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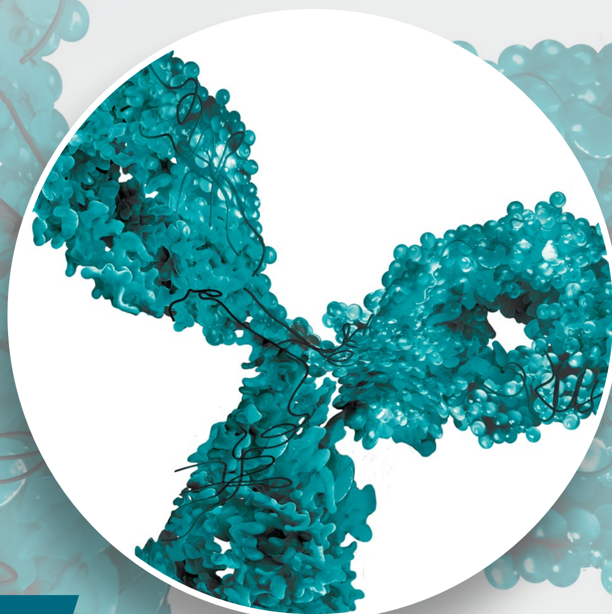

Peptide separation

Peptide separation on µPAC HPLC columns

Part 1: Cytochrome c tryptic digest

Keywords
Proteomics, tryptic digest, nano liquid chromatography (LC), chip based separation, low-flow, cytochrome c, µPAC

Goal

Demonstrate the gain in chromatographic performance that can be achieved for protein tryptic digest samples by using Thermo Scientific™ µPAC™ HPLC columns.

Introduction

Reversed-phase cytochrome c tryptic digest separations are routinely employed for the analysis/quality control of high performance liquid chromatography (HPLC) column performance towards proteomics applications. Easily detectable in UV and with few components, this sample proves to be an ideal tool in demonstrating column performance towards more complex digest separations requiring mass spectrometry (MS) detection. Not surprisingly, peak capacity values obtained for the separation of cytochrome c tryptic digest on reversed-phase liquid chromatography (LC) columns are often cited by different LC column vendors.

The unique properties of the µPAC HPLC columns enable very high separation resolution at moderate operating pressures.[1-2] The focus of this product spotlight is to illustrate the performance and versatility of a 200 cm µPAC column for peptide separations.

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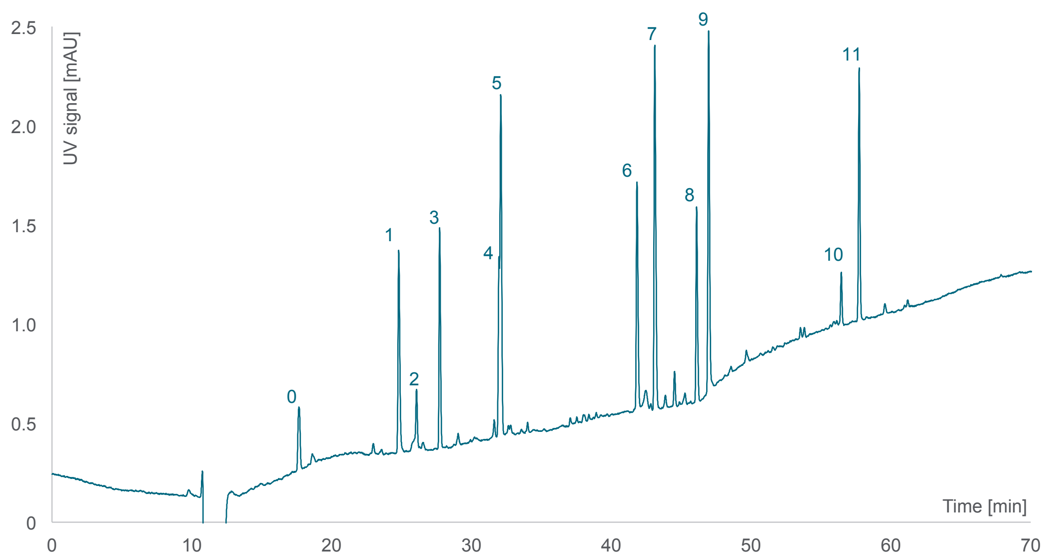

Figure 1. Example chromatogram of a cytochrome c digest separation on a 200 cm µPAC column. Sample: 0.5 µM cytochrome c tryptic digest; Gradient slope: 1–50% B in 60 min; flow rate: 900 nL/min; UV detection: 214 nm. The peptide amino acid sequence and theoretical monoistopic masses accompanying the identified peaks are listed in Table 1.

