APPLICATION NOTE 73911

Biosimilar sequence variant assessment and product quality attribute monitoring

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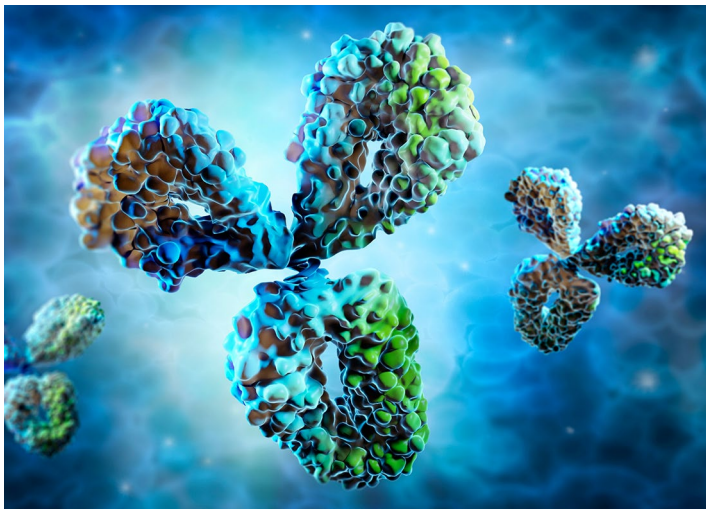

Key benefits

- Confident analysis of a biosimilar and its sequence variants through all stages of the HR-MAM workflow: creating the list of product quality attributes and monitoring thereof, new peak detection, and identification of newly found species.
- Easy-to-use, compliance-ready chromatography data solution (CDS) software with product quality monitoring and new peak detection capabilities provides a seamless workflow from data acquisition to data processing and reporting, allowing the HR-MAM workflow to be implemented in a QC laboratory.
- Full MS data acquisition using a high-resolution setting of 140,000 (FWHM) at *m/z* 200 at all stages of the workflow provides accurate results with very high confidence.

Goal

In this work, the applicability of the Thermo Scientific™ HR-MAM workflow for biosimilarity assessment is evaluated. The workflow was used to compare rituximab drug product (DP) with an investigational biosimilar produced in NIBRT's laboratories (BS). The study demonstrates the use of the workflow for sequence variant assessment and to monitor the variability of product quality attributes in biosimilar when compared to its originator.

Introduction

Analytical technologies in the biopharmaceutical sector are facing a growing challenge posed by the exponential increase of biopharmaceutical products on the market. Newly approved drugs include treatments based on novel types of antibody-based molecules, such as Fc-fusion protein and antibody-drug conjugates, drugs with increased complexity, such as bi-specific antibodies and an increasing number of biosimilars.

Biosimilars avail of a shortened approval pathway in regulatory agencies as established with the Biologics Price Competition and Innovation (BPCI) Act from 2009[1]. However, the reduced length and complexity for clinical trials are replaced by extensive analytical assessment of the similarity with their originator drugs. To reach this goal, in-depth knowledge of both originator and candidate biosimilar is required.

Data-rich analysis based on high-resolution accurate mass (HRAM) mass spectrometry (MS) experiments is key for a thorough characterization of biopharmaceuticals. Recently, HR-MS analysis has been introduced to the quality control laboratory to assess product attributes during bioprocess and lately for batch release. This is made possible through the implementation of a Multi-Attribute Method (MAM), which is a workflow based on peptide mapping analysis and the use of high-resolution mass spectrometry[2]. The Multi-Attribute Method is divided into a characterization phase, where the reference sample is analyzed through LC-MS/MS experiments that are processed to obtain a concise list of product quality attributes entailing their accurate masses and retention time information. These parameters are implemented in a processing method used in the second “monitoring” phase, where LC-MS experiments are performed on any following sample, acquiring high-resolution Full MS only. Compliant chromatography data software uses the processing method created in the first stage of data analysis to assess if the protein's product quality attributes (PQAs) are within an acceptable range. A second stage, the non-targeted MS processing step, performs a binary comparison of the sample compared to a reference, to verify that no new entities are present in the sample. This processing step is also commonly referred to as new peak detection (NPD).

In this work, the Thermo Scientific HR-MAM workflow[3,4] was used to evaluate product quality attributes in rituximab drug product versus an in-house produced biosimilar, with a special focus on differences in Fc N-glycosylation. Moreover, the in-house biosimilar is known to have several point mutations in all subunits. NPD was performed to verify the differences between the two samples and peptides only present in the biosimilar arising from these point mutations[5].

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Water, Optima™ LC/MS grade (Fisher Chemical, P/N 10505904)
- Acetonitrile with 0.1% formic acid (v/v), Optima™ LC/MS grade (Fisher Chemical, P/N 10118464)
- LC-MS grade formic acid (>99%, Pierce™, P/N 28905)
- Thermo Scientific™ Acclaim VANQUISH™ C18, 2.1 × 250 mm column (P/N 074812-V)
- 8.0 M guanidine hydrochloride solution (Sigma-Aldrich, P/N G7294-100ML)
- Invitrogen™ UltraPure™ 1 M Tris-HCl Buffer, pH 7.5 (P/N 15567027)
- DL-Dithiothreitol (DTT) BioXtra ≥99% purity (Sigma-Aldrich, P/N D-5545)
- Sodium Iodoacetate (IAC) BioUltra >98% purity (Sigma-Aldrich, P/N I-9148)
- Thermo Scientific™ Pierce™ Trypsin-Protease, MS grade (P/N 90058)
- Thermo Scientific™ Pierce™ BSA Protein Digest, MS grade (P/N 88341)
- Bio-Spin™ P6- Desalting Spin Columns (BioRad, P/N 732-6227)
- HiTrap™ Protein A (GE Healthcare)
- 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™ Horizon UHPLC system (P/N 5400.0105) consisting of:
  - Thermo Scientific™ Vanquish™ System Base (P/N VF-S01-A-02)
  - Thermo Scientific™ Vanquish™ Binary Pump H (P/N VH-P10-A-02)
  - Thermo Scientific™ Vanquish™ Split Sampler HT (P/N VH-A10-A-02)
  - Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A-02)
  - MS Connection Kit Vanquish (P/N 6720.0405)
- Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (P/N IQLAAEGAAPFALGMBDK)
- Thermo Scientific™ Nanodrop™ 2000 Spectrophotometer (P/N ND-2000)

