Poster # P-II-0440

Technological advancements in proteomics

Long-term stability and reproducibility of nano, capillary and micro-flow LC-MS separations

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Abstract

Purpose: Evaluate the reproducibility of nano and microLC-MS bottom-up proteomics data collected in three labs over several months of continuous operation using Thermo Scientific™ Vanquish™ Neo UHPLC systems, Thermo Scientific™ PepMap™ Neo or Thermo Scientific™ Acclaim™ PepMap™ columns, and Thermo Scientific™ Orbitrap Exploris™ 240, 480, or Thermo Scientific™ TSQ Altis™ triple quadrupole mass spectrometers.

Methods: Direct injection workflows in nano/cap or micro-flow configurations were used to evaluate long-term stability and reproducibility of low-flow LC-MS analysis with 75 µm and 1.0 mm ID columns.

Results: Vanquish Neo UHPLC systems and PepMap Neo columns deliver excellent chromatographic robustness and reproducibility for long-term nano- and micro-flow LC across multiple systems.

Introduction

NanoLC-MS analysis using long, narrow-bore columns and long gradients is well established as the gold standard for bottom-up discovery proteomics. Aside from the technical challenges associated with nanoLC, concerns remain around the day-to-day and system-to-system result reproducibility. High reproducibility is essential for generating the global data sets required to gain statistically significant insights for biomarker discovery and validation, cell line characterization, and drug development. Here, we evaluated the Vanquish Neo UHPLC system for robust and consistent long-term LC separation performance under typical nanoLC or microLC proteomics experimental conditions.

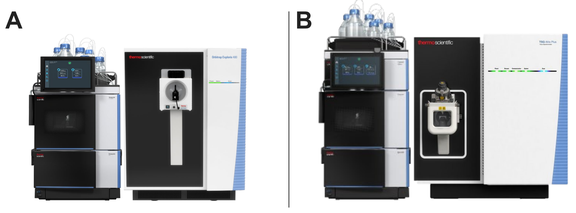

Figure 1. Vanquish Neo UHPLC system in the nano/cap-LC setup with an Orbitrap Exploris 480 mass spectrometer and EASY-Spray interface (A). Vanquish Neo UHPLC system in the micro-LC setup with a TSQ Altis mass spectrometer and Thermo Scientific™ OptaMax™ NG Ion Source (B).

Materials and methods

Sample preparation

Thermo Scientific™ Pierce™ HeLa Digest/PRTC Standard (A47996, 10 µg/vial) was reconstituted by adding 50 µL of 0.1% formic acid (FA) in water. The vial was subsequently sonicated for 2 min, followed by multiple sample aspiration and release cycles with a pipette to dissolve it completely. The final sample concentration was 200 ng/µL HeLa digest with 100 fmol/µL PRTC. Thermo Scientific™ Cytochrome C protein digest standard (1.6 nmol/vial, PN 161089) was reconstituted by adding 200 µL of 5% Acetonitrile/95% Water (v/v) with 0.1% formic acid (FA) in water. CAM-modified trypsin-digested BSA MS Standard (500 pmol/vial, New England Biolabs, PN P8108S) was reconstituted by adding 500 µL of 0.1% formic acid (FA) in water. The final sample concentration was 1 pmol/µL BSA protein digest.

Instrumentation

All experiments were performed using Vanquish Neo UHPLC systems interfaced to an Orbitrap Exploris 240 or 480 mass spectrometer (**Figure 1A**) operated in data-dependent acquisition (DDA) mode or TSQ Altis mass-spectrometer (**Figure 1B**) operated in MRM mode. Direct injection methods utilized EASY-Spray PepMap Neo columns for nanoLC-MS and Acclaim PepMap columns for microLC-MS. Long-term robustness was evaluated using the Vanquish Neo UHPLC system, thermostatted column compartment, and UV detector. A Thermo Scientific™ Double nanoViper™ PepMap™ Neo Column (75 µm × 500 mm, 2 µm) was used for nanoLC-UV robustness testing.

Methods

Experiments were performed with the following solvents: eluent A – 100% water, 0.1% formic acid; eluent B – 80% acetonitrile/20% water (v/v), 0.1% formic acid. Sample amount on the column was varied by altering the injection volume or sample concentration.

Data Processing and Analysis

Data files were processed with Thermo Scientific™ Proteome Discoverer™ Software (version 2.5) using a 2-step Sequest™ HT search algorithm and INFERYS rescoring node. The FDR was set <1% at the peptide and protein levels. LC-UV data were acquired and analyzed using the Thermo Scientific™ Chromeleon™ Chromatography Data System (version 7.2.10 MUd). Peak properties full width at half maximum (FWHM) and retention time (RT) were extracted for 8 BSA peptides using the Cobra peak detection algorithm.

