



Vydac Advances

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

Fall, 1999

New! Three SelectaPore™ C18 Columns for Pharmaceutical and Small-Molecule Analyses

- Three distinct selectivities
- Two C18 chemistries & pore sizes
- One unique analytical tool!

Over the years Vydac has offered many different reversed-phase columns, including covalently bonded C18 silica adsorbents designed for analytical applications including peptides, proteins, nucleic acids, vitamins, environmental pollutants, and drugs. In developing the SelectaPore product line, we focused specifically on the problem of purity analysis of novel small-molecule pharmaceuticals, where the need is somewhat different from routine analyses.

Long before a new drug is submitted for clinical trials and approval, analyses must reveal minor impurities, allowing them to be well characterized and the process to be modified as needed to produce high-purity product. Routine analyses must then be included in SOPs to ensure proper batch-to-batch process function. Of particular concern are by-products and contaminants co-eluting or nearly co-eluting with product from an HPLC column. Such contaminants may defy detection at the 1% level on a single HPLC column.

Recent advances in reversed-phase column technology have improved efficiency, speed, and peak symmetry. Vydac has been in the vanguard. But these developments, while improving the probability of detecting closely eluting impurities, do not solve the problem of co-eluting peaks. The solution to this problem must involve either orthogonal

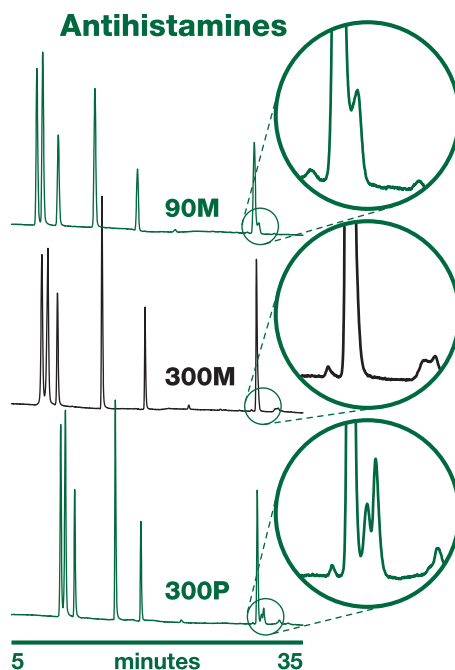


Figure 1. A mixture of antihistamines was separated on three 4.6mmID x 250mmL SelectaPore columns. Flow: 1.0 mL/min. Detection: 260 nm. SelectaPore 90M gradient: Linear, 12% to 42% ACN over 35 minutes in 0.1% TFA (v/v). SelectaPore 300M and 300P gradients: Linear, 5% to 32% ACN over 35 minutes in 0.1% TFA (v/v). The major peak in the area of detail is diphenylpyraline. The trailing shoulder peak seen on SelectaPore 90M was identified as promethazine. The other contaminant seen on SelectaPore 300P was not identified.

analyses using different methods or variations in reversed-phase selectivity. Finding a reversed-phase method with selectivity to reveal an impurity is preferable and simplifies later routine analyses.

Vydac's three new SelectaPore adsorbents offer a range of reversed-phase selectivities. All three are C18 reversed-phases, but take advantage of differences

in pore structure and bonding chemistry to produce retentivities that can vary differently with analyte structure.

SelectaPore 90M is C18 bonded with octadecyldimethylmonosilane to produce a "monomeric" bonded phase on a 90 Å pore-size silica.

SelectaPore 300M is also bonded with octadecyldimethylmonosilane, but on a 300 Å pore-size silica, which produces lower retentivity due to lower surface area per gram. This is advantageous for more hydrophobic compounds because they can be eluted with lower solvent concentrations to reduce solvent waste, and may also change relative retention of some analytes.

SelectaPore 300P is C18 bonded with a trifunctional octadecylsilane to produce a "polymeric" bonded phase on a 300 Å pore-size silica. The polymeric C18 surface chemistry is distinctly different from monomeric C18, and has been demonstrated to alter relative retention among many analytes. The combination of analyses on SelectaPore 300P and either of the other SelectaPore adsorbents is often the key to revealing minor peaks in the vicinity of a major analyte.

