ACE® HPLC Columns

Technical Report

# The Benefits of Ultra-Inert Stationary Phases for the Reversed Phase HPLC of Biomolecules

Chromatographers prefer inert stationary phases for reversed phase HPLC of ionic compounds because they minimize the negative affect of silanols on the separation. The result is improved peak shape and reproducibility when separating compounds that contain polar functional groups, especially amines. Now, a new generation of ultra-inert stationary phases, with extremely low silanol activity, has made it possible to achieve even better peak shape and reproducibility when separating these types of compounds. Scientists working with small molecules have been rapidly adopting this new technology and the recent introduction of wide-pore (300Å) ultra-inert phases makes the benefits of this technology available to those wanting to separate peptides and proteins by reversed phase HPLC.

## Ranking of 300Å Columns

To demonstrate the benefits of ultra-inert phases in biomolecule analysis, several commercially available 300Å pore-size reversed phase columns were tested using three different samples; neutral molecules to measure efficiency, pyridine/phenol to measure silanol activity and antidepressants to measure both silanol activity and metal content. These are the same test procedures typically used to evaluate standard pore columns (100Å) used for the analysis of small molecules in the chemical and pharmaceutical industries. Columns were ranked by efficiency, N, measured at 10% peak height. The value thus obtained not only measures overall efficiency, it also takes into consideration peak tailing usually caused by silanol interactions. Table 1 summarizes the performance of various columns as determined by each test along with a summary ranking based on all three tests.

## The Results

Overall column efficiency as measured in Test I is a reflection of how well a column is packed as well as particle size and particle size distribution. Although many columns performed similarly in this test, those with lower plate counts reflect poorer physical characteristics of the silica particle. In Test II, efficiencies for pyridine and phenol are a good measure of active silanols on the silica surface. Active silanols for the most part account for peak tailing and adsorptive losses of proteins. Since silanol activity is very hard to control in silica manufacture, columns exhibiting low silanol activity are most likely to give consistent results column-to-column and batch-to-batch. In addition, polar and basic compounds will have better peak shapes and hence greater sensitivity on columns with low silanol activity. Since most biomolecules are polar and many are basic, columns with low silanol activity are desirable. In Test III, N values for tricyclic-antidepressants, again measure active silanol activity as well as metal content. Amitriptyline, chromatographed at neutral pH is a standard test for measuring silica quality.

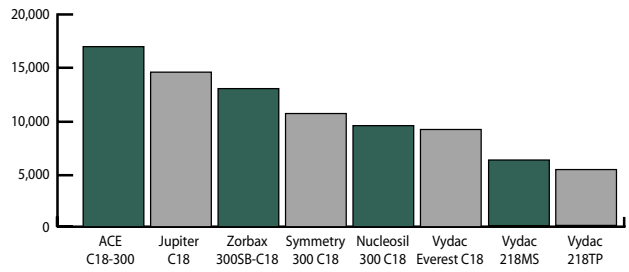
TABLE 1
**Efficiency Measurements (N) For Leading 300Å (5 µm, C18, 250 x 4.6mm) HPLC Columns**

| | TEST I | TEST II | TEST III | AVERAGE |
|---|---|---|---|---|
| ACE C18-300 | 23,400 | 14,400 | 14,000 | 17,300 |
| Jupiter C18 | 19,700 | 12,400 | 12,400 | 14,800 |
| Zorbax 300SB-C18 | 18,900 | 14,400 | 6,600 | 13,300 |
| Symmetry 300 C18 | 17,500 | 9,000 | 6,700 | 11,000 |
| Nucleosil 300 C18 | 20,300 | 6,700 | 400 | 9,100 |
| Vydac Everest C18 | 20,000 | 5,900 | 800 | 8,900 |
| Vydac 218MS | 14,600 | 1,300 | 1,400 | 5,800 |
| Vydac 218TP | 14,200 | 1,700 | 800 | 5,600 |

**Test I:** neutral molecule - toluene 80:20 MeOH/$H_2O$, 1.0ml/min
**Test II:** basic molecule 1 - pyridine 60:40 MeOH/$H_2O$, 1.0ml/min
**Test III:** basic molecule 2 - amitriptyline 80:20 MeOH/25mM $KH_2PO_4$ (pH 6.0), 1.0ml/min

The ranking of these 300Å columns shown in Figure 1 reflects their performance based on how well they are packed and also the activity (silanol and metal activity) of the stationary phase. Chromatographers with experience in HPLC of basic pharmaceuticals know that columns giving good results on these tests will perform the best on their samples. The benefits obtained from ultra-inert stationary phases are also important in wide-pore columns designed for the analysis of biomolecules.

