



Peptide Separations

All 300 Å Reversed-Phase Columns are Not the Same

Vydac was the first company, in the early 1980s, to produce an all-synthetic high-purity 300 Å HPLC silica. Covalently bonded reversed-phases derived from that material, Vydac TP silica, provided powerful tools for separating proteins, peptides, and oligonucleotides. Over the years Vydac TP adsorbents became the standard and most widely used materials for reversed-phase purification of synthetic peptides and oligonucleotides as well as isolation of proteins and separation of hydrolysis products for sequencing. Vydac's polymeric C4, C8, and C18 bonded phases provided excellent retention, selectivity, and chemical stability for long column life.

Building on experience with these materials, Vydac introduced additional adsorbent chemistries including a diphenyl phase and a monomeric C18 phase, both with somewhat unique selectivities.

More recently, Vydac introduced its 300 Å MS adsorbents which incorporate a specially treated base silica. The MS adsorbents maintain sharp, symmetrical peak shapes with lower concentrations of mobile phase modifiers such as trifluoroacetic acid (TFA) or heptafluorobutyrate (HFBA). They provide added mobile phase flexibility for analytical and preparative separations and can be especially useful for LC-MS analyses where high concentrations of TFA interfere with ion generation.

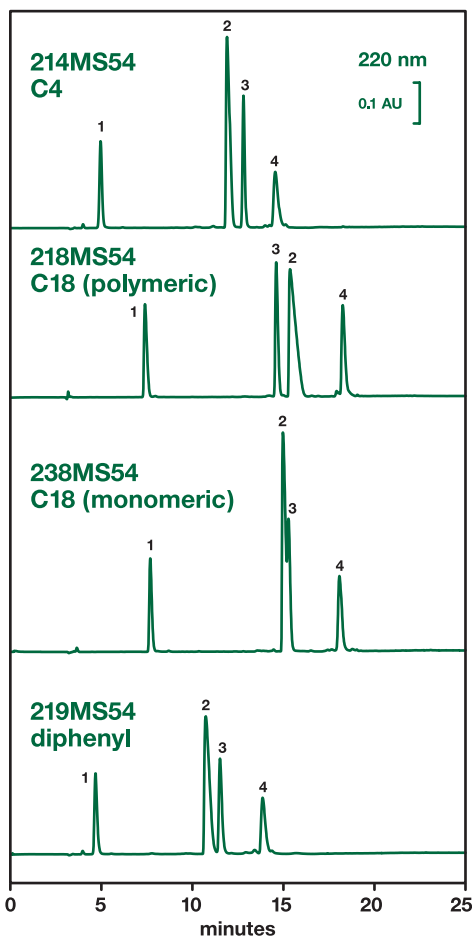


Figure 1. Separation of peptide mixture on 300 Å reversed-phase columns. All columns 4.6 mm ID x 250 mm L. Mobile phase: A = 0.02% TFA (v/v) in 5% ACN. B = 0.02% TFA (v/v) in 95% ACN. Flow rate: 1.0 mL/min. Gradient: Linear from 5 to 15 %B in 10 minutes. Hold 15 %B for 3 minutes. Then 15 to 20 %B in 1 minute and 20 to 40 %B in 10 minutes. Peaks: 1. neurotensin 1-8; 2. oxytocin; 3. angiotensin II; 4. neurotensin.

The various reversed-phase chemistries affect selectivity for proteins and peptides, sometimes in subtle ways. The existence of a variety of adsorbent chemistries provides a pool from which columns can be selected to resolve substances of interest, optimize separations, and maximize information from complex separations such as peptide fingerprints.

