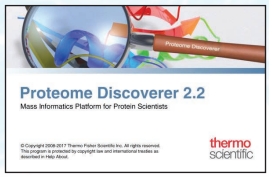
thermo scientific

APPLICATION NOTE 21744

Robust LC Separation

Optimized MS Acquisition

Comprehensive Data Informatics



A highly sensitive and robust 150 µm column to enable high-throughput proteomics

Authors

Xin Zhang,[1] Aaron Gajadhar,[2] Xuefei Sun,[1] Mike Baynham[3]

[1]Thermo Fisher Scientific, Sunnyvale, CA, USA

[2]Thermo Fisher Scientific, San Jose, CA, USA

[3]Thermo Fisher Scientific, Runcorn, UK

Keywords

Proteomics, stability, reproducibility, mass spectrometry, capillary LC, Data-Dependent Acquisition PLUS (DDA+), high-throughput proteomics, EASY-Spray, nanoLC, shotgun proteomics

Application benefits

- Easy, 'plug and spray' format for capillary LC
- Proven performance in retention time, back pressure, proteome coverage, and quantitative precision
- Robust, reliable column format; reproducible performance for >1000 injections of a complex sample with a consistent back pressure
- Maintains balance between sensitivity (>4500 proteins and ~30,000 peptides for HeLa digested sample) and robustness for large-scale studies

Goal

To demonstrate the robustness and reproducibility of the Thermo Scientific™ EASY-Spray™ PepMap™ C18 2 µm, 15 cm × 150 µm capillary column, which is designed for high-throughput and high-sensitivity proteomics

Introduction

Thermo Scientific™ EASY-Spray™ LC Columns provide a fully integrated and temperature-controlled column-emitter design with a single Thermo Scientific™ nanoViper™ connection between the LC and the MS ion source, which increases operational simplicity and provides accurate temperature control.

ThermoFisher SCIENTIFIC

The EASY-Spray PepMap C18 2 µm, 15 cm × 150 µm i.d. column enhances productivity through increased throughput capacity for current nanoLC users and increased sensitivity for analytical scale LC users. The increased loading capacity of this capillary column allows higher sample loading amounts to be analyzed while preserving the robustness and analytical stability necessary for demanding translational studies. The column is fully compatible with Thermo Scientific Capillary LC and Thermo Scientific mass spectrometry systems to offer a seamless integration of speed, sensitivity, and robustness required for high-throughput proteomics workflows.

The analysis was performed on the latest generation Thermo Scientific™ Q Exactive™ HF-X Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (MS), which provides the highest sensitivity and speed required for high-throughput proteomics. Data was collected using the new Data-Dependent Acquisition PLUS (DDA+) workflow, which takes the proven data-dependent acquisition method into an optimized complete workflow for the rigors of standardized label-free quantitation proteomics.[1]

The DDA+ label-free quantitation workflow on the Thermo Scientific™ Q Exactive™ HF-X Hybrid Quadrupole-Orbitrap™ MS is optimized for analytical robustness and reproducible precursor-based protein quantitation where the highest level of data precision is required. It builds on the proven, trusted approach of data-dependent MS acquisition and database-mediated identification utilized in peptide ID studies and applies the necessary analytical components to facilitate reproducible precursor-based protein quantitation. The DDA+ workflow leverages the capillary-flow LC system (capLC) and new EASY-Spray PepMap C18 2 µm, 15cm × 150 µm i.d. column in combination with the performance attributes of the latest generation Thermo Scientific MS to maximize robustness and up-time without sacrificing sensitivity. The DDA+ workflow and Q Exactive HF-X MS deliver more robust, highly reproducible and precise protein quantification for virtually all the proteins identified when combined the stable and sensitive EASY-Spray column. This precision becomes important when monitoring very subtle biological changes that require very high-fidelity quantitation.

