APPLICATION NOTE 22066

Use of a MAbPac RP capillary column to monitor glycosylation site occupancy in therapeutics

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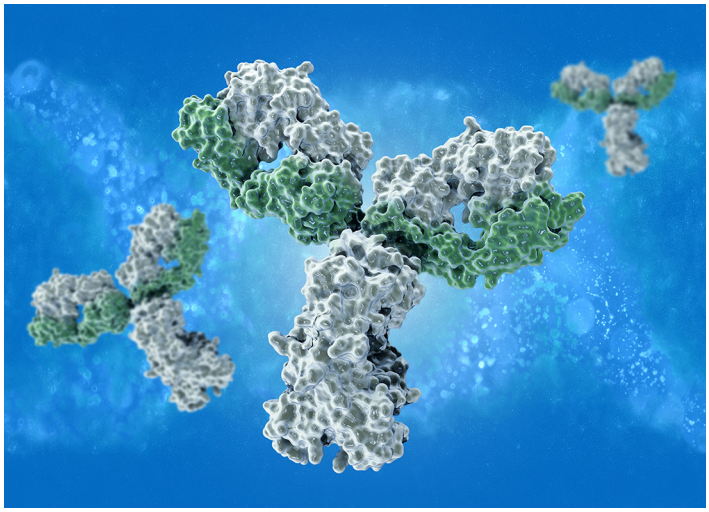


Goal

Introduce a new approach using the novel, easy-to-connect Thermo Scientific™ MAbPac™ RP capillary column for determining glycosylation site occupancy of therapeutic antibodies from the analysis of their tryptic digest.

Introduction

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. In a biotherapeutic drug, the glycan composition can dramatically affect the fuction and immunogenicity, and glycosylation is an important attribute in the optimization of glycoprotein drugs, such as monoclonal antibodies. Analyzing the glycans to understand and achieve the desired effector function of a drug candidate during clone selection and the following early stage of development is desirable as this critical attribute is required by regulatory agencies. Besides glycan analysis, measuring glycosylation site occupancy is also important to ensure consistency and efficacy during development. Clone selection and early optimization phase for a drug candidate requires monitoring protein attributes. This work addresses this analytical challenge to decipher glycosylation site occupancy at clone selection and early stages of product development where sensitivity is needed. The scarcity of sample available for analysis and the high frequency

of sampling necessitate the use of highly sensitive, high-throughput and robust methods delivering information-rich data at high turnover frequency. The MAbPac RP column in capillary format provides fast, highly reproducible separations of peptides. The robustness combined with high sensitivity makes this column a perfect tool to address the analytical challenge.

This application note describes highly reproducible glycosylation site occupancy measurement using a MAbPac capillary column analyzing 100 ng tryptic digests of three therapeutic antibodies. The use of heavy water ($H_2{}^{18}O$) during deglycosylation enables mass discrimination between the light ($H_2{}^{16}O$) and heavy peptide pool, and consequently site occupancy data can be generated in duplicate from one analytical run. Site occupancy data reported here matches well with those generated by targeted approaches such as Multiple Reaction Monitoring (MRM). Further advantages of the non-targeted, peptide-based approach is that it can be used for discovery glycoproteomics (when sequence is unknown). The method also distinguishes process development vs. chemical deamidation; the latter may be caused by sample preparation.

Experimental

Instrumentation

Capillary chromatography was performed using a Thermo Scientific™ UltiMate™ 3000 RSLCnano System (ULTIM3000RSLCNANO) consisting of the following:

- SR 3000 solvent rack (P/N 5035.9200)
- NCS 3500RS module (P/N 5041.0010A, featuring a NC pump, a loading pump and a column compartment)
- VWD-3400RS UV-VIS detector (P/N 5074.0010)
- WPS-3000TPL RS temperature controlled autosampler (P/N 5826.0020)

The NC pump was equipped with a Classic flow meter with a Capillary flow selector.

The chromatographic system was coupled to a Thermo Scientific™Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (IQLAAEGAAPFALGMBDK) using a Thermo Scientific™ EASY-Spray™ Source (ES081).

Consumables

A novel MAbPac RP capillary column with two Thermo Scientific™ nanoViper™ connections (4 µm, 0.15 mm × 150 mm, P/N 164947), a transfer line (50 cm × 20 µm), and capillary flow emitter (15 µm ID, P/N ES994) connected with Viper unions (P/N 6040-2304) were used in capillary chromatography experiments. Sample preparation and reactions were performed in either 0.2 mL volume reaction tubes (Applied Biosystems™ MicroAmp™ Reaction Tubes with cap, 0.2 mL, autoclaved, P/N N8010612) or 2 mL volume microfuge tubes (Invitrogen™, Nonstick, RNase-free Microfuge Tubes, 2.0 mL, P/N AM12475). If controlled temperature was needed, the tubes were incubated in a thermal mixer while digestion was performed with the Thermo Scientific™ SMART Digest™ Trypsin Kit (Thermo Scientific™, Thermal Mixer with 0.2 mL block, P/N 13687721).

