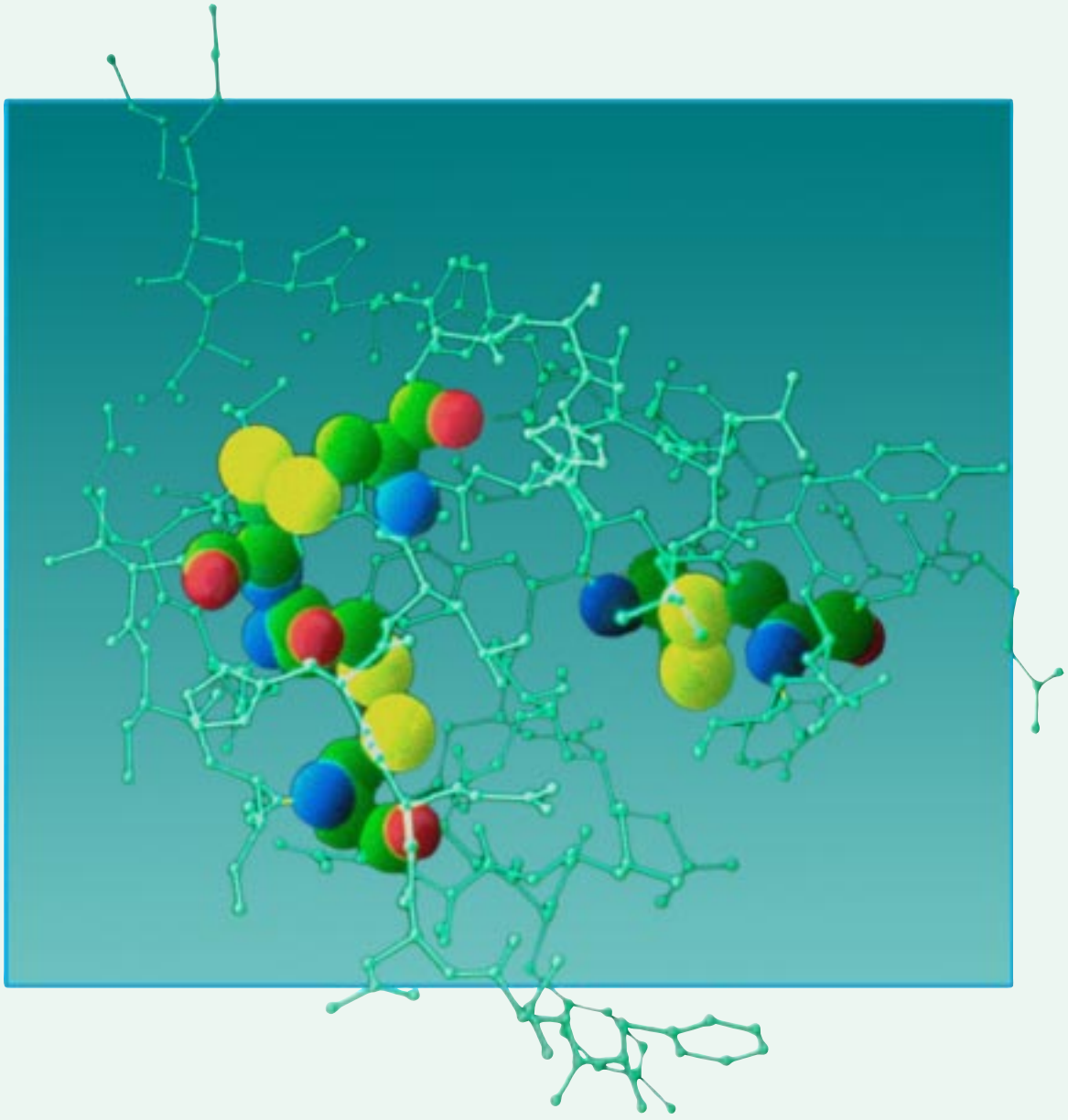
Principles and Applications of High-Performance Ion-Exchange Chromatography for Bioseparations



Introducing Vydac ***VHP***

High Performance Ion-Exchange Columns

VYDAC®

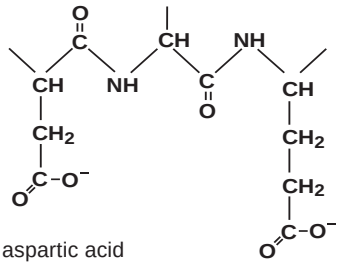
Principles of High-Performance Ion-Exchange Chromatography

Ion-Exchange Chromatography of Polypeptides

Ion-exchange chromatography separates proteins by charge primarily through electrostatic interactions between charged amino acid side chains and the surface charge of the ion-exchange resin.

Protein retention has been explained as a "net charge" phenomenon in which a protein is considered to be a point charge and retention is a function of the net charge of the protein at the pH of the mobile phase. Kopaciewicz and colleagues, however, have developed a more comprehensive mechanism of the ion-exchange chromatography of polypeptides by showing that significant retention of proteins often occurs at the pI, where the net charge is zero, and that the correlation between net charge and protein retention is often poor (Reference 1). They showed that charge assymetry better explains protein retention in ion-exchange chromatography and that this accounts for the fact that protein tertiary structure affects retention in ion-exchange chromatography. For instance, structural isomers with identical pI's sometimes can be separated by ion-exchange chromatography.

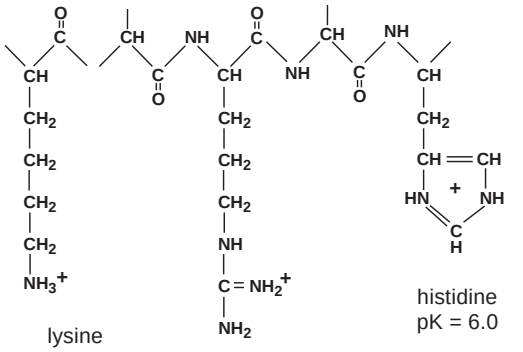
Figure 1. Protein anion exchange



aspartic acid
pK = 3.9

glutamic acid
pK = 4.3

Figure 2. Protein cation exchange



lysine
pK = 10.5

arginine
pK = 12.5

histidine
pK = 6.0

The pK's are given as the pK of the free amino acid. The actual pK of an amino acid sidechain inside a protein depends on the microenvironment of the protein where it is found.

Why use ion-exchange chromatography to purify proteins?

Ion-exchange chromatography separates proteins by charge under near physiological and non-denaturing conditions and ion-exchange resins generally have a high loading capacity. Polymeric based ion-exchange resins are very robust. They are stable in strong acid or base and are resistant to urea and guanidine-HCl. Ion-exchange chromatography is an excellent complement to such high resolution techniques as reversed-phase chromatography (see Reference 3 and Page 14 of this publication)

What is the effect of mobile phase pH?

Anion-exchange chromatography primarily retains biomolecules by the interaction of amine groups on the ion-exchange resin with aspartic or glutamic acid sidechains, which have pKs of ~ 4.4 (Figure 1). The mobile phase is buffered (see table of recommended buffers) at pH > 4.4, below which acid sidechains begin to protonate and retention decreases. Above pH 4.4 retention is largely dependent on the number of anionic sidechains present in the protein. Proteins containing the same number of anionic sidechains can often be separated by adjustment of the mobile phase pH between 7 and 10 where histidine is not protonated and lysine begins to deprotonate. Subtle changes occur to proteins in this pH region which affect the

Types of Ion-Exchange Resins

Type	Functional group	Common Term	Vydac Column
Cation exchange	sulfonic acid	S	400VHP Series
Anion exchange	quaternary amine	Q	300VHP Series
Anion exchange	tertiary amine	DEAE	301VHP Series

Contents

interaction of the protein with the resin and which allow fine-tuning of the anion-exchange separation. A mobile phase pH > 10 is not generally recommended because of possible protein degradation, such as deamidation, at higher pH's. Examples of adjusting pH and gradient conditions to optimize separations are shown in this publication on pages 7, 8, 9 and 11.

Cation-exchange chromatography retains biomolecules by the interaction of sulfonic acid groups on the surface of the ion-exchange resin with histidine (pK ~ 6.5), lysine (pk ~ 10) and arginine (pK ~ 12) (Figure 2). The mobile phase is buffered (see table of recommended buffers) to maintain the mobile phase below pH 6 or 7 in order to keep the basic sidechains protonated (Table 1). At higher pH the basic sidechains begin to deprotonate and retention decreases. Below pH 6 retention is dependent on the number of basic amino acids present in the protein.

Proteins with the same number of basic amino acids can often be separated by adjusting the mobile phase pH between 3 and 5 where aspartic acid and glutamic acid are partially protonated. Subtle changes occur to proteins in this pH region which affect the interaction of the protein with the resin and allow fine-tuning of the ion-exchange separation. Proteins differing in a single sidechain - for instance, aspartic acid in one versus isoaspartate in the other - can sometimes be separated by careful adjustment of the mobile phase around pH 4. Examples of adjusting pH and salt gradient conditions to optimize separations are shown in this publication on pages 7, 8, 9 and 11.

What is the effect of displacing ions?

Polypeptide separations are somewhat dependent on the displacer ion. Although sodium and choride are the most common displacer ions, differences in retention and selectivity for some polypeptides have been noted for various organic and inorganic anions in anion-exchange chromatography and various alkali and alkaline earth metals for cation-exchange chromatography (see Ref. 1 and 2).

How do hydrophobic interactions affect ion exchange separations?

