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Direct Comparison of 75 µm Nanocolumns to Analytical Columns Using a Waters® 2487 UV Detector Equipped With a Nanoliter Flow Cell Under Isocratic Conditions

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Introduction

Recently, nanoscale chromatography has gained much interest, as it is readily coupled to electrospray mass spectrometry allowing high sensitivity analysis of picomolar samples. This is especially apparent in the proteomics arena where 75 µm nanocolumns are routinely used to separate complex biological samples. Therefore, the optimization of nanocolumn performance is critical to obtaining meaningful chromatographic data. At this scale the use of more conventional UV detectors that are capable of handling nanoscale flow streams has been challenging. These nanocolumns are almost exclusively operated under gradient elution conditions, e.g. peptide analysis, where true column performance is not readily measured.

We have recently explored isocratic separations of small molecules in nanoliter flow regimes using 75 µm capillary columns packed with Waters Symmetry® C_{18} stationary phases using UV detection via a Waters 2487 UV detector with either a 1 nL or 25 nL flow cell. Thus a direct performance comparison can be made to an analytical scale Symmetry C_{18} (2.1 mm x 100 mm) column and a conventional UV flow cell.

In addition to nanocolumn evaluation, a thorough study of how chromatographic performance is affected by the type of unions employed and how band dispersion can be controlled was conducted. Finally, we performed preliminary investigations into the feasibility of using the new nanoliter flow cell UV prior to MS detection.

Experimental

Chromatographic Conditions

	Nanoscale	Analytical Scale
Column	3.5 µm Symmetry C_{18} 75 µm x 100 mm	3.5 µm Symmetry C_{18} 2.1 mm x 100 mm
System	Waters 2790 Alliance™ HT (2000:1 post-injector split)	Waters 2690 Alliance™
Flow Rate	~300 nL/min	250 µL/min
Detector	Waters 2487 UV with either a 1 or 25 nL flow cell	Waters 2487 UV

In all cases, an isocratic test mix of: (1) acetone, (2) ethyl paraben, (3) butyl paraben, and (4) naphthalene, was used (λ_{det} = 254 nm) to evaluate chromatographic performance. Mobile phases were comprised of either (a) 60:40 H_2O:ACN or (b) 40:60 H_2O:ACN. 75 µm nanocolumns were prepared in house.

Nanoliter Volume UV Flow Cells

Nanoliter flow cells were prepared in fused silica capillary and were adapted for use in a Waters® 2487 UV detector. Figure 1 presents a graphic image of a nano UV flow cell assembly. Flow cells with dimensions of 1 nL and 25 nL were fabricated.

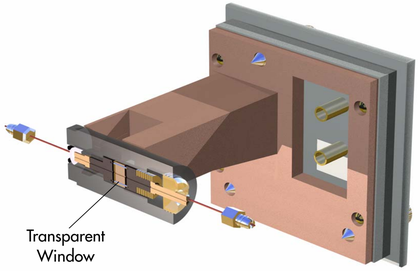

Figure 1. Graphic of a nano UV flow cell constructed in a single length of fused silica capillary.

Band Broadening Caused by UV Cell

A typical UV/MS experiment was simulated by placing a second nanoliter UV flow cell in line before the UV detector with an active 25 nL nanoliter flow cell. Sequential injections of acetone (2.5nL) were made both with and without the second flow cell in place, and the USP tailing factors of the resulting peaks were determined via Waters Millennium® 4.0 software. In both cases, a mobile phase composition of 60:40 H_2O:ACN was used at a flow rate of 400 nL/min and λ_{det} = 254nm.

Results and Discussion

Chromatography in the Nanoliter Flow Regime

Using this system, we are able to assess the overall chromatographic performance of Symmetry C_{18} nanocolumns in comparison to analytical scale columns. A direct comparison between the chromatography achieved using analytical and nanoscale systems is presented in Figure 2. From these data, it is apparent the 75 µm column performs comparably to an analytical column. The efficiencies of nanocolumns and analytical columns are shown in Figure 3. The nanoliter flow cell showed very good performance, Figure 2.

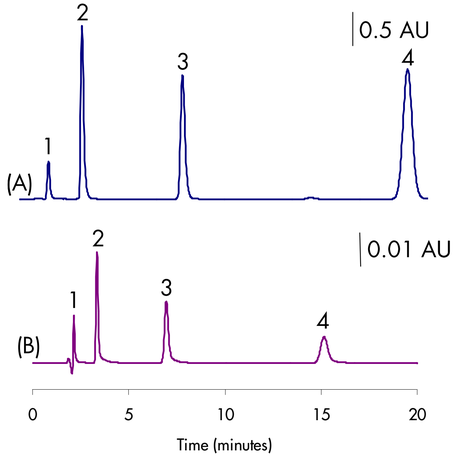

Figure 2. Separation of a small molecule mixture demonstrating the performance of a 2487 UV detector with (A) a standard flow cell and (B) a nanoliter flow cell.

