotted line demonstrates an ascending linear pH gradient with a slope of 0.15 pH units per column volume; The black dotted line demonstrates an descending linear pH gradient with a slope of 0.075 pH units per column volume. These pH gradients were developed in the absence of an ion exchanger. 1CV = 0.98 ml, Flow 1ml/min. The formation of ascending and descending pH gradients allows exquisite control over both cationic and anionic **pIsep** fractionation of proteins.

Influence of the Strong Ion Exchangers on the Controlled Formation of Linear pH Gradients



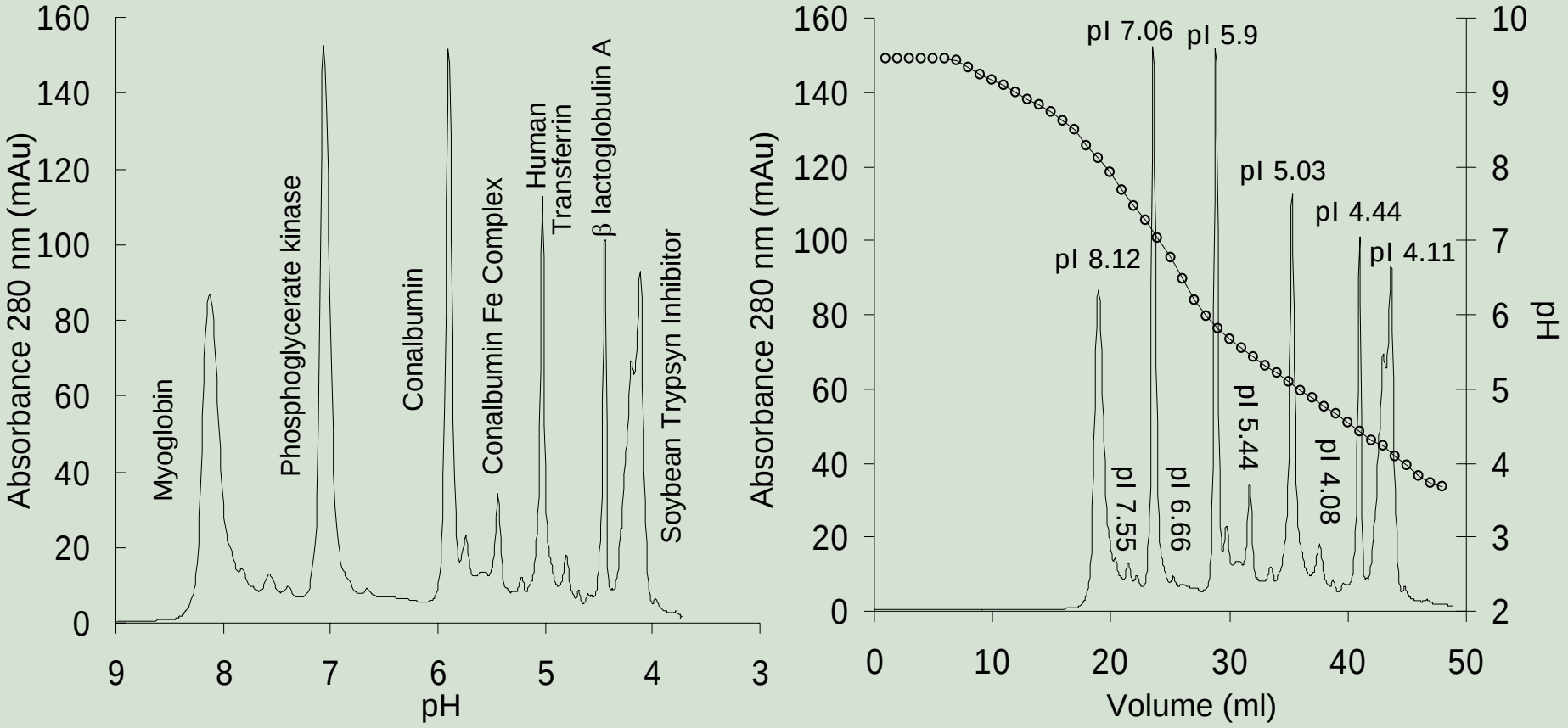


Together pIsep Buffers and pIsep Software Allow Controlled Formation of Nonlinear pH Gradients



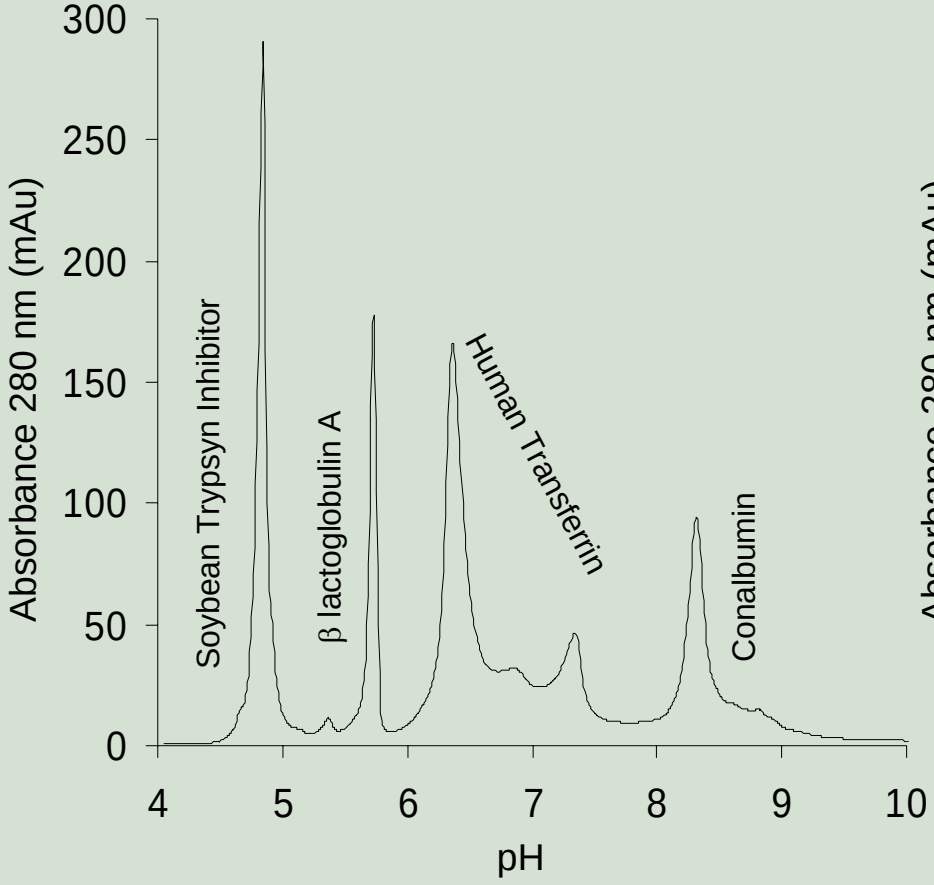
Example of a nonlinear pH gradient created in the pIsep software and reproduced using pIsep buffers on an ÄKTA FPLC. Black curve: a pH gradient generated using the **pIsep** software; Magenta curve - **pIsep** software-calculated percent of acidic **pIsep** buffer (%A) to be mixed with basic **pIsep** buffer (%B) in order to develop the software-generated pH gradient; Blue dots: programmed %A to be mixed with %B (green dots) in the Unicorn software of the ÄKTA FPLC that produced the experimentally observed nonlinear pH gradient (red dotted line). There is a delay of 4.5 CVs in the development of the experimentally observed pH gradient developed in the absence of an ion exchanger. 1CV = 0.98 ml, Flow 1ml/min.

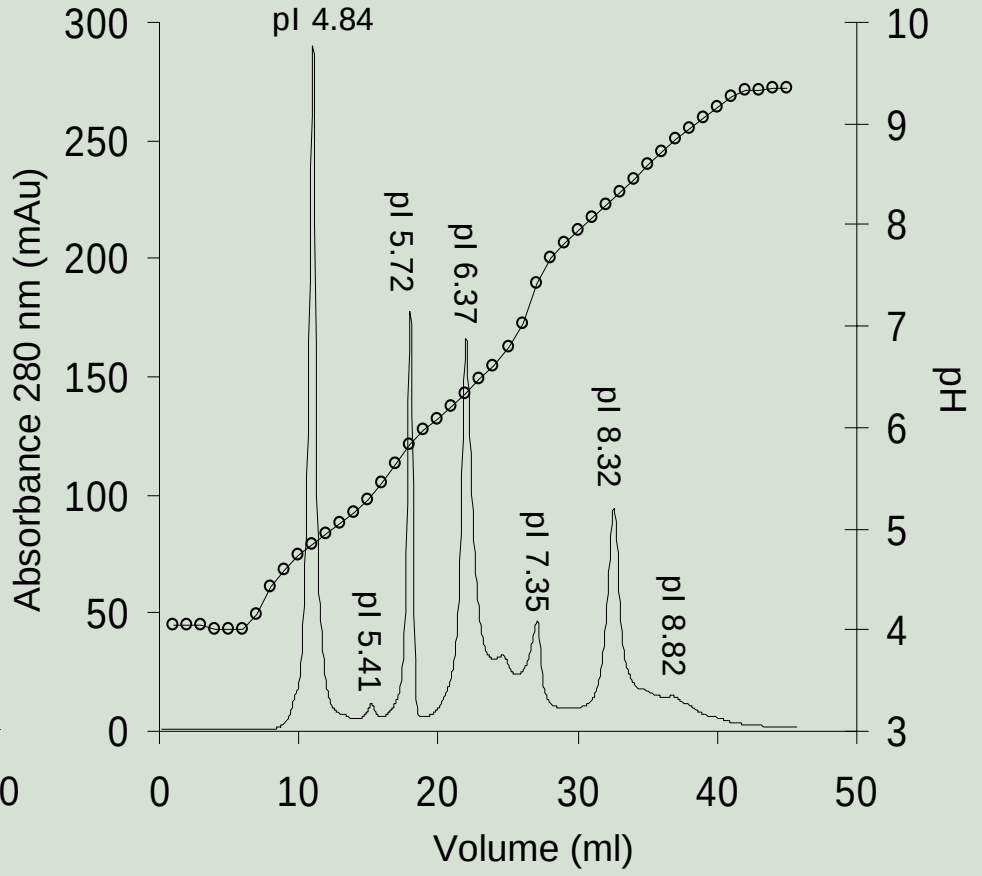
External pH Gradient Anionic pIsep of a Complex Mixture of Proteins



Separation of a complex mixture of proteins by external descending pH gradient pISep on a Mono Q HR 5/5 anion exchange column. The sample was loaded at pH 9.5 and eluted with an external pH gradient to pH 3.5. Despite the complexity of the sample, the closeness in **pIs** for several species and the wide pH range necessary to separate all components, excellent resolution is achieved.

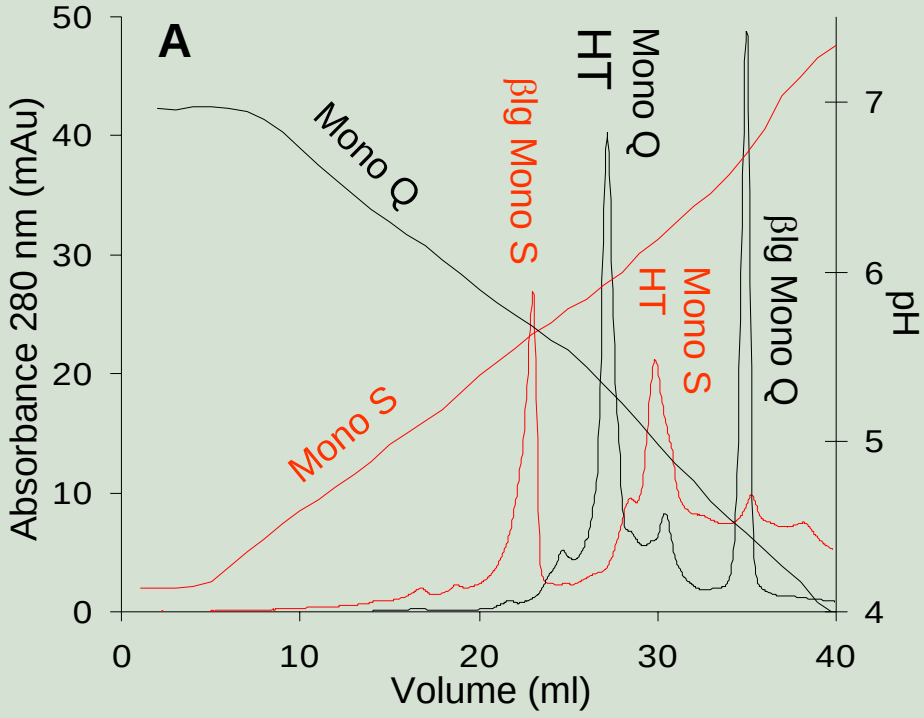
External pH Gradient Reverse, Cationic pIsep of a Complex Mixture of Proteins

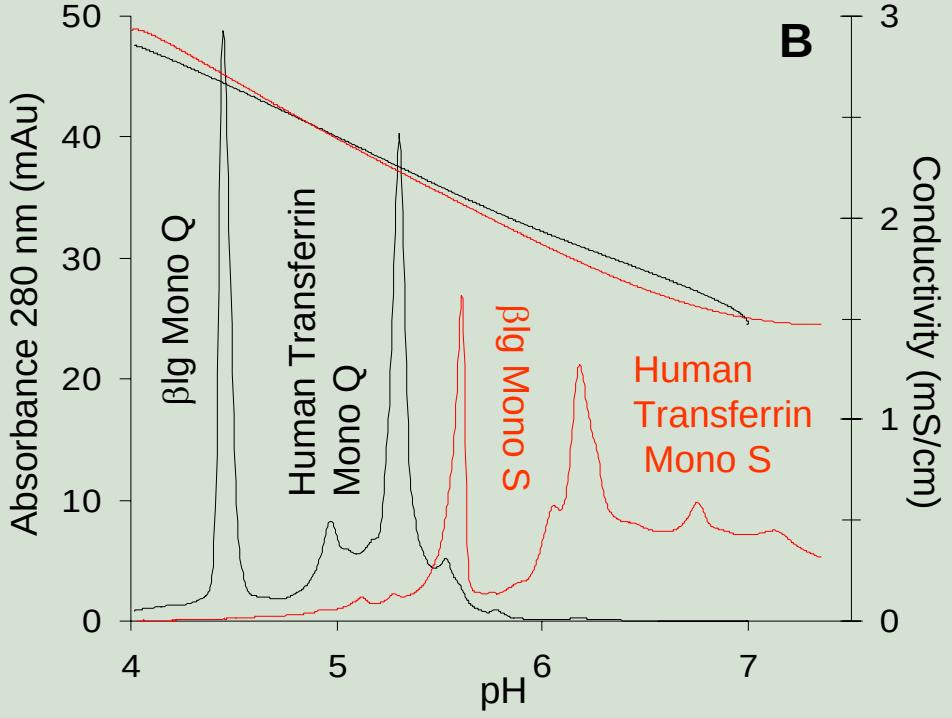




A mixture of proteins separated by external gradient, reverse, cationic pISep on a Mono S HR 5/5 column using a broad linear pH gradient from pH 4 to pH 9.5 (right panel dotted line). It is important to note that the STI and ß-lactoglobulin are very well separated by this reverse pH chromatographic technique compared to the external gradient anionic **pISep** separation over the same pH range presented in the previous example. This shows why it is important to have available both capabilities, external gradient **pISep** and external gradient reverse **pISep**.

