Thermo Scientific

ProPac HIC-10

Column Product Manual

P/N: 065093-04 December 2013

Thermo SCIENTIFIC

PRODUCT MANUAL

FOR

PROPAC HIC-10 COLUMNS

HIC-10 Analytical Column 2.1 x 100 mm (P/N 063653)
HIC-10 Analytical Column 4.6 x 100 mm (P/N 063655)
HIC-10 Analytical Column 7.8 x 75 mm (P/N 063665)

Thermo Scientific, 2013
Document No. 065093
Revision 04

TABLE OF CONTENTS

1. INTRODUCTION TO HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC) 5
1.1. Features of the HIC-10 Column 5
1.2. HIC-10 Typical Operating Conditions 5
1.2.1. HIC-10 Specifications - All Formats 6
1.3. Formats of the HIC-10 Column 7
1.3.1. HIC-10 Column Products 7
1.4. Key Applications of the HIC-10 Column 7

2. CHROMATOGRAPHY SYSTEM 8
2.1. Detectors 8
2.2. Pumps 8
2.3. Injectors 8
2.4. Column Oven 8

3. INSTALLATION 9
3.1. System Requirements 9
3.2. System Void Volume 9
3.3. The Injection Loop 9
3.4. Column Installation 9

4. OPERATION 10
4.1. Mobile Phase Selection 10
4.1.1. UV Detection 10
4.2. Quickstart for the HIC Column 10
4.3. Validating Column Performance 11
4.3.1. Procedure for Validating Column Performance 11
4.3.2. Standard Stock Solution 11
4.4. Data Collection 12
4.4.1. Data Processing and Specifications 12
4.5. Protein Test Using Gradient Conditions 13
4.5.1. Chromatographic Conditions 13
4.5.2. Components of Protein Mixture 13
4.5.3. Eluent Preparation 14
4.6. Examples of Protein Test Using Gradient Conditions 15
4.6.1. 2.1 x 100 mm Column Format 15
4.6.2. 4.6 x 100 mm Column Format 16
4.6.3. 7.8 x 75 mm Column Format 17

5. METHOD DEVELOPMENT 18
5.1. Ionic Strength 18
5.2. Organic Modifier 18
5.3. Gradient Methods for Different Formats 18
5.3.1. Stocks and Eluent Preparation 18
5.4. Example Gradient Methods for Applications 19
5.4.1. 2.1 x 100 mm Column 19
5.4.2. 4.6 x 100 mm Column 19
5.4.3. 7.8 x 75 mm Column 19
5.5. Dual Gradient Methods 20
5.5.1. 4.6 x 100 mm Column 20

6. APPLICATIONS 21
6.1. Mixture of Protein Standards 21
6.1.1. Mixture of Protein Standards: 2.1 x 100 mm Column 21
6.1.2. Mixture of Proteins: 4.6 x 100 mm Column 22
6.1.3. Mixture of Protein Standards: 7.8 x 75 mm Column 23
6.2. Separation of Bovine Serum Proteins 24
6.2.1. Separation of Bovine Serum Proteins: 4.6 x 100 mm Column 24
6.2.2. Separation of Bovine Serum Proteins: 7.8 x 75 mm Column 25
6.3. Separation of Snake Venom Proteins / Peptides 26
6.4. HIC Separation of Peptides - Proteomics Application 27
6.5. HIC Separation of Peptides - Proteomics Application (Dual Gradient) 28
6.6. HIC Separation of Monoclonal Antibody (MAb) 29
6.6.1. 7.8 x 75 mm Column Format – Dual Gradient 29
6.6.2. 7.8 x 75 mm Column 30
6.7. Separation of Degradation Products (Oxidation) in MAb Samples on Accelerated Stability 31
6.8. Separation of Casein Mixture 32
6.9. Thrombin Separation 33
6.10. Pancreatin Separation 34
6.11. 2D - Application-Human Skeletal Muscle Protein (HSMP) Separation 35

7. CARING FOR THE COLUMN 36

8. TROUBLESHOOTING GUIDE 37
8.1. High Backpressure 37
8.1.1. Finding the Source of High System Pressure 37
8.1.2. A Contaminated Column – Clean-up Procedure 37

9. REFERENCES 38

10. ATTACHMENTS 39

GUIDE TO IMPORTANT INFORMATION

SAFETY

Safety information can help prevent bodily harm.

WARNING

Warning information can help prevent equipment harm.

CAUTION

Caution information can help prevent problems.

NOTE

Note information can help with tips for improved use.

1. INTRODUCTION TO HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC)

Present Hydrophobic Interaction Chromatography (HIC) is based on the classical paper published by Tiselius in 1948[1] on "Adsorption separation by salting out". Tiselius noted that proteins and other substances which are precipitated by high salt concentrations are adsorbed strongly at lower concentrations than is required for precipitation. In addition, the same substances show little or no adsorption in salt-free solutions.

Hydrophobic interaction chromatography (HIC)[2-4] is a technique for separation of proteins, peptides, and other biomolecules by their degree of hydrophobicity. The mobile phase consists of a salting-out agent (for eg: ammonium sulfate), which, at high concentration, retains the protein by increasing the hydrophobic interaction between the solute and the stationary phase. The bound proteins are eluted by decreasing the salt concentration.

HIC differs from reverse-phase chromatography in the selection of solvent conditions. HIC does not denature proteins during purification as compared to reverse phase HPLC and preserves biological activity. HIC is ideal after salt precipitations and ion exchange chromatography.

HIC has been used extensively for purifying a variety of bio-molecules including serum proteins[4,8], membrane bound proteins[5], nuclear proteins[6], recombinant proteins[7], and receptors[9]. Several recent applications reported analysis of human α-thrombin[10], and purifications of various isotypes of caseins from milk[11]. HIC has also been used in the purification of monoclonal antibodies[12] and purification of enzymes[13-14]. These and various other applications have shown the general applicability of HIC as an important purification step at the initial, intermediate and/or final steps in the purification schemes of proteins.

We have developed a new silica based HIC stationary phase with multifunctional attachment of amide groups. The surface chemistry of this phase was optimized by inclusion of hydrophobic and hydrophilic ligands which were designed to improve the hydrolytic stability of silica support in the aqueous media. These modifications result in superior resolution and peak efficiencies of proteins/ peptides and provide a versatile column with sufficient longevity.

1.1. Features of the HIC-10 Column

- Ideal separation technique for separation of protein/peptides including complex mixtures
- Non-denaturing conditions to preserve biological activity
- Excellent resolution and high efficiencies
- High capacity column
- Compatible with organic solvents and can be used in separations of very hydrophobic proteins
- Broad range of applications

1.2. HIC-10 Typical Operating Conditions

Format	2.1 x 100 mm	4.6 x 100 mm	7.8 x 75 mm
Flow Rate Range	0.15 - 0.2 mL/min	0.5 - 1.0mL/min	1.0 - 1.5 mL/min
Typical Backpressure*	300 - 500 psi	700 - 900 psi	1000 - 1300 psi

*For applications (excluding system pressure).

For assistance, contact Technical Support for Dionex Products. In the U.S., call 1-800-346-6390. Outside the U.S., call the nearest Thermo Fisher Scientific office.

1.2.1. HIC-10 Specifications - All Formats

Shipping Solution: Acetonitrile
Storage Solution: Acetonitrile
Bonding: Proprietary

1.2.1.1. Silica Gel Characteristics

Characteristic	Value
Substrate	Ultra-pure silica
Phase	HIC
Substrate Particle Diameter	5-µm
Particle Shape	Spherical
Particle Size Distribution (40/90)	1.2
Metal Impurity (ppm) Na, Fe, Al	<10.0
Pore Volume (mL/g)	0.9
Endcapped	No
Pore Size	300 Å
Surface Area	100 m^2/g
Surface Chemistry	Amide / Ethyl
Solvent Limit	100%

1.2.1.2. Column Performance Limits

Test Parameter	Value
Mobile Phase Compatibility	Upto 3.0 M ammonium sulfate / 0.1 M phosphate salts, urea, guanidine hydrochloride, organic solvent (e.g. acetonitrile, acetone, methanol, etc.)
pH range	2.5 - 7.5
Max. Operating Pressure	< 3,500 psi
Operating Temperature Range	25 - 40 °C

1.2.1.3. Typical Performance

Test Parameter	Value
Protein Capacity-Dynamic Capacity	Varies for different proteins
Protein Capacity-Breakthrough Capacity	340 mg (7.8 x 75 mm column) for lysozyme

1.3. Formats of the HIC-10 Column

HIC-10 columns are available in 2.1 x 100 mm (P/N 063653), 4.6 x 100 mm (P/N 063655), or 7.8 x 75 mm (P/N 063665) formats. All are stainless steel columns.

1.3.1. HIC-10 Column Products

Part Number	Product Name	Dimensions
063653	ProPac HIC-10	2.1 x 100 mm
063655	ProPac HIC-10	4.6 x 100 mm
063665	ProPac HIC-10	7.8 x 75 mm

1.4. Key Applications of the HIC-10 Column

Hydrophobic Interaction Chromatography (HIC) is a general and powerful chromatography technique and can be used for proteins and peptides separations. The following applications have been successfully developed using the HIC-10 column. HIC-10 can also used for enzyme purifications (e.g. α-Amylase) and is a method of choice after ammonium sulfate precipitation step. These and various other applications have shown the general applicability of HIC-10 as an important purification step at the initial, intermediate, and/or final steps in the purification schemes of proteins.

Examples of separation / purification applications:

- Mixture of protein standards
- Serum proteins
- Snake venom proteins/peptides
- Trypsin digests of proteins
- Monoclonal antibody (MAb)
- Forced oxidation degradation products of MAb
- Thrombin
- Pancreatin
- 2D analysis of cell/tissue extracts (e.g. human skeletal muscle cell extract)

2. CHROMATOGRAPHY SYSTEM

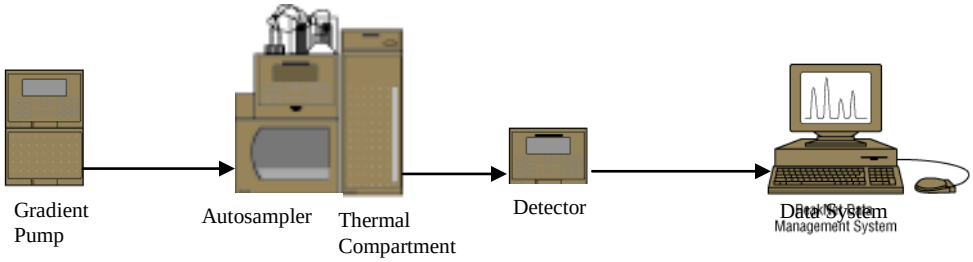


Figure 1
HPLC System Configuration

2.1. Detectors
UV detectors from various manufacturers can be used. However, UVD 340U from Dionex is highly recommended.

2.2. Pumps
Gradient LC pumps from any manufacturers can be used. However, 680 HPG (Stainless steel), DP/SP pump (ICS-3000) from Dionex Corp. is highly recommended.

2.3. Injectors
Either an auto-sampler (ASI 100 and WPS 3000 from Dionex Corp.) or a manual injector can be used for sample injection.

2.4. Column Oven
A column oven (TCC-100 from Dionex Corp.) is recommended. Optimal reproducibility of results can be achieved by regulating the temperature of the column using a column oven during chromatography.

3. INSTALLATION

3.1. System Requirements
HIC-10 columns can be run on any HPLC system, including Dionex HPLC systems.

3.2. System Void Volume
For best performance, minimize the lengths of all connecting tubing and remove all unnecessary switching valves and couplers. For assistance, contact Technical Support for Dionex Products. In the U.S., call 1-800-346-6390. Outside the U.S., call the nearest Thermo Fisher Scientific office.

3.3. The Injection Loop
Use a sample loop according to your sample injection volume requirement. Typically, for manual injections, a sample loop of the same size is recommended. For auto-samplers, 5, 10, or 20 µl injection with a 25 µl loop and 100 to 250 µl injections, with a 100 or 250 µl loop respectively, are recommended.

NOTE

Up to 250 µL can be loaded on 7.8 x 75 mm. The upper limit depends on the concentration of protein, type of sample, and the quality of separation desired. Higher than 250 µL volumes can be loaded if the peak shapes are not critical and the application is intended only to concentrate proteins from a dilute sample.

3.4. Column Installation
Connect the column with the direction arrow on the label pointing toward the detector.

4. OPERATION

Reliable, reproducible results require eluents that are free from impurities and prepared properly. Obtaining reliable, consistent, and accurate results require mobile phases that are free of ionic and spectrophotometric impurities. Chemicals, solvents, and de-ionized water used to prepare mobile phases should be of the highest purity available. Maintaining low levels of trace impurities and low particle contamination levels in mobile phases also help to protect your columns and system components.

