thermoscientific

High-throughput analysis with improved proteome coverage using new designed micro pillar array column (µPAC)

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ABSTRACT

Radial elongation of microfluidic pillars greatly enhances separation efficiency and therefore allows reducing the footprint needed to achieve sufficient resolution. Compared to µPAC columns with cylindrical pillar shapes, only a fraction of the separation length is needed to enable high flow rate flexibility and versatile operation. In this work, a new 5.5 cm long µPAC Neo High Throughput column with rectangular shaped pillar array was introduced. Capillary flow rates were applied on this short column due to its lower back pressure and narrower peak widths (FWHM<1.50 sec) were achieved when short gradient times (such as 5 min gradient) were implemented. Compared with the packed emitter column for high-throughput proteomics analysis (such as 180 and 100 sample per day (SPD)), this new µPAC column could identify 10-30% more peptides for different sample loading (50ng – 1µg HeLa digest) and better column-to-column and run-to-run reproducibility in terms of peptide retention time was achieved.

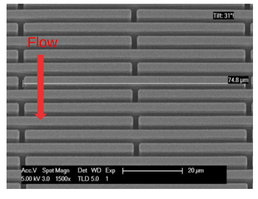
INTRODUCTION

LC-MS based proteomics has been an essential tool to analyze complex biological and clinical samples. Recent innovation in instrumentation, especially mass spectrometer, enhances the analysis speed, resolution, sensitivity, and robustness. As an important part of the separation core, the LC column plays a crucial role by reducing the sample complexity prior to injection into the MS. The micro pillar array column (µPAC) is an innovative LC column with unique pillars uniformly arranged in a microfluidic channel, which provides highly efficient separation and low back pressure. Here, a new µPAC column with radially elongated pillars and short channel length (Table 1 and Figure 1) was developed and used in combination with HRAM mass spectrometry to conduct high-throughput bottom-up proteomics analysis.

Table 1. µPAC Neo High Throughput column specifications.

Parameter	5.5cm µPAC Neo High Throughput
Pillar shape	Rectangular
Pillar dimension (µm)	75 x 3
Interpillar distance (µm)	2
Channel width (µm)	1850
Channel depth (µm)	25
Channel length (cm)	5.5
Surface morphology	Core-shell
Porous layer thickness (nm)	500
Pore size range	100-300
Surface functionalization	C18 + HMDS
Flow rate range (nL/min)	250-2500
Void volume (µL)	1.5
Maximum Pressure (bar)	450
Maximum Temperature (°C)	60

Figure 1. SEM image of pillar array in µPAC Neo High Throughput column.



MATERIALS AND METHODS

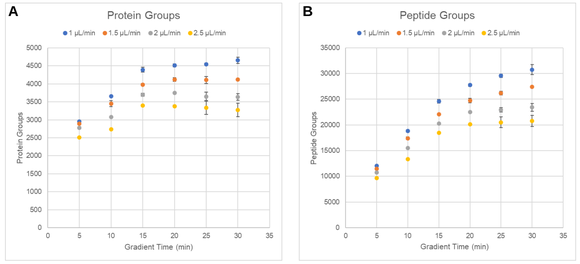
Sample Preparation – Thermo Scientific™ Pierce™ HeLa Protein Digest Standard was diluted using 0.1% formic acid (FA) to reach concentration of 200 ng/µL. Pierce retention time calibration peptide mixture (PRTC) was added to a final concentration of 100 fmol/µL.

Instrumentation and Methods – All experiments were performed using a Thermo Scientific™ Vanquish™ Neo UHPLC system operated in direct injection mode and coupled with a Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer operated in data-dependent acquisition (DDA) mode. The µPAC column was heated to 50 ºC in Sonation oven, and its outlet connected to 15 µm i.d. (ES994) or 20 µm i.d. emitter for electrospray ionization.

Data Analysis – Acquired proteomics data were processed using Thermo Scientific™ Proteome Discoverer™ (v3.0) and CHIMERYS™. The false discovery rate (FDR) was set as 1% on peptide and protein level. The peptide peak width (FWHM) was determined with apQuant.