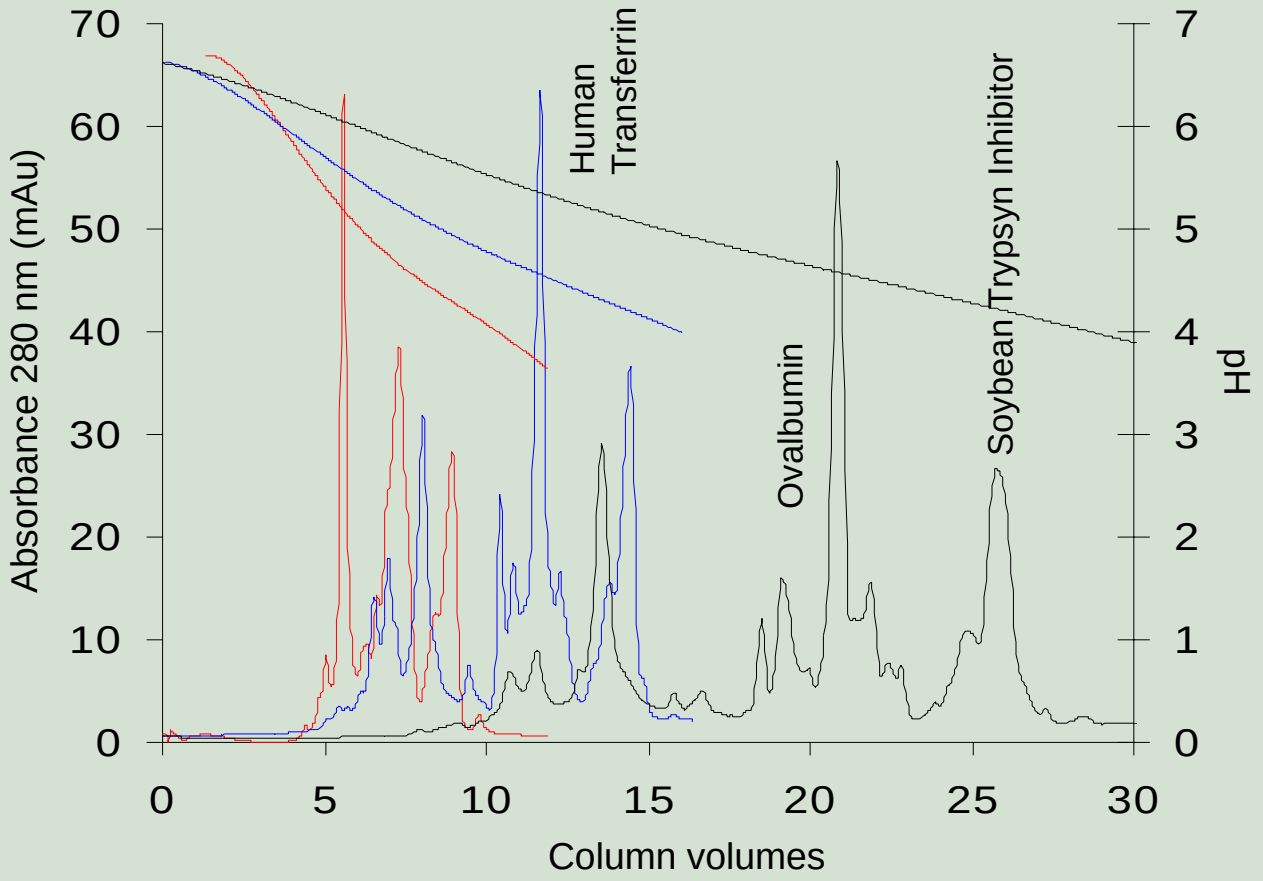
Combined pIsep is a New Technique for Separation of Proteins





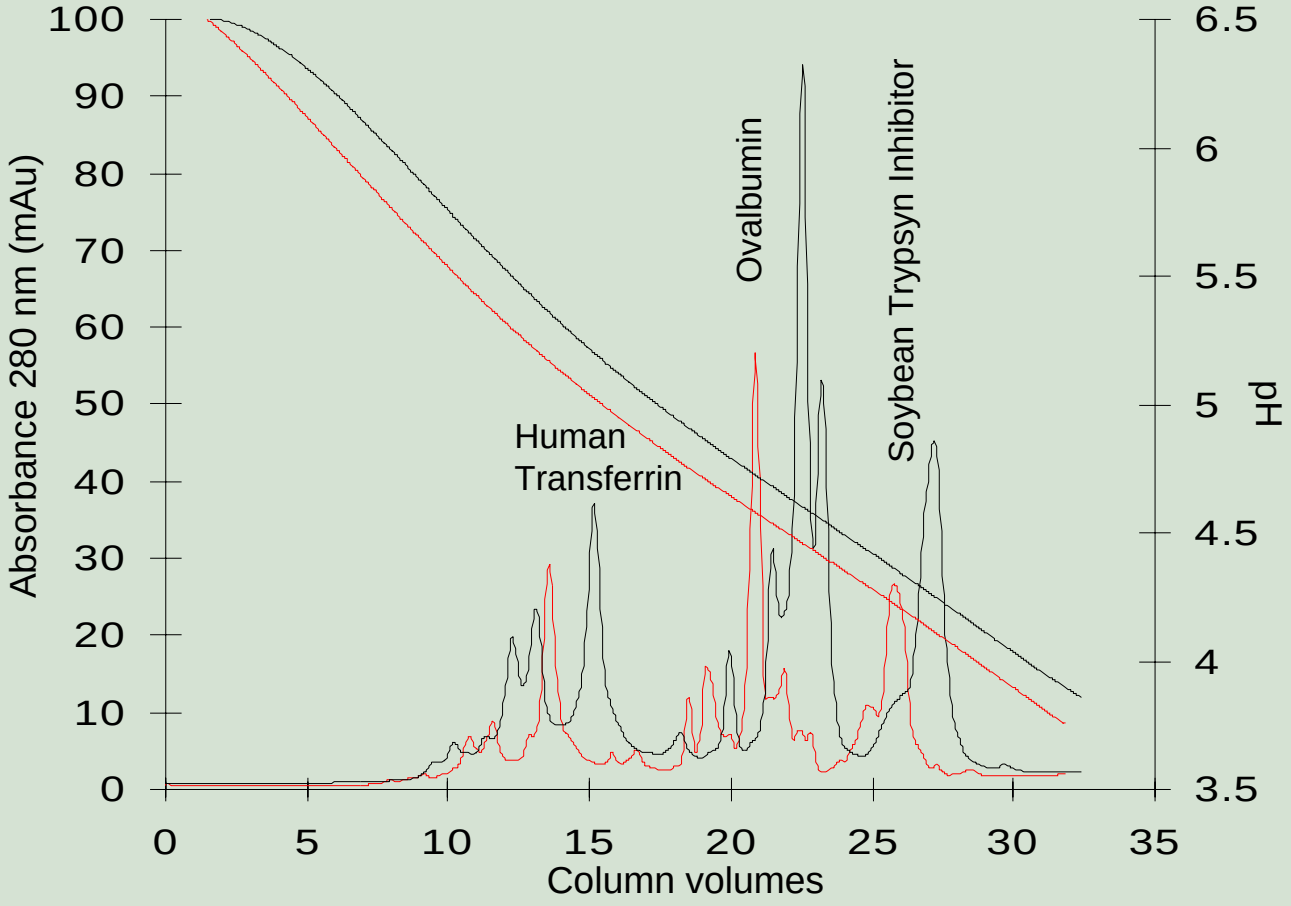
Combined pIsep: anionic pIsep followed by reverse, cationic pIsep. An anion exchange column (e.g. Mono Q) and a cation exchange column (e.g. Mono S) are connected in series. Proteins are initially applied to and bound by the Mono Q at the starting pH. As the external pH gradient is developed from pH 7 to pH 4, the proteins are separated and eluted from the Mono Q and immediately bound to the Mono S. On completion of these steps, the two columns are disconnected and the proteins are eluted from the cation exchanger by reverse **pIsep** with a pH gradient from pH 4 to pH 7.5. **Panel A** illustrates the changes in absorbance and pH during the initial anionic **pIsep** step (black lines) followed by the reverse cationic **pIsep** step (red lines). **Panel B** demonstrates the changes in absorbance and conductivity as a function of pH during the first anionic **pIsep** step (black lines) followed by the second reverse, cationic **pIsep** step (red lines) The combined technique could equally well be executed in reverse order starting with the cationic **pIsep** step and finishing with the anionic **pIsep** step.

In pIsep the pH Gradient Formation is Controllable: The Flatter the Gradient the Better the Separation



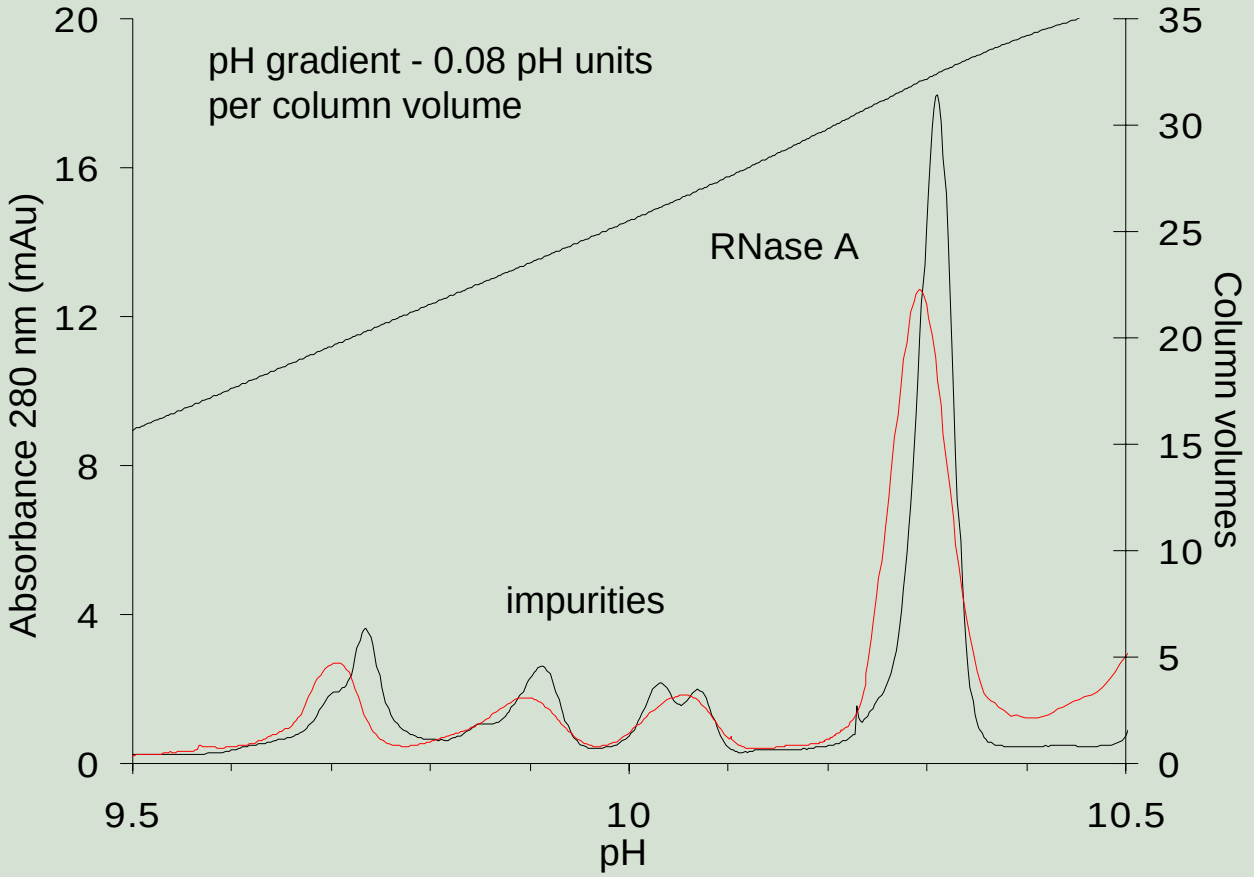
Anionic pISep separations of a mixture containing human transferrin, ovalbumin and soybean trypsyn inhibitor over the pH range 6.7-3.5. The effectiveness of the **pISep** fractionation is influenced by the slope of the pH gradient: red line – slope 0.25 pH units/CV; blue line – slope 0.15 pH units/CV and black line – slope 0.09 pH units/CV. Column: Mono Q HR 10/10, column volume 7.85ml, flow rate 4 ml/min. Proteins were purchased from Sigma and used without preliminary purification.

pIsep is Scalable



Analytical anionic pIsep (black lines) and 8 times scale-up separation (red lines) of a mixture containing human transferrin, ovalbumin and soybean trypsyn inhibitor over the pH range 6.5-3.5. The pH gradient was developed over 30 column volumes (0.09 pH units per CV) on both analytical Mono Q HR 5/5 and the scale-up Mono Q HR 10/10 column at flow rates 1 ml/min and 4ml/min respectively. Proteins were purchased from Sigma and used without preliminary purification.

