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APPLICATION NOTE 21849

Comparability study for the determination of post-translational modifications of infliximab innovator and biosimilars by automated high-throughput peptide mapping analysis

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Keywords

NIBRT, biopharmaceuticals, biotherapeutics, biosimilars, follow-on biologic, comparability studies, CQAs, monoclonal antibodies (mAbs), IgG1, infliximab, post-translational modifications (PTMs), peptide mapping, bottom-up, high throughput, Magnetic SMART Digest kit, KingFisher Duo Prime Purification System, Vanquish Flex Binary UHPLC System, Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer

Application benefits

- Rapid, automated digestion leading to highly reproducible results for innovator and biosimilar comparability studies with less hands-on time
- Simple reproducible protein digestion with minimal user intervention for peptide mapping analysis
- High confidence in workflow results with excellent quality data; excellent coverage and low levels of post-translational modifications (except for M34+oxidation site where noticeable levels were observed)

Goal

Biosimilars are an increasingly important area of interest for the pharmaceutical industry worldwide as patents for the first biologics derived from recombinant technology, including monoclonal antibodies (mAbs), are expiring. The development and demonstration of biosimilarity represents a significant challenge and is required to show the presence or absence of differences resulting from the manufacturing process by investigating the physicochemical and biological properties of a biosimilar candidate molecule compared to the corresponding reference product (innovator). This study evaluated the use of the Thermo Scientific™ SMART Digest™ Trypsin Kit, with Magnetic Bulk Resin option (Magnetic SMART Digest), protocol in combination with the Thermo Scientific™ KingFisher™ Duo Prime

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purification system to investigate PTMs in the innovator and biosimilar drug substances. The efficiency and reproducibility of the platform was evaluated with a specific focus on the determination of protein sequence coverage and identification of post-translational modifications (PTMs), including deamidation, oxidation, lysine clipping, glycation, and glycosylation.

Introduction

Monoclonal antibodies (mAbs) are an important class of therapeutic proteins and the fastest growing class of therapeutic agents due to their high specificity to target antigens, long serum half-life in humans, and ability to treat a wide range of ailments. Biologics are among the highest-cost treatments on the global market today, which implies the need for low-cost alternatives.[1] As the patents of many biologics expire, the development of biosimilar products with similar quality, safety and efficacy profiles to the original biologics should improve the accessibility of biotherapeutic drugs to patients. In emerging markets, biosimilars already offer more affordable prices, which are of high importance to economies where expensive treatments are not financially feasible.[2]

Regulatory bodies worldwide have already prepared guidelines to regulate the development of biosimilar products.[3,4] Biosimilars or follow-on biologics are biologic products that receive authorization based on an abbreviated regulatory application containing comparative quality and nonclinical and clinical data that demonstrate similarity to a licensed biological product. Regulatory authorities have generally reached the consensus that extrapolation of biosimilarity from one indication to other approved indications of the reference product can be permitted if it is scientifically justified[5] and is an important way to simplify biosimilar development.

The similarity between a proposed biosimilar product and the reference product (innovator) can be affected by many factors. MAb products exist as a mixture of heterogeneous variants due to post-translational modifications that arise during cell culture, purification, and storage. Some of these modifications can alter the biological activity, drug metabolism and pharmacokinetics (DMPK), and immunogenicity and thus may pose a risk to the patient. In this way, extensive analytical testing platforms are needed for in-depth characterization and to ensure product stability, proper in-process controls, safety, and efficacy. During the development and production of therapeutic monoclonal antibodies, characterization of structural variants is a critical challenge. The rigors of biotherapeutic development and analysis have clearly indicated a need for control over every stage of development. The biopharmaceutical industry requires fast and robust analytical platforms to fulfill regulatory requirements involved in the Biologics License Applications (BLA) process.

Infliximab is a tumor necrosis factor (TNF-α) blocker and a chimeric monoclonal IgG1 antibody composed of human constant (75%) and murine variable (25%) regions. It is produced by recombinant cell line cultured by continuous perfusion. TNF-α is a key proinflammatory cytokine involved in chronic inflammatory diseases.[6] Its hyperactivity and enhanced signaling pathways can be observed in inflammatory diseases where it activates further pro-inflammatory cascades. By binding to both the soluble subunit and the membrane-bound precursor of TNF-α, infliximab disrupts the interaction of TNF-α with its receptor and may also cause lysis of cells that produce TNF-α.[7]

Infliximab was first approved by the United States Food and Drug Administration (FDA) in 1998 as an intravenous injection. It is indicated for the treatment of various inflammatory disorders such as adult or pediatric Crohn's disease, adult or pediatric ulcerative colitis, rheumatoid arthritis in combination with methotrexate, ankylosing spondylitis, psoriatic arthritis, and plaque psoriasis.[8] In clinical trials, multiple infusions of infliximab resulted in a reduction of signs and symptoms of inflammatory diseases and induction of remission in patients who have had an inadequate response to alternative first-line therapies for that disorder.

In September 2013, the European Medicines Agency (EMA) approved the first two biosimilars of infliximab. Now, those two biosimilars together incur more sales than all the other biosimilars on the market combined.[9] In the US market, there are currently two biosimilars of infliximab available that demonstrate a high degree of similarity to the reference product. They are approved for all eligible indications of the reference product.[9,10]

This application note presents the benefits of using the recently developed Magnetic SMART Digest method to perform a comparability study of PTMs for infliximab innovator and biosimilars. An efficient approach that

combines automated enzymatic digestion using the Magnetic SMART Digest kit on a KingFisher Duo Prime purification system, analysis with the high-resolution, accurate-mass (HRAM) capabilities of the Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer, and high-resolution chromatographic separation with the Thermo Scientific™ Acclaim™ VANQUISH™ C18, 2.2 µm, 2.1 × 250 mm column on a Thermo Scientific™ Vanquish™ Flex binary UHPLC system. Thermo Scientific™ BioPharma Finder™ software was used to interrogate the high-quality data sets.

