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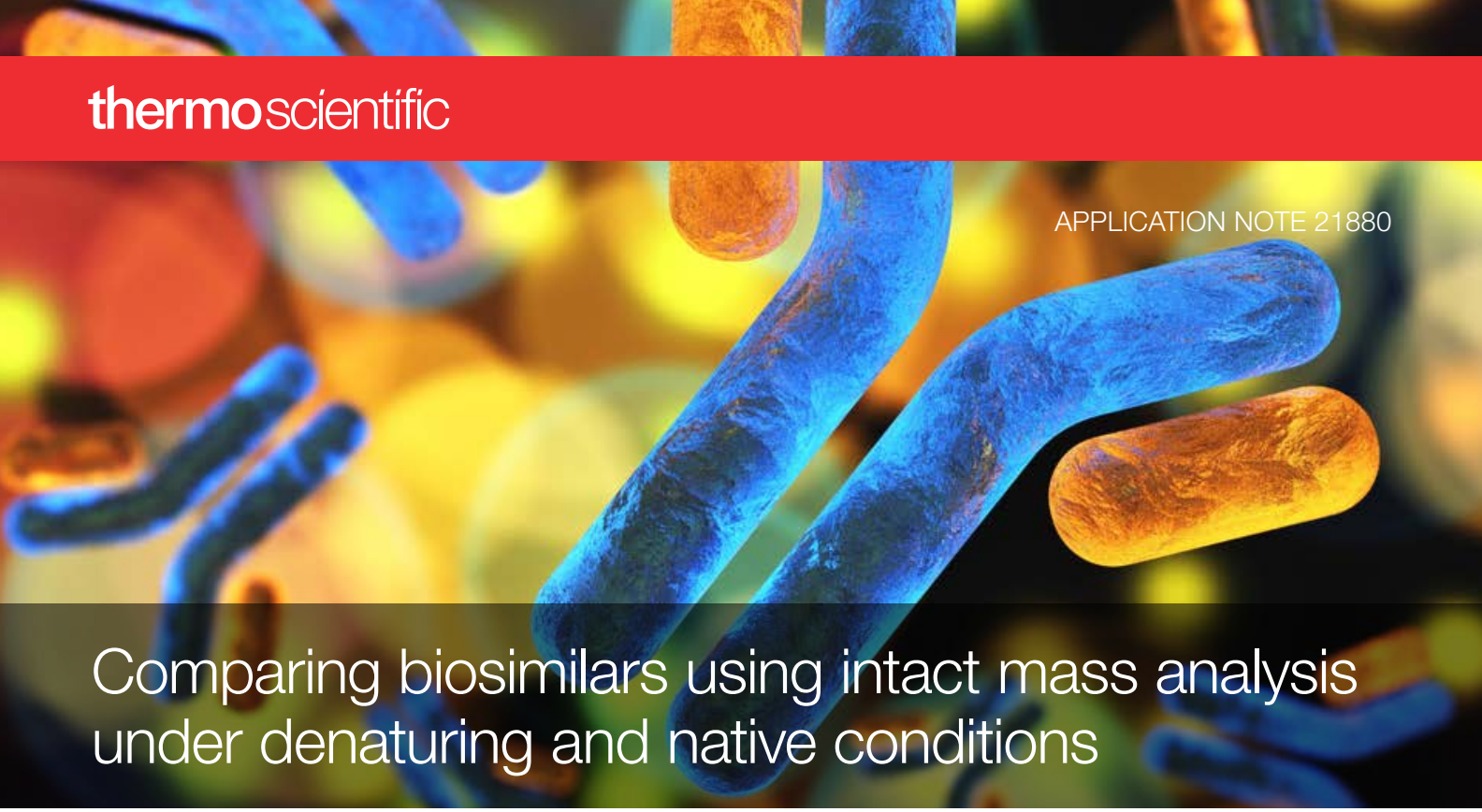
APPLICATION NOTE 21880



Comparing biosimilars using intact mass analysis under denaturing and native conditions

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Keywords

NIBRT, biopharmaceutical, biotherapeutic, monoclonal antibody (mAb), IgG, biosimilar, Intact analysis, *N*-glycan profile, native, denaturing, reversed phase, size exclusion chromatography, infliximab, MAbPac RP column, MAbPac SEC-1 column, Vanquish Flex Binary UHPLC system, Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer, BioPharma Option, BioPharma Finder

Application benefits

- Demonstrate the benefits of a rapid intact mass analysis workflow for the analysis of product quality while minimizing potential artefacts that may be generated by sample preparation.
- Demonstrate the applicability of Thermo Scientific™ MAbPac™ RP and Thermo Scientific™ MAbPac™ SEC-1 columns for intact protein applications in the denatured and native form.
- Highlight the benefits of using a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole- Orbitrap™ mass spectrometer with Biopharma Option and extended mass range for high-resolution accurate-mass MS analysis on different subunits of monoclonal antibodies.

Goal

To highlight the applicability of intact protein high-resolution accurate-mass (HRAM) spectrometry techniques for biotherapeutic characterization and biosimilar evaluation. To show the importance of HRAM MS techniques to characterize mAb variants on the intact level. To demonstrate that methods are easy to optimize, fast, and reproducible.