4.1. Mobile Phase Selection

4.1.1. UV Detection

Like any HPLC application detected by UV, the mobile phase should have a low UV background.

4.2. Quickstart for the HIC Column

1. Prepare the mobile phases. In-line degassing can be done by using Solvent Rack SOR-100 from Dionex Corp. or, alternatively, degas them under vacuum with sonication.
2. Install the mobile phases on the LC pump.
3. Turn on and program the detector.
4. Prime the pumps with eluents.
5. Install the column.
6. Wash the storage solution off from the column with 0.05 M ammonium acetate (pH 5.4) for about 15 minutes (0.2 mL/min for 2.1 x 100 mm format, 1.0 mL/min for 4.6 x 100 mm format, or 1.5 mL/min for 7.8 x 75 mm format).
7. Wash the the column with Eluent B (0.1 M NaH_2PO_4 , pH 7.0) for about 15 minutes (0.2 mL/min for 2.1 x 100 mm format, 1.0 mL/min for 4.6 x 100 mm format, or 1.5 mL/min for 7.8 x 75 mm format).
8. Equilibrate the column with mobile phase Eluent A for at least 10 minutes.
9. Inject 1–250 µL of sample (depending on the format).
10. Run the gradient of your choice, or start with a gradient as suggested in the manual.
11. At the end of the last run, flush the column with 0.05 M ammonium acetate (pH 5.4) for approximately 10 minutes.
12. Store the column in Acetonitrile.
13. Turn off the pump and detector.
14. Remove the column and plug the ends.

4.3. Validating Column Performance

Dionex recommends that you perform an efficiency test on your HIC-10 column before you use it. The purpose of column performance validation is to make sure that no damage is done to the column during shipping. Test the column using the stated conditions on the Quality Assurance Report (QAR) enclosed in the column box. Repeat the test periodically to track the column performance over time. Note that slight variations may be obtained on two different HPLC systems due to system electronic, plumbing, operating environment, reagent quality, column condition, and operator technique.

4.3.1. Procedure for Validating Column Performance

Each column is validated with an isocratic test. A QAR is provided with the column. To retest and qualify your column, you may perform the following isocratic test.

4.3.1.1. General Column Equilibration

- 15 minutes with equilibration buffer with the recommended flow rate.

Column	Flow Rate
2.1 x 100 mm	0.2 mL/min
4.6 x 100 mm	1.0 mL/min
7.8 x 75 mm	1.5 mL/min

- Equilibration Buffer: 0.1 M ammonium acetate (pH 5.4 ± 0.1) in 30/70 (v/v) acetonitrile/DI H_2O (see Mobile Phase Preparation).

4.3.1.2. Mobile Phase Preparation (0.1 M ammonium acetate (pH 5.4 ± 0.1) in 30/70 (v/v) acetonitrile/DI H_2O)

1. Mix 50 mL of 2 M ammonium acetate solution, pH 5.4 (Dionex P/N 033440) with 650 mL of DI water (18.2 megohm-cm) and degas on vacuum with sonication for 2 minutes. Check the pH.
2. Add 300 mL of acetonitrile to make up to 1liter.
3. Degas on vacuum with sonication for 30 seconds.

NOTE

The column should be stored in Acetonitrile for long term stability of the column. For next day usage, it can be stored in mobile phase pH≤ 7.0.

4.3.1.3. Test Temperature = 30 °C ± 0.1 °C

4.3.1.4. Detector Setting = Wavelength 230 nm (for all formats)

4.3.2. Standard Stock Solution

4.3.2.1. Standard Stock Solution Preparation

Stock Solution A (Cytosine)

1. Prepare of 2 mg/mL stock solution of cytosine in DI water (18.2 megohm-cm)

Stock Solution B (Mixture of 4-Butyl benzoic acid + Amitriptyline + Phenanthrene)

1. Weigh 1 mg each of 4-butyl benzoic acid, amitriptyline, and phenanthrene. Dissolve in 1 mL acetonitrile.
2. Mix well. Sonication (30 seconds) may be used to aid dissolution of the crystals.

4.3.2.2. Standard Injection Solution

- Cytosine: 0.025 mg/mL
- 4-Butyl benzoic acid: 0.025 mg/mL
- Amitriptyline: 0.025 mg/mL
- Phenanthrene: 0.025 mg/mL

4.3.2.3. Standard Injection Solution Preparation

Add 12.5 µL of stock solution A + 25.0 µL of stock solution B + 962.5 µL of mobile phase in a volumetric flask to make 1.0 mL.

4.4. Data Collection

Part No.	Description	HPLC Instrument Program	
063653	HIC-10 2.1x100 mm	Pump	Isocratic Flow = 0.20 mL/min Pre-run time = 2 min Run time = 5 min.
		Column Temp	30 °C ± 0.3
		Injection Loop	1 µL
		UV Detector	Wavelength = 230nm Acquisition 0-5 min
		Pump Pressure	Acquisition 0-5 min
063655	HIC-10 4.6x100 mm	Pump	Isocratic Flow = 1.0 mL/min Pre-run Time = 2 min Run Time = 5 min
		Column Temp	30°C ± 0.3
		Injection Loop	5 µL
		UV Detector	Wavelength = 230 nm Acquisition 0-5 min
		Pump Pressure	Acquisition 0-5 min
063665	HIC-10 7.8x75 mm	Pump	Isocratic Flow = 1.5 mL/min Pre-run time = 2 min Run time = 5 min.
		Column Temp	30 °C ± 0.3
		Injection Loop	10 µL
		UV Detector	Wavelength = 230 nm Acquisition 0-5 min
		Pump Pressure	Acquisition 0-5 min

4.4.1. Data Processing and Specifications

Process the data for desired parameters and compare with the specifications.
(Please see QAR sheet to get the actual specifications set for one of the small molecules, phenanthrene.)

4.5. Protein Test Using Gradient Conditions

Since HIC-10 is used routinely to separate proteins, a simple protein mixture separation may be performed periodically to track the column performance.

4.5.1. Chromatographic Conditions

Column:	2.1 x 100 mm, 4.6 x 100 mm, or 7.8 x 75 mm
Eluents:	A: 2 M $(NH_4)_2$ SO_4 in 0.1 M NaH_2PO_4 (pH 7.0)
	B: 0.1 M NaH_2PO_4 (pH 7.0)
Flow Rate:	0.2 mL / min for 2.1 x 100 mm
	1.0 mL / min for 4.6 x 100 mm
	1.5 mL / min for 7.8 x 75 mm
Temperature:	30 ˚C (or room temperature)
Injection volume:	1-2 µL for 2.1 x 100 mm
	5-10 µL for 4.6 x 100 mm
	15-30 µL for 7.8 x 75 mm
Detection:	UV at 214 nm
Analyte:	Protein Mixture standard containing Ribonuclease A and Lysozyme 1 mg/mL of each protein after 1:1 dilution with Eluent A.

4.5.2. Components of Protein Mixture

The proteins used are Ribonuclease A (Sigma: R-5125) and Lysozyme from chicken egg white (Calibiochem: 4403).

4.5.2.1. Standard Injection Solution Preparation of Ribonuclease A + Lysozyme Protein Mixture (1mg / mL)

1. Dissolve 2 mg of each of Ribonuclease A and Lysozyme in 1 mL of Eluent B.
2. Add 1 mL of Eluent A.
3. Use within 48 hours if kept at room temperature. If it is stored at 4 ºC, injection solution can be used up to a week.

4.5.3. Eluent Preparation

4.5.3.1. Eluent A: 2 M $(NH_4)_2$ SO_4 in 0.1 M NaH_2PO_4 (pH 7.0)

Eluent A Preparation: Dissolve 264.2 grams of ammonium sulfate (Sigma: A 4418) and 12 g of NaH_2PO_4 (Sigma: S8282) in 650 mL DI H_2O (18.2 megohm-cm) stock. Adjust the pH to 7.0 with 50% NaOH and volume to 1 liter with DI H_2O.

4.5.3.2. Eluent B: 0.1 M NaH_2PO_4 (pH 7.0)

Eluent B Preparation: Dissolve 12g of NaH_2PO_4 in 900 mL of DI H_2O (18.2 megohm-cm). Adjust the pH to 7.0 with 50% NaOH and the volume to 1 liter with DI H_2O.

4.5.3.3. Column Washing Solution: 0.05 M Ammonium Acetate pH 5.4 ± 0.1

Column Washing Solution Preparation:

1. Mix 25 mL of 2 M ammonium acetate solution, pH 5.4 (Dionex P/N 033440) with 975 ± 1 mL of DI water (18.2 megohm-cm).
2. Degas on vacuum with sonication for 5 minutes.

4.5.3.4 Column Storage Solution: Acetonitrile

4.5.3.5 Degassing the Eluent

Before use, the eluents should be degassed. The degassing can also be done in-line either by using the Dionex GP40 or GP50 pump degas function, or using a solvent rack SOR-100 degasser (Dionex), or off-line by using a vacuum pump. Vacuum degas the solvent by placing the eluent reservoir in a sonicator and drawing vacuum on the filled reservoir with a vacuum pump for 5-10 minutes.

4.5.3.6 Filtering the Eluent

To extend the lifetime of your column as well as your HPLC pump, the eluents should be filtered using a 0.22 µm membrane filter to remove insoluble contaminants.

4.6. Examples of Protein Test Using Gradient Conditions

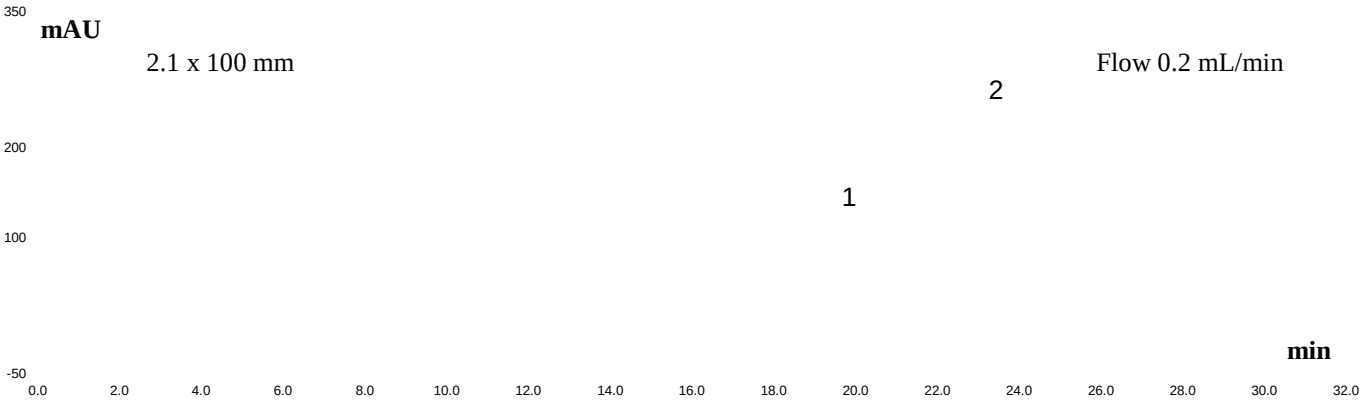
4.6.1. 2.1 x 100 mm Column Format

4.6.1.1. Gradient Method

Time	%A	%B	Comments
-45	0.0	100.0	Pre-wash with 100%B for 15 min. (This step is only needed for the first injection.)
-30	100.0	0.0	Preconditioning of the column before sample injection
0.0	100.0	0.0	Sample injection
2.0	100.0	0.0	Avoid gradients during sample injection
27.0	25.0	75.0	Gradient
27.1	0.0	100.0	Wash/Gradient
30.0	0.0	100.0	Wash
32.0	100.0	0.0	Re-equilibration to be ready for next sample injection

4.6.1.2. Test Chromatogram

WVL: 214 nm
Flow: 0.2 mL/min
Eluents: A: 2 M $(NH_4)_2 SO_4$ in 0.1 M NaH_2PO_4 (pH 7.0)
B: 0.1 M NaH_2PO_4 (pH 7.0)
Sample: Mixture of proteins (1 mg/mL each after 1:1 dilution with Eluent A)
Elution: 1. Ribonuclease A, 2. Lysozyme
Inj. Volume: 5 µl (Note that 1-2 µL is ideal for analytical runs.)



