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A dual-column, single-spray configuration for capillary and micro-flow LC-MS applications

Authors

Runsheng Zheng[1], Martin Rendl[1],
Alec C. Valenta[2], Christopher Pynn[1],
Yuan Lin[2], Maksim Daniliuk[3], Ece Aydin[1],
Robert van Ling[4], Lukas Taujenis[3],
Wim Decrop[1], Martin Samonig[5],
Anne Morgenstern[1]

[1]Thermo Fisher Scientific, Germany

[2]Thermo Fisher Scientific, USA

[3]Thermo Fisher Scientific, Lithuania

[4]Thermo Fisher Scientific, Netherlands

[5]Thermo Fisher Scientific, Austria

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Goal

Demonstrating a tandem LC-MS configuration for high-throughput proteomics and peptide quantification with near 100% MS utilization

Introduction

Nanoflow-based tandem direct injection workflows are highly effective at increasing sample throughput in high-sensitivity analysis where flow rates below 1 µL/min for columns with 50 and 75 µm internal diameters (I.D.) and lengths ranging from 15 to 75 cm are employed.[1] Nevertheless, the challenges associated with nano-column and/ or emitter clogging caused by specific sample matrices or higher sample loads remain. These limitations can, however, be overcome by adopting flow rates above 1 µL/min with capillary and micro-bore columns (150 µm – 1 mm I.D.), which offer advantages in terms of increased robustness and throughput.[2] The loss in sensitivity when moving to higher flow rates can be partially overcome both by injecting larger sample quantities and through enhanced mass spectrometer ion sampling efficiency.

Here we demonstrate the use of the Thermo Scientific™ Vanquish™ Neo UHPLC system tandem direct injection workflow for capillary and micro-flow LC separations to further increase throughput and MS utilization within this application space. This configuration, featuring two columns with a single electrospray ionization (ESI) emitter, offers several benefits. Firstly, it eliminates analytical variation arising from using two emitters, as is required for the nano/capillary (dual spray) configuration.[1] Secondly, the emitter lifetime is extended by flushing salts to waste and maintaining a constant flow rate through fast, offline column cleaning and equilibration. Similar to the tandem nano-flow direct

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injection workflow, this configuration also employs a dedicated pump for gradient delivery on both columns, ensuring higher inter-column retention time reproducibility. In addition, both nano-flow and capillary/micro-flow configurations incorporate an intelligent LC method design for simple sequence setup and automatic alignment of critical workflow parameters such as flow rate and pressure of the respective pumps, as well as appropriate timing for valve switching. This configuration is supported by standardized, optimal fluidics using Thermo Scientific™ nanoViper™ Fingertight fittings with 20 µm I.D. (1–5 µL/min) and 50 µm (5–100 µL/min) capillaries (Table 1).

To demonstrate the performance of this setup, we evaluated reproducibility using newly introduced Thermo Scientific™ double nanoViper (DNV) 150 µm × 150 mm Thermo Scientific™ PepMap™ Neo columns at a throughput of 180 samples/day. Subsequently, proof-of-concept studies were carried out using micro-bore columns with 300 and 1000 µm I.D. to further increase the throughput and expand the application range.

Overall, the tandem capillary and micro-LC configuration offers enhanced throughput and greater mass spectrometer utilization in high-sensitivity analysis, covering flow rates from 1 to 100 µL/min.

Experimental

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.

Consumables

- Water with 0.1% FA (v/v), Optima™ LC/MS grade, Thermo Scientific™ (P/N LS118-500)

- 80% ACN with 0.1% FA (v/v), Optima™ LC/MS grade, Fisher Chemical™ (P/N LS122500)

- Formic acid (FA), Optima™ LC/MS grade, Fisher Chemical™ (P/N 10596814)

- Isopropanol (IPA), Optima™ LC/MS grade, Fisher Chemical™ (P/N 10684355)