Software packages

- Thermo Scientific™ BioPharma Finder™ software, version 3.2
- Thermo Scientific™ Chromeleon™ Chromatography Data System software, version 7.2.10

Sample preparation

Rituximab biosimilar expression and purification

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 rituximab monoclonal antibody using lipid-based transfection system (Gibco). The vectors (pFUSEss-CHIg-hG1 and pFUSE2ss-CLIg-hk) were purchased from Invivogen. The cells were harvested and samples of clarified media were passed through a HiTrap Protein A column (GE Healthcare) using the ÄKTA™ Avant system (Cytiva), then washed with phosphate buffered saline before elution of mAbs from the Protein A column using 100 mM citric acid, pH 3.3.

Tryptic digest

50 µg of mAb was diluted to 0.5 mg/mL with 7.0 M guanidine hydrochloride, 100 mM Tris pH 8.3 to give a final volume of 100 µL. The reduction of disulfide bonds was performed with 2 µL of 500 mM DTT and incubation at room temperature for 30 minutes. Following reduction, samples were alkylated by the addition of 4 µL of 500 mM sodium iodoacetate and incubation for 20 minutes at room temperature in the dark. Alkylation was quenched by adding 4 µL of 50 mM DTT.

BioSpin-6 columns were conditioned by centrifugation at 1000 × g for 2 minutes with 50 mM Tris pH 7.9 (4 x 500 µL) and the flow-through discarded. 110 µL of the reduced and alkylated sample was added to the column bed and the flow-through was collected in a fresh 1.5 mL microcentrifuge tube by centrifugation at 1000 x g for 4 minutes. Pierce Trypsin-Protease was added to the samples at a 1:10 ratio and incubated at 37 °C for 30 minutes. Formic acid (10% v/v) was added at a 1:10 ratio in volume to halt digestion. Sample preparation was performed in individually digested triplicates. Samples were transferred to individual LC vials and placed into an autosampler at 5 °C.

LC-MS conditions

Following MS system calibration, a system suitability test was performed using the BSA Protein Digest. Peptide mapping analysis was carried out using an Acclaim VANQUISH C18 reversed-phase column (2.1 × 250 mm, 2.2 µm) on a Vanquish Horizon UHPLC system coupled to a Q Exactive Plus mass spectrometer. Mobile phase A was 0.1% formic acid in water and B was 0.1% formic acid in acetonitrile. The LC gradient is detailed in Table 1. Column temperature and the flow rate was maintained at 25 °C and 300 µL/min throughout the run.

Table 1. LC gradient applied for peptide mapping experiments

Time [min]	%B
0.0	2
100	40
100.5	80
104	80
104.5	2
110	40
110.5	80
114	80
114.5	2
120	2

Tune parameters were set to the following: sheath gas flow rate 40 arbitrary units (au), auxiliary gas flow rate 10 au, spray voltage was 3.8 kV, the capillary temperature was 320 °C, S-lens RF voltage was 50, and auxiliary gas heater temperature was 400 °C.

For the monitoring step, the MS method was set to acquire Full MS only scans in positive ionization mode with a default charge state of 2. Data were acquired at a resolution setting of 140,000 (FWHM) at *m/z* 200 with an acquisition gain control (AGC) target of 3×10^6 for a scan range of *m/z* 200–2,000 and a maximum injection time of 100 ms.

For the peptide mapping step, the method comprised of Full MS scans followed by data-dependent Top5 MS^2 scans with the Full MS scan parameters set as for the above Full MS only method. Data-dependent scans were acquired with a resolution setting of 17,500 with an AGC target of 5×10^5 and a minimum AGC threshold of 2×10^3. The maximum injection time was set to 200 ms with an isolation window of 1.2 Th. The normalized collision energy was set to 28 with a dynamic exclusion window of 7.0 seconds.

MS data processing

Peptide identification and PTM analysis were carried out using BioPharma Finder software version 3.2 applying parameters as summarised in Table 2. For the monitoring of PQAs throughout the time course of the cell culture process study, a target peptide workbook was generated with Biopharma Finder software, and components were filtered to consider peptides with up to only 1 missed cleavage (except for the peptide TKPREEQYNSTYR, which was also included because of their significant abundances). Peptides containing sodium or potassium adducts as well as those that correspond to non-specific protease activity, gas-phase generated ions, or unspecified modifications were excluded.

