

TheReporter

Reprinted from Volume 14, No. 6, 1995

T295036

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Hydrophobic Interaction Chromatography (HIC) Columns: Method Development

N. Lai, K. Pardue

Hydrophobic interaction chromatography is an effective method for protein separation under mild conditions. Method development is a crucial step for each separation by HIC. This article describes the effect of salt type and concentration, pH, and isopropanol additive on protein separations on phenyl ligands.

In hydrophobic interaction chromatography (HIC), as in reversed phase chromatography, protein separation is based on interaction of hydrophobic patches on the surface of the protein with hydrophobic ligands covalently attached to the base matrix (Figure A). HIC is a more gentle technique that lends to the use of salt-containing aqueous mobile phases that tend not to denature or unfold the protein.

A number of factors influence protein binding by HIC, including base matrix, ligand type and degree of substitution (1), salt type and concentration, pH, and mobile phase additives. These parameters must be established by experiment for each new separation. ■

The contribution of the base matrix cannot be overlooked. In the data that follow, we compare SigmaChrom™ HIC-Phenyl and Progel™-TSK Phenyl-5PW columns, representing the two most widely used types of support: hydrophilic crosslinked polysaccharide and copolymer. Selectivity, even with the same ligand, is not the same for the two supports. It is necessary to modify adsorption and elution conditions in switching from one support to the other.

Figure A. Proteins by Hydrophobic Interaction Chromatography

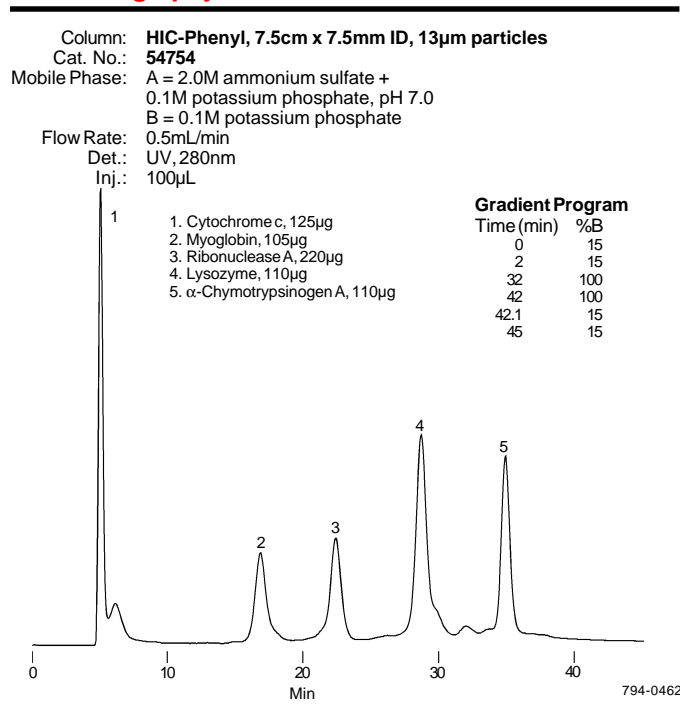


Table 1. Elution Volume of Proteins vs. Salt Type and Concentration

Protein	Ammonium Sulfate			Sodium Sulfate		Potassium Sulfate	
	1.0M	1.5M	2.0M	1.0M	1.5M	1.0M	1.5M
Progel-TSK Phenyl-5PW (7.5cm x 7.5mm ID, 10µm)							
Cytochrome c	2.55	2.70	7.95	2.40	5.37	2.25	2.55
Myoglobin	3.39	8.07	15.60	4.20	15.18	2.85	7.50
Ribonuclease A	3.90	13.50	19.80	9.00	15.18	2.85	7.50
Lysozyme	11.85	20.10	24.69	17.94	24.99	23.07	26.70
α-Chymotrypsin	23.10	27.18	29.58	25.20	29.58	23.07	26.70
α-Chymotrypsinogen A	26.70	30.06	32.10	28.86	32.28	26.22	29.49
SigmaChrom HIC-Phenyl (7.5cm x 7.5mm ID, 12-15µm)							
Myoglobin	2.55	3.50	8.00	2.55	12.60	2.50	12.00
Ribonuclease A	3.95	7.00	11.00	7.65	17.00	5.60	15.13
Lysozyme	6.75	11.50	14.50	8.85	21.00	10.30	17.25
α-Chymotrypsinogen A	13.00	16.00	17.00	14.00	22.00	14.90	19.00

Conditions:

Progel-TSK Phenyl-5PW, 1.0mL/min, 30 min linear gradient from 0% B to 100% B; A = 0.1M phosphate buffer + salt, pH 7.0, B = 0.1M phosphate buffer, pH 7.0.

