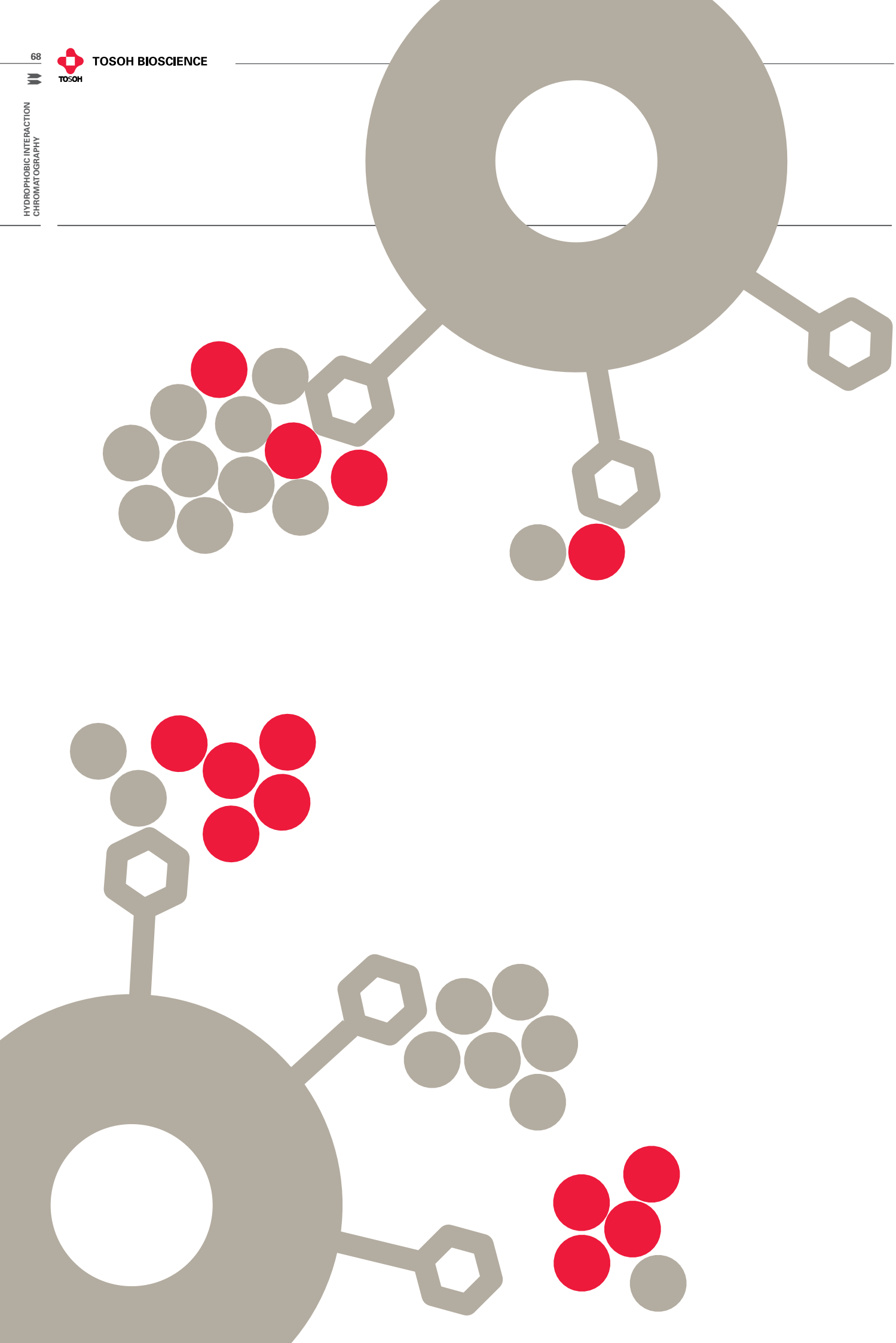


HIC
HYDROPHOBIC INTERACTION CHROMATOGRAPHY

HIC PRODUCTS

- TSKgel Ether-5PW
- TSKgel Phenyl-5PW
- TSKgel Butyl-NPR

TOSOH FACT

Tosoh Bioscience provides solutions for today's biological purification needs. In fact, some of the first commercial HIC products were manufactured by Tosoh. We take pride in our ability to design new products based on existing chemistries to solve specific customer applications.

