Vydac Advances

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

Vydac's New 218MR Column Runs Rings

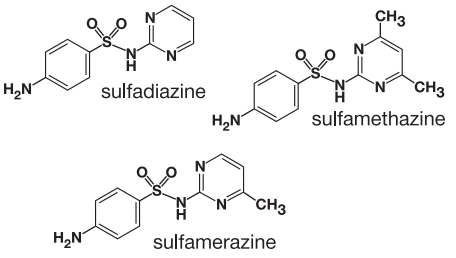
A Special 300Å Reverse-Phase Column for Multi-Ring Compound Separation

Many active pharmaceuticals have complex multiple-ring structures and are best separated by wide-pore reverse-phase chromatography. Vydac's new 218MR column, a 5µm spherical 300Å silica-based C18, is designed specifically for such separations. It is subject to quality control procedures that assure performance in accordance with USP specifications. The analyses shown here were done on a 218MR53 column. When compared to a 4.6mm ID, this 3.2mm ID column offers

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

A standard 4.6mm ID column is also available. (See page 2 for details.)

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



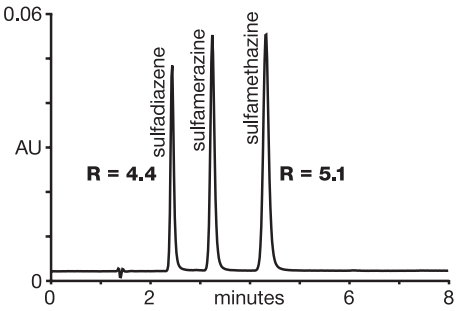
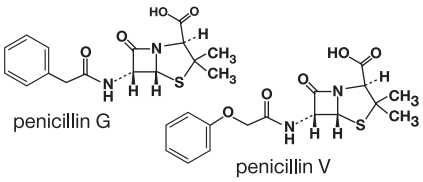


Figure 2. Trisulfapyrimidines on 218MR53. Conditions: 254nm, isocratic 86:13:1 water:ACN:glacial HOAc, 1.0 mL/min. USP requires R not less than 3.0.



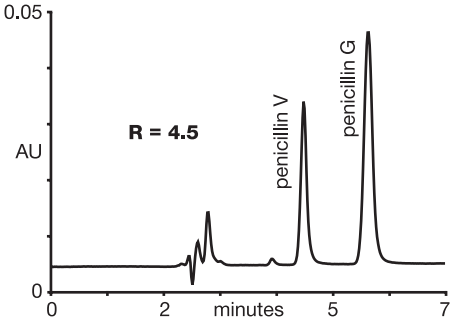
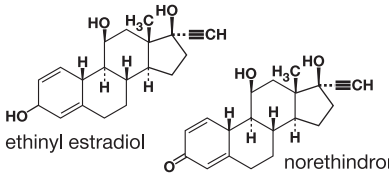


Figure 4. Penicillin V on 218MR53. Conditions: 254nm, isocratic 65:35 water:ACN + 1% acetic acid, 0.5 mL/min. USP requires R not less than 3.0.



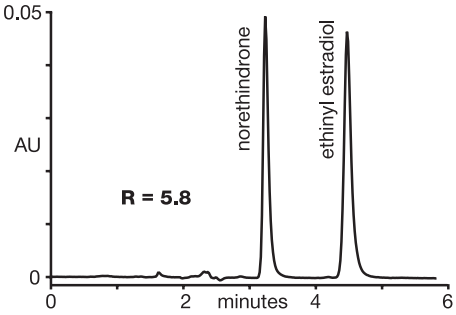
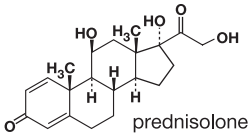


Figure 1. Norethindrone and ethinyl estradiol on 218MR53. Conditions: 200nm, isocratic 60:40 ACN:water at 0.5 mL/min. USP requires R not less than 2.0.



