ABSTRACT: This study demonstrates how the latest ultrahigh-performance liquid chromatography (UHPLC) technology can be combined with high-resolution accurate-mass (HRAM) mass spectrometry (MS) and long columns packed with fully porous particles to improve bottom-up proteomics analysis with nanoflow liquid chromatography−mass spectrometry (nanoLC-MS) methods. The increased back pressures from the UHPLC system enabled the use of 75 μm I.D. × 75 cm columns packed with 2 μm particles at a typical 300 nL/min flow rate as well as elevated and reduced flow rates. The constant pressure pump operation at 1500 bar reduced sample loading and column washing/equilibration stages and overall overhead time, which maximizes MS utilization time. The versatility of flow rate optimization to balance the sensitivity, throughput with sample loading amount, and capability of using longer gradients contributes to a greater number of peptide and protein identifications for single-shot bottom-up proteomics experiments. The routine proteome profiling and precise quantification of >7000 proteins with single-shot nanoLC-MS analysis open possibilities for large-scale discovery studies with a deep dive into the protein level alterations. Data are available via ProteomeXchange with identifier PXD035665.

KEYWORDS: bottom-up proteomics, nanoLC-MS, single-shot analysis, UHPLC, Orbitrap, high-resolution accurate-mass, long nanocolumns

INTRODUCTION

Due to advances in liquid chromatography−mass spectrometry (LC-MS) instrumentation, complex peptide mixtures can now be separated with the high resolving power of modern column technologies and detected based on the accurate mass measurement and high resolution of MS instruments. When coupled with the latest data processing tools and big data capability, large amounts of raw data can be analyzed and reported to understand biological processes better. While there is a growing interest in micro-LC-MS and advanced multidimensional techniques, today, proteome analyses are usually based on nanoLC-MS methods, with long columns and shallow gradients giving high peak capacity (the theoretical maximum number of fully resolvable peaks). A higher peak capacity indicates more peptides resolved and identified, meaning even low-level peptides can be detected and characterized within a single-shot bottom-up proteomics experiment. In order to improve the separation efficiency, three main approaches are currently deployed: (i) increasing the column length to boost the number of analyte distribution events between the mobile and stationary phases; (ii) decreasing the particle size to reduce analyte diffusion; (iii) utilizing long pillar array columns with the uniform structure to decrease eddy dispersion and peak broadening during the separation.
Although nanoLC-MS has undoubtedly driven considerable leaps in our understanding of proteomics, the throughput is often limited to only a few samples a day, and the MS utilization time is reduced while using longer columns. These issues are mainly due to the limits of LC technology and are especially relevant for direct-onto-separation column injection experiments where the loading flow rate is restricted by the high pressure of the long separation column. Moreover, the required concomitant column washing and equilibration further reduce the throughput of nanoLC-MS analysis.

With the constraints presented by current methods, the evidence suggests that peak capacity, sensitivity, proteome depth, and the number of protein and peptide identifications could be improved by advancing LC and column technology and optimizing separation and MS acquisition parameters. Increasing the column length is the most straightforward path toward increasing efficiency, as the two are directly proportional. The peak capacity can be improved by utilizing shallower and longer gradients, as long as the peak width increase and the reduction of peak height do not level out gains from the increased separation time. Increasing the sensitivity of the nanoLC-MS analysis can compensate for the reduction in peak height. Since the inverse relationship between the flow rate and electro spray ionization (ESI) efficiency is the most significant factor in increasing sensitivity, decreasing the flow rate during the separation phase should also help to increase the number of protein and peptide identifications.

Here we demonstrate how the low-flow UHPLC technology, with extended pressure capabilities, can be used to explore the previously challenging use of longer columns, enable optimized flow rates and gradient lengths, and maintain reproducible and robust separation profiles. We explored how increased separation efficiency affects protein and peptide identification on two MS platforms and studied the analytical variability of label-free quantification using different gradient lengths. These results show that quantitative bottom-up proteomics analysis can significantly improve by optimizing nanoLC chromatographic conditions.