Reagents, chemicals

The mobile phases were prepared using MS grade water (Fisher Scientific™ UHPLC-MS grade water, P/N W8-1), acetonitrile (Fisher Scientific UHPLC-MS grade acetonitrile, P/N A956-1), and formic acid (Fisher Scientific™ Optima™ UHPLC-MS grade formic acid, P/N A117-50).

mAbs were digested using the SMART Digest Trypsin Kit (P/N 60109-103), non-magnetic option) consisting of 96 SMART Digest tubes, SMART Digest buffer, a collection plate, and a Thermo Scientific™ SOLAµ™ SPE plate. Trifluoroacetic acid (TFA, Thermo Scientific™ Pierce™, P/N 28901) was used as an additive while cleaning up the tryptic digest on the SOLAµ SPE plate.

Ammonium bicarbonate and water-^{18}O were purchased from Sigma-Aldrich.

Deglycosylation of tryptic peptides was carried out using PNGase F enzyme from Rapid™ PNGase F kit (New England Biolabs Inc, Ipswich, MA). Upon completion of digestion, the digests were further reduced with Tris(carboxyethyl)phosphine (TCEP) (Thermo Scientific™,Bond-Breaker™ TCEP solution, neutral pH, P/N 77720) and alkylated with iodoacetamide (Acros Organics, Thermo Scientific™, P/N 122270250).

Therapeutic antibodies (mAbs)

Trastuzumab and Fab glycosylated mAb were acquired from Roche Diagnostics GmbH (Penzberg, Germany). Rituximab was donated by a local collaborating biopharma company.

Sample preparation

A) Preparing tryptic digests of mAbs

150 µL of SMART Digest buffer was dispensed into a SMART Digest reaction tube and 50 µL of mAb solution containing 100 µg of protein was added to the tube. Sample preparation was performed as described by the SMART Digest user guide with the inclusion of reduction and alkylation steps after digestion. Once the digestion was complete, 8.2 µL Bond-Breaker TCEP solution (500 mM) was added to the digested mAb solution and was further incubated at 50 °C for 30 min. The reduced peptides were then alkylated with iodoacetamide by setting the concentration to 20 mM and incubated for 1 h at room temperature in the dark.

B) Preparing samples for mapping deamidation and assessment of glycosylation site occupancy using water-^{18}O

Scheme 1 shows the detailed experimental procedure, which is based on the work published by Liu et al.[1]

C) Liquid chromatography of tryptic peptides

In the capillary chromatographic experiment, mobile phase A was water containing 0.1% formic acid and mobile phase B was 80/20 acetonitrile/water (v/v) containing 0.1% formic acid (by volume). The flow rate applied was 2 µL/min. The gradient program is described by Table 1. Separation of peptides were performed at 45 °C.

