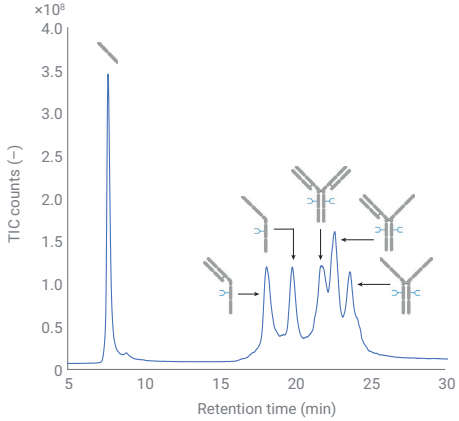
Application Note

Biopharma

Agilent InfinityLab

Monitoring Product-Related mAb Fragments

Intact protein analysis with the Agilent 1290 Infinity II Bio LC System enables UV and MS detection of low molecular weight species



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Abstract

Product-related impurities such as low molecular weight (LMW) or high molecular weight (HMW) species are considered critical quality attributes (CQAs) in therapeutic monoclonal antibody (mAb) products and need to be monitored across the drug production process. This application note developed an RPLC method based on the excellent performance of the Agilent 1290 Infinity II Bio LC System combined with the PEEK-lined Agilent PLRP-S column. By analyzing the reduced heavy and light chains of the NISTmAb, excellent relative retention time and area deviations were observed, even with extremely shallow gradient slopes. After method development, all relevant LMW fragments, such as two heavy chains (H2) or two heavy chains and one light chain (H2L), could be separated and detected. Due to the sequential coupling of the UV and MS detector, this method can be used in several areas of the biopharmaceutical production chain. The method also stands as an alternative to SDS-PAGE/CE-SDS with the possibility to analyze two CQAs – LMW species and post-translational modifications (PTMs) – in one run.

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Introduction

mAbs are a major product class of biopharmaceuticals and have been used successfully to tackle various diseases.[1] These biomolecules possess a conserved heterotetrameric structure, consisting of two heavy chains and two light chains connected by disulfide bonds. During manufacturing or improper storage, product-related impurities such as LMW species (see Figure 1) or HMW species (e.g., antibody dimers) can be formed. Those impurities can be present even after extensive purification steps, making it essential to monitor them as a CQA for a drug product. HMW species such as antibody dimers, trimers, or higher aggregates can routinely be analyzed and separated by size exclusion chromatography (SEC) with UV detection.[2] Coupling of SEC with MS detection can be performed to further characterize impurities regarding molecular weight and PTMs.[3] The analysis of LMW species such as heavy chain (H), light chain (L), or H2L fragments can be carried out by capillary electrophoresis-sodium dodecyl sulfate (CE-SDS).[4] Unfortunately, CE-SDS cannot be coupled to MS detection due to high ion suppression caused by SDS, and therefore proposed identities of LMW species are often based on empirical knowledge. This application note shows an alternative analysis of LMW species of mAbs based on the excellent performance of the 1290 Infinity II Bio LC and the PEEK-lined PLRP-S column. Due to the reversed-phase liquid chromatography (RPLC) mode, all relevant reduction-induced LMW fragments of the NISTmAb can be detected with UV and MS for routine or in-depth analysis as needed.

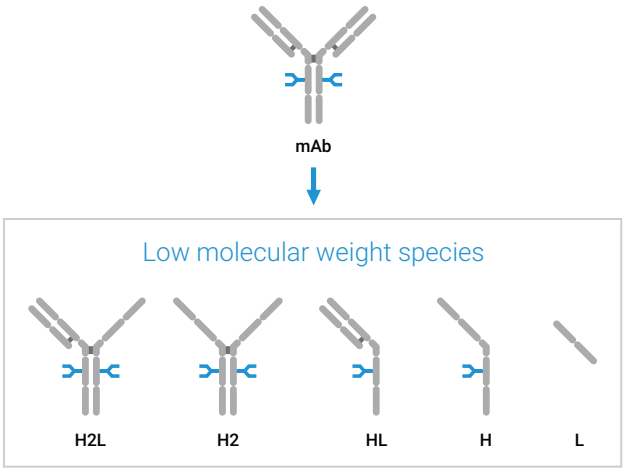

Figure 1. Schematic overview of reduction-induced LMW species of monoclonal antibodies (mAb). Abbreviations: H2L (two heavy chains and one light chain), H2 (two heavy chains), HL (one heavy chain and one light chain), H (heavy chain), and L (light chain).

