Technical note | 003308

Pharma / Biopharma

Compliance-ready LC-UV-MS-based monitoring of antibody quality attributes using a single quadrupole mass spectrometer

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Keywords

Post-translational modification (PTM), glycosylation, UHPLC, ISQ EM mass spectrometer, adalimumab, monoclonal antibody (mAb) quality control

Application benefits

- The Thermo Scientific™ ISQ™ EM Single Quadrupole Mass Spectrometer (SQMS) enables compliance-ready MS-based monitoring of antibody quality attributes for quality control (QC) labs.
- The use of a single quadrupole mass spectrometer enables targeted analysis of complex samples with the ability to confirm peak identity by mass-to-charge ratio (*m/z*) and retention time (RT).
- The Thermo Scientific™ SMART Digest™ Trypsin Kit in combination with the Thermo Scientific™ KingFisher™ Duo Prime Purification System provides fast and simple standardized protein digestion.
- The Thermo Scientific™ Hypersil GOLD™ Peptide column provides robust and reproducible peptide separation performance, allowing for reliable targeted quantification of monitored quality attributes.
- The system suitability test (SST) and intelligent run control (IRC) functionality in Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) allows for automated data analysis of standards and samples.

Goal

To demonstrate a targeted LC-UV-SQMS-based peptide monitoring workflow for QC labs, resulting in increased productivity in sample preparation, data analysis, and reporting.

Introduction

Therapeutic monoclonal antibodies (mAbs) are recombinant proteins, produced by highly complex, ectopic expression in cell culture and used as biotherapeutics. They are susceptible to a variety of chemical and enzymatic modifications during expression, purification, and storage. The integrity of protein therapeutics must be closely monitored at every stage of development and manufacturing to ensure quality, safety, and efficacy of the drug product.[1,2] During the development phase, a detailed characterization of the mAb is conducted to verify the amino acid sequence and to identify modifications that could constitute potential quality attributes. For this purpose, high-resolution accurate mass (HRAM) mass spectrometry and MS/MS analysis is applied to localize and quantify these post-translational modifications (PTMs) as well as stress-induced modifications on the peptide level. Once characterized, quality attribute monitoring can be transferred to a targeted MS1-only approach. This Multi-Attribute Method (MAM) most often uses HRAM instrumentation.[3] While MAM is suitable for QC, many QC labs shy away from complex and cost-intensive equipment. In order to achieve easy and reliable detection of the individual PTMs in a single run, a less comprehensive—but still fit-for-purpose and cGMP-compliant—approach is useful.

In this study, a liquid chromatography method with UV (LC-UV) detection was extended by coupling it to a single quadrupole mass spectrometer (SQMS) to monitor N-linked glycan structures for the IgG I class monoclonal antibody adalimumab. The antibody consists of two identical light and heavy chains, connected with inter- and intrachain disulfide bridges. To perform monitoring on the peptide level, the protein is digested into peptides. For this purpose, a reducing agent in combination with the SMART Digest Trypsin Kit was used. The endoprotease trypsin cleaves the peptide bonds at the C-terminal side of the amino acids arginine (R) and lysine (K). This results in an antibody-specific collection of peptides which is ready for subsequent analysis by LC-UV-SQMS.

Ideally, a monoclonal antibody reference standard is used as the SST standard, as it also supports monitoring of digestion performance. If no reference standard is available, the alternative use of a peptide retention time calibration standard helps to monitor the system performance, as demonstrated here (referred to as SET). Chromeleon CDS enables an automated workflow for unattended SET testing and sample analysis based on pre-defined parameters and actions using the SST/IRC functionality in the software. Immediately after the measurement, a report is generated that indicates whether the sample is within the required specifications, providing a simple “pass/fail” result. A schematic of the full workflow is illustrated in Figure 1.

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm, Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification (P/N 50136149)
- Acetonitrile, Optima™ LC/MS grade, Fisher Chemical™ (P/N A955)
- Methanol, Optima™ LC/MS grade, Fisher Chemical™ (P/N A456)
- Formic acid, Optima™ LC/MS grade, Fisher Chemical™ (P/N A117)
- SMART Digest Trypsin Kit (Magnetic Bulk Resin option with low pH buffer) (P/N 60109-101-MB-LPH)
- Thermo Scientific™ Bond-Breaker™ TCEP Solution, Neutral pH (P/N 77720)
- Thermo Scientific™ Pierce™ Peptide Retention Time Calibration (PRTC) Mixture (P/N 88321)
- Adalimumab (Humira™) solution, 10 mg/mL. Purchased from a reputable vendor

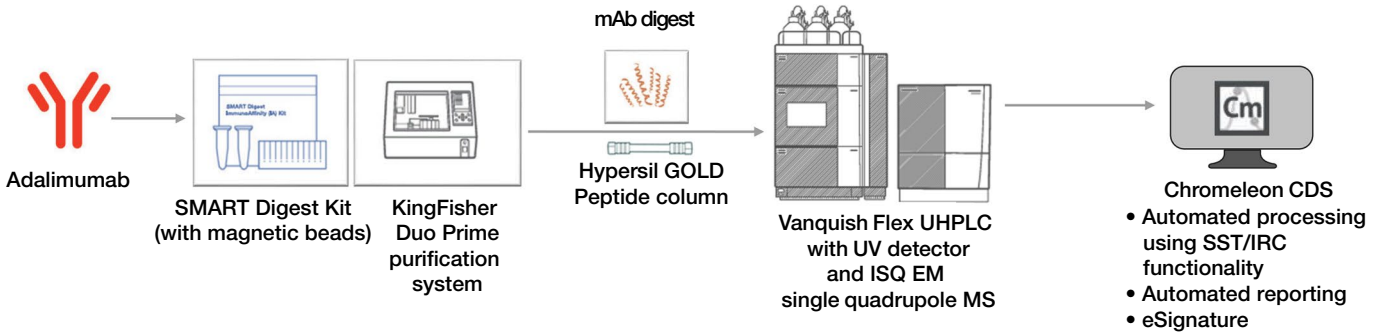


Figure 1. Schematic of the workflow including sample preparation, data acquisition, and data processing