We encourage you to have a confidential discussion with us about your specific needs. Whether it is a surface modification of an existing product or the creation of a new one, we encourage you to call on us to meet your needs for a customized solution.

INTRODUCTION TO TSKgel HIC COLUMNS

Hydrophobic Interaction Chromatography (HIC) is based on the interaction between hydrophobic groups on a protein and a hydrophobic ligand on the solid support. HIC offers a distinct advantage for easily denatured proteins; it can be run using moderate concentrations of ammonium sulfate, which favors the stability of many proteins.

The binding of proteins to a hydrophobic matrix is affected by a number of factors including (1) the type of ligand, (2) the ligand density on the solid support, (3) the backbone material of the matrix, (4) the hydrophobic nature of the protein, and (5) the type of salt used. All of these factors help to make HIC a powerful technique for the separation of biomolecules.

Tosoh Bioscience offers three different HIC column types in analytical format: TSKgel Phenyl-5PW, Ether-5PW and Butyl NPR. TSKgel Phenyl-5PW is also available in preparative column formats. See FIGURE 1 for the structure of the HIC resins.

COLUMN SELECTION

TSKgel HIC stationary phases are polymethacrylate-based with a choice of three ligands with varied hydrophobicities from low to high. TSKgel Ether-5PW and Phenyl-5PW are based on a porous base matrix with 100 nm pores and available with various particle sizes depending on column dimensions, while TSKgel Butyl-NPR is based on a 2.5 µm nonporous base particle. Nonporous resins (NPR) re typically used for high-speed analytical applications.

TSKgel ETHER-5PW is less hydrophobic than TSKgel Phenyl-5PW. It displays weaker interaction and thus shorter retention times compared to Phenyl-5PW.

TSKgel Ether-5PW is the best choice for the separation of very hydrophobic proteins such as membrane proteins or monoclonal antibodies.

The TSKgel PHENYL-5PW columns were the first commercially available, polymer-based columns for high performance HIC. These columns have been instrumental to the increase in popularity of this technique for analytical, preparative, and process scale separations of biopolymers.

TSKgel BUTYL-NPR is the least hydrophobic among the three TSKgel HIC columns and requires a higher salt concentration for binding. TSKgel Butyl-NPR columns provide fast and quantitative HIC, because smaller particles provide higher efficiency. By packing the 2.5 µm nonporous resin particles into shorter columns, typical analysis times are reduced to less than 10 minutes. Pore diffusion is often the rate-limiting step in the overall mass transport of large biomolecules through a porous column. Eliminating the pores provides higher resolution at higher flow rates. Another benefit of NPR resins is excellent mass recovery, allowing quantitation down to nanogram levels. These properties make TSKgel Butyl-NPR the preferred choice for process monitoring and quality control. TSKgel Butyl-NPR is getting increasingly popular for the analysis of antibody drug conjugates (ADCs) and is available in two dimensions: 3.5 cm length for high throughput and 10 cm length for high resolution.

TSKgel HIC columns are compatible with water soluble organic solvents at concentration below 50 % (20 % for Butyl-NPR).

FIGURE 1

Structure of TSKgel HIC resins

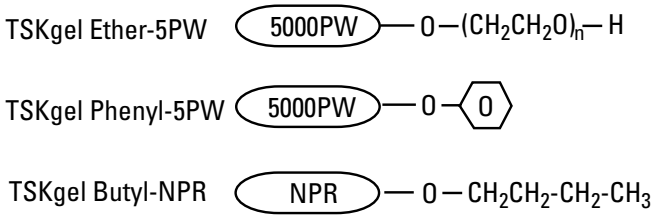


TABLE I

Column selection for the TSKgel HIC columns

Sample	MW range (Da)	TSKgel Column
Peptides	< 10,000	Butyl-NPR
Medium to large proteins	> 10,000	Phenyl-5PW Ether-5PW Butyl-NPR
DNA, RNA, and PCR products	> 500,000	Phenyl-5PW Butyl-NPR
Oligonucleotides	> 10,000	Phenyl-5PW Butyl-NPR

FEATURES

- Choice of three hydrophobic ligands (ether, phenyl or butyl)
- Rigid polymeric base resin
- Similar chemistry to TOYOPEARL resins
- TSKgel Phenyl-5PW offered in PEEK hardware
- Ether and Phenyl available in 2 mm ID format

BENEFITS

- Added flexibility during method development
- Wide pH range (2-12) enabling robust cleaning options
- Seamless scalability from analytical to preparative scaley
- Eliminates undesirable interactions with column hardware
- LC-MS applications

HIC

COMPARISON OF SELECTIVITY

FIGURE 2 compares the separation of standard proteins on the Ether, Phenyl, and Butyl supports under similar operating conditions.

