Vydac Advances

Quarterly Technical Newsletter on the Characteristics and Use of Vydac HPLC Columns *Fall, 1997*

Separating Complex Carbohydrates

Vydac 218TP and 238TP Reversed-Phase Columns Aid in Structure Research

Founded in 1985, the Complex Carbohydrate Research Center (CCRC) at the University of Georgia, Athens, is dedicated to advancing knowledge of the structure and functions of oligo- and polysaccharides. The Center employs about 100 scientists and staff and actively pursues research using a variety of techniques in an area regarded by many as the last frontier of molecular biochemistry. More information regarding the CCRC can be obtained by visiting the world wide web site at **http://www.ccrc.uga.edu**.

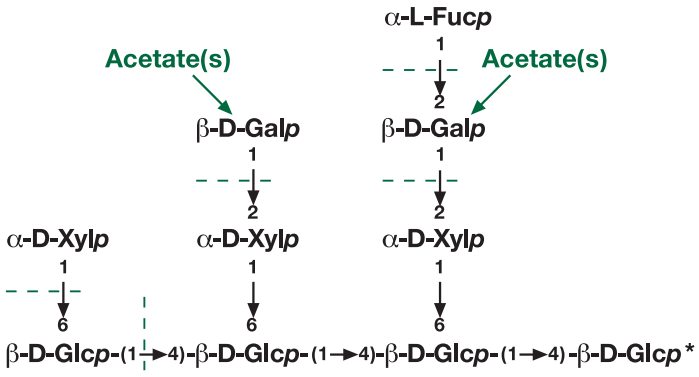


Figure 1. Maximum structure of a cell-wall-component xyloglucan oligosaccharide isolated from bean cell culture. An asterisk designates the reducing end. Underivatized variants of this structure differing in acetate position on the galactose residues or arising by cleavage separate in distinct retention-time groups on two Vydac reversed-phase columns.

Developing separation methodology is an integral part of the CCRC's research effort. Chromatographic separations of closely related oligosaccharides help elucidate structural details to obtain essential metabolic information. The structure and chromatograms in Figures 1 and 2 are examples of such separations.

In this case the object of study was the structural characterization of xyloglucan, a major hemicellulosic component of the primary cell wall of higher plants. Xyloglucan polymer obtained from the medium of a bean cell culture was digested with a fungal endo-β-1,4-glucanase, yielding a variety of xyloglucan oligosaccharides. The maximum structure of the oligosaccharides is depicted in Figure 1. The backbone of the molecule is a string of four glucopyranose residues. To the first three of these are linked successively xylose, galactose, and fucose residues in a stairlike arrangement. The galactose residues are often acetylated at the 3, 4, or 6 positions. In solution, acetyl groups migrate slowly between the three positions, reaching in about half a day an equilibrium that favors the 6 position (80%) with the remainder equally divided between 3 and 4. Smaller oligosaccharides obtained from the culture medium are indicated by dashed lines. They can be minus one or more residues including the fucosyl, galactosyl, the leftmost xylosyl, and the leftmost glucosyl. To structurally characterize the different oligosaccharides (particularly the location of the O-acetate) via spectroscopic methods (mass spectrometry, NMR, etc.) it was imperative to purify the native, underivatized oligosaccharides.

As shown by the chromatograms of Figure 2, reversed-phase chromatography on Vydac 218TP or 238TP columns can separate most of these molecules. The presence of the

(continued on Page 2)

