Continuous MS Utilization for Proteomics Data Acquisition Using a Novel Low-flow Tandem LC-MS Setup

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ABSTRACT

Purpose: Generation of high throughput intelligent low-flow LCMS methods which afford virtually continuous proteomics sample data acquisition (zero MS idle time)

Methods: The Thermo Scientific™ UltiMate™ 3000 RSLCnano system, Thermo Scientific™ EASY-Spray™ or Thermo Scientific™ Acclaim PepMap™ columns and Thermo Scientific™ Q Exactive™ HF-X Mass Spectrometer (MS) were used to run typical shotgun proteomics experiments and verify the performance and robustness of intelligent high-throughput low-flow LC-MS methods.

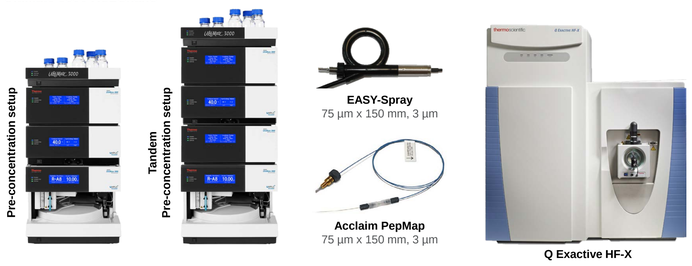
Results: The high-throughput low-flow pre-concentration LC method using a single low-flow pump required just eight minutes total cycle time whilst providing a peptide elution window (> 5 min), with good retention time stability and chromatographic peak shape. The novel tandem pre-concentration LC-MS setup further enhanced productivity by enabling a reduced seven minutes cycle time whilst delivering close to 100% MS utilization affording > 200 samples per day turnover with near zero carryover.

INTRODUCTION

State-of-the-art LC-MS platforms for shotgun proteomics represent a significant financial investment to the laboratory. Whilst low flow rates and long columns provide the necessary sensitivity and resolution required, they are typically associated with long washing and equilibration steps and long sample loading times. In combination with slow autosampler routines and sub-optimal separation, equilibration and washing conditions can account for up to 50% or more of the total LC-MS runtime. The common belief that low-flow LC-MS cannot deliver high-throughput analysis often results in applying of unnecessary long methods for analysis of relatively simple samples. Here we demonstrate novel and intelligent low-flow LC-MS methods that deliver high separation efficiency, high-throughput, extremely low carryover, and robust LC-MS results.

MATERIALS AND METHODS

UltiMate 3000 RSLCnano



LC System Setup

The UltiMate 3000 RSLCnano system in nano-flow pre-concentration configuration was used to separate peptides on an EASY-Spray (75 µm x 150 mm, 3 µm). In the novel pre-concentration tandem LC configuration peptides were separated on two Acclaim PepMap C18 (75 µm x 150 mm, 3 µm) columns. The flow rate of 1.5 µL/min was used for both setups. The micro-flow pump was exploited for fast sample loading at 150 µL/min onto a 0.3 mm x 5 mm, 5 µm Acclaim PepMap trap cartridge. The sample loop was washed with 80% acetonitrile (ACN) in water with 0.1 % formic acid (FA). The mobile phase A was water with 0.1 % FA and mobile phase B was 80% ACN with 0.1 % FA. The sample was loaded with 0.1 % FA in channel A of loading pump onto the trap cartridge. The trap cartridge was washed with 80 % ACN, 0.1 % FA in channel B of the loading pump .

MS Instrumentation and ESI Interface

The RSLCnano system in pre-concentration setup was connected to a Q Exactive HF-X mass spectrometer using the EASY-Spray source. The tandem LC configuration was coupled to MS via the Thermo Scientific™ Nanospray Flex™ source with metal emitter. The MS instrument was operated in Full-Scan and DDA modes.

Data Acquisition and Analysis

Data were acquired with Thermo Scientific™ Xcalibur™4.1. The RSLCnano system was controlled with Standard Instrument Integration (SII) 1.3 software. DDA data for HeLa cells protein digest were processed with Thermo Scientific™ Proteome Discoverer™ 2.2 software using Sequest HT search algorithm. The false discovery rate (FDR) for peptides and proteins was set at 1 %. The chromatographic peak characteristics of extracted ion chromatograms (EIC) of Cytcochrome C (CytC) and HeLa peptides were calculated using Chromeleon 7.2.