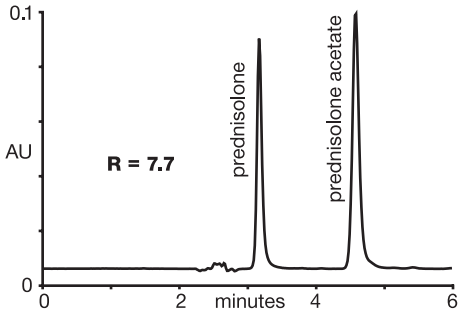
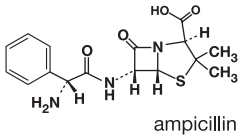


Figure 3. Prednisolone acetate on 218MR53. Conditions: 254nm, isocratic, 3:2 water:ACN at 1 mL/min. USP requires R not less than 2.0.



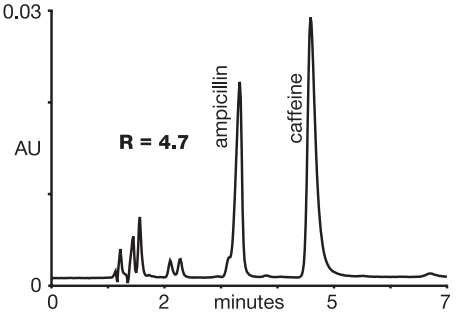
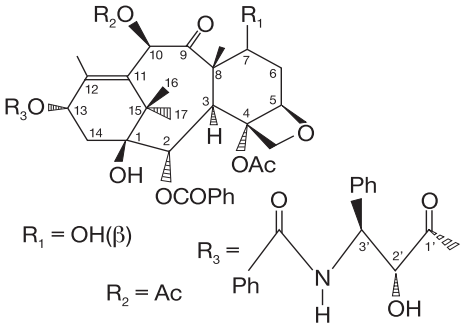


Figure 5. Ampicillin on 218MR53. Conditions: 254nm, isocratic 909:80:10:1 water:ACN:1M KH_2PO_4:1N HOAc at 1.0 mL/min. USP requires R not less than 2.0.

Taxol and Related Products

Two reverse-phases are better than one.

Taxol, also called paclitaxel, and related taxanes are complex multi-ring compounds for which reverse-phase analysis on Vydac's new 218MR column is ideally suited.



Originally isolated from bark of the Pacific Yew tree, *Taxus brevifolia*, taxol has engendered intense interest as an anti-cancer agent. Anti-tumor activity results from taxol's ability to stabilize microtubules and thereby inhibit mitosis. Its scarcity due to the unfavorable economics of Pacific Yew as a source, toxicity, and poor solubility have been problems. Research has focused on synthesis, potentially improved natural or engineered biological sources, and evaluation of analogues for enhanced anti-tumor activity and reduced side effects. In these efforts, chromatographic analysis and purification have key roles.

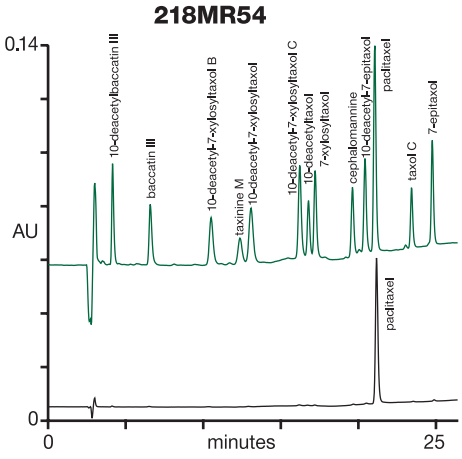


Figure 6. Separation of standard mixture and purified taxol on silica-based reverse-phase.
Column: 218MR54, 4.6mm ID x 250mm L.
Conditions: 1 mL/min, absorbance at 227 nm, (A=50mM NaOAc, pH 6.7, B=ACN) hold 34%B for 5 min, then linear gradients to 58%B in 16 min, and 70%B in 2 min. (Vertical scale on lower chromatogram is shown at 1/3 actual.)

