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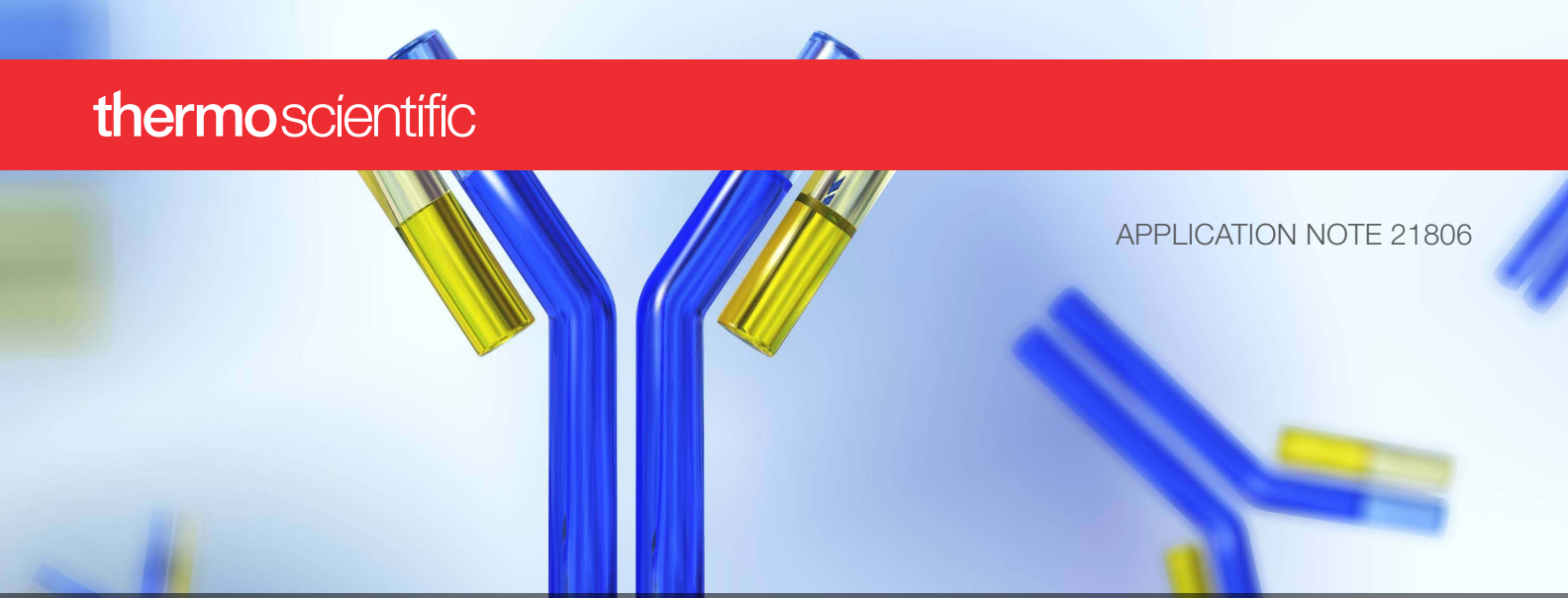

IdeS-cleaved mAb subunit analysis with LC-HRAM-MS: a quick and accurate comparison of biosimilar and originator biotherapeutics

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Keywords

NIBRT, biopharmaceutical, biotherapeutic, monoclonal antibody (mAb), IgG, middle-up, glycan analysis, MAbPac RP columns, Vanquish Flex Binary UHPLC system, Virtuoso Vial Identification System, Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer, IdeS, rituximab, trastuzumab, bevacizumab, BioPharma Finder

Application benefits

- Demonstrate the benefits of using a middle-up approach for biotherapeutics characterization
- Demonstrate the applicability of Thermo Scientific™ MAbPac™ RP columns for subunit 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 subunits of monoclonal antibodies to evaluate glycans and other micro-variants

Goal

To highlight the use of middle-up techniques for biotherapeutics characterization and biosimilar evaluation. To show the importance of high-resolution, accurate-mass MS techniques to characterize mAbs variants at middle-up level, obtaining structural data on glycoforms, lysine truncation and other micro-variants present on the product. To demonstrate the support that subunit analysis can give during product development. To demonstrate that the method is easy to optimize, fast, and reproducible.

