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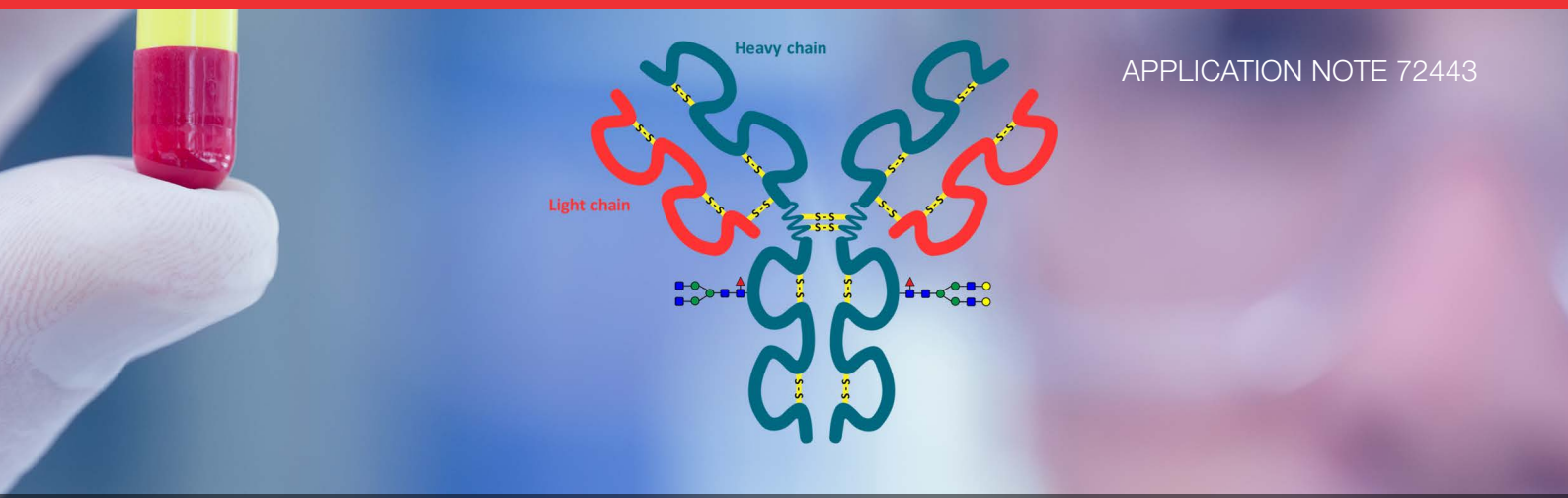

APPLICATION NOTE 72443

HILIC – an alternative separation technique for glycopeptides

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Keywords
Monoclonal antibodies, Accucore, Amide HILIC, Biocompatible UHPLC, SMART Digest, Protein Digest, Biotherapeutics characterization, Biopharma, Vanquish Flex UHPLC

Goal

Demonstrate the suitability of the Thermo Scientific™ Vanquish™ Flex UHPLC system and Thermo Scientific™ Accucore™ 150 Amide HILIC column for efficient and reliable separation of glycopeptides.

Introduction

In this work, an approach to efficiently separate peptides and glycopeptides of a monoclonal antibody (mAb) in a single run is demonstrated. The separation was achieved using hydrophilic interaction liquid chromatography (HILIC) with the Accucore 150 Amide HILIC column. Peptides and glycopeptides were obtained by digesting Infliximab using the Thermo Scientific™ SMART Digest™ kit. The reproducibility of the glycopeptide peaks was evaluated for repeated injections.

Peptide mapping is one of the routine methods for biotherapeutics characterization. This technique, combined with mass spectrometry, is utilized in research environments for the determination of the primary sequence of a mAb and the identification of post-translational modifications (PTMs). Glycosylation affects the potency and efficacy of the biotherapeutic.

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Therefore, during production process optimization, the levels of critical glycosylations are monitored and used as feedback to facilitate development, in order to acheive a given glycan pattern.

After enzymatic digestion, peptide mapping is typically run by reversed-phase (RP) chromatography.[1] However, unlike RP chromatography—which provides very low retention of glycopeptides—HILIC offers significantly more retention for glycopeptides than peptides. This results in a very distinct elution profile between the two, allowing for considerably more resolution than RP. In this study, a HILIC-UV/FLD method was developed and glycan identification was performed with LC-MS experiments. HILIC, paired with UV or fluorescence detection, provides a robust and unique detection tool for routine analysis (e.g. glycoform stability studies)—with retention times as qualitative and peak area as quantitative information.

