High Resolution Silica Hydrophobic Interaction Chromatography (HIC) Column for Protein/Peptide Separations with Improved Hydrolytic Stability

S. Rao, A. Bordunov, and C. Pohl, Dionex Corporation, Sunnyvale, CA, USA

DIONEX
Passion. Power. Productivity.

ABSTRACT

HIC is a powerful technique and is routinely used for protein purification. We have developed a new silica based HIC stationary phase with multifunctional amide group attachments. 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 resulted in superior resolution and peak efficiencies of proteins/ peptides and provided a versatile column with sufficient longevity.

HIC separates proteins, peptides and other biomolecules by taking advantage of surface exposed hydrophobicities. HIC complements ion-exchange chromatography and gel filtration chromatography for separation of various bio molecules. In this poster, we have demonstrated the use of ProPac® HIC-10 column for various applications. Some examples include 1) Mixture of proteins consisting of cytochrome c, myoglobin, ribonuclease A, lysozyme and chymotrypsinogen 2) Mixture of peptides 3) Trypsin digests of proteins 4) Monoclonal antibodies 5) Snake venom proteins 6) Serum proteins 7) Pancreatin. We have determined the loading capacity for proteins and for monoclonal antibodies. Also, the effect of addition of solvent to the eluent on the peak shape was evaluated.

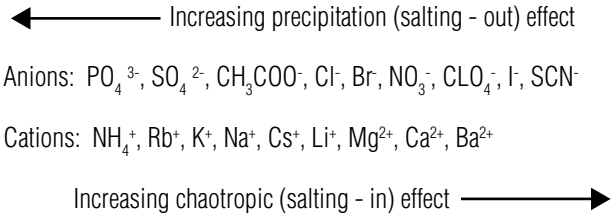
ProPac HIC-10 column exhibited excellent reproducibility and ruggedness. We have compared the ProPac HIC-10 column with other leading HIC columns for efficiency, peak shape, resolution, and capacity. The new ProPac HIC-10 column displayed high capacity and superior overall performance.

INTRODUCTION

Hydrophobic interaction chromatography (HIC)[1–3] 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 (e.g., 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.

The effects of salts in HIC can be accounted for:

1. The precipitation of proteins or
2. Their positive influence in increasing the molal surface tension of water.



Relative effects of salts on the molal surface tension of water are as follows. Sodium, potassium and ammonium sulphates produce relatively higher salting out or molal surface tension increment effects than sodium or lithium chloride.

$Na_2SO_4 > K_2SO_4 > (NH_4)_2SO_4 > Na_2HPO_4 > NaCl > LiCl \ldots > KSCN$

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 biomolecules, 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. 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.

In this study, we have presented results from a newly developed 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 that were designed to improve the hydrolytic stability of silica support in the aqueous media. These modifications resulted in superior resolution and peak efficiencies of proteins/peptides and provided a versatile column with sufficient longevity. We have compared our new ProPac HIC-10 with columns from leading vendors and the results indicated that the new HIC column showed high-capacity and superior overall performance.

MATERIALS

Chromatographic Components

- P680 HPG pump, UVD-340U detector, GINA50 autosampler were from Dionex Corporation
- Column oven TCC-100 from Dionex Corporation and L-306 is from SSI.
- Chromatography was controlled by Chromeleon Chromatography Management Software from Dionex Corporation.

Proteins, Peptides, Biomaterials, and Chemicals

Cytochrome c, myoglobin, ribonuclease A, chymotrypsinogen, bovine serum albumin, β–casein, bovine serum, thrombin, cobra venom, and HPLC peptide standard mixture were obtained from Sigma. MAb was a gift from Protein Design Labs, Fremont, CA. Ammonium sulfate and all other analytical-grade chemicals were also obtained from Sigma. Lysozyme is from Calbiochem. Immobilized trypsin was from Pierce.

