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Tailored high-throughput low-flow LC-MS methods for large sample cohort analysis

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Goal

Present five robust capillary-flow LC-MS methods affording a broad spectrum of proteome coverage and throughput ideal for large scale clinical research studies.

Introduction

Robust high-throughput low-flow LC-MS methods, for large sample cohorts

The availability of blood and its plasma and serum constituents, together with its comparative ease of sampling, makes it ideal for clinical research studies. Proteins as major players in catalytic, structural, and signaling functions are important research targets. While antibody-based assays are currently the method of choice for protein analysis, issues concerning their specificity, combined with the challenges related to multiplexing and the high cost in the development of new assays, pose significant challenges.

LC-MS based technologies, while enabling precise detection and quantification of multiple targets from a single analysis, have yet to fulfill their potential in the field of blood proteomics. Sticking points include challenges arising from both the complexity and dynamic concentration range of the constituent proteins, which demand both high resolution and robustness from the LC columns employed. On top of this is the need for high-throughput capabilities in order to cope with the number of samples involved, yet without sacrificing sensitivity. While sensitivity can, to an extent, be addressed using conventional nanoLC, this comes at the expense of the throughput.

We recently demonstrated the efficacy of a method that employs a cycle time of only 8 minutes, affording a throughput of 180 samples per day while at the same time offering high levels of sensitivity and robustness.[1] Here we complement this method with a complete low-flow LC-MS method portfolio, which yields analysis times ranging from the original 8 minutes to 60 minutes, which when taken together, provide a spectrum of analytical depth and quantitative dynamic range well suited to the fields of blood and cellular proteomics. These methods provide the flexibility to analyze samples of varying complexity and balance the throughput with the required proteome depth accordingly while maintaining very high levels of MS utilization.

Experimental

Consumables

- Fisher Scientific™ Water, LC-MS grade (P/N W6-212)
- Fisher Scientific™ Acetonitrile (ACN), LC-MS grade (P/N 10616653)
- Thermo Scientific™ Pierce™ Trifluoroacetic acid (TFA), LC-MS grade (P/N 85183)
- Fluidics and columns used to set up pre-concentration application

Samples

- Thermo Scientific™ Pierce™ HeLa protein digest (P/N 88328, 20 µg/vial) was reconstituted to a final concentration of 200 ng/µL in loading buffer.
- Serum samples were from SeraLab (UK) collected from healthy donors.

Extraction and digestion of crude serum

Crude serum samples were processed and enzymatically digested using a simple procedure that does not require reduction and alkylation of cysteine residues and is fully compatible with automated sample preparation (Figure 1).

Briefly, 200 µL methanol were added to 50 µL serum aliquots in an Eppendorf™ LoBind™ 96-well plate. The plate was vortexed for 5 minutes and subsequently centrifuged (1500 g, room temperature). After discarding the supernatant, the plate was inverted and allowed to dry for 15 minutes. Then, 200 µL digestion mix (pH 7) were added to each well and the plate vortexed until the pellet had re-dissolved. Another 250 µL digestion mix were added. The samples were then digested for 3 hours at 37 °C and 1250 rpm. The digestion was quenched using 50 µL of a 10% formic acid solution.

Table 1. Fluidics, columns, and consumable accessories required to run the application. All parts are contained within the Thermo Scientific™ UltiMate™ 3000 RSLCnano EASY-Spray™ connection kit (P/N 6720.0395) unless otherwise indicated. Consumables are from Thermo Fisher Scientific unless stated otherwise.

#	Item	P/N
a	Thermo Scientific™ EASY-Spray™ column, 3 µm, 75 µm i.d. × 15 cm or Thermo Scientific™ Acclaim™ PepMap™ 100 C18 column, 3 µm, 75 µm i.d., × 15 cm, 100 Å*	ES800A* (or 164568)**
b	300 µm i.d. × 5 mm packed with Acclaim PepMap 100 C18, 5 µm, (set of 5 cartridges) µ-Precolumn holder, 5 mm, with 30 µm i.d. connecting tubing, nanoViper fittings	160454 164649
1	Thermo Scientific™ nanoViper™ capillary FS/PEEK sheathed 1/32" i.d. × L 20 µm x 350 mm	6041.5240
2	Thermo Scientific™ nanoViper™ capillary FS/PEEK sheathed 1/32" i.d. × L 75 µm x 650 mm	6041.5775
3	Thermo Scientific™ nanoViper™ capillary FS/PEEK sheathed 1/32" i.d. × L 75 µm x 550 mm	6041.5760
	Thermo Scientific™ 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	Thermo Scientific™ nanoViper™ capillary FS/PEEK sheathed 1/32" i.d. × L 20 µm x 550 mm	6041.5260
6	Union Viper	6040.2304
	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#

