

Subunits analysis approach for the determination of fucosylation levels in monoclonal antibodies using LC-HRAM-MS

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Keywords

NIBRT, biopharmaceutical, biotherapeutic, monoclonal antibody (mAb), IgG, IdeS, EndoS, biosimilar, intact protein analysis, glycan analysis, fucosylation, MAbPac RP columns, Vanquish Flex Binary UHPLC System, Virtuoso Vial Identification System, Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer, BioPharma Finder

Application benefits

- Demonstrate the benefits of using middle-up analytical methods for the simultaneous quantification of mAb core afucosylation and high-mannose type glycans, which affect efficacy and drug clearance
- Demonstrate the applicability of Thermo Scientific™ MAbPac™ RP columns for mAbs subunits analysis
- Highlight the benefits of using the Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer for high-resolution, accurate-mass MS analysis on different domains of monoclonal antibodies

Goal

To highlight middle-up techniques for biotherapeutics *N*-glycan characterization. To show the importance of high-resolution, accurate-mass MS techniques to confidently quantify mAb *N*-glycoforms with a special focus on core afucosylation. To demonstrate that the method is easy to optimize, fast, and reproducible.

Introduction

There is a growing interest in the analysis of recombinant monoclonal antibodies (mAbs) and in the study of their biological interaction mechanisms. *N*-glycans present on the Fc region of the monoclonal antibody can affect monoclonal antibody stability but can also interfere with the intended mechanism of action of the drug. It has been demonstrated that when the monoclonal antibody presents an afucosylated chitobiose core, the affinity of the mAb for the FcγRIII expressed on natural killer cells is enhanced leading to an antibody-dependent cellular cytotoxicity (ADCC).^{1,2} As a consequence, antibody drug engineering has made much effort to enhance this feature, and an accurate quantification of glycan core fucosylation is often required.³

In this study, high-resolution, accurate-mass spectrometry (LC-HRAM-MS) was used in a middle-up approach for the quantification of core afucosylation on three mAbs. Samples were digested with IdeS and EndoS enzymes in non-reducing conditions. IdeS enzyme action cleaves the monoclonal antibody in the hinge region and generates two polypeptides (F(ab')₂ and 2 x scFc regions). EndoS enzyme specifically cleaves the chitobiose core leaving on the polypeptide an N-acetyl glucosamine with or without the core fucose (Figure 1). Moreover, EndoS showed a specific activity for complex glycan and does not digest high mannose type glycans.⁴ The use of LC-HRAM-MS allows an accurate quantification of core afucosylation.

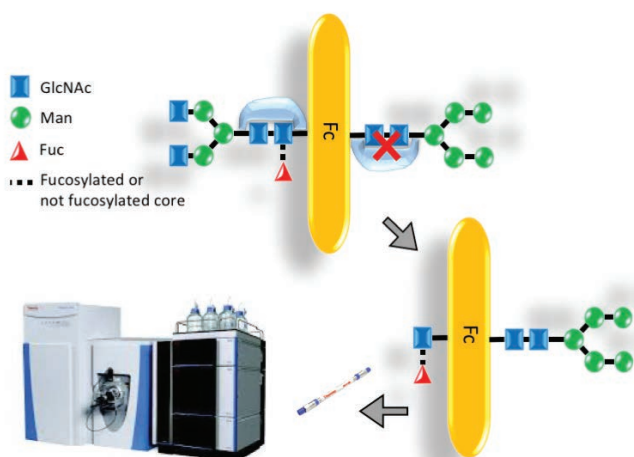


Figure 1. EndoS mechanism of action on Fc *N*-glycans. Complex type *N*-glycans are digested at chitobiose core level, leaving unaltered the linkage with core fucose, where present. EndoS is highly specific for complex type *N*-glycan so high-mannose containing glycans are not affected.