Table 1. Gradient program for peptide separation

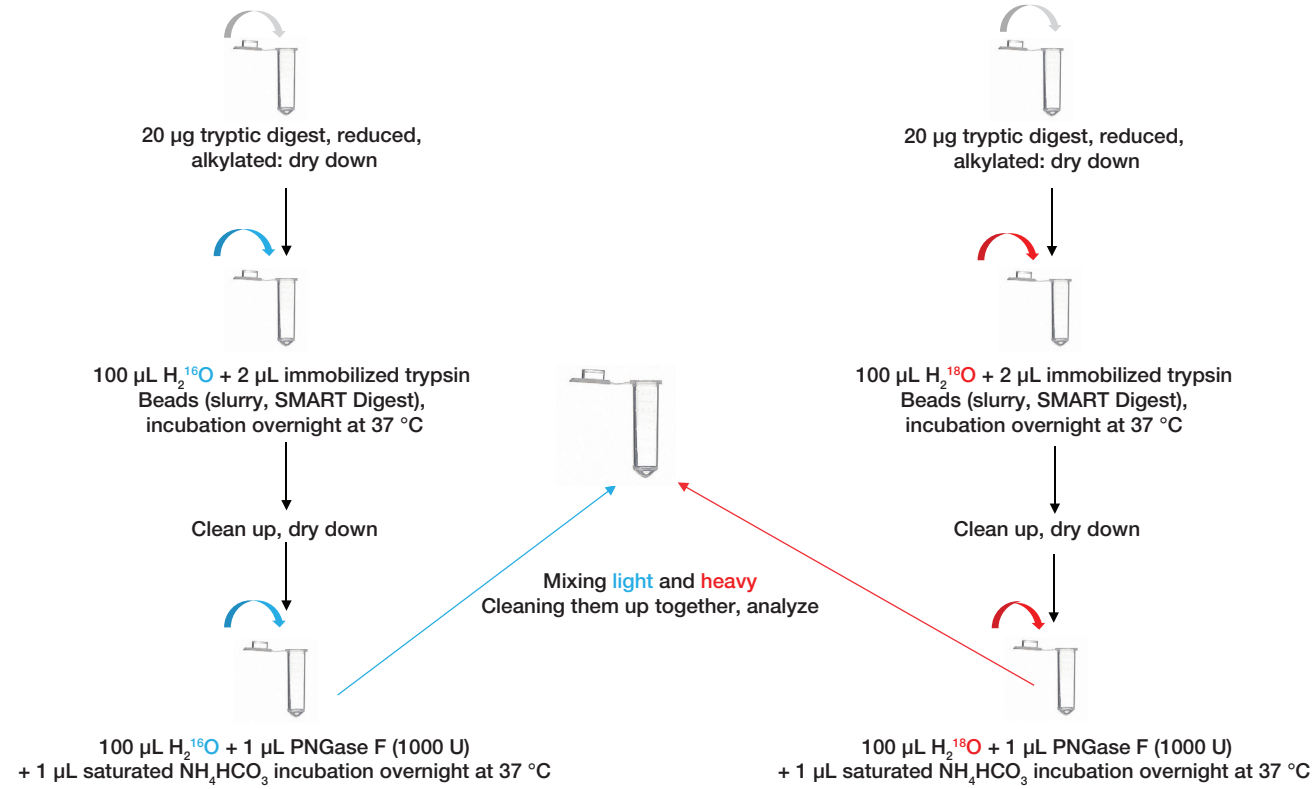
Time (min)	% B
0	5
30	50
30.5	5
38.5	5

D) Mass spectrometry conditions

Tryptic peptides were analyzed in data dependent acquisition mode (Full MS-ddTop5) in positive mode. Table 2 lists the experimental parameters.

Table 2. Full MS-Top5 DDA parameters

Parameters	Values
Capillary temperature	320 °C
Spray voltage	1.5 kV
Scan range (Full MS)	350–1200
Resolution (Full MS/MS^2)	70,000/17,500
AGC, Full MS/MS^2	$3 \times 10^6/2 \times 10^5$
Max injection time (Full S/MS^2)	60 ms/80 ms
Isolation window	1.9 T
Microscans (Full MS/MS^2)	1
NCE	28 kV
Minimum AGC	2×10^3
Exclude isotopes	On
Dynamic exclusion	15 s



Scheme 1. Sample preparation workflow for measuring glycosylation site occupancy

E) Connecting the column to the HPLC outlet and mass spectrometer

The inlet of the MAbPac capillary column (with two nanoViper fittings) was connected to the HPLC outlet using a Viper union by carefully tightening the nut finger-tight using the black knurled screw. The column outlet is connected to a second Viper union. The outlet of the second Viper union is connected to a transfer line (55 cm × 20 µm, with two nanoViper fittings, P/N 6041.5260), while the other end of the transfer line is connected to the capillary flow emitter. The emitter is easily plugged into EASY-Spray Source.

Results and discussion

Although the primary goal of this study was to determine glycosylation site occupancy of three therapeutic antibodies, using the approach described by Liu et al.[1] and Scheme 1 with modifications, it was also possible to distinguish chemical deamidation (happening spontaneously during the experimental procedure) from deamidated residues already present in the drug before the experiment. Figure 1 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.

Table 3 shows the sequence of these peptides. Peak 1 is detected as a triply charged doublet, with the difference of *m/z* 1.33 between light and heavy isotopologues. This corresponds to only a 4 Da difference between the heavy and light parent ions of the peptide. However, simulated digestion of trastuzumab *in silico* reveals that this peptide is expected to contain asparagine (N), contrary to the detected aspartic acid (D). This indicates asparagine had undergone deamidation and had been converted into aspartic acid before the experimental procedure was carried out. Hence, one can conclude this deamidation is attributable to process development of the drug or may have occurred during long-term storage.

