

## Analytical and Small Preparative Scale Protein Separations, Using Hydrophobic Interaction Chromatography

*Hydrophobic interaction chromatography employs mild conditions that do not denature proteins. A sample is introduced onto the column in an aqueous mobile phase containing a high salt concentration. Proteins are retained by mild interactions between hydrophobic patches on the protein molecules and hydrophobic ligands on the packing. Individual proteins are eluted sequentially by decreasing the salt concentration. In reversed phase chromatography, in contrast, denaturing, nonpolar solvents are required to elute the proteins from the packing. Ligand characteristics, salt type and concentration, and other variables influence protein binding by HIC. Several HIC columns are described in this bulletin, and example applications are shown.*

### Key Words

- proteins
- hydrophobic interaction chromatography
- HIC

Hydrophobic interaction chromatography (HIC) is an effective method for separating proteins under mild (nondenaturing) conditions. In HIC, as in reversed phase chromatography (RPC), separations are based on the interactions of hydrophobic patches on the surfaces of the protein molecules with hydrophobic ligands covalently attached to the base matrix. HIC and RPC differ, however, in the degree of protein binding. Proteins bind very strongly to the more highly substituted RPC materials, and nonpolar solvents are required to elute them. This often leads to loss of protein activity. HIC is a gentler technique, typically compatible with aqueous mobile phases that do not denature or unfold the proteins.

Modest experimentation with the separation parameters allows HIC to be a highly effective tool. A sample is introduced onto the HIC column in an aqueous mobile phase containing a high salt concentration. The proteins in the sample are retained by a mild hydrophobic interaction with the packing. Individual proteins are eluted sequentially by decreasing the salt concentration. Ligand type and degree of substitution, salt type and concentration, pH, mobile phase additives, and other variables influence protein binding by HIC. To maximize recovery of protein mass and activity, and ensure optimal use of salt additives, the general rule-

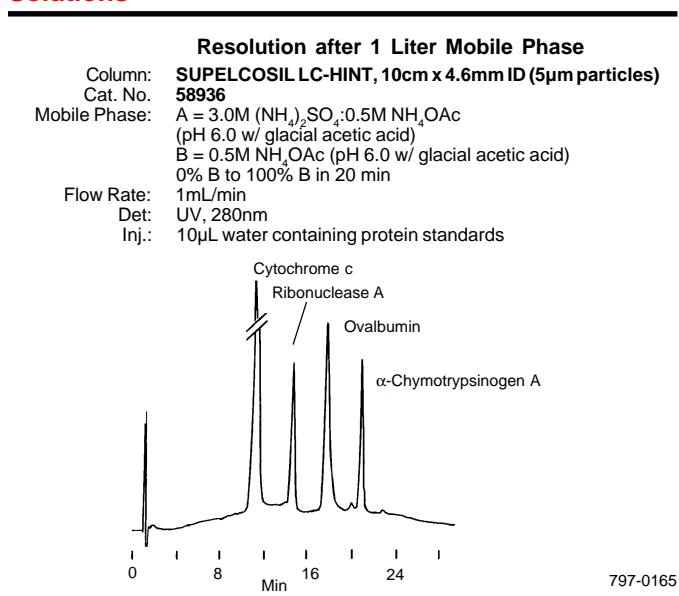
of-thumb is to separate less hydrophobic proteins with very hydrophobic ligands, and vice versa.

Table 1 summarizes the characteristics of several HIC columns. Because the columns are short, reequilibration between analyses is significantly faster than with 25cm or 30cm columns, yet these columns effectively separate proteins in milligram amounts, as well as on an analytical scale.

### SUPEL COSIL LC-HINT Columns

SUPEL COSIL™ LC-HINT columns contain a specially stabilized diol polar phase, bonded to spherical 5µm silica particles. The packing is physically and chemically very stable, even when exposed to high pressures or highly concentrated salt solutions. Figure A shows the separation of a common mixture of four globular proteins on a SUPEL COSIL LC-HINT column. Sample capacity was determined by injecting up to 10mg of protein in a constant volume of 100µL. Retention times for the separation shown in Figure A were unchanged for totals of up to 4mg of

**Figure A. SUPEL COSIL LC-HINT Columns Withstand Prolonged Exposure to Concentrated Salt Solutions**



**Table 1. Characteristics of HIC Columns**

Description	Matrix	Particle Size (µm)	Functional Group	Pore Size (Å)	Dimensions	pH Range
SUPEL COSIL LC-HINT	silica	5	diol	100	10cm x 4.6mm	2-7.5
TSKgel Butyl-NPR	silica	2.5	butyl	none	3.5cm x 4.6mm	2-7.5
TSKgel Ether-5PW	polymer	10	ether	1000	7.5cm x 7.5mm	2-12
TSKgel Phenyl-5PW	polymer	10	phenyl	1000	7.5cm x 7.5mm	2-12