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Introduction

The past decade has witnessed an exponential growth of approved biopharmaceutical drugs, with the trend likely to continue in the coming years. Most of the biopharmaceuticals approved by the United States Food and Drug Administration (FDA) or the European Medicines Agency (EMA) still have valid patents that cover the whole production process, from the cell line clone to the purification steps and fill finish. The expanding business of biopharmaceuticals leads competitor pharma companies to develop biosimilar products that are potentially more affordable, offering savings between 15 and 30% for patients.[1]

A biosimilar is defined as a drug presenting minimal variations from its originator in terms of physiochemical characterization data and clinical evaluation of pharmacological performance. Since a biopharmaceutical is inherently heterogeneous, it is likely that both originator and biosimilar may present the same variations but in a slightly different ratio that could potentially affect functions like antigen recognition, Fc binding, or product stability. As a consequence, the safety of the patient may be put at risk.[2]

The first monoclonal antibody (mAb) biosimilar that was approved by the FDA and introduced on the market was infliximab Inflectra® produced by Celltrion (Incheon, South Korea), which was developed on the basis of Remicade® from Janssen. Both products are produced in murine myeloma Sp2/0 cell lines using different clones and manufacturing processes. Minor differences between the two drugs have already been highlighted, especially in glycan profiles, levels of dimers, and basic charged variants.[1,2]

In this study, a quick comparison between originator biopharmaceuticals and their respective biosimilars using intact protein analysis supported by HRAM mass spectrometry was performed. Using a high-resolution Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer, it is possible to obtain rapid information about glycosylation profiles and C-terminal lysine loss based on high-resolution intact mass analysis. More importantly, this information is obtained without sample preparation that can cause modifications to the sample.

Here, an in-house-produced biosimilar of infliximab expressed in a CHO cell line was analyzed as well as the commercial drug product (DP1) and a commercial biosimilar (DP2). A comparative study between LC-MS characterization using denaturing and native conditions was performed. The intact mass analysis allowed a quick and complete evaluation of the quality of the product (Figure 1).

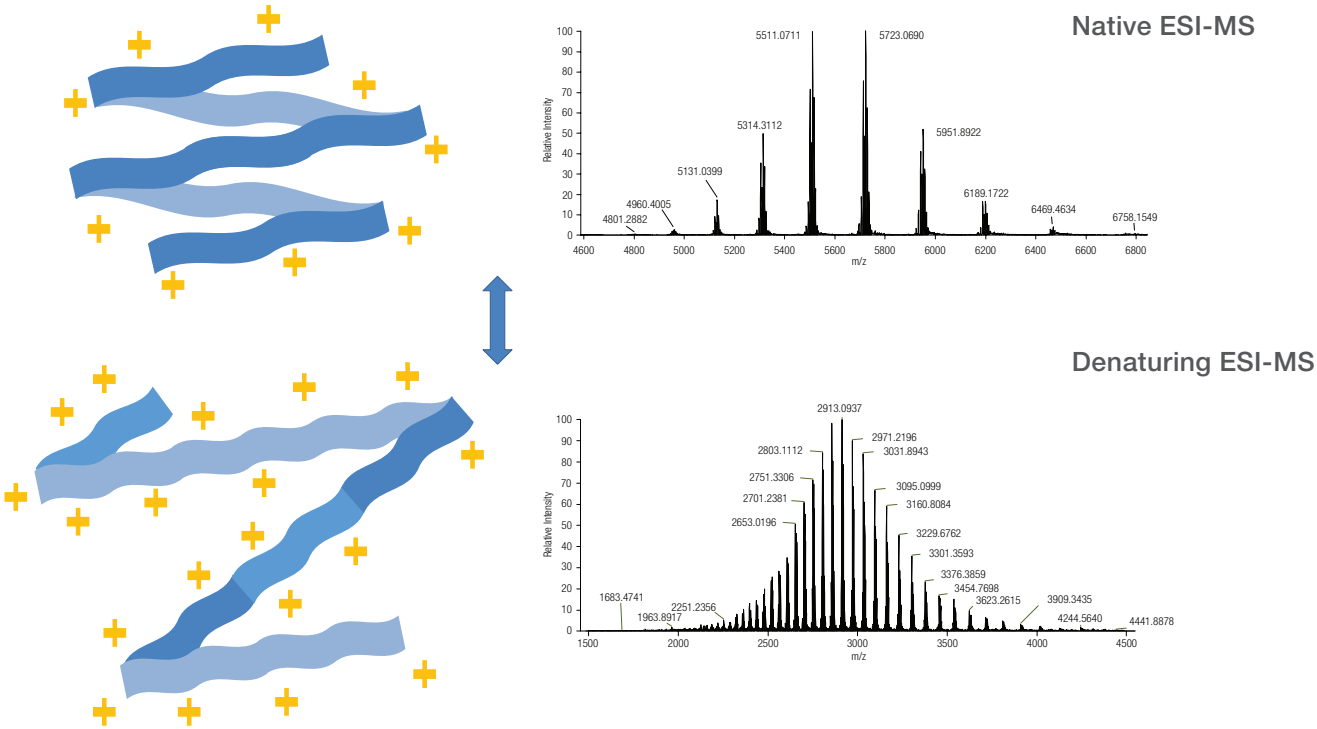


Figure 1. Schematic representation of intact mass analysis in both native and denaturing conditions (left); two examples of the respective typical charge envelopes (right)