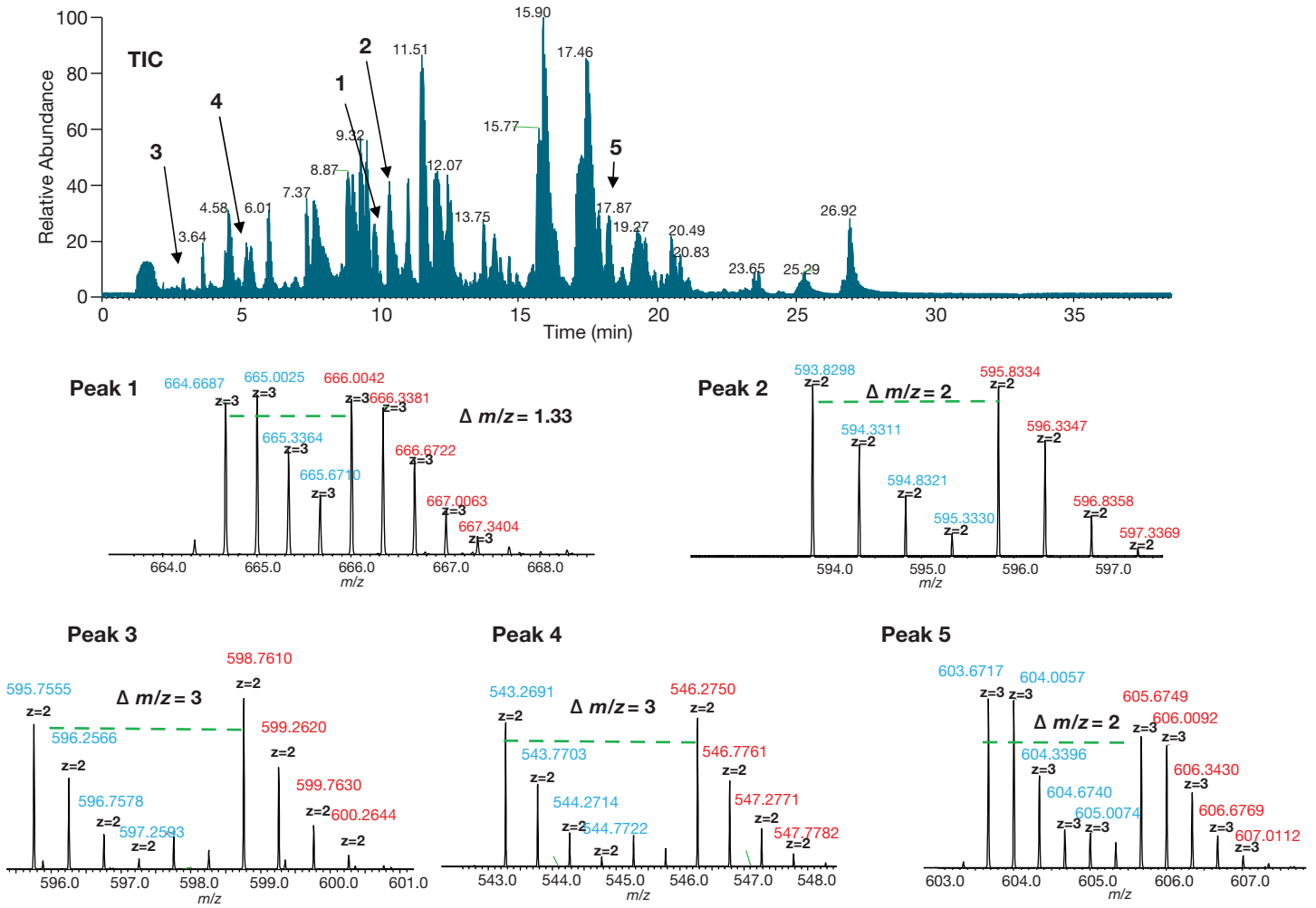


Figure 1. Labeling with $H_2{}^{18}O$ discriminates masses and helps identify glycosylation and deamidation sites.

Table 3. Assignment of deamidation and glycosylation based on mass shift

Peaks	Peptide sequence	Theoretical *m/z*	Experimental *m/z*	Accuracy (ppm)	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	6.56	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

In contrast, 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. It is important to note that in both peptides, a glycine (G) is located next to asparagine (N). It is well known, that deamidation proceeds faster if the amino acid susceptible to conversion is followed by a small, flexible residue like glycine, which decreases the steric hindrance and exposes the asparagine for conversion.[2] 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 Figures 2–4 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).

