# Cleaning of Reversed-Phase Columns Used for Synthetic Peptides

## Introduction

Cleavage of synthetic peptides from solid-phase resins generates reactive carbonium ions which are "scavenged" by anisole and thioanisole. The scavenger-carbonium-ion reactions yield large, organic-soluble, aromatic molecules that can foul reversed-phase columns during peptide purification. These contaminants are highly retained. Washing the column with 100% acetonitrile (ACN) or 100% methanol may not elute them. Occasionally washing with solvents will remove some contaminants which, if concentrated, produce a waxy precipitate that some have mistakenly assumed to be the C18 phase coming off the column.

## Procedure

A reversed-phase column needs to be cleaned when the peptide peak becomes broader than previous runs. To clean the column:

1) Reverse the column. Strongly retained molecules remain on top of the column bed and are most readily eluted by reverse flow.
2) Use half the normal flow rate, and set the detector to 260 nm.
3) Wash with 100% isopropanol (IPA) for at least three column volumes or until any eluted peak approaches baseline.
4) Wash with 100% methylene chloride ($CH_2Cl_2$) for at least three column volumes or until the eluant reaches baseline.
5) After the absorbance returns to baseline with methylene chloride, wash the $CH_2Cl_2$ out of the column with 100% IPA before returning to the usual solvent system.

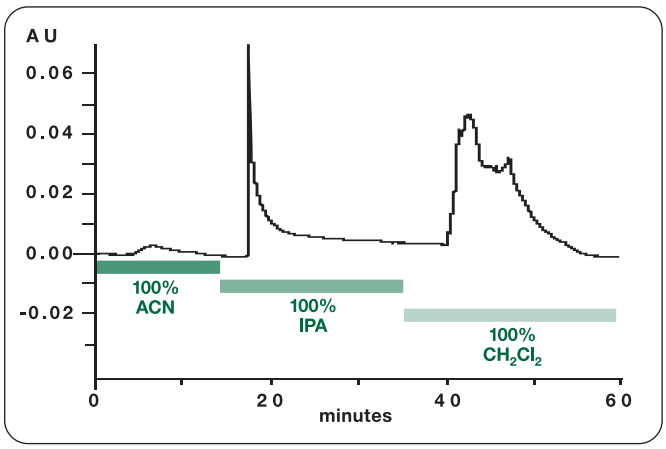


*Figure 1. Cleaning procedure performed on a Vydac 218TP1022 (C18, 10µm, 2.2 cm ID x 25 cm L) preparative column returned because peaks had become broader than initial runs on the new column. Absorbance peaks are due to aromatic molecules eluting.*

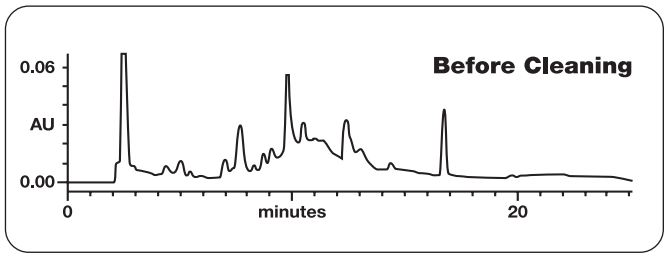


*Figure 2. Peptide chromatogram supplied by another customer returning a Vydac 218TP54 (C18, 5µm, 0.46 mm ID x 25 cm L) analytical column because peptide peaks had become broader than initial runs on the new column. Conditions: 1.5 ml/min. 220nm. A = 20% ACN/0.1% TFA. B = 60% ACN/0.1% TFA. Gradient: 0-100% B over 30 min. Inj: 20 µL of 1 mg/mL synthetic peptide.*

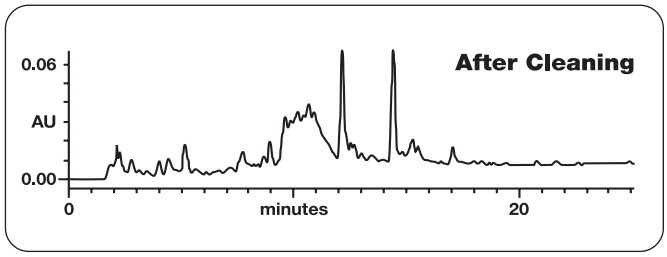


*Figure 3. Peptide chromatogram on same Vydac 218TP54 column as in Figure 2 after cleaning. Conditions as in Figure 2. The chromatogram shows significantly improved column performance. The peptide sample was provided by the company returning the column.*