The simple peptide separations shown in Figure 1 illustrate this point. A mixture of small peptide hormones is separated on four different Vydac 300 Å reversed-phase columns under identical conditions with a low TFA concentration (0.02%) in the mobile phase. The 214MS54 polymeric C4 column resolves all four peptides. The 218MS54 polymeric C18 column also resolves the peptides, but with longer retention times as might be expected. Interestingly, the elution order for oxytocin and angiotensin II is reversed – one of the subtle selectivity differences that makes variety in reversed-phase chemistries useful. Separation on the 238MS54 monomeric C18 column shows the same elution order as 214MS. Oxytocin and angiotensin II are only partially resolved, but adjustment of the gradient pattern might be expected to improve resolution in that region of the chromatogram. The 219MS54 diphenyl reversed-phase column produces good resolution and peak shapes, with shorter retention times.

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Contributed Article

Vydac encourages customers with interesting applications using Vydac columns and adsorbents to submit articles about their work for publication in *Vydac Advances*. The article on the next two pages was submitted by Dr. Gary Hathaway of the Beckman Institute at California Institute of Technology. We wish to express our appreciation to Dr. Hathaway and his associates for this article.

Peptide HPLC

(continued from page 1)

Two conclusions can be drawn from these results:

First, in determining whether a separation can be performed by reversed-phase HPLC, and in optimizing the separation, it can be useful to try several columns with different stationary-phase chemistries. The peptides in the mixture tested here are just useful markers. The observed retention differences are indicative of the unique selectivities available from Vydac.

Second, one should always exercise caution in changing reversed-phase columns for an established method, testing well to assure the replacement column performs the separation as desired, without significant differences in resolution or elution order.

Ordering Information

Cat. No.	Description
214MS54	LC/MS R-P Column. Polymeric C4. 300 Å. 5 µm. 4.6 mm i.d. x 250 mm.
218MS54	LC/MS R-P Column. Polymeric C18. 300 Å. 5 µm. 4.6 mm i.d. x 250 mm.
238MS54	LC/MS R-P Column. Monomeric C18. 300 Å. 5 µm. 4.6 mm i.d. x 250 mm.
219MS54	LC/MS R-P Column. Diphenyl. 300 Å. 5 µm. 4.6 mm i.d. x 250 mm.

Direct Gradient Elution of Peptides for Capillary LC/MS/MS

*Application To The ABRF-00SEQ Protein Sequencing Test Peptide
(A New Method for Forming Off-Line, Static, Microliter-Scale Gradients)*

The sensitivity achieved through the concentrating effect of a capillary column, the frugal use of sample provided by tandem elution, and the ability to analyze complex mixtures by "peak parking" with MS/MS analysis are valid reasons for coupling reversed-phase HPLC to electrospray mass spectrometry (1,2). Although mixing gradients for direct elution at nL/min flowrates can be problematical, direct-flow gradient elution is achievable with static gradients stored off-line (3-5). We investigated the feasibility of forming 15 µL, stored linear gradients without dynamic or static mixers by using an autosampler to draw small steps of

increasing organic composition into narrow (178 µm ID) tubing (Figure 2). A model based on laminar flow with radial diffusivity (6,7) was used to verify and optimize the experimental configuration (Figure 3). Gradients took less than 10 minutes to form, proved stable to storage for over 60 minutes, and gave acceptable resolution and accuracy (8)(Figure 4). To illustrate the usefulness of the method, our analysis of the ABRF-00SEQ† test peptide is shown in Figure 5. A 188 femtomole aliquot of the 5 picomole test sample was injected and the slowly eluting peak was analyzed by collision-activated dissociation and tandem mass