Experimental

Equipment

The Agilent 1290 Infinity II Bio LC System coupled to the Agilent 6545XT AdvanceBio LC/Q-TOF comprised the following modules:

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) equipped with a Standard Flow Quick
- Connect Bio Heat Exchanger (G7116-60071) and two Agilent Thermal Equilibration Devices (G7116-60013)
- Agilent 1290 Infinity II Variable Wavelength Detector (VWD) (G7114B), equipped with a Bio Micro Flow Cell VWD, 3 mm, 2 µL, RFID.
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6545XT)

Software

- Agilent MassHunter workstation data acquisition (B.09.00 or later)
- Agilent MassHunter Qualitative Analysis (10.0 or later)
- Agilent MassHunter BioConfirm (10.0 or later)

Columns

Agilent PLRP-S 5 µm 1000 Å, 2.1 × 100 mm PEEK-lined (part number PL1912-2502PK)

Chemicals

Agilent InfinityLab Ultrapure LC/MS acetonitrile (part number 5191-4496) and the Agilent-NISTmAb (part number 5191-5744) were used. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). DL-dithiothreitol (DTT) was purchased from Merck (Darmstadt, Germany).

Sample preparation

To partially reduce the NISTmAb, 40 µg were incubated with 1 mM DTT in an amber glass vial directly in the 1290 Infinity II Bio Multisampler at 4 °C. Full reduction into heavy chain (H) and light chain (L) was achieved by incubating 40 µg of NISTmAb with 10 mM DTT at 60 °C for 30 minutes. Injection concentration was 1 mg/mL NISTmAb or reduced NISTmAb.

Table 1. LC method for analyzing the intact NISTmAb and corresponding LMW species with the Agilent 1290 Infinity II Bio LC.

Parameter	Value
Column	Agilent PLRP-S 5 µm 1,000 Å, 2.1 × 100 mm PEEK-lined
Solvent	A) Water + 0.1% formic acid B) Acetonitrile + 0.1% formic acid
Gradient	0.00 min – 25% B 9.00 min – 30% B 34.00 min – 38% B 34.01 min – 100% B 36.00 min – 100% B 36.01 min – 25% B 40.00 min – 25% B
Flow Rate	0.400 mL/min
Temperature	60 °C with thermal equilibration devices installed
UV Detection	VWD: 280 nm, 10 Hz/MS: see Table 2
Injection	Injection volume: 0.3 µL Sample temperature: 4 °C Wash: 3 s with water (flush port)

Table 2. Source and MS parameters for the analysis of the intact NISTmAb and corresponding LMW species.

Parameter	Value
Instrument	Agilent 6545XT AdvanceBio LC/Q-TOF
Gas Temperature	350 °C
Drying Gas Flow	12 L/min
Nebulizer	35 psig
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
VCap	4,000 V
Nozzle Voltage	2,000 V
Fragmentor	180 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Acquisition Mode	Positive, extended (*m/z* 10,000) mass range
Mass Range	*m/z* 100 to 10,000
Acquisition Rate	1 spectrum/sec
Reference Mass	*m/z* 922.0098

Results and discussion

The analysis of biopharmaceuticals throughout the production process from manufacturing to quality control demands the best performance possible from LC systems. To evaluate the 1290 Infinity II Bio LC's performance regarding the analysis of mAb fragments, the NISTmAb was entirely reduced with DTT, resulting in H and L fragments. Figure 2 shows the relative retention time and area standard deviations (RSD) based on seven consecutive injections. It shows that the retention time and area precision of the 1290 Infinity II Bio LC coupled to the 6545XT AdvanceBio LC/Q-TOF is excellent and perfectly suited for analyzing mAb fragments with shallow gradients. Even though the LC method consists of two linear gradient steps with slopes of 0.32 and 0.55 %B/min, the RSD values remain low with 0.190% (L) and 0.056% (H) for the retention time and 0.530% (L) and 0.744% (H) for the area precision.

