



# Vydac Advances

Quarterly Technical Newsletter on the Characteristics and Use of Vydac HPLC Columns

Summer, 1999

## Vydac Introduces New SP (Small-Pore) Silica for Pharmaceutical and Vitamin Analysis

- 90 Å pore size
- 5 µm particle diameter
- C18 reversed-phase (201SP replaces 201HS)
- C8 reversed-phase (208SP replaces 208HS)

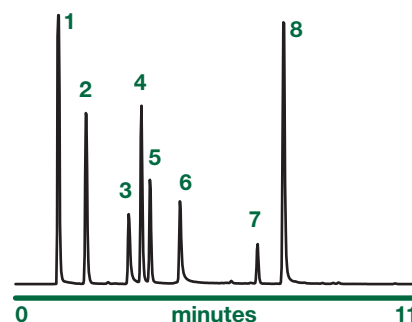
Customers can now order Vydac's new SP small pore silica-based reversed-phase columns for the analysis of pharmaceuticals and water-soluble vitamins. Two reversed-phase chemistries are available: 201SP, a C18 phase, and 208SP, a C8. Both are monomerically bonded and exhaustively end-capped. SP columns have inherently high performance that does not depend on deactivation or solvent modifiers. They are very stable, with long lifetimes in normal use.

201SP and 208SP are entirely new adsorbents designed to replace Vydac's older 201HS and 208HS columns. The SP base material is similar to HS in its 90Å pore size and consequent high surface area, however it is produced by a more robust process.

Performance of SP columns is similar to their HS predecessors. They undergo identical quality tests and meet the same specifications. Tests include packed column efficiency, selectivity for water soluble vitamins, and selectivity for tricyclic antidepressants (208SP only).

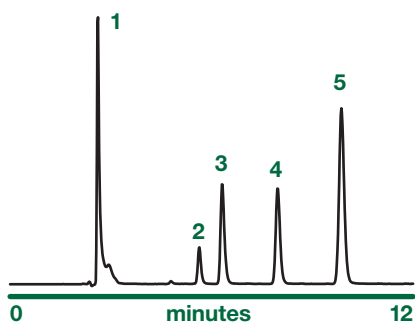
In addition to the applications actually tested, Vydac believes the new SP silica columns will be suitable for virtually all applications previously performed on

(continued on page 2)

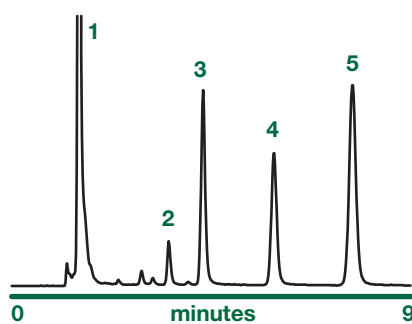


**Figure 3. Vitamin selectivity test for 201SP.** The test is performed on a 4.6mmID x 250mmL column (Vydac Catalog No. 201SP54).

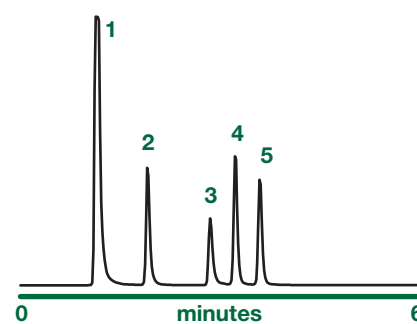
**Mobile phase:** A = 0.1M KOAc adjusted to pH 4.9 to 5.2 with formic acid. B = 50:50 ACN:water. **Gradient:** 5% to 60% B over 15 minutes. **Flow rate:** 1.5 mL/min. **Detection:** 254 nm. **Peaks:** (1) ascorbic acid (C), (2) nicotinic acid (Niacin), (3) pyridoxine (B6), (4) thiamine (B1), (5) nicotinamide (B3), (6) folic acid (M), (7) cyanocobalamin (B12), (8) riboflavin (B2). All peaks must be baseline resolved.



**Figure 1. Efficiency test for 201SP (C18).** **Column:** Vydac 201SP54 (4.6mmID x 250mmL). **Mobile phase:** Isocratic. 70:30 ACN:water. **Flow rate:** 1.0 mL/min. **Detection:** 254 nm. **Peaks:** (1) uracil, (2) benzene, (3) dimethylaniline, (4) naphthalene, (5) biphenyl. Efficiency is measured for the well-retained biphenyl peak and must exceed 64,000 theoretical plates per meter. The N,N-dimethylaniline peak must also meet standards for retention, efficiency, and symmetry. The dimethylaniline test verifies column performance for very basic compounds.