All the analyses have been performed on a high-resolution analytical platform consisting of a Thermo Scientific™ Vanquish™ Flex Binary UHPLC and Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer. Since scFc region (~25 KDa) and intact F(ab')₂ region (~100 KDa) are generated, the analysis will involve obtaining MS information from two polypeptides with very different mass range; consequently, different mass settings and tune files need to be used (Tables 2 and 3). In this way, high-quality, high-resolution data is obtained on the scFc region, without losing information from Fab' region.

For the three mAbs (rituximab, bevacizumab, and trastuzumab), both originator and an in-house produced biosimilar were analyzed. Deconvoluted spectra from the six samples were obtained using Thermo Scientific™ BioPharma Finder™ 3.0 software for intact mass analysis.

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ·cm resistivity Water, Fisher Chemical™ Optima™ LC/MS grade (P/N 10505904)
- Water with 0.1% formic acid (v/v), Fisher Chemical™ Optima™ LC/MS grade (P/N 10188164)
- Acetonitrile with 0.1% formic acid (v/v), Fisher Chemical™ Optima™ LC/MS grade (P/N 10118464)
- HiTrap® Protein A (GE Healthcare)
- Ammonium hydrogen carbonate, Acros Organics™ (P/N 393212500)
- FabRICATOR® (Genovis) (P/N A0-FR1-020)
- IgGZERO® (Genovis) (P/N A0-IZ1-010)
- Amicon® spin filter units, 10 KDa MWCO MAbPac RP column, 4 μm, 2.1 × 50 mm (P/N 088648)
- Thermo Scientific™ Virtuoso™ vial, clear 2 mL kit with septa and cap (P/N 60180-VT405)
- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)

Sample handling equipment

- Thermo Scientific™ Vanquish™ Flex Binary UHPLC System including:
 - Binary Pump F (P/N VF-P10-A)
 - Column Compartment H (P/N VH-C10-A)
 - Split Sampler FT (P/N VF-A10-A)
 - System Base Vanquish Horizon (P/N VH-S01-A)
- Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer (P/N IQLAAEGAAPFALGMBDK)
- Thermo Scientific™ Nanodrop™ 2000 Spectrophotometer (P/N ND-2000)

Sample pre-treatment

ExpiCHO-S™ Cells (Gibco, #A29127) were derived from a non-engineered subclone that has been screened and isolated from Chinese hamster ovary (CHO) cells. Cells were cultured in suspension in serum-free, chemically defined media (Gibco), and transiently transfected with plasmid DNA encoding particular monoclonal antibody using lipid-based transfection system (Gibco). The vectors (pFUSEss-CH1g-hG1 and pFUSE2ss-CL1g-hk) were purchased from Invivogen. Following transfection, the cells were harvested, and samples of clarified media were passed through a Protein A column, then washed with phosphate buffered saline before elution of mAb from the Protein A column using 100 mM citric acid, pH 3.2. MAb solutions were buffered exchanged in PBS and protein concentration was evaluated with the Nanodrop 2000 Spectrophotometer.

Sample preparation - subunit analysis of IdeS/EndoS-digested mAb

Forty micrograms of each mAb was diluted to 1 mg/mL using 50 mM ammonium bicarbonate pH 7.0. Then, 0.5 µL of the IdeS enzyme and 2 µL of EndoS enzyme was combined with the mAb solution. Samples were incubated at 37 °C for 2 hours at 500 rpm. Following incubation, samples were reduced to dryness via vacuum centrifugation and reconstitution in 40 µL of MS-grade water with 0.1% FA.

LC conditions

Mobile phase A:	Water with 0.1% formic acid (v/v)
Mobile phase B:	Acetonitrile with 0.1% formic acid (v/v)
Flow rate:	0.3 mL/min
Column:	MabPac RP, 4 µm, 2.1 × 50 mm
Column temperature:	80 °C
Pre-column heater:	80 °C
Autosampler temperature:	5 °C
Injection volume:	1 µL
Injection wash solvent:	Methanol/water, 10:90
Needle wash:	Enabled pre-injection
Gradient:	See Table 1 for details

