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BioBasic® SEC Columns

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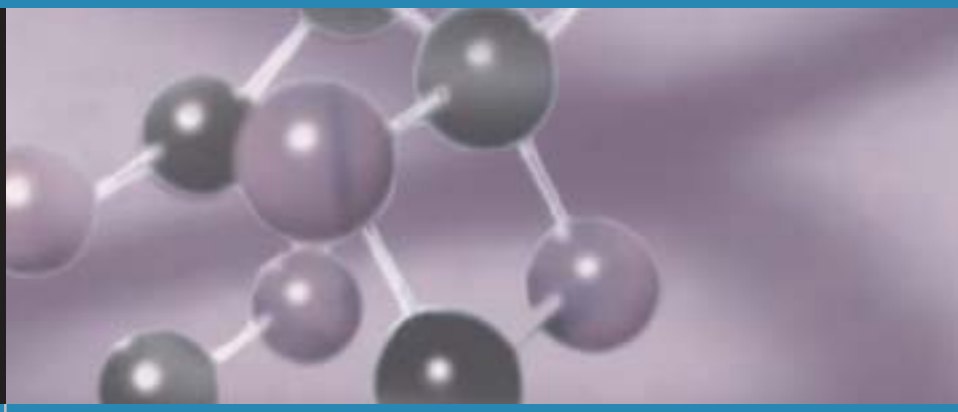

Silica-based size exclusion columns for superior resolution and efficiency

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Size-exclusion chromatography (SEC) is the accepted technique for determining the molecular mass distribution of polymers. BioBasic SEC columns give high efficiency separations for a wide range of molecular weight samples. The columns are offered in a wide range of pore sizes and employ polymer-coated silica to ensure highest efficiency and accurate molecular weight data.

- **Silica-based size exclusion columns for superior resolution of water-soluble compounds**
- **Pore sizes from 60 to 1000Å for a wide range of sample molecular weights**
- **Easy and straight-forward method development**
- **Superior column lifetimes and efficiencies**

Applications:

- **Proteins**
- **Polyethylene Gylcols/Oxides**
- **Polysaccharides/Pullulans**

Advantages of Silica SEC Columns

Polymer based columns are popular for size exclusion separations. However, polymer columns can be compressed, and shrink or swell with changes in solvent composition. These characteristics limit their operating pressure and can reduce separation efficiency.

In contrast, BioBasic SEC columns are based on chromatographic silica, which is mechanically rigid, does not swell or shrink with changes in solvent and shows higher efficiencies than polymer-based columns (minimum 70,000 plates/meter).

Figure 1 shows the separation of proteins with a wide range of molecular weights using a BioBasic SEC 300 (300Å pore size) column.

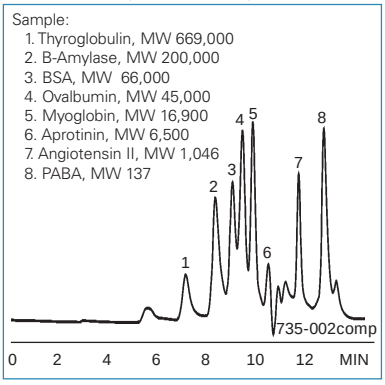
BioBasic SEC Columns

BioBasic SEC columns are available in four pore sizes (60, 120, 300 and 1000Å) to cater for separation of samples from 100 to 10,000,000 molecular weight.

To provide accurate data, size exclusion columns must separate sample molecules strictly on the basis of their size in solution. Secondary ionic or hydrophobic interactions must be minimized, as they will degrade the size-based separation. BioBasic SEC columns employ highly base-deactivated 5µm silica, which is coated with a hydrophilic polymer to ensure separation occurs only on the basis of sample size.

BioBasic SEC columns are ideal for high efficiency gelfiltration separation of proteins and other biological molecules where the absence of secondary interactions, such as adsorption, is essential for accurate analysis (Figure 1).

figure 1. Separation of proteins



BioBasic SEC 300, 5µm, 300x7.8 mm
Eluent: 0.1M KH_2PO_4, pH 7
Flow: 1.0 ml/min
Detector: UV @ 280 nm

How Retention is Achieved

In size exclusion, retention is determined by the accessibility of the sample molecule to the pores. Maximum retention occurs if the sample can fully access the pores. Minimal retention occurs if the sample is larger than the pores and elutes with the solvent. Hence, samples elute in order of size, with the highest molecular weight samples eluting first.

