Routine proteome analysis using 50 cm µPAC columns

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High flow rate flexibility: 100 to 2000 nL/min—corresponding column backpressures of respectively 12.5 and 250 bar—maximum operating pressure is 350 bar.

Increased column robustness: Each column has been manufactured by etching channels out of a solid piece of silicon, the column is perfectly bidirectional and contains no particles nor frits.

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

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

Improved proteome coverage: Higher peak capacity translates into better separation of tryptic peptides and hence allows for more proteins to be identified within a single run.

Introduction

The practice of bottom-up proteomics relies to a large extent on the separation performance that can be achieved with nano liquid chromatography mass spectrometry (LC-MS) equipment. Depending on the sample complexity or the instrument time that can be dedicated to a certain sample, different LC columns and corresponding LC-MS/MS methods are often required. When aiming for comprehensive proteome analysis with deep coverage, relatively long columns (lengths up to 75 cm) are typically operated with long and shallow solvent gradients, delivering the highest chromatographic performance. This is indeed a good strategy if very complex samples need to be analyzed and when as much information as possible needs to be retrieved from these samples. However, daily routine proteome analysis often deals with much less complex samples or demands increased sample throughput, making total analysis times above 120 min undesirable or even impossible.

Thermo Scientific™ µPAC™ HPLC columns are micromachined nano LC chip columns offering an alternative to scientists doing proteomics research. The inherent high permeability and low 'on-column' dispersion obtained by the perfect order of the separation bed makes µPAC based chromatography unique. The peak dispersion originating from heterogeneous flow paths in the separation bed is eliminated (no A-term contributions) and therefore components remain much more concentrated during separation.[1] The freestanding nature of the pillars also leads to much lower backpressure allowing the use of very long columns with exceptional peak capacities.[2]

Complementary to its landmark 200 cm long column which is ideally suited to perform comprehensive and sometimes time consuming proteome research, the Thermo Scientific 50 cm long µPAC column can be used in a more routine research setting. With an internal volume of 3 µL, this column is perfectly suited to perform high throughput analyses with shorter gradient solvent times (30, 60, and 90 minute gradients) and it can be used over a wide range of flow rates, between 100 and 2000 nL/min.

Experimental

The separation performance of two packed bed (PB) nano LC columns (150 × 0.075 mm, sub-2 µm porous silica particles obtained from 2 different vendors) and a 50 cm long µPAC column were evaluated by analyzing 500 ng of a tryptic digest originating from a human cell lysate. For every column, separation was performed using three different gradient times (30, 60, and 90 min), and this in triplicate as illustrated in Table 1.

Sample preparation and experimental set-up

Thermo Scientific™ Pierce™ HeLa Protein Digest Standard (P/N 88328) in lyophilized form was used. 20 µg of lyophilized peptide material was reconstituted in 40 µL of 0.1% formic acid in LC-MS grade water to a concentration of 500 ng/µL. Samples were spiked with Thermo Scientific™ Pierce™ Retention Time Calibration (PRTC) mixture (0.5 pmol/µL; P/N 88320) to a final concentration of 50 fmol/µL. Freshly prepared protein digest standard was used for each column type.

All columns were positioned in the column compartment and maintained at 50 °C during the entire experiment. The nano LC system was configured to perform direct injection of 1 µL sample onto the column. All columns were operated at a flow rate of 300 nL/min. In addition, the 50 cm µPAC column was operated at a flow rate of 1000 nL/min as much lower flow resistance is observed for this type of columns. A non-linear gradient from 1% to 50% of solvent B (0.1% formic acid in 80% LC-MS grade acetonitrile) was applied in respectively 30, 60, and 90 min.

For these experiments, the nano LC system was coupled to a high resolution mass spectrometer. All columns were connected to a New Objective™ PicoTip® emitter and for the 50 cm µPAC column, a grounded connection was provided between the outlet union and the mass spectrometer. For all columns, the voltage required for electro spray ionization (ESI) was applied on a 50 µm through-bore stainless steel union through a liquid junction.

Results

One of the main goals of this experiment was to benchmark the separation performance of the 50 cm µPAC column against two commonly used packed bed columns obtained from different vendors. Rather than aiming for extremely deep proteome coverage, the 50 cm µPAC column is designed for proteome research where improvements in reliability and throughput are needed aside excellent chromatographic performance.

Table 1. Experimental design.