Table 1. List of identified peptide peaks with their corresponding amino acid sequence and theoretical monoisotopic mass.

Analytical columns	
Thermo Scientific µPAC HPLC	200 cm bed length, 18 µm pillar length
Packed bed	75 µm × 15 cm C18, 2 µm, 100Å

Peak number	Amino acid sequence	Theoretical mass (monoisotopic)
0	Ac-GDVEK	588.3
1	YIPGTK	677.4
2	KYIPGTK	805.5
3	IFVQK	633.4
4	KTGQAPGFSYTDANK	1538.8
5	TGQAPGFSYTDANK	1455.7
6	MIFAGIK	778.4
7	TGPNLHGLFGR	1167.6
8	GEREDLIAYLKK	1433.8
9	EDLIAYLKK	963.5
10	GITWGEETLMEYLENPKK	2137.0
11	GITWGEETLMEYLENPK	2137.0

Results and discussion

500 fmol of cytochrome c tryptic digest was injected on a 200 cm µPAC column and separated using five different gradient slopes at flow rates ranging from 300 to 900 nL/min. A chromatogram obtained for a 60 min gradient separation at a flow rate of 900 nL/min is shown in Figure 1. Peak analysis demonstrates that peaks with an excellent symmetry (asymmetry value 1.2) and average widths (4σ) as low as 0.22 min can be obtained on the µPAC columns.

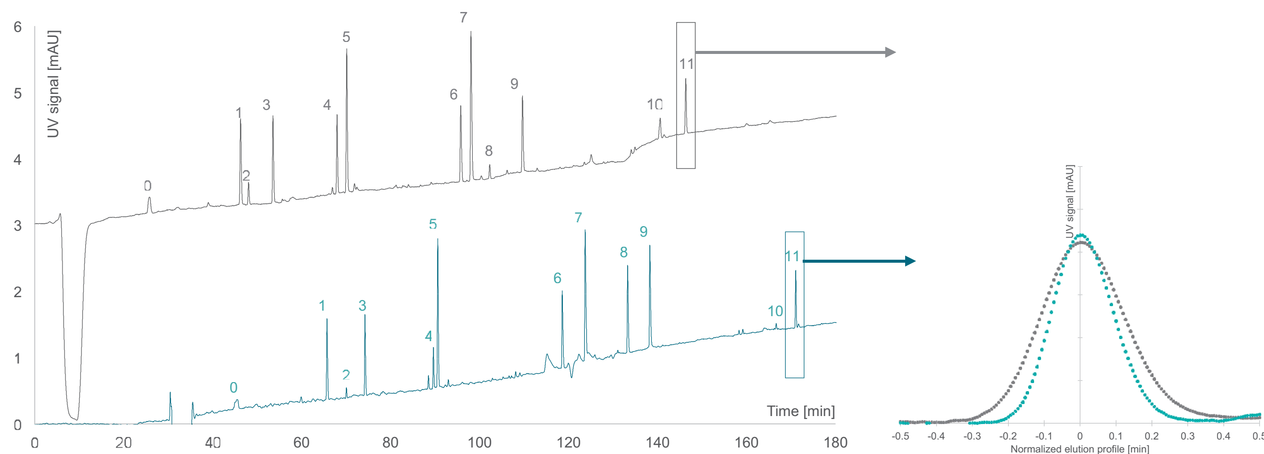

Figure 2. Comparison of a cytochrome c digest separation performed on a packed bed (grey) and on a µPAC (teal) column. Sample: 0.5 µM cytochrome c tryptic digest; gradient slope: 1–50% B in 180 min; flow rate: 300 nL/min; UV detection: 214 nm. The peptide amino acid sequence and theoretical monoistopic masses accompanying the identified peaks are listed in Table 1. The normalized elution profile of peak 11 is shown to the right for both column types.