HIGH-THROUGHPUT LOW-FLOW LC-MS

High-throughput Low-flow LC-MS: Theoretical Considerations

Classic nanoLC proteomics methods provide depth of coverage and sensitivity via long nano columns (typically with 50-75µm I.D.). Whilst clearly the authority on uncompromising protein coverage and PTM analysis, it is not appropriate for applications where throughput and robustness are paramount. The demand for increased productivity is linked to biomarker validation, population biomonitoring, fast quality assessment for samples procured for biobanking, serum/plasma proteome profiling, assays development in clinical research. In contrast to common belief, low-flow LC systems, particularly the UltiMate 3000 RSLCnano, are well suited for developing fast LC-MS methods due to low pump delay volume, fast response to changes in flow or mobile phase composition, integrated micro-flow pump for fast sample loading, and high-pressure capabilities. The high-throughput can be achieved by increased linear velocities on columns with low internal diameter or/and on short columns. In this work we used 75 µm x 15 cm columns at 1.5 µL/min flow rate to maintain high ESI MS sensitivity that strongly depends on the flow rate as we showed previously (Technical Note 72277).

Fast Low-flow LC-MS Pre-concentration Method Explained

Using the standard pre-concentration setup (Figure 1A) based on Thermo Scientific™ nanoViper™ fingertight connections with 20 µm ID first peptide elutes from the column after ca. one minute at flow rate 1.5 µL/min. The LC method includes peptides elution from the column and column washing (Figure 1B). The column is equilibrated, when the autosampler executes the injection routine of ca one minute (Figure 2). Then, the sample is loaded onto the trap cartridge within six seconds using loading pump at 150 µL/min. In order to minimize carryover, the injection loop is washed in parallel with gradient elution . Additionally, the trap cartridge is washed at the end of the gradient to remove strongly retaining components. Then, the trap cartridge is re-equilibrated with solvent A and ready for the next sample loading (Figure 2).

Figure 1. The standard pre-concentration nanoLC setup with calculated delay volumes, volume of trap cartridge and column (A); and gradient conditions (B). The column is re-equilibrated during sample injection routine (see Figure 2).

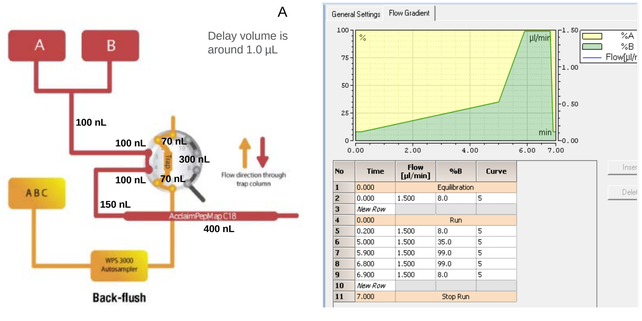
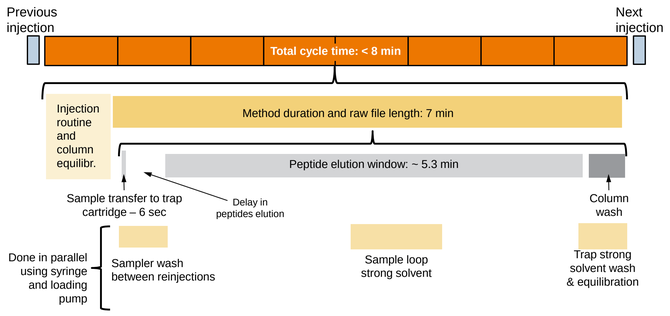


Figure 2. The scheme of high-throughput low-flow LC-MS method with eight minute total cycle time, fast sample loading and intensive fluidics wash in parallel with sample analysis for minimal carryover.



FAST LOW-FLOW LC-MS METHOD PERFORMANCE

The fast low-flow LC-MS method was tested during 24 hours (180 injections) by sequential injections of HeLa and CytC protein digests, and blanks (Figure 3A). The method resulted in near zero carryover (Figure 3B) and delivered robust peak performance as well as reproducible results for DDA bottom-up proteomics (Figure 4). The standard deviation of retention times for HeLa peptides was below 0.1 min (Figure 4A). The peak area variation did not exceed 10 % for all selected peptides (Figure 4B). The method provides excellent separation, extremely sharp peptide peaks with PWHM below three seconds (Figure 4C) and low carryover < 0.2% (Figure 3B). While using fast DDA acquisition with TOP40 MS2 events at 7.5K resolution and injection time of 14 milliseconds more than 10K of MS/MS events were acquired that resulted in around 6200 PSMs and 1000 protein groups (1 % FDR) per single injection (Figure 4D).

