High Peak Capacity Nano LC Peptide Separations Using Long Packed Columns

INTRODUCTION

Precise information of the proteome and identification of biomarkers is necessary to monitor dynamic changes in living organisms and predict the onset of an illness. One of the methods is called shot gun proteomics, where the proteins are digested, the resulting peptides are separated by high-performance liquid chromatography, and identification is performed with tandem mass-spectrometric detection. Digestion of proteins may lead to a very large number of peptides. For example, it has been estimated that digestion of a cell lysate may produce up to 500,000 peptides. Therefore, the separation of highly complex peptide samples is one of the major challenges of current analytical chemistry.

Porous silica particles have become the preferred stationary phase in liquid chromatography. This is due to their superior purity, large surface area, and outstanding mechanical strength, among other features. Acclaim® PepMap™ column technology allows high-efficiency separations of tryptic peptides, and is available from 75 µm I.D. up to 1 mm I.D. formats. The capillary format of these columns (75 µm I.D.) ensures high mass sensitivity, which is typically a factor of 4000 higher than 4.6 mm I.D. columns operating at conventional flow rates.

This technical note describes a method for optimizing peak capacity for the separation of complex peptide mixtures in nano LC. In particular, the effects of gradient time and column length on the resolution are demonstrated. Finally, run-to-run retention time repeatability when applying long gradient times are determined.

EXPERIMENTAL

LC system	UltiMate® 3000 Nano LC System
Columns	Acclaim PepMap 3 µm C18, 100Å 75 µm I.D. x 15 cm (p/n 160321), 25 cm (p/n 164261), and 50 cm (p/n 164451)
Mobile Phase A	0.05% TFA in water
Mobile Phase B	0.04% TFA in 80:20% acetonitrile:water
Gradient	4–55% B gradient, 5 min wash at 90% B, 25 min equilibration
Flow Rate	300 nL/min
Inj. Volume	1 µL (1 pmol sample)
Flow Cell	3 nL
Detection	UV at 214 nm
Data Collection Rate	2.5 Hz
Response Time	1 s
Temperature	60 °C
Sample	Tryptic digest of transferrin, bovine serum albumin, β-galactosidase, alcohol dehydrogenase, lysozyme and cytochrome c (protein mix digest, PMD, p/n 161089)

RESULTS AND DISCUSSION

A good measure for the separation performance is peak capacity (n_c), which is defined as the theoretical maximum number of peaks that can be separated with a resolution of 1 and elute within the applied gradient window. The formula for peak capacity is:

Equation 1: $$n_c = 1 + \frac{t_G}{W}$$

where t_G is the gradient time and W the peak width measured at 4σ (13.4% of the peak height). An example of the effect of gradient time (t_G) on the peak capacity is shown on a 15 cm long column in Figure 1. The separations of a tryptic digest of a six protein mixture (PMD) were obtained at gradient times of 15, 30, and 60 min, respectively. A flow rate of 300 nL/min was applied, which is a typical flow rate when executing nano LC-ESI-MS/MS experiments. This flow rate allows the formation of a stable electrospray and high detection sensitivity is obtained. Figure 1 clearly shows that the number of resolved peptides is much higher when applying longer gradients.

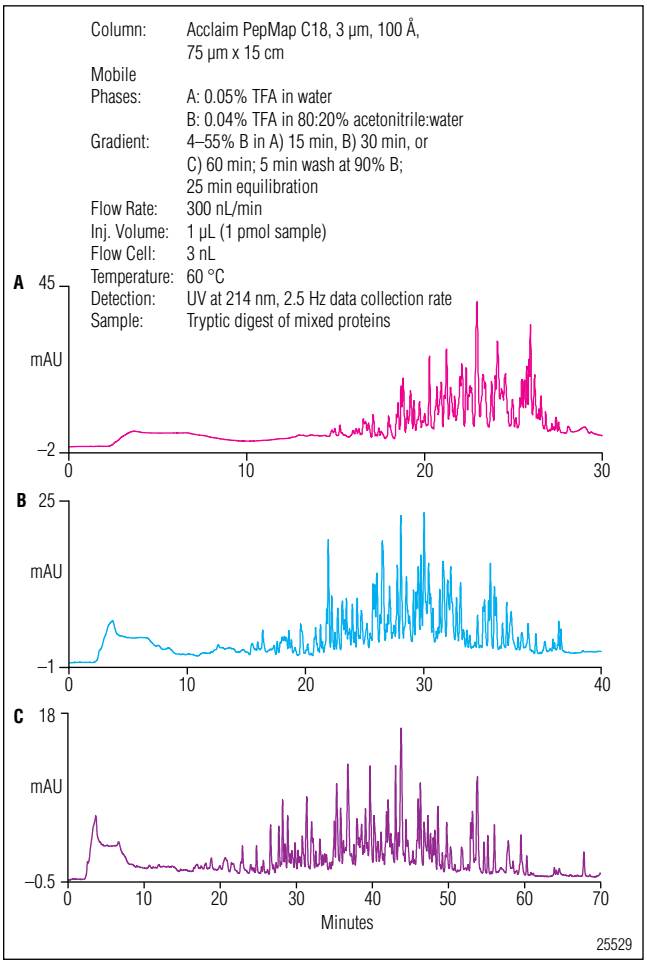

Figure 1. Separations of a tryptic digest of six proteins performed at gradient times of A) 15 min, B) 30 min, and C) 60 min. 75 µm x 15 cm Acclaim PepMap column packed with 3 µm silica C18 particles.