Gradient time (min)	50 cm µPAC column (300 nL/min)	50 cm µPAC column (1000 nL/min)	15 cm PB1 (300 nL/min)	15 cm PB2 (300 nL/min)
30 min	repl 1	repl 1	repl 1	repl 1
	repl 2	repl 2	repl 2	repl 2
	repl 3	repl 3	repl 3	repl 3
60 min	repl 1	repl 1	repl 1	repl 1
	repl 2	repl 2	repl 2	repl 2
	repl 3	repl 3	repl 3	repl 3
90 min	repl 1	repl 1	repl 1	repl 1
	repl 2	repl 2	repl 2	repl 2
	repl 3	repl 3	repl 3	repl 3

Elution profiles for tryptic digest sample

MS basepeak chromatograms obtained for 60 min gradient separations (i.e. 90 min total run time) of 500 ng HeLa protein digest standard are shown in Figure 1. Even though very similar relative abundances were found for the conventional packed bed columns (1.5E+08), the highest value (2.5E+08) was found for the 50 cm µPAC operated at a flow rate of 300 nL/min. A slightly lower overall relative abundance was found for the 50 cm µPAC column when operated at 1000 nL/min (1.0E+08), but a substantial reduction of the column void time can be achieved by increasing the flow rate (Table 2). Because of the low column backpressure that is inherent to µPAC column backbones, chromatographic separations on 50 cm µPAC columns can be performed at elevated flowrates up to 2000 nL/min. As shown in Figure 2, the maximum column backpressure of the µPAC column (even when operated at 1000 nL/min) is exceptionally low compared to those observed for the packed bed columns. With stated backpressures around 40 bar, this represents a more then 6-fold reduction of the column backpressure for the 50 cm µPAC column.