Figure 3. Typical TIC ad BPC profiles of CytC and HeLa protein digests obtained using fast low-flow LC-MS method (A) and EIC of HeLa peptides for 200 ng HeLa sample followed by blank injection (blue traces of EIC) (B).

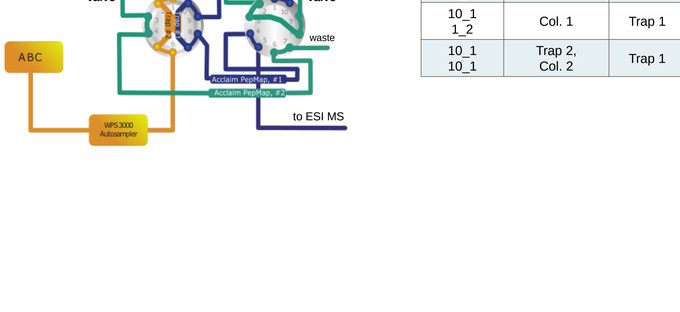
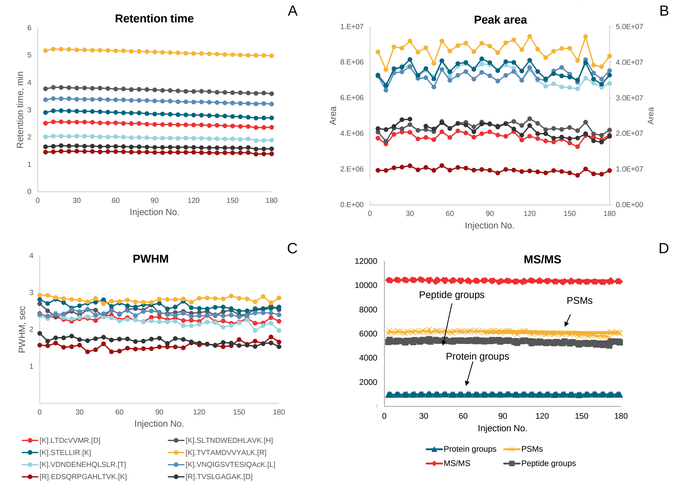


Figure 4. Robustness of fast low-flow LC-MS method over 180 consecutive injections.
(A) stability of retention times. (B) peak area stability of extracted HeLa peptides; right axis: STELLIR, TVTAMDVVYALK; left axis: LTDcVVMR, SLTNDWEDHLAVK, VDNDENEHQLSLR, VNQIGSVTESIQAcK, EDSQRPGAHLTVK, TVSLGAGAK. (C) peak width at half maximum (PWHM). (D) the number of MS/MS events, PSMs, protein and peptide groups identified at 1 % FDR.



NOVEL TANDEM LOW-FLOW LC-MS SETUP

The presence of fluidic connections and column void volume limits users to achieve continuous MS utilization using typical setups with one separation column due to the time required for the sample to pass from the autosampler through the column to ESI interface. The problem can be solved by performing the next sample injection routine, sample transferring onto the trap cartridge and separation column, while the gradient analysis of previous injection is still running. The novel low-flow tandem LC setup presented here (Figure 5) fulfills all of these requirements and has advantages of (i) using the same separation pump for peptides elution on column 1 and column 2 which eliminates any small differences caused by slight variations in pump gradient delivery and calibration; (ii) trap cartridges washing by the a loading pump gradient program; (iii) next injection preparing while the current injection is running; (iv) parallel washing and re-equilibration of the second separation column. Low-flow tandem LC setup allows to direct the flow from the separation pump, equilibration pump and microflow loading pump to each of trap cartridge or separation column by switching left and/or right valve installed in the RSLCnano system column compartment (Table 1).

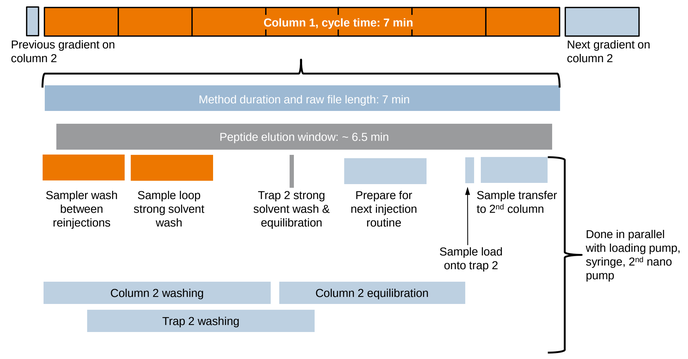
Figure 5. The novel tandem low-flow LC setup fluidics scheme requires two low-flow pumps and two 10-port 2-position switching valves in the column compartment.

