Product spotlight | 000475

Proteomic profiling

Bottom-up proteomic profiling with µPAC HPLC columns

Keywords
Micro pillar array column, µPAC, microfabrication, capillary LC, low-flow LC, RPLC-MS/MS, gradient separation, column performance, peak capacity, bottom-up proteomics

Abstract
In this product spotlight, the performance of Thermo Scientific™ µPAC™ HPLC columns is evaluated in a typical capillary-flow bottom-up proteomics workflow. 500 ng of tryptic digest sample (HeLa cell lysate) was injected and separated using reversed-phase liquid chromatography over a range of flow rates (2.5–10 µL/min) and solvent gradients (15–60 min). Excellent chromatographic performance could be achieved resulting in an average increase in protein identifications of 20% compared to packed bed alternatives.

Key features of µPAC columns

Flow rate flexibility
1 to 15 µL/min—corresponding column backpressures of respectively 19 and 300 bar—maximum operating pressure is 350 bar.

Column robustness
Each column has been manufactured by etching channels out of a solid piece of silicon, the column is perfectly bi-directional and contains neither particles or frits.

Column-to-column reproducibility
Each column is manufactured using the same lithographic mask, making every column identical.

Separation performance
Peak capacity values above 200 can be obtained with short (30–90 min) gradient separations.

Introduction

Mass spectrometry-based proteomics has become an essential tool in biological, biomedical, and biopharmaceutical research. Due to the increased sensitivity that can be achieved compared to analytical flow liquid chromatography mass spectrometry (LC-MS), the majority of LC-MS based proteomics research is performed within the nanoflow liquid chromatography regime, with flow rates typically below 1 µL/min and total run times exceeding 60 min. Compared to the LC-MS methods employed in other 'omics' fields, the need for high separation power often prevails over sample throughput, making relatively long separations the gold standard in LC-MS based proteomics. Capillary and microflow LC-MS solutions are gaining interest as the demand for large quantitative proteomics studies that require increased throughput and robustness is growing.[1,2] By working at higher flow rates, the impact of gradient delay and sample loading volumes on the total analysis time can be reduced and low flow rate or column dimension related technical errors (such as electrospray instability, column clogging or the presence of void volumes in the analytical flow path) can be minimized.

A micro pillar array column based solution for capillary-flow LC-MS is presented in this product spotlight. In contrast to packed bed LC columns that contain randomly packed beads as their stationary phase, micro-chip based pillar array chromatography columns have a separation bed of perfectly ordered and freestanding pillars obtained by lithographic etching of a silicon wafer. The regular mobile phase flow pattern through these micro-chip pillar array columns adds very little dispersion to the overall separation, resulting in better peak resolution, sharper elution and increased sensitivity.[3] The free-standing nature of the pillars also leads to much lower back pressure buildup, and makes it possible to operate longer columns.[4]

Experimental

The separation performance of a µPAC column and a commercial packed bed (PB) capillary-flow LC column (150 × 0.300 mm, 2 µm porous silica particles) was evaluated by injecting 500 ng of a tryptic run digest originating from a human cell lysate. For every column, three different gradient times (15, 30, and 60 min) were performed in triplicate and at different flow rates (10, 7.5, 5, 2.5 µL/min).

Analytical columns	
Thermo Scientific µPAC HPLC	50 cm bed length, 28 µm pillar length
Packed bed capillary-flow LC	0.300 mm × 150 mm, 2 µm particles

