Optimizing Particle Size and Column Length: What Is the Best Way to Utilize Nano UHPLC in Proteomics?

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Introduction

The development of UHPLC capabilities on the nano LC scale has opened up new ways to provide proteomics researchers with the peak capacity required for their work. UHPLC principles involve the use of smaller particles which allow faster analysis and/or better resolution, usually focused on throughput in small molecule analysis. However, in proteomics analysis speed is of lesser concern compared to peak capacity. The increased pressures in UHPLC can be used to support increased column lengths as well. With 25 and even 50 cm columns being implemented in routine applications, the one-meter barrier has become visible. Here, the full potential of nano UHPLC is utilized to determine the effects of smaller particles and column length on protein identification.

Theory

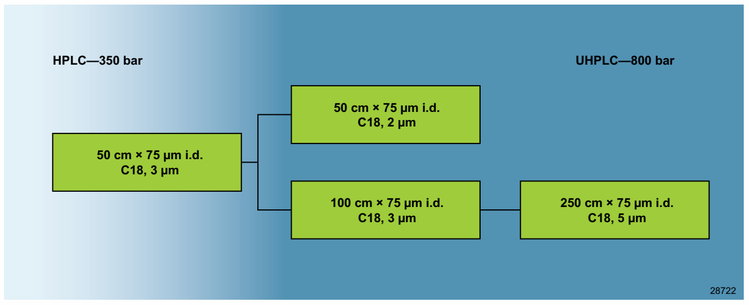
For many years, nano LC equipment in proteomics has been used with reversed-phase (RP) columns packed with 3 µm diameter particles. The most commonly used column length is 15 cm, but longer columns of 25 cm and even 50 cm length can be operated easily within the limited pressure range (350–400 bar) of most nano LC equipment.

Whereas conventional-scale UHPLC equipment is used at its full potential, the emerging nano UHPLC instrumentation is generally used as a replacement for the same analysis. As a result, the full potential of nano LC is not utilized, exploiting the pressure capabilities to support smaller particles or increased column lengths.

The backpressure of columns increases proportionally to the square of the particle size. This means that the available UHPLC nano column of 50 cm length with 2 µm particles should be comparable to a 9/4 longer column packed with 3 µm particles. When using a 5 µm particle, a column length 25/4 times longer should produce a similar backpressure.

Figure 1 shows how the pressure potential of a nano UHPLC system can be fully utilized by using the same column length with a smaller particle, or by using the same particle with increased length. All columns have been designed to operate around the maximum pressure of the systems. Naturally, the packing of columns with such length presents enormous challenges.

FIGURE 1. How to use the full potential of nano UHPLC: use the same column length with a smaller particle, or use the same particle with a longer column.



Experimental

The 50 cm columns used are commercially available products, but the 1 and 2.5 m columns were prototypes prepared specially for this project. All separations were performed on a Thermo Scientific Dionex UltiMate 3000 RSLCnano system with an upper pressure limit of 800 bar. The columns were connected using Thermo Scientific Dionex nanoViper fingertight fittings to ensure ability to withstand UHPLC pressures without damaging the columns due to overtightening.

Column Performance

The column performance was tested with an isocratic separation of a two-component mixture (uracil and pyrene) under various flow rates. The peak shape and retention time were used to calculate the plate number and height for the tested column. This determined how well the columns were packed and how their performance corresponded.

Separation Performance

The separation performance was tested by separating 500 fmol bovine serum albumin (BSA) tryptic digest with varying gradient lengths (120, 240, 360, and 480 min) at the same flow rate (275 nL/min). Standard mobile phases—A) water and B) 80% acetonitrile—with TFA as an ion-pairing agent, were used for the separation. TFA was chosen because it is generally accepted to provide the best separation performance.

LC-MS/MS analysis was performed with the 2.5 m long column and an *E. coli* tryptic digest with 360 and 600 min gradients.