**EXPERIMENTAL PROCEDURES**

**LC and MS Instrumentation**

The Vanquish Neo UHPLC system was coupled with Orbitrap Exploris 480 mass-spectrometer or Orbitrap Eclipse Trivid mass spectrometer for nanoLC-MS bottom-up proteome profiling. The EASY-Spray source was used to interface LC and Orbitrap Exploris 480 MS and Nanoflex Spray with FAIMS Pro interface used for Orbitrap Eclipse Trivid MS. Peptides were separated on EASY-Spray or Double nanoViper PepMap Neo columns 75 μm I.D. × 75 cm (P/N ES75750PN or DNV75750PN) or 75 μm I.D. × 50 cm (P/N ES75500PN or DNV75500PN) with the maximum pressure of 1500 bar. The nanoLC-MS system was controlled with Standard Instrument Integration (SII) for Xcalibur software. All hardware and data acquisition software were from Thermo Fisher Scientific.

**NanoLC-MS Analysis**

The Vanquish Neo UHPLC system was configured for direct injection analysis. HeLa protein digest peptides were separated with two-step linear solvent gradients, performed at flow rates 200 to 500 nL/min with gradient lengths of 90, 120, 180, and 240 min. The mobile phase A and weak wash liquid was water with 0.1% FA (P/N LS118−500), and the mobile phase B and strong wash liquid was 80% acetonitrile with 0.1% FA (P/N LS112.500). All solvents were from Thermo Fisher Scientific. The autosampler temperature was 7 °C, and the column temperature was 50 °C. The sample was injected with Fast Loading set to ‘Enabled’ with Pressure Control at 1500 bar. The column Fast Equilibration function was set to ‘Enabled’ with Pressure control at 1500 bar, and the equilibration factor was set to 2. Vial bottom detection was set to ‘Enabled’.

The Orbitrap Exploris MS was operated in DDA mode using a full scan with m/z range 375−1200, Orbitrap resolution of 60,000, normalized target value 300%, and maximum injection time set to Auto. The intensity threshold for precursor was set to 1 × 10^6. MS/MS spectra starting from 120 m/z were acquired in data-dependent acquisition (DDA) mode with 25 dependent scans, where the precursors were isolated in a window of 2.0 Da and subsequently fragmented with HCD using an NCE (normalized collision energy) of 26%. Orbitrap resolution was set to 15,000. The normalized AGC target was 50%, and the maximum injection time was Auto.

The Orbitrap Eclipse MS was operated in DDA mode, using a full scan with m/z range 375−1500, Orbitrap resolution of 120,000, normalized target value 250%, and maximum injection time set to Auto. FAIMS compensation voltages of −45, −55, and −75 V were combined in a single run with a total cycle time of 3 s. The intensity threshold for precursors was set to 5 × 10^5. MS/MS spectra were acquired in an ion trap analyzer and fragmented by stepped HCD using normalized collision energy (NCE) of 30%. Precursors were isolated in a window of 1.0 Da. The linear ion trap acquired spectra in the Turbo mode ranged from 200 to 1400 m/z. The normalized AGC target was set to 300%, and the maximum injection time was 15 ms.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD035665.

**Sample Preparation**

Pierce HeLa Digest/PRTC Standard (Thermo Fisher Scientific, 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 aspirating and releasing 10 times with a pipet to reconstitute the sample fully. The sample was subsequently transferred to another vial of HeLa Digest/PRTC Standard, which was again sonicated for 2 min and mixed in the same way to produce a final sample concentration of 400 ng/μL HeLa containing 200 fmol/μL PRTC.

**Data Processing**

Acquired Orbitrap Exploris 480 .raw DDA files were processed with Proteome Discoverer 2.5 software (Thermo Fisher Scientific) using a two-step Sequest HT search algorithm and INFERYS rescoring node. Further analysis was done using R scripts. The Skyline software package was used to extract and analyze peptide peak areas.

**RESULTS**

We thoroughly studied the influence of (i) column length, (ii) flow rate, (iii) gradient length, and (iv) sample loading on the number of identifications and quantification precision in nanoLC-MS bottom-up proteomics experiments. These variables provided insights for selecting optimal nanoLC conditions for deep, single-shot proteome profiling on fully
The Effect of Column Length on NanoLC Separations

The 40 to 50 cm column lengths, operated at flow rates between 0.2 and 0.3 μL/min, have become the standard for deep, single-shot nanoLC-MS proteomics.22 This is due to the maximum available LC pressure ratings of 800–1200 bar and the complexity associated with reproducible packing of the longer columns.23 With the increased 1500-bar pressure available with the UHPLC system in this experiment, both 75 μm I.D. × 50 and 75 cm columns could be tested, while achieving decreased sample loading and column equilibration times, by setting the pump to constant pressure operation mode. The sample load time, column washing, and equilibration times for a 75 μm I.D. × 50 cm column using the most frequently utilized constant flow (0.3 μL/min) mode is around 50 min with a 25 μL loop for direct injection setup. The constant pressure 1500 bar sample loading and column washing/equilibration reduce overhead time to less than 20 min. The 1500 bar LC pressure capabilities also allow longer, 75 cm nanocolumns to safely explore the practical benefits of deeper proteome profiling without the risk of overpressure and sequence stop frequently occurring during the sample loading step or column washing step when the elute composition and viscosity are rapidly changing.

