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Technical Note 106

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Ultrahigh-Efficiency LC-MS/MS Separations of Proteolytic Digests on PepSwift Monolithic Columns

INTRODUCTION

Shotgun proteomics, in which proteins are digested and the resulting peptides are separated by high-performance liquid chromatography (HPLC) and detected by tandem mass-spectrometry (MS/MS), has become the standard approach in proteomics research.[1,2] The number of peptides present in a single proteolytic digest can be in the 100,000s and the concentration range can encompass many orders of magnitude.[3,4] When analyzing complex peptide mixtures, the identification and quantification of low abundance peptides by MS is often hindered by insufficient separation and ion-suppression effects.To improve the separation of complex proteomic samples, increasing effort is directed to the development of novel column technology.[5,6]

Macroporous polymer-based monolithic separation materials were first developed in the late 1960s, and recent developments have now made them a viable alternative to packed column technology.[7,8] The morphology of the polymer monolith features macropores (flow-through pores) and polymer microglobules as shown in Figure 1. This material is well suited to perform large-molecule separations because mass transfer is driven by convection, rather than by diffusion, as in the case of porous particles.

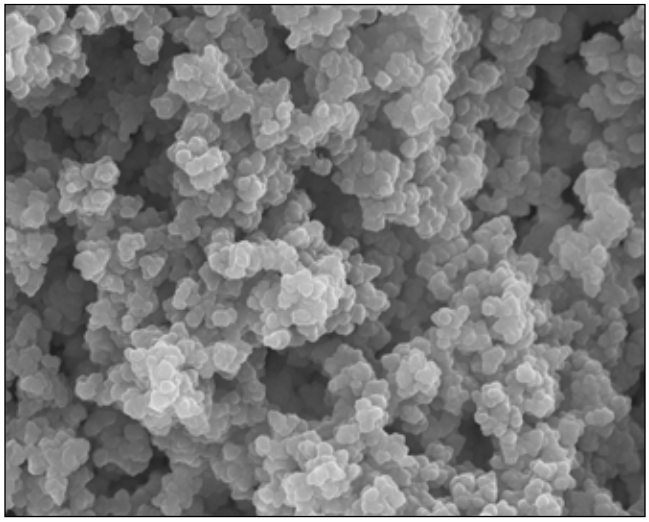

Figure 1. Typical interconnected porous structure of a PepSwift™ polymer monolithic column.

In addition, the size of the macropores and microglobules can be tuned independently to optimize monolith morphology to yield high-efficiency separations.[9] Due to the monolith's continuous structure that can be attached to the capillary wall, the columns are highly robust.

In this study, the LC performance of 50 mm, 250 mm, and 1 m capillary poly(styrene-co-divinylbenzene) monolithic columns is evaluated for LC-MS/MS peptide-mapping experiments. The optimization of LC conditions (including flow rate and gradient time) and column length for LC-MS performance is discussed. The potential of 1 m monolithic columns in one-dimensional LC coupled to tandem mass-spectrometry detection is demonstrated by the analysis of tryptic-digest proteomic samples of varying complexity.

EQUIPMENT

UltiMate® 3000 nano LC system including:

- On-line degasser
- Dual gradient pump (two)
- Thermostatted flow manager
- Temperature-controlled well-plate sampler

Chromeleon® Chromatography Data System (CDS) software Version 6.8 was used to control the HPLC system.

The UltiMate 3000 system was coupled on-line to a high-capacity ion-trap mass spectrometer (HCTultra™) from Bruker Daltonics GmbH.

CHEMICALS

Acetonitrile (CH_3CN) and trifluoroacetic acid (TFA, ≥99.95%) were purchased from Biosolve (Valkenswaard, The Netherlands).

Water was purified using a Milli-Q® Gradient A10 system from Millipore (Molsheim, France).