**Figure 2. Efficiency test for 208SP (C8).** **Column:** Vydac 208SP5415 (4.6mmID x 150mmL). **Mobile phase:** Isocratic. 60:40 ACN:water. **Flow rate:** 1.0 mL/min. **Detection:** 254 nm. **Peaks:** (1) uracil, (2) benzene, (3) dimethylaniline, (4) naphthalene, (5) biphenyl. The biphenyl peak must have a measured efficiency of at least 64,000 theoretical plates per meter and N,N-dimethylaniline must meet standards for retention, efficiency, and symmetry as described for 201SP in Figure 1.



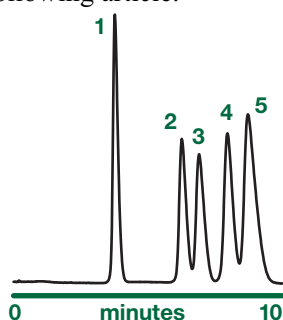
**Figure 4. Vitamin selectivity test for 208SP.** The test is performed on a 4.6mmID x 150mmL column (Vydac Catalog No. 208SP5415). **Mobile phase:** A = 0.1M KOAc adjusted to pH 4.9 to 5.2 with formic acid. B = 50:50 ACN:water. **Gradient:** 5% to 60% B over 15 minutes. **Flow rate:** 1.5 mL/min. **Detection:** 254 nm. **Peaks:** (1) ascorbic acid (C), (2) nicotinic acid (Niacin), (3) pyridoxine (B6), (4) thiamine (B1), (5) nicotinamide (B3). All peaks must be baseline resolved.

## New SP Silica

(continued from page 1)

HS columns. As always, performance should be validated for your application by specific suitability testing. Vydac agrees to accept for return and refund the purchase price if the SP column does not meet your requirements.

In addition to quality assurance procedures illustrated in figures 1 through 5, performance of SP reversed-phase columns for separations of barbiturates has been validated as described by the following article.



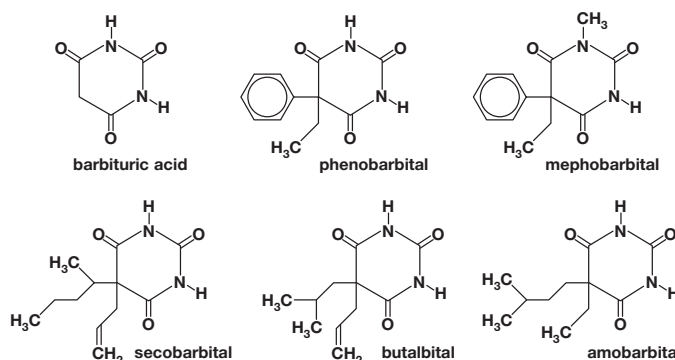
**Figure 5. Tricyclic antidepressant selectivity test for 208SP.** The test is performed on a 4.6mmID x 50mmL column (Vydac Catalog No. 208SP5405). **Mobile phase:** Isocratic. 0.025M sodium phosphate, pH 3, 25% ACN. **Flow rate:** 2.0 mL/min. **Detection:** 210 nm. **Peaks: (1)** doxepin, **(2)** desipramine, **(3)** imipramine, **(4)** nortriptyline, **(5)** amitriptyline.

## Ordering Information

Cat.No.	Description
<b>201SP54</b>	<b>90Å, C18:</b> Column, Reversed-Phase C18, 90Å, 5µm 4.6mm ID x 250mm L
<b>201SP5415</b>	<b>90Å, C18:</b> Column, Reversed-Phase C18, 90Å, 5µm 4.6mm ID x 150mm L
<b>208SP54</b>	<b>90Å, C8:</b> Column, Reversed-Phase C8, 90Å, 5µm 4.6mm ID x 250mm L
<b>208SP5415</b>	<b>90Å, C8:</b> Column, Reversed-Phase C8, 90Å, 5µm 4.6mm ID x 150mm L
<b>201SP5405</b>	<b>90Å, C8:</b> Column, Reversed-Phase C8, 90Å, 5µm 4.6mm ID x 50mm L
<b>218TP54</b>	<b>300Å, C18:</b> Column, Reversed-Phase polymeric C18, 300Å, 5µm 4.6mm ID x 250mm L
<b>238TP54</b>	<b>300Å, C18:</b> Column, Reversed-Phase monomeric C18, 300Å, 5µm 4.6mm ID x 250mm L

Other column sizes are available for analytical and preparative applications.