For intact mass analysis, denaturing conditions are usually employed to allow access to most amino acid and potential modification sites. The gold standard for this analysis is to use reversed-phase columns as they work with eluents compatible with MS analysis.[3] Under these conditions the eluents cause protein denaturation and as a result, the generated spectrum show ions with a high number of charges (in a range between 20 and 70) and generally appears in the range of *m/z* 2,000–3,000 for mAbs. Generation of highly informative spectra, particularly when the analysis of mAb micro-variants is considered, requires high mass resolution.[3] Recently, advances have been made in the use of native LC conditions hyphenated with mass spectrometry thanks to the improvements of ESI source design and the introduction of new volatile mobile phases. Under these native conditions, the resulting spectrum will appear at higher *m/z* values, generally >5,000 *m/z*, due to the mAb exhibiting lower charge states based on only the surface charge of the native structure. This provides greater spectral spatial resolution of resulting features[3] and less overlap of variants from one charge state with variants in the adjacent charge state. The BioPharma Option available on the Q Exactive Plus MS is a fundamental tool that enables the acquisition of high-resolution mass spectra at higher *m/z* values required for analysis of large mAbs under native conditions.

A comparison of denatured and native intact mass analysis was performed in this study to highlight the flexibility of the Q Exactive Plus BioPharma platform for intact mass applications.

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ•cm resistivity
- Water, Optima™ LC/MS grade (Fisher Chemical) (P/N 10505904)
- Water with 0.1% formic acid (v/v), Optima™ LC/MS grade (Fisher Chemical) (P/N 10188164)
- Acetonitrile with 0.1% formic acid (v/v), Optima™ LC/MS grade (Fisher Chemical) (P/N 10118464)
- HiTrap™ Protein A column
- Amicon™ spin filter units, 10 KDa MWCO
- Phospate buffered saline (PBS) buffer solution pH 7.0
- Ammonium acetate
- MAbPac SEC-1 column, 4.0 × 300 mm
- 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)
- Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (P/N IQLAAEGAAPFALGMBDK)
- Thermo Scientific™ Nanodrop™ 2000 Spectrophotometer ND-2000

Sample pretreatment

Gibco™ 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 a particular monoclonal antibody using 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 then washed with phosphate buffered saline before elution of mAb from the column using 100 mM citric acid, pH 3.2. Protein concentration was evaluated with a Nanodrop 2000 Spectrophotometer.

Sample preparation

Commercially available drugs (DP1 and DP2) were diluted to 3 mg/mL solution with LC/MS grade water. In-house biosimilars were buffer exchanged to PBS pH 7 and the concentration was adjusted to 3 mg/mL.

LC conditions

Intact denaturing analysis

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:	70 °C (pre-heater active)
Autosampler temp.:	5 °C
Injection volume:	3 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 intact denaturing analysis

Time (minutes)	% A	% B	Curve
0	75	25	5
2.5	55	45	5
3.0	20	80	5
4.0	20	80	5
4.2	75	25	5
8.0	75	25	5

Intact native analysis

Mobile phase:	50 mM ammonium acetate, isocratic
Flow rate:	0.3 mL/min
Column:	MAbPac SEC-1, 5 µm, 4.0 × 300 mm, pore size 300 Å
Column temperature:	30 °C
Autosampler temp.:	5 °C
Injection volume:	3 µL
Injection wash solvent:	Methanol/water, 10:90
Needle wash:	Enabled pre-injection

MS conditions

Detailed MS method parameters are shown in Tables 2 and 3.

Table 2. Summary of used tune parameters

MS Source Parameters	Setting for Intact Denaturing Analysis	Setting for Intact Native Analysis
Source	HESI	HESI
Sheath gas pressure	35 au	20 au
Auxiliary gas flow	10 au	5 au
Probe heater temperature	175 °C	275 °C
Source voltage	3.8 kV	3.8 kV
Capillary temperature	275 °C	275 °C
S-lens RF voltage	80	200

Table 3. Summary of used MS parameters

General	Setting for Intact Denaturing Analysis	Setting for Intact Native Analysis
Runtime	0 to 8 min	0 to 28 min
Polarity	Positive	Positive
Full MS parameters		
Full MS mass range	1500–4500 *m/z*	2500–8000 *m/z*
Resolution settings	35,000	35,000
Mode on	Protein	HMR
AGC target value	3e6	3e6
Max Injection time	100 ms	200 ms
SID	100	150
Microscans	10	10

MS data processing

Thermo Scientific™ BioPharma Finder™ 3.0 software was used for data analysis; detailed parameter settings are shown in Table 4.

Table 4. Biopharma Finder 3.0 software parameter settings for analysis of intact proteins in denaturing and native conditions

Intact Analysis ReSpect™ Algorithm	Settings Denaturing	Settings Native
Charge	10 to 70	10 to 50
Mass range (*m/z*)	1500–4500	4500–8000
Scan offset	1	1
Output mass range	140,000 to 160,000 Da	140,000 to 160,000 Da
Deconvolution mass tolerance (ppm)	20	20
Minimum adjacent charges (low and high model mass)	6	3

Results and discussion

Protein analysis in the biopharmaceutical characterization laboratory relies on MS data for identification. To analyze monoclonal antibody variants, it is important that the mass spectrometer is capable of providing high resolution data on the three most-used levels of analysis; i.e. peptide level, subunit, and intact protein analysis. The BioPharma option available on the Q Exactive Plus Orbitrap MS provides three modes of operation and a higher upper mass range, allowing increased flexibility across the three levels of analysis mentioned above.