- Fluidics and columns used to run the Vanquish Neo UHPLC system tandem direct injection workflow for the respective capillary and microflow based applications are given in Table 1 and Figure 1.
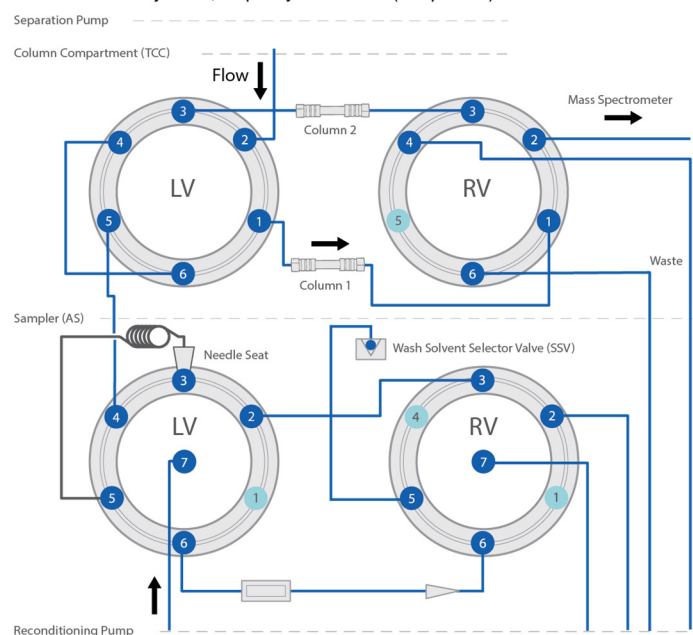

Figure 1. Fluidic configuration for tandem capillary and micro-flow LCMS applications

Table 1. Fluidic connections and accessories for tandem capillary and micro-flow LC-MS configurations

Flow regime	Fluidic connection	Separation column	MS ion source	Emitter
Capillary flow (1–5 µL/min)	20 µm I.D. nanoViper capillary	PepMap Neo 150 µm I.D. × 150 cm (P/N DNV150150PN)	Nanospray Flex ion source	Stainless steel emitter (ES542)
			Easy-Spray / Nanospray Flex ion source	Bullet emitter (ES994) / Stainless steel emitter (ES542)
Micro-flow (5–100 µL/min)	50 µm I.D. nanoViper capillary	PepMap 300 µm I.D. × 15 cm (P/N 164537)	OptaMax NG ion source	Low-flow needle insert* (OPTON-30139, 50 µm I.D.)
		PepMap 1,000 µm I.D. × 5 cm (P/N 164454)		
		PepMap 1,000 µm I.D. × 15 cm* (P/N 164711)		

*Included in Tandem Workflow Kit

Tandem capillary and micro LC hardware and fluidic configurations

- Vanquish Neo UHPLC system (P/N VN-S10-A + Vanquish Display P/N 6036.1180)

- Thermo Scientific™ Vanquish™ Column Compartment N (P/N VN-C10-A)

- Two 2-position 6-port low-dispersion switching valves (P/N 6250.1520)

- Thermo Scientific™ Vanquish™ Binary Pump N (P/N VN-P10-A)

- Tandem Workflow Kit, Vanquish Neo (P/N 6250.1030)

- Thermo Scientific™ Nanospray Flex™ (w/ ES542 emitter), Thermo Scientific™ EASY-Spray™ (w/ ES994 emitter), or Thermo Scientific™ OptaMax NG™ (w/ OPTON-30139 low-flow needle) ion sources

- Vanquish Neo system driver 1.5 for tandem workflow execution

This configuration supports the tandem direct injection workflow using nanoViper fingertight fittings for fluidic connections (20 μm I.D. for capillary flow and 50 μm for micro-flow applications) and is optimized for maximum separation performance.

LC solvents

The recommended solvents are listed in Table 2.

Installing and connecting the capillary and micro-columns

Two columns of identical dimensions were installed between two low dispersion switching valves in the column compartment using either 20 μm or 50 μm I.D. capillaries according to the workflow instructions provided on the Vanquish User Interface (VUI). The outlet capillary from the post-column valve was then connected to a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer via the ion sources and emitters described in Figure 1. Intelligent workflows ensure that the pre- and post-column switching valve positions are synchronized to exclusively deliver the active peptide elution window from each column to the mass spectrometer (Figure 2).

MS acquisition

MS data were acquired using an Orbitrap Exploris 240 mass spectrometer in data-independent acquisition (DIA) mode. All MS acquisition settings are available for download in the Thermo Scientific™ AppsLab Library of Analytical Applications for all reported methods.

Data acquisition and processing

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. The data processing templates are available for download from the AppsLab Library.