One of the major challenges when analyzing LMW species with RPLC is the insufficient resolving power to separate antibody fragments such as H2 or H2L due to their similarity in hydrophobicity compared to the actual mAb. These fragments can occur in the fermentation process or by partial reduction in the final product. However, these fragments can also be generated artificially by partial reduction over time with a low amount of DTT and decreased temperature. With this technique, an RPLC method based on the PEEK-lined PLRP-S and the 1290 Infinity II Bio LC was developed.

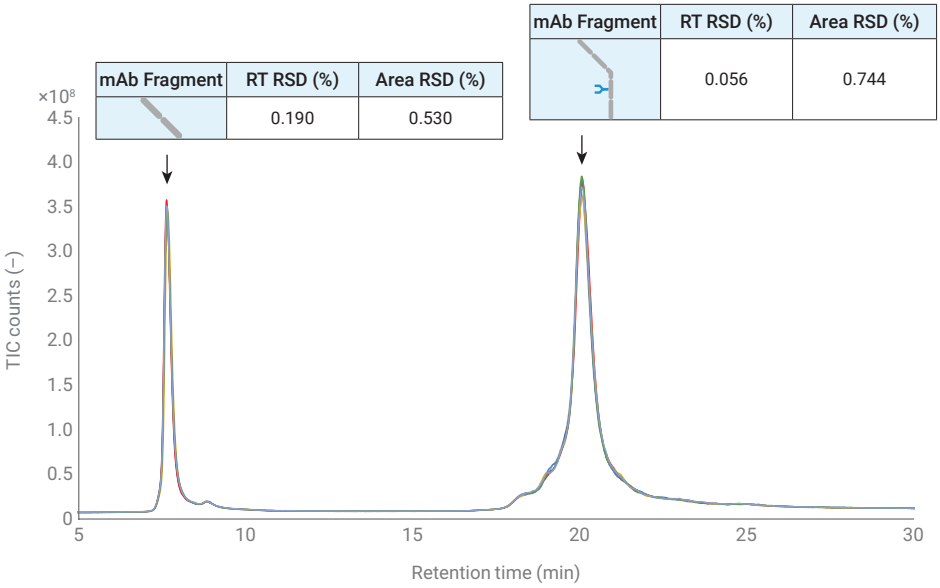

Figure 2. Relative retention time and area precision (RSD, n = 7) values for the Agilent 1290 Infinity II Bio LC analyzing heavy and light chain fragments derived by reduction of the NISTmAb.

The dynamic reduction of the NISTmAb in the 1290 Infinity II Bio Multisampler can be seen in the chromatogram of Figure 3. All of the relevant mAb fragments depicted in Figure 1 can be nicely resolved and change over time due to the addition of DTT. In particular, the separation of the H2, H2L fragments, and the NISTmAb is exceptionally good for RPLC, rendering the combination of the PEEK-lined PLRP-S column and the 1290 Infinity II Bio LC the method of choice for the analysis of LMW. Thanks to the RPLC mode, the 1290 Infinity II Bio LC System can easily be coupled to the 6545XT AdvanceBio LC/Q-TOF, and MS data can be analyzed in Agilent MassHunter BioConfirm. After deconvolution, the spectra in Figure 3 depict the main glycoforms of the respective fragments. The characteristic glycosylation of the NISTmAb shows that it is possible to analyze PTMs of the different fragments easily with this method. Additionally, Figure 4B shows the extracted ion chromatograms (EIC) of representative ions for the fragments clustering around the mAb peak. These EICs also offer good peak shape owing to the resolving power of the PEEK-lined PLRP-S column.

Since the instrumentation setup comprises the 1290 Infinity II Variable Wavelength Detector and the 6545XT AdvanceBio LC/Q-TOF in sequence, UV and MS detection is possible in one run with little to no band broadening and convenient method transfer from process development to quality control (Figure 4A).