Table 2. BioPharma Finder software parameter settings for peptide mapping data analysis

Component detection	**Setting**
Absolute MS signal threshold	2.0×10^4 counts
Typical chromatographic peak width	0.3
Relative MS signal threshold (% base peak)	1.0
Relative analog threshold (% of highest peak)	1.0
Width of Gaussian filter (represented as 1/n of chromatographic peak width)	3.0
Minimum valley to be considered as two chromatographic peaks	80.0%
Minimum MS peak width (Da)	1.2
Maximum MS peak width (Da)	4.2
Mass tolerance (ppm for high-res or Da for low-res)	4.0
Maximum retention time shift (min)	0.50
Maximum mass (Da)	30,000
Mass centroiding cutoff (% from base)	15
Identification	**Setting**
Maximum peptide mass	7,000
Mass accuracy	8 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	Lys
Side chain	Deamidation (NQ) Succinimide (N) Oxidation (MW)

The target workbook (.wbpf) was imported into Chromeleon CDS and the data were analyzed using the ICIS MS peak detection algorithm. Table 3 summarizes parameter settings for PQA quantitation and Table 4 details the MS component table for the studied PQAs.

Non-targeted MS processing for New Peak Detection (NPD) in Chromeleon CDS was used for the analysis of sequence variants. The NPD settings used for this study are provided in Table 5, after optimization of most critical parameters such as frame time width, *m/z* range, RT range, and peak intensity threshold.

Table 3. Chromeleon CDS parameter settings for peptide mapping data processing and PTMs quantitation

MS detection	Setting
Extracted ion chromatogram	MS default detection settings
Detection algorithm	ICIS
Area noise factor	5
Peak noise factor	10
Baseline window	40
Noise method	INCOS
Min peak width	3
Multiplet resolution	10
Area tail extension	5
Area scan window	0
MS settings	**MS chromatogram settings**
Mass precision	4 decimal places
Mass tolerance (manually defined)	5.0 ppm
Smoothing	None
Peptide table	**Setting**
Imported from BPF3.1 peptide workbook	-
Composite scoring	**Setting**
Pass score if at least	2 criteria passed
Fail score if less than	1 criterion passed
MS criteria	General MS
Isotopic dot product	≥0.9000
Mass accuracy	≤5.00 ppm
Peal apex alignment	≤0.50 min

Table 5. Chromeleon CDS new peak detection parameter settings for sequence variant analysis

Global settings	
Fixed reference injection	Commercial IgG1 DP
Max threads	8
Alignment parameters	
Alignment bypass	False
Alignment min intensity	1,000
Correlation bin width	1
Max RT shift	1
RT limits for alignment	True
Tile size	300
Frame parameters	
Frame time width (min)	1
m/z max	1,800
m/z min	200
m/z width (ppm)	10
Maximum number of frames	5,000
Peak intensity threshold	1,000
Retention time start-stop (min)	1.0–90 .0
Scan filter(s)	FTMS + p ESI Full MS (200-2,000)
Filter settings	
PR element	= 0
PR size	>1
Charge	is between 2 and 3
Ratio	≥1,000,000

Table 4. List of the PQAs monitored for rituximab DP

Modification	Peptide sequence	Charge states
A1G0 A2G1 M3 M9 A1G0F A2G1F M4 A1G0M5 A2G2F M5 A1G1F A2S1G0F M6 A1S1F A2S1G1F M7 A2G0 A2S2F M8 A2G0F A3G1F Aglycosylation	EEQYN[301]STYR TKPREEQYN[301]STYR	+2, +3, +4 +3, +4
Deamidation, succinimide	GFYPSDIAVEWESN[388]GQPEN[393]N[394]YK	+2, +3, +4
Methionine oxidation	SSSTAYM[81]QLSSLTSEDSAVYYCAR	+2, +3, +4
	DTLM[256]ISR PKDTLM[256]ISR	+1, +2 +3
	WQQGNVFSCSVM[432]HEALHNHYTQK WQQGNVFSCSVM[432]HEALHNHYTQKSLSLSPG	+2, +3, +4, +5 +3, +4, +5

Results and discussion

Biosimilarity assessment relies on the in-depth characterization of originator drug and evaluation of product quality attributes for the comparison with the investigational biosimilar. For those post-translational modifications that are considered critical for product safety and efficacy, this is even more important during the evaluation of mAb biosimilarity.

In this work, a MAM workflow based on PQAs relative to rituximab drug product (Figure 1) was applied to our in-house investigational biosimilar to evaluate differences present between the two mAbs. From the previous analysis, it was known that the amino acid sequence of our biosimilar differed from the originator for 11 point mutations located in 5 tryptic peptides.

Characterization phase and workbook generation

Following the tryptic digestion of originator and biosimilar samples, LC-MS/MS experiments were performed only on the originator, while tryptic digestion of our biosimilar was analyzed only in Full MS mode.

LC-MS/MS spectra were used to evaluate which PQAs need to be included in the monitoring step of our MAM workflow (Table 4). Subsequently, a target peptide workbook file was generated in BioPharma Finder software containing the list target peptides (Figure 2) with relevant information required for monitoring: the peptide sequence, theoretical masses, and detected charge states, the *m/z* values of the isotopes, and retention times.

Monitoring phase and PQA evaluation

Upon import of the Target Peptide workbook file into Chromeleon CDS, a processing method was created to process the LC-MS experiments from the biosimilar according to the parameters provided in Table 3.

