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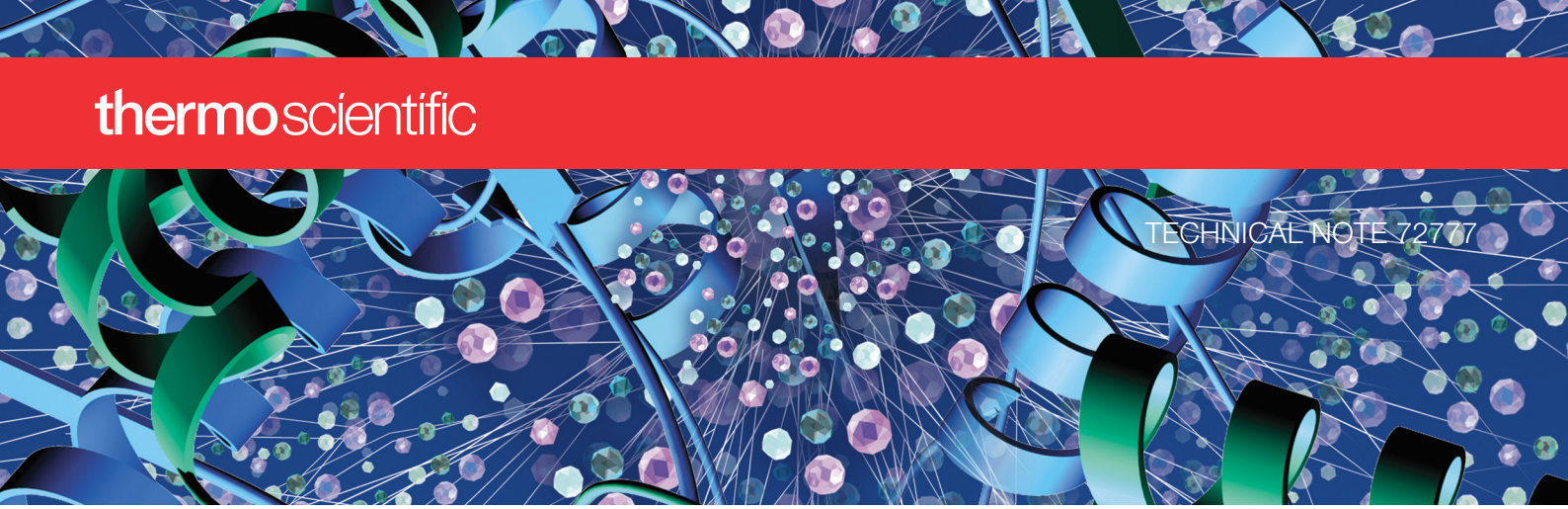

High-throughput capillary-flow LC-MS proteomics with maximum MS utilization

Authors

Alexander Boychenko[1], Christopher Pynn[1], Bart van den Berg[1], Tabiwang N. Arrey[2], Mike Baynham [3], Wim Decrop[1], Martin Ruehl[1]

[1]Thermo Fisher Scientific, Germering, Germany

[2]Thermo Fisher Scientific, Bremen, Germany

[3]Thermo Fisher Scientific, Runcorn, United Kingdom

Keywords

High-throughput low-flow LC, LC-MS based proteomics

Goal

Develop a high-throughput, robust, capillary-flow method for LC-MS based proteomics

Contents

1. Introduction 2
   - 1.1 The need for speed in low-flow proteomics applications 2
   - 1.2 High-throughput LC methods that afford near 100% MS-utilization 2
2. Experimental 2
   - 2.1 Consumables 2
   - 2.2 Samples 2
   - 2.3 LC-MS configuration and separation conditions 2
   - 2.4 MS conditions 5
   - 2.5 Data acquisition and processing 5
3. Results and discussion 6
   - 3.1 High-throughput low-flow LC method explained 6
   - 3.2 LC-MS and chromatographic performance, method robustness 6
4. Conclusions 9
5. References 10

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1. Introduction

1.1 The need for speed in low-flow proteomics applications

Conventional nano LC based bottom-up "discovery" proteomics methods provide depth of coverage and sensitivity by exploiting the high peak capacity achieved with long nano UHPLC columns (typically 50–75 µm i.d. with ≤ 2 µm particles) long gradients (≥ 2 hours) and nano-flow rates (≤ 300 nL/min). While this is still considered to be state-of-the-art for uncompromising protein coverage and PTM analysis, it is not well suited for applications where high throughput, as well as a high degree of sensitivity are required.[1]

Demands for high-throughput, low-flow LC-MS methods have been predominantly driven by translational proteomics applications ranging from biomarker validation, population biomonitoring, fast quality assessment for samples procured for biobanking, to serum/plasma proteome profiling and assay development in precision medicine research. However, high-throughput low-flow LC-MS solutions are also gaining traction in other market areas, for example the food and beverage sector.[2]

Optimal high-throughput, low-flow LC methods need to strike a fine balance between facilitating increased linear velocity without compromising ESI sensitivity, which has been shown to decline rapidly at flow rates above 5 µL/min.[3] As such, preferred flow rates for high-throughput methods are around 3 to 5 times higher compared to conventional nano LC methods (i.e. 1.5 µL/min for 75 µm i.d. columns) and around 3 to 5 µL/min for capillary-LC methods (run on 150 µm columns). Furthermore, all such applications are run on short LC columns: from 2 to 15 cm in length.