To demonstrate the possibility of implementing denaturing and native protein mass spectrometry analysis on the same instrument, both types of analyses have been performed on innovator and biosimilar infliximab drug products and a biosimilar of infliximab produced in-house and expressed in a CHO cell line. All results show good agreement with peptide mapping data obtained previously (Application Note 21849).[4]

Each mAb sample was diluted to 3 mg/mL solution and analyzed on a MAbPac RP, 4 µm, 2.1 × 50 mm column and a MAbPac SEC-1, 4 × 300 mm column for intact mass analysis under denaturing and native conditions, respectively. Analyses were performed in technical triplicates injecting 10 µg for all the samples, while MS settings used are reported in Tables 2 and 3.

Collected data were deconvoluted and analyzed using BioPharma Finder 3.0 software using the ReSpect™ algorithm with the sliding window processing method according to settings reported in Table 4. The main variants obtained in the deconvoluted spectra belong to basic variants containing two, one or no lysines on the heavy chain C-terminus and the glycoforms present at asparagine N300 in the Fc region of the antibody (Figure 2). Theoretical masses are listed in Table 5. Since in-house-produced infliximab presents a different primary sequence, theoretical masses for this product are listed as well.

The data obtained from intact mass analysis in denaturing conditions showed that in infliximab drug product 1, the most prominent proteoforms present were the variants containing both C-terminal lysines and a fucosylated complex glycan on both heavy chains (27.74% abundance). Further analysis of this peak in the deconvoluted spectrum shows variants lacking both or one C-terminal lysines (21.62% and 12.04%, respectively). In addition to these three main basic variants, more glycovariants are present, showing one or two terminal galactoses.

As already reported,[2] for drug product 2 higher levels of lysine loss were observed (54.72% abundance, Figure 3C), while the most abundant glycoform carried a terminal galactose (41.47%, Figure 3A). In general, higher abundance of terminal galactose (72.45% DP2 vs. 31.71% DP1) and lower levels of high mannose *N*-glycans were revealed for DP2 (0% vs. 5.49% in DP1).

Table 5. Theoretical average molecular weight of infliximab drug product and biosimilar. Main basic variants and *N*-glycan pairs are taken into account. For all species 16 disulfide bonds were considered as fixed modifications.

	DP1/2 Theoretical Average MW	In-house Biosimilar Theoretical Average MW
1xG0F_G0F	148768.91	148658.85
1xG0F_G1F	148931.05	148820.99
1xG1F_G1F	149093.19	148983.13
1xG1F_G2F	149255.33	149145.27
1xLysLoss,1xG0F_G0F	148640.73	148530.67
1xLysLoss,1xG0F_G1F	148802.87	148692.82
1xLysLoss,1xG1F_G1F	148965.02	148854.96
1xLysLoss,1xM5_M5	148184.24	148074.18
1xM5_M5	148312.41	148202.36
2xLysLoss,1xG0_G0	148220.29	148110.23
2xLysLoss,1xG0_G1	148382.43	148272.37
2xLysLoss,1xG0_G0F	148366.42	148256.36
2xLysLoss,1xG0F_G0F	148512.56	148402.51
2xLysLoss,1xG0F_G1F	148674.70	148564.65
2xLysLoss,1xG1F_G1F	148836.84	148726.79
2xLysLoss,1xG1F_G2F	148998.99	148888.93
2xLysLoss,1xM5_M5	148056.06	147946.04

In comparison with the commercially available products, the biosimilar produced in our laboratories showed a reduced complexity in the deconvoluted spectrum with a high predominance of lysine loss variants (88.79%, Figure 3C). Intact protein analysis revealed a percentage of glycoforms with terminal galactose similar to the one found in DP1, while increased levels of high-mannose glycans were found (12.13%, Figure 3A). In a similar way, afucosylated glycans showed a relative abundance that was double that of DP1 (Figure 3B).

