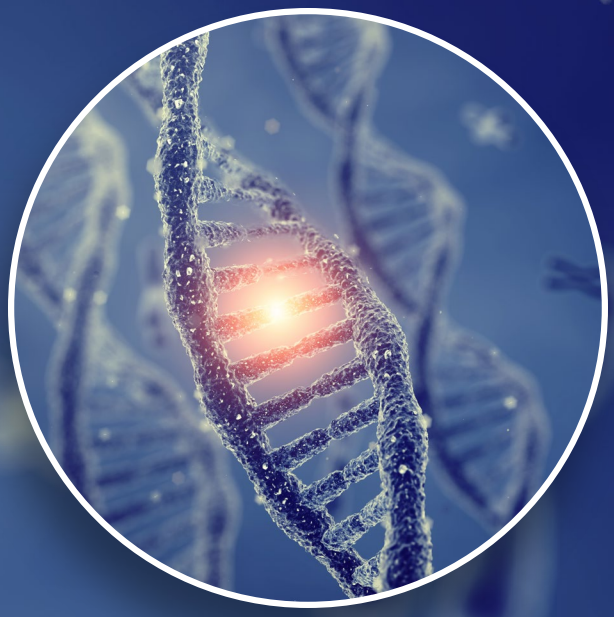
Product spotlight | 000476



Proteome analysis

High throughput, routine and comprehensive proteome analysis using a µPAC HPLC column based capillary-flow LC-MS workflow

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

Abstract

In this product spotlight, a variety of liquid chromatography mass spectrometry (LC-MS) based proteome analysis needs are described. Thermo Scientific™ µPAC™ HPLC columns are evaluated for different user needs ranging from 12 samples per day in a comprehensive mode and maximizing the data throughput to as high as 65 samples per day for high sample throughput analyses. 2 µg of tryptic digest sample (HeLa cell lysate) was injected and separated using reversed-phase liquid chromatography over a range of flow rates (2–10 µL/min) and solvent gradients (15–120 min).

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 and contains neither particles or frits.

Column-to-column reproducibility

Each column is manufactured using the same lithographic mask, making every column nearly identical.

Separation performance

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

Introduction

A series of LC-MS proteomics workflows with capillary-flow micro pillar array (µPAC) based chromatographic separation columns are presented. The high operational flexibility and unique chromatographic properties of this stationary phase format allow precise tuning of high-performance liquid chromatography (HPLC) methods according to the analytical requirements (sample throughput, separation performance and sensitivity). At present, the vast majority of LC-MS based proteomics research is still performed within the nanoflow liquid chromatography (LC) regime, using 75 µm inner diameter LC columns operated at sub µL/min flow rates.[1,2] The driving force to use nano liquid chromatography for proteomics research has been the huge increase in detection sensitivity that can be achieved by downscaling column dimensions (column and particle diameter). The relative in peak dilution of an analyte is inversely related to the column's inner diameter, and therefore a given amount of injected sample material will produce more concentrated analyte peaks on columns with reduced internal diameters. Besides, another important benefit of working with nanoflow LC is the improved ionization efficiency due to the use of electro spray emitter tips with internal diameters in the low micrometer range.[3]

There is a growing demand for large quantitative proteomics research studies that combine increased throughput, excellent robustness and high sensitivity, therefore capillary and microflow LC-MS solutions are gaining more and more interest. Recent publications using LC-MS technology report on several approaches to tackle the challenges associated with these large proteomic sample sets.[8–12] However, they all use silica particle based packed bed LC columns (column internal diameters ranging from 0.15 to 1.0 mm) as an essential part of their LC-MS workflow, limiting the use of strategies that rely on the transfer of accurate retention times, as no particle packed column can ever be identical to another. In this product spotlight, we present a multi-purpose capillary-flow LC column that is produced using an entirely different fabrication process. Nanometer precision 2D designs are transferred onto silicon wafers and transformed into an extremely uniform array of superficially porous silicon pillars using deep reactive ion etching processes that were initially developed for the microelectronics industry. Apart from eliminating virtually any column-to-column variability, precise positioning of these 5 µm diameter silicon pillars creates a stationary phase support that introduces minimal dispersion (or dilution of the samples) into the separation process.[13, 14] These columns can also be operated at LC pump pressures that are significantly lower than what is needed to operate the current in packed bed capillary-flow LC columns (sub 2 µm particles), hereby reducing the shear force on LC pump components and positively affecting their lifetime. Three different LC workflow scenarios—high throughput, routine and comprehensive proteome analysis – are presented that have been optimized in terms of MS acquisition time, chromatographic performance and proteome coverage.