1.2 High-throughput LC methods that afford near 100% MS-utilization

Other facets of conventional discovery proteomics methods employing long columns and gradients are the associated protracted column washing and equilibration steps which, combined with slow autosampler routines and long sample loading and elution times, can account for 50% or more of the total LC-MS run.

Here we present a novel high-throughput capillary-flow LC method that affords a short sample analysis cycle time of only 8 minutes while enabling a high degree of MS utilization. Significantly, 6 minutes out of each 8-minute analysis cycle are dedicated not only to gradient run time but to actual peptide MS-data acquisition resulting in as yet unseen levels of MS productivity.

2. Experimental

2.1 Consumables

- Fisher Scientific™ LC-MS grade water (P/N W6-212)
- Fisher Scientific™ LC-MS grade acetonitrile (P/N 10616653)
- Thermo Scientific™ Pierce™ LC-MS grade trifluoroacetic acid (TFA), (P/N 85183)
- Thermo Scientific™ Pierce™ LC-MS grade formic acid (P/N 28905)
- Fluidics and columns used to set up pre-concentration application shown in Table 1 and Figure 1.

2.2 Samples

- Thermo Scientific™ Pierce™ HeLa protein digest (P/N 88328, 20 µg/vial) reconstituted to a final concentration of 200 ng/µL in loading buffer (see Table 2 for details).
- Thermo Scientific™ Dionex™ Cytochrome C digest (P/N 161089, 1.6 nmol/vial) reconstituted to a final concentration of 1 pmol/µL.

2.3 LC-MS configuration and separation conditions

Measurements were carried out using a Thermo Scientific™ UltiMate™ 3000 RSLCnano system,[4] equipped with a Thermo Scientific™ ProFlow™ flow meter (P/N 6041.7850). The system was configured (Figure 1, Table 1) using the Thermo Scientific™ EASY-Spray™ connection kit (P/N 6720.0395) and EASY-Spray ES800 column as described in the UltiMate 3000 RSLCnano Standard Applications Guide (Document No. 4820.4103). An EASY-Spray ES800 column was chosen for these experiments because its relatively short bed length (15 cm) and 3 µm particle size render it capable of capillary flow rates without generating backpressures that exceed the upper pressure limit of the system while still affording high resolution chromatography (see Figures 4A, B, and C).

Table 1. Fluidics, columns, and consumable accessories required to run the application. All parts are contained within the UltiMate 3000 RSLCnano EASY-Spray connection kit (P/N 6720.0395) unless otherwise indicated. The letter and number assignments are given in Figure 1. Note: consumables are from Thermo Fisher Scientific unless stated otherwise.

#	Item	P/N
a	EASY-Spray column, 15 cm × 75 µm i.d., Thermo Scientific™ Acclaim™ PepMap™ 100 C18 column, 3 µm, 100 Å or Thermo Scientific™ Acclaim™ PepMap™, RSLC column, C18, 3 µm, 100 Å, 75 µm × 15 cm	ES800* (or 164568)**
b	300 µm i.d. × 5 mm packed with Acclaim PepMap 100 C18, 5 µm, (set of 5 cartridges)	160454
	µ-Precolumn holder, 5 mm, with 30 µm i.d. connecting tubing, Thermo Scientific™ nanoViper™ fittings	164649
1	nanoViper capillary FS/PEEK sheathed 1/32" i.d. × L 20 µm x 350 mm	6041.5240
2	nanoViper capillary FS/PEEK sheathed 1/32" i.d. × L 75 µm x 650 mm	6041.5775
3	nanoViper capillary FS/PEEK sheathed 1/32" i.d. × L 75 µm x 550 mm	6041.5760
	nanoViper sample loop 20 µL, FS/PEEK sheathed	6826.2420
4	PTFE tubing, 500 µm i.d. 100 cm, used as waste tubing	6720.0077
5	nanoViper capillary FS/PEEK sheathed 1/32" i.d. x L 20 µm × 550 mm	6041.5260
	Union Viper	2261.5061
	1/16" Universal Fingertight fitting, one-piece design, extra-long thread, 4 pieces	6720.0015
	Polypropylene vials for WPS with glass insert, 250 µL, 25 pieces	6820.0027
	Polypropylene caps for WPS vials, 25 pieces	6820.0028
	Cytochrome C digest, 1.6 nmol, lyophilized	161089
	Transport vial including cap and seal (5 vials)	6820.0023#

*P/N ES800 must be ordered separately.
** P/N 164568 is a linear Acclaim PepMap column (must be ordered separately) and can be used as an alternative to the EASY-Spray column variant.
# Included in the accessory kit (P/N 5820.8910, delivered with the Thermo Scientific™ Dionex™ UltiMate™ WPS-3000 TPL RS module.

