Increased sensitivity and throughput with easy-to-connect capillary MAbPac RP columns

Benefits

- Robust column design for top-down and middle-down proteomics
- Simple-to-connect design for MS compatibility
- Higher sensitivity compared with analytical scale liquid chromatography (LC)
- Easy-to-use format for capillary LC
- Directly connected to capillary-flow and nano-flow emitters
- Thermo Scientific™ EASY-Spray™ format

Introduction

The Thermo Scientific™ MAbPac™ RP 4 µm 15 cm × 150 µm capillary column has been specifically designed for low-flow, highly sensitive top-down, middle-down, and monoclonal antibodies (mAbs) analysis. The novel chemistry is fully compatible with Thermo Scientific™ capillary LC and Thermo Scientific™ Orbitrap™ mass spectrometer systems.

Equipped with a Thermo Scientific™ nanoViper™ finger tight connector at inlet and out ends, this column is easy to connect and simple to use. Also, the column is available in a convenient EASY-Spray design which delivers ultimate ease-of-use by taking advantage of the “plug and spray” to low-flow LC.

The new EASY-Spray column comes with an integrated heater column and a newly optimized emitter tip design for performance and longevity at capillary flow.

Column technology

The Thermo Scientific™ MAbPac RP 15 cm x 150 µm column is packed with Thermo Scientific™ MAbPac™ RP 4 µm diameter polymeric material, with 1500 Å pores.

It is used for the separation of mAbs, proteins and peptides that are analyzed in proteomic and biopharmaceutical experiments. The capillary MAbPac RP column is designed for direct compatibility with mass spectrometry detectors.

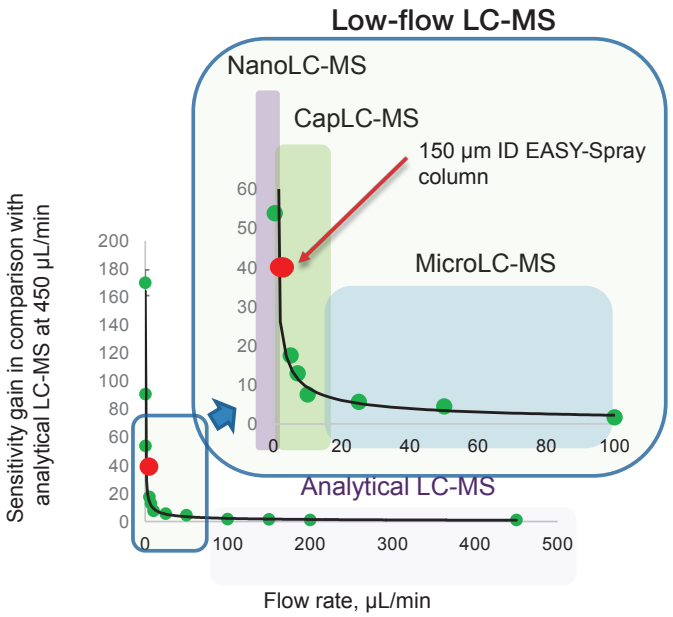
Applications

Improved sensitivity

There is a major sensitivity improvement to be gained by using capillary flow rates compared to analytical flow rates. This improvement is shown using the hyperbolic function in Figure 1.

The increase in sensitivity when analyzing biomolecules is particularly useful given that often the amount of sample is limited and a maximum level of information is required.

Figure 2 demonstrates these benefits by comparing the 50 mm × 3 mm analytical MAbPac RP column operating at a 500 µL/min flow rate to the 150 mm x 0.15 mm capillary column packed with the same resin, running at 2 µL/min flow rate.



*Sensitivity gain or loss is dependent on compound and application.

Figure 1. The increase in sensitivity experienced with low-flow LC-MS compared to analytical flow LC-MS