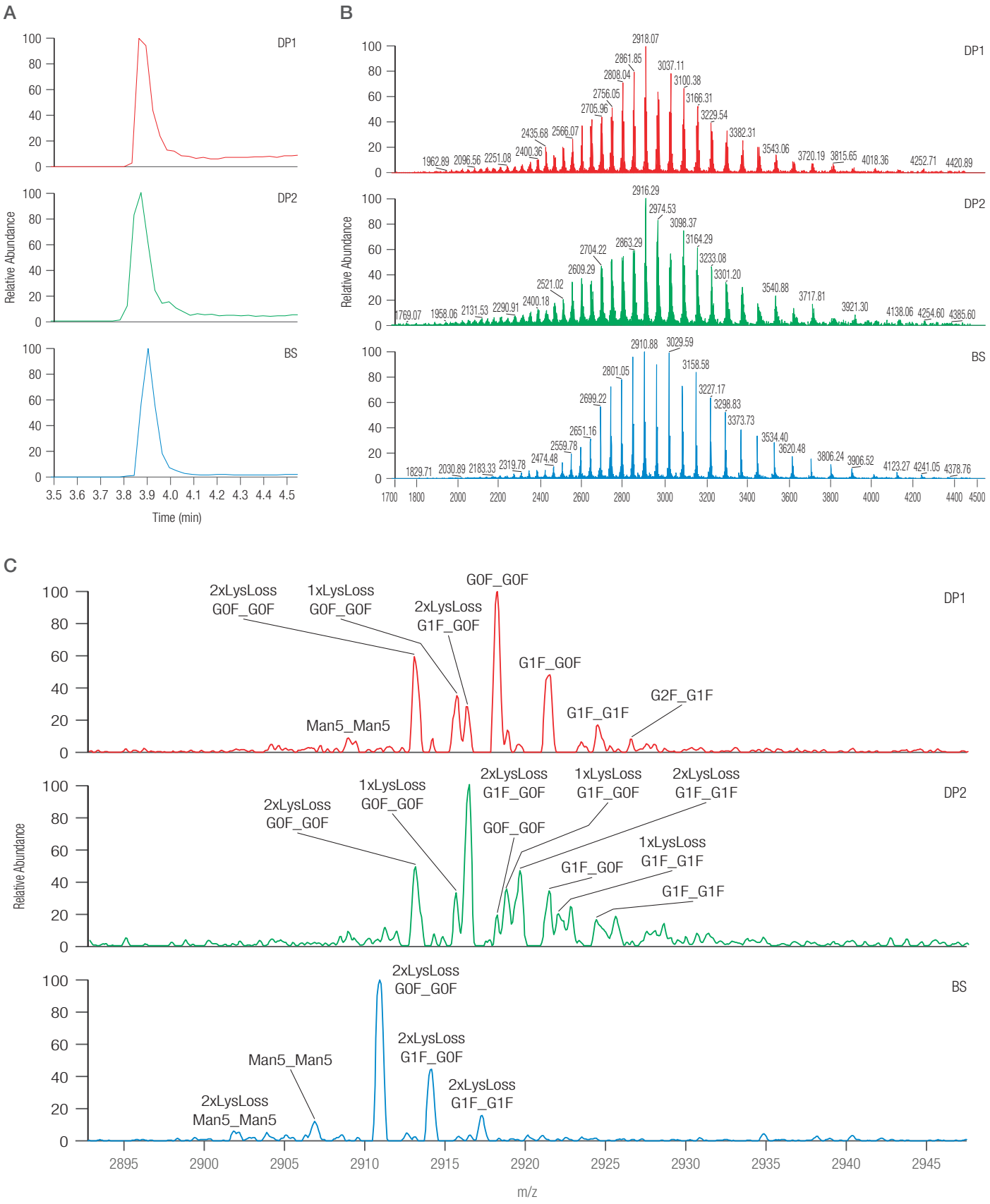


Figure 2. Intact LC/MS analysis in the denatured and native form. RP-MS analysis on infliximab drug products and biosimilar. Section A shows the base peak chromatograms for the two drug products and in-house-produced biosimilar. In B the three charge envelopes are presented while section C represents the zoom for the mass range between 2890 and 2950 *m/z* showing charge state +51. The most abundant species are assigned for each mAb sample.

