Vydac® Advances

Quarterly Technical Newsletter on the Characteristics and Use of Vydac HPLC Columns *Spring, 2001*

Separating Small Molecules on 300 Å Reversed Phase

Although it is generally accepted that small-molecule analyses by reversed-phase HPLC are performed on small-pore (60 -100 Å) adsorbents, this is in some respects a historical accident. During the early development of HPLC technology, wide-pore 300 Å silica-based adsorbents were the last to become available.

When Vydac developed its 300 Å pore-size synthetic silica in the early 1980s, it was introduced as an alternative HPLC silica with advantages over existing small-pore packings. Although the new 300 Å material rapidly caught the attention of protein and nucleic acid chemists, small-molecule HPLC applications were already established on small-pore reversed-phase silicas. As a consequence of that, Vydac's 300 Å silica received less attention from HPLC chemists doing small-molecule analyses. Nonetheless, 300 Å silica-based reversed-phase columns can be used and often provide advantages for small molecule separations. For example, Figure 1 shows a small-molecule separation on a 300 Å pore-size C18 column that succeeds in resolving two sample components that coelute on a 90Å C18 adsorbent. In addition, the 300 Å adsorbent

Benefits of Vydac 300 Å columns for small molecule HPLC:

- **Better selectivity**
- **Savings on solvent use and disposal**
- **Faster separations**

Grace Acquires Vydac

Vydac/The Separations Group is pleased to announce its recent acquisition by W.R. Grace & Co. (NYSE: GRA). Vydac's trademark and operating capabilities will be maintained as a unit of Grace's Davison Silica Products business. With annual sales of approximately $1.6 billion, over 6,000 employees, and operations in nearly 40 countries "the resources available within the Grace organization will enhance Vydac's ability to serve its customers, especially in global markets and the highly critical biopharmaceutical process purification segment," stated Shawn Williams, Business and Operations Manager. "With its expertise in silica materials, Grace is ideally positioned to contribute in separation science. Vydac's bonded-silica technology combines well with Grace's position as a leading global silica gel supplier."