Results

Long-term nanoLC-MS robustness

The Vanquish Neo UHPLC system was run continuously using a single PepMap Neo column for a period of 6 months. The BSA protein digest was separated using a 90-minute nano-flow gradient (120-min cycle time) typical for bottom-up proteomic experiments. Chromatographic parameters were evaluated for 8 selected BSA peptide peaks using UV detection (**Figure 2**).

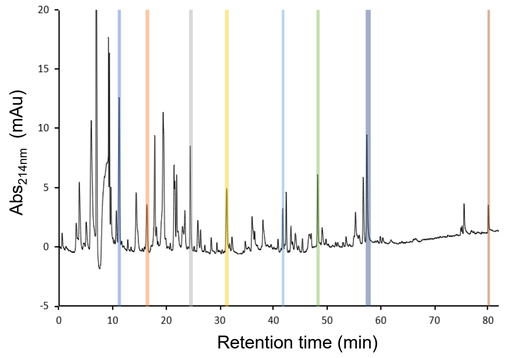

Figure 2. Representative LC-UV chromatogram for a 1 pmol injection of BSA protein digest onto a 75 µm × 50 cm PepMap Neo column. The 8 selected peaks are highlighted.

Peptide retention times were demonstrated to be stable across all 1,600 injections (**Figure 3**). The retention time standard deviation was below 0.3 min over the entire 6 months period for each set of 100 injections.

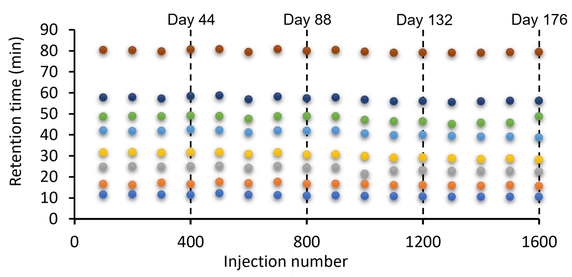

Figure 3. The retention time for 8 selected peptides from 1,600 injections of BSA protein digest over 176 days (approximately 6 months). Retention time values are the means per set of 100 injections.

Peak FWHM was assessed for the same set of peptides as an indicator of chromatographic reproducibility (**Figure 4A**). Consistent FWHM was observed for each peptide and no trends were observed. In contrast, the "random" FWHM variation present for some of the peptides was attributed to overlapping peaks, which in some instances could not be resolved by UV.

Reproducible gradient delivery and column robustness in the study are attributed to controlled flow ramping during fast sample loading and column equilibration and Thermo Scientific™ SmartInject™ for minimizing column pressure shock. An integrated needle seat filter frit prevents debris accumulation on the column head, further improving column lifetime. Separation robustness is reflected by the stable column backpressure (Δ < 25 bar) over the 6 month analysis period (**Figure 4B**).

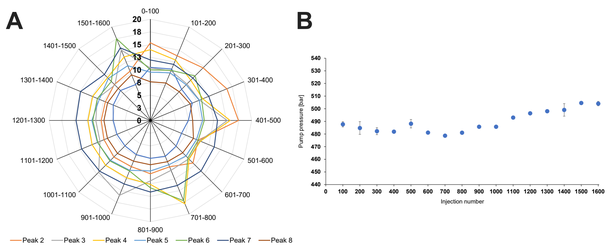

Figure 4. Mean peak FWHM for 8 BSA peptides over 1,600 replicate injections shown in seconds averaged across 100 injections (A). Column backpressure measured 1 min after gradient start at 300 nL/min and 50ºC column temperature on a PepMap Neo column per 100 injections (B).

System-to-system nanoLC-MS Reproducibility

High RT reproducibility is required for label-free quantitative nanoLC-MS analysis where peptide retention times are used to increase ID confidence and to compare results across LC-MS instrumentation. The results for 4 replicate injections on 6 systems showed excellent RT precision (<0.2% RSD) for all selected Cytochrome C digest peptides. Mean RT (**Figure 5**) is shown below for each peptide across all 6 Vanquish Neo UHPLC systems.

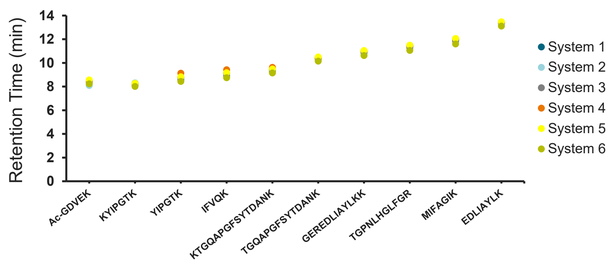

Figure 5. Mean RT values for Cytochrome C protein digest peptides separated on 6 Vanquish Neo UHPLC systems using a 20 min nanoLC method (N = 4).