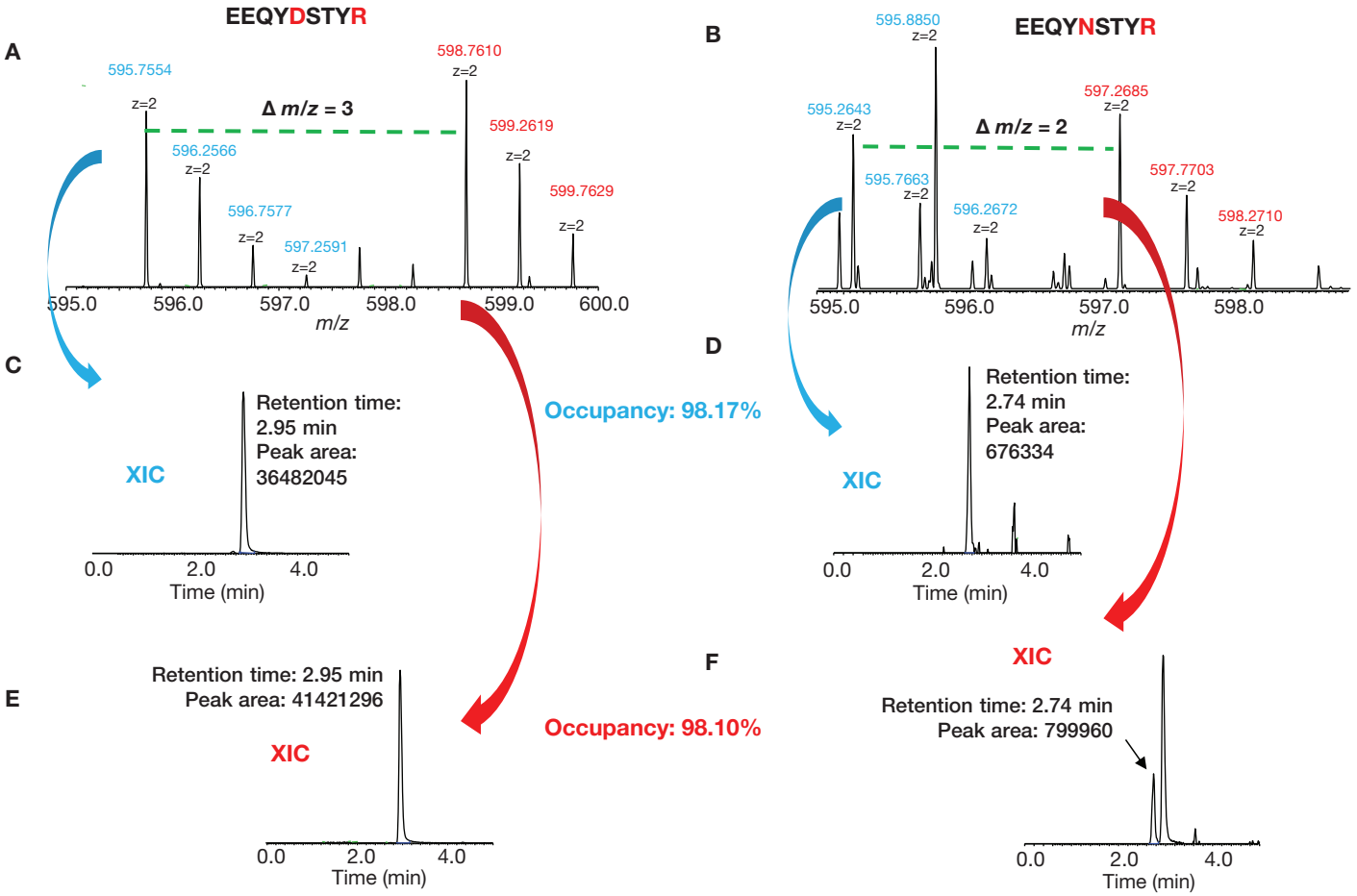


Figure 2. Calculation of site occupancy of N300 in trastuzumab

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 displayed by Figures 2–4 and listed in Table 4. %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 4. Retention times of EEQYDSTYR and EEQYNSTYR peptides in the three mAbs

	Retention time of EEQYDSTYR	Retention time of EEQYNSTYR
Trastuzumab	2.95 min.	2.74 min
Rituximab	2.94 min	2.73 min
Fab glycosylated mAb	2.93 min	2.72 min

Figures 2–4/A show the doublets after deglycosylation with the difference of 6 Da between the light and heavy isotopologue. Figures 2–4/C are the XIC of the light version of these peptide, Figures 2–4/E show the XIC of the heavy isotopologue of the deglycosylated peptide. A small portion of the original, asparagine containing peptide was not glycosylated in the therapeutic antibody (Figures 2–4/B). As proven by the mass shift (4 Da), this peptide was only labeled in the C-terminus and not susceptible to chemical deamidation (asparagine is followed by a serine residue with the bulkier hydroxymethyl group).