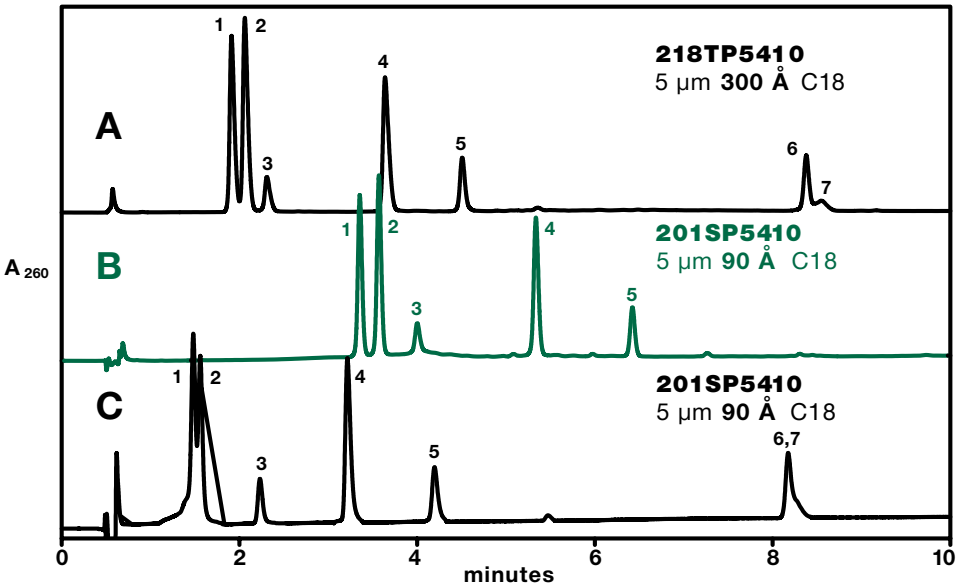

Figure 1. Separation of antihistamines on Vydac large-pore and small-pore reversed-phase columns. Column dimensions: All 4.6 mm i.d. x 100 mm L. Flow: 2.5 mL/min. Mobile phase: A = 0.1% (v/v) TFA in H_2O. B = 0.1% (v/v) TFA in ACN. Gradient, A & B: 5% to 35% B in 10 minutes. Gradient, C: 10% to 40% B in 10 minutes. Peaks: 1) pheniramine; 2) doxylamine; 3) methapyraline; 4) chlorpheniramine; 5) orphenadrine; 6) diphenylpyraline; 7) promethazine. The 300 Å polymeric C18 column provides better resolution of peaks 6 and 7 compared to the 90 Å reversed-phase material. Eluting all peaks in similar time on the 90 Å column (chromatogram C) requires 25% more organic solvent, a significant cost in routine analytical HPLC.

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300 Å Separations

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produces the separation in the same overall run time with less organic solvent in the mobile phase.

Wide-Pore Advantages

Wide-pore reversed-phase adsorbents do not replace small-pore adsorbents. They do complement small-pore adsorbents by providing alternative reversed-phase media that can more successfully accomplish certain separations and analytical objectives. Their potential advantages fall into three basic areas:

(1) The larger base-silica pore size allows added variety in bonding chemistries. Polyfunctional silanes can be used to produce polymeric reversed-phase layers (Figure 2). With small-pore silicas monomeric bonded phases are generally used because the more complex polymeric phases require more space, tend to block the smaller pores and limit chromatographic performance. On 300 Å silica, polymeric phases provide practical adsorbents with subtly different selectivities for small molecules as well as proteins and peptides. Phases with bulky rigid substituents such as diphenyl are also possible, providing another variant in selectivity. Figure 3 shows the same antihistamine separation as in Figure 1, this time run on two different 300 Å reversed-phase columns – one with a polymeric C18 bonded phase and one with a monomeric C18 phase. This demonstrates that the greater selectivity for diphenylpyraline and promethazine is truly due to the polymeric bonded phase, and not pore size.

(2) It should also be remembered that not all small molecules, in the sense of non-polymers, are really "that small." Many non-polymeric molecules of interest, particularly in biochemistry and pharmaceutical sciences, are actually quite large – steroids, certain antibiotics, retinoids, taxanes and other natural products to name a few. These "large" small molecules often show better retention and improved selectivity on 300 Å reversed-phase adsorbents because they are able to more freely enter the pores for access to the entire adsorbent surface.

(3) The lower total surface area of a 300 Å pore silica – roughly 70 m^2/g vs. 250 m^2/g for a 90 Å pore silica – produces lower retentivity on similar surface chemistry while maintaining selectivity. This means that less organic solvent is needed in the mobile phase to elute analytes over a given range of retention times. Methods on 300 Å adsorbents generally reduce solvent usage, and with that comes reduced solvent cost.

Fast LC

Extending the solvent-reduction advantages of wide-pore adsorbents, Vydac now also offers its 300Å reversed-phase materials in 3 µm particle diameters. The smaller 3 µm particles improve mass transfer between the mobile phase and stationary phase in the column by decreasing the distances that analyte molecules must diffuse within the pores to interact with adsorbtive surfaces. Improved mass transfer allows separations to be performed more rapidly using a combination of shorter columns, higher mobile-phase flow rates and faster gradients without sacrificing peak sharpness or resolution. With modern high-efficiency chromatographic systems, the use of 3 µm adsorbents in shorter 100 mm or 50 mm long columns for *"Fast LC"* provides not only faster analyses, but also additional reductions in solvent use, cost, and importantly, reduced disposal cost for spent mobile phases.

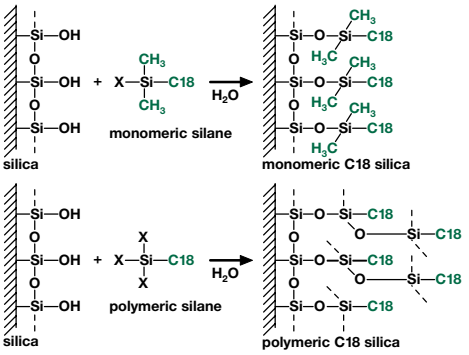

Figure 2. Monomeric vs. polymeric C18 bonding. Use of trifunctional bonding reagent results in a more complex multilayered C18 bonded phase.