**Table 2. Elution Volume of Proteins vs. Salt Type and Concentration: TSKgel Phenyl-5PW Column (7.5cm x 7.5mm ID, 10µm)**

Protein	Ammonium Sulfate			Sodium Sulfate		Potassium Sulfate	
	1.0M	1.5M	2.0M	1.0M	1.5M	1.0M	1.5M
Cytochrome c	2.6	2.7	8.0mL	2.4	5.4mL	2.2	2.6mL
Myoglobin	3.4	8.1	15.6	4.2	15.2	2.8	7.5
Ribonuclease A	3.9	13.5	19.8	9.0	15.2	2.8	7.5
Lysozyme	11.8	20.1	24.7	17.9	25.0	23.1	26.7
α-Chymotrypsin	23.1	27.2	29.6	25.2	29.6	23.1	26.7
α-Chymotrypsinogen A	26.7	30.1	32.1	28.9	32.3	26.2	29.5

Conditions:

A = 0.1M phosphate buffer + salt, pH 7.0; B = 0.1M phosphate buffer, pH 7.0; 0% B to 100% B in 30 min, 1.0mL/min.

Data courtesy of TosohHaas.

protein. When the total protein concentration exceeded 4mg, retention times decreased and peak shape deteriorated. These experiments were conducted using water as the sample mobile phase. Since most proteins are more soluble in water than in high salt phases, it may be necessary to increase the sample volume when injecting proteins in salt solutions.

### TSK-GEL HIC Columns

TSK-GEL® 5PW HIC columns contain porous polymer particles with phenyl or oligoethyleneglycol (ether) groups. The high porosities of TSKgel Phenyl-5PW and TSKgel Ether-5PW packings allow very large proteins to enter the internal pore structure, affording high capacity for such compounds. TSKgel Ether-5PW packing is less hydrophobic than TSKgel Phenyl-5PW packing, and is better for separating hydrophobic proteins. Column capacity varies from 0.5mg to 1mg, depending on the protein.

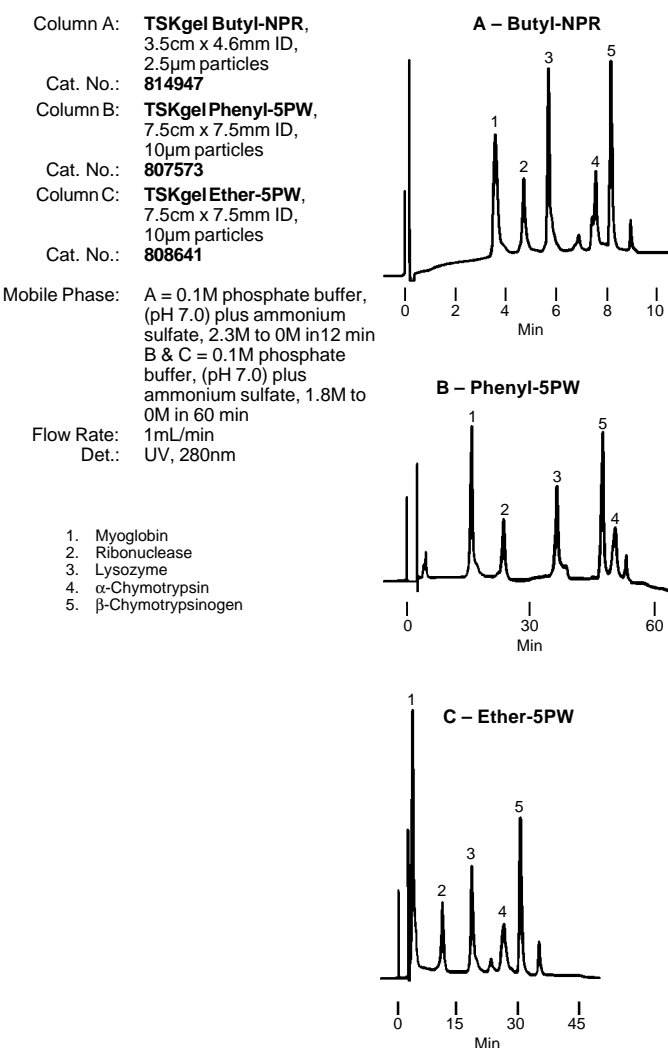
TSKgel Butyl-NPR columns provide fast, quantitative HIC analyses – typical analysis times are less than 15 minutes. Excellent mass recovery, due to the nonporous nature of the silica support, allows quantification down to the nanogram range. Relative to TSKgel Ether-5PW and TSKgel Phenyl-5PW materials, protein retention is intermediate (Figure B). Since almost all the surface area of a porous particle is inside the pores, the capacity of the NPR column is much smaller than that of the porous 5PW columns. An NPR column's capacity for a pure protein is 2mg, but sample loading for crude samples can be as much as 100mg.