Table 1. Mobile phase separation for subunit analysis

Time (min)	%A	%B	Curve
0	80	20	5
2	80	20	5
16	55	45	5
17	20	80	5
18	20	80	5
18.5	80	20	5
28.0	80	20	5

MS conditions

Table 2. Summary of used tune parameters

MS Source Parameters	Tune File scFc 0.0 to 10.0 min	Tune File Fab' 10.1 to 28.0 min
Source	HESI	HESI
Sheath gas pressure	25	35
Auxiliary gas flow	10	10
Probe heater temperature	150 °C	175 °C
Source voltage	3.6 kV	3.8
Capillary temperature	320 °C	275 °C
S-lens RF voltage	60.0	80.0

Table 3. Summary of used MS parameters

General	scFc	F(ab') ₂
Runtime	0 to 10.00 min	10.01 to 28 min
Polarity	Positive	Positive
Full MS Parameters		
Full MS mass range	600–3000 <i>m/z</i>	1000–4000 <i>m/z</i>
Mode	Protein	HMR
Resolution settings	140,000	70,000
AGC target value	3e6	3e6
Max injection time	200 ms	200 ms
SID	0.0 eV	40.0 eV
Microscans	5	5

MS data processing

Detailed parameter settings are shown in Table 4. Only MS data related to scFc regions were deconvoluted.

Table 4. Biopharma Finder 3.0 software parameter settings for analysis of scFc region after Ides and EndoS digestion

Parameter	Value
Algorithm	Xtract™
Output mass range	10,000–30,000
Output mass	M
S/N threshold	3.00
Rel. abundance threshold	0.00
Charge range	5–50
Min. num. detected charges	3
Isotype table protein	Protein
Fit factor (%)	80
Remainder threshold (%)	25
Consider overlap	Yes
Resolution at 400 <i>m/z</i>	Raw file specific
Charge carrier	H ⁺
Minimum intensity	1
Expected intensity error	3
<i>m/z</i> range	600–3000
Chromatogram trace type	TIC
Sequence matching mass tolerance	20.00 ppm
Mass tolerance	10.0 ppm
RT tolerance	1.000 minutes
Minimum number of required occurrences	1

Results and discussion

Product development in the biopharmaceutical industry is focused on understanding the relationship between drug structure and its function when in contact with the patient. During the complex and numerous mechanisms activated by monoclonal antibody interaction with cells, the role of post-translational modifications (PTMs) is crucial; the exact pattern of modification can determine specific biological activity.⁵ PTMs must be controlled to avoid adverse effects that potentially threaten patient safety but also present an opportunity to manipulate the biological activity in a particular desired direction.

Glycosylation is by far the most complex of the PTMs. For IgG1 mAbs, the glycosylation site is located in the C_H2 domain of the Fc region and plays a critical role in mAb effector functions, which can include complement activation or interaction with FcγR receptors. Core fucosylation has been proved to have a key role in the interaction of mAbs with FcγRIIIA, inducing activation of antibody-dependent cell cytotoxicity (ADCC), activation that is desired when targeting malignant cells.^{1,2} Recently, mAb engineering has focused on the production of low level or completely afucosylated mAbs. Similarly, the levels of high-mannose present on the Fc region of mAbs can influence binding to FcγRs and increase antibody clearance from the serum, decreasing circulating half-life.⁶ For both product development and quality control, accurate and quick monitoring of both fucosylation and high-mannose quantitation is important.

In this study, three biopharmaceuticals (rituximab, trastuzumab, and bevacizumab, (DP)) have been tested and compared with their in-house produced biosimilars (BS). A simultaneous digestion with IdeS and EndoS enzymes produced 2 × scFc regions and an intact F(ab')₂ region; technical triplicates were prepared. Samples were analyzed with a Vanquish Flex Binary UHPLC system equipped with a MabPac RP 4 μm, 2.1 × 50 mm column. A Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer was used for high-resolution, accurate-mass detection (Figure 2).