Sample handling

- Fisherbrand™ Mini Centrifuge (P/N 12-006-901)
- Fisherbrand™ Mini Vortex Mixer (P/N 14-955-152)
- Thermo Scientific™ mySPIN™ 6 Mini Centrifuge (P/N 75-004-061)
- Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipettes: 100–1,000 µL (P/N 4641100N)
- Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipettes: 10–100 µL (P/N 4641070N)
- Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipettes: 1–10 µL (P/N 4641030N)
- Fisherbrand™ Locking-Lid Microcentrifuge Tubes with Polypropylene Snap-Cap™ (P/N 02-681-300)
- Thermo Scientific™ SureSTART™ 2 mL Polypropylene Snap Top Microvials for <2 mL Samples, Level 1 Everyday Analysis (P/N 6ERV11-03PPCF)
- Thermo Scientific™ SureSTART™ 11 mm Snap Caps, Level 2 High-throughput Applications (P/N 6ARC11ST1)
- Thermo Scientific™ KingFisher™ Duo Prime Purification System (with Thermo Scientific™ BindIt™ software 4.0) (P/N 5400110)
- Thermo Scientific™ KingFisher™ 96 deep-well plate (P/N 95040450)
- Thermo Scientific™ KingFisher™ 12-tip comb, for 96 deep-well plate (P/N 97003500)

Instrumentation

- Thermo Scientific™ Vanquish™ Flex UHPLC system with SQMS consisting of:
  - System Base Vanquish Horizon/Flex (P/N VF-S01-A-02)
  - Vanquish Binary Pump F (P/N VF-P10-A-01)
  - Vanquish Split Sampler FT (P/N VF-A10-A-02)
  - Vanquish Column Compartment H (P/N VH-C10-A-03)
  - Vanquish Variable Wavelength Detector F (P/N VF-D40-A)
  - ISQ EM mass spectrometer (P/N ISQEM-ESI)

Sample preparation

The peptide retention time calibration (PRTC) mixture (mixture of 15 synthetic peptides), which covers a wide range of hydrophobicity, was used as the peptide SET sample. It was transferred directly from the original tube into an HPLC vial and measured. The peptide sequences and masses in the sample are shown in Table 1.

Table 1. Peptide sequences, monoisotopic masses, and hydrophobicity factors from PRTC sample

#	Peptide sequence	Monoisotopic mass	Hydrophobicity factor (HF)
1	SSAAPPPPPR	985.5220	7.56
2	GISNEGQNASIK	1224.6189	15.50
3	HVLTSIGEK	990.5589	15.52
4	DIPVPKPK	900.5524	17.65
5	IGDYAGIK	843.4582	19.15
6	TASEFDSAIAQDK	1389.6503	25.88
7	SAAGAFGPELSR	1171.5861	25.24
8	ELGQSGVDTYLQTK	1545.7766	28.37
9	GLILVGGYGTR	1114.6374	32.18
10	GILFVGSGVSGGEEGAR	1600.8084	34.50
11	SFANQPLEVVYSK	1488.7704	34.96
12	LTILEELR	995.5890	37.30
13	NGFILDGFPR	1144.5905	40.42
14	ELASGLSFPVGFK	1358.7326	41.18
15	LSSEAPALFQFDLK	1572.8279	46.66

The digest was conducted in the heatable incubation lane A of a KingFisher 96 deep well plate. For each digest reaction, 150 µL low pH SMART Digest Trypsin buffer was aliquoted into a corresponding well and supplemented with 2 µL neutral TCEP solution 0.5 M (5 mM final concentration in 200 µL). Water and 200 µg antibody were added to reach a final volume of 200 µL. SMART Digest magnetic Trypsin bead stock slurry was diluted 15:85 (add 15 µL bead slurry to 85 µL buffer), and 100 µL of this working stock solution was pipetted each into the two bead reservoirs per reaction, using, for example, lane C and D of the deep well plate. The 12-well tip comb was placed into lane B.

The digest can be conducted in an automated fashion using the KingFisher Duo Prime method as outlined in Table 2. A two-temperature regime is used starting with an initial digest temperature of 70°C (offset 74°C) for 15 minutes. SMART Digest beads are removed, and the digest solution is cooled to 37°C (offset 40°C). Fresh SMART Digest beads are added, and the digestion is continued for another 30 minutes at 37°C. Beads are removed and the digest solution is cooled to 10°C.

150 µL digest solution is added to 50 µL 4% formic acid and directly used for LC-UV-SQMS analysis.

Table 2. KingFisher Duo Prime method to perform automated sample digestion

Step	Lane	Step	Beginning of step	Mixing/heating	End of step
1	B	Pick-up tip comb	n/a	n/a	n/a
2	C	Collect beads	n/a	10 s, half mix	Collect beads
3	A	Digest 1	Release beads, fast	15 min, medium, 74°C	Collect beads, 3x 15 s
4	C	Release beads	5 s, fast	n/a	n/a
5	A	Cool 40°C	n/a	10 min, paused, above well, 40°C	Post-temperature 40°C
6	D	Collect beads	n/a	10 s, half mix	Collect beads
7	A	Digest 2	Release beads, fast	30 min, medium, 40°C	Collect beads, 3× 15 s
8	D	Release beads	5 s, fast	n/a	n/a
9	A	Cool 10°C	n/a	10 min, paused above well, 10°C	Postmix, 5 s bottom mix; collect beads 3× 15 s, post temp. 10°C
10	B	Leave tip comb	n/a	n/a	n/a