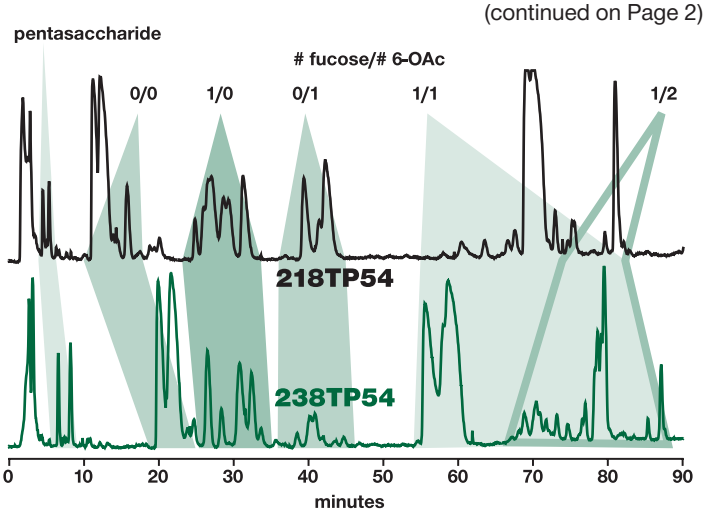


Figure 2. Separation of oligosaccharides represented by the decasaccharide structure of Figure 1 on two Vydac reversed-phase columns. Presence of fucose and/or at least one acetate at the 6 position on galactose produce major retention effects. **Columns:** 218TP54 "polymeric" reversed-phase and 238TP54 "monomeric" reversed-phase, both with 5µm spherical packing in 4.6mm ID x 250mm L columns. **Flow rate:** 1 mL/min. **Mobile phase:** methanol in water. **Gradient:** linear, 7% to 10% MeOH in 60 minutes, then to 25% at 90 minutes. **Detection:** ELSD. The logarithm of the detector signal has been plotted to accentuate smaller peaks.

Complex Carbohydrates

(continued from Page 1)

fucose residue and acetylation of galactose at the 6 position markedly increase retention, causing the molecules to emerge within the indicated time groups. Within these groups, presence or absence of additional sugar residues and acetylation of galactose at the 3 or 4 positions have less dramatic effects, but are nonetheless sufficient to produce resolution in most instances. The samples were chromatographed without prior derivatization, and peaks were detected using a SEDEX 55 evaporative light scattering detector (ELSD) purchased from Richard Scientific, Novato, CA. Peaks were collected and were further analyzed by mass spectrometry and NMR after evaporation of the volatile solvents.

The Vydac 218TP and 238TP are both C18 reversed-phase columns, but produced using "polymeric" and "monomeric" bonding chemistries, respectively. These two columns provide subtly different selectivities, and one or the other may produce better resolution of specific analyte pairs.

Information and chromatograms for this article were generously provided by

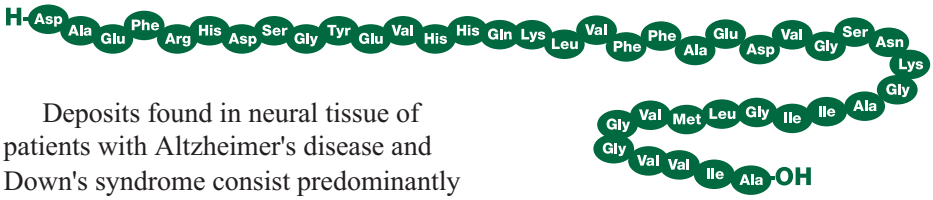
Markus Pauly
CCRC, University of Georgia
220 Riverbend Road
Athens, GA 30602
(706) 542-4419
mpauly@ccrc.uga.edu

Ordering Information

Cat. No.	Description
238TP54	Column, Octadecyl (C18), **Monomeric**, 5µm, 300Å, 4.6mm ID x 250mm L
218TP54	Column, Octadecyl (C18), **Polymeric**, 5µm, 300Å, 4.6mm ID x 250mm L
259VHP54	Column, **Polymer Reversed-Phase**, 5µm, 300Å, 4.6mm ID x 150mm L

Other analytical and preparative column dimensions available upon request.

Reversed-Phase Purification of Recombinant β-Amyloid Peptide



Deposits found in neural tissue of patients with Altzheimer's disease and Down's syndrome consist predominantly of a 42-amino-acid peptide, the β-amyloid protein. This peptide, the subject of intense research, has been synthesized chemically and produced as a fusion protein. In either case, contaminating materials arising from the production milieu must be removed. But purification of β-amyloid protein can be a challenging affair, due to its tendency to aggregate and its inherent low solubility.