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ•cm resistivity
- Water, Optima™ LC/MS grade (Fisher Chemical) (P/N 10505904)
- Acetonitrile, Optima™ LC/MS grade (Fisher Chemical) (P/N 10001334)
- 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)
- Trifluoroacetic acid (TFA) (Fisher Chemical) (P/N 10294110)
- SMART Digest Trypsin Kit, Magnetic Bulk Resin option (P/N 60109-101-MB)
- Thermo Scientific™ Pierce™ DTT (Dithiothreitol), No-Weigh™ Format (P/N 20291)
- Iodoacetic acid, sodium salt, 99% (IA) (Acros Organics) (P/N 10235940)
- Thermo Scientific™ KingFisher™ Deepwell, 96 well plate (P/N 95040450)
- Thermo Scientific™ KingFisher™ Duo 12-tip comb (P/N 97003500)
- Acclaim VANQUISH C18 column, 2.2 µm, 2.1 × 250 mm (P/N 074812-V)
- 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)

Equipment

- KingFisher Duo Prime Purification system (P/N 5400110)
- 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 (P/N ND-2000)

Sample preparation

Commercially available infliximab monoclonal antibody products (DP1 and DP2) were supplied at different concentrations and two biosimilar (BS1 and BS2) were produced in house using CHO and HEK expression systems, respectively (Table 1). Monoclonal antibody samples were prepared in triplicate.

Table 1. Monoclonal antibodies used in the study

Drug	Specifications	Concentration	Type
Infliximab DP1	Sp2/0 cell line	10 mg/mL	Recombinant chimeric IgG1 mAb
Infliximab DP2	Sp2/0 cell line	10 mg/mL	Recombinant chimeric IgG1 mAb
Infliximab BS1	In-house CHO expressed	9.3 mg/mL	Recombinant chimeric IgG1 mAb
Infliximab BS2	In-house HEK expressed	10.7 mg/mL	Recombinant chimeric IgG1 mAb

Infliximab biosimilar in-house production

ExpiCHO-S™ Cells (Gibco, #A29127) were derived from a non-engineered subclone that has been screened and isolated from Chinese hamster ovary (CHO) cells. Expi293F™ Cells (Gibco, #A14527) were derived from the human HEK293 cell line, adapted to suspension culture and high-density growth. 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 (PBS). mAbs were eluted from the Protein A column using 100 mM citric acid, pH 3.2. mAbs solutions were buffered exchanged in PBS and protein concentration was evaluated with a Nanodrop 2000 Spectrophotometer.

Sample preparation using a SMART Digest trypsin kit, magnetic bulk resin option (Magnetic SMART Digest)

Samples were diluted to 2 mg/mL in water. For each sample digest, sample and buffers were added to each lane of a KingFisher Deepwell 96 well plate as outlined in Table 2. Bead "wash buffer" was prepared by diluting SMART Digest buffer 1:4 (v/v) in water. Bead buffer was neat SMART Digest buffer. Digestion was performed using the Kingfisher Duo Prime Purification System with Thermo Scientific™ BindIt™ software (version 4.0), using the protocol outlined in Table 3. Samples were incubated for 45 minutes at 70 °C on medium mixing speed (to prevent sedimentation of beads), with post-digestion cooling carried out to 10 °C. Following digestion, disulfide bond reduction was performed with 10 mM DTT for 30 minutes at 57 °C and subsequently alkylated with 20 mM IA in darkness for 30 minutes. The reaction was quenched with 15.45 µL of 100 mM DTT followed by 15.64 µL 10% TFA (final concentration 11 mM DTT and 1% TFA). Samples were then injected immediately into the LC-MS (3 µg).

Table 2. KingFisher Duo Prime plate layout utilized for sample preparation. Reagents and associated volumes placed in each well are outlined.

Lane	Content	Volume Applied to Each Well (µL)
A	SMART Digest buffer	150
	Sample (2 mg/mL)	50
B	Tip Comb	
C	Empty	
D	Magnetic SMART Beads	15
	Bead Buffer (SMART Digest buffer)	100
E	Bead Wash Buffer (SMART Digest buffer 1:4 (v/v))	200
F	Waste Lane (Water)	250

Table 3. Protocol for automated peptide digestion with the KingFisher Duo Prime system

Step	Release Bead	Mixing	Collect Beads	Temp	Lane
Collect Bead	–	10 s Bottom Mix	3 count, 1 s	–	D
Bead Wash	Yes	1 min Medium Mix	3 count, 1 s	–	E
Digest and Cool	Yes	45 min Medium Mix	3 count, 15 s	During digestion: 70 °C Post-digestion: 10 °C	A
Release Beads	Yes, Fast	–	–	–	F

LC conditions

Column:	Acclaim VANQUISH C18, 2.2 µm, 2.1 × 250 mm
Mobile Phase A:	0.1% formic acid aqueous solution
Mobile Phase B:	0.1% formic acid solution in acetonitrile
Flow Rate:	0.3 mL/min
Column Temperature:	25 °C (Still air mode)
Autosampler Temp.:	5 °C
Injection Volume:	10 µL
Injection Wash Solvent:	MeOH:H_2O, 10:90 (v/v)
Needle Wash:	Enabled pre-injection
Gradient:	See Table 4 for details

Table 4. Mobile phase gradient for UHPLC separation of peptides

Time (minutes)	Flow (mL/min)	% Mobile Phase B	Curve
0.00	0.30	2.0	5
45.0	0.30	40.0	5
46.0	0.30	80.0	5
50.0	0.30	80.0	5
50.5	0.30	2.0	5
65.0	0.30	2.0	5

MS conditions

Detailed MS method parameters are shown in Tables 5 and 6.

Table 5. MS source and analyzer conditions

MS Source Parameters	Setting
Source	Thermo Scientific™ Ion Max source with HESI-II probe
Sheath Gas Pressure	40 psi
Auxiliary Gas Flow	10 arbitrary units
Probe Heater Temperature	400 °C
Source Voltage	3.8 kV
Capillary Temperature	320 °C
S-lens RF Voltage	50 V

Table 6. MS method parameters utilized for peptide mapping analysis

General	Setting	MS^2 Parameters	Setting
Run Time	0 to 65 min	Resolution Settings	17,500
Polarity	Positive	AGC Target Value	1.0×10^5
Full MS Parameters	**Setting**	Isolation Width	2.0 *m/z*
Full MS Mass Range	200–2,000 *m/z*	Signal Threshold	1.0×10^4
Resolution Settings	70,000	Normalized Collision Energy (HCD)	28
AGC Target Value	3.0×10^6	Top-N MS^2	5
Max Injection Time	100 ms	Max Injection Time	200 ms
Default Charge State	2	Fixed First Mass	–
SID	0 eV	Dynamic Exclusion	7.0 s
Microscans	1	Loop Count	5

Data processing and software

Thermo Scientific™ Xcalibur™ software version 4.0.27.13 (Cat. No. OPTON-30487) was used for data acquisition and analysis. For data processing, Thermo Scientific™ Biopharma Finder™ software version 3.0 was applied. Detailed parameter settings are shown in Table 7.