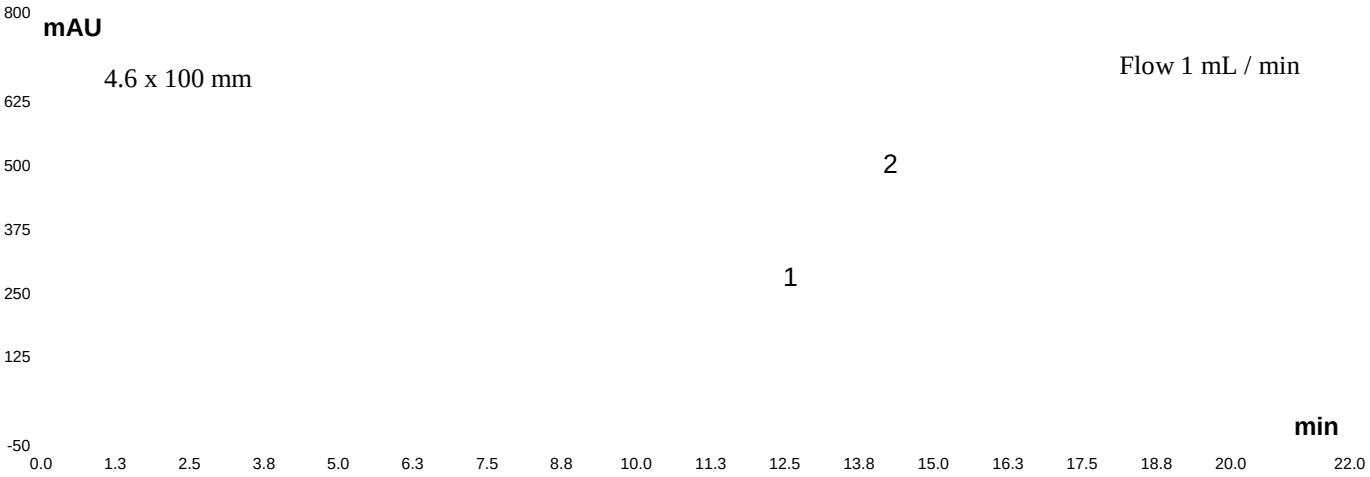
4.6.2. 4.6 x 100 mm Column Format

4.6.2.1. Gradient Method

Time	%A	%B	Comments
-25.0	0.0	100.0	Pre-wash with 100%B for 15 minutes. (This step is only needed for the first injection.)
-10.0	100.0	0.0	Preconditioning of the column before sample injection
0.0	100.0	0.0	Sample injection
2.0	100.0	0.0	Avoid gradients during sample injection
17.0	25.0	75.0	Gradient
17.1	0.0	100.0	Wash/Gradient
20.0	0.0	100.0	Wash
22.0	100.0	0.0	Re-equilibration to be ready for next sample injection

4.6.2.2. Test Chromatogram

WVL: 214 nm
Flow: 1 mL/min
Eluents: A: 2 M $(NH_4)_2 SO_4$ in 0.1M NaH_2PO_4 (pH 7.0)
B: 0.1 M NaH_2PO_4 (pH 7.0)
Sample: Mixture of proteins (1 mg/mL each after 1:1 dilution with eluent A)
Elution: 1. Ribonuclease A, 2. Lysozyme
Inj. Volume: 10 µl



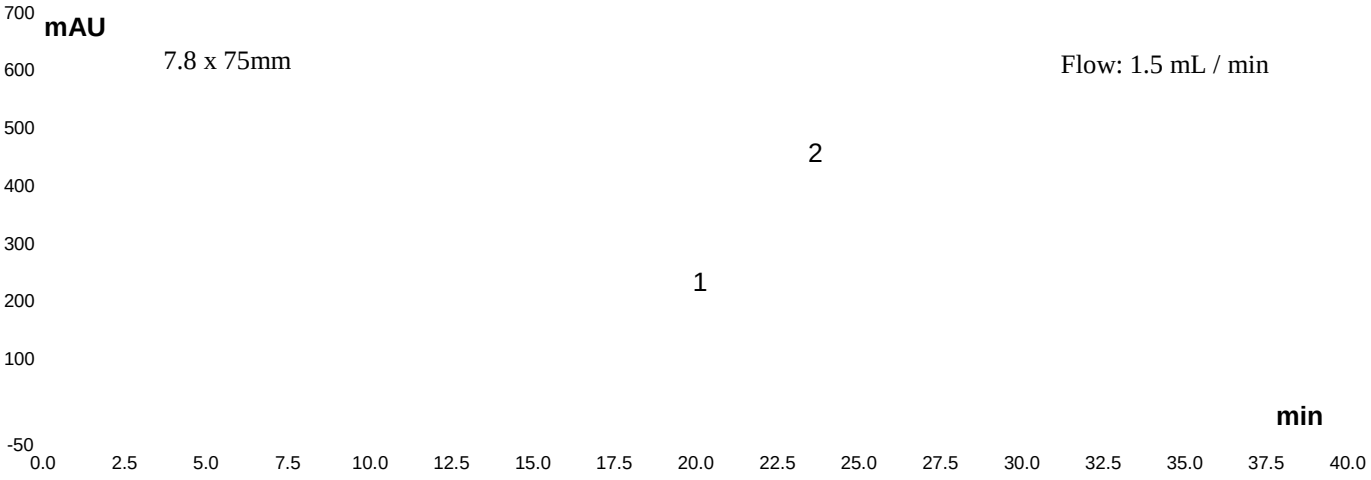
4.6.3. 7.8 x 75 mm Column Format

4.6.3.1. Gradient Method

Time	%A	%B	Comments
-25.0	0.0	100.0	Pre-wash with 100%B for 15 min. (This step is only needed for the first injection.)
-10.0	100.0	0.0	Preconditioning of the column before sample injection
0.0	100.0	0.0	Sample injection
2.0	100.0	0.0	Avoid gradients during sample injection
32.0	25.0	75.0	Gradient
33.0	0.0	100.0	Wash/Gradient
38.0	0.0	100.0	Wash
40.0	100.0	0.0	Re-equilibration to be ready for next sample injection

4.6.3.2. Test Chromatogram

WVL: 214 nm
Flow : 1.5 mL/min
Eluents: A: 2 M $(NH_4)_2 SO_4$ in 0.1 M NaH_2PO_4 (pH 7.0)
B: 0.1 M NaH_2PO_4 (pH 7.0)
Sample: Mixture of proteins (1 mg/mL each after 1:1 dilution with Eluent A)
Elution: 1. Ribonuclease A, 2. Lysozyme
Inj. Volume: 20 µL



5. METHOD DEVELOPMENT

5.1. Ionic Strength

Increasing ionic strength in the mobile phase (Eluent A) will result in increased retention times for analytes. If a decreased retention time is desired, reduce the ionic strength of Eluent A. First, start with 2 M $(NH_4)_2$ SO_4 in 0.1 M NaH_2PO_4 (pH 7.0). If the analyte eluted much later in the gradient, or did not elute at all, try reduced ionic strength 1 M or 0.5 M of $(NH_4)_2$ SO_4 in 0.1 M NaH_2PO_4 (pH 7.0). If $(NH_4)_2$ SO_4 in 0.1 M NaH_2PO_4 (pH 7.0) is not suitable for any particular application, one can use NaCl or other salts in the eluents.

5.2. Organic Modifier

Increasing organic modifier in the mobile phase generally results in decrease in retention for all analytes.

5.3. Gradient Methods for Different Formats

5.3.1. Stocks and Eluent Preparation

5.3.1.1. Eluent A

2 M $(NH_4)_2$ SO_4 in 0.1 M NaH_2PO_4 (pH 7.0)

Dissolve 264.2 g of ammonium sulfate (Sigma: A 4418) and 12 g of NaH_2PO_4 (Sigma: S8282) in 650 mL DI H_2O (18.2 megohm-cm). Adjust the pH to 7.0 with 50% NaOH and volume to 1 liter with DI H_2O.

5.3.1.2. Eluent B

0.1 M NaH_2PO_4 (pH 7.0)

Dissolve 12 g of NaH_2PO_4 in 900 mL of DI H_2O (18.2 megohm-cm). Adjust the pH to 7.0 with 50% NaOH and the volume to 1 liter with DI H_2O.

5.3.1.3. Eluent C

DI H_2O, or 0.05 M Ammonium Acetate (pH 5.4)

Mix 25 mL of ammonium acetate solution (2M stock solution), pH 5.4 (Dionex P/N 033440) with 975 mL of DI H_2O (18.2 megohm-cm) and adjust the volume to1 liter with DI H_2O.

5.3.1.4. Eluent D

30/70 (v/v) Acetonitrile/DI H_2O or
0.05 M Ammonium Acetate (pH 5.4 ± 0.1) in 30/70 (v/v) Acetonitrile/DI H_2O

Mix 25 mL of ammonium acetate (2 M stock solution), pH 5.4 (Dionex P/N 033440) with 675 mL with DI H_2O (18.2 megohm-cm).Degas by vacuum for 2 minutes with sonication, and add 300 mL of acetonitrile, and degas for 30 seconds.

5.3.1.5. Eluent E

70/30 (v/v) Acetonitrile/DI H_2O or
0.05 M Ammonium Acetate (pH 5.4 ± 0.1) in 70/30 (v/v) Acetonitrile/DI H_2O

Mix 25 mL of ammonium acetate (2 M stock solution), pH 5.4 (Dionex P/N 033440) with 275 mL with DI H_2O (18.2 megohm-cm). Degas by vacuum for 2 minutes with sonication, and add 700 mL of acetonitrile, and degas for 30 seconds.

5.4. Example Gradient Methods for Applications

5.4.1. 2.1 x 100 mm Column

Flow rate = 0.2 mL / min.

5.4.1.1. Gradient Method*

Time	%A	%B	Comments
-50.0	0.0	100.0	Pre-Wash (This step is only needed for the first injection.)
-30.0	100.0	0.0	Equilibration
0.0	100.0	0.0	Avoid gradients during sample injection
2.0	100.0	0.0	Sample injection
27.0	0.0	100.0	Gradient Elution
32.0	0.0	100.0	Wash
33.0	100.0	0.0	Re-equilibration to be ready for next sample injection

*Gradient conditions may be changed accordingly for different analytes to get desired separations.

5.4.2. 4.6 x 100 mm Column

Flow rate = 0.5 mL/min.

5.4.2.1. Gradient Method*

Time	%A	%B	Comments
-50.0	0.0	100.0	Pre-Wash (This step is only needed for the first injection.)
-30.0	100.0	0.0	Equilibration
0.0	100.0	0.0	Avoid gradients during sample injection
2.0	100.0	0.0	Sample injection
22.0	0.0	100.0	Gradient Elution
27.0	0.0	100.0	Wash
30.0	100.0	0.0	Re-equilibration to be ready for next sample injection

* Flow rates and gradient conditions may be changed accordingly for different analytes to get desired separations. Flow rates up to 1.0 mL/min can be used and run times can be shortened accordingly (for 4.6 mm x 100 mm).

5.4.3. 7.8 x 75 mm Column

Flow rate = 1 mL/min.

5.4.3.1. Gradient Method*

Time	%A	%B	Comments
-50.0	0.0	100.0	Pre-Wash (This step is only needed for the first injection.)
-30.0	100.0	0.0	Equilibration
0.0	100.0	0.0	Avoid gradients during sample injection
2.0	100.0	0.0	Sample injection-
62.0	0.0	100.0	Gradient Elution
67.0	0.0	100.0	Wash
70.0	100.0	0.0	Re-equilibration to initial conditions

* Flow rates and gradient conditions may be changed accordingly for different analytes to get desired separations. Flow rates up to 1.5 mL/min can be used and run times can be shortened accordingly (for 7.8 x 75 mm).

5.5. Dual Gradient Methods

WARNING

a) It is very important to note that "Step to Wash the high salt buffer" is absolutely needed before the second solvent gradient (from Eluents C and D) is started. Since, high salt concentration is used in Eluent A, if you are using a dual pump (even when you are using an individual pump for each eluent), the mixing of high salt containing eluents with organic solvents should be avoided.

b) DO NOT USE 100% ORGANIC SOLVENTS for the second gradient.
Unlike reverse phase columns, HIC-10 is a weak hydrophobic column; therefore often ≤ 30% (V/V) solvent concentration is sufficient to elute tightly bound proteins (if present).

c) The above gradient program is just an example; the program can be altered according to the protein of interest.

d) Flow rates and gradient conditions may be changed accordingly for different analytes to get desired separations. Flow rates up to 1.0 mL/min (for 4.6x 100 mm) can be used and run times can be shortened accordingly.

5.5.1. 4.6 x 100 mm Column

Flow rate* = 0.5 mL/min.