SigmaChrom HIC-Phenyl, 0.5mL/min, 30 min linear gradient from 0% B to 100% B, A = 0.1M potassium phosphate, monobasic + salt, pH 7.0, B = 0.1M potassium phosphate, monobasic, pH 7.0.

Progel-TSK data courtesy of TosoHaas.

Figure B. Effect of Salt Type and Concentration

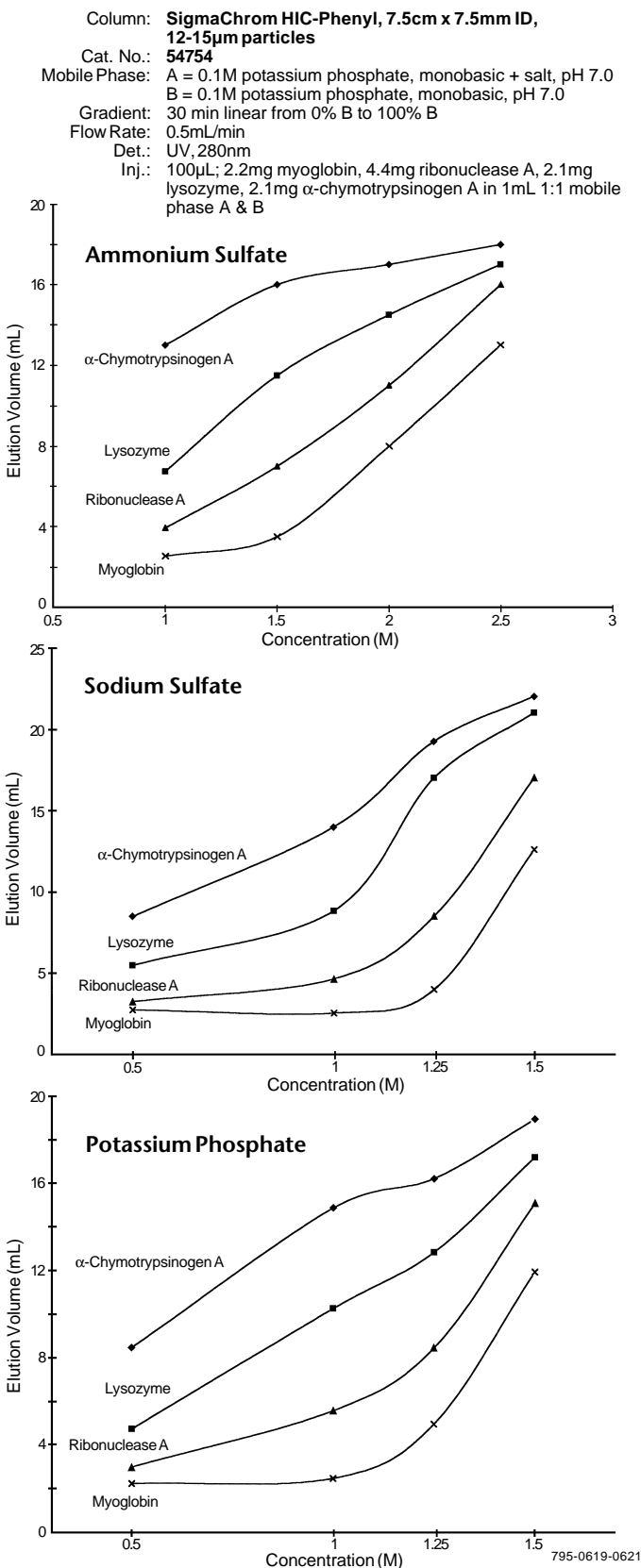
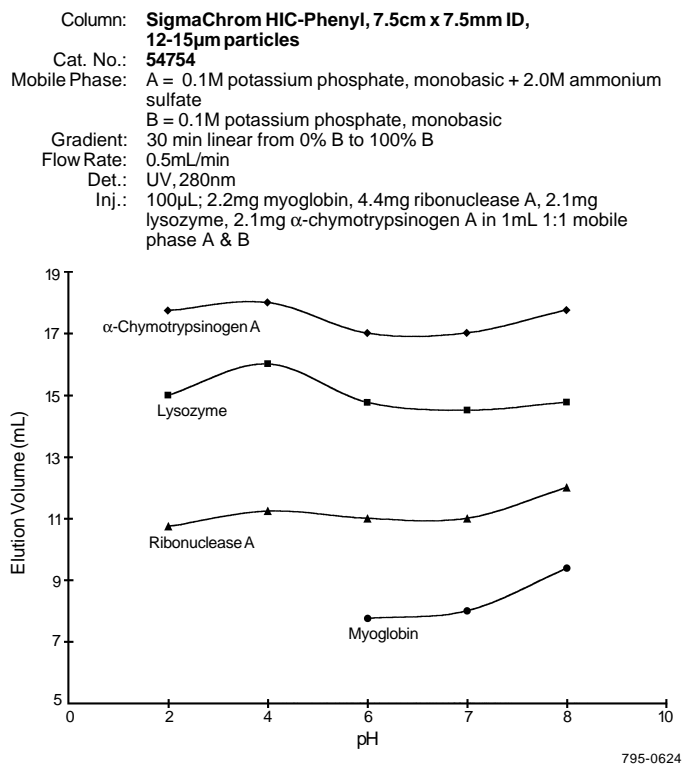