Experimental

The column was operated at flow rates of respectively 2, 5, and 10 µL/min. A non-linear gradient from 1 to 45% solvent B (0.1% formic acid in 80% LC/MS grade acetonitrile) in respectively 108, 54, and 17.5 min. The LC solvent gradient profiles can be found in Figure 1.

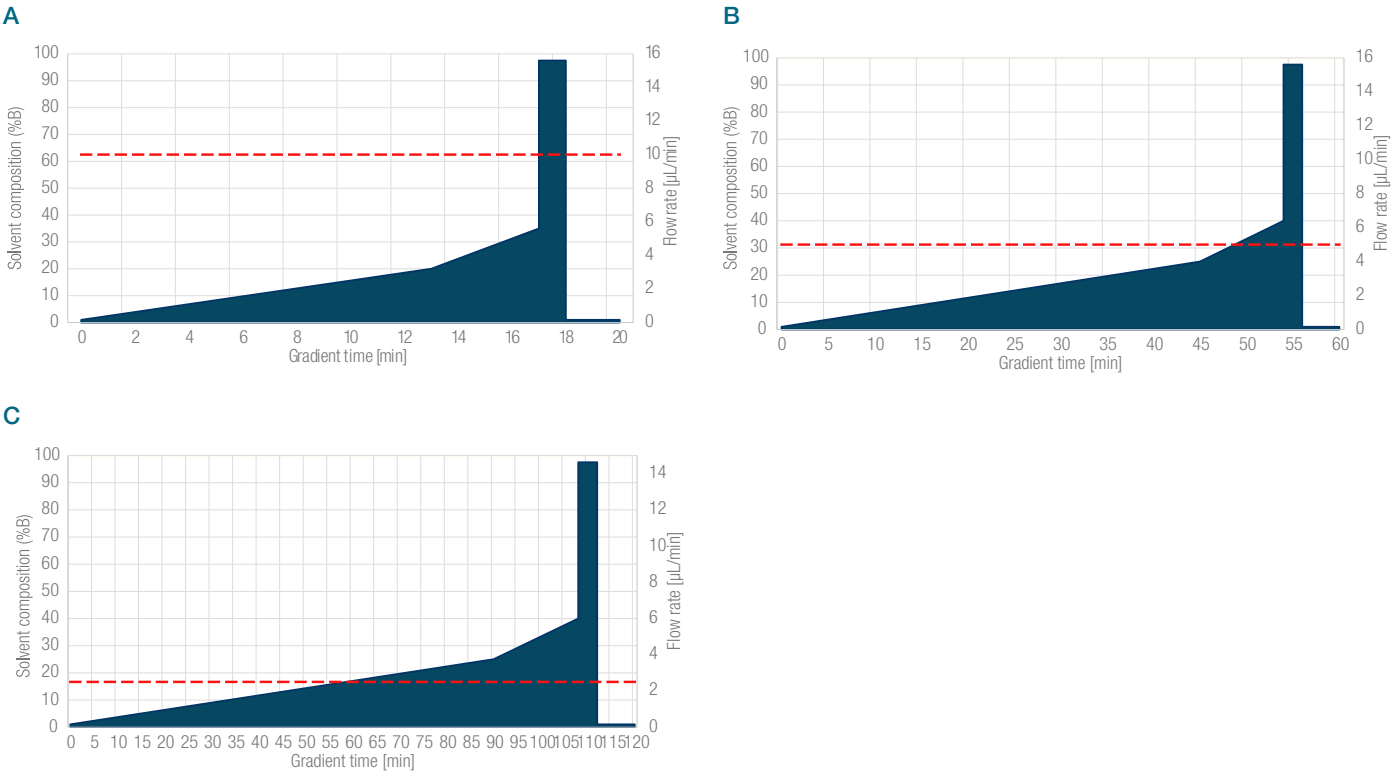


Figure 1. Optimal LC solvent gradient profiles for 3 defined needs in LC-MS proteome research. A) High throughput method, 10 µL/min, total run time (with sample pick-up) 20.68 min. B) Routine method, 5 µL/min, total run time (with sample pick-up) 60.68 min. C) Comprehensive method, 2 µL/min, total run time (with sample pick-up) 120.68 min. Solvent A: water (100%) with 0.1% (v/v) formic acid. Solvent B: water/acetonitrile (20/80) with 0.1% (v/v) formic acid.