Figure 1 shows an example of the power of differing C18 selectivities on the three SelectaPore columns. The area of detail surrounds the diphenylpyraline peak in a mixture of antihistamines obtained commercially. Gradient conditions were adjusted as indicated in the figure legend, causing diphenylpyraline to emerge at 31 minutes on all three columns.

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"SelectaPore" is a trademark of The Separations Group, Inc.

ISO 9001
Certified

VYDAC®

leaders in synthetic-silica technology
serving chromatographers with quality packings and bonding chemistry since 1971

SelectaPore

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All three SelectaPore C18 columns are based on silica that is modified by a proprietary process to reduce silanols before bonding, contributing to excellent peak symmetry for basic and polar analytes. All three SelectaPore C18 columns are also extensively end-capped using trimethylsilane reagent after C18 bonding.

SelectaPore columns are available in a convenient kit containing one 25-cm-long analytical column of each type at a reduced price.

Ordering Information

Cat.No.	Description
SelectaPore 90M (90Å, monomeric C18):	
201SP54	Column, 4.6mm ID x 250mm L
201SP5415	Column, 4.6mm ID x 150mm L
201SP52	Column, 2.1mm ID x 250mm L
201SP5215	Column, 2.1mm ID x 150mm L
SelectaPore 300P (300Å, polymeric C18):	
218WP54	Column, 4.6mm ID x 250mm L
218WP5415	Column, 4.6mm ID x 150mm L
218WP52	Column, 2.1mm ID x 250mm L
218WP5215	Column, 2.1mm ID x 150mm L
SelectaPore 300M (300Å, monomeric C18):	
238WP54	Column, 4.6mm ID x 250mm L
238WP5415	Column, 4.6mm ID x 150mm L
238WP52	Column, 2.1mm ID x 250mm L
238WP5215	Column, 2.1mm ID x 150mm L
SelectaPore Kit:	
200SPK54	Includes one of each 4.6mmID x 250mmL column (201SP54, 218WP54, & 238WP54)

Other analytical and preparative column and particle sizes available on request.

SelectaPore Column Evaluated for Cat's Claw Analysis

Sales of herbal preparations are currently estimated at over \$12 billion per year worldwide and growing rapidly. Traditional herbal concoctions escape much regulation because they are classified as natural food supplements, not drugs. With claims supported by tradition and folklore, herbal products are often viewed by consumers as more "natural" and "organic" than pharmaceuticals. Their increasing use raises issues relating to efficacy, identification, ecology, side effects, and interactions. They also represent a potent area of investigation for discovery of new defined pharmaceuticals. Chromatographic analysis and purification play an important role in identification and research.

Cat's claw (*Uncaria tomentosa*) is a woody vine native to Peruvian Amazon forests. The bark and stems have been used traditionally by the Ashaninka indians for treatment of digestive complaints and arthritis. Increasingly, cat's claw preparations are being sold in world markets. These products are said to stimulate immunity, suppress inflammation, and "promote well-being." Stimulatory effects on cellular immunity as well as possible inhibitory effects on formation of amyloid plaque related to Alzheimer's disease have been reported in research publications. Environmental concerns have arisen regarding depletion of cat's claw vines due to harvesting by Peruvian farmers as a source of income alternative to cultivating coca.

The active components of cat's claw are believed to be one or more of six stereoisomeric pentacyclic oxindole alkaloids. Separation of the six isomers by reversed-phase HPLC has been reported (Ref. 1). A second naturally occurring chemotype has been reported to contain in addition two tetracyclic oxindole alkaloids which affect the central nervous system and act as antagonists to the immunostim-

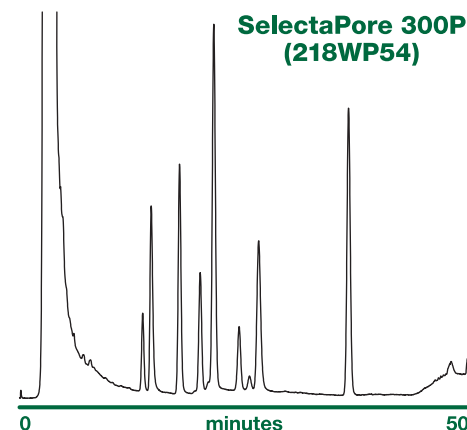


Figure 2. Separation of cat's claw extract on SelectaPore 300P. Column: Vydac 218WP54, polymeric C18, 300 Å, 5 µm, 4.6mmID x 250mmL. Detection: 245 nm. Flow: 1.0 mL/min. Mobile phase: A = 20% ACN/10% MeOH/10 mM KH₂PO₄, pH 6.66. B = ACN. Gradient: Hold 8% B first 10 minutes. Then linear ramp to 13% B over 20 minutes. Then ramp to 23% B over 10 minutes. Then to 100%B over 7 minutes and hold. Peak identities unknown.

ulatory effects of the pentacyclics (Ref. 2.). The plant used for medicinal purposes by the Ashaninkas contains only pentacyclic alkaloids. In view of these differences, chromatographic analysis can be important for type identification.

