

# Ultrahigh-Performance Nano LC-MS/MS Analysis of Complex Proteomic Samples



Evert-Jan Sneekes, Bjorn de Haan, and Remco Swart  
Dionex Corporation, Amsterdam, The Netherlands

## INTRODUCTION

Determination of the proteome and identification of biomarkers are required to monitor dynamic changes in living organisms and predict the onset of an illness. One popular method to tackle contemporary proteomic samples is called shotgun proteomics, in which proteins are digested, the resulting peptides are separated by high-performance liquid chromatography (HPLC), and identification is performed with tandem mass spectrometry. Digestion of proteins typically leads to a very large number of peptides. For example, digestion of a cell lysate easily generates 500,000 peptides. The separation of these highly complex peptide samples is one of the major challenges in analytical chemistry.

The main strategy to improve the efficiency of packed columns is either to increase column length or to decrease the size of the stationary phase particles. However, to operate these columns effectively, the LC conditions need to be adjusted accordingly. Naturally, the on-line coupling to MS systems has to be taken into account in the optimization process.

Here, the authors report on the performance of nano LC columns operating at ultrahigh pressure. The effects of column parameters (particle size and column length) and LC conditions (gradient time, flow rate, column temperature) were investigated with reversed-phase (RP) gradient nano LC. High-resolution LC-MS separations of complex proteomic peptide samples are demonstrated by combining long columns with 2  $\mu\text{m}$  particles and long gradients. The effects of LC parameters on performance and the influence on peptide identification are discussed.

## EXPERIMENTAL

All experiments were performed on an UltiMate<sup>®</sup> 3000 RSLCnano system (Dionex Germering, Germany) connected to an HCTultra<sup>™</sup> ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany).

Mobile Phase A:	Water, 0.05% TFA
Mobile Phase B:	20% Water, 80% Acetonitrile, 0.04% TFA
Loading Solvent:	98% Water, 2% Acetonitrile, 0.05% TFA
Trap Column:	Acclaim <sup>®</sup> PepMap <sup>™</sup> Nanotrap, 75 $\mu\text{m}$ i.d. $\times$ 2 cm, packed with 3 $\mu\text{m}$ C18 particles
Nano Column:	Acclaim PepMap RSLC column, packed with 2.2 or 3 $\mu\text{m}$ C18 particles, see legend for details
Sample:	<i>E. coli</i> tryptic digest

## COLUMN PERFORMANCE

Figure 1 compares the isocratic performance of two 75  $\mu\text{m}$   $\times$  15 cm nano columns packed with either 2.2  $\mu\text{m}$  or 3  $\mu\text{m}$  particles. The smaller particles provide faster, more efficient separations, allowing for higher peak capacities. However, decreasing the particle size from 3  $\mu\text{m}$  to 2.2  $\mu\text{m}$  will also double the column backpressure; therefore, these columns present new challenges in preparation and operation.

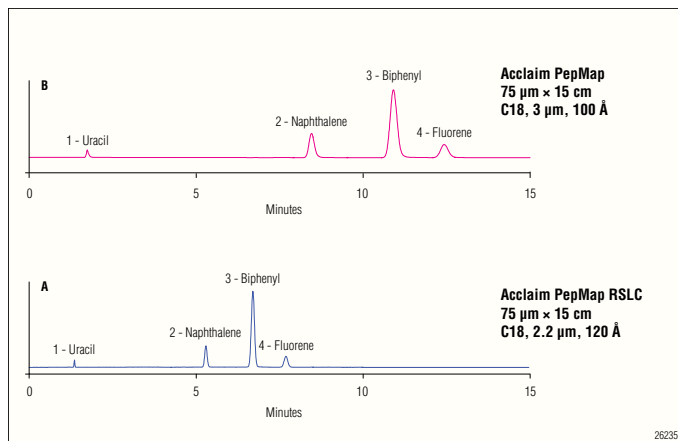


Figure 1. Isocratic column performance tests for a 75  $\mu\text{m}$  i.d.  $\times$  15 cm column packed with A) 2.2  $\mu\text{m}$  particles or B) 3  $\mu\text{m}$  particles.

Figure 2 compares the efficiency in plates/meter for different lengths of nano columns packed with 2.2  $\mu\text{m}$  particles and shows that columns up to 50 cm in length can be prepared as efficiently as shorter columns.