Experimental

Consumables and apparatus

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity
- Thermo Scientific™ Pierce™ 0.1% Formic acid (v/v) in acetonitrile, LC-MS grade (P/N 85174)
- Thermo Scientific™ Pierce™ 0.1% Formic acid (v/v) in water, LC-MS grade (P/N 85171)

Standards

- Thermo Scientific™ Pierce™ HeLa protein digest standard (P/N 88328)
- Thermo Scientific™ Pierce™ Peptide retention time calibration mixture (P/N 88321)

LC method

The 15 cm × 150 µm EASY-Spray column was used in profiling of proteomics samples with the Thermo Scientific Capillary LC-MS platform, an optimal flow rate of 1.2 µL/min was shown to be ideal, with average back pressure at 200 bar (Table 1).[2] The flow rate was 3 µL/min when used for high-throughput, targeted analysis with a typical back pressure around 450 bar (Table 2).[2]

Table 1. Low flow gradient conditions

Low flow gradient 1.2 µL/min for HeLa digest	Mobile Phases A: Water with 0.1% formic acid B: Acetonitrile with 0.1% formic acid
Time (min)	%B
0	5
5	5
50	40
51	95
53	95
54	5
59	5

Table 2. High flow gradient conditions

High flow gradient 3 µL/min for PRTC	Mobile Phases A: Water with 0.1% formic acid B: Acetonitrile with 0.1% formic acid
Time (min)	%B
0	5
1	5
11	35
11.5	90
13	90
13.1	5
15	5

Instrumentation

A capillary-flow Thermo Scientific™ UltiMate™ 3000 RSLCnano system was set up for pre-concentration mode using a 300 µm × 5 mm trap column in back-flush configuration at 40 °C (P/N 6720.0315). An EASY-Spray 15 cm × 150 µm column (P/N ES806A) was connected to the system and coupled to the mass spectrometer with an EASY-Spray source (P/N ES081) operating at 40 °C.

MS detection conditions

MS analysis was performed on a Q Exactive HF-X mass spectrometer in Data-Dependent Acquisition+ mode (DDA+).

Source parameter

Spray voltage	2 kV
Capillary temperature	275 °C

Full MS parameters for HeLa analysis

Resolution	120,000
AGC target	3e6
Maximum IT	60 ms
Scan range	350–1400 *m/z*

MS/MS parameters for HeLa analysis

Resolution	7,500
AGC target	1e5
Maximum IT	11 ms
Normalized collision energy (NCE)	28
Isolation window	1.2 *m/z*
Dynamic Exclusion	20.0 s
Charge state screening	Enable to reject unassigned and single charged ion

Software

Spectral.raw files were analyzed using Thermo Scientific™ Proteome Discoverer™ software, version 2.2. Label free quantitation was conducted using Feature Mapper and Precursor Ions Quantifier nodes in the Consensus Step and Minora Feature Detector node in the Processing Step with default settings.

Software parameter

Precursor mass tolerance	10 ppm
Fragment mass tolerance	0.02 ppm
Static modification	Carbamidomethylation (+57.021 Da)
Dynamic modification	Oxidation (+15.995 Da) of methionine
Percolator data database	SwissProt complete human database
Criteria	1%

Results and discussion

Column-to-column reproducibility

Previous studies have shown the robustness of an EASY-Spray 15 cm × 150 µm column over an extended injection sequence with cytochrome C protein digestion separation. Over eight days of operation, an excellent retention time stability (Relative Standard Deviation (RSD) < 1%) was observed for 350 injections and a very stable peak area (RSD < 10%) for 150 consecutive injections (day 5 and 8) was obtained even without internal standard correction during long term testing.[2]

In this study, three columns were tested using standard Peptide Retention Time Calibration Mixture (PRTC) for column-to-column reproducibility. A flow rate of 3 µL/min with a 15 min LC gradient for high-throughput, targeted analysis method was applied in this evaluation (Table 2). The peak width at half maximum (PWHM) was approximately 3 s with a peak capacity of approximately 300. All peptides eluted out within 10 min.

Figure 1A shows the PRTC chromatograms from three separate 15 cm x 150 µm columns and Figure 1B shows the back pressure from the individual columns. Very consistent inter-column retention (RSD <3%) and back pressure (all back pressures within 3% RSD) were obtained with all three columns tested.