Cat's claw powder provided by a Vydac customer was extracted with acetonitrile and the resulting sample separated on SelectaPore 300P (Fig. 2). Nine resolved peaks were detected by absorbance at 245 nm. Previous analyses by reversed-phase HPLC have detected eight main peaks in Cat's claw preparations. Separation on SelectaPore 90M (results not shown) produced stronger retention with some peaks running into the high-solvent wash. SelectaPore 300M was not tried, but might be expected to produce different selectivity.

References

1. Stuppner, H., Sturm, S., and Konwalinka, G., "HPLC Analysis of the Main Oxindole Alkaloids from *Uncaria tomentosa*." *Chromatographia*, **34**, 597-530 (1992)
2. Reinhard, K.H., "Uncaria tomentosa (Willd.) D.C.: cat's claw, una de gato, or saventaro." *J. Altern. Complement. Med.*, **2**, 143-151 (1999)

Peptide Separations on a C18 LC/MS Column

Optimizing the Effects of Mobile-Phase Modifiers

In *Vydac Advances* for Summer, 1998, we discussed varying mobile phase conditions to optimize separation of synthetic peptides on three Vydac C18 reversed-phase columns. Since then, introduction of new Vydac LC/MS columns has permitted efficient peptide separations with reduced concentrations of mobile phase modifiers such as TFA. This is particularly beneficial for electrospray MS detection because it reduces the suppressive effect of TFA and improves peak amplitudes without special post-column procedures.

The fact that sharp, symmetrical peaks can be obtained on an LC/MS column with lower modifier concentrations does not mean that modifiers have no effect. On the contrary, as shown here for a test mixture of six small peptides, changing modifiers and modifier concentrations can significantly affect not only peak shapes, but retention and selectivity.

The column is Vydac's new 238MS54, a monomerically bonded C18 on 5 μ m, 300 Å base silica with a proprietary treatment that reduces the concentrations of modifier required. All separations were performed with a 10% to 40% linear gradient of acetonitrile over 30 minutes. As is evident from the chromatograms of Figure 3, significant differences in selectivity arise depending on the concentrations and identities of modifier ions present in the mobile phase, and perfluorinated acidic modifiers even at very low levels (0.005% w/v) can increase retention.

For this particular mixture, the best spread of peaks was obtained with heptafluorobutyric acid (HFBA) at 0.02% (w/v). However, this might be expected to differ for other peptides.