Table 2. Solvents for Vanquish Neo operation in tandem configuration

|	Solvent	Composition
Binary Pump N (Upper pump / separation pump)	Mobile phase A	H_2O with 0.1% FA
	Mobile phase B	80/20 (v/v) ACN / H_2O with 0.1% FA
Binary Pump N (Lower pump / reconditioning pump)	Mobile phase A	H_2O with 0.1% FA
	Mobile phase B	80/20 (v/v) ACN / H_2O with 0.1% FA
Split Sampler NT Metering Device	Weak wash liquid	H_2O with 0.1% FA
	Strong wash liquid	80/20 (v/v) ACN / H_2O with 0.1% FA
Split Sampler NT Wash Port	Weak wash liquid	H_2O with 0.1% FA
	Strong wash liquid	ACN with 0.1% FA
Binary Pump N and Split Sampler NT	Rear seal wash buffer	25/75 (v/v) H_2O / IPA with 0.1% FA
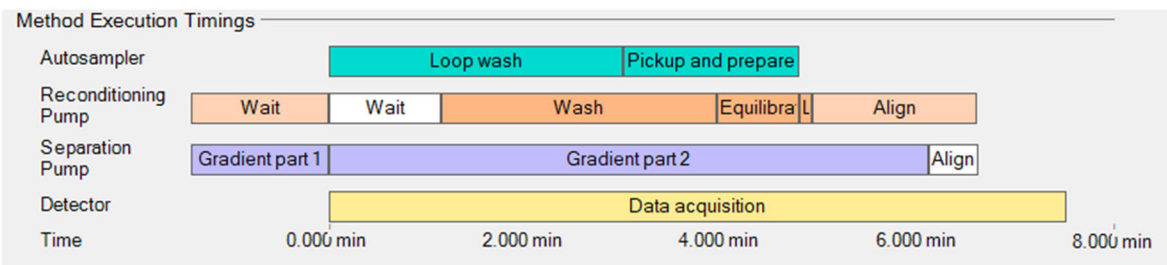
Figure 2. Example workflow execution schematic as displayed in the instrument method editor for the 180 samples/day method (8 min cycle time)

Results and discussion

Configuring the tandem workflow and creating methods

The Vanquish Neo UHPLC system must first be configured to run the correct workflow configuration on the Vanquish System Controller (VSC) via the Vanquish User Interface (VUI). The configuration is set using the A00 script of the VUI. Settings are given in Figure 3 below. Care should also be taken to assure that the pumps are correctly assigned—lower (reconditioning pump) vs. upper (separation) pump. Note that in the example shown here, the "switching valve connected to detector" setting is set to "right." In this case, the post-column capillary is connected between the right valve of the column compartment and the MS ion source and should be applied when the LC system is positioned to the left hand side of the MS ion source to allow for the shortest possible post-column connection. If the LC system is positioned on the right-hand side of the source the user should select "left" from the "switching valve connected to detector drop down menu.

The script guides the user through the entire fluidic configuration process, finishing with a summary page detailing which scripts should be run next, including setting solvent types (assuming they differ from those previously selected), running the auto start up script, and setting the separation column specifications. The final script in the list is B06 – condition columns.

The column resistance is determined as part of the B06 script. Determination of the column resistance is essential for proper tandem operation as it is used to define the maximum flow rate available for column washing, equilibration, and sample loading.

The user must define three parameters before starting the script (Figure 4). In particular, the column temperature must be set to the temperature that will be used to run the application. If the columns have already been in use and column reconditioning is not required, setting the "Redetermine Column Resistance Only" toggle to "on" will skip the column conditioning portion of the script, speeding up the script execution time.

The system uses the column resistance values to determine specific method boundary conditions which are automatically uploaded into the instrument method wizard / editor, such as the minimum possible cycle time.

Once the instrument has been configured, the instrument method wizard / editor is used to program parameters such as flow rate, gradient length, and gradient composition. Here the column wash type and number of wash cycles are also defined. The instrument driver then auto-optimizes the method accordingly.

Details on understanding and optimizing the tandem workflow using the instrument method editor can be found in the Vanquish Neo User Guide version 2.0 or higher.[3]

For convenience, all method templates presented in this document have been uploaded to the AppsLab Library. The corresponding method overview is also shown in Tables 3 and 4.

Figure 3. Configuring tandem capillary flow LC on the Vanquish User Interface

Figure 4. Measuring column resistance values to determine optimal flow rate for applications

A practical configuration consideration of optimal flow rates for chromatographic performance

Based on the literature[4] and findings from a 75 µm I.D. × 15 cm column (Figure 5), it was determined that the post-column dispersion effect (20 µm I.D. × 55 cm tubing) is not significant enough to impact the chromatogram at flow rates greater than 1 µL/min. This discovery allows for the installation of two columns equipped with two nanoViper fittings in the column compartment between two switching valves, presenting an alternative configuration to the dual-column dual-spray setup that is specifically optimized for nano-flow applications below 1 µL/min.[1]

Evaluating LC-MS performance using Double nanoViper PepMap Neo columns for high-throughput applications