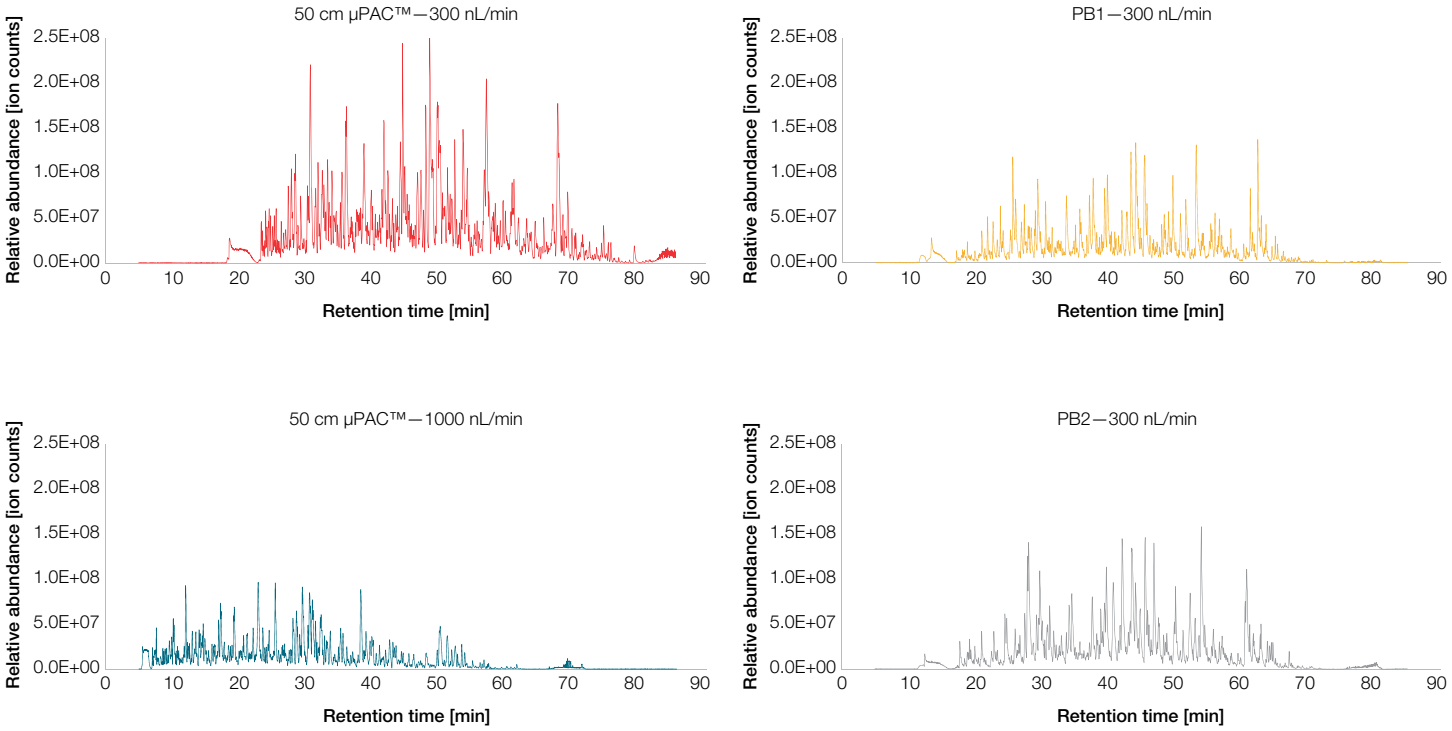

Figure 1. Basepeak MS chromatograms of 500 ng HeLa cell digest for 60 min gradients on 50 cm µPAC column operated at 300 nL/min (red), 50 cm µPAC column operated at 1000 nL/min (blue), 150 mm × 75 µm packed bed column 1 (PB1) (yellow) and 150 mm × 75 µm packed bed column 2 (PB2) (grey). See experimental section for LC gradient and MS conditions.

Table 2. Column void times and accompanying backpressures observed for three different LC columns.

	Flowrate (nL/min)	Void time (min)	Backpressure (bar)
PB column A	300	11.4	249
PB column B	300	11.3	296
50 cm µPAC column	300	18.1	40
50 cm µPAC column	1000	5.4	122

