Vydac Advances

Quarterly Technical Newsletter on the Characteristics and Use of Vydac HPLC Columns *Winter, 1998*

New!

Vydac 3µm 300Å Reversed-Phase Columns Speed Protein and Peptide Analyses

Fast, High-Resolution Separations Using Popular Vydac Chemistries

Decreasing the particle size of an HPLC packing facilitates rapid chromatography by speeding diffusion between the mobile phase and adsorptive surfaces of the stationary phase. For small molecule separations this leads to

- **sharper peaks**
- **resolution on shorter columns**
- **reduced analysis time**
- **reduced solvent consumption.**

In fact, much of the performance gain of modern HPLC over earlier chromatographic techniques is the result of improvements in the manufacture and use of small-particle adsorbents.

Unlike small molecules, peptides and proteins typically do not partition by repeatedly adsorbing and desorbing from the stationary-phase as they move through a chromatographic column. Instead, separation is based on mobile-phase gradients. Strong adsorption is followed by selective release when the concentration of organic component reaches a specific value for each analyte. Retention of proteins and peptides is more an all-or-nothing phenomenon.

The main effect of reducing adsorbent particle size for this type of separation is to allow mobile phase molecules to move rapidly in and out of the packing. This permits a quick and uniform release of each adsorbed analyte as the critical mobile-phase composition is attained. Performance advantages are somewhat different from those for small molecules. Much protein and peptide chromatography is already run on short columns with 5µm to 10µm packings. The 3µm particle size speeds exchange between the mobile phase and packing, and allows you to

- **increase the mobile phase flow rate**
- **run a shallower gradient on a per-volume basis during the same run time.**

This results in the fast, high resolution separations shown here with Vydac's new 3µm 300Å reversed phases.

Figure 1 shows a comparison of separations of five standard proteins run on a 100 mm long 3µm reversed-phase column (Vydac 238TP3410) with a flow rate of 2.5 mL/minute, and using the same gradient timing on a 50 mm long column with the same packing (Vydac 238TP3405) at a higher flow rate of 4.0 mL/minute. The higher flow rate is made possible by the lower back pressure of the shorter column. Note the faster analysis time together with improved resolution on the shorter column due to the shallower elution gradient.

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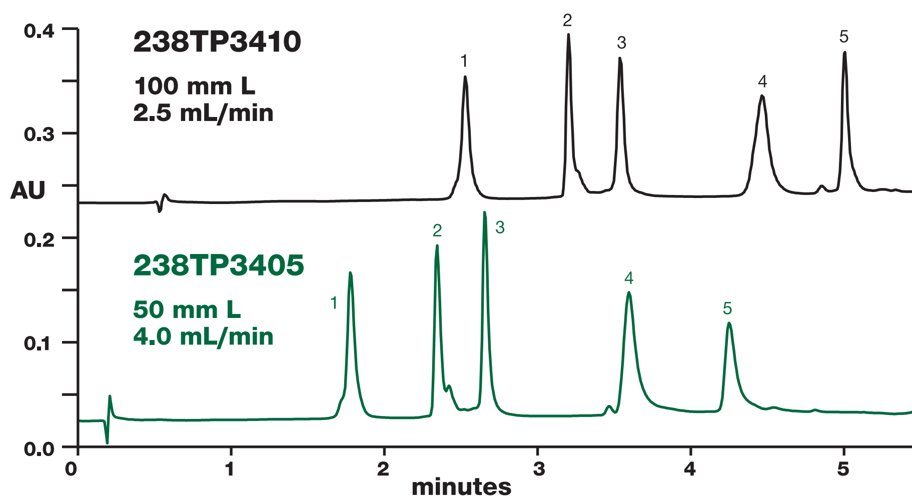

Figure 1. Comparison of protein separations on 3µm 300Å reversed-phase columns of 100 mm and 50 mm lengths. Note the higher flow rate on the 50 mm column which produces a shallower mobile-phase composition gradient on a per-volume basis. **Columns:** Vydac 238TP3410 "monomeric" C18 reversed-phase, 3µm particle size, 300Å pore size, 4.6mmID x 100mmL, and Vydac 238TP3405, identical packing in 4.6mmID x 50mmL column. **Flow rates:** 2.5 mL/min and 4.0 mL/min, respectively. **Mobile phase:** A = 20% acetonitrile (v/v) in water with 0.1% TFA (w/v). B = 45% acetonitrile (v/v) in water with 0.1% TFA (w/v). **Gradient:** 0% to 100% B in 4 minutes for both columns. **Detection:** 215nm. **Sample:** Standard protein mixture. **Peaks:** (1) ribonuclease, (2) insulin, (3) cytochrome C, (4) BSA, and (5) myoglobin.