FIGURE 1
**300Å C18 Columns Ranked by Average Efficiency**



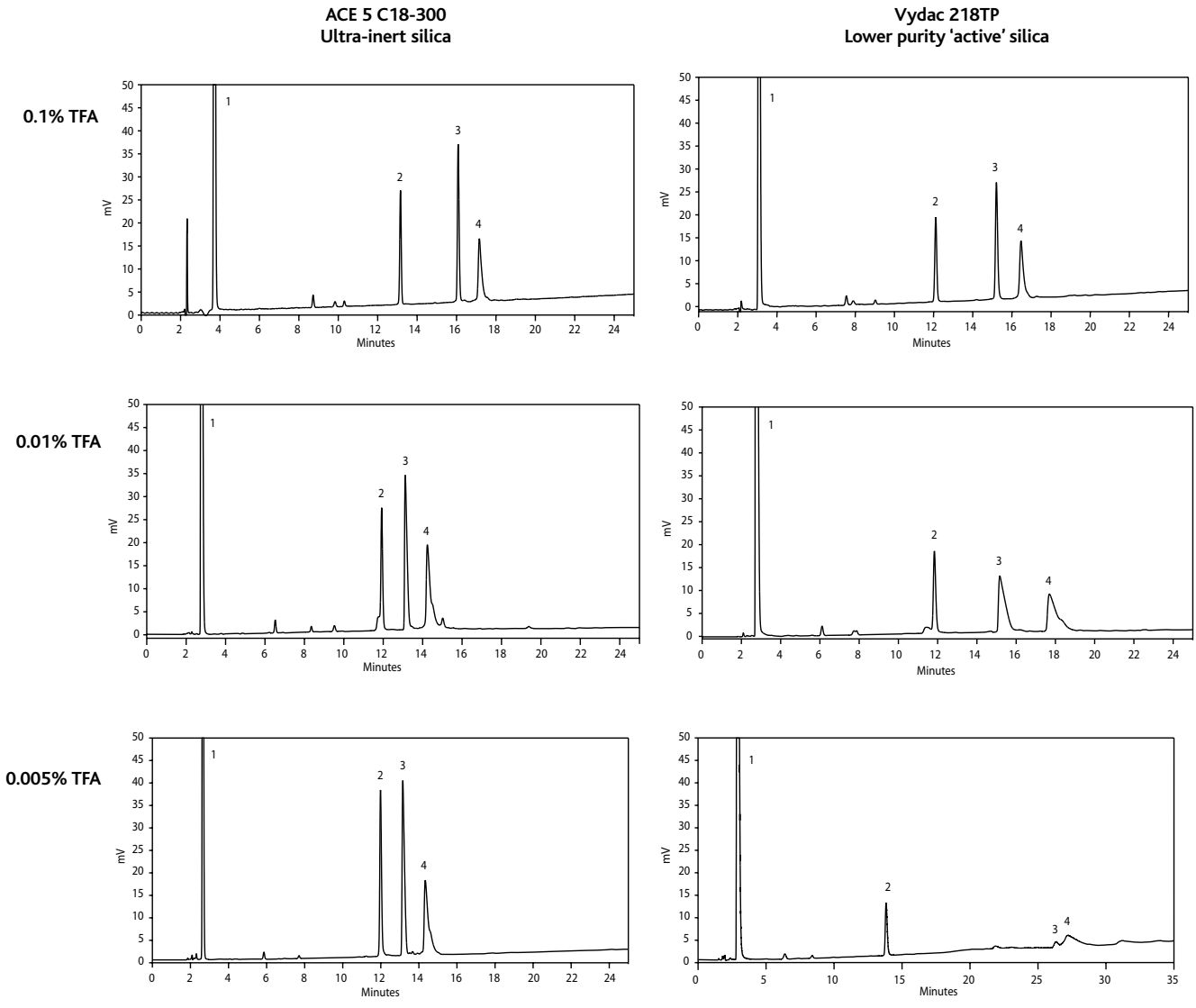

By averaging N values obtained by each column in Tests I, II and III, columns can be ranked by overall quality, reflecting packing integrity and inertness.

## Mobile Phase Additives - Effect on Sensitivity

TFA or trifluoroacetic acid has long been used as an additive to the mobile phase for reversed phase separation of peptides and proteins. This additive is typically used to improve both the peak shape and resolution of complex mixtures of peptides and proteins. As shown in Figure 2, the use of 0.1% TFA in the mobile phase enables a column packed with an active stationary phase to give peak widths comparable to those obtained from a new generation column made from ultra-inert stationary phase. As we lower TFA concentration, however, to 0.01% and finally 0.005%, peak widths on the ultra-inert phase stay the same, but degrade on the active stationary phase. But why would you want to lower TFA levels if 0.1% gives good performance? Having the ability to chromatograph peptides and proteins at very low levels of TFA is a real benefit where high sensitivity detection with a mass spectrometer is required. TFA complexes with polypeptides and can enhance selectivity. However this same complexation lowers sensitivity in the mass spectrometer.