The letter and number assignments are given in Figure 2. *P/N ES800A must be ordered separately. **P/N 164568 is a linear Thermo Scientific™ PepMap™ column and can be used as an alternative to the EASY-Spray variant. #Five pieces are included in the Autosampler accessories kit (P/N 5820.8910).

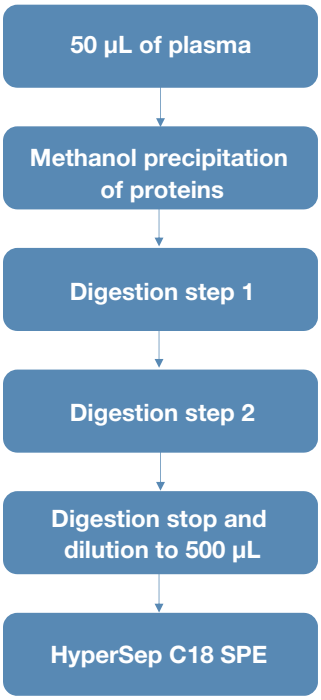

Figure 1. Serum extraction and digestion workflow

Finally, 100 µL of the digest were loaded onto a Thermo Scientific™ HyperSep™ C18 cartridge (100 mg bed weight, 1 mL capacity, P/N 60108-302), washed with 400 µL 0.1% formic acid (FA), eluted with 400 µL 0.1% FA in 50% ACN, dried, and resuspended in 200 µL of 0.1% FA. Then, 160 µL was transferred to a polypropylene vial with glass insert.

LC-MS configuration and separation conditions

Measurements were carried out using a Thermo Scientific™ UltiMate™ 3000 RSLCnano system,[2,3] equipped with a ProFlow™ flow meter (P/N 6041.7850), with the recommended flow range from 50 to 1500 nL/min. The system was configured (Figure 2, Table 1) using the EASY-Spray connection kit (P/N 6720.0395) and EASY-Spray ES800A column as described in the UltiMate 3000 RSLCnano Standard Applications Guide[4]. An EASY-Spray ES800A column was chosen for these experiments because its relatively short bed length (15 cm) and 3 µm particle size render it compatible with a broad range of flow rates without generating backpressures that exceed the upper pressure limit of the UHPLC system while affording high resolution chromatography (Figure 2).

The number and letter descriptions for each of the fluidic components (in black) are given in Table 1. The values given in blue in Figure 2 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. Method files containing full LC-MS parameters are available for download from the Thermo Scientific AppsLab Library.