Whereas traditional packed bed nano liquid chromatography (LC) columns are limited in separation length due to instrument pressure limitations, the unique µPAC column format allows operating columns with an exceptional length at moderate pressures. The benefit of this increase in separation length becomes apparent when long gradient times are applied. This is demonstrated in Figure 2, where an 180 min gradient separation on a µPAC column is being compared with a commercial column routinely used for peptide analyses.

A clear comparison in performance can be made by plotting the average peak width as a function of gradient time (Figure 3). The rate at which peptide peak width increases as a function of gradient time is much lower on the µPAC column. For long gradient times (>120 min), this results in a 19% reduction of the average peptide peak width.

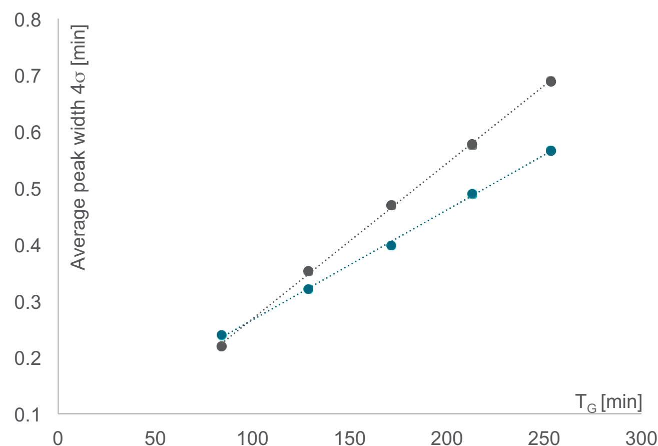

Figure 3. Comparison of the 0.8 average peak widths (4σ) obtained for gradient separations of cytochrome c tryptic digest. Grey: packed bed column, teal: µPAC column. Peak 1, 3, 4, 5, 6, 7, 9, and 11 listed in Table 1 have been used to calculate the average peak width (n=8). Sample: 0.5 µM cytochrome c digest; gradient slope: 1–50% B in 60, 120, 180, 240 and 300 min; flow rate: 300 nL/min; UV detection: 214 nm.

The high permeability of the µPAC column format also permits operation over a wide range of flow rates with limited LC pump pressure requirements (<300 bar), whereas the pressure required for packed bed columns sets substantial restrictions on their operating versatility and potential towards future generations. This is clearly demonstrated in Figure 4, where LC pump pressures needed to operate the different columns are compared.

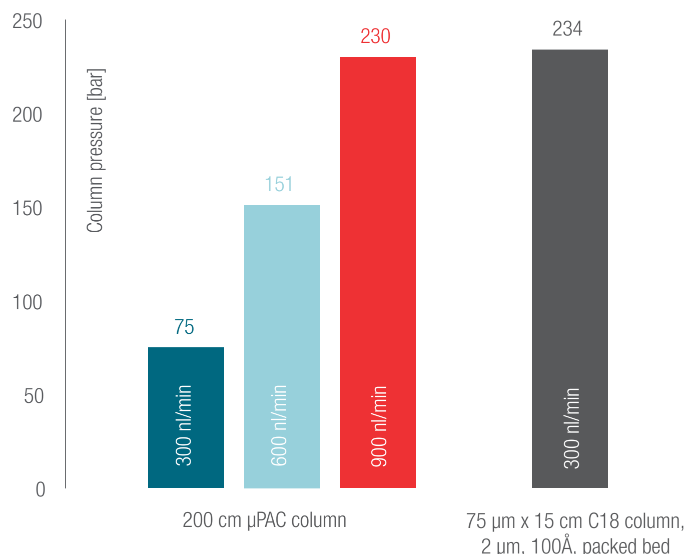

Figure 4. Comparison of the maximum column pressure needed to perform a gradient separation. The packed bed column (grey) operated at 300 nL/min. The µPAC column operated at three different flow rates: 300, 600, and 900 nL/min.