Table 7. Biopharma Finder 3.0 software parameter settings for analysis of peptide mapping data

Component Detection	Setting
Absolute MS Signal Threshold	8.0 x 10^4 counts
Typical Chromatographic Peak Width	0.3 s
Relative MS Signal Threshold (% base peak)	1.00
Relative Analog Threshold (% of highest peak)	1.00
Width of Gaussian Filter (represented as 1/n of chromatographic peak width)	3
Minimum Valley to Be Considered as Two Chromatographic Peaks	80.0
Minimum MS Peak Width (Da)	1.20
Maximum MS Peak Width (Da)	4.20
Mass Tolerance (ppm for high-res or Da for low-res)	4.00
Maximum Retention Time Shift (min)	1.69
Maximum Mass (Da)	30,000
Mass Centroiding Cutoff (% from base)	15
Identification	**Setting**
Maximum Peptide Mass	7,000
Mass Accuracy	5 ppm
Minimum Confidence	0.8
Maximum Number of Modifications for a Peptide	1
Unspecified Modification	-58 to +162 Da
N-Glycosylation	CHO
Protease Specificity	High
Static Modifications	**Setting**
Side Chain	Carboxymethylation
Variable Modifications	**Setting**
N Terminal	Gln→Pyro Glu
C Terminal	Loss of lysine
Side Chain	Deamidation (N) Deamidation(Q) Glycation (K) Oxidation (MW)

Results and discussion

Biosimilar medicines must be produced in accordance with the specific requirements established by the regulatory agencies to prove comparability to the reference product in terms of efficacy, quality, and safety. Thus, the biosimilar is authorized for all or some of the indications approved for the reference product. Dosage and route of administration must be the same as the innovative biological. Biosimilars are complex biological molecules and the required studies to prove biosimilarity are much more challenging than those requested for a generic drug. Comparability studies naturally arise because subtle manufacturing variations may yield micro-heterogeneities that can affect product potency and/or toxicity. Extensive physicochemical characterization of innovator products and the proposed biosimilar should be performed.[10]

Through the physicochemical and functional level comparisons emerged the 'fingerprint' concept from FDA guidelines on biosimilars (2012).[11] This concept is the candidate biosimilar should be highly similar with fingerprint-like similarity. The bases of this program are a set of analytical assessments able to demonstrate that the quality attributes for the biosimilar candidate are highly similar to the quality attributes for the reference product. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) established guidelines in 2004 that describe a comparability exercise to ascertain whether a biologically manufactured product retained its quality despite changes in manufacturing.[12,13] The 1999 ICH Q6B guideline describes how to use analytical procedures to establish acceptance criteria for proteins made by cell culture expression systems.[14] Guidelines outline the basic underlying principles used to set specifications, including characterization, analysis, and manufacturing controls.[15] Further, prespecified quantitative parameters, e.g., primary structure, glycosylation, disulfide structure, charge variants, size variants, biophysical characterization (secondary structure, tertiary structure, and thermal stability), and biological characterization must be identified to determine acceptability of raw materials, excipients and final product.[16]

Two candidate biosimilar mAbs were compared to two commercially available chimeric IgG1 products by peptide mapping analysis using the magnetic SMART trypsin digestion with subsequent LC-MS analysis of the generated peptides. This provided a powerful method for PTMs characterization to ensure mAb quality.

A peptide map is a characteristic fingerprint of a protein and the end product of several processes that provide a comprehensive understanding of the protein being analyzed. Peptide mapping is a routine analysis for the characterization of monoclonal antibodies; however, it often involves tedious sample preparation steps, e.g., pre-treatment, digestion, and clean-up procedures, which might reduce reproducibility due to differences among techniques, technicians, or different partner labs. This variation can be specially challenging when it is necessary to compare different product batches or biosimilar products across months or years. As the data quality is imperative, variation in results might jeopardize product quality, affecting the ultimate product efficacy and safety.

Using the Magnetic SMART Digest kit automated with the KingFisher Duo Prime system simplifies the process and reduces the time needed for peptide mapping sample preparation. This approach provides significant improvements in reproducibility over existing protocols, which results in fewer sample failures, higher throughput, and the ability to more easily interrogate data. Figure 1 shows four chromatograms of peptides from infliximab drug products and biosimilars, digested with the Magnetic SMART Digest kit. Obtained base peak chromatograms are very similar but show distinct differences (Figure 1). Each protein to be mapped presents unique characteristics that must be well understood so that the validated development of a peptide map provides sufficient specificity for characterization.

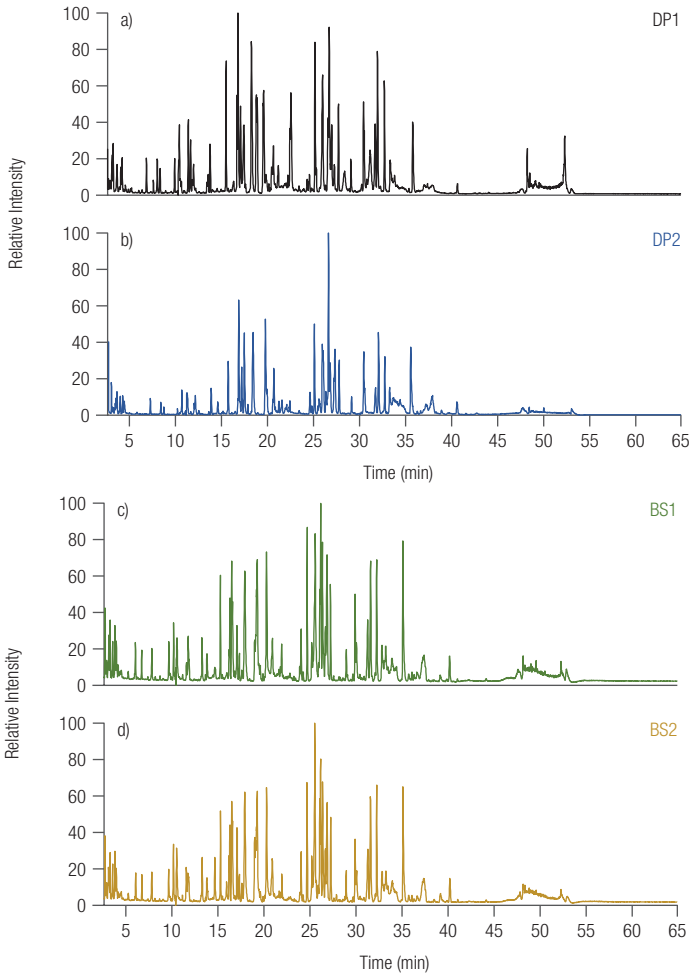

Figure 1. Base peak chromatograms (BPCs) obtained from peptide mapping experiments of a) DP1, b) DP2, c) in-house CHO expressed infliximab, and d) in-house HEK expressed infliximab, after Magnetic SMART digestion with the KingFisher Duo Prime system