Ion-exchange resins with hydrophobic character may result in multiple-mode separations. Multiple mode separations are sometimes beneficial but are more likely to be complicating or detrimental to ion-exchange chromatography. In addition hydrophobic adsorption may lead to reduced recovery, band broadening and/or protein denaturation. Many proteins, such as bovine serum albumin and ovalbumin, are particularly sensitive to ion-exchange resins with hydrophobic character. To minimize hydrophobic adsorption and avoid mixed-mode separations in protein ion-exchange chromatography, Vydac developed the VHP matrix by modifying PS-DVB beads with a hydrophilic surface (see Page 3).

What is the effect of temperature?

Temperature affects ion-exchange chromatography separations through its effect on the structure of the protein. Although temperature does not affect the electrostatic interaction, it often affects the structure of a protein and therefore the interaction of the protein with the ion-exchange resin. Subtle variations in selectivity with temperature may result from temperature induced changes in protein structure.

Recommended Buffers for Polypeptide Ion-Exchange Chromatography

A wide range of buffers are available for use with ion-exchange chromatography. Recommended buffers for various ranges of pH are listed below.

Anion-Exchange Chromatography Buffers

Buffers for anion exchange are generally basic amines.

Buffer	Concentration	Anion	pKa	Buffering Region
L-histidine	20 mM	Cl-	6.15	5.5 - 6.8
bis-Tris	20 mM	Cl-	6.50	5.8 - 7.0
bis-Tris propane	20 mM	Cl-	6.80	6.4 - 7.3
Triethanolamine	20 mM	Cl-	7.77	7.3 - 8.2
Tris	20 mM	Cl-	8.16	7.5 - 8.8
diethanolamine	20 mM	Cl-	8.88	8.4 - 9.4

Cation Exchange Chromatography Buffers

Buffers for cation-exchange chromatography are acids.

Buffer	Concentration	Cation	pKa	Buffering Region
formate	20 mM	Na+	3.75	3.3 - 4.3
acetate	20 mM	Na+	4.76	4.2 - 5.2
MES	20 mM	Na+	6.15	5.5 - 6.7
phosphate	20 mM	Na+	2.1/7.2	2.0 - 7.6
HEPES	20 mM	Na+	7.55	7.6 - 8.2

References

1. Retention Model for High-Performance Ion-Exchange Chromatography, W. Kopaciewicz, M.A. Rounds, J. Fausnaugh and F.E. Regnier, *J. Chrom. 266, 3-21 (1983)*

2. Characterization of the influence of displacing salts on retention in gradient elution ion-exchange chromatography of proteins and peptides, G. Malmquist and N. Lundell, *J. Chrom. 627, 107-124 (1992)*

3. Two-Dimensional Liquid Chromatography of Peptides: An Optimization Strategy, N. Lundell and K. Markides, *Chromatographia 34, 369-375 (1992)*

Vydac VHP Ion-Exchange Columns

Vydac VHP Ion-exchange columns are high-performance columns for the ion-exchange separation and purification of polypeptides and polynucleotides. VHP ion-exchange columns combine spherical polystyrene-divinylbenzene (PS-DVB) copolymer beads with a robust hydrophilic surface and stable derivatization chemistry to produce three types of high performance ion-exchange resins for bioseparations.

- 400VHP sulfonic acid (strong - 'S' type) cation exchange (Page 5)
- 300VHP quaternary amine (strong - 'Q' type) anion exchange (Page 6)
- 301VHP tertiary amine (moderate - 'DEAE' type) anion exchange (Page 6)

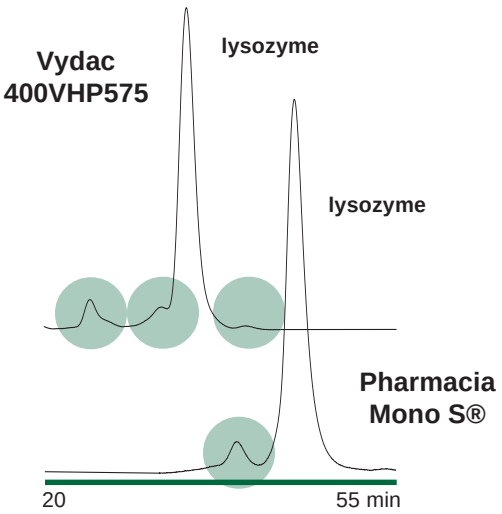
VHP Ion-exchange columns offer superior resolution

The selectivity resulting from Vydac's unique surface chemistry is illustrated by the separation of small impurities in a lysozyme sample.

- The Vydac 400VHP (strong cation-exchange) column separates three impurities from lysozyme
- .... versus only one impurity on the traditional protein ion-exchange column.

Conditions

Vydac 400VHP575 (5 µm,
Strong cation exchange, 7.5 x 50 mm)
Pharmacia MonoS® (10 µm,
Strong cation exchange, 5 x 50 mm)
Eluent: 10 mM phosphate, pH 4.7,
gradient from 0 to .25 M NaCl
Sample: lysozyme



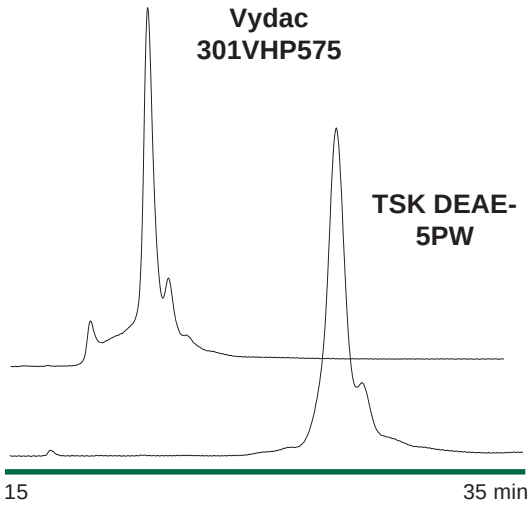
VHP Ion-exchange columns offer superior column efficiency

Vydac VHP Ion-exchange columns are the first truly high-performance ion-exchange columns for the separation of proteins and polypeptides.

Five or eight micron beads and excellent surface chemistry combine to offer higher separation efficiencies than obtained with traditional protein ion-exchange columns. In this example, VHP column selectivity and efficiency resolved impurities in conalbumin better than the traditional ion-exchange column. Not only did the VHP column show better efficiency but conalbumin was also eluted with 25 mM less sodium chloride than on the traditional ion-exchange column.

Conditions

Vydac 301VHP575 (5 µm,
DEAE anion exchange, 7.5 x 50 mm)
TSK DEAE-5PW (10 µm,
DEAE anion exchange,
7.5 x 50 mm)
Eluent: 10 mM Tris-HCL,
pH 8.0, gradient from
0 to .1 M NaCl in 40 min.
Sample: conalbumin

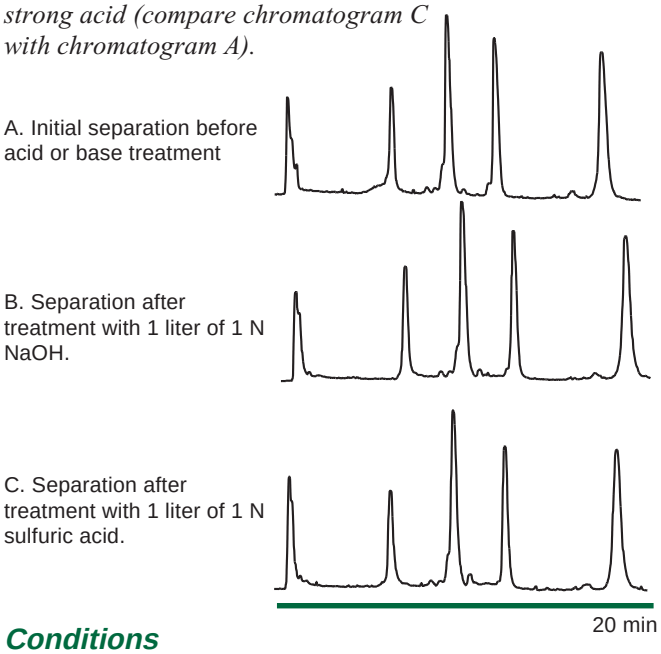


Vydac VHP columns are robust.

The useful pH range is 0 to 14 and the maximum pressure is 3000 psi!

Because the PS-DVB beads, hydrophilic surface and derivatization chemistries of VHP resins are resistant to attack by acid or base, the pH operating range of VHP ion-exchange columns is from pH 0 to pH 14. This means that protein separations can be optimized by adjusting the mobile phase to any pH within the operating range for ion-exchange chromatography (pH 2 to 8 for cation exchange and pH 4.5 to 10 for anion exchange). **It also means that VHP ion-exchange columns can be cleaned using either a strong base such as 1 N sodium hydroxide or a strong acid such as 1 N sulfuric acid or hydrochloric acid to remove contaminants.** Highly crosslinked PS-DVB beads permit the use of VHP columns to a maximum of 3000 psi.

The acid and base stability of VHP columns is highlighted in the separation of a series of proteins before and after washing with strong base and strong acid. Selectivity is not affected by washing the column with strong base (compare chromatogram B with chromatogram A) nor by washing with strong acid (compare chromatogram C with chromatogram A).