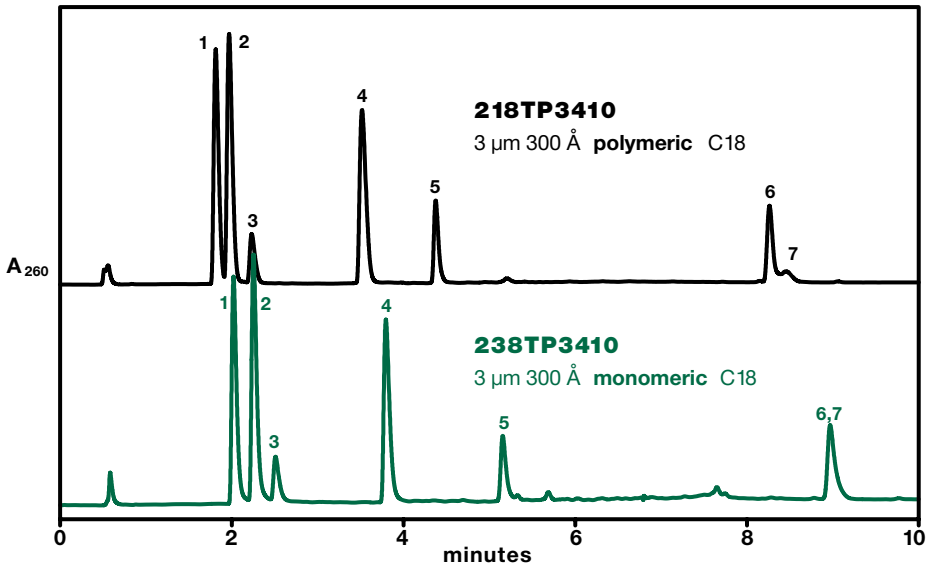

Figure 3. Comparison of antihistamine separations on monomeric and polymeric C18 phases on 300 Å silica-based adsorbents. Column dimensions: Both 4.6 mm i.d. x 100 mm L. Flow: 2.5 mL/min. Mobile phase: A = 0.1% (v/v) TFA in H_2O. B = 0.1% (v/v) TFA in ACN. Gradients: 5% to 35% B in 10 minutes. Peaks: Same as Figure 1. Resolution of peaks 6 and 7 is better on the polymeric bonded phase than on the monomeric phase under the same conditions.

The advantages of fast LC on shorter columns packed with 3 µm 300 Å reversed-phase adsorbents are clearly demonstrated in the separations of Figures 4 through 6. For complex separations such as the screening of Cats Claw extracts for alkaloids (Figure 4) and analysis of taxanes (Figure 5), time savings of 50% or more and solvent savings of 33% or more are clearly possible. For simple routine analyses such as the prednisolone QC separation of Figure 6, very high analytical throughputs can be achieved.

