

HPLC columns

Thermo Scientific μ PAC HPLC Column robustness in bottom-up proteomics

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Keywords

Microfluidics, bottom-up proteomics, tryptic digest, nano LC, column lifetime, sample contaminants

Goal

Demonstrate micro pillar array column (μ PAC) life time for bottom-up proteomics as well as resilience towards challenging samples often encountered in this field.

Introduction

Being one of the main workhorses in today's analytical research, the output of many laboratories is dominated by the amount of time that liquid chromatography mass spectrometry (LC-MS) systems are running smoothly. With a lot of determining factors, it is often quite a challenge to keep these systems up and running. Next to technical issues that can occur with the LC-MS system's hard- and software, LC column failure is one of the most frequent causes of LC-MS system down time. Typical observations of LC column failure are an increase in column backpressure, deviating peak shapes or gradual shifts in retention time.

An alternative to classical packed-bed LC columns are the Thermo Scientific™ μ PAC™ HPLC Columns, which are distinguished by several features. The inherent high permeability and low 'on-column' dispersion obtained by the perfect order of the separation bed makes μ PAC (micro pillar array columns)-based chromatography unique. The peak dispersion originating from heterogeneous flow paths in the separation bed is eliminated (no A-term contributions) and therefore components remain much more concentrated (sharp peaks) during separation.¹ The freestanding nature of the pillars also leads to much lower backpressure allowing the use of very long columns.² The result is a top performing nano LC column that is very robust and is much less prone to sample related column failure.

To demonstrate this, a single μ PAC column was operated under standard bottom-up proteomics conditions over a period of 6 months. Sequential injection of a HeLa cell tryptic digest, a blank and a Cytochrome C tryptic digest sample was performed to evaluate column robustness. In addition, several sample sets that are perceived as challenging have been injected to demonstrate the column's resilience to sample related column failure.

Column lifetime

UV chromatograms obtained for the separation of 100 ng HeLa cell digest sample on a 200 cm μ PAC column are shown in Figure 1. In this Figure, HeLa cell digest injection 1 to 1000 have been displayed at an interval of 100 injections. In between each HeLa cell digest injection, a blank sample and a Cytochrome C

digest sample have been injected to monitor the chromatographic performance over the entire experiment. The UV chromatograms obtained for the separation of Cytochrome C digest are shown in Figure 2. The numbers below the chromatograms indicate the 6 peptide peaks that have been used to evaluate column stability and performance.

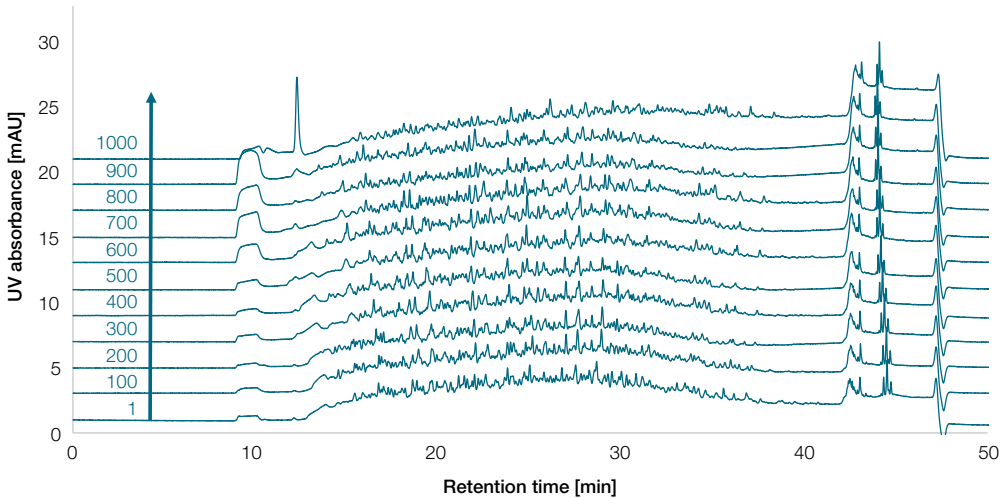


Figure 1. UV chromatograms obtained for the separation of 100 ng tryptic HeLa cell digest. Injection 1 to 1000 are displayed at an interval of 100 injections.

