Poster # P-II-0696

Efficient human plasma proteome profiling at up to 240 samples per day

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Abstract

Purpose: Develop and validate high throughput capillary flow LC-MS methods for use in translational research.

Methods: Neat human plasma protein digests were prepared and separated using capillary flow LC on a Thermo Scientific™µPAC Neo High throughput column hyphenated to Orbitrap MS for detection. Data was acquired in data independent acquisition.

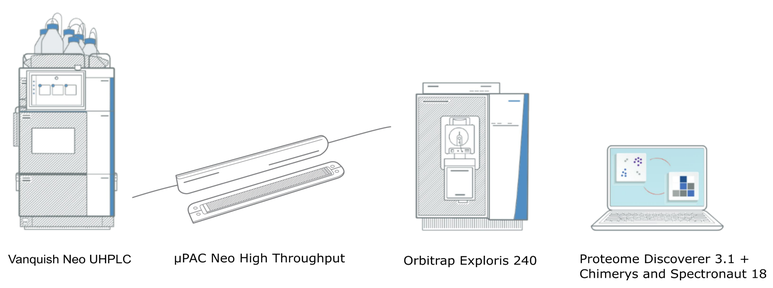
Results: At a sample throughput rate of 170 samples per day, 274 protein groups could be identified of which 248 could be quantified at precision levels below 20% CV.

Introduction

LC-MS-based proteomics stands as a vital tool for the analysis of intricate biological and clinical samples. Within the separation core, the LC column assumes a pivotal role by reducing sample complexity before its injection into the MS. The micro pillar array column (µPAC™) emerges as an innovative LC column featuring uniformly arranged pillars within a microfluidic channel, offering both highly efficient separation and minimal back pressure. In this study, we used a 5.5 cm long µPAC Neo High Throughput column that has channels filled with rectangular-shaped silicon pillars, coupled with HRAM mass spectrometry. Our goal was to expedite bottom-up proteomics profiling of neat human plasma samples while leveraging pillar arrays' benefits, such as high reproducibility and minimal sample carry-over.

Materials and methods

Figure 1. Experimental setup



Sample preparation

Pooled human blood plasma (Sigma Aldrich) underwent S-Trap™ mini spin column (Protifi) digestion, followed by resuspension in 0.1% TFA to 1000 ng/µL.

LC-MS configuration

Neat plasma digests were analyzed using a Thermo Scientific™ Orbitrap™ Exploris 240 mass spectrometer coupled to a Thermo Scientific™ Vanquish™ Neo UHPLC system. Separation occurred on a µPAC Neo High Throughput column in direct injection mode. The outlet of the LC column was connected to a 30 µm ID stainless steel emitter for optimal performance and stability at capillary flow rates. A dilution series from 10 to 2000 ng of neat plasma digests was injected by adjusting the injection volume from 10 nL to 2 µL and consequently separated using various flow rate methods. The mass spectrometer was operated in data independent acquisition (DIA) mode, with MS1 and MS2 resolution set at respectively 30 and 15K. Using an isolation window of 10 Th, precursor mass ranges were set from 500-700, 450-700 and from 400-800 m/z, yielding respectively 20, 25 and 40 scan events for the 240, 170 and 100 samples per day methods.

Data analysis

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

Table 1. Three Different Gradients for 240 SPD, 170 SPD and 100 SPD on Thermo Scientific™ µPAC Neo 5.5cm Columns. Using Optimized LC Settings on the Vanquish Neo System and Mass Spectrometric Method on Orbitrap Exploris 240.

