Reducing carryover in high-sensitivity low-flow LC-MS analysis: the comprehensive study of multi-wash, ZebraWash, and large volume injections

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ABSTRACT

Purpose: Demonstrate the superior performance of the ZebraWash procedure in Thermo Scientific™ Vanquish™ Neo UHPLC systems for rapid and effective reduction of the trap column carryover in the trap-and-elute workflow for low-flow LC-MS applications and evaluate the large volume injection capabilities with multi-draw injection procedure.

Methods: The Thermo Scientific™ Vanquish™ Neo UHPLC system, Thermo Scientific™ PepMap™ Neo columns, and Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer were coupled using Thermo Scientific™ EASY-Spray™ interface. Nano/cap trap-and-elute injection workflow configuration was used to assess the efficiency of carryover removal using ZebraWash procedure. The multi-draw injections with up to 500 µL were tested with different loading buffers to ensure high peptide recovery.

Results: The ZebraWash procedure of the Vanquish Neo UHPLC system sets a new standard for efficient reducing of trap column carryover. This results in higher confidence using the trap-and-elute workflow. The independent and parallel washing of separation and trap columns delivers high-quality results without requiring intermediate wash runs, thus increasing sample throughput. The high-volume loading with multi-draw allows to inject up to 500 µL of sample onto the trap cartridge without peptide loss when the trifluoroacetic acidic aqueous solution is used as loading buffer.

INTRODUCTION

Two main workflows are used in low-flow LC-MS proteomics experiments: (i) direct injection of the sample onto the separation column and (ii) trap-and-elute injection when the sample is first injected onto the shorter trap column and then the separation gradient is delivered through the trap and separation column. The limitation of the direct injection workflow in low-flow LC analysis is the long loading times that are a result of the requirement to load relatively large sample volumes (several microliters) onto the very narrow (75 and 150 µm internal diameter) and often long (15–75 cm) columns. The overhead time increases significantly if the liquid chromatography (LC) system cannot accelerate sample loading with elevated pressure capabilities due to LC hardware limitations. The trap-and-elute workflow allows substantial acceleration of the sample loading. In this case, the sample is loaded onto the short trap column (5–20 mm) with the same or slightly larger internal diameter than the separation column in higher flow rates. As a result, the sample loading onto the trap column takes seconds rather than minutes as required for the direct injection setup.

Figure 1. Nano/cap LC-MS setup with Vanquish Neo UHPLC system and Orbitrap Exploris 480 mass-spectrometer coupled via Thermo Scientific™ EASY-Spray™ interface

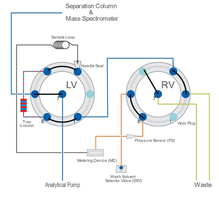

Figure 2. Schematic representation of the ZebraWash washing of the trap column with repeating plugs of strong and weak wash liquids

While adding the trap column to the flow path has many advantages, it also has some drawbacks that should be considered. Sample loading onto the short trap column might result in losses of very hydrophilic peptides if the loading volume or loading buffer are not optimized. Adding fluidic lines and a column with different inner dimensions and lengths might lead to increased carryover if washing is not done properly. Carryover—resulting from sample overloading, insufficient washing, nonspecific sample binding, or column chemistry—can prevent having consistent results for each injection. In the past, to reduce the carryover on the trap column and injection fluidics, users ran matrix blanks between sample runs or manually created special washing procedures using an external pump. This action reduced the sample throughput and increased the method's complexity. Thus, a fully automated, optimized, and easy-to-use solution to remove carryover caused by the trap column while maintaining sample throughput and confidence in results is required. Here, we developed and introduced an innovative ZebraWash procedure for rapid and effective reduction of the trap column carryover in trap-and-elute workflows.

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.

Instrumentation

All experiments were performed using Vanquish Neo UHPLC systems interfaced to an Orbitrap Exploris 480 mass spectrometer operated in data-dependent acquisition (DDA) mode (Figure 1). The system was configured for trap-and-elute nanoLC injections. Thermo Scientific™ EASY-Spray™ PepMap™ Neo UHPLC column, 75 µm × 500 mm, 2 µm, 1,500 bar and Thermo Scientific™ Acclaim™ PepMap™ C18 column, 3 µm, 75 µm × 150 mm, 20 mm packing bed separation and trap columns were used for carryover studies. The shorter EASY-Spray PepMap Neo column (75 µm ID x 15 cm, ES75150PN) and Thermo Scientific™ PepMap™ Neo Trap Cartridge (0.3 mm x 5 mm, 5 µm) were used for evaluation of multi-draw capabilities for large volume injections.