Results

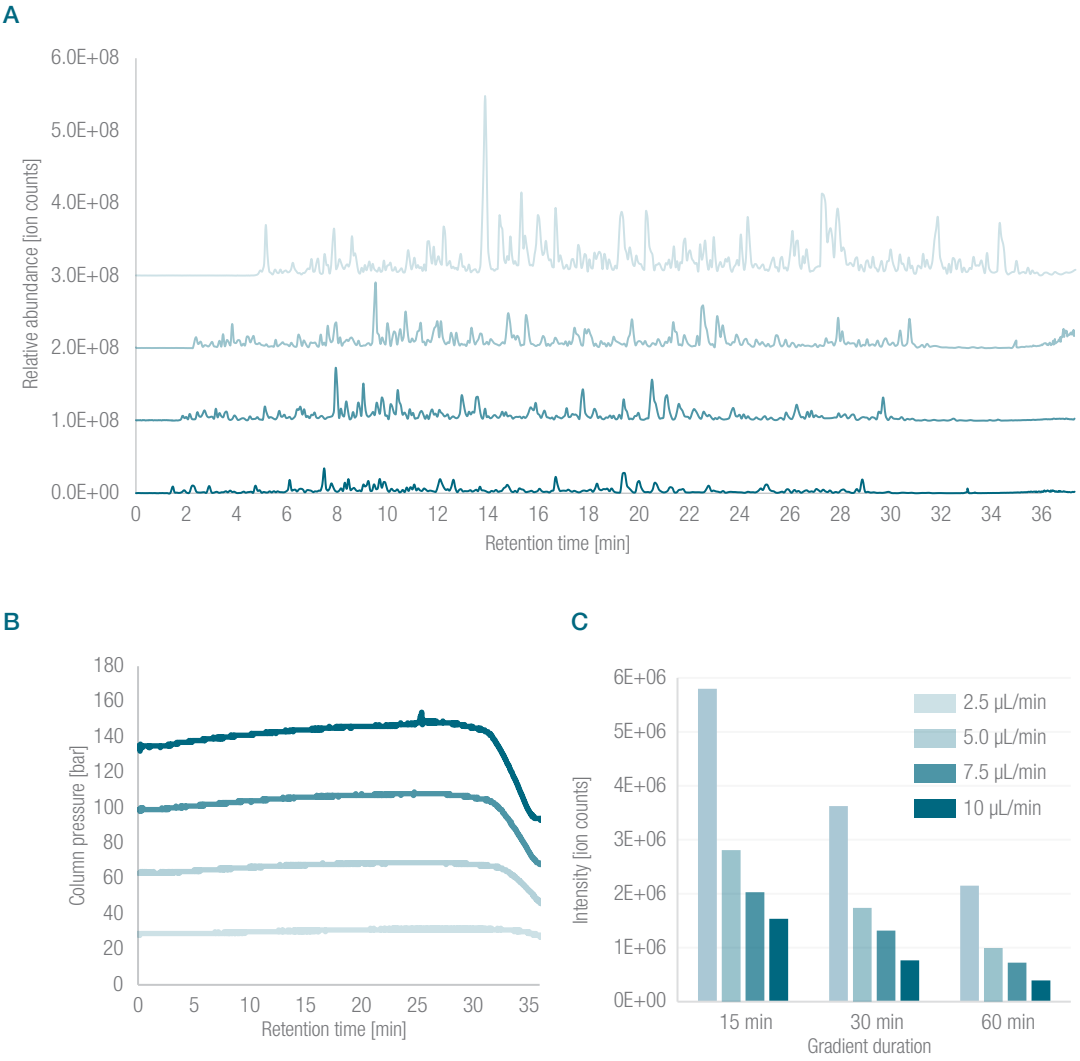


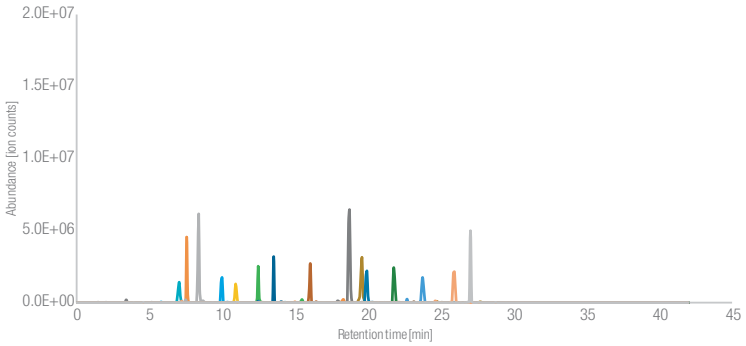
Figure 1. A) Basepeak MS chromatograms of 500 ng of HeLa cell digest for a 30 min gradient separation on a µPAC column at flow rates of 2.5, 5, 7.5, and 10 µL/min (from top to bottom). B) Column backpressure profiles of 30 min gradient separations on a µPAC column at 2.5, 5, 7.5, and 10 µL/min (from top to bottom). C) Mean ion intensities of all identified peptides for 15, 30, and 60 min gradient separations of 500 ng HeLa cell digest performed at 2.5 (light blue), 5, 7.5, and 10 (dark blue) µL/min flow rates on a µPAC column.