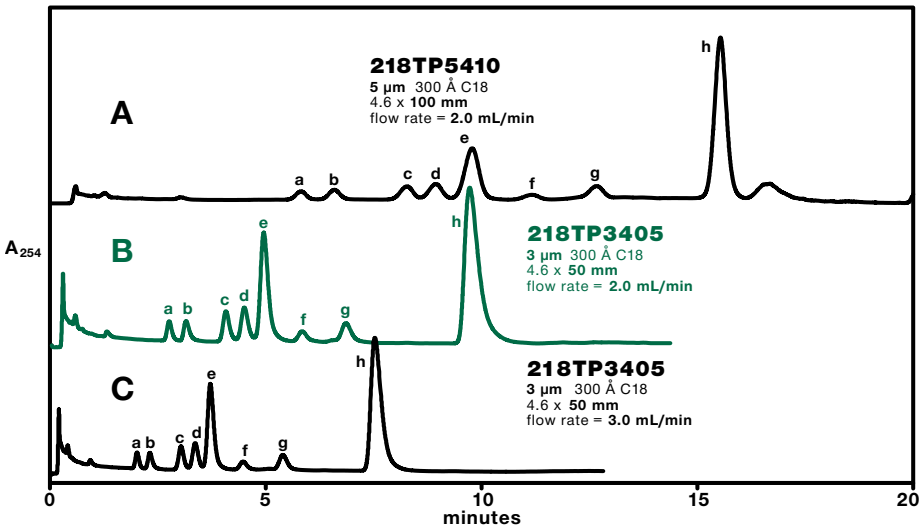

Figure 4. Fast screening of extract of Cats Claw. Mobile phase: A = 10 mM KH_2PO_4, pH 6.6, 20% ACN, 10% MeOH. B = 100% ACN. Gradient: 0% to 5%B in 5 minutes, then ramp to 13%B in 5 minutes, and to 100%B in 3 minutes. An excellent example of the benefits of short, fast columns: Elution of the eight alkaloids in chromatogram A takes about 15 minutes. In chromatogram B on a shorter column, the separation is accomplished in 10 minutes with a time and solvent savings of 33%. Increasing flow rate in chromatogram C saves another 25% in time while increasing solvent consumbtion by only 12.5% compared to B.

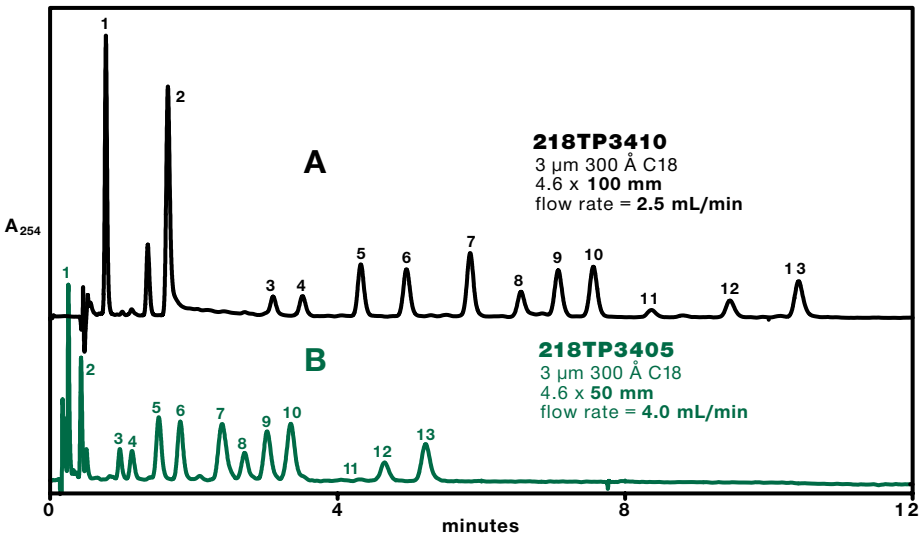

Figure 5. Fast screening of taxanes. Mobile phase: A = 50 mM NaOAc, pH6.7. B = ACN. Gradient: 30% to 40%B in 10 minutes, then hold for 2 minutes. Peaks: 1) 10-deacetylbaccatin III; 2) baccatin III; 3) 10-deacetyl-7-xylosyltaxol B; 4) taxinine M; 5) 10-deacetyl-7-xylosyltaxol; 6) 10-deacetyl-7-xylosyltaxol C; 7) 7-xylosyltaxol; 8) cephalomannine; 9) 10-deacetyl-7-epitaxol; 10) paclitaxel; 11) 10-deacetyltaxol; 12) taxol C; 13) 7-epitaxol. In chromatogram B, a complex separation of thirteen compounds is achieved in less than six minutes. By increasing the flow rate to 4.0 mL/min and reducing the column length to 50 mm, the time savings in approximately 50%. Not so obvious is the solvent savings, which is about 5.5 mL per run, or 20%.