Remsima® and Inflectra® were the first mAb biosimilars to be approved in the European Union. Remsima and Inflectra are both infliximab biosimilars to the originator Remicade® (Janssen).[2] During their evaluation, as per the ICH Q6B and EMA guidelines[3], these mAbs had to meet a significant number of strict criteria to be granted biosimilarity by the EMA (European Medicines Agency).[4] The evaluation of the glycosolated sites found on the heavy chain of mAbs are among the most critical to characterize, as glycosylation of these sites affects the potency and efficacy of the biotherapeutic.

Many conditions during the up- and downstream processing affect the manifestation of PTMs. Glycosylation is characterized with an array of chromatographic techniques. Depending on the scope of the analysis, glycans may be cleaved from the protein and then analyzed either natively or after fluorescent labelling, mostly with MS or fluorescence detection, respectively. Additionally, glycans can be analyzed at the glycopeptide level after enzymatic digestion of the protein with MS detection.

The column was operated by the Vanquish Flex Quaternary UHPLC system. The Vanquish Flex Quaternary system features a low pressure mixing pump for highest application flexibility. In addition, all Vanquish UHPLC systems feature SmartInject technology. SmartInject technology significantly improves the retention time precision, thereby increasing the confidence in peak assignment.[5]

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Thermo Scientific Accucore Amide HILIC, 2.6 µm, 150 Å, 2.1 × 150 mm (P/N 16726-1520130)
- Thermo Scientific SMART Digest kit (P/N 60109-101)
- Thermo Scientific™ Pierce™ DTT (Dithiothreitol), No-Weigh™ Format (P/N 20291)
- Thermo Scientific™ Pierce™ C18 spin columns (P/N 89870)
- Thermo Scientific™ formic acid, LC-MS Grade (for pH adjustment) (P/N 85178)
- Thermo Scientific™ 9 mm MS certified clear screw thread kit: (P/N C4000-LV1W)
- Sample vials, with insert and 9 mm vial screw caps with pre-assembled septa
- Fisher Scientific™ trifluoroacetic acid, Optima™ LC-MS Grade (P/N 1015347)
- Fisher Scientific™ ammonium formate, Optima™ LC-MS Grade (P/N A11550)
- Fisher Scientific™ LC-MS grade acetonitrile (P/N A955-212)
- Fisher Scientific™ Fisherbrand™ Premium Microcentrifuge Tubes: 1.5 mL (P/N 05-408-129)

Recommended lab equipment

- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)
- Virtuoso 9 mm Wide Opening SureStop™ Screw Thread Vial Convenience Kit (P/N 60180-VT405)
- Thermo Scientific™ Digital Heating Shaking Drybath (P/N 88880028)
- Thermo Scientific™ Orion Star™ A211 pH Benchtop Meter (P/N1 3-645-519)
- Fisher Scientific™ Microcentrifuge (Benchtop) (P/N 3722L)
- Fisher Scientific™ Fisherbrand™ Mini Vortex Mixer (P/N 14-955-152)

Sample preparation

A commercially available mAb, infliximab drug product (Hospira UK Limited, Leamington Spa, United Kingdom), was supplied at a concentration of 10 mg/mL in formulation buffer. The sample was digested for 45 minutes at 70°C at 1,200 rpm using the Heating Shaking Drybath in conjunction with the SMART Digest kit. Upon completion of the enzymatic digestion, the peptides were reduced of all disulfide linkages by the addition of 5 mM DTT in the final volume. Reduction took place for thirty minutes at room temperature under cover from any source of light radiation. A solid phase extraction purification of the reduced peptides was performed using the C-18 Spin Columns. After each column was washed with 50% acetonitrile and equilibrated with 5% acetonitrile with 0.5% trifluoroacetic acid (TFA), the samples were loaded (150 µL per column) and washed with 5% acetonitrile with 0.5% TFA to remove the SMART Digest buffer and excess DTT. The peptides were finally eluted and solvated in 80% acetonitrile, which provided a sufficiently non-polar solution, suitable for HILIC chromatographic starting conditions allowing effective loading retention and subsequent gradient separation. Diluted samples were aliquoted into sample vials and stored at 4°C in the Vanquish autosampler prior to analysis.

