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IMPORTANCE OF CHROMATOGRAPHIC EFFICIENCY IN ANALYSIS OF COMPLEX MATRICES BY LC/MS

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OVERVIEW

We systematically evaluated parameters that contribute to chromatographic performance and ruggedness of 75 µm ID nano HPLC columns. The effect of packing pressure on column performance and lifetime was evaluated. Column permeability and chromatographic efficiency were measured on columns packed at different pressures, and results were compared to similarly packed 4.6 mm ID columns.

The effect of non-ideal connections on HPLC performance was measured by systematically varying the unswept dead volume in the connections. The effect of these extra-column contributions on peak broadening, tailing, peak capacity and sensitivity were measured in both isocratic and gradient analyses.

Robustness was evaluated by LC/MS after subjecting 75 µm columns to extended methods designed to stress the packed bed.

INTRODUCTION

Capillary/nanoscale HPLC columns are ideally suited for LC/MS applications in proteomics, where limited sample quantities are available for analysis. HPLC column miniaturization creates several practical problems that must be overcome. Reliable low volume connections between columns and electrospray emitters are particularly important in maintaining high performance separations. An efficient HPLC 75 µm ID column will generate chromatographic peaks of 50 nL, thus only a few nanoliters of extra-column volume can be tolerated. Other factors which must be considered when developing reproducible nanoscale columns are that the packing procedures must be able to generate stable, long-lived columns that can withstand the rigors of routine shipping, handling and repeated pressure surges which occur during sample injection.

EXPERIMENTAL

Isocratic

HPLC Column	Waters® Symmetry® C_{18}, 3.5 µm, 4.6 mm x 100 mm or Waters NanoEase™ C_{18}, 3.5 µm, 75 µm x 100 mm
HPLC System	Waters Alliance® 2695 Separations Module
Flow rate	1 mL/min (analytical), 10 µL/min split 40:1 to 250 nL/min* (capillary)
Detector	Waters CapLC® 2487 UV Detector @ 254 nm (10 nl cell)
Mobile Phase	60/40 Acetonitrile/Water
Software	Waters Empower™

*Flow rate was measured using a flow sensor which was connected to the exit to the UV detector. The sensor consisted of a custom-made bellows attached to a linear variable displacement transformer (LVDT) (Trans-Tek Inc., Ellington, CT) and core (Schaevitz Sensors, Fairfield, NJ). Signal conditioning was performed with a Schaevitz conditioning unit. Labview software was written to provide real-time flow rate data.

Gradient UV

HPLC Column	Waters NanoEase Atlantis™ dC_{18}, 3.5 µ, 75 µ x 150 mm
HPLC System	Waters CapLC with split flow
	2487 UV Flow (3 nl cell) modified
HPLC Conditions	Gradient: 3-65%B @ ~ 200 nl/min
Mobile Phase	Eluent A: 0.1% TFA in water
	Eluent B: 0.1% TFA in acetonitrile
Samples	BSA Digest (2 pmol/µL)
Software	MassLynx™ 3.5

Gradient Mass Spectrometry

HPLC Column	Waters NanoEase Symmetry C_{18}, 3.5 µ, 75 µ x 150 mm
HPLC System	Waters CapLC
HPLC Conditions	Gradient: 3-60%B in 60 min @ ~ 200 nl/min
MS	Waters Micromass® LCT™
MS Conditions	Cone Voltage: 35 V
	Source Temperature: 80 °C
Mobile Phase	Eluent A: 0.1% TFA in water
	Eluent B: 0.1% TFA in acetonitrile
Samples	BSA Digest (2 pmol/µL)
	Enolase Digest (2 pmol/µL) prepared inhouse
Software	MassLynx 4.0

RESULTS AND DISCUSSION

Packed Bed Density Study

A 75 µm ID x 100 mm column was slurry packed with 3.5 µm Symmetry C_{18} particles at a range of packing pressures. Column permeability was measured using Darcy's Law, and compared to 4.6 x 100 mm columns packed with similar packing material. Chromatographic efficiency was determined by isocratic HPLC.