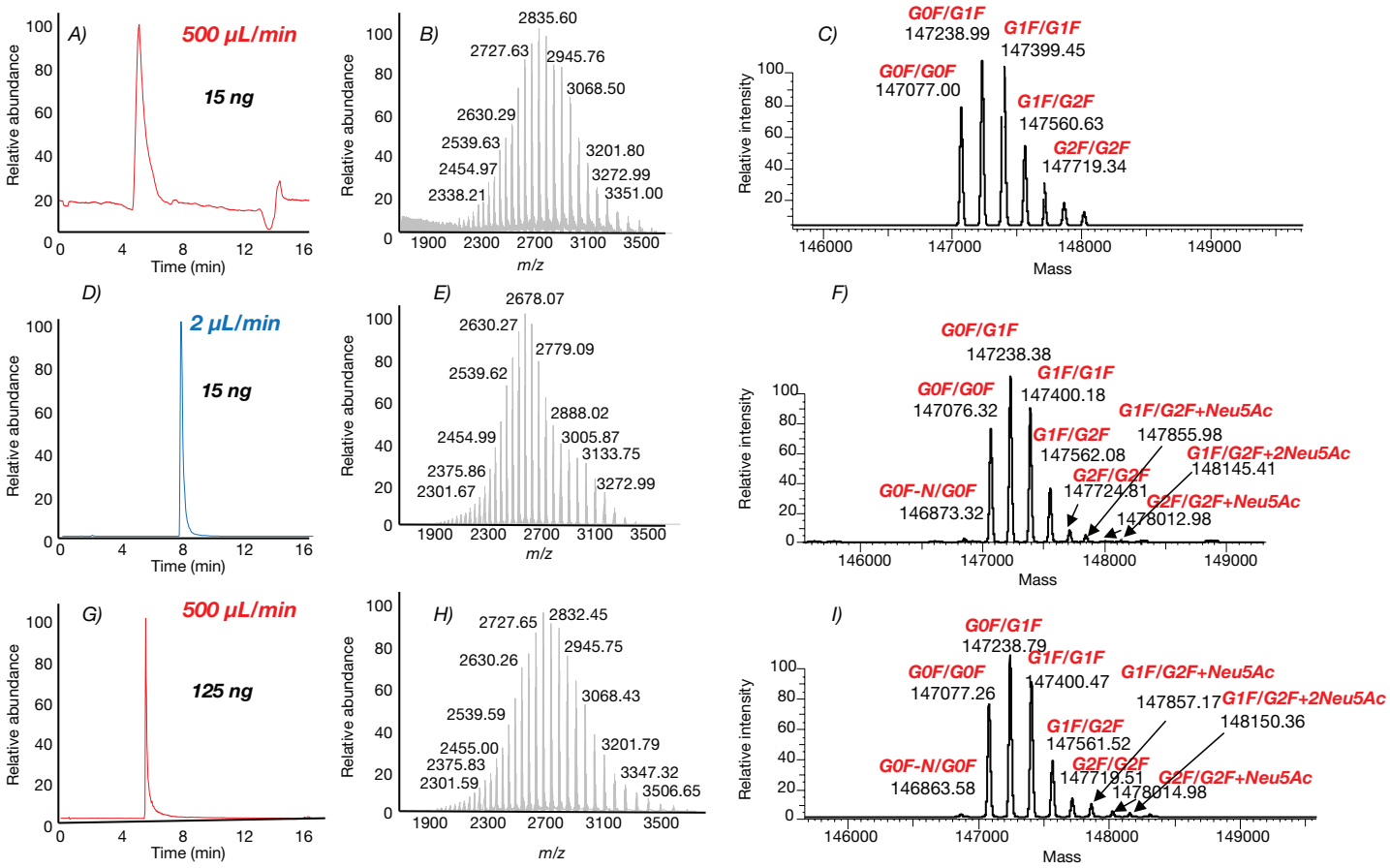


Figure 2. The effect of the flow rate on sensitivity/glycoform distribution

Injecting a relatively low amount of intact mAb (rituximab) on the high flow column results in a broad peak due to dispersion caused by using a 20 times larger column i.d. (Figure 2A) which in turns leads to a low quality spectra being obtained (Figure 2B) and a low signal-to-noise (S/N) ratio of 17.

Analysis of the same mass of mAb on the capillary column (Figure 2D) significantly improved peak shape and reduced noise level, leading to a good quality spectrum (Figure 2E) and S/N ratio (S/N 626 – Figure 2F) being observed.

Figure 2G is the peak obtained by injecting 125 ng onto the analytical column as a control and clearly shows that an injection of nearly 8 times the mass onto the analytical column would be required to obtain similar sensitivity to the capillary column.

Intact protein and top-down analysis

Liquid chromatography-mass spectrometry (LC-MS) based workflows of intact proteins include the intact mass and top down approaches. Figure 3A shows the TIC of six recombinant proteins acquired in protein mode. Due to the high resolution applied, four proteins (peaks 1, 3, 4, and 5) could be isotopically resolved as demonstrated by Figure 3B where charge states are assigned to each peak of the protein charge envelope.

