Poster # P-II-0441

High-throughput LC-MS

A tandem capillary and micro-flow LC workflow for high-throughput quantitative proteomics at near 100% mass spectrometer utilization

Runsheng Zheng[1], Martin Rendl[1], Christopher Pynn[1], Alec Valenta[2], Ece Aydin[1], Maksim Daniliuk[3], Robert van Ling[4], Wim Decrop[1], Martin Samonig[5], Anne Morgenstern[1]
Thermo Fisher Scientific, [1]Germany; Thermo Fisher Scientific, [2]USA; Thermo Fisher Scientific, [3]Litiuanija; Thermo Fisher Scientific, [4]Netherlands; Thermo Fisher Scientific, [5]Austria

Abstract

Purpose: Evaluate a Thermo Scientific™ Vanquish™ Neo UHPLC system tandem capillary/micro-flow LC workflow for maximizing mass spectrometer (MS) utilization in bottom-up proteomics.

Methods: Capillary and micro-flow method performance was evaluated using 150 - 1,000 µm inner diameter (I.D.) columns at sample throughputs from 48 – 277 samples/day (SPD).

Results: We demonstrated that flow rates >1 µL/min were compatible with a post-column valve without a significant impact on peak widths (FWHM), simplifying workflow implementation. At 180 SPD using a 150 µm x 150 mm column, from 3,111 - 4,281 protein groups were identified in 50 - 1,000 ng of HeLa digest. Across 30 injections and 8 columns the median RT and protein abundance RSD for 200 ng digest were 4% and 6%, respectively, while an average of >3,200 protein group IDs had CV < 10%. We then evaluated column carryover using extended washing cycles without impacting cycle time, yielding ~0.03% for 200 ng HeLa digest at 180 SPD. Throughput was increased to 240 SPD using a 300 µm x 15 cm column, yielding 2,282 protein groups from 200 ng digest. Decreasing column length to 5 cm column enabled 277 SPD, with 2,400 protein groups identified from 1 µg digest.

Introduction

Nano-flow based tandem direct injection (TDI) workflows are highly effective at increasing sample throughput where flow rates < 1 µL/min are utilized for 50 - 75 µm I.D columns.[1] Although nano-flow LC-MS provides high sensitivity, column and emitter robustness presents a major challenge. In addition, sample throughput is limited by the high backpressures when using 50 - 75 cm columns. Such limitations are overcome using flow rates >1 µL/min with capillary and micro-bore columns (150 µm - 1 mm I.D.).[2] The decrease in sensitivity when using higher flow rates can be partially mitigated by injecting more sample on column.

Here we demonstrate the use of the Vanquish Neo UHPLC system TDI workflow for capillary and micro-flow LC (1 – 100 µL/min) to increase throughput and MS utilization. This configuration, featuring two columns with a single electrospray ionization (ESI) emitter, offers several benefits. First, it eliminates variation arising from using multiple emitters, as is required for the nano/capillary (dual spray) configuration.[1] Second, emitter lifetime is extended by flushing salts to waste and maintaining a constant flow rate through offline column cleaning and equilibration. Similar nano-flow, it employs a dedicated gradient pump for high intercolumn retention time reproducibility. Both configurations incorporate intelligent method design for sequence setup and automatic alignment of workflow parameters such as flowrate and pump pressure, as well as appropriate timing for valve switching.

Materials and methods

Sample preparation

Thermo Scientific™ Pierce™ HeLa Digest/PRTC Standard(P/N A47996, 10 µg/vial) was reconstituted by adding 50 µL of 0.1% formic acid (FA) in water with 2% acetonitrile (ACN). The vial was subsequently sonicated for 5 min, followed by aspirating and releasing 10 times with a pipette to fully reconstitute the sample.

Hardware and fluidic configuration

TDI capillary/micro-flow configuration:

- Vanquish Neo UHPLC system
- Thermo Scientific™ Vanquish™ Column Compartment N
- Two 2-position 6-port low-dispersion switching valves
- Thermo Scientific™ Vanquish™ Binary Pump N
- Tandem Workflow Kit, Vanquish Neo
- Thermo Scientific™ Nanospray Flex™, Thermo Scientific™ EASY-Spray™, or Thermo Scientific™ OptaMax NG™ ion sources
- Vanquish Neo system driver 1.5 for tandem workflow execution
- Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer operated in data independent acquisition (DIA) mode

TDI methods (**Tables 1-2**) were performed using the following capillary and micro-flow columns: Thermo Scientific™ Double nanoViper PepMap™ Neo UHPLC columns (150 µm I.D. x 15 cm) or Thermo Scientific™ Acclaim PepMap™ 100 HPLC columns (300 µm x 15 cm or 1 mm I.D. x 5-15 cm). For capillary (1 - 5 µL/min) and micro-flow (1 - 5 µL/min) methods, the Thermo Scientific™ EASY-Spray™ capillary emitter (ES994, 15 µm I.D.) and low-flow metal needle insert (50 µm I.D.) were used, respectively. Column pairs were place within the column compartment (**Figure 2**).