Equation 1 shows that peak capacity is also influenced by the peak width (W), which is directly related to the column efficiency. To enhance diffusion in the mobile and stationary phases, and consequently obtain narrower peaks, the separations were performed at a column temperature of 60 °C. At this temperature peak capacities were typically 50% higher than when applying a column temperature of 25 °C.

Figure 2 shows the trend of the effect of gradient time on peak capacity for 15 cm long nanocolumns. A steep initial increase in peak capacity is observed when increasing the gradient time from 5 to 40 min. At longer gradient times the rate of increase is less steep, and peak capacity tends to reach a maximum at approximately 120 min. This is caused by the increase in average peak width of peptides at longer gradient times.

In order to further increase the number of peptide resolved in a one-dimension LC run the effect of column length on peak capacity was investigated. Typically, when increasing the column length (e.g., by a factor of 2), the plate number increases by the same factor, influencing peak capacity. Figure 3 shows the separation of the PMD on a 15, 25, and 50 cm long column, respectively, applying a gradient time (t_G) of 120 min and a column temperature of 60 °C. When comparing the LC performance in the different chromatograms, it can be observed the number of resolved peptides is much higher on the longer column. Table 1 summarizes the peak capacities obtained on 15, 25, and 50 cm columns. A maximum peak capacity of 443 was generated on a 50 cm column applying a 120 min gradient, while the increase in column pressure was easily accommodated by the nano LC pump. An additional advantage of the use of long columns is that mass loadability increases with column length. This can be especially beneficial when fingerprinting very low-abundant peptides and a high mass loadability is required.

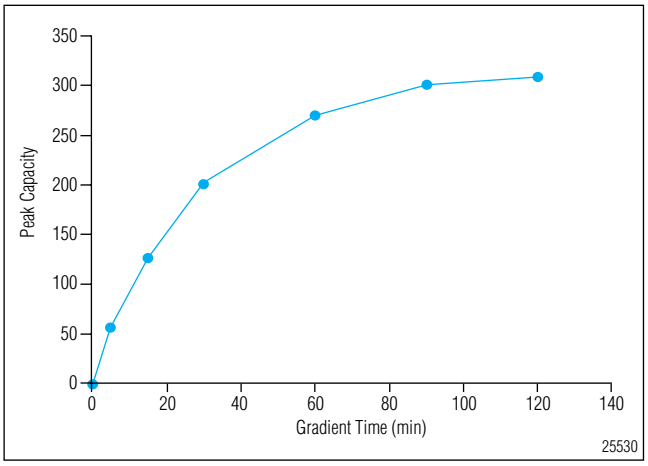

Figure 2. Effect of gradient time on peak capacity of tryptic peptides. 75 µm x 15 cm Acclaim PepMap column packed with 3 µm silica C18 particles.