Instrumentation

The separation was achieved in HILIC mode by using the Accucore 150 Amide HILIC column. The column was operated by the Vanquish Flex Quaternary UHPLC system (Table 1 and Table 2). Detection was performed using the Thermo Scientific™ Vanquish™ Diode Array Detector HL with a LightPipe™ 10 mm standard flow cell or the Thermo Scientific™ Vanquish™ Fluorescence Detector F with a 2 µL micro bio flow cell.

- The Vanquish Flex UHPLC system consisted of the following:
  - Flex System Base (P/N VF-S01-A)
  - Quaternary Pump (P/N VF-P20-A)
  - Column Compartment H (P/N VH-C10-A)
  - Split Sampler FT (P/N VF-A10-A) with 25 µL and 100 µL sample loops
  - Diode Array Detector HL (P/N VH-D10-A) with LightPipe 10 mm standard flow cell (P/N 6083.0100)
  - Fluorescence Detector F (P/N VF-D50-A) with 2 µL micro bio flow cell (P/N 6079.4330)
  - Static Mixer for 200 µL mixing volume (P/N 6044.5110)

The Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer was used for MS detection. The detailed MS source and method parameters are given in Tables 3 and Table 4.

Separation conditions

Table 1. Chromatographic conditions.

UHPLC Experimental Conditions	
Column:	Accucore Amide 150 Å HILIC 2.1 × 150 mm
Mobile Phase:	A: Acetonitrile/water (90:10 v/v) with 10 mM ammonium formate B: 10 mM Ammonium formate, pH 4.4 Buffers filtered through 0.2 µm filter membrane before use.
Gradient:	Full & Short as described in Table 2.
Flow Rate:	0.5 mL/min
Temperature:	50°C still-air
Injection Volume:	24–50 µL of ~ 0.25 µg/µL digested infliximab sample
UV Detection:	280 nm, DAD
FLD Detection:	280 nm excitation 304 nm emission (filter wheel, auto)

Table 2. Chromatographic gradient conditions.

Gradient (Full)		Gradient (Short)	
Time (min)	B	Time (min)	B
0	10%	0	25%
45	60%	1	30%
46	80%	15	35%
50	80%	16	80%
51	10%	20	80%
70	10%	21	25%
		40	25%

Table 3. Mass spectrometer source conditions.

Source:	Ion Max source with HESI-II probe
Sheath Gas Pressure:	25 psi
Auxiliary Gas Flow:	10 arbitrary units
Probe Heater Temperature:	350°C
Capillary Temperature:	320°C
S-Lens RF Voltage:	60 V
Source Voltage:	3.5 kV

Table 4A. Mass spectrometer conditions (full MS).

Full MS Parameters	
Full MS Mass Range:	*m/z* 400–2000
Resolution Settings:	120,000 (FWHM at *m/z* 200)
Target Value:	3e6
Max Injection Time:	100 ms
Default Charge State:	2
SID:	0 eV

Table 4B. Mass spectrometer conditions (MS^2).

MS^2 Parameters	
Resolution Settings:	15,000 (FWHM at *m/z* 200)
Target Value:	5e5
Isolation Width:	2.0 *m/z*
Signal Threshold:	1e4
Normalized Collision Energy (HCD):	27
Top-N MS2:	3
Max Injection Time:	250 ms
Dynamic Exclusion:	5.0 s

Data processing

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), version 7.2 SR5, was used for data acquisition and Thermo Scientific™ BioPharma Finder™ software, version 2.0, was used for data analysis.

Results and discussion

The separation of a tryptic digest of infliximab was obtained with a 45 minute gradient and a total analysis time of 75 minutes, including column wash with high buffer content and re-equilibration at initial conditions. Figure 1 shows a UV chromatogram with an overlay of five subsequent 24 µL injections of infliximab tryptic digest, with the glycopeptide region highlighted between 28.0 and 38.0 minutes. Note the separation of the two structural isomers A2G1Fa and A2G1Fb at approximately 32.0 to 32.5 minutes. The repeatability was assessed using the retention time standard deviation (SD) and relative standard deviation (RSD) of all glycopeptides automatically calculated by Chromeleon CDS. The RSD was below 0.05% for all four main glycopeptides (Table 5). All retention time SDs were in the range of 0.013 to 0.017 minutes. This data shows excellent flow delivery and composition precision of a long shallow gradient. It has been previously demonstrated[5,6] that with repeatable gradient delivery as well as improved retention time and high area precision, LC-UV-MS (or LC-FLD-MS) can be used to identify and quantitate peptide mapping results, which can then be easily transferred to an LC-UV (or LC-FLD) QA/QC stability monitoring application. Of course, if FLD is to be used, it is important to ensure that the monitored peptides contain at least one of the three fluorescent amino acids (tryptophan, tyrosine, or phenylalanine).

