Application note | 002651

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Proteomics

Efficient and sensitive peptide mapping approach by µPAC columns with ultralow sample loading

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Keywords

Peptide mapping, µPAC Neo HPLC columns, monoclonal antibodies, mAb, low-flow LC-MS, high throughput proteomics, low load, single cell proteomics

Goals

- Demonstrate reproducibility of peptide mapping of biopharmaceuticals using the Thermo Scientific™ µPAC™ Neo HPLC column
- Show the utility of the µPAC Neo HPLC column for mapping post-translational modification (PTM) analysis such as oxidation, deamidation, and glycopeptides

Introduction

Peptide mapping is one of the key analytical workflows for the characterization of monoclonal antibodies to obtain information about their sequences, post-translational modifications, and mutations. Traditional packed columns are usually used for peptide mapping at analytical flow rates and high sample loading. However, low-flow chromatography has become the preferred LC method when increased sensitivity is needed. Microfabricated pillar array columns (µPAC) were introduced as an innovative technology for low-flow LC-MS. The µPAC column design employs perfectly organized micrometer sized silicon structures modified with C18, eliminating any Eddy dispersion and increasing column permeability. It also provides high peak capacity separations at low-flow rates with enhanced ionization sensitivity. In this application note, peptide mapping is conducted using the 50 cm µPAC Neo column and the 5.5 cm µPAC Neo High Throughput columns, demonstrating outstanding performance.

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Experimental

Instrumentation

- Thermo Scientific™ Vanquish™ Neo UHPLC system (P/N VN-S10-A-01)
- Thermo Scientific ™ Orbitrap Exploris™ 480 mass spectrometer (P/N BRE725533)

Consumables

- 50 cm µPAC Neo HPLC column (P/N COL-NANO050NEOB)
- µPAC Neo High Throughput HPLC column (P/N COL-CAPHTNEOB)
- Thermo Fisher™ PepMap™ Neo trap cartridge (P/N 174500)
- Thermo Fisher™ PepMap™ Neo trap cartridge holder (P/N 174502)
- Fisher Scientific™ 0.1% Formic acid in water, Optima™ LC-MS grade (P/N LS118-212)
- Fisher Scientific™ 0.1% Formic acid in acetonitrile, Optima™ LC-MS grade (P/N LS120-212)
- Thermo Scientific™ Water, UHPLC-MS grade (P/N W8-1)
- Thermo Scientific™ Pierce™ Trifluoroacetic acid (TFA), Sequencing grade (P/N 28903)
- NISTmAb tryptic digestion standard (NC1645948)
- Thermo Scientific™ EASY-Spray™ nano and capillary emitters (P/N ES993 and ES994)
- Thermo Scientific™ SureSTART™ 0.2 mL TPX Screw Top Microvial with Glass Insert (P/N 60180-1655) and Thermo Scientific™ SureSTART™ 9 mm Screw Caps, Level 3 High Performance (P/N 6PSC9STB1)

Sample preparation

For the regular sample without TFA, the 40 µg of NISTmAb tryptic digestion standard was reconstituted in 100 µL of 0.1% FA in water as stock, then diluted to 80 ng/µL for LC-MS injection.

For the sample with TFA, the 40 µg of NISTmAb tryptic digestion standard was reconstituted in 100 µL of water, then the sample at 80 ng/µL with 0.1% TFA was made by diluting with 0.125% of TFA.

Liquid chromatography of tryptic peptides

In the chromatographic experiments, mobile phase A was water containing 0.1% formic acid and mobile phase B was 80/20 acetonitrile/water (v/v) consisting of 0.1% formic acid (by volume). Table 1 shows the 5- and 15-minute elution method as examples; for the 10- and 30-minute elution methods, the durations of %B changing from 4% to 22.5%, then to 35% and finally to 45% are proportionally altered. The 5 min elution method flowing at 1.5 µL/min and 10 min elution method flowing at 1 µL/min were performed on the 5.5 cm µPAC Neo High Throughput column; the 15 min elution method flowing at 0.5 µL/min and the 30 min elution method flowing at 0.2 µL/min were performed on the 50 cm µPAC Neo column. To get stable spray, the 15 µm ID emitter (P/N ES994) and the 10 µm ID emitter (P/N ES993) were used for the 5.5 cm µPAC Neo High Throughput column and the 50 cm µPAC Neo column, respectively. To increase retention of hydrophilic peptides on the trapping column, the sample was diluted with 0.1% TFA in water when a trap-and-elute workflow was used. The loading flow rate used for trapping was 60 µL/min.