Chromatographic conditions

Table 3. Chromatographic conditions

Parameter	Value
Column	Hypersil GOLD Peptide 150 × 2.1 mm, 1.9 µm, 175 Å (P/N 26002-152130)
Mobile phase A	Water + 0.1% formic acid
Mobile phase B	Acetonitrile + 0.1% formic acid
Gradient	Time (min) % B 0.0 2.0 0.5 2.0 1.0 9.0 22.0 35.0 23.0 90.0 26.0 90.0 27.0 2.0 45.0 2.0
Flow rate	0.25 mL/min
Column temperature	50°C (with active pre-heater at 50°C, forced-air mode, fan speed 5)
Post column cooler temperature	40°C
Autosampler temperature	6°C
Needle wash solution	10/90 (v/v) acetonitrile/water + 0.1% formic acid
Needle wash mode	Before Draw
Injection volume	6.7 µL
Wavelength	214 nm
Data collection rate, response time	20.0 Hz, 0.20 s

MS settings

First, a full scan run of the samples was carried out to determine the mass-to-charge ratio (*m/z*) and retention time (RT) of each peptide. Finally, the measurements were conducted in component mode (SIM mode) by entering the respective RT window of the corresponding *m/z* into the instrument method.

Table 4. Instrument and scan settings for the ISQ EM mass spectrometer

Parameter	Value
Ionization mode	HESI
Polarity (spray voltage)	Positive (+3,000 V)
Method type: Component mode	*m/z* and retention time windows are listed in Table 5 and Table 6
Resulting total scan time	0.500 s
SIM scan width	0.10 amu
Lowest dwell time	0.046 s
CID voltage	20 V
Vaporizer temperature	144°C
Ion transfer tube temperature	300°C
Gas flow pressures	Sheath gas: 32.3 psig Auxilliary gas: 3.6 psig Sweep gas: 0.5 psig

Chromatography Data System

The Thermo Scientific™ Chromeleon™ 7.3.2 MUa CDS was used for data acquisition and processing.

Table 5. Mass-to-charge ratio (*m/z*) and RT windows of the PRTC sample

Name	Start time [min]	End time [min]	*m/z*
SSAAPPPPPR	4.0	6.0	493.8
GISNEGQNASIK	4.5	6.5	613.3
HVLTSIGEK	5.5	7.5	496.3
IGDYAGIK	6.0	8.5	422.7
DIPVPKPK	7.0	9.0	451.3
TASEFDSAIAQDK	8.0	10.0	695.8
SAAGAFGPELSR	9.5	11.5	586.8
ELGQSGVDTYLQTK	10.0	12.0	773.9
SFANQPLEVVYSK	13.0	15.0	745.4
GLILVGGYGTR	13.5	15.5	558.3
GILFVGSGVSGGEEGAR	13.5	15.5	801.4
LTILEELR	16.0	18.0	498.8
NGFILDGFPR	16.5	18.5	573.3
ELASGLSFPVGFK	17.5	19.5	680.4
LSSEAPALFQFDLK	19.0	21.0	787.4

Table 6. Mass-to-charge ratio (*m/z*) and RT windows of the adalimumab glycopeptides (5a = zero missed cleavage, 5b = one missed cleavage)

Name	Start time [min]	End time [min]	*m/z*
5a_N301	3.8	5.8	595.3
5a_N301_A1G0	3.5	5.5	1143.0
5a_N301_A2G0	3.5	5.5	830.0
5a_N301_A2G0F	3.5	5.5	878.7
5a_N301_A2G1F	3.5	5.5	932.7
5a_N301_A2G2F	3.4	5.4	986.7
5a_N301_M3	3.5	5.5	1041.4
5a_N301_M5	3.2	5.2	1203.5
5b_N301_A2G0	3.2	5.2	743.3
5b_N301_A2G0F	3.2	5.2	779.8
5b_N301_A2G1F	3.2	5.2	820.4
5b_N301_A2G2F	3.2	5.2	860.9
5b_N301_M5	3.2	5.2	722.8

Results and discussion

Monitoring of adalimumab glycopeptides was performed using an LC-UV-SQMS method. Regularly monitored glycan modification was selected as an example to demonstrate the utility of adding a mass spectrometer where the LC-UV method does not provide sufficient separation capability and selectivity.