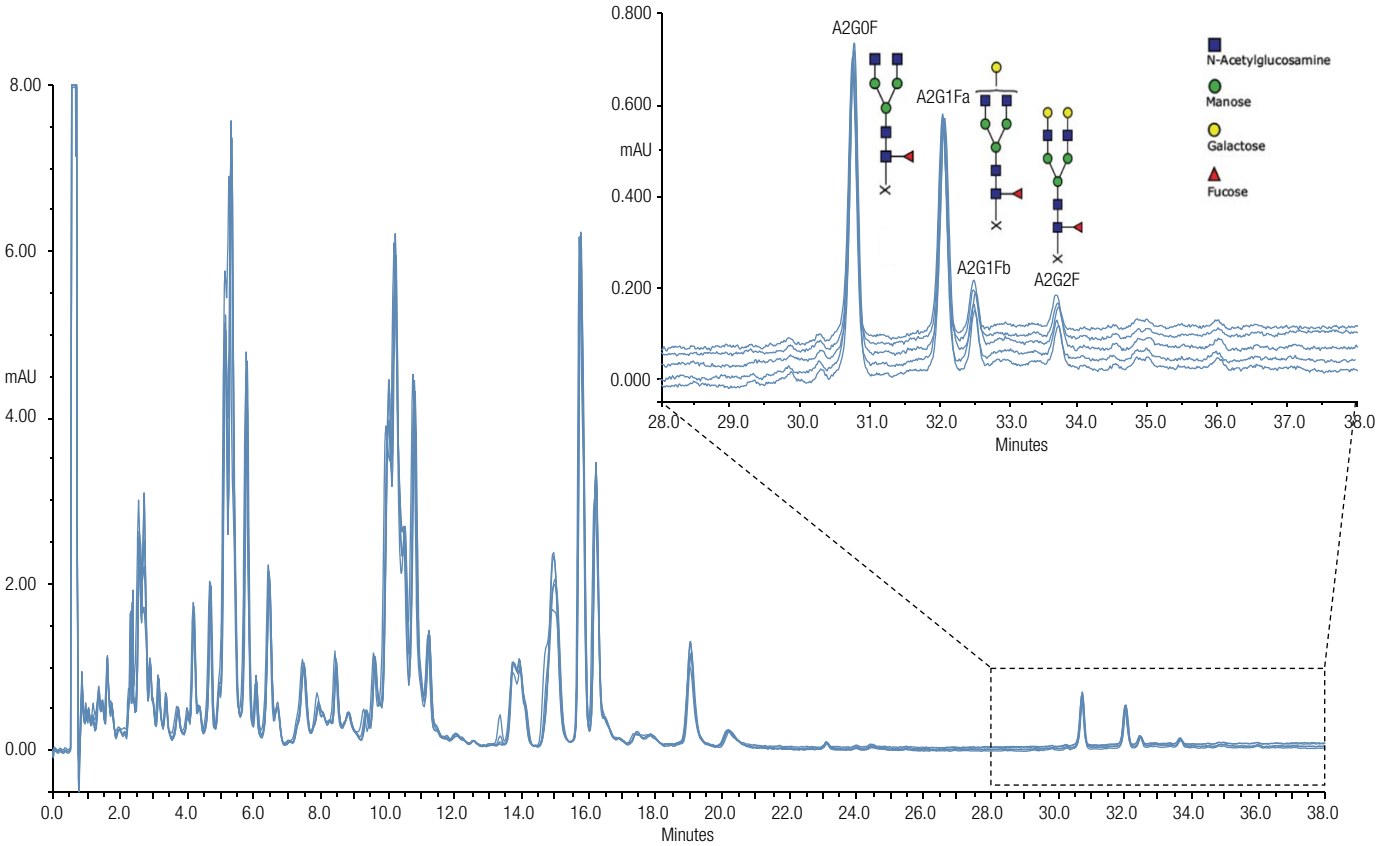


Figure 1. UV chromatogram overlay of five subsequent (24 µL) injections of infliximab tryptic digest, highlighting the excellent retention time precision and the distinct glycopeptide region showing the four most abundant glycoforms.