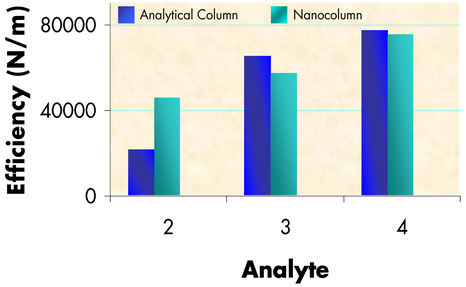

Figure 3. Bar graph comparing the efficiencies of an analytical Symmetry C_{18} column and a Symmetry C_{18} nanocolumn of the same bed length (100 mm). Efficiencies were calculated by dividing the plate height at 5σ by the column length.

Comparison of 1 nL and 25 nL UV Flow Cells

In addition to comparing nano-LC to analytical LC, an evaluation of 1 nL and 25 nL cells was conducted as illustrated in Figure 4. In both cases, acceptable S/N and detector response are observed.

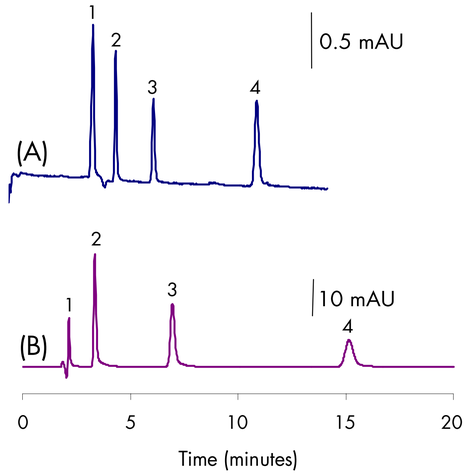

Figure 4. UV detection of a small molecule mixture using (A) a 1 nL flow cell and (B) a 25 nL flow cell.

Impact of Band Dispersion

We have found that the connection of both the nanocolumn and detector capillary has a significant effect on the chromatography. Tailing factors (USP) were dramatically improved by using end-to-end connections of capillaries in a single PEEK sleeve vs. a zero dead volume (ZDV) connection as reported in Table 1.

Connection Type	Cleavage Method	Tailing Factor for Acetone
ZDV	Manual Cutting	2.56
PEEK Sleeve		1.53
ZDV	Mechanical Cutting	0.78
PEEK Sleeve		1.01

Table 1. USP tailing factors for acetone obtained using different connection types and cleavage methods.

The impact of a nanoliter UV flow cell on band broadening and tailing in a LC-UV/MS application was determined. No significant change in tailing factor is observed as detailed in Table 2.

Configuration	USP Tailing Factor
without inactive cell	1.05
with inactive cell	1.09

Table 2. USP tailing factors for injections of acetone in configurations with and without an inactive inline nanoliter UV flow cell.

After optimization of nanocolumn performance and connections, complex separations of biological samples was performed using LC/MS (TOF) as shown in Figure 5.

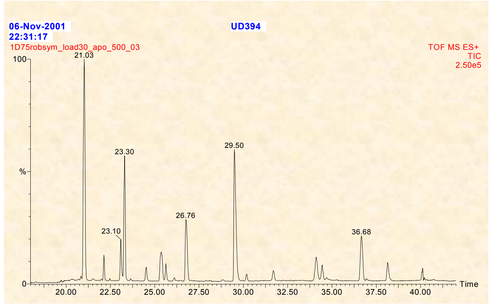

Figure 5. TIC plot for the separation of a tryptic digest of apomyoglobin digest using a 75 µm x 100 mm nanocolumn. The column was eluted at an approximate flow rate of 200nL/min with a gradient of 2-50% ACN:H_2O (0.1% formic acid) over 30 minutes.

Conclusions

- Nanocolumns can be prepared with similar performance to that of an analytical column.
- Nanoliter UV flow cell has facilitated isocratic testing of the 75 µm nanocolumns.
- Critical consideration of column connections has significantly improved the nano-LC.
- Use of UV detection with a nanoliter flow cell prior to MS detection should not dramatically effect the chromatographic peak shape.