Columns from Dionex Corporation

ProPac HIC-10 are Silica columns based on proprietary ethyl/amide based chemistry

P/N	Description	Dimensions
063653	ProPac HIC-10 5 µm, 300 Å	2.1 × 100 mm
063655	ProPac HIC-10 5 µm, 300 Å	4.6 × 100 mm
063665	ProPac HIC-10 5 µm, 300 Å	7.8 × 75 mm

Columns from Commercial Vendors for Comparison

- Biosuite Phenyl (Polymer, 10 µm, 1000 Å, 7.5 × 75 mm) from Waters Corp.
- Column-Phenyl 5PW (Polymer, 10 µm, 1000 Å, 7.5 × 75 mm) from Tosoh Corp.
- Ethyl (Silica, 5 µm, 300 Å, 4.6 × 100 mm) from PolyLC Inc.

METHODS

Capacity test by breakthrough experiment

After equilibrating the column with eluent A, either lysozyme (0.3 mg/mL, Figure 10) or MAb (0.18 mg/mL, Figure 11) was added to eluent A with mixing. Continue pumping the column with eluent A (containing the protein) and monitor the UV trace to record the capacity.

Stability of the ProPac HIC-10 bonded phase and mechanical ruggedness

An isocratic small molecule test was performed between the repeated gradient runs to access the ruggedness of the ProPac HIC-10 column.

The test mixture contained cytosine, 4-butyl benzoic acid, amitriptyline, and phenanthrene; each of 25 µg/mL. This 4 small molecule mixture was injected (5 µL) interspersed by 10 blank methanol gradient runs (25%–95%). The total run time for blank is 6 min. The total run time for the small molecule mixture is 10 min including 5 min pre-injection equilibration. A total of 310 cycles were performed including 21 runs of small molecule injections to test the ruggedness of the ProPac HIC-10 column. The data was plotted for one of the small molecules (phenanthrene) as injection number vs. retention time, asymmetry, peak width (50%) and efficiency (plates).

Gradients

2.1 × 100 mm format: 0–100%B in 25 min

4.6 × 100 mm format: 0–100%B in 20 min

7.5 × 75 mm format: 0–100%B in 60 min

7.8 × 75 mm format: 0–100%B in 60 min

Or, as described in figures.

Dual Gradients

Dual gradients include an initital aqueous gradient followed by an organic solvent gradient.

SAMPLE PREPARATION

- **Protein mixture**
  Mixture of 5 proteins (1 mg/mL each final after 1:1 dilution with eluent A). (1) cytochrome c, (2) myoglobin, (3) ribonuclease A, (4) lysozyme, (5) chymotrypsinogen
- **Peptide mixture**
  Mixture of 5 peptides 1:1 diluted with eluent A. (1) Gly-Tyr, (2) Val-Tyr-Val, (3) Methionine Enkephalin Acetate (Tyr-Gly-Gly-Phe-Met), (4) Leucine Enkephalin (Tyr-Gly-GLy-Phe-Leu), (5) Angiotensin II acetate (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)
- **Bovine serum**
  Bovine serum (1:2 diluted with D.I. 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)
- **Cobra venom**
  Naja venom 5 mg/mL in PBS and diluted 1:1 with 1 M $(NH_4)_2\ SO_4$ in 0.1 M NaH_2PO_4 (pH 7.0)
- **Pancreatin**
  25 mg + 500 µL D.I. H_2O + 500 µL eluent A, mixed well and spun at 14,000 rpm for 20 min. Supernatant was used as Pancreatin sample.
- **Proteomics applications; Separation of peptides**
  (1) 1.2 mg of cytochrome c was digested with immobilized trypsin (Pierce). The digest was mixed with equal volume of eluent A before applying onto the column. See Figure 8.
- **Monoclonal antibody (MAb)**
  MAb was prepared as described in Figure 9 and was separated with 10% MeCN in Eluent B (Figure 9B) or without MeCN in eluent B (Figure 9A).

PROTEIN/PEPTIDE STANDARDS

Figure 1. Separation of a Mixture of Proteins

Column: 7.5 × 75 mm (Waters and Tosoh); 7.8 × 75 mm (Dionex ProPac HIC-10)
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: Mixture of proteins (1mg/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