Vydac 259VHP, a high-performance chemically resistant polymer-based adsorbent, permits reversed-phase separations to be performed under a variety of aggressive conditions including strong acid, strong base, presence of denaturants, and elevated temperatures. Also, 259VHP columns can be cleaned and sanitized by aggressive solvents without detriment to column performance.

Recombinant β-amyloid (1-42) was synthesized as a fusion protein. The peptide was cleaved from the fusion using a restriction protease under aggregate-forming conditions. The pellet was solubilized and loaded on a Vydac 259VHP54 column at 60°C (Figure 3). Peak 1 was determined to be the full length peptide by amino acid sequence and MALDI. Samples from four different runs exhibited the correct molecular weight (4514.8 - 4518.9, expected 4514.4) indicating that no chemical modification (oxidation of methionine, etc.) had occurred. Peak 2 represented a truncated deletion (6-42) of the peptide which probably occurs due to nonspecific activity of the protease. Chromatography at lower temperatures (35°C) gave 80-85% yield (peak area) compared to 60°C. However, the peaks were broader. Average recovery of the peptide at 60°C was 70-75%.

Courtesy of

Menon, N.K., Przybyla, A.E., Neuhaus, E.B. and R.A.Makula
Fermentation Research Facility
Department of Biochemistry
University of Georgia
Athens, GA 30602

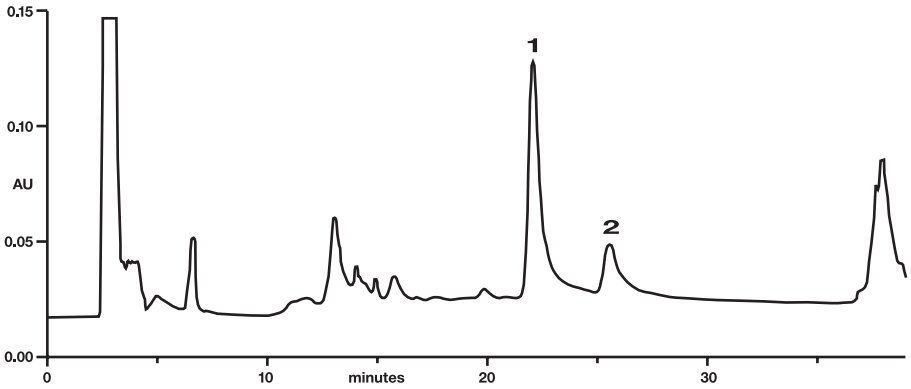


Figure 3. Purification of β-amyloid protein from solubilized aggregate. **Column:** Vydac 259VHP54, polymer reversed-phase, 4.6mm ID x 250mm L. **Conditions:** 1 mL/min at 60°C. Solvent A = 5% acetonitrile, 5mM ammonium acetate, pH 7.0. Solvent B = 10% isopropanol, 80% acetonitrile, 5mM ammonium acetate, pH 7.0. **Gradient:** 0 to 24%B in 5 minutes, 24-27%B in 25 minutes, then 27-100%B in 5 minutes.

Purification of a Synthetic 135-Base Oligonucleotide on a Vydac C4 Reversed-Phase Column

Compliments are always welcome, and Vydac recently received one from Joe Kosmoski at Washington State University. In a letter dated July 24, 1997, Joe wrote:

> I have recently purchased a Vydac C4 (Cat# 214TP1010) reversed-phase column for the purification of synthetic oligonucleotides. When discussing my options with your technical staff, there was some uncertainty as to the size limit for purification with this column. Nonetheless, I felt confident that we could isolate the desired 135-base-long product. At the time, there was considerable interest on the part of Vydac as to the results of our experiment.
>
> Since then, I have successfully purified oligos ranging from 20 to 135 bases in length. Enclosed you will find chromatograms of the crude 135-base oligo with the 5'Dimethoxytrityl, and subsequent purification of the deprotected oligo. I was pleasantly surprised at the quality of separation. In addition, I have included data from polyacrylamide gel electrophoresis as a quality control. Feel free to share this information as you see appropriate. I hope you and your customers find this information helpful.
>
> In conclusion, I would like to thank Vydac for producing such a high quality product. After over a decade of HPLC experience behind me, I can say with great confidence that Vydac makes the best product for my separation needs.