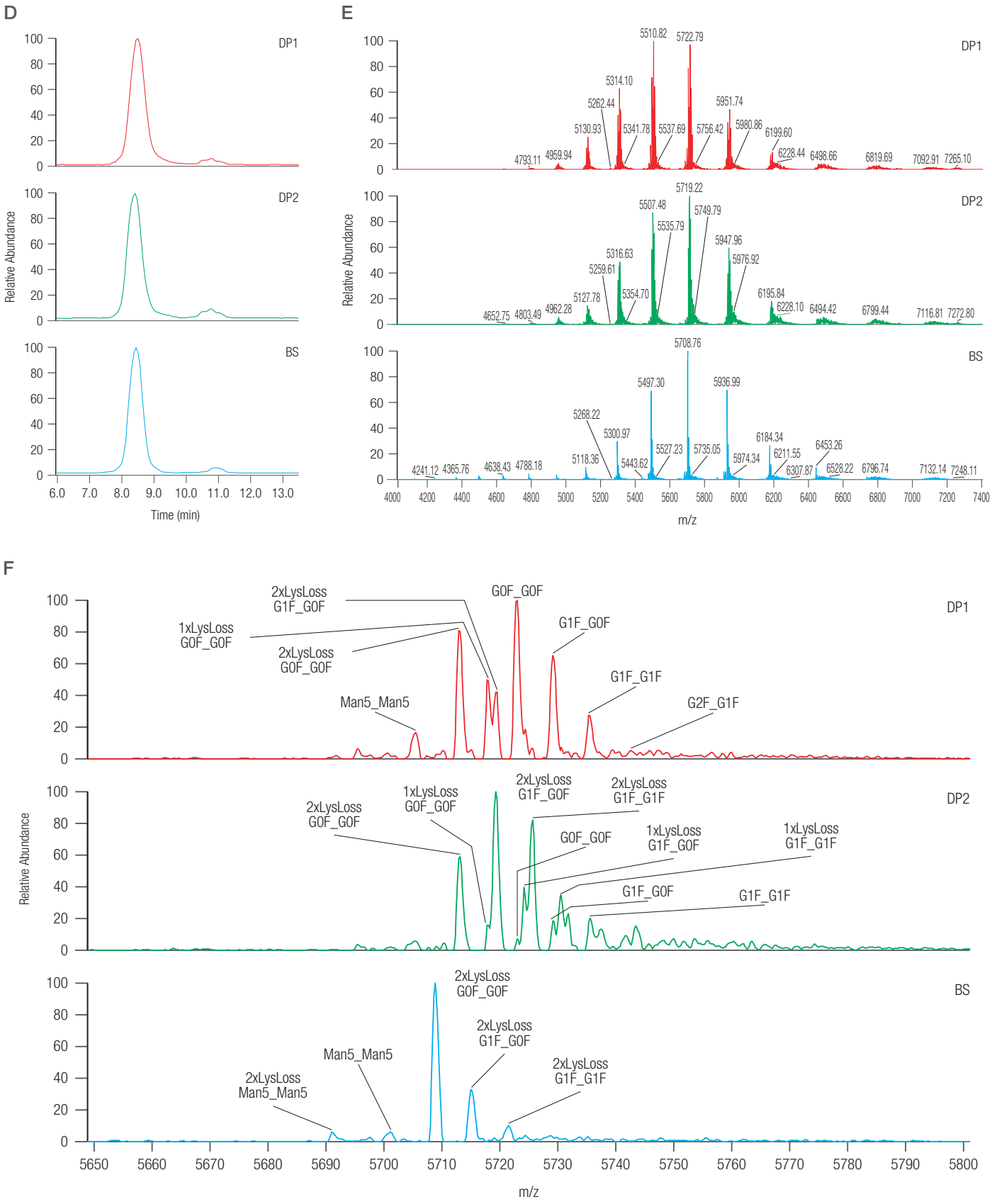


Figure 2 (Continued). For the SEC-MS analysis on infliximab drug products and biosimilar, section D shows the base peak chromatograms for the two drug products and in-house-produced biosimilar. In E the three charge envelopes are presented while section F represent the zoom for the mass range between 5600 and 5860 *m/z* showing charge state +26. The most abundant species is assigned for each mAb sample. The biosimilar sample shows different *m/z* values, which relate to a slightly different sequence and native molecular weight.

Table 6. Relative abundance (%) for the main variants obtained from infliximab DP1, DP2, and biosimilar in both denaturing and native conditions. All the measurements were obtained on technical triplicates.

	Relative Abundance (%, N=3)	Standard Deviation (n=3)	Mass Accuracy (ppm, mean value)	Relative Abundance (%, N=3)	Standard Deviation (n=3)	Mass Accuracy (ppm, mean value)
	Drug Product 1, Denaturing Conditions			Drug Product 1, Native Conditions		
1xG0F_G0F	27.74	1.05	10.2	27.37	1.44	7.4
1xG0F_G1F	17.79	1.19	8.8	19.26	0.74	6.7
1xG1F_G1F	6.56	0.20	9.3	8.03	0.21	6
1xG1F_G2F	1.63	0.40	3.5	–	–	–
1xM5_M5	5.49	0.62	4.5	3.92	0.38	11.6
1xLysLoss,1xG0F_G0F	12.04	1.19	17.8	9.98	0.82	15.4
1xLysLoss,1xM5_M5	1.38	0.32	9.4	–	–	–
2xLysLoss,1xG0_G0F	–	–	–	0.60	0.09	6.4
2xLysLoss,1xG0F_G0F	21.62	1.11	12.4	19.05	0.37	2
2xLysLoss,1xG0F_G1F	5.73	4.84	20.2	10.54	1.43	22.2
2xLysLoss,1xM5_M5	–	–	–	1.23	0.33	15.3
	Drug Product 2, Denaturing Conditions			Drug Product 2, Native Conditions		
1xG0F_G0F	5.14	0.26	3.4	–	–	–
1xG0F_G1F	8.64	0.15	4	3.21	0.48	9.1
1xG1F_G1F	6.61	0.46	15.7	5.40	0.88	14.8
1xLysLoss,1xG0F_G0F	6.15	0.31	15.8	–	–	–
1xLysLoss,1xG0F_G1F	9.60	1.33	11.7	8.83	0.43	27.7
1xLysLoss,1xG1F_G1F	9.14	0.41	5.7	10.42	0.70	7
1xLysLoss,1xM5_M5	–	–	–	0.61	0.14	13
2xLysLoss,1xG0F_G0F	16.26	0.69	6.6	16.32	1.29	7.9
2xLysLoss,1xG0F_G1F	23.22	0.88	9	29.49	1.06	3.6
2xLysLoss,1xG1F_G1F	15.24	0.60	8.8	19.45	1.10	12.3
2xLysLoss,1xG1F_G2F	–	–	–	6.27	0.71	13.6
	In-house Biosimilar, Denaturing Conditions			In-house Biosimilar, Native Conditions		
1xG0F_G0F	2.47	0.21	20.1	–	–	–
1xG0F_G1F	0.94	0.12	18.2	–	–	–
1xM5_M5	7.80	0.28	18.2	3.65	0.39	16.9
2xLysLoss,1xG0_G0	1.99	0.40	9	2.09	0.37	10.4
2xLysLoss,1xG0_G1	–	–	–	1.08	0.17	25.8
2xLysLoss,1xG0F_G0F	54.96	0.71	9.1	57.88	1.93	2.1
2xLysLoss,1xG0F_G1F	21.74	0.50	9.5	24.49	1.43	3.7
2xLysLoss,1xG1F_G1F	5.77	0.25	10.7	8.02	0.37	7.6
2xLysLoss,1xM5_M5	4.33	0.31	10.5	3.47	0.61	17.9

Similar values were obtained for intact mass experiments performed in native conditions (Figures 3D and 4). It is possible to observe that the results for the two techniques are similar for DP1 and biosimilar, with only some small discrepancies found in the results for DP2 (Figures 3C, 3D, and 4B). Variants containing both lysines are detected in a slightly lower amount in native conditions for DP2 with respect to denaturing conditions, while mAbs with 2 lysine loss show the opposite trend.