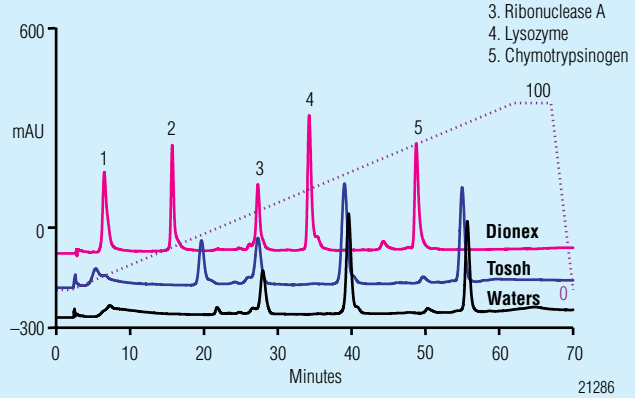


Figure 2. Separation of a Mixture of Proteins

Column: 4.6 × 100 mm (PolyLC) 4.6 × 100 mm (Dionex ProPac HIC-10)
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)
Sample: Mixture of 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

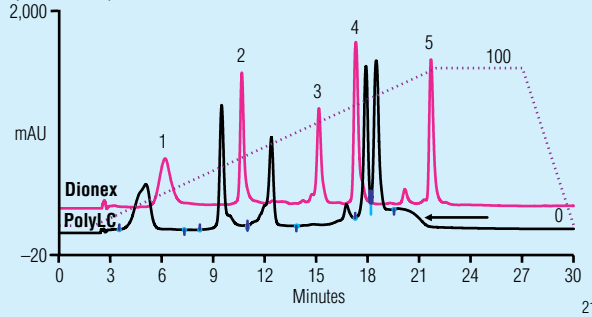


Figure 3. Separation of a Mixture of Proteins

Column: 2.1 × 100 mm (Dionex ProPac HIC-10)
Flow: 0.2 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: Mixture of proteins (1 mg/mL each final after 1:1 dilution with eluent A)
Inj. Volume: 5 µL
Order of elution: 1. Cytochrome c 2. Myoglobin 3. Ribonuclease A 4. Lysozyme 5. Chymotrypsinogen

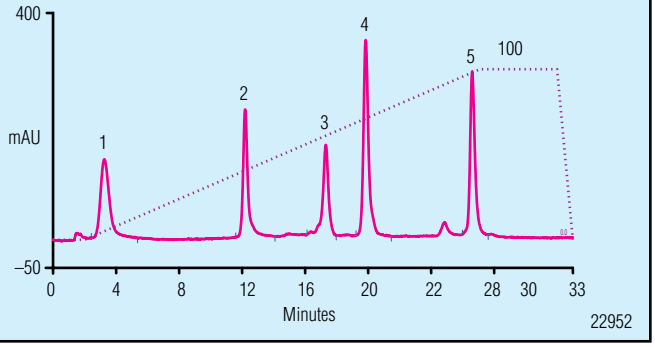
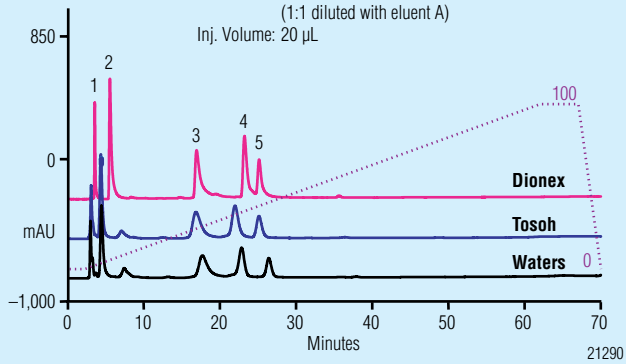


Figure 4. Separation of Peptide Mixture

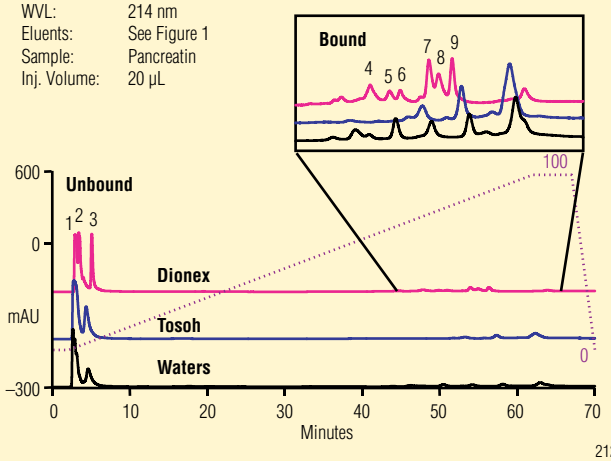
Column: 7.5 × 75 mm (Waters and Tosoh)
7.8 × 75 mm (Dionex ProPac HIC-10)
Flow: 1.0 mL/min
WVL: 214 nm
Eluents: (A) 2 M $(NH_4)_2SO_4$ in 0.1 M NaH_2PO_4 (pH 7.0)
(B) 0.1 M NaH_2PO_4 (pH 7.0)
Sample: 5 Peptide mixture from Sigma
(1:1 diluted with eluent A)
Inj. Volume: 20 µL