We're including the chromatograms and electrophoretic gel scan here for the interest of others involved in synthetic oligonucleotide purification. By first running the 5'-DMTr oligonucleotide, which is more strongly retained, and then the detritylated oligo with a shallower gradient, this two-step procedure favors a high degree of purification from extraneous compounds.

Our appreciation to

Joseph Kosmoski and
Dr. Michael Smerdon
Dept. of Biochemistry and Biophysics
Washington State University
Pullman, WA 99164-4660
(509) 335-7457
kosmo@mail.wsu.edu

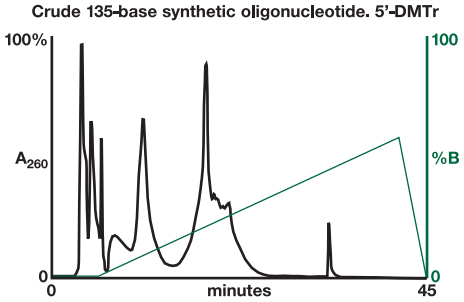


Figure 4. Purification of crude 5'-dimethoxytrityl 135-mer oligonucleotide. The last peak, at approximately 33 minutes, is the desired product. **Column:** Vydac 214TP1010, C4 reversed-phase, 10µm spherical, 10mm ID x 250mm L. **Conditions:** 5 mL/min. 260 nm. Solvent A = 0.1M Triethylammonium acetate, pH 7.0. Solvent B = acetonitrile. **Gradient:** linear, 0 to 60% B from 5 to 40 minutes.

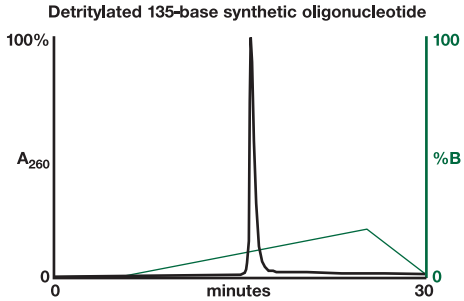


Figure 5. Purification of deprotected 135-mer oligonucleotide. **Column:** Vydac 214TP1010, C4 reversed-phase, 10µm spherical, 10mm ID x 250mm L. **Conditions:** 5 mL/min. 260 nm. Solvent A = 0.1M Triethylammonium acetate, pH 7.0. Solvent B = acetonitrile. **Gradient:** linear, 0 to 20% B from 5 to 25 minutes.

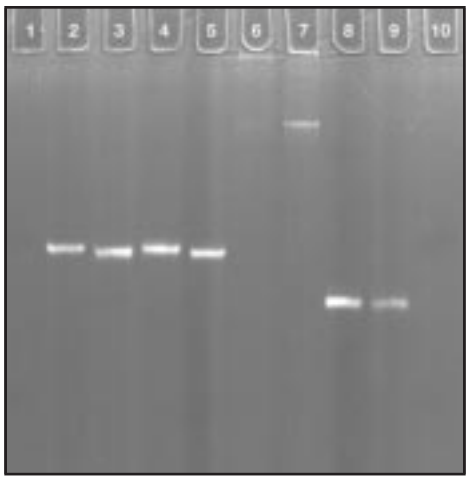


Figure 6. Phosphorimaging UV-shadow scan of HPLC-purified oligonucleotides on 12% polyacrylamide 7M urea denaturing gel. Lanes 1 & 10 contained dye markers that do not shadow. Lanes 2 & 3: a synthetic 30-mer and its complement. Lanes 4 & 5: a different 30-mer and its complement. Lane 6: unknown. Lane 7: the purified 135-mer. Lanes 8 & 9: a purified 20-mer and its complement.