Darcy's Law Equation

$$B^0 = \frac{4F\eta L}{\Delta P \pi d^2}$$

B^0= permeability
F= flow rate
n= viscosity
L= column length
P= backpressure
d= column I

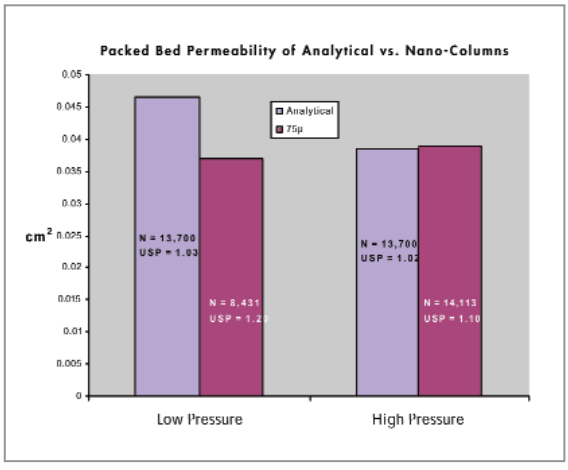

Figure 1. Nano column packed bed permeability is equivalent to analytical columns when packed at high pressure.

EXTRA COLUMN EFFECT STUDY

Observed bandspreading of peaks in chromatography is due to contributions from all fluidic components. Peak variance is a sum of each of the contributions:

$$\sigma^2_{obs} = \sigma^2_{injector} + \sigma^2_{column} + \sigma^2_{tubing} + \sigma^2_{connectors} + \sigma^2_{detector}$$

Most of the fluidic components introduce symmetric bandspreading. However, if poor connections are made that have unswept dead volume, peak tailing typically results. Such bandspreading can be fit to an exponential decay curve. In order to measure the contributions due to poor connections, a systematic study was conducted to determine the effect of tubing connections on bandspreading. One end of the capillary transfer tubing was cut at an angle of 0°, 15°, 30°, and 45°, then butted against the HPLC column which had been diamond polished (see Figures 5&6). In this way, a fixed, known dead volume could be introduced into the connection. A sample of acenaphthene was injected on the column, and the resulting chromatograms were fit to an exponentially modified Gaussian curve:

$$f(t) = A + \frac{B}{2\tau}\exp\left[\frac{\sigma^2}{2\tau^2} - \frac{t - t_r}{\tau}\right] \times erfc\left[\frac{\sigma}{\sqrt{2}\tau} - \frac{t - t_r}{\sqrt{2}\sigma}\right]$$

Where:

A= Offset

B= Amplitude

τ= Exponential decay time constant

σ= Gaussian time constant

t= Time

t_R= Retention time

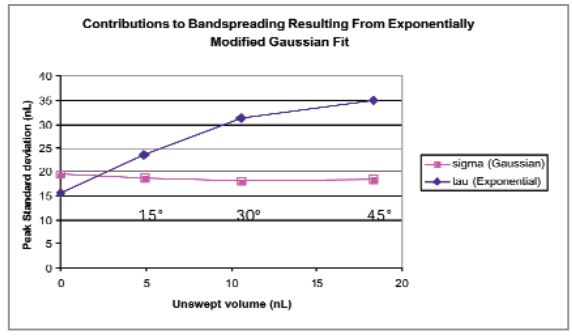

Figure 2. Results from fitting chromatographic data to exponentially modified Gaussian curve. Data shows that, as expected, adding extra-column bandspreading effects only the exponential component of the curve.

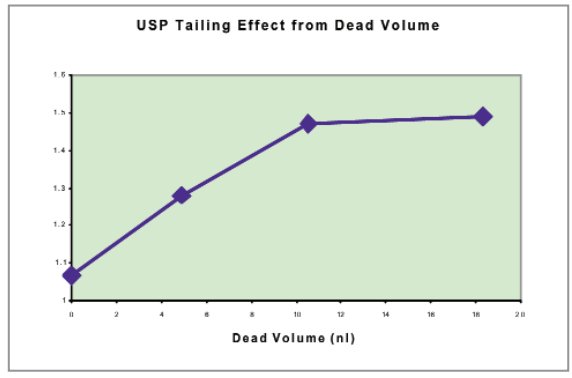

Figure 3. The graph shows the negative effect of dead volume on USP tailing factors.

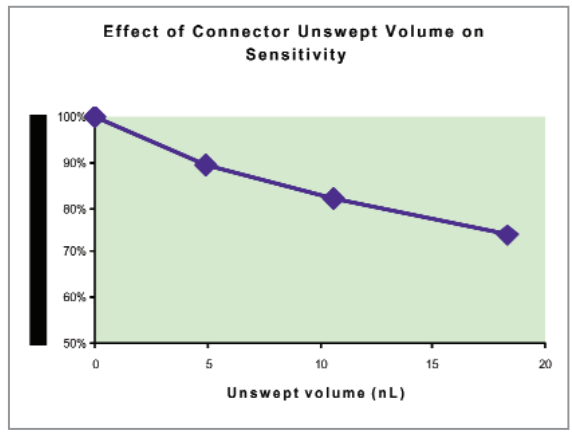

Figure 4. One significant effect of extra-column volume is the deleterious effect it has on signal intensity. In this experiment, adding an unswept volume of only a few nanoliters to one of the connections has a strong negative impact on sensitivity.

