thermo scientific

TECHNICAL NOTE 72602

Capillary-flow LC-UV sample quality control for low-flow LC-MS based proteomics

Authors

Christopher Pynn,[1] Martin Samonig,[1] Gabriela Krssakova,[2,3] Karl Mechtler,[2,3] Wim Decrop,[1] Remco Swart[1]

[1]Thermo Fisher Scientific, Germering, Germany

[2]Research Institute of Molecular Pathology (IMP), Protein Chemistry, Vienna, Austria

[3]Institute of Molecular Biotechnology (IMBA), Protein Chemistry, Vienna Biocenter (VBC), Vienna, Austria

Keywords

Capillary-flow LC-UV, quality control (QC), robustness, throughput, monolithic columns, LC-MS based proteomics

Goal

To develop a robust, high-throughput method for proteomics sample quality assessment prior to low-flow LC-MS.

Introduction

Low-flow LC-MS-based technologies are now an essential part of the proteomics analytical toolkit. Advancements in sensitivity, robustness, separation power, mass analyzer accuracy, and increased data acquisition speed make them almost indispensable in modern-day proteomics research. Furthermore, the improved ease-of-use and access to instrumentation, e.g. through interactions with core facilities, have made such platforms attractive to non-specialists.

State-of-the art LC-MS systems are highly sensitive, not only to the peptides or proteins of interest, but also to sample matrix components (e.g. salts, lipids, and DNA) along with a number of the reagents required in the sample preparation steps. Low-flow chromatography systems can automate some aspects of the sample clean-up process, for example through online desalting (trap-and-elute setup). However, other reagents commonly involved in sample processing, such as detergents, can cause analyte signal suppression and irreversible damage to separation columns and may take days to remove from the system. Similarly, attempted analyses of incomplete sample digests for bottom-up proteomics experiments can lead to column blockage and costly unplanned system downtime.

ThermoFisher SCIENTIFIC

To avoid such issues, particularly for facilities offering open access LC-MS proteomic analyses, a simple sample quality screening test is recommended prior to low-flow (nano)LC-MS. Such methods need to be easy to set up, robust, sensitive, high-throughput, and resistant to the common interferences associated with inadequate sample preparation. Furthermore, they should be impervious to reagents commonly used in proteomics sample workups, which are nevertheless incompatible with low-flow LC-MS. Capillary-flow applications have gained significant traction in recent years, due to their ability to unite the demands of higher sensitivity with increased throughput while delivering robustness equivalent to that of analytical-flow LC-MS.[1] Here we describe a robust sample QC method for low-flow LC-MS suitability screening based on a pre-concentration capillary-flow LC-UV setup using Thermo Scientific™ PepSwift™ monolithic columns. The method was used to monitor the digestion time course of a commercially available antibody. LC-UV-MS analysis was carried out to characterize peptide components observed exclusively at either early or late sample digestion time points. Example data acquired by a core facility are also presented, which highlight the power of the method for detecting detergent contamination that would be detrimental to nanoLC-MS analysis.

Experimental

Consumables

- Fisher Scientific™ LC-MS grade water (P/N W6-212)
- Fisher Scientific™ LC-MS grade acetonitrile (P/N 10616653)
- Thermo Scientific™ Pierce™ trifluoroacetic acid (TFA), LC-MS grade, (P/N 85183)
- Thermo Scientific™ Pierce™ heptafluorobutyric acid (HFBA) (P/N 11811385)
- Thermo Scientific™ SMART Digest™ Trypsin Kits (P/N 60109-101)
- Thermo Scientific™ UltiMate™ 3000 RSLCnano Pre-concentration monolithic LC kit (P/N 6720.0320) composed of the elements in Table 1.

Sample preparation

A commercially available monoclonal antibody infliximab drug product (Hospira® UK Limited, Leamington Spa, United Kingdom) was supplied at a concentration of 10 mg/mL in a formulation buffer containing 0.05 mg/mL polysorbate 80, 50 mg/mL sucrose, 0.22 mg/mL monobasic sodium phosphate monohydrate, 0.61 mg/mL dibasic sodium phosphate dihydrate, and sterile water adjusted to pH 7.2 using sodium hydroxide or hydrochloric acid.

