Maximizing low sample load coverage with 100 nL/min elution using 50 cm long mesoporous pillar array columns

Jeff Op de Beeck[1], Natalie Van Landuyt[1], Robert Van Ling[2], Bernard Delanghe[3] ,Paul Jacobs[1]

[1]Thermo Fisher Scientific, Ghent, Belgium, [2]Thermo Fisher Scientific, Breda, The Netherlands, [3]Thermo Fisher Scientific, Bremen, Germany

Abstract

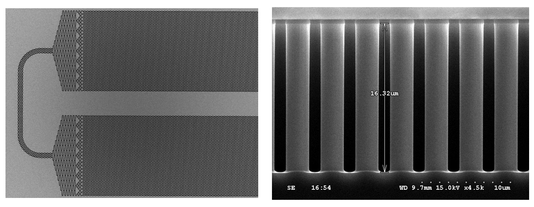
Purpose: Evaluate the impact of using an optimized LC-MS set-up with reduced post column volumes for high sensitivity low nanoflow proteomics experiments.

Methods: Dilution series of human cell lysate protein digest standards were separated using nanoflow LC on a 50 cm µPAC Neo High column hyphenated to Orbitrap MS for detection. Data was acquired in data independent acquisition.

Results: Reduction of median eluting peak widths by 20% resulted in an increase in proteome coverage of up to 27%.

Introduction

Figure 1. Scanning Electron Microscopy images of the pillar bed used in the 50 cm µPAC™ column. Column properties in table.

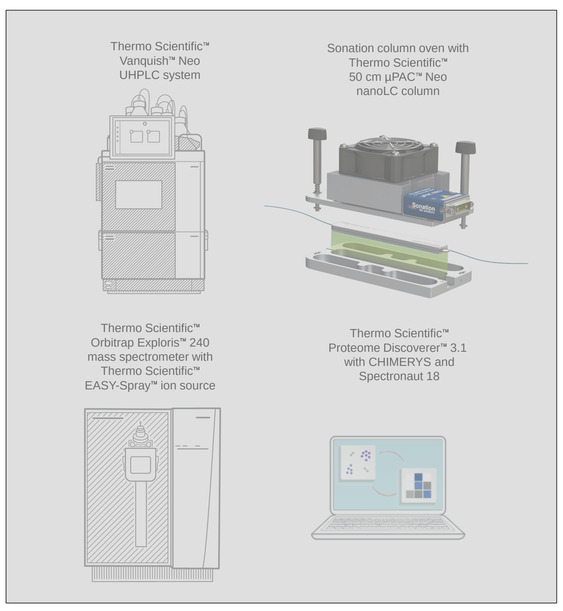


50 cm µPAC™ Neo Column	
Pillar shape	Cylindrical
Pillar diameter [µm]	5
Interpillar distance [µm]	1,25
Channel width [µm]	180
Channel depth [µm]	16
Column length [cm]	50
Column volume [µl]	1,5
Surface morphology	Core shell
Porous layer thickness [nm]	400
Pore size range [A]	100 - 300
Surface functionalization	C18 + HMDS

LC-MS-based proteomics relies on HRAM mass spectrometers for resolution, speed, and sensitivity, critical for accurate identification and quantification after LC separation. To match the increasing speed of MS instruments, nanoLC column development has focused on reducing dead volume and ensuring optimal elution, particularly at ultra-low flow rates. Pillar array technology, with its precise order and high permeability, demonstrates outstanding performance, although connectivity quality can significantly impact results. An optimal strategy is proposed to enhance connectivity and diminish post-column dead volumes, resulting in notable performance enhancements and enabling more thorough proteomic analysis. Moreover, its unique capabilities facilitate high-throughput, ultra-sensitive analysis by swiftly adapting to programmed flow rates, enabling 100 samples per day at elution rates as low as 100 nL/min.

Materials and methods

Figure 2. Experimental set-up.



Sample preparation

Mammalian cell digests were resuspended in 0.1% TFA, 10% DMSO to obtain stock solutions of respectively 100 ng\µL Further dilution (1:20) was done in 0.1% TFA, 1% ACN to obtain to a final sample concentration of 5 ng/µL.