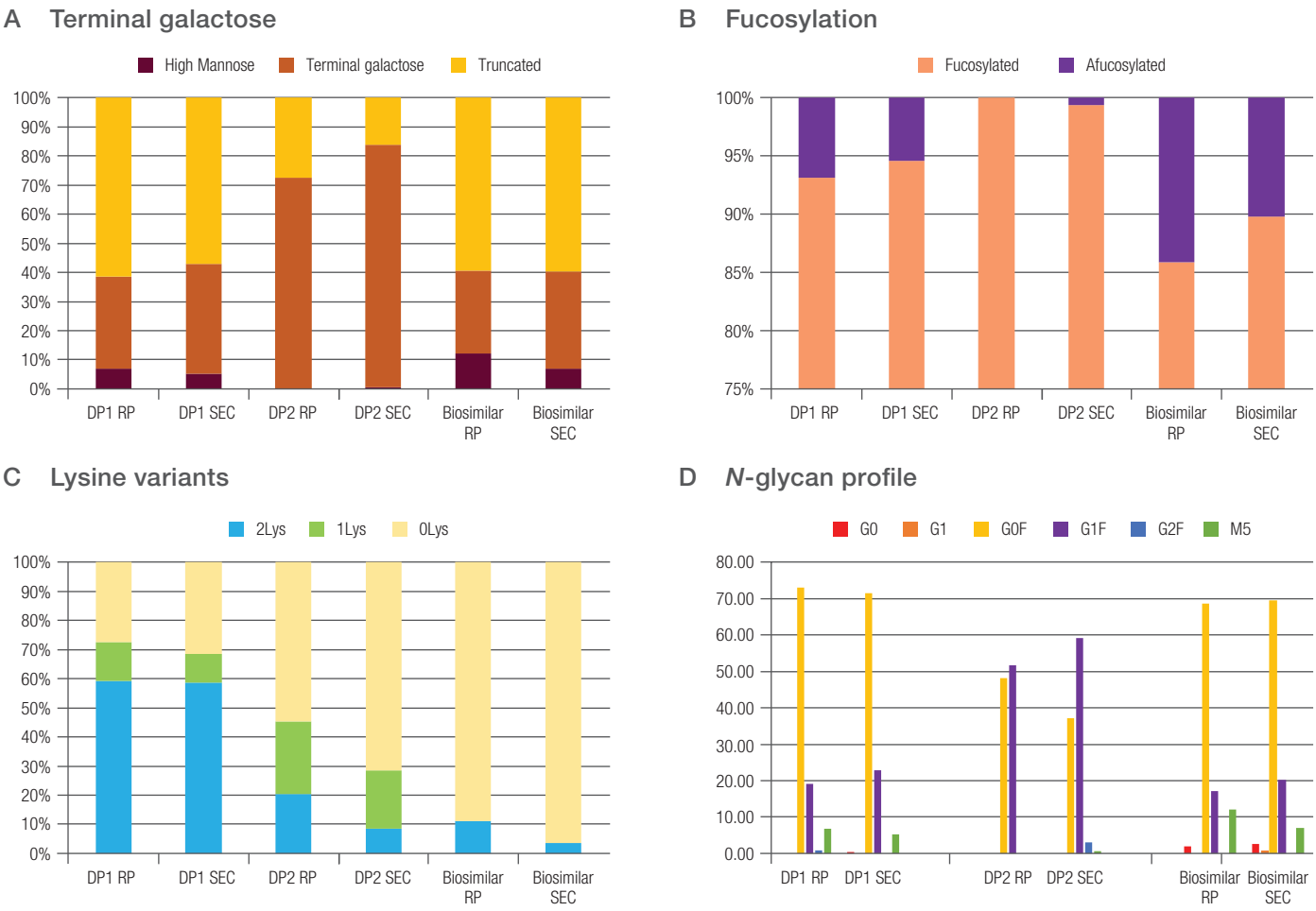
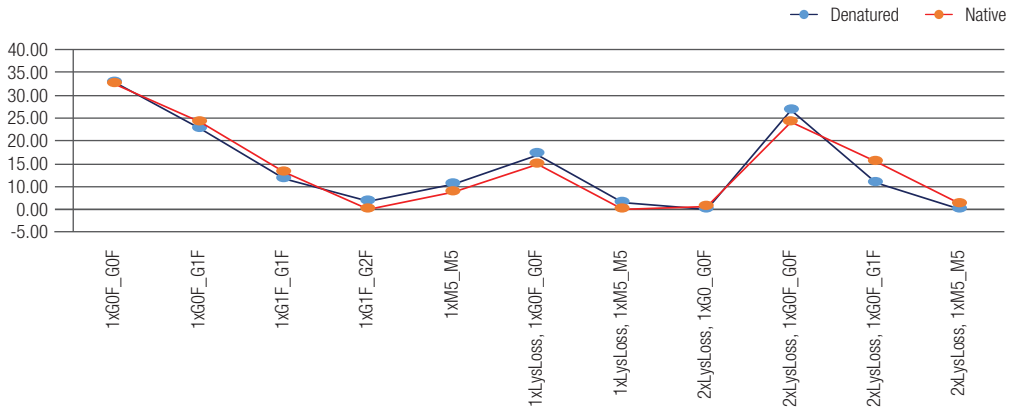


Figure 3. Statistical analysis of infliximab drug products and biosimilar variants relative abundancies. The graph on the top left (A) shows how the *N*-glycan variants are grouped when considering high-mannose or complex *N*-glycan (with a truncated structure or presenting a terminal galactose). In graph (B) *N*-glycans are grouped according to presence or absence of a core fucose. Lysine variants distribution is shown in graph (C), while relative abundancies of *N*-glycan structures are shown in graph (D).