Table 1. UltiMate 3000 RSLCnano pre-concentration monolithic LC kit contents (P/N 6720.0320). The letter and number assignments are given in Figure 1.

#	Item	P/N
a	PepSwift Monolithic Capillary Column, 200 µm I.D. × 5 cm (PS-DVB), Thermo Scientific™ nanoViper™	164557
b	PepSwift Monolithic Trap Column, 200 µm × 5 mm (PS-DVB), set of 2, nanoViper	164558
1	nanoViper capillary FS/PEEK sheathed 1/32" I.D. × L 50 µm × 350 mm	6041.5540
2	nanoViper capillary FS/PEEK sheathed 1/32" I.D. × L 75 µm × 650 mm	6041.5775
3	nanoViper capillary FS/PEEK sheathed 1/32" I.D. × L 75 µm × 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
	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

Note: Consumables are from Thermo Fisher Scientific unless stated otherwise.

The 50 µL infliximab sample, adjusted to 2 mg/mL with water, was diluted 1:4 (v/v) with the SMART Digest buffer provided with the kit. It was then transferred to a reaction tube containing 15 µL of the SMART digest resin slurry, corresponding to 14 µg of heat-stabile, immobilized trypsin. A time-course experiment was performed and tryptic digestion was allowed to proceed at 70 °C for 5, 10, 20, and 45 min at 1400 rpm. After the digestion, the reaction tube was centrifuged at 7000 rpm for 2 min, the supernatant was transferred to a new tube, and the centrifugation step was repeated.

Samples were diluted in loading solvent (water + 0.05% HFBA) to a final concentration of 25 ng/µL.

LC-UV configuration and separation conditions

Measurements were carried out using a capillary flow UltiMate 3000 RSLCnano system (5200.0356) consisting of:

- SRD-3400 (P/N 5035.9245)
- NCS-3500RS CAP (5041.0020) equipped with a classic flow meter (P/N 6041.7902A) and capillary-LC flow selector (P/N 6041.0003)
- Low dispersion 2 position, 10-port valve (P/N 6041.0001)
- WPS-3000TPL RS (P/N 5826.0020)
- RSLCnano preconcentration capillary kit (P/N 6720.0315)
- VWD-3400RS (P/N 5074.0010)
- UZ-View™ flow cell, 3 nL (P/N 6074.0270)

The system was configured using the 200 µm monolithic pre-concentration column kit (P/N 6720.0320) as described in the UltiMate 3000 RSLCnano standard applications guide (Document No. 4820.4103, Figure 1 and Table 1).

Fluidic Setup

Reagents and analysis conditions were used as described in Table 2.

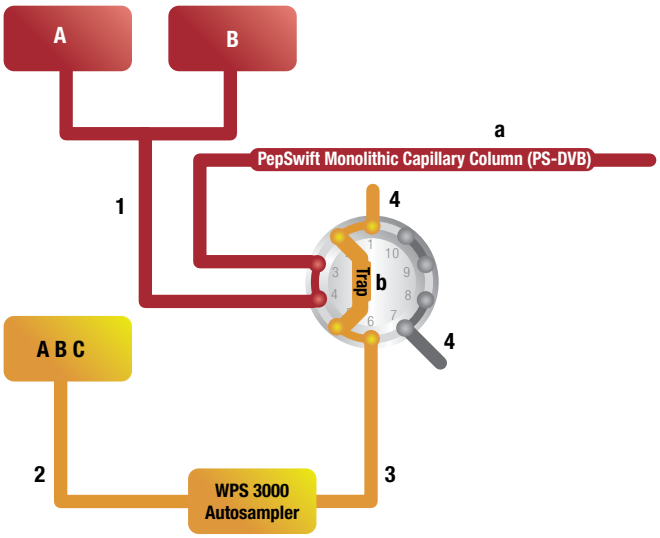

Figure 1. Fluidic connections for pre-concentration of sample onto a monolithic column. Note: The number and letter descriptions for each of the fluidic components are given in Table 1.

Table 2. LC Reagents and conditions for sample QC screening. TFA = Trifluoroacetic acid, ACN = Acetonitrile, HFBA = Heptafluorobutyric acid.

