High-throughput proteomics

At the intersection between chromatographic performance, ESI efficiency and instrument productivity: nano to capillary flow LC/MS on long µPAC columns

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Abstract

Purpose: Exploration of the maximum throughput achievable with improved 110 cm Thermo Scientific™ µPAC™ Neo Plus Columns at capillary flow rates. Evaluation of the impact of post-column dispersion in single emitter and dual-column tandem configurations.

Methods: HeLa cell digests were resuspended in 0.1% TFA and 1% ACN to create a 200 ng/µL stock solution, sonicated, diluted in 0.1% TFA, and vortexed. A Thermo Scientific™ Vanquish™ Neo UHPLC instrument was configured for either a single analytical column in a trap-and-elute setup or two columns in tandem. For single column experiments, a PepMap™ Trap Cartridge and a 110 cm µPAC Neo Plus column on a Thermo Scientific™ EASY-Spray™ Source were used. LC methods with flow rates from 200 to 1000 nL/min were tested for throughputs ranging from 16 to 50 samples per day. The column was positioned in an external heating device mounted directly onto the ionization source and connected to an ESI emitter via a 20 µm ID voltage spacer. For tandem column experiments, two 110 cm µPAC Neo Plus columns were placed in the Thermo Scientific™ Vanquish™ Neo column compartment between two low-dispersion 6-port switching valves. A 20 µm ID Thermo Scientific™ nanoViper™ line connected the eluents to an ESI emitter with an integrated liquid junction in an EASY-Spray source. Data were acquired using a Thermo Scientific™ Orbitrap Exploris™ 240480 mass spectrometer in data-independent acquisition mode.

Results: Optimal LC methods with throughput rates of 50, 40, 30, and 16 SPD were evaluated, identifying up to 8585 protein groups for the longest method. For gradients under 30 minutes, a capillary flow rate of 1 µL/min was optimal, identifying 6814 protein groups from 500 ng, with 90% quantified below 20% CV. This flow rate minimized the impact of a low dispersion valve, allowing tandem column operation with minimal chromatographic performance loss. Tandem operation increased productivity by over 25%, identifying up to 6848 protein groups at true 50 SPD with a median CV on protein abundance of 7.2% across 5 different columns.

Introduction

LC-MS-based proteomics commonly employs low-flow liquid chromatography (LC) to separate tryptic peptides, which helps reduce sample complexity before MS/MS detection and protein identification. This technique benefits from minimal dilution on miniaturized columns and increased ionization efficiency via nanoflow electrospray ionization (ESI). As mass spectrometry (MS) devices become more sensitive and faster, there is an increasing need to boost throughput. The challenge lies in maintaining or increasing proteome coverage while simultaneously reducing analysis time. Typically, higher flow rates improve instrument productivity but may compromise sensitivity. In this study, the impact of eluting flow rates was assessed across various throughput ranges to identify the optimal balance between productivity and proteome coverage. Additionally, the transition to a dual-column single emitter configuration was evaluated to determine its effects on productivity and coverage. This approach aims to enhance LC-MS performance, ensuring high throughput without sacrificing the depth of proteome analysis.

Materials and methods

Sample preparation

HeLa cell digests were resuspended in 0.1% TFA and 1% ACN to obtain a 200 ng/µL stock solution, sonicated, diluted, and vortexed before use.

MS configuration and data acquisition settings

All experiments were conducted using a 10 µm ID fused silica emitter with integrated liquid junction (ES993) and an EASY-Spray source coupled to an Orbitrap Exploris mass spectrometer with either 240 or 480 max resolution.

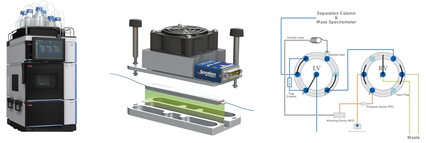
Data independent acquisition (DIA) parameters were as follows: MS1 resolution = 120k, MS2 resolution = 30k, MS1 AGC = 300%, MS2 AGC = 800%, precursor mass range = 525-825 Th, MS1/MS2 maxIT = auto. Isolation widths were varied based on gradient length: 16 SPD = 8Th or 4Th, 30 SPD = 8Th, 40 SPD = 12Th, 50SPD = 15Th.