Table 1. Gradient program for peptides separation

50 cm µPAC Neo HPLC column		5.5 cm µPAC Neo High Throughput HPLC column		
Duration (min)	Flow (µL/min)	Duration (min)	Flow (µL/min)	%B
0	0.5	0	1.5	1.0
0.1	0.5	0.1	1.5	4.0
9.9	0.5	3.3	1.5	22.5
3.75	0.5	1.3	1.5	35.0
1.25	0.5	0.4	1.5	45.0
0.9	0.5	0.9	1.5	99.0
6.1	0.5	2	1.5	99.0

Mass spectrometry parameters

Tryptic peptides were analyzed in data-dependent acquisition mode (full MS-ddTop 8) in positive mode. The details MS parameters are listed in Table 2.

Table 2. MS parameters for peptide mapping

	50 cm µPAC Neo column	5.5 cm µPAC Neo High Throughput column
Spray voltage	2 kV	2 kV
Resolution	30K	30K
m/z range	200–1,600	200–1,600
RF lens %	50	50
AGC (%)	100	100
Max IT	40	5
Microscan	1	1
Intensity threshold	5.00E+03	5.00E+03
Charge state	1–7	1–7
Dynamic exclusion	10	3
Top N	8	8
Isolation window	2	2
HCD	30	30
Resolution	15K	7.5K
First mass	120	120
AGC (%)	100	200
Max IT	30	12
Microscan	2	1

Data analysis parameters

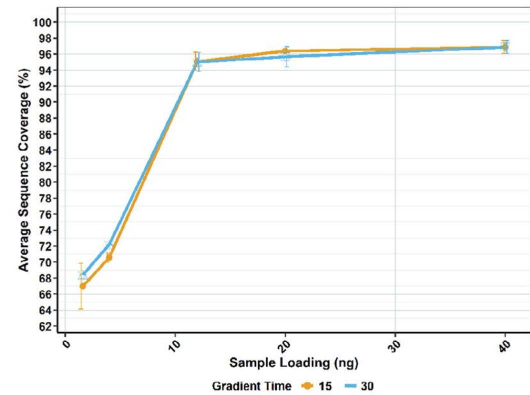
Raw data were analyzed by Thermo Scientific™ BioPharma Finder™ 5.1 software with automatic parameter values. Peptides with |ppm error| ≤ 10 ppm, identified only by MS^2, MS area ≥ 1E5, and Miss cleavage ≤ 2 were selected to calculated sequence coverage. Glycopeptides were manually confirmed by MS^2 spectrum.

Results and discussion

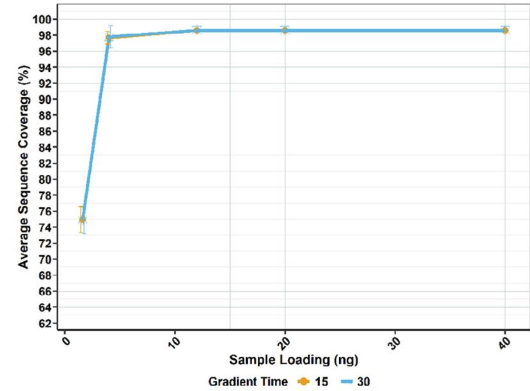
Impact of sample loading and elution duration

To fully evaluate the lowest possible sample loading for µPAC columns on the application of peptide mapping, extreme low amounts of NISTmAb Tryptic Digestion Standard (1.6 ng, 4 ng, 12 ng, 20 ng, and 40 ng) were loaded on either a 50 cm µPAC Neo column or a 5.5 cm µPAC Neo High Throughput column with different elution methods. Sequence coverages obtained for increasing loading amounts are plotted in Figure 1, with (a) and (b) obtained with a 50 cm µPAC Neo column, and (c) and (d) obtained with a 5.5 cm µPAC Neo High Throughput column. As the sample loading increases from 1.6 ng to 12 ng, the sequence coverages of both heavy (HC) and light chain (LC) increased significantly, whereas no substantial improvement is observed when increasing sample loading from 12 ng to 40 ng.

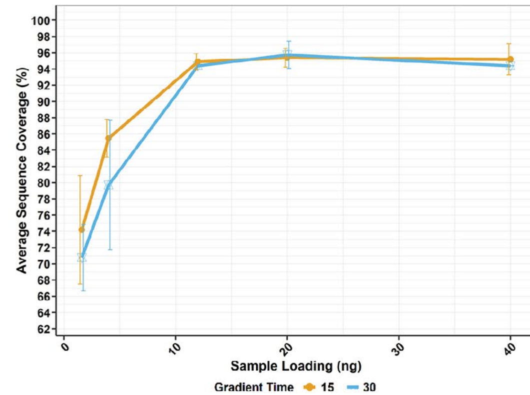
(a) 50 cm µPAC column peptide mapping for Heavy Chain