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Introduction

Recombinant monoclonal antibodies (mAbs) are the fastest growing class of human therapeutics. Their success in the biopharmaceutical industry and the introduction of a growing number of biosimilar therapeutics go along with the need for reliable and fast characterization methods to establish drug quality and safety. Indeed, a biosimilar is a drug presenting minimal variations from its originator due to several factors like the expression system and growing conditions, the purification steps, or the final formulations. It is of critical importance to monitor and quantify these variations to correlate them to any potentially different *in vivo* activity, as different clearance time or other interactions within the patient.

To spot these differences, full sequence coverage and post-translational modifications (PTMs) are usually obtained with a bottom-up approach employing a combination of several LC-MS/MS datasets derived from different and orthogonal enzymatic digestions of the protein. Top-down or middle-up approaches have the potential to minimize sample handling and artefacts and to give quicker or complementary information.

In this study, liquid chromatography hyphenated with high-resolution, accurate-mass spectrometry (LC-HRAM-MS) was used in a middle-up approach for the comparison of three commercially available mAb drug substances (DS) and their respective biosimilar obtained in house (BS). Samples were digested with IdeS enzyme, cleaving the monoclonal antibody in the hinge region and generating, after reduction of disulfide bonds, two pairs of polypeptides from the heavy chain along with the light chain portion (Figure 1).[1]

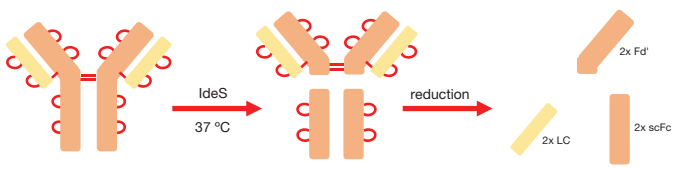

Figure 1. IdeS digestion scheme. IdeS enzyme cleaves the monoclonal antibody below the hinge region allowing the separation of the Fc region and $F(ab')_2$ region. Following treatment with a reducing agent, *intra-* as well as *inter*-molecular disulfide bonds are reduced, generating 2× scFc, 2× LC and 2× Fd' polypeptides.

Analysis was 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. Data were analyzed using the platform for intact mass analysis within Thermo Scientific™ BioPharma Finder™ 3.0 software. The quality of the data together with the quick analysis allowed a confident identification and sequence verification of light chain and Fd' region and a rapid analysis of Fc region variants, including glycoform and N-terminal lysine loss. Moreover, BioPharma Finder 3.0 software is able to rapidly provide a comparison with previously acquired data used as reference; this provides a rapid and confident way for the determination of batch-to-batch variations in analytical laboratories.

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)
- Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), Thermo Scientific™ Pierce™ (P/N 20490)
- Ammonium hydrogen carbonate, Acros Organics™ (P/N 393212500)
- FabRICATOR® (Genovis) (P/N A0-FR1-020)
- Amicon® spin filter units, 10 KDa MWCO
- 8M Guanidine-HCl, Thermo Scientific™ Pierce™ (P/N 10167783)
- Thermo Scientific™ 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 preparation

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 a lipid-based transfection system (Gibco). The vectors (pFUSEss-CHIg-hG1 and pFUSE2ss-CLIg-hk) were purchased from Invivogen. Following transfection, the cells were harvested, and samples of clarified media were passed through a HiTrap Protein A column (GE Healthcare), then washed with phosphate buffered saline before elution of mAb from the Protein A column using 100 mM citric acid, pH 3.2. MAbs solutions were buffer exchanged in PBS and protein concentration was evaluated with the Nanodrop 2000 Spectrophotometer.

Middle-up analysis of IdeS-digested mAb

For middle-up analysis, 160 µg of each mAb in phosphate buffered saline (PBS) or formulation buffer (2 µg/µL solution) were combined with 2 µL of the IdeS digestion solution (67 units IdeS/µL in Optima grade water) and incubated at 37 °C for 2 hours at 500 rpm. For the reduction of disulfide bonds, 4M guanidine hydrochloride and 50 mM TCEP for 45 minutes at 56 °C were used. Following incubation, samples were reduced to dryness via vacuum centrifugation and reconstituted in 0.1% formic acid prior to LC-MS analysis.