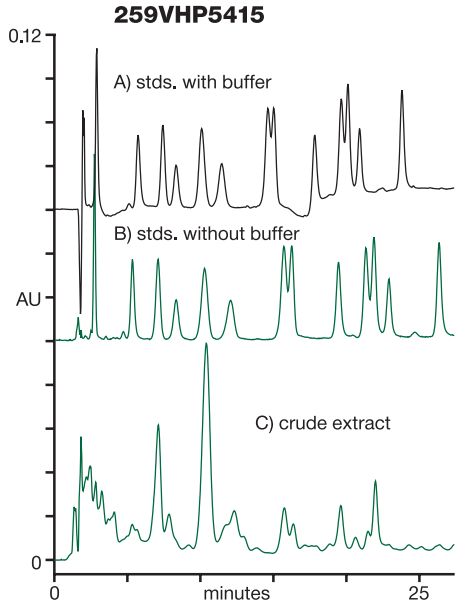


Figure 7. Standard mixture (A,B) and crude yew-bark extract (C) on polymer-based reverse-phase.
Column: 259VHP5415, 4.6mm ID x 150mm L.
Conditions: Same as in Figure 6, except mobile phase A = pure water for chromatograms B and C. (Vertical scale on chromatogram C is 1/3 actual.)

In Figure 6, the Vydac 218MR 300Å silica-based reverse-phase column provides excellent resolution of all 13 components in a standard mixture of taxol and taxol analogues (obtained from Hauser Chemical).

In Figure 7, a Vydac 259VHP polymer reverse-phase column separates the same standard mixture and a crude extract of bark from a yew tree. This column also separates the 13 components. (NOTE: A 150mm-long column was used in this work. Using a 250mm column would provide 40% more theoretical plates for these partitioning molecules.) The polymer reverse-phase provides two distinct advantages for analysis and purification of crude samples:

(1) Resolution does not depend on including buffer in the mobile phase.

(2) The acid- and base-resistant column can be easily cleaned to remove contaminants between runs.

The silica-based 218MR column is best for rapid, sensitive analyses of purified samples and synthetic mixtures, whereas the 259VHP column is for crude extracts.

Cat. No.	Description
218MR54	Column, Octadecyl (C18), Silica-Based, 5µm, 300Å, 4.6mm ID x 250mm L
259VHP5415	Column, Polymer Reverse-Phase, 5µm, 300Å, 4.6mm ID x 150mm L
259VHP54	Column, Polymer Reverse-Phase, 5µm, 300Å, 4.6mm ID x 250mm L

Larger diameters are available for preparative applications.

Effects of Column Length in Ion-Exchange of Peptides

In ion-exchange methods, salt concentration and pH are typically the most important factors affecting selectivity and resolution. Column length beyond that necessary to assure efficient sample adsorption appears to have little effect. Thus we tend to think of ion exchange as an all-or-nothing phenomenon: use a short column, load sample at low salt to assure retention of desired components, run a gradient of increasing salt, each component releases from the column and emerges at a characteristic ionic strength. End of story.

But is it really? In our last issue, we described separations of tryptic peptide digests by cation exchange on a 25mm-long 400VHP column using shallow salt gradients. The shallow gradients got us thinking. A shallow gradient actually approximates an isocratic condition at any given time during the run. Is it possible that significant dynamic exchange between the stationary and mobile phases could occur in this case? If so, a longer column might improve resolution.

That's exactly what we found. But with an added surprise! The chromatograms of Figure 8 compare separation of the same tryptic digest of lactoperoxidase under identical conditions on two 400VHP cation exchange columns, the 25mm long column used previously and a longer 100mm column of approximately the same diameter. The longer column resolves more peaks.

Attempts to compare the chromatograms directly lead to frustration! Not only is there a difference in resolution, but significantly altered selectivity as well. On reflection, it's to be expected. The shorter column provides selectivity dominated by the rate of desorption. On the longer column, adsorption rates in the dynamic exchange play a greater role. The ratio of the two rates of course will be a function of peptide structure, affecting the partition coefficient differently for each peptide in the digest.