(b) 50 cm µPAC column peptide mapping for Light Chain



(c) µPAC Neo High Throughput column peptide mapping for Heavy Chain



(d) µPAC Neo High Throughput column peptide mapping for Light Chain

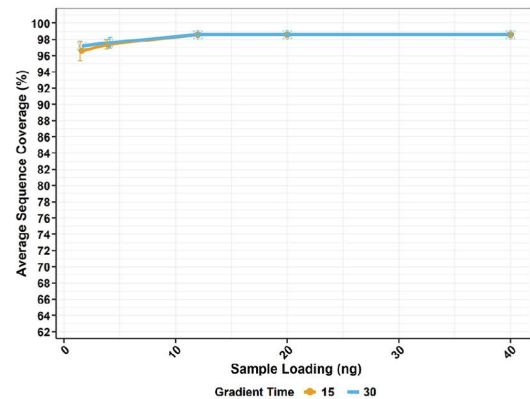


Figure 1. Sequence coverages results at different sample loading and elution durations. (a) and (b) are from 50 cm µPAC Neo columns; (c) and (d) are from 5.5 cm µPAC Neo High Throughput columns.

Even though higher peak capacities are obtained by increasing the gradient length, no significant effect on sequence coverage was observed when longer gradients were used, justifying the use of the shortest methods (15 min elution method on the 50 cm µPAC Neo column and a 5 min elution method on the 5.5 cm µPAC Neo High Throughput column) for further evaluation as this combines high data productivity with high sequence coverage.

Total ion chromatograms (TIC) for a NISTmAb digest at different sample loading amounts are shown in Figure 2. Each row represents different sample loadings. As the sample loading increases, so does the intensity of the TIC. The signal increment of the TIC is not significant after 12 ng of sample loading, which is consistent with the trending of sequence coverage. To save time and introduce less deviation, 20 ng of sample was chosen for providing optimal and consistent results and preventing overloading the column.

Sequence coverage results on direct workflow

Figure 3 shows an illustration of the Vanquish Neo UHPLC system direct injection workflow, delivering the sample directly onto the separation column before performing gradient separation.

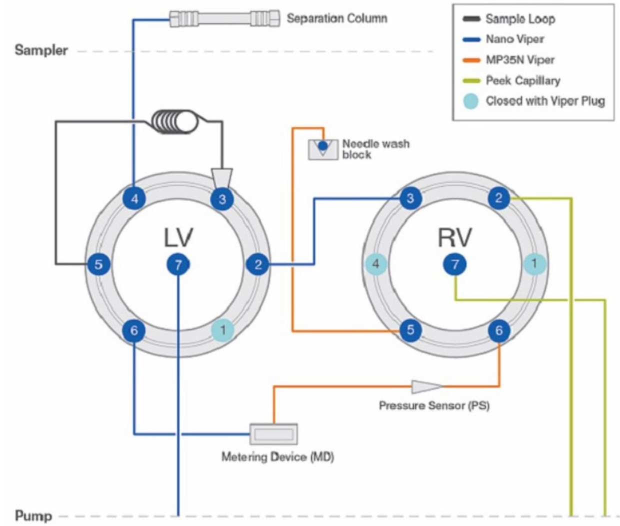
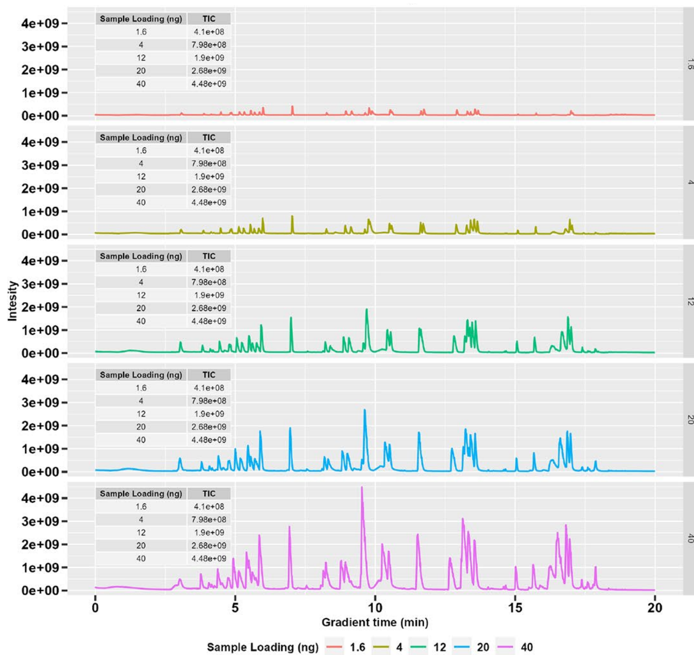


Figure 3. The Vanquish Neo UHPLC system direct workflow. Sample was directly loaded on the separation column after loading on the sample loop.