The deconvoluted spectrum (Figure 3C) displays the highly accurate masses of the proteins. The workflow does not show the masses of peak 2 and 6; they are larger proteins and therefore they cannot be deconvolved with isotopic resolution. Hence, it is not possible to determine their monoisotopic masses. Similar to the approach already shown (Figure 2) for an intact mAb, low resolution was applied to obtain the average masses of the charge states of these proteins.

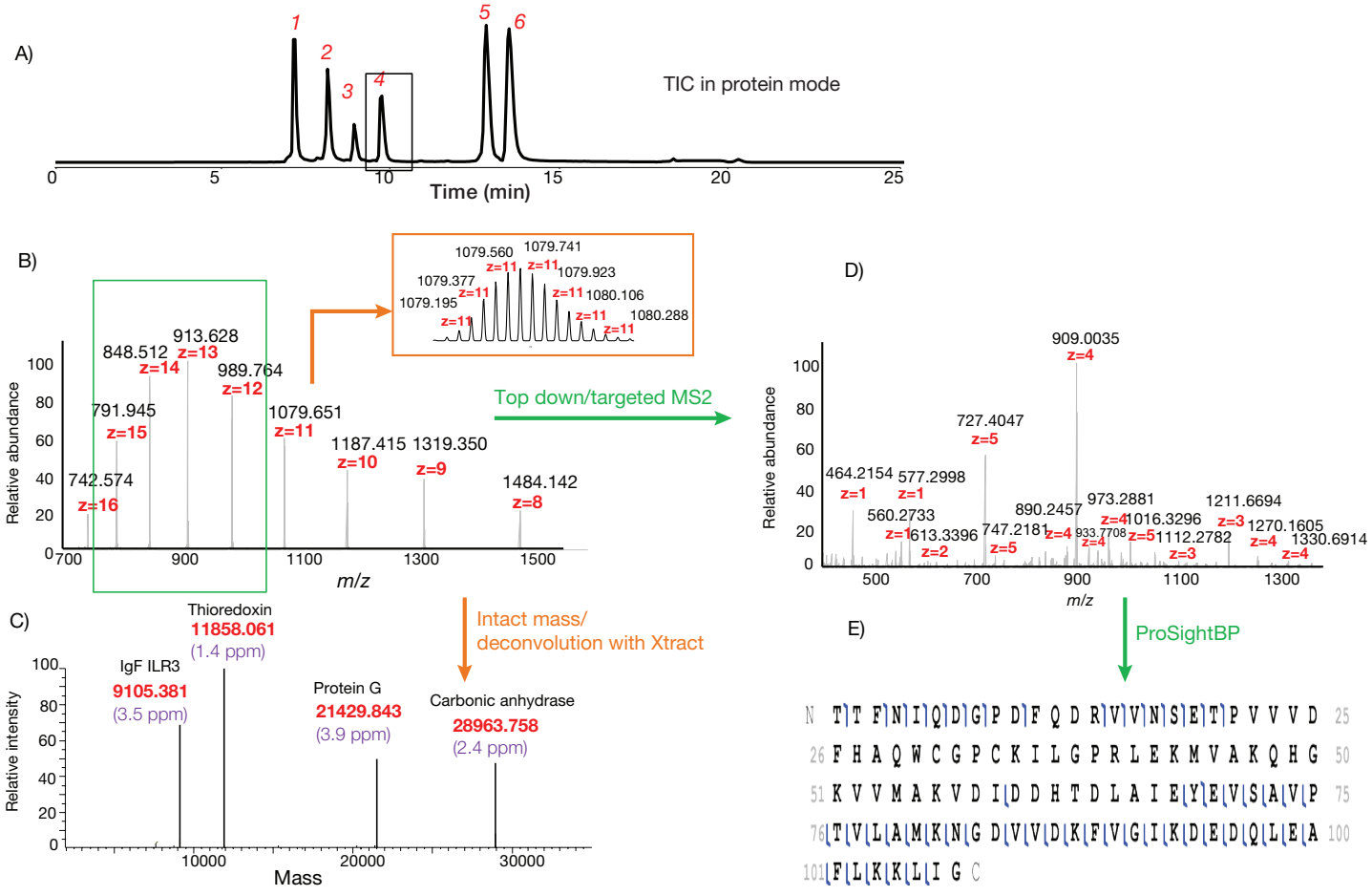


Figure 3. Workflows of intact proteins—intact mass and top down strategies

Top-down experiments using a targeted MS2 method were employed to confirm the sequence of proteins. The first charge state was selected and fragmented in the HCD cell. These product ions were stored until all charge states were isolated and fragmented. All the product ions were then detected in the Orbitrap mass analyzer, resulting in one fragment ion spectrum (Figure 3D). The fragment ions were assigned to the sequence of the protein, thus creating a fragment map with residue cleavages (Figure 3E).