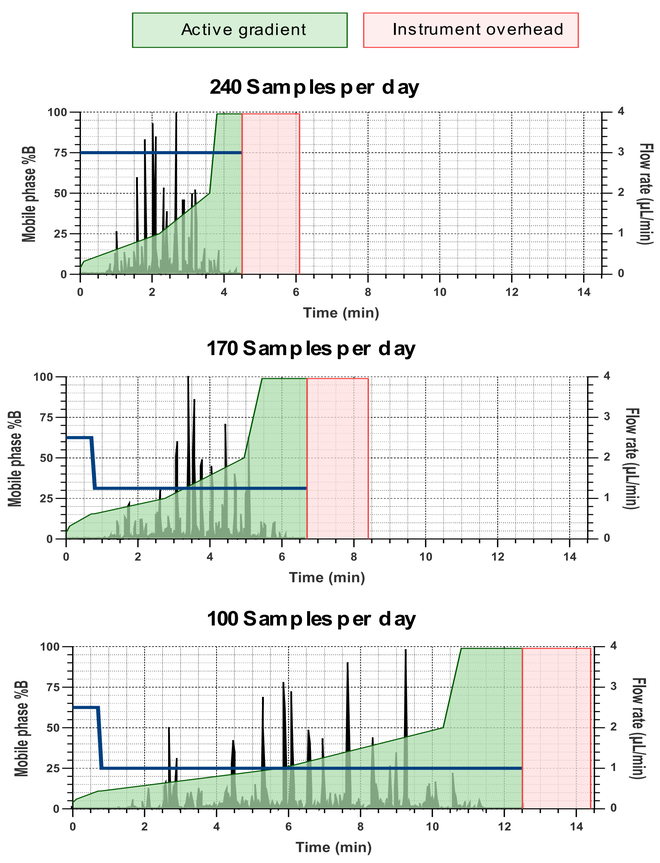
240 Samples per day			170 Samples per day			100 Samples per day		
Time (min)	*Flow rate (µL/min)*	*%B*	*Time (min)*	*Flow rate (µL/min)*	*%B*	*Time (min)*	*Flow rate (µL/min)*	*%B*
0	3.0	4	0	2.5	4	0	2.5	4
0.1	3.0	8	0.1	2.5	8	0.1	2.5	6
2.2	3.0	25	0.7	2.5	15.5	0.7	2.5	11
3.6	3.0	50	0.8	1.25	15.6	0.8	1.0	11.1
3.8	3.0	99	2.75	1.25	25	5.8	1.0	25
4.5	3.0	99	4.95	1.25	50	10.3	1.0	50
			5.45 – 6.7	1.25	99	10.8 - 2.5	1.0	99

Mass Spectrometric Method for All Gradients			
Global Parameters		***MS parameters***	
Polarization mode	Positive	Resolution MS1/DIA	30k/15k
Spray voltage	1,900V	Scan range MS1 (m/z) (240–170 / 100 SPD)	450-700/400-800
Ion transfer tube	300° C	RF lens (%)	70
Expected LC peak width	10 s	AGC target MS1/DIA (%)	300
APD	True	Isolation width (240/200/96 SPD)	10Th
Default charge state	2	Window overlap	1 Th
		Window placement optimization	On
		NCE (%)	28

Results

Three distinct capillary flow LC methods for high throughput have been devised to integrate sample throughput and sensitivity. Employing a direct injection approach, just under 2 minutes of the method are allocated to sample loading and column equilibration. Since the initial flow rate of the LC method permits complete column equilibration within half a minute, this procedure can proceed concurrently with the sample uptake phase. By employing this strategy, instrument productivity levels of 57%, 65%, and 76% were respectively attained at sample throughput rates of 240, 170, and 100 samples per day.

Figure 2. Gradient and flow rate profiles used to achieve 240, 170 and 100 sample per day throughput separations using a direct injection approach. Basepeak chromatograms obtained for the separation of 100 ng crude plasma digest sample has been overlaid.



Plasma proteome coverage

Using a 3.5-minute gradient 240 SPD method, the amount of protein groups that could be identified from neat human plasma steadily increased according to the sample load, up to a load of 200 ng plasma on column, where a plateau of about 250 protein groups was achieved.

Figure 3. Average number of protein groups identified (1% FDR- technical triplicates) from neat human plasma digest samples using LC methods with respective sample throughput of 240, 170 and 100 samples per day.