50 cm µPAC Neo column peptide mapping (15 min with 0.5 µL min)
Total ion chromatogram



µPAC Neo High Throughput column (5 min with 1.5 µL min)
Total ion chromatogram

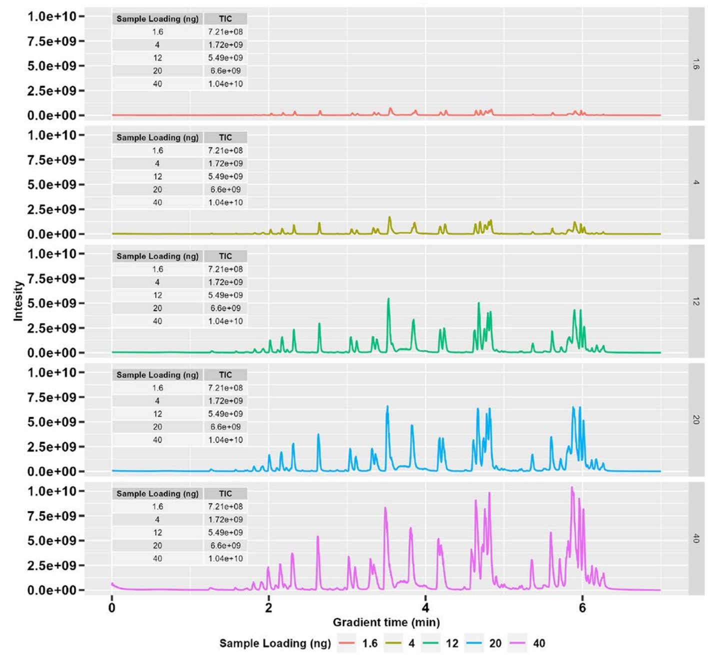


Figure 2. TIC for the 50 cm µPAC Neo column at a 15 min elution method (left) and for the 5.5 cm µPAC Neo High Throughput column at a 5 min elution method with different sample loading (right)

The sequence coverages for HC and LC with a 15 min elution method flowing at 0.5 µL/min on a 50 cm µPAC Neo column are shown in Figures 4a and 4b; those of a 5 min elution method flowing at 1.5 µL/min on a 5.5 cm µPAC Neo High Throughput column are shown in Figures 4c and 4d. For both columns, the best sequence coverage for HC is 96.4% and for LC is 98.6%. Considering the ultra-low sample loading of 20 ng and short elution period, this high sequence coverage is outstanding for rapid sequence coverage evaluation.

EICs of peptides with PTMs

The extracted ion chromatograms (EICs) of “DTLMISR” and its oxidized form (with methionine sulfoxide residue) are shown in Figures 5a and 5b; its oxidized form eluted earlier as it is more hydrophilic. The oxidized position was confirmed by the MS^2 fragments such as y4 and y5 with a 64 Da mass loss associated with the loss of methanesulfenic acid. The EICs of “NQVELTCLVK” and its deamidated form are shown in Figures 5c and 5d.

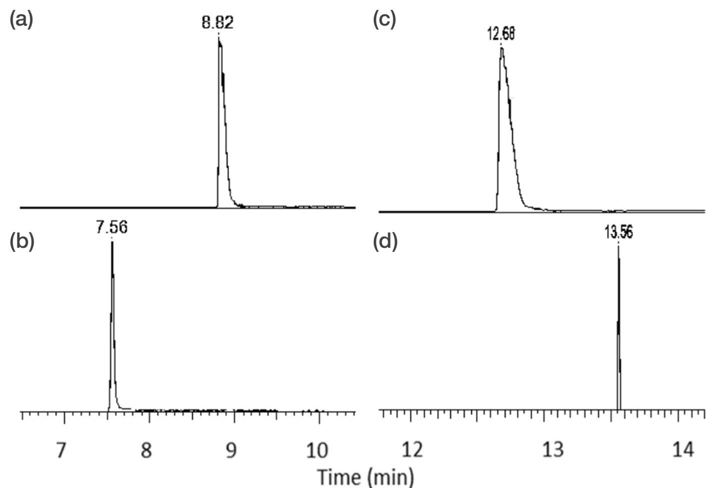


Figure 5. EICs of “DTLMISR” and its oxidized form (with methionine sulfoxide residue) (a) and (b); EICs of “NQVELTCLVK” and its deamidated form (c) and (d). All data are from the 50 cm µPAC Neo column.

(a)

(b)

(c)

(d)

Figure 4. Sequence coverages for HC and LC. (a) and (b) used a 15 min elution method flowing at 0.5 µL/min on a 50 cm µPAC Neo column; (c) and (d) used a 5 min method flowing at 1.5 µL/min on a 5.5 cm µPAC Neo High Throughput column.