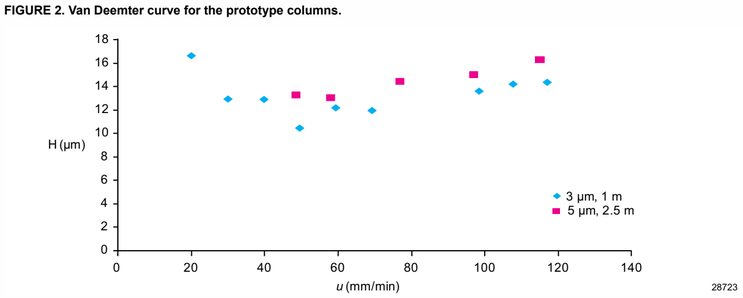
Results

Column Performance

Efficient packing of nano columns is a difficult task, particularly when very long formats (50 cm and longer) are required. In this work, the quality of the columns was assessed by isocratic elution of test compounds. The measurements demonstrated the efficiency of the column packing and found the average plates/meter for the columns packed with 5, 3, and 2 µm particles to be 120,000, 150,000, and 180,000 respectively. For the 2.5 m column, up to 288,000 theoretical plates were measured, which demonstrates that even very long columns can be packed with excellent quality (values obtained with unretained compound).

Figure 2 shows the plate height number of a retained test compound (retention factor = 1.15) measured close to the optimum flow. The minimum plate height numbers were measured within 40 and 90 mm/min, which correspond roughly to 100–275 nL/min.

FIGURE 2. Van Deemter curve for the prototype columns.

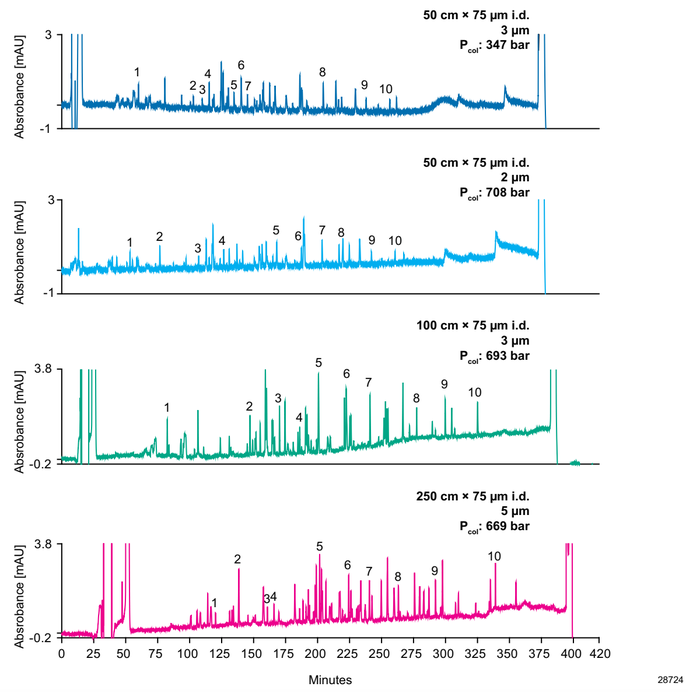


Gradient Performance

The column performance evaluation shows the potential of long columns, but only in their true application can this potential be realized. A measure for gradient separation efficiency is a parameter such as peak capacity, which represents the number of peaks that can fit in a certain gradient window.

Figure 3 shows the 500 fmol BSA tryptic digest separation on the four different columns (Figure 1) with the 360 min gradient. In each chromatogram 10 peaks were chosen for the evaluation of peak capacity. The peaks were selected based on their peak shape to exclude a bias caused by coeluting peaks.

FIGURE 3. Comparison of 360 min gradients with varying column lengths and particle sizes (described in the figure). The pressures are the maximum values that were observed during the analysis.