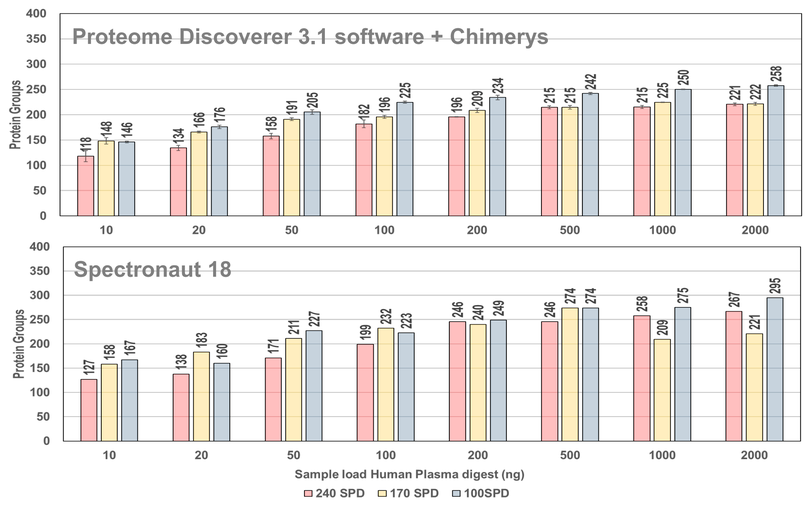
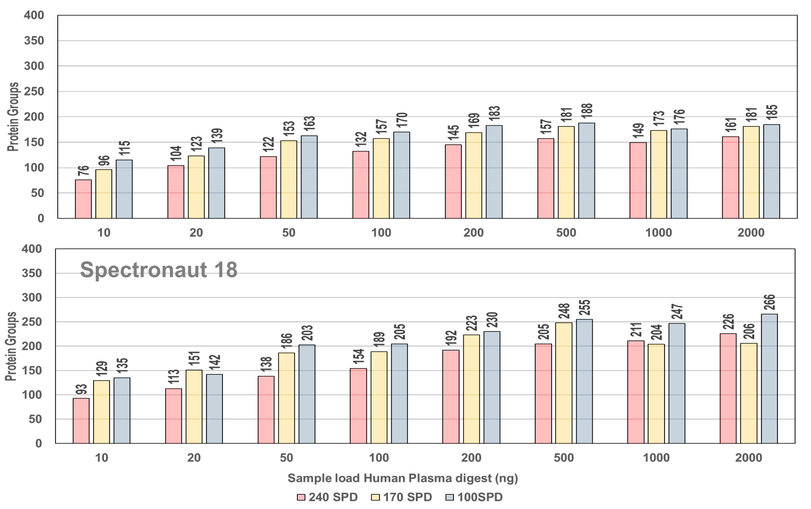
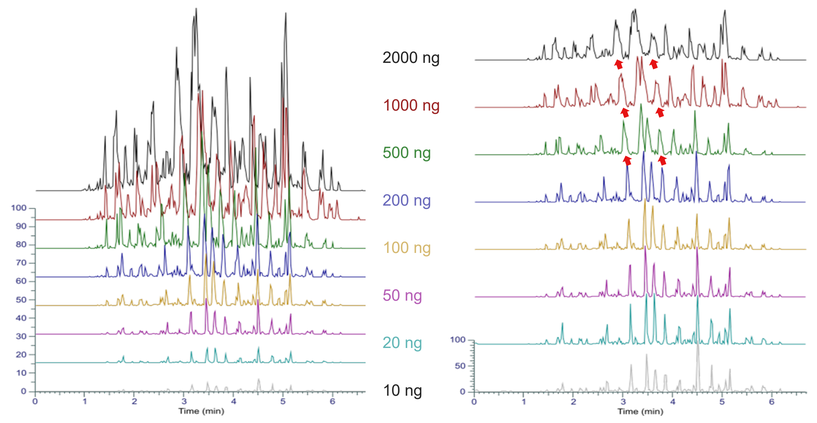


Figure 4. Proteins quantified at CV≤ 20% from neat human plasma digest samples using LC methods with respective sample throughput of 240, 170 and 100 samples per day.



Increasing beyond 200 ng didn't significantly improve protein identification, suggesting 200 ng is sufficient for favorable outcomes in this timeframe. Similar observations were done across different SPD settings (170, 100), where increasing sample loading beyond 500 ng did not increase proteome depth. This was also confirmed by checking the basepeak chromatograms and overlaying the different sample loads, where 200 ng was identified as the onset of overloading. Given the extremely high throughput of this method, fair quantitation could be achieved resulting in up to 205 protein groups quantified at CV below 20% for 500 ng of plasma analyzed in triplicate.

Figure 5. Basepeak chromatograms obtained for the separation of neat human plasma digest samples at a sample throughput rate of 170 samples per day. Dilution series from 10 to 2000 ng digest on column. Left: no signal normalization. Right: Intensity normalized for each sample load.



By extending the gradient length to respectively 5.5 and 11 minutes, increased depth and especially an increase in number of quantifiable proteins could be obtained. The µPAC Neo High Throughput column achieved a pragmatic compromise at 180 SPD, quantifying 248 protein groups (below 20% CV). The impact of extending gradients beyond the 5.5 min used for the 170 SPD method appeared to be very limited, as barely any depth could be gained. When comparing the results with those obtained with low capillary flow packed bed alternatives, significantly more proteins could be precisely quantified from technical triplicates (ranging from 60% to 5% for sample loads below 500 ng on column).

Figure 6. Comparison of µPAC Neo High throughput to Packed bed column alternatives. Proteins quantified at CV≤ 20% from neat human plasma digest samples using an LC method with a sample throughput of 170 samples per day.