Bar graphs of the monitored PQAs are displayed in Figure 3a, representing results obtained for oxidation, deamidation, and succinimide formation hotspots, showing comparable levels in DP and BS. Figure 3b summarizes the results obtained for the N-glycan distribution showing different relative abundances of the main glycoforms. In particular, for rituximab DP, galactosylated glycoforms were

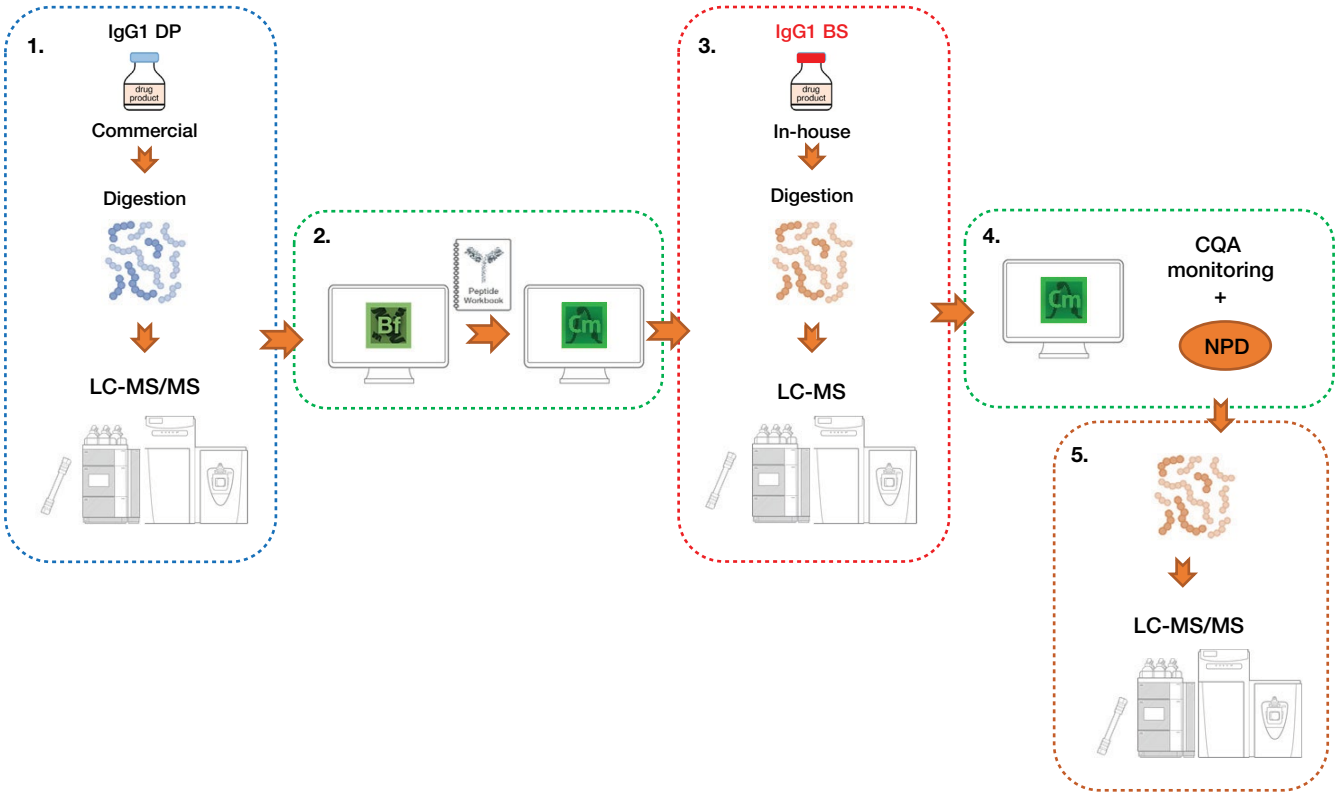

Figure 1. Thermo Scientific HR-MAM workflow for biosimilarity assessment. 1) Characterization phase; reference drug product is digested and analyzed via LC-MS/MS. 2) LC-MS/MS data are moved into BioPharma Finder software to generate a target peptide workbook that is exported from BioPharma Finder and imported in a Chromeleon Processing Method. 3) Biosimilar sample is digested and analyzed through LC-MS experiments. 4) Data are acquired and analyzed in Chromeleon software for PQA monitoring and new peak detection analysis. 5) If new species are found, the biosimilar sample is re-analyzed by LC-MS/MS for their identification.