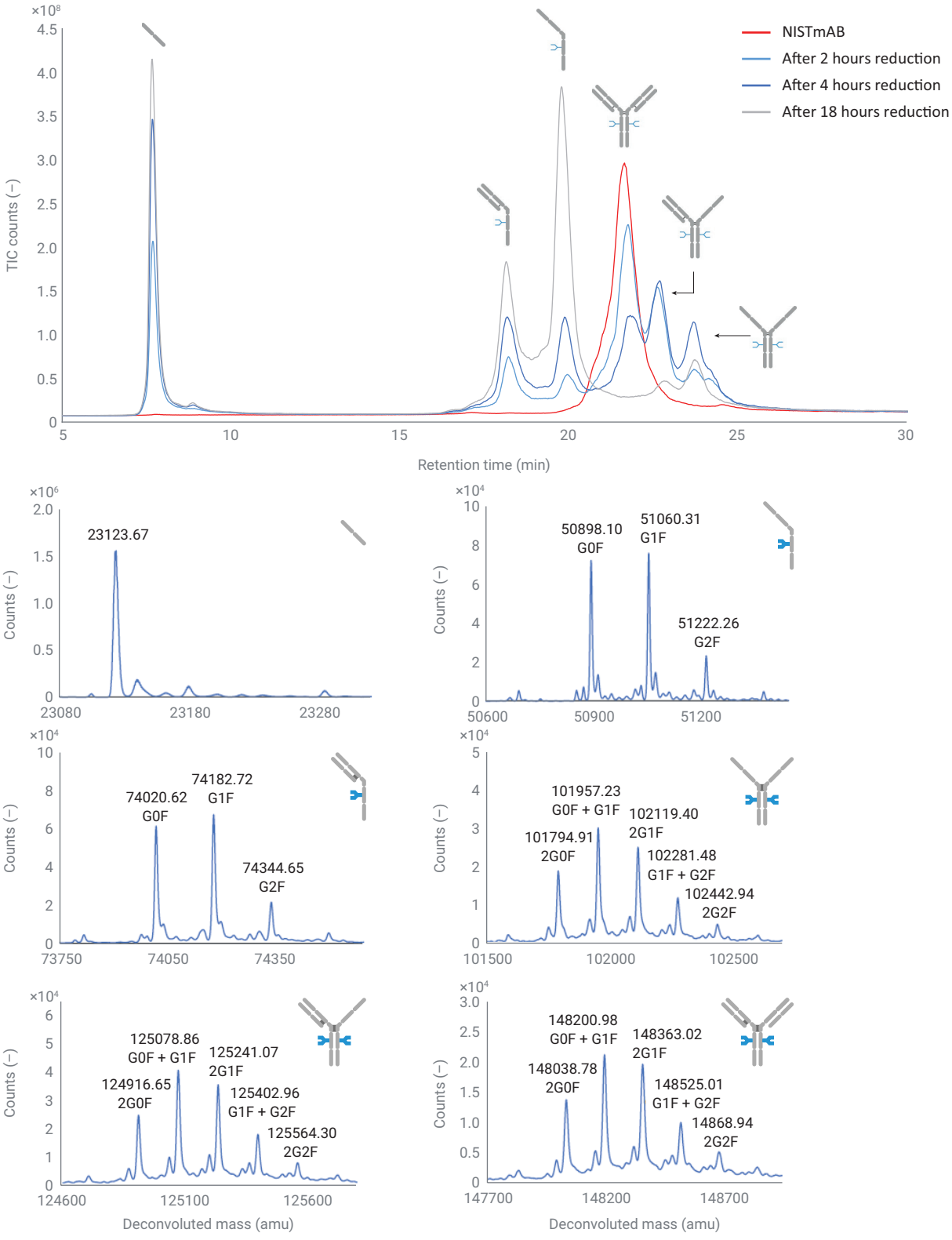

Figure 3. Chromatograms of the dynamic partial reduction of the NISTmAb separated by the Agilent 1290 Infinity II Bio LC and detected with the Agilent 6545XT AdvanceBio LC/Q-TOF. Corresponding extracted spectra of the respective fragments show the characteristic glycosylation of the NISTmAb.