Despite of the overlapping isotopologue of these two forms, with µPAC columns, they can be well separated. The b2+ ion of 243.1085 Th and b2+ ion of 244.0927 Th confirm the identification of "NQVELTCLVK" and its deamidated variant at the MS^2 level. Thus, µPAC columns show great selectivity for oxidized and deamidated peptide variants versus their unmodified forms. This significantly improves confidence of detection and identification of oxidized and deamidated peptides by eliminating overlapping between mass spectra.

Glycosylation is also an important PTM for antibodies. The EIC of three glycoforms on "EEQYNSTYR" are shown in Figure 6. In Figure 6a, for the 50 cm µPAC Neo column, +G0F and two other glycoforms are separated by 2.4 s in a 15 min elution duration, but the other two glycoforms +G1F and +G2F are coeluting. On the other hand, EICs for the 5.5 cm µPAC Neo High Throughput column are shown in Figure 6b. The +G0F and other two glycoforms are separated by 1.22 s, indicating that these three glycoforms can barely be separated with 5 min elution methods.

Better separation of hydrophilic peptides with trap-and-elute workflow

To further investigate the recovery of hydrophilic peptides that are missed in HC coverage, trifluoroacetic acid (TFA) was introduced in the sample preparation to act as an ion pairing agent. As TFA will suppress the ionization efficiency of electrospray ionization, a trap-and-elute workflow rather than direct injection workflow is applied to minimize the transfer of TFA into the mass spectrometer. Due to the ion pairing effect of TFA, hydrophilic peptides will experience increased retention on the trap and analytical column, which we anticipate will result in better resolution and recovery of these peptides. Figure 7 illustrates the fluidics of the workflow on the Vanquish Neo UHPLC system, where analytes are first loaded onto the trap column (backflush mode), and then transferred and further separated on the analytical column. The sequence coverages obtained for the trap-and-elute workflow (Figure 8) are similar to those obtained

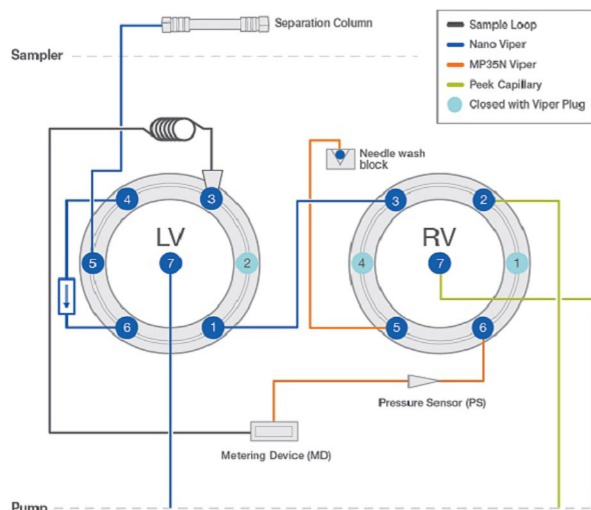


Figure 7. The Vanquish Neo UHPLC system trap-and-elute workflow. Sample was first loaded on the trapping column and then separated on the separation column.

with the direct injection workflow, confirming that no peptides are lost by loading the sample onto the trap column. This is again confirmed when comparing base peak chromatograms (BPC) of the direct and the trap-and-elute workflows (Figure 9) with similar BPC intensities for both workflows. The alternative elution order of some peptides is due to the addition of a trap column in backflush mode. The EICs of "EEMTK" and "ADYEK" from the direct workflow (Figures 10a and 10c) are eluting before the first 0.5 or 0.8 min on both columns, which shows poor separation with wide peaks. Figures 10b and 10d, on the other hand, show that these two peptides elute at 4.46 min and 4.51 min on a 50 cm µPAC Neo column and elute at 1.75 min and 1.76 min on a 5.5 cm µPAC Neo High Throughput column, which indicates that with the addition of TFA, these two peaks became sharper and can be partially separated. Thus, the addition of 0.1% TFA can help to retain the hydrophilic peptides on the trap column, resulting in improved recovery and better resolution during the subsequent analytical separation on µPAC columns.