21290

Figure 6. Separation of Pancreatin

Column: 7.5 × 75 mm (Waters and Tosoh);
7.8 × 75 mm (Dionex ProPac HIC-10)
Flow: 1.0 mL/min
WVL: 214 nm
Eluents: See Figure 1
Sample: Pancreatin
Inj. Volume: 20 µL

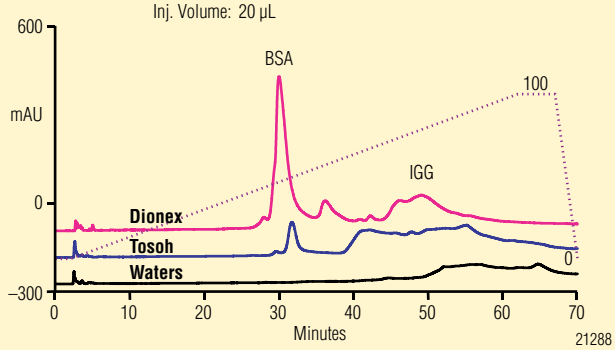


21292

APPLICATIONS

Figure 5. Separation of Bovine Serum Proteins

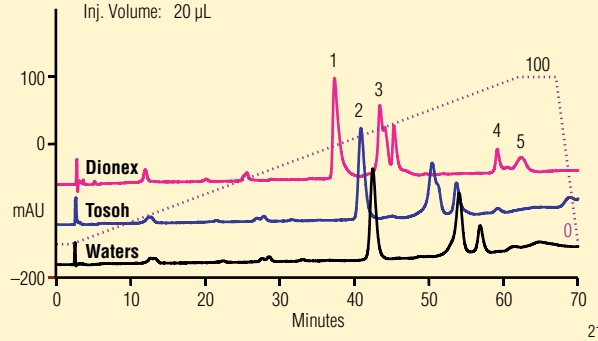
Column: 7.5 × 75 mm (Waters and Tosoh)
7.8 × 75 mm (Dionex ProPac HIC-10)
Flow: 1.0 mL/min
WVL: 214 nm
Eluents: See Figure 1
Sample: Bovine serum (1: 2 dilited with D.I. H_2O
and mixed with equal volume of
1 M $(NH_4)_2SO_4$ in 0.1 M NaH_2PO_4 (pH 7.0)
Inj. Volume: 20 µL



21288

Figure 7. Separation of Snake Venom Proteins/Peptides

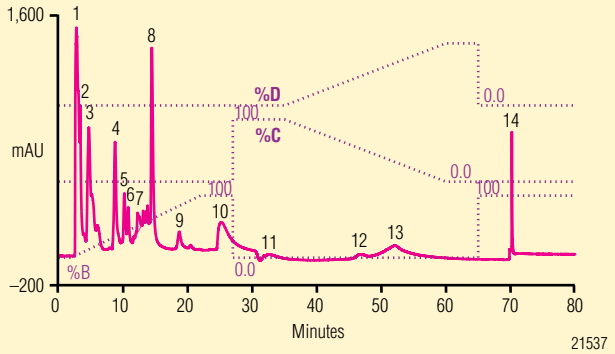
Column: 7.5 × 75 mm (Waters and Tosoh)
7.8 × 75 mm (Dionex ProPac HIC-10)
Flow: 1.0 mL/min
WVL: 214 nm
Eluents: (A) 2 M $(NH_4)_2SO_4$ in 0.1 M NaH_2PO_4 (pH 7.0)
(B) 0.1 M NaH_2PO_4 (pH 7.0)
Sample: Naja venom from Sigma [1:1 diluted with 1 M $(NH_4)_2SO_4$
in 0.1 M NaH_2PO_4 (pH 7.0)]
Inj. Volume: 20 µL