Utilizing the new Double nanoViper 150 µm × 150 µm PepMap Neo column (P/N DNV150150PN) with a maximum pressure rating of 1,500 bar, methods were developed with throughputs ranging from 48 to 225 samples/day. Using flow rates from 1.5 to 3.8 µL/min afforded a minimum MS utilization of 90% (Table 3 and Figure 6).
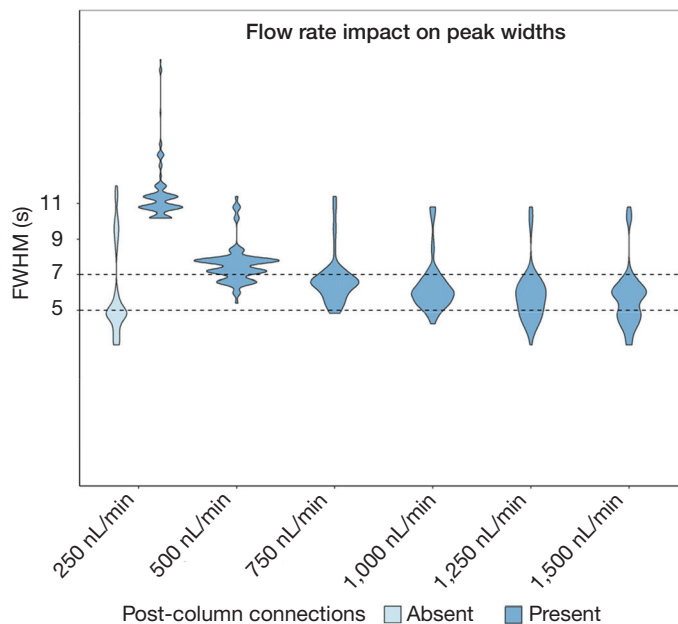

Figure 5. Evaluating the impact of post column tubing and connections on peak widths. Data represent the average of 15 PRTC peptides.

Separation column	MS ion source	Emitter	Flow rate (µL/min)	Throughput (samples/day)	Cycle time (min)	Elution window (min)	MS utilization (%)
PepMap Neo 150 µm I.D. x 15 cm	Nanospray Flex	Stainless steel emitter (ES542)	3.8	225	6.4	5.76	90
	Easy-Spray	Bullet emitter (ES994)	1.6	180	8	7.2	90
			1.5	100	14.4	13.4	93
			1.5	60	24	23	96
			1.5	48	30	29.2	97

Table 3. Ion source configurations and overview of the five high-throughput tandem capillary flow methods (flow rate range 1–5 µL/min). The 10 µL sample loop (P/N 6252.1960) is required for sample throughputs ≥180 samples/day.
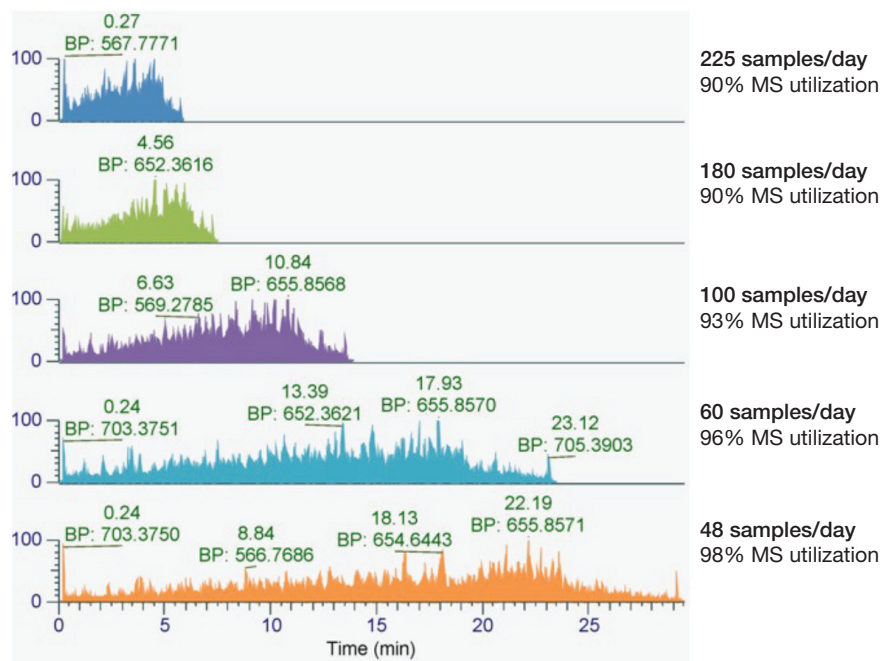

Figure 6. Separation profiles for capillary flow methods ranging from 48 to 225 samples/day (200 ng HeLa digest)