Property	Setting
Mobile phase A:	Water + 0.05% TFA
Mobile phase B:	Water/ACN (50/50 v/v) + 0.04% TFA
Loading solvent:	Water + 0.05% HFBA
Sample:	25 ng/µL, Note: *The sample must be diluted in the loading solvent.*
Injection volume:	1 µL (injection mode: microliter pickup)
UV detection:	214 nm
Loading time:	3 min (may vary with different injection volume/routine)
Gradient	1% to 70% B in 8 min, 90% B for 2 min, 8.5 min equilibration at 1% B
Oven temp.:	60 °C
Sample temp.:	5 °C
Loading flow rate:	10 µL/min
Gradient flow rate:	3.0 µL/min (capillary flow selector)
Switching program for low-dispersion valve:	
	Time (min) — *Valve positions*
	0 — 1-2
	3 — 10-1
	17 — 1-2

LC-UV-MS for characterization of sample digest fragments

For LC-UV-MS experiments, the UltiMate 3000 RSLCnano system was connected to a Thermo Scientific™ Q Exactive™ HF mass spectrometer using a Thermo Scientific™ EASY-Spray™ ion source (P/N ES081) and EASY-Spray transfer line (P/N ES791). The connection between the outlet of the UV cell and the EASY-Spray transfer line was realized using a MicroTight® union (P/N 00109-02-00055) as described on page 14 of "The Complete and Easy guide to configuring your Thermo Scientific™ Nano LC for Mass Spectrometric Analysis" (*Doc No. PP-64583-LC-MS-NanoLC-Complete-Easy-Guide-PP64583-EN.pdf*).

MS conditions

Table 3. MS source and data acquisition settings.

MS Conditions	
Source:	EASY-Spray source
Capillary temp.:	280 °C
S-lens voltage	60 V
Source voltage	1.75 kV
Full MS mass range	250–2000 *m/z*
Full MS parameters	
Resolution settings:	240,000
Target value:	3×10^6
Max injection time:	200 ms
Microscans:	1
SID:	10 eV

Data acquisition and processing

LC-UV data were acquired using Thermo Scientific™ Chromeleon™ 7.2 SR5 Chromatography Data System software.

LC-UV-MS data were acquired by running parallel sequences using Thermo Scientific™ Xcalibur™ software version 4.0 to obtain the MS data and Chromeleon 7.2 SR5 to run the LC and acquire the UV data.

Note: Full LC-UV-MS system control and data acquisition is also readily available through Xcalibur software using the Thermo Scientific Standard Instrument Integration (SII) for Xcalibur (≥1.2) to interface the LC with the MS.

Peptide component identification was done using Thermo Scientific™ BioPharma Finder™ software, version 3.0.

Results and discussion

Cap LC-UV for the evaluation of sample digests

The efficacy of a capillary-flow LC-UV method for sample screening was assessed using a digestion time-course for the commercially available monoclonal antibody infliximab. For the purposes of this particular experiment, an MS system was connected to the LC-UV setup. This was done for the purpose of demonstration, so that any missed cleavages, as would be expected from very short digests, could be characterized.

Infliximab, digested for only 5 minutes, was found to contain large protein fragments (Figure 2a, red UV trace, and Figure 2b). In contrast, analysis of the 45 minute incubation revealed single peptides that were not detectable in the 5 minute sample digest (Figure 2a, blue UV trace, and Figure 2c).