LC-MS configuration

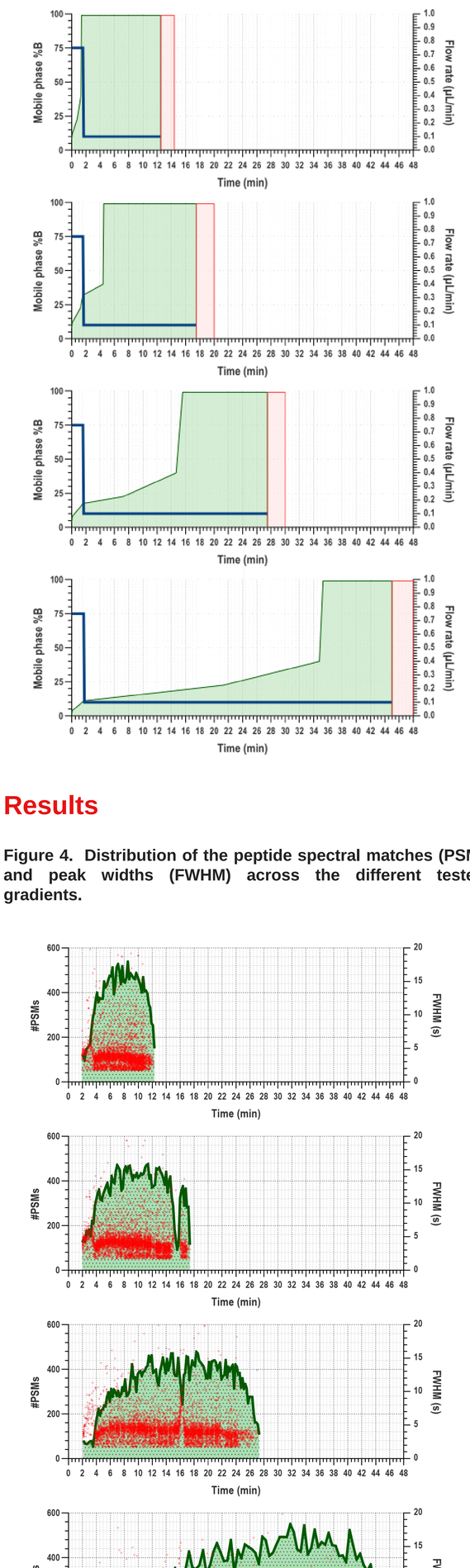
The separation was performed on a Thermo Fisher™ Vanquish™ Neo UHPLC instrument using a 50 cm long µPAC™ Neo column in a direct injection workflow. The LC column was positioned in an external heating device (Sonation, PRSO-V2_PF) directly mounted onto a Thermo Fisher™ EasySpray™ source that was coupled to a Thermo Fisher™ Orbitrap™ Exploris 240 mass spectrometer. Variable flow rate LC methods with initial flow at 750 nL/min and eluting flow rates of 100 nL/min were used. Data independent acquisition (DIA) parameters used are listed below.

Full Scan		MS² scan			
Resolution	Max IT	Resolution	Precursor mass range	Isolation window	Max IT
120k	Auto	60k	375-675 m/z	30 Th	118 ms
120k	Auto	120k	375-675 m/z	60 Th	246 ms

Data analysis

LC-MS data were analyzed either using Proteome Discoverer 3.1 with Chimerys or with Spectronaut 18. Results shown have been filtered to a 1% FDR.

Figure 3. Gradient and flow rate profiles used for low input LC-MS evaluation. 96, 72, 48 & 30samples per day throughput with elution at 100 nL/min. The actual data acquisition frame is displayed in green, the instrument overhead in red.



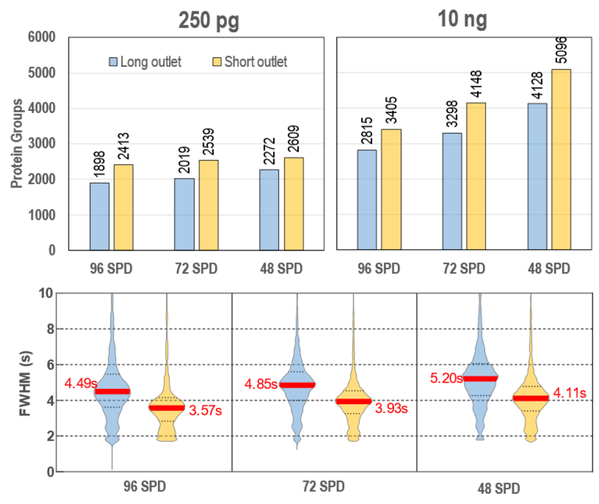
Results

Figure 4. Distribution of the peptide spectral matches (PSM) and peak widths (FWHM) across the different tested gradients.