Data analysis

Acquired .raw files were processed using the CHIMERYS™ DIA node (MSAID) or Spectronaut™ 19 software (Biognosys AG). The false discovery rate (FDR) was set below 1% at the peptide and protein level.

Table 1. Capillary-flow method overview

Column	Flow Rate (µL/min)	Throughput (samples/day)	Elution Window (min)	MS utilization (%)
PepMap Neo 150 µm x 15 cm	1.5	48	29.2	97
		60	23	96
		100	13.4	93
	1.6	180	7.2	90
	3.8	225	5.76	90

Table 2. Micro-flow method overview

Column	Flow Rate (µL/min)	Throughput (samples/day)	Elution Window (min)	MS utilization (%)
PepMap 300 µm x 15 cm	15	60	23.45	98
		100	13.85	96
		240	5.46	91
PepMap 1 mm x 5 cm	50	180	7.6	95
		240	5.6	93
	100	277	4.4	85
PepMap 1mm x 15 cm	50	60	23.15	96
		100	13.68	95
		120	11.35	95

Tandem Direct Injection workflow

The Vanquish Neo TDI workflow utilizes 2 LC pumps and 2 columns to eliminate method overhead (**Figure 1A**). The system performs column loading, washing, and equilibration on column 2 in parallel to acquisition on column one, decreasing cycle times and maximizing mass spectrometer utilization (**Figure 1B**).

Figure 1. Overview of LC system hardware (A) and operational principal (B) of TDI workflow.

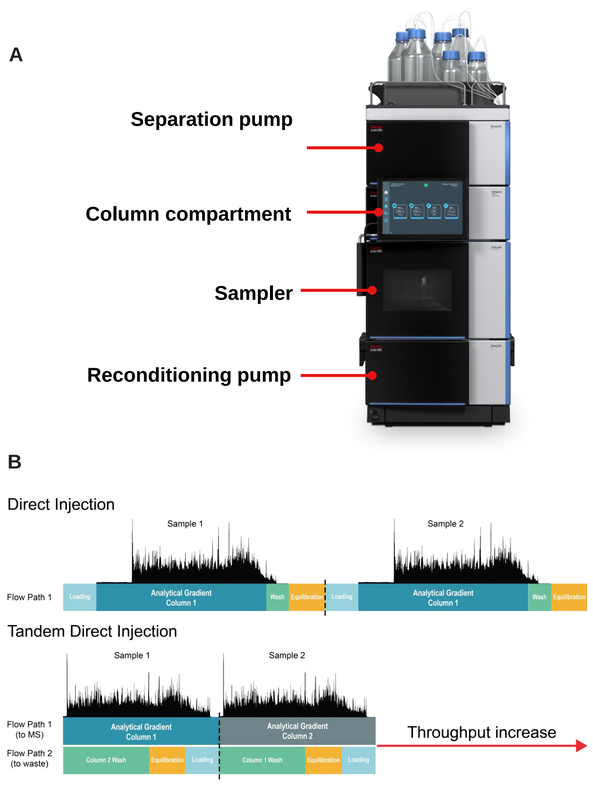
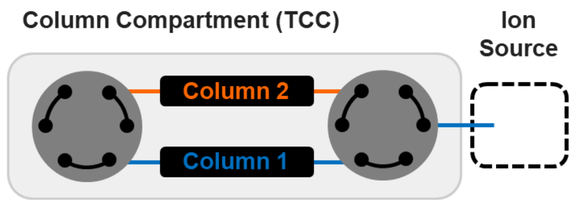


Figure 2. Each pair of identical columns was house within the column compartment. A post-column valve was utilized for switching between active columns. During loading, washing, and equilibration the inactive column was flowed to waste, extending ESI emitter lifetime.