Injection volume: 1 μ L
Flow rate: 1 μ L/min
Gradient conditions: 1-50% B in 30 min
Mobile phase composition: A – H₂O + 0.1% TFA, B – 80% Acetonitrile + 0.1% TFA
Column temperature: 35 °C
Detection: UV 214 nm

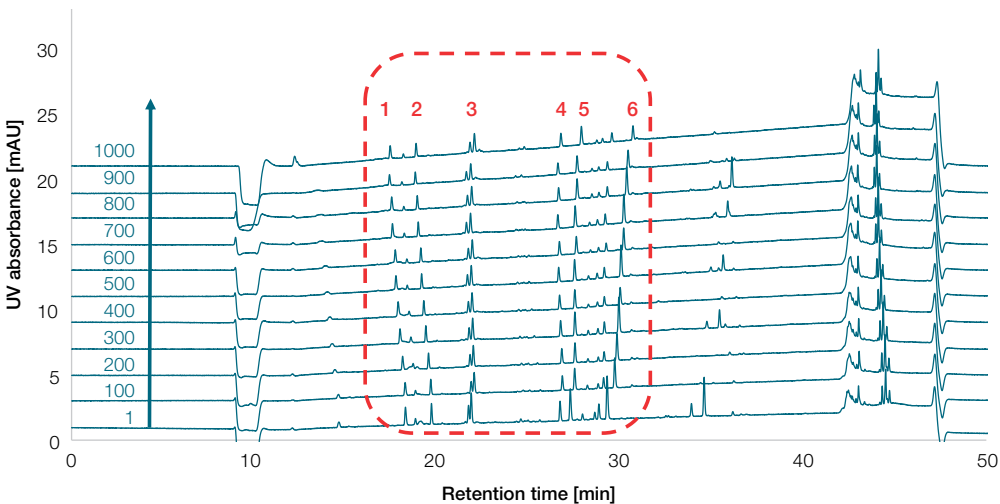


Figure 2. UV chromatograms obtained for the separation of 0.5 pmol Cytochrome C tryptic digest. Injection 1 to 1000 are displayed at an interval of 100 injections. Peptides that are used to evaluate column stability and performance are indicated by the numbers in orange.

Injection volume: 1 μ L
Flow rate: 1 μ L/min
Gradient conditions: 1-50% B in 30 min
Mobile phase composition: A – H₂O + 0.1% TFA, B – 80% Acetonitrile + 0.1% TFA
Column temperature: 35 °C
Detection: UV 214 nm

The retention time for each of these peptide peaks has been plotted as a function of the total number of HeLa cell digest injections in Figure 3a. Accompanying peak widths, peak asymmetry values and column pressures have been plotted

in Figure 3b, c, and d respectively. Averaged values and their corresponding coefficients of variance have been summarized in Table 1.