In our comparison, the 75 cm column outperformed the 50 cm column in a 90 min gradient by reducing the full width at half-maximum (FWHM) by 12% on average (Figure 1). This is due to the constant pressure 1500 bar sample loading and column washing/equilibration reducing the time to less than 20 min. The 1500 bar LC pressure capabilities also allow longer, 75 cm nanocolumns to safely explore the practical benefits of deeper proteome profiling without the risk of overpressure and sequence stop frequently occurring during the sample loading step or column washing step when the elute composition and viscosity are rapidly changing.

In our comparison, the 75 cm column outperformed the 50 cm column in a 90 min gradient by reducing the full width at half-maximum (FWHM) by 12% on average (Figure 1). That is equivalent to approximately 1.2 s, based on extracted ion chromatograms (EIC) of PRTC peptides spiked into the HeLa protein digest. Since 75 cm nanocolumns reduced FWHM, we continued exploring the flow rate effect to maximize achievable proteome depth.

The Effect of Flow Rate on Single-Shot NanoLC-MS Proteome Profiling

The back pressure provided by the UHPLC system allowed flow rates between 0.2 and 0.5 μL/min to be tested on the 75 μm I.D. × 75 cm columns while achieving up to 1450-bar pressure during the analysis. By reducing the flow rate to 0.2 μL/min, the ESI-MS ionization efficiency was improved, and the peak height of spiked PRTC peptides increased by 60% on average, compared with the typical nanoLC-MS at 0.3 μL/min flow rate (Figure 2A). The flow rate increase results in a drop in ionization efficiency and a subsequent decrease in peak height.24 We observed on average a 30% peak height reduction for 0.5 μL/min, compared to the 0.3 μL/min flow rate (Figure 2A).

Critically, the lower flow rate increased the MS1 intensity but almost did not change (±3%) the number of MS/MS events, the number of peptide-spectrum matches (PSMs), or the number of peptide or protein identifications (Figure 2B). This could indicate that 1 μg loading provided sufficient peak intensity at all tested flow rates to achieve the maximum number of identifications. However, the versatility of flow rate adjustment during the gradient separation phase might become a valuable tool for analyzing lower injection amounts when higher ESI MS sensitivity is required.

Proteome Depth Dependency on the Gradient Length

Increasing the gradient length can achieve a higher peak capacity since it is proportional to the gradient time.25,26 However, the increase in gradient length also leads to broader chromatographic peaks (Figure 3A). The FWHM of PRTC peptides for the same amount of injected material increases almost linearly with an increase in the length of the gradient (Figure 3A). However, FWHM increases more slowly vs gradient length, so the peak capacity continues to increase up to the 240 min gradient length (Figure 3A).

The FWHM increase proportionally reduces peak height (Figure 3B), which may negatively affect results if the peak intensity is insufficient to generate high-quality MS and MS/MS spectra. Therefore, matching the gradient length, sample injection amount, and column length are essential to achieve maximum protein and peptide identifications. The peak height losses can, to some extent, be compensated by loading larger sample amounts (Figure 3B), but this approach is limited by peak broadening due to the column overloading that consequently increases FWHM. Nevertheless, we observed a proportional increase of peak intensity with increased loading amount onto the column (Figure 3B), indicating high column loading capacity.

Interestingly, the number of peptides and proteins identified did not increase significantly with the increased sample amount, indicating that MS sensitivity can accommodate a reduction in intensity caused by peak broadening for studied injection amounts (Figure 4). A 240 min gradient with a 1 μg HeLa protein digest identified more than 7100 high confidence proteins and approximately 80,000 peptides. The number of identifications correlated with a peak capacity indicated the significance of chromatographic performance for the single-shot bottom-up nanoLC-MS proteome profiling.