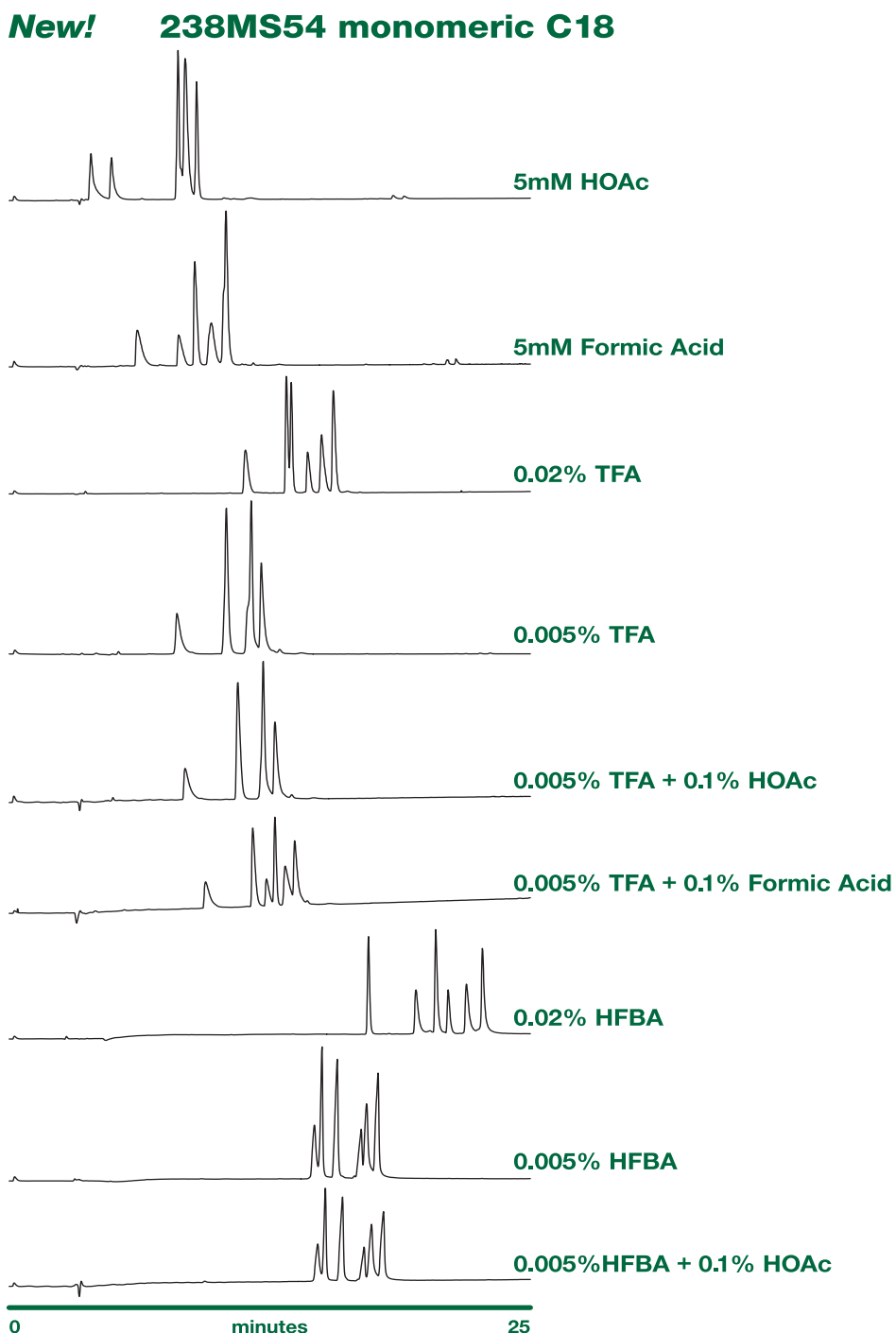


Figure 3. Separation of six small standard peptides on a Vydac C18 LC/MS column under varying mobile phase conditions. Sample components: oxytocin, bradykinin, angiotensin II, eledoisin-related peptide, neurotensin, and angiotensin I. Column: Vydac 238MS54 monomeric C18, 5 μ m, 300 Å, 4.6mmID x 250mmL. Flow: 1.0 mL/min. Detection: 220 nm. Gradient: Linear, 10% to 40% ACN over 30 minutes for all chromatograms. Mobile phase modifiers as indicated (w/v).

The new monomeric 238MS54 C18 column produced excellent peak shapes for these peptides and was markedly superior in this regard to the previously reported polymeric 218MS54 C18 LC/MS column (results not shown) with this peptide mixture.

The important lesson: In choosing conditions for HPLC of peptides, a few trial runs with different mobile phases are well worth the effort to determine the best conditions to use for a specific separation.

Vydac LC/MS columns are also available with C4 reversed-phase for proteins and hydrophobic peptides. Column diameters include 1.0mmID for low-flow HPLC with direct feed to MS detectors.

Ordering Information

Cat.No.	Description
Monomeric C18:	
238MS54	Column, LC/MS, C18 Reversed Phase, 300Å, 5µm, 4.6mm ID x 250mm L
238MS52	Column, LC/MS, C18 Reversed Phase, 300Å, 5µm, 2.1mm ID x 250mm L
238MS51	Column, LC/MS, C18 Reversed Phase, 300Å, 5µm, 1.0mm ID x 250mm L
Polymeric C18:	
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
Polymeric C4:	
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

Other column sizes are available for analytical and preparative applications.

New 2000/2001 Vydac Catalog Coming Soon!