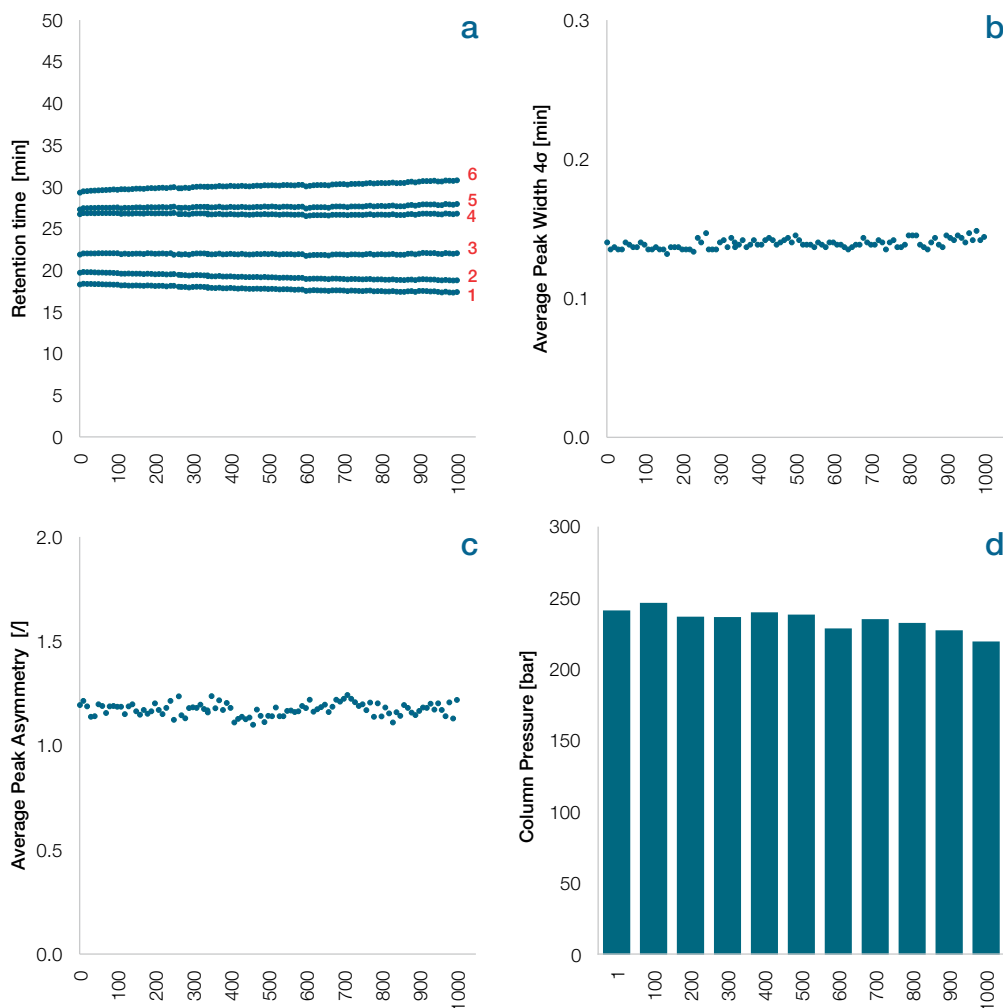


Figure 3. a) Retention time of 6 reference peptides from the Cytochrome C tryptic digest plotted as a function of the total number of HeLa cell digest injections. b) The average peak width obtained for all 6 reference peptides plotted as a function of the total number of HeLa cell digest injections. c) The average peak asymmetry obtained for all 6 reference peptides plotted as a function of the total number of HeLa cell digest injections. d) The average column pressure plotted as a function of the total number of HeLa cell digest injections.

Table 1. Chromatographic metrics obtained for Cytochrome C reference peptides.

	Value	Standard deviation	% CV
Retention Time Peptide 1 [min]	17.76	0.30	1.72
Retention Time Peptide 2 [min]	19.17	0.31	1.59
Retention Time Peptide 3 [min]	21.91	0.07	0.32
Retention Time Peptide 4 [min]	26.70	0.08	0.31
Retention Time Peptide 5 [min]	27.59	0.13	0.48
Retention Time Peptide 6 [min]	30.12	0.35	1.15
Average Peak Width [min]	0.14	0.01	10.20
Average Peak Asymmetry [I]	1.16	0.12	10.24
Average Column Pressure [bar]	235	7	3

With an overall retention time variation below 2% CV, excellent retention time stability is clearly observed over the entire 6 months of column operation. During this period, no significant effect on peak width nor peak asymmetry have been observed, again confirming the excellent column stability and performance. The column pressure was also found to be very stable over the entire period of operation, with an average of 235 bar (3400 psi) for a flow rate of 1 μ L/min.

Blank and reference sample injections included, these 6 months of continuous operation equal a total of 3526 injections, and a total of 195.36 mL or 21707 column volumes that have been flushed through the column.

Table 2. Summary.

	Value
Total operation time	6 months
Number of HeLa digest injections	1000
Total number of injections	3526
Total volume through column	195.36
Total amount of column volumes	21707

Sample related column failure

Standard sample preparation steps that are required in bottom-up proteomics experiments are: extraction of proteins from tissue or cells, fractionation to remove contaminants and proteins that are not of interest, enzymatic digestion of proteins into peptides and post-digestion separation to increase sample homogeneity. At this point, the sample is ready for separation and subsequent analysis by LC and MS respectively.³ Even though several separation or clean-up steps have been carried out before the analytical separation, it is not unlikely that some contaminants or potential hazards for the analytical column persist in the sample. A common source of contaminants are the reagents which are used for cell lysis or detergents used to isolate certain protein fractions. If present in substantial concentrations, these detergents can suppress the MS signal of the analytes of interest and cause interference with reversed-phase separation, sometimes even damaging instruments and irreversibly ruining columns.⁴ LC column damage has also been observed when injecting samples where a certain degree of aggregation or precipitation is observed. This generally results in a clogged LC column, preventing further use and urging column replacement.

3 different sets of “challenging” bottom-up proteomics samples were used to evaluate the resilience of the μ PAC column towards sample related failure. When previously injecting these samples onto a classical packed-bed nano LC column, column failure was observed and the analytical column had to be replaced. Each set of samples consists of 6 samples which have been injected in duplicate. In between sample injections, a blank injection and injection of a reference standard in duplicate has been carried out to evaluate μ PAC column performance and stability. The goal of this technical note is to demonstrate the ability of the μ PAC column to withstand or survive a series of “dirty” sample injections, it is however not excluded that the nature of these samples would interfere with proper MS functioning. Therefore UV detection at 214 nm wavelength was used for all experiments.