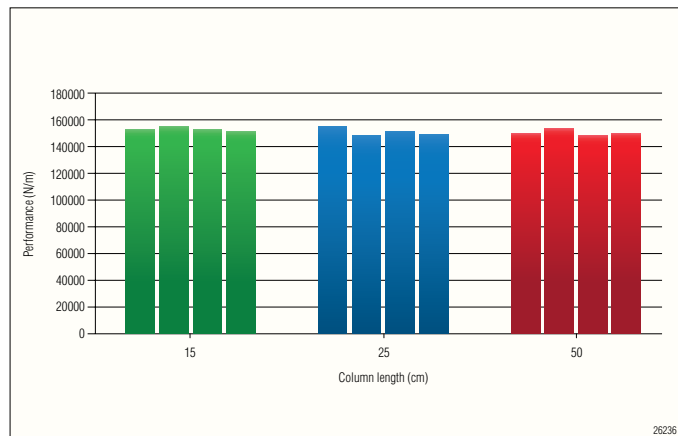


Figure 2. Comparison of column performance in plates per meter for various lengths of columns shows that columns of different lengths can be efficiently packed with 2.2  $\mu\text{m}$  particles.

## APPLICATION OF HIGH RESOLUTION NANO COLUMNS

Along with providing the highest peak capacity possible, proteomics separation strategies must be MS compatible. Long columns packed with 2.2  $\mu\text{m}$  C18 particles operated in preconcentration mode fulfill both these requirements.

Figure 3 shows the traditional preconcentration setup and the vented column setup. The traditional method uses a trapping column that is switched in-line or out-of-line during analysis. This allows complete separation of the loading and separation phases, and is ideal for samples that require extensive washing.

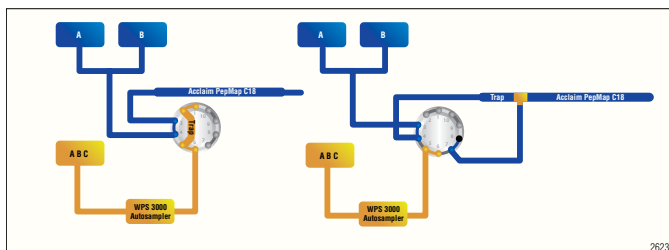


Figure 3. Two fluidic configurations for preconcentration. A) Traditional setup where the trap column is placed in line with the nano column after sample loading and washing. B) Vented column setup where trap and analytical column are connected directly.

The vented column configuration has the trap and analytical column connected directly using a T connector. This minimizes dead volume and allows the user to physically move the column away from the switching valve and place it closer to the MS. One important consideration with this method is that during every run the nano column must be pressurized and depressurized; with long columns packed with small particles, this pressure differential can adversely affect column lifetime, as pressure changes of up to 700 bar can occur.

## RESULTS

The RSLCnano system has a high-pressure limit of 800 bar, and can cope easily with the higher pressures associated with the use of smaller particles and longer columns, therefore, a 50 cm nano column packed with 2.2  $\mu\text{m}$  particles was used to separate an *E. coli* tryptic digest with various gradient lengths.

Figure 4 shows extracted ion chromatograms of two peptides ( $m/z$  902.9 and 1059.6) for the 30 min and 300 min gradient. Clearly, the spread between the peptides in relationship to the peak widths is more favorable for the 300 min gradient. Note that while the gradient is increased 10-fold, the average peak width increases only 3 times, resulting in a significant increase in peak capacity. Calculation shows that the peak capacity between the peaks shown is 40 for the 30 min gradient, and 300 for the 300 min gradient.

The spread between the peptides increases more than linear with the gradient time. This supports the theory that long columns are only effective when operated with long gradients. The gradient volume of the 30 min gradient at 270 nL/min is insufficient to effectively use the complete column. The clean mass spectra for the  $m/z$  1059.6 below the chromatogram clearly show that the longer gradient has the better separation.