Figure 2 shows the intra-column (20 runs for each column) and inter-column retention time consistency. Excellent reproducibility was obtained with run-to-run (intra-column) average RSD of <0.5% compared to the inter-column retention RSD of <3%.

Figures 3 and 4 show the analysis of a HeLa digest used to represent a typical shotgun proteomics experiment of a complex sample. Data-dependent acquisition analysis were carried out in a 60 minutes total analysis time (Table 1) with 4 µg of HeLa digest sample on three separate columns. Figure 3 shows both the total ion count (TIC) chromatogram and base peak traces for the tested three columns. Very consistent MS profiles were observed and similar normalized signal level (NL) obtained (chromatograms scaled to 3.3–3.5 E10 for TIC and 6.5–8.5 E9 for base peak trace) with both chromatograms. The consistency was further quantified by label free quantitation (LFQ) protein and peptide identifications using Proteome Discoverer 2.2 software. Excellent column-to-column consistency was observed, including coverage of >4500 proteins and ~30,000 peptides with both RSDs <2% among different columns (Figure 4).

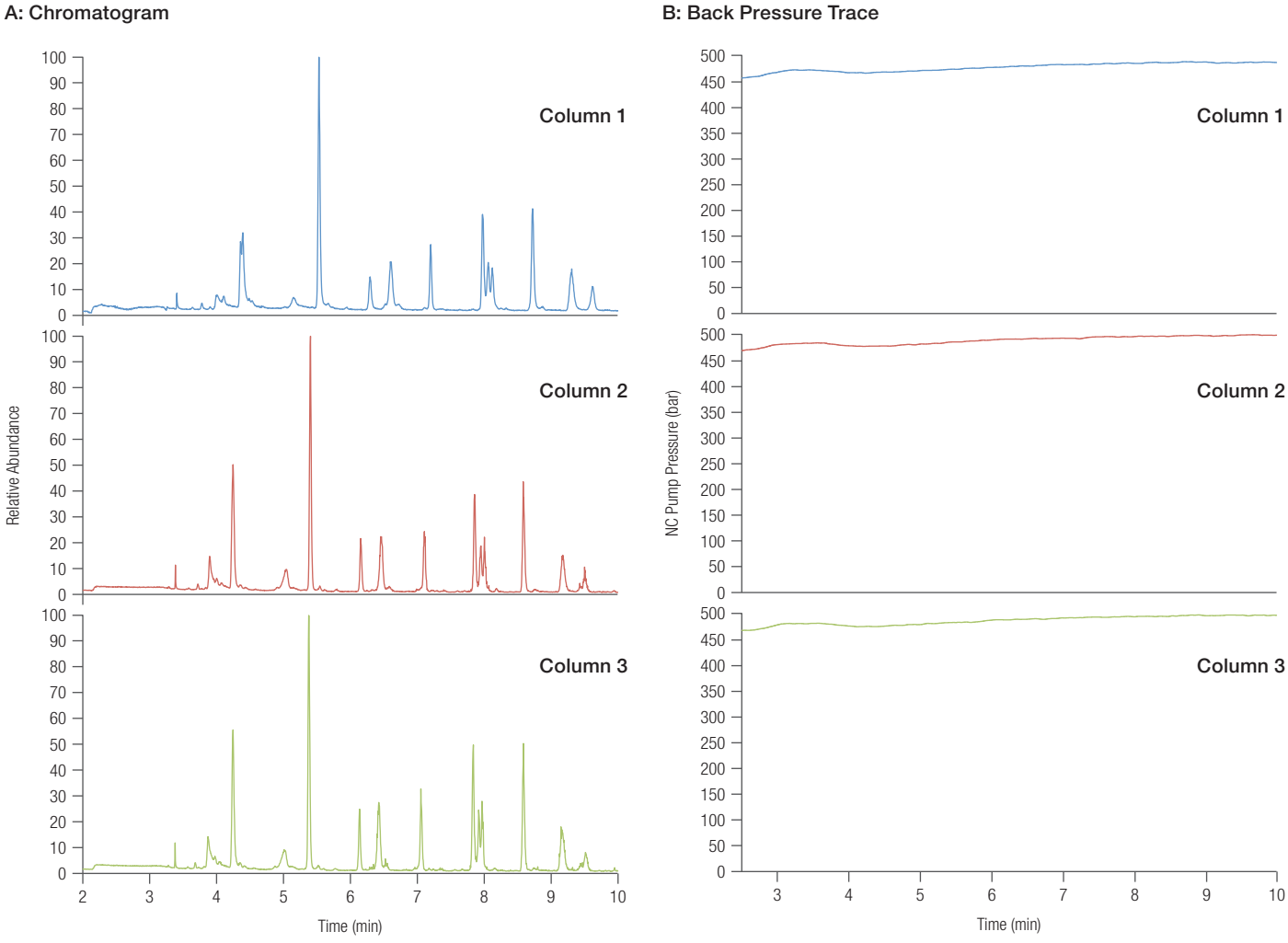


Figure 1. Representative chromatograms from three different columns indicating column-to-column retention and resolution reproducibility. (A) Chromatogram of Peptide Retention Time Calibration mixture with high flow gradient, (B) Back pressure traces recorded with different columns.

Retention time Consistency (n=20)

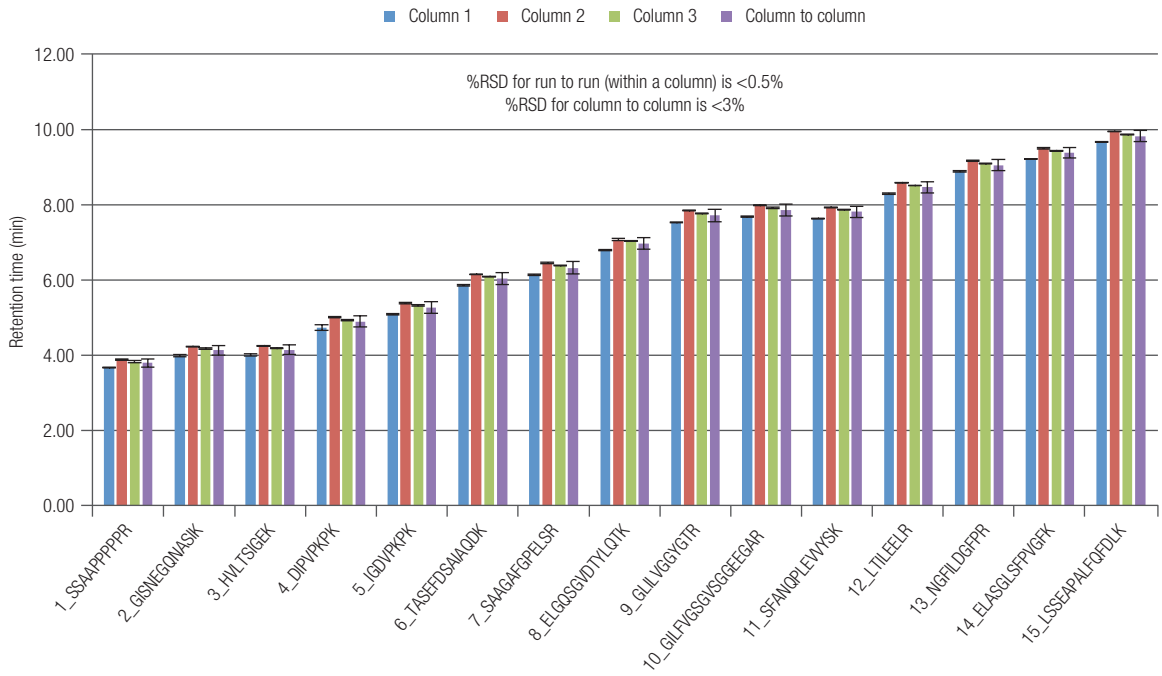


Figure 2. Intra and inter-column reproducibility of representative peptides from a PRTC standard, n = 20 injections