The physical properties of the silica particles require tight quality control, as it is the volume of pores with diameters between the inclusion and exclusion limits of the analytes that determine resolution.

The physical properties of BioBasic SEC columns are shown in Table 1.

table 1

Column	Pore Size	Pore Volume	Particle Size
BioBasic SEC 60	60Å	0.7ccg^{-1}	5µm
BioBasic SEC 120	120Å	1.0ccg^{-1}	5µm
BioBasic SEC 300	300Å	0.9ccg^{-1}	5µm
BioBasic SEC 1000	1000Å	0.9ccg^{-1}	5µm

Elution order is based on whether or not the analyte can enter the pores. If the analyte cannot enter the pores it passes through the column in the channels between the particles. Analytes that can enter the pores, either partially or completely, elute later.

In a packed column the interstitial volume outside the particles (i.e. the volume associated with the channels between the particles) is given as V_o. The volume inside the particles (inside the pores) is denoted as V_i.

The total volume of the mobile phase inside the column (V_m) is then (1):

$$V_m = V_o + V_i \quad (1)$$

The degree of permeation is denoted by K_D (the distribution co-efficient) of the analyte. The retention volume of any analyte(V_R)is expressed as (2):

$$V_R = V_o + K_D V_i \quad (2)$$

For completely excluded analytes K_D=0, while for totally permeating analytes K_D=1. (3):

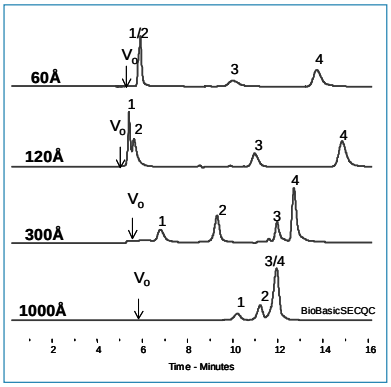
$$K_D = \frac{V_R - V_o}{V_m - V_o} \quad (3)$$

Equation 1 illustrates that pore volume is a very important variable of retention in SEC. A measure of porosity is the ratio of pore volume to void volume (V_i/V_o). The larger the ratio, the more volume available for the separation to occur, giving better resolution of more peaks. However, supports with a large V_i / V_o ratio are more fragile, which may compromise efficiency. Table 2 shows the ratio of V_i / V_o for BioBasic SEC columns.

table 2

Column Pore Size	V_i/V_o
60Å	1.40
120Å	1.77
300Å	1.16
1000Å	0.96

figure 2. Effect of Pore Size on Resolution



Columns: BioBasic SEC 300 x 7.8mm, 5µm
Eluent: 0.1M KH_2PO_4 pH7
Flow: 1.0 mL/min
Detector: UV @ 254
Injection: 20µL
Sample 1. Thyroglobulin (MW 669,000)
2. Ovalbumin (MW 45,000)
3. Angiotensin II (MW 1,046)
4. PABA (V_m) (MW 137)

Column Selection

Column selection should be lead by sample molecular weight, as the elution volume has a linear relationship to the log of molecular weight for a series of molecules of similar shape, due to pore exclusion/inclusion effects. Figure 2 shows the effect of various pore sizes in separating a set of proteins with a wide range of molecular weights. The smallest pore size (60Å) gives the highest resolution between the smallest pair of peptides, but fails to resolve the largest two proteins. The larger pore size (1000Å) resolves the large proteins, whilst giving less resolution for the smaller molecules.

Since all of the compounds are unretained and the entire separation takes place between the exclusion volume and the mobile phase volume, the ratio of these volumes is a measure of the peak capacity of the packing (Table 2). Table 3 shows the recommend molecular weight range for each BioBasic SEC pore size.

Molecules are eluted based on their size in solution. Linear or rod-like molecules will elute before globular molecules of the same molecular weight.