Conditions

Vydac 400VHP575 (5 µm, Strong cation exchange, 7.5 x 50 mm)
Eluent: 10 mM Tris-HCL, pH 7.34, gradient from 0 to .5 M NaCl
Proteins: 1. myoglobin; 2. conalbumin; 3. α-chymotrypsinogen A; 4. cytochrome c; 5. lysozyme

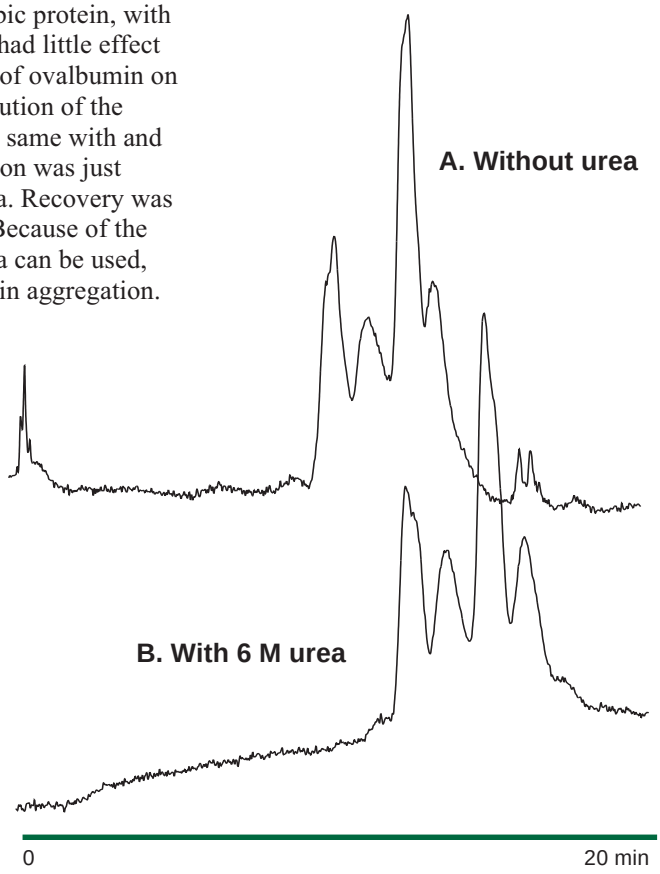
VHP columns are free from hydrophobic interactions.

Elution of a "sticky" protein

The hydrophilic surface on VHP resins virtually eliminates problems with hydrophobic or "sticky" proteins separated by ion-exchange chromatography. Urea or other mobile phase components to avoid hydrophobic adsorption are not needed with VHP ion-exchange columns. This is illustrated by the anion-exchange separation of ovalbumin, a hydrophobic protein, with and without urea. Urea had little effect on the chromatography of ovalbumin on the VHP column. Resolution of the isoforms was nearly the same with and without urea and retention was just slightly longer with urea. Recovery was the same in each case. Because of the robust VHP matrix, urea can be used, however, to avoid protein aggregation.

Conditions

Column: Vydac 301VHP575 (5 µm, DEAE-type anion exchange, 7.5 x 50 mm)
Eluent: 20 mM Tris, pH 8.0, gradient from 0 - 100 mM NaCl in 100 min at a flow rate of 1.0 ml/min.
A. Without urea
B. With 6 M urea.
Detection: UV at 280 nm
Sample: ovalbumin



For additional details regarding the VHP matrix please request a reprint of:

Characterization of a novel stationary phase derived from a hydrophilic polystyrene-based resin for protein cation-exchange high-performance liquid chromatography, Y-B. Yang, K. Harrison and J. Kindsvater, *J. Chrom. 723, 1-10 (1996)*

High Performance Bioseparations: Cation Exchange

Vydac 400VHP ('S' type) Columns

Description

Vydac 400VHP protein cation-exchange resins are spherical polystyrene-divinylbenzene (PS-DVB) beads with a chemically attached hydrophilic surface derivatized to form sulfonic acid groups.

Vydac 400VHP cation-exchange columns:

- Contain a strong or 'S' type cation-exchange resin
- Offer superior selectivity resulting in high-resolution protein separations
- Are robust, even under extreme operating conditions
- Exhibit superior column efficiency
- Are available for small to large-scale preparative purification
- Are compatible with FPLC® (using available M6 adapter).

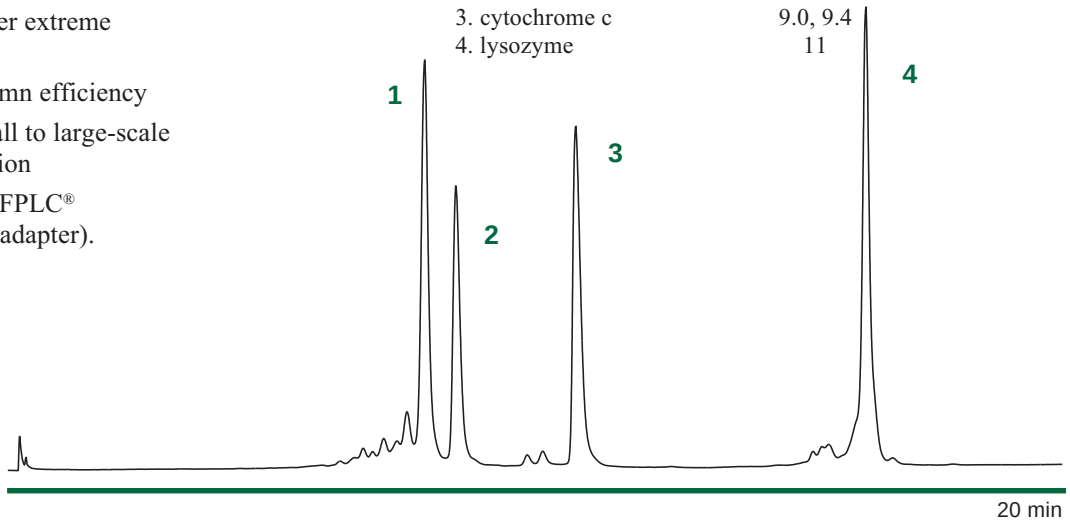
Separation of Proteins by High Performance Cation-Exchange Chromatography

Strong cation-exchange columns, such as Vydac 400VHP columns, are excellent for separating proteins with medium to high pI values.

Conditions

Column: 400VHP575 (5 µm, 'S' type cation exchange, 7.5 x 50 mm)
Eluent: 10 mM phosphate, pH 6.5 with a gradient from 0 to .5 M NaCl in 50 min.

Proteins:	**pI**
1. α-chymotrypsinogen A	8.8, 9.2, 9.6
2. ribonuclease	8.8
3. cytochrome c	9.0, 9.4
4. lysozyme	11



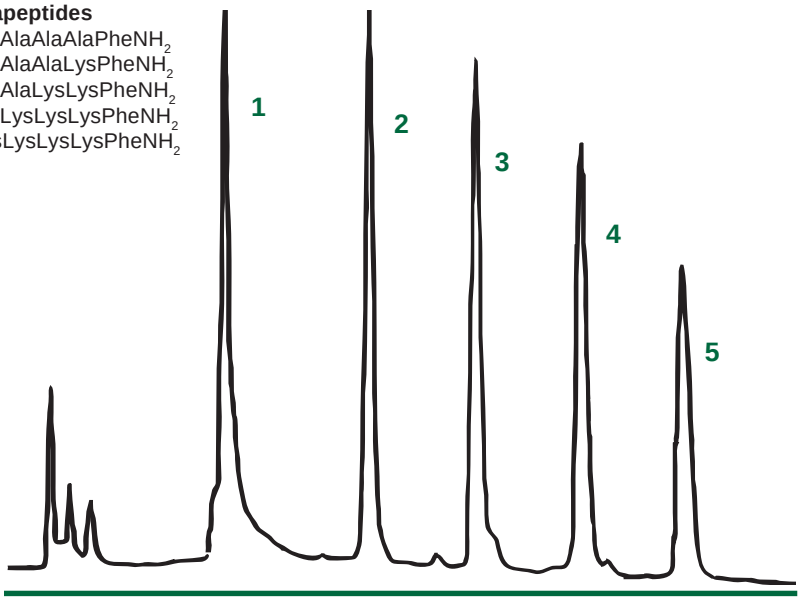
Separation of Peptides by Cation Exchange

Five pentapeptides with one to five positive charges (at low pH) were synthesized and separated on a 400VHP575 column. Separation between adjacent peptides and peak shape were excellent.

Conditions

Column: Vydac 400VHP575,
(S-type Cation Exchange, 5 µm, 7.5 x 50 mm).
Buffer A: 8 mM phosphate in 20% acetonitrile/water, pH 4.0
Buffer B: Buffer A with 0.4 M sodium chloride
Gradient: 1 min hold at 0% B,
0-100% B over 10 min.
Flow rate: 2.5 ml/min
Detection: UV at 220 nm
Sample: about 30 mgrams of each pentapeptide
Data courtesy of Mike Giles, Zeneca Pharmaceuticals

Pentapeptides
1. $AlaAlaAlaAlaPheNH_2$
2. $AlaAlaAlaLysPheNH_2$
3. $AlaAlaLysLysPheNH_2$
4. $AlaLysLysLysPheNH_2$
5. $LysLysLysLysPheNH_2$



Vydac 300VHP ('Q' type) and 301VHP ('DEAE' type) Columns

Description

Vydac 300VHP and 301VHP Protein Anion-exchange resins are spherical polystyrene-divinylbenzene (PS-DVB) beads with a chemically attached hydrophilic surface derivatized to form quaternary amine (300VHP) or tertiary amine (301VHP) groups.

Vydac VHP Anion-Exchange Columns:

- Contain a strong 'Q' type anion-exchange resin (300VHP), or a moderate 'DEAE' type anion-exchange resin (301VHP)
- Offer superior selectivity resulting in high-resolution protein separations
- Are robust, even under extreme operating conditions
- Exhibit superior column efficiency
- Are available for small to large scale preparative purification
- Are compatible with FPLC® (using available M6 adapter).

Comparison of 300VHP (Quaternary amine) and 301VHP (Tertiary amine) columns.