SAMPLES

Tryptic digest of six proteins: transferrin, bovine serum albumin, β-galactosidase, alcohol dehydrogenase, lysozyme, and cytochrome *c* (P/N 161088)

Tryptic digest of *E.coli* proteins (Bio-Rad)

CONDITIONS

Columns: PepSwift RP monolithic capillary columns,
0.2 × 50 mm length (P/N 164557)
0.2 × 250 mm length (P/N 164542)

Eluent: A: 0.05% aqueous TFA
B: 50:50% Water: CH_3CN + 0.05% TFA

Typical Gradient:

Time (min)	*%A*	*%B*
0	0	100
60	55	45
61	90	10
65	90	10
66	0	100
75	0	100

Flow Rates: 2 µL/min on 50 and 250 mm column
0.5 µL/min on 1 m monolith (coupled 250 mm columns)

Column Temp.: 60 °C

Inj. Volume: 1 µL (full loop)

ESI interface: Bruker nanospray ESI interface (metal needle in the electrospray source was replaced by a 20 µm i.d. × 90 µm o.d. fused-silica capillary)

MS Detection: Ion-trap mass spectrometry operating in MS/MS mode

MS Settings: Positive ionization mode
Scan range from 300–1600 *m/z*
Two precursor ions were selected for MS/MS fragmentation
Scan range for precursor ions of 100–2800 *m/z*
Scan speed in MS and MS/MS mode of 26,000 $m/z.s^{-1}$

RESULTS AND DISCUSSION

Peak Capacity in Gradient LC

A good performance criterion for the gradient separation of peptides is peak capacity (PC), which is defined as the maximum number of peaks that can be separated with a resolution of 1 and elute in the applied gradient window:

$$PC \approx \frac{t_G}{W} + 1 = \frac{t_G \cdot \sqrt{L}}{4 \cdot t_0(1+k_e) \cdot \sqrt{H}} + 1 \qquad (1)$$

where t_G is the gradient time and W the average 4σ peak width, L the column length, t_0 the column holdup time, k_e the retention factor of the analyte at the moment of elution (which—to a first approximation—may be assumed equal for all analytes), and H the plate height.

Equation 1 shows that maximizing the separation performance involves optimization of column technology, such as the morphology (reflected in the plate height) and the column length and tuning LC conditions, including gradient time and composition window (affecting k_e), and flow rate and column temperature (affecting k_e as well as H).

Figure 2 shows the effect of t_G on the separation of a tryptic digest of a mixture of six proteins (transferrin, bovine serum albumin, β-galactosidase, alcohol dehydrogenase, lysozyme, and cytochrome *c*) using a 50 mm × 0.2 mm capillary monolithic column. The peak capacity was experimentally calculated from MS data by averaging the 4 sigma peak width for at least five peptides and applying Equation 1. With increasing gradient time, the peak capacity increased significantly from 240 applying a 15 min gradient to 360 when using a gradient duration of 80 min.