Mobile phases should be selected to minimize interaction with the chromatographic surface. Usually a moderate buffer concentration of 0.05 - 0.2M is used for protein separations.

table 3

Molecular Weight (kDaltons)			
Pore Size	Proteins	Pullulans	Polyethylene Oxides/ Glycols
60Å	0.1-6	0.3-6	0.1-4
120Å	0.1-50	0.3-12	0.4-10
300Å	1-500	1-100	2-100
1000Å	20-4000	20-›1000	Not recommended

Ionic strength is a key parameter when controlling retention of biomolecules during gel filtration. Mobile phases should be selected to minimize possible interactions that could occur between the protein andsurface silanols present in their de-protonated form. This interaction is easily overcome by increasing the ionic strength of the mobile phase. Typically 0.05mol/L of salt is sufficient to eliminate this interaction. However, if the ionic strength is too high, hydrophobic interactions are enhanced, leading to increased retention.

Method development consists of selecting a mobile phase compatible with the sample type, and a column or columns with pore sizes that provide resolution for the molecular weight range of the sample.

Column Lifetime and Performance Stability

BioBasic SEC columns have been shown to be stable over 6000 to 7000 injections (column volumes) before a loss in efficiency is observed using a 0.1M KH_2PO_4 mobile phase at pH 7.

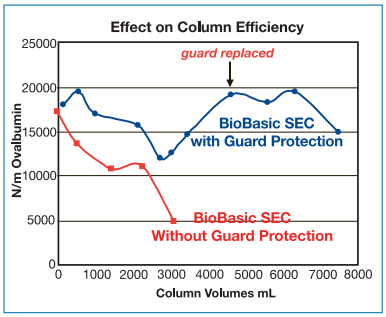
The number of injections performed before column performance deteriorates can be significantly increased by using a guard column to protect the analytical column from mobile phase contaminants, as well as sample impurities and particulates.

Figure 3 shows column efficiencies after 6000 column volumes of 0.1M KH_2PO_4 mobile phase. Efficiency was measured without and with a guard column.

Molecular Weight Calibration

Log molecular weight is plotted against elution volume to create a distribution co-efficient (calibration curve). The curve is usually linear for distribution co-efficients between 0.2 and 0.8. The gradient of the curve is sharp near the exclusion limit of large molecules and near total permeation of small molecules.

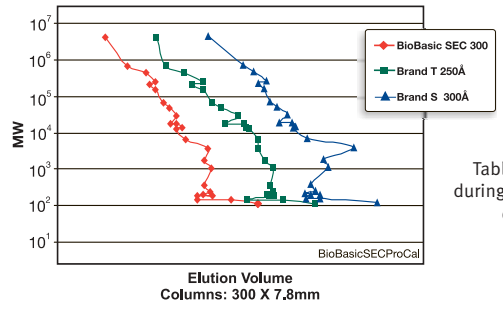
figure 3. Effect of Guard Column on Lifetime



To determine the molecular weights of unknown samples, the elution volume of the sample can be compared directly with that of standards of known molecular weight.

A smooth calibration curve is achieved when the separation is based only on sample size. Secondary interations of the sample with the silica surface will increase sample retention (higher elution volume) than otherwise expected from molecular weight calculations. Figure 4 shows a protein calibration curve for BioBasic SEC 300 and two competitor columns.
The effect of secondary interaction mechanisms can be seen between MW 1000 and MW10,000 for the Brand S

figure 4. Protein Calibration Curve



Calibration curves are offset for visual clarity.

Table 4 shows the data obtained during a molecular weight calibration of proteins and peptides.

table 4

Protein	MW	Protein	MW
1. DNA	4,000,000	14. Aprotinin	6,500
2. Thyroglobulin	669,000	15. Chain B Insulin	3,496
3. Ferritin	440,000	16. Neurotensin	1,673
4. Catalase	232,000	17. Angiotensin II	1,046
5. b-Amylase	200,000	18. Oxytocin	1,007
6. Alcohol deyhdrogenase	150,000	19. 5-AMP	347
7. BSA	66,000	20. GL-tyrosine hydrate	238
8. Ovalbumin	45,000	21. Triglycine	191
9. Carbonic anhydrase	29,000	22. Tyrosine	180
10. b-Lactoglobulin	17,500	23. L-Arginine	174
11. Myoglobin	16,900	24. D-Glutamic acid	147
12. Ribonuclease A	13,700	25. p-Aminobenzoic acid	137
13. Cytochrome C	12,500	26. Benzyl alcohol	108

New columns should be calibrated before injecting unknown samples. Performing a calibration ensures accurate quantification, as some solvent conditions may encourage secondary interactions between the sample and column, which may alter molecular weight calculations.