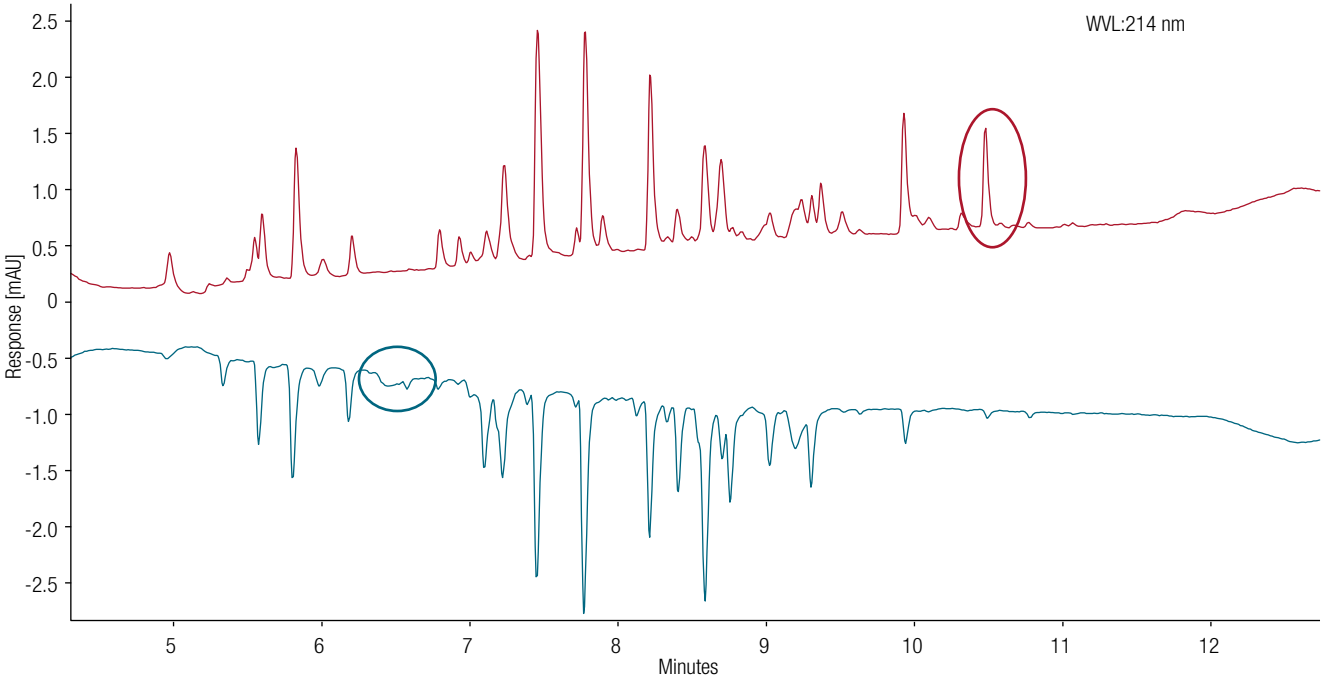

Figure 2a. Mirrored chromatograms for the UV trace at 214 nm comparing a 5 minute (red) partial digest with a 45 min (blue) complete digest. An example protein fragment resulting from the partially digested sample is circled in red. A peptide component only detectable after sufficient digestion time has elapsed is circled in blue. The identification of the respective components is given below in Figures 2b and 2c.