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 x 50 mm
Column temperature:	80 °C (active pre-heater) (Still air)
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
Valve:	First two minutes to waste
Intact Protein Mode:	On
Trapping gas:	0.2

Table 1. Mobile phase separation for middle-up analysis

Time (min)	%A	%B	Curve
0	75	25	5
1	75	25	5
16	68	32	5
17	20	80	5
18	20	80	5
18.5	75	25	5
28.0	75	25	5

MS conditions

Table 2. Summary of used tune parameters

MS Source Parameters	Setting for Middle-up Analysis
Source	HESI
Sheath gas flow rate	25
Auxiliary gas flow	10
Probe heater temperature	150 °C
Source voltage	3.8 kV
Capillary temperature	320 °C
S-lens RF voltage	60.0

Table 3. Summary of used MS parameters

General	Setting Middle-up Analysis
Runtime	0 to 28 min
Polarity	Positive
Full MS Parameters	
Full MS mass range	600–2400 *m/z*
Resolution settings	240,000
Protein mode	On
AGC target value	3e6
Max injection time	200 ms
SID	0.0 eV
Microscans	5

MS data processing

Detailed parameter settings are shown in Table 4.

Table 4. Biopharma Finder 3.0 software parameter settings for analysis of IdeS subunits. Default Xtract™ – Average over selected region method used.

Component Detection	
Output mass range	1,000 to 60,000
Output mass	M
S/N threshold	3.00
Rel. abundance threshold	0.00
Charge range	5 to 50
Min. num. detected charge	3
Isotope table	Protein
Fit factor	80%
Remainder threshold	25%
Consider overlap	Yes
Resolution at 400 *m/z*	Raw File Specific
Charge carrier	H+ (1.00727663)
Minimum intensity	1
Expected intensity error	3
m/z range	600.00 to 2400.00
Chromatogram trace type	TIC
Sensitivity	High
Rel. intensity threshold (%)	1
Identification	
Sequence matching mass tolerance	20.00 ppm
Mass tolerance	10.00 ppm
RT tolerance	1.000 min
Min. number of required occurrences	1

Results and discussion

The importance of quick and reliable analytical methods to characterize monoclonal antibody variants and modifications has been highlighted already. In an analytical laboratory it is important to evaluate lot-to-lot consistency or to investigate potential problems present along the production pipeline. In addition, reliable analytical methods are relevant to R&D laboratories where biosimilar products are developed to establish comparability with their innovator.

To investigate structural differences of our biosimilar products with commercially available monoclonal antibodies, a subunit analysis was performed. All monoclonal antibodies were expressed in Chinese Hamster Ovary (CHO) cell lines. The mAbs were digested with IdeS (FabRICATOR, Genovis). IdeS is a cysteine protease with high specificity for the hinge region of IgG1. After disulfide bond reduction and alkylation, three polypeptide populations are generated: the intact light chain (LC), a heavy chain fragment containing the N-terminus (Fd'), and the remaining heavy chain region containing the glycosylation site and the C-terminus (scFc). For LC separation, a MabPac RP, 4 µm 2.1 × 50 mm, column was used. Following LC-MS analysis, data was processed using the Xtract™ algorithm in BioPharma Finder 3.0 software, which is specific for the isotopically resolved data obtained with 25 kDa proteins at 240,000 high resolution. In the TIC trace three species corresponding to the different subunits were present. Figure 2 shows the TIC traces and charge envelope profiles for each peak for the in-house expressed biosimilar of bevacizumab. In Table 5 the experimental values for the three subunits of each DS and BS are compared with theoretical values for LC, Fd', and scFc, taking into account the most prominent variants. All variants were identified within an error of ±3 ppm. All the analyses were performed in triplicate.

The great advantage of using the Q Exactive platform is clearly visible from the spectra obtained for the three subunits (Figure 2); using "protein mode" and high-resolution settings for the MS analysis, it is possible to observe isotopically resolved signals that distinguish between protein variants with very close masses.