## Separation of Barbiturates on Small-Pore and Wide-Pore Reversed-Phase Columns

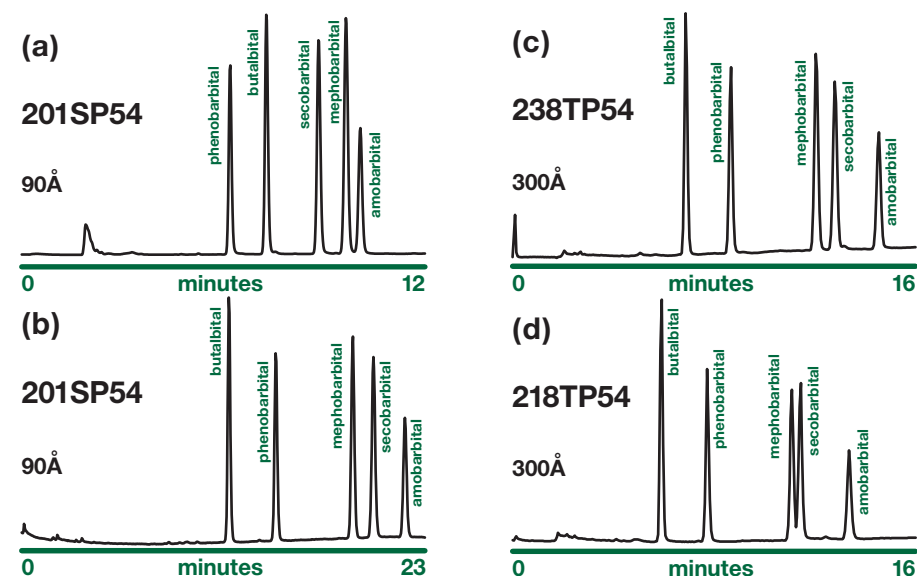


Barbiturates are derivatives of barbituric acid. They act as sedatives, hypnotics, and anticonvulsants by exerting depressant effects on the central nervous system. They are valued as therapeutics, but also common as drugs of abuse.

The five active barbiturates shown above were separated on a Vydac 201SP54 column (C18, 5µm, 90Å, 4.6mmID x 250mmL) with a simple acetonitrile:water mobile phase (Figure 6a). With an acidic phosphate buffered mobile phase (Figure 6b) the five compounds also separated on this column. The order of elution was reversed

for phenobarbital/butalbital and for secobarbital/mephobarbital due to differences in the mobile phase. Differences in counterions and pH will affect reversed-phase selectivity.

The five barbiturates were also separated with the acidic mobile phase on a monomerically bonded C18 reversed-phase column with 300Å pore size (Figure 6c). When the same separation was done on a polymerically bonded 300Å C18 column (Figure 6d), subtle differences in selectivity were observed.



**Figure 6. Separation of five barbiturates on Vydac reversed-phase columns.** **Columns:** (a & b) Vydac 201SP54 (monomeric C18, 90Å, 5µm, 4.6mmID x 250mmL). (c) Vydac 238TP54 (monomeric C18, 300Å, 5µm, 4.6mmID x 250mmL). (d) Vydac 218TP54 (polymeric C18, 300Å, 5µm, 4.6mmID x 250mmL). **Mobile phases:** (a) A = water. B = ACN. Gradient from 30% to 60%B over 20 minutes. (b,c,&d) A = 50mM KH<sub>2</sub>PO<sub>4</sub>, pH 2.51, 20% ACN. B = 90% ACN. Gradient from 0% to 20%B over 20 minutes.