A. TIC MS chromatogram

B. Base peak traces

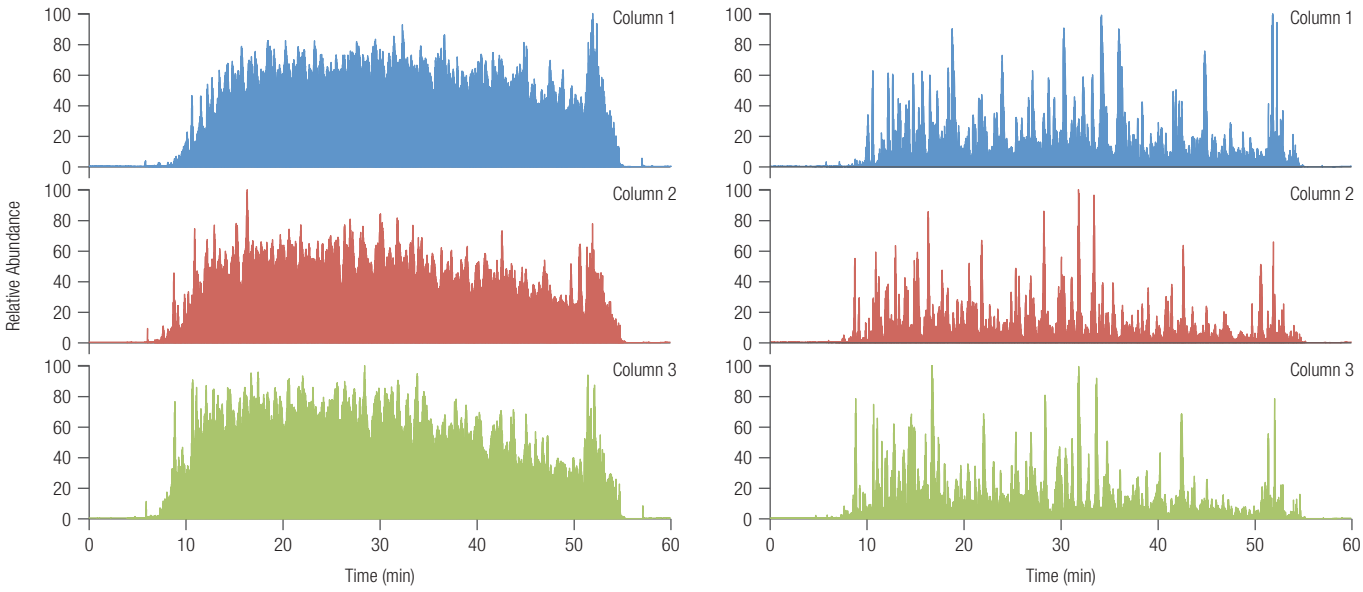


Figure 3. EASY-Spray Capillary column-to-column chromatogram reproducibility. (A) TIC MS chromatograms for three different columns, (B). Base peak traces for three columns.

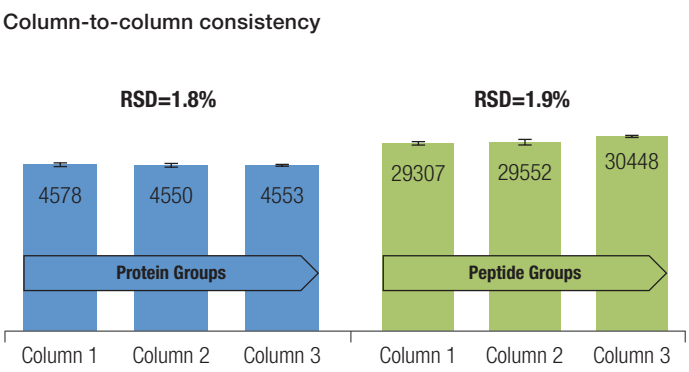


Figure 4. Unique and consistent peptide and protein group identifications for 3 columns

Column lifetime – column robustness test

Column lifetime is an important issue when developing an LC method. It can be affected by sample complexity, mobile phase composition, operating temperature, column conditioning, cleaning, and storage. In proteomics research, samples are usually very complex or crude with barely any sample pretreatment and can be a major challenge for low flow column lifetime. In addition, translational proteomics initiatives and large-scale sample cohort profiling requires durable, reliable column performance to maximize uptime and analytical consistency.