Table 5. Experimental and theoretical masses (Da) obtained for the three investigated drug products and their biosimilars

Chain (modifications)	Experimental Mass	Theoretical Monoisotopic Mass	Mass Δ (ppm)	Experimental Mass	Theoretical Monoisotopic Mass	Mass Δ (ppm)
	Bevacizumab Drug Substance			Bevacizumab Biosimilar		
LC	23436.4647	23436.4337	1.3	23436.4787	23436.4337	1.9
Fd'	25929.7278	25929.6590	2.7	25929.7166	25929.6590	2.2
scFc-Man5 No C-term Lys	–	–	–	24992.3997	24992.3524	1.9
scFc-G0F No C-term Lys	25220.4842	25220.4634	0.8	25220.5239	25220.4634	2.4
scFc-G0 No C-term Lys	25074.4234	25074.4055	0.7	–	–	–
scFc-G1F No C-term Lys	25382.5245	25382.5162	0.3	25382.5552	25382.5162	1.5
scFc-G2F No C-term Lys	25544.5858	25544.5690	0.7	–	–	–
	Rituximab Drug Substance			Rituximab Biosimilar		
LC	23042.3317	23042.3437	-0.5	23104.2915	23104.3052	-0.6
LC Pyro-Glu Q1	23025.3486	23025.3171	1.4	23087.3344	23087.2786	2.4
Fd'	25329.3602	25329.3663	-0.2	25357.4164	25357.3976	0.7
Fd' Pyro-Glu Q1	25312.3939	25312.3397	2.1	25340.4451	25340.3710	2.9
scFc-Man5 No C-term Lys	–	–	–	24992.3947	24992.3524	1.7
scFc-G0F No C-term Lys	25188.5186	25188.4913	1.1	25220.5144	25220.4634	2.0
scFc-G1F No C-term Lys	25350.5776	35350.5441	1.3	25382.5446	25382.5162	1.1
scFc-G2 No C-term Lys	25366.5267	25366.5390	-0.5	–	–	–
scFc-G2F No C-term Lys	25512.6200	25512.5970	0.9	–	–	–
	Trastuzumab Drug Substance			Trastuzumab Biosimilar		
LC	23428.5659	23428.5238	1.8	23428.5729	23428.5238	2.1
Fd'	25367.5717	25367.5174	2.1	25367.5752	25367.5174	2.3
scFc-Man5 No C-term Lys	24992.4013	24992.3524	2.0	24992.3892	24992.3524	1.5
scFc-G0F No C-term Lys	25220.5049	25220.4634	1.6	25220.5093	25220.4634	1.8
scFc-G0 No C-term Lys	25074.4149	25074.4055	0.4	–	–	–
scFc-G1F No C-term Lys	25382.5514	25382.5162	1.4	25382.5179	25382.5162	0.1
scFc-G2F No C-term Lys	25544.6259	25544.5690	2.2	–	–	–