21289

Figure 8. Separation of Peptides—Proteomics Application

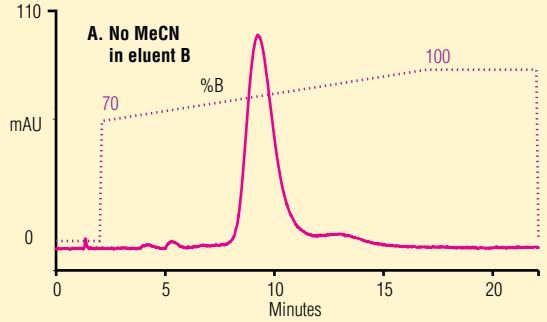
Column: 4.6 × 100 mm (Dionex ProPac HIC-10)
Flow: 0.5 mL/min
WVL: 214 nm
Eluents: (A) 2 M $(NH_4)_2SO_4$ in 0.1 M NaH_2PO_4, pH 7.0 (B) 0.1 M NaH_2PO_4, pH 7.0
Eluents: (C) D.I. H_2O (D) 70% MeCN
Sample: Trypsin digest (1.2 mg) of Cytochrome c (Immobilized trypsin was used)
Inj. Volume: 40 µL
Dual gradient: 0–100% B in 20 min. After equilibration: 0–100% D in 25 min



21537

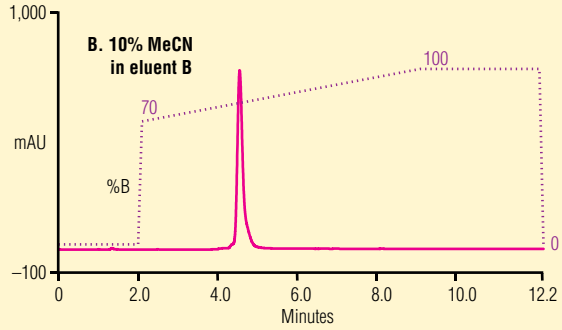
Figure 9. Separation of Monoclonal Antibody (MAb)

Column: Dionex ProPac HIC-10 (4.6 × 100 mm)
Flow: 1 mL/min
WVL: 280 nm
Sample: MAb 50 µL (50 mg/mL) + 450 µL Eluent A
Eluents: (A) 0.5 M $(NH_4)_2SO_4$ in 0.1M NaH_2PO_4 (pH 7.0)
(B) 0.1 M NaH_2PO_4 (pH 7.0)
Inj. Volume: 5 µL (25 µg)
Gradient: 70–100% B in 15 min



Sample: MAb
Inj. Volume: 20 µL (100 µg)
Gradient: 70–100% B in 7 min.