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

Depending on the time that can be used to perform an analysis, a good combination of flow rate and gradient time should be considered to maximize the chromatographic performance. When high throughput is needed with very short analysis times (≤20 min), micro-flow rates above 5 µL/min are advised. Medium capillary-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 2).

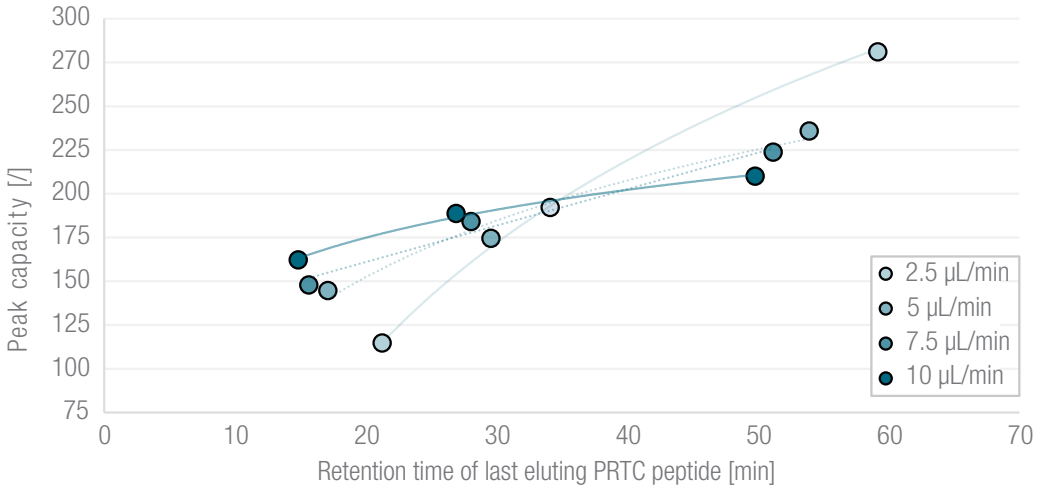


Figure 2. Peak capacity (n_c) values obtained using the average FWHM of 15 PRTC peptides plotted as a function of the time at which the most hydrophobic PRTC peptide elutes. Each color represented a different flow rate between 2.5 (light) and 10 (dark) µL/min.

High throughput proteome analysis

For short LC-MS methods where high sample turnover is required, the relative time that is spent on sample injection and column equilibration can become a crucial factor affecting data productivity. Working at higher flow rates is considered a valid strategy to reduce the impact of these processes. However, there's a delicate balance between flow rate and detection sensitivity that should not be neglected when setting up LC-MS methods. A high throughput capillary flow LC method at 10 µL/min has been defined that provides optimal MS time use within a time frame of nearly 21 min (Figure 1). When traditional reversed-phase LC-MS compatible solvents (acetonitrile/water) are used, approximately 200 to 250 bar of pump pressure will be needed to generate a flow rate of 10 µL/min, which is well below the maximum allowed column pressure of 350 bar. Figure 3 summarizes the metrics that could be obtained for a tryptic HeLa cell digest sample.

Base peak chromatograms obtained for triplicate injections of 2 µg HeLa cell digest clearly indicate the high level of chromatographic repeatability that can be achieved. In order to minimize the overhead time, a direct injection method (5 µL volume sample loop) was configured where sample pick-up parameters have been optimized. Up to 83% of the total time was effectively used to identify peptides at a sample turnover rate of 65 samples per day, making this among the most effective ever reported within capillary/microflow LC-MS based proteomics. Recent literature reports on percentages between 65 and 80% for similar sample turnover workflows.[8,9,11] The peptide full width half maximum (FWHM) was determined using the Thermo Scientific™ Proteome Discoverer™ software, version 2.4 compatible apQuant node,[15] resulting in an average value of 3.97s based on all peptide-spectrum matches (PSMs). The retention time reported for each PSM that was detected in all 3 replicates was used to calculate the average variation in retention time (Figure 3G-H). With values down to 0.12% CV or 0.75 s average variation in retention time, an exceptionally reproducible and robust high throughput LC-MS based proteomics workflow is demonstrated.