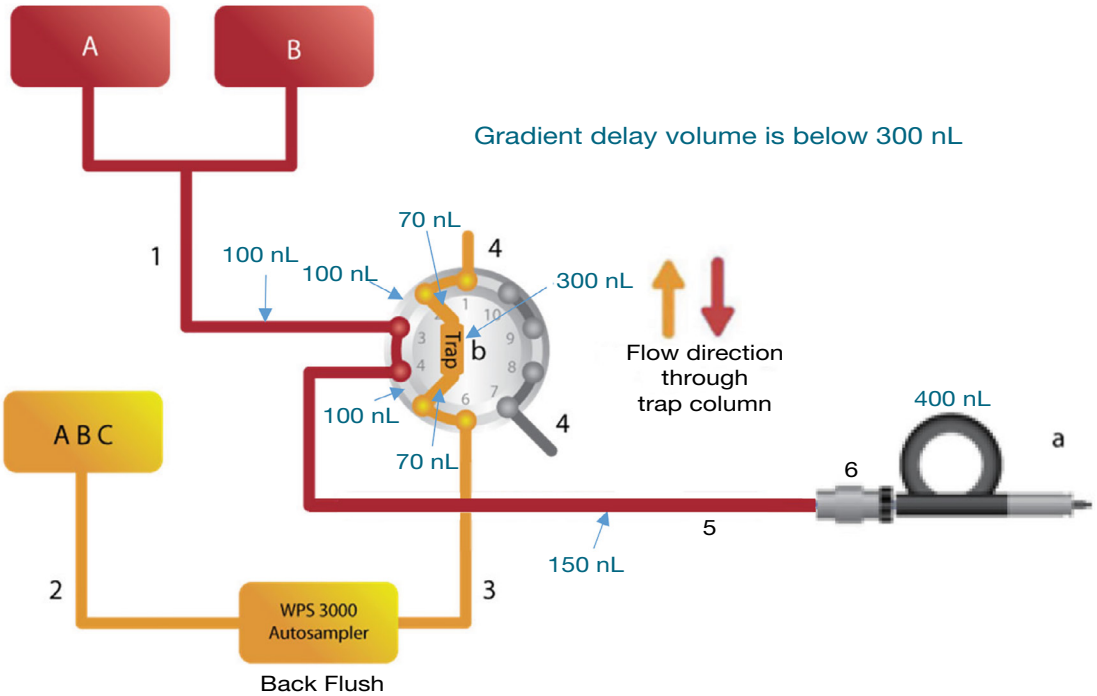

Figure 2. Fluidic setup used for a pre-concentration of sample onto a nano column experiment. Note: The number and letter descriptions for each of the fluidic components (in black) are given in Table 1. The values given in blue represent all the volumes from mixing of solvents A and B at the pump outlet until the emitter of the EASY-Spray column.

Table 2. 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)	100% Water + 0.05% TFA
Sample	HeLa digest (200 ng/µL) and crude serum digest (50 µL aliquot)
Sampler wash solvent (also used for trap cartridge wash)	100% ACN + 0.1% FA
Oven temperature / Easy-Spray column temperature	60 °C
Sample temperature	5 °C

MS acquisition parameters

MS data were recorded on either a Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer or a Thermo Scientific™ Q Exactive™ HF-X Hybrid Quadrupole-Orbitrap™ mass spectrometer. The MS tune and data acquisition parameters are available for download for each method on AppsLab.

Data acquisition and processing

Data were acquired using Thermo Scientific™ Xcalibur™ software. The UltiMate 3000 RSLCnano system was controlled using Standard Instrument Integration (SII). Chromatographic peak characteristics of extracted ion chromatograms (EICs) of peptides from cytochrome C (CytC) protein digest and HeLa cell protein digest was evaluated using Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), version 7.2.10. DDA data for HeLa cell protein 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 the protein level.

Results and discussion

Versatile low-flow LC methodology

The standard pre-concentration onto a trap cartridge UltiMate 3000 RSLCnano setup (Figure 2, Table 1) as well as the pre-concentration nanoLC kit (P/N 6720.0310) is most commonly associated with nanoLC-MS experiments with long columns to dive deep into the proteome. However, the versatility of the RSLCnano system permits this fluidic configuration to be adopted for the robust high-throughput methods shown here. Importantly, **no hardware or fluidic capillary changes are required** to switch between “deep dive” proteomics and high-throughput low-flow LC. The only change required is the column.

The core attributes and peak characteristics for each of the five methods are given in Table 3.

High-throughput LC-MS analysis with the UltiMate 3000 RSLCnano and the Orbitrap Exploris 480 system

The fast, low-flow LC-MS methods were evaluated using 200 ng HeLa injections. Peptides identified ranged from nearly 6000 to over 25,000 for the 8-minute and 48-minute methods, respectively (Figure 3a). Similarly, protein groups rose from 1000 to approximately 3000 for the 8-minute and 48-minute methods (Figure 3b).

Fast low-flow LC-MS profiling of crude serum extracts

Crude serum digests, prepared as described in the section *Extraction and digestion of crude serum,* were analyzed using each of the five high-throughput methods (Figure 4).

The methods afforded a wide and even distribution of peptides across the elution window with high levels of MS utilization.