Quaternary amine (300VHP) and tertiary amine (301VHP) ion-exchange columns have similar functional groups, however there are often subtle differences in protein resolution between the two. The differences are illustrated by the separation of carbonic anhydrase at two different pH's on a 300VHP and a 301VHP column. At pH 8.0 the 300VHP ('Q') column has sharper peaks than the 301VHP ('DEAE') column and partially resolves a minor peak. At pH 8.5, however, the 301VHP column partially separates components of the major peak which co-elute on the 300VHP column. Column selection is empirical depending on the separation requirement.

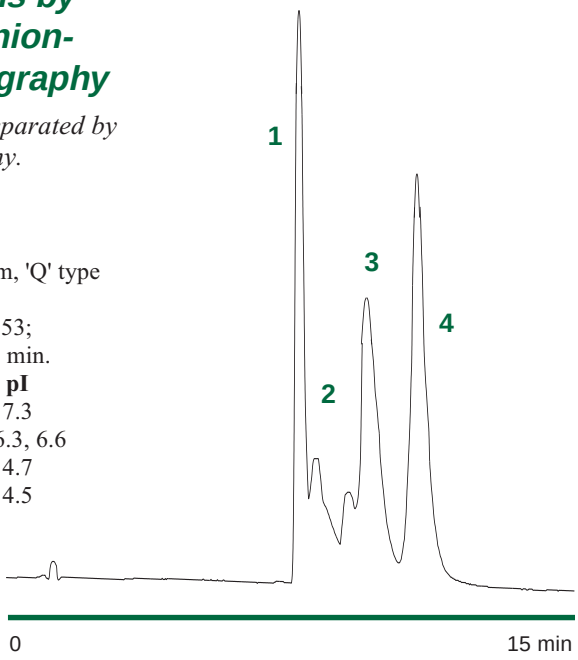
Separation of Proteins by High Performance Anion-Exchange Chromatography

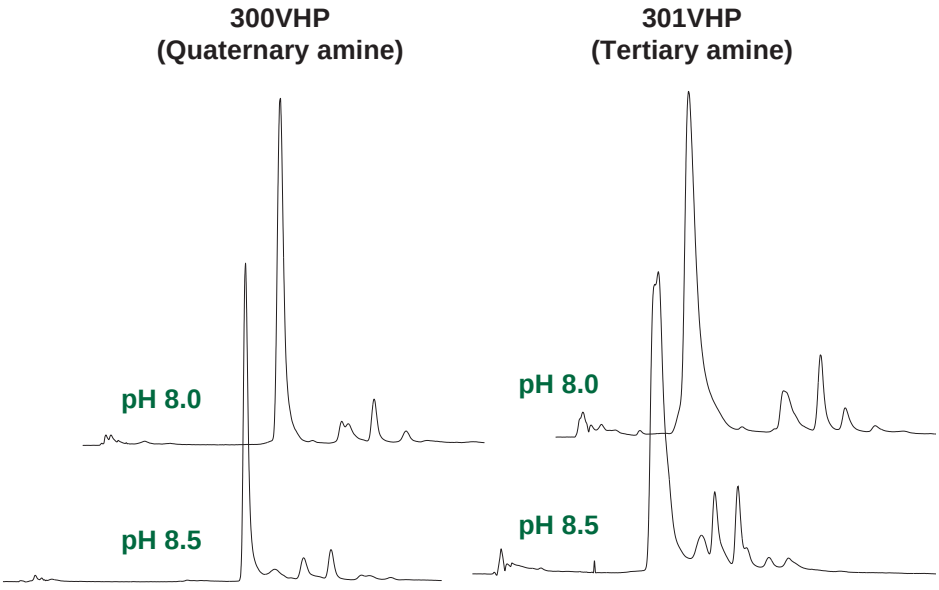
Proteins with low pI's can be separated by anion-exchange chromatography.

Conditions

Column: Vydac 300VHP575 (5 µm, 'Q' type anion exchange, 7.5 x 50 mm)
Eluent: 10 mM CHES/TEA, pH 9.53; gradient from 0 to .5 M NaCl in 20 min.

Proteins	**pI**
1. bovine carbonic anhydrase	7.3
2. conalbumin	6, 6.3, 6.6
3. ovalbumin	4.7
4. soybean trypsin inhibitor	4.5





Conditions

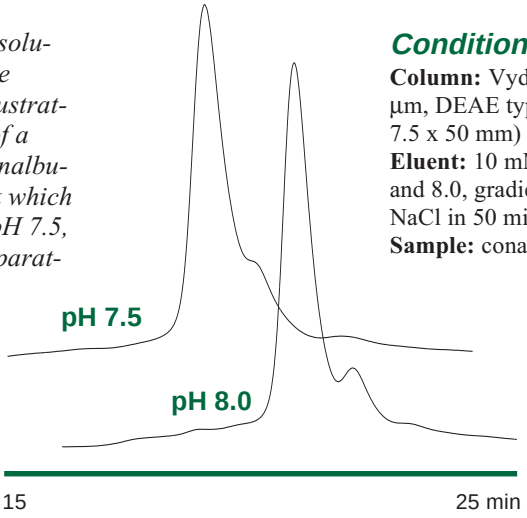
Columns:
Vydac 300VHP575 (5 µm, Q type anion exchange, 7.5 x 50 mm)
Vydac 301VHP575 (5 µm, DEAE type anion exchange, 7.5 x 50 mm)
Eluent: 10 mM Tris-HCl, pH 8.0 or 8.5, gradient from 0 to 150 m M NaCl in 60 minutes.
Sample: carbonic anhydrase

The influence of mobile phase pH on protein ion-exchange separations

Anion-exchange chromatography

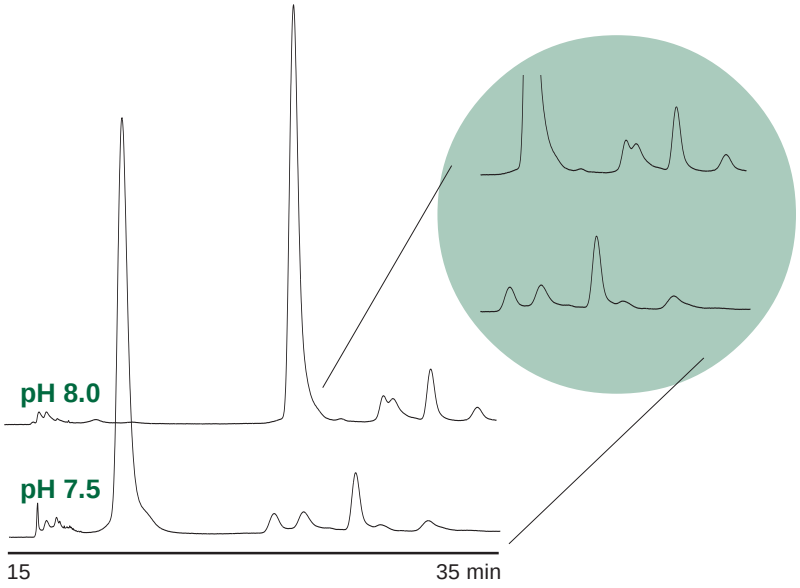
Protein ion-exchange separations can be optimized by careful adjustment of the pH of the mobile phase. Adjusting the pH between 7.5 and 8.5 affects the protonation of histidine and changes the resolution between proteins by anion-exchange chromatography.

The effect of pH on resolution in anion-exchange chromatography is illustrated by the separation of a small peak trailing conalbumin. The trailing peak which is only a shoulder at pH 7.5, is more completely separated at pH 8.0.



Conditions

Column: Vydac 301VHP575 (5 µm, DEAE type anion exchange, 7.5 x 50 mm)
Eluent: 10 mM Tris-HCL, pH 7.5 and 8.0, gradient from 0 to .5 M NaCl in 50 min.
Sample: conalbumin



In the anion-exchange separation of impurities in carbonic anhydrase, reducing the pH from 8.0 to 7.5 resulted in decreased retention and better resolution.

Conditions

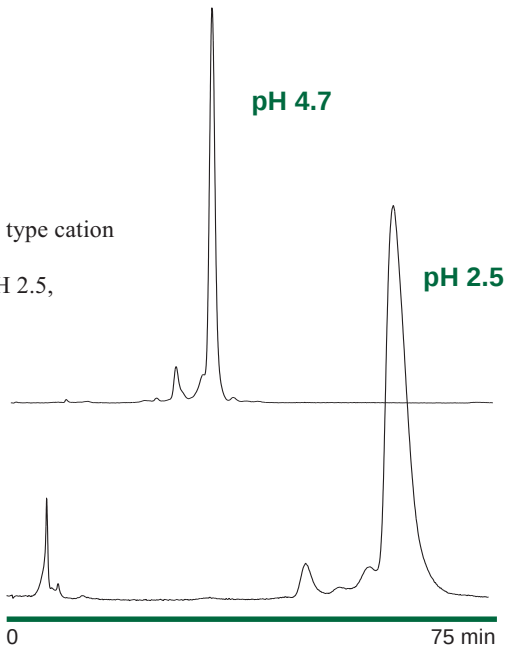
Column: Vydac 300VHP575 (5 µm, DEAE-type anion exchange, 7.5 x 50 mm)
Eluent: 10 mM Tris-HCL, pH 7.5 and 8.0, gradient from 0 to .15 M NaCl in 60 min.
Sample: carbonic anhydrase

Cation-exchange chromatography

Protein structure and charge change as aspartic acid and glutamic acid become protonated between pH 2.5 and 5.0. This affects the cation-exchange separation of proteins. Although the acid sidechains are not directly involved in the cation-exchange interaction, they affect the charge, charge density and, possibly, the tertiary structure of the proteins. In this example impurities in lysozyme are partially resolved from lysozyme at pH 4.7 but are more fully resolved at pH 2.5, where the aspartic and glutamic acid sidechains are protonated.