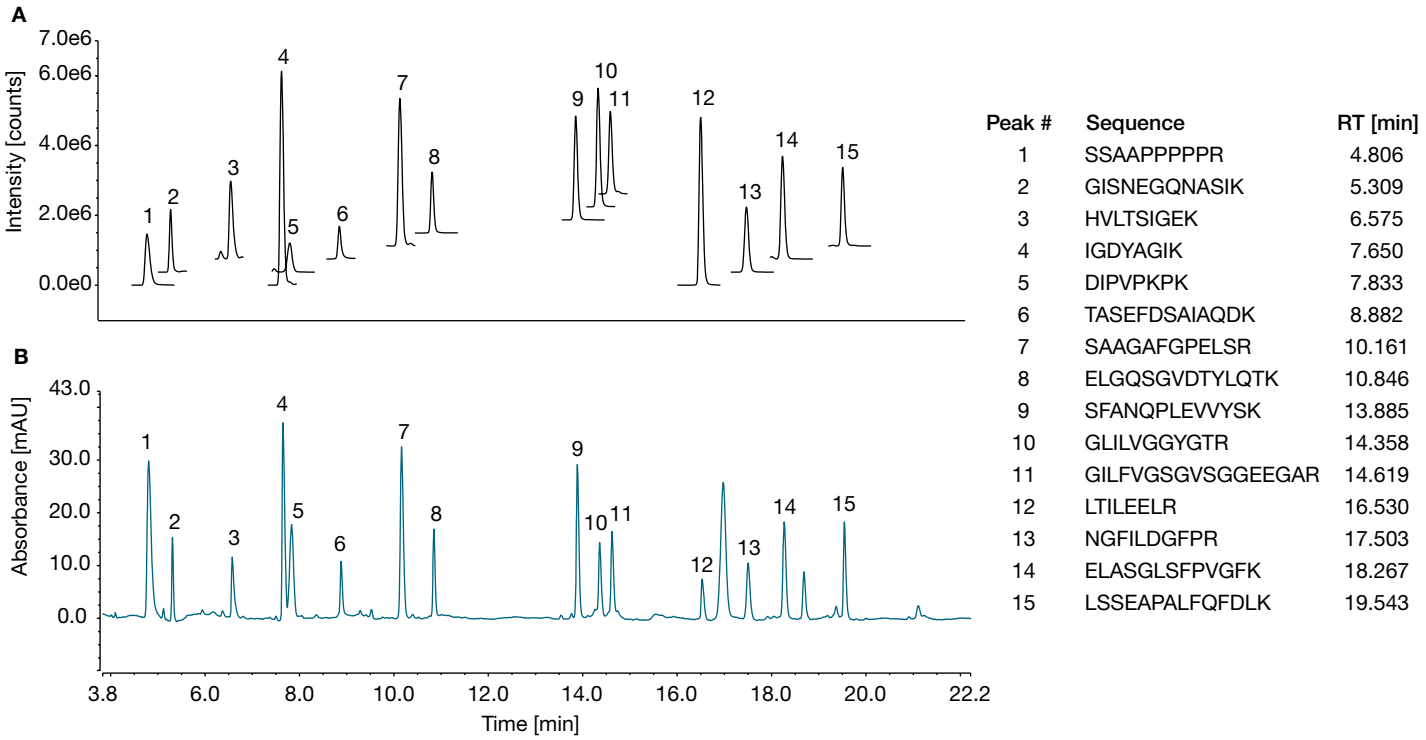
System Performance Evaluation Test (SET) using SST functionality in Chromeleon CDS

The PRTC sample consists of 15 synthetic peptides. Chromatograms for UV and MS channels are shown in Figure 2. All target compounds were detected in both UV at 214 nm and MS in single ion monitoring (SIM) chromatogram (named in Chromeleon CDS as MS Quantitation channel). Target peaks are labeled with the respective peptide sequence and retention time. Unassigned peaks in the UV channel belong to impurities of the synthetic peptide mixture but do not interfere with analytes of interest.

Integrated SST functionality in Chromeleon CDS enables automated data processing while the sequence is running. Prior to the measurements, individual test cases and performance criteria are defined and included in the processing method.

Nineteen test cases, as outlined in Figure 3 in column *"Name",* are performed using UV and MS channels to confirm that the analytical system is operating within specifications (see column(s) *"Ref value(s)"* in Figure 4), suitable to the samples to be examined. Peak asymmetry is assessed on three components: peak 1 (*SSAAPPPPPR),* peak 8 *(ELGQSGVDTYLQTK),* and peak 15 *(LSSEAPALFQFDLK).* Resolution is tested for two critical peak pairs, and relative standard deviation of retention times (RSD RT) and peak areas (RSD peak area) on both channels are evaluated for three of those with the lowest signal in each channel.

While the measurements run, the Chromeleon CDS automatically checks these criteria and executes commands depending on the result and the action entered in the method after one injection is completed. The creation of a test case is wizard-driven and comprises a total of five steps. Figure 4 shows the test case for "resolution" as one example. Under the "Evaluation" tab, the criteria for the "resolution" parameter must be defined, here as ≥2. The component on which this parameter is to be tested is defined in the "Peak/ Channel" tab. In case of a failed result (e.g., a resolution of 1.5 obtained) for the peak *GLILVGGYGTR,* the queue is aborted, and no further sample is injected.



Peak #	Sequence	RT [min]
1	SSAAPPPPPR	4.806
2	GISNEGQNASIK	5.309
3	HVLTSIGEK	6.575
4	IGDYAGIK	7.650
5	DIPVPKPK	7.833
6	TASEFDSAIAQDK	8.882
7	SAAGAFGPELSR	10.161
8	ELGQSGVDTYLQTK	10.846
9	SFANQPLEVVYSK	13.885
10	GLILVGGYGTR	14.358
11	GILFVGSGVSGGEEGAR	14.619
12	LTILEELR	16.530
13	NGFILDGFPR	17.503
14	ELASGLSFPVGFK	18.267
15	LSSEAPALFQFDLK	19.543

Figure 2. Chromatograms of MS Quantitation channel (A) and UV channel at 214 nm (B). Peak numbers correspond to respective peptide sequences and retention times.

A

System Suitability Test and Intelligent Run Control

Group Area — Drag a column header here to group by that column.

#	Name	Statistics	Eval. Formula	Operator	Ref.Value 1	Ref.Value 2	Channel	Peak	Inj.Condition	Result
1	Interferences/ Carry-Over UV	None	peak.retention_time	not between	4.5	24	UV_VIS_1	All Identified Peaks	injection.name = "Solvent Blank (98/2 A/B)"	NA -> Passed
2	Interferences/ Carry-Over MS	None	peak.retention_time	not between	4.5	24	TIC	All Identified Peaks	injection.name = "Solvent Blank (98/2 A/B)"	NA -> Passed

B

System Suitability Test and Intelligent Run Control

Group Area — Drag a column header here to group by that column.