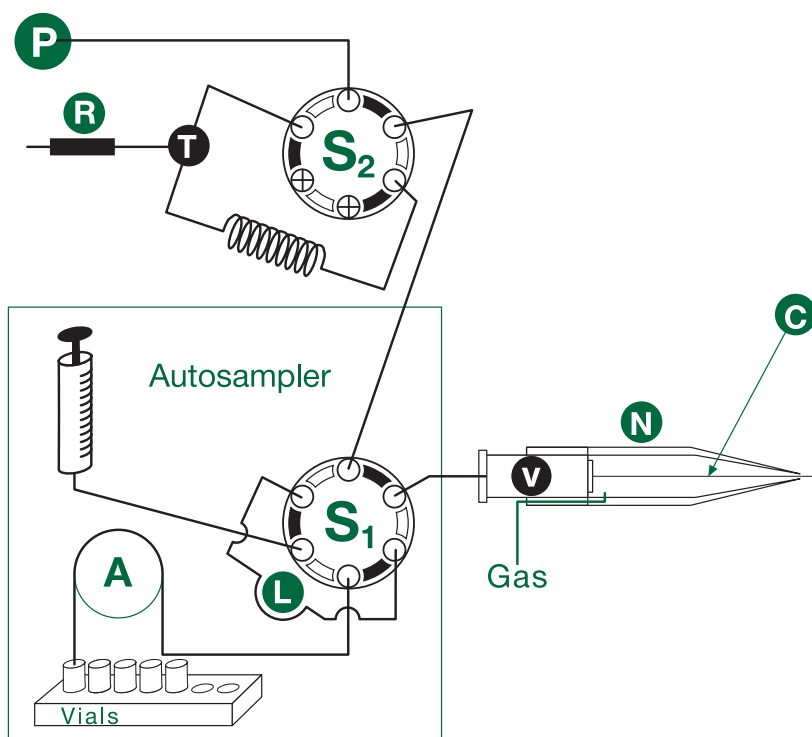


Figure 2. Sketch of Experimental Setup. (P) single syringe drive pump; (A) syringe needle of the autosampler; (S1) autosampler switching valve; (S2) peak parking valve; (T) "T" splitter; (R) restrictor; (V) Valco stainless steel union; (C) column in pulled fused silica capillary; (N) nebulizer/sprayer body; (L) 178 µm ID peak gradient loop. The "peak parking" technique consists of momentarily switching valve S1 as the peak emerges from the column in order to reduce the flow rate and provide increased time for peak analysis.

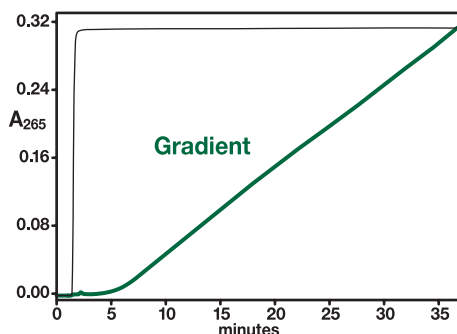


Figure 3. UV absorbance tracing of 30-minute gradient. This gradient was formed from 10 steps of 1.5 μ L each from 2% to 54% acetonitrile/0.02% TFA/0.1% acetic acid. The gradient profile was obtained using an ABI detector equipped with an LC Packings 35 nL flowcell recording at 265nm using acetonitrile doped with ~3% acetone. The black trace is for a full loop injection at 54% acetonitrile to calibrate the absorbance scale to the final gradient concentration.

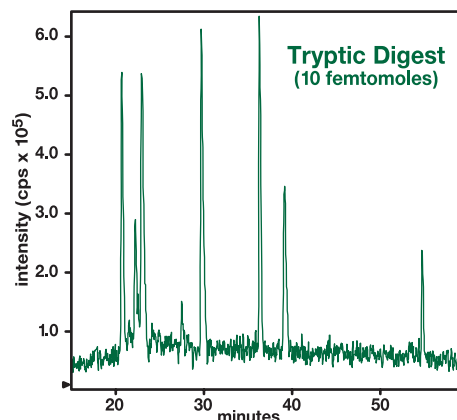


Figure 4. Separation of 10 femtomoles of a tryptic digest of horse apomyoglobin. Flow rate: 200 nanoliters/min. Mobile phase: Gradient formed as shown in Figure 3, except of 75 minutes duration. Column: 100 μ m ID, 3 μ m, 300 \AA Vydac 238TP C18 reversed phase. Detection: MS, total count.