Glycosylation site occupancy

Glycosylation is one of the most important post-translational modifications (PTMs) and plays a pivotal role in the folding of proteins into their final three-dimensional, biologically active conformation and cell-to-cell adhesion. The MAbPac RP column in capillary format provides fast, highly reproducible separations of peptides and its robustness, combined with high sensitivity makes this column a perfect tool to address the analytical challenge of deciphering glycosylation site occupancy. In this case, the glycosylation site occupancy of three therapeutic antibodies was determined.

As an example, Figure 4 displays the TIC of trastuzumab peptides after pooling 20 µg of tryptic digests labeled and deglycosylated in the presence of "light" ($H_2{}^{16}O$) and "heavy" ($H_2{}^{18}O$) water. In the TIC, there are five peaks assigned; their mass spectra are shown below.

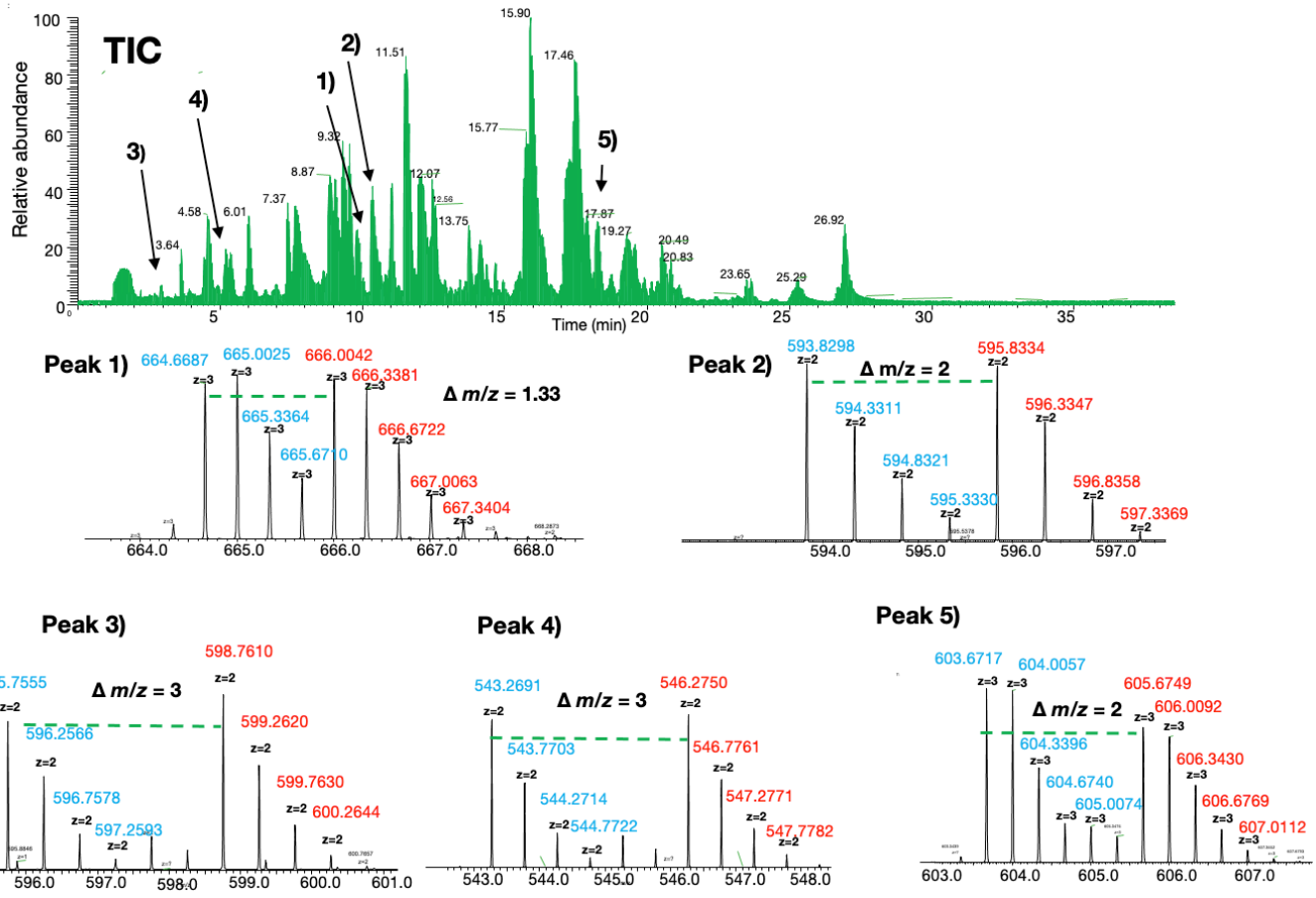


Figure 4. Labeling with $H_2{}^{18}O$ discriminates masses and helps identify glycosylation and deamidation sites

Table 1 shows the sequence of these peptides. Peaks 4 and 5 both exhibit a 6 Da difference between the light and heavy parent ions in the corresponding doublets, indicating that the conversion of asparagine into aspartic acid took place during the experimental procedure, resulting in the incorporation of an additional oxygen.