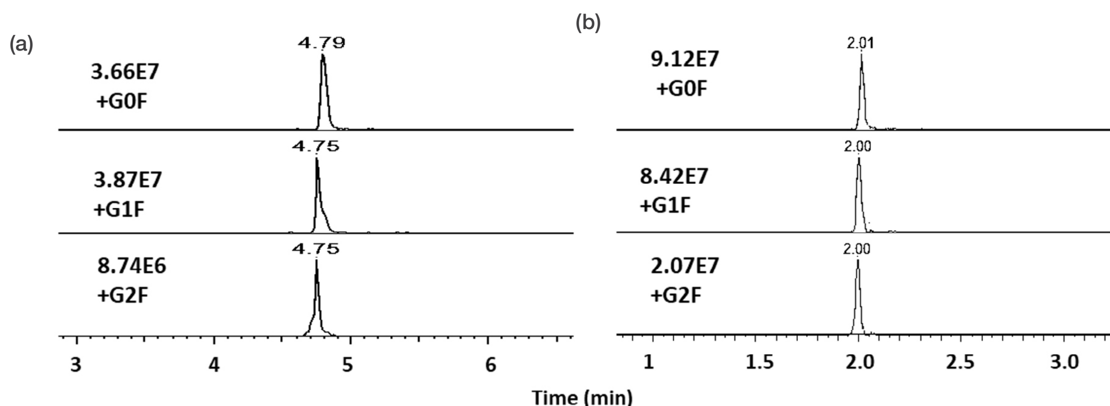


Figure 6. EICs of glycopeptides for (a) 50 cm µPAC Neo column with a 15 min elution method, (b) 5.5 cm