Figure 5 illustrates typical calibration curves for BioBasic SEC 300 for proteins, pullulans and polyethylene glycols/oxides over a broad range of molecular weights.

figure 5. Molecular Weight Calibration Curves

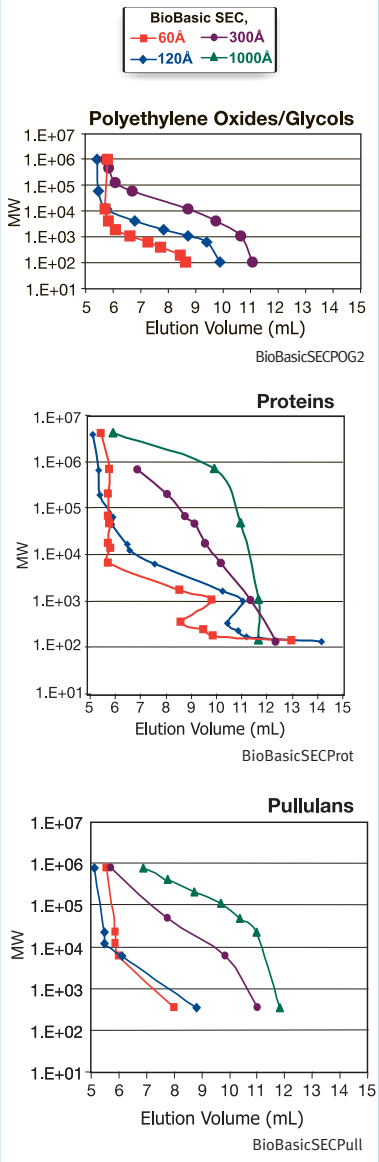
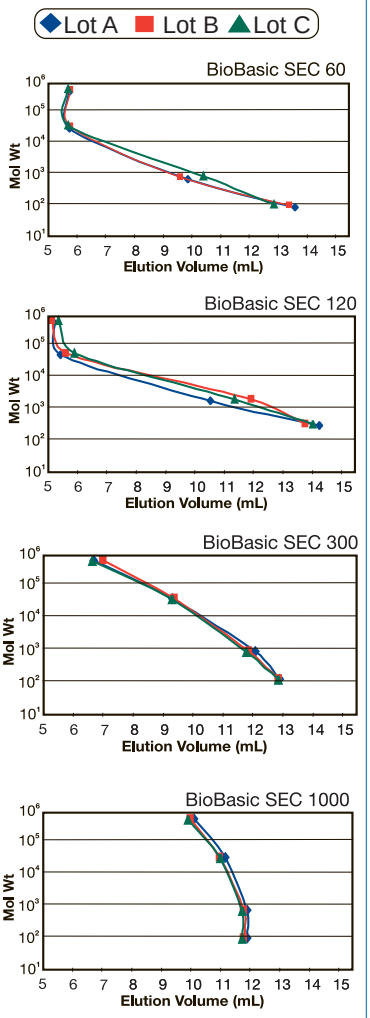


figure 6. Lot -to-lot reproducibility

Columns: BioBasic SEC 5µm, 300x7.8mm
Eluent: 0.1M KH_2PO_4 pH7
Flow rate: 1.0 mL/min
Detector: UV @ 254

Protein	MW
Thyroglobulin	669,000
Ovalbumin	45,000
Angiotensin II	1,046
PABA (V_m)	137