To ensure optimal TDI operation the instrument method editor aligns the execution timings of each module within the LC system **(Figure 3)**. Boundary conditions for all Method parameters are determined by the system controller based on column pressure and flow specifications including flow resistance, which is determined by running script A00.

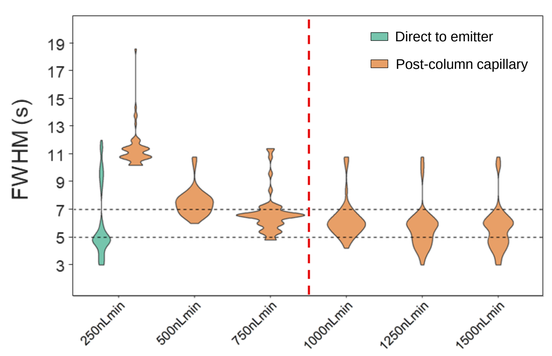
Figure 3. Method execution timings for 180 SPD method.

Results

Optimizing chromatographic performance

The impact of post-column system dispersion was evaluated at flow rates from 250 - 1,500 nL/min. It was determined that post-column dispersion did not have a major impact on PRTC peptide peak FWHM at flow rates >1 µL/min (**Figure 4**).

Figure 4. Impact of post-column dispersion (20 µm x 55 cm capillary) on average peak width for 15 PRTC peptides.

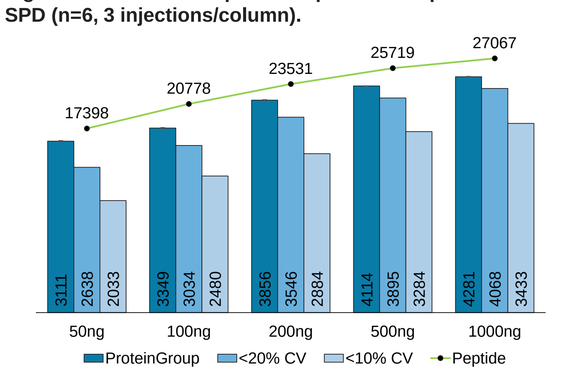


Evaluating PepMap Neo Double nanoViper columns for High-throughput applications

Using the new Double nanoViper 150 µm x 15 cm PepMap Neo column, methods were developed ranging from 48 - 225 SPD. Using flow rates from 1.5 - 3.8 µL/min provided MS utilization from 90 - 97%. At 180 SPD, we identified 3,111 - 4,281 protein groups from 50 - 1,000 ng (250 - 2,500 nL injection) HeLa digest in DIA mode (**Figure 5**). This resulted in > 3,500 protein groups quantified in 200 ng with CV < 20% (**Figure 5**).

Note that the above method provides a 7.2-minute elution window. In contrast, direct injection and T&E workflows require ~4 and 2 minutes of additional overhead time, respectively, for a comparable peptide elution window. Accordingly, the tandem direct injection workflow yields a 25–50% increase in throughput for capillary flow methods.[3]

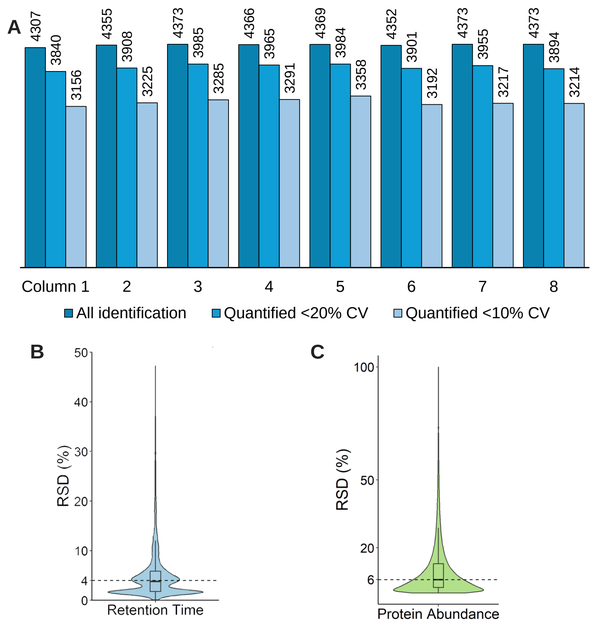
Figure 4. Proteome depth and quantitative precision at 180 SPD (n=6, 3 injections/column).