Peak 3 is the peptide containing the glycosylation site (N300). As such, when deglycosylating with PNGase F, this asparagine was converted into aspartic acid (6 Da difference between the light and heavy labeled peptides). XICs of the light and heavy versions of the peptide were used to calculate glycosylation site occupancy of this therapeutics.

Calculation of site occupancy shown by Figure 5 is performed from the ratio of peak areas of both the light and heavy EEQYDSTYR (the peptide which was originally glycosylated) and EEQYNSTYR (the peptide which was not originally glycosylated in the drug material).

Excellent reproducibility on a MAbPAc RP capillary column is highlighted by the reproducibility of retention times of both the deaminated (originally glycosylated, aspartic acid containing) and the non-glycosylated (asparagine containing) peptides listed in Table 2. %CV values of the retention times of these peptides occurring in the digests of the three mAbs were found to be 0.34% (for D-containing peptide) and 0.37% (for the N-containing peptide).

Table 1. Assignment of deamidation and glycosylation based on mass shift.

Peaks	Peptide sequence	Theoretical *m/z*	Experimental *m/z*	Error	Location
1	ASQDVDTAVAWYQQKPGK*	1990.9736	1990.9826	4.52	Light chain
2	GPSVFPLAPSSK	1185.6380	1185.6438	4.89	Heavy chain
3	EEQYDSTYR (from EEQYN(300)STYR)	1189.4874	1189.4952	5.56	Heavy chain
4	IYPTDGYTR (from IYPTN(55)GYTR)**	1084.5176	1084.5224	4.43	Heavy chain
5	VVSVLTVLHQDWLDGK (from VVSVLTVLHQDWLN(318)GK)	1807.9820	1807.9914	5.20	Heavy chain