Table 1. Effect of Column Length on Peak Capacity*

Column Length (cm)	Peak Capacity
15	308
25	338
50	443

*LC conditions as described in Figure 3

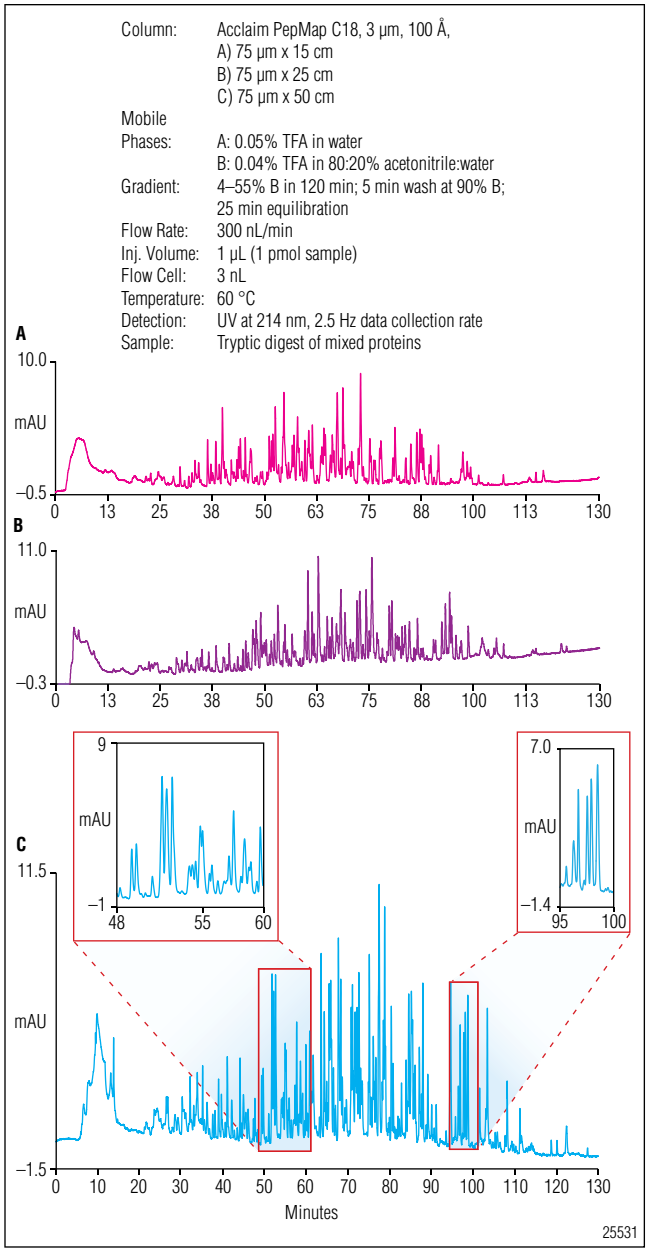

Figure 3. Effect of column length on LC performance for the separation of a tryptic digest of six proteins, performed at a gradient time of 120 min. Column length: A) 15 cm, B) 25 cm, and C) 50 cm.

A good run-to-run retention time repeatability is essential in fields such as label-free proteomics and biomarker validation, as this enables mapping differences between complex peptide samples. The retention time stability and pressure drop repeatability was determined during five consecutive separations of a digest of the PMD sample, using a single 25 cm column and applying a gradient time of 120 min (Figure 4). The standard deviation for the retention time of a peptide eluting at a retention time of 107.15 min was 10.2 s, which corresponds to an RSD value of only 0.17%. The column pressures recorded at the start of the gradient were used to determine pressure stability. The variation in column pressure was characterized by an RSD value of 0.28%.

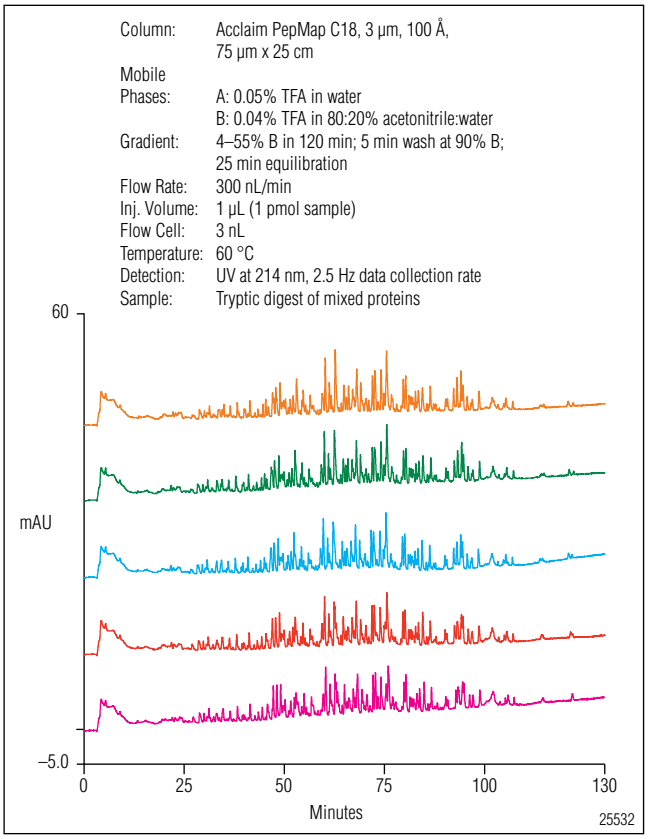

Figure 4. Run-to-run repeatability for the separation of a complex peptide sample on a 25 cm packed column, applying a gradient time of 120 min.

CONCLUSION

Peak capacity in one-dimensional LC was maximized by using a long gradient time in combination with long nano LC columns, and applying a high column temperature. A maximum peak capacity of 443 was obtained on a 50 cm Acclaim Pepmap nanoLC column applying a gradient time of 120 min and a column temperature of 60 °C.

The greater resolving power obtained on longer columns yields a higher detection sensitivity (peaks become narrower and intensity increases, while the gradient volume stays the same), and minimizes ion-suppression effects with mass-spectrometric detection, which is essential for the analysis for very low-abundant peptides.

ORDERING INFORMATION

In the U.S., call 1-800-346-6390, or contact the Dionex Regional Office nearest you. Outside the U.S., order through your local Dionex office or distributor. Refer to the following part numbers:

PRODUCT DESCRIPTION	PART NUMBER
Acclaim PepMap100, 3 µm, 100Å	
75 µm i.d. × 5 cm	160316
75 µm i.d. × 15 cm	160321
75 µm i.d. × 25 cm	164261
75 µm i.d. × 50 cm	164451

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

North America
U.S. (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

LPN 2111 PDF 7/16