Example of a Dual Gradient Method

Time	%A	%B	%C	%D	Comments
-50.0	0.0	100.0	0.0	0.0	Pre-Wash (This step is only needed for the first injection.)
-30.0	100.0	0.0	0.0	0.0	Equilibration
0.0	100.0	0.0	0.0	0.0	Avoid gradients during sample injection
2.0	100.0	0.0	0.0	0.0	Sample Injection
22.0	0.0	100.0	0.0	0.0	First Gradient
27.0	0.0	100.0	0.0	0.0	Step to Wash the high salt buffer
27.01	0.0	0.0	100.0	0.0	Getting ready for solvent wash
35.0	0.0	0.0	100.0	0.0	Getting ready for solvent wash
60.0	0.0	0.0	0.0	100	Second Gradient
65.0	0.0	0.0	0.0	100	Solvent wash
65.01	0.0	0.0	100.0	0.0	Remove solvent from column
75.0	0.0	0.0	100.0	0.0	Remove solvent from column
75.01	0.0	100.0	0.0	0.0	Getting ready for the next sample
80.0	0.0	100.0	0.0	0.0	Getting ready for the next sample
80.01	100.0	0.0	0.0	0.0	Ready for the next injection

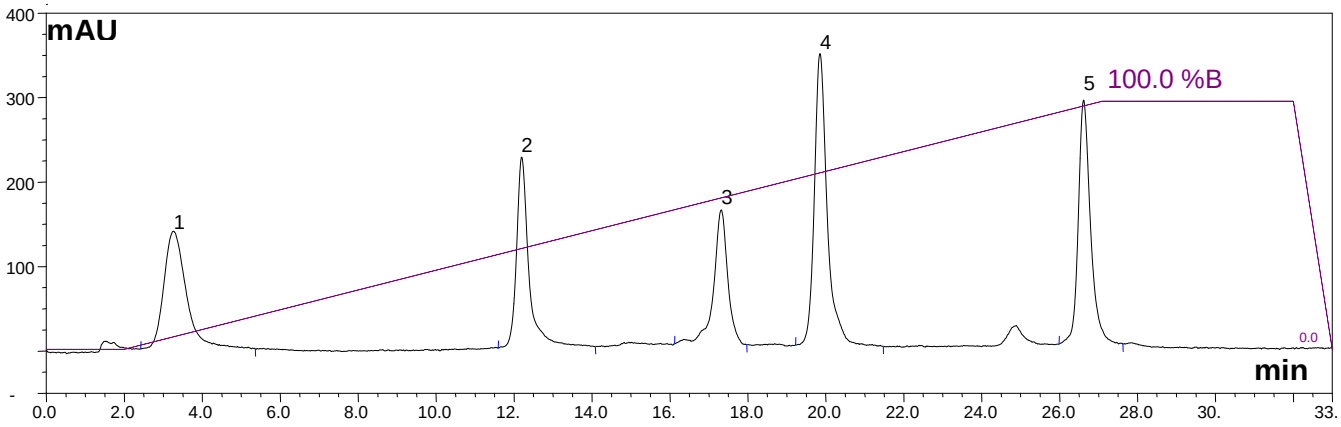
* 1. Flow rates and gradient conditions may be changed accordingly for different analytes to get desired separations.
  2. Similar dual gradients can be used for other formats. But make sure that the flow rate is compatible for the format used.

6. APPLICATIONS

6.1. Mixture of Protein Standards

6.1.1. Mixture of Protein Standards: 2.1 x 100 mm Column*

Column: 2.1 x 100 mm
WVL: 214 nm
Flow: 0.2 mL/min
Eluents: A: 2 M $(NH_4)_2 SO_4$ in 0.1 M NaH_2PO_4 (pH 7.0)
B: 0.1 M NaH_2PO_4 (pH 7.0)
Sample: Mixture of five proteins (1 mg/mL each final after 1:1 dilution with Eluent A)
Inj.Vol.: 5 µl

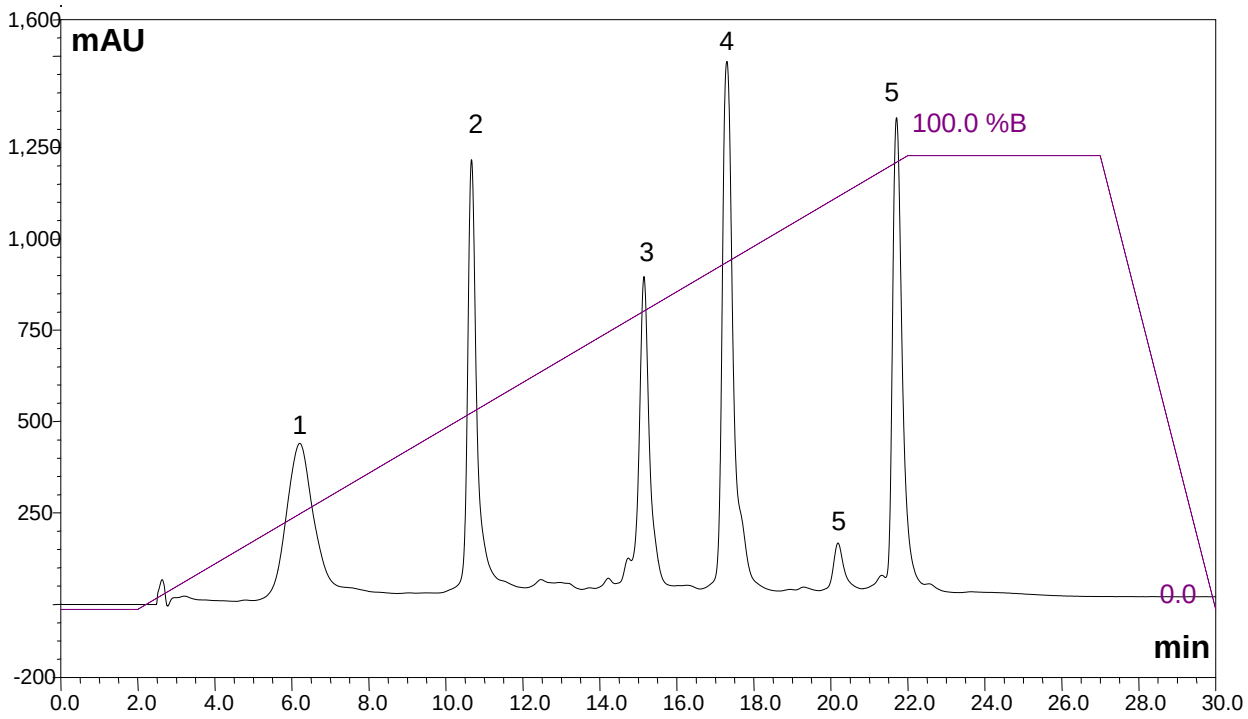


Order of Elution:
1. Cytochrome C
2. Myoglobin
3. Ribonuclease A
4. Lysozyme
5. Chymotrypsinogen

*Gradient conditions may be changed accordingly for different analytes to get desired separations. The ideal sample injection volume is 1-2 µl for analytical runs.

6.1.2. Mixture of Proteins: 4.6 x 100 mm Column*

Column: 4.6 x 100 mm
WVL: 214 nm
Flow: 0.5 mL/min
Eluents: A: 2 M $(NH_4)_2 SO_4$ in 0.1 M NaH_2PO_4 (pH 7.0)
B: 0.1 M NaH_2PO_4 (pH 7.0)
Sample: Mixture of five proteins (1 mg/mL each final after 1:1 dilution with Eluent A)
Inj. Volume: 20 µl



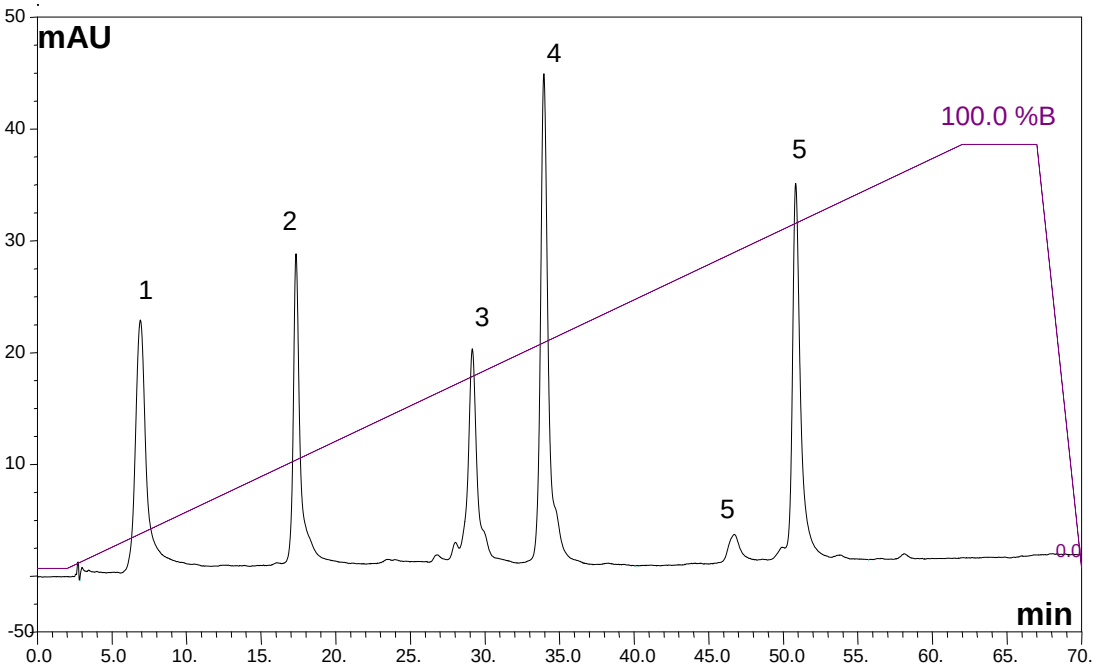
Order of Elution:

1. Cytochrome C
2. Myoglobin
3. Ribonuclease A
4. Llysozyme
5. Chymotrypsinogen

*Flow rates and gradient conditions may be changed accordingly for different analytes to get desired separations. Flow rates up to 1.0 mL/min (for 4.6 x 100 mm) can be used and run times can be shortened accordingly.

6.1.3. Mixture of Protein Standards: 7.8 x 75 mm Column*

Column: 7.8 x 75 mm format
WVL: 214 nm
Flow: 1.0 mL/min
Eluents: A: 2 M $(NH_4)_2$ SO_4 in 0.1 M NaH_2P_{O4} (pH 7.0)
B: 0.1 M NaH_2PO_4 (pH 7.0)
Sample: Mixture of five proteins (1 mg/mL each final after 1:1 dilution with Eluent A)
Inj. Volume: 20 µl



Order of Elution:

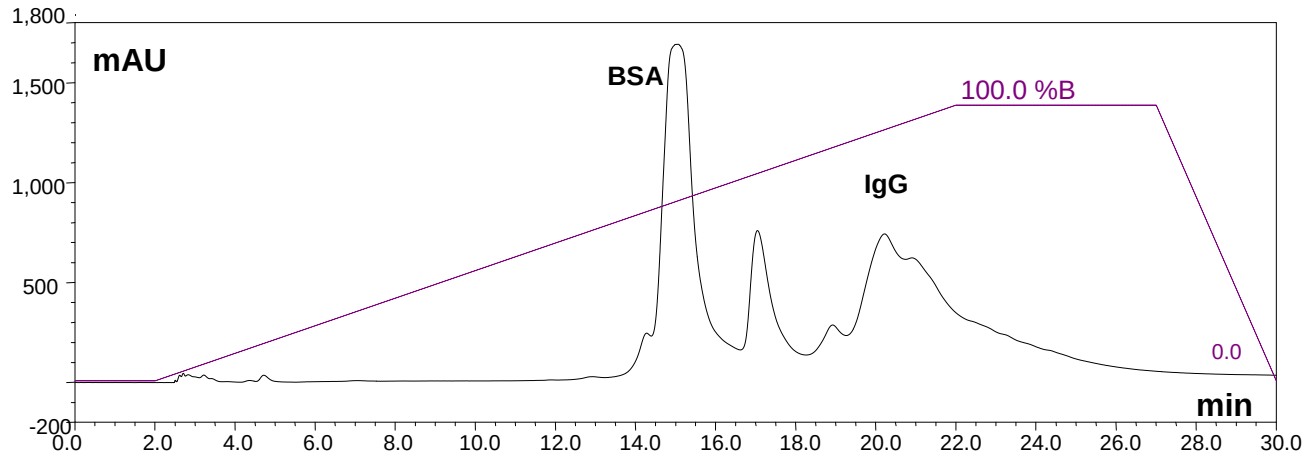
1. Cytochrome C
2. Myoglobin
3. Ribonuclease A
4. Lysozyme
5. Chymotrypsinogen

*Flow rates and gradient conditions may be changed accordingly for different analytes to get desired separations. Flow rates up to 1.5 mL/min (for 7.8 x 75 mm) can be used and run times can be shortened accordingly.

