

Improving resolution in nanoLC separations for proteomics using ultra high pressures

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Abstract

Eksigent Technologies has developed a splitless nanoLC system that addresses three of the main demands for high resolution separations—low gradient delay volume, reproducible gradient delivery, and operating pressures up to 10,000 psi.

The use of this system for increasing peak capacity of peptide separations are demonstrated, and the effect of gradient slope and columns length are discussed.

Introduction

Nanoflow liquid chromatography coupled with nanoelectrospray mass spectrometry (nanoLC-MS) has proven to be an invaluable tool for sensitive peptide and protein analysis. As the demand for more complex proteomic analyses grows, instrumentation and experimental methods that support higher resolution peptide separations are required. We present new hardware developments and columns for improving the resolution of nanoLC separations, while maintaining the benefits of reproducible separations and high mass sensitivity.

Methods

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

Separations: 75 μ m ID nanoLC columns of different lengths, packed with 3 μ m particles with 120Å pores (ChromXP C18CL, Eksigent Technologies) were used. Mobile phases were water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). Linear gradients from 3-30% B were run between mobile phases A and B.

Samples: Separations were conducted on either a peptide test mixture (Eksigent) that contains ILY-TYR, VAL-TYR-VAL, Methionine Enkephalin Acetate, Leucine Enkephalin, and Angiotensin II Acetate (1 ng/ μ l each), or a tryptic digest of BSA (Michrom Bioresources, Inc.) (0.2 pmol/ μ l). Injection volume was 1 μ l.

MS Instrumentation: An LCQ ion trap mass spectrometer (Thermo Fisher, San Jose, CA) was used in MS mode with a Picoview nanospray source with 20 μ m ID capillary /10 μ m ID tips (New Objective, Woburn, MA).

High Pressure nanoLC pump design

The new 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 (see diagram). A fast-response pressure source (a pneumatic amplifier) is used to maintain the programmed flow rates. Using a new design for the pressure source, along with changes to the flow rate measurement and control system, the operating pressure range for the system has been significantly increased. 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.

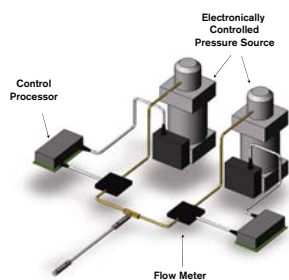


Figure 1. Schematic of the Microfluidic Flow Control (MFC) system for gradient generation.

Based on the measurements of flow meters in each mobile phase, a microprocessor based controller makes real-time adjustments to the electronically controlled pressure sources to maintain accurate and reproducible flow rates and gradient composition profiles.

Reproducible separations at up to 10,000 psi

Retention time reproducibility in nanoLC-MS is becoming increasingly important when using techniques such as Accurate Mass and Time tags and biomarker validation using scheduled MRM's. Retention time reproducibility was evaluated using a peptide standard with a 10 cm column and an additional restrictor (figure 2), and a BSA digest on a 80 cm long column (figure 3). At pressures up to 10,000 psi reproducibility was better than 0.3% RSD.

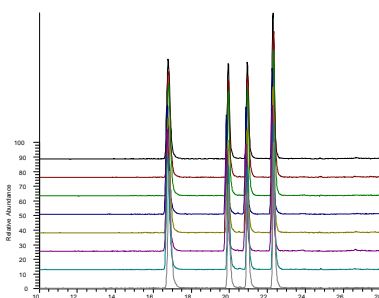


Figure 2. Reproducibility for a 5-35%B gradient in 15 min of the peptide mix standard at 200 nl/min flow rate.

Total maximum backpressure after the injection valve was 9,000 psi (restrictor + column). Reproducibility was < 0.3% for all peptides.

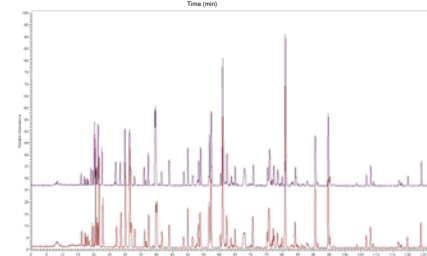


Figure 3. Reproducibility for a 3-30%B gradient in 80 min of the BSA tryptic digest. Column used was 75 μ m x 80 cm; flow rate 300 nl/min and maximum back pressure 9,800 psi.