Table 3. Key attributes and peak characteristics for each of the tailored LC-MS methods

	Flow, µL/min	Samples per 24 hr	MS utilization, %	Average PWHM, s	Average PW base, s	Asymmetry
60 min	0.300	24	95	10	19	1.23
48 min	0.600	30	90	9	18	1.21
24 min	0.800	60	87	7	13	1.17
14.4 min	1.000	100	85	4	7	1.13
8 min	1.500	180	75	3	6	1.16

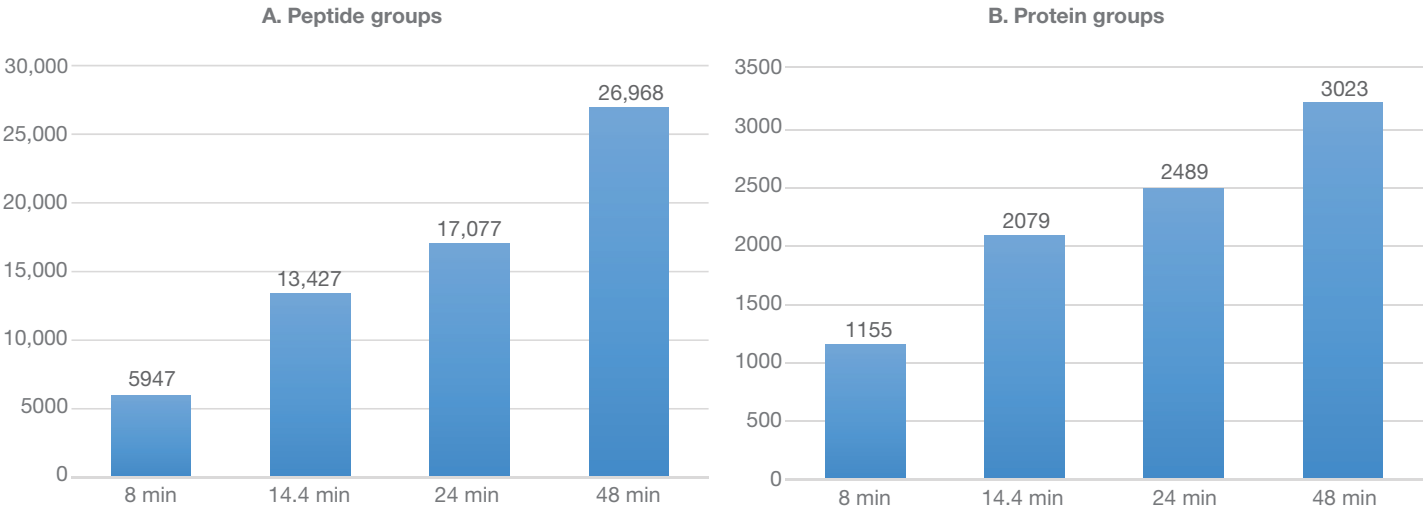

Figure 3. The number of peptide groups (A) and protein groups (B) identified with a 1% FDR from 200 ng HeLa protein digest 8, 14.4, 24, and 48 min long low-flow for LC-MS methods