The column lifetime test was carried out using a proteomics profiling method with an optimal flow rate of 1.2 µL/min, 60 minutes LC gradient (Table 1) and PRTC-spiked HeLa digest sample. Three PRTC peptides (early, middle, and late eluting) (5_IGDYAGI**K**, 9_GLILVGGYGT**R** and 14_ELASGLSFPVGF**K**) were extracted from HeLa injections #002, 102, 202, 302 and 402 (Figure 5). Consistent retention times were observed with <3% RSDs indicating the robustness of the capillary EASY-Spray column. Table 3 summarizes the results achieved to demonstrate the longevity and robustness of the EASY-Spray 15 cm × 150 µm column.

Column lifetime performance was further evaluated in a simulated large-cohort analysis following 1000 injections of HeLa digest sample using the DDA+ label-free quantitation workflow. The long-term quantitative performance was not compromised either as comparable proteome coverage or as quantitative precision observed throughout the 1000 injection experiment (Figure 6). Both %RSDs for the overall and quantitation at RSD <20% protein groups are <5%, indicating the robustness of the capillary EASY-Spray column. This dependable, robust performance is essential for large-scale proteomics initiatives where hundreds of injections at a time are commonplace.

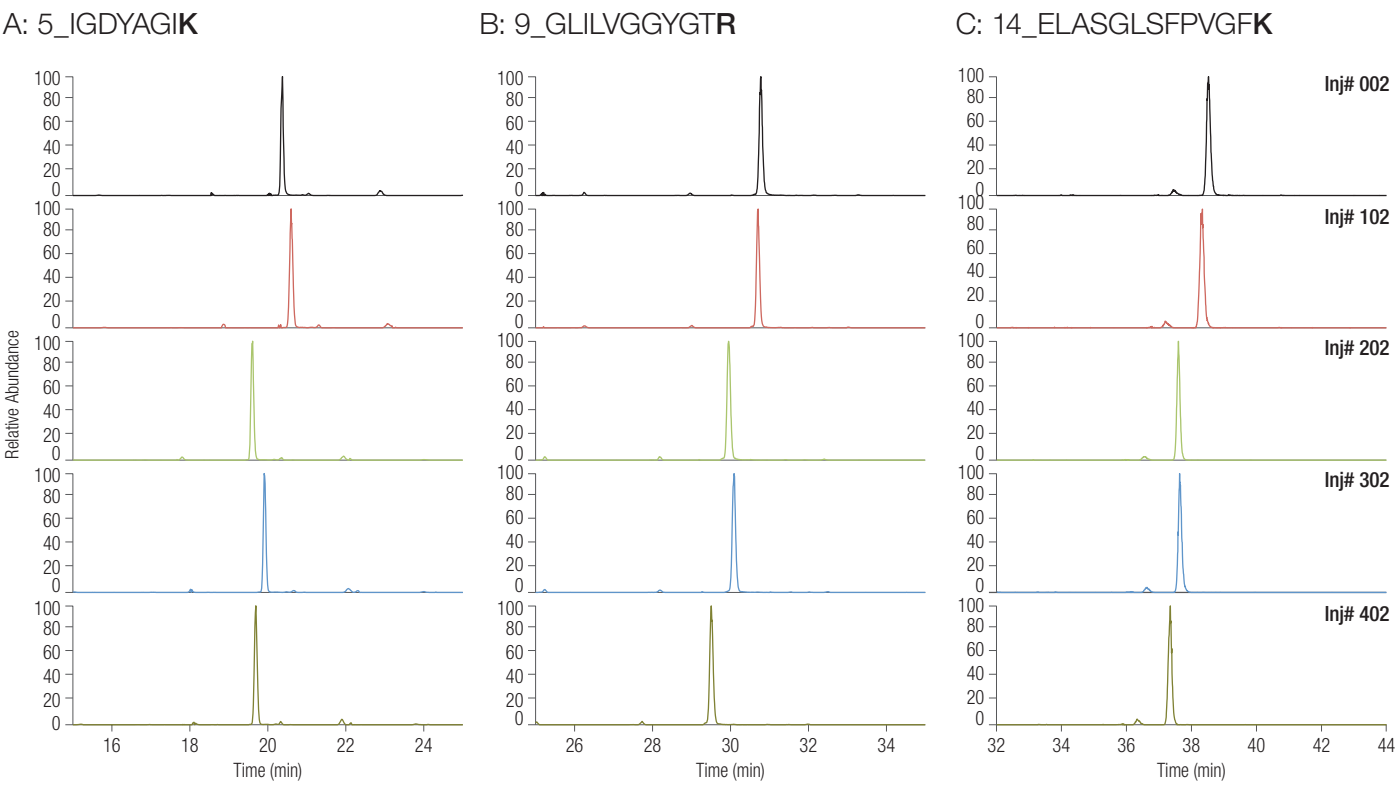


Figure 5. PRTC peptide extracted chromatograms from HeLa digest injections #002, #102, #202, #302, and #402

Table 3. Retention time reproducibility: extracted PRTC peptides from PRTC-spiked HeLa digest sample

Injection #\ Retention time	5_IGDYAGI**K**	9_GLILVGGYGT**R**	14_ELASGLSFPVGF**K**