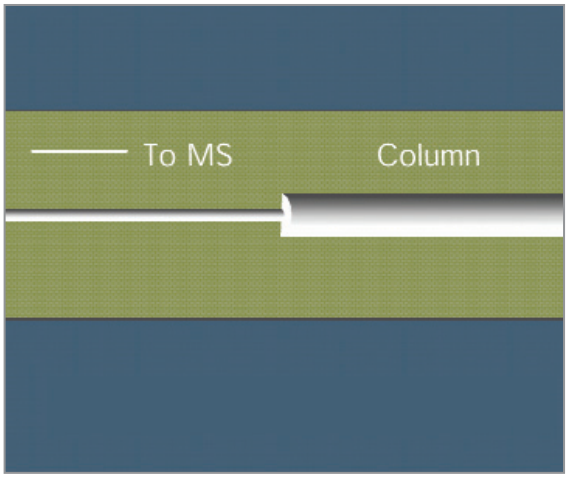

Figure 5. No dead volume at connection.

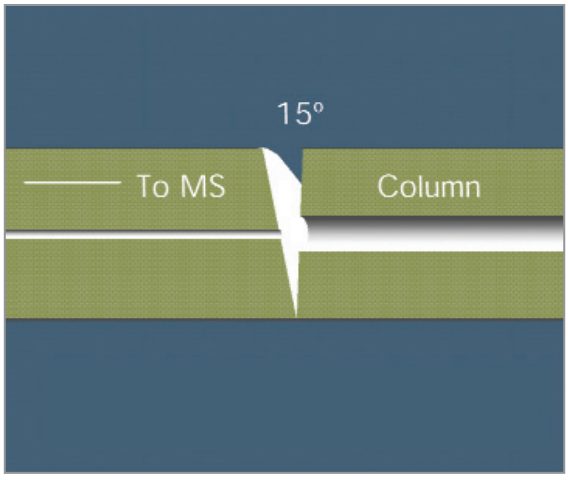

Figure 7. Fixed dead volume at connection.

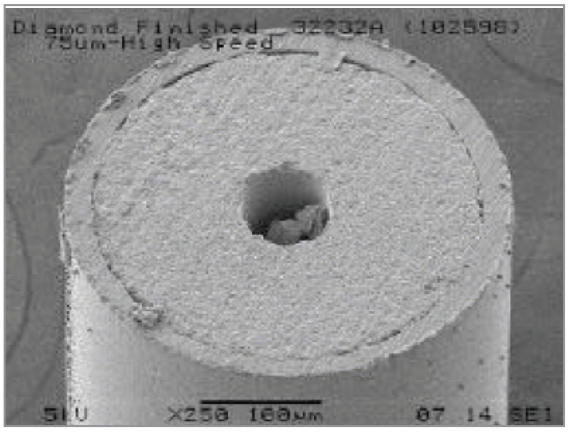

Figure 6. Diamond polished capillary.

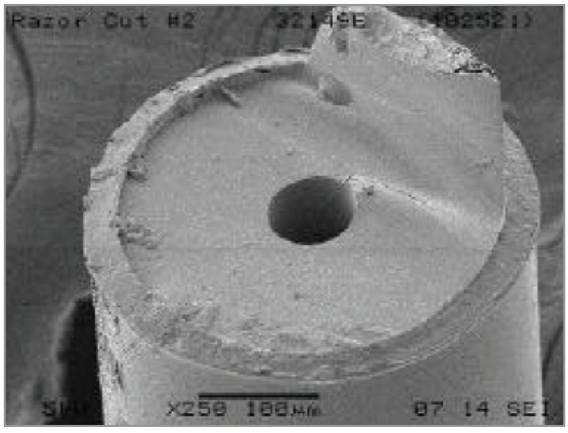

Figure 8. Capillary cut with ceramic tool.