Due to the different nature and size of scFc and F(ab')₂ regions (~25 KDa and ~100 KDa, respectively), a combined MS method was created with two different MS settings (Table 3) and using two different MS tune files (Table 2). Raw files were processed using BioPharma Finder 3.0 software to deconvolute MS spectra and obtain signal intensity to use for quantitation purposes.

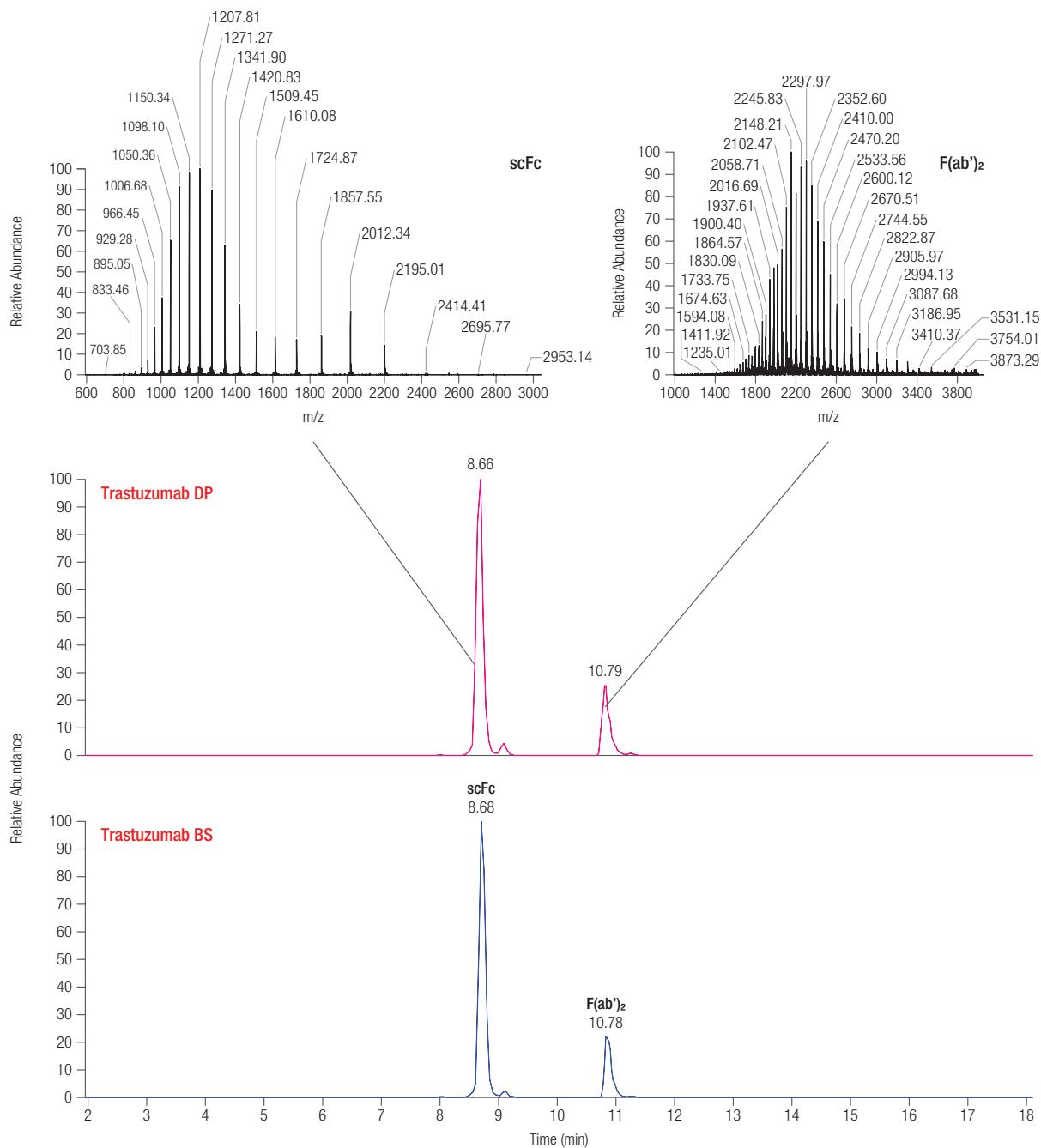


Figure 2. BP (Base Peak) chromatograms of trastuzumab drug product [DP] and biosimilar [BS] after IdeS and EndoS digestion. Separation was obtained on a MabPac RP 4 μ m, 2.1 \times 50 mm column.