SAMPLE CAPACITY

One definition of sample capacity is the amount of pure compound injected onto the column at which the peak width is 10% larger than the peak width under low loading conditions. Using this definition, the capacity of a 7.5 mm ID x 7.5 cm L TSKgel Phenyl-5PW column varies from 0.1 to 1 mg of protein. Resolution and peak width are dependent on sample loading, as shown in FIGURE 3. Therefore, sample loading should be kept within 0.1 - 0.5 mg in order to obtain the highest resolution.

Separations on TSKgel Ether-5PW columns usually take 30 - 60 minutes. 0.5 mg of pure protein can be purified from a 5 - 10 mg crude protein mixture using a 7.5 mm ID x 7.5 cm L column.

Since almost all of the surface area of a porous particle is inside the pores, the capacity of the 4.6 mm ID x 3.5 cm L TSKgel Butyl-NPR column is significantly less than that for the 7.5 mm ID x 7.5 cm L Phenyl-5PW column. Capacities for the Butyl-NPR column are 100 µg for crude sample and 2 µg for pure sample.

CHEMICAL STABILITY

TSKgel 5PW-type HIC columns are physically and chemically stable in water soluble organic solvents (at < 50% methanol, ethanol, ACN, DMF, DMSO or < 30 % chloroform). Change the solvent gradually by reducing the flow rate (preferably with a gradient) because rapid change may cause degradation of column efficiency. Note: When changing to an organic solvent, reduce the salt concentration to prevent precipitation of the salt on the column. Also, chaotropic agents (urea, SDS, etc.) will reduce the adsorption of biomolecules; therefore, use low levels of these agents (<2 mol/L).

Polymer-based columns are stable when cleaning at alkaline pH. All TSKgel HIC columns can be routinely operated from pH 2-12. TABLE II shows that the phenyl groups on the TSKgel Phenyl-5PW are stable for more than 10 days upon exposure to 0.5 mol/L NaOH or 0.5 mol/L acetic acid.

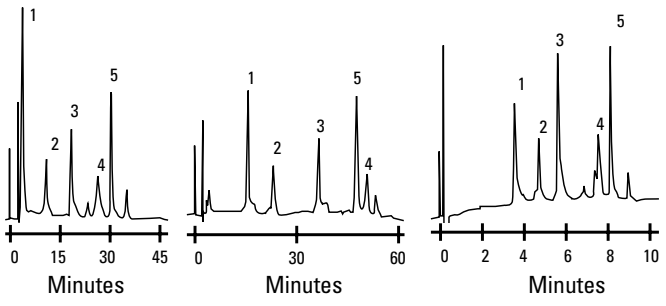
TABLE II

Long-term exposure of TSKgel Phenyl-5PW to acid and base

Acid/base	Phenyl content (mmol/mL - resin)	
	Before exposure	*After 10 days exposure*
0.5 mol/L CH_3COOH	0.105	0.106
0.5 mol/L NaOH	0.105	0.104