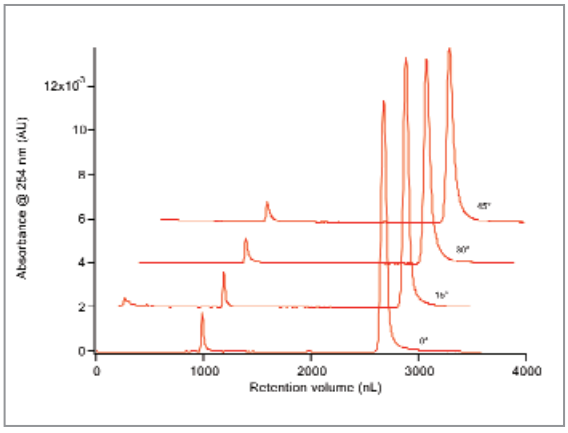

Figure 9. Effect of angle in connection tubing on peak shape. Isocratic elution of acetone/ acenaphthene. HPLC conditions described in experimental section.

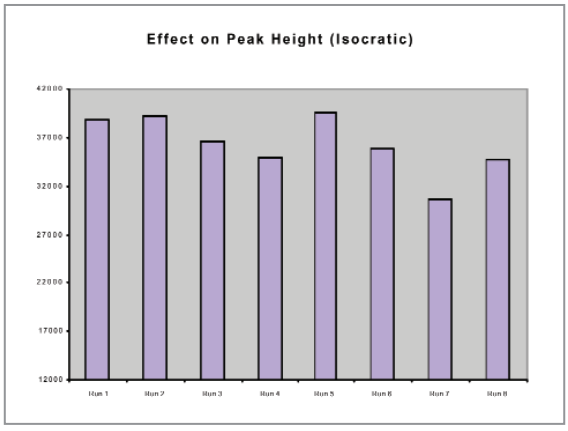

Figure 10. Effect of peak height under isocratic solvent switching conditions. Column ruggedness is maintained throughout multiple runs.

COLUMN RUGGEDNESS STUDY

A study was performed to investigate the ruggedness of a 75 µm columns packed with either 3.0 µ Atlantis dC_{18} or Symmetry C_{18}. The column efficiency was tested and then used for the gradient separation of a BSA Digest.

The column was subjected to multiple solvent switch cycles to stress the packed bed construction. An isocratic efficiency test was performed after each cycle. After multiple cycles, the BSA Digest gradient was repeated.

A 75 µ x 100 mm Symmetry C_{18} column was tested for ruggedness by LC/MS with a BSA Digest. The gradient digest was repeated 37 times with identical conditions.

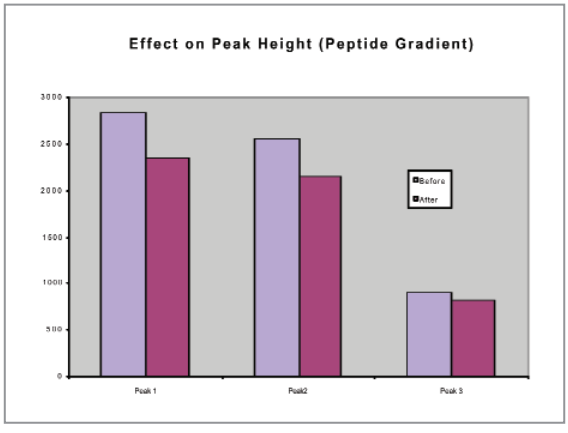

Figure 11. Effect of peak height for a gradient peptide separation before and after the column was subjected to solvent switching. The column experienced minor loss of peak height for peaks at the beginning, middle, and end of the gradient.

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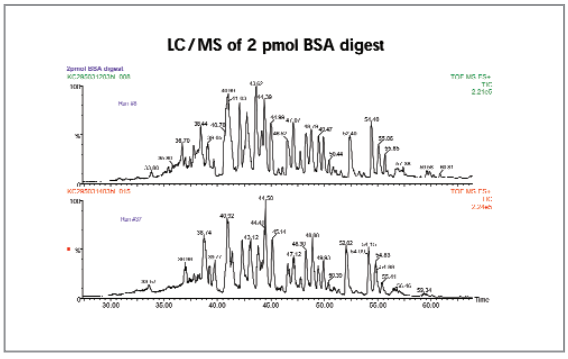

Figure 12. BSA Digest with a 75 µ x 100 mm Symmetry C_{18}. Column robustness is maintained after multiple runs under identical conditions.

CONCLUSIONS

- 75 µm ID columns can be packed to have the same packed bed permeability as analytical column.
- Extra column bandspread effects are of significant importance to nano flow LC/MS systems.
- The addition of dead volume at a nano column outlet degrades the separation performance of 75 µm HPLC separations.
- The 75 µm x 150 mm Atlantis dC_{18} column maintained chromatographic performance after being subjected to multiple stress tests.
- The 75 µm x 100 mm Symmetry C_{18} column maintained performance after 37 injections of BSA Digest.

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