Table 5. Table of retention time standard deviations of a Vanquish Flex system for the glycopeptide analysis of an Infliximab tryptic digest

Peak	Average Retention Time (min)	Standard Deviation of Retention Time (min)	Relative Standard Deviation of Retention Time (%)
A2G0F	30.761	0.013	0.042
A2G1Fa	32.058	0.016	0.050
A2G1Fb	32.503	0.017	0.052
A2G2F	33.701	0.015	0.045

An optimized short gradient method (Table 2) was also developed to enable the efficient separation of the glycopeptides in the corresponding region of the chromatogram with a significant loss in resolution of the non-glycosylated peptides. Figure 2 shows non-glycosylated peptides eluting between 0.0 and 7.0 minutes followed by the glycopeptide region starting at 7.0 minutes. The short gradient allows the glycosylation to be characterized in a shorter time, saving time and solvents, without a loss in glycopeptide peak resolution.

For further identification of the glycan structures, a 100 µL loop was installed and injections of 50 µL were loaded onto the column. This allowed more peaks to be resolved with UV and sufficient sensitivity for MS1 and MS2 identification. The corresponding UV trace and extracted ion chromatograms (XIC) are shown in Figure 3. Glycopeptides were analysed using high-energy collision dissociation (HCD) fragment ion spectra, which contain ions exclusively representing sequential loss of glycan residues and no fragment ions representing the decomposition of the peptide. All 18 glycopeptide peaks could be identified on the MS1 as well as MS2 level using the Biopharma Finder software (Table 6) and are visualized with XICs in Figure 3. All peaks could also be detected with UV, however quantitation is limited to the four most abundant peaks in the chromatogram.