The main goal of this product spotlight is to demonstrate the separation performance of the µPAC column and to benchmark the performance against a commonly used conventional packed bed capillary-flow LC column. Rather than aiming for deep proteome coverage, the µPAC column is designed for bottom-up proteomics research where improvements in robustness and throughput are needed aside excellent chromatographic performance.

Capillary-flow LC-MS

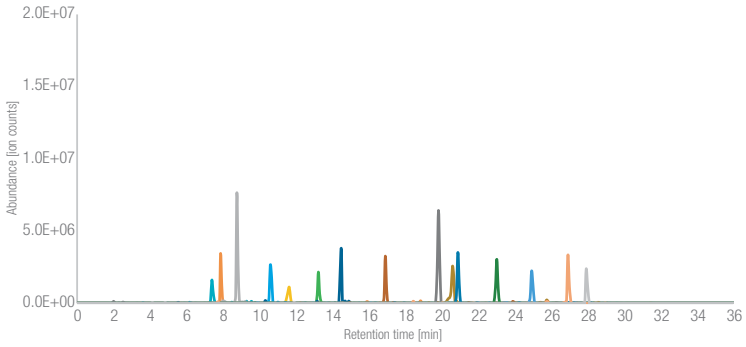
As a consequence of the low column backpressure inherent to µPAC column backbones, chromatographic separation can be performed over a wide range of flow rates (1 to 15 µL/min). MS basepeak chromatograms of 30 minute gradient separations (500 ng HeLa protein digest standard) are shown for 4 different flow rates (2.5, 5, 7.5, and 10 µL/min) (Figure 1A). Whereas the first eluting peptide peaks are observed at a retention time of 5 min at 2.5 µL/min, operating the column at 10 µL/min allows reducing the column void time down to 1 min, which can result in a significant increase of sample throughput for bottom-up proteomics experiments.

A



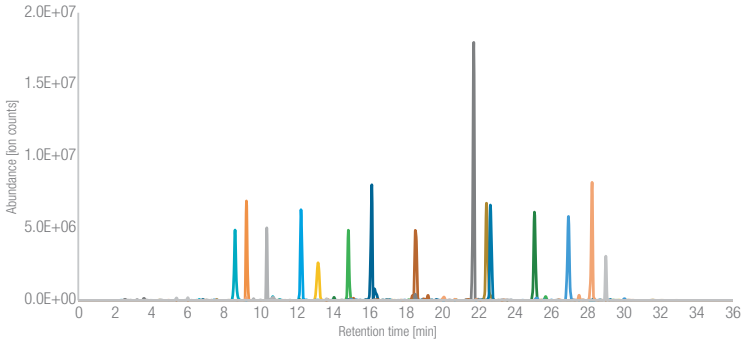
Mass (+2)	RT [min]	10 µL/min FWHM [min]	Area
493.7683	6.50	0.11	1.40E+05
613.3167	7.03	0.07	1.83E+05
496.2867	7.81	0.10	1.61E+06
451.2834	5.96	0.11	3.10E+05
422.7363	7.59	0.10	1.30E+06
695.8324	11.98	0.08	1.40E+05
586.8003	13.17	0.13	6.54E+05
773.8955	15.65	0.11	3.55E+05
558.3259	18.37	0.09	2.68E+05
801.4115	19.23	0.13	4.36E+05
745.3924	19.53	0.14	3.56E+05
498.8018	21.49	0.12	1.73E+05
573.3025	23.41	0.15	2.43E+05
680.3735	25.60	0.08	2.60E+05
787.4212	26.93	0.08	1.35E+05
		0.11	4.38E+05