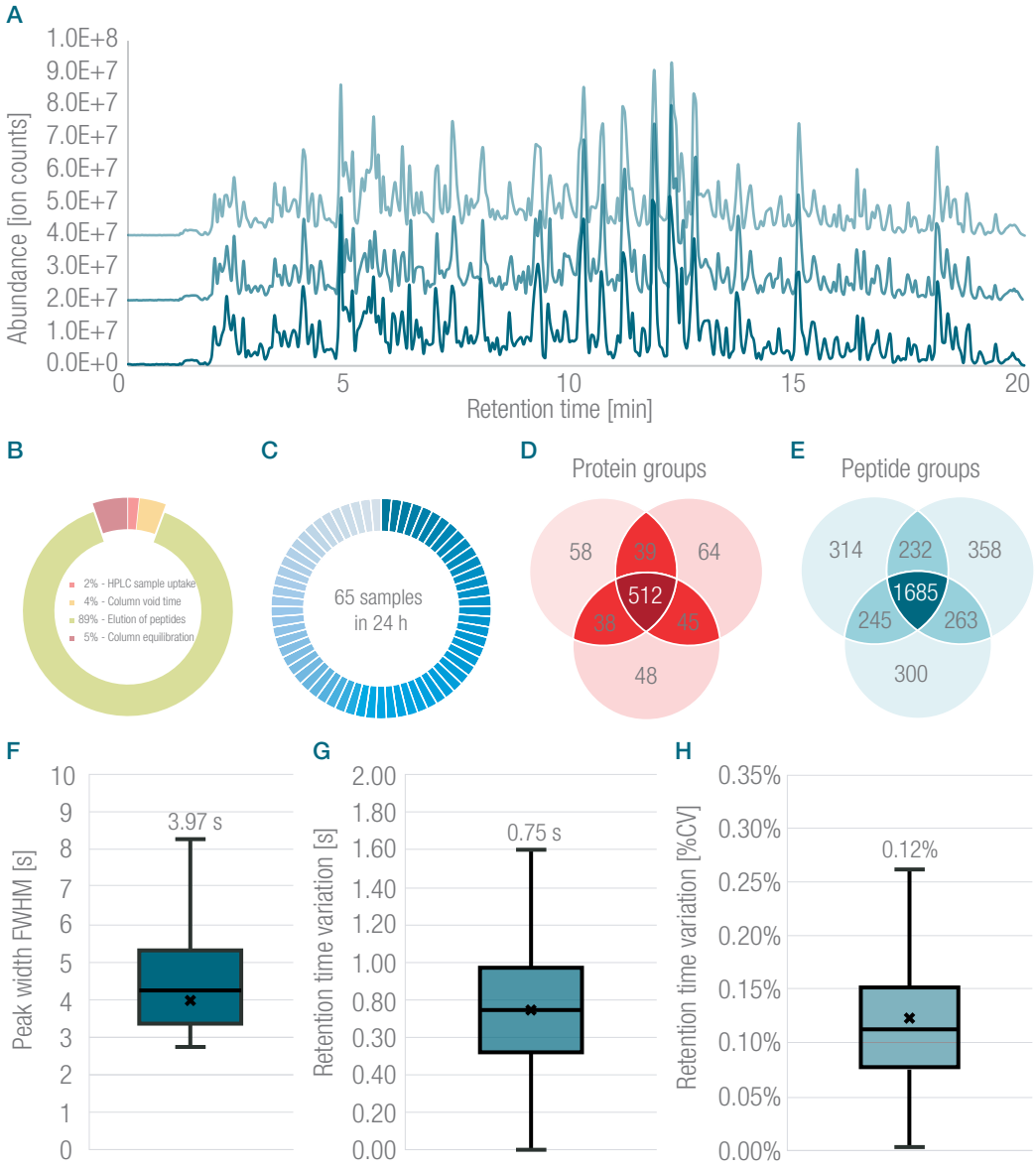


Figure 3. High throughput proteome analysis with a µPAC column. 2 µg of HeLa cell digest was injected using a direct injection mode. A) Basepeak chromatograms obtained for triplicate analysis. B) Relative time use of the instrument. C) Sample turnover rate. D) Number of identified protein groups. E) Number of identified peptide groups. F) Average peak widths (FWHM) for all PSMs. G) Retention time variation (absolutes) observed for all peptides shared in triplicate runs. H) Retention time variation (relative %CV) observed for all peptides shared in triplicate runs.