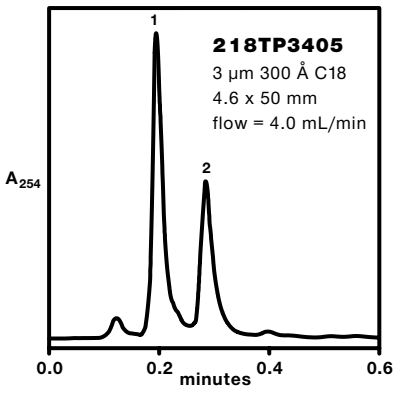

Figure 6. Fast QC of prednisolone. Mobile phase: Isocratic. 40% ACN in water. Peaks: 1) prednisolone acetate; 2) prednisolone. In this day of high-throughput screening, short fast columns are the way to go. This separation was done at a flow of 4.0 mL/min in less than half a minute using isocratic elution. Using an automated system, at 0.5 minutes per analysis, this would result in 960 analyses in an eight hour working day. (Over lunch, add another 120.)

Columns for Fast LC

Cat. No.	Description
	50 mm long columns:
238TP3405	Column, Octadecyl (C18), Monomeric, 3µm, 300Å, 4.6mm ID x 50mm L
218TP3405	Column, Octadecyl (C18), Polymeric, 3µm, 300Å, 4.6mm ID x 50mm L
214TP3405	Column, Butyl (C4), Polymeric, 3µm, 300Å, 4.6mm ID x 50mm L
	100 mm long columns:
238TP3410	Column, Octadecyl (C18), Monomeric, 3µm, 300Å, 4.6mm ID x 100mm L
218TP3410	Column, Octadecyl (C18), Polymeric, 3µm, 300Å, 4.6mm ID x 100mm L
214TP3410	Column, Butyl (C4), Polymeric, 3µm, 300Å, 4.6mm ID x 100mm L

Exhibit Schedule

Come visit Vydac's booth and speak with us at the following meetings:

ASMS
Chicago, IL May 27-31 Booth #70

17th American Peptide Symposium
San Diego, CA Jun. 9-14 Booth #207

HPLC 2001
The Netherlands Jun. 17-22 Booth #81

ACS Fall 2001
Chicago, IL Aug. 27-29 Booth #1240

Biotechnica
Hannover, FRG Oct. 9-11

AAPS 2001
Denver, CO Oct. 22-24 Booth #2035

LC/MS/MS

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verified that the sequence of this peptide was SLSLILYSR.

Clearly a very powerful technique for protein analysis and characterization, the resolution and reproducibility of Vydac's 300 Å reversed-phase adsorbent contributes to the successful application of LC/MS technology to biomedical research.

Courtesy of:

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MS Adsorbents

Vydac MS adsorbents incorporate a proprietary surface modification that reduces the levels of modifiers such as TFA required to obtain good symmetrical peaks in protein and peptide HPLC. They were specifically developed with the needs of mass spectroscopists in mind.

In addition to standard 4.6 mm i.d. HPLC columns, Vydac MS adsorbents are also available pre-packed in narrow-bore 2.1 mm and 1.0 mm i.d. columns, as well as 0.5 mm and 0.3 mm i.d. capillary columns for microflow applications. Ordering information for capillary columns appears below. For larger diameter columns, please consult the Vydac catalog or the shopping section of Vydac's website at **www.vydac.com**.

Modifier Effects in Peptide Separations

Reversed-phase peptide separations are sensitive to presence and differences in concentration of mobile phase modifiers. Here are two examples.

In Figure 11, a crude 22-amino-acid synthetic peptide is purified on a 300 Å C4 reversed-phase column. Chromatogram A, with 0.3% TFA (v/v) in the mobile phase, achieves clear separation of the desired product from its closest contaminant. In chromatogram B, when the same separation is performed with 0.1% TFA (v/v), the two peaks coelute. It is also interesting to note that decreasing TFA concentration, all else constant, generally decreases peptide retention times.

