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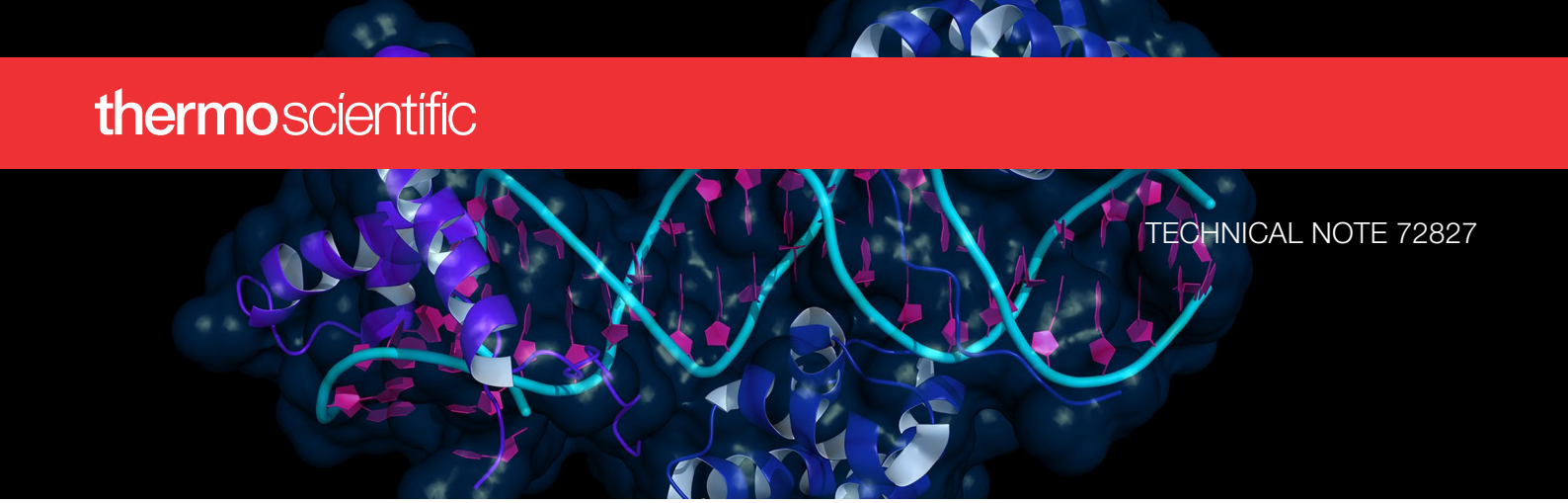

TECHNICAL NOTE 72827

High-throughput tandem capillary-flow LC-MS for maximum MS utilization

Authors

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Keywords

Tandem high-throughput, low-flow LC, capillary-flow, continuous MS utilization, proteomics

Goal

Create tandem high-throughput capillary-flow LC-MS based proteomics methods to permit fast sample data acquisition with maximum MS utilization time.

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1. Introduction

1.1 The next level in high-throughput low-flow proteomics applications

While nano-flow LC-MS proteomics applications yield unsurpassed depth of coverage and sensitivity, they do not lend themselves well to high-throughput workflows and are commonly associated with high levels of MS idle time, especially when long columns are employed. In contrast, capillary-flow LC-MS methods offer a unique blend of sensitivity and throughput.[1] We recently published a capillary-flow LC-MS method that exploits both of these attributes while also facilitating previously unseen levels of MS utilization using a Thermo Scientific™ UltiMate™ 3000 RSLCnano system configured for routine nanoLC applications (TN72777).[2] By optimizing the sample injection routine and taking full advantage of the versatility of the UltiMate 3000 RSLCnano system, we created robust reproducible methods with a complete cycle time of only 8 minutes. Moreover, the MS utilization time (time during which the MS is accumulating sample peptide data) was 75%, equating to a peptide elution window of 6 out of every 8 minutes.[2]

In this technical note, we demonstrate how MS productivity can be further increased to almost 100% without sacrificing MS sensitivity or depth of proteome coverage using the UltiMate 3000 RSLCnano system in tandem low-flow LC configuration coupled with intelligent post-column flow diversion and "look ahead" injection routines.

1.2 Tandem LC-MS for continuous peptide data acquisition

For high throughput applications such as that described in TN72777,[2] the presence of fluidic connections and column void volume limits the possibility to further increase MS utility when using just one separation column. This is due to the time required for the sample to pass from the autosampler, through the connecting capillaries and column, to the MS. This problem can be overcome by employing a tandem LC setup in which a second analytical separation column is incorporated into the system. A continuous sample feed to the MS is achieved by running temporally offset alternating gradients on each of the columns (Figure 1).