Cat. No.	Description
400VHP5410	Column, Strong Cation Exchange, 5µm, 900Å, 4.6mm ID x 100mm L
400VHP552	Column, Strong Cation Exchange, 5µm, 900Å, 5.0mm ID x 25mm L

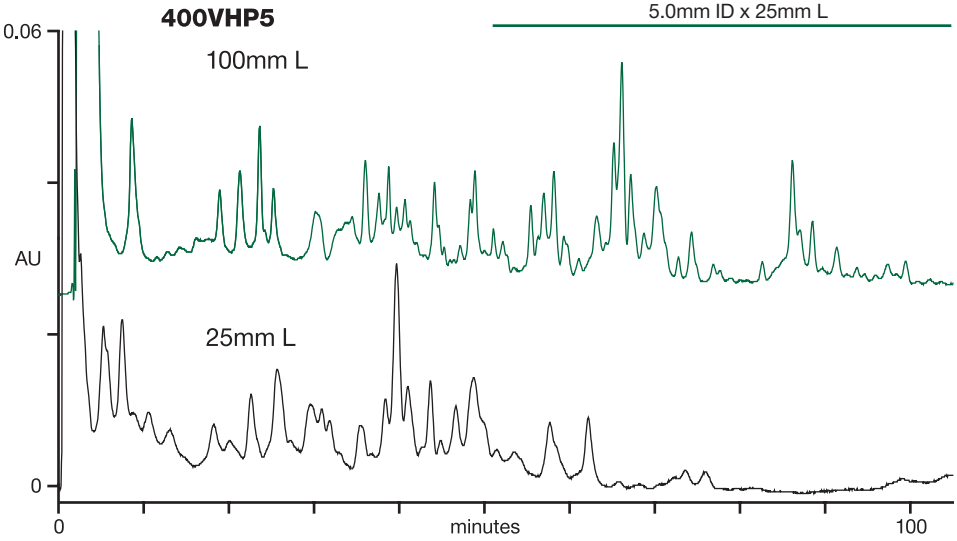


Figure 8. Comparison of cation-exchange separation of peptide digest on columns of different length. Sample: Tryptic digest of lactoperoxidase. **Conditions:** 0.5 mL/min. Buffer A = 0.1% TFA (w/v) in 50:50 water:ACN. Buffer B = 100 mM $NaClO_4$ in A. Gradient: 0% to 100% B in 100 minutes.

Vydac's Unique Silica

Reproducibility is important, and with silica-based packings control of the substrate is essential. Much attention has focused on silica surface area and pore characteristics. The importance of chemical impurities is finally being appreciated, especially for protein and peptide separations.

It was concern about chemical purity that first led Vydac to pioneer synthetic silica processes as opposed to silica sol methods used by other manufacturers. Silica sols are alkaline suspensions produced from naturally occurring silica. In addition to silicon and oxygen, they contain sodium ions and other impurities. Silica gels are produced when sols are acidified with sulfuric acid, leaving traces of sulfate and ion-exchange character.

In the late '70s, Vydac began making silica in a new way, starting with purified organic silicates. Perfection of this technology led to chromatographic adsorbents of exceptional performance. Consistent with this heritage, at Vydac chromatographic reproducibility is our credo, providing assurance of dependable chromatography, time after time.

Tech Tips

Are ghost peaks haunting your chromatograms?

The Vydac guide to chromatographic exorcism

Ghost peaks in gradient chromatogram blank runs, with no sample, are the most frequent problem described by callers to Vydac's technical staff. What causes them? There are several possible answers. Systematic, logical investigation will pinpoint the cause.

RI Effects

Rapid changes in solvent composition can create false peaks due to refractive index (RI) sensitivity with some detectors. This is similar to the baseline blip that results from sample injection. If there is a rapid composition change in the gradient, try making it more gradual. This should broaden an RI ghost into mere baseline drift. All other ghosts result from contamination.

The Column

The easiest source to rule out is the column itself. Because it is a finite source, column contamination will decrease with time if the column is washed with solvents stronger than needed to elute the ghost peak(s).