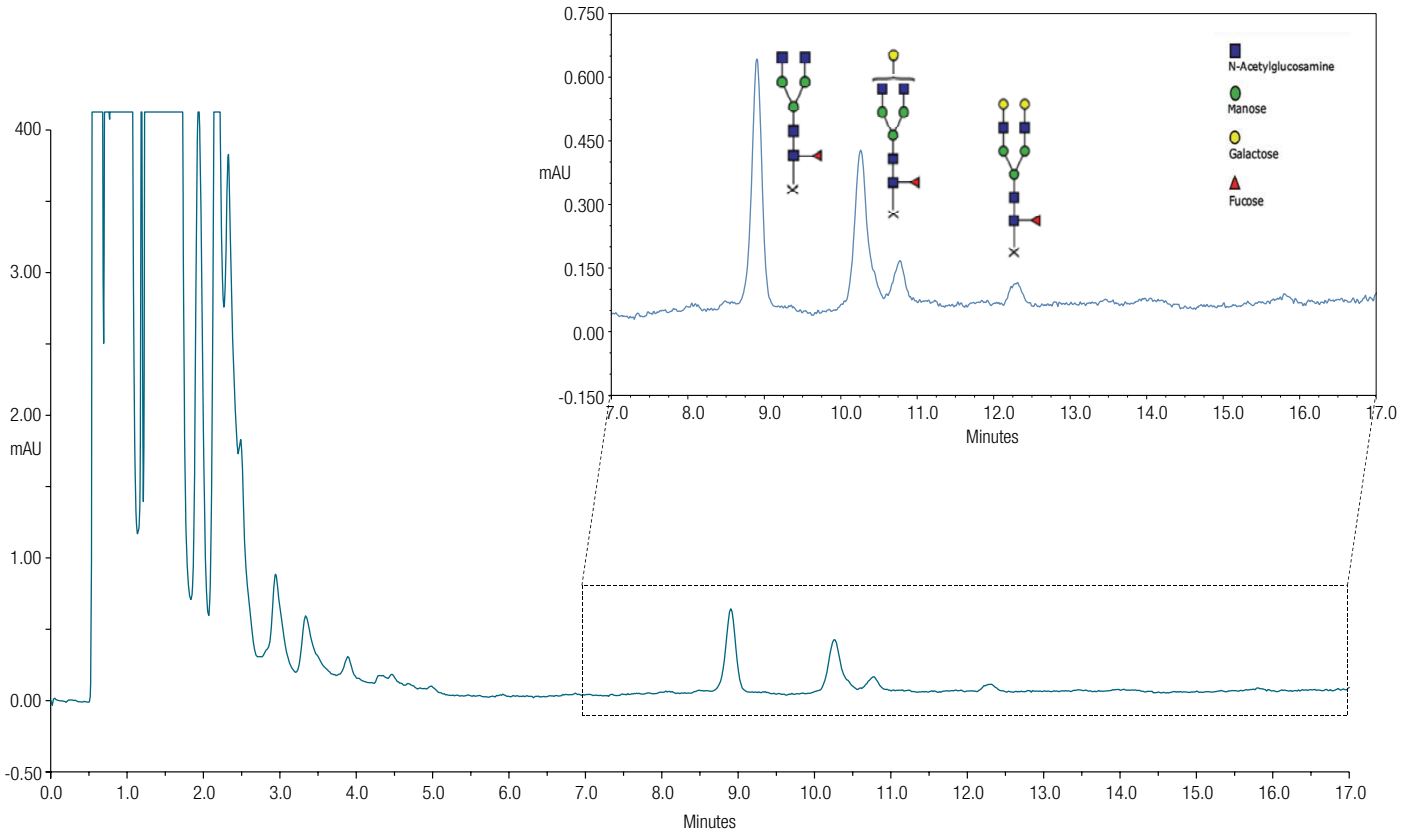


Figure 2. Chromatogram illustrating the short gradient separation of the glycopeptides from the non-glycosylated peptides (24 µL injection).

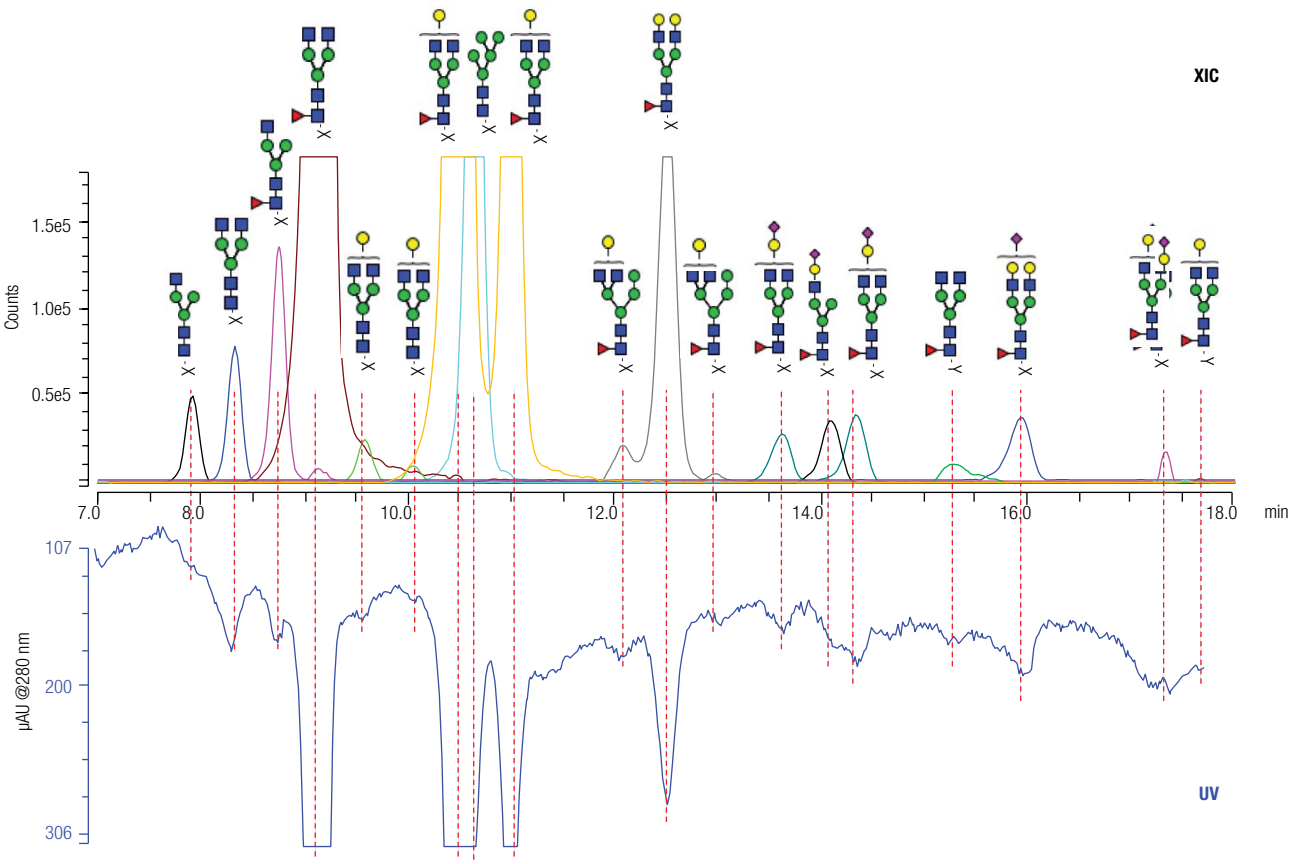
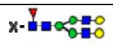
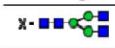
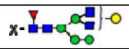
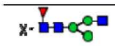
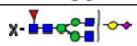
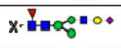
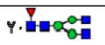
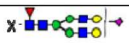
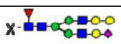
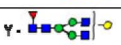
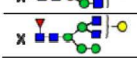