B



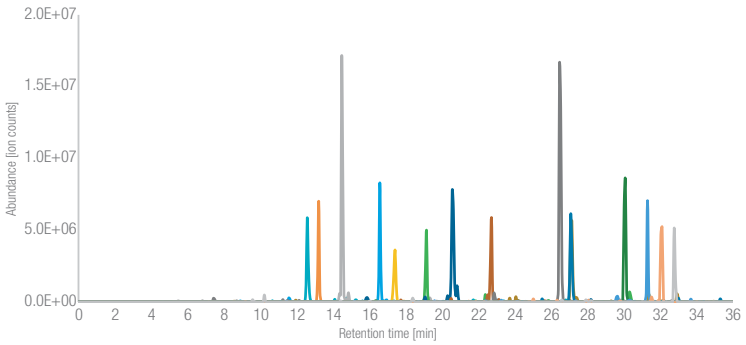
Mass (+2)	RT [min]	7.5 µL/min FWHM [min]	Area
493.7683	7.48	0.12	2.10E+05
613.3167	8.02	0.08	2.76E+05
496.2867	8.84	0.11	2.08E+06
451.2834	7.06	0.10	4.12E+05
422.7363	8.68	0.11	1.73E+06
695.8324	13.33	0.11	1.83E+05
586.8003	9.16	0.12	1.95E+06
773.8955	17.01	0.06	3.30E+05
558.3259	19.92	0.13	6.11E+05
801.4115	20.72	0.13	6.33E+05
745.3924	21.03	0.13	6.16E+05
498.8018	23.21	0.08	1.97E+05
573.3025	24.34	0.14	4.33E+05
680.3735	27.08	0.09	6.40E+05
787.4212	28.07	0.06	2.40E+05
		0.10	7.03E+05

C



Mass (+2)	RT [min]	5 µL/min FWHM [min]	Area
493.7683	9.03	0.08	7.42E+05
613.3167	9.63	0.09	1.31E+06
496.2867	10.57	0.12	4.57E+06
451.2834	8.71	0.07	6.71E+05
422.7363	10.44	0.14	4.39E+06
695.8324	15.12	0.06	5.90E+05
586.8003	10.88	0.10	4.42E+06
773.8955	18.66	0.07	1.27E+06
558.3259	21.84	0.11	7.37E+05
801.4115	22.54	0.08	1.10E+06
745.3924	22.77	0.12	2.08E+06
498.8018	25.14	0.11	1.53E+06
573.3025	27.09	0.14	1.54E+06
680.3735	28.70	0.09	1.84E+06
787.4212	29.59	0.07	4.72E+05
		0.10	1.82E+06

D



Mass (+2)	RT [min]	2.5 µL/min FWHM [min]	Area
493.7683	13.33	0.08	9.78E+05
613.3167	13.92	0.09	1.53E+06
496.2867	15.18	0.09	4.23E+06
451.2834	13.09	0.11	1.76E+06
422.7363	15.08	0.11	5.97E+06
695.8324	19.84	0.10	1.06E+06
586.8003	15.44	0.11	4.85E+06
773.8955	23.45	0.06	1.71E+06
558.3259	19.67	0.10	1.06E+06
801.4115	27.86	0.14	3.32E+06
745.3924	27.85	0.13	3.29E+06
498.8018	30.83	0.12	2.60E+06
573.3025	31.66	0.11	1.91E+06
680.3735	33.32	0.10	2.57E+06
787.4212	34.12	0.09	8.44E+05
		0.10	2.51E+06

Figure 2. MS extracted ion chromatograms (EIC) obtained from 15 reference Thermo Scientific PRTC mix peptides that have been spiked at 50 fmol/µL into a background of 500 ng HeLa protein digest standard and separated following a 30 min gradient at respectively 10 (D), 7.5 (C), 5 (B) and 2.5 (A) µL/min. Chromatographic metrics have been listed in the tables to the right.