Intercolumn precision

Column-to-column reproducibility was evaluated using 150 µm x 15 cm PepMap Neo columns (8) at 180 SPD. We observed ~4% retention time RSD over 30 injections with 200 ng HeLa digest (**Figure 5B**). The low RT variability provided reproducible identification of >4,300 protein groups (**Figure 5A**) with <6% CV of median protein abundance when enabling match-between-run (**Figure 5C**).

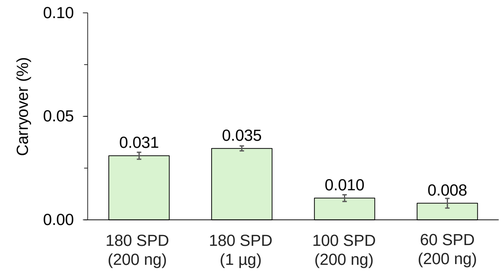
Figure 5. Intercolumn precision including proteome depth (A), retention time (B), and protein abundance (C) (n=8).



Reducing column carryover

Compared to a direct injection workflow, TDI enables increased column washing without impacting cycle time. In addition, pre-programmed washing patterns simplify method creation for a variety of different sample types. Using the trapezoidal wash and methods from 60 - 180 SPD, we evaluated the carryover for 200 ng and 1 µg HeLa injections. This resulted in low quantified peptide peak area for high-throughput methods, reaching 0.35% carryover for 1 µg of at 180 SPD (**Figure 6**).

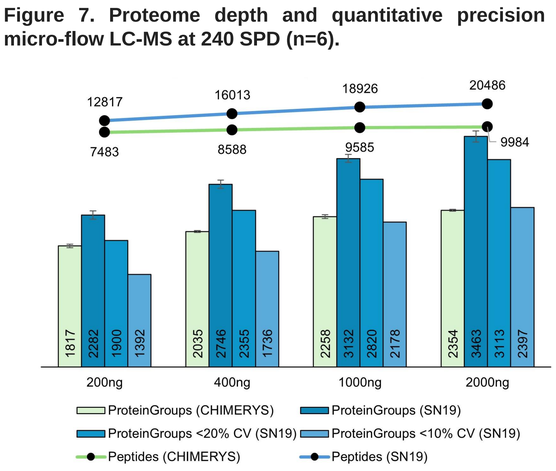
Figure 6. Column carryover quantified as percent of HeLa digest peak areas (n=6).



Increasing throughput with micro-bore columns

TDI is compatible with micro-bore columns (0.3 - 1 mm I.D.) for increased throughput and robustness. Using a 300 µm x 15 cm column at 15 µL/min increased throughput to 240 SPD at 91% MS utilization (**Table 2**). With this method, >2,200 proteins were identified from 200 ng HeLa digest (**Figure 7**, Spectronaut™).

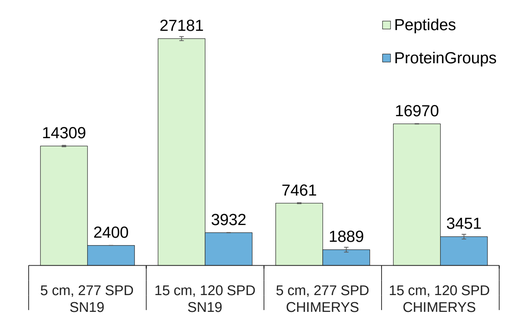
Figure 7. Proteome depth and quantitative precision for micro-flow LC-MS at 240 SPD (n=6).



Ultra-robust proteome profiling with 1 mm columns

Utilizing either a 1 mm x 5 cm or 1 mm x 15 cm PepMap column enabled 277 and 120 SPD with 2,400 and 3,932 protein groups identified, respectively (**Figure 8**, Spectronaut™ 19).

Figure 7. Proteome depth for micro-flow LC-MS at 120 and 277 SPD (1 µg digest, n=6).



Conclusions

A new workflow was developed and evaluated for high-throughput capillary and micro-flow proteome profiling. The workflow offers:

- >90% MS utilization across a variety of columns, flow rates, and sample throughputs
- Intelligent workflow execution and optimized chromatographic performance
- High intercolumn reproducibility
- Low carryover through extended washing cycles

References

1. Zheng, R.; et al. Thermo Fisher Scientific, Technical Note 003335 (2024).
2. Bian, Y.; et al. Robust, reproducible and quantitative analysis of thousands of proteomes by micro-flow LC–MS/MS. Nature Communication 2020, 11,157.
3. Zheng, R.; et al. Thermo Fisher Scientific, Technical Note 000138 (2021).

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