

The effect of chromatographic resolution on peptide identification.

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Abstract

Eksigent Technologies has developed a splitless nanoLC system that allows for reproducible gradient delivery, up to 10,000 psi.

The use of this system for increasing peak capacity of peptide separations is demonstrated. The effect of gradient slope, column length and particle size are discussed, and the effect of increased peptide separation on the identification of proteins in complex samples is investigated.

Introduction

Nanoflow liquid chromatography coupled with nanoelectrospray (nanoLC-MS) is the method of choice for sensitive peptide and protein analysis for proteomics research.

We have demonstrated previously the theoretical and practical increase of peak capacity using longer columns in nanoLC/MS analysis of peptides¹. In order to get the most out of using longer columns, gradient length has to be increased proportionally with column length. In this presentation we will compare peak capacities on columns up to 60 cm length packed with 3µm particles, with a 25 cm column packed with sub-2 µm particles. In addition we will investigate the effect of increased resolution separations on the identification of proteins in a complex sample.

Methods

LC Instrumentation: A direct flow nanoLC system (NanoLC-Ultra 2D, Eksigent Technologies) was used for all experiments.

Separations: 75 µm ID nanoLC columns of different lengths, packed with 3µm particles with 120Å pores (ChromXP C18CL, Eksigent Technologies) were used. A 25 cm nanoLC column packed with 1.7 µm 128 Å pore size particles was obtained from Waters (Milford, MA, USA). Mobile phases were water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). Linear gradients from 3-35% B were run between mobile phases A and B. Flow rate was 250 nL/min.

Samples: Separations were conducted on either a tryptic digest of BSA (Michrom, Auburn, CA, USA) (0.2 pmol/µl), or a tryptic digest of E. coli (0.5 µg/µl). Injection volume was 1 µl.

MS Instrumentation: An LTQ ion trap mass spectrometer (Thermo Fisher, San Jose, CA, USA) was used with a Picoview nanospray source with 20µm ID capillary /10µm ID tips (New Objective, Woburn, MA, USA). The MS method for the E. coli samples consisted of one MS scan followed by 4 MS/MS scans on the 4 most intense precursors. Dynamic exclusion was used (repeat count 2, 30 sec. duration, 15 sec. exclusion)

Data analysis: Peaklists we generated using Mascot Distiller (Matrix Sciences, London, UK). Database searching was performed using ProteinPilot (Applied Biosystems, Foster City, CA, USA) against the UniProt database.

High Pressure nanoLC pump

The nanoLC-ultra pump used for the work presented in this poster builds on previous designs using Microfluidic Flow Control (MFC). In MFC, real-time feedback control is used to maintain accurate flow rates at nanoliters per minute to generate accurate and reproducible gradients for chromatography. Flow meters in each mobile phase path continuously monitor flowrate and feed a signal back to a microprocessor control system. The NanoLC-Ultra system can be used to generate highly reproducible gradients at flow rates from 50-500 nL/min with column pressures as high as 10,000 psi. Retention time reproducibility is illustrated for a BSA digest on a 80 cm long column (figure 1). At pressures up to 10,000 psi reproducibility was better than 0.3% RSD.



Figure 1. Reproducibility for a 3-35%B gradient in 80 min of a BSA tryptic digest on a 80 cm column. Maximum backpressure was 9,800 psi

Maximizing Peak Capacity

When maximum peak capacity is required in gradient separations, gradient slope is reduced (i.e., the total gradient time is increased), and/or column length is increased. In order to determine the optimal gradient length for a certain column length, we have derived earlier¹ the relationship between peak capacity and gradient length for different column lengths and particle sizes.

The dependence of peak capacity n_c on column length L and plate height H can be calculated with $n_c = \sqrt{(L/H) / (1 + a \cdot L/t_g)}$ where $a = S\Delta\phi/u$ with S is solvent strength, $\Delta\phi$ is change in gradient composition, u is mobile phase velocity and t_g is gradient time.

Figure 2 graphs peak capacity as a function of gradient time for various column lengths and particle sizes. $H = 7.5 \mu\text{m}$ for columns packed with 3 µm particles and 4.5 µm for columns packed with 1.7 µm particles. For "a" a value of 2 sec/mm was used. From this figure the gain in peak capacity with longer columns is shown, and it can be seen that gradient length needs to be adjusted to fully benefit from using longer columns. In addition it can be seen that a 25 cm column packed with 1.7 µm particles is the best choice for gradients up to 50 min length, while for longer gradients a 60 cm column packed with 3 µm particles offer better peak capacity.