For the 180 samples/day method (8 minute cycle time), we identified 3,111–4,281 protein groups from 50–1,000 ng (250–2,500 nL injection) HeLa digest in DIA mode (Figure 7). From these, more than 3,500 protein groups were quantified with less than 20% coefficient of variation (CV) from 200 ng digest (Figure 7).
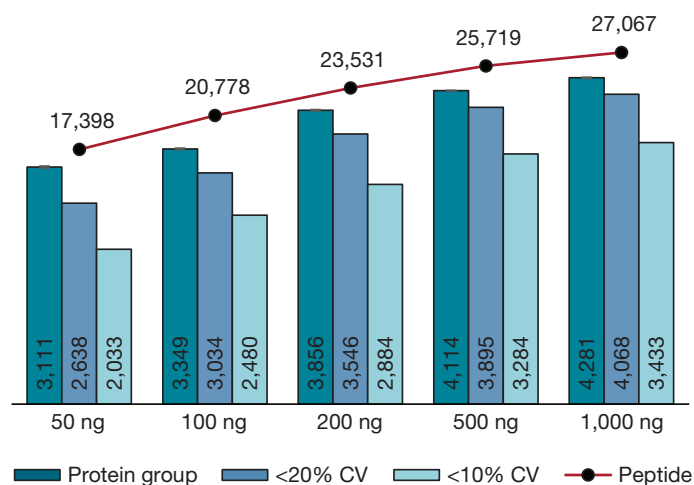

Figure 7. Proteome depth and quantitative precision using the 180 samples/day capillary flow method (n=6, 3 per column)

Note that the above tandem method affords a 7.2-minute elution window. In comparison, the direct injection and trap-and-elute workflows require approximately 4 and 2 minutes of additional overhead time, respectively, to achieve a comparable peptide elution window. Accordingly, the tandem direct injection workflow yields a 25–50% increase in throughput for capillary flow methods.[5]

Run-to-run and column-to-column reproducibility

A column-to-column reproducibility study was performed utilizing eight 150 μm × 15 cm PepMap Neo columns at a throughput of 180 samples/day. We observed a variation of only 4% in retention time over 30 injections with 200 ng HeLa digest (Figure 8A). The low retention time variability allowed for confident and reproducible identification of over 4,300 protein groups (Figure 8B) with less than 6% coefficient of variation (CV) of the median protein abundance when enabling match-between-run (Figure 8C). The reproducible chromatographic performance and proteome coverage highlight the utility of the tandem direct injection workflow for confident analysis of complex samples.

Evaluating increased throughput with micro-bore (300 and 1,000 μm I.D.) columns

The tandem direct injection workflow configuration can also be applied to micro-flow LC (1,000 μm × 15 cm) PepMap columns offering the advantage of increased sample throughput for the same peptide elution window, compared to the standard configuration. For example, we took a benchmark 100 samples per day method, which has a cycle time of 14.4 minutes at 50 μL/min,[2,6] and migrated this to a tandem configuration. For the tandem workflow we achieved the same peptide elution window while increasing the sample throughput to 120 samples per day, corresponding to a reduced cycle time of 12 minutes (Figure 9C and Table 4). This represents a 20% increase in sample throughput and a high MS utilization rate of 94%.
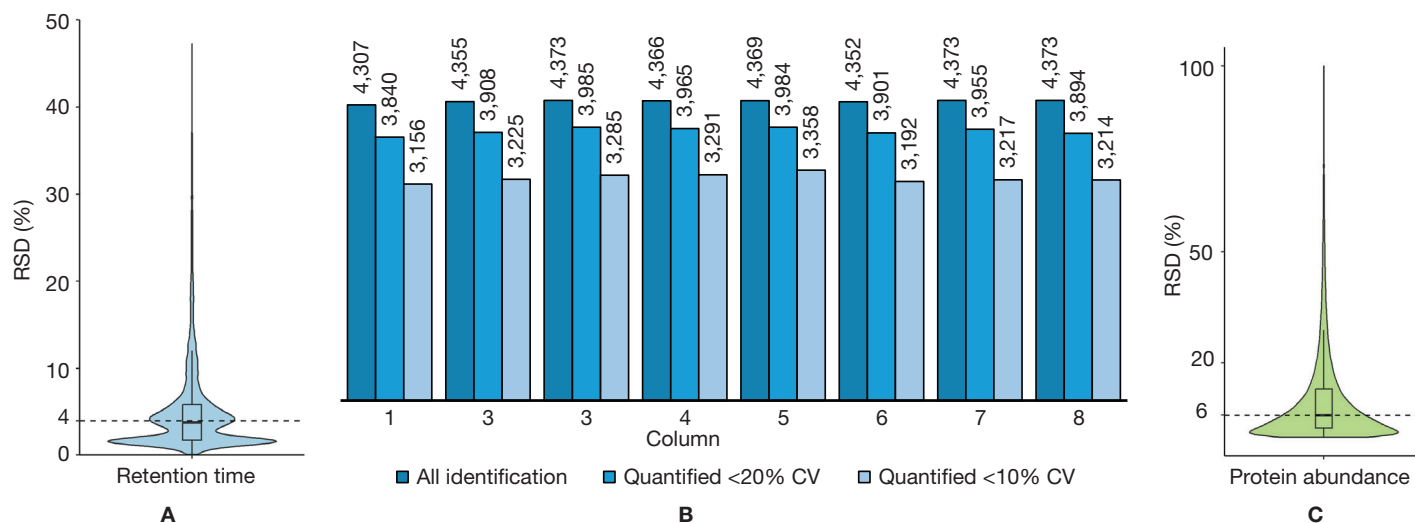