Table 1. The flow direction from equilibration pump, micro-flow loading pump and separation pump for different combinations of left and right valve positions.

Left / Right valve position	Equilibration pump	Loading pump	Separation pump / MS
1_2 10_1	Col. 2	Trap 2	Trap 1, Col. 1
1_2 1_2	Trap 1, Col. 1	Trap 2	Col. 2
10_1 1_2	Col. 1	Trap 1	Trap 2, Col. 2
10_1 10_1	Trap 2, Col. 2	Trap 1	Col. 1

We carefully optimized the duration for processes that are completed in parallel with separation gradient including the use of the “PrepareNextInjection” command that automatically starts the injection routine for the next sample in the sequence prior to the end of the current run (Figure 6). The creation of the running sequence using tandem LC method doesn’t differ from usual experience as only one tandem method is required for both columns, and switching of left and right valves is completed using intelligent conditioning commands in the method. The developed tandem low-flow LC-MS method has a cycle time of seven minutes, however further reduction is possible if higher-throughput for the analysis of less complex samples is required.

Figure 6. The scheme of tandem low-flow LC method with seven min total cycle time, automated switching between columns, near 100% MS utilization, sample injection and pre-loading onto separation column, intensive washing and re-equilibration of injection fluidics, trap cartridges, separation column in parallel with running separation gradient.



CONTINIOUS MS UTILIZATION WITH TANDEM LC-MS

We tested the tandem pre-concentration low-flow LC-MS method using HeLa and CytC protein digests. Tryptic peptides were well distributed over the entire seven minutes cycle time and new .raw file was generated each seven minutes (Figure7), allowing > 200 samples to be analyzed within 24 hours. The distribution of PSMs over the entire seven minute runtime confirms near 100% utilization of MS time (Figure 8A). The wider elution window for tandem low-flow LC in comparison with pre-concentration method results in more MS/MS events and protein groups identification (Figure 8B). Noticeably, the peak width increased up to two times using the tandem LC setup compared to pre-concentration setup. This is mainly related to post-column dispersion using the Acclaim PepMap columns, which is almost negligible when using EASY-Spray columns.

Figure 7. The typical BPC profiles of HeLa protein digest on column 1 and 2 sequentially collected using tandem low-flow LC-MS method.

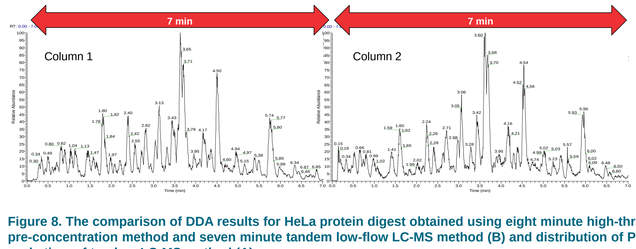
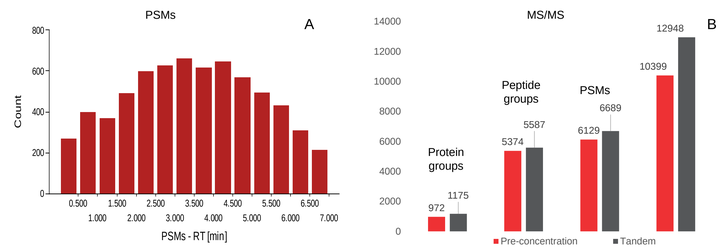


Figure 8. The comparison of DDA results for HeLa protein digest obtained using eight minute high-throughput low-flow LC-MS pre-concentration method and seven minute tandem low-flow LC-MS method (B) and distribution of PSMs over seven minute cycle time of tandem LC-MS method (A).



CONCLUSIONS

We developed a fast low-flow LC method with eight minutes total cycle time and > five minute peptide elution window using standard pre-concentration setup. We also proposed a novel tandem low-flow LC setup for near 100% MS utilization and high-throughput analysis.

- An intelligent use and versatility of UltiMate 3000 RSLCnano system allows to develop low-flow LC-MS methods with throughput comparable to analytical flow LC-MS
- The novel easy-to-setup tandem low-flow LC method eliminates MS instrument idle time and significantly boosts LC-MS productivity and throughput
- In contrast to conventional tandem LC setups, the first low-flow analytical pump in the novel tandem configuration is used exclusively for gradient elution, whilst the second pump is used for sample loading and column reconditioning
- The execution of fluidics, column and trap cartridges washing steps in parallel with the separation gradient achieves fast LC cycle times and near zero carryover of tryptic peptides

TRADEMARKS/LICENSING