FIGURE 2

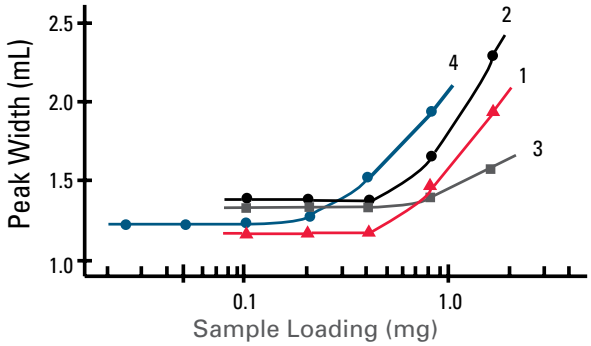
Comparing HIC columns



Column: TSKgel Ether-5PW & TSKgel Phenyl-5PW, 7.5 mm ID x 7.5 cm L TSKgel Butyl-NPR, 4.6 mm ID x 3.5 cm L; Sample: 1. myoglobin, 2. ribonuclease A, 3. lysozyme, 4. α-chymotrypsin, 5. α-chymotrypsinogen; Injection: 5PW-type columns: 100 µL (50-100 µg), NPR-type column: 20 µL (1.5-40 µg); Elution: 60 min linear gradient from 1.8 mol/L to 0 mol/L $(NH_4)_2SO_4$ in 0.1 mol/L phosphate buffer, pH 7.0, for 5PW-type columns; 12 min linear gradient from 2.3mol/L to 0 mol/L $(NH_4)_2SO_4$ in 0.1 mol/L phosphate buffer, pH 7.0 for TSKgel Butyl-NPR; Flow rate: 1.0mL/min; Detection: UV @ 280 nm

FIGURE 3

Dependence of peak width on sample loading in the separation of proteins



Column: TSKgel Phenyl-5PW, 7.5 mm ID x 7.5 cm L; Sample: 1. myoglobin; 2. ribonuclease A; 3. ovalbumin; 4. α-chymotrypsin; concentration: 0.025 % to 1.6 %; Elution: 60 min linear gradient of $(NH_4)_2SO_4$ from 1.5 mol/L to 0 mol/L in 0.1 mol/L phosphate buffer (pH 7.0); Flow rate: 0.5 mL/min; Temperature: 25 °C; Detection: UV @ 280 nm

APPLICATIONS - TSKgel HIC COLUMNS

ANTIBODY FRAGMENTS

FIGURE 4 shows the separation of Fab and Fc fragments of an antibody on TSKgel Butyl-NPR. The appearance of additional Fc fragments is due to the oxidation of methionine residues by 0.10% t-butylhydroperoxide (tBHP). The numbers above the Fc peaks correspond to the number of oxidized residues in each fragment.

ANTIBODY AGGREGATES

The use of a short TSKgel Butyl-NPR column for the separation of a monoclonal antibody and its high molecular weight aggregates is shown in FIGURE 5. The total aggregate content of this sample is about 11 %, which was also confirmed by SEC on TSKgel G3000SW$_{XL}$ (5 micron, 7.8 x 300 mm) the current industrial standard for mAb aggregate analysis. Because of the high efficiency of the nonporous particles of TSKgel Butyl-NPR only low sample amounts are needed for aggregate analysis.

ANTIBODY DRUG CONJUGATES (ADCs)

ADCs are becoming an increasingly important class of therapeutic agents for treatment of cancer. One of the most important quality attributes of an ADC is the drug to antibody ratio (DAR), the average number of drugs that are conjugated. This determines the amount of "payload" that can be delivered to the tumor cell.* Aditya Wakankar and others described the analysis of an ADC on TSKgel Butyl NPR that yielded five predominant peaks that corresponding to mAb containing zero, two, four, six and eight drugs.

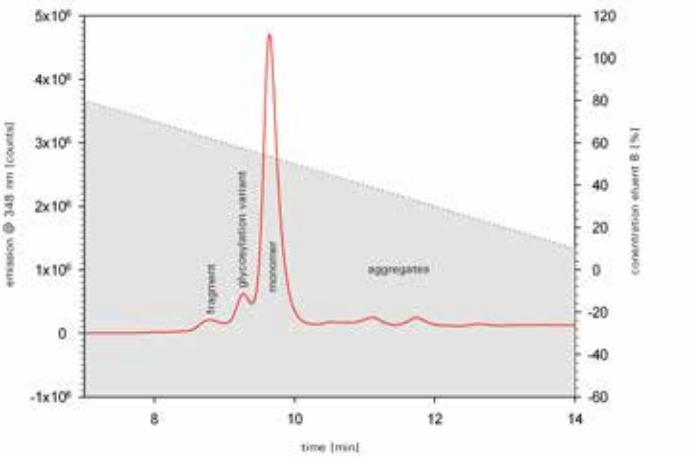
TSKgel ETHER-5PW

ANTIBIOTICS

The TSKgel Ether-5PW column was used to determine the relative purity of the antibiotic components C-1027 and C-1027-AG (FIGURE 6). Antibiotic C-1027 is composed of a protein consisting of many hydrophobic and hydroxyamino acids with a non-protein chromophore. Antibiotic C-1027-AG is composed of the hydrophobic and hydroxyamino acids without the chromophore.