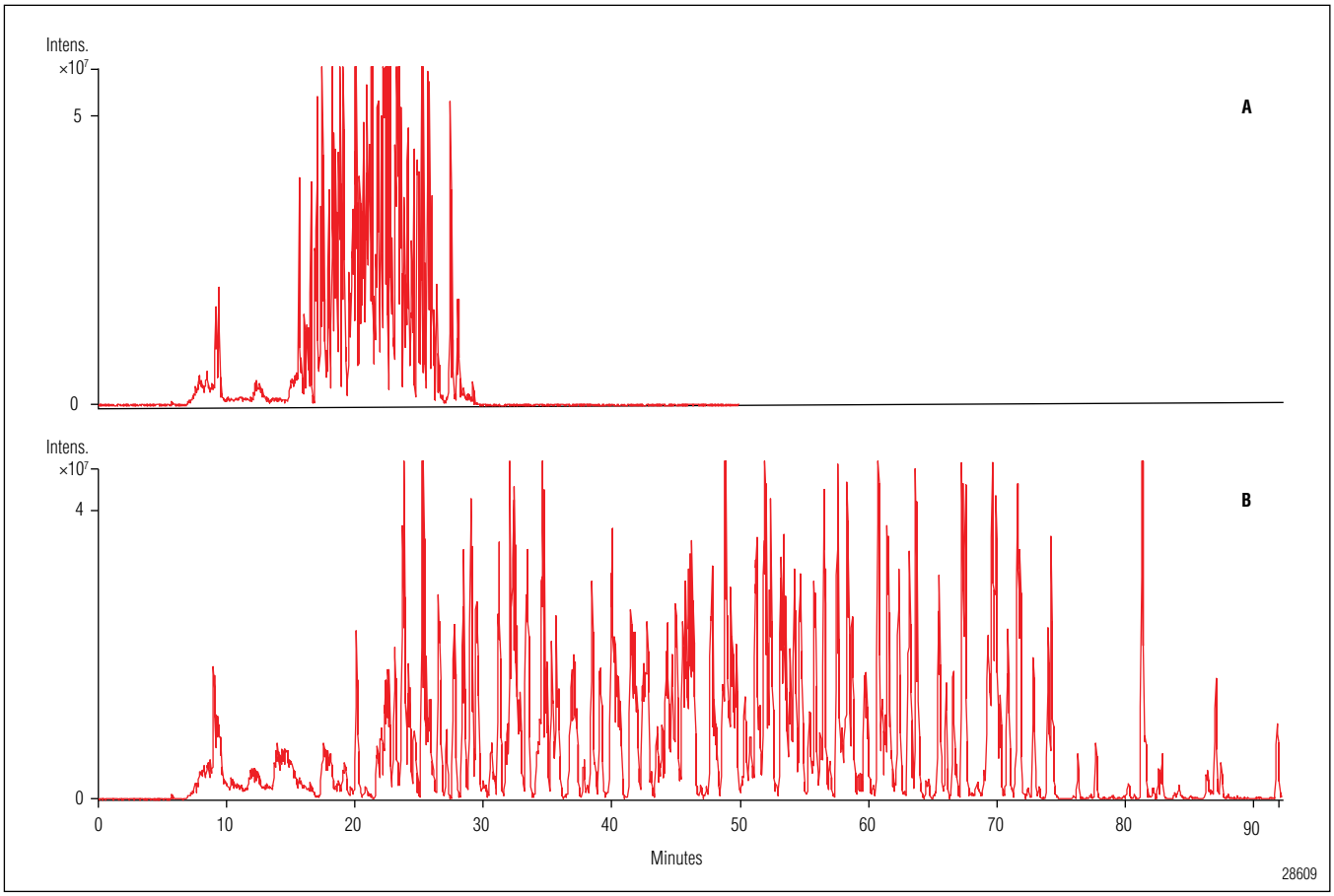

Figure 2. High-resolution separations (base-peak chromatograms) obtained on a 50 mm PepSwift monolithic column applying a 15 min (Figure 2A) and 80 min gradient (Figure 2B).

Effect of Column Length on Separation Performance

Figure 3 shows the effect of gradient time normalized for the column holdup time on peak capacity measured on 50 mm and 250 mm monolithic capillary columns. This representation ensures that gradient steepness is comparable. Typically, the peak capacity increased rapidly for short gradients and appeared to level off, approaching a maximum at longer gradient times. This was caused by the linear increase in peak width with increasing gradient time. The maximum peak capacity of 400, obtained with a 50 mm column, increased to approximately 500 when using the 250 mm column and scaling the gradient duration with respect to column length.

When comparing the column performance of different column lengths at the same gradient slope, note that the total analysis time ($t_0 + t_{delay} + t_{gradient} + t_{eq}$) may dramatically differ. Figure 4 shows a comparison between the LC performance of a 50 and 250 mm monolith taking into account the total analysis time. For separation with analysis times below 80 min, the 50 mm monolith provides superior separations. For more demanding separations (peak capacities >370), use of 250 mm monoliths is recommended.

For more complex mixtures, the requirements of the HPLC separation are much more stringent. Figure 5 shows the base-peak chromatogram of a separation of *E. coli* peptides on a 1 m monolithic column (four 250 mm columns connected in series) applying a 600 min gradient. The peak capacity exceeded 1000, resulting in a high-resolution separation and extending the dynamic range of the method because fewer peptides were influenced by ion-suppression effects.

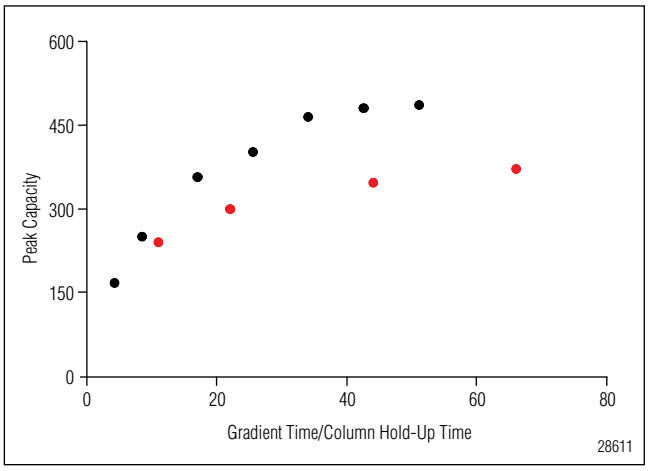

Figure 3. Effect of gradient time on peak capacity using 50 (red) and 250 mm (black) monolithic capillary columns.