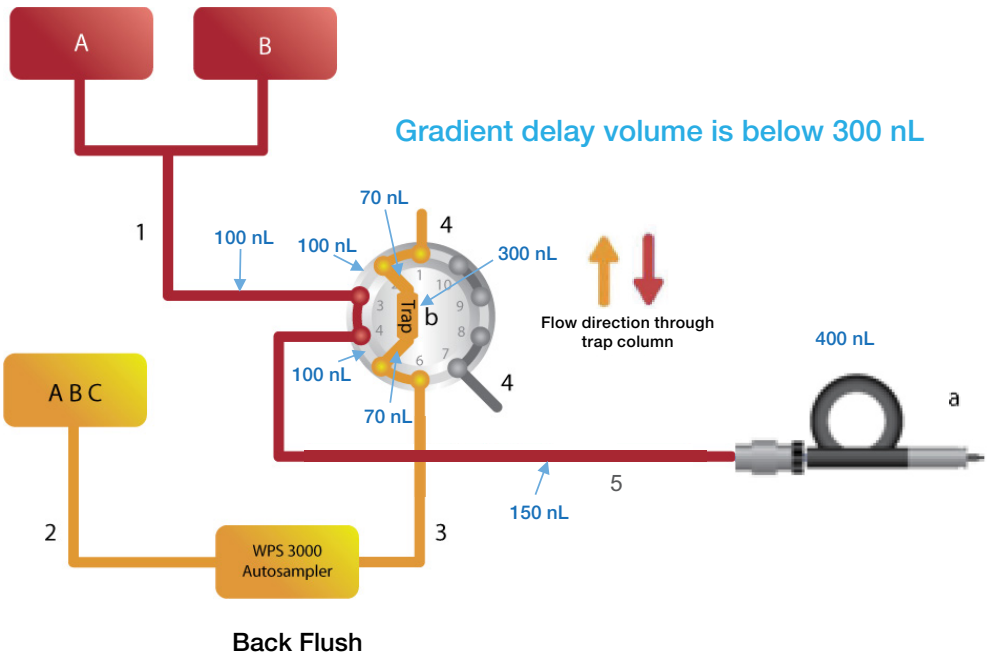

Figure 1. Fluidic setup used for a pre-concentration of sample onto a nano column experiment. Note: The number and letter descriptions for each of the fluidic components (in black) are given in Table 1. The values given in blue represent all the volumes from mixing of solvents A and B at the pump outlet until the emitter of the EASY-Spray column.

Solvents and analysis conditions were used as described in Tables 2 and 3.

Table 2 (A). LC solvents and conditions for high-throughput low-flow analysis. FA= Formic acid, TFA = Trifluoroacetic acid, ACN = Acetonitrile

Property	Setting
Mobile phase A:	100% Water + 0.1% FA
Mobile phase B:	20%/80% Water/ACN + 0.1% FA
Loading solvent (Loading Pump A):	100% Water + 0.05% TFA
Sample:	Cytochrome C digest (1 pmol/µL) and HeLa digest (200 ng/µL)
Sampler wash solvent (also used for trap cartridge wash):	100% ACN + 0.1% FA
Injection volume:	1 µL (air-flanked microliter pickup)
Loading time:	0.1 min
Gradient flow rate:	1.5 µL/min (ProFlow flow meter)
Gradient:	*Time* *%B* 0 8 0.2 8 5.0 35 5.9 99 6.8 99 6.9 8
Column oven / EASY-Spray column temp.:	60 °C
Sample temp.:	5 °C
Loading flow rate:	150 µL/min (reduced to 10 µL/min when the trap cartridge and analytical column are in line) *Time* *Flow, µL/min* 0 150 0.3 150 0.5 10 6.6 10 6.7 150 7 150

Table 2 (B). Switching program for low-dispersion valve in column compartment

Time	Valve positions
0	1-2
0.1	10-1
6.8	1-2

Table 2 (C). Commands manually inserted into the method Script Editor

Time	Command
4	Sampler.InjectValveToLoad
4.1	Sampler.Wash
6.9	Sampler.InjectValveToInject