#	Name	Statistics	Eval. Formula	Operator	Ref.Value 1	Ref.Value 2	Channel	Peak	Inj.Condition	Result
3	Peak Asymmetry	None	peak.asymmetry("ep")	between	0.8	1.8	UV_VIS_1	SSAAPPPPPR	injection.number = 8	Passed
4	Peak Asymmetry	None	peak.asymmetry("ep")	between	0.8	1.8	UV_VIS_1	ELGQSGVDTYLQTK	injection.number = 8	Passed
5	Peak Asymmetry	None	peak.asymmetry("ep")	between	0.8	1.8	UV_VIS_1	LSSEAPALFQFDLK	injection.number = 8	Passed
6	Resolution (USP)	None	peak.resolution("usp","next")	>=	2		UV_VIS_1	SSAAPPPPPR	injection.number = 8	Passed
7	Resolution (USP)	None	peak.resolution("usp","next")	>=	2		UV_VIS_1	GLILVGGYGTR	injection.number = 8	Passed
8	RSD of Peak Retention Times UV	Relative Standard Deviation	peak.retention_time	<=	0.5		UV_VIS_1	HVLTSIGEK	injection.name = "Peptide SET sample"	Passed
9	RSD of Peak Retention Times UV	Relative Standard Deviation	peak.retention_time	<=	0.5		UV_VIS_1	TASEFDSAIAQDK	injection.name = "Peptide SET sample"	Passed
10	RSD of Peak Retention Times UV	Relative Standard Deviation	peak.retention_time	<=	0.5		UV_VIS_1	NGFILDGFPR	injection.name = "Peptide SET sample"	Passed
11	RSD of Peak Retention Times MS	Relative Standard Deviation	peak.retention_time	<=	0.5		MS Quantitation	DIPVPKPK	injection.name = "Peptide SET sample"	Passed
12	RSD of Peak Retention Times MS	Relative Standard Deviation	peak.retention_time	<=	0.5		MS Quantitation	TASEFDSAIAQDK	injection.name = "Peptide SET sample"	Passed
13	RSD of Peak Retention Times MS	Relative Standard Deviation	peak.retention_time	<=	0.5		MS Quantitation	GISNEGQNASIK	injection.name = "Peptide SET sample"	Passed
14	RSD of Peak Area UV	Relative Standard Deviation	peak.area	<=	2		UV_VIS_1	HVLTSIGEK	injection.name = "Peptide SET sample"	Passed
15	RSD of Peak Area UV	Relative Standard Deviation	peak.area	<=	2		UV_VIS_1	TASEFDSAIAQDK	injection.name = "Peptide SET sample"	Passed
16	RSD of Peak Area UV	Relative Standard Deviation	peak.area	<=	2		UV_VIS_1	NGFILDGFPR	injection.name = "Peptide SET sample"	Passed
17	RSD of Peak Area MS	Relative Standard Deviation	peak.area	<=	5		MS Quantitation	DIPVPKPK	injection.name = "Peptide SET sample"	Passed
18	RSD of Peak Area MS	Relative Standard Deviation	peak.area	<=	5		MS Quantitation	TASEFDSAIAQDK	injection.name = "Peptide SET sample"	Passed
19	RSD of Peak Area MS	Relative Standard Deviation	peak.area	<=	5		MS Quantitation	GISNEGQNASIK	injection.name = "Peptide SET sample"	Passed

Figure 3. SST test cases for solvent blank (A) and PRTC sample (B)

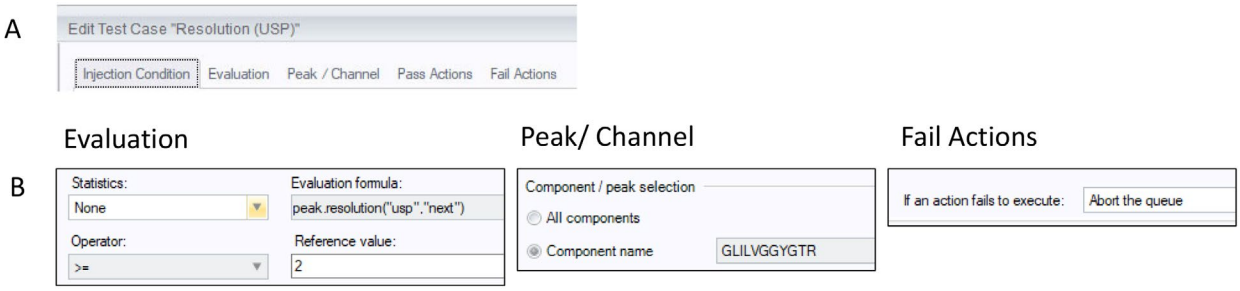


Figure 4. Wizard for test case "Resolution (USP)". (A) Five steps in the wizard; (B) detailed parameters in the tabs “evaluation”, “peak/ channel”, and “fail actions”

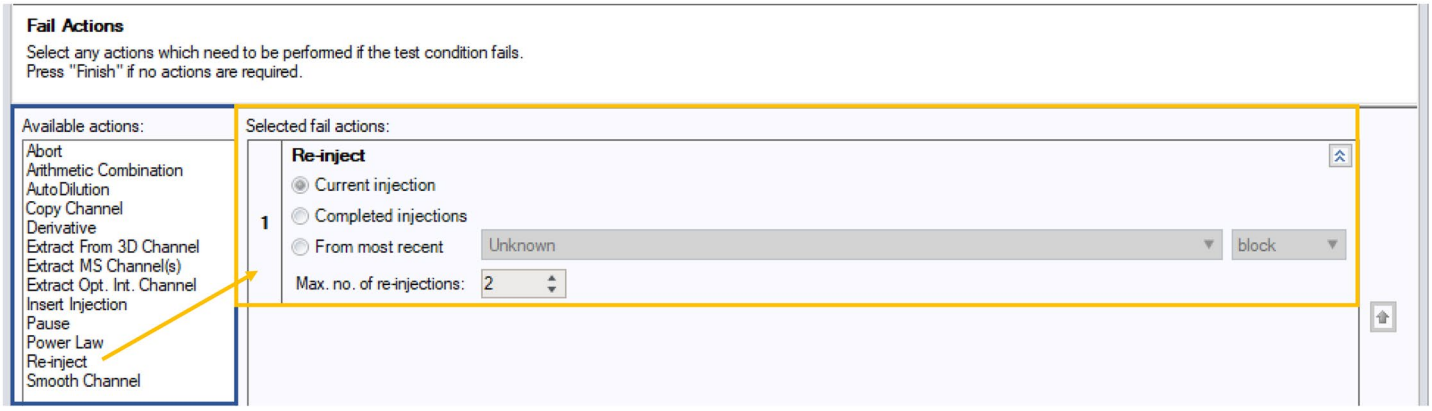


Figure 5. Available actions in the "Fail Actions" tab (blue box) with the selection of the Re-inject option (orange box)

Another option is to use the “Re-Inject” action instead of “Abort”. This automatically adds as many injections to the sequence as the user has defined (Figure 5). The corresponding entries can be found in the audit trail so that the automatically inserted injections can be tracked as required for a regulated environment. The “Re-Inject” action is used in this study for the “Interferences/ Carry-Over UV and MS” test cases, with a maximum re-injection number of 2.