### HiTrap HIC Test Kit

The HiTrap® HIC Test Kit contains five media – Phenyl Sepharose® High Performance, Phenyl Sepharose 6 Fast Flow (low sub), Phenyl Sepharose 6 Fast Flow (high sub), Butyl Sepharose 4 Fast Flow, and Octyl Sepharose 4 Fast Flow – packed in ready-to-use 2.5cm x 7mm, 1mL cartridges. Differences in hydrophobicity and ligand density influence binding, resolution, selectivity, and analyte recovery (Figure C). Because screening experiments must be performed for each new separation, the kit is ideal for optimizing separation conditions. The plastic cartridges have syringe and pump connections.

### Applications

**Figure B. Proteins on TSK-GEL Hydrophobic Interaction Chromatography Columns**

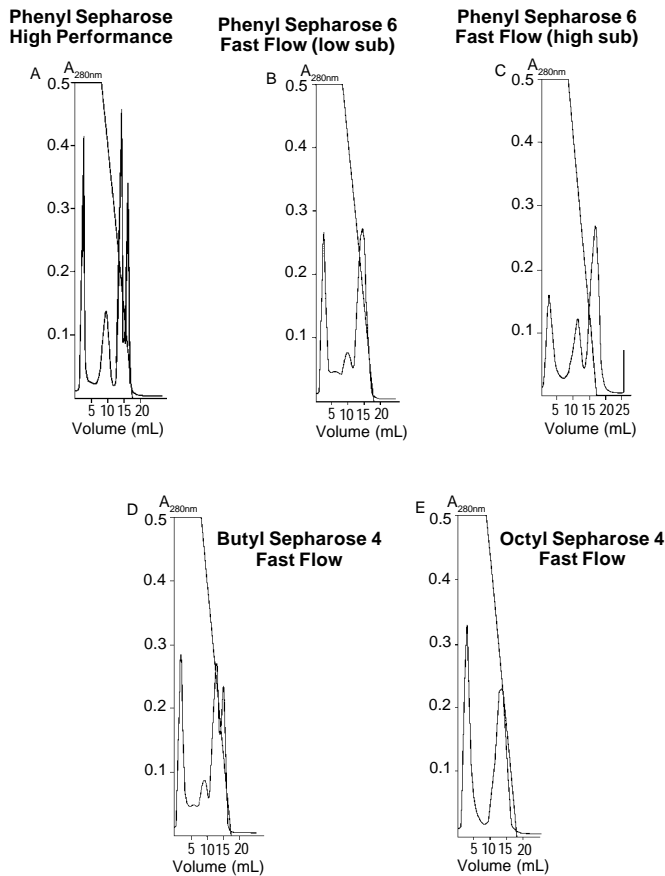


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The applications in Figures D-F demonstrate the benefits of HIC for protein separations. Human serum proteins must be resolved in the preparation of monoclonal antibodies. Figure D shows that these proteins can be resolved in small to relatively large quantity on a SUPELCOSIL LC-HINT column. Investigators at The Pennsylvania State University used a SUPELCOSIL LC-HINT column to isolate 5-lipoxygenase from a partially purified potato extract (Figure E). They were able to process about 5mg of protein at a time, and obtained a 280% increase in specific enzymatic activity. Figure F shows a purification of lipoxidase on a TSK-GEL column.

### Figure C. Selectivity of Media in HiTrap HIC Test Kit

Columns: **HiTrap HIC, 1mL**  
 Cat. No.: **54814** (HiTrap HIC Test Kit)  
 Starting Buffer: 0.1M Na<sub>2</sub>HPO<sub>4</sub>/1.7M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, pH 7.0  
 Elution Buffer: 0.1M Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0  
 Gradient: 0% to 100% elution buffer over 10mL  
 Flow Rate: 1.0mL/min (150cm/hr)  
 Det.: UV, 280nm (FPLC system)  
 Inj.: 1mL starting buffer containing cytochrome c, ribonuclease A, lysozyme, α-chymotrypsinogen, 1:3:1:1 (6mg total)

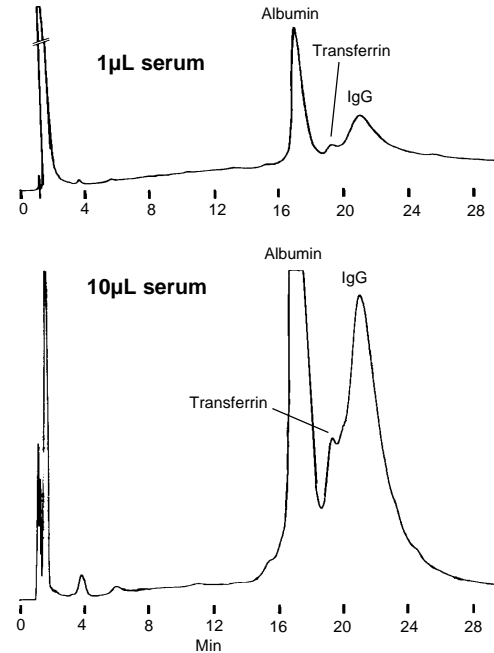


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### Figure D. Human Serum Proteins by HIC