The first set of samples was obtained from a human cell line where solubilization and enrichment of integral and membrane-associated proteins was performed using Triton X-114. Even though several detergent depletion steps were performed prior to injection onto the analytical column, substantial amounts of detergent were still present. Apart from interfering with MS detection, packed bed LC column properties were affected urging for column replacement. UV chromatograms obtained for the separation of these samples on a 200 cm μ PAC column are shown in Figure 4a. UV chromatograms obtained for the separation of a Cytochrome C digest before and after each challenging sample are shown in Figure 4b.

For the second set of samples, another sample with detergent contamination was used. In this case, NP-40 was used for cell lysis of plant material. The sample set consisted of two controls, where NP-40 had not been removed, and four samples where NP-40 had been removed using Thermo Scientific™ HiPPR™ Detergent Removal Spin Column Kit. The bottom two UV chromatograms shown in Figure 5a were obtained for the control samples containing NP-40. As can be seen from the reference chromatograms shown in Figure 5b, injection of samples containing NP-40 does not change the chromatographic properties of the μ PAC column.

The final set of samples was obtained with an *in vitro* TnT® Quick Coupled Transcription/Translation System. After elaborate sample preparation, these samples contained a black precipitate. When injecting these samples onto a standard packed bed nano LC column, sudden increase in column backpressure prevented further use of this column. Again, UV chromatograms obtained for the set of challenging samples are shown in Figure 6a, UV chromatograms for intermediate Cytochrome C reference separations are shown in Figure 6b.

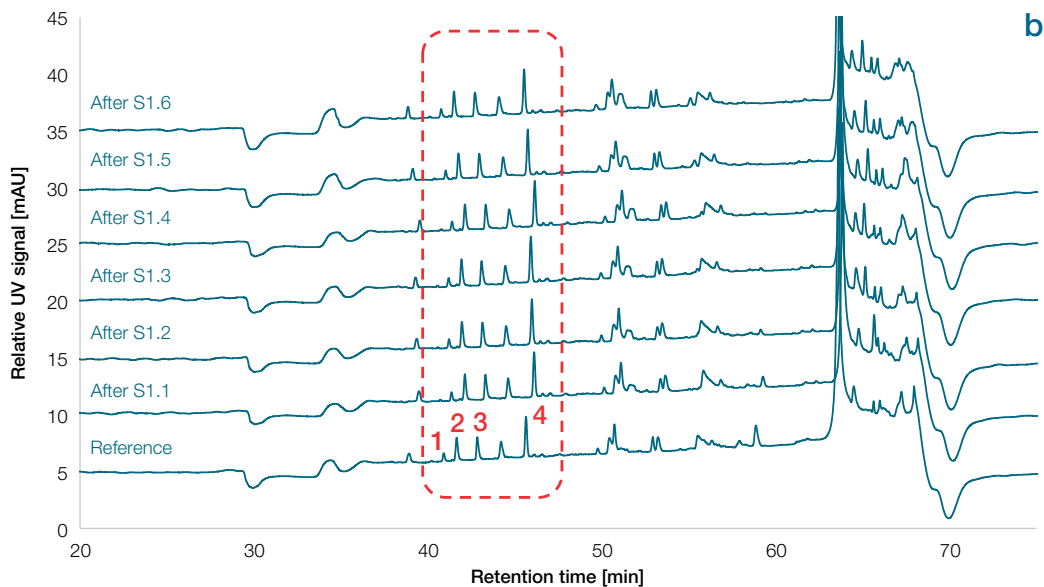
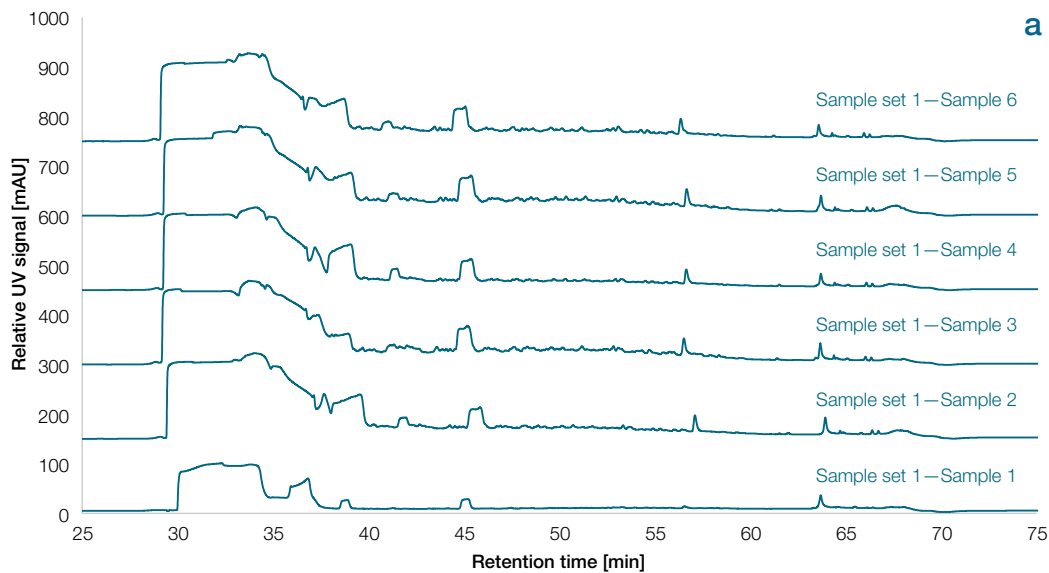