Conditions

Column: Vydac 400VHP575 (5 µm, 'S' type cation exchange, 7.5 x 50 mm)
Eluent: 10 mM phosphate, pH 4.7 or pH 2.5, gradient from 0 to .25 M NaCl
Sample: lysozyme

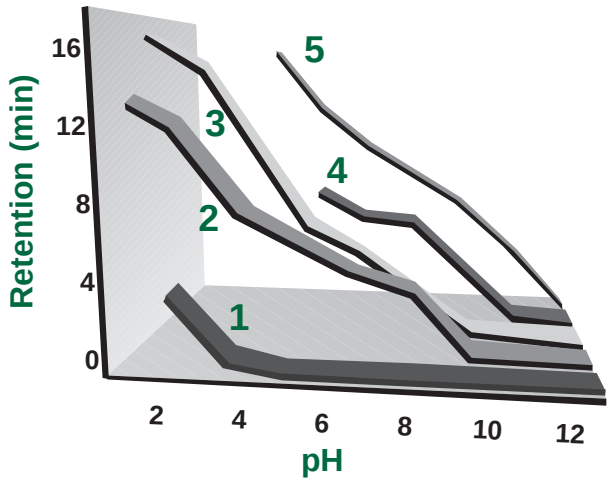


Protein Retention Map on 400VHP: Retention versus pH.

A map of retention versus pH will show the optimum pH range for the separation of a set of proteins. As the pH increases, histidine, then lysine then arginine lose their positive charge and retention decreases on the cation-exchange resin.

Column: Vydac 400VHP575
(5 µm, 'S' type cation exchange, 7.5 x 50 mm)
Eluent: buffer used depended on pH;
NaCl gradient from 0 - .5 M

Protein	Source	Mw	pI
1. myoglobin	horse muscle	17500	6.47-7.76
2. a-chymotrypsinogen A	bovine pancreas	25000	8.8, 9.2, 9.6
3. ribonuclease A	bovine	13683	8.8
4. cytochrome c	horse heart	12200	9.0, 9.4
5. lysozyme	chicken egg	13930	11



Optimization of the Separation of Mouse Hemoglobins.

The pH of the mobile phase has the greatest effect on protein separations and is the first parameter to adjust. Several pH's were tested while developing a method for the separation of mouse hemoglobins. pH 6.2 was determined to be optimum. The NaCl gradient slope was reduced and the flow rate increased to arrive at the final separation conditions.

Mouse hemoglobins co-eluted at pH 7.34.

Conditions: 10 mM Tris-HCl at pH 7.34, 0 - .5 M NaCl in 20 min. at 1.0 ml/min

Lowering the pH to 6.2 resolved several components.

Conditions: 10 mM (NH4)2HPO4 at **pH 6.2**, 0 - .5 M NaCl in 20 min. at 1.0 ml/min

Reducing the gradient slope improved resolution.

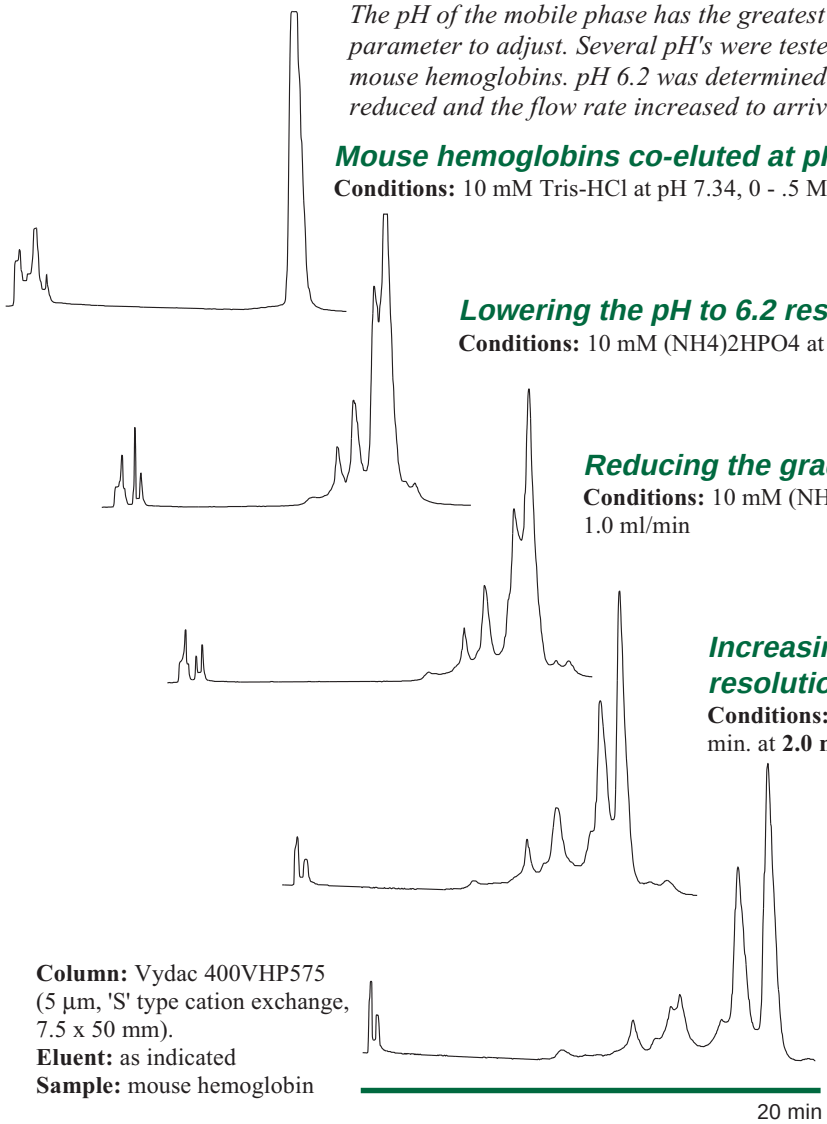
Conditions: 10 mM (NH4)2HPO4 at pH 6.2, **0 - .2 M NaCl** in 20 min. at 1.0 ml/min

Increasing the flow rate further improved resolution and reduced the separation time.

Conditions: 10 mM (NH4)2HPO4 at pH 6.2, 0 - .2 M NaCl in 20 min. at **2.0 ml/min**

Reducing the gradient slope further resulted in optimum resolution.

Conditions: 10 mM (NH4)2HPO4 at pH 6.2, **0 - .1 M NaCl** in 20 min. at 2.0 ml/min

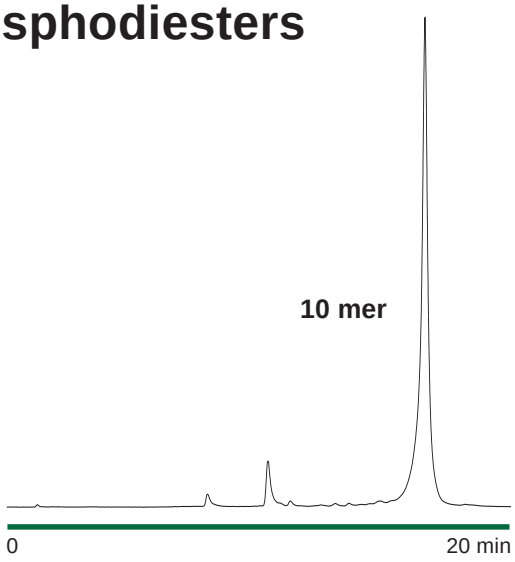


Column: Vydac 400VHP575
(5 µm, 'S' type cation exchange, 7.5 x 50 mm).
Eluent: as indicated
Sample: mouse hemoglobin

Oligonucleotides: Phosphodiesters

Purification of a synthetic oligonucleotide

Synthesis of phosphodiester oligonucleotides result in high yields of the target oligonucleotide and lesser amounts of n-1, n-2, etc. in addition to reaction products. High performance anion-exchange chromatography is able to separate oligonucleotides differing by a single base (see insert of oligonucleotide "ladder"). The separation of impurities from a 10 mer synthetic oligonucleotide is shown here.

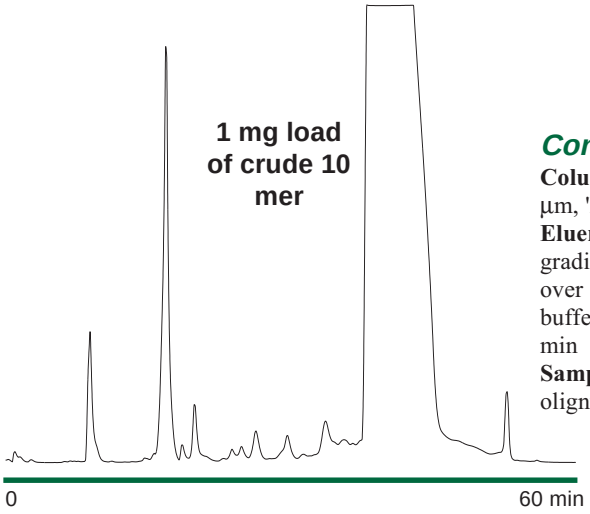


Conditions

Column: Vydac 301VHP575 (5 µm, 'DEAE' type AX, 7.5 x 50 mm)
Eluent: 25 mM TEAA, pH 8.0, gradient from 0 to 500 mM NaCl over 25 min.
Sample: 10 mg crude synthetic 10-mer olignucleotide

Scaleup of synthetic oligonucleotide purification

One mg of a synthetic 10 mer phosphodiester oligonucleotide was purified on a 7.5 x 50 mm Vydac anion-exchange column (301VHP575). Typical of scale-up separations, the NaCl gradient slope was reduced a factor of five from the analytical run to improve resolution when loading the larger sample. Further reduction in the NaCl gradient would allow even larger sample loading. Since loading capacity is dynamic, actual sample capacity depends on purity and yield requirements, however loads of > 5 mg are possible with the 7.5 x 50 mm column. Semi-preparative and preparative VHP columns are also available for even higher sample loading.