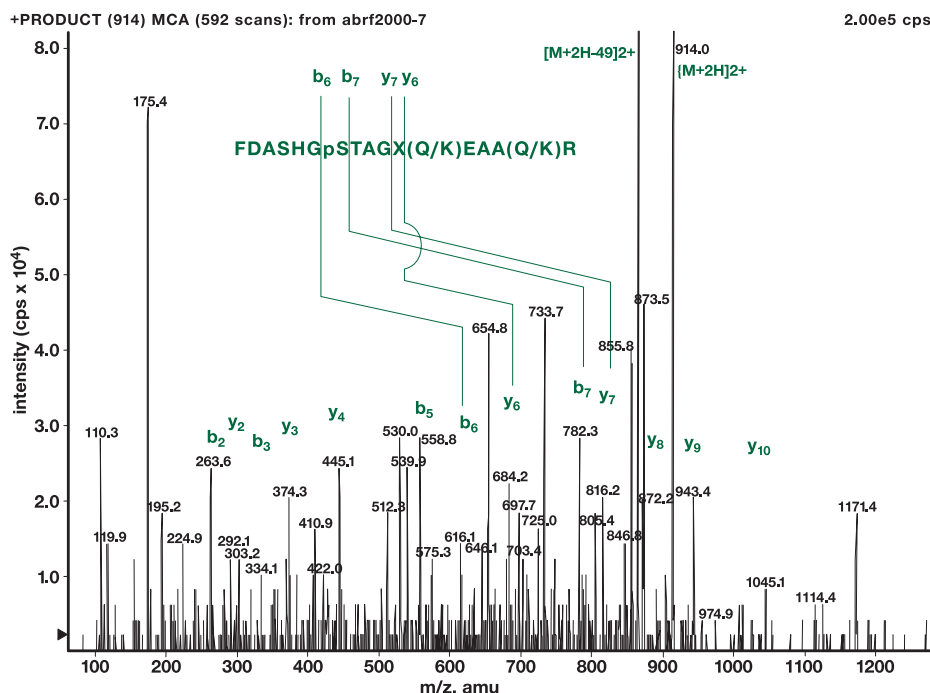


Figure 5. LC/MS/MS analysis of 188 femtomoles of ABRF00-SEQ test peptide parked at a flow rate of <10 nL/min. Peaks corresponding to a,b and y series ions allowed identification of phosphoserine (pS). Column and conditions were as described in the legend to Figure 4.

spectrometry. Much of the sequence, including the critical phosphoserine at position 7 and the C-terminus were assigned from the fragmentation data. Importantly, the small amount used by the analysis spared the remaining sample for use in the less sensitive chemical sequencing analysis needed to identify residues 11 (hydroxyproline), and 12 (glutamine). Our chemical sequencer failed to detect residue 16 leaving Gln/Lys as the only ambiguity (Gln has the same nominal mass as Lys; Hyp the same nominal mass as Leu/Ile).

† The Association of Biomolecular Resource Facilities

References

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3. D. Ishii, T. Takeuchi and A. Wada, in Introduction to Microscale High-Performance Liquid Chromatography, D. Ishii, (ed.), VCH Publishers, Inc., New York, pp 33-69 (1988).
4. M.T. Davis, D.C. Stahl and T.D. Lee, *J. Am. Soc. Mass Spectrom.* 6, 571-577 (1995).
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6. G. Taylor, *Proc. Roy. Soc. London Ser. A* 219, 186-203 (1953).
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8. Zhou, J., Rusnak, F., Colonius, T., and Hathaway, G.M. (2000) *Rapid Commun. Mass Spectrom.* 14, (6): 432-438.

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Chromatographers Need Columns and Information

Vydac Delivers!

We know we're not the only company in the HPLC column business. However, we do believe Vydac is unique in many ways, and ideally suited to serving the needs of chromatographers for quality HPLC columns and the information necessary to apply them successfully.