Figure 8. Evaluation of intercolumn precision including retention time (A), proteome depth (B), and protein abundance (C) (n=8)

Next, when using the 300 µm I.D. × 15 cm PepMap column we achieved a throughput of 240 samples per day with a cycle time of 6 minutes (Figures 9B and 10; Table 4). Operating at a flow rate of 15 µL/min, more than 2,200 protein groups were consistently identified from a 200 ng HeLa digest within a 5.4-minute elution window (90% MS utilization). When injecting higher sample quantities, such as 2,000 ng, the number of identified protein groups increased to over 3,400. This demonstrates the potential of the system for robust and higher throughput applications, particularly for non-limited sample quantities.

When transitioning to a 1 mm × 5 cm PepMap column operated at 100 µL/min, we achieved an increase in throughput up to 277 samples/day (Table 4, Figure 9A), while maintaining a high MS utilization rate of 95% and approximately 2,400 protein groups from 1 µg HeLa digest (Figure 11).

Enhancing separation column lifetime using inline trap columns as guard columns in the tandem direct injection workflow

Trap-and-elute workflows generally provide the benefits of accelerating sample loading and protecting the separation column from the sample matrix. In combination, this leads to higher sample throughput and a longer column lifetime. While sample loading times are eliminated using a tandem direct injection workflow, the challenge of preserving column lifetime remains. By employing a trap column as a guard column directly in line with the separation column, this aim can be partially achieved.
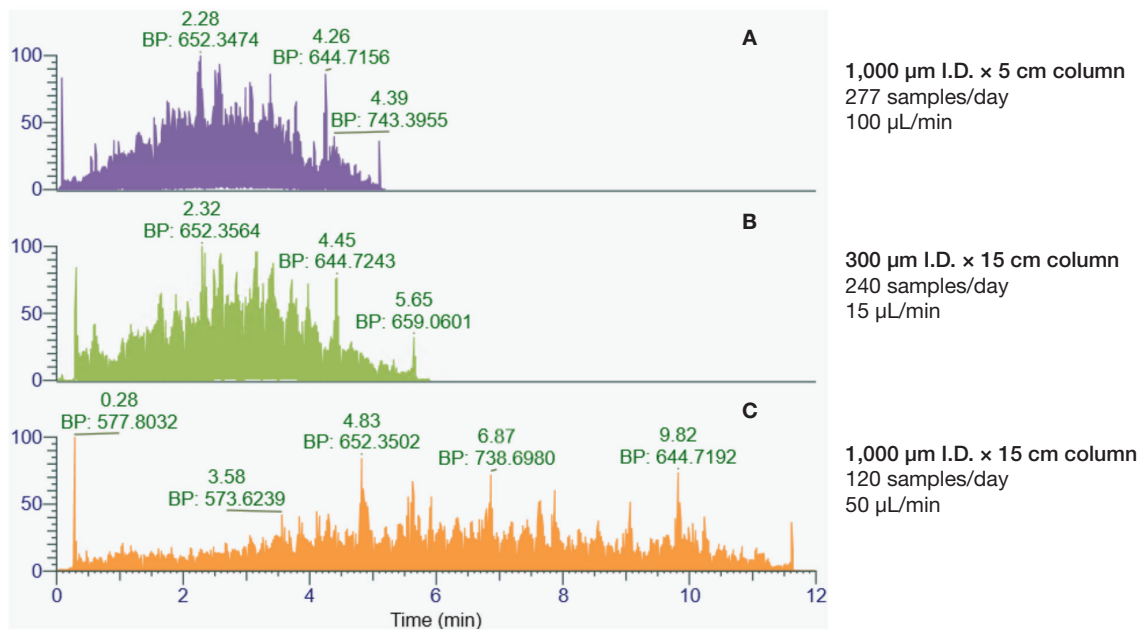