Faster MS/MS Acquisition Speed and Ion-Mobility Fractionation Impact

While there is a clear benefit to running longer gradients on longer columns to increase the proteome depth in single-shot experiments, the throughput requirements often limit the maximum run time. Therefore, we investigated if faster OT/IT MS acquisition and ion-mobility filtering would benefit from increased separation efficiency using a 90 min gradient (Figure 5). On average, the number of peptide identifications increased by 19%, and the number of protein groups increased by 18% by adding ion-mobility fractionation and faster OT/IT acquisition.
Figure 2. Effect of flow rate from 0.2 to 0.5 μL/min on the PRTC peak height normalized to peak height at 0.3 μL/min (n = 3) (A) and the relative change in the number of identified and quantified peptides and proteins with 90 min gradient and 1 μg loaded onto the column of HeLa protein digest (B).

Figure 3. Dependency of the FWHM (A) and peak height (B) on the gradient length using the constant flow rate (250 nL/min) and different loading amounts (n = 3).

Figure 4. Number of high-confidence master proteins (A) and peptides (B) that were identified and quantified (with match-between-run) using gradient lengths from 90 to 240 min and loading amounts from 1 to 4 μg. The match between runs was used for three replicates, each gradient length and loading amount (n = 3).
Quantification and Analytical Variability

Proteome profiling studies often aim to discover changes in protein abundances in cases and controls. So, it is critical to confidently identify a protein and quantify as many proteins as possible with high precision. We used replicates to assess the analytical variability of protein quantification for different gradient lengths (Figure 6A). Additionally, we checked the correlation between the loading amount and estimated protein abundance (Figure 6B).

Independent of the gradient length, 90% of all quantified proteins showed less than 25% relative standard deviation (RSD) for protein abundance, which provides high confidence in the analytical results’ quality (Figure 6A). Additionally, the proportional increase of protein abundance with the increased injection amount for each gradient length confirms the wide dynamic range of nanoLC-MS analysis using long columns (Figure 6B). A slight trend toward reduced quantification precision was observed for larger loading amounts for the longest, 240 min gradient (Figure 6A). This might be related to a more challenging estimation of peak boundaries for broader chromatographic peaks, as shown in Figure 3.

CONCLUSION

The UHPLC system with high-pressure capabilities, when coupled with highly sensitive HRAM mass spectrometry, provides the basis for improved bottom-up proteomics with nanoLC-MS analysis using long columns. The increased back pressure capabilities provide flexibility for optimization of gradient length and flow rate during peptide separation while accelerating sample loading and column washing/equilibration using elevated flow rates. In this study, using a 75 μm I.D. × 75 cm column reduced the FWHM by >10% of the peptide peaks compared to the 75 μm I.D. × 50 cm equivalent. Notably, the increased throughput can be achieved for direct injection analysis with automated constant pressure sample loading workflows and concomitant washing and equilibration stages.

This study also demonstrated that the flow rate reduced to as little as 0.2 μL/min delivers a 60% sensitivity gain compared to the equivalent study at a flow rate of 0.3 μL/min. As expected, increasing the gradient length did provide a higher peak capacity when a long 75 cm nanocolumn was used. A 90 min gradient was shown to identify approximately 6200 protein groups with OT/OT acquisition and approximately 7300 with adding ion-mobility fractionation and deploying faster OT/IT MS acquisition. The highest number (approximately 80 000) of peptides were identified with a 240 min gradient. The nanoLC-MS analysis on the long column also provided low analytical variability with more than 90% of proteins quantified below 25% RSD with DDA mode.

The versatility of low-flow UHPLC, longer columns, optimized flow rates, and increased gradient lengths can all be utilized, each delivering higher, quantitative proteome depth. Ultimately, this can lead to greater numbers of protein and peptide identifications that may be left undiscovered by other methods.

Figure 5. Number of protein groups and peptides identified (n = 3, without match-between-run) with 90 min gradients. One μg HeLa protein digest loaded onto 75 μm I.D. × 75 cm, 2 μm column with DDA OT/OT and FAIMS, OT/IT MS/MS acquisition.

Figure 6. Frequency of protein distribution vs the abundance RSD % for three replicates of HeLa protein digest (A) and protein abundances for different injection amounts (B) (n = 3).
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Notes
The authors declare the following competing financial interest(s): Dr. Runsheng Zheng, Dr. Christopher Pynn, Dr. Alexander Boychenko are employees of Thermo Fisher Scientific. The other authors declare no competing financial interests.

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