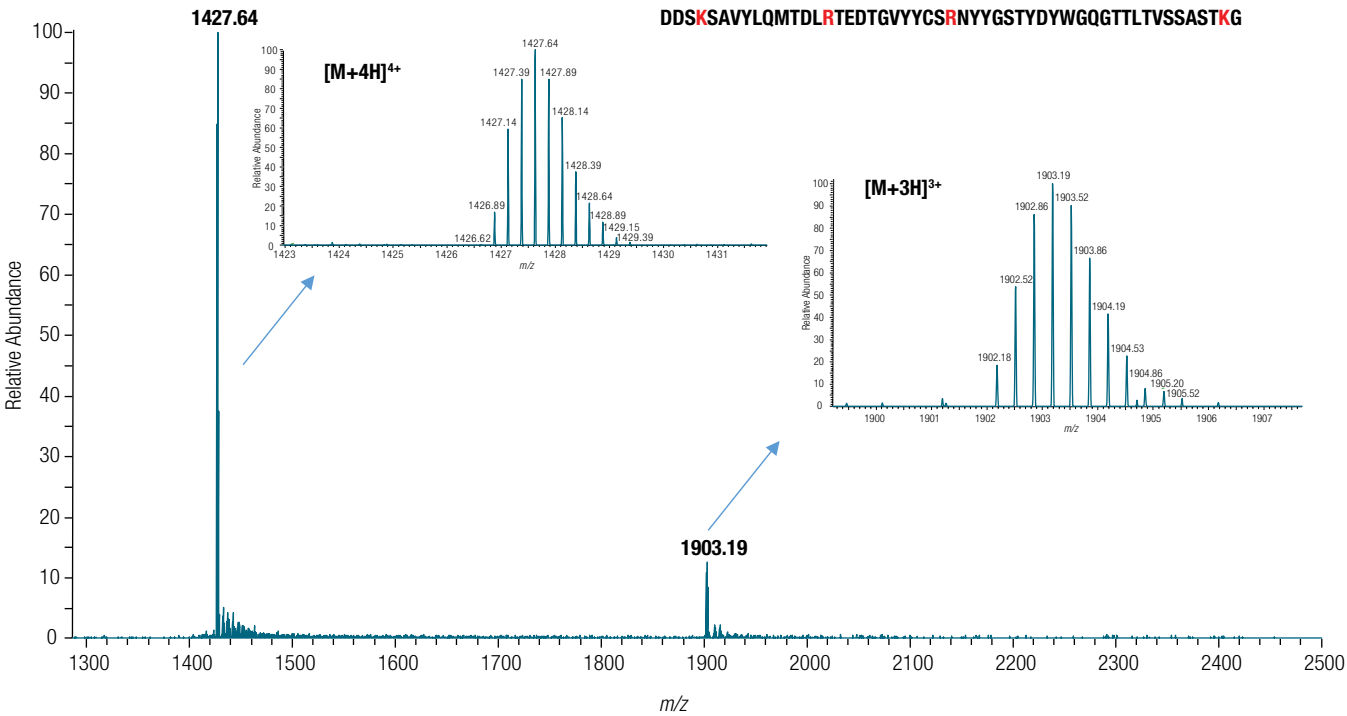

Figure 2b. Example mass spectrum of the red component highlighted in Figure 2a reveals a 5,7 kDa polypeptide with four missed cleavage sites here present as $[M+4H]^{4+}$ and $[M+3H]^{3+}$ species. Missed cleavage sites are denoted in red.