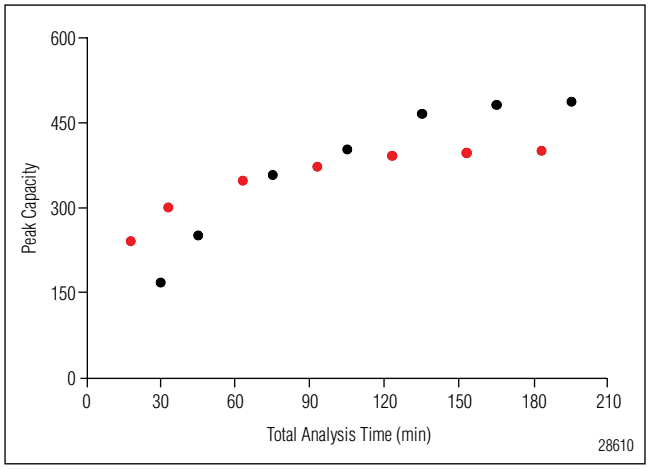

Figure 4. Peak capacity vs total analysis time obtained on 50 (red symbols) and 250 mm monoliths (black symbols). Conditions as described in Figure 2. $t_{0,\ 50\ mm\ column}$ *= 0.6 min,* t_{delay} *= 1.4 min,* $t_{eq,\ 50\ mm\ column}$ *= 2 min,* $t_{0,\ 250\ mm\ column}$ *= 2 min, and* $t_{eq,\ 250\ mm\ column}$ *= 10 min.*

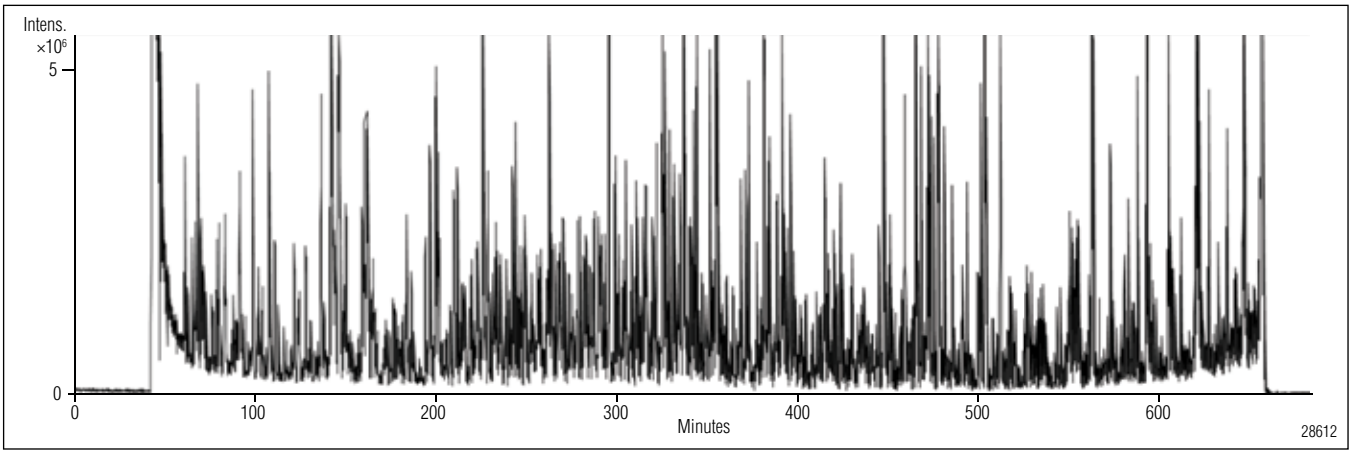

Figure 5. Base-peak chromatogram of a tryptic digest of E. coli *using a 1 m monolithic column (four 250 mm columns connected in series) operating at a flow rate of 0.5 µL/min and a gradient time of 600 min.*

CONCLUSION

In this study, high-efficiency LC-MS/MS separations of complex proteolytic digests are demonstrated using 50 mm, 250 mm, and 1 m PepSwift monolithic capillary columns. The robustness of these high-efficiency monolithic materials, covalently bonded to the capillary wall, and their excellent retention-time stability makes this column technology very attractive in comparison to packed capillary columns.

Maximizing peak capacity involves optimizing LC conditions (including flow rate, gradient time, and column temperature) and column technology (i.e., column length and morphology). Peak capacities in excess of 1000 can easily be obtained using PepSwift column technology on the UltiMate 3000 nano LC system.

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