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 derived the relationship between peak capacity and gradient length for different column lengths.

Peak capacity, n_c , is given by $n_c = 1 + (t_{r,last} - t_{r,first})/W$, where t_r is retention time and W is the average peak width. From Linear Solvent Strength Theory [1] the retention time can be estimated from $t_r = t_0 + (t_0/b) \ln(1+bk_0)$. Here t_0 is the retention time of an unretained component, k_0 is the capacity factor at gradient starting conditions, and $b = S\Delta\phi / t_0$ where S is the solvent strength, $\Delta\phi$ the change in gradient composition and t_0 the gradient time. Peak width can be estimated from $W = 4t_0(1+k_0/(1+bk_0)) / \sqrt{N}$, where N is the number of plates of the column. For $bk_0 \gg 1$, the peak capacity can be estimated from $n_c = \sqrt{N} \ln(k_{0,last} / k_{0,first}) / (4(1+b))$.

For a given set of compounds and a given velocity, the peak capacity approaches a maximum of $\sqrt{N} \ln(k_{0,last} / k_{0,first}) / 4$ for $t_0 \gg S\Delta\phi$ (that is, for $b \ll 1$).

Both N and t_0 are proportional to column length. To maximize peak capacity for a given column length, the gradient time needs to be increased to a point that there is minimal increase in peak capacity (thus satisfying the condition $t_0 \gg S\Delta\phi$). Doubling the column length offers a $\sqrt{2}$ increase in peak capacity and doubles t_0 . To achieve this increase in peak capacity requires doubling the gradient time.

With $N = L/H$ and $t_0 = L/u$, with u is the mobile phase velocity, the functional dependence of peak capacity on column length can be seen by plotting $n_c' = \sqrt{(L/H)} / (1 + aL/t_0)$ where $a = S\Delta\phi/u$. Figure 4 graphs n_c' as a function of gradient time for various column lengths and is shown plotted for typical values of $H = 7.5 \mu$ m and $a = 2$ sec/mm. 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.

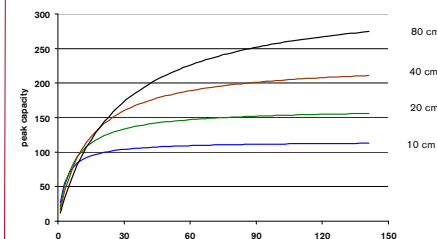


Figure 4. Peak Capacity plotted as a function of gradient length for different length columns.

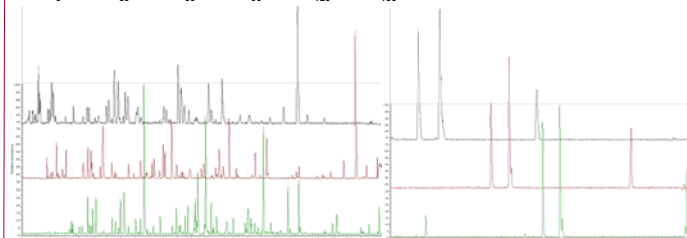


Figure 5a. Separation of a BSA digest using a 120 min gradient on a 10 cm (top trace, 40 cm (middle trace) and 80 cm (lower trace) column.

Figure 5b. Extracted ion chromatograms from 5a. See text for details.

Figure 5a shows the gain in peak capacity that can be obtained going from a 10 cm to a 80 cm column using a 120 min gradient. The observed peak capacities are in good correlation with the theoretical values that are plotted in figure 4. In figure 5b extracted ion chromatograms are shown for three main components (m/z 582.7, 474.8 and 1015.5) and two less abundant species (468.7 and 1057.4) It can be seen that peak widths are decreasing going from the 10 cm to the 80 cm column, and that species with low abundance not separated on the 10 cm column can be resolved using the longer columns.

Conclusions

A splitless nanoLC system that can deliver precise gradients at 10,000 psi backpressure has been constructed.

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.

NanoLC columns up to 80 cm in length in combination with increased gradient length can separate low abundance species that cannot be separated with shorter columns.

The effect of peptide resolution on peptide ID in complex mixtures is under investigation.

[1] L.R. Snyder, J.J. Kirkland, J.L. Glajch, *Practical HPLC Method Development*, second ed., Wiley-Interscience, New York, 1997.