Routine proteome analysis

Taking the previously observed column performance characteristics into account (Figure 2), an optimal µPAC method was developed that can be used to maximize the output of routine proteome analyses with run times in the order of 60 min. Operating the column at a flow rate of 5 µL/min will approximately need 100 to 150 bar of pump pressure and produces the highest peak capacity (and thus the sharpest peptide peaks) for this gradient time. Using this method, the first and last peptide peak elute respectively around 3.4 and

57.7 min, generating an elution window of nearly 55 min and nearly yielding an effective MS time use of 90% (Figure 4). An average FWHM of 8.25 s was calculated from the apQuant node, equivalent to a peptide peak width at the base of 14 s or 0.24 min and delivering a peak capacity value close to 250. As can be expected for methods that give an increased elution window, the relative variation in retention time drops even further (0.08% CV) even though a slight increase in absolute value is observed (going from 0.75 s for the high throughput method to 1.13 s for the routine method).

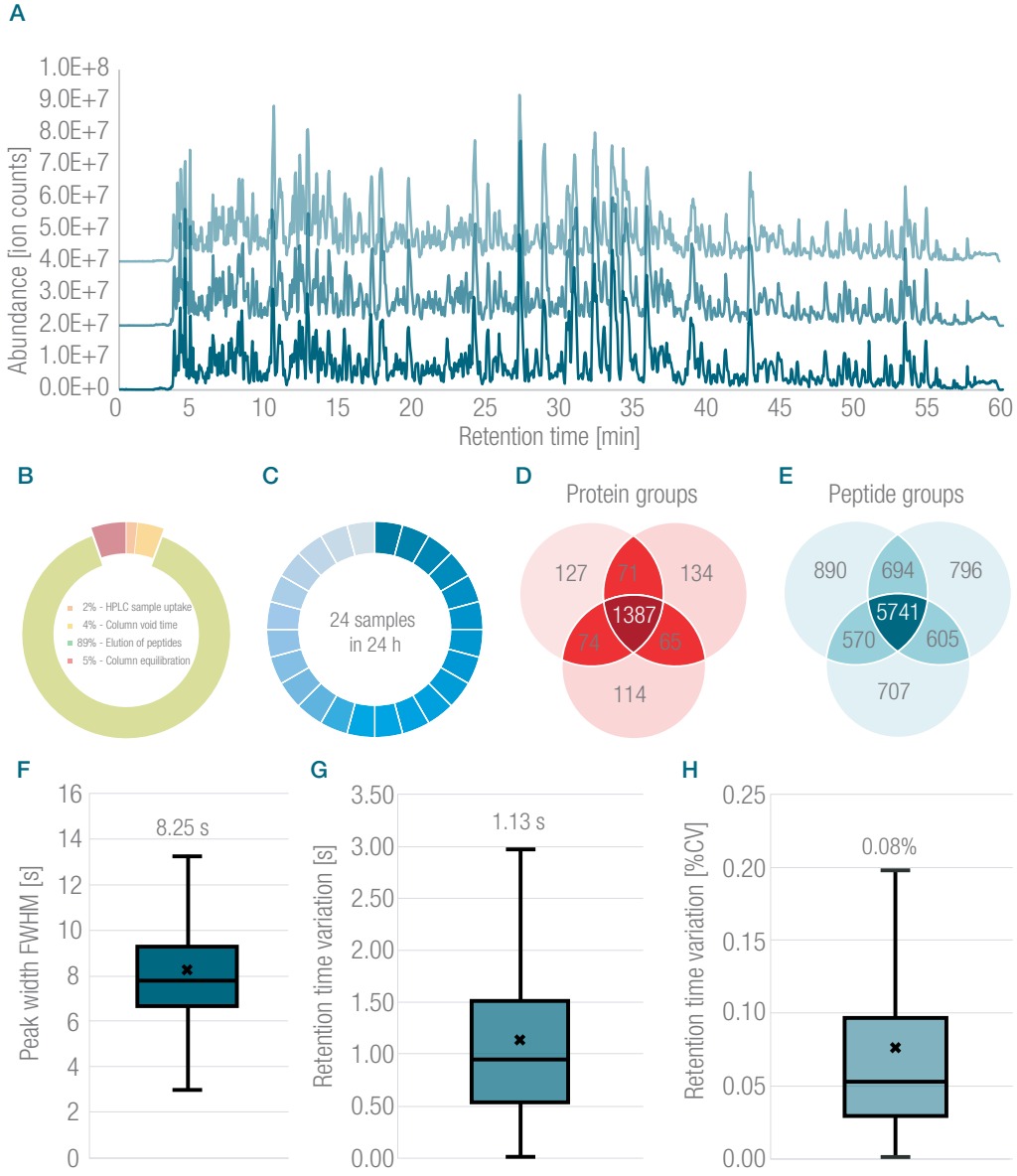


Figure 4. Routine proteome analysis with a µPAC column. 2 µg of HeLa cell digest was injected using a direct injection mode. A) Basepeak chromatograms obtained for triplicate analysis. B) Relative time use of the instrument. C) Sample turnover rate. D) Number of identified protein groups. E) Number of identified peptide groups. F) Average peak widths (FWHM) for all PSMs. G) Retention time variation (absolute - s) observed for all peptides shared in triplicate runs. H) Retention time variation (relative %CV) observed for all peptides shared in triplicate runs.

Comprehensive proteome analysis