Table 3. General settings for fast autosampler routines

Property	Setting
Draw speed:	1 µL/s
Draw delay:	2 s
Dispense speed:	8 µL/s
Dispense delay:	2 s
Dispense to waste speed:	8 µL/s
Sample height:	2 mm
Puncture depth:	8 mm
Wash volume:	25 µL
Wash speed:	8 µL/s
Flush volume:	5 µL

2.4 MS conditions

Table 4. MS tune settings

Parameters / Components	Settings / Details
Source settings	
ESI source:	EASY-Spray emitter
Polarity:	Positive
Ion transfer tube temperature:	300 °C
Spray voltage positive ion:	1.9 kV
Ion Funnel RF level:	40

Table 5. MS settings for Full MS experiments

Parameters / Components	Settings / Details
MS instrument:	Thermo Scientific™ Q Exactive™ HF-X
Acquisition mode:	Full MS / DDA
Full MS	
Resolution:	120,000
AGC target:	3e6
Maximum IT:	50 ms
Scan range:	375–2000 *m/z*

Example data files complete with method parameters are available for download: https://appslab.thermofisher.com/App/4167/fast-lowflow-lcms-proteomics

Table 6. MS settings for DDA experiments

Parameters / Components	Settings / Details
MS 1 resolution:	60,000
AGC target:	3e6
Maximum IT:	25 ms
Scan range:	350–1500 *m/z*
DDA	
MS2 resolution:	7,500
AGC target:	2e5
Maximum IT:	14 ms
TopN:	40
Isolation window:	1.4 *m/z*
Fixed first mass:	100 *m/z*
NCE:	27
AGC target:	1e3
Charge exclusion:	Unassigned, 1, 7, 8, >8
Peptide match:	Preferred
Dynamic exclusion:	5 s

2.5 Data acquisition and processing

Data were acquired using Thermo Scientific™ Xcalibur™ software version 4.1. The UltiMate 3000 RSLCnano system was controlled using Standard Instrument Integration (SII) 1.3 software. Chromatographic peak characteristics of extracted ion chromatograms (EICs) of peptides from Cytochrome C (CytC) proteome digest and HeLa cell proteome digest were evaluated using Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software version 7.2.8. DDA data for HeLa cell proteome digest were processed with Thermo Scientific™ Proteome Discoverer™ software version 2.2 using the SEQUEST™ HT search algorithm. The false discovery rate (FDR) was below 1% at the peptide and protein level.

3. Results and discussion

3.1 High-throughput low-flow LC method explained

Several LC method components were optimized to enable the short analysis cycle time (see Figure 2), ensure high performance, and reduce carryover to near zero:

- Autosampler injection and dispense speeds were increased to afford an injection routine of less than one minute without compromising injection reproducibility (see Table 3 for setting details).
- The analytical column was washed and equilibrated independently of the trap cartridge.
- The equilibration of the analytical column was completed during the autosampler injection routine.
- The trap was switched off-line at the end of the gradient, washed with strong solvent stored in the sample loop, and equilibrated using the loading pump prior to the next injection.
- Sample transfer onto the trap cartridge and online desalting were reduced to 6 seconds by adopting a loading pump flow rate of 150 µL/min.

To minimize carryover, the following steps were implemented during the run:

- Air flanked microliter pickup injection was used with an empty transport liquid vail and a 5 µL flush volume
- After the gradient elution had commenced, the injection needle was washed with organic solvent using the autosampler syringe.
- At 4 minutes run time, the inject valve was switched to the 'load' position and the autosampler wash procedure was triggered. This ensured that the injection loop was thoroughly washed and filled with a 20 µL plug of 100% acetonitrile/0.1% FA solution. The commands that must be manually inserted into the method script to achieve this functionality are given in Table 2.
- The analytical column was washed at the end of the gradient with 99% of solvent B.
- The trap column was switched back in-line with the loading pump during the column wash phase of the run (at 6.8 minutes).
- By switching the loop back to 'inject' at the end of the run (6.9 minutes), the plug of acetonitrile contained in the loop was pushed to the trap cartridge to provide intense washing.

3.2 LC-MS and chromatographic performance, method robustness

The fast, low-flow LC-MS method was evaluated for chromatographic data quality and robustness through continuous operation equivalent with over 180 injections per day. The sample sequence consisted of repeat cycles of CytC and HeLa cell protein digests (see Figure 3A) as well as blank injections (Figure 3B).