FIGURE 5

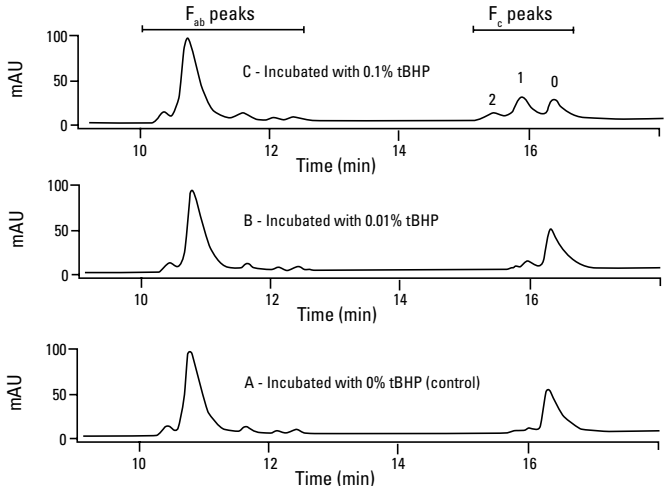
Analysis of monoclonal antibody and aggregates using a TSKgel Butyl-NPR column



Column: TSKgel Butyl-NPR, 2.5 µm, 4.6 mm ID × 3.5 cm L,
Mobile phase: A: 3 mol/L NaCl, B: H_2O, Gradient: 0-100%B in 10 min
Flow rate: 1.0 mL/min, Detection: flourescence, Ex: 280 nm, Em: 348 nm
Injection vol.: 5 µg, Sample: IgG_1

FIGURE 4

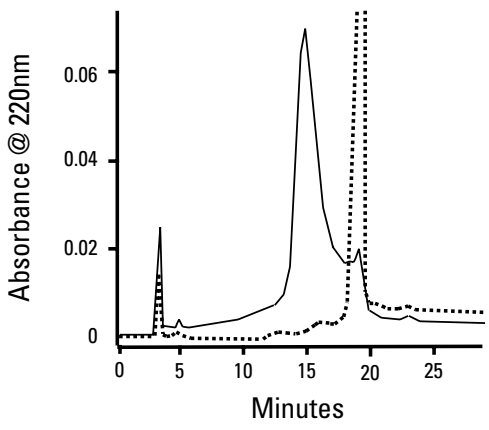
Separation of Fab and Fc fragments on TSKgel Butyl-NPR



Column: TSKgel Butyl-NPR, 4.6 mm ID x 3.5 cm L; Elution: Buffer A: 2 mol/L $(NH_4)_2SO_4$, 20 mmol/L Tris, pH 7, Buffer B: 20 mmol/L Tris, pH 7; Gradient: linear from 10 % B to 100 % B in 34 minutes; Flow rate:1 mL/min; Temperature: 30°C

FIGURE 6

Purification of anti-tumor antibiotic



Column: TSKgel Ether-5PW, 7.5 mm ID x 7.5 cm L; Sample: C-1027, C-1027-AG concentration: 1 mg/mL; Injection: 20 µL; Elution: linear gradient from 1.5 mol/L to 0 mol/L $(NH_4)_2SO_4$ in 0.1 mol/L phosphate buffer, pH 7.0; Flow rate: 0.8 mL/min; Detection: UV @ 220 nm

* *Aditya Wakankar et. al. 'Analytical methods for physicochemical characterization of antibody drug conjugates', mAbs 3:2, pages 161-172; March/April 2011.*

HIC

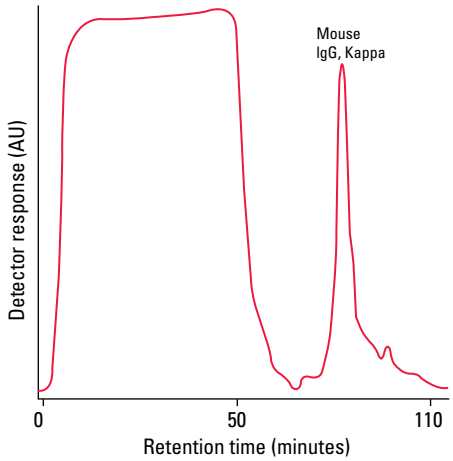
APPLICATIONS - TSKgel HIC COLUMNS

MONOCLONAL ANTIBODIES

Monoclonal antibodies (mAbs) play a part in many research, diagnostic, and therapeutic applications. Monoclonal antibodies are generally the most hydrophobic proteins in ascites fluid and cell culture supernatant.