Ordering Information

(for columns on pages 3 & 4)

Cat. No.	Description
214TP1010	Column, C4, 10µm, 300Å, 10mm ID x 250mm L
214TP54	Column, C4, 5µm, 300Å, 4.6mm ID x 250mm L
214TP5415	Column, C4, 5µm, 300Å, 4.6mm ID x 150mm L

Other analytical and preparative column dimensions available upon request.

Real-World Vydac Applications Wanted!

Vydac seeks partners in promoting better chromatography

The value of Vydac's high-performance packings and columns is their ability to solve separation problems that are important to scientist in a variety of laboratories. We work hard to provide the best products and are always interested in sharing success stories about how they have been used by our customers. The articles in this issue of *Vydac Advances* are examples.

Do you have a novel application or technical tip you would like to see featured in a future issue? If so, please contact Vydac's technical support staff by phone, mail, or E-mail. Publication in *Vydac Advances* is easy! We'll assist in any way we can with writing and graphic production to minimize the time you spend while making useful information available to others.

Assessing Polypeptide Integrity: Reversed-Phase HPLC vs. PAGE

In pursuit of well-characterized therapeutics

Sensitive, reproducible analytical methods are essential to development of well-characterized pharmaceuticals. For polypeptides, polyacrylamide gel electrophoresis (PAGE) and reversed-phase HPLC are commonly used. Here we offer a comparison of both methods by analyzing a common polypeptide, chymotrypsin, after exposure to conditions expected to cause degradation.

Chymotrypsin, purchased commercially, was dissolved at 1 mg/mL in water, PBS, and 0.1% TFA/5% ACN. Portions of each solution were incubated as indicated, then analyzed by reversed-phase chromatography (Figure 7) and PAGE (Figure 8). Sample 1 was the original aqueous solution held at 4°C for comparison. Lane 0 of the gel is a standard polypeptide mixture.

Results

Both methods clearly show decomposition in the incubated samples and formation of aggregates. However, some decomposition products visible in chromatograms 6 and 7 were not seen with PAGE. With reference to polypeptide purity and integrity, PAGE provided no extra information over that available from reversed-phase analysis alone. Reversed-phase HPLC, on the other hand, provided additional information and a more quantitative picture of purity.