Data Analysis

LC-MS data were analyzed either using a trial version of Thermo Scientific™ Proteome Discoverer™ 3.3 Software with CHIMERYS or with Spectronaut® 19. Results shown have been filtered to a 1% FDR.

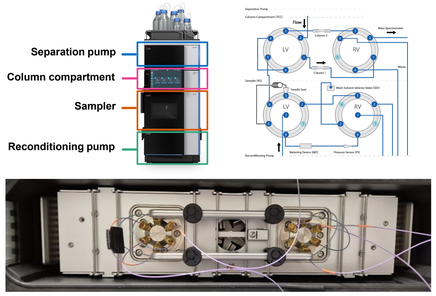
LC configuration (1) - Trap and Elute workflow

- The 110 cm µPAC Neo Plus configuration minimizes pre- and post-column dispersion.
- The new columns are fully bidirectional, featuring equal-length capillaries on both ends.
- Utilizing a voltage spacer (20 µm ID, ACCVSP2006) for grounding prevents electrical current from interfering with the separation behaviour of multiply charged species and reduces on-column oxidation.
- Columns are placed in an external heating device (Sonation, PRSO-V2_PF) mounted onto the ionization source.
- Sample loading onto a PepMap Neo trap cartridge in backflush trap-and-elute mode minimizes overhead and enhances workflow robustness.



LC configuration (2) - Tandem Direct Injection workflow

- Two 110 cm µPAC Neo Plus columns were installed between two 6-port valves in the column compartment of the Tandem Vanquish Neo.
- Connection to a 10 µm ID fused silica emitter (ES993) was facilitated by a 550 mm long, 20 µm ID nanoViper capillary (6250.5260).
- Additional grounding of the 110 cm µPAC Neo Plus columns in TDI is unnecessary, as it is achieved via the column compartment valves.



Results

Optimized flow rate methods for 50, 40, 30 & 16 SPD

Figure 1. Left: Evolution of peak width as a function of gradient length for 3 different flow rates. Right: Observed void time versus flow rate (blue) and column pressure versus flow rate (red).

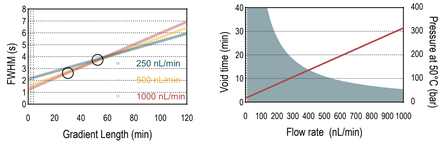
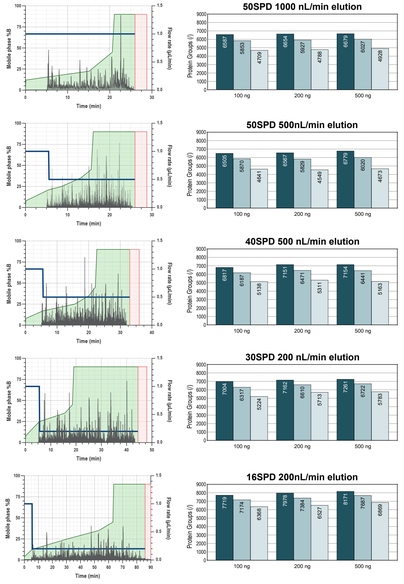


Figure 2. Left: Gradient and flow rate profiles for LC methods with throughput rates of 50, 40, 30, and 16 SPD, overlaid with the basepeak chromatogram for the separation of 200 ng HeLa digest. Right: Protein groups identified (dark teal) and quantified (teal < 20% CV, light teal < 10% CV) from technical triplicates and dilution series ranging from 100 to 500 ng of HeLa digests.



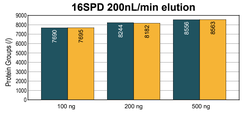


Figure 3. Protein groups identified from 200ng HeLa digests (n=3) acquired with isolation windows of 4Th. Spectronaut 19 (blue) versus trial version of Proteome Discoverer 3.3 with CHIMERYS (yellow).

Dual column single emitter operation at 50 SPD

Figure 4. Impact of a low-dispersion 6-port valve in the post-column plumbing. Left: Peak width versus flow rate. Right: Protein groups identified versus flow rate.