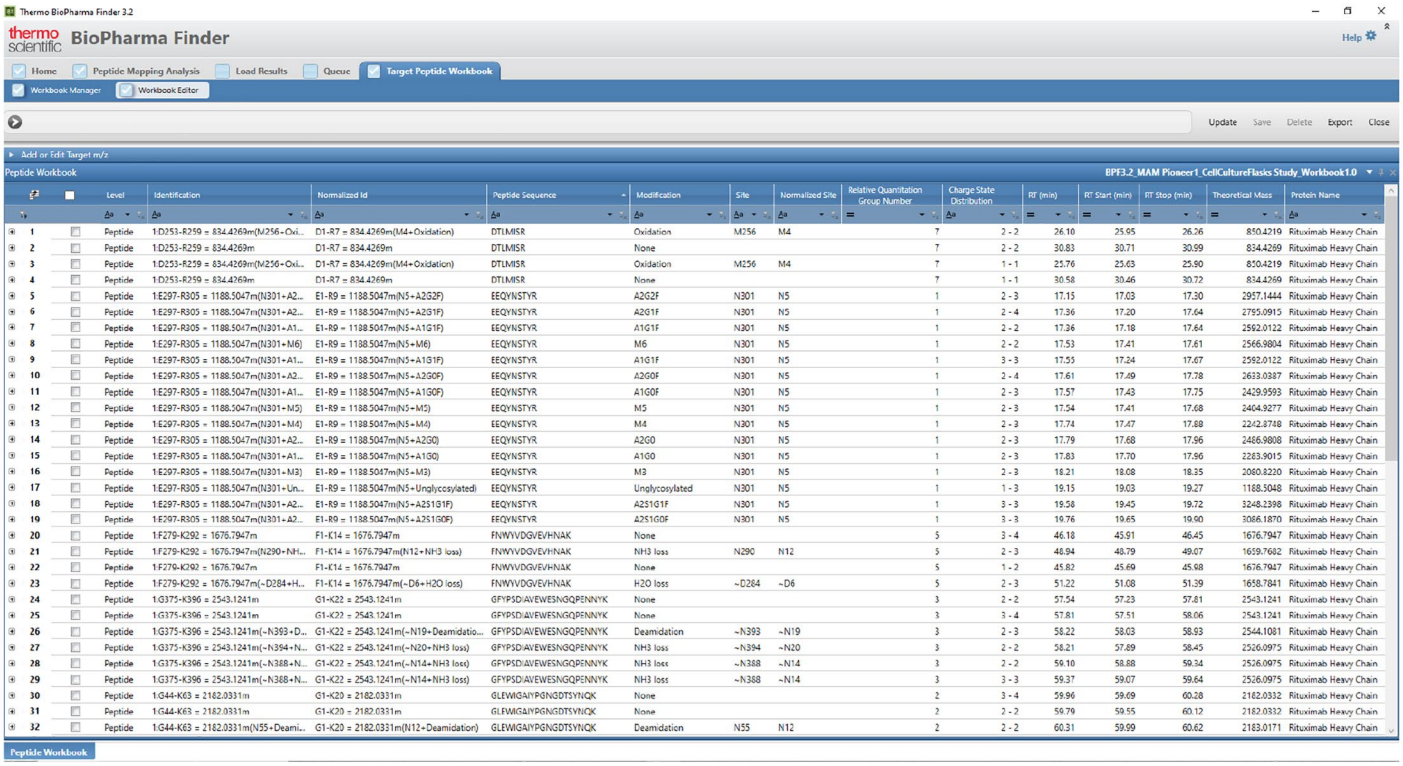

Figure 2. BioPharma Finder target peptide workbook. The workbook is generated in BioPharma Finder software and saved as a .wbpf file that is being imported in Chromeleon CDS for targeted data processing.

found at higher abundance with respect to the biosimilar, with A2G1F being the most abundant N-glycan (41.9%) while the same N-glycan reaches 25.3% of the total in the biosimilar IgG1. This difference could have a consequence on the effector function and thus needs to be monitored[6]. Another important difference in the glycosylation profile is the presence of a higher level of high mannose N-glycans in the biosimilar product (17.3% vs 1.7%, in the BS and DP, respectively), with high levels of high mannoses influencing rates of clearance of the IgG1 from the patient organism[7].

New peak detection and identification of new components

Following PQA analysis, the non-targeted MS processing step was applied to the data obtained for the triplicate biosimilar samples to evaluate the presence of new components with the originator analysis set as a reference. For detailed parameter settings, refer to Table 5. As a result, for this analysis step, eleven frames were detected representing differences between the biosimilar and originator samples (Figure 4), and that required further analysis for their identification. The sequence variants differences present in the biosimilar could explain the presence of the detected frames as the mirror plot of the total ion chromatograms representing Full MS spectra each of the originators and the biosimilar samples is shown in Figure 5. The comparison of both traces clearly shows several distinct differences that were likely caused by the sequence variations present in the biosimilar sample, causing a difference in chromatographic separation profile as well as ionization efficiency. Yet, not all 11 frames that were picked up in the new peak detection step could be caused by the expected sequence variations, which required the next step aiming to identify all peaks representing the 11 frames.

Identification of new peaks

To identify the new species that were found in the biosimilar, the biosimilar sample was rerun with an LC-MS/MS method. Data analysis in BioPharma Finder software, matching the acquired spectra against the originator sequence, revealed only incomplete sequence coverage (98% for the heavy chain and 73.7% for the light chain) due to the different peptides present in the biosimilar. When searching the data again using the amino acid sequence of the biosimilar, 100% sequence coverage was achieved for both heavy and light chain, and the components identified for the missing peptides showed a mass corresponding to some of the frames detected from the non-targeted MS processing (Figure 4). MS/MS spectra for these components are allowed to confirm their identity with high confidence (Figure 6).