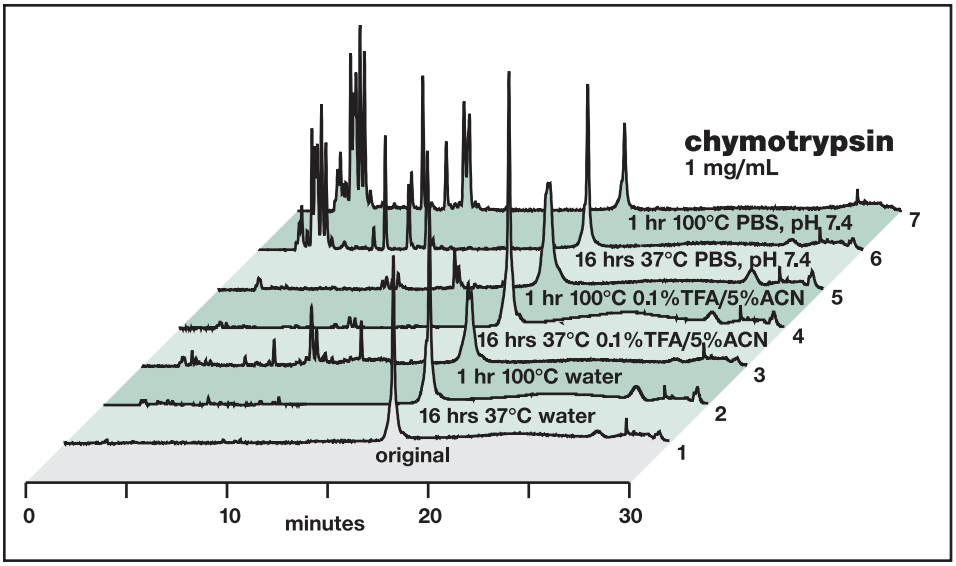


Figure 7. Reversed-phase HPLC of chymotrypsin samples. Column: Vydac 214TP54 C4 5µm 4.6mmID x 250mmL. **Conditions:** 1 mL/min. 280nm. Solvent A = 0.1% TFA (w/v) in water. Solvent B = 0.09% (w/v) TFA in ACN. **Gradient:** 20% to 60% B in 40 minutes.

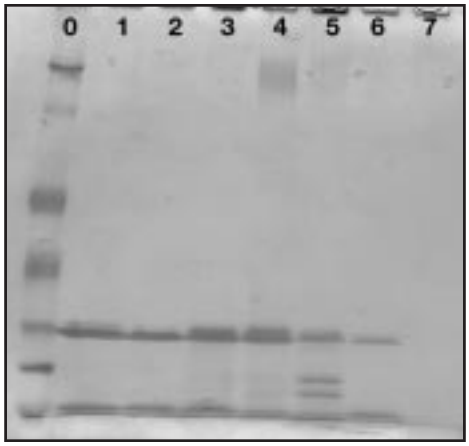


Figure 8. PAGE analysis of chymotrypsin samples. A Novex 12% tris glycine gel, 1.5 mm thick, was run under reducing conditions and silver stained. Numbered lanes correspond to chromatograms of Figure xa.

Courtesy of

Tim Malaney
Telios Pharmaceuticals, Inc.
San Diego, CA

More Protein Characterization

Retention on reversed-phase depends on the hydrophobic "footprint" of a protein, which will vary with tertiary and quarternary structure since hydrophobic residues tend to face inward in aqueous solution. When several stable configurations exist they can often be separated by reversed-phase chromatography.

Coupling reversed-phase separation with laser light scattering detection can be particularly useful for characterizing protein oligomers, as shown by this chromatogram (Figure 9) provided by Wyatt Technology Corporation. It allows one to measure absolute molar masses directly, and to observe the properties of the protein in solution. By varying the degree of oxidation, larger bFGF oligomers, from trimers to pentamers, have also been detected.

For more information, contact

Wyatt Technology Corporation
802 E. Cota Street
Santa Barbara, CA 93103

Phone: (805) 963-5904
Fax: (805) 965-4898
Web: http://www.wyatt.com

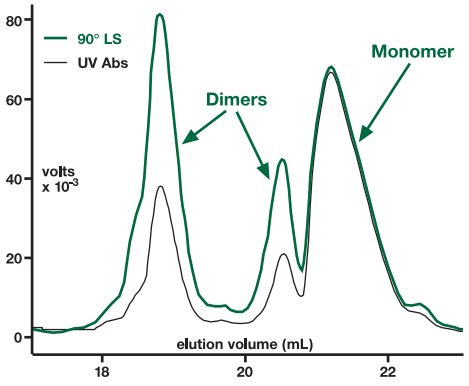


Figure 9. Portion of reversed-phase chromatogram of basic fibroblast growth factor (bFGF) at degree of oxidation produced by 0.2 equivalents of DTNB. Column: Vydac 214TP5415, C4, 5µm, 300Å, 4.6mmID x 150mmL. **Flow:** 0.7 mL/min. **Detection:** UV diode-array and miniDawn™ laser light scattering.

New!