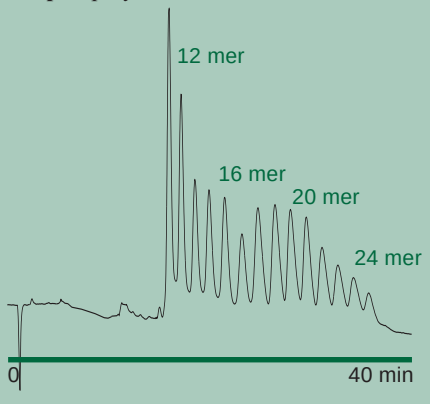
Conditions

Column: Vydac 301VHP575 (5 µm, 'DEAE' type AX, 7.5 x 50 mm)
Eluent: 25 mM TEAA, pH 8.0, gradient from 0 to 200 mM NaCl over 50 min. Final wash with buffered 500 mM NaCl in A at 60 min
Sample: 1 mg. synthetic 10-mer olignucleotide

Oligonucleotide "Ladder"

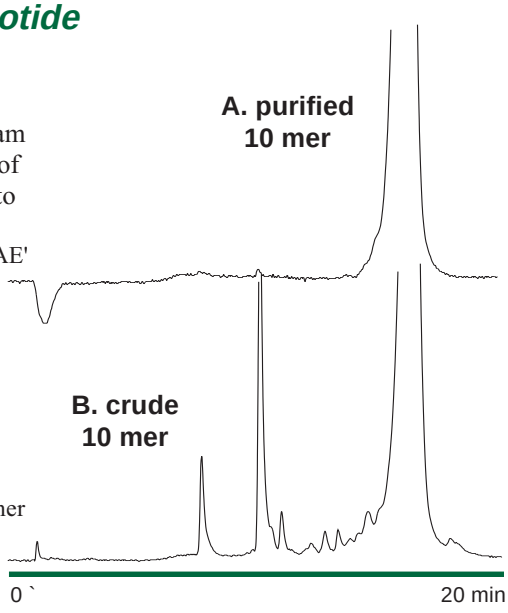
Conditions

Column: Vydac 301VHP575 ('DEAE' type ion exchange, 5 µm, 7.5 x 50mm)
Eluent: 1.0 mL/min. 10mM Tris-HCL, pH 8.0, gradient from 0 to 0.2M NaCl in 5 minutes, then 0.2 - 0.3M NaCl in 40 min.
Sample: poly dT, 12 - 24 mer



Verification of oligonucleotide purification

An anion-exchange analysis of the purified oligonucleotide (Chromatogram A) is compared with a chromatogram of the crude material (chromatogram B) to verify the purify.
Column: Vydac 301VHP575 (5 µm, 'DEAE' type AX, 7.5 x 50 mm)
Eluent: 25 mM TEAA, pH 8.0, gradient from 0 to 500 mM NaCl over 25 min.
Sample:
A. purified (99% pure) synthetic 10-mer olignucleotide from1 mg. scaleup (after desalting on 218TP54 reversed-phase column)
B. 10 mg crude (86% pure) synthetic 10-mer oligonucleotide

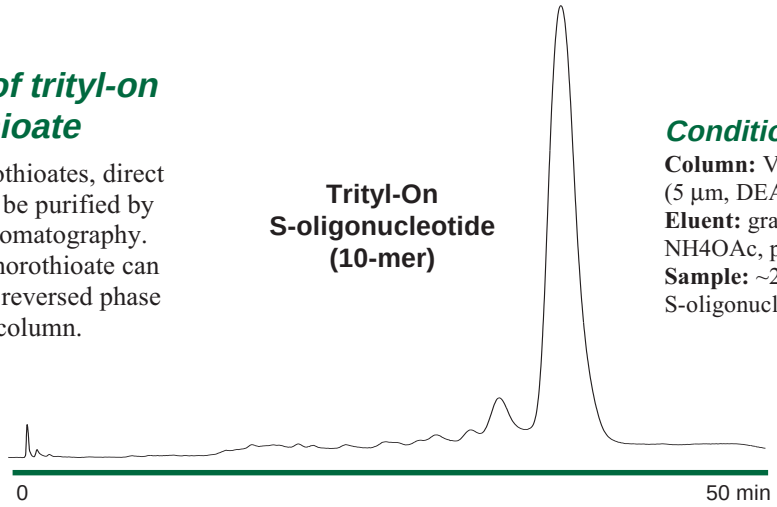


Oligonucleotides: Phosphorothioates

Phosphorothioate oligonucleotides, a new type of "anti-sense" therapeutic likely to be approved by the FDA in the near future for the treatment of viral diseases, are difficult to separate because they are very "sticky" and they aggregate. Phosphorothioate oligonucleotides have previously been separated using high pH mobile phases in order to avoid aggregation. We found that by incorporating 50% isopropanol (IPA) in the eluting buffer, **both trityl-on and trityl-off** phosphorothioates can be separated by anion-exchange chromatography at near neutral pH on a Vydac 301VHP column.

Purification of trityl-on phosphorothioate

Trityl-on phosphorothioates, direct from synthesis, can be purified by anion-exchange chromatography. The purified phosphorothioate can then be desalted by reversed phase on a Vydac 214TP column.



Conditions

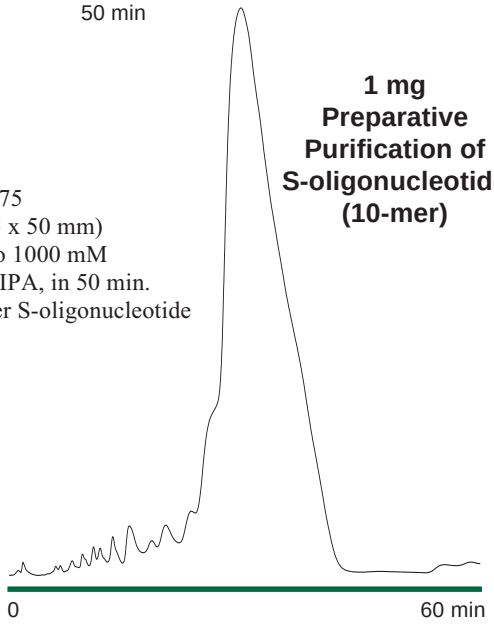
Column: Vydac 301VHP575
(5 µm, DEAE- type AX, 7.5 x 50 mm)
Eluent: gradient from 20 to 1000 mM NH4OAc, pH 8, with 50% IPA, in 50 min.
Sample: ~23 mg crude 10-mer S-oligonucleotide

Scale-up of phosphorothioate purification

The purification of a phosphorothioate oligonucleotide can be scaled up to at least 1 mg on a 7.5 x 50 mm anion-exchange column (Vydac 301VHP575). Reducing the slope of the NaCl gradient would allow even larger sample loading. Since loading capacity is dynamic, actual sample capacity depends on purity and yield requirements, however loads of > 5 mg are possible with the 7.5 x 50 mm column. Semi-preparative and preparative VHP columns are also available for even higher sample loading.

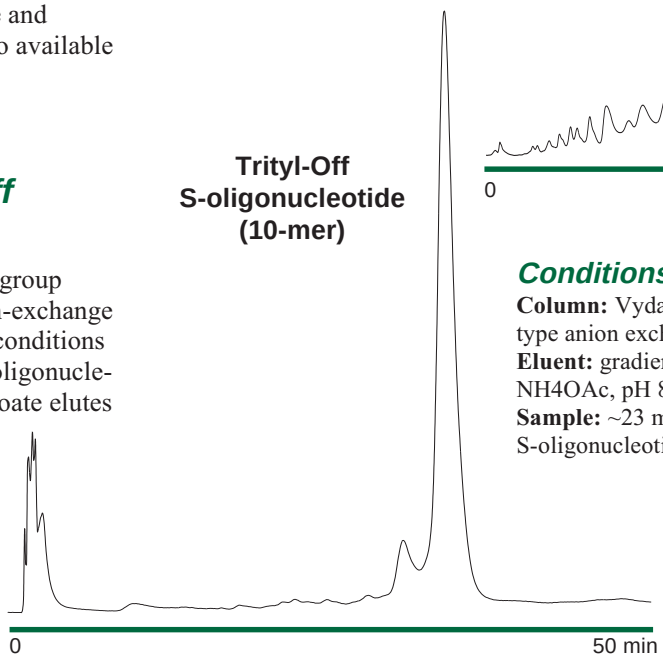
Conditions

Column: Vydac 301VHP575
(5 µm, DEAE type AX, 7.5 x 50 mm)
Eluent: gradient from 20 to 1000 mM NH4OAc, pH 8, with 50% IPA, in 50 min.
Sample: 1 mg crude 10-mer S-oligonucleotide



Purification of trityl-off phosphorothioate

Phosphorothioates with the trityl group removed can be purified by anion-exchange chromatography under the same conditions as purification of the trityl-on S-oligonucleotide. The trityl-off phosphorothioate elutes slightly earlier than the trityl-on S-oligonucleotide. The trityl group elutes near the void volume.



Conditions

Column: Vydac 301VHP575 (5 µm, DEAE-type anion exchange, 7.5 x 50 mm)
Eluent: gradient from 20 to 1000 mM NH4OAc, pH 8, with 50% IPA, in 50 min.
Sample: ~23 mg trityl-off S-oligonucleotide.