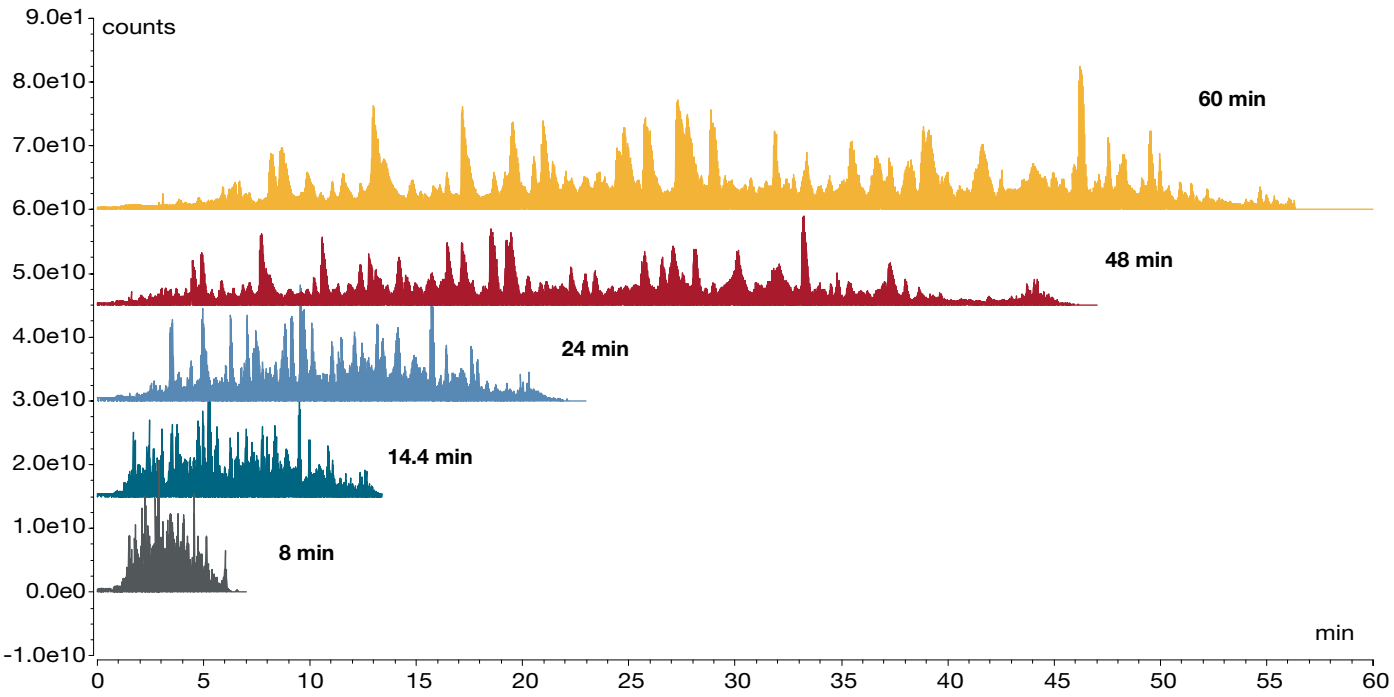

Figure 4. Typical TIC profiles of crude serum digests analyzed using the 8, 14.4, 24, 48, and 60-minute methods

Method robustness was assessed through the consecutive analysis of 200 crude serum extracts over three days using the 14.4-minute method (Figure 5).

The continuous repeat analysis of crude serum had no impact on either system robustness, the quality of the chromatography, or the MS sensitivity.

Using the tailored low-flow LC-MS methods we were able to identify from above 1000 peptide groups and 150 protein groups within 8 minutes to more than 2700 peptide groups and 250 protein groups using the 60-minute method (Figures 6a and 6b). The further (up to 30%) improvements in proteome coverage can be achieved with Proteome Discoverer version 2.4 by using “Precusor Detector Node” and Spectral Library Search.

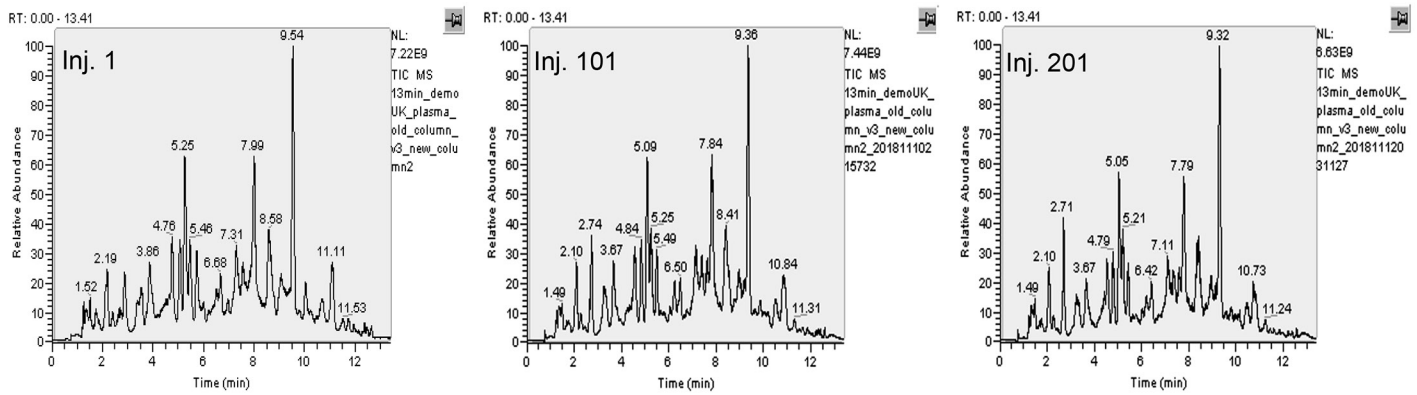

Figure 5. Representative TIC chromatograms selected from the continuous measurement of >200 crude serum samples over a 3-day period