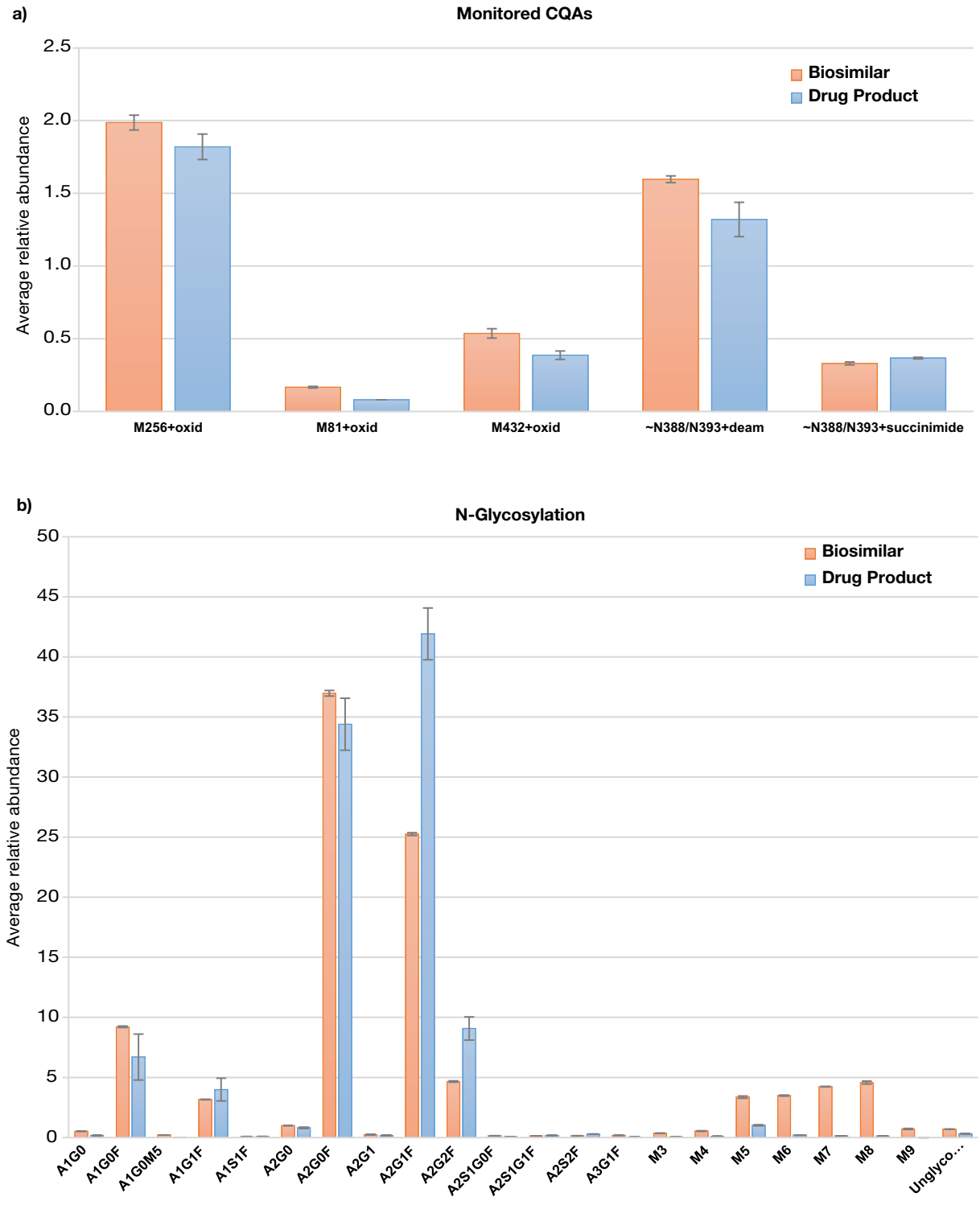

Figure 3. PQAs for rituximab drug product and an in-house biosimilar. Average relative abundances were obtained from triplicate independent tryptic digests via HR-MAM workflow using Chromeleon CDS.

Frame peak #	Mass (*m/z*)	RT (min)	Molecular weight (*m/z*)	Identification
1	300.689	2.84	599.3637	KVEPK
2	236.642	3.21	471.2684	VEPK
3	319.146	5.45	636.2772	EEMTK
4	310.141	5.45	618.266	EEMTK, H2O loss
5	752.355	35.64	2255.0445	ASSSVSYMHWYQQKPGSSPK
6	708.385	38.66	1414.755	Nonspecific peptide IYAPSNLASGVPAR
7	851.386	39.59	1700.757	ASSSVSYMHWYQQK
7	567.930	39.60	1700.7681	ASSSVSYMHWYQQK
8	751.399	48.90	2252.1723	PGSSPKPWIYAPSNLASGVPAR
9	566.970	53.37	1697.8873	PWIYAPSNLASGVPAR
9	849.950	53.43	1697.885	PWIYAPSNLASGVPAR
10	578.845	56.81	1155.6747	not identified
11	1470.140	62.59	2939.2701	VEAEDAATYYCQQWSFNPPTFGAGTK