Figure 4. a) UV chromatograms obtained for the separation of a “challenging” bottom-up proteomics sample. Sample set 1 containing Triton X-114. 6 different samples have been injected. b) UV chromatograms obtained for intermediate reference separations of Cytochrome C digest. Peptides that were used to evaluate column performance and stability are indicated.

Injection volume: 1 μL

Flow rate: 1 $\mu\text{L}/\text{min}$

Gradient conditions: 1-50% B in 30 min

Mobile phase composition: A – H_2O + 0.1% FA, B – 80% Acetonitrile + 0.1% FA

Column temperature: 35 $^\circ\text{C}$

Detection: UV 214 nm

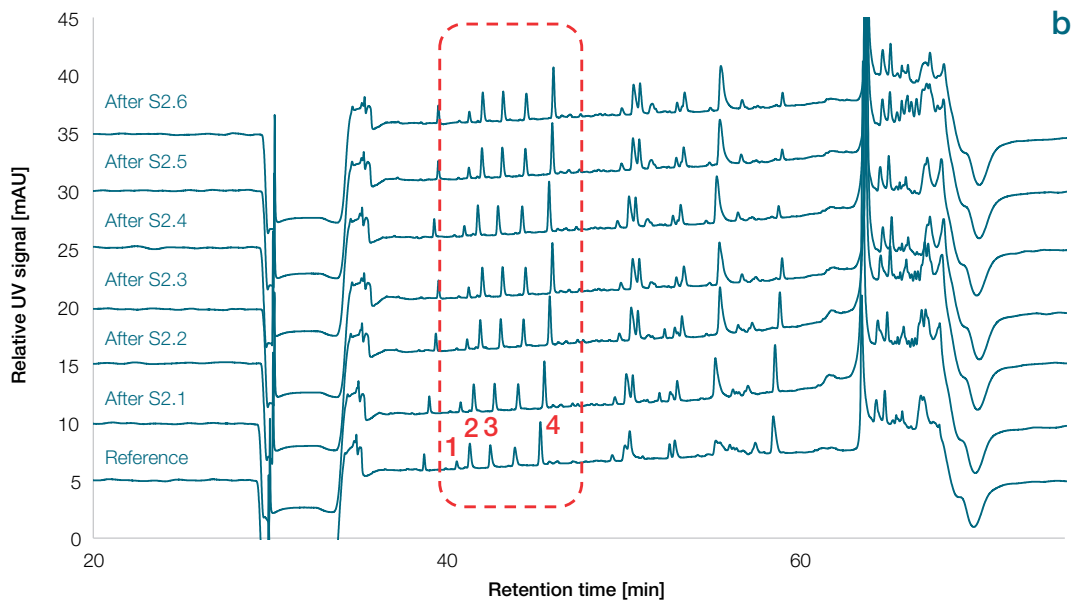
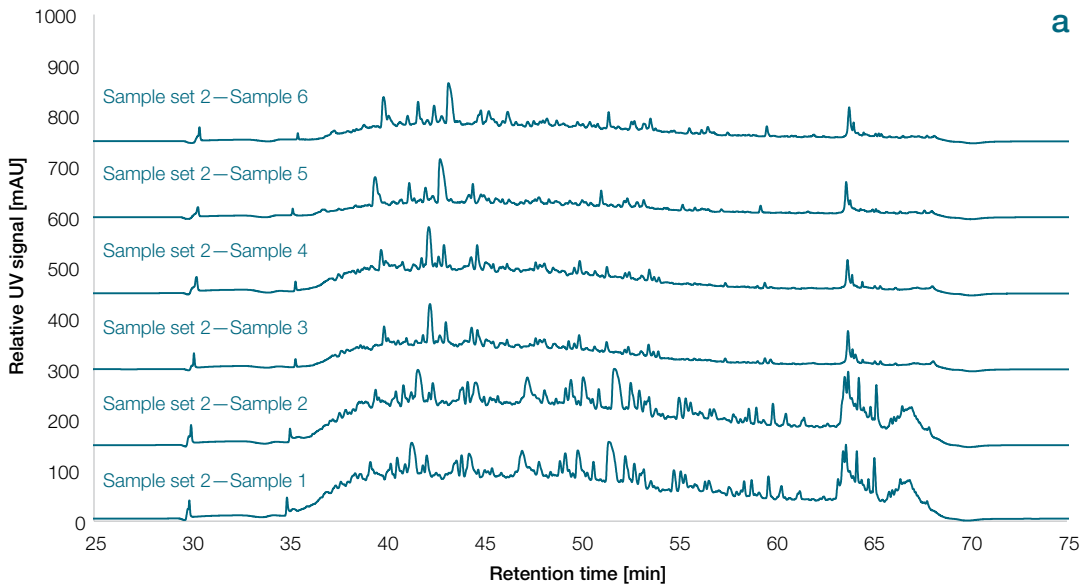