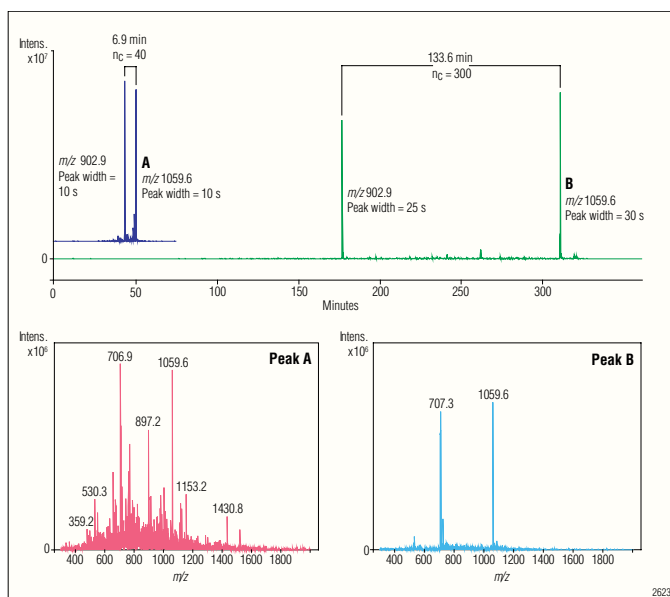


Figure 4. Extracted ion chromatograms for two peptides ( $m/z$  902.9 and 1059.6) from an *E. coli* cell lysate digest, separated using a 50 cm nano column packed with 2.2  $\mu\text{m}$  particles with 30 min (blue) and 300 min (green) gradients. The spread between the peaks increases greatly as the gradient time increases, while the peak width increase is far less significant. Consequently, the peak capacity ( $n_c$ ) between the peaks increases significantly for the longer gradient.

Figure 5 shows the base-peak chromatogram of a 10 hour gradient of the *E. coli* tryptic digest, resulting in an overall peak capacity of 750.

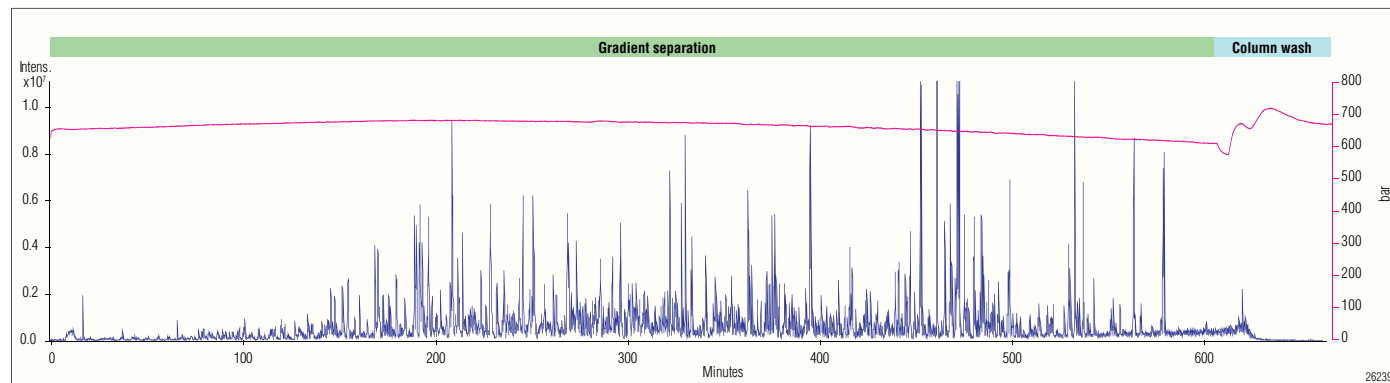


Figure 5. Ten hour gradient separation of an *E. coli* tryptic digest on a 50 cm nano column packed with 2.2  $\mu\text{m}$  particles operated at 270 nL/min. A peak capacity of 750 was determined using these conditions.

## REPEATABILITY

Repeatability of an analytical method is critical, especially in quantitative analysis on large proteomics sample sets by MRM analysis. In Figure 6, an overlay of 5 consecutive 120 min gradient separations of PMD using a vented column configuration is shown. Table 1 shows the average spread deviation between the five chromatograms is only 4.64 s.