µPAC Neo High Throughput column with a 5 min elution method

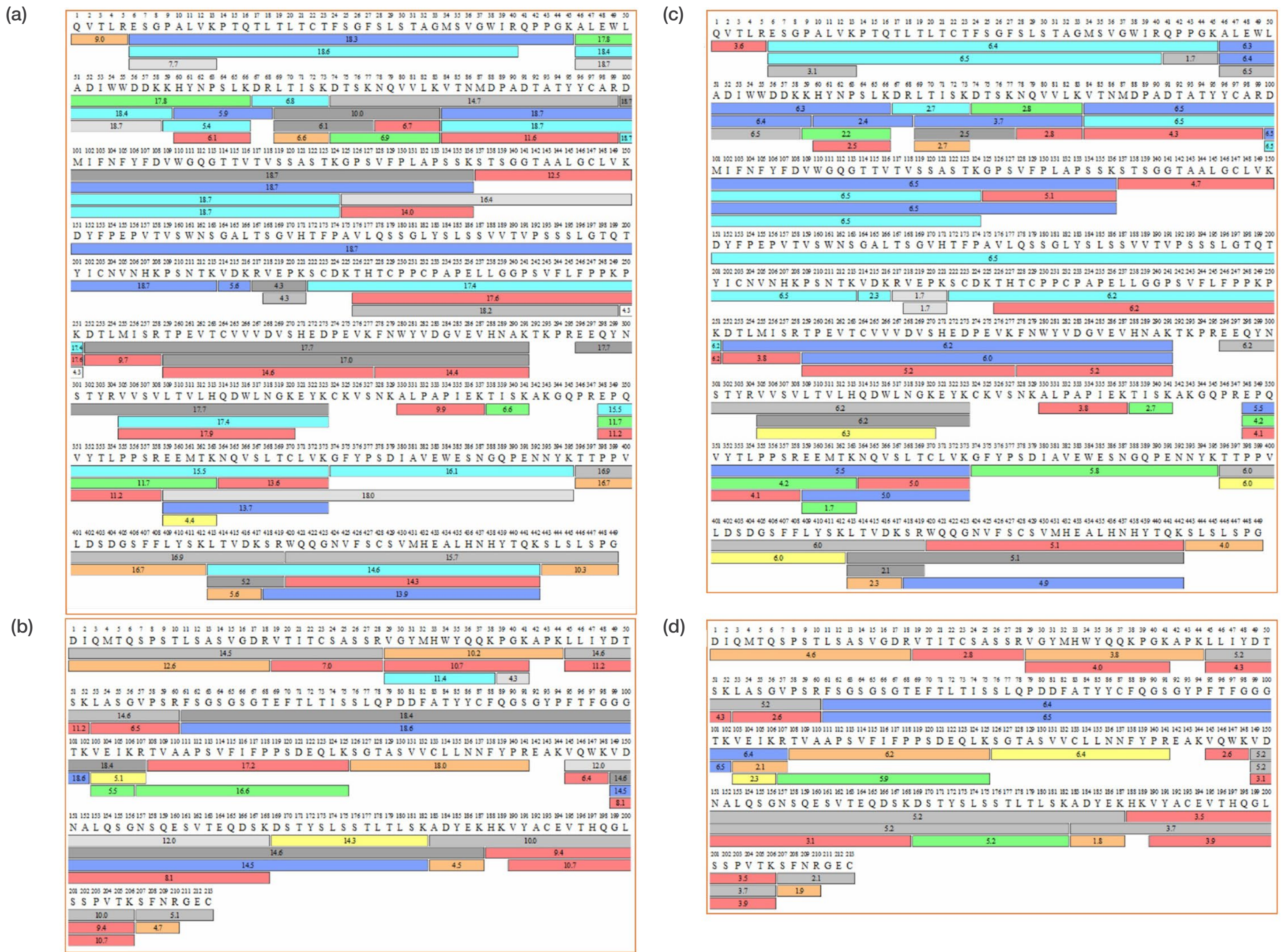
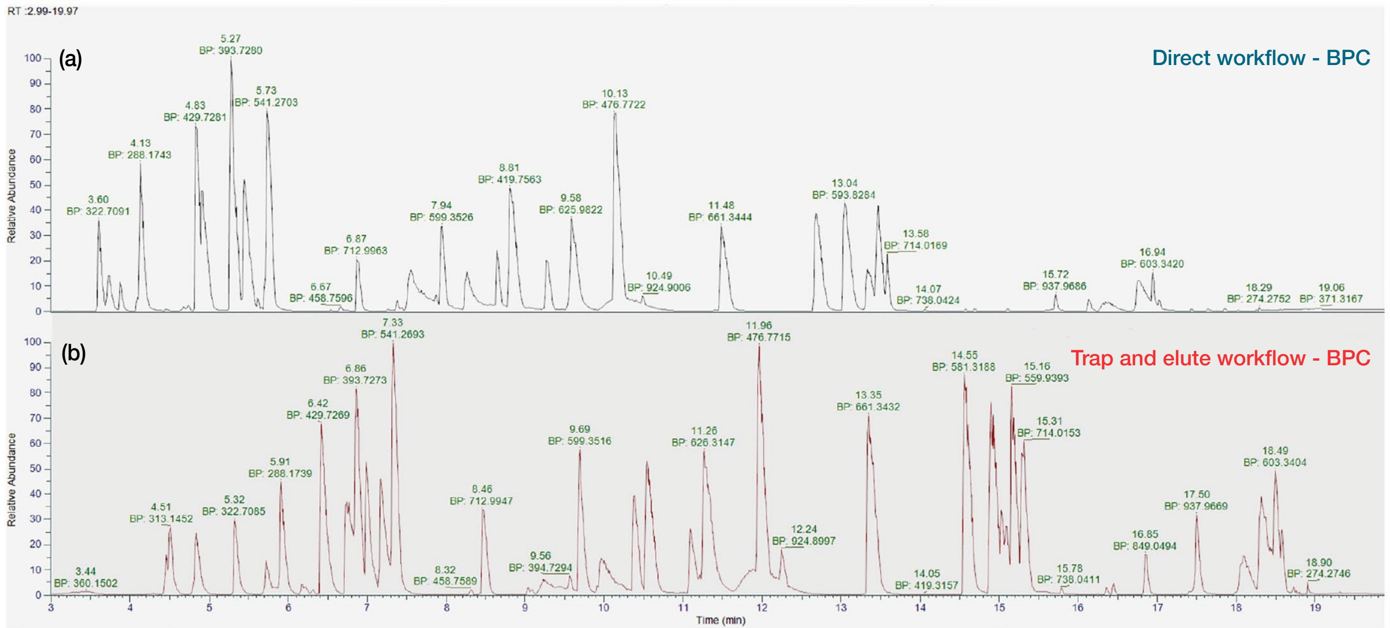