ISO 9001 Certified VYDAC® *serving chromatographers with quality packings and bonding chemistry for over 26 years*

3µm 300Å Reversed-Phase Columns for Proteins and Peptides

(continued from Page 1)

The same five proteins (Figure 2) and also a mixture of six standard peptides (Figure 3) were run under the fast separation conditions on 3µm 50 mm long columns with three different reversed-phase chemistries for a comparison of selectivities. The 218TP and 238TP packings are both octadecyl (C18) phases on the same 300Å silica base, but with polymeric and monomeric bonding chemistries, respectively. (See *Vydac Advances*, Spring, 1997, for details.) The 214TP packing is a polymerically bonded butyl phase.

Good separations of the five proteins were obtained on all three columns, with the butyl (C4) column providing the best peak shape and resolution.

In the case of the peptide mixture, the C4 column (separation not shown) failed to resolve peaks 2 and 3. Both C18 columns were effective in resolving all components, with the monomeric bonding chemistry (238TP) providing the best resolution and peak shapes.

These results suggest that Vydac's 238TP monomeric C18 may be the best all-around choice for both peptide and protein analyses. Still, for mixtures of uncertain composition the ability to screen on a variety of reversed-phase chemistries with varying selectivity provides added assurance that all components will be seen.

Vydac's new 3µm columns make rapid analysis possible and are ideal for:

- **in-process testing**
- **combinatorial library screening**
- **screening of recombinant clones**
- **studies of reaction kinetics**

Ordering Information

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

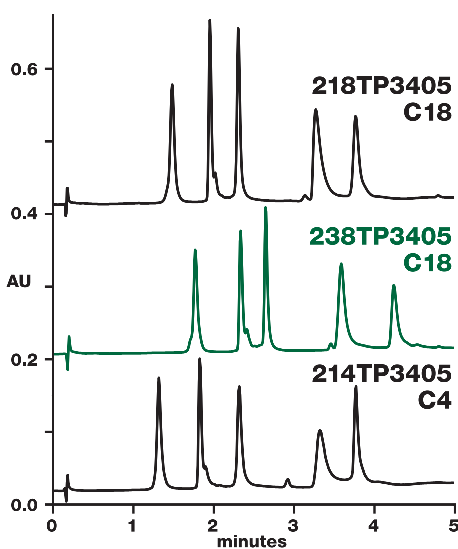

Figure 2. Comparison of protein separation on three 3µm 300Å reversed-phase columns. Flow rate: 4.0 mL/min. **Columns:** Vydac 218TP3405 "polymeric" C18, 238TP3405 "monomeric" C18, and 214TP3405 C4, all 4.6mmID x 50mmL. **Conditions:** As described for Figure 1. **Sample:** Standard protein mixture. **Peak order:** Same as Figure 1.

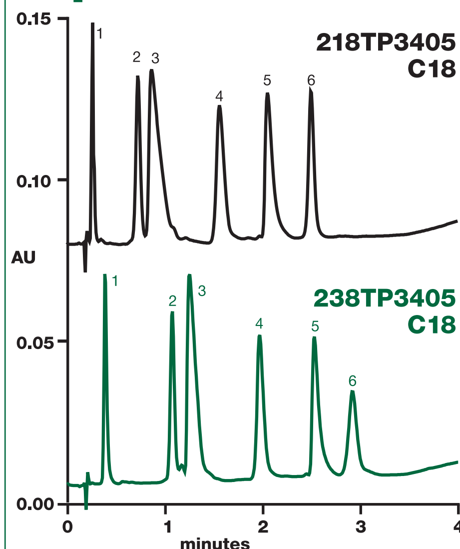

Figure 3. Comparison of peptide separation on two 3µm 300Å reversed-phase columns. Flow rate: 4.0 mL/min. **Columns:** Same as Figure 2. **Mobile phase:** A = 15% acetonitrile (v/v) in water with 0.1% TFA (w/v). B = 25% acetonitrile (v/v) in water with 0.1% TFA (w/v). **Gradient:** 0% to 100% B in 3 minutes. **Detection:** 215nm. **Sample:** Peptide mixture. **Peaks:** (1) neurotensin fragment 1-8, (2) oxytocin, (3) neurotensin fragment 8-13, (4) angiotensin II, (5) neurotensin, and (6) angiotensin I.