After generating the XICs from both light and heavy versions (Figures 2–4/D and Figures 2–4/F), corresponding ratios were obtained by dividing deglycosylated peak areas by the summed deglycosylated and unglycosylated areas result in site occupancy. Using heavy and light pairs of deglycosylated/unglycosylated peptides, the site occupancy can be calculated twice from one experiment (twoplexing). Since deamidation and deglycosylation are accompanied by a mass increase of 0.984 Da, such calculations are difficult to achieve without the use of heavy water to discriminate masses and making the modifications more detectable.

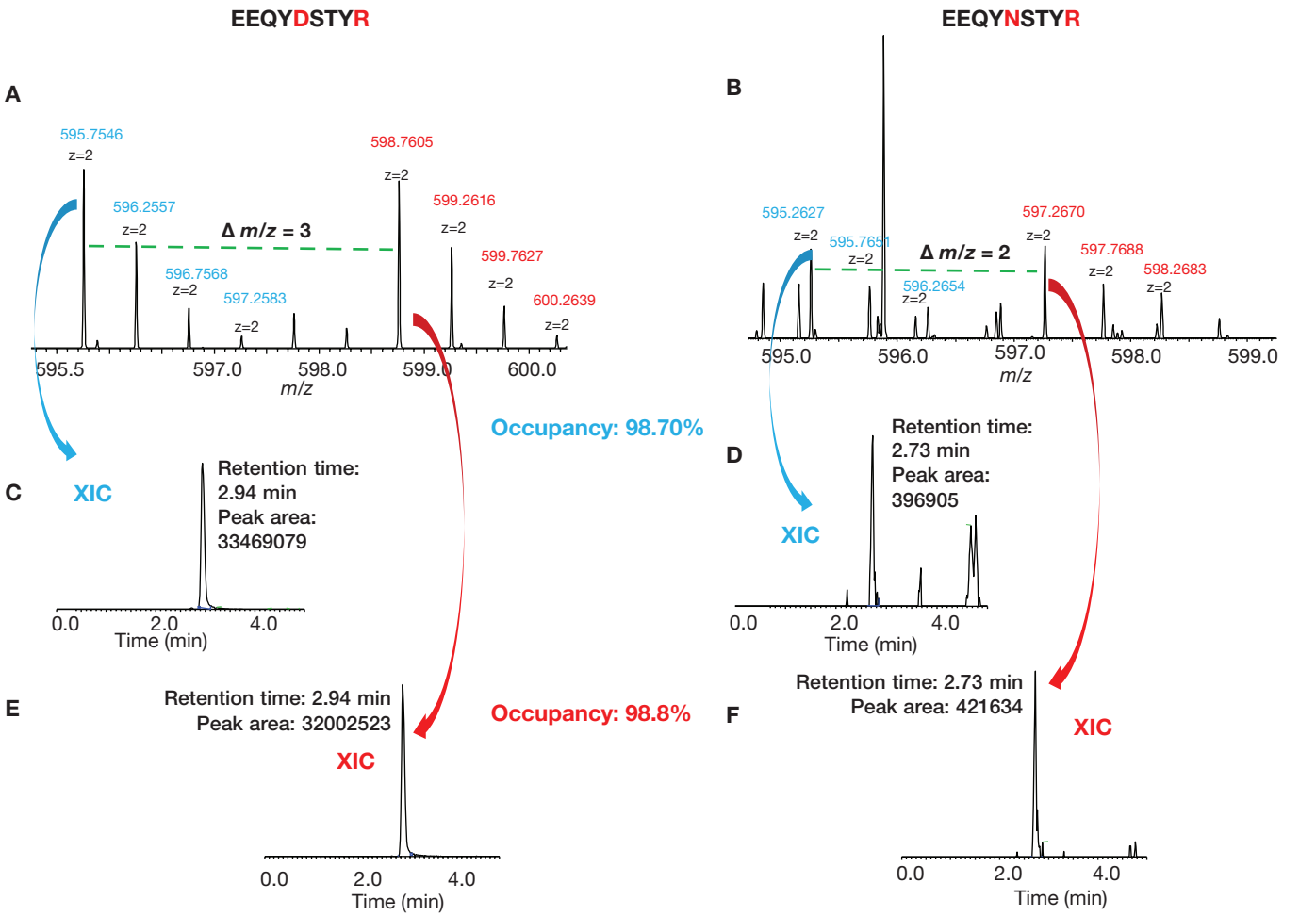


Figure 3. Calculation of site occupancy of N301 in rituximab

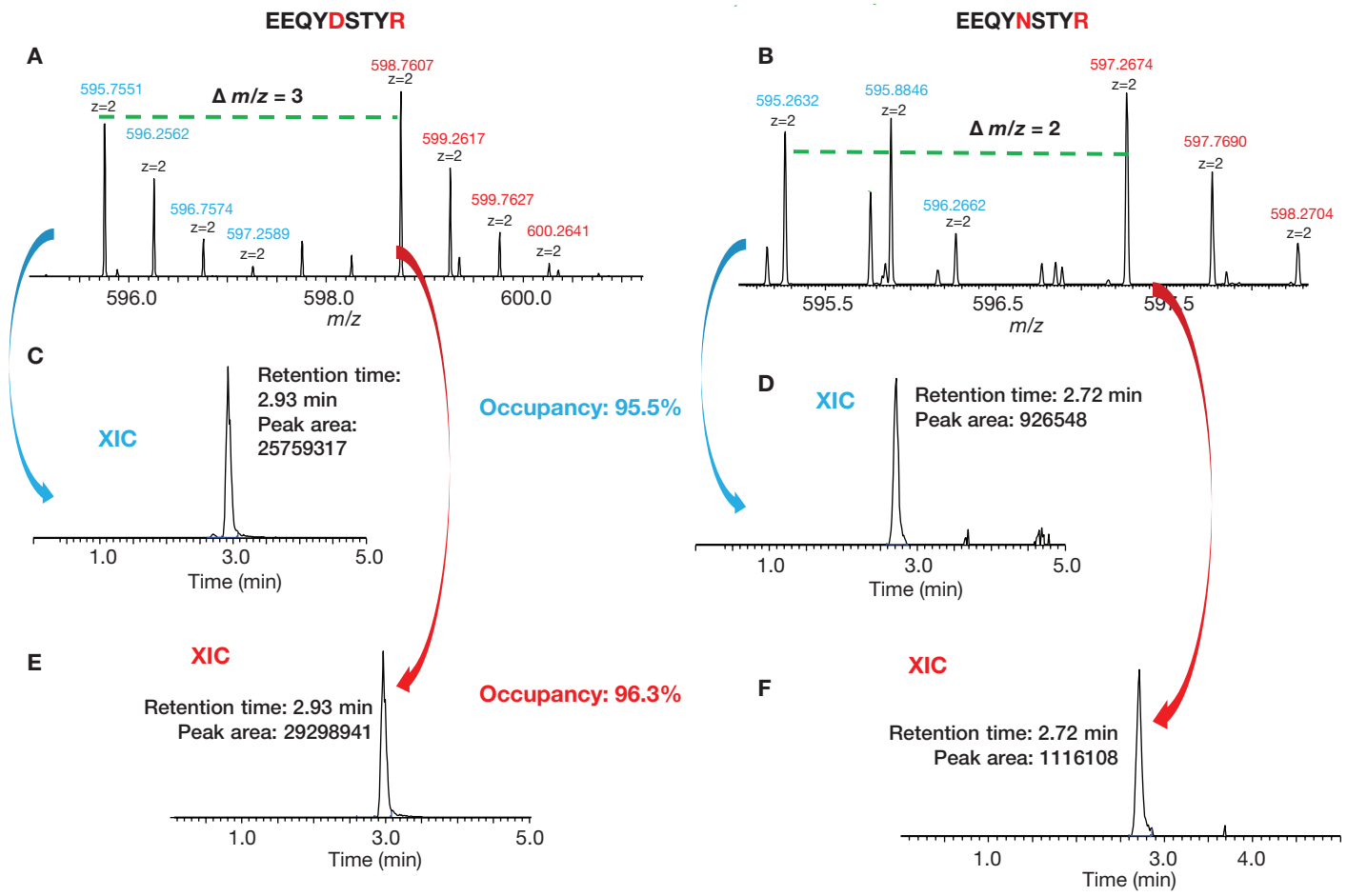


Figure 4. Calculation of site occupancy of N306 in Fab glycosylated mAb

Site occupancy of the Fc glycosylation site is expected to be high and comparable to the values published in literature, wherein MRM was performed to determine site occupancy of therapeutic proteins.[3] Site occupancy values of trastuzumab, rituximab and the Fab glycosylated mAb on the Fc portion were calculated from both the light and heavy glycosylated/unglycosylated peptide pairs of the same experiment and compared against the data reported by literature, except for Fab glycosylated mAb (Table 5).

Table 5. Fc site occupancy of mAbs determined by different approaches

mAb	Site occupancy determined by MRM	Site occupancy determined by peptide mapping
Trastuzumab	99.02	98.13
Rituximab	99.57	98.75
Fab glycosylated mAb	n.a.	96.40

Figure 5 displays the calculation of site occupancy of the N52 glycosylation site located in the variable portion of the Fab. This value was found to be 82.25% (averaging twoplexing data), considerably lower than Fc glycosylation site occupancy. Somewhat similar yet not accurate data from % distribution of glycosylated vs. unglycosylated peptide confirms this funding in so far as 73% of occupancy was calculated. This can only be considered as an approximation due to the higher ionization efficiency of unglycosylated peptides compared to glycosylated ones.