Digested adalimumab sample analysis using LC-UV-SQMS

UV detection is widely used for peptide monitoring for targeted PTMs; however, unresolved peptides with low abundance are not detectable by UV. In this study, adalimumab glycopeptides are monitored in a single analysis by LC-UV-SQMS. Because the chromatogram of a digested mAb sample can be highly complex, as shown in Figure 6, data analysis based on the UV signal alone can be inconclusive. Coupling the UV detector to a SQMS leads to more reliable peak confirmation and quantitation.

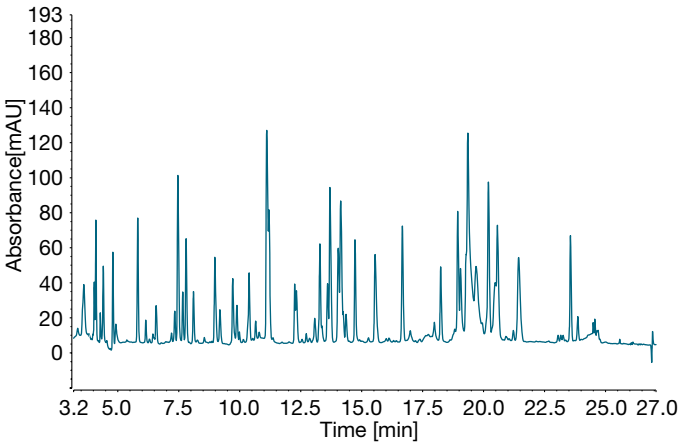


Figure 6. UV chromatogram obtained from a tryptic digest of adalimumab, acquired at 214 nm wavelength

The ISQ EM mass spectrometer operates in a mass range of *m/z* 10–2,000. Targeted *m/z* of PTMs are included in the acquisition method with a retention time (RT) window (refer to Table 6). Beside the UV chromatogram (Figure 6), a SIM chromatogram of the monitored glycopeptides is obtained, as shown in Figure 7.

The most prevalent glycan moieties of adalimumab and their corresponding glycopeptides were monitored. Glycans found in mAbs are composed of a common core structure (A2) that is extended with galactose (G) and sialic acid (S) and other mono-saccharide moieties (Figure 8). The distal GlcNac of the A2 core structure can be linked with fucose (F). This results in a characteristic glycan profile for each mAb and in a mixture of glycopeptides that co-elute in a close retention time range. Due to the lack of chromatographic separation, the monitoring of these glycopeptides with UV signal only is not feasible. Using the ISQ EM mass spectrometer reduces the need to chromatographically resolve all peptides and enables mass confirmation.

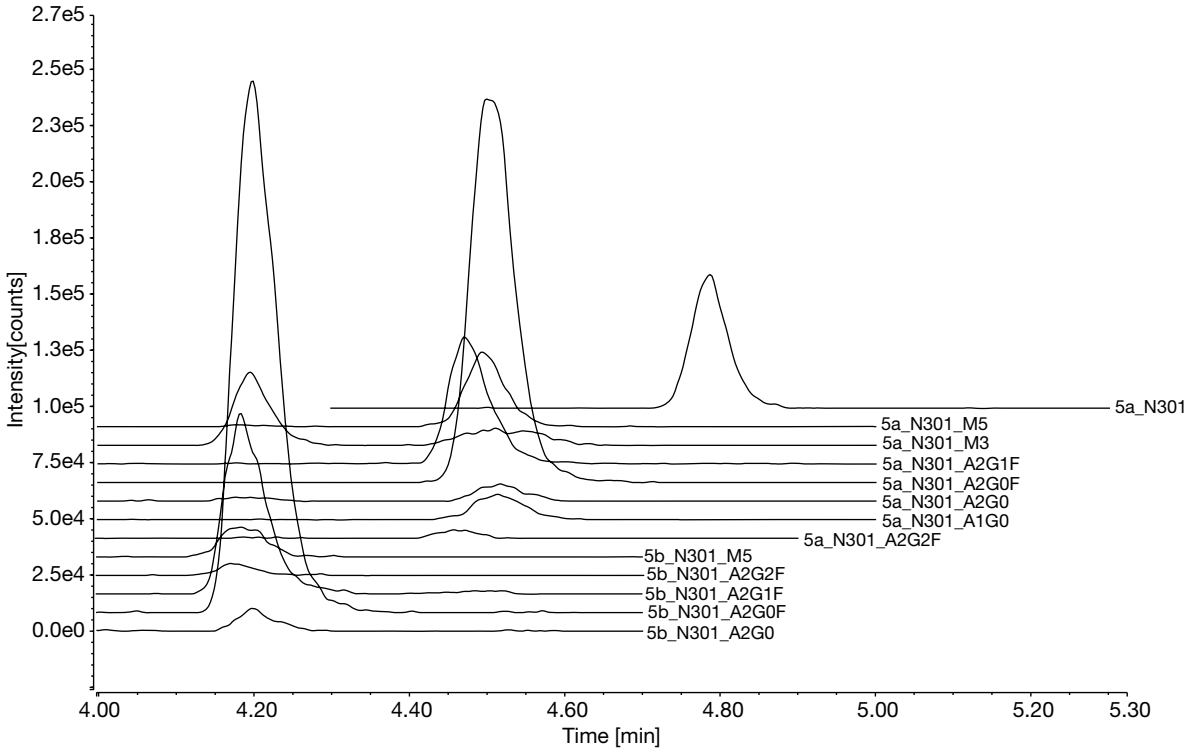


Figure 7. MS Quantitation channel for adalimumab glycopeptides (5a = EEQYN[glycan]STYR, 5b = TKPREEQYN[glycan]STYR)