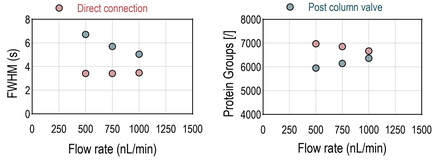
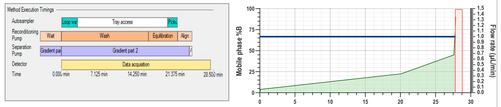
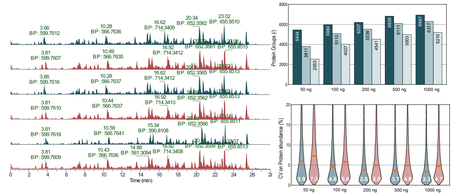


Figure 5. Top: LC gradient profiles and tandem workflow execution timings for the 50 SPD throughput method. Bottom: Productivity comparison between single column direct injection set-up and trap and elute workflow.



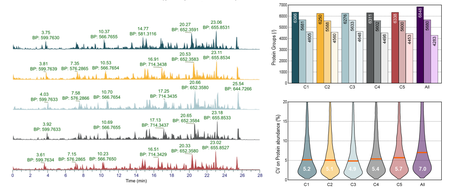
50 Samples per day	Active Elution (min)	Overhead (min)	Total (min)	Productivity
Direct Injection	18.8	10	28.8	65%
Trap and Elute	19.8	9	28.8	69%
Tandem Direct Injection	27.3	1.5	28.8	95%

Figure 6. Left: Base peak chromatograms obtained for the separation of 200 ng HeLa digest using a 50SPD method and run on 2 columns in tandem. (Blue = column 1, Red = column 2) Top Right: Protein group identifications. Total IDs (dark teal), IDs with CVs < 20% (teal) and IDs with CVs < 10% (light teal), n=6. Bottom Right: Violin plots of CVs on protein group level for single column (teal, n=3) versus dual column (red, n=6). Red mark indicates median CV.



Reproducibility between columns

Figure 7. Left: Basepeak chromatograms for the separation of 500 ng HeLa digest using a 50 SPD method on 5 tandem columns. Top Right: Protein group identifications for each column (n=6) and combined (n=30), showing total IDs (dark), IDs with CVs < 20% (medium), and IDs with CVs < 10% (light). Bottom Right: Violin plots of CVs at the protein group level for single columns (C1-C5, n=6) versus all columns combined (purple, n=30). Red mark indicates median CV.



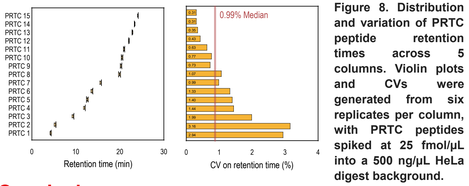


Figure 8. Distribution and variation of PRTC peptide retention times across 5 columns. Violin plots and CVs were generated from six replicates per column, with PRTC peptides spiked at 25 fmol/µL into a 500 ng/µL HeLa digest background.

Conclusions

- Increasing the flow rate to 1 µL/min significantly increases instrument productivity. For gradient times below 30 minutes, 1 µL/min performs slightly better than lower flow methods, identifying up to 6679 protein groups at 50 SPD.
- For gradient lengths over 50 minutes, a 200 nL/min flow rate provides superior chromatographic metrics and proteome coverage, resulting into a maximum of 8556 protein groups identified at 16 SPD.
- The negative impact of a low-dispersion 6-port valve's impact scales inversely with flow rate. At 1 µL/min, peak width increases by 40%, yielding 5% lower proteome coverage but 26% higher productivity.
- Optimized dual column single-emitter methods identified up to 6947 protein groups at 50 SPD with 3-6% CVs for the same column and 4-8% between columns (n=6).
- Column reproducibility was shown across 5 columns, with an ID rate variation of 0.7% for HeLa digests and a median RT CV of 0.99% for spiked-in PRTC peptides.

References

R Zheng, M Rendl, AC Valenta, C Pynn, Y Lin, M Daniliuk, E Aydin, R van Ling, L Taujenis, W Decrop, M Samonig, A Morgenstern; *A dual-column, single-spray configuration for capillary and micro-flow LC-MS applications*, TN003314, Thermo Fisher Scientific

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