## Results

The chromatograms in Figure 2 and Figure 3 look very different and demonstrate the effect of proper column cleaning. Before cleaning the column, one of the larger peaks eluted with a broad irregular peak early in the gradient. After cleaning, the two largest peaks elute after the broad peak.

The broad peak on the peptide analysis is probably a mixture of smaller protecting-group/scavenger reaction products that will elute with an ACN gradient. If these are small molecules, then this broad peak should elute earlier on a C4 column than it does on a C18 column. The small molecules should also elute isocratically with the initial conditions because small molecules partition, while peptides adsorb.

Figure 4 shows the same peptide sample used for Figure 3, but separated on a Vydac C4 reversed-phase column. Delaying the onset of the gradient is seen to have the expected effect. The small molecules elute at about the same time because these molecules partition. The peptide peaks elute ten minutes later because these macromolecules adsorb rather than partition.

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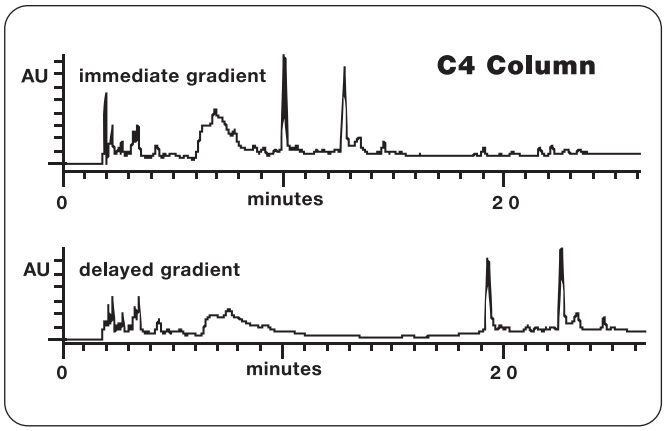


*Figure 4. Peptide chromatograms on a Vydac 214TP54 column (C4, 5µm, 0.46 mm ID x 25 cm L). Conditions: Top chromatogram same as Figures 2 and 3. For the bottom chromatogram, the onset of the gradient was delayed 10 minutes to allow an initial isocratic separation at 20% ACN. The broad peak is probably a mixture of small molecules and elutes earlier on the less hydrophobic C4 column than on the C18 column. The small molecules will also elute isocratically with the initial conditions because small molecules partition while peptides adsorb.*

## Conclusions

1) Reversed-phase columns used for crude synthetic peptides need occasional cleaning with strong solvents like IPA and $CH_2Cl_2$.
2) Peptides show similar retention on both C4 and C18 columns due to the adsorption/desorption effect.
3) Small molecules are not as strongly retained on C4 as on C18 due to partitioning.

C4 columns should be used for most synthetic peptides. Retention will be very similar to C18, and the C4 column will be easier to clean. Only if the peptide does not bind to C4 should a C18 be considered.

## Note:

For more information on a powerful separation method for synthetic peptides request Application Note #9601: "Strong Cation Exchange and Reversed Phase Purification of a Synthetic Peptide".

## ORDERING INFORMATION:

| Vydac Cat. # | Description |
|---|---|
| | **Analytical Columns:** |
| 218TP54 | C18 Reversed-Phase Column, 5µm, 4.6 mm ID x 250 mm L |
| 214TP54 | C4 Reversed-Phase Column, 5µm, 4.6 mm ID x 250 mm L |
| | **Preparative Columns:** |
| 218TP1022 | C18 Reversed-Phase Column, 10µm, 22 mm ID x 250 mm L |
| 214TP1022 | C4 Reversed-Phase Column, 10µm, 22 mm ID x 250 mm L |

Other column sizes are also available.

To place an order, contact The Nest Group toll-free at 800-347-6378.