Column: **SUPELCOSIL LC-HINT, 10cm x 4.6mm ID (5µm particles)**  
 Cat. No.: **58936**  
 Mobile Phase: A = 3.0M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>:0.5M NH<sub>4</sub>OAc (pH 6.0 w/ glacial acetic acid)  
 B = 0.5M NH<sub>4</sub>OAc (pH 6.0 w/ glacial acetic acid)  
 0% B to 100% B in 20 min  
 Flow Rate: 1mL/min  
 Det.: UV, 280nm



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### Figure E. 5-Lipoxygenase Isolated from Extract of Potato Tubers

Column: **SUPELCOSIL LC-HINT, 10cm x 4.6mm ID, 5µm particles (with 0.5µm screen filter)**  
 Cat. No.: **58936**  
 Mobile Phase: A = 2.0M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in 50mM K phosphate, pH 6.3  
 B = 50mM K phosphate/50mM NH<sub>4</sub>OAc  
 0% B (3 min), to 75% B in 7 min, hold 10 min, then to 100% B in 5 min  
 Flow Rate: 1mL/min  
 Temp.: 25°C  
 Det.: UV, 280nm  
 Inj.: 500µL 50mM K phosphate, pH 6.3, containing 5mg crude extract

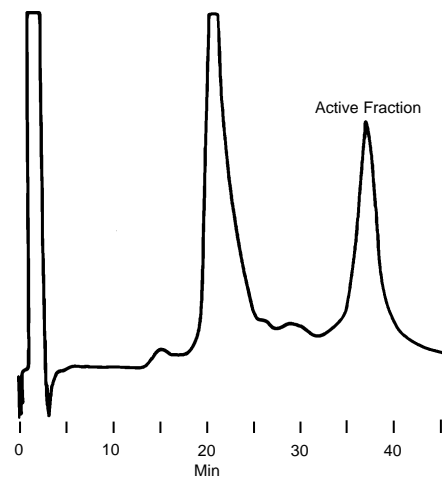
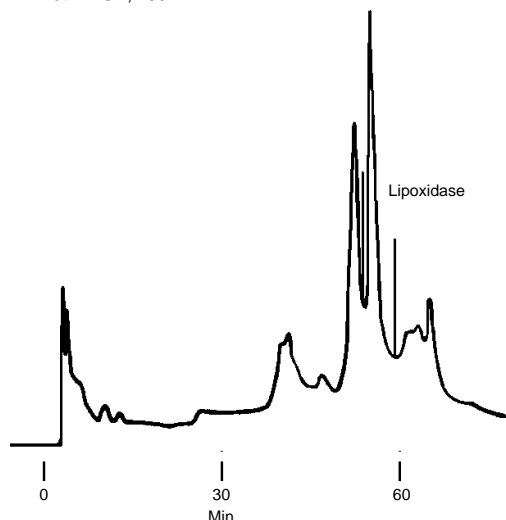


Figure provided by P. Reddanna and C. Channa Reddy, Center for Air Environmental Studies, Department of Veterinary Science The Pennsylvania State University, University Park, PA USA.

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## Figure F. Purification of Commercial Lipoxidase

Column: **TSK-GEL Phenyl-5PW, 7.5cm x 7.5mm ID, 10µm particles**  
 Cat. No.: **807573**  
 Mobile Phase: A = 0.1M phosphate buffer, pH 7.0 + 1.5M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>  
 B = 0.1M phosphate buffer, pH 7.0  
 linear gradient, 0% B to 100% B over 60 min  
 Flow Rate: 0.5mL/min  
 Det.: UV, 280nm



794-0320

## Ordering Information:

Description	Cat. No.
HiTrap HIC Test Kit	54814
SUPELCO SIL LC-HINT column	58936
Supelguard™ LC-HINT guard column kit	59637
Replacement guard columns, pk. of 2	59638
TSK-GEL Butyl-NPR column	814947
TSK-GEL Ether-5PW column	808641
TSK-GEL Ether-5PW guard column kit	808643
TSK-GEL Ether-5PW packing (5mL)	808644
TSK-GEL Phenyl-5PW column	807573
TSK-GEL Phenyl-5PW guard column kit	807652
TSK-GEL Phenyl-5PW packing (5mL)	807651

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HIC applications are often scaled-up to low pressure media. For a complete listing of our HIC media, see the current Supelco catalog.

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