Table 5 summarizes the data obtained for both drug product and biosimilar drug for the scFc regions.

The data revealed different species for the scFc regions (Figure 3) derived from EndoS digestion bearing the first *N*-acetylglucosamine of the chitobiose core with or without the core fucose residue. The EndoS enzyme has no effect on high-mannose type glycans, so peaks corresponding to scFc region bearing Man5 or Man6 glycoforms are visible. On the polypeptide backbone, only a low amount of species containing C-terminal lysine

were present. A very small amount of unglycosylated scFc region was present for some of the samples (<0.5%).

As reported in Figures 4, 5, and 6 relative abundancies were calculated using the intensities obtained in the deconvoluted spectra and DP samples are plotted and compared with in-house produced biosimilar.

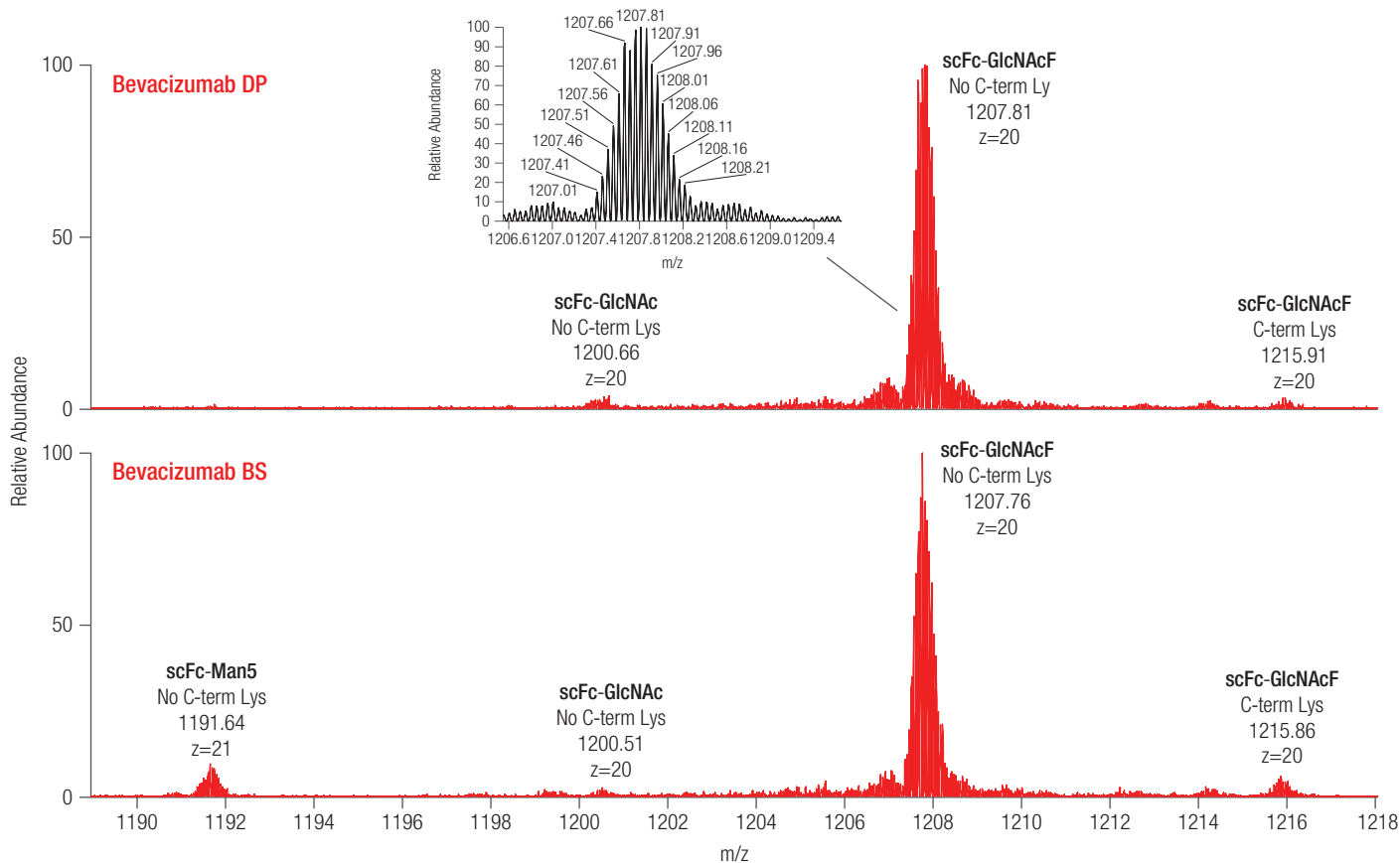


Figure 3. MS spectra from bevacizumab DP and BS scFc regions; zoom between 1190 and 1220 m/z. In DP, three peaks for charge state +20 are visible for the scFc region. The biosimilar mass spectrum shows a peak with charge state +21 that is derived from the scFc region, bearing Man5 N-glycan.

Table 5. Experimental and theoretical masses (Da) obtained for the three investigated drug substances and their biosimilars. Disulfide bonds located on the scFc region have been considered when calculating theoretical masses.

Chain (modifications)	Experimental Mass	Theoretical Monoisotopic Mass	Mass Δ (ppm)	Experimental Mass	Theoretical Monoisotopic Mass	Mass Δ (ppm)
Bevacizumab	Drug Product			Biosimilar		