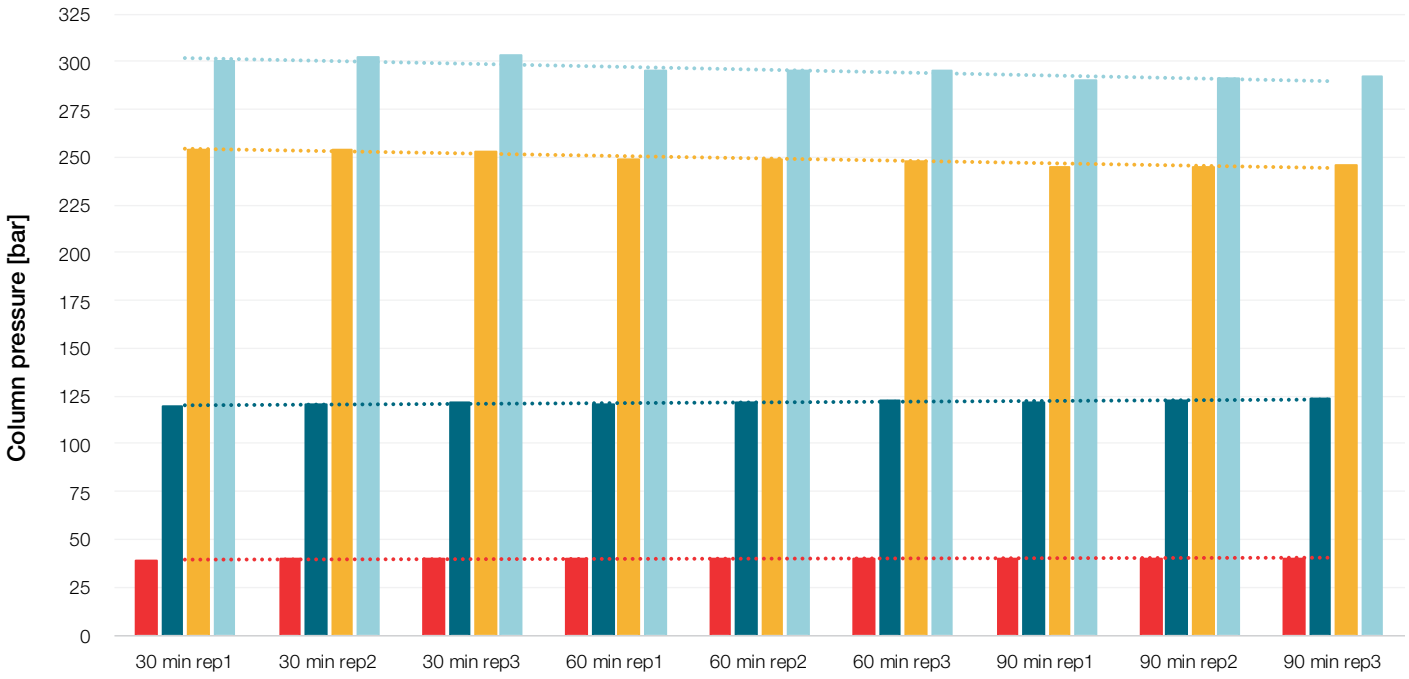

Figure 2. Column pressures (bar) of each replicate (n=3) for 30, 60, and 90 min gradient separations for 50 cm µPAC column operated at 300 nL/min (red), 50 cm µPAC column operated at 1000 nL/min (teal), 150 mm × 75 µm packed bed column 1 (PB1) (yellow) and 150 mm × 75 µm packed bed column 2 (PB2) (light blue). Dotted lines show the evolution of the backpressure for each of the columns over the entire experiment.

Chromatographic performance observed for peptide retention time standards

Besides a high flow rate flexibility (100–2000 nL/min) and low column backpressure, excellent peak shapes were found for the 50 cm µPAC column. Based on the 15 reference peptides from the PRTC mixture, average peak widths ranging from 0.13 to 0.22 min (measured at 13.5% height or 4σ) were observed, which is remarkably lower if compared to both packed bed column types (Figure 3). This trend is even more pronounced when running the µPAC column at a flow rate of 1000 nL/min.

Calculation of the peak capacity (nC) according to Neue[3] revealed higher numbers for the µPAC column compared to both conventional nano LC columns, and this for all gradient durations that were tested (Figure 4). The exceptional peak capacity value of 226 obtained for a 30 min gradient separation at a flow rate of 1000 nL/min highlights the position of this 50 cm µPAC column as a valid improvement to the traditional packed bed columns that are frequently used in daily routine proteomics research.

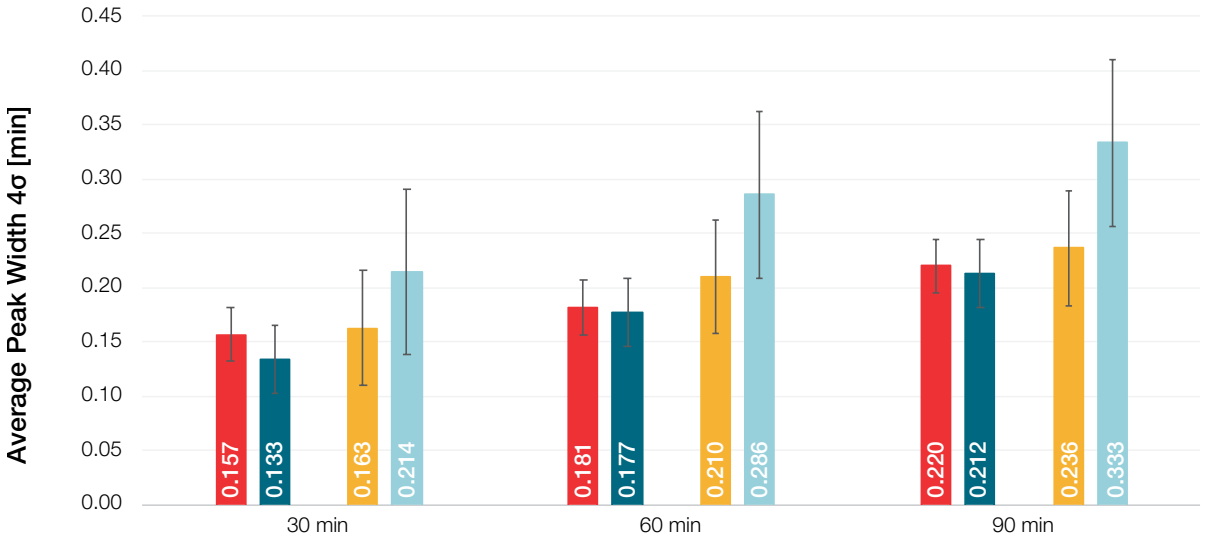

Figure 3. Average peak widths measured at 4σ, obtained for 15 reference peptides (Thermo Scientific PRTC mixture) that have been spiked at 50 fmol/µL into 500 ng HeLa cell lysate for 30, 60, and 90 min gradient separations. The 50 cm µPAC column operated at 300 nL/min is shown in red, 50 cm µPAC column operated at 1000 nL/min in teal, 150 mm × 75 µm packed bed column 1 (PB1) in yellow and the 150 mm × 75 µm packed bed column 2 (PB2) is shown in light blue.