The first drug product (DP1) can be identified with 100% sequence coverage while DP2, BS1, and BS2 can be identified with 100% sequence coverage for HC and 98.60% for LC (Table 8). The missing peptide corresponds to a tripeptide 2:E143-K145 (sequence EAK), which has probably not been detected due to poor column retention and low intensity signals where confirmatory MS2 data could not be obtained. The same peptide was identified successfully for DP1 as part of a missed cleavage peptide 2:R108-K145, eluting at 24.7 minutes with low signal intensity <4.9e+04.

A sequence coverage map (Figure 2) shows the overlap of the different peptides identified with different intensities and the different lengths due to missed cleavages. As an example, the sequence coverage map is shown for the drug product DP1. The colored bars show the identified peptides, and the numbers in the bars reflect the retention time. The different colors indicate the intensity of the peptide in the MS1 scan.

The number of detected MS peaks in the samples varied between 304 and 535 for light chain and 804 and 1397 for heavy chain. The chimeric commercially available biosimilar DP2 showed the biggest number of detected MS peaks for both heavy and light chain.

Table 8. Sequence coverage for the studied recombinant IgG1 mAbs

Proteins	Sample	Number of Peaks	Sequence coverage (%)
Recombinant Chimeric IgG1			
Heavy Chain	DP1	1116	100.00
	DP2	1473	100.00
	BS1	864	100.00
	BS2	914	100.00
Light Chain	DP1	487	100.00
	DP2	573	98.60
	BS1	312	98.60
	BS2	326	98.60

Heavy chain

Light chain

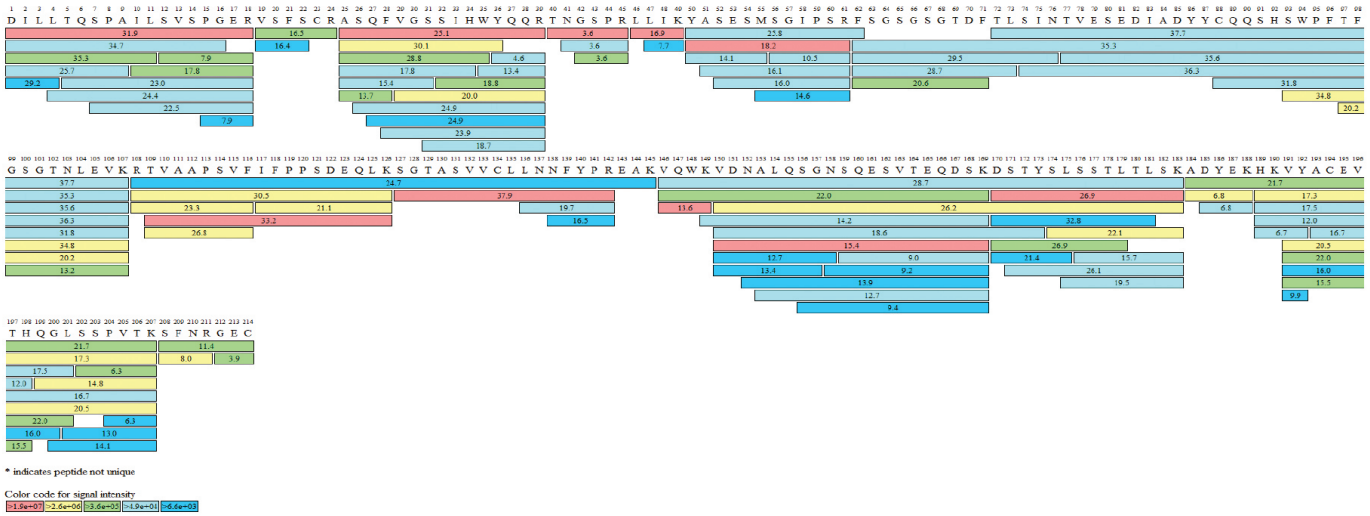

Figure 2. Sequence coverage map of DP1 heavy (top) and light chain (bottom), obtained using a 65 min gradient for peptide separation on an Acclaim VANQUISH C18, 2.2 µm, 2.1 x 250 mm column. The colored bars show the identified peptides, with the number in the bars reflecting the retention time (min) and the intensity of the peptide in the MS1 scan: red = high abundant >1.9e+07; yellow >2.6e+06; green >3.6e+05; light blue >4.9+04; cyan=low abundant >6.6e+03.

In regard to the peptide identification with high confidence, all matched peptides were expected to have ≤ 5 ppm and ≥ 5 ppm of MS mass error, confidence score ≥ 95, and/or confirmatory MS/MS spectra. Figure 3 shows an example of the selected ion chromatograms (SIC) and corresponding MS/MS spectra for the selected peptide LEESGGGLVQPGGSMK present on the four infliximab heavy chains, which elutes at 19.3 min. The combination of high-quality MS and MS/MS data provides a more confident peptide