To achieve maximum sensitivity and throughput in liquid chromatography (LC), the design of LC methods with variable flow rates is essential. Initially, a high flow rate can be used to rapidly push analytes through the column. Upon breakthrough, drastically reducing the flow rate enhances nano-electrospray ionization efficiency, thereby maximizing sensitivity without compromising throughput. For the 50 cm µPAC Neo column, which has a volume of 1.5 µL and a maximum allowable flow rate of 750 nL/min (resulting in approximately 300 bar pressure at 50° C), peptide breakthrough is expected around 2 minutes post-injection. Reducing the flow rate at this point increases sensitivity. However, the gradient must be optimized so that the %B evolution remains linear as a function of total pumped volume, not just time. Figure 3 illustrates examples of these methods, showing modulation of flow rate and %organic mid-gradient. For the highest throughput methods, the entire active gradient can be formed at high flow rate and subsequently pushed isocratically through the column. The duration of this method is limited by the volume between the injector and detector. For instance, a 1.5 µL volume could yield active elution for up to 15 minutes at a 100 nL/min flow rate.

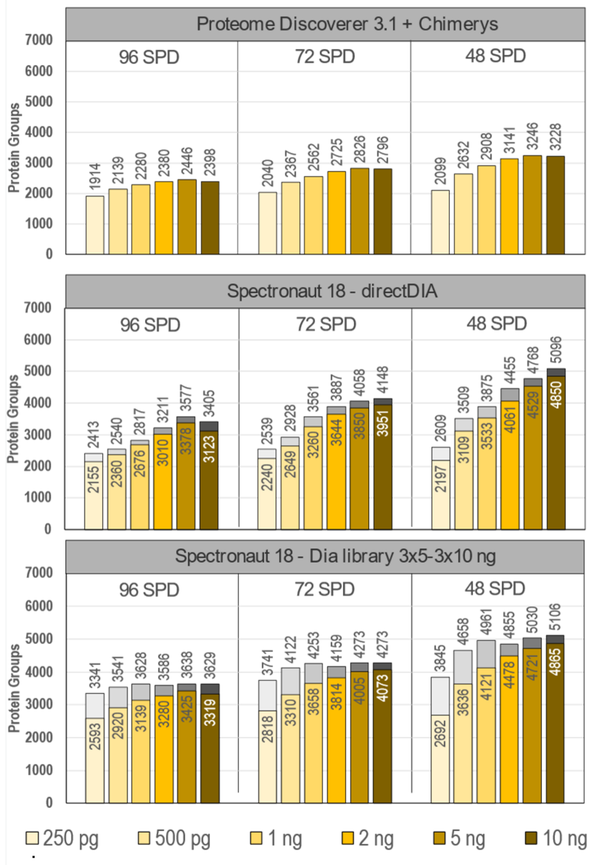
Utilizing a column heating device directly attached to the ESI source of the mass spectrometer enabled close integration of the nanoLC column, reducing the post-column volume by a factor of 3 to as little as 50 nL. Ensuring a low dead volume connection from the outlet fitting to a suitable nano electrospray emitter preserved the "on-chip" separation performance, resulting in significantly improved chromatography and deeper proteome coverage.

Figure 5. Top: Average number of protein groups identified from 0.250 and 10 ng of human cell lysate digest standard with 50 cm µPAC Neo columns. Operated either with the 50 cm (long outlet – blue) or the 15 cm line (short outlet – yellow) as an outlet (Data independent acquisition – processing using Spectronaut 18 - 1% FDR - technical triplicates searched together). Bottom: Violin plots showing the respective peak width distribution obtained for each method and with both fluidic configurations.



Anticipating the greatest performance gains at lower flow rates, we conducted benchmarking with sample loads ranging from 250 pg to 10 ng. Methods were optimized for elution at 100 nL/min, achieving sample throughput rates of 96, 72, and 48 samples per day with instrument productivities of 70%, 77.5%, and 85%, respectively. Post-column volume reduction led to a performance increase of up to 20% (reducing average FWHM by approximately 1 s) and increasing proteome coverage by up to 27% on the protein group level and identifying up to 2609 protein groups from as little as 250 pg using a library free data processing approach.

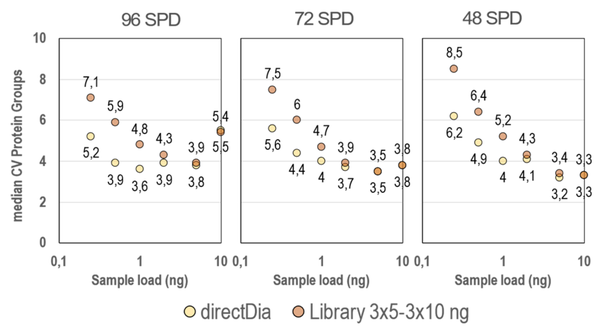
Figure 6. Proteins identified and quantified at CV ≤ 20% from human cell lysate digest standards using LC methods with respective sample throughput of 96, 72 and 48 samples per day. Top: Average number of protein groups identified using Proteome discoverer 3.1 and Chimerys. Middle: Average number of protein groups identified and quantified at CV ≤ 20% using a directDIA approach in Spectronaut 18. Bottom: Average number of protein groups identified and quantified at CV ≤ 20% using a library-based search approach in Spectronaut 18.