RESULTS

Figure 2. Protein (A) and peptide (B) group IDs obtained for the separation of 200 ng HeLa digest on µPAC Neo High Throughput column at different capillary flow rates (1 µL/min – 2.5 µL/min) and different gradient times (5 min – 30 min); and median peptide peak width (C) determined with apQuant under these conditions.



µPAC Neo High Throughput column has a unique rectangular shape pillar array, which provides higher separation efficiency. So, this column with a short channel length could achieve sufficient resolution and much lower back pressure. Therefore, it is feasible to employ higher flow rate on the column without compensation on resolving power. Figures 2A and 2B show the identified protein and peptide group numbers at different capillary flow rates and gradient times on this column for the separation of 200 ng HeLa digest. ES994 of 15 µm i.d. emitter was used. In general, more peptides could be identified with increase in gradient time. But when flow rate is too high, such as 2.5 µL/min, the peptide group IDs reach a plateau at longer gradient time (>20 min) and even the protein group IDs starts to decrease, which mainly results from the combination of the broader peak width and the lower electrospray ionization efficiency at high flow rate. Within the investigated flow rate range (1 µL/min – 2.5 µL/min), more proteins and peptides were identified at lower flow rates. But if the gradient time is short, like 5 min, the difference among them is not significant, which is due to similar peak widths (FWHM<1.5 sec) obtained in all cases (Figure 2C).

Figure 3. Protein (A) and peptide (B) group IDs obtained for 5 min gradient time separation of 200 ng HeLa digest on µPAC Neo High Throughput column at different flow rates (0.2 µL/min – 2.5 µL/min).

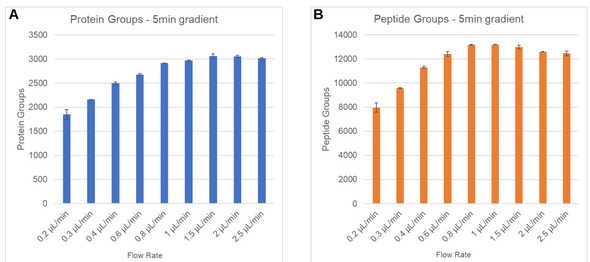


Figure 4. Protein (A) and peptide (B) group IDs obtained for 30 min gradient time separation of 200 ng HeLa digest on µPAC Neo High Throughput column at different flow rates (0.2 µL/min – 2.5 µL/min).

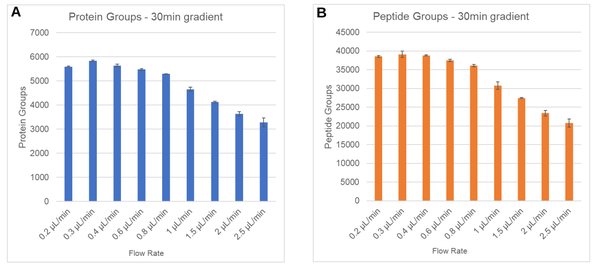


Figure 3 shows the protein (A) and peptide (B) group IDs identified on µPAC Neo High Throughput column for the separation of 200 ng HeLa digest in 5 min gradient time at different flow rates (0.2 µL/min – 2.5 µL/min). In this test, a 20 µm i.d. emitter was used. When flow rate increases from nano flow rate to capillary flow rate (>1 µL/min), both protein and peptide IDs increase and reach the maximum at the flow rate range of 1 – 1.5 µL/min. It indicates capillary flow rates could be applied with short gradient time on this µPAC column to achieve high throughput analysis without sacrificing the separation performance.

The same experiments were conducted using 30 min gradient method and the results were presented in Figure 4. Compared with 5 min gradient method, significantly more proteins and peptides are identified, but opposite trend is observed. With increase in flow rate from nano to capillary flow, identified peptide and protein numbers decrease. Therefore, for deep dive proteomics analysis, longer gradient methods are beneficial and nano flow rate (such as 300 nL/min) can be used to combine the better separation with higher electrospray ionization efficiency.