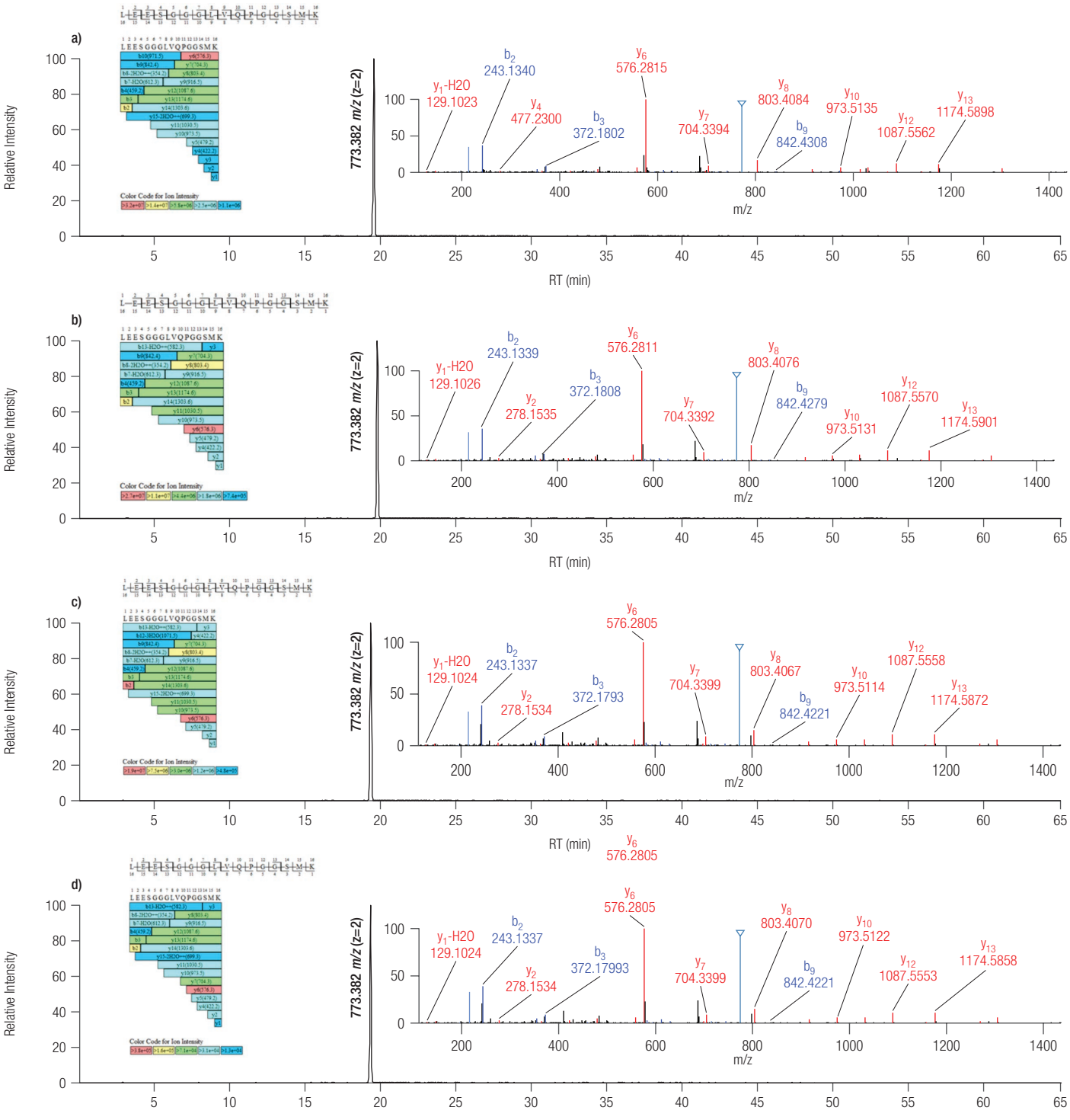

Figure 3. Representative extracted ion chromatogram (XIC) of HC peptide LEESGGGLVQPGGSMK, MS/MS spectra and fragment coverage map from digested (a) DP1, (b) DP2, (c) BS1, and (d) BS2 biotherapeutics. NL indicates normalized level (NL) intensity. The color for the lines and labels for the identified ions in the experimental spectrum vary based on the ion type, as follows: dark blue for "b" ions with a charge on the N-terminal side and red for "y" ions with a charge on the C-terminal side.

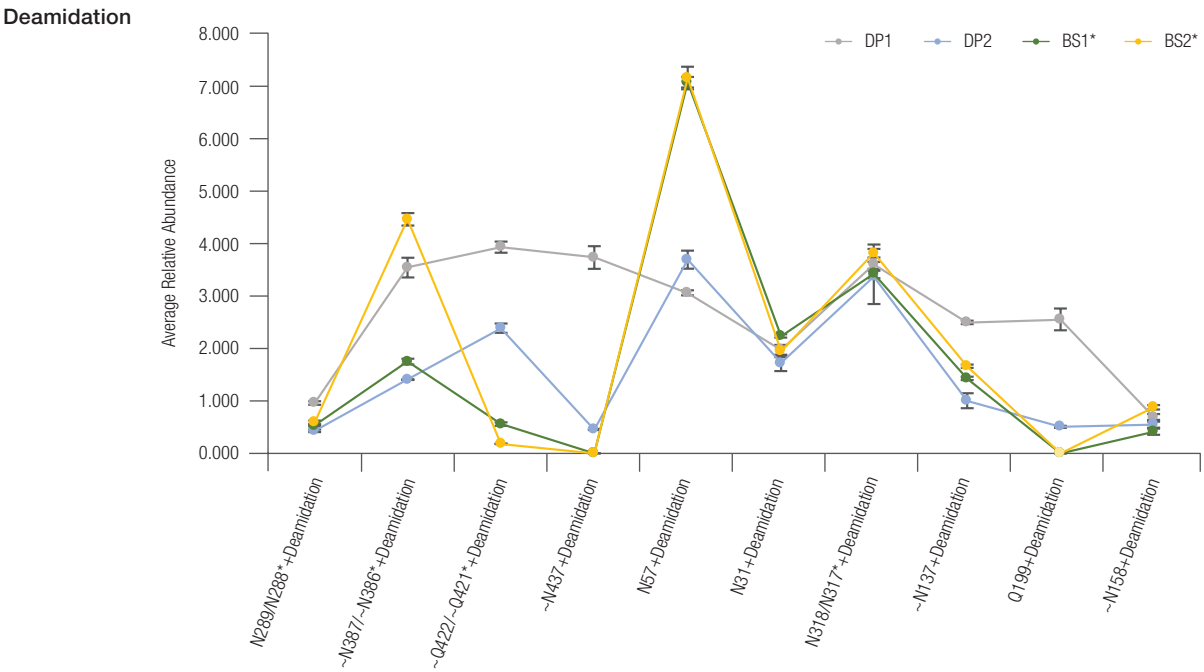

Figure 4. Average relative abundance (n=3) of 10 identified deamidation modifications for infliximab DP1, DP2, and in-house biosimilars BS1 and BS2, respectively. * indicates the different position in biosimilars of corresponding modification in the drug products.

match. The experimental MS/MS spectrum displays an inverted triangle marker at the top of the spectral line for the theoretical precursor ion (773.382 *m/z*, z=2). The labels appear in color for the identified peaks and show their fragment ion assignments and charge states, for example, “b2”, “y6”, or “y12”. The peptide fragment coverage map displays the peptide sequence with corresponding modification, and the peptide sequence with the numbered amino acid sequence and the identified fragment lines, and finally the identified fragment ions using a color-coded code for ion intensity (red, yellow, green, cyan, and blue), with red as most intense and blue as the least intense.