While separation column 1 is running a gradient, separation column 2 is washed and equilibrated (Figure 1). Post-column valve switching is used to ensure

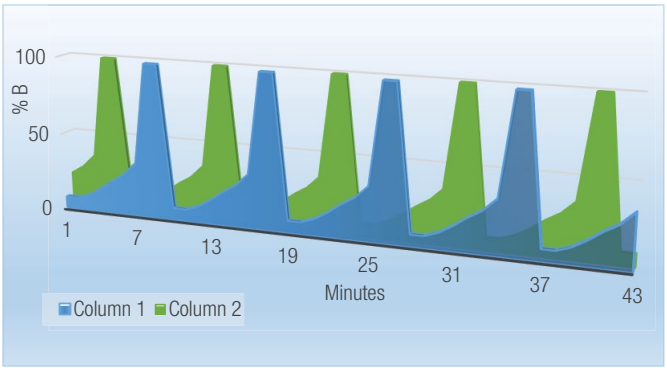

Figure 1. Flow-gradient diagram depicting high-throughput low-flow tandem LC operation affording >200 samples per day

the eluting peptides from column 1 are delivered to the MS and the effluent from column 2 is diverted to waste. After the sample separation on column 1 is complete, the post-column valve is switched and gradient 2 delivers the gradient separation while column 1 is washed and equilibrated. Through the use of intelligent valve switching and "look ahead" injections using the "PrepareNextInjection" command (see Table 2 for details), the method can be optimized such that there is virtually no delay between the end of sample 1 elution into the MS and the beginning of sample 2 (for details see section 3.1).

Running a novel tandem LC low-flow setup on the UltiMate 3000 RSLCnano system requires the addition of a high-pressure gradient low-flow pump module, a second 2-position, 10-port valve to the column oven, and a second separation and trap column (Figure 2). The single stack setup ensures that both the system footprint as well as the length of connecting capillaries, and hence delay volume, are minimized.

2. Experimental

2.1 Consumables

- Fisher Scientific™ Water, LC-MS grade (P/N W6-212)
- Fisher Scientific™ Acetonitrile, LC-MS grade (P/N 10616653)
- Thermo Scientific™ Pierce™ Trifluoroacetic acid (TFA), LC-MS grade (P/N 85183)
- Thermo Scientific™ Pierce™ Formic acid (FA), LC-MS grade (P/N 28905)

Fluidics and columns used to set up the application are listed in Table 1.

Table 1. Fluidics, columns, and consumable accessories required to run the application. The letter and number assignments are given in Figure 2A.

#	Quantity	Item	P/N
a	2	Thermo Scientific™ Acclaim™ PepMap™ 100, C18, 3 µm, 100 Å, 75 µm × 15 cm	164568
b	2	300 µm I.D. × 5 mm packed with Acclaim PepMap 100 C18, 5 µm, (set of 5 cartridges)	160454
	2	µ-Precolumn holder, 5 mm, with 30 µm I.D. connecting tubing, Thermo Scientific™ nanoViper™ fingertight fittings	164649
1	4	Thermo Scientific™ nanoViper™ capillary FS/PEEK sheathed 1/32" I.D. × L 20 µm × 350 mm	6041.5240
2	1	Thermo Scientific™ nanoViper™ capillary FS/PEEK sheathed 1/32" I.D. × L 75 µm × 650 mm	6041.5775
3	1	Thermo Scientific™ nanoViper™ capillary FS/PEEK sheathed 1/32" I.D. × L 75 µm × 550 mm	6041.5760
		Thermo Scientific™ nanoViper™ sample loop 20 µL, FS/PEEK sheathed	6826.2420
4	2	PTFE tubing, 500 µm I.D., 100 cm, used as waste tubing	6720.0077
5	3	NanoLC column to MS tubing I.D. × A.D. × L 20 µm × 280 µm × 1 m	6041.5292*
6	1	nanoViper capillary FS/PEEK sheathed 1/32" I.D. × L 20 µm × 150 mm	2261.5061
	1	Polypropylene vials for WPS with glass insert, 250 µL, 25 pieces	6820.0027
	1	Polypropylene caps for WPS vials, 25 pieces	6820.0028
	1	Transport vial including cap and seal (5 vials)	6820.0023**
	1	Cleavage stone	6720.0016
	2	2-position, 10-port switching valve (installed in column compartment)	6041.0001A

* This capillary must be cut to the appropriate length using the cleavage stone.
** Included in the accessories kit (P/N 5820.8910) of the autosampler.
Note: Consumables are from Thermo Fisher Scientific unless stated otherwise.