TECH TIP

Reversed-Phase Column Cleaning: SDS is OK!

Vydac's *Handbook of Analysis and Purification of Peptides and Proteins by Reversed-Phase HPLC* cautions (page 40) about the adverse effects of residual surfactant in samples. Even trace amounts of SDS can noticeably degrade a reversed-phase polypeptide chromatogram.

Also noted, however, is that surfactants appear to be removed by the gradient and do not permanently harm a column. This leads to a question: "Can SDS be used for cleaning contaminated reversed-phase columns?" The answer, as shown by the chromatograms of Figure 4, is "Yes!" Separation of six polypeptides on a C18 column (A) became degraded (B) after contamination of the column by protecting group and scavenger products from a peptide synthesis. We cleaned the column by injecting 500 µL of 1% SDS

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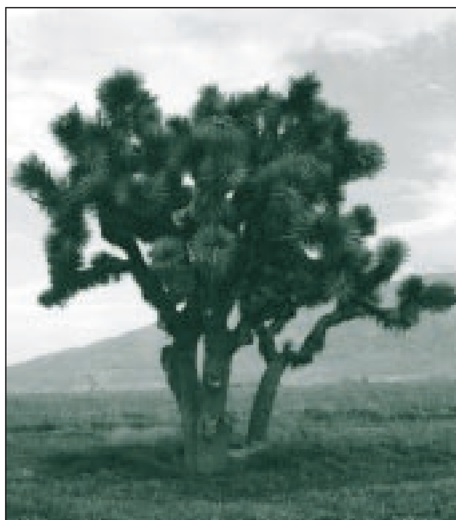
What Is That Thing?

Vydac's Neighbor

The Joshua Tree (*Yucca brevifolia*) is a unique plant found only in the Mojave Desert of Southern California, Arizona, Nevada, and Utah. It is the largest yucca and grows to heights of 15-40 feet. It shares a unique synergy with a particular species of moth (*Tegeticula)*, the females of which have evolved special organs that collect pollen and deposit it on yucca flowers. The moth lays her eggs in the flower's ovary where the larvae hatch and feed on yucca seeds. The yucca moth is the only species that can transfer pollen from one yucca flower to another. Without the moth, the Joshua Tree could not produce seeds. Without the seeds, the larvae would not survive.

Like the Joshua Tree, Vydac resides in the Mojave and enjoys a unique synergy – with its customers. By providing high performance separation tools, Vydac empowers chromatographers and biochemists to better analyze and purify molecules important to their research, quality, and manufacturing activities. Customers provide our livelihood and give us information that helps us better understand their needs, improve products, and develop useful new columns.



Over the years, we've made the Joshua Tree a symbol. We've used it in our catalogs and other publications, in photographs and graphic representation, to represent Vydac, our location in the high desert of California, and the relationship we perceive with our customers.

For more about Joshua Trees and the Mojave Desert, visit URL **http://www.desertusa.com/jtree/josh_month.html** with your web browser.

Get Vydac Technical Publications Fast on the Web!

Vydac's World Wide Web site is up and running at **http://www.vydac.com**.

There you'll find information about our company, how to contact us, and how to purchase Vydac products around the world. We've also provided a variety of technical information including tips for better chromatography and the answers to frequently asked questions about Vydac products and applications.

The full text of all recent Vydac technical publications, including application notes, column care and use guides, and previous issues of *Vydac Advances* are available. Publications can be downloaded as HTML files for viewing with popular web browsers including Netscape, Internet Explorer, and AOL. Or you can download PDF files containing the original printed format for viewing with Adobe's Acrobat Reader, Version 3.0 or higher.

If you are internet connected, keep an eye on Vydac's web site. New information and publications will frequently appear there before you receive them in the mail.