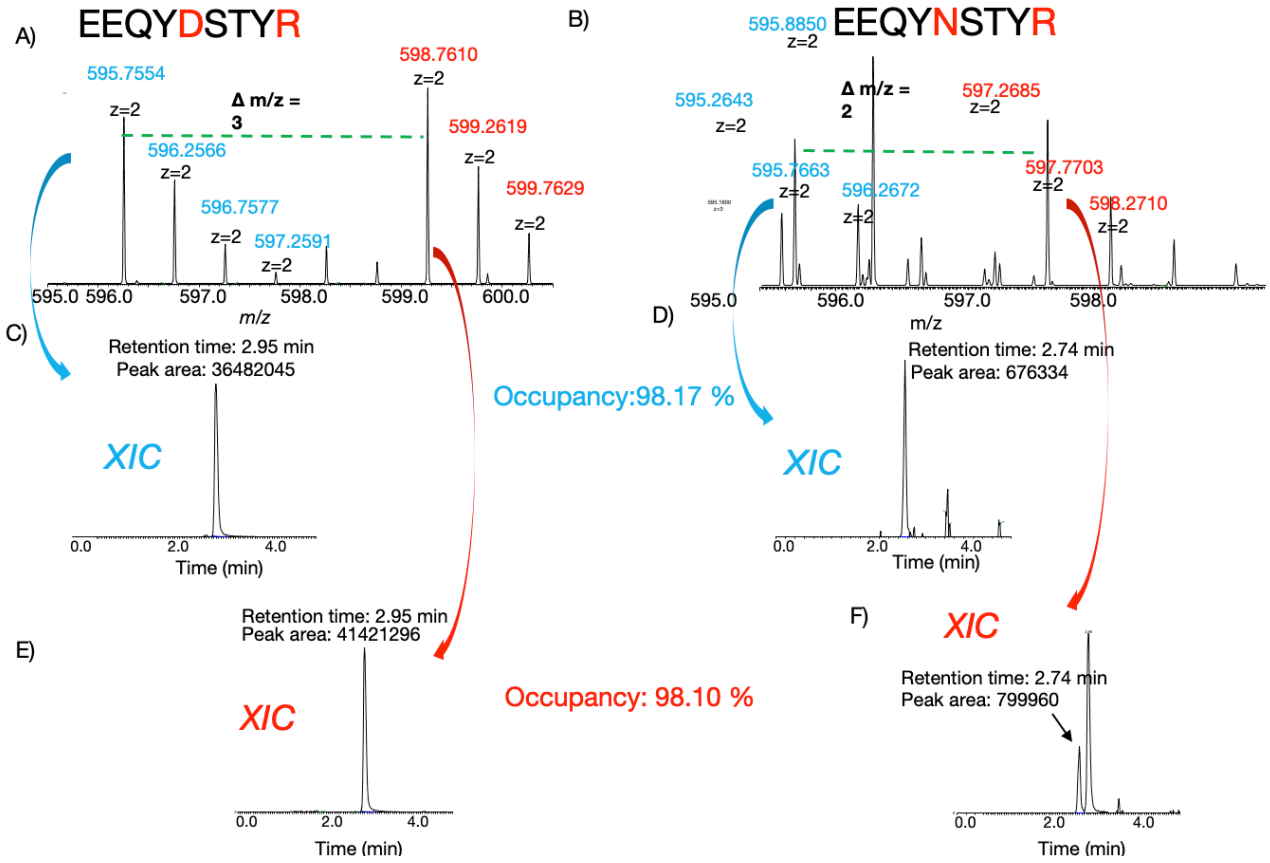


Figure 5. Calculation of site occupancy of N300 in trastuzumab

Table 2. Site occupancy of two mAbs determined by different approaches.

mAbs	Site occupancy determined by MRM	Site occupancy determined by peptide mapping
Trastuzumab	99.02	98.13
Rituximab	99.57	98.75

Conclusion

The use of the MAbPac RP capillary column offers utmost sensitivity and excellent selectivity for the mass spectrometric analyses of intact proteins and mAb subunits. It supports all the intact protein workflows using small amounts of sample. Miniaturizing the column using the robust hardware design ensure that the excellent resolving power of the high flow MAbPac RP columns for proteins is maintained. The workflows demonstrated above are easily applied to discovery proteomics when samples are available at limited quantity.

We have demonstrated that the MAbPac RP capillary column is a useful tool for analyzing tryptic peptides, which is due to its moderately hydrophobic resin and has been shown to be particularly useful when uncovering an important protein attribute, the glycosylation site occupancy, as shown in the workflow presented here. When compared to analytical flow rates, the capillary format results in highly reproducible peptide mapping from 50 to 100 ng digest of therapeutic antibodies and the column format (EASY-Spray format or double nanoViper fittings) makes the use and connection of this column very simple for the operator.

thermo scientific

Ordering information

Description	Particle size	Part number
EASY-Spray MAbPac RP 15 cm x 150 µm	4 µm	ES907
MAbPac RP Cap 4 µm 15 cm x 150 µm 1200 bar DNV	4 µm	164947
Nano EASY Emitter 10 µm, without transfer line	-	ES993
Capillary EASY Emitter 15 µm, without transfer line	-	ES994

Operational specifications for MAbPac RP capillary columns

Parameter	Recommendation
Typical applications	Intact protein and/or high MW peptide analysis
Base material	PSDVB (Polystyrene Divinylbenzene)
Particle size	4 µm
Pore size	1500 Å
Column dimensions	150 µm i.d. x 15 cm
Recommended flow	1.0 – 3.0 µl/min
Recommended temperature	40 – 60 °C PN ES907 max 60 °C PN 164947 max 110 °C
Maximum pressure	600 bar
pH stability	0 to 9
Solvent compatibility	All common RP solvents

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

ThermoFisher SCIENTIFIC