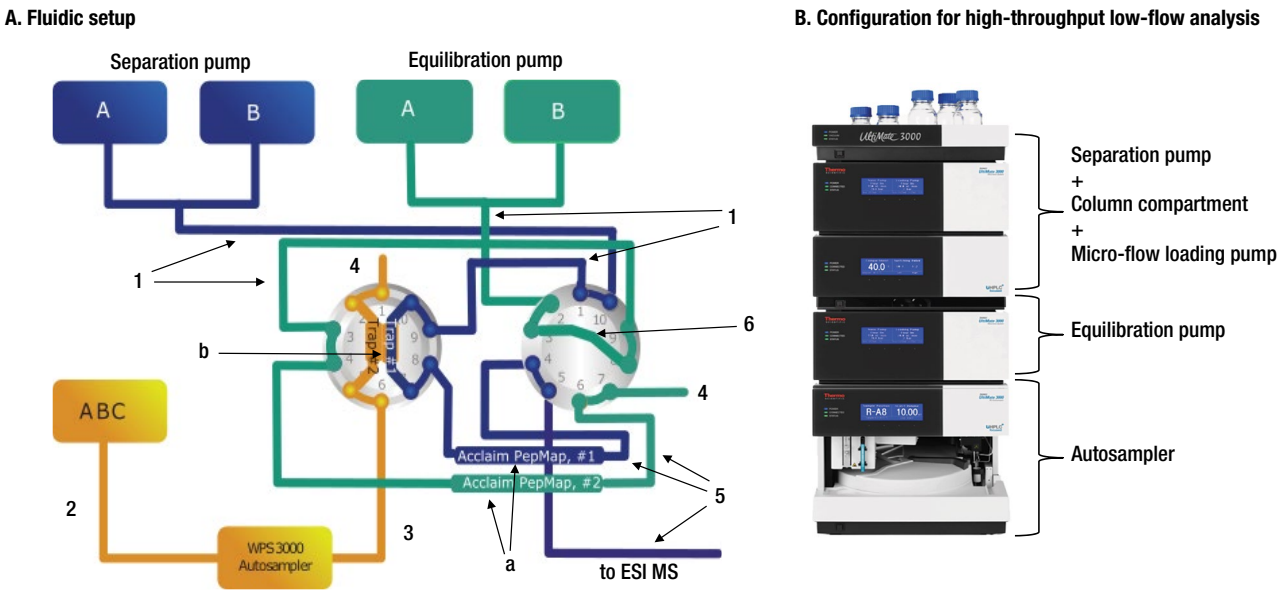

Figure 2. Fluidic (A) and UltiMate 3000 RSLCnano (B) configurations for the high-throughput low-flow tandem pre-concentration LC setup. Note: The number and letter descriptions for each of the fluidic components (in black) are given in Table 1.

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 2A for details).
- Thermo Scientific™ Dionex™ Cytochrome C (CytC) 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 an UltiMate 3000 RSLCnano system,[3] comprising an UltiMate NCS-3500RS pump, Thermo Scientific™ UltiMate™ NCP-3200RS pump, and Thermo Scientific™ UltiMate™ WPS-3000TPL RS pulled loop autosampler (Figure 2B). Both NCS-3500RS and NCP-3200RS pumps were configured with a ProFlow™ flow meter. The device names and signals of the NCP-3200RS pump were modified through the addition of the number "2" at the end of the standard instrument property fields in the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) instrument configuration manager. For example, the main device name was changed from "PumpModule" to "PumpModule2" to avoid a conflict in the signal names. The system fluidics were configured as shown in Figure 2A and Table 1. In order to interface the respective linear Acclaim PepMap column outlet with the 10-port valve on the right-hand side of the column oven, the fused silica outlet supplied with the columns was removed and replaced with the "nano column to MS tubing" (#5 in Table 1). The column outlet is connected to the "nano column to MS tubing" using a nano connector supplied with the column. A description of how to make the connection is given in the UltiMate 3000 RSLCnano Standard Applications Guide[4] section 2.1.2. Before the capillary is installed it must first be **cut to a length of 30 cm** using the cleavage stone. The LC system was interfaced with the MS using the Thermo Scientific™ Nanospray Flex™ ion source (P/N ES071) using stainless steel nanobore emitters (P/N ES542).