In Figure 11C the concentration of TFA was 0.1% (w/v). Note that retention times are further reduced and that the order of the major peak and contaminant is reversed from that in A. This underscores the fact that TFA concentrations specified by weight are <u>not</u> equivalent to concentrations specified by volume. TFA measurement by volume gives a higher concentration due to the density of TFA. For method reproducibility, it is always important to specify which method was used to measure TFA concentration.

Figure 12 shows the effect of adding 10% methanol to the mobile phase. Conditions are otherwise the same as for Figure 11B. In this instance, adding methanol achieved separation of the contaminating peak as in Figure 11A, but without the higher TFA concentration.

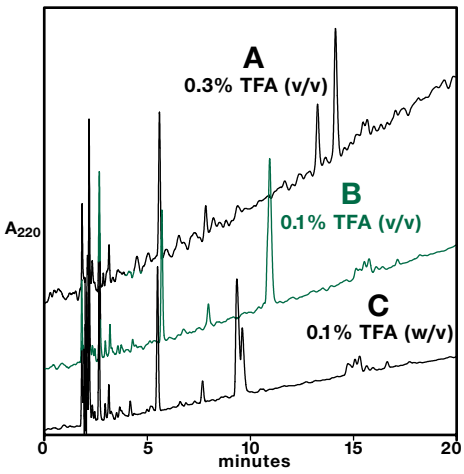

Figure 11. Effect of TFA in peptide separation. Column: Vydac 214TP54, 5 µm, 300 Å, C4 reversed phase, 4.6 mm i.d. x 250 mm L. Flow: 1.5 mL/min. Mobile phase: A = TFA as indicated in H_2O. B = TFA as indicated in ACN. Gradient: 40% to 60%B in 20 minutes.

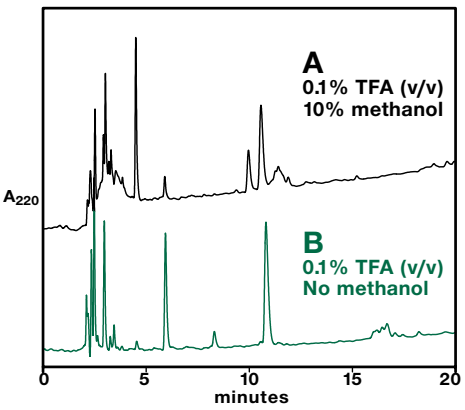

Figure 12. Effect of methanol in peptide separation. Column: Vydac 214TP54, 5 µm, 300 Å, C4 reversed phase, 4.6 mm i.d. x 250 mm L. Flow: 1.5 mL/min. Mobile phase: A = 0.1 % TFA (v/v) in H_2O. B = 0.1 % TFA (v/v) in ACN. C = 0.1% TFA (v/v) in MeOH. Gradient: 40% to 60%B in 20 minutes. 10%C constant in chromatogram A.

Pre-Packed Reversed-Phase Capillary Columns for LC/MS

Cat. No.	Description
214MS5.305	LC/MS R-P Column. Polymeric C4. 300 Å. 5 µm. 0.3 mm i.d. x 50 mm.
214MS5.310	LC/MS R-P Column. Polymeric C4. 300 Å. 5 µm. 0.3 mm i.d. x 100 mm.
214MS5.505	LC/MS R-P Column. Polymeric C4. 300 Å. 5 µm. 0.5 mm i.d. x 50 mm.
214MS5.510	LC/MS R-P Column. Polymeric C4. 300 Å. 5 µm. 0.5 mm i.d. x 100 mm.

Cat. No.	Description
218MS5.305	LC/MS R-P Column. Polymeric C18. 300 Å. 5 µm. 0.3 mm i.d. x 50 mm.
218MS5.310	LC/MS R-P Column. Polymeric C18. 300 Å. 5 µm. 0.3 mm i.d. x 100 mm.
218MS5.505	LC/MS R-P Column. Polymeric C18. 300 Å. 5 µm. 0.5 mm i.d. x 50 mm.
218MS5.510	LC/MS R-P Column. Polymeric C18. 300 Å. 5 µm. 0.5 mm i.d. x 100 mm.