Perform several blank gradient runs in quick succession. If possible, incorporate extended washes with strong eluant. If ghost-peak sizes remain the same from run to run, the source is not the column. If their size decreases, the source may be protein aggregates or other difficult-to-dissolve contaminants in the column. Ion exchange columns with purely aqueous buffers are suspect for bacterial contamination. Larger peaks may appear again after an idle period. In this case, clean the column as recommended in the maintenance guide.

Solvent A

If ghost peaks remained of fairly constant size during successive gradients, the source is probably a mobile phase component. Contaminants in the weak solvent ("A") can accumulate on the column during equilibration and the early part of a gradient. Try varying equilibration time before successive runs. Figure 9 shows an example. If the size of the peaks changes in direct relation to equilibration time, the probable source is solvent A.

Solvent B

Contaminants from the strong solvent ("B") can also accumulate on the column during early portions of a gradient. In this case, ghost-peak size should be independent of equilibration time. Try equilibrating the column with a weak mixture of B in A for a time before pure A. This should increase the size of the peaks if B is the source, as shown in Figure 10.

The Autosampler

Are you using an autosampler? Contamination in blank or wash solution from an autosampler can also cause ghosts. If the peak-size tests have not isolated the problem, try bypassing the sampler to see if this cures it.

In Most Cases

Our experience with customers who encounter ghost peaks is that in almost every case the source is mobile phase, usually solvent A. Invariably, the solvent-pickup filter is also contaminated. A labyrinthine haven for bacterial growth, it should be replaced frequently, along with the solvent.

Reverse-phase protein and peptide separations where A is water with TFA but no acetonitrile are especially problematic. We suggest including at least a 5% ACN concentration in solvent A for several reasons:

(1) Reverse-phase protein chromatography works better with a little ACN.

(2) There's less outgassing during the run if solvent and water are premixed.

(3) Better reproducibility, especially with single-pump gradient systems.

(4) ACN inhibits microbial growth.

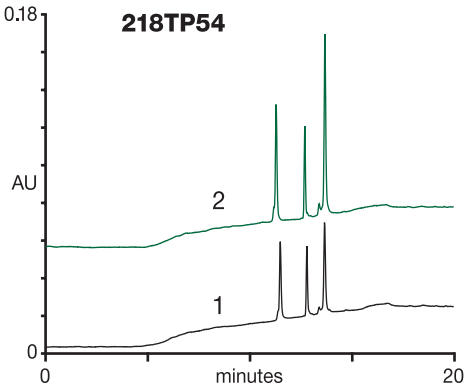


Figure 9. Effect of varying equilibration times with contaminated Solvent A. Column: 218TP54, 5µm, 300Å, C18 reverse phase, 4.6mm ID x 250mm L. **Sample:** none. **Conditions:** 214 nm. 1.5 mL/min. A=10% ACN and 0.1% TFA (v/v) in water, spiked with 25ng/mL insulin, RNase, and lysozyme. B=90% ACN and 0.1% TFA (v/v). Gradient from 100% A to 100% B in 20 min. **Equilibration:** (1) 10 min. (2) 30 min.

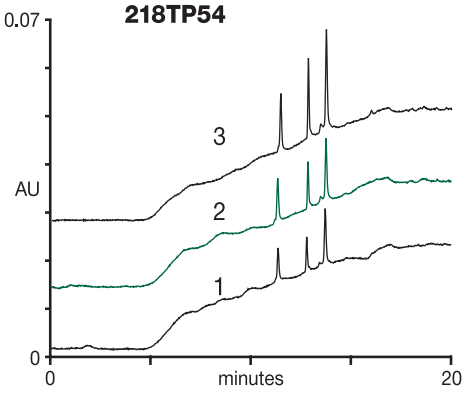


Figure 10. Effect of varying equilibration times with contaminated Solvent B. Column and Conditions: Same as Figure 9, except A was clean and B was spiked at 50ng/mL. **Equilibration:** (1) 10 min. (2) 30 min. (3) 30 min with 10%B, then 10 min with A.