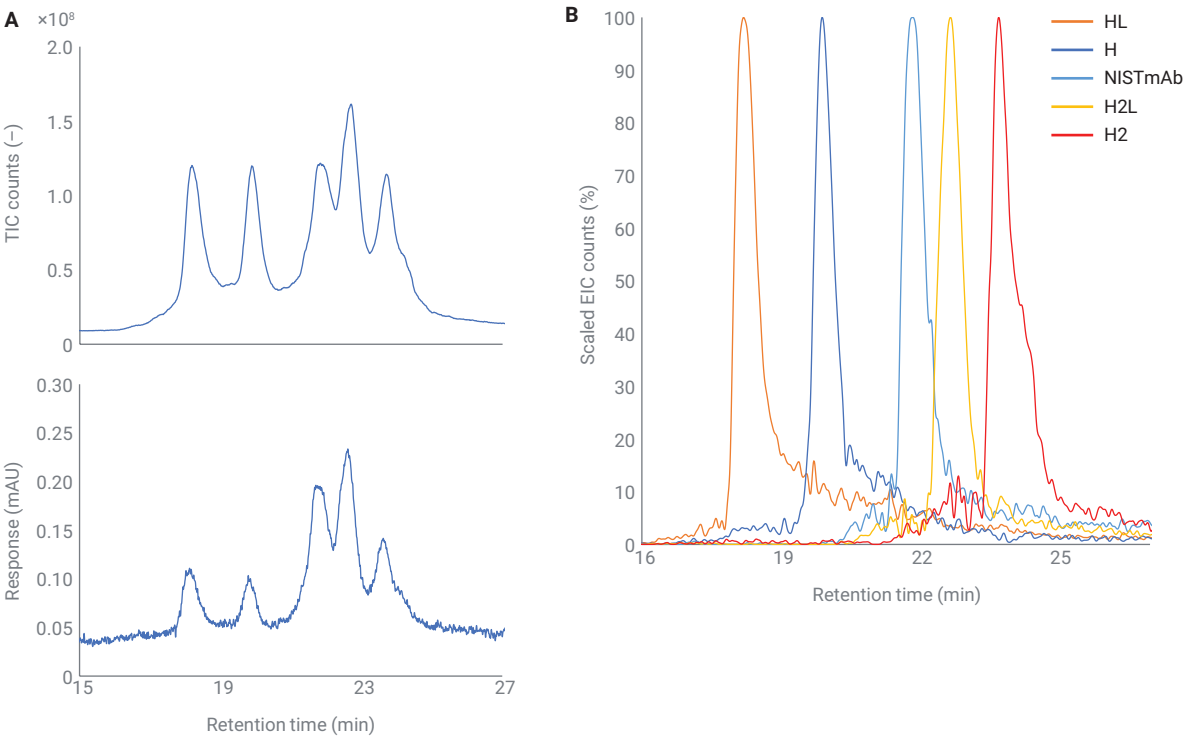

Figure 4. MS and UV chromatogram of the fragments clustering around the NISTmAb acquired in one run (A). Extracted ion chromatograms of NISTmAb fragments showing excellent peak shape (B).

Conclusion

Traditional SDS-PAGE and the modern equivalent CE-SDS are widely used to analyze product-related impurities like LMW and HMW species. However, structural identification of LMW species with these methods has been challenging and primarily based on empirical knowledge. This application note presents an RPLC method capable of separating all relevant reduction-induced LMW species of the NISTmAb. The 1290 Infinity II Bio LC showed excellent retention time and area precision values based on the heavy chain and light chain fragment analysis. Dynamic reduction of the NISTmAb in the 1290 Infinity II Bio Multisampler and subsequent detection with the 6545XT AdvanceBio LC/Q-TOF showed the potential of the method to analyze post-translational modifications. When combined with fragment analysis, this capability can accelerate biopharmaceutical development. That is why the PEEK-lined PLRP-S column and the 1290 Infinity II Bio LC are a future-proof combination for the analysis of biopharmaceuticals across the production process up to final quality control.

References

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DE44181.2356018519

Printed in the USA, January 29, 2021
5994-3021EN

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