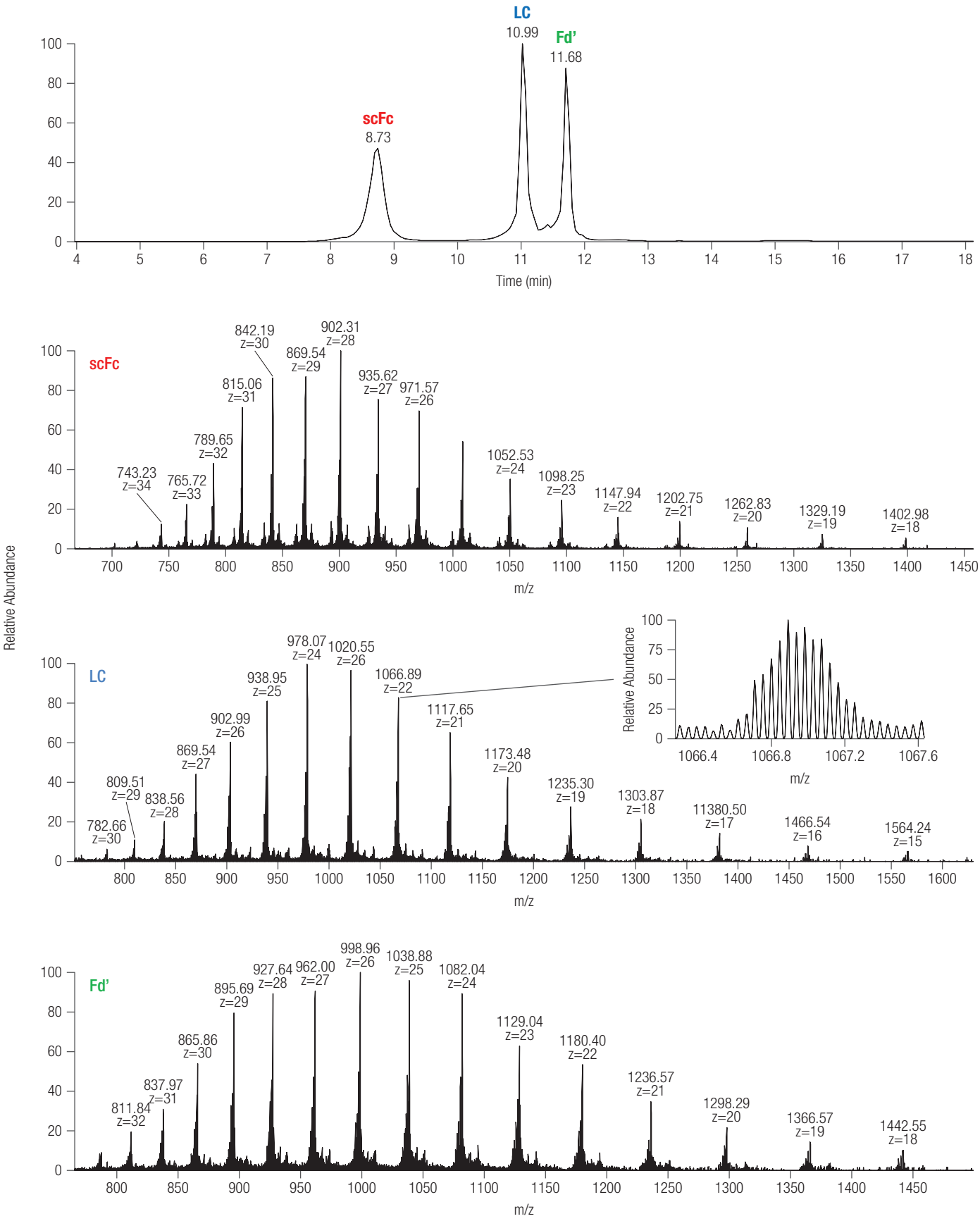

Figure 2. LC-MS analysis of IdeS digested bevacizumab biosimilar. In the top panel the TIC trace shows the three subunits obtained after enzymatic digestion and separated on a MabPac RP, 2.1 × 50 mm column. Bottom panels show the charge envelope profiles obtained for the three subunits using the protein mode on the Q Exactive Plus mass spectrometer; in the zoom it is possible to observe fully isotopically resolved peaks for +22 charge state of the LC region.

As an example, thanks to high resolution and mass accuracy obtained on the Q Exactive Plus Hybrid Quadrupole Platform, it was possible to monitor pyroglutamic acid formation on the Q1 residue for both heavy and light chain of rituximab drug product, prove that the same modification was present on the biosimilar, and quantify the two variants (Figure 3). As well, BioPharma Finder 3.0 software returns a table containing identified variants after deconvolution together with their total signal intensities; using this data a quick comparison of variants abundances and ratio can be performed.