FIGURE 2

**Sensitivity and Peak Shape as a Function of TFA Concentration**




Column: 250 x 4.6mm, 5µm C18 300Å
Mobile Phase: A: TFA in $H_2O$
B: TFA in MeCN
(% TFA as specified above)
10% to 55% B in 37.5 mins.

Flow Rate: 1.5ml/min
Detection: UV 200nm
Compounds: 1. Gly-Tyr
2. Oxytocin
3. Angiotensin II
4. Neurotensin

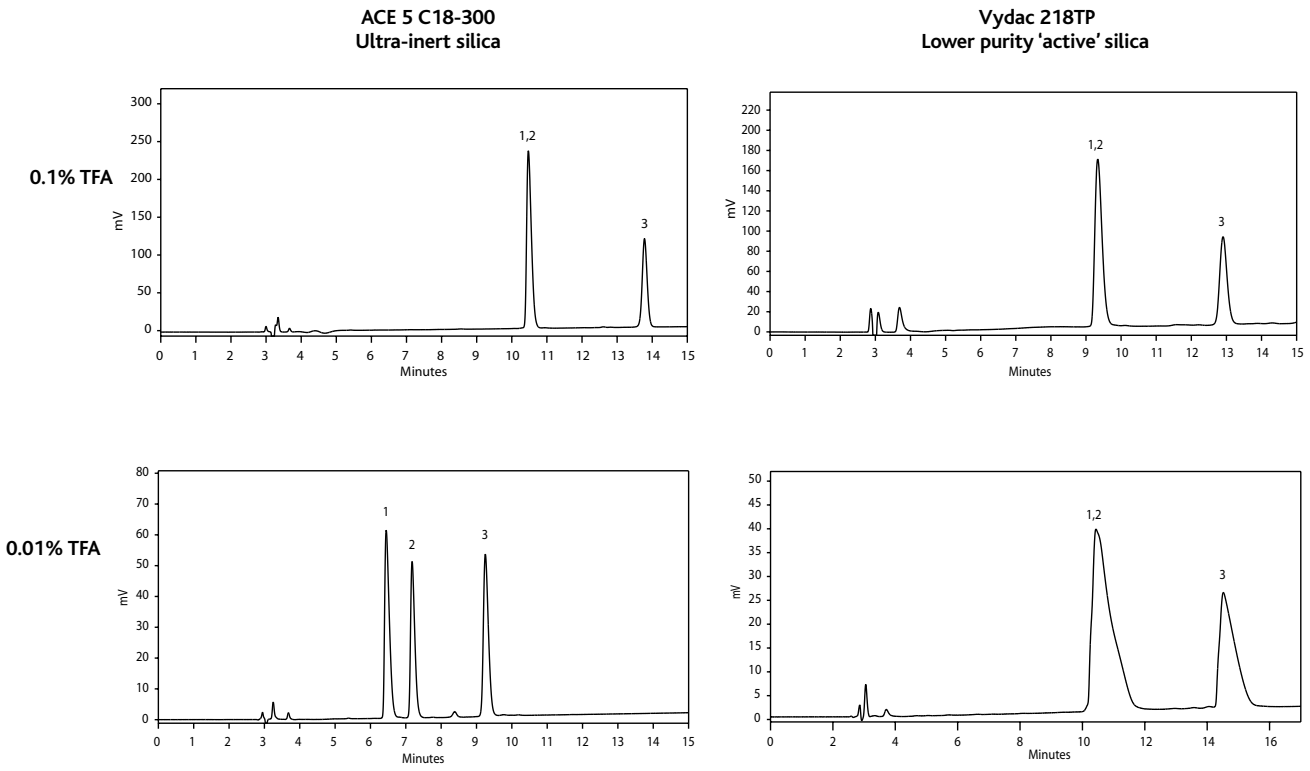
**Columns based on lower quality silica (right chromatograms) show a dramatic loss in performance as TFA concentration is lowered. Columns from ultra-inert silica such as ACE (left chromatograms) maintain performance when TFA concentration is decreased.**

## Mobile Phase Additives - Effect on Selectivity

The ability of TFA and other mobile phase additives to complex with peptides and proteins can be used to adjust selectivity and improve resolution. As shown in Figure 3, lowering TFA from 0.1% to 0.01% enabled the resolution of angiotensin II & III. In the case of the ultra-inert ACE column, peak shape and sensitivity remained constant with this change, as resolution improved dramatically. In the case of the Vydac column, packed with a more active stationary phase, resolution improved, but peak shape was severely degraded.