# Cation-Exchange Chromatography of Peptides

## Effects of pH and organic modifier on retention and selectivity

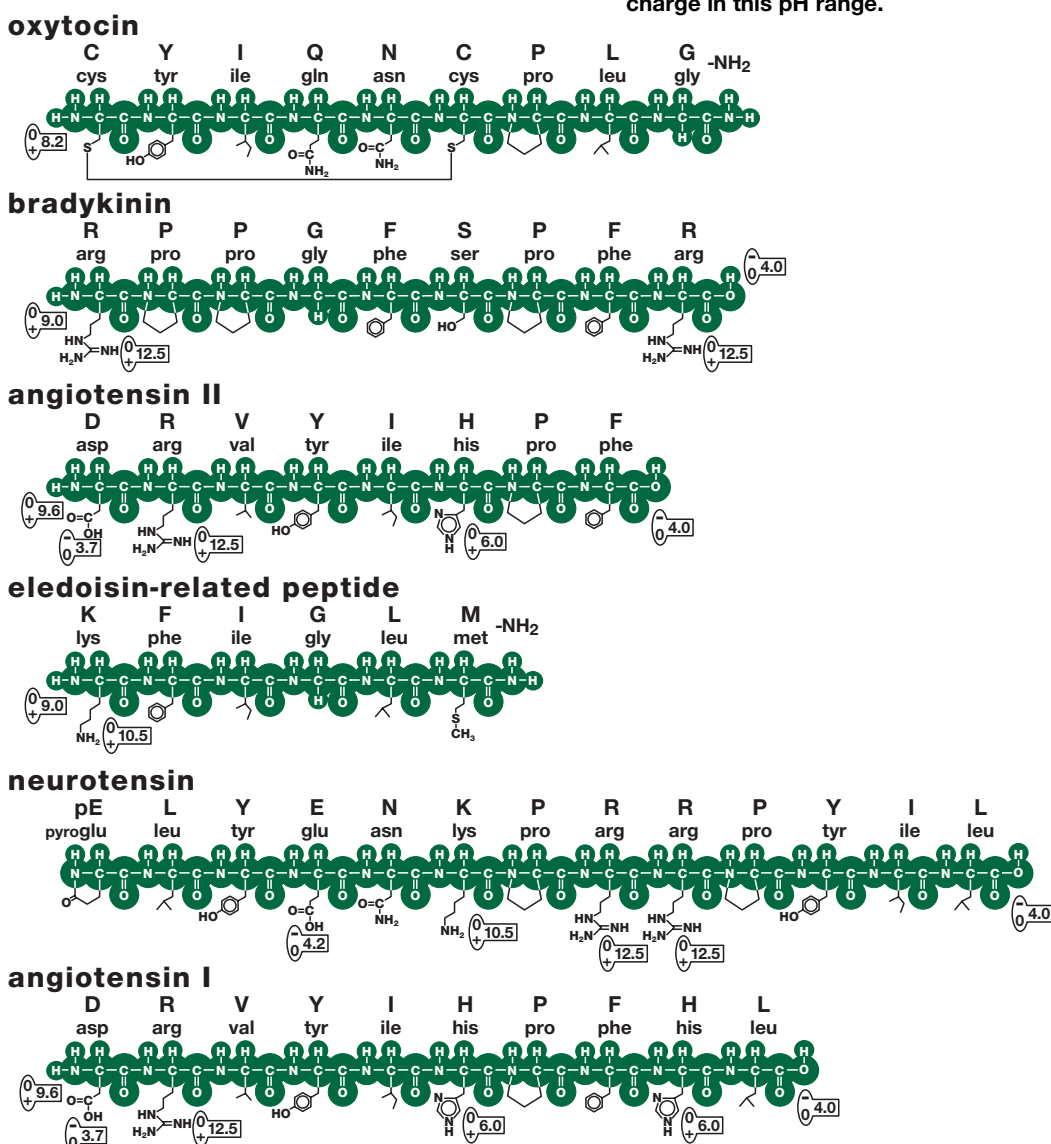
Aqueous separations on ion exchange columns are driven primarily by charge differences. But in the presence of organic solvent, hydrophilic differences can also play a role. This is illustrated by the separation of six peptides of known structure on Vydac 400VHP, a high-performance sulfonic-acid-type strong cation exchanger based on a hydrophilic-coated polymer matrix with 5  $\mu\text{m}$  particle size and 900  $\text{\AA}$  pore size.

### Calculating Net Charge

The net charge on a peptide in solution varies with pH. The approximate charge at any given pH can be calculated from examination of the peptide structure. Figure 7 shows the structures of the test peptides used in this work. Approximate  $\text{pK}_a$  values for functional groups active in the range pH 2 to pH 8 are shown in flags adjacent to their locations in side chains and termini.