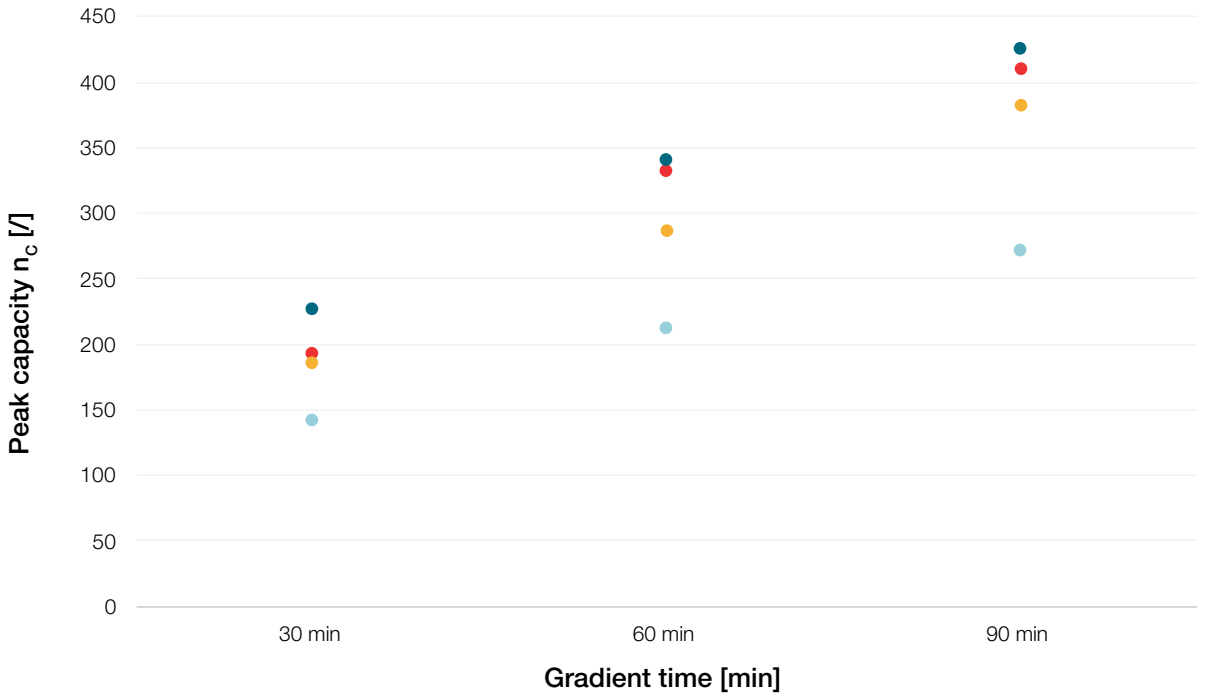

Figure 4. Peak capacities (nC) of respectively 30, 60, and 90 min gradient separations for a 50 cm µPAC column operated at 300 nL/min (red), 50 cm µPAC column operated at 1000 nL/min (teal), 150 mm × 75 µm packed bed column 1 (PB1) (yellow) and 150 mm × 75 µm packed bed column 2 (PB2) (light blue).

Increased proteome coverage

Acquired MS^2 spectra were screened against the human reference database (UniProt) with a false discovery rate (FDR) of 0.1% by using the Thermo Scientific™ Proteome Discoverer™ software, version 2.2. More than 1.700 protein groups (based on 6.100 peptide groups) could be identified with the 50 cm µPAC column by using a 60 min gradient separation profile (Figure 5).

Compared to the two conventional packed bed columns, this is an average increase of 40% in protein and 60% in peptide ID's. In line with the improved chromatographic performance (reduced peak width and increase in peak capacity) that is observed when working with the µPAC column, higher proteome coverage, both at the protein and the peptide level, was observed for all conditions tested within this experiment.