Peptide mapping analysis also facilitates identification and quantitation of PTMs. Many common PTMs cause a shift in reversed-phase LC retention relative to the native peptide. In combination with direct MS and MS/MS analysis it can be used to interrogate modifications with relatively large mass shifts, such as C-terminal lysine (128 Da), glycation (162 Da) and small mass differences such as deamidation (1 Da), oxidation (16 Da), and others.

Table 10 summarizes the identification and relative quantification of a subset of monitored modifications across the infliximab innovator and biosimilar candidates studied. PTMs such as deamidation, oxidation, glycation, C-terminal lysine clipping, and glycosylation are confidently identified based on MS1 and/or MS/MS spectra. A tilde (~) before the modification indicates the modification was found on the parent tryptic peptide but could not be localized on a specific amino acid within the MS/MS spectra. The relative abundance of the detected modifications in the four infliximab products has a relative standard deviation < 10% in most cases. Overall, the method shows that important information regarding PTMs can be obtained reproducibly and accurately.

Deamidation of asparagine (Asn, N)[17] and glutamine (Gln, Q)[18] residues is a common degradation of proteins and it can significantly impact protein structure and function. The rate of deamidation depends on protein sequence and conformation, as well as external factors such as temperature, pH, and time. Figure 4 shows the average relative abundance of eight of the most abundant deamidation modifications for the infliximab products studied. N387/N386 and N57 residues were detected for all the studied mAbs and seem to be more susceptible to PTM showing the most abundant deamidation levels (4.5% for the infliximab biosimilar HEK expressed and around 7% for both studied biosimilars).

In relation to glutamine (Q) deamidation, residue Q422/Q421 of HC seems to be the most susceptible to PTMs, being present at a low level in the biosimilars (<0.5%) and in higher abundances for the drug products between 3.93 and 2.38%.

Microheterogeneity can sometimes be attributed to oxidation of tryptophan (W) or methionine (M) residues. This is another common PTM observed in proteins and peptides. Oxidation of methionine occurs in mAbs during purification, formulation, and storage processes.[19] Oxidation can also occur from frequent freeze-thawing cycles. *In vivo* oxidation is caused by oxygen radicals and other biological factors (e.g., exposure to certain oxidizing drugs or other compounds). *In vitro* oxidation can be due to conditions encountered during purification or formulation. Protein chemists in process development and quality control are concerned with oxidation as it can adversely impact the activity and stability of biotherapeutics.[20] The studied infliximab products in the present work show low oxidation levels between 0.2 and 3.0% except for M34 which is potentially susceptible to oxidation (Figure 5). This was a common modification for all the four infliximab studied products, showing highest levels for the biosimilars produced in-house by CHO and HEK expression systems (32.47% and 27.99%, respectively) and for DP1 (13.66%). It is noteworthy to mention higher levels have been found in the literature for the studied DP infliximab products, which could be attributed to the fact that this PTM is susceptible to change and varies between production batches. Pisupati *et al.*[21] have recently published a multidimensional analytical comparison of Remicade and the biosimilar Remsima/Inflectra where the levels of oxidation were remarkably similar for both mAbs, showing highest oxidation levels for the M34 site (26.44% and 28.69%, respectively), M255 site (20.80% and 21.53%, respectively), and M18 (6.71% and 6.94%, respectively).

Oxidation

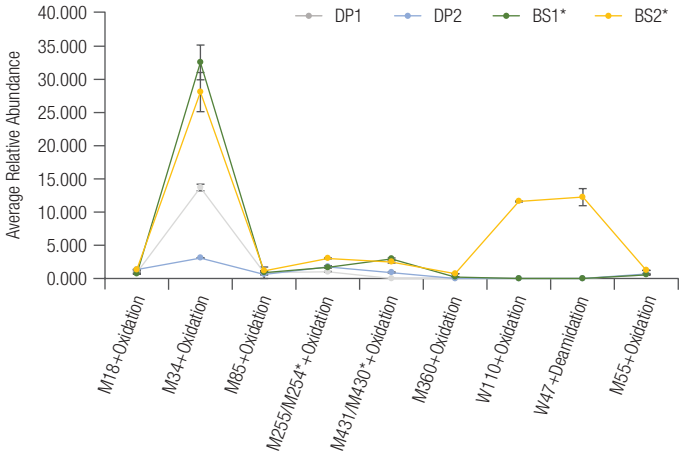

Figure 5. Average relative abundance (n=3) of nine identified oxidation modifications for infliximab drug products (DP1 and DP2) and two biosimilars produced in-house by CHO (BS1) and HEK (BS2) expression systems, respectively. * indicates the different position in biosimilars of corresponding modification in the drug products.

These differences highlight the importance of monitoring CQAs along the production batches.

Some of the most noted PTMs for therapeutic mAbs are their varied *N*-linked glycan structures, which include galactosylation, fucosylation, mannosylation, and sialylation. Glycosylation is a highly variable and heterogeneous process that depends on such factors as clonal variation, production cell line, media, and culture conditions[22-24] and their characterization and quantification are critical to ascertain therapeutic efficacy and safety of the drug. *N*-glycans have important structural functions as they stabilize the CH2 domain of IgGs. Deglycosylation makes mAbs thermally less stable and more susceptible to unfolding and they are more prone to aggregation. Moreover, functionality of the IgG is influenced by the attached *N*-glycans and their size.[25] MAbs produced by Chinese hamster ovary (CHO) cells typically have complex biantennary structures with no bisecting *N*-acetylglucosamine (GlcNAc) and a high level of core fucosylation. Overexpression of *N*-acetylglucosaminetransferase III in such cell lines increases bisecting GlcNAc and nonfucosyated oligosaccharides on mAbs and thus raises ADCC (antibody-dependent, cell-mediated cytotoxicity).[26]