Figure 5. a) UV chromatograms obtained for the separation of a “challenging” bottom-up proteomics sample. Sample set 2 containing NP40. 6 different samples have been injected, only sample 1 and 2 contained NP40. b) UV chromatograms obtained for intermediate reference separations of Cytochrome C digest. Peptides that were used to evaluate column performance and stability are indicated.

Injection volume: 1 μL

Flow rate: 1 $\mu\text{L}/\text{min}$

Gradient conditions: 1-50% B in 30 min

Mobile phase composition: A – H_2O + 0.1% FA, B – 80% Acetonitrile + 0.1% FA

Column temperature: 35 $^\circ\text{C}$

Detection: UV 214 nm

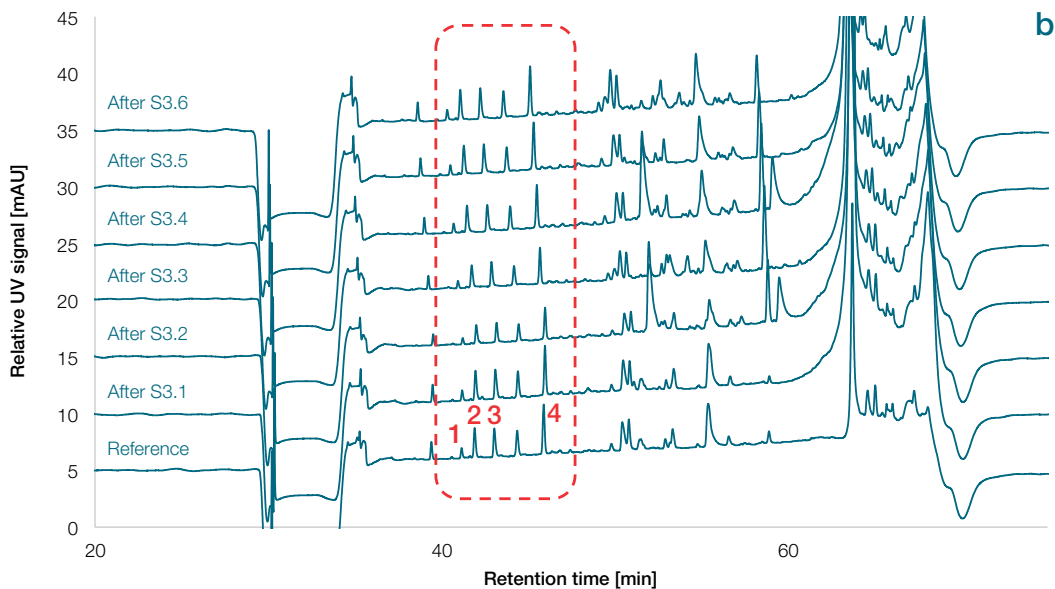
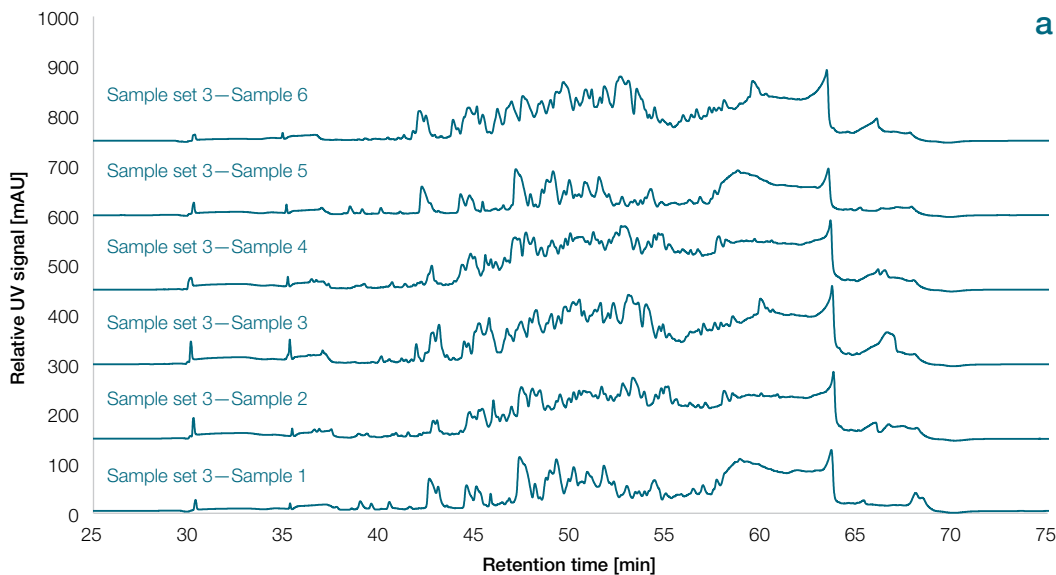