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

Table 2A. LC solvents and conditions for high-throughput low-flow analysis

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 channel: Loading Pump B channel:	 100% Water + 0.05% TFA ACN + 0.1% FA		
Sample:	Cytochrome C digest (1 pmol/µL) and HeLa digest (200 ng/µL)		
Sampler wash solvent:	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		
Gradient:	Low-flow separation pump		
	Time, min	*%B*	
	0	8	
	5.3	35	
	6.7	70	
	6.7	8	
	7	8	
Gradient:	Equilibration pump		
	Time, min	*%B*	
	0	99	
	3.0	99	
	3.3	8	
	7	8	
Oven temperature:	60 °C		
Sample temperature:	5 °C		
Loading and washing gradient:	Micro-flow loading pump		
	Time, min	*Flow, µL/min*	*%B*
	0	150	95
	0.5	150	95
	0.6	150	0
	5.6	150	0
	6.7	150	95
	7	150	95

Table 2B. Switching program and commands for high-throughput low-flow analysis

Property	Setting		
Intelligent switching program for 10-port valves in the column compartment. These commands must be inserted manually into the script editor:	*Time, min*	*Command*	*Valve positions*
	0		
	If		
		ColumnOven.ValveLeft	1_2
		ColumnOven.ValveRight	10-1
	Else		
		ColumnOven.ValveLeft	10-1
		ColumnOven.ValveRight	1-2
	End If		
	6		
	If	ColumnOven.ValveLeft	1_2
		ColumnOven.ValveLeft	10_1
	Else		
		ColumnOven.ValveLeft	1_2
	End If		
Commands manually inserted into the method script editor:	*Time, min*	*Command*	
	1.5	Sampler.InjectValveToLoad	
	1.6	Sampler.Wash	
	3.4	Sampler.InjectValveToInject	
	3.6	Sampler.PrepareNextInjection	
	5.8	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

2.4 MS conditions

Table 4. MS tune settings

Parameters / Components	Settings / Details
Source settings	
ESI Source:	Nanospray Flex
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 Hybrid Quadrupole-Orbitrap™ mass spectrometer
Acquisition mode:	Full MS / DDA
Full MS	
Resolution:	120,000
AGC target	3e6
Maximum IT:	50 ms
Scan range:	375–2000 *m/z*

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	
MS 2 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™ 4.1 software. The UltiMate 3000 RSLCnano tandem system was controlled using Standard Instrument Integration (SII) 1.3 software. Chromatographic peak characteristics of extracted ion chromatograms (XICs) of peptides from the HeLa cell protein digest were evaluated using Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) 7.2.8. DDA data for HeLa cell protein digest were processed with Thermo Scientific™ Proteome Discoverer™ 2.2 software 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 Tandem high-throughput low-flow LC method explained

An optimized LC method is used for tandem LC operation that enables ~100% MS sample data acquisition while maintaining a short analysis cycle time and high sensitivity (Figure 3). This method contains the following attributes:

- Simultaneous washing of separation column 2 and trap column 2 parallel to gradient elution on separation column 1.
- Parallel washing of injection fluidics and sample loop during sample elution.
- Look ahead injections: sample 2 is picked up, loaded onto the trap column 2, concentrated, and transferred to the separation column 2 while sample 1 is undergoing gradient elution in parallel (Table 2B).
- The delay in sample peptide elution is removed using post-column flow diversion.
- Intelligent valve switching commands enable LC control for both columns using a single LC method (Table 2B).
- High retention time reproducibility via use of one dedicated low-flow (NCS-3500RS) pump for gradient elution while the second (NCP-3200RS) pump is used for column washing and equilibration.

An example data file containing complete LC-MS method parameters is available for download at https://appslab.thermofisher.com/App/4176/tandem-lowflow-lcms.

3.2 Continuous MS utilization with tandem LC-MS

Optimized high-throughput tandem LC-MS methodology using one single pump for gradient elution on both analytical columns enabled reproducible chromatography with peptide data elution covering more than 6.8 of 7 minutes of each sample run (Figure 4), equivalent to over 97% MS utility with a throughput of >200 samples per day.

Furthermore, the even distribution of PSMs over the entire elution window (Figure 5A) ensures a constant sample data feed at a complexity level that can be handled by the Q Exactive HF-X mass spectrometer.