***N*-Glycosilation**

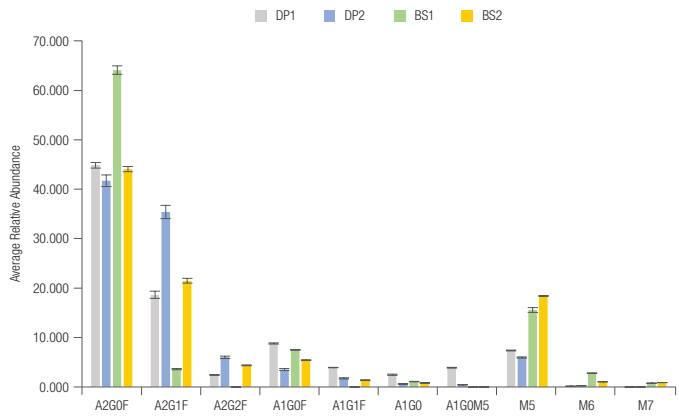

Figure 6. Average relative abundance (n=3) of identified *N*-glycosylation on the Fc region for infliximab drug products and two biosimilars produced in-house by CHO and HEK expression systems, respectively

High abundance of glycosylation of the heavy chain is also observed for the four studied infliximab products in the Fc region at position N300 (DP1 and DP2) or N299 (in-house produced infliximab biosimilars expressed by CHO and HEK cell lines), where the main glycans are complex biantennary oligosaccharides containing from 0 to 2 non-reducing galactoses with fucose attached to the

reducing end of *N*-acetylglucosamine (A2G0F, A2G1F, A2G2F, and A1G0F). Also present at lower levels are afucosylated biantennary (A1G0) and high mannose (M5, M6, and M7) structures (Figure 6). Core fucosylation is relatively quantified between 75.16% (biosimilar produced in-house CHO expressed) and 88.31% (DP2).

Glycosylation of the Fc region is also important for maintaining a long catabolic half-life.[27] IgGs containing high-mannose glycans have shown increased serum clearance,[28] and in addition to terminal sialic acid leads to upregulation of the surface expression of the FcγRIIb on inflammatory cells, thereby initiating the anti-inflammatory cascade.[29] Mannose-5 (M5) N-glycans are detected in high abundance for infliximab biosimilars produced in-house by CHO (15.58%) and HEK (18.38%) cell lines. BS1 and BS2 also contain low levels of higher mannose structures such as M7 and M8 (0.33–0.86%), which have not been detected for DP1 and DP2 (Table 10). According to the literature, high levels of M5 were observed during development of a therapeutic mAb produced in CHO cell line and correlated to the increase of cell culture medium osmolality levels and culture duration.[30] *N*-acetylneuraminic acid (Neu5Ac or NANA) is detected only for DP1 and DP2 at levels < 0.5%. Those infliximab samples also showed low levels of immunogenic *N*-glycolylneuraminic acid (Neu5Gc or NGNA) between 0.10% and 2.88%. The presence of Neu5Gc in recombinant therapeutic proteins expressed in nonhuman cell cultures may be immunogenic and potentially relevant to half-life, efficacy, and adverse events.[31,32] Figure 7 shows the comparison between high mannose, non-galactosylated and galactosylated glycans relatively quantified for the four studied infliximab drug products. Non-galactosylated biantennary *N*-glycan structures are detected with the highest relative abundances (60.76% for DP1 and 76.21% for infliximab biosimilar produced in-house CHO expressed), while the highest levels of galactosylation are detected for infliximab drug product DP2 (45.17%) and infliximab biosimilar produced in-house HEK expressed (27.81%). Regarding high mannose content there is a noticeable variability between the four studied samples, DP2 contained the lowest high mannose content (6.86%) and infliximab biosimilars in-house CHO and HEK expressed with the highest levels (20.03% and 20.75%, respectively).

More detailed information on the glycosylation of infliximab can be found in Application 3648 in the Thermo Scientific™ AppsLab Library of Analytical Applications

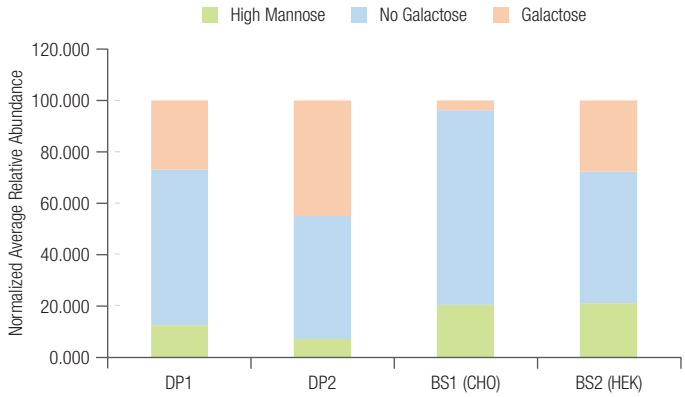

Figure 7. Normalized average relative abundance (n=3) of identified high-mannose, non-galactosylated and galactosylated *N*-glycans for infliximab drug products, and two biosimilars produced in-house by CHO and HEK expression systems, respectively

where a detailed *N*-glycosylation from commercial chimeric IgG1 mAb (infliximab) is shown.[33] The Vanquish Horizon UHPLC system was used for the high-resolution determination of 2-AA (anthranilic acid) labelled N-glycans released from infliximab DP. Separation was performed on a 150 mm Thermo Scientific™ Accucore™ 150 Amide HILIC column with fluorescence detection giving separation in less than 30 minutes.

The C-terminal lysine (Lys) variant is a very common modification observed in monoclonal antibodies and recombinant proteins. Although the effect this variation has on protein activity does not seem to impact potency or safety profile,[34] the degree of heterogeneity of C-terminal Lys variants reflect the manufacturing consistency and should be monitored for product consistency. Lys loss is detected in only three of the four infliximab samples and at different levels (Table 10), with the lowest % of modification for DP1 (38.89%) and the highest level of modification detected for the infliximab biosimilars produced in-house from CHO (94.21%) and HEK (98.77%).

Other commonly targeted modifications are lysine (K) glycations, which are listed in Table 10. In total between 3 and 14 lysine glycations could be identified and relatively quantified for DP1 (<0.13%) and DP2 (< 0.84%), while infliximab biosimilar produced from CHO shows the highest levels of glycation (<2.98%) with more than 20 lysine residues modified. Interestingly, this modification was not observed for infliximab biosimilar from HEK.

Table 10. Comparison of the deamidation, oxidation, glycation, lysine-loss, and glycosylation modifications identified for the different infliximab drug products and biosimilars studied. (n=3). * indicates the different position in biosimilars of corresponding modification in the drug products.