Methods and data processing

MS data were acquired with an Orbitrap Exploris 480 mass spectrometer in data-dependent acquisition (DDA) mode. Acquired .raw files of HeLa sample were processed with Thermo Scientific™ Proteome Discoverer™ 2.5 software using a 2-step SEQUEST™ HT search algorithm and INFERYS rescoring node. The false discovery rate (FDR) was set below 1% at both the peptide and the protein level. Further data analysis and plotting were performed with R script.

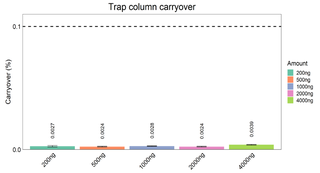

Figure 3. Trap column carryover after injection of HeLa protein digest with amounts from 200 to 4,000 ng, 3 replicates for each injection amount. The carryover was estimated based on the sum intensity of all quantified HeLa peptides.

RESULTS

Working principle of ZebraWash

The automated ZebraWash wash procedure was developed to reduce the carryover with samples containing strongly adsorbing analytes or contaminants. Each switch between the strong and weak solvent is defined as one ZebraWash cycle (Figure 2). It consists of 3 µL strong wash liquid and 3 µL of weak wash liquid. The number of cycles can be adjusted in the instrument method editor. The integrated system intelligence automatically controls the entire washing procedure. A maximum number of wash cycles of 2, 4, and 16 are allowed for 10, 25, and 100 µL sample loops, respectively.

Decoupling the LC fluidics, trap column, and separation column carryover

The overall carryover in the LC-MS analysis combines contributions from the fluidics, trap column, and separation column. In the standard trap-and-elute workflow in the Vanquish Neo UHPLC system, the washing of the trap column is completed independently from the washing of the separation column. The system automatically switches the trap column offline to wash and re-equilibrate it using the metering device at the end of the gradient separation while the separation column is washed with the separation pump.

As ZebraWash minimizes the carryover on the trap column, we developed an experimental procedure to decouple the carryover of the separation and trap columns. The standard nanoLC-MS run was followed by two consecutive gradients through the separation column, while the trap column was washed with the ZebraWash procedure. As the fluidics were not switched and the trap column stayed offline, the blank injection afterward was used to measure the carryover that originated from the trap column and injection fluidics. The ZebraWash procedure was tested with 16 cycles (maximum supported number of cycles with a 100 µL loop) and achieved an ultra-low level of carryover (< 0.004%) on the trap column independent of the injection amount (Figure 2). In total, ZebraWash pushed 48 µL of strong wash liquid and 48 µL of weak wash liquid through the trap column during the washing procedure.

Impact of sample amount and wash cycles on overall carryover

Minimal overall carryover levels from run to run prevent biased analysis. While ZebraWash reduces the carryover from the trap column, the contribution of the separation column becomes the major carryover source. Using the standard Fast Wash (FW) procedure or ZebraWash (ZW) resulted in overall carryover levels below 0.05% for injection amounts up to 4,000 ng (Figure 3). The increased number of ZebraWash cycles gradually reduced the contribution of trap column carryover, as seen from the intensity levels and the number of identified peptides in blank runs (Figures 4 and 5). The higher loading amounts lead to higher carryover levels, e.g., the carryover for the 4,000 ng sample was approximately 3–4 times higher for the 200 ng sample, which could be attributed to the overloading of the separation column. The washing of the trap column with standard Fast Wash is also an efficient method of carryover reduction.

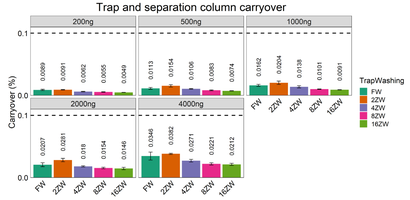

Figure 4. The level of overall carryover for different injection amounts and trap wash procedures, 3 replicates for each injection amount. The carryover was estimated based on the sum intensity of all quantified HeLa peptides.

The volume of the strong wash liquid with the Fast Wash procedure (12 µL) is two times larger than with 2 cycles of ZebraWash, which explains the slightly more efficient trap column washing (Figure 3). When consuming the same volume of strong wash liquid, the four cycles of ZebraWash consistently reduced more carryover than the Fast Wash procedure. It must be noted that the washing duration is dependent on the trap column dimension. The nano trap used here permits approximately 12 µL/min at 800 bar and room temperature (23 ° C) during the washing procedure. As a result, it takes around 6 min to thoroughly wash and equilibrate the trap column with four cycles of ZebraWash and an equilibration factor of 2. Therefore, with a 25 µL sample loop in the standard configuration of the Vanquish Neo UHPLC system, it is recommended to have four cycles of ZebraWash for trap column washing. Users might use the other trap columns with larger i.d. for faster washing when considering more cycles for more comprehensive washing.