FIGURE 7

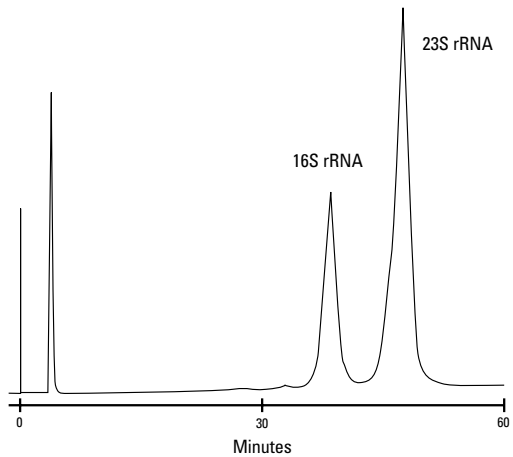
Monoclonal antibody purification



Column: TSKgel Ether-5PW, 10 µm, 8.0 mm ID × 7.5 cm, glass
Mobile phase: 67.5 min isocratic load and wash with 1 mol/L $(NH_4)_2SO_4$ in 1 mol/L glycine, 0.5 mol/L phosphate buffer, pH 7.0, followed by a 37.5 min linear gradient from 1.0 mol/L to 0 mol/L $(NH_4)_2SO_4$ in 1.0 mol/L glycine, 0.05 mol/L phosphate, pH 7.0; Flow rate: 1.0 mL/min; Detection: UV @ 280 nm, 3.0 AUFS; Injection vol.: 50 mL; Sample: 25 mL raw cell culture supernatant, 200 mg total protein, 15 mg total antibody diluted to 50 mL with initial elution buffe

FIGURE 8

Retain large RNAs on TSKgel Phenyl-5PW



Column: TSKgel Phenyl-5PW, 7.5 mm ID x 7.5 cmL;
Sample:16S and 23S rRNA from E. coli, 0.05 mg in 0.1 mL; Elution: 0 min linear gradient from 2 mol/L to 0 mol/L $(NH_4)_2SO_4$ in 0.1mol/L phosphate buffer, pH 7.0; Flow rate: 60.5 mL/min; Detection:UV @ 280 nm

FIGURE 7 shows typical results from the screening of two mAbs using a TSKgel Ether-5PW column.

TSKgel PHENYL-5PW

RNAs

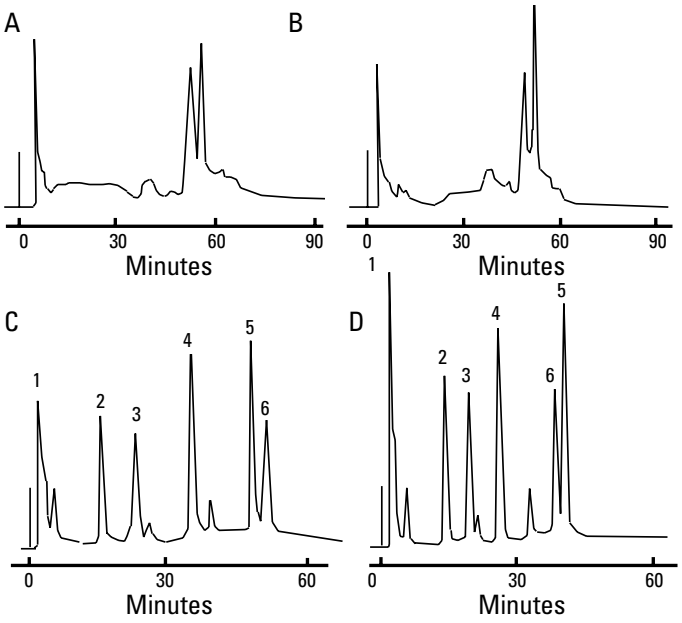
FIGURE 8 illustrates the separation of 16S and 23S ribosomal RNA on a TSKgel Phenyl-5PW column. The approximate molecular weights of these RNAs are 560,000 and 1,100,000 Da, respectively.