scFc-GlcNAcF No C-term Lys	24121.0534	24121.0355	0.7	24121.0534	24121.0355	0.7
scFc-GlcNAcF C-term Lys	24249.1191	24249.1305	-0.5	24249.0689	24249.1305	-2.5
scFc-GlcNAc No C-term Lys	23974.9751	23974.9776	-0.1	23974.9501	23974.9776	-1.1
scFc-Man5 No C-term Lys				24988.3171	24988.3211	-0.2
scFc No C-term Lys	23771.8913	23771.8982	-0.3			

Table 5 (continued). Experimental and theoretical masses (Da) obtained for the three investigated drug substances and their biosimilars. Disulfide bonds located on the scFc region have been considered when calculating theoretical masses.

Chain (modifications)	Experimental Mass	Theoretical Monoisotopic Mass	Mass Δ (ppm)	Experimental Mass	Theoretical Monoisotopic Mass	Mass Δ (ppm)
Rituximab	Drug Product			Biosimilar		
scFc-GlcNAcF No C-term Lys	24089.0953	24089.0634	1.3	24121.0718	24121.0355	1.5
scFc-GlcNAcF C-term Lys	24217.1425	24217.1584	-0.7	24249.1316	24249.1305	0.0
scFc-GlcNAc No C-term Lys	23943.0272	23943.0055	0.9	23974.9859	23974.9776	0.3
scFc-Man5 No C-term Lys				24988.3644	24988.3211	1.7
scFc-Man6 No C-term Lys				25150.3826	25150.3739	0.3
scFc-Man5 C-term Lys				25116.4546	25116.4160	1.5
Trastuzumab	Drug Product			Biosimilar		
scFc-GlcNAcF No C-term Lys	24121.0538	24121.0355	0.8	24121.0723	24121.0355	1.5
scFc-GlcNAcF C-term Lys	24249.1164	24249.1305	-0.6			
scFc-GlcNAc No C-term Lys	23974.9968	23974.9776	0.8	23974.9965	23974.9776	0.8
scFc-Man5 No C-term Lys	24988.3353	24988.3211	0.6	24988.3401	24988.3211	0.8
scFc-Man6 No C-term Lys				25150.4324	25150.3739	2.3
scFc No C-term Lys	23771.9178	23771.8982	0.8			