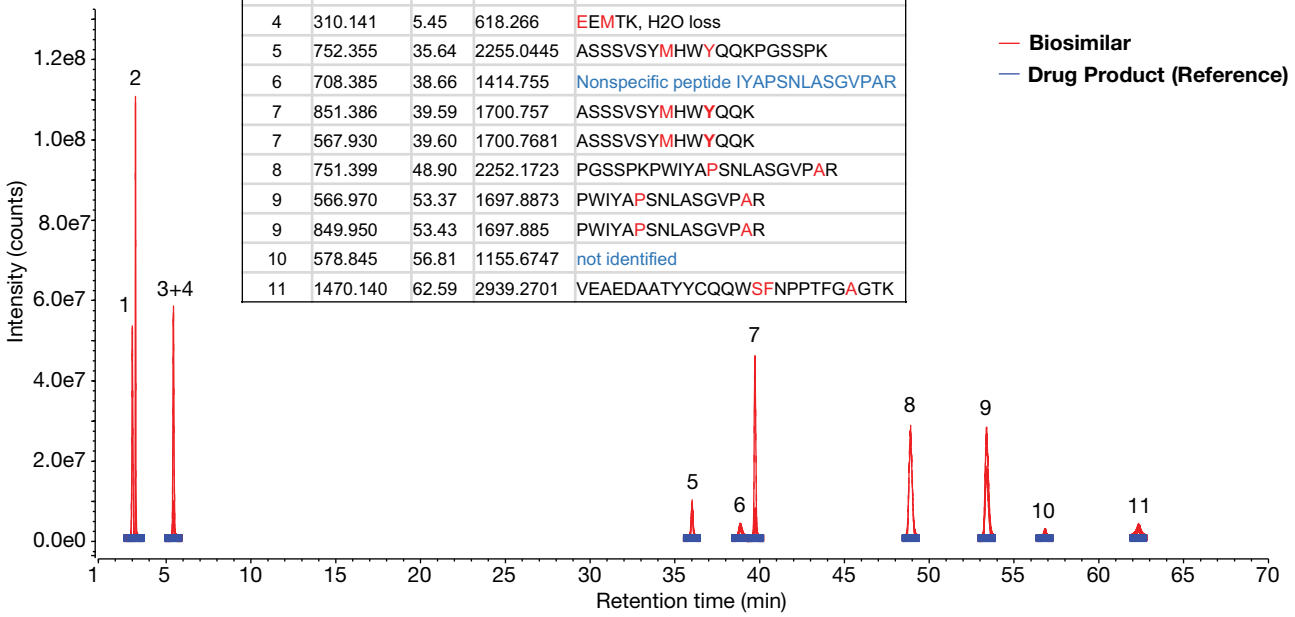

Figure 4. Frame plot from NPD analysis performed on the biosimilar drug product using the originator drug product as a reference. The table shows the main parameters of the detected frames (*m/z*, retention time) as well as peptide sequence identifications based on analysis of LC-MS/MS data analyzed in BioPharma Finder software.

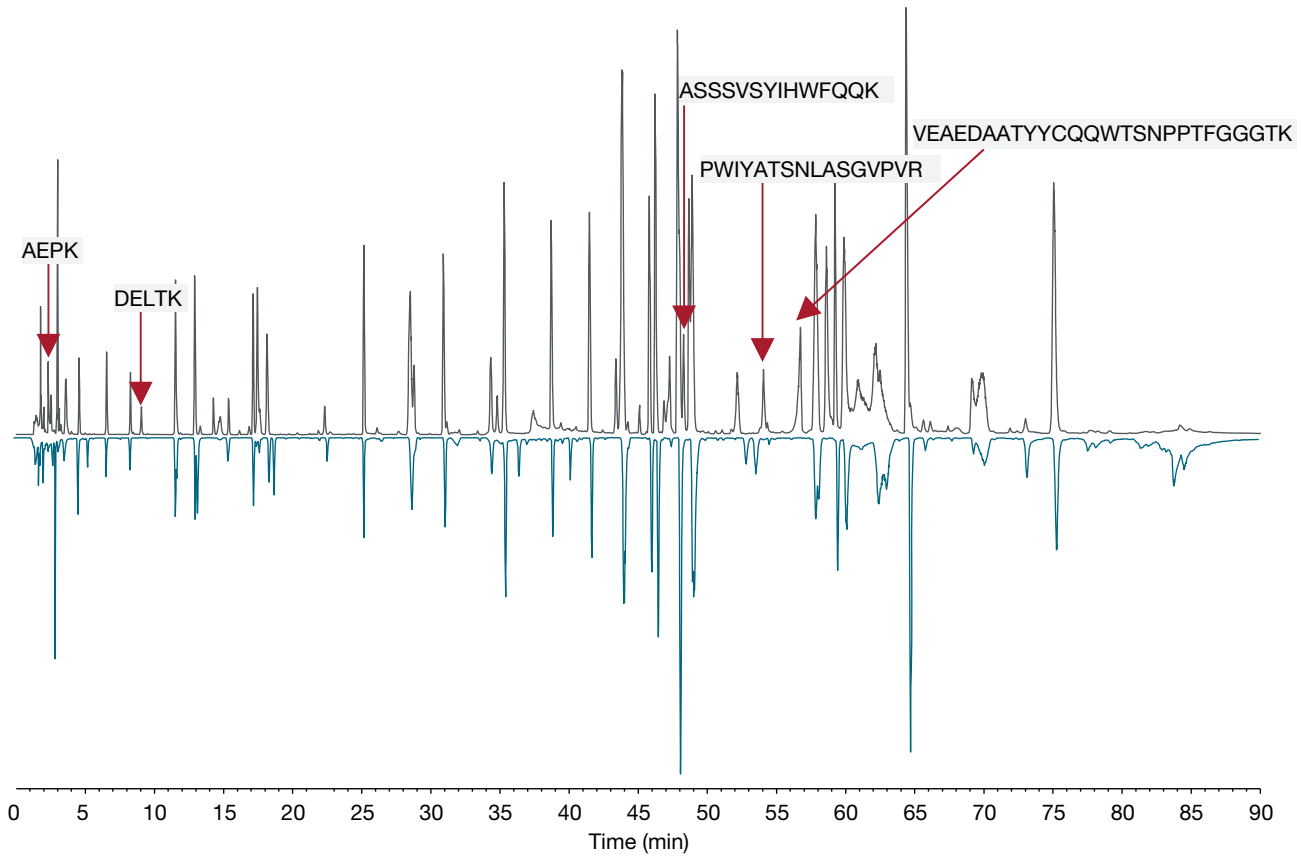

Figure 5. TIC mirror plot obtained from peptide mapping experiments of commercial IgG1 DP (black trace) and investigational IgG1 BS (blue trace). The red arrows highlight peaks representing peptides only detected for IgG1 DP.