Figure 6. a) UV chromatograms obtained for the separation of a “challenging” bottom-up proteomics sample. Sample set 3 containing a dark/black precipitate. 6 different samples have been injected. b) UV chromatograms obtained for intermediate reference separations of Cytochrome C digest. Peptides that were used to evaluate column performance and stability are indicated.

Injection volume: 1 μ L

Flow rate: 1 μ L/min

Gradient conditions: 1-50% B in 30 min

Mobile phase composition: A – H₂O + 0.1% FA, B – 80% Acetonitrile + 0.1% FA

Column temperature: 35 °C

Detection: UV 214 nm

To evaluate the influence of these “challenging” samples on the chromatographic properties of the μ PAC column, several parameters have been monitored throughout the entire experiment. An important parameter which is indicative for column clogging or contamination is the backpressure it generates for a certain flow rate. In Figure 7a, the column backpressure at the end of each run has been plotted as a function of the amount of injections. No significant increase in column backpressure was observed, with pressures ranging from 115 to 130 bar for a flow rate of 300 nL/min.

Four tryptic peptides in the Cytochrome C reference sample have been used to monitor retention time stability and column performance. Excellent retention time stability with a variation in retention time below 1% CV was observed for all four reference peptides (Figure 7b), and this throughout the entire experiment.

In addition, no effect on peptide peak width was observed (Figure 7c), with peak widths ranging from 0.17 to 0.27 min.

This excellent robustness and resilience towards “challenging” samples can be attributed to several unique features of the μ PAC column. The chromatographic bed is entirely microfabricated and consists of an array of freestanding silicon pillars with 2.5 μ m spacing in between them. Compared to packed bed columns (packed with sub-2 μ m particles), the flow through pores are 4 times larger and thus 4 times less likely to clog when samples containing particulate matter or debris are introduced. Additionally, the porous shell nature of the pillars ensures that virtually no column related sample carry over is observed. This makes column equilibration and cleaning steps much more effective as compared to fully porous material typically used to perform chromatography.