Hot Refs, Cool Apps

tRNA mixtures: High-resolution separations on 214TP 300Å C4 reverse-phase using reverse gradients of sodium formate and $(NH_4)_2SO_4$ are described in

1. Zhang, S-B., P.M. Bronskil, Q-S. Wang, and J.T-F. Wong, *J. Chromatog.*, **360** (1986), 282-287.
2. Xue, H., W. Shen, R. Giege, and J.T-F. Wong, *J. Biol. Chem.*, **288** (1993), 9316-9322.
3. Auld, D.S., and P. Schimmel, *EMBO Journal*, **15** (1996), 1142-1148.

Cat.No.	Description
214TP104	Column, Protein-Peptide R-P C4, Silica-Based, 10µm, 300Å, 4.6mm ID x 250mm L

Genetic Research

Oligonucleotide Purification and Analysis with Vydac's 301VHP Column

A tool of unique value for molecular geneticists

Phosphodiesters

Syntheses of phosphodiester oligonucleotides often result in high yields of the target oligonucleotide and lesser amounts of n-1, n-2, etc. High performance anion-exchange chromatography on a Vydac 301VHP column will separate oligonucleotides differing in length by one nucleotide residue. The separation of impurities from a synthetic 10-mer is shown below.

easily resolves oligos differing by one residue

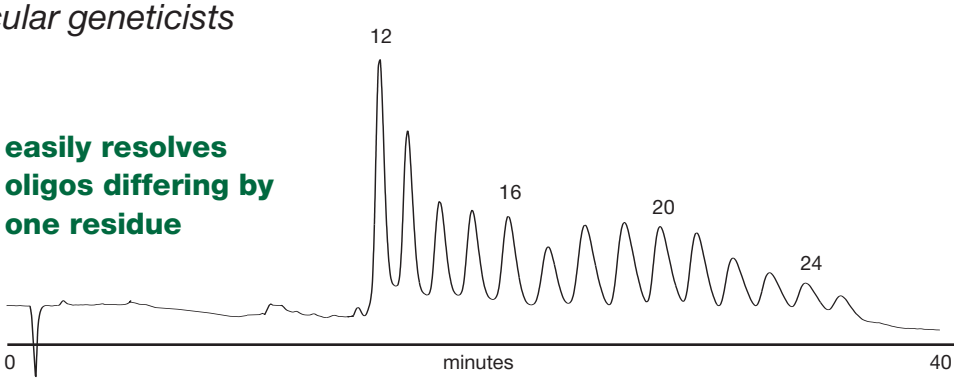


Figure 11. Oligonucleotide "Ladder". Separation of poly-dT 12-25-mer mixture. **Column:** Vydac 301VHP575, 7.5mm ID x 50mm L. **Conditions:** 1.0 mL/min. 10mM Tris-HCL, pH 8.0, gradient from 0 to 0.2M NaCl in 5 minutes, then 0.2 - 0.3M NaCl in 40 min.

■ **analyze**

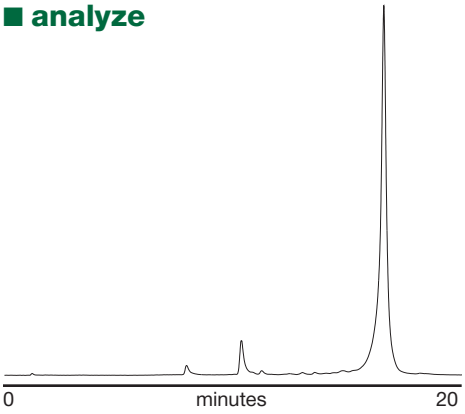


Figure 12. Analysis by anion exchange. **Sample:** 10µg of crude product from the synthetic reaction for a 10-mer oligonucleotide. **Column:** Vydac 301VHP575, 7.5mm ID x 50mm L. **Conditions:** 1.0 mL/min. 25mM TEAA, pH 8.0, gradient from 0 to 500mM NaCl over 25 minutes.