Figure 3. A mirrored overlay of XIC (top) and UV (bottom) detection methods. XIC and UV resulting after 50 µL on-column injections of infliximab tryptic digest, both utilizing the short gradient. Peptide X= EEQYNSTYR and (missed-cleaved) peptide Y = TKPREEQYNSTYR.

Table 6. Glycan structure, name, retention time, and their *m/z* detected.

Structure	Glycan	M+2H	Structure	Glycan	M+2H
	A1G0	1142.9614		A2G2F	1479.5829
	A2G0	1244.5010		A2G1M4F	1479.5839
	A1G0F	1215.9895		A2Sg1G0F	1552.1024
	A2G0F	1317.5295		A1Sg1F	1450.5619
	A2G1	1325.5273		A2Sg1G0F	1552.1024
	A2G1	1325.5273		A2G0Fmc	1558.6803
	A2G1F	1398.5566		A2Sg1G1F	1633.1296
	M5	1203.4742		A2Sg1Ga1F	1714.6578
	A2G1F	1398.5566		A2G1Fmc	1720.7315
	A2G1M4F	1479.5839			

LC-UV or LC-FLD can be used when in-depth characterization is not needed (or has already been performed), for instance in stability studies. The data interpretation of these experiments is based on retention time as qualitative and peak area as quantitative information. For this reason—paired with the fact that the tryptic digest of Infliximab contains glycopeptides with fluorescent amino acids (Y - tyrosine)—fluorescence detection was also performed to compare to the LC-UV data. It is easy to overlook that peptides containing tryptophan, tyrosine or phenylalanine can be detected with FLD as well as with UV. The main advantage of FLD over UV detection is increased signal-to-noise at low analyte concentration in addition to a homogeneous response relative to the number of a specific fluorescent amino acid in the peptide sequence. This equates to increased signal for lower abundance peaks and—in particular in this case—since each glycopeptide contains two tyrosine amino acids, enables accurate quantitation using the FLD detector. UV detection is more generally applicable, however the important difference between the homogeneous response of the FLD compared to the inhomogeneous response of UV (because different molecules and functional groups absorb UV light to varying degrees) can be beneficial and should be understood. For this reason, fluorescence detection can be of particular use in many biopharma applications where homogeneous response is needed for quantitation. Figure 4 shows an overlay of two 24 µL injections, one with UV and the other with FLD detection. It is clear that the signal-to-noise ratio is larger for the FLD when compared to the UV detector.

Given the excellent precision displayed with this method, you can have confidence in your qualitative and quantitative information gathered with LC-UV or LC-FLD for the four main glycans, without the need for continued MS (Figures 1, 2, and 4).

As can be seen in Figure 4, when the signal intensities are normalized between FLD and UV detectors for the main four peaks (noting the slightly different responses between the homogeneous and inhomogeneous detectors as described earlier), it is clear that the level of noise is considerably higher for the UV detector. This means that after peaks have been confidently identified using MS, additional peaks can be confidently monitored in stability studies with FLD compared to UV detection.