Figure 4 shows the data related to the scFc region for the three investigated mAbs. Quantitative analysis was performed on all the samples using three distinct sample preparations. The data revealed an overall similarity between the complex *N*-glycan profiles of bevacizumab drug product and the in-house produced biosimilar. In the latter, high-mannose glycan with five hexoses residues (Man5) was expressed only in the biosimilar product (9.3%) and the abundancies of the remaining glycoforms were uniformly lower in BS, accordingly. As for rituximab and trastuzumab, a lower level of terminal galactose was obtained in the biosimilar products. Indeed, in all the samples G0F glycoform is the most abundant but its abundance has values around 50% in both drug products but it goes up to 80–90% in the biosimilars. Glycoforms containing one terminal galactose are strongly expressed in the drug products with values of 42.3% and 35.2% for rituximab and trastuzumab, respectively. Fully galactosylated glycans are also present in these biotherapeutics with percentages of 10.6% for rituximab (accounting for both G2 and G2F glycoforms) and 4.6% for trastuzumab. Their corresponding biosimilars show only one glycoform containing galactose (G1F) and their abundance is as low as 7.2% for rituximab and 6.4% for trastuzumab. In an opposite way, the abundance of Man5 glycoform is high in biosimilars (15.0% and 6.4% for rituximab and trastuzumab, respectively) and close to zero in drug products. All these data can be potentially related to the mAbs *in vivo* and *in vitro* activity; it has been shown that afucosylated glycans can enhance the antibody-dependent cellular cytotoxicity (ADCC) as the binding affinity of the Fc region of mAbs to the Fcγ receptor increases.[2] As well, higher levels of galactosylation can impact mAb effector function[3], while a different type of activity is attributed to high mannose glycans.[4] It is clear that glycan analysis is of crucial importance in biotherapeutic analysis for both quality control and product development to ensure product safety and maximize desired effects.

Other peaks in the deconvoluted spectra for the three subunits are present in low abundance; these can be associated with the presence of lysine on the C-terminus (+128 Da), glycation (+162 Da), or minor glycoforms with only one N-acetylglucosamine on the core (-203 Da).

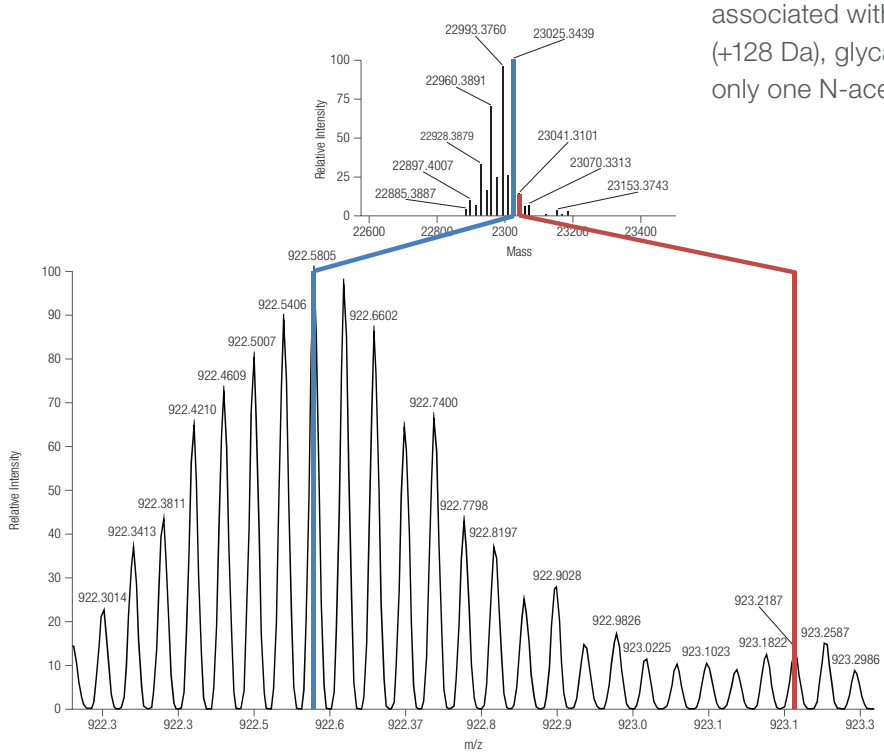

Figure 3. Light chain analysis of rituximab drug product. The MS and deconvoluted spectra show the peaks processed to obtain light chain (red) and light chain modified at position Q1 with a pyroglutamic acid (blue).