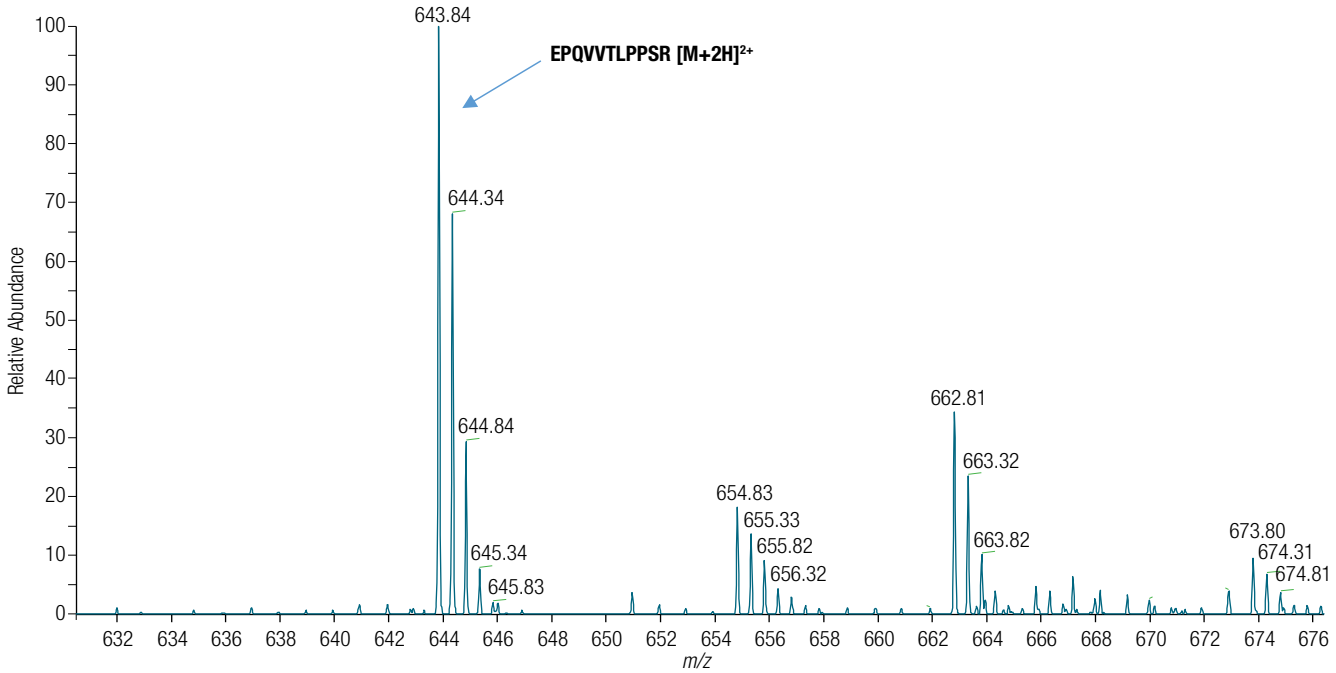

Figure 2c. Example mass spectrum of the blue component highlighted in Figure 2a reveals a doubly charged peptide at *m/z* 643 [M+2H]$^{2+}$.

Comparison of column backpressure for samples from each of the digestion time points revealed no significant differences between the partial and complete digests (Figure 3). Even the analysis of the fully intact antibody resulted in only a slight pressure increase between 9 and 12 minutes (Figure 3, turquiose pressure trace). While a fully undigested sample would be detrimental to a nano-LC column, (by causing a blockage), the resilience of the monolithic column to pressure change, even when exposed to large undigested proteins such as antibodies, highlights the suitability and robustness of the method for dealing with incomplete sample digests.

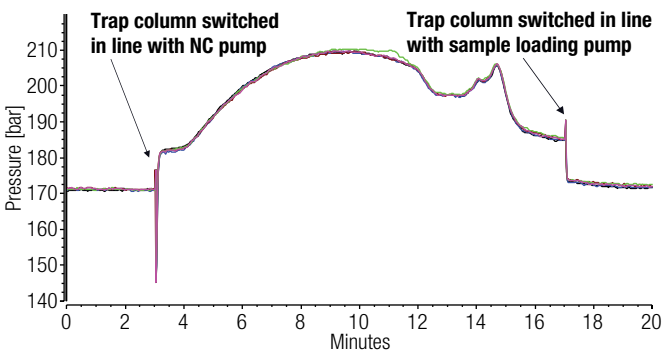

Figure 3. An overlay of five NC pump backpressure profiles corresponding to the analysis of 5 min (brown), 10 min (pink), 20 min (dark blue), and 45 min (black) antibody digests. The pressure profile for the analysis of the intact antibody is also shown for reference (turquoise).

Screening for detergent contamination

Protein preparation for MS analysis is accomplished by a variety of methods that are selected according to the source of the proteins in question, for example cells, tissue, or plasma, and the analytical question to be answered, for example protein-protein interaction or identification and quantification. Accessing the proteins of interest commonly require lysis and extraction steps that utilize detergents. The majority of detergents are incompatible with both reversed-phase chromatography separations and electrospray mass spectrometers and can potentially damage instruments and irreversibly impair columns.[3]

Detergent contamination detected by LC-UV screening on a monolith column (Figure 4) reveals a protein digest that is not suitable for low-flow LC-MS analysis in its current state due to excessive detergent content. In this case, the detergent must first be removal prior to LC-MS analysis (for example using Thermo Scientific™ Pierce™ detergent removal spin columns P/N 87777).