Antibody Purification

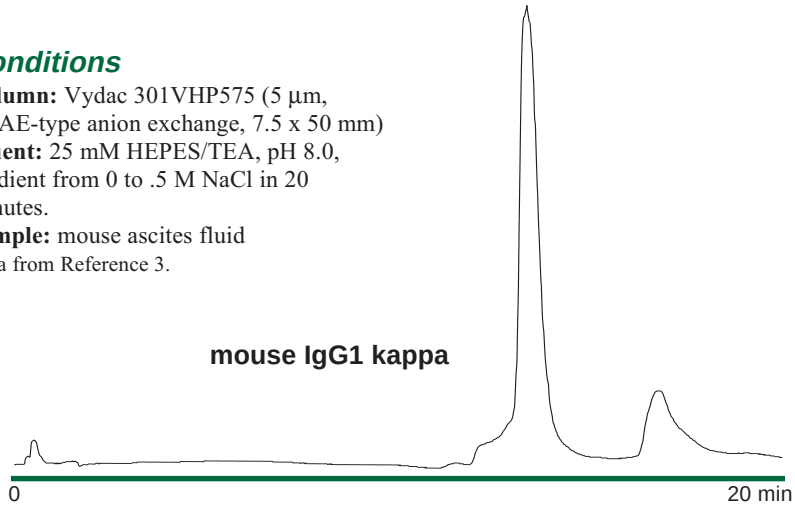
High-performance ion-exchange chromatography is an effective tool in the separation, analysis and purification of immunoglobulins. Although affinity chromatography may be used for the purification of antibodies, it is costly, subject to leaching and is not effective in separating sub-classes. High-performance ion-exchange chromatography avoids the practical problems of affinity chromatography, with high resolution separations.

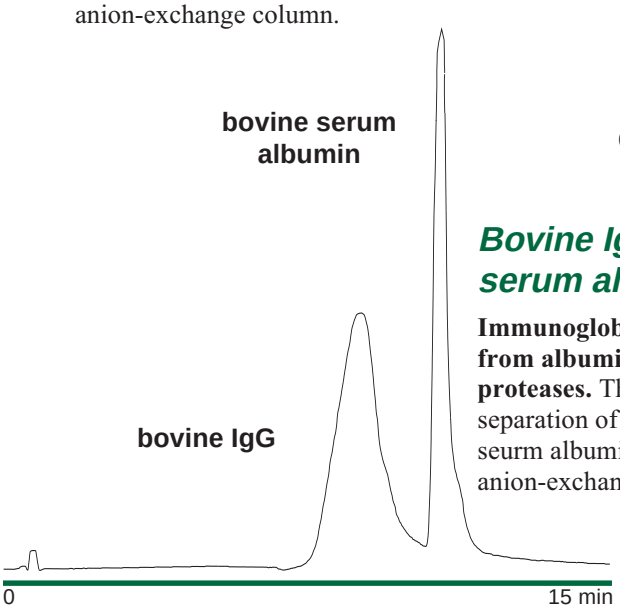
Monoclonal mouse IgG1 kappa in ascites fluid.

Monoclonal antibodies produced in mouse ascites can be separated from other components of the ascites fluid. This is illustrated in the separation of mouse IgG1 from ascites fluid by high performance anion-exchange chromatography using a Vydac 301VHP575 (DEAE) anion-exchange column.

Conditions

Column: Vydac 301VHP575 (5 µm, DEAE-type anion exchange, 7.5 x 50 mm)
Eluent: 25 mM HEPES/TEA, pH 8.0, gradient from 0 to .5 M NaCl in 20 minutes.
Sample: mouse ascites fluid
Data from Reference 3.





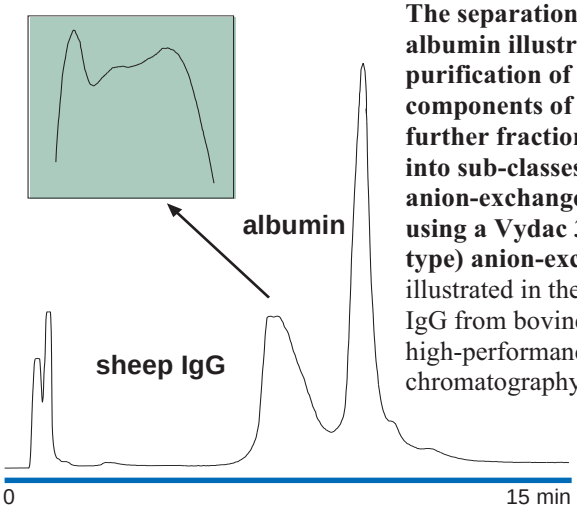
Bovine IgG and bovine serum albumin

Immunoglobulins can be separated from albumin, transferrins and proteases. This is illustrated in the separation of bovine IgG from bovine seurm albumin by high-performance anion-exchange chromatography.

Conditions

Vydac 300VHP575 (5 µm, Q-type anion exchange, 7.5 x 50 mm)
Eluent: 10 mM CHES/TEA, pH 9.53, gradient from 0 to .5 M NaCl in 20 minutes.
Sample: bovine IgG and bovine serum albumin
Data from Reference 3.

Expanded view of antibody peak showing fractionation of the polyclonal antibody into sub-classes.



Separation of antibodies in sheep serum

The separation of sheep serum from albumin illustrates not only the purification of antibodies from other components of the sample but also the further fractionation of the antibodies into sub-classes by high-performance anion-exchange chromatography using a Vydac 301VHP575 (DEAE type) anion-exchange column. This is illustrated in the separation of bovine IgG from bovine seurm albumin by high-performance anion-exchange chromatography.

Conditions

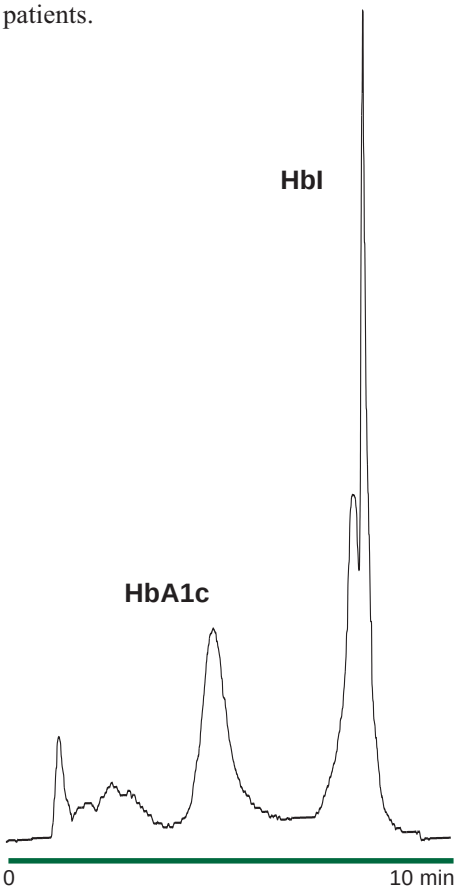
Vydac 301VHP575 (5 µm, DEAE-type anion exchange, 7.5 x 50 mm)
Eluent: 10 mM HEPES/TEA, pH 8.0, gradient from 0 to .5 M NaCl in 20 minutes.
Sample: sheep serum
Data from Reference 3.

For more details about the separation and purification of antibodies request a reprint of Reference 3:

Influence of Column Types and Chromatographic Conditions on the IEX Chromatography of Antibodies, Y. Yang and K. Harrison, *Presented at the 15th I.S.P.P.P., Boston, MA, USA, Nov 18-20, 1995. J. Chrom. A, 743, 171-180 (1996)*

Analysis of Glycosylated Hemoglobin

The quantity of glycosylated hemoglobin in the blood stream reflects average blood sugar levels, an important parameter in maintaining the health of diabetic patients.



Conditions

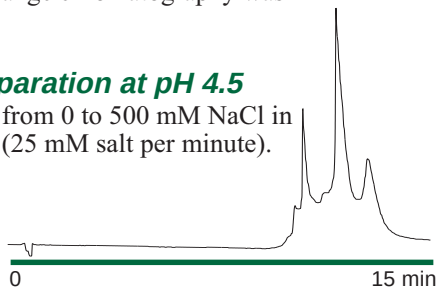
Column: Vydac 400VHP575 (SCX, 5 µm, 7.5 x 50 mm).
Buffer A: 5 mM malonate, pH 5.7, with 37.6 mM NaCl and 0.10 g/L sodium azide
Buffer B: 9.8 mM malonate, pH 5.7, with 339.8 mM NaCl and 0.20 g/L sodium azide
Gradient: Complex from 5 - 70% Buffer B over nine min.
Flow rate: 1.0 ml/min
Detection: UV at 230 nm, 0.1 AUFS
Sample: Blood from diabetic patient with HbI Philadelphia - anticoagulated with EDTA and mixed into hemolysis reagent.
Data courtesy of G.A. van den Berg, H.J.J. Wenselaar and A.J. Bakker, Dept of Clinical Chemistry, KCL Foundation, Leeuwarden, The Netherlands

Separation of Standard Human Hemoglobins

Hemoglobins can be separated by high-performance cation-exchange chromatography using the Vydac 400VHP575 strong cation-exchange column. Human hemoglobins are separated here using a citrate buffer at pH 5.53. The initial separation by cation-exchange chromatography was at pH 4.5 Increasing the pH slightly to 4.9 improved resolution while reducing the retention time. Increasing the pH to 5.5 improved resolution further. Decreasing the rate of increase in salt concentration (shallower gradient) fully resolved the standard hemoglobins.

Initial separation at pH 4.5

Gradient is from 0 to 500 mM NaCl in 20 minutes (25 mM salt per minute).