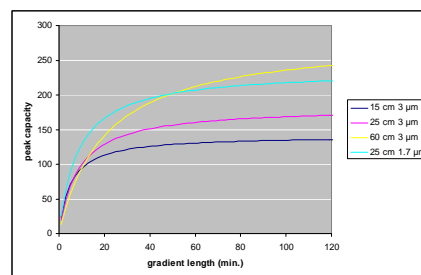


Figure 2. Calculated Peak Capacity plotted as a function of gradient length for different length columns and particle sizes

Figure 3 demonstrates the gain in peak capacity that can be obtained using different columns lengths and particle sizes using a 50 min gradient. The observed peak capacities are in good correlation with the theoretical values that are plotted in figure 2. In figure 3b extracted ion chromatograms are shown for two closely eluting peptides (m/z 582.5, and 474.2). It can be seen that peak widths are decreasing using a longer column or column packed with smaller size particles.

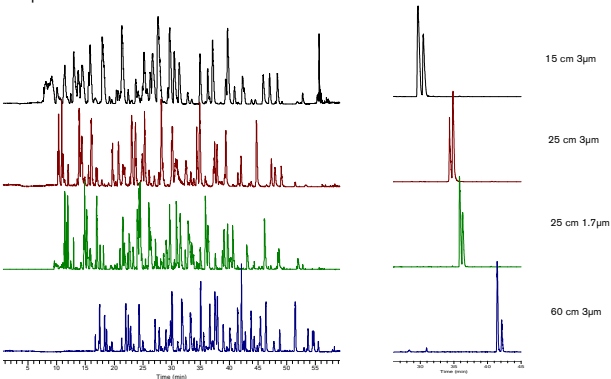


Figure 3a. Separation of a BSA digest using a 50 min gradient on different length and particle size columns

Figure 3b. Extracted ions m/z 582.5 and 474.2 from 3a.

Effect of resolution on peptide identification

To study the effect of increased peptide separation an E.coli digest was analyzed on columns of different length and particle size. Both the 25 cm 1.7 µm column and 60 cm 3 µm column required a max. pressure of ca. 7,000 psi. Gradients from 3-35%B with lengths of 30 min, 50 min and 120 min were used. The results of the database searches are summarized in figure 4. Keeping gradient length constant, the number of peptides and proteins identified increases going from a 15 cm 3 µm column to a 25 cm 1.7 µm column. The 60 cm 3 µm column, although providing similar peak capacity as the 25 cm 1.7 µm when tested with a BSA digest using the 50 min gradient, did result in a lower number of peptides and proteins identified. The 25 cm 1.7 µm column gave the highest number of identifications with each of the gradients tested.

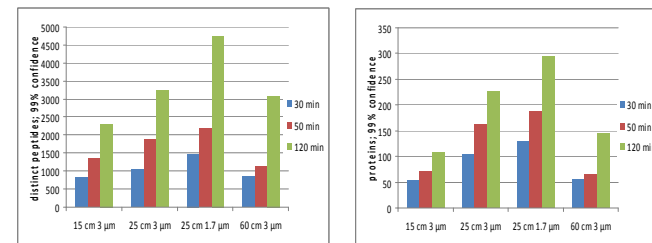


Figure 4 Number of distinct peptides and proteins identified by ProteinPilot with different column lengths/particles and gradient lengths

Conclusions

Theory shows that in order to maximize the increase in peak capacity for reversed phase peptide separation using longer columns, gradient length needs to be increased proportionally with the increase in column length.

Experimental results are in good correlation with the theoretically derived peak capacities for different length columns and particle sizes

Using the same length gradient (and MS acquisition time), increasing the column length from 15 to 25 cm and/or decreasing particle size clearly increases the number of identified peptides. When compared to a 25 cm column packed with 1.7µm particles, a 60 cm column packed with 3 µm particles produced fewer peptide and protein ID's, even though the peak capacity appeared to be similar. The reasons for this are under investigation.

Acknowledment: The authors would like to thank Christie Hunter at Applied Biosystems (Foster City, CA, USA) for the E.coli tryptic digest sample preparation and assistance with the ProteinPilot database searches.

1) Remco van Soest*, David W. Neyer; Jia Eng Siow, and Phil Paul, Eksigent Technologies, Dublin, CA; poster ASMS 2008: "Improving resolution in nanoLC separations for proteomics using ultra high pressures".