MODULATION OF SELECTIVITY

The addition of organic solvents or chaotropic agents in the final buffer can improve separations. However, relative elution positions may change. Therefore, add chaotropic agent and organic solvent in small quantities. See FIGURE 9 for the effect of chaotropic agents and organic solvents on the HIC separation of two different samples.

FIGURE 9

Effect of urea and isopropanol on the separation of commercial lipoxidase and a standard protein mixture



Column: TSKgel Phenyl-5PW, 7.5 mm ID x 7.5 cm L;
Sample: A & B: commercial lipoxidase, C & D: protein mixture: 1. cytochrome C, 2. myoglobin, 3. ribonuclease A, 4. lysozyme, 5. α-chymotrypsinogen, 6. α-chymotrypsin; Elution: A: 60 min linear gradient from 0.1 mol/L phosphate buffer containing 1.5 mol/L $(NH_4)_2SO_4$ (pH 7.0) to 0.1 mol/L phosphate buffer (pH 7.0), B: 60 min linear gradient from 0.1 mol/L phosphate buffer containing 1.5mol/L $(NH_4)_2SO_4$ (pH 7.0) to 0.1 mol/L phosphate buffer containing 2 mol/L urea (pH 7.0), C: 60 min linear gradient from 0.1 mol/L phosphate buffer containing 1.8 mol/L $(NH_4)_2SO_4$ (pH 7.0) to 0.1 mol/L phosphate buffer (pH 7.0), D: 60min linear gradient from 0.1 mol/L phosphate buffer containing 1.8 mol/L $(NH_4)_2SO_4$ (pH 7.0) to 0.1 mol/L phosphate buffer (pH 7.0) containing 7% isopropanol; Flow rate : A & B: 0.5 mL/min; C & D: 1.0 mL/min;
Temp.: 25°C; Detection: UV @ 280 nm

ORDERING INFORMATION

Part #	*Description*	*ID (mm)*	*Length (cm)*	*Particle size (µm)*	*Number theoretical plates*	*Flow rate (mL/min) range*	*Maximum pressure drop (MPa)*
TSKgel Glass columns							
0014013	Ether-5PW Glass, 100 nm	5.0	5.0	10.0	≥ 600	0.5 - 0.8	2.0
0014014	Ether-5PW Glass, 100 nm	8.0	7.5	10.0	≥ 1,000	0.5 - 1.0	2.0
0013063	Phenyl-5PW Glass, 100 nm	5.0	5.0	10.0	≥ 600	0.5 - 0.8	2.0
0008804	Phenyl-5PW Glass, 100 nm	8.0	7.5	10.0	≥ 1,000	0.5 - 1.0	2.0
TSKgel Stainless Steel Columns							
0018760	Ether-5PW, 100 nm	2.0	7.5	10.0	≥ 1,000	0.05 - 0.1	0.6
0008641	Ether-5PW, 100 nm	7.5	7.5	10.0	≥ 1,000	0.5 - 1.0	2.0
0018759	Phenyl-5PW, 100 nm	2.0	7.5	10.0	≥ 1,000	0.05 - 0.1	0.8
0007573	Phenyl-5PW, 100 nm	7.5	7.5	10.0	≥ 1,000	0.5 - 1.0	2.0
0007656	Phenyl-5PW, 100 nm	21.5	15.0	13.0	≥ 3,000	4.0 - 6.0	2.0
0014947	Butyl-NPR, nonporous	4.6	3.5	2.5		0.5 - 1.0	20.0
0042168	Butyl-NPR, nonporous	4.6	10.0	2.5	> 4,000	0.5 - 1.0	20.0
TSKgel PEEK columns							
0020023	BioAssist Phenyl, 100 nm	7.8	5	10.0	≥ 1,000	0.5 - 1.0	2.0

Guard column products		**ID (mm)**	**Length (cm)**	**Particle size (µm)**	
0014025	Ether-5PW Guardgel Kit, Glass			20.0	For P/Ns 0014013 and 0014014
0008643	Ether-5PW Guardgel Kit			20.0	For P/N 0008641
0007652	Phenyl-5PW Guardgel Kit			20.0	For P/N 0007573
0016095	Phenyl-5PW Prep Guardgel Kit			20.0	For P/N 0007656

HIC