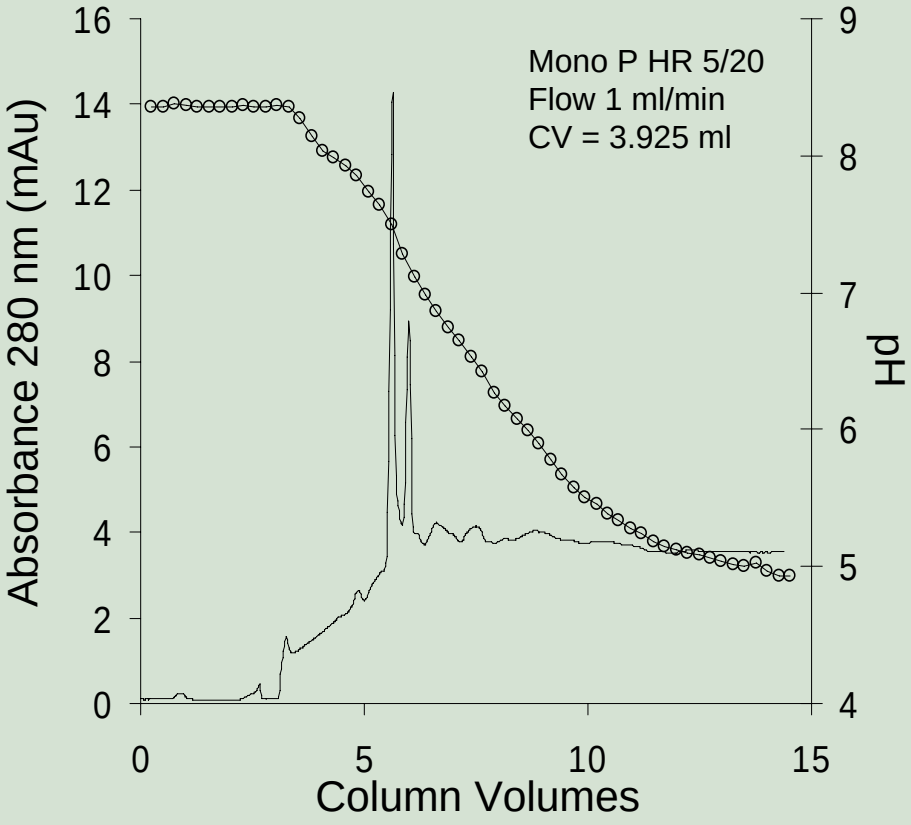
Cheap, Large Preparative pIsep Scale-UP is Feasible: Mono S vs. Source 15S

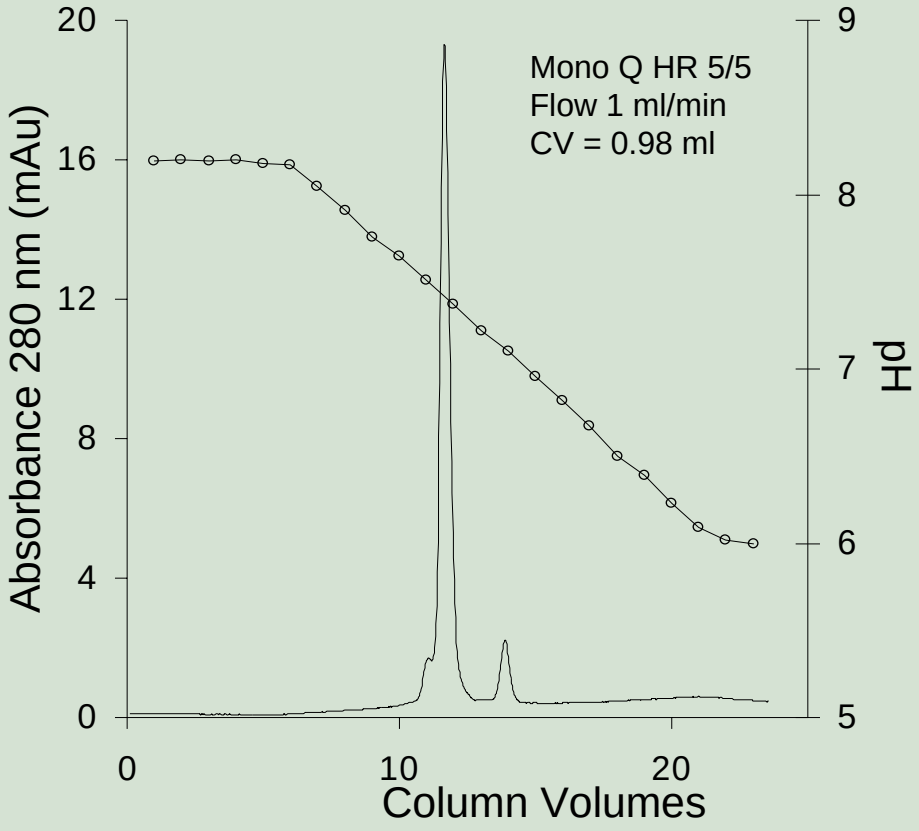


Mono S and Source 15S are strong cation exchange resins trade mark of Amersham Biosciences

Quality of reverse pIsep purification of RNase A conducted on Mono S (black line) and Source 15S (red line). Mono S is a costly, highly efficient cation exchanger with 10 µm particles. Source 15S is a 10 times cheaper, highly scalable, high resolution cation exchanger with 15 µm particles. The comparison of the chromatograms suggests that a cost effective, easy, large preparative cationic **pIsep** scale up is feasible on Source 15S without compromising the quality of separation.

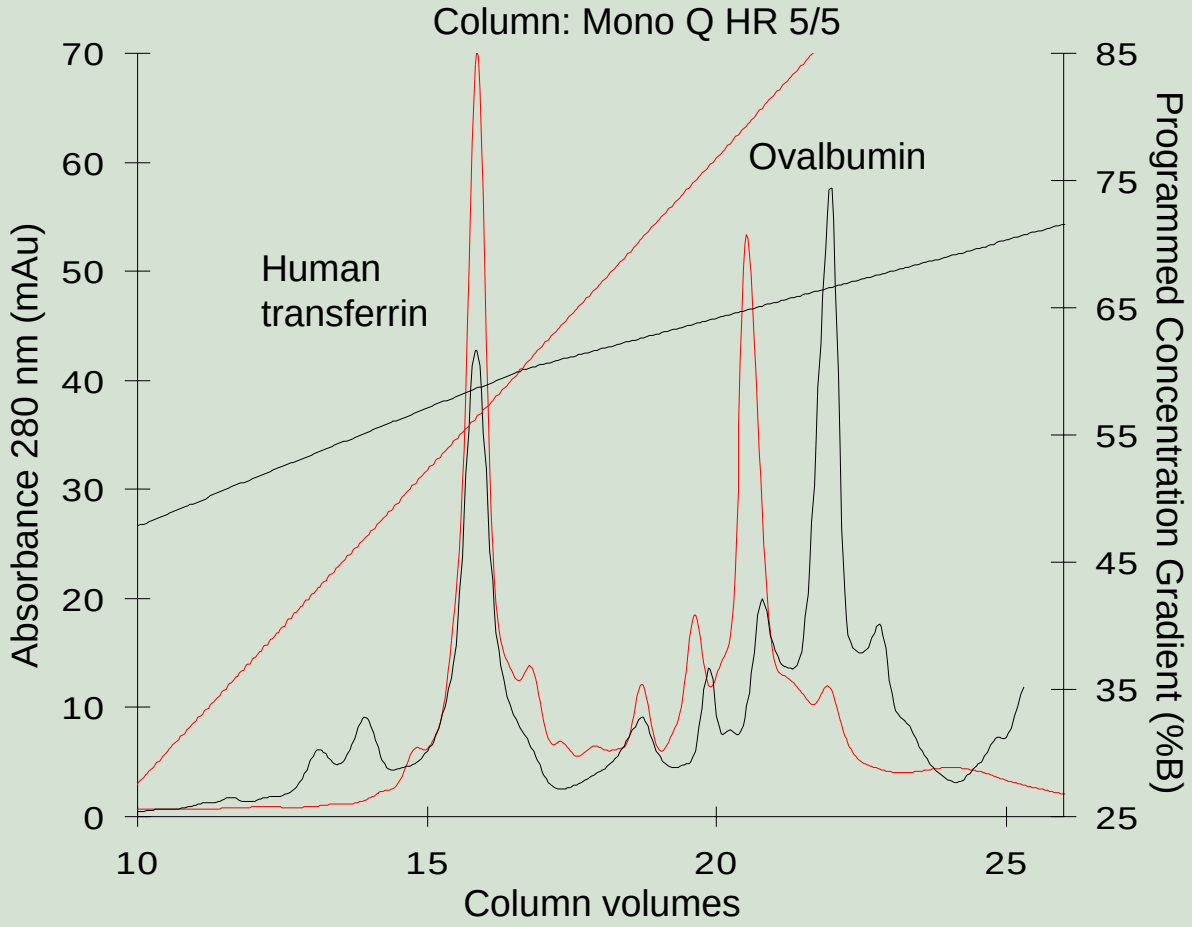
Anionic pIsep Separates Proteins Better than Chromatofocusing with Polybuffers





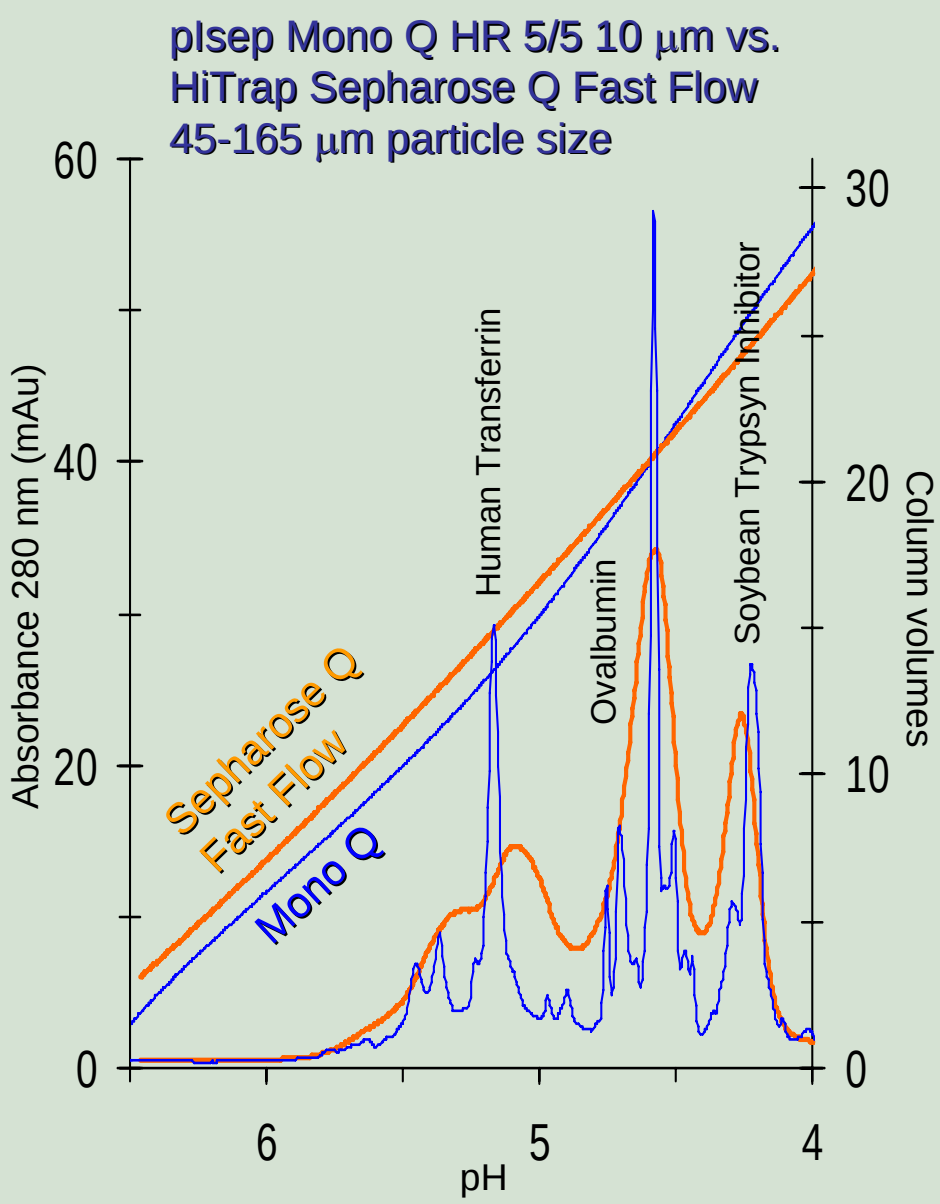
Left Panel - Separation of two variants of phosphoglycerate kinase utilizing a chromatofocusing system commercially marketed by GE Healthcare which uses a weak anion exchange column Mono P HR 5/20 and Polybuffers. Start buffer: 25 mM TE iminodiacetic acid pH 8.3, elution buffer: 6 ml Polybuffer 96 + 14 ml Polybuffer 74, iminodiacetic acid pH 5 diluted to 200 ml. Note the small separation between peaks. **Right Panel** – The same variants of phosphoglycerate kinase fractionated with an external pH gradient generated by CryoBioPhysica **pISep** buffers. Note the very linear effluent pH gradient and the superior separation of the protein species.