To have a quick overview of the differences between samples, a mirror plot can be used. By storing data from a reference sample in the BioPharma Finder library it is possible to perform a direct comparison with new samples, without the need to reprocess old data files. As an example, in Figure 4 the mirror plot for the scFc regions of the drug product vs. the corresponding biosimilar is shown. The most intense variations are soon visible as well as differences in the primary sequence of amino acids that can cause a shift of the mass values in the deconvoluted spectra. This is possible to observe in the mirror plot associated with rituximab in Figure 4; the primary sequence of the rituximab BS presents two modifications at D360 and L362 that are substituted by a glutamic acid and a methionine, respectively, confirmed by peptide mapping analysis [not shown]. This shifts the deconvoluted mass spectra of 32 Da (Figure 4).

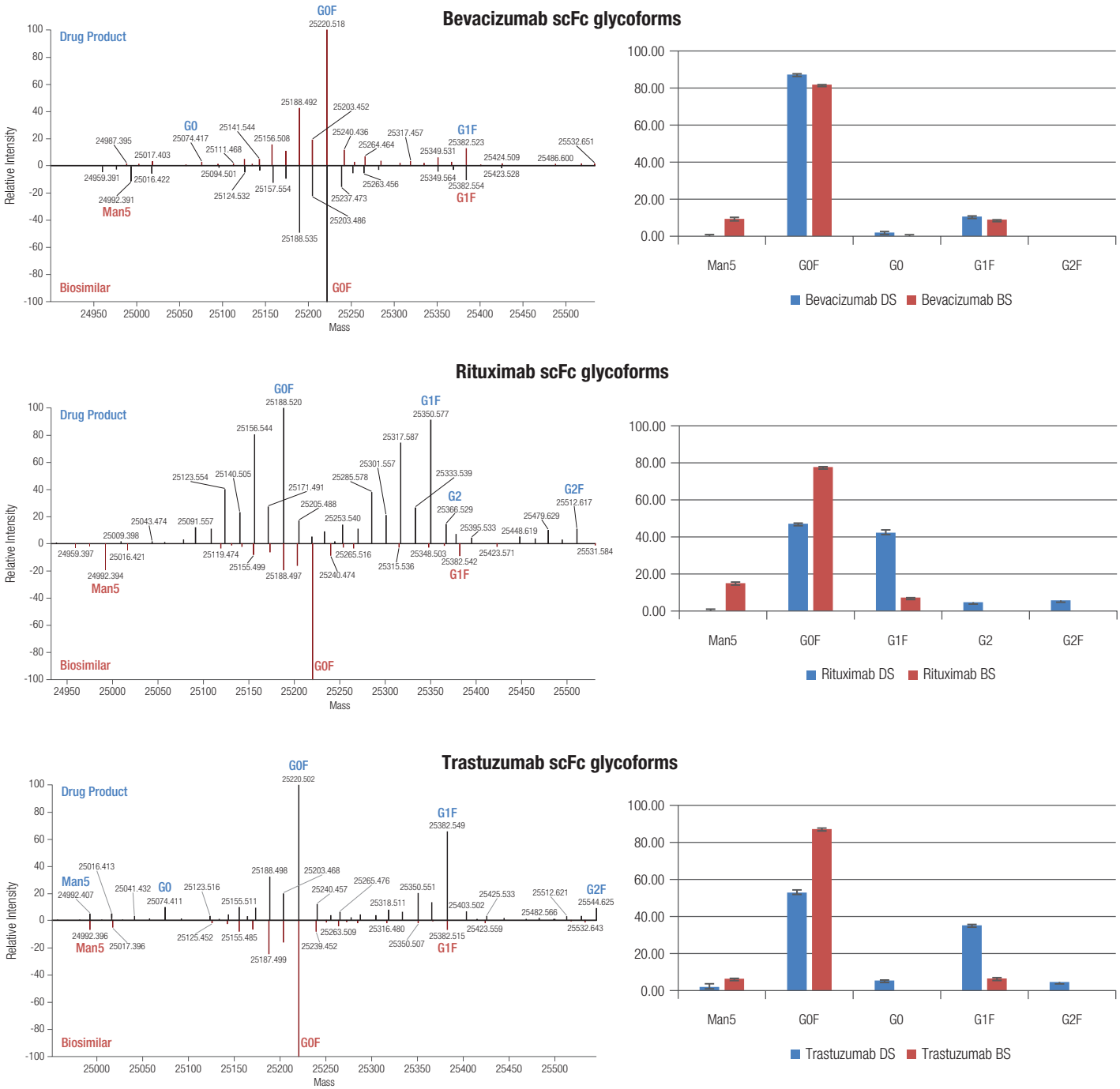

Figure 4. On the left, a mirror plot of deconvoluted mass spectra of scFc regions from drug products and their respective biosimilars. On the right, abundancies of the most abundant glycoforms were compared for drug products and biosimilars. All data were obtained in triplicate and standard deviation values are represented on the error bars.