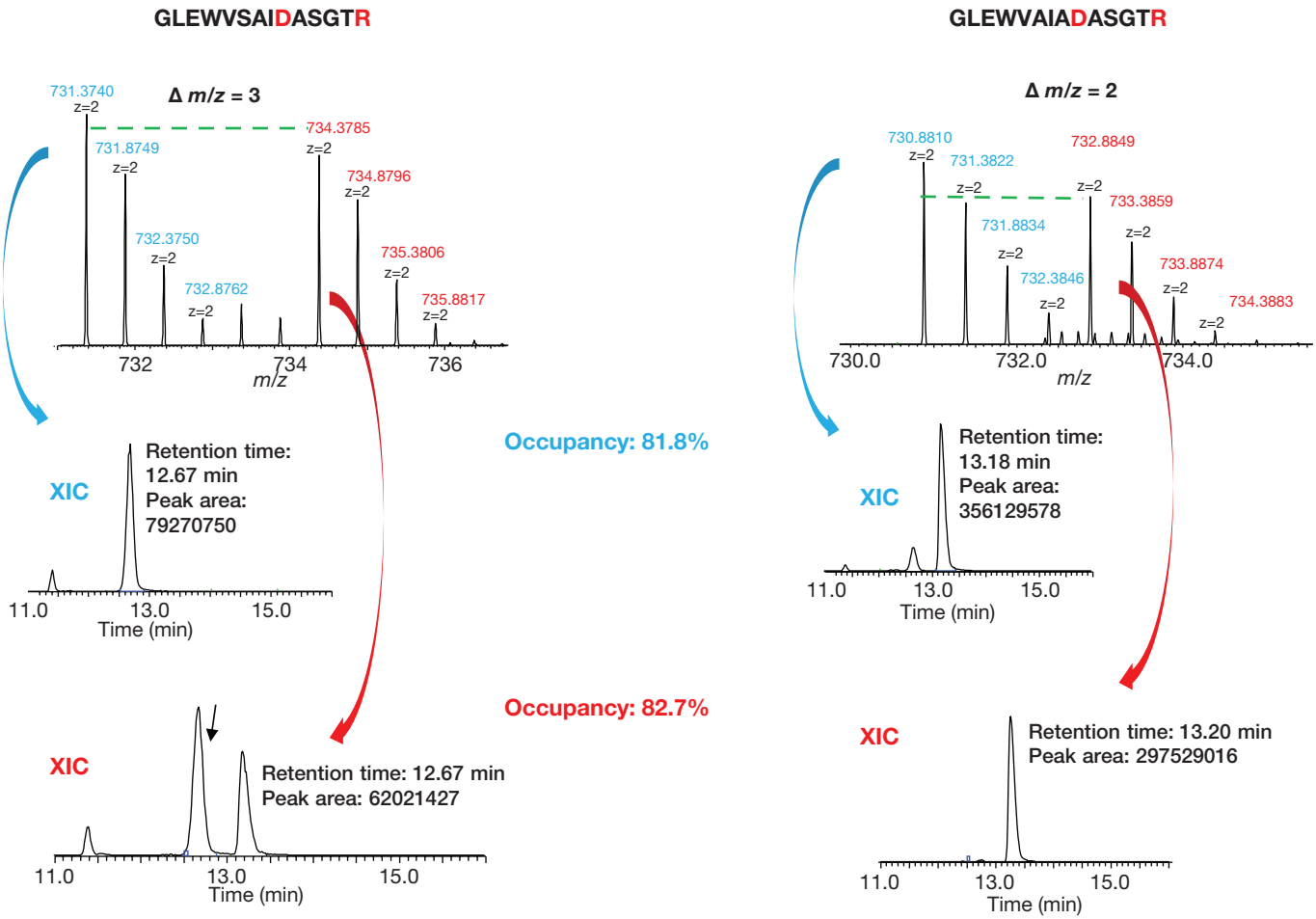


Figure 5. Calculation of site occupancy of N52 in Fab glycosylated mAb

Conclusion

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 is demonstrated 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 (Thermo Scientific™ EASY-Spray™ format or double nanoViper fittings) makes the use and connection of this column very simple for the operator. The short but reproducible separations, as well as the high sensitivity attributable to capillary flow, highlight the applicability of this column in the clone selection and early optimization phases of biopharmaceutical development.

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