Figure 9. Typical elution profiles for tandem microflow applications with a (A) 1,000 µm × 5 cm PepMap column yielding 277 SPD, (B) 300 µm × 15 cm PepMap column yielding 240 SPD, and (C) 1000 µm × 15 cm PepMap column in tandem micro-flow LC-MS configuration

Separation column	MS ion source	Emitter	Flow rate (µL/min)	Throughput (samples/day)	Cycle time (min)	Elution window (min)	MS utilization (%)
PepMap 300 µm I.D. x 15 cm	OptaMax NG Ion Source	Low-flow needle insert (50 µm ID)	15	240	6	5.46	91
			15	100	14.4	13.85	96
			15	60	24	23.45	98
PepMap 1000 µm I.D. x 5 cm			100	277	5.2	4.4	85
			50	240	6	5.6	93
			50	180	8	7.6	95
PepMap 1000 µm I.D. x 15 cm			50	120	12	11.35	95
			50	100	14.4	13.68	95
			50	60	24	23.15	96

Table 4. Ion source conditions and overview of high-throughput tandem micro-flow (5–100 µL/min) methods employing 300 µm and 1 mm I.D. columns at flow rates ranging from 15–100 µL/min. The 10 µL sample loop (P/N 6252.1960) is required for sample throughputs ≥240 samples/day.
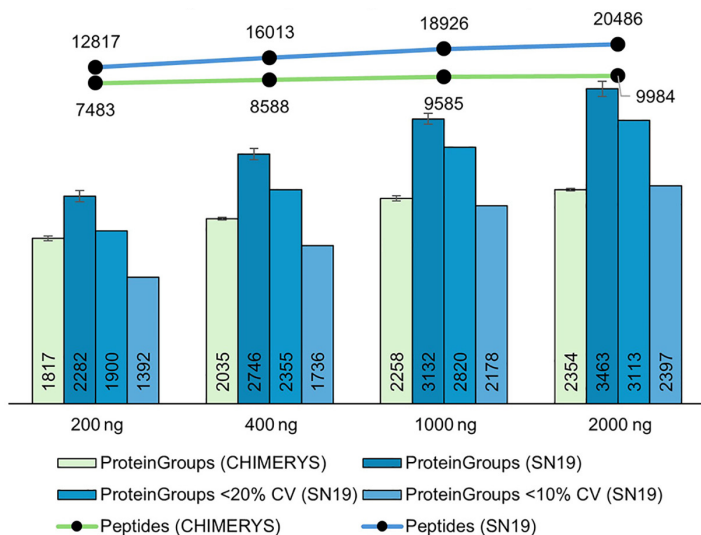
Figure 10. Proteome depth and quantitative precision for micro-flow LC-MS at 240 samples/day (300 µm I.D. × 15 cm, 15 µL/min, n=6)
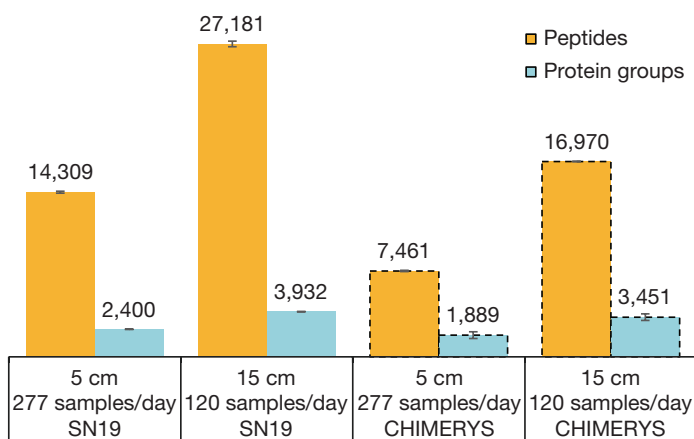

Figure 11. Proteome depth for LC-MS at 120 and 277 samples/day (1 µg sample, n=6)
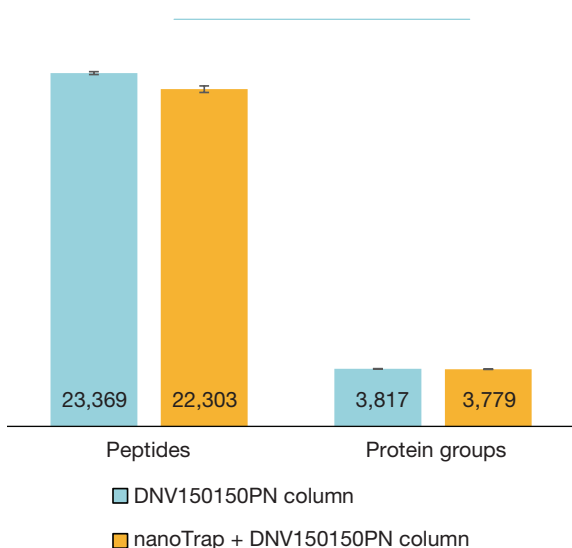