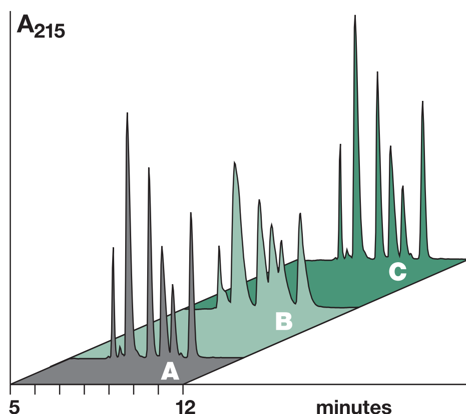


Figure 4. Polypeptide chromatograms. A. Original. B. Contaminated column. C. After SDS cleaning. **Column:** Vydac 218TP54, C18, 4.6 x 250 mm. **Mobile phase:** A = 15% ACN in water. B = 27% ACN in water. Both with 0.1% (v/v) TFA. **Gradient:** 0 to 100% B in 15 minutes.

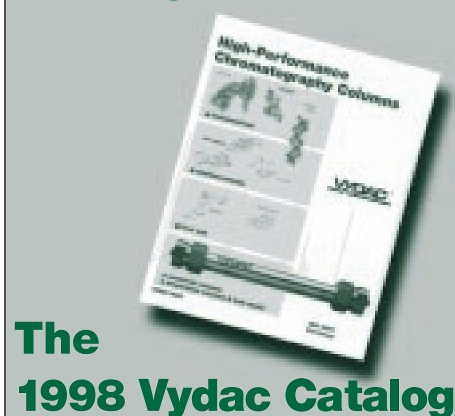
Column Cleaning with SDS

(continued from Page 2)

solution at 1 mL/min, followed by a 10-minute gradient from 5% to 95% acetonitrile with 0.1% (v/v) TFA. After equilibrating to starting conditions, the peptide separation was restored (C).

This demonstrates that even large amounts of SDS are effectively removed by a solvent gradient. While there's no guarantee it will restore all columns, SDS is a reasonable agent to try for cleaning. As with any cleaning procedure, periodic routine application is preferable to catastrophic intervention after heavy contamination has accumulated.

Coming Soon!



The 1998 Vydac Catalog

New! 64 pages of HPLC columns, bulk media, applications and technical tips. Coming in February!

Designing Separations for Difficult Peptides

Solubility Trials Can Be the Key.

Hydrophobic peptides pose a challenge in developing purifications. They are often difficult to dissolve, with low solubility in pure aqueous or organic solvents, but soluble at intermediate compositions. Dissolution studies can provide valuable information for chromatography. Find a solvent mixture that dissolves the peptide. Dilute with the aqueous component, but not so much as to cause the peptide to fall out of solution. Then use that as a starting point for the chromatographic mobile phase.

Direct dissolution of hydrophobic peptides in aqueous solvent mixtures can be slow. Frequently a peptide will appear to be insoluble when it is actually the kinetics of dissolution that are causing difficulty. If you encounter apparent insolubility problems, try the following procedure with a selection of likely solvents and trial samples of the peptide.

(1) Always add the pure solvent, i.e., the organic component, first. This overcomes wetting problems and brings hydrophobic parts of the peptide into the liquid phase first.

(2) Next add concentrated buffer components. This sets the pH, which adjusts the charges on ionic amino acids.

(3) Finally, add the aqueous portion of the mixture. In many cases the peptide will not dissolve until this final step, but a peptide that appeared insoluble will dissolve rapidly when these steps are followed. Here is an example:

A difficult synthetic peptide provided by Dr. Don Diamond (City of Hope National Medical Center, Duarte, CA) was known to contain 25 amino acid residues and two attached fatty acids. Approximately one milligram of peptide was added to each of six 6 x 50 mm test tubes, and the dissolution sequences in Table I were attempted.

Based on the results of Table I, 10 mg of the peptide was solubilized by adding 3.6 mL of *n*-propanol, then 200 µL of glacial acetic acid, and finally 0.6 mL of 5% acetic acid in water. The peptide does not dissolve until the 5% acetic acid is added. The resulting solution contains 2.27 mg/mL peptide in 86% *n*-propanol/5% acetic acid. When an aliquot was diluted 8/1 with 0.5% acetic acid, the peptide remained in solution.