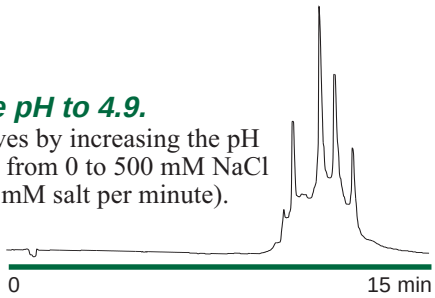


Conditions

Vydac 400VHP575 (5 µm, cation exchange, 7.5 x 50 mm)
Eluent: 20 mM TEA citrate, pH as indicated, NaCl gradient as indicated
Sample: standard human hemoglobin
Data from Reference 2.

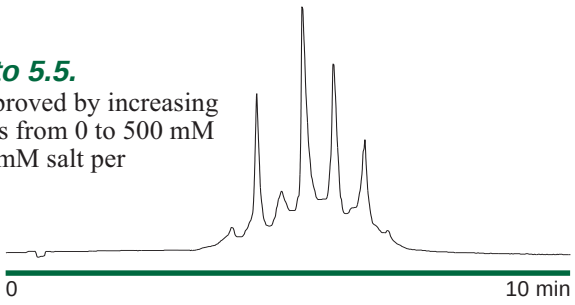
Increasing the pH to 4.9.

Resolution improves by increasing the pH to 4.9. Gradient is from 0 to 500 mM NaCl in 20 minutes (25 mM salt per minute).



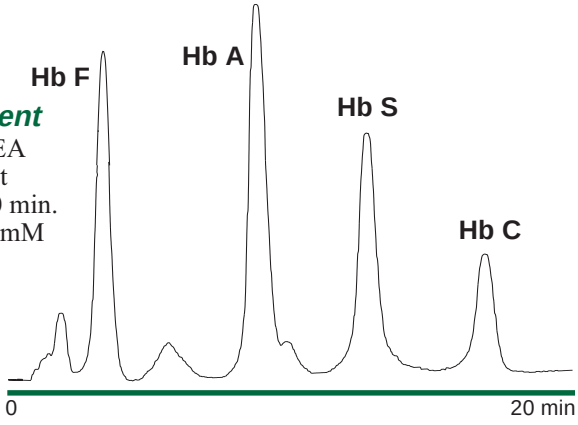
Increasing the pH to 5.5.

Resolution is further improved by increasing the pH to 5.5. Gradient is from 0 to 500 mM NaCl in 20 minutes (25 mM salt per minute).



Using a shallower gradient

Final elution is with 20 mM TEA citrate at pH 5.5 with a gradient from 25 to 125 mM NaCl in 20 min. Increasing the initial salt to 25 mM and making the gradient more shallow at 5 mM increase per minute fully resolves the standard human hemoglobins.



Complementary Bioseparation Techniques:

Ion Exchange and Reversed-Phase Chromatography

Ion exchange and reversed-phase chromatography are complementary. Ion-exchange chromatography separates on the basis of charge; reversed phase separates on the basis of hydrophobicity. Used in series, these complementary separation techniques offer better purification than can be achieved with either one alone; in parallel they offer mutual confirmation of analytical results.

Characteristics of High-Performance Ion-Exchange Chromatography

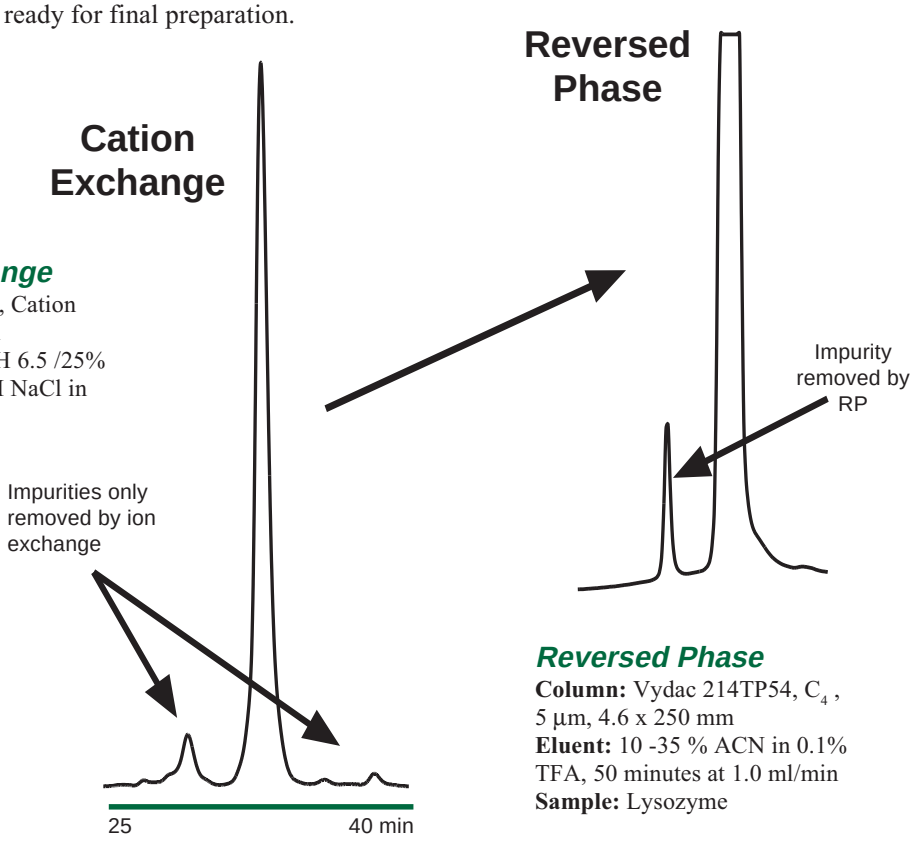
- Operation under near-physiological, non-denaturing conditions
- High loading capacity
- Resistant to reagents such as strong base, strong acid and Gu-HCl
- Use of urea or other chaotropes to break-up complexes
- Optimization of elution selectivity by adjustment of pH

Characteristics of Reversed-Phase Chromatography

- High resolution separations based on differences in hydrophobicity
- Use of volatile buffers or ion-pairing agents
- Removal of salt or buffers from ion-exchange chromatography step

Ion Exchange and Reversed-Phase Chromatography used in series to remove Impurities in lysozyme

Ion-exchange chromatography is generally the first step in the purification of proteins. The partially purified polypeptide, containing salts and buffers from the ion-exchange separation, is then loaded onto a reversed-phase column. Purification based on hydrophobicity or conformation then takes place and the collected sample elutes in a volatile solution, ready for final preparation.



Strong Cation Exchange

Column: Vydac 400VHP575, Cation exchange, 5 µm, 7.5 x 50 mm
Eluent: 10 mM phosphate, pH 6.5 /25% ACN; gradient from 0 - 0.1 M NaCl in 25 min at 1.0 ml/min

Reversed Phase

Column: Vydac 214TP54, C_4 , 5 µm, 4.6 x 250 mm
Eluent: 10 -35 % ACN in 0.1% TFA, 50 minutes at 1.0 ml/min
Sample: Lysozyme

For more details about reversed-phase HPLC in the separation of polypeptides consult:
The Vydac Handbook of Analysis and Purification of Peptides and Proteins by Reversed-Phase HPLC
Available on request from Vydac or download at ***http://www.vydac.com***.

Protein Ion-Exchange Column Selection Guide

	300VHP	301VHP	400VHP
Functional Group:	Quaternary amine	Tertiary amine	Sulfonic acid
Type:	'Q' type anion	'DEAE' type anion	'S' type cation

Particle size	Column size			
5 micron	***Analytical***			
	5 x 25 mm	300VHP552	301VHP552	400VHP552
	7.5 x 50 mm	300VHP575	301VHP575	400VHP575
	7.5 x 50 mm (PEEK)	300VHP575P	301VHP575P	400VHP575P
8 micron	***Semipreparative / Preparative***			
	10 x 100 mm	300VHP81010	301VHP81010	400VHP81010
	22 x 100 mm	300VHP82210	301VHP82210	400VHP82210

Technical Specifications

	300VHP	301VHP	400VHP
Type of ion exchange	anion exchange	anion exchange	cation exchange
Functional group	triethylamine	diethylamine	sulfopropyl
Type of ion exchange	Q	DEAE	S
Pore diameter	900 A	900 A	900 A
Particle size:	5 or 8 micron	5 or 8 micron	5 or 8 micron
Maximum pressure	3000 psi	3000 psi	3000 psi
pH Stability	0 - 14	0 - 14	0 - 14
Protein capacity (frontal)	29 mg/ml (ovalbumin)	33 mg/ml (ovalbumin)	40mg/ml (lysozyme)

Typical Protein Mass Recovery on VHP Ion-Exchange Columns

Protein	Amount Injected	Recovery (%)
myoglobin	6.25 mg	87.1
conalbumin	62.5 mg	106.7
a-chymotrysinogen A	50.0 mg	99.9
cyctochrome c	18.75 mg	99.9
lysozyme	37.5 mg	96.9

References

1. Characterization of a novel stationary phase derived from a hydrophilic polystyrene-based resin for protein cation-exchange high-performance liquid chromatography, Y-B. Yang, K. Harrison and J. Kindsvater, *J. Chrom. 723, 1-10 (1996)*

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3. Influence of Column Types and Chromatographic Conditions on the IEX Chromatography of Antibodies, Y. Yang and K. Harrison, *Presented at the 15th I.S.P.P.P., Boston, MA, USA, Nov 18-20, 1995.*

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5. Analytical and Preparative Separation of Anionic Oligosaccharides by Weak Anion-Exchange High-Performance Liquid Chromatography on an Inert Polymer Column, G.Guile, S.Y.C. Wong and R.A. Dwek, *Anal. Biochem. 222, 231-235(1994)*

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