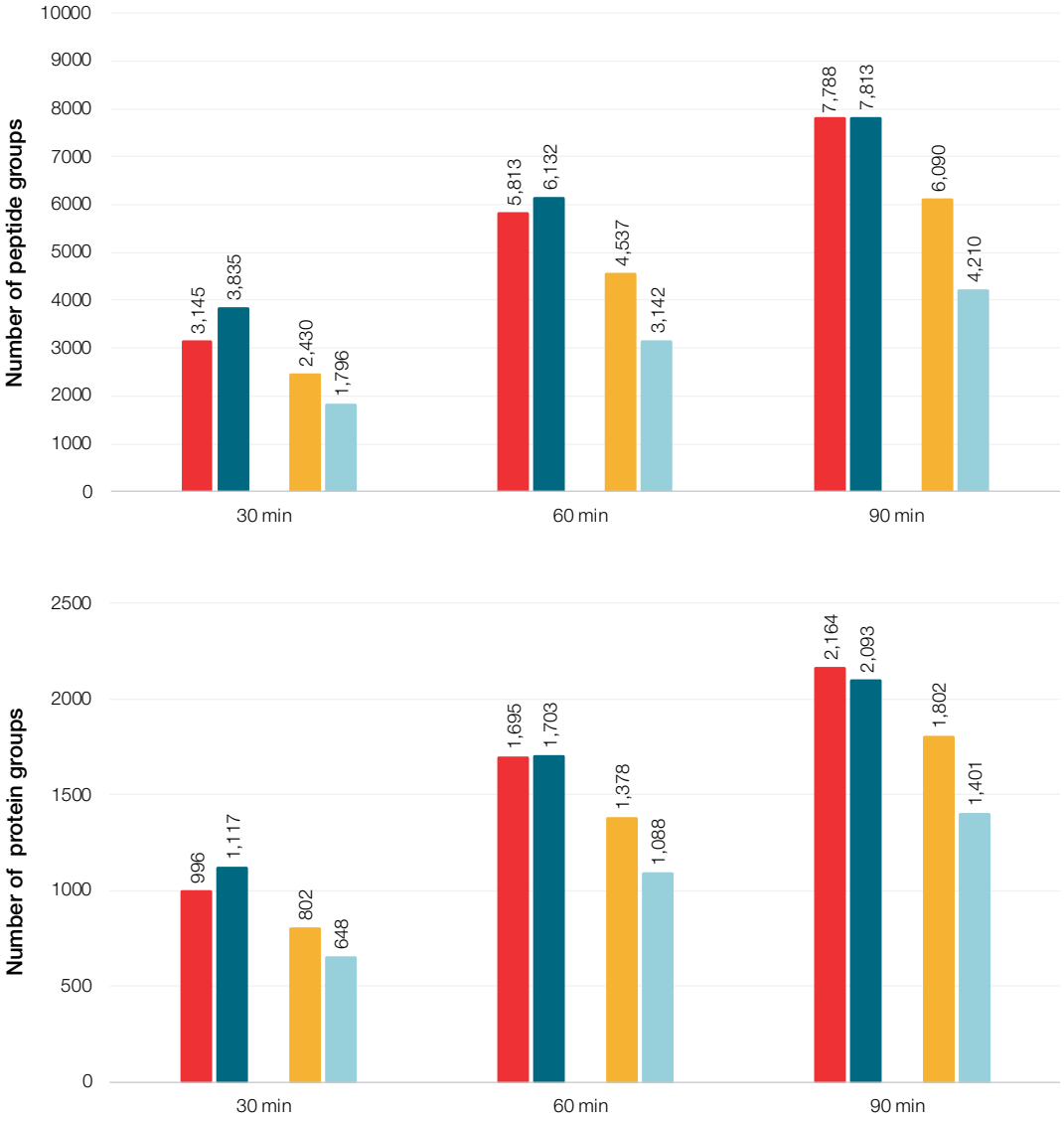

Figure 5. Average numbers of identified protein and peptide groups for 30, 60, and 90 min gradient separations performed on a 50 cm µPAC column operated at 300 nL/min (red), 50 cm µPAC column operated at 1000 nL/min (teal), 150 mm × 75 µm packed bed column 1 (PB1) (yellow) and 150 mm × 75 µm packed bed column 2 (PB2) (light blue).

Conclusions

µPAC technology clearly offers several benefits regarding robustness, high operational flexibility and excellent separation performance compared to conventional packed bed column technology. When aiming for comprehensive proteome analysis with deep coverage, the 200 cm µPAC column which delivers unprecedented separation performance is the best choice. However, the true benefits of using a long 200 cm µPAC column will only come into full play when working with long solvent gradient times (>120 min). With an internal column volume of approximately 3 µL and an increased operational flexibility (flow rates up to 2000 nL/min), the 50 cm µPAC column serves those who are looking for increased separation performance in daily routine proteome analysis settings where shorter gradient times (<120 min) and increased throughput are desired.

References

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