6.2. Separation of Bovine Serum Proteins

6.2.1. Separation of Bovine Serum Proteins: 4.6 x 100 mm Column*

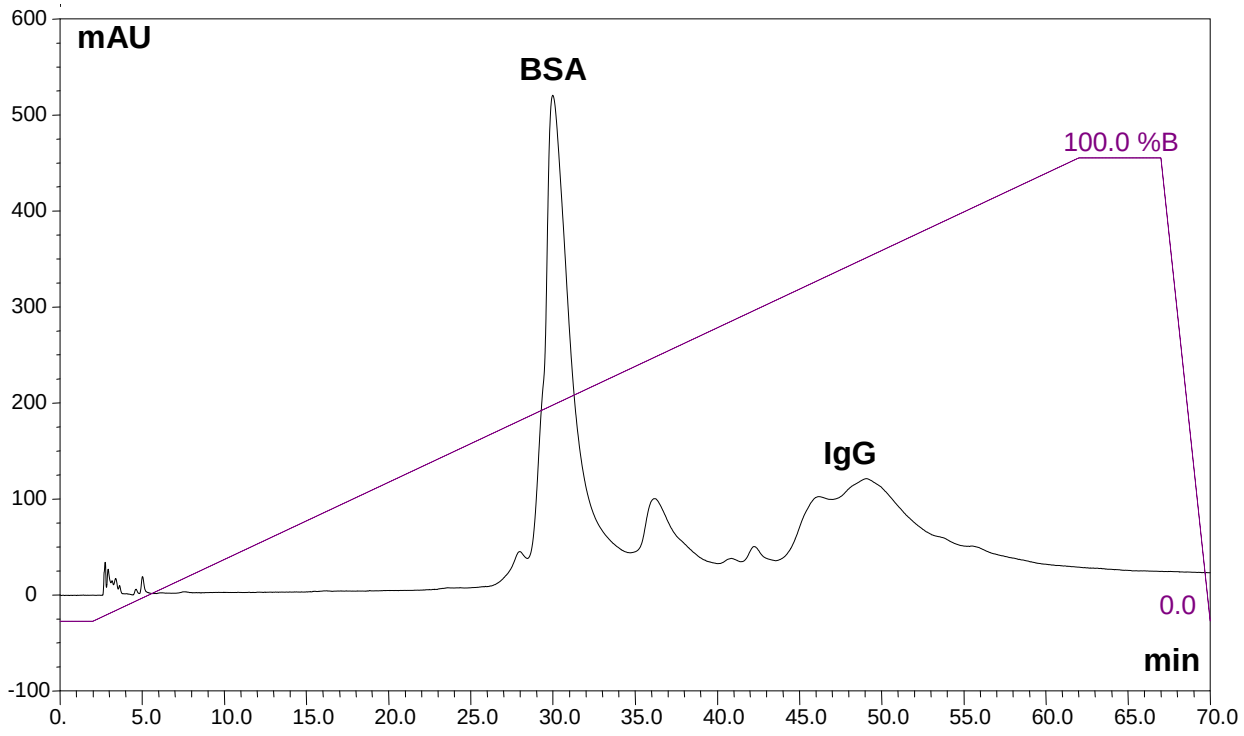
Column:	4.6 x 100 mm
WVL:	214 nm
Flow:	0.5 mL/min
Eluents:	A: 2 M $(NH_4)_2$ SO_4 in 0.1 M NaH_2PO_4 (pH 7.0) B: 0.1 M NaH_2PO_4 (pH 7.0)
Sample:	Bovine serum (1:2 diluted with DI H_2O and mixed with equal volume of 1 M $(NH_4)_2$ SO_4 in 0.1 M NaH_2PO_4 (pH 7.0)
Inj. Volume:	20 µl



*Flow rates and gradient conditions may be changed accordingly for different analytes to get desired separations. Flow rates up to 1.0 mL/min (for 4.6 x 100 mm) can be used and run times can be shortened accordingly. In this example column is overloaded with the sample.

6.2.2. Separation of Bovine Serum Proteins: 7.8 x 75 mm Column*

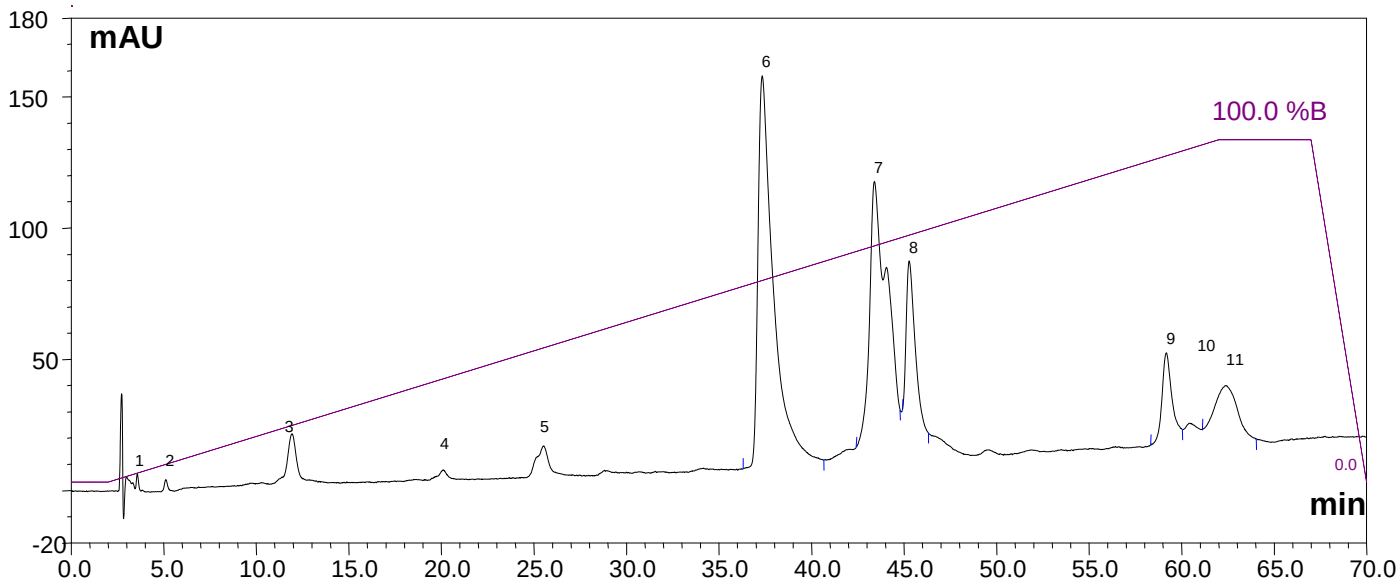
Column:	7.8 x 75 mm
WVL:	214 nm
Flow:	1.0 mL/min
Eluents:	A: 2 M $(NH_4)_2 SO_4$ in 0.1M NaH_2PO_4 (pH 7.0) B: 0.1 M NaH_2PO_4 (pH 7.0)
Sample:	Bovine serum (1:2 diluted with DI H_2O and mixed with equal volume of 1 M $(NH_4)_2 SO_4$ in 0.1 M NaH_2PO_4 (pH 7.0)
Inj. Volume:	20 µl



*Flow rates and gradient conditions may be changed accordingly for different analytes to get desired separations. Flow rates up to 1.5 mL/min (for 7.8 x 75 mm) can be used and run times can be shortened accordingly.

6.3. Separation of Snake Venom Proteins / Peptides*

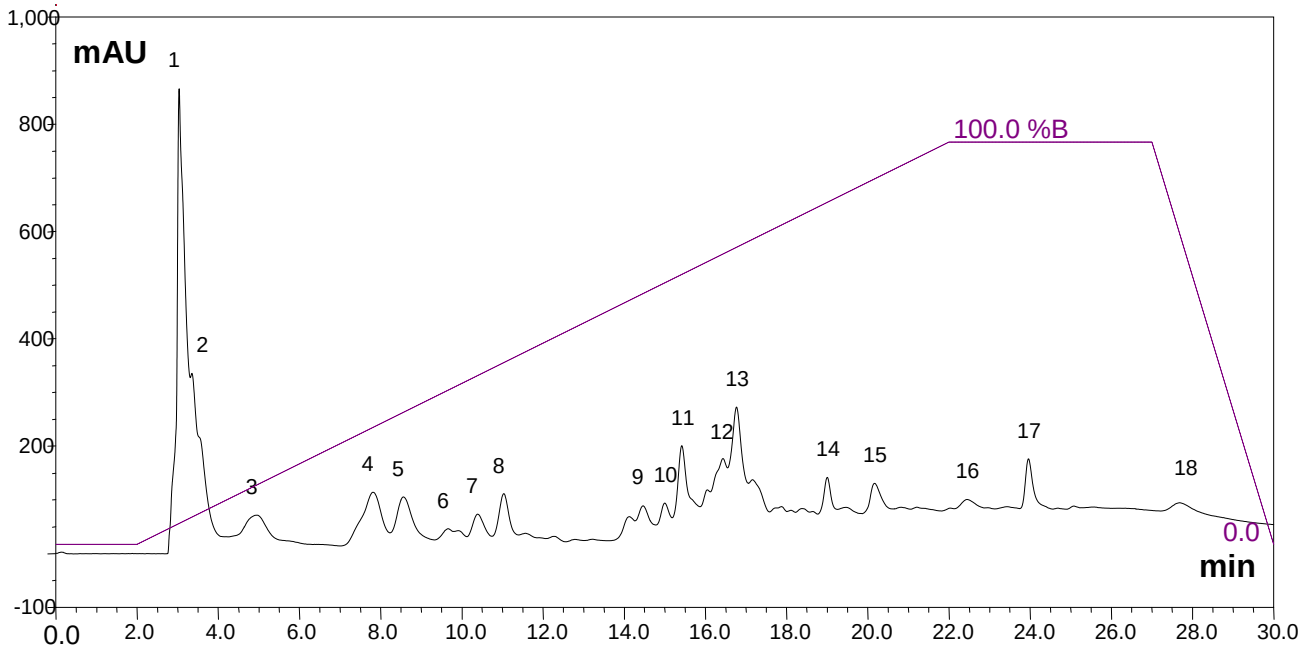
Column:	7.8 x 75 mm format
WVL:	214 nm
Flow:	1.0 mL/min
Eluents:	A: 2 M $(NH_4)_2$ SO_4 in 0.1M NaH_2PO_4 (pH 7.0)
	B: 0.1 M NaH_2PO_4 (pH 7.0)
Sample:	Naja venom from Sigma [1: 1 diluted with 1 M $(NH_4)_2$ SO_4 in 0.1M NaH_2PO_4 pH 7.0)]
Inj. Volume:	20 µl



*1. The identities of individual peaks are not assigned.
2. Flow rates and gradient conditions may be changed accordingly for different analytes to get desired separations. Flow rates up to 1.5 mL/min (for 7.8 x 75 mm) can be used and run times can be shortened accordingly.

6.4. HIC Separation of Peptides - Proteomics Application*

Column: 4.6 x 100 mm
Flow: 0.5 mL/min
WVL: 214 nm
Eluents: A: 2 M $(NH_4)_2 SO_4$ in 0.1 M NaH_2PO_4 (pH 7.0)
B: 0.1 M NaH_2PO_4 (pH 7.0)
Protein Sample: Trypsin digest (720 µg) of BSA (Protein /Trypsin 100:1)
Injection volume: 160 µl



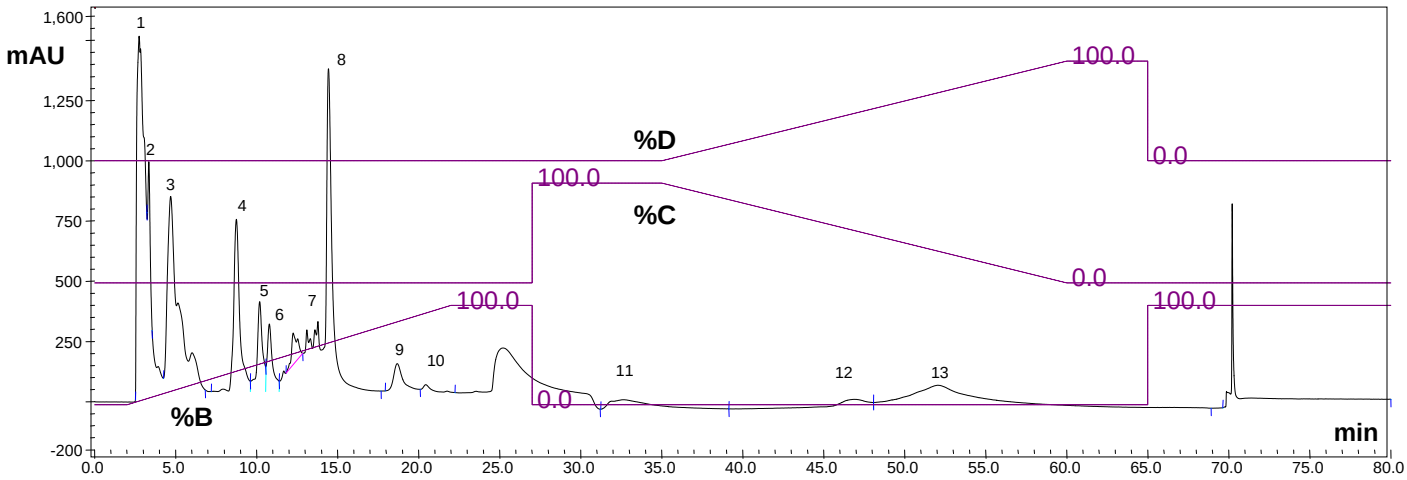
*1. The identities of individual peaks are not assigned.
2. Flow rates and gradient conditions may be changed accordingly for different analytes to get desired separations. Flow rates up to 1.0 mL/min (for 4.6 x 100 mm) can be used and run times can be shortened accordingly.