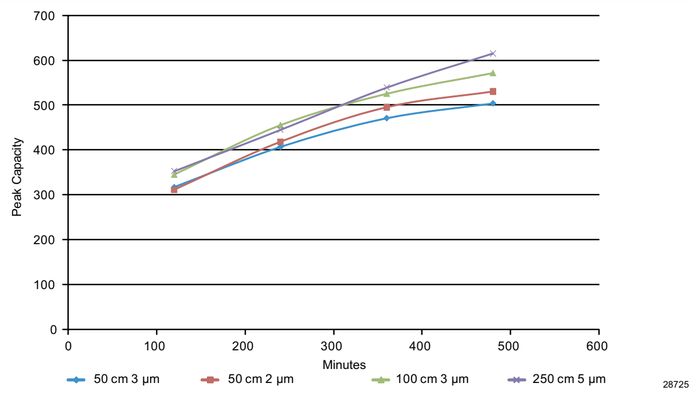


The peak capacity (nc) is based on the gradient time and the peak width at 13.4% height, generally applied in the relationship: nc = 1 + gradient time/peak width. The average peak widths at 13.4% of the peak height were 45.7, 43.7, 41.2, and 40.1 seconds for the runs depicted in Figure 3.

The peak capacities for these columns were determined for various gradient lengths, and the overview is given in Figure 4. It is known that gradient length should be matched to column length, therefore 120 min is considered the short gradient time for these long columns.

Figure 4 shows that overall the column length is the larger contributor to separation power. Even with the short gradients, the difference is visible between 50 cm and longer columns. The difference between the 100 cm and 250 cm column only becomes visible with very long gradients. Peak capacity plots generally show a parabolic curve, where the longer gradients will reach a plateau in peak capacity. The additional column length will allow a longer gradient before this plateau is reached.

FIGURE 4. Comparison of obtained peak capacities with gradient variation on different column lengths.



The gradients could be extended more for the 100 and 250 cm column to allow more differentiation, but this would provide gradient lengths that are beyond practical use. In real life applications, the connection to the mass spectrometer data file size, repetition of analysis, and throughput have to be considered as well.

Dilution Effects

Another, often overlooked aspect in peak capacity evaluation is peak dilution. The peak widths will increase with gradient lengths, and this increase in peak width with the same injected amount will result in dilution of the eluting peak. This can affect sensitivity in sample-limited cases. Figure 5 compares the same column with various gradient lengths. The intensity scale remains constant to illustrate the effect of peak dilution. Figure 6 shows the average peak height and peak area values of the marked peaks. The peak height clearly decreases, whereas the peak area remains relatively constant. This proves the injected amount is the same, whereas intensity is dropping.

FIGURE 5. Separation of BSA digest with 120, 240, 360, and 480 min gradient on the 100 cm long column. Peak numbers mark the peaks used for data evaluation.

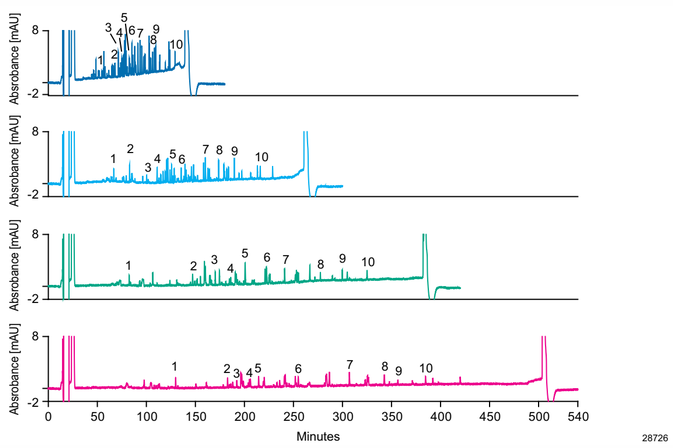
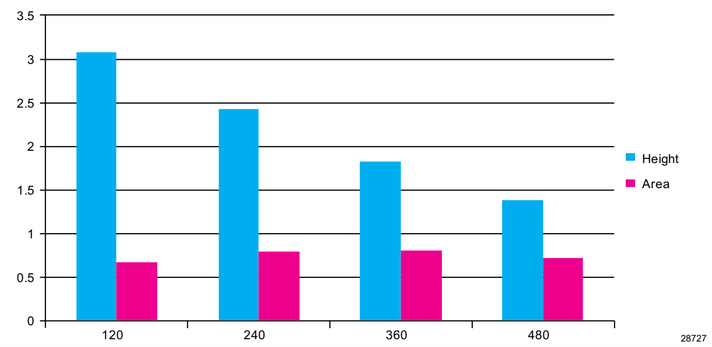


FIGURE 6. Comparison of average peak height and width for the different gradient separations depicted in Figure 5.