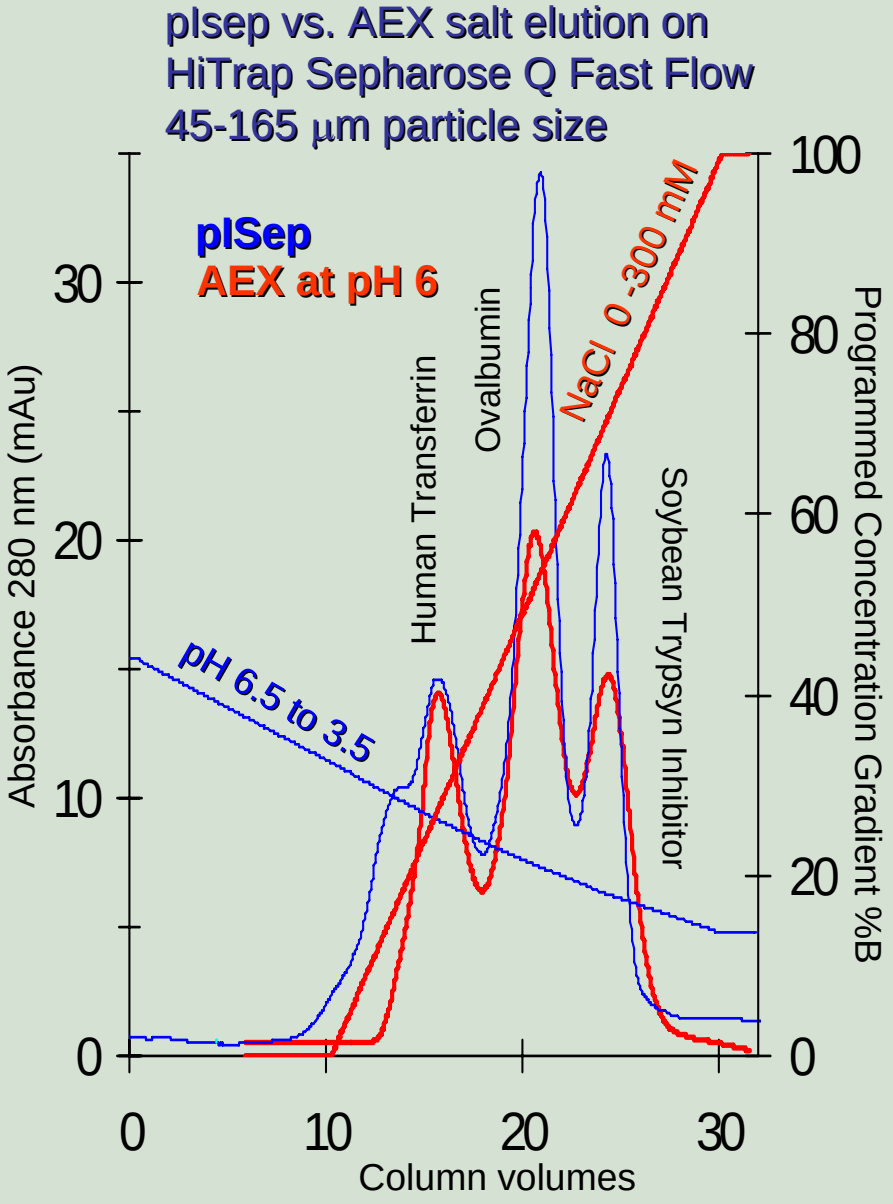
Anionic pIsep Separates Proteins Better than AEX with NaCl Gradient Elution on High Resolution Gels



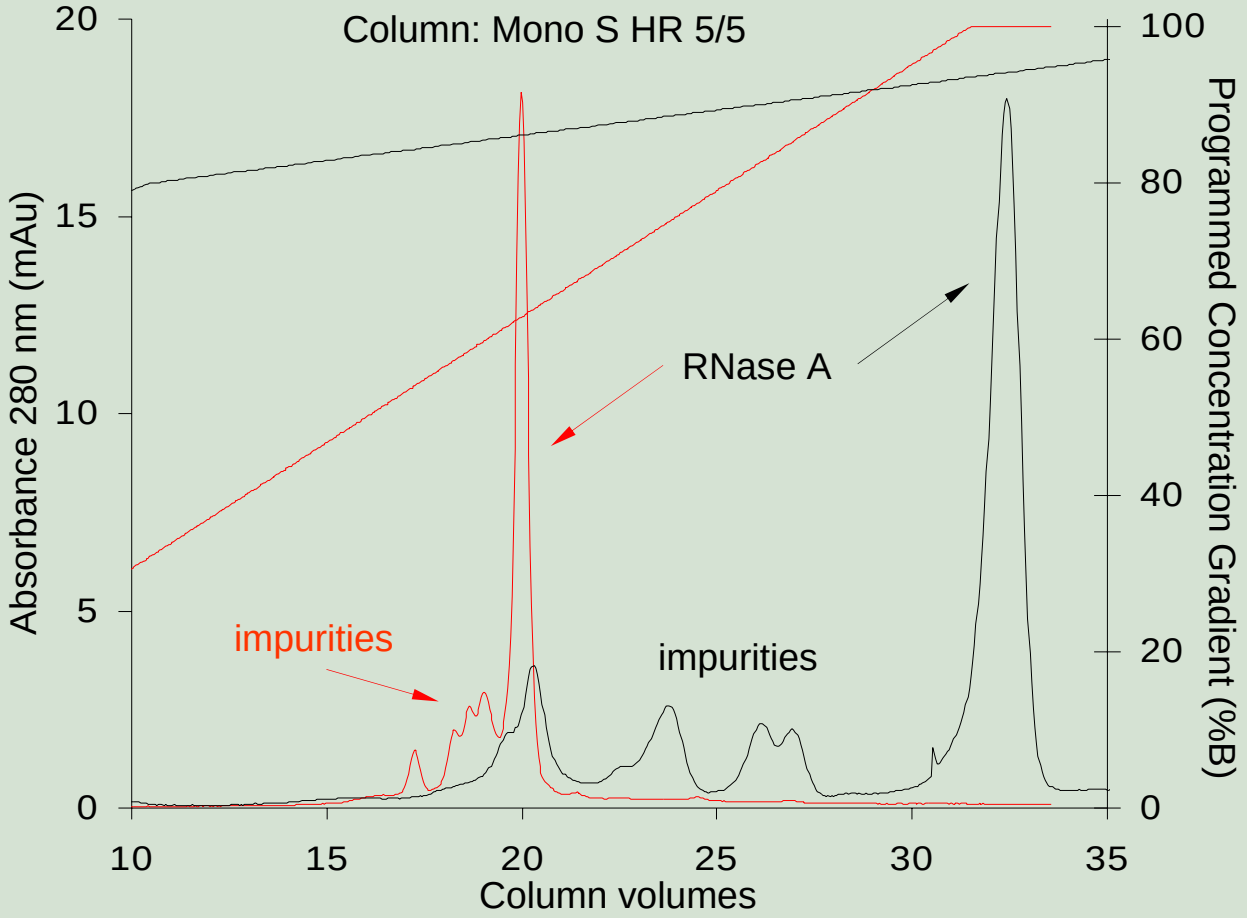
pIsep versus anion exchange chromatography (AEX). Black lines: **pIsep** fractionation of a mixture of human transferrin and ovalbumin: absorbance and profile of the external pH gradient, 0.098 pH units/column volume from pH 6 to pH 4; Red lines: AEX salt elution chromatography of the same proteins - absorbance and profile of the salt gradient, 9.4 mM NaCl/column volume from 0 to 0.3 M NaCl at pH 6.

Anionic pIsep Separates Proteins Better than AEX with NaCl Gradient Elution on Less Effective Gels



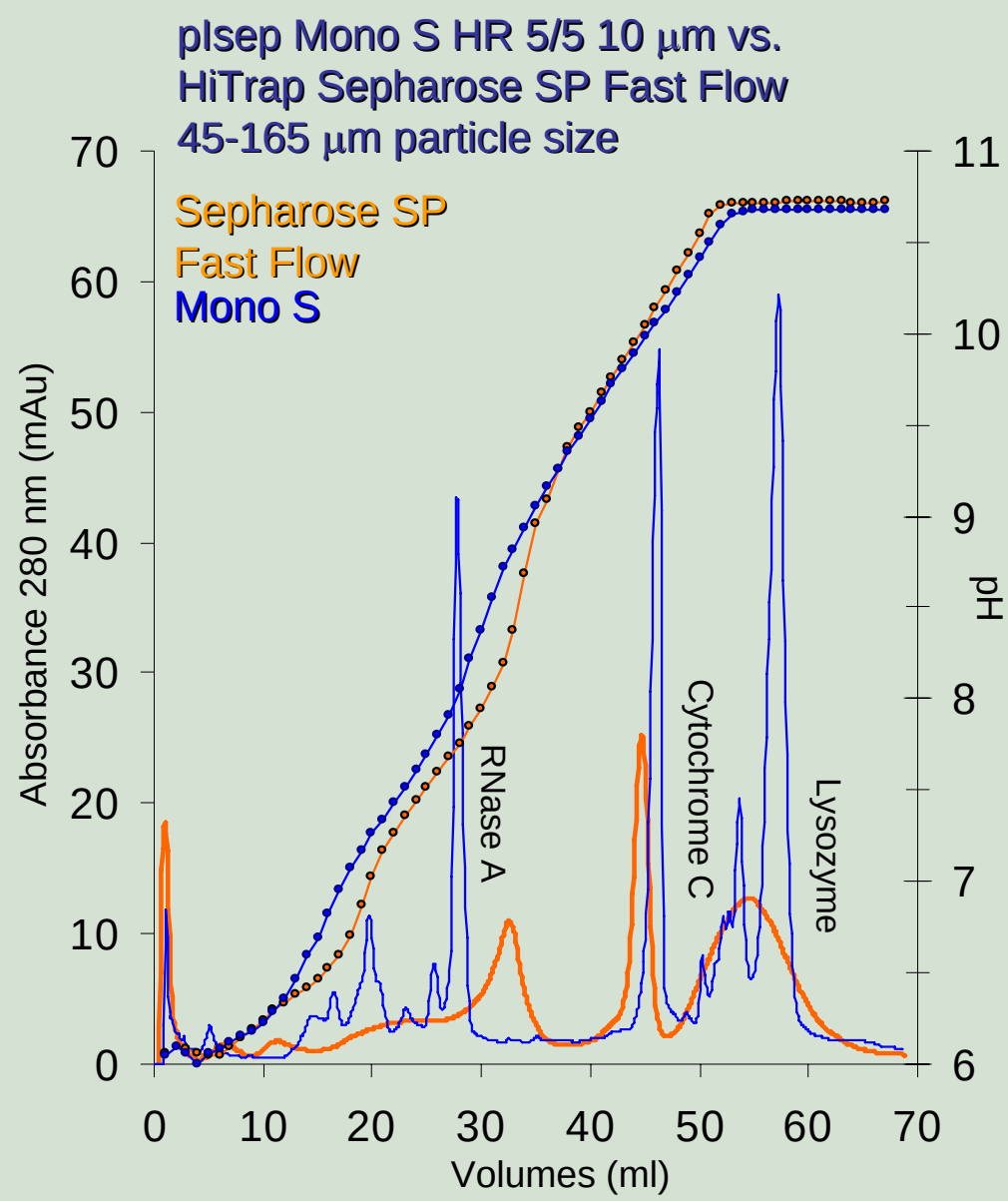


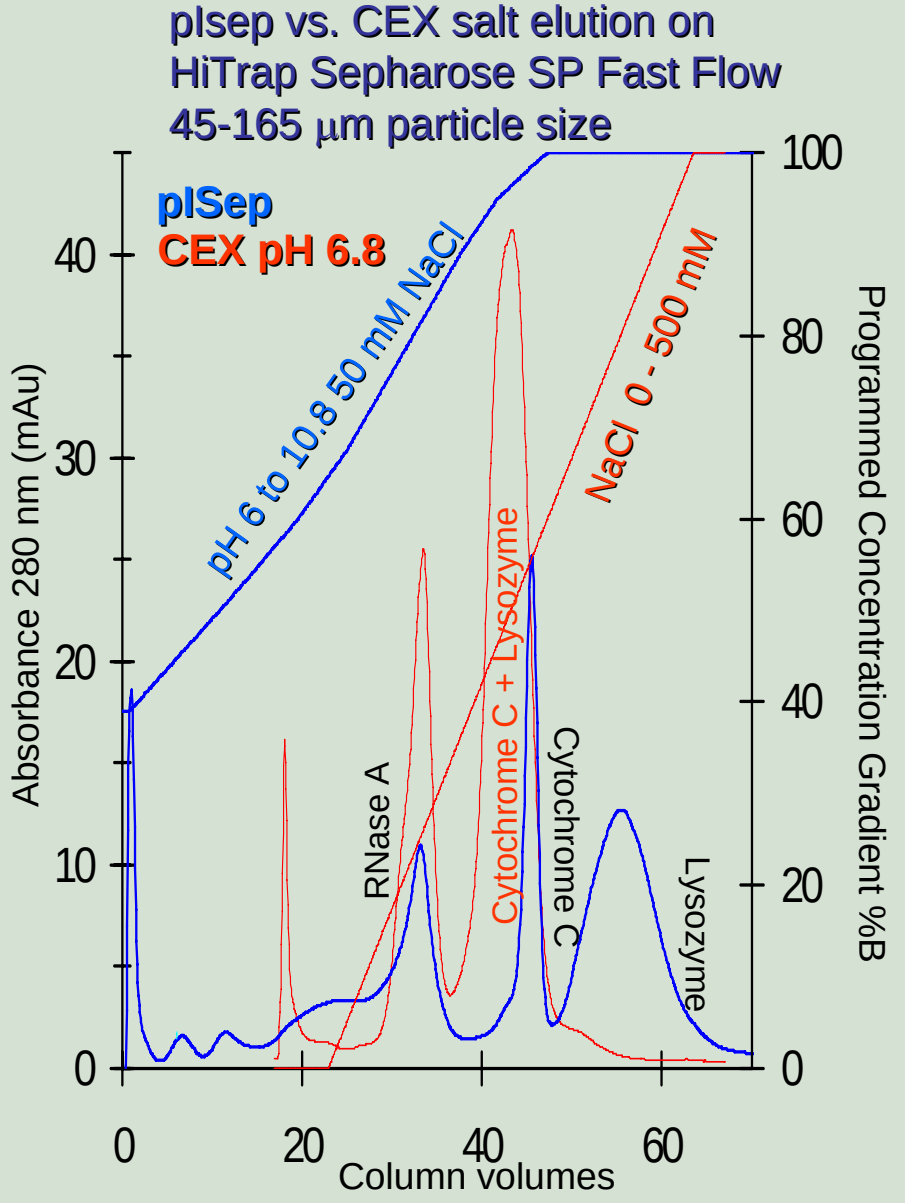
Reverse pIsep Separates Proteins Better than CEX with NaCl Gradient Elutionon on High Resolution Gels