Figure 12. Evaluation of capillary flow LC-MS performance when using a nanoViper trap column in line with the separation column (n=6)

In this instance, we installed a 1,200 bar 75 µm × 2 cm nanoViper trap column (P/N 164946) prior to each of the two 150 µm × 15 cm columns while using the 180 samples/day method. This configuration introduces a slight delay of approximately 0.3 minutes in elution, which is equivalent to the volume of the nanoViper trap columns at 1.6 µL/min. Ultimately, the proteome depth achieved using this configuration is comparable to the tandem direct injection workflow (Figure 12).

While the use of a guard column can be used to extend column lifetime with minimal impact on proteome depth, it does not provide online desalting, unlike the standard trap-and-elute workflow. Therefore, users are advised to utilize the trap-and-elute workflow when online desalting is required.

Overall, this configuration offers users an alternative approach to achieve the accelerated sample throughput of tandem direct injection while protecting the separation column.

Tandem direct injection reduces column carryover without impacting MS utilization

Another facet of the tandem direct injection workflow compared to its single system counterpart is the capacity to carry out more thorough column washing without impacting sample throughput or MS utilization. The Vanquish Neo tandem workflow incorporates four pre-programmed washing patterns, catering to different applications, sample types, or user preferences (for details see Reference 1). The 150 µm × 15 cm columns were used to investigate column carryover at throughputs ranging from 60 to 180 samples/day using the trapezoidal wash pattern. This resulted in extremely low column carryover (based on all quantified HeLa peptide peak areas) for high-throughput methods, reaching levels of approximately 0.03% for the 180 samples/day method in DIA mode with 200 ng and 1 µg digest on column. Notably, for longer gradients even lower carryover levels were observed (0.008% carryover at 60 samples/day), as the longer cycle times permitted increased washing volumes (Figure 13).
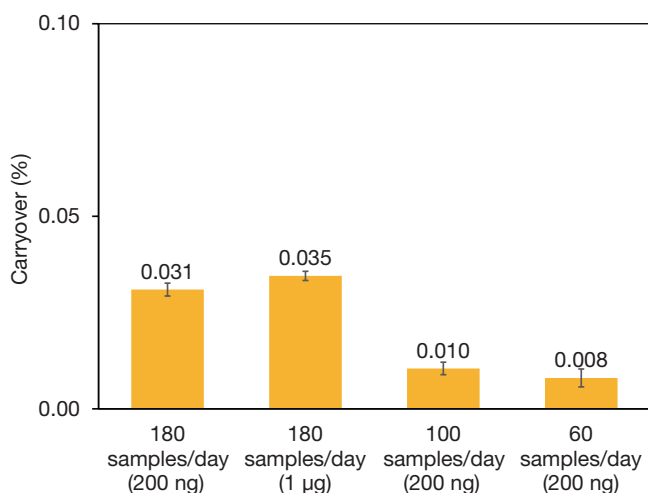
Figure 13. Column carryover for high-throughput DIA applications using the tandem direct injection workflow (n=6)

Conclusions

The introduction of a tandem direct injection workflow supporting capillary and micro-flow applications (1–100 µL/min) affords optimal LC-MS performance using columns with I.D. of 150, 300, and 1,000 µm. This workflow ensures that the overall MS utilization is maintained above 90%, maximizing the active acquisition window within a given cycle time and offers several key attributes:

• **Ease-of-configuration:** The system is designed with standardized fluidic connections, making it straightforward to set up and configure.

• **Intelligent workflow execution:** The workflow is intelligently designed to simplify the process of method creation, enhancing user-friendliness.

• **Maximized MS utilization:** The workflow permits maximum MS utilization without sacrificing throughput, ensuring efficient use of instrument time.

• **Single emitter:** The use of a single emitter with stable spray run-to-run enables robust ionization and reduces the chance of analytical variation.

• **Improved sample throughput:** The tandem configuration supports an increase in sample throughput across all the supported columns, accommodating a range of sample volumes and flow rates.

• **Low column carryover:** The configuration provides extended washing routines to decrease carryover without impacting cycle time, enhancing the confidence in identification and quantification.

• **Low column-to-column variation:** The configuration demonstrates low variation between columns, resulting in reproducible chromatograms and consistent proteome coverage.

Overall, the Vanquish Neo tandem direct injection workflow offers a versatile and efficient solution for a wide range of LC-MS applications, providing ease of use, high MS utilization, robust performance, and reproducible results.

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