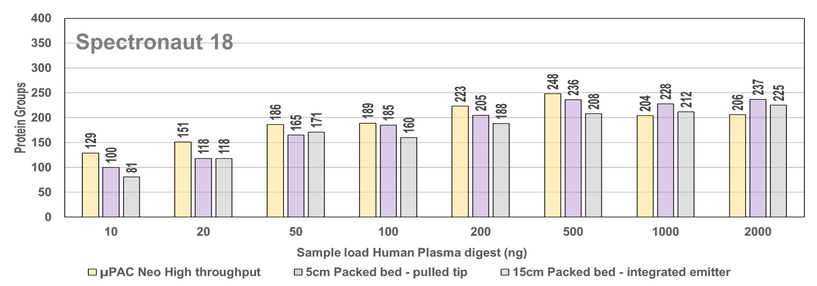
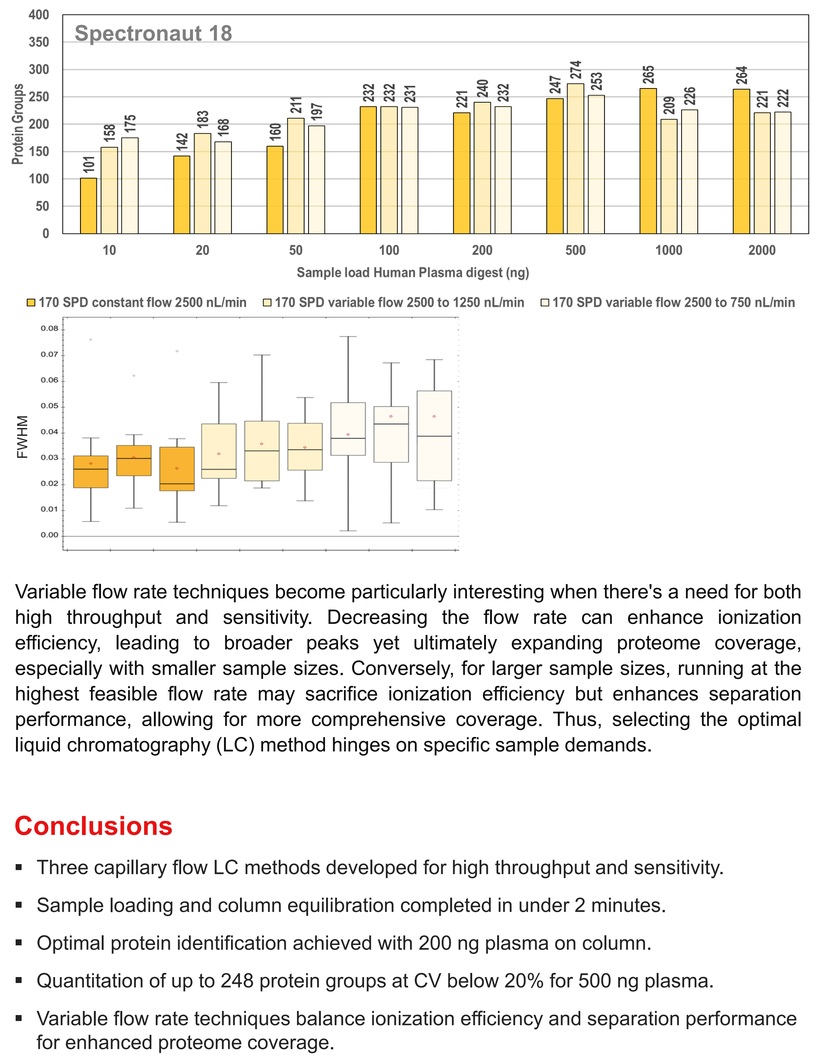


Figure 7. Top: Average number of protein groups identified (1% FDR- technical triplicates) from neat human plasma digest samples using three different LC methods with a sample throughput of 170 samples per day. Constant flow rate method versus variable flow rate method. Bottom: Respective peak width distributions obtained using 3 different 170 samples per day methods.



Variable flow rate techniques become particularly interesting when there's a need for both high throughput and sensitivity. Decreasing the flow rate can enhance ionization efficiency, leading to broader peaks yet ultimately expanding proteome coverage, especially with smaller sample sizes. Conversely, for larger sample sizes, running at the highest feasible flow rate may sacrifice ionization efficiency but enhances separation performance, allowing for more comprehensive coverage. Thus, selecting the optimal liquid chromatography (LC) method hinges on specific sample demands.

Conclusions

- Three capillary flow LC methods developed for high throughput and sensitivity.
- Sample loading and column equilibration completed in under 2 minutes.
- Optimal protein identification achieved with 200 ng plasma on column.
- Quantitation of up to 248 protein groups at CV below 20% for 500 ng plasma.
- Variable flow rate techniques balance ionization efficiency and separation performance for enhanced proteome coverage.

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