FIGURE 3

**Selectivity as a Function of TFA Concentration**




Column: 250 x 4.6mm, 5µm C18 300Å

Mobile Phase: A: TFA in H2O

B: 80% TFA in MeCN
20% TFA in $H_2O$
(% TFA as specified above)
25% to 40% B in 15 mins.

Flow Rate: 1.0ml/min

Detection: UV 215nm

Compounds: 1. Angiotensin II
2. Angiotensin III
3. Angiotensin I

**Resolution has increased by lowering the TFA concentration. Columns made from lower quality silica show decreased performance.**

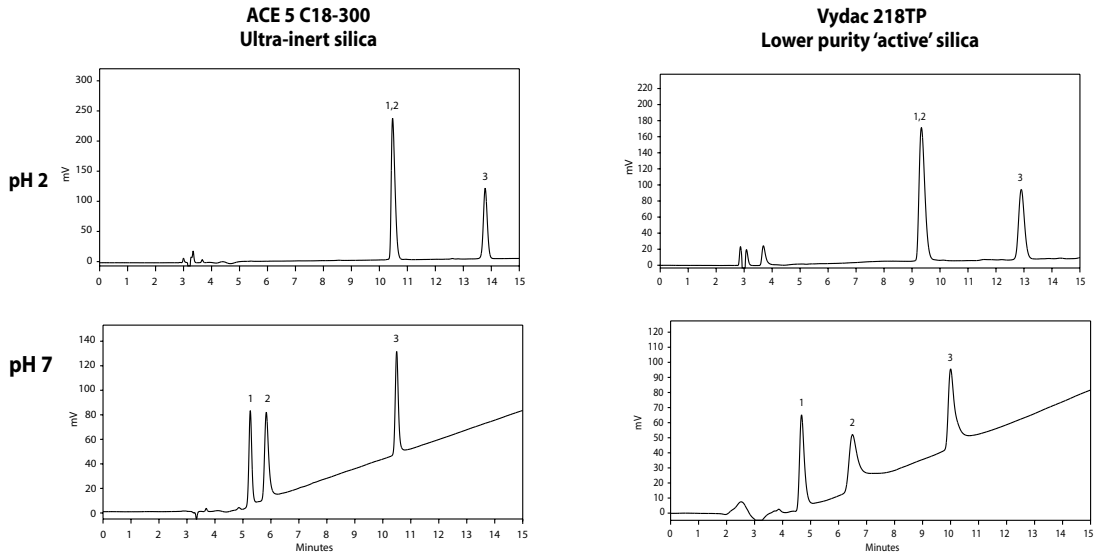
## Mobile Phase pH - Effect on Peak Shape, Sensitivity and Selectivity

Most biomolecules are charged. Peptides and proteins have numerous charges. We know from experience with small molecules, that mobile phase pH can be a powerful tool for changing retention and thus optimizing the resolution of charged compounds. The same is true for peptides. Again using angiotensin II and III as an example, Figure 4 shows no resolution of these two peptides at pH 2 on either the ACE ultra-inert column or a column packed with a more active stationary phase. By raising the pH to 7, both columns now give good resolution. The difference is, the ACE ultra-inert column maintained good peak shape whereas the more active column showed poorer peak shape and a loss in performance. This phenomenon is observed in most reversed phase applications with polar compounds. At high pH, silanol interactions are more prevalent and hence peak tailing becomes more apparent on active stationary phases.

## Summary - The Benefits of Ultra-Inert Stationary Phases for the Reversed Phase HPLC of Biomolecules

The chromatography of biomolecules, in particular peptides and proteins can be improved by using HPLC columns packed with ultra-inert stationary phases. These types of columns will have much less silanol and metal activity to interfere with the separation. In addition, ultra-inert stationary phases perform well even when using low amounts of TFA in the mobile phase. Using reduced levels of TFA in the mobile phase not only improves mass spec detection, but also provides a means of increasing selectivity and resolution. Mobile phase pH is another powerful means for improving selectivity and resolution. Ultra-inert columns, such as ACE, show no loss in performance at higher pH. Methods developed on ultra-inert columns will be more rugged over time as these columns are more reproducible column-to-column and lot-to-lot.

FIGURE 4
**Effect of Mobile Phase pH on Resolution**




Column: 250 x 4.6mm, 5µm C18 300Å
Mobile Phase: pH 2 A: 0.1% TFA in $H_2O$
B: 0.1% TFA in MeCN
pH 7 A: 10mM $NH_4OAc$ in $H_2O$
B: MeCN
25% to 40% B in 15 mins.

Flow Rate: 1.0ml/min
Detection: UV 215nm
Compounds: 1. Angiotensin II
2. Angiotensin III
3. Angiotensin I

**Adjusting the pH of the mobile phase is a powerful tool for increasing selectivity. Only columns based on ultra-pure silica will maintain performance at higher pH values.**

# For more information contact:



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