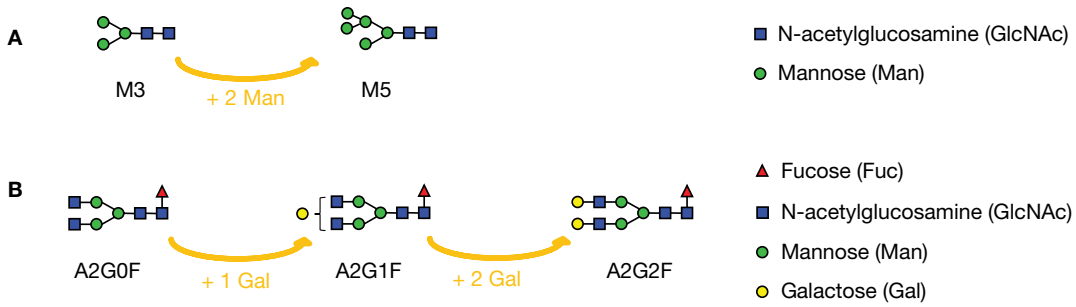


Figure 8. Glycan structures with different numbers of mannose (A) or terminal galactoses (B)

MS data in Chromeleon CDS can be processed the same way as UV data. Therefore, the MS channels can be processed by using the intelligent run control (IRC) functionality in the software for automated data evaluation and reporting. Depending on the sample requirements, any number of IRC test cases can be entered into the processing method to analyze the target samples. In this study, IRC test cases for the confirmation of the target peaks in a certain retention time window are applied to the target samples. This allows for a rapid assessment of the presence of target peaks for further evaluation, such as relative quantitation of glycopeptides based on relative abundance compared to a reference sample.

For a fully automated workflow, results of the IRC test cases included in the processing method can be presented in a report template of Chromeleon CDS. The report contains clear visualization of all test cases, test values, and pass/ fail results (Figure 9). The user benefits from the fact that data evaluation can be fully automated not requiring any manual intervention, adding consistency while removing potential user bias, and that results can be seen at first glance. Furthermore, electronic signature approval is incorporated into Chromeleon CDS, ensuring 21 CFR 11 compliance throughout the process.

The eWorkflow™ containing the processing method and reporting file can be found in the AppsLab entry for this technical note in the "Downloads" section.

Method evaluation

Digested adalimumab samples were prepared in triplicate and consecutively injected three times each. To assess specificity of the method a mock digest (sample without enzyme) was prepared and analyzed. No signals were observed, demonstrating the high specificity of the method (data not shown).

Relative quantitation of the selected glycan modification was evaluated by summing the peak areas of each variant and dividing by the total sum of the peak areas of all variants including the unglycosylated form. Figure 10 shows the % relative abundance obtained from the MS Quantitation channel. The error bars indicate the standard deviation of relative abundance for the three consecutive injections of each digested sample. The relative standard deviation (%RSD) of the relative abundances were ≤9.4% for values below 5% relative abundance and ≤3.2 for values higher than 5% relative abundance when comparing all three digests.

The results of this study demonstrate the efficency of employing the SMART Digest Trypsin Kit for a standardized sample preparation, along with the Hypersil GOLD peptide column and a single quadrupole MS. This combination results in reliable detection and monitoring of adalimumab peptides from a tryptic digest, offering identity confirmation based on mass and retention time. Furthermore, it facilitates accurate relative quantification of the targeted attribute, ensuring robust analytical outcomes. The use of automated data processing and reporting in the CDS results in fast and efficient data analysis and rapid insight into sample pass/fail results.

A

SST Results							
No.	Injection Name	Evaluation Formula	Peak Name	RT (DETECTED)	Ref. RT MIN	Ref. RT MAX	Test Result
1	Sample	peak.retention_time	5a_N301	4.79	4.7	5	Passed
2	Sample	peak.retention_time	5a_N301_A1G0	4.51	4.4	4.65	Passed
3	Sample	peak.retention_time	5a_N301_A2G0	4.52	4.4	4.65	Passed
4	Sample	peak.retention_time	5a_N301_A2G0F	4.50	4.4	4.65	Passed
5	Sample	peak.retention_time	5a_N301_A2G1F	4.47	4.4	4.65	Passed
6	Sample	peak.retention_time	5a_N301_A2G2F	4.46	4.4	4.65	Passed
7	Sample	peak.retention_time	5a_N301_M3	4.51	4.4	4.65	Passed
8	Sample	peak.retention_time	5a_N301_M5	4.49	4.4	4.65	Passed
9	Sample	peak.retention_time	5b_N301_A2G0	4.20	4.1	4.4	Passed
10	Sample	peak.retention_time	5b_N301_A2G0F	4.20	4.1	4.4	Passed
11	Sample	peak.retention_time	5b_N301_A2G1F	4.18	4.1	4.4	Passed
12	Sample	peak.retention_time	5b_N301_A2G2F	4.17	4.1	4.4	Passed
13	Sample	peak.retention_time	5b_N301_M5	4.18	4.1	4.4	Passed
		13	Total Result:				Passed