A new Vydac Catalog is in the works and expected to be available for mailing during the month of January, 2000. We've enjoyed serving your HPLC column needs during the present millenium, and we look forward to continuing to do so in the next!

The new catalog will be up to date and packed with information about Vydac columns and applications. Be sure to request a copy from your local Vydac distributor.

Recombinant Alzheimer's Proteins (continued from page 5)

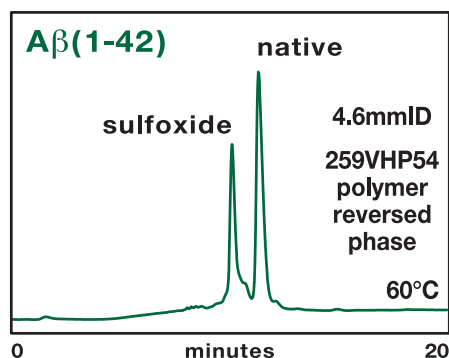


Figure 7. Separation of pure Ab(1-42) peptide, native form and sulfoxide form on polymer-based reversed-phase.

Column: Vydac 259VHP54 5µm, 300Å, 4.6mmID x 250mmL. Conditions: 60°C, 1.0 mL/min. Mobile phase: A = 0.05% TFA in 5% MeCN. B = 0.05% TFA in 90% MeCN. Gradient: Linear 0 to 28% B over 8 minutes. Then to 40% B over 24 minutes.

Ordering Information

Cat.No.	Description
259VHP54	Column, Polymer Reversed Phase, 300Å, 5µm, 4.6mm ID x 250mm L
259VHP5415	Column, Polymer Reversed Phase, 300Å, 5µm, 4.6mm ID x 150mm L
259VHP822	Column, Polymer Reversed Phase, 300Å, 8µm, 22mm ID x 250mm L

Ordering information for silica-based low-TFA C4 and C18 LC/MS reversed-phase columns appears at left.

Purified Aβ proteins contain a methionine at position 35 that can become oxidized under certain conditions to yield a sulfoxide form which bears one additional negative charge. The Vydac 259VHP54 column was also able to separate the sulfoxide form from the native form of Aβ(1-42), as shown in Figure 7. (Peak identities confirmed by MS.)

Information and chromatograms for this article courtesy of

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Vydac Reversed-Phase Columns Aid in Purification of Recombinant Alzheimer's Proteins

University of Georgia Group Produces Pure A β (1-42) in Milligram Quantities

The neural deposits (plaque) characteristic of Alzheimer's disease consist predominantly of a 42-amino-acid-residue protein, A β (1-42), and to a lesser extent a 40-residue truncated version, A β (1-40). Milligram quantities of these proteins in purified form would surely be useful for studying Alzheimer's plaque formation. Although both have been synthesized chemically, issues related to batch-to-batch reproducibility, presence of blocking groups, and variable peptide lengths make routine production by chemical synthesis undesirable.

At the Prep '99 Meeting in San Francisco this past May, researchers from the University of Georgia reported using a recombinant system to produce A β (1-42) and A β (1-40) as fusion proteins in *E. coli*. The fusion proteins are expressed at high levels (average 40 mg/L of culture) and can be rapidly purified by metal-chelating chromatography.

Once the fusion protein has been purified, A β (1-42) or A β (1-40) is released by cleavage with Factor Xa protease. The digestion yields the desired protein plus a lesser amount of a truncated protein, A β (6-42) or A β (6-40), produced by nonspecific activity of the protease. Some undigested fusion protein may also remain.

A single purification step by reversed-phase chromatography is sufficient for isolation of highly purified product from the cleavage mixture. For A β (1-40), reversed-phase chromatography was performed under acidic conditions at 60°C on a Vydac low-TFA silica-based C4 or C18 column (Fig. 4).