As shown in Figures 1A and C, the highest relative ion abundancies were found when operating the column at 2.5 µL/min. When calculating the mean abundance of all identified peptides (Figure 1C), the highest value (5.80E+06) was found for the 15 min gradient performed at lowest flow rate of 2.5 µL/min, while the lowest value (3.92E+05) was found at the 60 min gradient at 10 µL/min. When looking at this from a chromatographic point of view, the relative in-peak concentration of analytes (peptides in the current case) is inversely related to the gradient length, but also depends on the peak dispersion generated by the LC column. Higher relative in-peak concentrations will be observed when working at 2.5 µL/min, as this approaches the optimal flow rate of 1 µL/min.[5] When combining LC with MS detection, another important factor affects the sensitivity that can be achieved. An increase in ionization efficiency is typically observed when reducing the flow rate. In addition to eluting at a higher relative peak concentration, more efficient ionization will further increase the MS signal obtained for the separations in the lower flow rate range (2.5 µL/min). This illustrates the importance of finding the right balance between sample throughput and detection sensitivity.

Chromatographic performance

Besides a flow rate flexibility and low column backpressure, excellent peptide peak shapes were obtained with the µPAC column. Based on 15 reference peptides from the Thermo Scientific™ Pierce™ Retention Time Calibration (PRTC) mixture, average peak widths measured at half maximum (FWHM) between 0.10 and 0.11 min were achieved for the 30 min gradient separations at all flow rates. Elution profiles of all 15 PRTC peptides are shown in Figure 2, chromatographic metrics can be found in the accompanying tables. Average PRTC peptide peak widths have been determined for each condition (flow rate × gradient length) and were plotted as a function of the retention time at which the last PRTC peptide elutes (Figure 3A). For short gradient durations, sharper peptide peaks are observed at higher flow rates, favoring the use of high flow rates when high throughput is required. However, high flow rates do not result in better separation for all gradient lengths. Figure 3A clearly demonstrates how the rate at which peak width increases with gradient length is flow rate dependent. The closer to the optimal column flow rate, the less peak width is affected by gradient elongation.

In gradient elution mode, chromatographic performance is commonly expressed as peak capacity n_C, a measure that takes both the elution window or gradient duration and the average peak width of the analytes into account.[6] When aiming for the highest peak capacity within a given time frame, each column has an ideal flow rate and gradient length combination. This is clearly illustrated in Figure 3B, where the peak capacity obtained with the µPAC column is plotted for all flow rates that were tested. When high throughput is needed with very short analysis times (≤20 min), flow rates above 5 µL/min are advised. Medium flow rates (2.5–5 µL/min) will give the highest peak capacity for separations that generate an elution window between 20 and 40 min and lower end capillary-flow (≤2.5 µL/min) can be used to get maximum peak capacity for long gradient separations with elution windows beyond 40 min.