B

Summary								
Peak Group(s)	Retention Time min	Area counts*min	Sum Group peak area counts*min	Total peak area all identified peaks counts*min	Relative Abundance %	Relative Abundance MIN	Relative Abundance MAX	Relative Abundance CHECK
A1G0	4.514	855.95	855.95	44006.06	1.9	1.5	3	PASS
A2G0	4.197	565.98		44006.06				
A2G0	4.517	473.13	1039.11	44006.06	2.4	1.5	3	PASS
A2G0F	4.199	15486.34		44006.06				
A2G0F	4.500	11489.50	26975.84	44006.06	61.3	55	65	PASS
A2G1F	4.183	4936.34		44006.06				
A2G1F	4.470	3140.32	8076.65	44006.06	18.4	15	20.5	PASS
A2G2F	4.168	310.30		44006.06				
A2G2F	4.457	203.15	513.45	44006.06	1.2	0.5	2	PASS
M3	4.510	866.16	866.16	44006.06	2.0	0.5	2	PASS
M5	4.184	934.66		44006.06				
M5	4.495	1355.26	2289.92	44006.06	5.2	5	8	PASS
unglycosylated	4.787	3388.97	3388.97	44006.06	7.7	5	8	PASS

Figure 9. Reporting: IRC confirmation of presence of target peaks in a certain RT window (A) and report of relative quantitation results for glycopeptides in the sample (B)

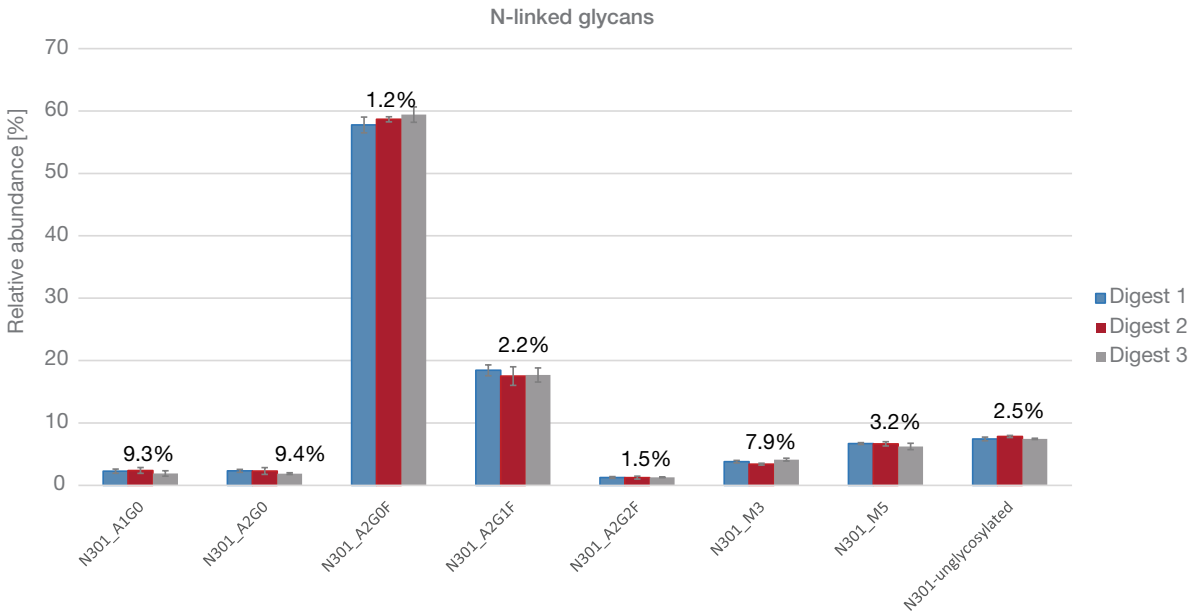


Figure 10. Relative abundance [%] for adalimumab glycopeptides of three prepared digests with error bars indicating the standard deviation for three consecutive injections for each digested sample. Numbers above the bars represent %RSD values obtained across the three digests.

Conclusion

In this study, we demonstrated a simple LC-UV-SQMS workflow for peptide monitoring in which MS complements UV detection for mass confirmation of peptides including co-eluting or poorly separated components. Study highlights are as follows:

- Nineteen SET test criteria were entered into the processing method and evaluated automatically during the measurements without any user interaction. The report showed that all test criteria passed.
- While the UV chromatogram for the adalimumab sample is complex, peak confirmation is easily and reliably achieved using the ISQ EM MS.
- Similar to the SST functionality, the IRC function in Chromeleon CDS is used for on-the-fly decision making in sample analysis while the sequence is running. A clear reporting and the electronic signature support full 21 CFR 11 compliance.
- An eWorkflow allows automation of analysis from sample injection to reporting.
- Using the SMART Digest Trypsin Kit for automated sample digestion in combination with the Hypersil GOLD peptide column for chromatographic separation, good reproducibility with RSD for relative abundance ≤9.4% (relative abundance below 5%) and RSD ≤3.2% (relative abundance above 5%) for adalimumab glycopeptides was achieved.

This workflow allows QC laboratories to monitor targeted PTMs in mAb samples simultaneously, and consequently increase productivity.

Acknowledgements

The authors are very grateful to Kai Scheffler, Carina Soetebier, and Matthias Hrobarsch for scientific advice.

References

1. Gucinski, A.C.; Boyne, M.T. Evaluation of intact mass spectrometry for the quantitative analysis of protein therapeutics. *Analytical Chemistry,* **2012**, *84*(18), 8045–8051
2. Jefferis, R. Posttranslational Modifications and the immunogenicity of Biotherapeutics. *Journal of Immunology Research,* **2016,** 1–15
3. Millan-Martin, S.; Jakes, C.; Carillo, S.; Bones, J. Multi-attribute method (MAM) to assess analytical comparability of adalimumab biosimilars, *Journal of Pharmaceutical and Biomedical Analysis,* **2023,** *234,* 115543, https://doi.org/10.1016/j.jpba.2023.115543

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