Table I

Step 1. Strong (Organic) Component	Step 2. Buffer	Step 3. Weak (Aqueous) Component	Success?
CHCl3	TEAA pH6	acetone	NO
CHCl3 + *n*-propanol	TEAA	water	NO
THF + *n*-propanol	TEAA	water	NO
100µL *n*-propanol + 100µL THF	100µL HOAc	100µL water	YES
200µL *n*-propanol	100µL HOAc	100µL water	YES
200µL *n*-propanol	10µL HOAC	100µL 5%HOAc in water	YES

Initial attempts at chromatography using a gradient from 5% to 85% *n*-propanol in 0.5% HOAc on a C4 column (Vydac 214TP54) resulted in no elution of peptide (measured as A_{280}). The same procedure on a diphenyl column (Vydac 219TP54) produced the chromatogram of Figure 5.

Based on these results, a much larger sample load of peptide was applied to the diphenyl column equilibrated at 29% *n*-propanol in 0.5% HOAc. This produced the chromatogram of Figure 6, the starting point for a preparative method.

Further development will include adding ACN to the mobile phase, trying Vydac's 259VHP polymer reversed-phase column, and attempting ion-exchange in 50% *n*-propanol/acetic acid as an orthogonal separation step.

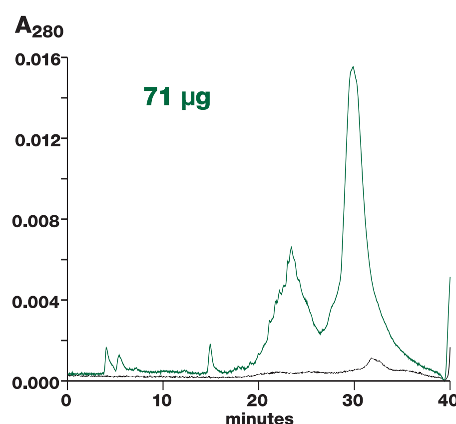


Figure 5. Initial trial chromatogram of lipid peptide. Column: Vydac 219TP54 phenyl reversed-phase, 4.6mmID x 250mmL. **Sample:** 250 µL of 8:1 diluted peptide solution = 71 µg. **Flow rate:** 0.75 mL/min. **Mobile phase:** A = 5% *n*-propanol/0.5% HOAc. B = 85% *n*-propanol/0.5% HOAc. **Gradient:** 0% to 100% B in 30 minutes. The black trace is a post-run blank.

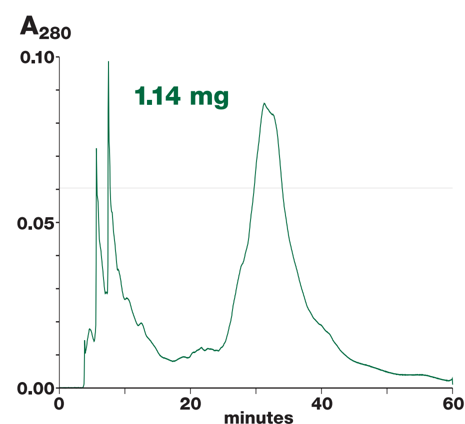


Figure 6. Initial prep chromatogram of lipid peptide. Column: Same as Figure 5. **Sample:** 1.5 mL of 3:1 diluted peptide solution = 1.14 mg. Three 500 µL injections at 1.5 minute intervals. **Flow rate:** 0.75 mL/min. **Mobile phase:** A = 29% *n*-propanol/0.5% HOAc. B = 61% *n*-propanol/0.5% HOAc. **Gradient:** Hold 100% A for 6 minutes. Then, 0% to 100% B in 30 minutes. Black trace is post-run blank.

Vydac Ion-Chromatography Column Proves Ideal Tool for Nucleotide Analysis

Ion and Reversed-Phase Effects Couple with Environmentally Friendly Mobile Phase

Here's a unique bioresearch application for Vydac's 302IC4.6 column, originally designed for separating inorganic ions in environmental analysis. The packing, a low-capacity quaternary amine anion exchanger based on high-purity 10µm large-pore silica, separates organic ions by a mixture of ionic and reversed-phase (hydrophobic) interactions.