Column Reproducibility

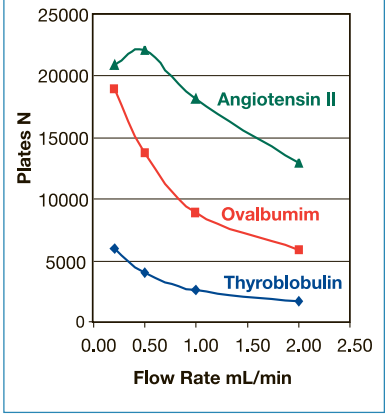
Column-to -column reproducibility is of key importance to guarantee robust method development. Every BioBasic SEC silica lot is tested chromatographically with a range of proteins to confirm accuracy of retention volumes. Figure 6 shows the excellent lot-to-lot reproducibility of BioBasic SEC columns. Every column also receives an efficiency test to confirm compliance with efficiency specifications. BioBasic SEC 60, 120 and 300 columns have a guaranteed minimum efficiency of 70,000 plates/meter. BioBasic SEC1000 columns have a guaranteed minimum efficiency of 60,000 plates/meter.

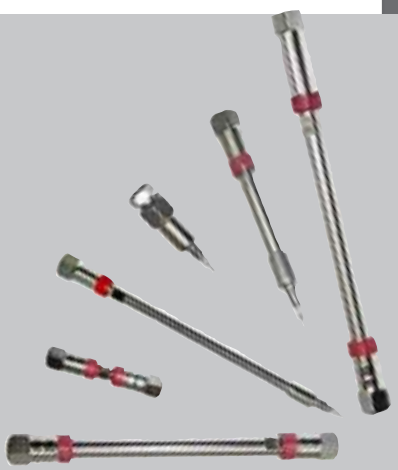
Each column is shipped with a test certificate containing both the silica lot test and the column efficiency test data.

Optimizing SEC Separations

The effect of operating parameters such as flow rate, temperature and buffer concentration on performance is shown in Figures 7, 8, and 9.

figure 7. Effect of Flow Rate





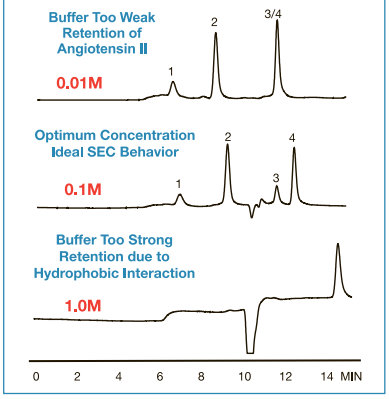
Flow rate and temperature affect diffusion rates, and hence affect mass transfer, which is the primary mechanism by which sample molecules move in and out of the pores. The impact of mass transfer is greatest for smaller analytes, as larger proteins have slower kinetics responsible for diffusion and mass transfer. Hence, maximum efficiency is obtained at low flow rates for large proteins (Thyroglobulin and Ovalbumin).

Figure 8 shows the change in retention seen with different strength buffers.

Figure 9 shows how temperature control can increase peak efficiency, as elevated temperatures assist mass transfer. While elevated temperature can be used to increase sensitivity by improving peak efficiency, it should be noted that temperatures above 40°C may denature proteins or compromise other sample types.

figure 8. Effect of Buffer Concentration

Protein	MW
Thyroglobulin	669,000
Ovalbumin	45,000
Angiotensin II	1,046
PABA (V_m)	137



Columns: **BioBasic SEC 300 5µm, 300x7.8mm**
Eluent: KH_2PO_4 ,pH7
Flow rate: 1.0 mL/min
Detector: UV @ 254
Injection: 20µL

Sample Clean-up From Biological Matrices

Since BioBasic SEC columns are designed to elute proteins with high recoveries, they can be used for direct automated analysis of serum or urine samples.

Direct Serum Injection

Small pore size columns (60Å) can be used for drug analysis with direct serum injection. Figures 10 and 11 demonstrate the use of a single SEC column and column switching techniques to separate protein serum matrices from smaller drug compounds.