Bevacizumab

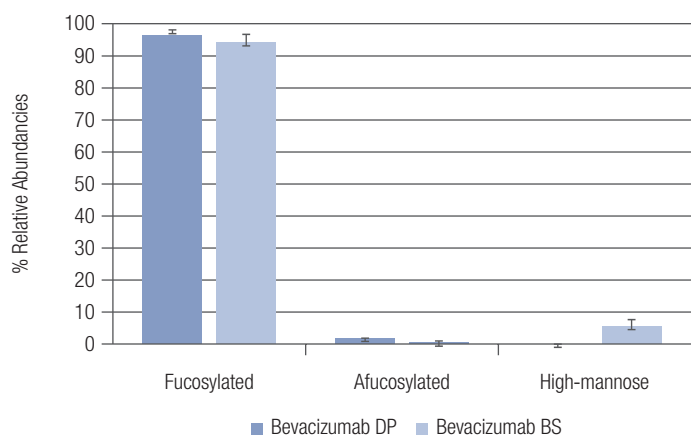


Figure 4. Summary table of fucosylation levels on bevacizumab drug product and biosimilar. Standard deviation was calculated on technical triplicates.

Rituximab

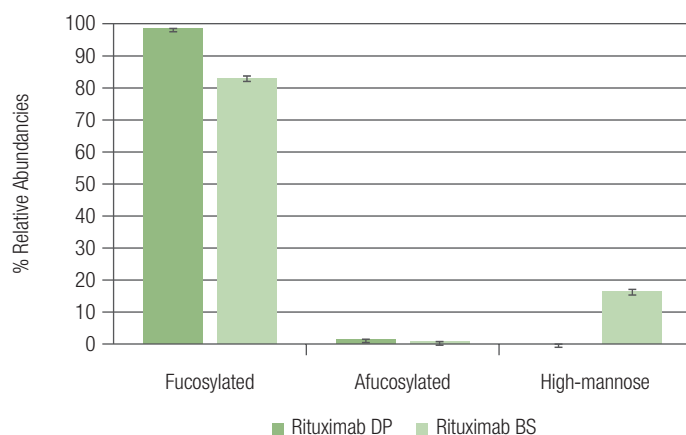


Figure 5. Summary table of fucosylation levels on rituximab drug product and biosimilar. Standard deviation was calculated on technical triplicates.

Trastuzumab



Figure 6. Summary table of fucosylation levels on trastuzumab drug product and biosimilar. Standard deviation was calculated on technical triplicates.

The main difference between drug products and biosimilars is found in high-mannose abundancies. Between the three drug products, high-mannose species were only detected in trastuzumab (0.7% abundance), while in the biosimilars they have relative abundancies ranging from 5% to 16%. These values account for the major difference between DP and BS samples in rituximab and bevacizumab as afucosylated complex *N*-glycan have a minimal contribution to the glycan pool (1.8–0.3%). For trastuzumab DP the percentage of afucosylated *N*-glycan reaches 8% of abundance in contrast with the 0.9% of biosimilar product; for this reason, fucosylated species are more abundant in the BS sample.

Thanks to the workflow we applied in this study, a quick and accurate profile of *N*-glycan afucosylation and mannosylation was obtained. This screening method can help to assess *N*-glycan critical attributes in approximately one hour to help quality check or product development.

Conclusions

- The Q Exactive Biopharma platform equipped with MabPac RP columns resulted in quick and highly accurate data sets on IdeS plus EndoS digested samples.
- IdeS/EndoS digested samples gave an important snapshot of the glycosylation profile of mAbs focusing on the fucosylation level of *N*-glycan core structure with minimal sample preparation compared to glycan release and labeling.
- BioPharma Finder 3.0 software allowed quick deconvolution of the spectra and confident identification of the subunits and their variations, giving accurate quantitative information.

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