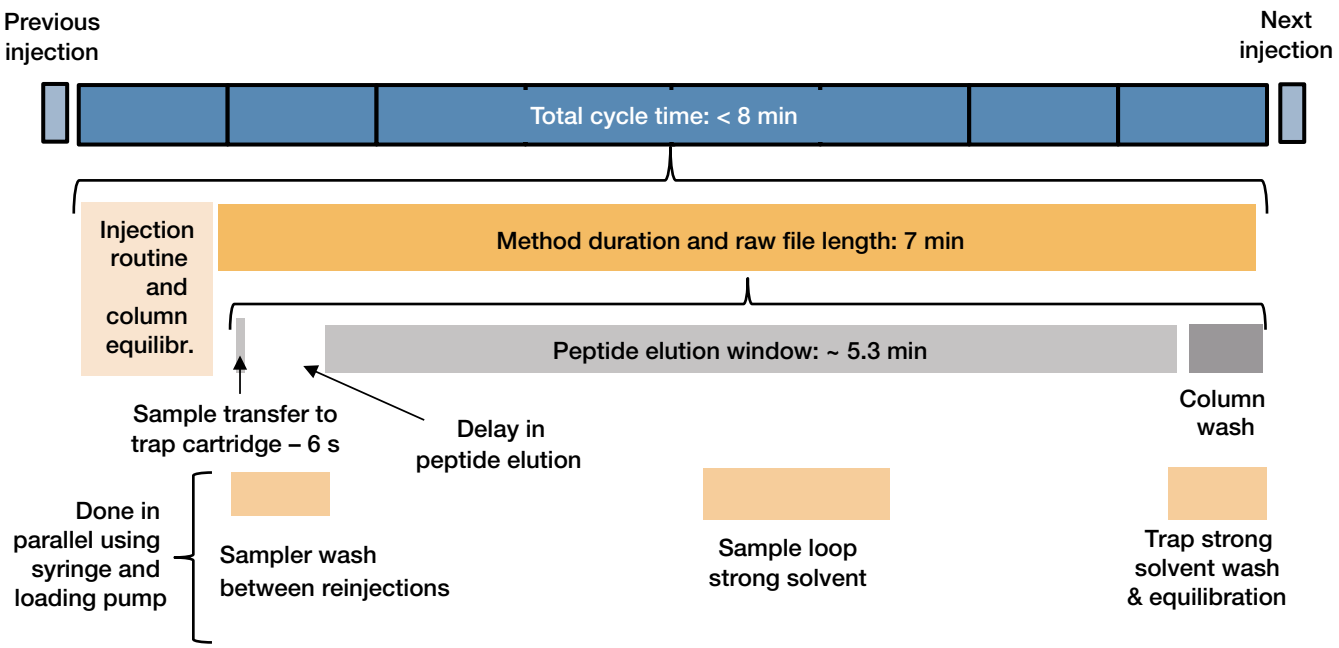

Figure 2. Schematic of the high-throughput low-flow LC-MS method

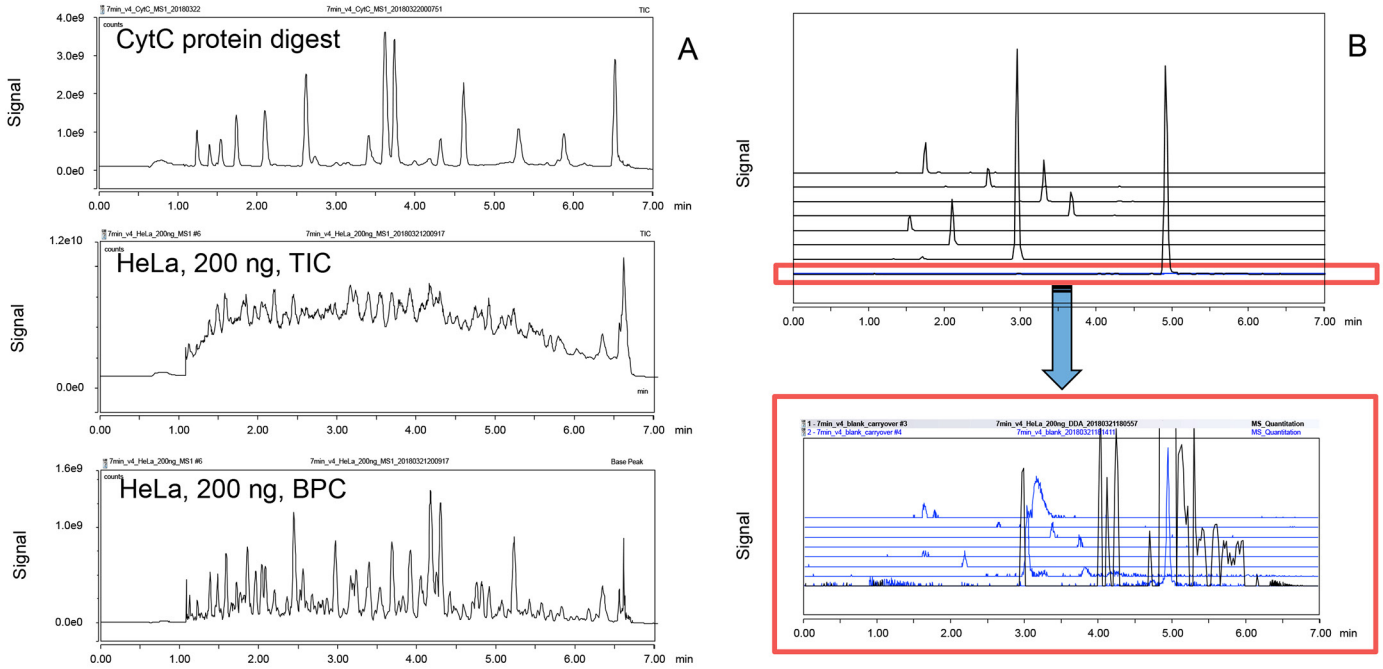

Figure 3. Typical TIC and BPC profile of CytC and HeLa protein digests obtained using the high-throughput low-flow LC-MS method (A), and EICs of 200 ng HeLa peptide digest injections (black EIC traces) followed by blank injections (blue EIC traces) (B)

The delay in peptide elution at the beginning of each run of approximately 1 minute arises from the sum of the void volumes resulting from the trap cartridge, analytical column, and corresponding connections (Figure 1) that in total equates to approximately 1 µL.

The gradient delay volume from the point at which the gradient is formed at the flow meter outlet until the trap cartridge is below 300 nL in this configuration. This is equivalent to 12 seconds of gradient delay time at a flow rate of 1.5 µL/min.

Eight peptides from HeLa cell protein digests exhibiting retention times that spanned the entire peptide elution window were selected to evaluate retention time stability, carryover, and chromatographic peak characteristics.

Standard deviation of retention times for HeLa peptides were below 0.1 minutes (Figure 4A), and peak area variation did not exceed 10% for all selected peptides (Figure 4B). Furthermore, FWHM for selected peptides was below 3 seconds for all peptides (Figure 4C) while low sample carryover (<0.2%, Figure 3B) was exhibited across the entire range.

The fast acquisition DDA capabilities of the Q Exactive HF-X MS with TOP40 MS2 events at 7.5K resolution and an injection time of 14 milliseconds yielded more than 10,000 MS/MS events and around 6200 PSMs per single 200 ng HeLa injection (Figure 4D).