figure 9. Effect of Temperature

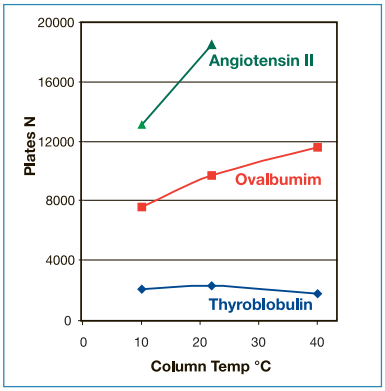
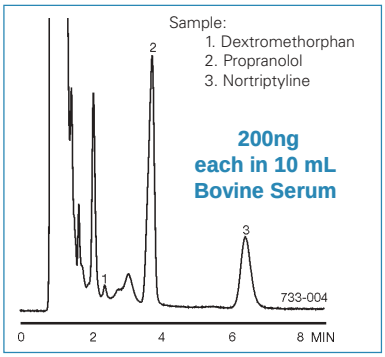


figure 10. Direct Serum Injection Drug Analysis



Column: **BioBasic SEC 60, 5µm, 150x3.0mm**
Eluent: 90% 0.05M KH_2PO_4 pH 3.5/10% ACN
Flow: 0.5mL/min
Detector: UV @ 220

Figure 10 shows a single column application, where the slight hydrophobic nature of the polymer coating and the large surface area of the small pore size are used to retain small moderately polar drugs.

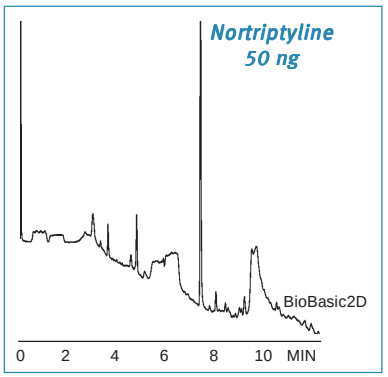
Two-Dimensional (2-D) Sample Clean-Up

Alternatively, column switching can be used to transfer the desired drug fraction eluted from the BioBasic SEC column onto a reversed phase column, where gradient elution is used to analyze the sample.

For the more hydrophobic drug (Nortriptyline), the fraction eluted from the BioBasic SEC 60 column with a high aqueous mobile phase is re-focused onto a BetaBasic 18 (C18) column prior to gradient elution. This combination provides the most sensitive analysis.

Please note that while serum proteins are eluted to waste, both columns should be protected by the corresponding guard column to prevent contamination and maximize analytical column lifetimes.

figure 11. 2D SEC Sample Clean-Up



Column 1: **BioBasic SEC 60, 5µm, 150 x 3mm**
Eluent: 90% 0.05M KH_2PO_4 pH3.5 / 10%ACN
Flow: 0.5mL/min

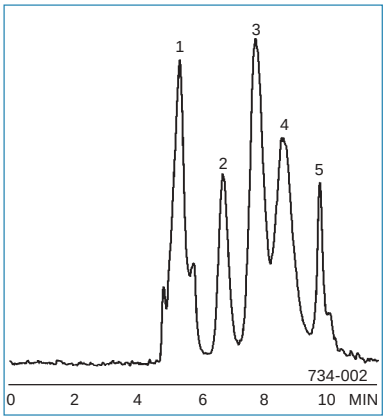
Column 2: **BetaBasic 18, 3µm, 100 x 4.6mm**
Mobile phase: A=90% 0.05M KH_2PO_4 pH3.5/10%ACN
B=75%ACN/25%Water
Gradient: 0-90%B in 10 minutes
Injection: 10µL Calf Serum x 5µg/mL
Detector: UV @ 215

BioBasic SEC Columns

- Silica-based size exclusion columns for superior resolution and efficiency
- Offered in four pore sizes to cater to a wide range of sample molecular weights
- Provide rapid isocratic size-based separation of water-soluble polymers and proteins
- Optimized for fast and easy method development due to minimal secondary interactions
- Give superior column lifetimes for thousands of injections, particularly when protected by a guard column