■ **purify**

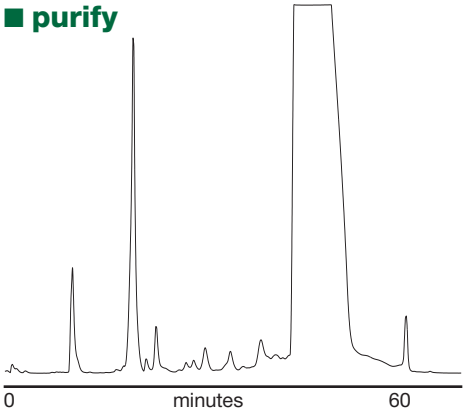


Figure 13. Scale-up for purification. **Sample:** 1mg of crude synthetic 10-mer oligo. **Column:** Vydac 301VHP575, 7.5mm ID x 50mm L. **Conditions:** 1.0 mL/min. 25mM TEAA, pH 8.0, gradient from 0 to 200mM NaCl over 50 minutes. Final wash with 500mM NaCl at 60 minutes.

■ **verify**

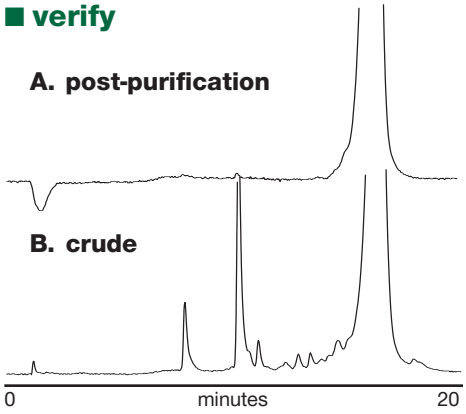


Figure 14. Verification of purity. **Sample:** (A) Purified synthetic 10-mer oligo from 1mg scale-up separation at left, after desalting on 218TP54 reverse-phase column. (B) 10µg crude synthetic 10-mer. **Column:** Vydac 301VHP575, 7.5mm ID x 50mm L. **Conditions:** As in Figure 12.

Typical of scale-up separations, NaCl gradient slope was reduced by a factor of five to improve resolution with the larger sample. Further reduction in gradient slope permits even larger sample loads, up to 5 mg or more with the 7.5 x 50 mm column.

Phosphorothioates

Phosphorothioate oligonucleotides have previously been separated using high-pH mobile phases to avoid aggregation. We found that incorporating 50% isopropanol (IPA) in the eluting buffer allows **both trityl-on and trityl-off** phosphorothioates to be separated by anion exchange at near neutral pH on a Vydac 301VHP column. The trityl-off phosphorothioate elutes slightly earlier than the trityl-on S-oligonucleotide. The trityl group elutes near the void volume.

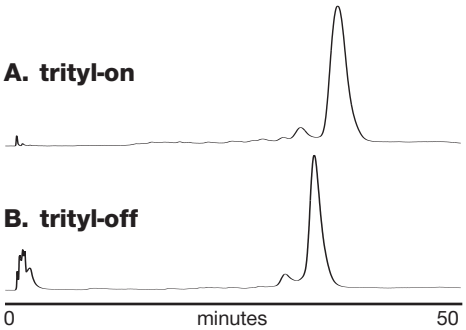


Figure 15. Phosphorothioate oligonucleotides. **Column:** 301VHP575, 7.5 x 50 mm. **Eluent:** 0.7 mL/min. Gradient, 20 to 1000mM NH_4OAc, pH 8, with 50% IPA, in 50 min. **Samples:** ~23µg, 10-mer.

Products

Cat. No.	Description
For high-resolution oligonucleotide separations:	
301VHP575	Column, Anion-Exchange, Tertiary Amine, 'DEAE' type, 5µm, 900Å, 7.5mm ID x 50mm L
For desalting by reverse-phase chromatography:	
218TP54	Column, Octadecyl (C18), Polymeric, 5µm, 300Å, 4.6mm ID x 250mm L

Larger columns are also available.