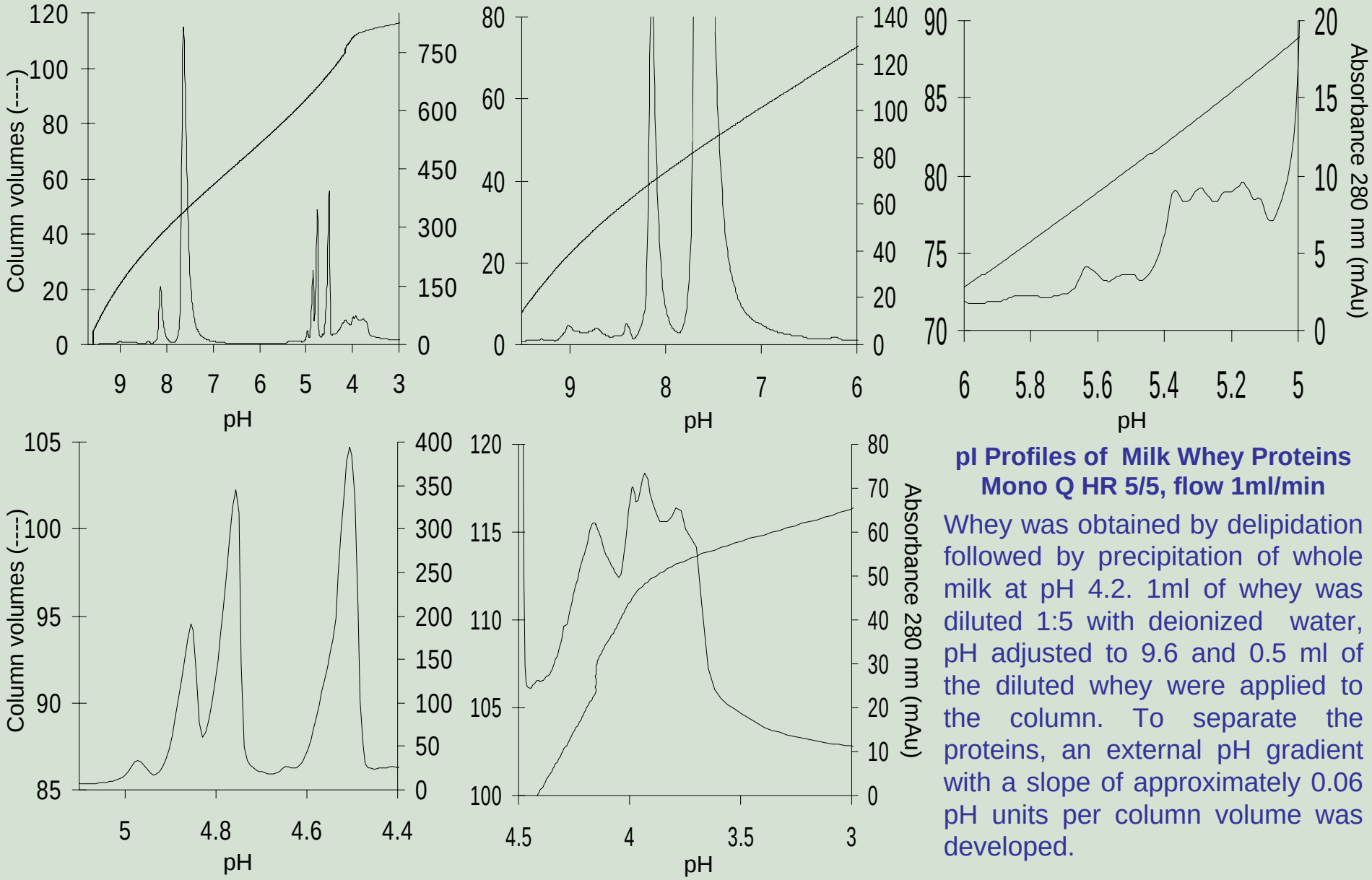
Comparison of reverse, cationic pIsep and cation exchange (CEX) purification of RNase A. Black lines: reverse **pIsep -** programmed pH gradient of 0.08 pH units/column volume, from pH 8.5 to 10.5; Red lines: CEX salt elution - programmed salt gradient of 15.3mM NaCl/column volume, from 0 to 0.5 M NaCl at pH 5. Farther flattening of the salt gradient does not improve the CEX separation. RNase A purchased from Sigma and used without preliminary purification.

Cationic pIsep Separates Proteins Better than CEX with NaCl Gradient Elution on Less Effective Gels





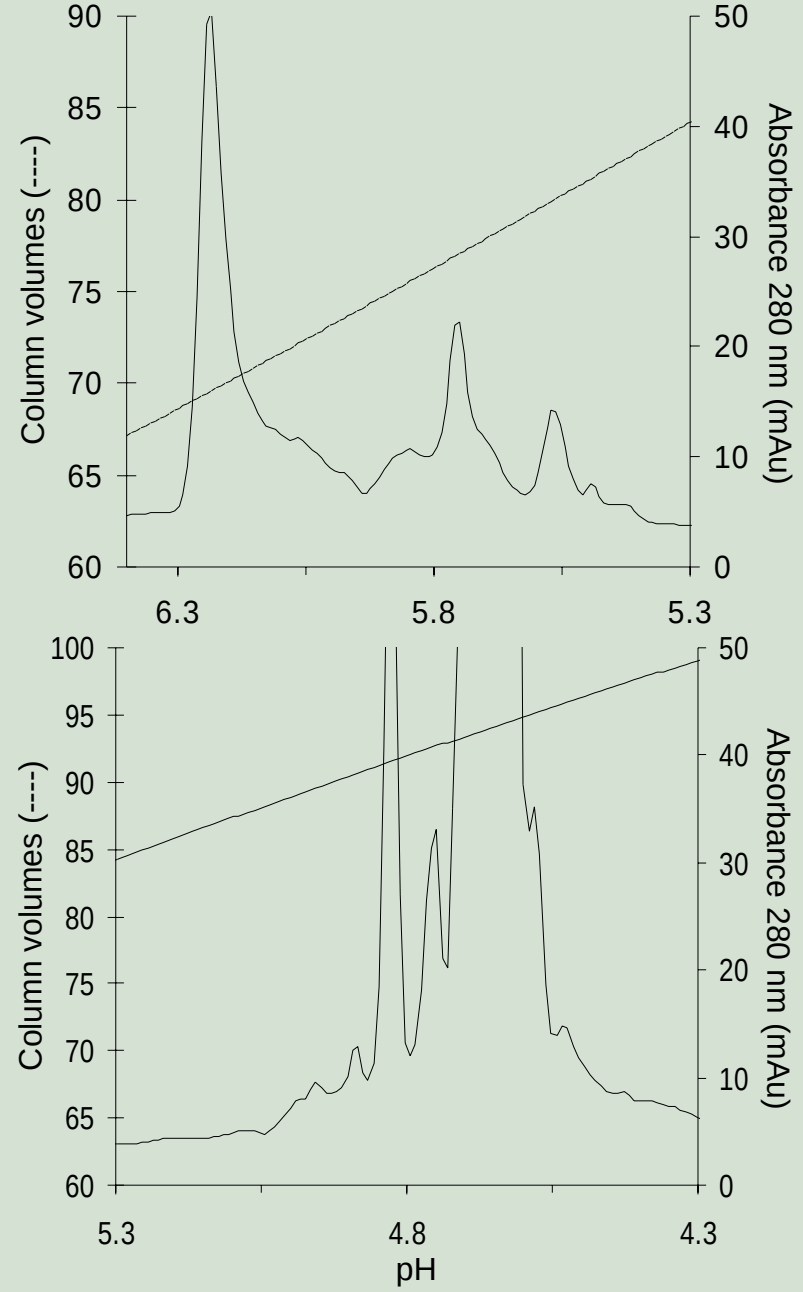
Anionic pIsep Profile of Milk Whey Proteins

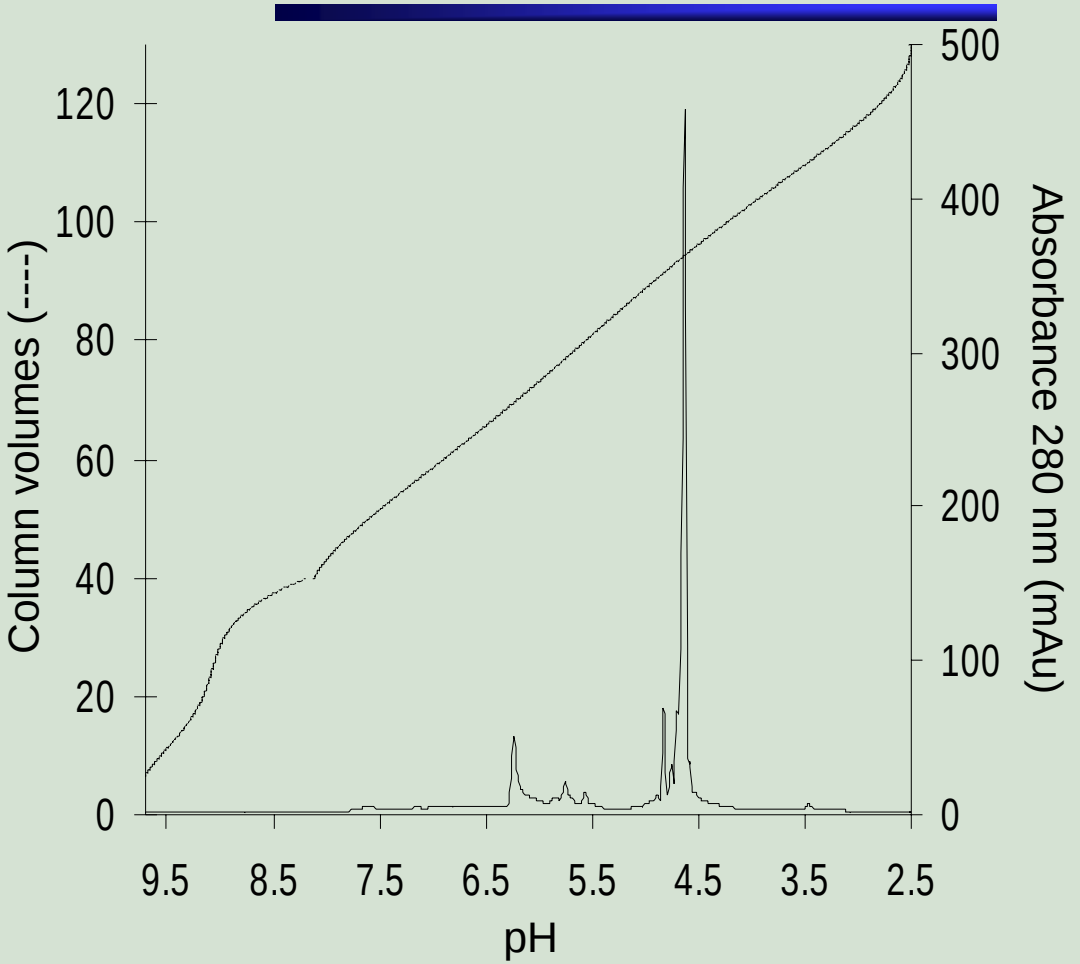


pI Profiles of Milk Whey Proteins
Mono Q HR 5/5, flow 1ml/min

Whey was obtained by delipidation followed by precipitation of whole milk at pH 4.2. 1ml of whey was diluted 1:5 with deionized water, pH adjusted to 9.6 and 0.5 ml of the diluted whey were applied to the column. To separate the proteins, an external pH gradient with a slope of approximately 0.06 pH units per column volume was developed.