6.5. HIC Separation of Peptides - Proteomics Application (Dual Gradient)*

Column:	4.6 x 100 mm
Flow:	0.5 mL/min
WVL:	214 nm
Eluents:	A: 2 M $(NH_4)_2$ SO_4 in 0.1 M NaH_2PO_4 (pH 7.0)
	B: 0.1 M NaH_2PO_4 (pH 7.0)
	C: DI H_2O**
	D: 70/30 (V/V) MeCN / DI H_2O
Sample:	Trypsin digest (1.2 mg) of Cytochrome C (50 µl protein digest +
	50 µl 8 M urea/Tris pH 8.0+100 µl PBS + 100 µl Eluent A)
Inj. Volume:	40 µl

**For better reproducibility and for the stability of the phase, 0.05 M ammonium acetate (pH 5.4) may be used instead of DI H_2O.

Dual Gradient: 0-100%B in 20 min – After Equilibration- 0-100%D in 25 min



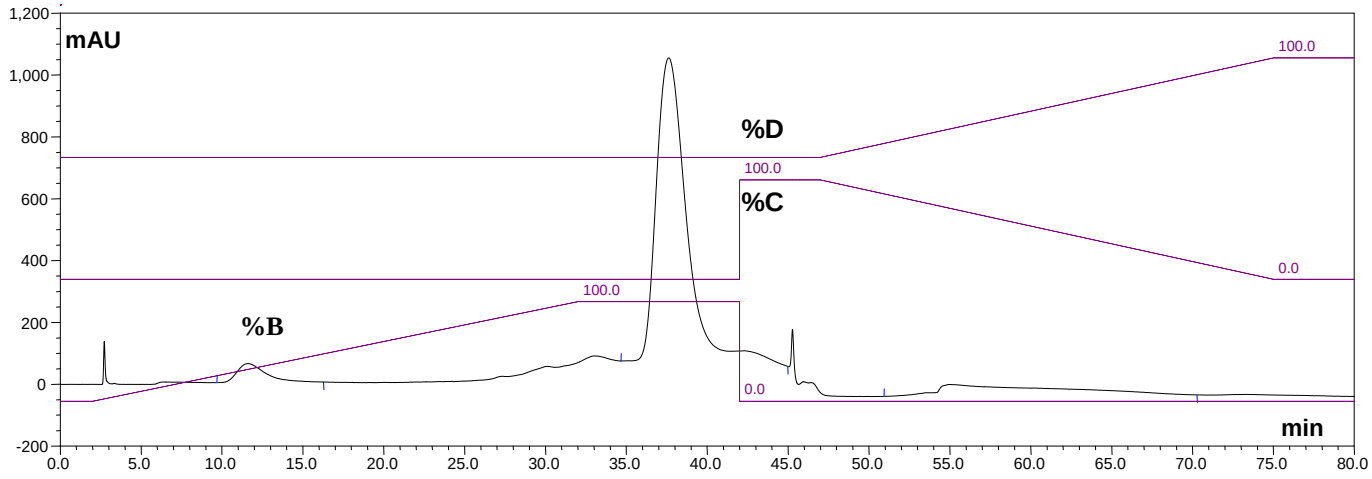
*1. The identities of individual peaks are not assigned.
2. Flow rates and gradient conditions may be changed accordingly for different analytes to get desired separations. Flow rates up to 1.0 mL/min (for 4.6 x 100 mm) can be used and run times can be shortened accordingly.

6.6. HIC Separation of Monoclonal Antibody (MAb)*

6.6.1. 7.8 x 75 mm Column Format – Dual Gradient

Column:	7.8 x 75 mm
Flow:	1 mL/min
WVL:	214 nm
Eluents:	A: 1 M $(NH_4)_2$ SO_4 in 0.1M NaH_2PO_4 (pH 7.0)
	B: 0.1 M NaH_2PO_4 (pH 7.0)
	C: DI H_2O**
	D: 35/65 (V/V)MeCN / DI H_2O
Sample:	Monoclonal Antibody (MAb) 100 µl (50 mg/mL) + 200 µl PBS +
	300 µl 2M $(NH_4)_2$ SO_4 in 0.1 M NaH_2PO_4 (pH 7.0)
Inj. volume:	20 µL

Dual Gradient: 0-100%B in 30 min –After Equilibration: 0-100%D in 28 min

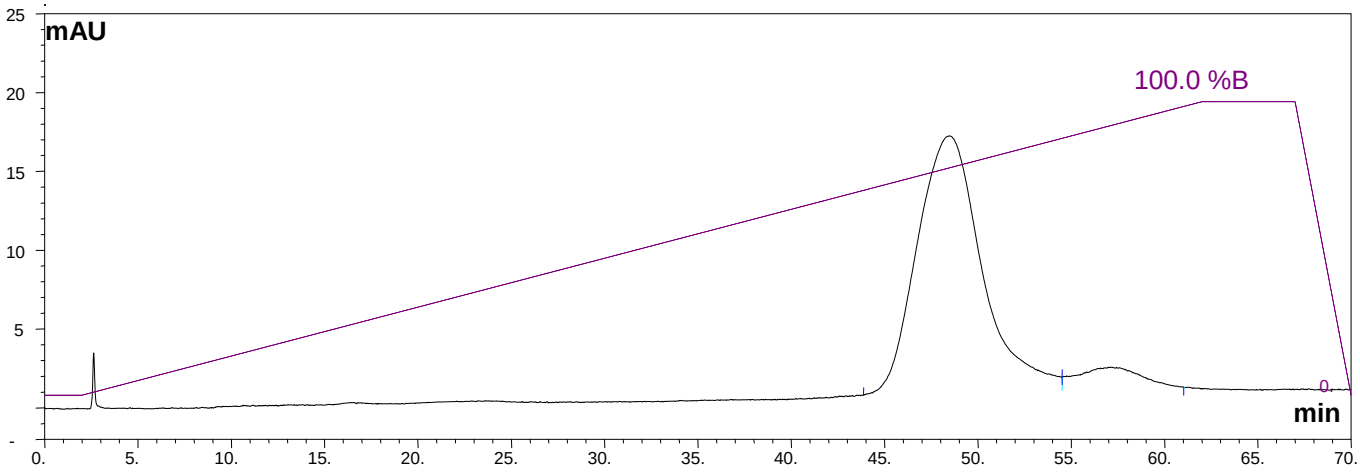


*Flow rates and gradient conditions may be changed accordingly for different analytes to get desired separations. Flow rates up to 1.5 mL/min (for 7.8 x 75 mm) can be used and run times can be shortened accordingly.

**For better reproducibility and for the stability of the phase 0.05 M ammonium acetate (pH 5.4) may be used instead of DI H_2O.

6.6.2. 7.8 x 75 mm Column*

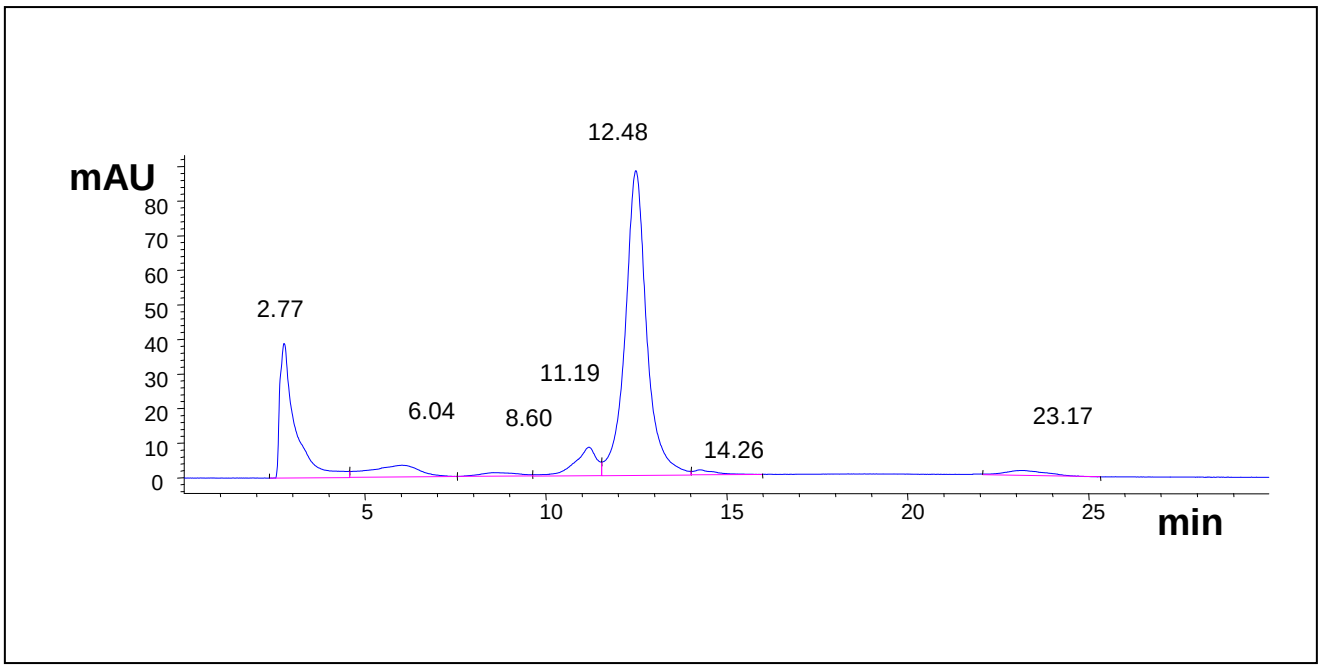
Column:	7.8 x 75 mm
Flow:	1 mL/min
WVL:	214 nm
Eluents:	A: 0.5 M $(NH_4)_2$ SO_4 in 0.1 M NaH_2PO_4 (pH 7.0) B: 0.1 M NaH_2PO_4 (pH 7.0)
Sample:	Monoclonal Antibody (MAb) MAb50 µL (50 mg/mL) + 450 µL DI H_2O + 500 µL 1 M $(NH_4)_2SO_4$ in 0.1 M NaH_2PO_4 (pH 7.0)
Inj. volume:	20 µL (50 µg)
Gradient:	0-100%B in 60 min.



*Flow rates and gradient conditions may be changed accordingly for different analytes to get desired separations. Flow rates up to 1.5 mL/min (for 7.8 x 75 mm) can be used and run times can be shortened accordingly.