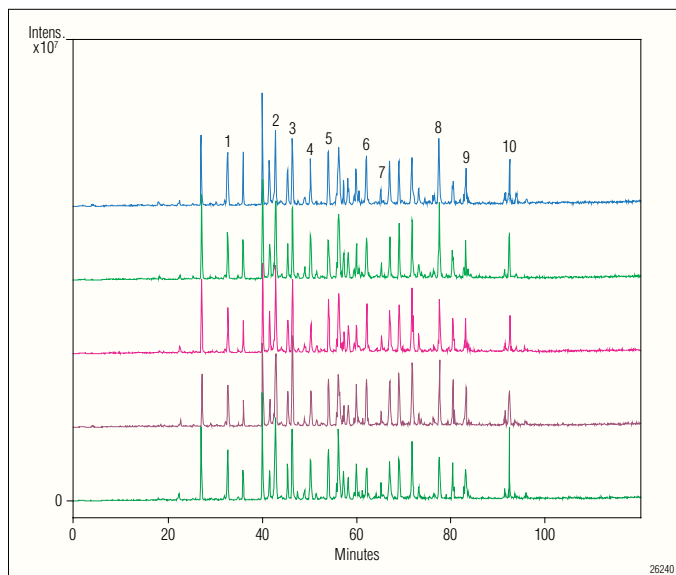


Figure 6. Overlay of five consecutive PMD separations on a vented column configuration.

## Incorporation in Proteomics Workflows

Despite the high peak capacities that can be achieved with the instrumentation and column technology currently available, most proteomics analyses are multistep processes. Gel electrophoresis (GE), free-flow electrophoresis (FFE), liquid chromatography (LC), and many other techniques are typically used upstream of the final step of RP-LC-MS/MS. The full potential of a proteomics analysis can only be achieved when efficiency has been maximized at all steps. The optimized LC-MS/MS separation shown here can easily be applied as the final step in almost any proteomics workflow.

## CONCLUSIONS

- Ultrahigh-performance LC-MS/MS can be achieved in proteomics by utilizing smaller particle sizes in conjunction with a high-pressure nano LC system.
- Only the combined effect of small particles, longer columns, and increased gradient time will maximize the result for proteomics experiments.
- Under optimized conditions, a 50 cm long nano column operated at ~700 bar can achieve a peak capacity of 750 in 10 h.
- The LC-MS/MS analysis presented here is easy to perform and demonstrates excellent reproducibility. For more complex samples it can be incorporated as the final step in a multidimensional LC proteomics workflow.
- The UltiMate 3000 RSLCnano allows the user to fully exploit the possibilities of state-of-the-art column technology.

Table 1. Reproducibility and Absolute Spread for Peaks Indicated in Figure 5

Peak	1	2	3	4	5	6	7	8	9	10	Average
Tr (min)	34.2	44.6	46.8	51.6	55.0	61.2	62.9	76.6	82.4	94.9	
RSD	0.13%	0.07%	0.04%	0.07%	0.07%	0.04%	0.06%	0.04%	0.03%	0.03%	0.06%
Spread (s)	6	4	2.8	4.8	4.8	4.4	5.6	5.2	4	4.8	4.64

HCTultra is a trademark of Bruker Daltonics.  
PepMap is a trademark and Acclaim and UltiMate are registered trademarks of Dionex Corporation.

Speed • Simplicity • Solutions



### Dionex Corporation

1228 Titan Way  
P.O. Box 3603  
Sunnyvale, CA  
94088-3603  
(408) 737-0700

### North America

U.S./Canada (847) 295-7500

### South America

Brazil (55) 11 3731 5140

### Europe

Austria (43) 1 616 51 25 Benelux (31) 20 683 9768 (32) 3 353 4294  
Denmark (45) 36 36 90 90 France (33) 1 39 30 01 10 Germany (49) 6126 991 0  
Ireland (353) 1 644 0064 Italy (39) 02 51 62 1267 Sweden (46) 8 473 3380  
Switzerland (41) 62 205 9966 United Kingdom (44) 1276 691722

### Asia Pacific

Australia (61) 2 9420 5233 China (852) 2428 3282 India (91) 22 2764 2735  
Japan (81) 6 6885 1213 Korea (82) 2 2653 2580 Singapore (65) 6289 1190  
Taiwan (886) 2 8751 6655

[www.dionex.com](http://www.dionex.com)



LPN 2824-01 04/11  
©2011 Dionex Corporation