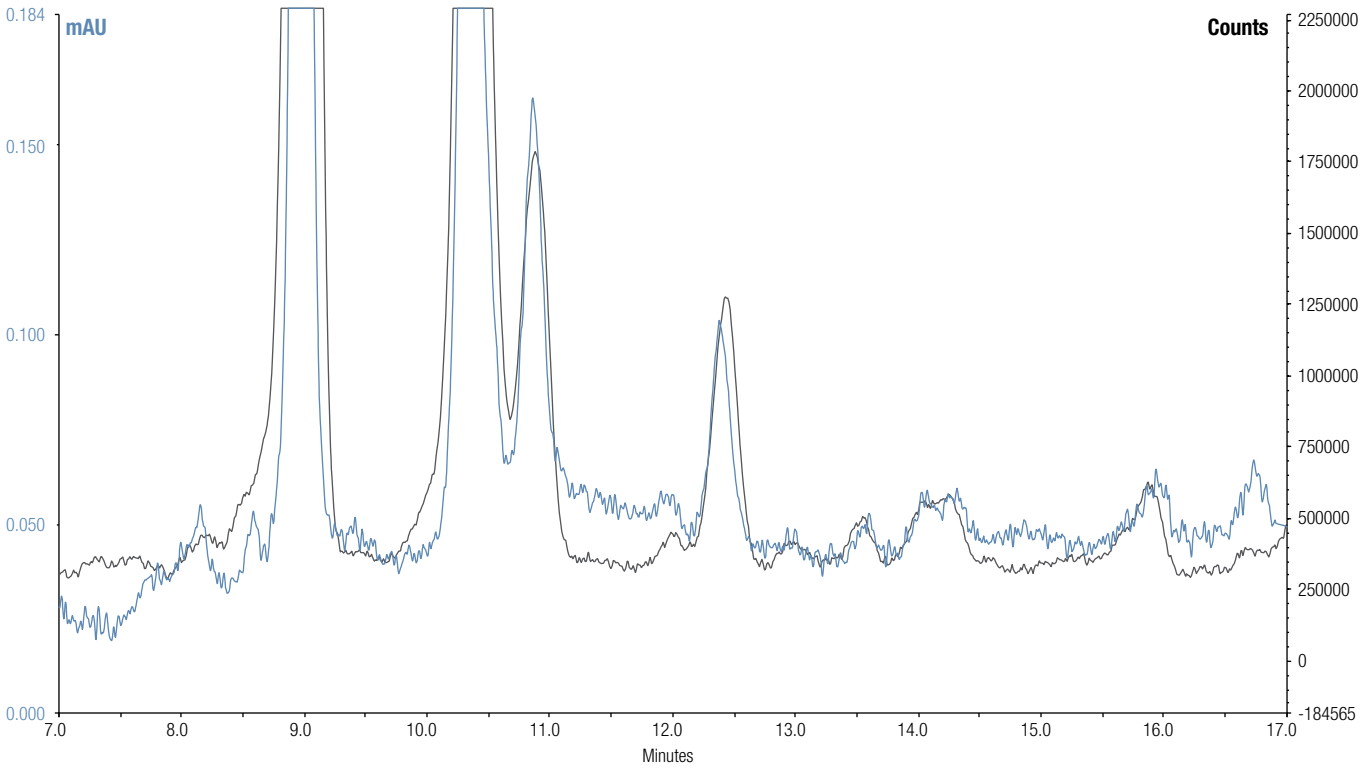


Figure 4. An overlay of UV (blue) and FLD (black) detection methods, both after 24 µL on-column injections of infliximab tryptic digest, both utilizing the short gradient.

Conclusion

- The Vanquish Flex UHPLC system combined with UV or FLD detection and coupled to the Q Exactive HF MS, provides a robust LC-MS setup to characterize and monitor glycosylation on the peptide level of mAbs.
- Using reversed-phase (RP) chromatography, the differences in hydrophobicity are negligible between two glycopeptides with the same amino-acid structure, leading to poor separation. HILIC mode has the considerable advantage in the separation of glycopeptides when compared to traditional RP chromatography, because differences in a given peptide's hydrophilicity—due to differing glycan structures—can be separated with increased resolution. Additionally, with HILIC mode the glycosylated peptides are well separated from the unglycosylated peptides because of the significant difference in hydrophilicity. This allows the unglycosylated peptides to be discarded, by means of method optimization, reducing analysis time and increasing throughput without a loss of resolution.
- A similar glyco-profile was achieved, with a much simplified sample preparation effort (when compared to labelled glycan analysis).
- This method is widely appropriate for the analysis of N-glycans found in many mAbs. However, for more complex glycoproteins (e.g. Enbrel® - etanercept)—which contain many sites of glycosylation including N- and O-linked glycans—may be too complicated to interpret. In these situations, it is worth considering released N-glycan analysis and subsequent O-glycan analysis or consider a post-digestion middle-down mass spectrometry approach.
- The effective glycopeptide separation from other non-glycosylated tryptic peptides of a monoclonal antibody (mAb) has been shown with the use of the Accucore 150 Amide HILIC column. The column provides an effective mode to separate the glycoforms based on their hydrophilic interactions.

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