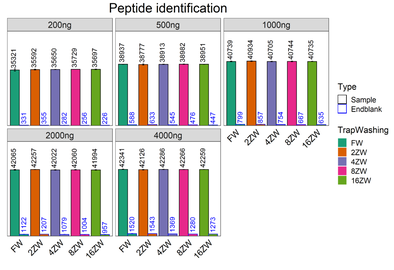

Figure 5. The number of peptides identified with the injection of 200 to 4,000 ng of HeLa protein digest and a blank injection afterward, 3 replicates. The trap column was washed with Fast Wash or ZebraWash (2, 4, 8, 16 cycles).

LARGE VOLUME INJECTIONS: MULTI-DRAW

Vanquish Neo UHPLC system allows to make highly precise and reproducible injections for a wide variety of injection volumes — from 10 nL to 500 µL — without the need to draw excess sample volume or use a transfer liquid. Multi-draw functionality supports larger injection volumes for trap-and-elute workflows through iterative sample pick-up. After each aspiration, the respective sample volume is transferred to the trap column. This is repeated until the full sample volume has been completely transferred to the trap column. The loading of the same amount of concentrated (200 ng/µL) and diluted sample (5 ng/µL) resulted in similar signal intensity and chromatographic profiles (Figure 6). Additionally, the number of peptides and proteins identified was also similar that indicates that comparable amount of sample was loaded onto the trap cartridge with 5 µL as well as 500 µL injection volume (Figure 6B).

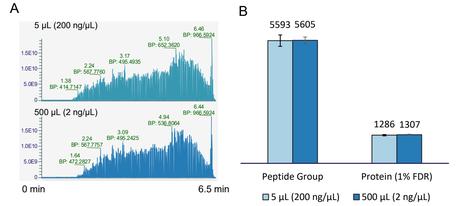

Figure 6. Total ion chromatograms (TICs) of 1 µg HeLa protein digest obtained after injection of 5 µL of 200 ng/µL sample and 500 µL of 2 ng/µL diluted sample onto the same trap cartridge (A) and the number of peptides and proteins identified from both injections using 180 samples per day (SPD) method (B).

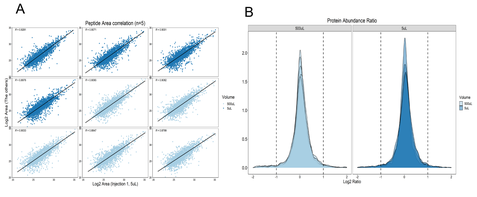

Figure 7. The correlation between peptide areas for 5 injection replicates of HeLa protein digest with 500 µL sample (5 ng/µL) and 4 injection of 5 µL sample (200 ng/µL) vs. the first injection of 5 µL of concentrated sample (A) and distribution of log2 protein abundance ration for the same injection replicates (B)

Quantitative performance of large-volume injections.

The high correlation of peptide peak areas between replicates with large volume injections of diluted sample and low volume injections of concentrated sample proves that there is no systematic bias with peptides breakthrough during the loading process if the aqueous solution of TFA is used as a loading buffer (Figure 7A). Additionally, similar correlation coefficients were obtained for all combinations that indicate the high reproducibility of results. The distribution of protein abundance ratios also did not show systematic biases (Figure 7B) that provides additional confidence in the quantitative results when multi-draw is used to load large volumes of diluted samples onto the trap column.

CONCLUSIONS

The ZebraWash procedure of the Vanquish Neo UHPLC system sets a new standard for efficient reducing of trap column carryover. This results in higher confidence using the trap-and-elute workflow. The independent and parallel washing of separation and trap columns delivers high-quality results without requiring intermediate wash runs, thus increasing sample throughput. The number of ZebraWash cycles and the composition of strong wash liquid can be optimized for different proteomics samples to ensure long-term robustness with minimal cross-contamination between samples.

The multi-draw of diluted protein digests and loading them onto the trap cartridge using aqueous solution of trifluoroacetic acid allows to avoid sample losses and provides the great tool to analyze samples directly after immunoprecipitation or dilution after solid-phase extraction without the need to dry and lyophilize sample.

TRADEMARKS/LICENSING

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