Figure C. Effect of pH



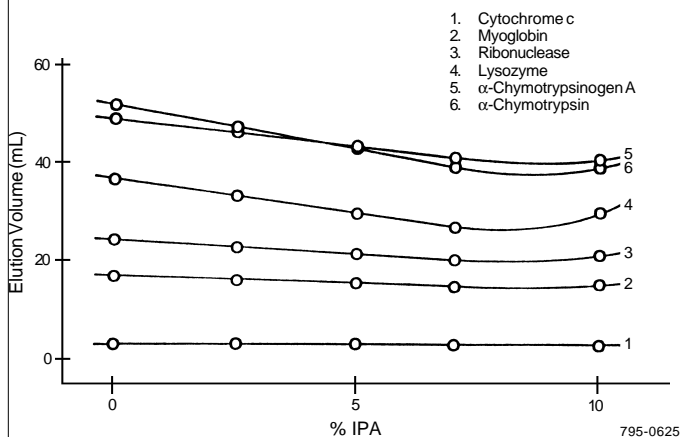
As salt concentration increases, the quantity of bound proteins increases. Proteins elute during a decreasing salt gradient. The salts most frequently used in HIC are ammonium sulfate, sodium sulfate, and potassium sulfate. Of these, ammonium sulfate is the most popular because of its high solubility (up to 4M) and its inhibition of microbial growth. In our experience, sodium sulfate must be limited to lower concentrations (up to 1.5M) due to lower solubility. Figure B shows the effect of salt type and concentration on the elution volume of four proteins on an HIC-Phenyl column. Ammonium sulfate had the smallest elution volumes, followed by sodium sulfate and potassium phosphate.

The HIC-Phenyl column is less hydrophobic than the Phenyl-5PW column (Table 1). Thus, less salt is needed when using the HIC-Phenyl column, which would function well for more hydrophobic proteins. Generally, very hydrophobic ligands are used with less hydrophobic proteins, and vice versa. These situations allow for optimum mass recovery and activity, while minimizing the concentration of salt additives.

The effect of pH is not always apparent. A lower pH will, in theory, increase hydrophobic interactions. HIC applications typically are run between pH 5 and pH 7. We varied the pH from 2 to 8 for separation of four proteins on an HIC-Phenyl column (Figure C). The different pH values did not have a significant effect on elution volumes. (Myoglobin is not soluble at pH 2 or 4.) The same trends were observed on a Phenyl-5PW column (data not shown).

Figure D. Effect of Isopropanol

Column: **Progel-TSK Phenyl-5PW, 7.5cm x 7.5mm ID, 10µm particles**
 Cat. No.: **807573**
 Mobile Phase: A = 0.1M phosphate + 1.8M ammonium sulfate, pH 7.0
 B = 0.1M phosphate + IPA, pH 7.0
 Det.: UV, 280nm
 Flow Rate: 1mL/min
 Gradient: 60 min linear from 0% B to 100% B



Mobile phase additives, such as isopropanol (IPA), usually decrease protein-ligand interactions. Nonpolar portions of alcohols and detergents compete with the proteins for ligand sites. Chaotropic salts affect the ordered structure of water and bound proteins. However, at high concentrations, the additives may cause protein aggregation and/or denaturation. Therefore, the effect of additives is not always straight-forward. The trend of elution volume vs. %IPA on a Phenyl-5PW column (Figure D) was similar to that on an HIC-Phenyl column (data not shown). On the Progel-TSK Phenyl-5PW column, several of the proteins showed a slight increase in elution volume from 7% IPA to 10% IPA.

Ordering Information:

Description	Cat. No.
SigmaChrom HIC-Phenyl Column 7.5cm x 7.5mm ID	54754
Replacement Frits, pk. of 2	54770
Progel-TSK Phenyl-5PW Column 7.5cm x 7.5mm ID	807573
Progel-TSK Phenyl-5PW Guard Column Kit	807652

Suggested Reading

Hydrophobic Interaction Chromatography: Principles and Methods
23582

Reference

1. *The Reporter*, Vol. 14, No. 4, pp. 8-10.