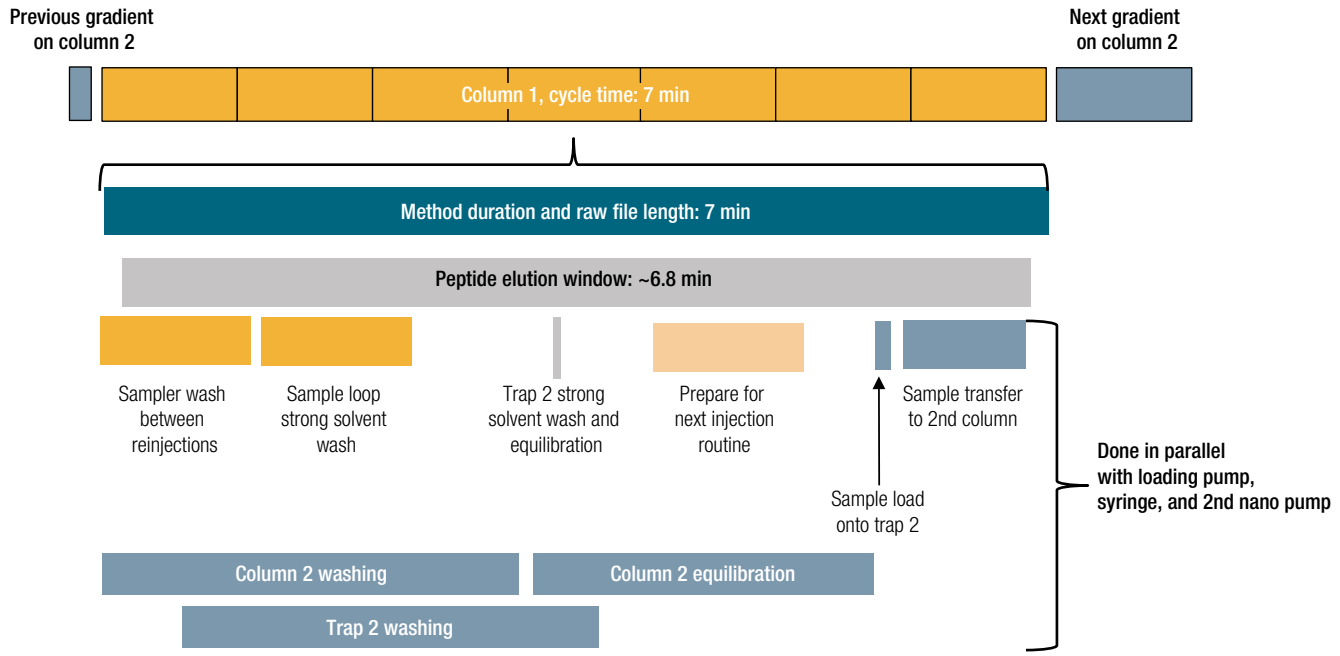

Figure 3. Schematic of the pre-concentration tandem high-throughput low-flow LC method

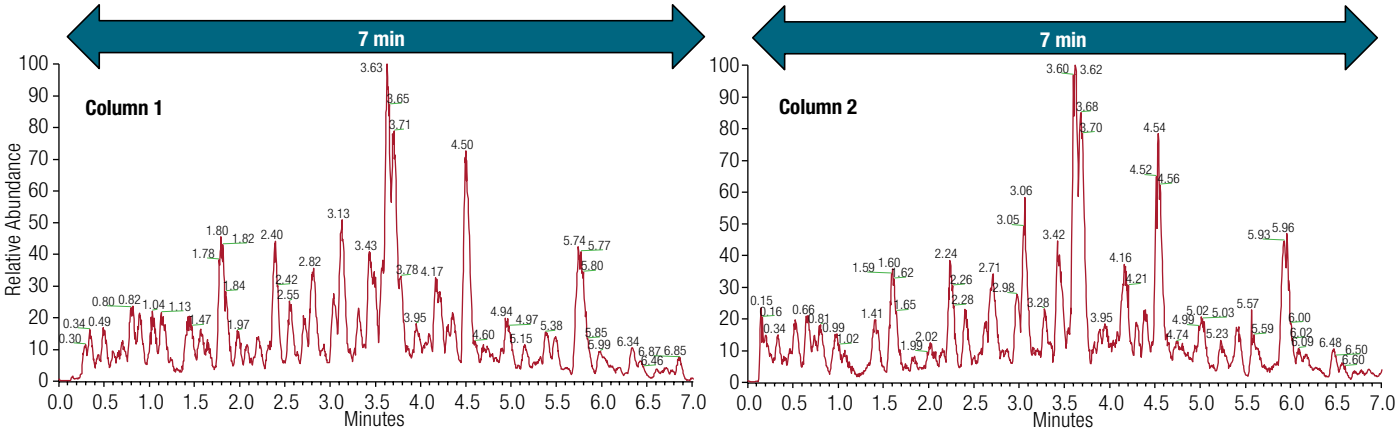

Figure 4. Typical BPC profile of HeLa protein digests for data sequentially collected on column 1 and 2 using the tandem low-flow LC-MS method