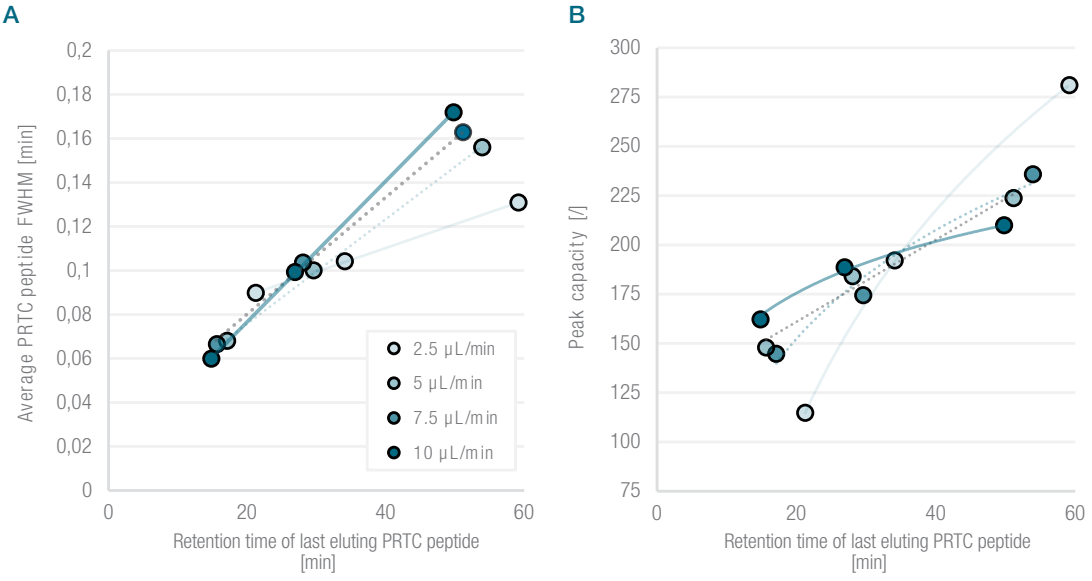


Figure 3. A) Average peak widths (FWHM) of all 15 peptides from the PRTC mixture that was spiked (50 fmol/µL) into a human cell line digest plotted as a function of the retention time (RT) of the last eluting peptide and this for different flow rates between 2.5 (light) and 10 (dark) µL/min. B) Peak capacity (n_C) values obtained using the average FWHM of all 15 PRTC peptides plotted as a function of the time at which the most hydrophobic PRTC peptide elutes. Each color represent a different flow rate between 2.5 (light) and 10 (dark) µL/min.

When comparing the µPAC column to a packed bed alternative, striking differences in retention time stability were observed (Figure 4). Whereas an average coefficient of variation on retention times (including all 15 PRTC peptides) of 0.73% was observed for the packed bed column, variation in retention time was reduced almost 3-fold (down to 0.26% CV) by working with the µPAC column. This highlights again the positioning of the µPAC column as a robust and reliable alternative to the traditional packed bed capillary-flow columns frequently used in high-throughput proteomics research.

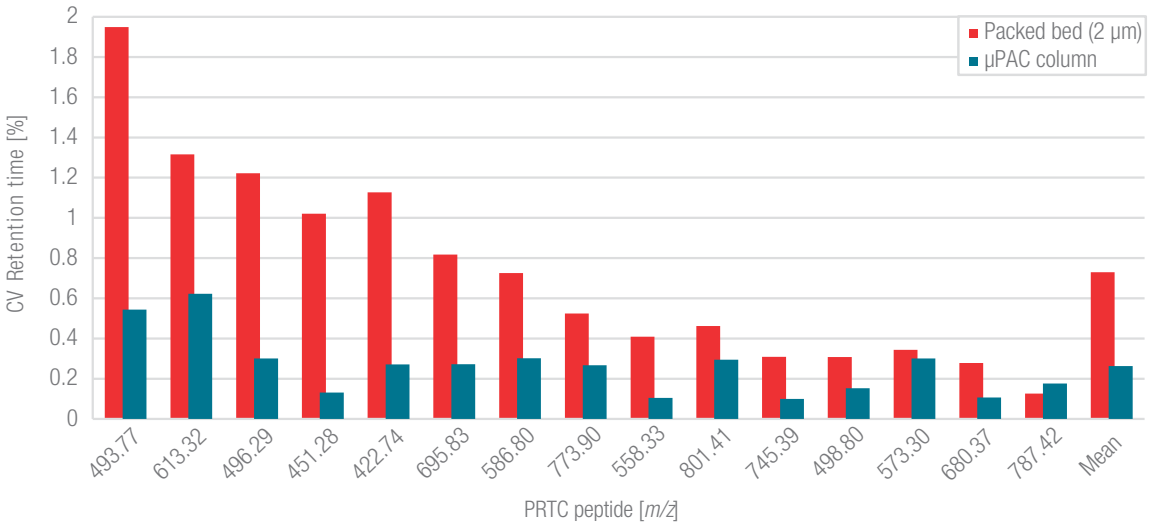


Figure 4. Bar chart diagram of coefficient of variation (CV) values of mean retention times obtained for 15 reference peptides Thermo Scientific PRTC mixture that were spiked (50 fmol/µL) into a human cell line digest and separated using a 30 min gradient at a flow rate of 7.5 µL/min. Blue represents a µPAC column. Red represents the conventional packed bed capillary-flow column (150 × 0.300 mm, 2 µm).