Cat. No.	Description
238MS5.305	LC/MS R-P Column. Monomeric C18. 300 Å. 5 µm. 0.3 mm i.d. x 50 mm.
238MS5.310	LC/MS R-P Column. Monomeric C18. 300 Å. 5 µm. 0.3 mm i.d. x 100 mm.
238MS5.505	LC/MS R-P Column. Monomeric C18. 300 Å. 5 µm. 0.5 mm i.d. x 50 mm.
238MS5.510	LC/MS R-P Column. Monomeric C18. 300 Å. 5 µm. 0.5 mm i.d. x 100 mm.

Vydac Adsorbent Aids in LC/MS/MS Analyses

Our appreciation to Dr. Susan Weintraub of the Department of Biochemistry at the University of Texas Health Science Center in San Antonio for this article.

Vydac's application-dedicated 218MS 5 µm diameter polymeric-C18 reversed-phase adsorbent was packed in a New Objective PicoFrit™ fused-silica capillary column (75 µm i.d. x 6 cm L). A Michrom MAGIC 2002 micro HPLC coupled to a Thermo Finnigan LCQ ion trap mass spectrometer was used for these studies. To verify column performance, a 300-femtomole sample of a tryptic digest of myoglobin was separated using a gradient of acetonitrile in water/HOAc/TFA at a flow rate of 0.4 µL/min and detected by the mass spectrometer (Figure 7).

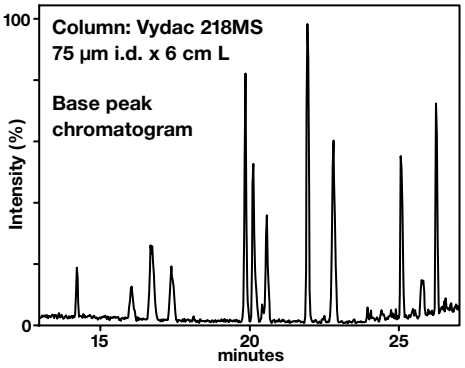

Figure 7. HPLC separation of tryptic digest of myoglobin. Column: PicoFrit™ (New Objective) 75 µm i.d. packed to 6 cm with Vydac 218MSB5 5 µm 300 Å C18 reversed-phase adsorbent. Mobile phase: A = 0.5% HOAc/0.005% TFA. B = 0.5% HOAc/0.005% TFA/90% ACN. Flow: 0.4 µL/min. Programmed gradient: 2% B to 72% B in 10 min. Delay time from pump to column approximately 10 minutes. Detection: MS.

In a recent study conducted by Dr. Jonathan King in the laboratory of Dr. Merle Olson at the Health Science Center in San Antonio, it was observed that there is increased transport of protein into bile in septic animals. It was of interest to identify the major bile proteins in order to ascertain if any new species were being secreted during sepsis. To accomplish this, rat bile proteins were separated by two-dimensional gel electrophoresis and a spot corresponding to a 55-kilodalton protein was subjected to in-gel tryptic digestion. A sample of the digest was applied to the 218MS capillary column and eluted with a gradient of acetonitrile in water/HOAc/TFA, resulting in the base peak chromatogram shown in Figure 8A. A typical selected ion retrieval trace (*m/z* 526.4, $[M+2H]^{2+}$ for a 1050.8-Da peptide) is shown in Figure 8B. A second injection of the digest was analyzed by a data-dependent MS/MS protocol followed by database searching with the program SEQUEST which revealed the identity as a rat vitamin D binding protein. To illustrate the spectra obtained during this process, in Figure 9 is the mass spectrum for the peptides eluting from 31.65 to 31.75 minutes and in Figure 10 is the MS/MS spectrum of the doubly-charged ion at *m/z* 526.4. The SEQUEST search results