For A β (1-42), purification on silica-based reversed-phase columns was problematic due to low solubility, strong retention, and a tendency to

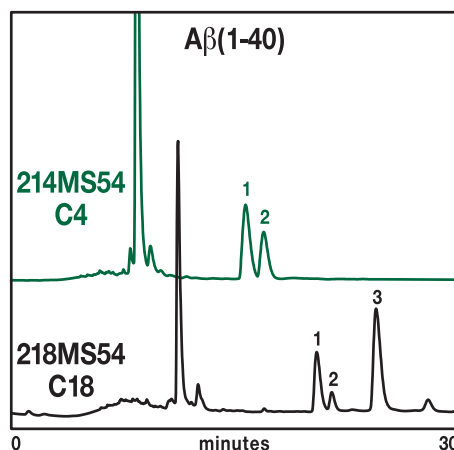


Figure 4. Separation of digested A β (1-40) fusion on silica-based low-TFA reversed phase. Columns: Vydac 214MS54 C4 and 218MS54 C18. Both 5 μ m, 300Å, 4.6mmID x 250mmL. Conditions: 60°C, 1.0 mL/min. C4 mobile phase: A = 0.05% TFA in 5% MeCN. B = 0.05% TFA in 95% MeCN. Gradient: Linear 0 to 20% B over 8 minutes. Then 20 to 24% B over 24 minutes. C18 mobile phase: A = 0.075% TFA in 5% MeCN. B = 0.075% TFA in 95% MeCN. Gradient: Linear 0 to 25% B over 8 minutes. Then to 29% B over 24 minutes. Peaks: 1. A β (1-40); 2. A β (6-40); 3. uncut fusion.

aggregate. These problems could be counteracted by raising the temperature to 80°C in the mildly acid mobile phase, but the high temperature makes the method difficult to scale up for preparative separations. Alternatively, pH above neutrality reduces retention and helps prevent aggregation, but high-pH mobile phases are detrimental to silica-based columns. A Vydac 259VHP54 polymer-based reversed-phase column provided the answer, by virtue of its resistance to high pH. Separation with good yields could be performed at pH 8 and either 60°C or 40°C (Fig. 5). Polymer-based 259VHP columns also have the advantage that they can be cleaned by more aggressive washing solutions.

Finally, the reversed-phase separation on 259VHP was scaled up to a 22mmID preparative column. A sample

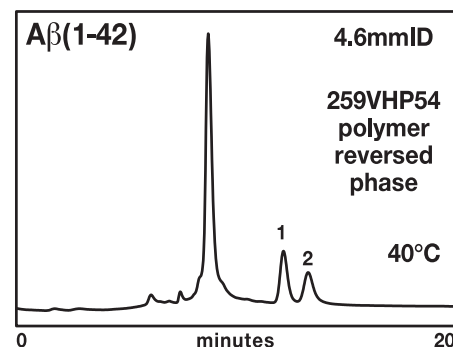


Figure 5. Separation of purified digested A β (1-42) fusion on polymer-based analytical reversed phase. Column: Vydac 259VHP54 5 μ m, 300Å, 4.6mmID x 250mmL. Conditions: 40°C, 1.0 mL/min. Mobile phase: A = 5mM potassium acetate, pH 8.0 in 5% MeCN. B = 5mM potassium acetate, pH 8.0 in 90% MeCN. Gradient: Linear 0 to 20% B over 8 minutes. Then to 26% B over 12 minutes. Peaks: 1. A β (1-42); 2. A β (6-42).

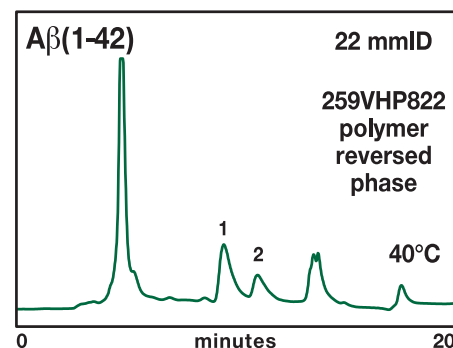


Figure 6. Separation of purified digested A β (1-42) fusion on polymer-based preparative reversed phase. Column: Vydac 259VHP822 8 μ m, 300Å, 22mmID x 250mmL. Conditions: 40°C, 15 mL/min. Mobile phase: A = 5mM potassium acetate, pH 8.0 in 5% MeCN. B = 5mM potassium acetate, pH 8.0 in 90% MeCN. Gradient: Linear 0 to 20% B over 7 minutes. Then to 25% B in 8 minutes. Peaks: 1. A β (1-42); 2. A β (6-42).

load of 15 mg total protein on that column resulted in a yield of 2 mg of purified A β (1-42) protein (Fig. 6). Purity of final product was determined by MALDI/TOF mass spectrometry with each product yielding a single peak of the correct molecular weight.

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