When aiming for highest peak capacity that can be achieved with this column, it is advised to operate the column in the lower range of the capillary flow rate regime (1–3 µL/min). Even though the contribution of overhead time to the total analysis time becomes more significant and broader peptide peaks are observed for short gradients, the combination of a low flow rate and long gradient time will be most beneficial when comprehensive proteome analysis is required. For long gradients (>60 min), the dispersion observed for peptides will be at its minimum when working at reduced flow rates. On the other hand, an increase in ESI efficiency is observed by

lowering the flow rate down to several µL/min. The combined effect produces a substantial increase in detection sensitivity (3 fold) which allows identifying peptides over a higher dynamic concentration range and hereby maximizing the amount of features identified in a single run. Using this method, the first and last peptide peak elute respectively around 8.3 and 110 min, generating an elution window of over 100 min and nearly yielding an effective MS time use of 84% (Figure 5). Performing these long gradient separations at elevated flow rates (e.g., 10 µL/min) would allow increasing the effective MS time use up to 97%. An average FWHM of 11.58 s was calculated from the apQuant node, equivalent to a peptide peak width at the base of 19.7 s or 0.33 min and delivering a peak capacity value of 335 (measured at the peak base). In accordance with the observations when comparing the high throughput and routine method, a decrease in relative retention time variation is observed (0.07% CV) even though the absolute retention time variation increases to 1.81 s (based on all shared PSMs).