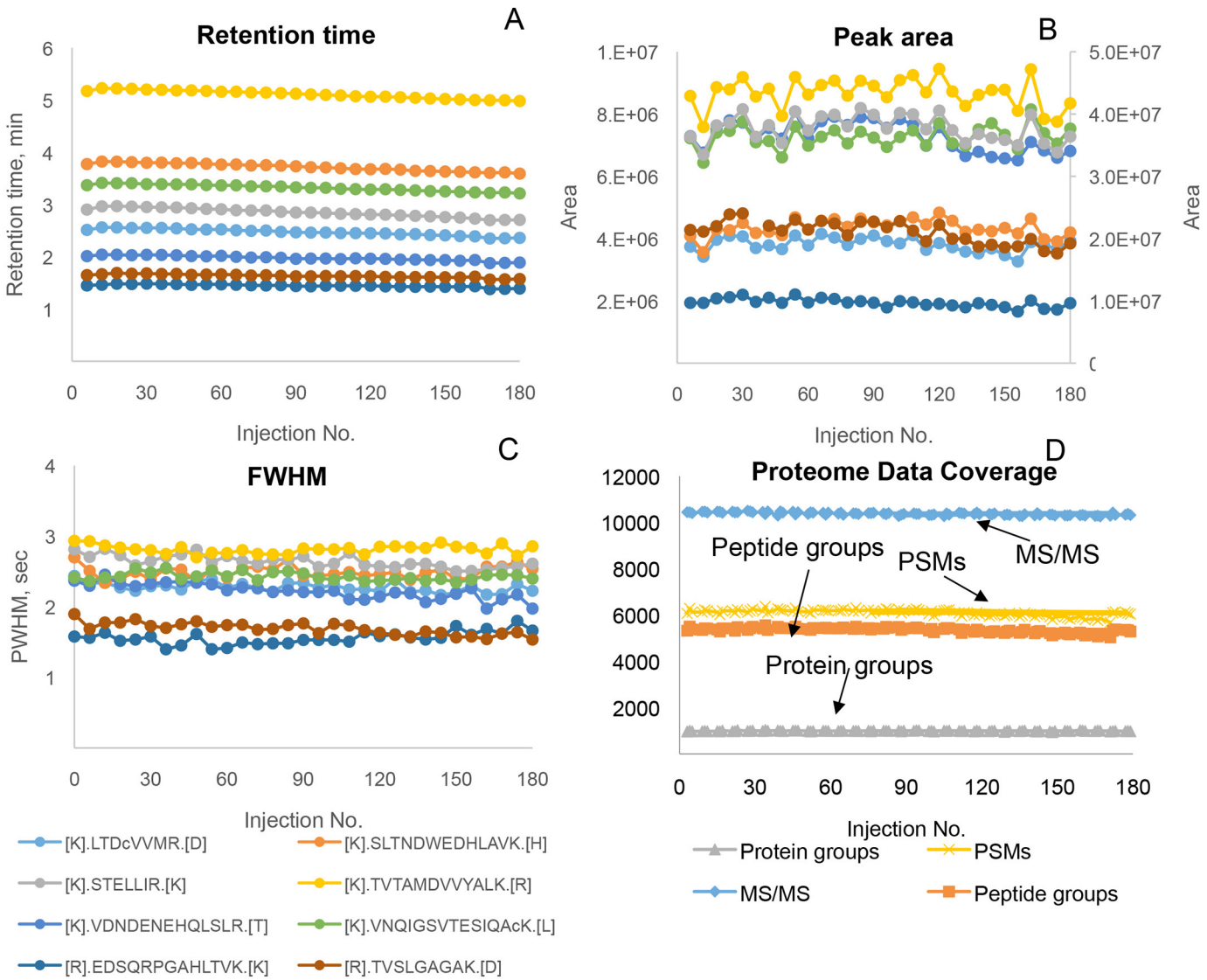

Figure 4. Chromatographic performance and method robustness assessment using the peak properties of eight peptides (A, B, and C) and proteome data coverage over a 24-hour period from 200 ng HeLa cells protein digest (D). A: retention time stability; B: peak area stability (left axis peak areas for LTDcVVMR, SLTNDWEDHLAVK, VDNDENEHQLSLR, VNQIGSVTESIQAcK, EDSQRPGAHLTVK, TVSLGAGAK, right axis peak areas for STELLIR and TVTAMDVVYALK). C: Full width at half maximum (FWHM) D: the number of MS/MS events, PSMs, and protein and peptide groups identified at 1% FDR.

For comparison's sake, example data were acquired using a high-throughput LC method run on a recently released low-flow LC system from vendor 'E' that employs solid phase extraction (SPE) for off-line sample pre-concentration (trapping) and desalting (Figure 5) prior to elution online.

While the peptide elution profile is around 6 minutes on both systems, there is an elution delay of around 4 minutes on vendor E's system, bringing the total analysis time to 11 minutes. Furthermore, vendor E cites an overhead of 3 minutes, bringing the run time to around 14 minutes per sample. In terms of throughput per day, the method developed for the UltiMate 3000 RSLCnano system yields almost double the throughput compared with vendor E (Figure 5). Furthermore, the MS utilization time (the proportion of time, when the MS is able to deliver actual sample related data) is about 75% for the UltiMate 3000 RSLCnano system, while it is only 45% for vendor E.