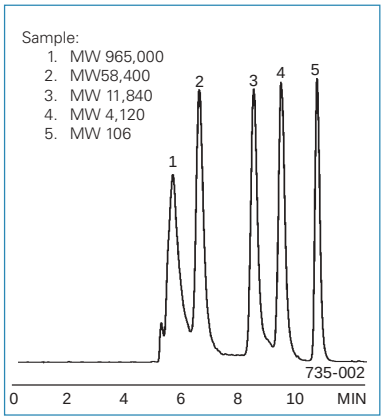
Applications

Polyethylene Glycols/Oxides - 120Å pore size



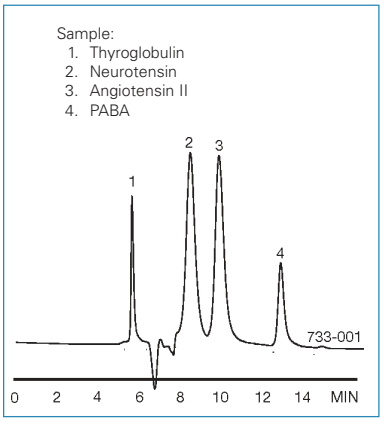
Column: BioBasic SEC 120, 5µm, 300x7.8mm
Eluent: 100% Water
Flow: 1.0 mL/min
Detector: ELSD

Polyethylene Glycols/Oxides - 300Å pore size



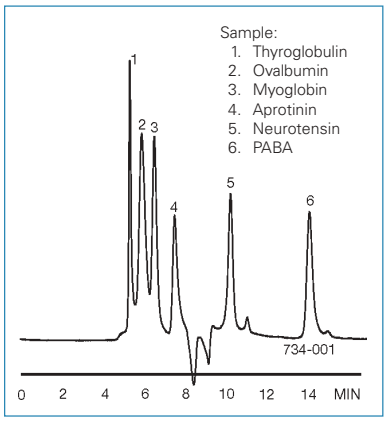
Column: BioBasic SEC 300, 5µm, 300x7.8mm
Eluent: 100% Water
Flow: 1.0 mL/min
Detector: ELSD

Proteins/Peptides - 60Å pore size



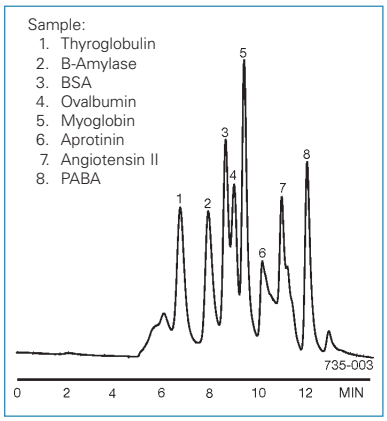
Column: BioBasic SEC 60, 5µm, 300x7.8mm
Eluent: 0.1M KH_2PO_4 pH 7
Flow: 1.0 mL/min
Detector: UV @ 280

Proteins/Peptides - 120Å pore size



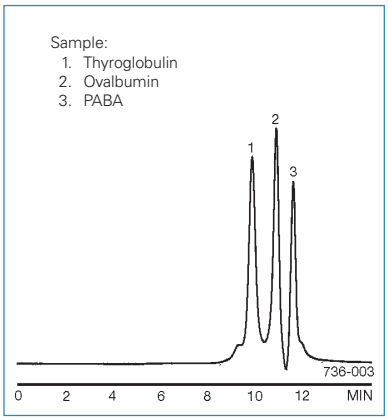
Column: BioBasic SEC 120, 5µm, 300x7.8mm
Eluent: 0.1M KH_2PO_4 pH 7
Flow: 1.0 mL/min
Detector: UV @ 280

Proteins/Peptides - 300Å pore size

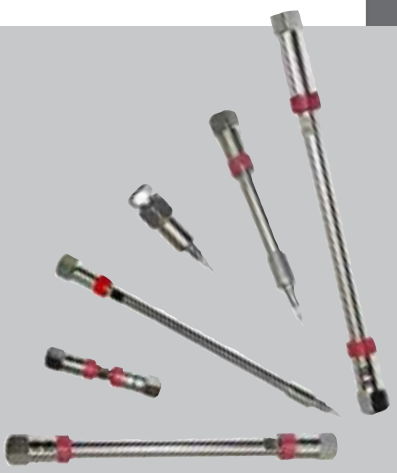


Column: BioBasic SEC 300, 5µm, 300x7.8mm
Eluent: 0.1M KH_2PO_4 pH 7
Flow: 1.0 mL/min
Detector: UV @ 254

Proteins/Peptides - 1000Å pore size



Column: BioBasic SEC 1000, 5µm, 300x7.8mm
Eluent: 0.1M KH_2PO_4 pH 7
Flow: 1.0 mL/min
Detector: UV @ 280



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