6.7. Separation of Degradation Products (Oxidation) in MAb Samples on Accelerated Stability

Column:	4.6 x 100 mm
Flow:	0.75 mL/min
WVL:	220 nm
Eluents:	A: 1 M $(NH_4)_2$ SO_4 in 0.1 M NaH_2PO_4 (pH 7.0)
	B: 0.1 M NaH_2PO_4 (pH 7.0)
Sample:	MAb sample
Inj. volume:	100 µL (50 ug)

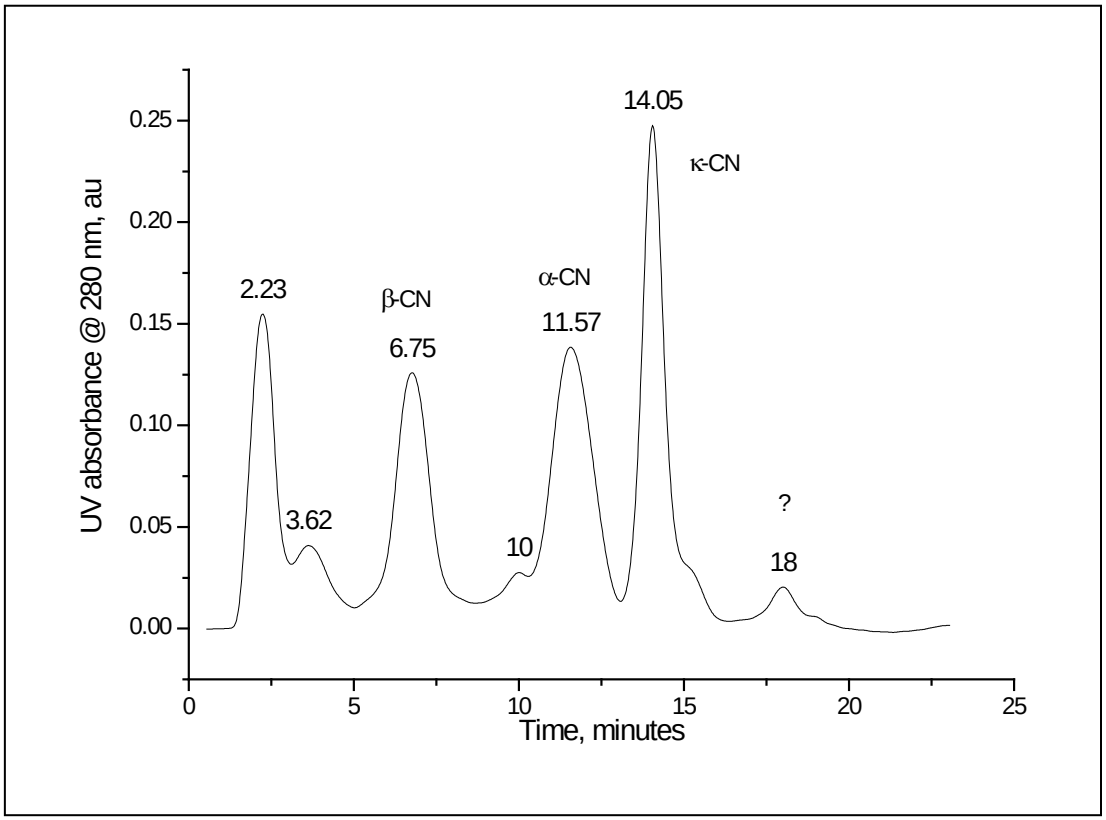


6.8. Separation of Casein Mixture*

Column: 7.8 x 75 mm
WVL: 280 nm
Flow: 1.5 mL/min
Eluents: **A**: 2 M $(NH_4)_2$ SO_4 in 0.1 M NaH_2PO_4 (pH 7.0) and 8 M Urea
B: 0.1 M NaH_2PO_4 (pH 7.0) and 8M urea
Inj. Vol: 20 µL
Temperature: 23 ± 1 °C
Sample: Commercial casein mixture (alfa, beta, kappa-casein 1 mg/mL each)
All the samples were diluted 1:10 in 70% solvent A- 30%-solvent B before injection.

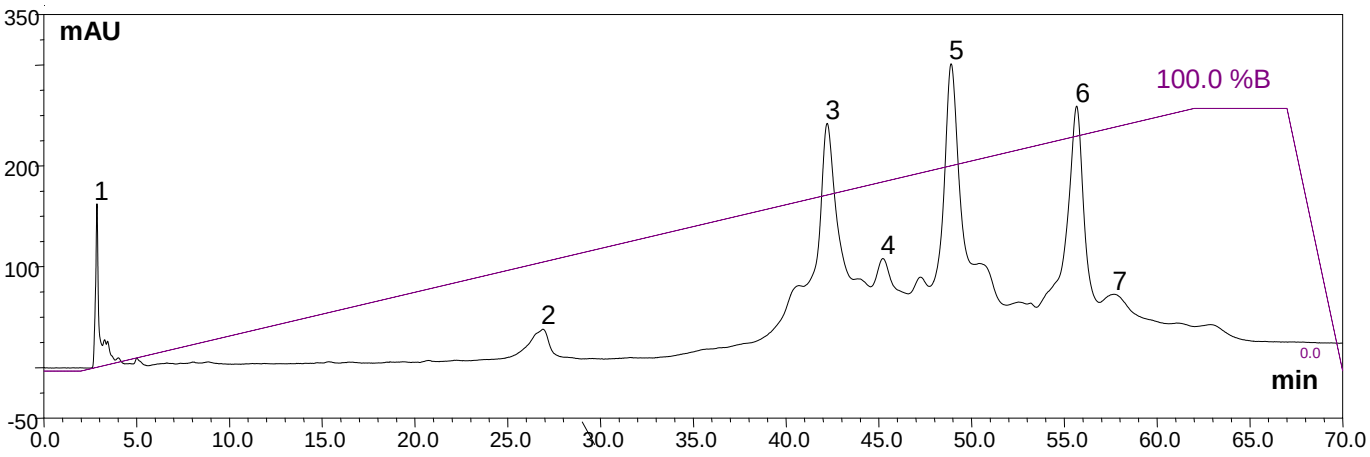
* These data are provided by Dr.Emilia Bramanti, Laboratory of Instrumental Analytical Chemistry, Institute for Chemical and Physical Processes (IPCF), CNR 56124 PISA Italy

Elution Conditions:
3 min isocratic elution in 70% A-30% B + 5 min linear gradient to 50%A-50%B, isocratic elution for 3 min in 50%A-50%B + linear gradient to 100% B.



6.9. Thrombin Separation*

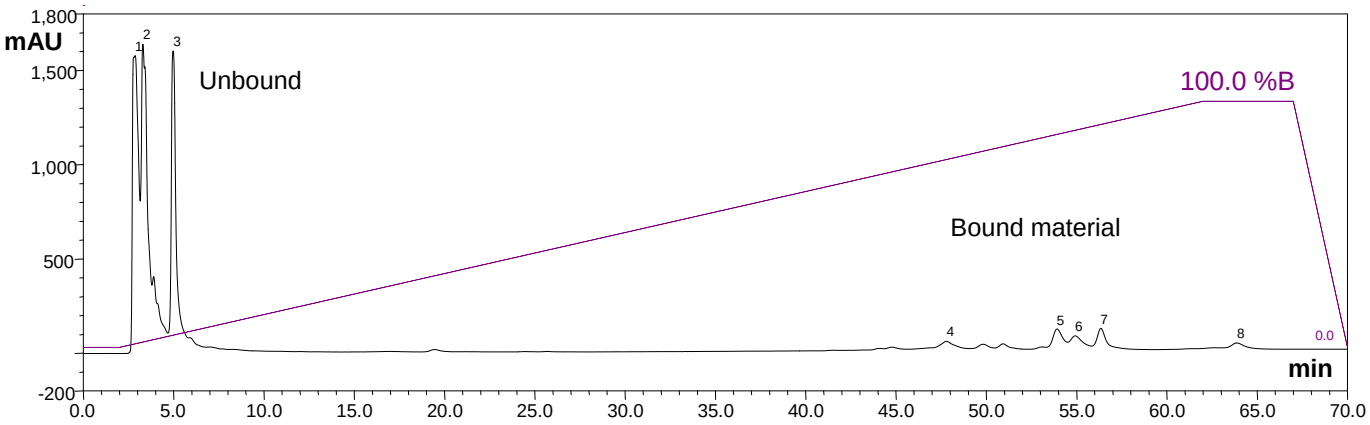
Column:	7.8 x 75 mm
Flow:	1.0 mL/min
WVL:	214 nm
Eluents:	A: 2 M $(NH_4)_2$ SO_4 in 0.1 M NaH_2PO_4 (pH 7.0)
	B: 0.1 M NaH_2PO_4 (pH 7.0)
Inj. volume:	20 µl
Sample:	Thrombin 3 mg/mL (Sigma): Thrombin is a serine proteinase that converts fibrinogen to fibrin monomers in the blood coagulation process. It activates Factor XIII and interacts with other coagulation factors, initiates platelet secretion and aggregation. α-thrombin is biologically active. β and γ are not.



*1. The identities of individual peaks are not assigned.
2. Flow rates and gradient conditions may be changed accordingly for different analytes to get desired separations. Flow rates up to 1.5 mL/min (for 7.8 x 75 mm) can be used and run times can be shortened accordingly.

6.10. Pancreatin Separation*

Column:	7.8 x 75 mm
Flow:	1.0 mL/min
WVL:	214 nm
Eluents:	A: 2 M $(NH_4)_2$ SO_4 in 0.1 M NaH_2PO_4 (pH 7.0), B: 0.1 M NaH_2PO_4 (pH 7.0)
Sample:	Pancreatin (Sigma) (25 mg/ml supernatant) [Complex protein mixture from pancreas containing many enzymes including amylase, trypsin, lipase, ribonuclease and proteases]
Inj. volume:	20 µL



*1. The identities of individual peaks are not assigned.
2. Flow rates and gradient conditions may be changed accordingly for different analytes to get desired separations. Flow rates up to 1.5 mL/min (for 7.8 x 75 mm) can be used and run times can be shortened accordingly.

6.11. 2D - Application-Human Skeletal Muscle Protein (HSMP) Separation

Columns: First dimension: 4.6 x 100 mm (ProPac HIC-10)
Second dimension: 2.0 x 250 mm (ProPac WCX-10, from Dionex)
Individual fractions from HIC applied on to 2 mm x 250 mm (WCX-10, from Dionex Corp.) in the second dimension. (Data not shown.)

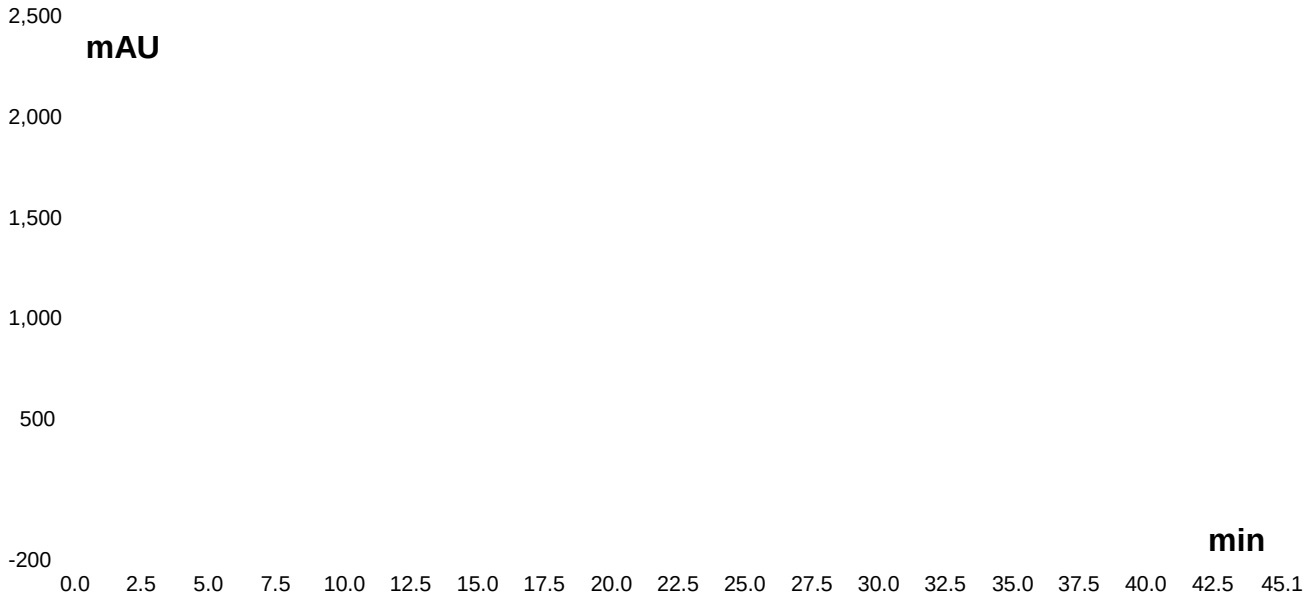
First Dimension Chromatography on HIC-10 Column

Flow: 0.4 mL/min
WVL: 214 nm
Eluents: A: 2 M $(NH_4)_2$ SO_4 in 0.1 M NaH_2PO_4 (pH 7.0)
B: 0.1 M NaH_2PO_4 (pH 7.0)
Sample: HSMP 2.5mg
Inj. Volume: 250 µl

3 minute fractions were collected, desalted by centricon filter units (10 kDa cut off).

Individual fractions are applied on to the ion-exchange column to separate further in the second dimension. Further, each fraction from the second dimension will be desalted, digested with trypsin and identify the proteins by fingerprinting using Mass Spectrometry.

HIC Separation in the First Dimension



7. CARING FOR THE COLUMN

These guidelines should be followed to ensure the high performance of the HIC-10 column.

Never load turbid / precipitated samples without filtering.

1. Always degas and filter mobile phases through a 0.22-µm membrane filter.
2. Always filter the samples with 0.22 µm syringe filter.
3. When switching to a new mobile phase, the column should be equilibrated as suggested in the method.
4. The pH range is from pH 2.5 to 7.5. However, it is preferred that the column be used at pH range 5.0-7.0 to achieve a longer column life time.
5. We recommend washing the column with an aqueous buffer after use (with 0.05 M ammonium acetate, pH 5.4, or other buffer, pH 5-7) instead of washing with DI water alone to achieve a longer column life time.
6. We recommend that the column be stored in 100% Acetonotrile, Acetronitrile/DI H_20 for overnight as well as long time storage to achieve longer column life time.
7. Be sure that solvents are miscible when changing mobile phases. This is especially important when using dual gradients. Since HIC uses high salt concentrations in Eluent A, it is very, very important to avoid mixing solvent with high salt solution as precipitation occurs.
8. The recommended operating temperature is ambient room temperature (<40 °C).
9. The recommended maximum backpressure limit is 3500 psi.