System-to-system reproducibility of peptide and protein IDs was evaluated using the 90 min gradient method on injections of 200 ng HeLa cell protein digest under standard MS acquisition conditions. An average of 33,000 peptides from 4,400 protein groups were identified in a single-shot nanoLC-MS analysis (**Figure 6**). As little as 4.1% variation in peptide groups and 2.2% in protein groups was observed for the 6 Vanquish Neo UHPLC systems coupled to an Orbitrap Exploris 240 mass spectrometer.

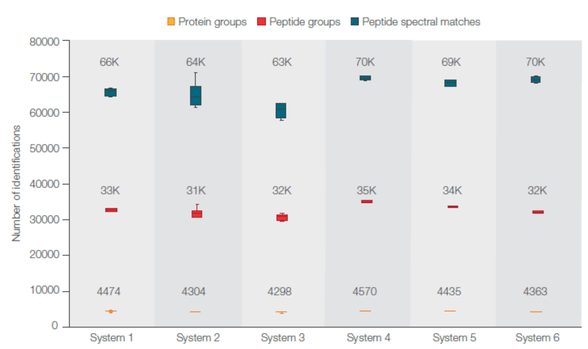

Figure 6. Comparison of HeLa protein digest profiling with 6 Vanquish Neo UHPLC systems coupled to an Orbitrap Exploris 240 mass spectrometer. Separation on 75 µm × 50 cm, 2 µm EASY-Spray PepMap Neo columns, 300 nL/min.

System-to-system nanoLC-MS Reproducibility

A high-throughput micro-flow LC-MS (14.4-min cycle time, 100 samples per day) method was developed based on previously published work[1,2]. A total of 760 injections was run over 7.5 days to test the result reproducibility. The sequence was a repetition of the following injections: 3 sample, 3 blank, and 3 matrix injections resulting in a total of 254 HeLa digest/PRTC injections. Method robustness was evaluated using pressure trace consistency, RT stability, and peak area reproducibility of the 12 PRTC peptides. Pressure traces were reproducible (not shown), with run-to-run variation of < 3 bar across the sequence, including the initial flow rate ramp (0.2 - 2 min) and column washing.

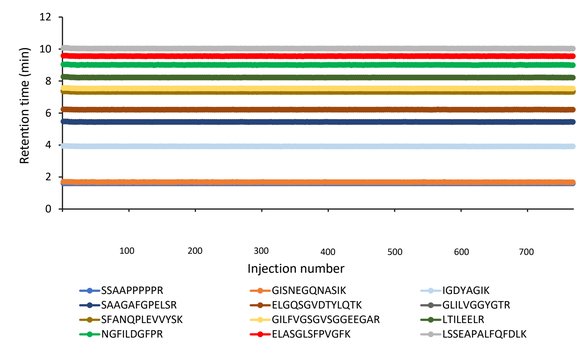

Figure 7. Retention time stability of 12 PRTC peptides for 760 injections.

High retention time precision was observed in **figure 7**, where the retention time was stable for all 12 peptides. Relative standard deviation was well below 0.5% for all peptides during seven days of system operation. Ten peptides had an RSD < 0.1%, one was 0.14%, and one was 0.31%. Minimal retention time reduction was observed (≤ 3 s or ≤ 1%) for the first 20 injections. The initial retention time shift was most likely due to stationary phase conditioning.

Conclusions

We evaluated the long-term chromatographic stability and result reproducibility generated with the Vanquish Neo UHPLC system coupled to either HRAM or QqQ mass spectrometers. Evaluation under nano- and micro-flow LC conditions revealed highly-consisten results and rugged system operation. Taken together, these data provide evidence of the reliability and performance of state-of-the-art low-flow UHPLC systems and consumables.

- The Vanquis Neo UHPLC system and PepMap Neo columns deliver robustness and result reproducibility for long-term operation under maximum performance conditions.
- Retention times and IDs were highly-consistent among 6 Vanquish Neo UHPLC systems and PepMap Neo columns.
- A micro-flow LC-MS method (14.4 min) was developed and evaluated, showing high reproducibility across 760 injections.

References

1. Bian, Y., Zheng, R., et al. Robust, reproducible and quantitative analysis of thousands of proteomes by micro-flow LC–MS/MS. Nat. Comm. 2020, 11, 157.
2. Bian, Y., Bayer, F. P. et al. Robust Microflow LC-MS/MS for Proteome Analysis: 38000 Runs and Counting. Anal. Chem. 2021, 93, 8, 3686-3690.

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