Figure 5. Median peptide peak width comparison between 0.3 µL/min and 1.5 µL/min flow rates obtained on µPAC Neo High Throughput column.

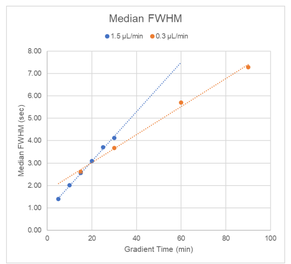


Figure 5 shows the median peptide peak width comparison between nano flow rate (300 nL/min) and capillary flow rate (1.5 µL/min) using different gradient time methods on µPAC Neo High Throughput column. Good linear relationship between peak width and gradient time was obtained for both flow rates. But the slope for 300 nL/min is smaller than 1.5 µL/min. It is clearly to see narrower peak width is achieved for nano flow rate when longer gradient time is implemented. But for short gradient method, it is expected to get narrower peak width using the higher flow rate (1.5 µL/min) method.

By taking advantage of the extended flow capabilities of the µPAC Neo High Throughput column shown above, a set of robust high-to-medium throughput LC-MS methods was developed on the Vanquish Neo UHPCL system coupled with the orbitrap mass spectrometer. Variable flow rate was employed during the gradient formation to push peptide elution forward and allow for more effective peptide separation. For example, in the 180 SPD method, 2.5 µL/min flow rate is used in the beginning for 0.6 min to push sample on the column and then 1.5 µL/min flow rate is applied for 6 min gradient separation.

Figure 6 shows the benchmarking results between µPAC Neo High Throughput column and traditional packed emitter column (150 µm × 50 mm, 1.7 µm) using the high throughput methods (180 and 100 SPD) with different sample loads (50 ng – 1 µg HeLa). µPAC Neo High Throughput column can identify 2.6% - 7.2% more proteins and 8.1% - 28.6% more peptides than packed emitter column.

Figure 6. Comparison of identified protein (A) and peptide (B) group IDs between µPAC Neo High Throughput column (blue) and packed emitter column (orange, 150 µm x 50 mm, 1.7 µm) using 180 SPD and 100 SPD methods. Bar color change from light to dark indicates different sample loads: 50 ng, 100 ng, 200 ng, 500 ng, 1 µg.

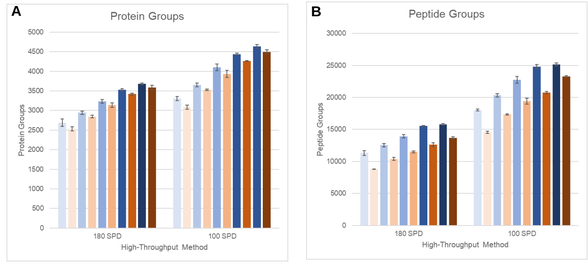


Figure 7. RPTC peptide peak retention time comparison between three µPAC Neo High throughput columns and two packed emitter columns.

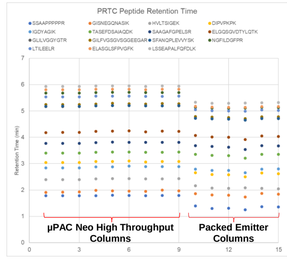


Figure 7 plots 15 PRTC peptides retention times eluted on three µPAC Neo High Throughput columns and two packed emitter columns. Triplicate runs were conducted for each column. For µPAC Neo High Throughput column, most of the PRTC peptides retention time variation is less than 1% cross different columns and multiple runs. But for packed emitter column, the first 8 PRTC peptides retention time %RSD is larger than 1.4%. This indicates µPAC Neo High Throughput column could provide better column-to-column and run-to-run reproducibility.

CONCLUSIONS

- New µPAC Neo column enables high throughput analysis, such as 180 SPD, by employing capillary flow rate and short gradient time.
- µPAC Neo High Throughput column shows better performance for high throughput proteomics analysis compared with packed emitter column.
- µPAC Neo High Throughput column has better column-to-column and run-to-run reproducibility in terms of peptide retention time.

TRADEMARKS/LICENSING

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