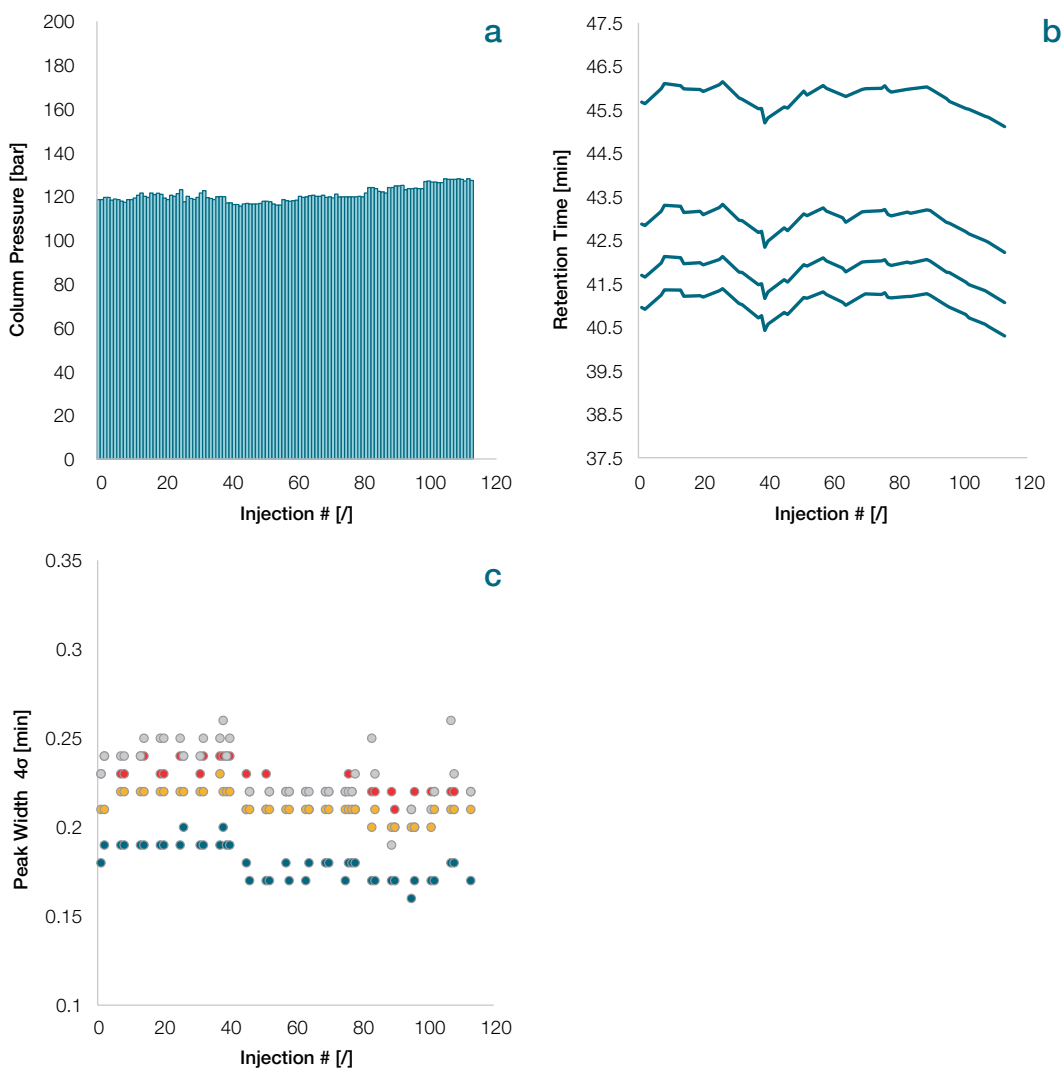


Figure 7. a) The average column pressure plotted as a function of the total number of injections. b) Retention time of 4 reference peptides from the Cytochrome C tryptic digest plotted as a function of the total number injections. c) The average peak width obtained for all 4 reference peptides plotted as a function of the total number injections.

Table 3. Chromatographic metrics obtained for Cytochrome C reference peptides.

	Value	Standard deviation	% CV
Retention Time Peptide 1 [min]	41.04	0.28	0.69
Retention Time Peptide 2 [min]	41.79	0.28	0.68
Retention Time Peptide 3 [min]	42.96	0.28	0.66
Retention Time Peptide 4 [min]	45.79	0.27	0.59
Peak Width Peptide 1 [min]	0.18	0.01	5.48
Peak Width Peptide 2 [min]	0.23	0.01	4.30
Peak Width Peptide 3 [min]	0.23	0.02	7.54
Peak Width Peptide 4 [min]	0.21	0.01	3.42
Average Column Pressure [bar]	121	3	3

Conclusions

- Next to technical issues that can occur with the LC-MS system's hardware and software, LC column failure is one of the most frequent causes of LC-MS system down time.
- Due to the unique fabrication procedure, μ PAC columns have the potential to be much less prone to sample related column failure and to withstand more sample injections without losing performance.
- Continuous operation of a 200 cm μ PAC column over a period of 6 months did not affect the chromatographic properties of the column.
- After a total of 3526 injections, of which 1000 tryptic HeLa cell digest injections, the overall retention time for a reference standard (Cytochrome C digest) was found to be very stable with a coefficient of variance below 2% for all reference peptide peaks.
- Several "challenging" bottom-up proteomics samples were subsequently injected onto the μ PAC column, and again no impact on column performance nor retention has been observed.

References

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