Figure 8. Trap-and-elute workflow with 0.1% TFA in the sample with sequence coverages for HC and LC with a 15 min elution method flowing at 0.5 µL/min on a 50 cm µPAC Neo column (a) and (b), and with a 5 min method flowing at 1.5 µL/min on a 5.5 cm µPAC Neo High Throughput column (c) and (d)

50 cm µPAC Neo column (15 min with 0.5 µL/min)



µPAC Neo High Throughput column (15 min with 1.5 µL/min)

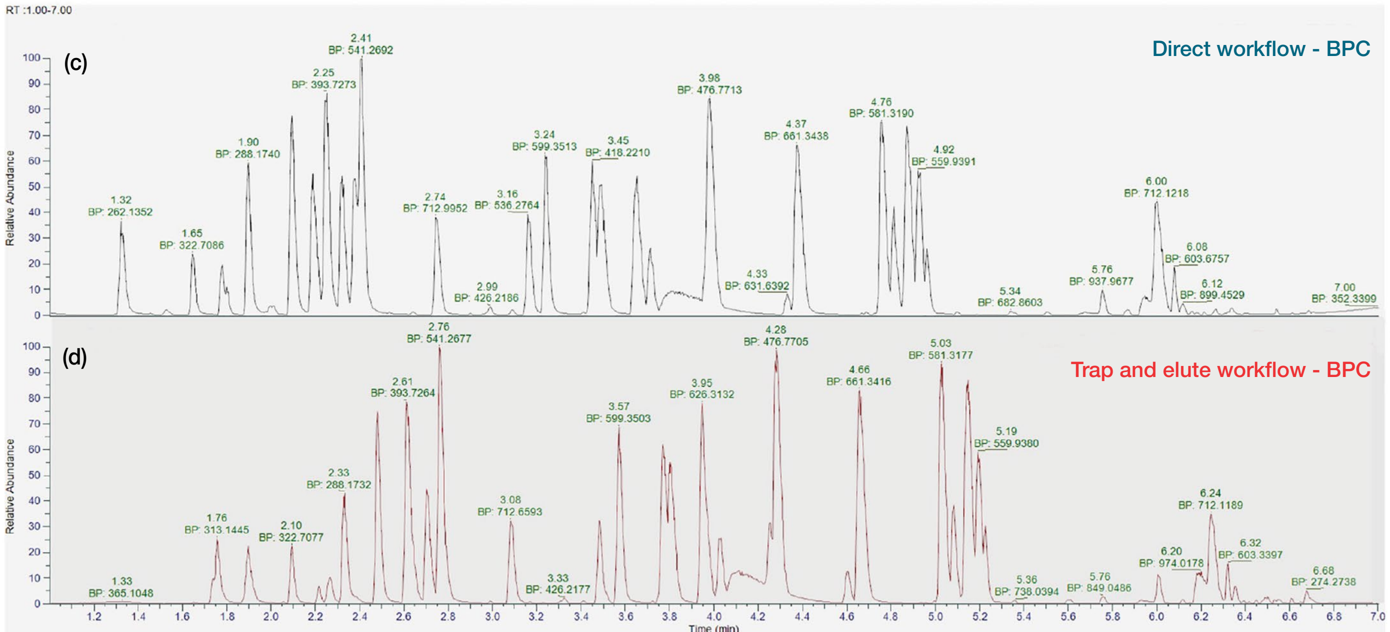


Figure 9. Base peak comparison (BPC) comparison between direct workflow (a) and (c) and trap-and-elute workflow (b) and (d). (a) and (b) are from a 50 cm µPAC Neo column. (c) and (d) are from a 5.5 cm µPAC Neo High Throughput column.

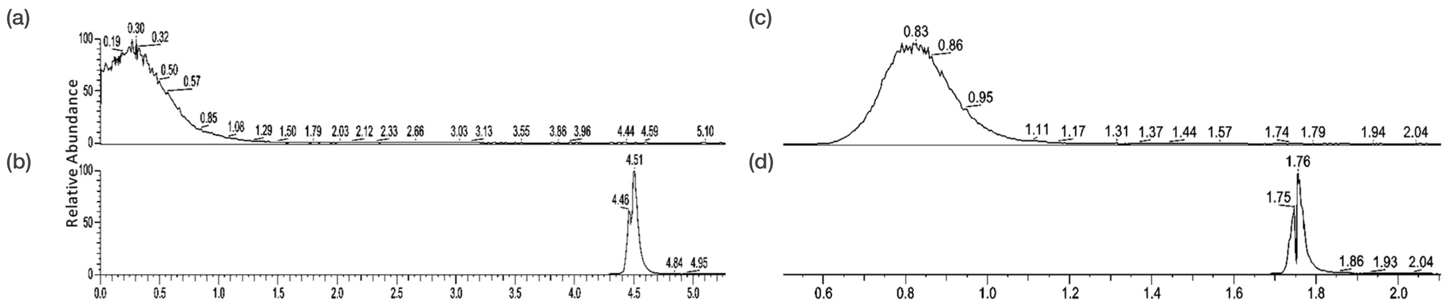


Figure 10. EICs of "EEMTK" and "ADYEK" from direct workflow (a) and (c) and trap-and-elute workflow (b and d). (a) and (b) are from a 50 cm µPAC Neo column. (c) and (d) are from a 5.5 cm µPAC Neo High Throughput column.

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Conclusion

Excellent sequence coverage was obtained for very low amounts of NISTmAb Tryptic Digestion Standard using high-throughput low flow LC-MS on µPAC Neo columns. In summary, 96.4% and 98.6% sequence coverage could be achieved for HC and LC NISTmAb, respectively, with only 20 ng of tryptic digest loaded and separated using a 15 min elution method and a 50 cm µPAC Neo column. Due to the increased separation path length of this column, improved resolution for PTMs was observed (e.g., glycopeptides). Similar sequence coverage levels (96.4% and 98.6% for HC and LC, respectively) could be achieved with the µPAC Neo High Throughput column even though the total run-to-run time was reduced by 2.5 times, and a 3 times higher flow rate was used, making this set-up suitable for high throughput sequence coverage screening purposes. Small tryptic peptides such as TKPR and VSNK in HC chain are not covered because their MS^1 signals are too low to be detected in a 20 ng sample loading. With the addition of 0.1% TFA, the same sequence coverage was achieved, but it improved the separation and recovery of small hydrophilic peptides. Overall, µPAC Neo columns provide high sequence coverages at ultra-low sample loading, opening opportunities to reduce sample collection effort for clinical mAb samples.

Learn more at **thermofisher.com/lowflowhplccolumns**

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