- ◆ Protonated amino groups at the N-terminus, in lysine side-chains, the guanidino moiety of arginine, and the imidazole ring of histidine contribute positive charges.
- ◆ Deprotonated carboxyl groups at the C-terminus and in side chains of glutamic and aspartic acid residues produce negative charges.
- ◆ Amidated carboxyls, the N-terminal pyroglutamate (a cyclic amide) of neurotensin, cysteine sulfhydryls (under reducing conditions), and the phenolic hydroxyl of tyrosine are not sufficiently acidic or basic to carry charge in this pH range.

net charge			
pH 2	pH 4.1	pH 6	pH 8
+1	+1	+1	+0.5
+3	+2.5	+2	+2
+3	+2	+0.5	0
+2	+2	+2	+2
+3	+2	+1	+1
+4	+3	+1	0



**Figure 7. Calculating the net charge on a peptide as a function of pH.**

Flags adjacent to ionizable functional groups in the structure indicate  $\text{pK}_a$  values together with the predominant charge state of that functional group for pH values above the  $\text{pK}_a$  (upper symbol) and for pH values below the  $\text{pK}_a$  (lower symbol). For  $\text{pH} = \text{pK}_a$ , charge is 1/2.

Included with the  $pK_a$  value in each flag are two symbols for charge states (+, -, or 0). The upper symbol indicates the predominant charge at pH values above  $pK_a$  and the lower symbol the predominant charge at pH values below  $pK_a$ . At pH differing from  $pK_a$  by one or more, the group will carry a full charge of the sign indicated. At pH equal to  $pK_a$  the group should be counted as 1/2 charge. The sum of contributions from all charged groups in the structure at each pH gives the approximate net charge on the peptide, shown in the table for four typical pH values.

### Results and Conclusions

Separations of the six peptides carried out at pH 2 and pH 4.1 are shown in Figure 8. In each case the sample was loaded at pH 2. For the pH 4.1 separations, a 15 minute rinse at pH 4.1 was followed by a gradient of salt concentration.

Figure 8a shows the separation in buffers containing 50% ACN with a salt gradient from 0 to 100mM NaClO<sub>4</sub> over 50 minutes. Significant observations are:

- ◆ Oxytocin, with a net charge of +1 is retarded slightly but not retained on this column.
- ◆ Three peptides all carrying a net charge of +2 are retained and baseline separated. Hydrophilic effects are responsible for the resolution of these peaks.
- ◆ Conversely, two peptides carrying significantly different net charges (bradykinin, +2.5; angiotensin I, +3) elute together. Hydrophilic effects are powerful enough to overcome the charge difference.

The separation of Figure 8b is similar. However, a shallower salt gradient, from 0 to 50mM NaClO<sub>4</sub> over 50 minutes, was able to partially resolve bradykinin and angiotensin I.

Figure 8c shows the separation run with the same gradient as 8a, but with 25% ACN instead of 50% ACN. Organic modifiers are known to strengthen ionic interactions resulting in complex effect on peptide retention (Ref. 1). As expected for small peptides, all components of the test mixture were less strongly retained in the lower ACN

concentration due to weaker ionic interaction with the stationary phase. In fact, most of the peaks emerged during the period of change to pH 4.1, before the onset of the salt gradient. Interestingly, bradykinin and angiotensin I are completely resolved under these conditions, possibly as a result of hydrophilic effects, or possibly because separation has occurred during the period before complete pH equilibration.

Finally, Figure 8d shows the separation run at pH 2 throughout with the higher ACN concentration and same salt gradient as 8a. The baseline break indicates the point at which elution times have been shifted 16 minutes to compensate for the fact that the salt gradient was commenced immediately (without the initial hold for pH shift). It can be seen that at pH 2 and 50% ACN good retention and complete resolution were obtained for all peaks. However, the elution of angiotensin II and neurotensin are reversed, a phenomenon that cannot be explained by charge effects.

The order of elution for neurotensin, angiotensin II, and bradykinin in Figure 8d, at a pH where all three carry similar net charge, is the best evidence that hydrophilic effects are responsible for their separation. It is the reverse of the order of elution seen on a reversed-phase column where retention is due to hydrophobic interactions (Ref. 2).