Anionic pIsep Profile of Egg White Proteins

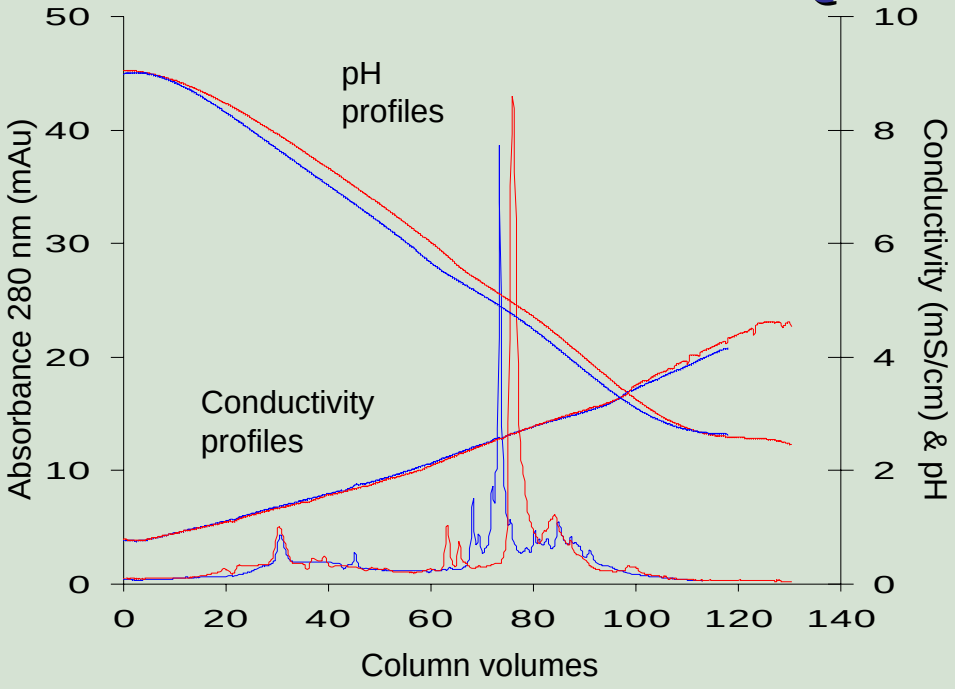


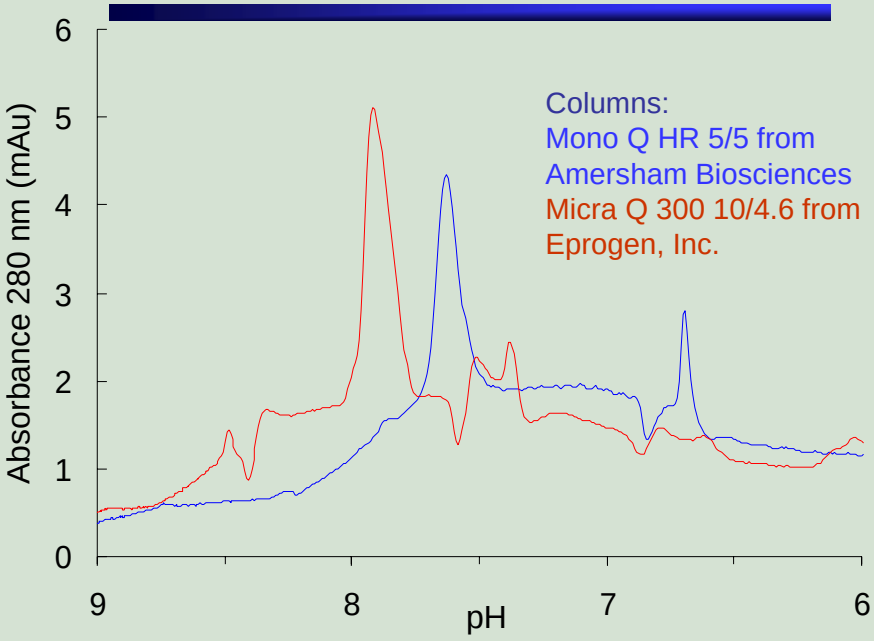


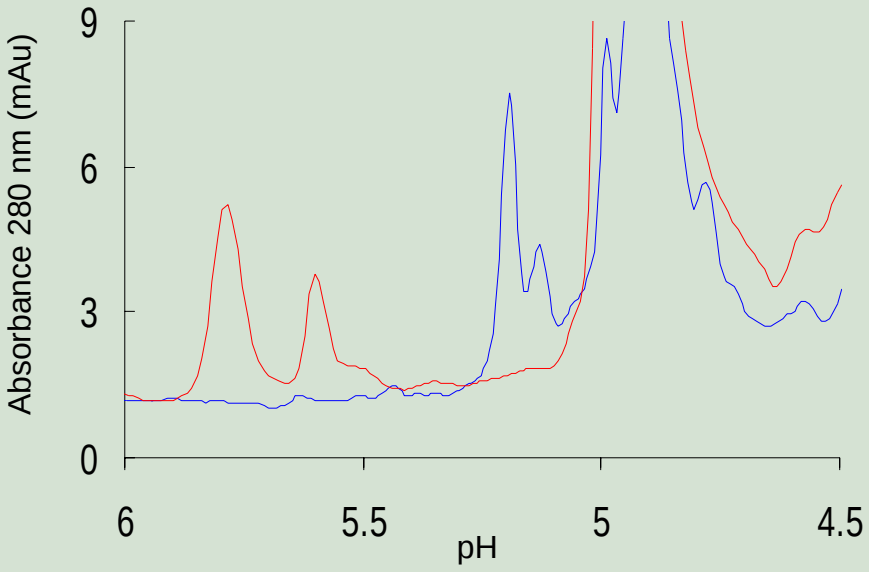
Column Mono Q HR 5/5, flow 1ml/min

pI profiles of egg white proteins: 20 µl of egg white were diluted 1:50 with starting buffer pH 9.7 and applied to the column. To separate the proteins an external pH gradient with a slope of approximately 0.07 pH units per column volume was developed.

Anionic pIsep Profiles of Human Serum Proteins
Micra Q300 vs. Mono Q





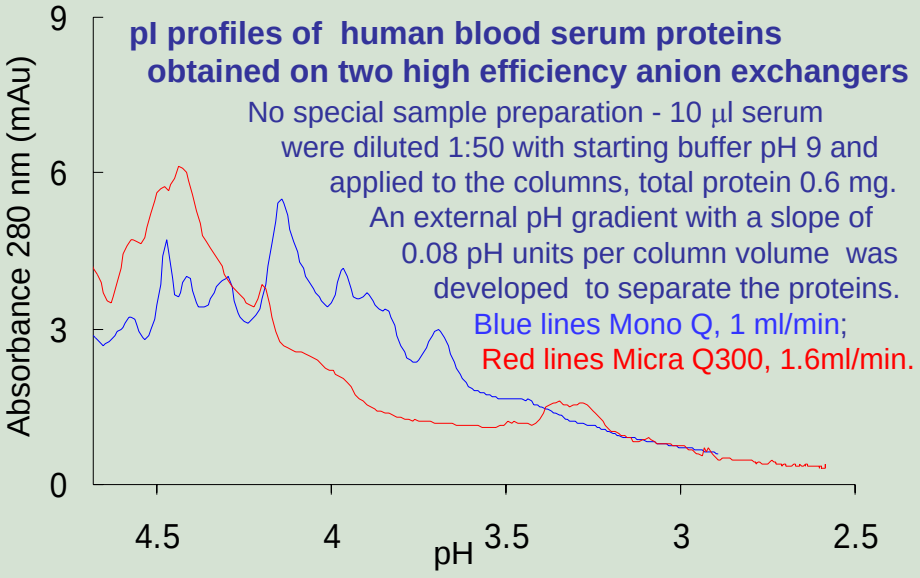


pI profiles of human blood serum proteins obtained on two high efficiency anion exchangers

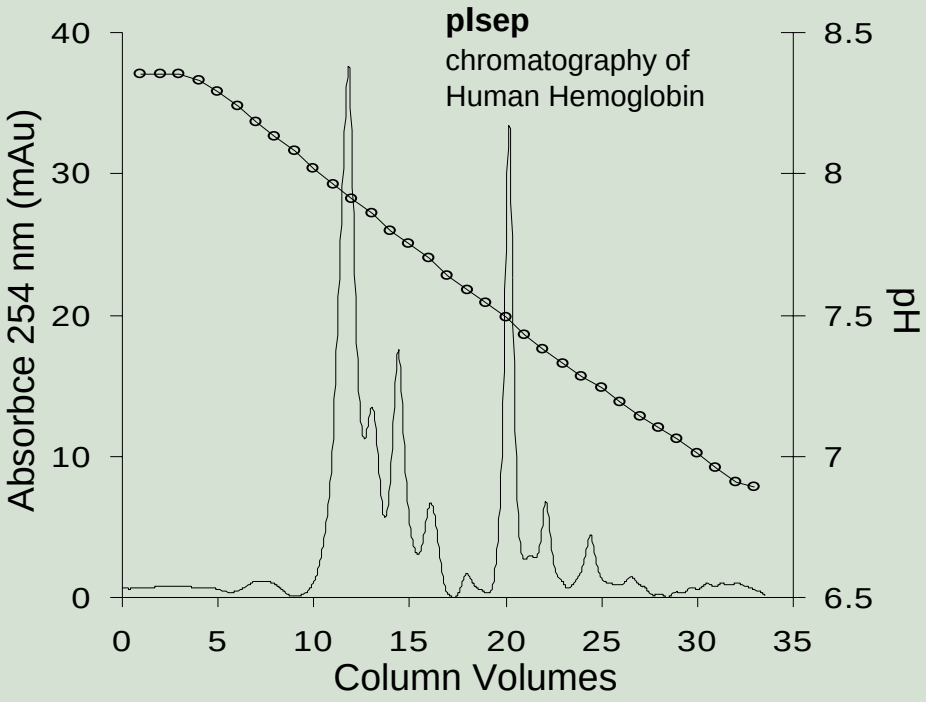
No special sample preparation - 10 μl serum were diluted 1:50 with starting buffer pH 9 and applied to the columns, total protein 0.6 mg. An external pH gradient with a slope of 0.08 pH units per column volume was developed to separate the proteins.

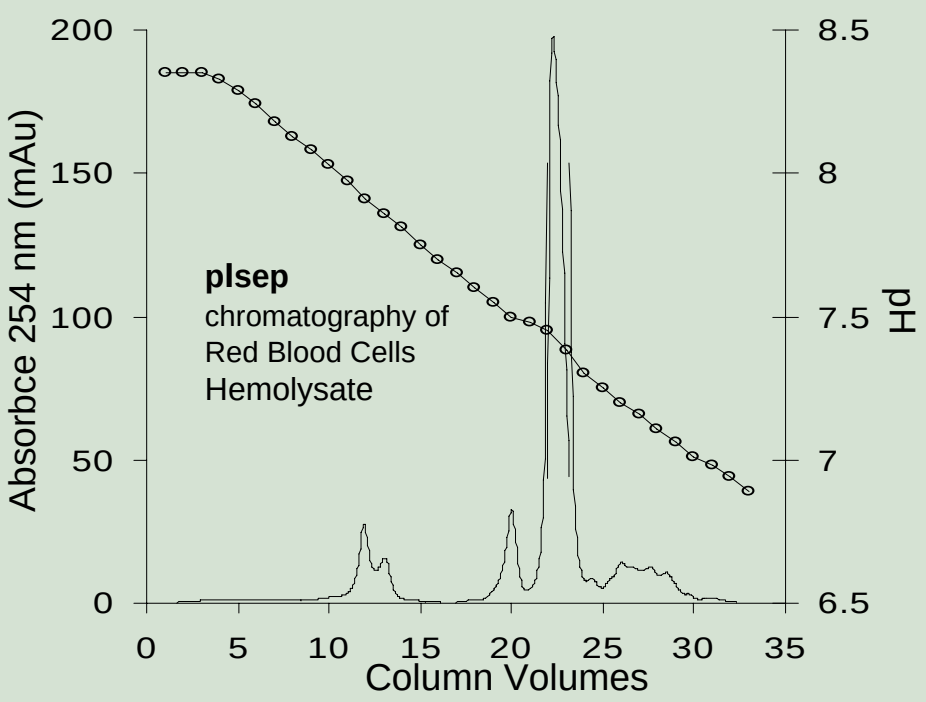
Blue lines Mono Q, 1 ml/min;

Red lines Micra Q300, 1.6ml/min.