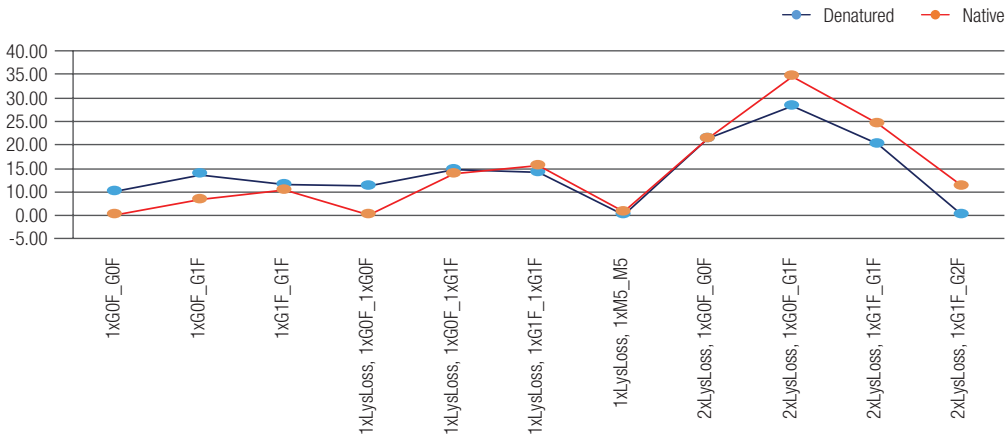
The results obtained for DP1, DP2, and our in-house produced biosimilar show an overall similarity between the *N*-glycan structures expressed. DP1 and our in-house produced biosimilar, although expressed in different cell lines, show more similarities. DP2, expressed in the same cell line as DP1, contains more of the differing features. Moreover, galactosylation levels suggest a potential impact on DP2 effector functions.[5] DP2 also shows lower levels of high-mannose which can lead to changes in the clearance rate of the drug in the patient body.[6] It has been reported and shown that therapeutics with higher afucosylation show increased ADCC activity, however the same levels were shown here for DP1 with respect to DP2.[2] It is important to say that levels of glycosylation, especially galactosylation, are usually reported as a range of values and they can vary across different production batches.[1,2] For this study only one lot was investigated per product.

For lysine content, the three analyzed infliximab products showed very different profiles with DP1 having the most basic characteristics and the in-house biosimilar showing the opposite trend with truncation levels only around 10%. Nevertheless, this modification is not considered critical for infliximab pK or activity, as both lysine residues on the C-term are rapidly cleaved *in vivo*.[7]

A DP1



B DP2



C Biosimilar

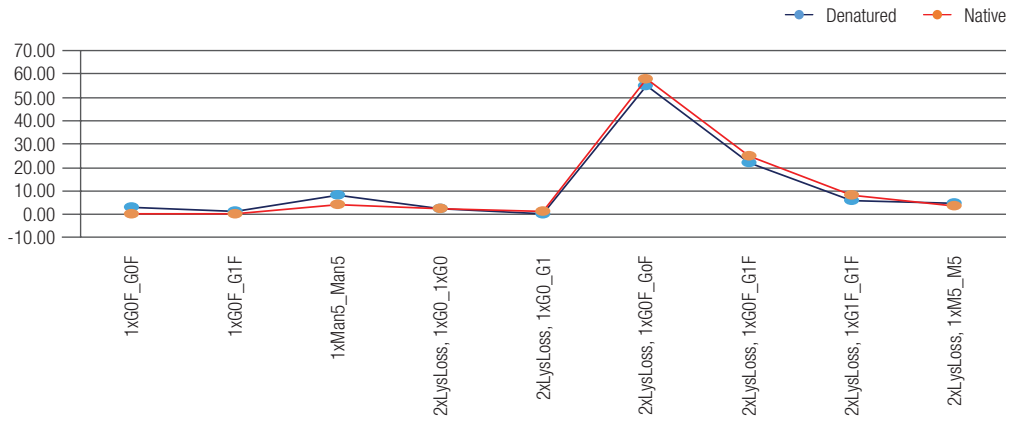


Figure 4. MAb variants analyzed through intact MS analysis in denaturing (blue) and native (red) conditions.
The profiles for DP1, DP2, and a biosimilar of infliximab are shown in each graph (A, B, C, respectively).

Conclusions

- The Q Exactive Plus with BioPharma option platform demonstrates excellent performance for both denaturing and native intact mass experiments, highlighting the flexibility of this platform. The BioPharma option allowed acquisition of high-resolution MS data in native conditions and confident identification of mAb variants on the intact protein level.

- BioPharma Finder software, using the ReSpect algorithm and sliding window processing method, allowed quick deconvolution and identification of the data in less than 30 minutes for each sample set.

- Vanquish Flex UHPLC systems include Thermo Scientific™ SmartInject technology with quaternary or binary pumps producing highly robust and reproducible chromatography over thousands of runs on a column, while maintaining full biocompatibility. The ability to obtain high peak purities through robust and precise chromatographic separations using the MAbPac RP and MAbPac SEC-1 columns has a positive influence on MS data quality.

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