Products

Service begins with good products. Vydac's twenty-nine years as a developer and manufacturer of HPLC adsorbents and columns has given us proprietary technology and experience that is especially valuable in the areas of biotechnology and pharmaceutical development. Our original 300 Å wide-pore reversed-phase adsorbents are based on Vydac TP silica. This was the first chromatographic silica to be manufactured using synthetic-silica technology. Synthetic silicas are produced from purified organosilicate starting materials rather than natural silica sols. In addition to reduced levels of undesirable contaminants, this process gave us better control over silica reproducibility. Since its development in the early 1980s, Vydac's synthetic-silica manufacturing process has been improved and refined to produce chromatographic silica of even greater purity and consistency.

Applications

Beginning shortly after its introduction, Vydac TP 300 Å reversed-phase adsorbents found ready application in the rapidly developing field of biotechnology where the benefits of high resolution separations in isolation, purification, and characterization of proteins, peptides, and oligonucleotides, whether natural or synthetic in origin, are virtually endless. Vydac's new adsorbents were ideally suited for these uses because of their purity and

their large pore size, which provides efficient access for macromolecules to adsorptive surfaces. As applications developed, so did Vydac's experience in the field, making us uniquely capable in advising customers regarding how to use our products most effectively. A number of valuable publications emerged from this activity, most notably Vydac's comprehensive monograph, *The Handbook of Analysis and Purification of Peptides and Proteins by Reversed-Phase HPLC*, still current and available free for the asking. The *Handbook* can also be downloaded in PDF form from Vydac's website.

Concurrent with the development of biotechnology applications, Vydac also gained significant experience with many pharmaceutical, food, and environmental applications for which references can be found in our catalog. A variety of specific application notes have been published. They can also be found on the website.

Preparative and Process Capability

Our capability as a manufacturer of the base silica and polymer matrices used in our adsorbents allows us to offer preparative columns and bulk quantities from one kilogram to hundreds of kilograms for process applications at favorable prices. Vydac preparative reversed-phase adsorbents are currently used in the production of several FDA-approved biopharmaceutical products. Regulatory support data can be provided for GMP applications.

Rapid Delivery from Stock

Our capability as a manufacturer also gives us control over supply, and allows us to keep virtually all of the products we offer in stock, ready for shipment. We

pride ourselves on rapid delivery in response to customer orders. When you need a column, we don't think you should have to wait a month to get it. The vast majority of customer orders to Vydac are shipped the same day we receive them. On request, expedited overnight shipping will often put the column in your laboratory the next morning for emergency requirements.

Qualified Technical Staff

Our Technical Department, staffed by qualified application chemists, provides direct consultation to help Vydac customers with problems, recommendations, application development, and any other issues that may arise. Vydac technical support people are chemists and biochemists by training with real-world experience using HPLC to address the same situations experienced by many of our customers. A phone call to our Technical Department at **800-247-0924** or **USA 760-244-6107** will put you in touch, not just with a placeholder, but with an experienced individual who can offer real insight into solving problems. Our Technical Support department can also be reached via email to **experts@vydac.com**. Members of our support team are also available selectively on request for in-house seminars on a variety of HPLC application topics.

Fast Track!

The Direct Path to Vydac Products and Applications

All of Vydac's product and application publications, as well as complete online ordering capability, are available on Vydac's website. If you haven't been there, it's worth a visit.

<http://www.vydac.com>

EPA Methods 531 and 8330

The US Environmental Protection Agency specifies use of reversed-phase chromatography on C18 columns for determination of N-methylcarbamoyloxime and N-methylcarbamate pesticides in water (Method 531) and for trace analysis of nitroaromatic and nitramine explosive residues in water, soil, or sediment (Method 8330). The chromatograms of Figures 6 and 7 show a representative selection of substances separated as specified by these methods on Vydac's 201SP54 90 Å pore-size C18 reversed-phase column.