Anionic pIsep Profile of Human Hemoglobin





Buffer A: pIsep acidic buffer pH 2.4, **Buffer B: pIsep** basic buffer pH 10.7

Sample: Left Panel - 50 µl Freeze-dried human hemoglobin from Sigma Cat # H7379, (8mg/ml) pH 8.5 Right Panel - 25 µl Erythrocyte Hemolysate (28mg/ml) pH 8.5. Samples were applied, and the column washed and equilibrated with starting buffer 67.4%B pH 8.4

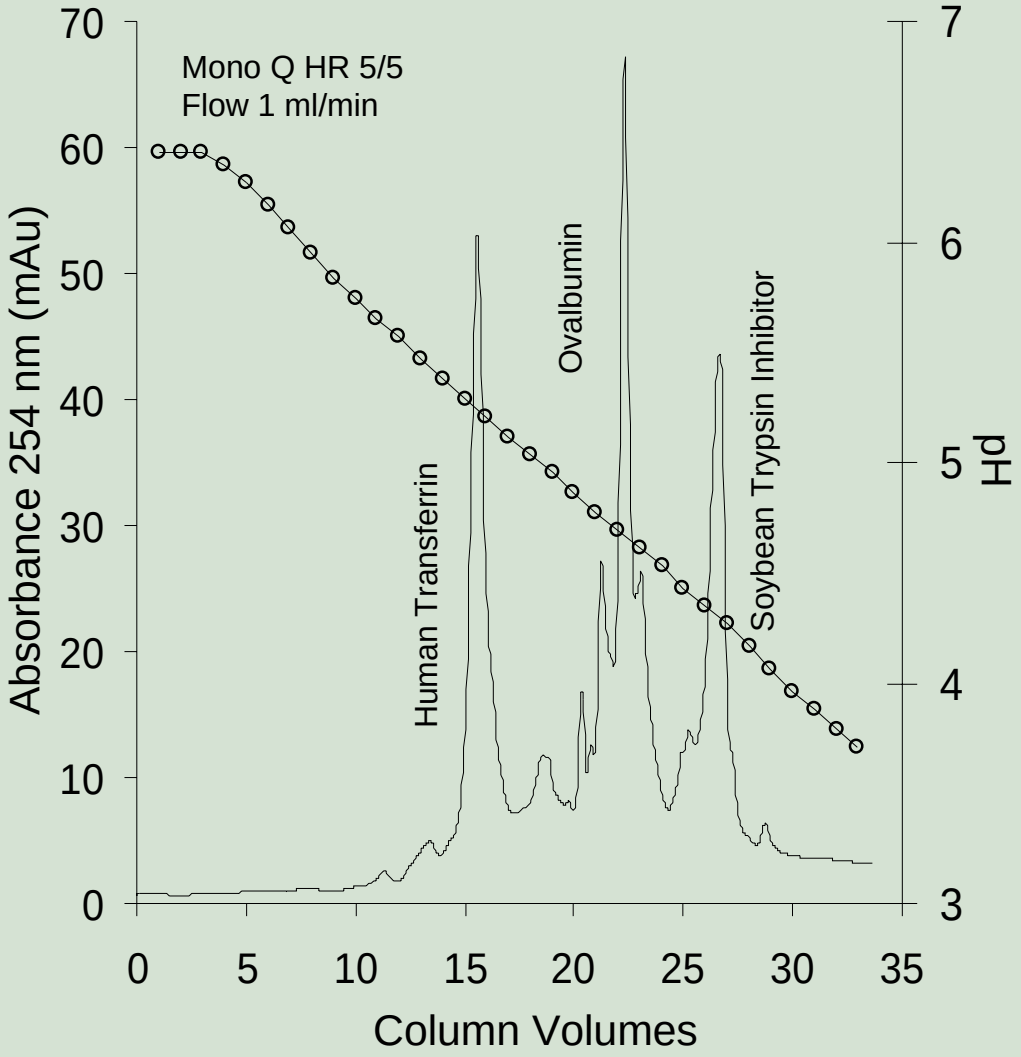
pH gradient: slope 0.05 pH units/CV, from pH 8.4 to pH 6.9 in 30 CVs. Protocol: **1.** 67.4%B to 60.5%B in 10 CVs; **2.** 60.5%B to 56%B in 7.6 CVs; **3.** 56%B to 49.5%B in 12.4 CVs; **4.** at 49.5%B 3 CVs.

Column: Mono Q HR 5/5 - column volume 0.98 ml (Amarsham Pharmacia)

Flow rate: 1 ml/min

Detection: 254 nm

pIsep Protocol for Testing the Separation Efficacy of an Anion Exchange Column

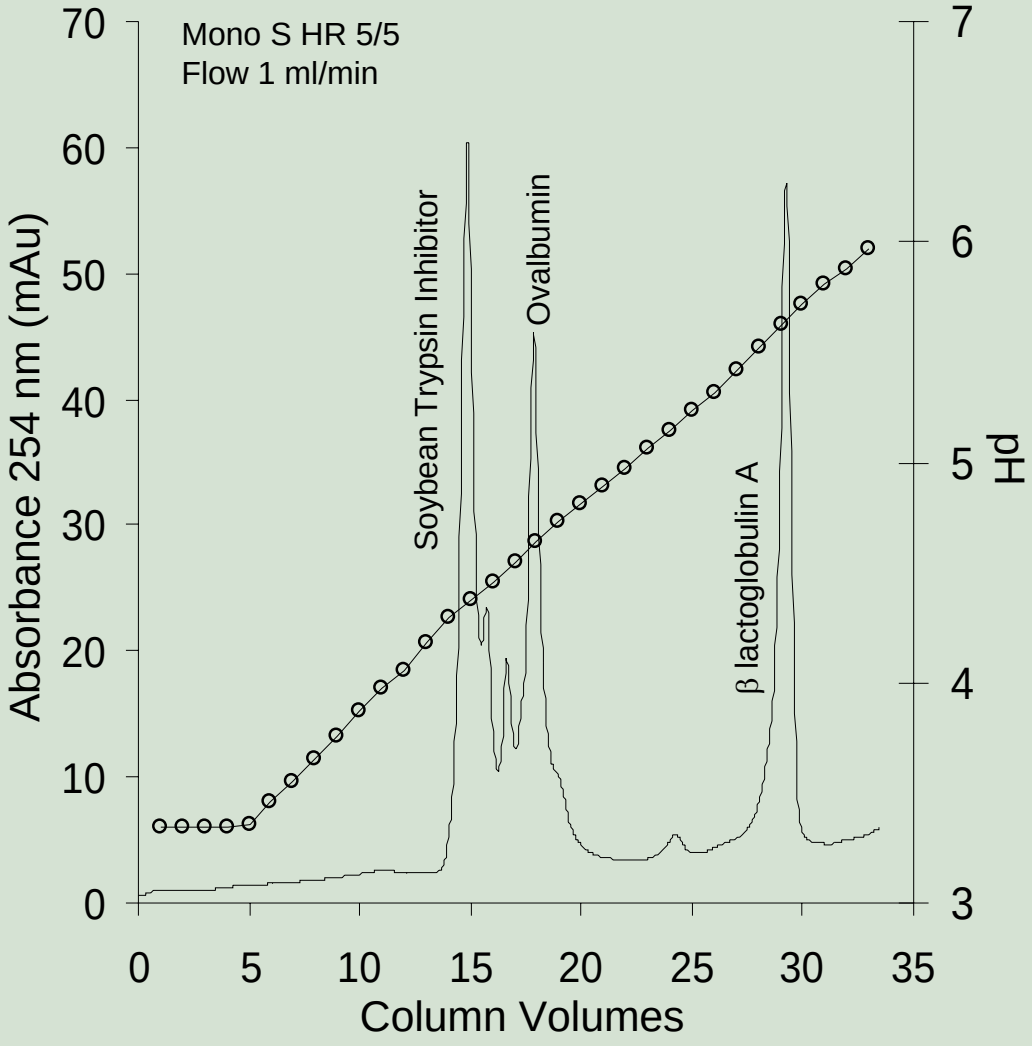


External descending pH gradient pIsep

Buffer A: pIsep acidic buffer, pH 2.4
Buffer B: pIsep basic buffer, pH 10.7
Sample: Human Transferrin 2mg/ml, Ovalbumin 4mg/ml, Soybean Trypsin inhibitor 2mg/ml, 100 µl solution pH 6.5
Apply the sample, wash and equilibrate the column with start buffer containing 44.5% B.
pH gradient: slope, 0.1 pH units/CV, pH 6.5 - 3.5 in 30 CVs.
1. from 44.5% B to 20.4% B in 22 CVs
2. from 20.4%B to 13.8%B in 8 CVs
3. at 13.8%B 2 CVs.
Flow rate: as recommended for the column
Detection: 280 nm to 254 nm

CryoBioPhysica resolution pIsep test conducted on Mono Q HR 5/5 high efficiency anion exchange column from GE Healthcare (formerly Amersham Biosciences)

pIsep Protocol for Testing the Separation Efficacy of a Cation Exchange Column



External ascending pH gradient pISep

Buffer A: pIsep acidic buffer pH 2.4

Buffer B: pIsep basic buffer pH 10.7

Sample: Soybean Trypsin inhibitor 2mg/ml, Ovalbumin 4mg/ml, β lactoglobulin 2mg/ml, 100 µl solution pH 3.4

Apply the sample, wash and equilibrate the column with start buffer containing 13.8%B

pH gradient: slop 0.1 pH units/CV, pH 3.5 - 6.5 in 30 CVs.

1. from 13.8% to 22.4%B in 10 CVs

2. from 22.4%B to 44.5%B in 20 CVs

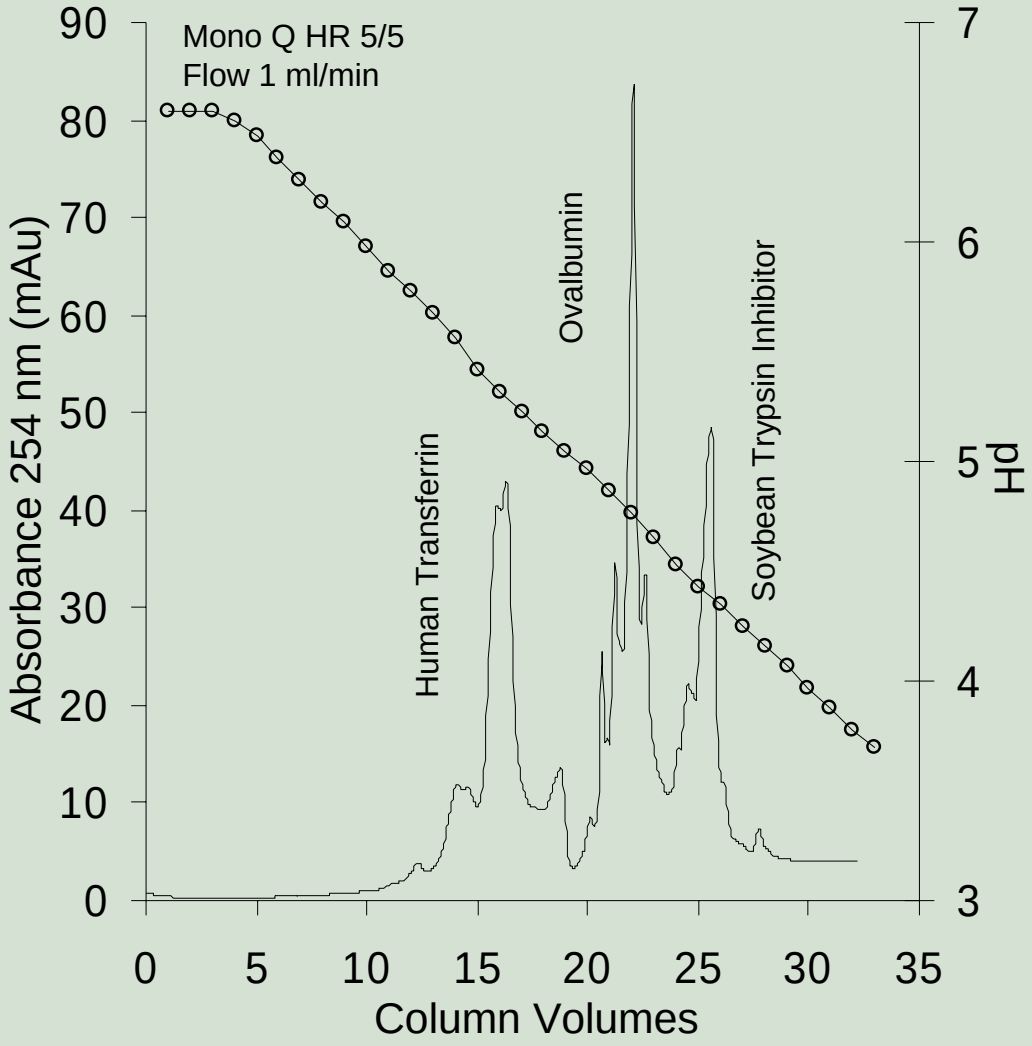
3. at 44.5%B 2 CVs.

Flow rate: as recommended for the column

Detection: 280 nm to 254 nm

CryoBioPhysica resolution pIsep test conducted on Mono S HR 5/5 high efficiency cation exchange column from GE Healthcare (formerly Amersham Biosciences)

pIsep Can Separate Hydrophobic Proteins and Proteins Having Low Solubility



External descending pH gradient pISep, resolution test, 2 M urea

Buffer A: pIsep acidic buffer+2 M urea pH 2.3

Buffer B: pIsep basic buffer+2 M urea pH 10.8

Sample: mixture of Human Transferrin 2mg/ml, Ovalbumin 4mg/ml, Soybean Trypsin inhibitor 2mg/ml 100 µl solution pH 6.5. Apply the sample, wash and equilibrate the column with starting buffer 66.3%B

pH gradient: slop 0.1 pH units/CV, pH 6.5 - 3.5 in 30 CVs.

1. from 65.3%B to 60%B in 8 CVs

2. from 60%B to 50%B in 13 CVs

3. from 50%B to 44.5%B in 9 CVs

4. at 44.5%B 2 CVs.