Vydac's 218MR Column for USP-Compliant Separations of Multi-Ring Pharmaceuticals

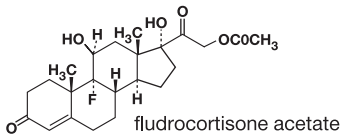
A Solvent-Saving 3.1mm ID Column Provides High Sensitivity

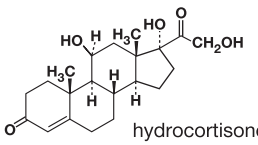
In our last issue, we featured Vydac's new 218MR reversed-phase columns, specially quality controlled to assure performance in accordance with USP specifications for complex multi-ring pharmaceuticals. In addition, compared to standard 4.6mm ID columns our 218MR53 3.2mm ID columns provide

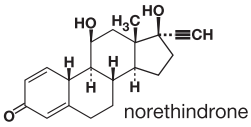
- **twice the sensitivity**
- **half the solvent consumption**

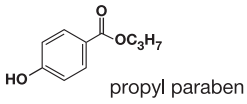
Here are two more examples showing 218MR to be an excellent choice for pharmaceutical analyses. Hydrocortisone was run under two solvent conditions. Chromatogram B provides a faster analysis and higher sensitivity, while still meeting system suitability requirements. Although not specified for suitability, norethindrone would make a better internal standard.

Cat. No.	Description
218MR53	Column, Octadecyl (C18), Silica-Based, 5µm, 300Å, 3.2mm ID x 250mm L









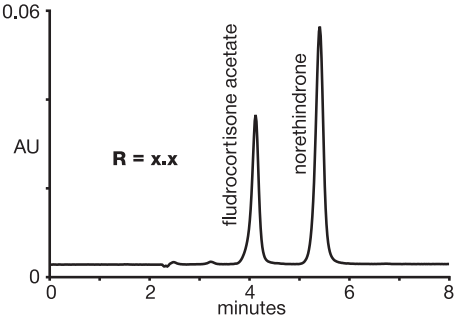


Figure 10. Fludrocortisone on 218MR53. Conditions: 254nm, isocratic 45:55 ACN:water at 0.5 mL/min. USP requires R not less than 2.5.

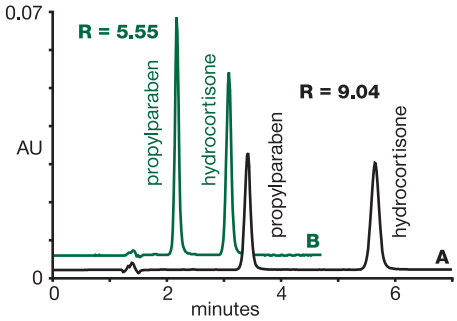
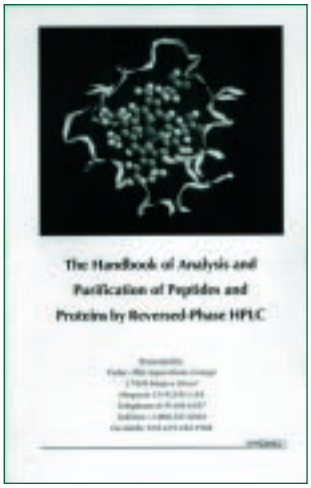


Figure 11. Hydrocortisone on 218MR53. Conditions: (A) 254nm, isocratic 20:20:60 MeOH:ACN:water at 0.5 mL/min. (B) 254nm, isocratic 25:25:50 MeOH:ACN:water at 0.5 mL/min. USP requires R not less than 2.0.

Vydac Wrote the Book on Peptide and Protein Reversed-Phase HPLC



The second edition of Vydac's publication, *The Handbook of Analysis and Purification of Peptides and Proteins by Reversed-Phase HPLC*, is a compendium of essential information regarding this technique that no protein or peptide chemist should be without. From basic principles of reversed-phase interaction to specific applications, scale-up recommendations, and answers to frequently asked questions, this 64-page monograph engenders both basic and advanced understanding.

It's yours for the asking at no charge. Simply request a copy from Vydac or from the Vydac distributor in your area.