### Summary

- Net charges on peptides can be manipulated by changing pH.
- An organic-solvent modifier increases ionic retention and hydrophilic effects.
- Hydrophilic effects play a role in peptide separation by cation exchange.
- Optimizing a peptide separation may require manipulating first pH and then solvent concentration.

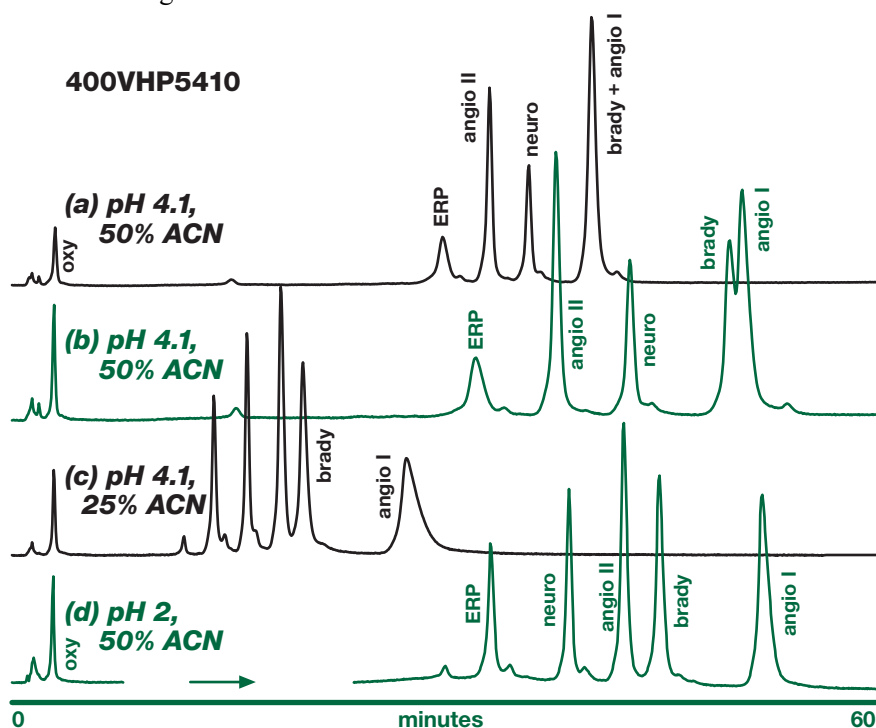
### References

1. Vydac Application Note #9808.
2. Vydac Handbook of Analysis and Purification of Peptides and Proteins by Reversed-Phase HPLC, Figure 9, page 10.

### Ordering Information

Cat.No.	Description
400VHP5410	Column, Cation-Exchange Sulfonic Acid, 900Å, 5µm 4.6mm ID x 100mm L

Other column sizes are available for analytical and preparative applications.



**Figure 8. Separation of six peptides by cation exchange chromatography.**

**Column:** Vydac 400VHP5410 (4.6mmID x 100mmL). **Flow:** 0.7 mL/min. **Detection:** 220 nm. **Mobile phase:** (a) A = 20mM TEAP, pH2, 50% ACN. B = 20mM TEAP, pH4.1, 50% ACN. C = 100mM NaClO<sub>4</sub> in B. Gradient: 100%A to 100%B in 1 minute. Hold 100%B for 15 minutes. Then linear 100%B to 100%C over 50 minutes. (b) Same as (a), except linear 100%B to 50%B:50%C over 50 minutes. (c) Same as (a), except 25% ACN. Gradient: 100%A to 100%B over 15 minutes. Then linear 100%B to 100%C over 50 minutes. (d) A = 20mM TEAP, pH2, 50% ACN. B = 100mM NaClO<sub>4</sub> in A. Gradient: 100% A to 100%B over 50 minutes. Chromatogram (d) displaced 16 minutes at break to compensate for absence of pH change period.

## New LC/MS columns perform well with no TFA!

High-performance separations of tryptic-digest peptides in 5mM HOAc

In the Spring, 1999, issue of *Vydac Advances* we introduced two new column chemistries designed specifically for applications in LC/MS analysis of proteins and peptides. High levels of TFA (0.1% or more) in chromatographic eluates suppress ion generation and reduce coupled mass spectrometer response. Vydac 214MS C4 reversed-phase and 218MS C18 reversed-phase columns are based on 300Å synthetic silica with a proprietary surface modification. They reduce the requirement for TFA in the mobile phase, producing sharp, symmetrical peptide and protein peaks with TFA concentrations down to 0.01%, which greatly simplifies MS detection and improves sensitivity.