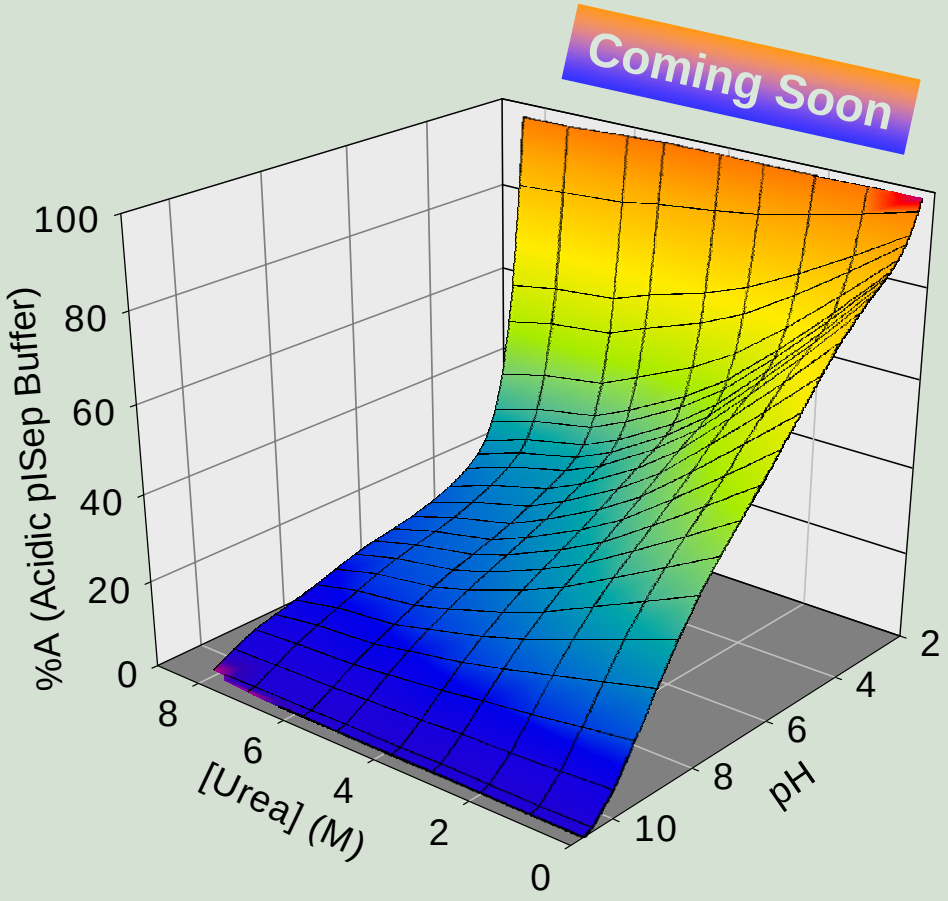
Flow rate: as recommended for the column

Detection: 280 nm to 254 nm

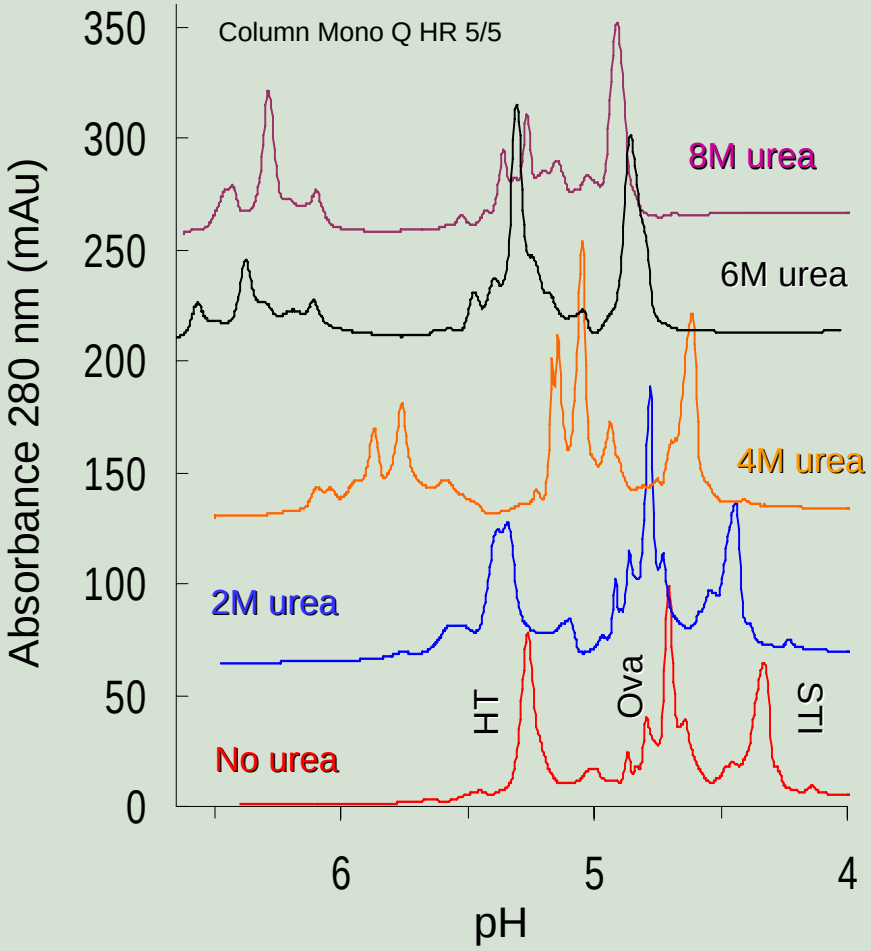
The chromatographer can resolve protein solubility issues by utilizing nonionic or zwitterionic detergents. Urea concentrations up to 8 M do not compromise the pISep separation resolution.

The pIsep pH Gradient Maker^{Plus} Software, Limitless Ability to Form Linear and Nonlinear pH Gradients with pIsep Buffers at any User Defined Urea Concentration in the Range 0 to 8 M

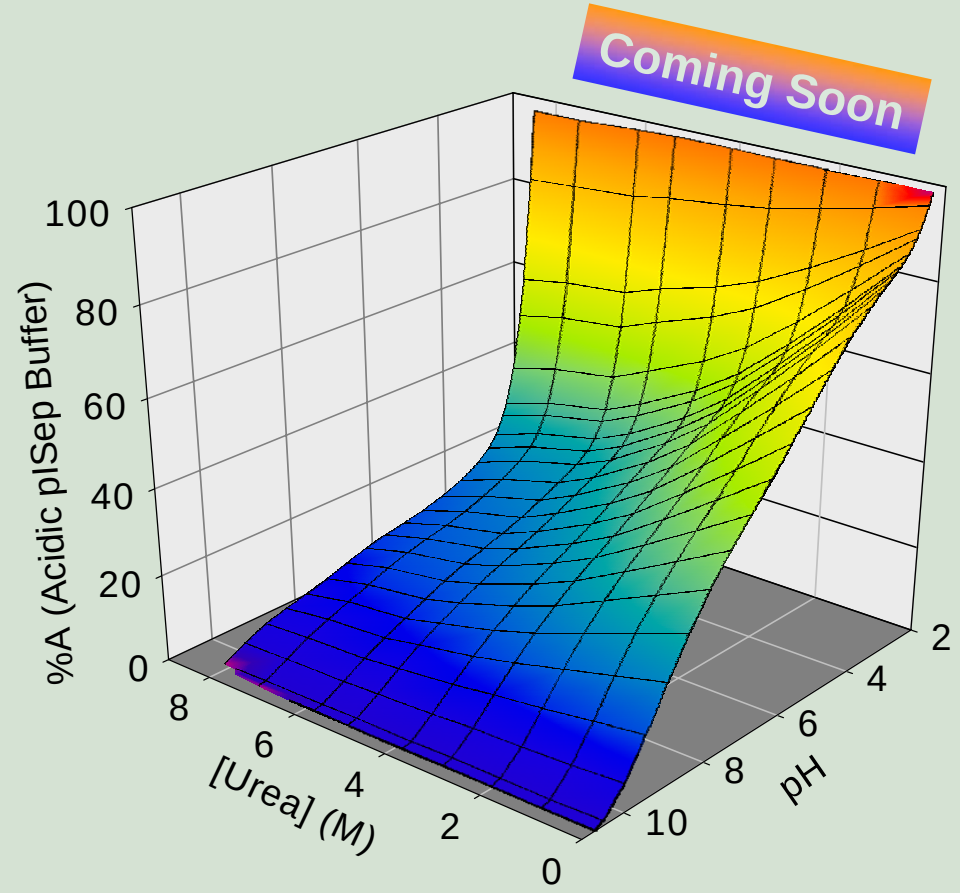
pISep is a new tool for analyzing and separating denatured states of proteins generated by urea



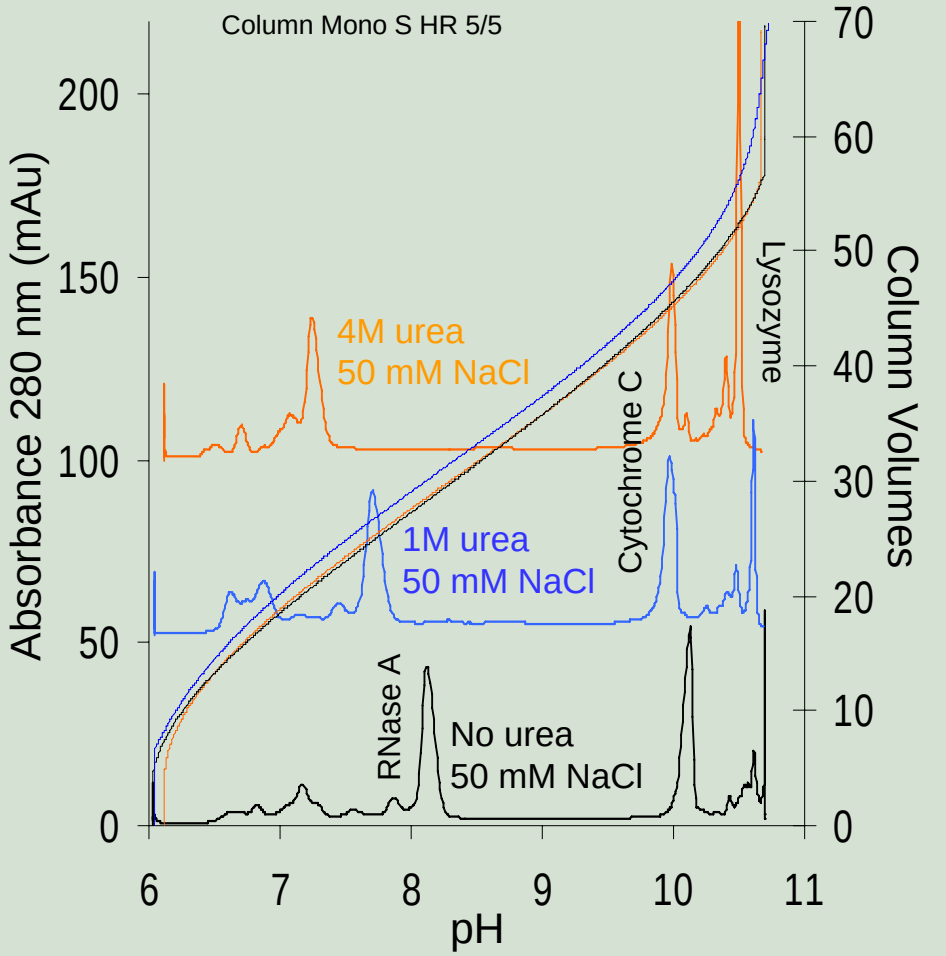
Separations of Human Transferrin, Ovalbumin and Soybean Trypsin Inhibitor by anionic pIsep in presence of different urea concentrations



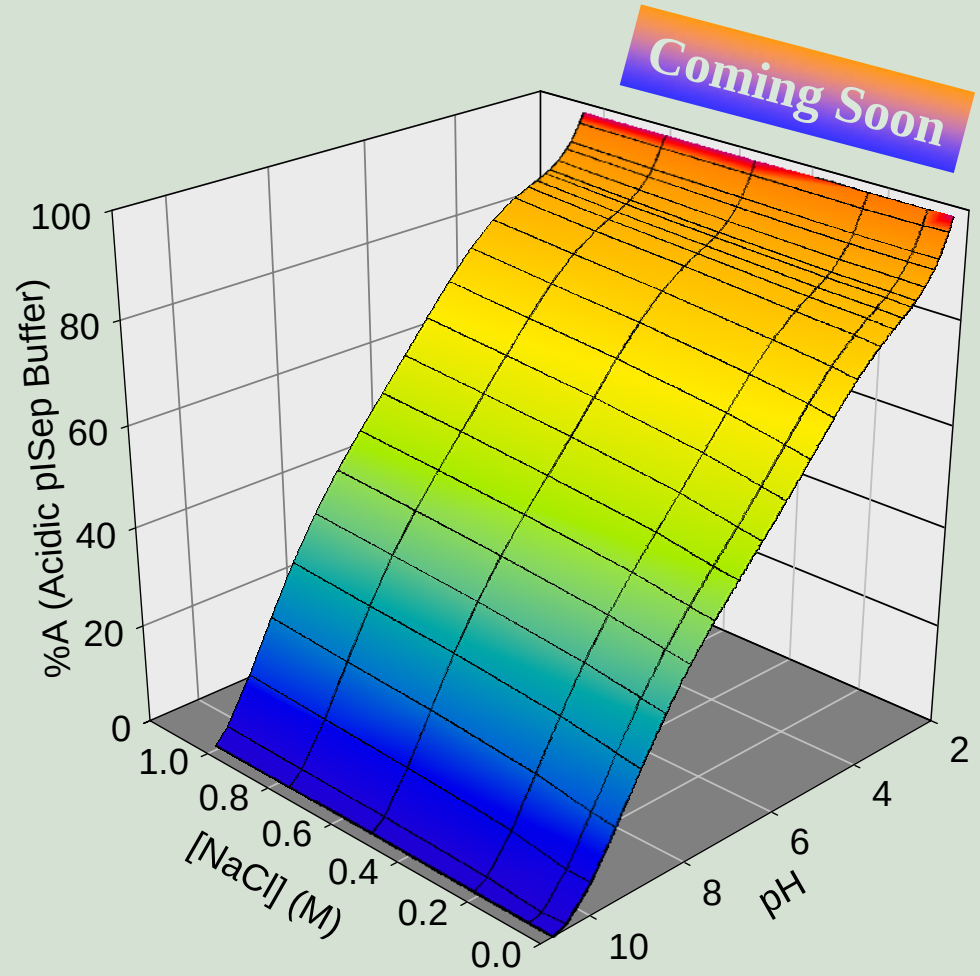
The pIsep pH Gradient Maker^{Plus} Software, Limitless Ability to Form Linear and Nonlinear pH Gradients with pIsep Buffers at any User Defined Urea Concentration in the Range 0 to 8 M



Separations of of RNase A, Cytochrome C and Lysozyme by cationic pIsep in presence of different urea concentrations



The pIsep pH Gradient Maker^{Plus} Software, Limitless Ability to Form Linear and Nonlinear pH Gradients with pIsep Buffers at any User Defined NaCl Concentration in the Range 0 to 1 M



Separations of RNase A, Cytochrome C and Lysozyme by cationic pIsep in presence of different NaCl concentrations