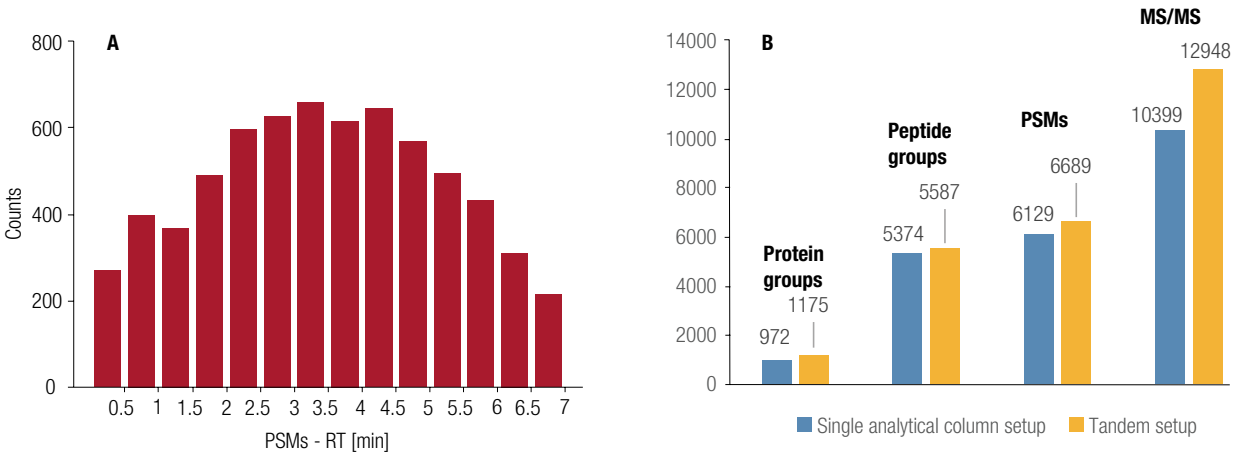

Figure 5. Distribution of PSMs from 200 ng HeLa cells protein digest eluted using the 7-minute tandem LC-MS method (A) and a comparison of the DDA results from an 8-minute single analytical column LC high-throughput pre-concentration method with the 7-minute tandem pre-concentration method

The proteomics data collected using the tandem LC-MS method were compared with data collected using the recently described high-throughput pre-concentration low-flow method employing a single analytical column.[2] The wider elution window afforded by the tandem low-flow LC resulted in more MS/MS events and subsequently more protein group identifications when compared to the single analytical column-based method (Figure 5B). This was despite the fact that the peak width was up to twice the width for the tandem method. The wider peak width observed for the tandem LC method can be attributed to post-column dispersion that happens during post-column flow guiding using the switching valve. This effect is virtually non-existent using a Thermo Scientific™ EASY-Spray™ LC column used in the single analytical column setup.[2] Nevertheless, our results clearly show that the benefits of the increased peptide elution window afforded by the tandem LC method (which covers almost the entire 7 minutes of runtime) vs. 6 out of 7 minutes for the single analytical column method outweighs any negative impact caused by the lower chromatographic resolution achievable with the tandem LC setup for very short gradients.

4. Conclusions

The versatility of the UltiMate 3000 RSLCnano system provides the flexibility necessary to meet the individual demands of modern proteomics research and beyond. The system shows superior performance both in terms of retention time precision when employed for long shallow gradient elution experiments typical of deep-dive proteomic analysis[6] as well as short gradient high-throughput methodology, making maximum use of MS time with the added benefit that both types of experiments can be performed without any changes required in either LC hardware or capillary fluidics.

Here we show that with modest changes to the hardware and fluidics, MS utilization can be still further increased to permit constant MS sample data acquisition with high throughput and more protein group identifications. We demonstrate the feasibility to detect over 1100 protein groups in 7-minute cycles using very strict database search criteria. These methods can easily be adapted for shorter (or longer) gradients according to the application needs, while always maintaining virtually 100% MS utilization.

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