During product development it is also important to monitor correct expression of the biotherapeutic, e.g. correct cleavage of the signal peptide. Indeed, with the subunit analysis performed on the in-house produced biosimilars, it was possible to monitor the presence of five additional amino acids on the light and heavy chains. These correspond to the first five aminoacids (LVTNS) belonging to the signal peptide on the N-terminus. This signal is present in all samples with a very low percentage (<1%) except for trastuzumab biosimilar, where the presence of this peptide reaches 10% on the heavy chain and represents the most abundant species on the light chain, while the correctly expressed light chain has an intensity around 75% of the most abundant peak (Figure 5). The high-resolution, accurate-mass spectrometry data allowed to unambiguously identify this variant with a mass deviation (Δ = 1.6 ppm).

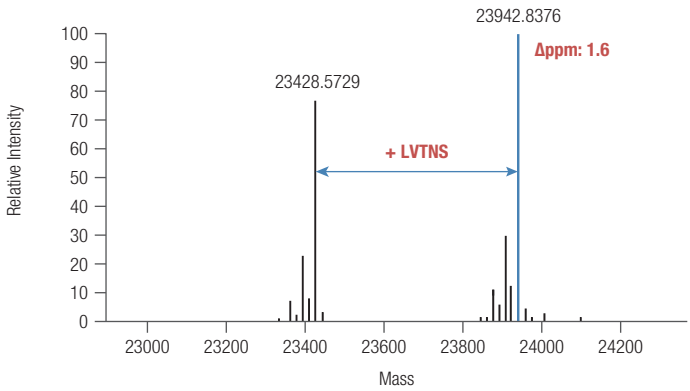

Figure 5. Deconvoluted mass spectrum of trastuzumab biosimilar light chain region. An intense signal at 23942.8376 Da is visible; this corresponds to the addition of five amino acids belonging to the signal peptide only partially cleaved from the antibody. Accurate mass (Δ < 2 ppm) allowed the unambiguous identification of this variant.

Conclusions

- High quality data was obtained for IdeS subunit analysis using the Q Exactive Biopharma platform and MabPac RP columns.
- The combination of the IdeS digestion with the high resolution of LC-HRAM-MS analysis allows fast and efficient data generation of important details on mAbs structure, like glycosylation profile, lysine truncation, and primary sequence modifications.
- BioPharma Finder 3.0 software allows quick deconvolution of the spectra and confident identification of the subunits and their variations. The integrated tool for data comparison can give an overview of differences between samples.
- The new and complete workflow for IdeS subunits analysis is fast and efficient with a simple sample preparation. The LC-MS method is easily optimized and can be used for routine data analysis.

References

1. Sjogren, J.; Olsson, F.; Beck, A. Rapid and improved characterization of therapeutic antibodies and antibody related products using IdeS digestion and subunit analysis. *Analyst*, 2016, 141, 3114-3125, doi:10.1039/C6AN00071A.
2. Upton, R. et al. Orthogonal Assessment of Biotherapeutic Glycosylation: A Case Study Correlating N-Glycan Core Afucosylation of Herceptin with Mechanism of Action. *Analytical Chemistry*, 2016, 88, 10259-10265, doi:10.1021/acs.analchem.6b02994.
3. Raju, T. S.; Jordan, R. E. Galactosylation variations in marketed therapeutic antibodies. *mAbs*, 2012, 4, 385-391, doi:10.4161/mabs.19868.
4. Bowden, T. A. et al. Chemical and Structural Analysis of an Antibody Folding Intermediate Trapped during Glycan Biosynthesis. *Journal of the American Chemical Society*, 2012, 134, 17554-17563, doi:10.1021/ja306068g.

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