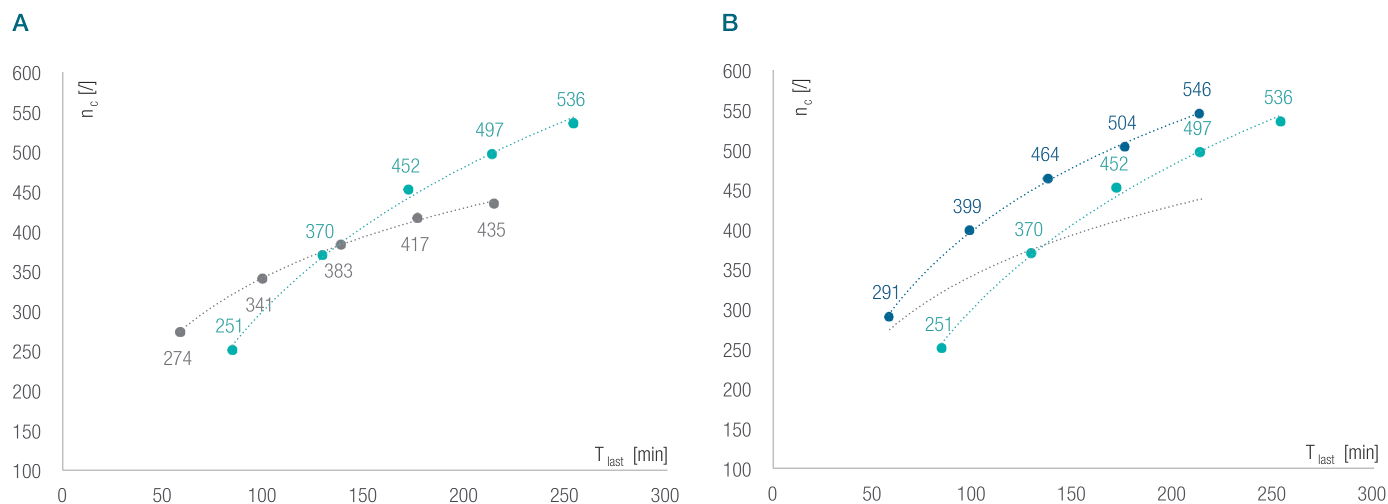

Figure 5. Comparison of the peak capacity (n_c) obtained for the gradient separation of cytochrome c digest. n_c has been plotted as a function of T_{last}, the time at which the last peptide peak elutes. A) A packed bed column (grey) is compared to a µPAC column (green) at a flow rate of 300 nL/min. B) n_c values obtained on a µPAC column at 900 nL/min (blue) is added to the curve. Peak capacity is calculated according to equation (1), based on peaks 1, 3, 4, 5, 6, 7, 9, and 11 listed in Table 1. Sample: 0.5 µM cytochrome c digest; gradient slope: 1–50% B in 60, 120, 180, 240, and 300 min; flow rate: 300 and 900 nL/min; UV detection: 214 nm.

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By plotting peak capacity as a function of the elution time of the last component (t_{last}), a fair comparison towards chromatographic performance per time unit can be made between column types and flow rates. For each condition, peak capacities were calculated according to equation (1) using the peak width of 8 baseline separated tryptic peptides (peptide 1, 3, 4, 5, 6, 7, 9, and 11, listed in Table 1).

$$n_c = 1 + \frac{t_g}{(\sum_1^i 4.\sigma_i)/i} = 1 + \frac{t_g}{\overline{w}_{13\%}}$$

A significant gain in peak capacity per time unit can be observed at a flow rate of 300 nL/min, with an increase up to 14% (Figure 5A). When the time needed to achieve a certain peak capacity is taken into account, an even more striking gain in peak capacity per time unit can be achieved by increasing the flow rate to 600 or 900 nL/min. At these flow rates, a net gain in peak capacity up to 25% can be obtained (Figure 5B).

Conclusions

µPAC column technology is presented as a versatile and high performant alternative for the separation of complex peptide samples by reversed-phase liquid chromatography.

- A significant decrease in average peak width is observed for long gradient times (>120 min)
- An increase in peak capacity of 25% can be obtained compared to 15 cm long packed bed alternatives
- High column permeability allows operation over a wide range of flow rates (100–1000 nL/min)
- Peak capacity per time unit can be boosted by operating at elevated flow rates

References

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