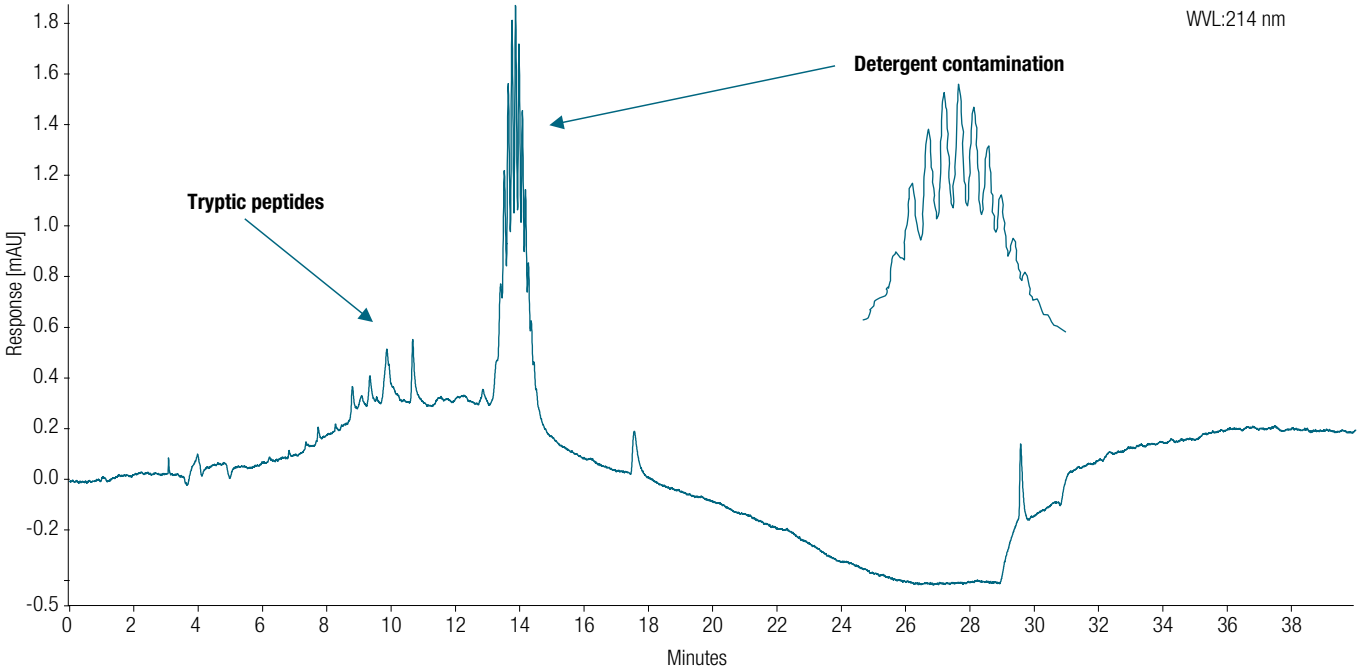

Figure 4. UV chromatogram of a BSA digest heavily contaminated with detergent (Triton™).

Conclusions

Proteomics-based LC-MS platforms capable of unparalleled sensitivity are all the more susceptible to both sample and matrix components as well as to reagents required for sample extraction and work up. For laboratories offering open access analytics (e.g. core facilities) to remain viable, the need for robust high-throughput sample screening to eradicate unscheduled system downtime is clear.

Capillary-flow LC-UV analyses using PepSwift monolithic capillary columns combine the advantages of low-flow LC sensitivity with the robustness of polymer-based monolithic phase substrates, which confer both high pH stability and the ability to separate a broad range of analytes in a single run. Furthermore, their unique open channel design along with their lack of frit requirements means that they are less prone to blockages. Such instrumental setups are ideal for pre-screening of proteomics samples to guard against unnecessary and expensive nanoLC-MS system downtime caused by incomplete or inadequate proteomics sample workup.

For detailed information on other Thermo Fisher Scientific reversed-phase capillary-flow LC solutions, please refer to Reference 4.

References

1. Meding, S.; Boychenko, A. Capillary Flow LC-MS Unites Sensitivity and Throughput, *Chromatography Today* **2016,** June, *03,* 43–45.
2. UltiMate 3000 RSLCnano System – Versatility and Performance – The Ultimate Solution for All Separation Workflows, BR-71898 (2016).
3. Zhou, J-Y.; Dann, G.P.; Shi,T.; Wang,L.; Gaio, X.; Su,D.; Nicora, C.D.; Shukla, A.K.; Moore, R.J.; Liu, T.; et al. Simple sodium dodecyl sulfate-assisted sample preparation method for LC-MS-based proteomics applications. *Anal.Chem.* **2012,** *84,* 2862–2867.
4. Boychenko, A.; Meding, S.; Decrop, W.; Ruehl, M.; Swart, R. Capillary-flow LC-MS: combining high sensitivity, robustness and throughput, TN-72277-LC-MS-Capillary-Flow-TN72277-EN (2017).

For Research Use Only. Not for use in diagnostic procedures.

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

ThermoFisher SCIENTIFIC