When dealing with low sample amounts, it was reported that utilizing a project (and method)-specific library generated from these limited samples produced more favorable outcomes compared to a library-free approach. By employing a spectral library-based approach, specifically utilizing a DIA library consisting of 3 x 5 ng and 3 x 10 ng DIA runs (totalling 47,236 precursors), we were able to identify 3845 protein groups from 250 pg HeLa protein digests. Furthermore, when working with sample loads above 1 ng, we successfully identified nearly 5,000 proteins using this approach.

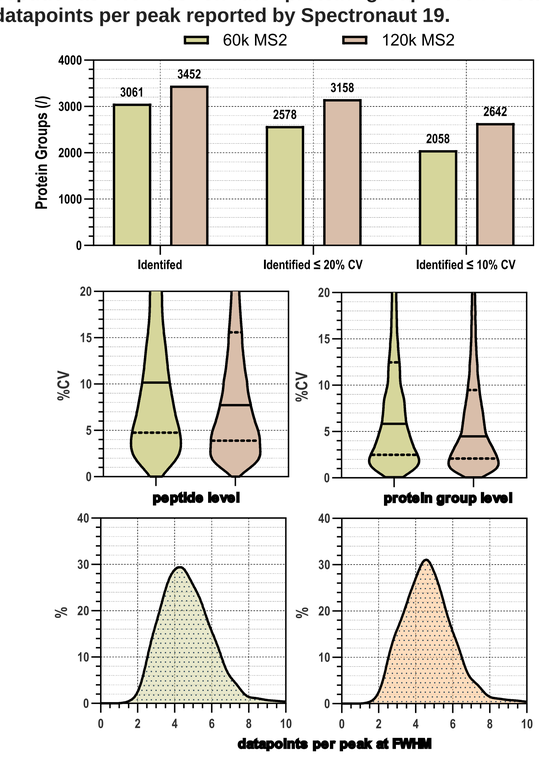
The library-based approach had the most significant impact on the lowest sample amounts, resulting in a relative increase of identifications of up to 47% compared to the library-free approach. It is worth noting that while the use of a library-based approach may slightly reduce overall quantification precision (as shown in Figure 7), there is still an absolute increase in the number of proteins quantified below a coefficient of variation of 20%. For the lowest sample amounts, this gain can reach up to 26%.

Figure 7. Median coefficients of variation reported on the protein group level as a function of amount injected. Comparison of library-free and library-based approach.



Subsequently, we performed a comprehensive evaluation to assess the impact of extending the gradient time, which consequently reduced sample throughput to 30 samples per day (SPD). Additionally, we examined the effects of increasing the MS2 resolution to 120,000 and expanding the isolation window from 30 to 60 m/z, while ensuring consistent scan cycle times. These modifications were achieved by increasing the maximum injection time to 246 ms and reducing the number of isolation windows from 10 to 5. Despite these adjustments, identical scan cycle times and data points per peak were maintained for both settings. Our results demonstrated a significant 11.5% increase in the number of identified protein groups. Furthermore, there was an improvement in quantitation, as evidenced by lower coefficients of variation (CVs) at both the peptide and protein group levels. This led to the quantification of 3,158 protein groups below 20% CV, compared to 2,578 protein groups at 60,000 MS2 resolution.

Figure 8. Comparison of DIA acquisition settings for technical triplicates of 250 pg bulk HeLa digest, separated at a throughput of 30SPD. Top: Identified and quantified protein groups. Middle: Coefficients of variation obtained on both the peptide as well as on the protein group level. Bottom: datapoints per peak reported by Spectronaut 19.



Conclusions

- Operating the column in a heating device at the ESI source improved chromatography and proteome coverage by reducing post-column volume and preserving separation performance.
- Peak widths reduced by 20% at 100 nL/min eluting flow rates.
- Proteome coverage impact was highest at the lowest flow rates, with up to 27% increase in identified proteins.
- Identified up to 2,609 protein groups from 250 pg of HeLa digest using a library-free processing approach.
- Identified up to 3,845 protein groups using a project-specific library from 3 x 5 ng and 3 x 10 ng DIA runs.
- Extending gradient time and increasing MS2 resolution increased identified protein groups by 11.5% and improved quantitation accuracy, with more protein groups quantified at below 20% CV.

Trademarks/licensing