Peptide and protein identification

Considering the overall number of identified protein and peptide groups, it becomes clear that the µPAC column outperforms the traditional packed bed column (Figure 5). For a 30 min gradient at a flow rate of 7.5 µL/min, a relative gain of more than 20% at the protein level and more than 40% at the peptide level was found for the µPAC column. But also for the other applied gradient times (15 and 60 min) and flow rates (2.5–10 µL/min) the µPAC column outperforms conventional packed bed column, resulting in a higher proteome coverage (both at protein and peptide level).

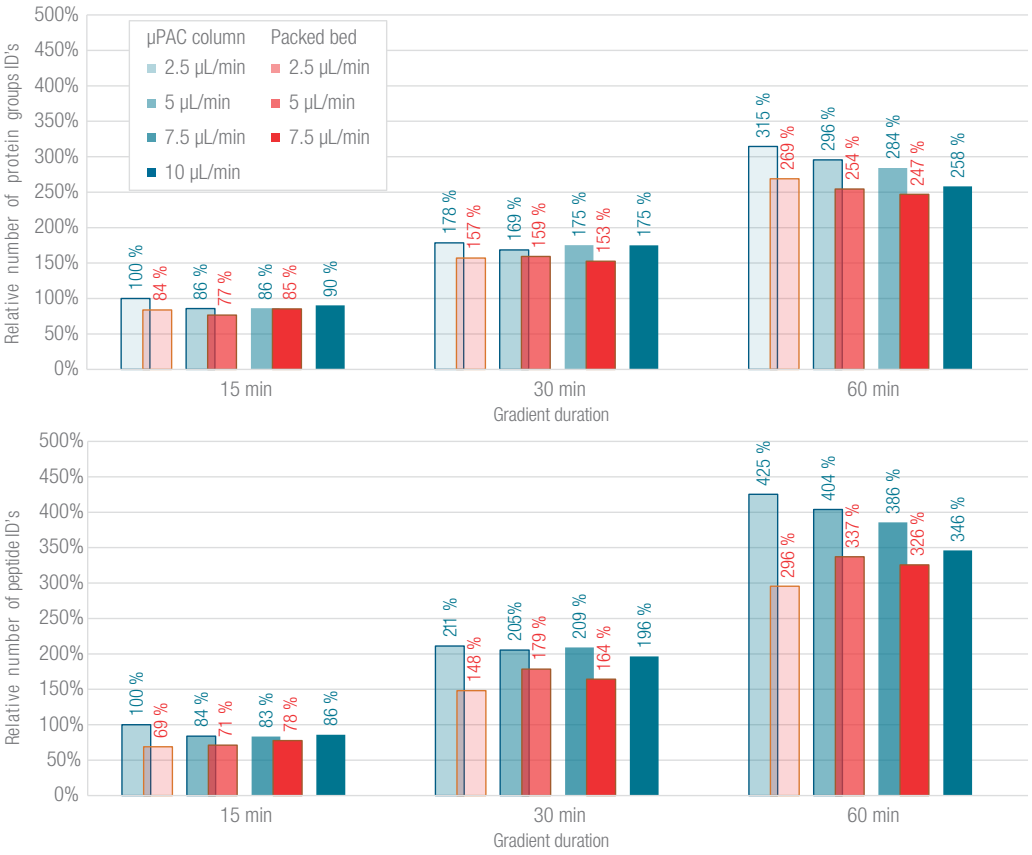


Figure 5. Relative number of identified protein and peptide groups for 15, 30, and 60 min gradient separations performed on both a µPAC column (blue) as well as on a conventional 0.300 × 150 mm packed bed column (red) which were operated at flow rates between 2.5 µL/min (light) and 10 µL/min (dark).

Conclusions

Capillary-flow µPAC column is presented that offers unprecedented performance over a wide range of LC flow rates (1–10 µL/min), and that, given its low pressure-drop, can even be used to flow rates up to 15 µL/min. Compared to packed bed alternatives that operate in the same flow rate range and have similar column volumes, significantly lower pump pressures are needed and better separation is achieved resulting in a steady increase in identified features (45% at the peptide level, 20% at the protein group level). As a result of the solid nature of the µPAC column backbone, extremely reproducible separation of tryptic digest samples can be obtained with a variation in retention time down to 0.26% CV.

References

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