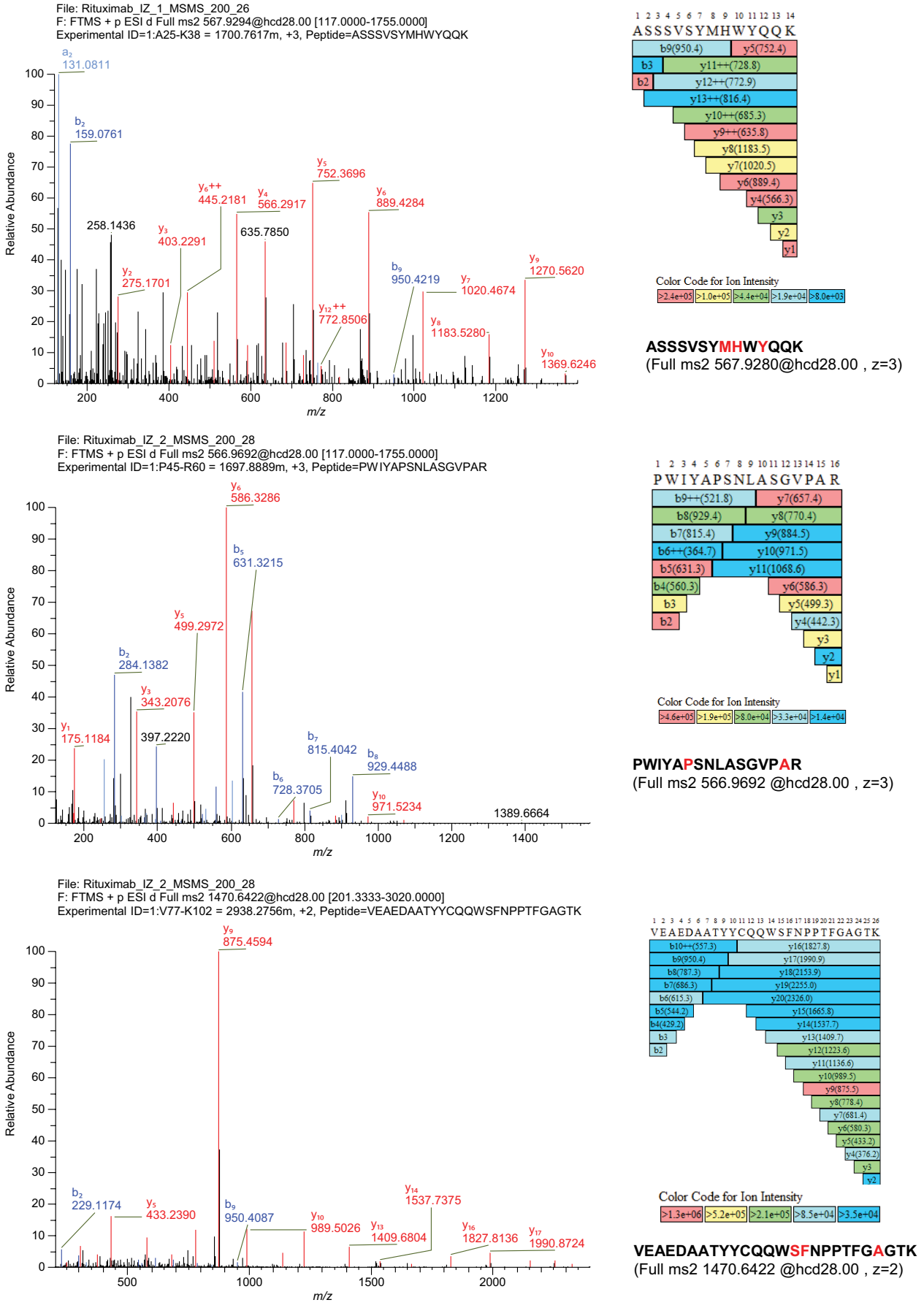

Figure 6. Annotated MS/MS spectra of three frames arising from NPD on the biosimilar and identified as peptides belonging to the light chain with several amino acid mutations. Peak assignments and fragment coverage maps were obtained from BioPharma Finder software.

However, only 9 of the 11 frames were assigned to new peptides arising from the sequence variants. For the remaining two frames, one was identified as a peptide deriving from a non-specific activity of trypsin (Frame #6, Figure 4), while the last frame (Frame #10, Figure 4) remained unidentified in the peptide mapping results obtained for searching either the originator or biosimilar amino acid sequence in BioPharma Finder software. Despite its rather low abundance, the obtained MS/MS spectrum was of high quality and allowed for *de novo* sequencing, obtaining the peptides sequence TTIEEIIPIK, matching the detected precursor ion with a mass accuracy of 0.9 ppm. This peptide is associated with an uncharacterized protein, thus further analysis will be required to confirm its presence in the sample and possible origin.

Conclusions

- This study demonstrates the applicability of the Thermo Scientific HR-MAM workflow for the analysis of biosimilar and PQA evaluation.
- All stages of the HR-MAM workflow were applied: protein characterization and selection of product quality attributes for monitoring, generation of a target peptide workbook in BioPharma Finder software and subsequent transfer into Chromeleon CDS, targeted data processing based on full MS only data, second round processing using the non-targeted MS approach by comparing the data files originating from biosimilar versus originator, and lastly identification of frames representing new peaks.
- All LC-MS data acquisition as well as PQA monitoring and non-targeted MS data analysis were performed in Chromeleon CDS with a seamless transition to BioPharma Finder software via the use of a target peptide workbook.
- The HR-MAM workflow successfully supported the evaluation and reporting across oxidation, deamidation, and succinimide formation, as well as N-glycosylation levels that resulted in significantly different levels between the originator and biosimilar samples.
- The non-targeted MS processing was successfully applied to highlight the differences of the biosimilar compared to its originator, most—yet not all—of which were associated with small sequence variants present in the heavy and light chains.
- Overall, the HR-MAM workflow was found to be an excellent analytical setup to carry out mAb comparability studies in compliance-ready software.

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