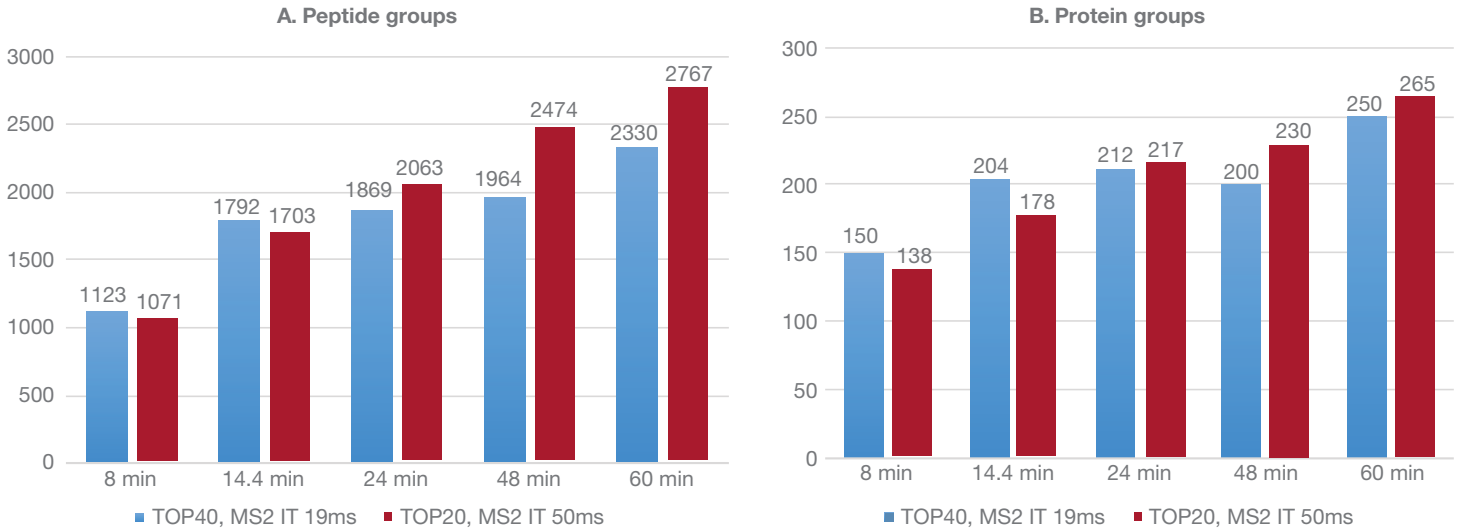

Figure 6. The number of peptide groups (A) and protein groups (B) identified with a 1% FDA in crude serum protein digests using the tailored high-throughput methods

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Conclusions

The requirement for high-throughput multi-analyte assays is of primary importance for clinical scientific research, including the rapidly emerging field of personalized medicine. Although immunoaffinity-based assays yield unsurpassed levels of sensitivity, specificity, and multiplexing capabilities, development costs and reproducibility of results continue to pose a significant challenge.

LC-MS analysis of body fluids poses has yet to fulfill its potential. Challenges include the high dynamic range of proteins, the presence of high-abundant proteins that cover >99% of total proteome, and the significant amount of hydrophobic small molecules, e.g., phospholipids, that can irreversibly absorb to the column stationary phase.

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 nanoLC-MS proteomics applications as well as high-throughput low-flow LC-MS analyses.

Here we introduced a set of novel high-throughput low-flow LC-MS methods for the research-based analysis of biological samples, including biofluids, which yield the following attributes.

- High MS utilization (>80%)
- Multiple analyte quantification capabilities
- Broad analyte concentration range spanning five orders of magnitude
- Compatibility with challenging sample matrices
- Negligible carryover

These methods afford a range of cycle times and analytical depth measurements tailored to suit a wide range of clinical research applications without the need to change any of the hardware or fluidics. Furthermore, they are fully compatible with the latest Orbitrap Exploris 480 and Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometers.

References

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