E. coli Separations

The BSA tryptic digest is an ideal sample to determine column characteristics, although it does not resemble the typical samples analyzed in proteomics. Therefore an *E. coli* tryptic digest was separated on the 2.5 m long column with various gradients. Gradients of 360, 600, and even 1440 min (24 h) were used to separate the sample. This immediately presented one of the practical limitations: the MS data file from the 24 h gradient was so large (cut off over 2 GB) that it could not be opened and processed. However, from the UV data, a peak capacity in excess of a 1000 was determined for this separation.

The 360 and 600 min gradients were searched against the Swissprot database with a 99% confidence and minimum of two peptides identified. The resulting protein and peptide identifications are shown in Figure 7.

FIGURE 7. Venn diagrams for the protein and peptide identifications.

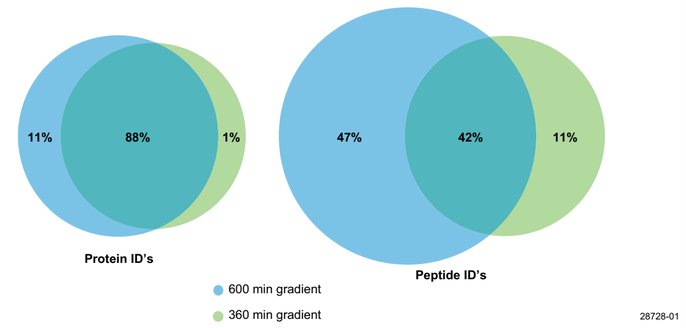
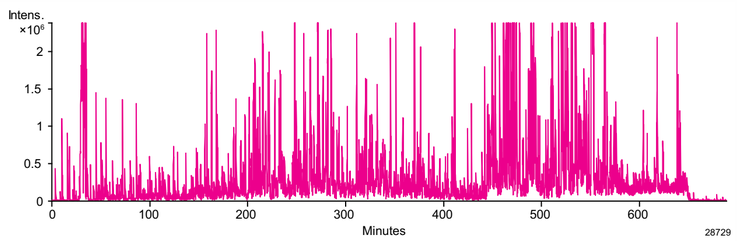


Figure 7 shows that although there are more proteins identified in the 600 min gradient, the major difference is found in the peptide identifications. The improved separation resulted in a higher sequence coverage, which was on average 77% higher for the longer gradient. This phenomenon is observed more often and is easily explained by the natural abundance differences in the sample and peak dilution effects. Peptides from a high-abundance protein will be affected less by peak dilution.

Figure 8 below shows the base peak chromatogram of the 600 min gradient of the *E. coli* tryptic digest.

FIGURE 8. Base peak chromatogram of the separation of *E. coli* tryptic digest on the 250 cm column with a gradient from 4–65% B in 600 min at a flow rate of 275 nL/min.



Conclusions

- Nano UHPLC instrumentation is optimally used with longer columns rather than smaller particles if separation power is the primary focus. Smaller particles can be used to increase throughput.
- Extremely long columns of more than 1 m provide excellent separations, but are generally outside practical usability for most applications due to their equally long run times and data file sizes.
- Extending the gradient will improve separation power, but will result in peak dilution, which must be considered when developing a method. Samples with relatively equal abundance of species will benefit the most from the improved separation power.
- All presented nano UHPLC separations operate at pressures close to or exceeding 700 bar; nanoViper™ fingertight fittings offer easy configuration and reliable operation under these conditions.

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