8. TROUBLESHOOTING GUIDE

The following instructions should help you to locate and eliminate problems traceable to hardware and chemistry issues. It also provides a selection of cleanup and reconditioning procedures that have been found effective by many users. Please keep in mind that some problems may be due to other reasons, such as sample contamination, poor water quality, etc. If you cannot solve your problem with the help of this manual, please contact the DIONEX North America Technical Center at 1-800 DIONEX-0 or the nearest DIONEX office (see, "DIONEX Worldwide Offices" on the Dionex Reference Library CD-ROM).

8.1. High Backpressure

8.1.1. Finding the Source of High System Pressure

First, find out whether the backpressure is caused by the column or not by simply checking the system pressure without using the column. If the system pressure is excessively high, determine the cause of the high pressure. Make sure that the pump is set to the correct flow rate. Higher than recommended mobile phase flow rates will cause higher pressure. Measure the pump flow rate if necessary with a stop watch and graduated cylinder.

1. Determine which part of the system is causing the high pressure. It could be a piece of tubing that has plugged, collapsed tubing walls from over tightening, an injection valve with a plugged port, a column with particulates plugging the bed support, a plugged High-pressure In-line filter, or the detector cell. To identify which part of the chromatographic system is causing the problem, disconnect the pump fluid line from the injection valve and turn the pump on. Watch the pressure; it should not exceed 50 psi (0.34 MPa). Continue adding the system components back into the fluid path, one by one, while watching the system pressure.
2. Check the mobile phases for contamination.

8.1.2. A Contaminated Column – Clean-up Procedure

WARNING

Do not clean the column with NaOH or any high pH (>pH 7.5) eluents.

1. If a contaminated column is causing the backpressure, it can be cleaned by using various cleaning procedures.
2. However, it can be successfully cleaned with organic solvents or using aqueous solvents with proteolytic enzymes. Disconnect and reverse the column assembly, so that the mobile phase enters the column 'outlet end' and the wash passes from the 'inlet end' and goes directly into waste. First wash the column with 50 mM NH_4HCO_3 (pH 7.0) and inject pepsin (sigma) repeatedly and see whether backpressure is lowered. One can load a mixture of proteolytic enzymes (100 µg) in the column and let it stand overnight, followed by a thorough wash with the eluent and later with 0.05 ammonium acetate, pH 5.4.
3. Hydrophobic contaminants may be cleaned with an acetonitrile or methanol or acetone gradient.
4. Reassemble the column back into the correct orientation.

9. REFERENCES

1. A. Tiselius. *Arkiv for Kemi, Mineralogi Geologi* **26B** 1 (1948)
2. S. Shaltiel and Z. Er-el. *Proc. Nat. Acad. Sci. US*., **70**: 778 (1973)
3. Z. Er-el, Y. Zaidenzaig and S. Shaltiel. *Biochem. Biophys. Res. Commun*., **49**: 383 (1972)
3. A.J. Alpert. *J. Chromatogr*. **359**: 85 (1986)
4. Z. Hrkal, J. Rejnkova. *J. Chromatogr*. **242**: 385 (1982)
5. R.D. McNair, A.J. Kenny. *Biochem. J*. **179**: 379 (1979)
6. D.E. Comings, A.G. Miguel, H.H. Lesser. *Biochim. Biophys. Acta* **563**: 25 (1979)
7. M. Belew, M. Yafang, L. Bin, J. Berglof, J.-C. Janson. *Bioseparation* **1**: 397 (1991)
8. S.C. Goheen and R. S. Matson. *J. Chromatogr* **326**: 235 (1985)
9. B.S. Warren, P. Kusk, R.G. Wolford, G.L. Hager. *J. Biol. Chem*. **271**: 11434 (1996)
10. G. Karlsson. *Prot. Exp. and Purif.* **27**:171 (2003)
11. E. Bramanti, C. Sortino, M. Onor, F. Beni and G. Raspi. *J. Chromatogr A*: **994**: 59 (2003)
12. Y. Yamasaki, T. Kitamura and Y. Kato. Poster #420 presented at the 9th International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Philadelphia, 6-9, Nov (1989)
13. C. Su and E.H. Oliw. *J. Biol. Chem*. **273**: 13072 (1998)
14 E. Eichhorn, J.R. Van Der Ploeg and T. Leisinger. *J. Biol. Chem*. **274**: 26639 (1999)

10. ATTACHMENTS

ProPac® HIC-10
Bonded Silica
5µm 300Å (4.6 x 100 mm)
Product No. 063655

Date: 07-Jul-05 07:56
Serial No. : 000002
Lot No. : 05-03-006

Mobile Phase: 0.10 M Ammonium acetate, pH 5.4 ± 0.1 in 30/70 (v/v) Acetonitrile/DI H2O
Flow Rate: 1.00 mL/min **Temperature:** 30 °C
Detection: UV, 230 nm **Injection Volume:** 5.0 µL

Storage Solution: 0.05 M Ammonium acetate, pH 5.4 ± 0.1 in 80/20 (v/v) Acetonitrile/DI H2O

No.	Peak Name	Ret.Time (min)	Asymmetry (EP)	Efficiency (EP)	Concentration
1	Cytosine	1.31	1.39	8978	0.025 mg/mL
2	Amitriptyline	1.43	1.33	7792	0.025 mg/mL
3	4-Butylbenzoic acid	2.03	1.33	6955	0.025 mg/mL
4	Phenanthrene	3.10	1.25	6687	0.025 mg/mL

QA Results:

Analyte	Parameter	Specification	Results
Phenanthrene	Efficiency	>=4,050	Passed
Phenanthrene	Asymmetry	0.95-1.65	Passed
Phenanthrene	Retention Time	2.98-3.42	Passed
	Pressure	<=935	635

The ProPac HIC-10 column is a silica-based product. Care must be taken to ensure the operating conditions do not exceed the limitations of bonded-silica material, specifically the pH must be maintained between pH 2.5 to 7.5. All warranties are made void if this product is exposed to mobile phase conditions outside of this range.

Production Reference
Datasource: SILICA1_local
Sequence: 063655_HIC_46X100_COL_PACK_TES
Sample No.: 7

6.70 Build 1820 (Demo-Installation)

Chromeleon® Dionex Corp. 1994-2005

ProPac® HIC-10
Bonded Silica
5µm 300Å (2.1 x 100 mm)
Product No. 063653

Date: 19-Aug-05 13:04
Serial No. : 001001
Lot No. : 05-03-006

Mobile Phase: 0.10 M Ammonium acetate, pH 5.4 ± 0.1 in 30/70 (v/v) Acetonitrile/DI H2O
Flow Rate: 0.20 mL/min **Temperature:** 30 °C
Detection: UV, 230 nm **Injection Volume:** 1.0 µL

Storage Solution: 0.05 M Ammonium acetate, pH 5.4 ± 0.1 in 80/20 (v/v) Acetonitrile/DI H2O

No.	Peak Name	Ret.Time (min)	Asymmetry (EP)	Efficiency (EP)	Concentration
1	Cytosine	1.46	n.a.	5801	0.025 mg/mL
2	Amitriptyline	1.57	n.a.	4886	0.025 mg/mL
3	4- Butyl benzoic acid	2.11	1.10	5306	0.025 mg/mL
4	Phenanthrene	3.05	1.27	4511	0.025 mg/mL

QA Results:

Analyte	Parameter	Specification	Results
Phenanthrene	Efficiency	>=3,420	Passed
Phenanthrene	Asymmetry	0.95-1.65	Passed
Phenanthrene	Retention Time	2.98-3.42	Passed
	Pressure	<=715	490

The ProPac HIC-10 column is a silica-based product. Care must be taken to ensure the operating conditions do not exceed the limitations of bonded-silica material, specifically the pH must be maintained between pH 2.5 to 7.5. All warranties are made void if this product is exposed to mobile phase conditions outside of this range.

Production Reference:
Datasource: SILICA1_local
Sequence 063653_HIC_21x100_Col_Pack_Test
Sample No.: 71

6.70 Build 1820 (Demo-Installation)

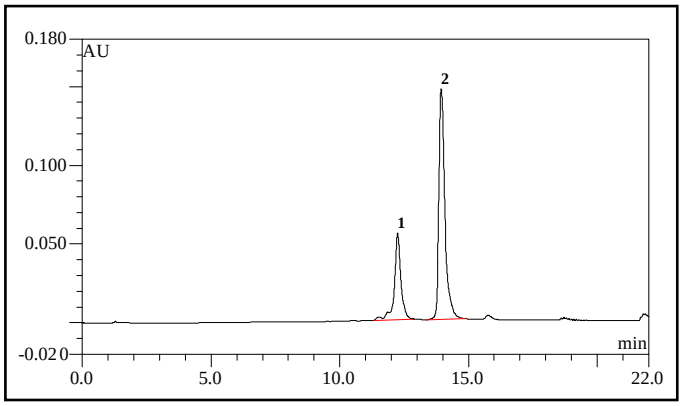
Chromeleon® Dionex Corp. 1994-2005

Lot Validation
ProPac® HIC-10
Bonded Silica
(Using 4.6 x 100 mm)

Date: 26-Jul-05 09:55
Lot No. : 05-03-006

Flow Rate: 1.00 mL/min
Detection: UV, 214 nm
Temperature: 30 °C
Sample Ribonuclease A + Lysozyme (1 mg/mL each)
Injection Volume: 10 µL

Eluent Composition
%A: 2.0 M (NH4)2SO4 in 0.1 M NaH2PO4, pH 7.0 ± 0.1
%B: 0.1 M NaH2PO4, pH 7.0 ± 0.1



Eluent Profile

Time	%A	%B	Comment
-25.00	0	100	*Pre-wash 100%B for 15 min
-10.00	100	0	Preconditioning
0.00	100	0	Sample injection
2.00	100	0	Avoid gradients during sample injection
17.00	25	75	Gradient
17.10	0	100	Wash/Gradient
20.00	0	100	Wash
22.00	100	0	Re-equilibration to be ready for sample injection

*Pre-wash step is not needed for the second run after equilibration

No.	Peak Name	Ret.Time (min)	Concentration
1	RibonucleaseA	12.24	1.0 mg/mL
2	Lysozyme	13.94	1.0 mg/mL

Production Reference
Datasource SILICA1_local
Sequence 063655_HIC_46X100_COL_PACK_TEST
Sample No.: 38

6.70 Build 1820 (DemoInstallation)

Chromeleon® Dionex Corp. 1994-2005

ProPac® HIC-10
Bonded Silica
5µm 300Å (7.8 x 75 mm)
Product No. 063665

Date: 28-Jul-05 12:51
Serial No.: #3
Lot No.: 05-03-006

Mobile Phase: 0.10 M Ammonium acetate, pH 5.4 ± 0.1 in 30/70 (v/v) Acetonitrile/DI H2O
Flow Rate: 1.50 mL/min **Temperature:** 30 °C
Detection: UV, 230 nm **Injection Volume:** 10.0 µL

Storage Solution: 0.05 M Ammonium acetate, pH 5.4 ± 0.1 in 80/20 (v/v) Acetonitrile/DI H2O

No.	Peak Name	Ret.Time (min)	Asymmetry (EP)	Efficiency (EP)	Concentration
1	Cytosine	1.85	1.13	8948	0.025 mg/mL
2	Amitriptyline	2.02	1.14	7699	0.025 mg/mL
3	4-Butyl benzoic acid	2.90	1.27	6861	0.025 mg/mL
4	Phenanthrene	4.45	1.19	5703	0.025 mg/mL

QA Results:

Analyte	Parameter	Specification	Results
Phenanthrene	Efficiency	>=3,060	Passed
Phenanthrene	Asymmetry	0.95-1.87	Passed
Phenanthrene	Retention Time	4.23-4.78	Passed
	Pressure	<=550	460

The ProPac HIC-10 column is a silica-based product. Care must be taken to ensure the operating conditions do not exceed the limitations of bonded-silica material, specifically the pH must be maintained between pH 2.5 to 7.5. All warranties are made void if this product is exposed to mobile phase conditions outside of this range.

Production Reference:
Datasource: SILICA1_local
Sequence: 063665_HIC_78X75_COL_PACK_TES
Sample No.: 7

6.70 Build 1820 (Demo-Installation)

Chromeleon® Dionex Corp. 1994-2005