	Relative Abundance			
Modification	**DP1 (n=3)**	**DP2 (n=3)**	**BS1* (n=3)**	**BS2* (n=3)**
Q13+Deamidation	1.071	0.779	0.156	-
N31+Deamidation	1.972	1.718	2.236	1.945
Q39+Deamidation	0.833	0.639	-	0.212
N57+Deamidation	3.057	3.688	7.066	7.146
Q84+Deamidation	0.905	0.628	0.142	0.262
N101+Deamidation	0.882	-	0.969	1.259
Q112+Deamidation	0.997	-	-	-
N162/N161*+Deamidation	0.599	0.787	-	0.432
N279+Deamidation	0.142	-	-	-
N289/ N288*+Deamidation	0.960	0.430	0.529	0.590
N318/N317*+Deamidation	3.603	3.368	3.424	3.811
N328+Deamidation	-	0.159	-	-
N364/ N363*+Deamidation	1.728	0.939	0.240	1.240
Q365/ Q364*+Deamidation	2.626	0.458	0.995	0.154
~N387/~N386*+Deamidation	3.537	1.404	1.748	4.456
~N392+Deamidation	-	1.985	-	-
~Q422/~Q421*+Deamidation	3.926	2.384	0.558	0.183
~N437+Deamidation	3.729	0.456	-	-
Q6+Deamidation	1.152	0.734	0.128	0.231
~Q27+Deamidation	-	-	0.115	-
~Q37+Deamidation	1.668	1.269	0.261	0.671
~N137+Deamidation	2.494	1.003	1.428	1.660
Q147+Deamidation	0.864	0.449	-	0.169
~N158+Deamidation	0.694	0.547	0.413	0.878
~Q166+Deamidation	1.833	1.109	-	-
Q199+Deamidation	2.551	0.506	-	-
N210+Deamidation	0.255	0.217	-	-
~Q155+Deamidation	-	1.075	-	0.247
M18+Oxidation	0.875	1.324	0.679	1.310
M34+Oxidation	13.660	3.062	32.446	27.992
M85+Oxidation	0.861	0.605	0.898	1.142
M255/M254*+Oxidation	0.991	1.745	1.652	3.017
~M360+Oxidation	-	-	0.232	0.723
M431/M430*+Oxidation	-	0.905	2.934	2.487
M55+Oxidation	0.566	0.702	0.543	1.217
W47+Oxidation	-	-	-	12.214
W110+Oxidation	-	-	-	11.564
K67+Glycation	0.027	0.201	0.878	-
K149+Glycation	0.100	0.842	2.266	-
K188+Glycation	0.129	0.152	2.983	-
K43+Glycation	-	0.063	0.286	-
K54+Glycation	-	0.037	0.327	-
K136/K135*+Glycation	-	0.290	1.272	-
K150+Glycation	-	0.419	-	-
K251/~K250*+Glycation	-	0.080	0.521	-
~K291/~K290*+Glycation	-	0.235	1.306	-
K320/K319*+Glycation	-	0.140	0.403	-
K329/K328*+Glycation	-	0.341	1.377	-
K49+Glycation	-	0.660	1.468	-
K169+Glycation	-	0.188	0.637	-

Table 10 (continued). Comparison of the deamidation, oxidation, glycation, lysine-loss, and glycosylation modifications identified for the different infliximab drug products and biosimilars studied. (n=3). * indicates the different position in biosimilars of corresponding modification in the drug products.

Relative Abundance				
Modification	**DP1 (n=3)**	**DP2 (n=3)**	**BS1* (n=3)**	**BS2* (n=3)**
K3+Glycation	-	-	0.235	-
K19+Glycation	-	-	0.082	-
K78+Glycation	-	-	0.383	-
K207+Glycation	-	-	0.336	-
K224+Glycation	-	-	0.517	-
K322+Glycation	-	-	0.238	-
K336+Glycation	-	-	0.239	-
K362+Glycation	-	-	0.195	-
K145+Glycation	-	-	0.268	-
K190+Glycation	-	-	0.305	-
K450/K449*+Lys Loss	38.892	-	94.212	98.768
N300/N299*+A1G0	2.466	0.551	1.065	0.800
N300/N299*+A1G0F	8.785	3.523	7.471	5.410
N300+A1G0M4	1.705	0.241	-	-
N300+A1G0M5	3.866	0.417	-	-
N300+A1G0M5F	1.831	-	0.568	-
N300+A1G1F	3.908	1.713	3.573	1.359
N299+A2G1FB	-	-	-	0.282
N300+A1G1M5	1.359	-	-	-
N300+A1G1M5F	0.524	-	-	-
N300+A1S1M5	0.512	0.400	-	-
N300+A1Sg1	0.318	0.099	-	-
N300+A1Sg1F	2.100	0.983	-	-
N300/N299*+A2G0	1.660	0.822	0.818	0.204
N300/N299*+A2G0F	44.860	41.700	64.118	44.047
N300+A2G1	0.370	0.422	-	-
N300/N299*+A2G1F	18.605	35.393	3.573	21.467
N300/N299*+A2G2F	2.401	5.985	-	4.349
N299+A3G0F	-	-	1.295	-
N300+A2Sg1G0F	0.757	2.628	-	-
N300+A2Sg1G1F	-	2.881	-	-
N299*+A2S1G1F	-	-	-	0.238
N300+A2Ga1G1F	-	0.344	-	-
N300+A2Sg2F	-	0.260	-	-
N300+M4	0.094	0.053	-	-
N300/N299*+M5	7.329	5.917	15.581	18.378
N300/N299*+M6	0.121	0.208	2.767	1.036
N299/N299*+M7	-	-	0.756	0.861
N299/N299*+M8	-	-	0.537	0.326
N300/N299*+Unglycosylated	0.390	0.517	1.222	0.738

Conclusions

- The Magnetic SMART Digest kit provides simple and rapid protein digestion for peptide mapping analysis and PTM investigations for comparability studies between innovator and biosimilar monoclonal antibodies.
- Analysis of four infliximab samples gave excellent quality data with high confidence in the results.
- Excellent sequence coverage (~100%) was observed.
- Low levels of post-translational modifications (PTMs) were observed with the Magnetic SMART Digest kit, except for the M34+oxidation site, where noticeable levels were relatively quantified.
- Peptide mapping was easily automated, resulting in less sample handling, increased productivity, and improved reproducibility, even with peptides at low levels. This will allow confident transfer of methods between laboratories.
- Infliximab biosimilarity of the primary structure and PTMs was achieved successfully by the analytical approach using automated magnetic SMART digestion for peptide generation and subsequent LC-MS analysis. The Thermo Scientific peptide mapping workflow provided reproducible results with excellent mass accuracy and high sensitivity.
- BioPharma Finder software can provide automatic data processing, peptide sequence matching, and protein sequence coverage mapping accurately and with high confidence.

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