002 (New column)	20.82	30.78	38.77
102 (after 100 inj)	20.60	30.71	38.33
202 (after 200 inj)	19.61	29.95	37.60
302 (after 300 inj)	19.91	30.10	37.63
402 (after 400 inj)	19.69	29.51	37.35
AVG	20.13	30.21	37.94
%RSD	2.73	1.77	1.56

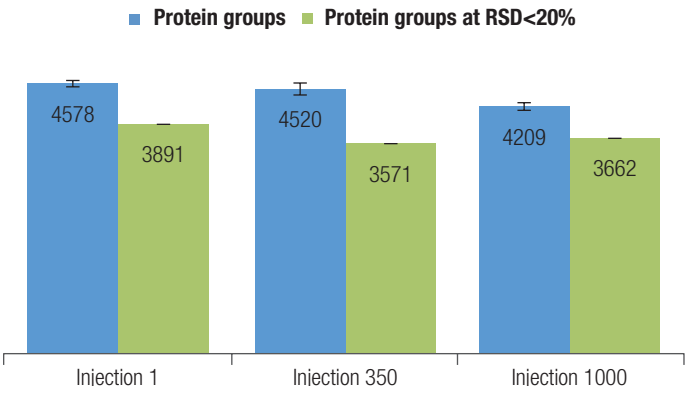


Figure 6. Durable long-term quantitative performance over 1000 injections: protein groups at Inj #1, #350, and #1000

Conclusions

A new configuration of the EASY-Spray column in 15 cm x 150 µm format was developed with all the advantages of the "plug and spray" approach, but with an emphasis on throughput and robustness for cap-flow applications. Great column-to-column reproducibility, sensitivity, and robustness (up to 1000 Hela digest injections) ensure results consistency necessary for successful large-scale proteomics studies. With this new configuration, both high-flow (flow rate up to 3 µL/min) and high-throughput (running time as short as 15 min) analytical performance are made possible for proteomics. When combined with the highly sensitive Q Exactive HF-X Hybrid Quadrupole-Orbitrap mass spectrometer and new DDA+ quantitative workflow, the EASY-Spray 15 cm × 150 µm column provides a reliable, robust chromatography solution to empower quantitative, standardized, high-throughput proteomic needs.

References

1. Thermo Fisher Scientific, **2017**, Pushing the leading edge in protein quantitation: Integrated, precise, and reproducible proteomic workflows
2. Thermo Fisher Scientific, **2017**, Increased sample throughput with a capillary EASY-Spray column

Find out more at **thermofisher.com/EASYSpray**

AN21744EN 0419S

ThermoFisher SCIENTIFIC