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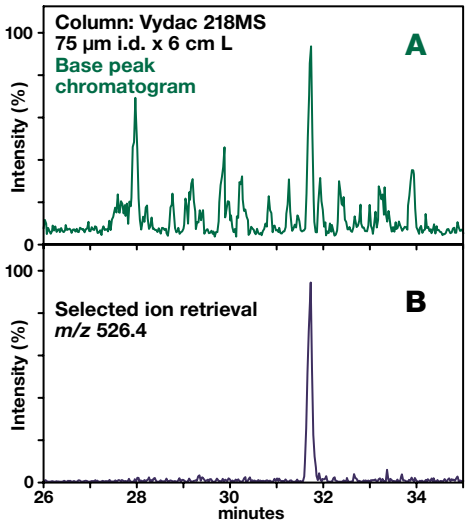

Figure 8. HPLC separation of in-gel tryptic digest of 55-kDa rat bile protein. Column: PicoFrit™ (New Objective) 75 µm i.d. packed to 6 cm with Vydac 218MSB5 5 µm 300 Å C18 reversed-phase adsorbent. Mobile phase: A=0.5% HOAc/0.005% TFA. B = 0.5% HOAc/0.005% TFA/90% ACN. Flow: 0.3 µL/min. Programmed gradient: 2% B to 72% B in 10 min. Delay time from pump to column approximately 13 minutes. Detection: MS.

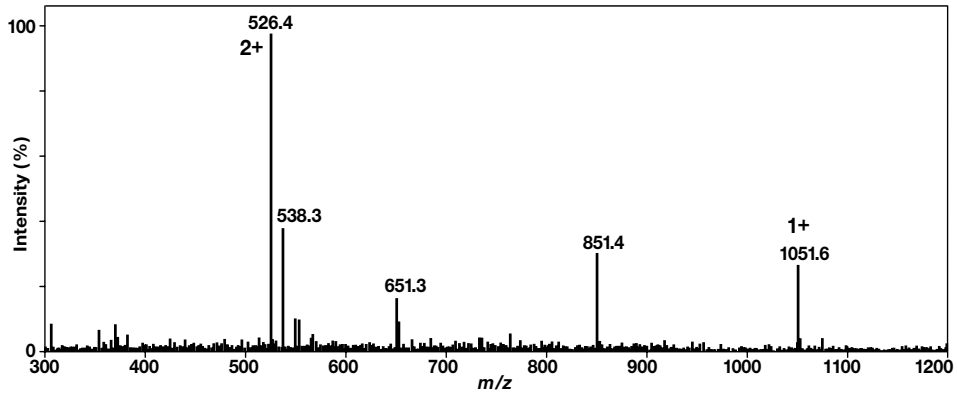

Figure 9. Mass spectrum of peptides eluting from 31.65 – 31.75 min (Figure 8).

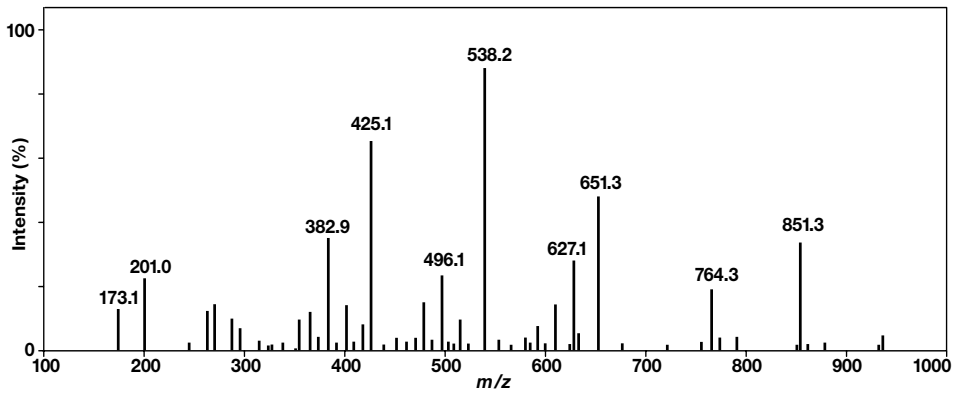

Figure 10. MS/MS spectrum generated by collision-induced dissociation of the ion at *m/z* 526.4 (RT 31.7 min).