Easy Mobile-Phase Disposal

The nucleotide separation shown in Figure 7 was performed with a mobile phase that is atypical by reversed-phase standards because it contains no organic solvent, thus simplifying disposal. Instead, the acetic acid used for pH adjustment takes the place of the organic solvent in facilitating the reversed-phase portion of the mixed-mode separation.

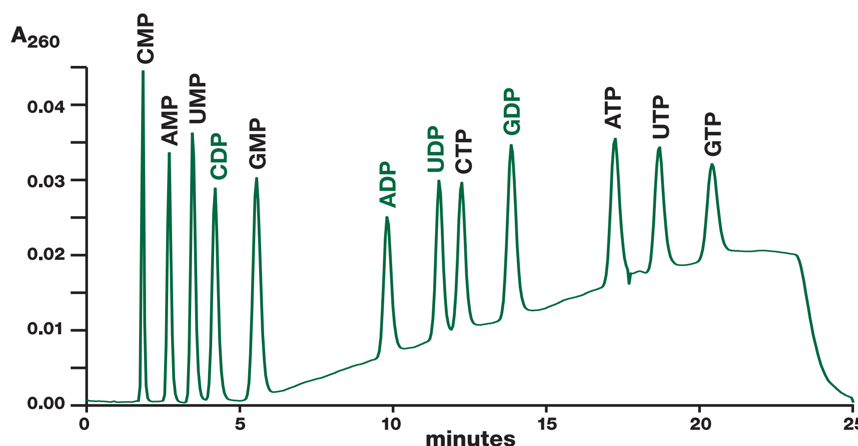
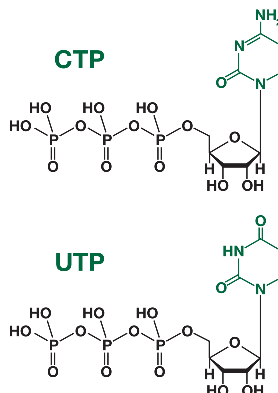
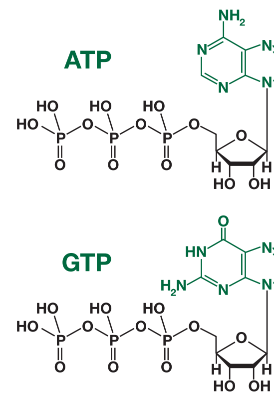


Figure 7. Separation of twelve common nucleotides on Vydac ion chromatography column. **Column:** Vydac 302IC4.6, 4.6mmID x 250mmL. **Flow rate:** 2.0 mL/min. **Detection:** 260nm. **Mobile phase:** A = NaH_2PO_4/Na_2HPO_4 (1:1 molar ratio) at 25mM total concentration in water, adjusted to pH 2.8 with acetic acid. B = NaH_2PO_4/Na_2HPO_4 (1:1 molar ratio) at 125mM total concentration in water, adjusted to pH 2.9 with acetic acid. **Gradient:** 0% B for 2 minutes, then linear from 0% to 100% B in 17 minutes, hold 100% for 2 minutes, return to 0% B in 0.1 minute. **Sample:** 3µL containing 0.25µg of each nucleotide.

pyrimidine nucleotides



purine nucleotides



Retention and Mobile-Phase Effects

As indicated by the elution order, ionic interactions due to the negative charges of the phosphates appear to play a major role, separating mono-, di-, and tri-phosphate nucleotides as groups. Within each group, differing properties of the bases result in separation of individual nucleotides. Hydrophobic interactions vary with size of the heterocyclic ring structure and tend to cause purine nucleotides to be retained longer than pyrimidine nucleotides.

However, the bases also carry positive charges at the mobile-phase pH, thereby moderating ionic retention. Uracil is the least basic, reflecting its lack of an amino substituent on the heterocyclic ring. This accounts for longer retention of uridine nucleotides and their apparent anomalous elution between the corresponding purine nucleotides.

The 1:1 proportion of dibasic and monobasic phosphate salts in the mobile phase was important in achieving conditions for this separation. Using only dibasic phosphate eluted all peaks too early, probably because more acetic acid was needed to adjust the pH. Using exclusively monobasic phosphate eluted all peaks much later.

Ordering Information

Cat. No.	Description
302IC4.6	Column, Ion Chromatography, Quarternary Amine, 10µm, 4.6mm ID x 250mm L