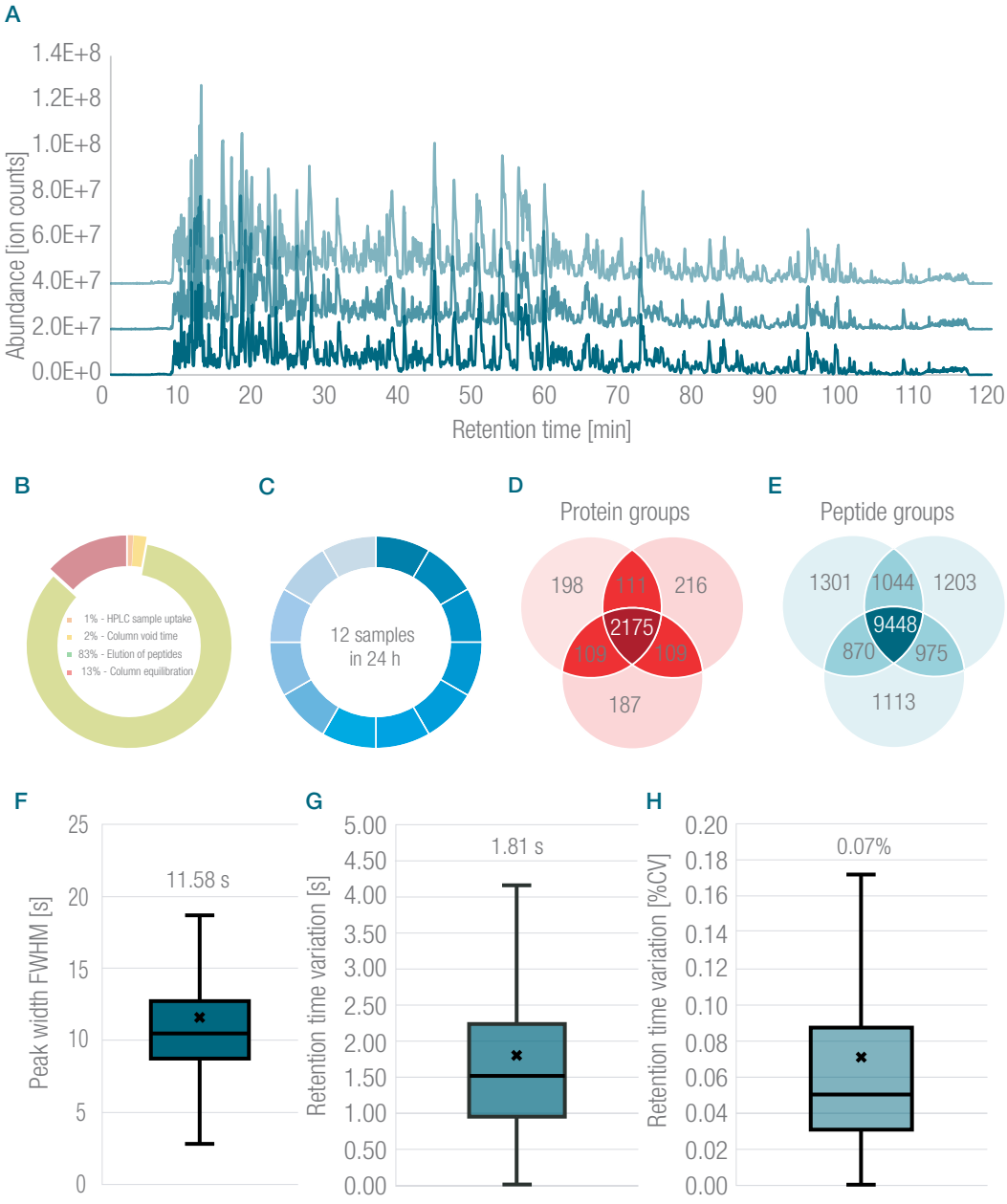


Figure 5. Comprehensive proteome analysis with a µPAC column. 2 µg of HeLa cell digest was injected using a direct injection mode. A) Basepeak chromatograms obtained for triplicate analysis. B) Relative time use of the instrument. C) Sample turnover rate. D) Number of identified protein groups. E) Number of identified peptide groups. F) Average peak widths (FWHM) for all PSMs. G) Retention time variation (absolutes) observed for all peptides shared in triplicate runs. H) Retention time variation (relative %CV) observed for all peptides shared in triplicate runs.

Conclusions

Using optimal LC methods for a variety of LC-MS based proteome analysis needs, the µPAC column provides versatile output in terms of separation performance and throughput. As a consequence of the low column back pressure, the column can be operated over a range of flow rates, enabling analytical scientists to tune LC methods according to the sample complexity or to the size of the sample set. Three common separation needs in LC-MS proteome research have been identified and tailored LC methods with optimal output in terms of data productivity and sensitivity have been developed. For large sample sets where maximum throughput is required, a high flow rate method with a sample turnover rate of 65 samples per day is advised. By operating the µPAC column at a flow rate of 10 µL/min and running a 17.5 min solvent gradient, total overhead time (including sample pick up, column void time and column equilibration) can be reduced to 17% of the total analysis time, leaving 83% of the total time available for data generation. When looking for medium throughput but maximum data productivity, a routine LC program is advised that combines increased sensitivity with increased separation power. At a flow rate of 5 µL/min, column void time increases, but the relative contribution of overhead time can be reduced by applying longer solvent gradients (54 min), which yields a sample turnover rate of 24 samples per day with 89% of effective MS time use. By increasing the gradient duration even further, this could even approach 97%. However, the maximum outcome in terms of chromatographic performance and subsequent peptide/protein group identifications would not be achieved. When comprehensive analysis with no limit on time consumption per sample is at hand, lowering the flow rate even further to 2 µL/min will give the best results. Comprehensive proteome analysis using a 108 min solvent gradient at a sample turnover rate of 12 samples per day results in maximum peak capacity (335 measured at peak base) and the highest sensitivity (more efficient electrospray ionization at lower flow rates). In addition to the flexible operation benefits, the perfect order and unique control of stationary phase design result in excellent chromatography at the highest level of LC reproducibility. With peptide retention time variation of respectively 0.75 s (0.12%), 1.13 s (0.08%), and 1.81 s (0.07%), a set of exceptionally reproducible, robust and versatile LC-MS based proteomics workflows is demonstrated.

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