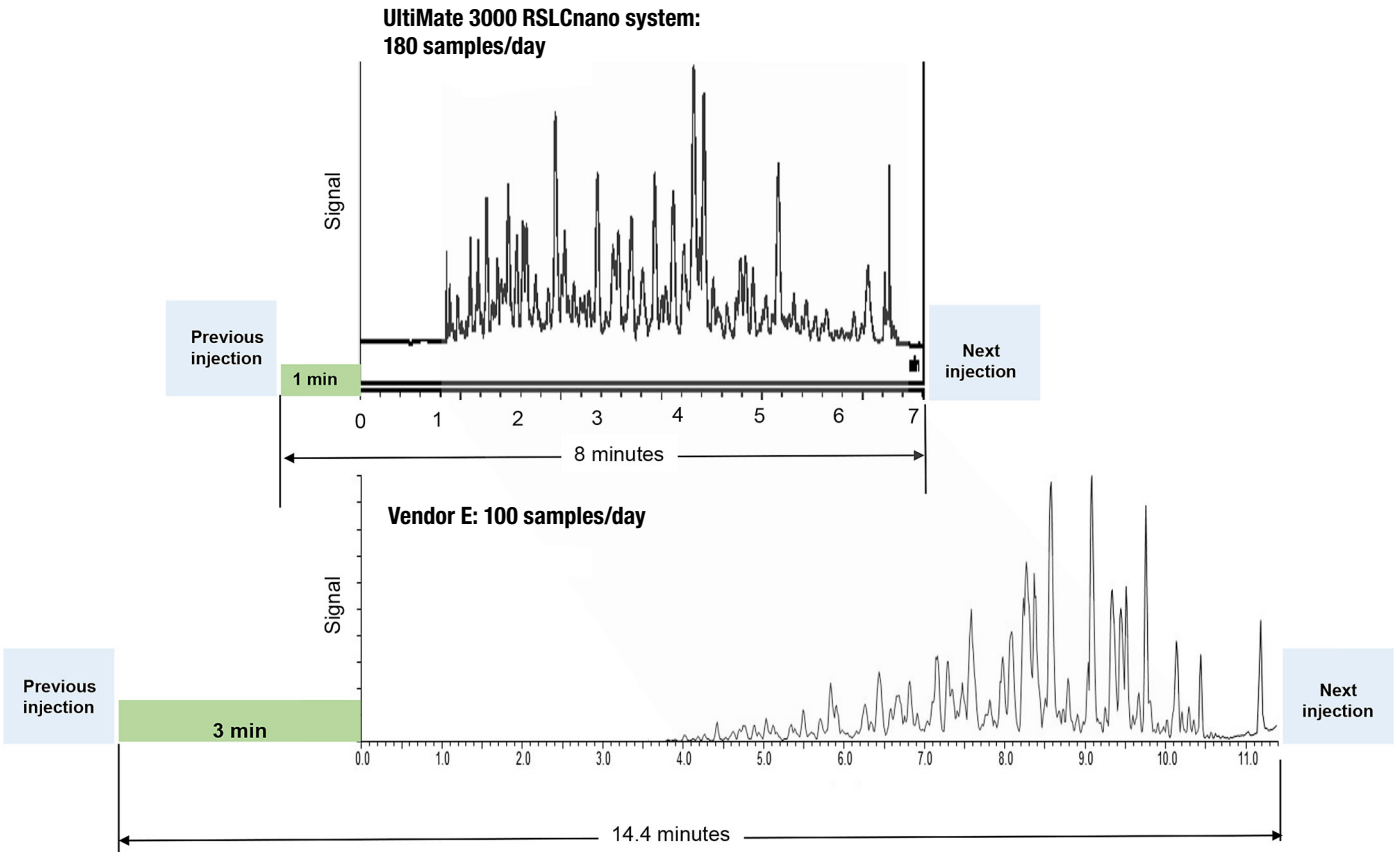

Figure 5. Examples of base-peak chromatograms of HeLa cell lysate protein digest comparing the high-throughput LC method described in this work with a pre-defined 'locked' (non-editable) high-throughput method run using the LC system from vendor E

4. Conclusions

There is an increasing demand for robust low-flow LC-MS based instrument methodology in the fields of industrial and translational proteomics and beyond. This is being met by a rise in the number and type of (capillary and/or micro) low-flow LC solutions, tailored to meet such needs. The UltiMate 3000 RSLCnano system is a versatile platform with a proven track record for servicing the requirements of both the most demanding deep-dive nano LC-MS proteomics applications as well as high-throughput low-flow LC-MS analysis carried out at capillary- and micro-flow rates.

Here we demonstrate a novel high-throughput capillary flow LC-MS method on an UltiMate 3000 RSLCnano LC system that solely makes use of fluidic components and hardware routinely adopted for conventional nano-LC type applications.

The UltiMate 3000 RSLCnano system is uniquely capable of the execution of such high-throughput LC-MS methods due to its:

- Low NC pump gradient delay volume of approximately 25 nL
- Wide pressure / flow footprint
- Ability to respond quickly to changes in mobile phase composition
- Integrated micro-flow pump for high speed online sample loading and desalting as well as trap / sample loop washing and column equilibration
- Flexible software control enabling the creation of intelligent customizable LC methods that can reduce overhead runtimes to less than a minute while affording adequate column equilibration enabling on-line high-speed loading and desalting
- Robust pressure stable fluidic components complete with industry-leading nanoViper connections
- High capacity (up to 8 µg on column), low back pressure, durable trapping cartridges that afford very fast sample loading, desalting, washing, and re-equilibration for multiple sample use, even with complex sample matrices such as serum or plasma

By exploiting all these advantages, we were able to create a high-throughput LC-MS method with an 8-minute run-to-run time and 6-minute peptide elution window.

Each 8-minute cycle comprises:

- Online sample loading, pre-concentration, and desalting
- Thorough column and sample loop wash, affording stabile retention times and minimal sample carryover
- High-resolution chromatography across the entire gradient
- Minimal loss of costly MS sample analysis time (75% MS utilization)

The UltiMate 3000 RSLCnano system is a safe investment that provides:

- Freedom to optimize and tailor LC methods according to individual requirements
- The ability to switch between high-throughput screening applications and discovery proteomics (long column/gradient) workflows without any hardware changes
- Low-flow LC-MS compatibility for low solvent consumption, environmentally friendly, and sensitive analytics
- The capacity to run a variety of application types from proteomics to HILIC-based metabolomics methods or ternary gradient analytical LC separations on up to 1 mm or even 2.1 mm i.d. columns using flow rates up to 2.5 mL/min[5]

The UltiMate 3000 RSLCnano system with the next-generation high-resolution, accurate-mass Orbitrap™ analyzers represents the state-of-the-art in low-flow LC-MS applications.

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