23144

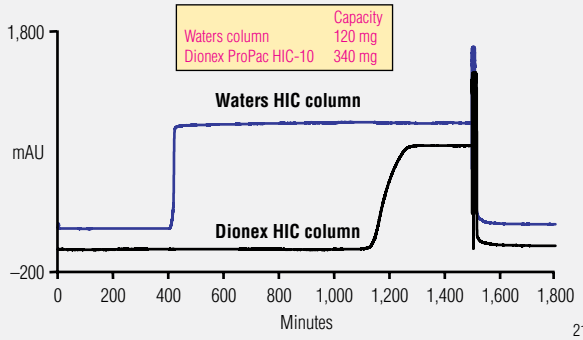


21538

COLUMN CAPACITY

Figure 10. Capacity of HIC Columns, Lysozyme

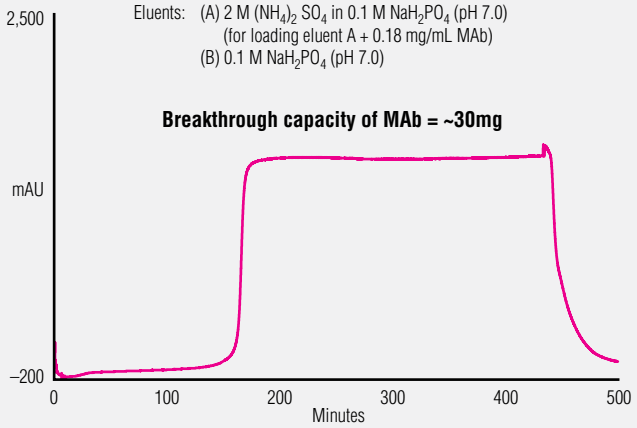
Column: 7.5 × 75 mm, (Waters)
7.8 × 75 mm, (Dionex ProPac HIC-10)
Flow: 1.0 mL/min
WVL: 214 nm
Eluents: (A) 2 M $(NH_4)_2SO_4$ in 0.1 M NaH_2PO_4 (pH 7.0)
(For loading eluent A + 0.3 mg/mL lysozyme)
(B) 0.1 M NaH_2PO_4 (pH 7.0)



21297

Figure 11. Capacity of HIC Columns, MAb

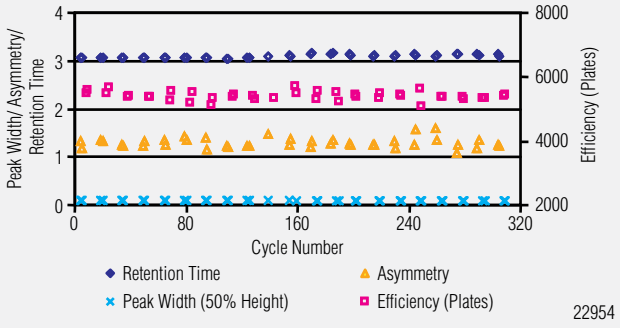
Column: 4.6 × 100 mm (Dionex ProPac HIC-10)
Flow: 1.0 mL/min
WVL: 214 nm
Eluents: (A) 2 M $(NH_4)_2SO_4$ in 0.1 M NaH_2PO_4 (pH 7.0)
(for loading eluent A + 0.18 mg/mL MAb)
(B) 0.1 M NaH_2PO_4 (pH 7.0)



COLUMN STABILITY

Figure 12. Stability of the Bonded Phase and Mechanical Ruggedness

Column:	4.6 × 100 mm, (Dionex ProPac HIC-10)
Flow:	1 mL/min
Eluent (Isocratic):	30% CH_3CN/0.1 M Amm. Acetate pH 5.4
Eluents (Gradient):	Eluent A: 25 mM Ammonium acetate pH 5.4
	Eluent B: 100% Methanol
Sample:	Small molecule mixture was injected every 10th cycle



22954

CONCLUSION

We introduced a new Hydrophobic Interaction Chromatography column (ProPac HIC-10) for the separation of proteins and peptides.

We used our proprietary silica bonding technology for improved hydrolytic stability.

The new silica ProPac HIC-10 column displayed excellent resolution and stability, and high capacity. Breakthrough capacity for lysozyme is 340 mg (7.8 × 75 mm) and for MAb is 30 mg (4.6 × 100 mm).

We compared the performance of the ProPac HIC-10 column with other HIC columns for various applications. The ProPac HIC-10, under the same conditions exhibited higher resolution and better overall performance.

ACKNOWLEDGEMENTS

We thank A. Heckenberg for her contributions to the development of ProPac HIC-10.

REFERENCES

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

Passion. Power. Productivity.

DIONEX

Dionex Corporation
1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
(408) 737-0700

North America
Sunnyvale, CA (408) 737-8522 Westmont, IL (630) 789-3660
Houston, TX (281) 847-5652 Atlanta, GA (770) 432-8100
Marlton, NJ (856) 596-0600 Canada (905) 844-9650

www.dionex.com

Europe
Austria (43) 1 616 51 25 Belgium (32) 3 353 4294
Denmark (45) 36 36 90 90 France (33) 1 39 30 01 10
Germany (49) 6126 991 210 Italy (39) 06 66 51 5052
The Netherlands (31) 161 43 43 03
Switzerland (41) 62 205 99 66
United Kingdom (44) 1276 691722

Asia Pacific
Australia (61) 2 9420 5233 China (852) 2428 3282
India (91) 22 28475235 Japan (81) 6 6885 1213
Korea (82) 2 2653 2580

LPN 1864-01 06/06