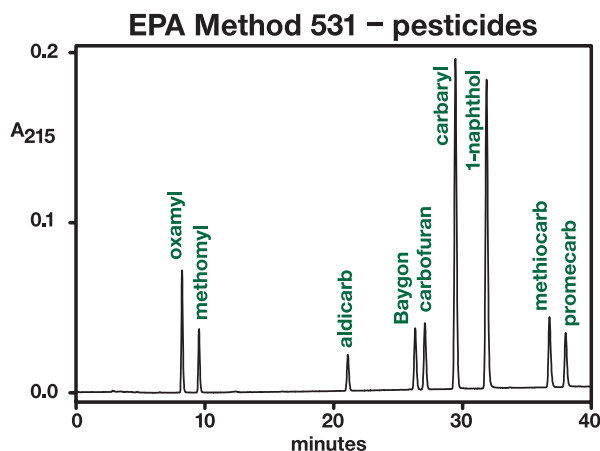


Figure 6. Separation of N-methylcarbamoyls oximes and N-methylcarbamates according to EPA Method 531. Column: Vydac 201SP54 90 Å, 5 µm, C18 reversed-phase, 4.6 mm ID x 250 mm L. Mobile phase: A = 5% acetonitrile/95% water (v/v). B = 90% acetonitrile/10% water (v/v). Flow rate: 1.0 mL/min. Gradient: Linear from 10% to 60% B over 35 minutes.

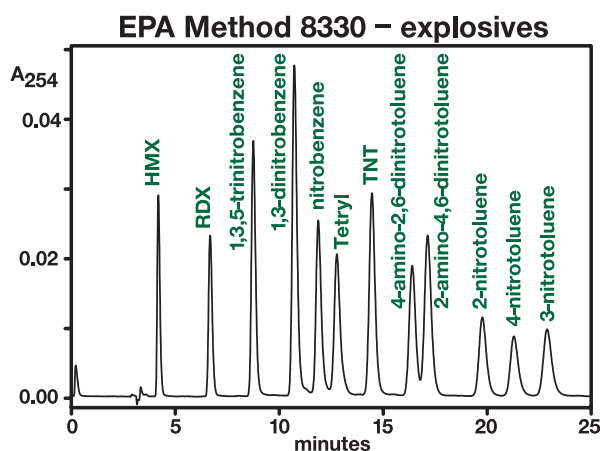


Figure 7. Separation of nitroaromatics and nitramines according to EPA Method 8330. Column: Vydac 201SP54 90 Å, 5 µm, C18 reversed-phase, 4.6 mm ID x 250 mm L. Mobile phase: Isocratic. 50% methanol/50% water (v/v). Flow rate: 1.0 mL/min.

Ordering Information

Cat. No.	Description
201SP54	Reversed-Phase Column. Monomeric C18. 90 Å. 5 µm. 4.6 mm i.d. x 250 mm.

Meetings & Exhibits

You will find the Vydac booth and Vydac technical personnel for consultation in the exhibitor areas at the following upcoming meetings. Come talk with us about your analytical, preparative, and process HPLC requirements. That's why we'll be there. We'd love to see you!

Protein Symposium

San Diego, CA Aug. 5-9 Booth 518

ACS Fall

Washington, DC Aug. 21-23 Booth 223

Biotechnology

San Diego, CA Sept. 28

AAPS

Indianapolis, IN Oct. 29-Nov. 2 Booth 1210

Positions Available

Vydac is seeking two additional application chemists for our Technical Support Department. These positions require a degree and HPLC experience. Excellent oral communication and writing skills are important, as well as the ability to work independently and take projects from inception to completion. Responsibilities include application support and development, seminar and poster presentations, exhibits and customer contact. Some overnight travel is required.

Qualified applicants should contact Michael Li, VP, Business Development, at **800-247-0924** or via email to mli@vydac.com.