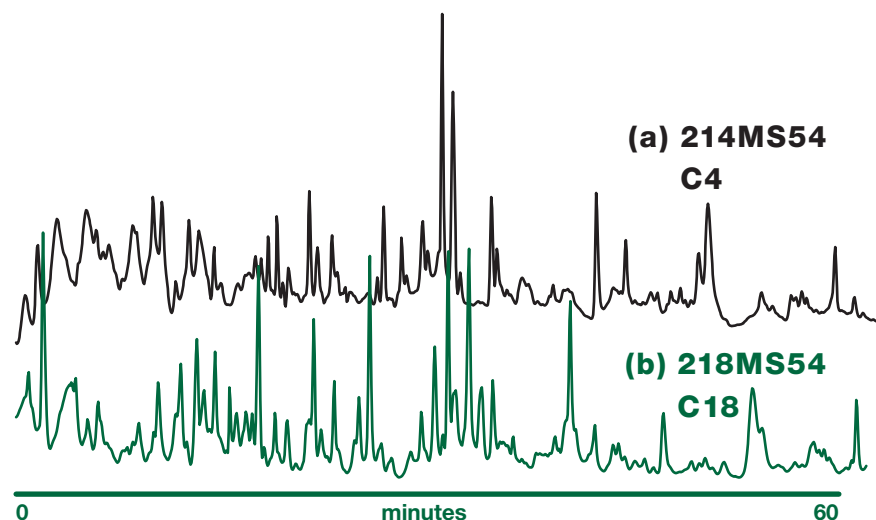
Here we show separations (Figure 9) of a tryptic digest of apotransferrin performed with mobile phases containing 5mM acetic acid, but no TFA. As can be seen, peaks are sharp with minimal tailing, providing the fine detail typical of a good high-performance digest separation.

With obvious advantages for LC/MS, use of acetic acid as an alternative to TFA provides a safe evaporative mobile phase with potential benefits for preparative applications as well.

### Ordering Information

Cat.No.	Description
<b>C4:</b>	
214MS54	Column, LC/MS, C4 Reversed Phase, 300Å, 5µm, 4.6mm ID x 250mm L
214MS52	Column, LC/MS, C4 Reversed Phase, 300Å, 5µm, 2.1mm ID x 250mm L
214MS51	Column, LC/MS, C4 Reversed Phase, 300Å, 5µm, 1.0mm ID x 250mm L
<b>C18:</b>	
218MS54	Column, LC/MS, C18 Reversed Phase, 300Å, 5µm, 4.6mm ID x 250mm L
218MS52	Column, LC/MS, C18 Reversed Phase, 300Å, 5µm, 2.1mm ID x 250mm L
218MS51	Column, LC/MS, C18 Reversed Phase, 300Å, 5µm, 1.0mm ID x 250mm L

Other column sizes are available for analytical and preparative applications.



**Figure 9. Separations of tryptic digest of apotransferrin with zero-TFA mobile phase.**  
**Columns:** (a) Vydac 214MS54 (C4, 5µm, 300Å, 4.6mmID x 250mmL). (b) Vydac 218MS54 (C18, 5µm, 300Å, 4.6mmID x 250mmL). **Flow:** 1 mL/min. **Detection:** 220 nm. **Mobile phase:** Gradient from 0 to 30% ACN in 5mM HOAc over 60 minutes.

## RamPak™ now available

Axial-compression system for preparative scale-up



- easy to use
- versatile
- reliable and cost-effective

Through a partnership with Varian Instruments, Inc., Vydac is proud to offer RamPak column packing systems.

These unique, pneumatically controlled axial-compression systems facilitate easy, reliable on-site packing of high-performance preparative and process columns. They are ideal for use with Vydac bulk adsorbents with chemistries identical to analytical and semipreparative columns for reliable scaleup.

A selection of column dimensions, from 41 mm to 150 mm ID, provides versatility to meet current and future requirements. Varying the amount of packing slurry determines the column length, up to 350 mm. RamPak columns can be tailored to maximize throughput while reducing costs.

For more information and quotations, contact Vydac Technical Support at [experts@vydac.com](mailto:experts@vydac.com).

"RamPak" is a trademark of Varian Instruments.