Application Note

Pharmaceutical and Biopharmaceutical

Improved Analysis of Intact Proteins and Peptides by Reversed Phase HPLC Using the Altura Ultra Inert PLRP-S 1000 Å Column

Authors

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Abstract

When considering the separation of large biomolecules, challenges with traditional stainless-steel column hardware are common, such as missing peaks or the need to precondition columns before use by making repeated injections. Additionally, undesirable interactions between chemically complex biomolecules (proteins, peptides, oligonucleotides, etc.) and metal ions contained in column hardware can significantly hamper resolution between closely eluting components. In this application note, we demonstrate how to achieve a significant gain in LC/MS performance by upgrading to Agilent Altura Ultra Inert coated column technology for intact mAb and ADC analysis.

Introduction

Intact protein analysis by reversed-phase HPLC (RP-LC) plays a vital role in quality control for determining the purity of biotherapeutic proteins and improving the identification and quantification of critical quality attributes (CQAs). When coupled to mass spectrometric detection, it allows several things. First, an accurate molecular weight to be determined; second, the identification of variants containing post-translational modifications (PTMs) and proteoforms; and third, the antibody-to-drug ratio in the case of antibody drug conjugates (ADCs).[1]

While many RP columns have a silica-based stationary phase to enable broader functionalization, residual acidic silanol groups can interact with basic residues in peptides, proteins, and other biomolecules, resulting in significant peak tailing and loss of chromatographic performance. Polystyrene/divinyl benzene (PS/DVB)-based stationary phases do not contain unintentional adsorption sites. Additionally, larger pore sizes have demonstrated superior chromatographic efficiency through less diffusion restriction into the pore volume and reduction of size exclusion mechanisms, degrading the RP-based selectivity.[2]

Wide-pore columns like the PLRP-S 1000Å provide an exceptional performance with biomolecules, particularly when combined with MS-compatible mobile phase conditions such as formic acid.

Even so, some undesirable interactions may be observed when using stainless-steel column hardware. Molecules that possess multiple negative charges (including oligonucleotides, phosphorylated peptides and proteins, and proteins with low isoelectric point in general) may interact with exposed metal ions in stainless-steel column hardware. This can result in the need to "deactivate" new columns by making multiple injections of sample until reproducible results are obtained.

This application note demonstrates how the new Altura Ultra Inert PLRP-S 1000 Å column serves as an effective high-performance workhorse in the analysis of large, intact biomolecules. Developed to effectively block nonspecific adsorption during separations, Ultra Inert technology reduced passivation time 15-fold and delivered ~ 10% greater recovery for mAbs and ADCs and up to 100% for smaller biomolecules like GLP-1s and small globular proteins.

Experimental

Reagents and chemicals

Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). LC/MS grade acetonitrile (ACN) was purchased from Agilent Technologies (p/n 5191-5101-001) and LC/MS formic acid was purchased from Sigma-Aldrich.

All formulated mAbs and the ADC sacituzumab govitecan were purchased from Evidentic at 10 mg/mL. Exenatide acetate (AT27767-25MG) and lyophilized lysozyme were purchased from Sigma-Aldrich.

Instrumentation

- Agilent 1290 Multicolumn thermostat (G7116B)
- Agilent 1290 Bio multisampler (G7137A)
- Agilent 1290 Bio high-speed pump (G7132A)
- Agilent 6530 Q-TOF MS system

Software and data processing

- Agilent MassHunter Workstation, version 10.1
- Agilent MassHunter Qualitative Analysis software, version B.07
- Agilent DAR calculator, version B01.01

Sample preparation

All samples were dissolved to 1 mg/mL in 0.1% formic acid.

Analytical methods

Table 1. LC conditions.

Ion-Pair Reversed-Phase LC/MS	
Column	– Agilent PLRP-S 1000 Å, 2.1 × 50 mm, 5 µm (p/n PL1912-1502) – Agilent Altura PLRP-S 1000 Å, 2.1 × 50 mm, 5 µm with Ultra Inert technology (p/n PL1912-1502A)
Mobile Phase	Eluent A) 0.1% formic acid Eluent B) ACN + 0.1% formic acid
Flow Rate	0.21 mL/min
Column Temperature	65 °C
Injection Volume	1.0 µL
Total Run Time	10 min
Gradient	Time (min) %A %B 0 75 25 5 75 25 10 50 50 10.01 1 99 12 1 99 12.01 75 25 15 75 25

Table 2. MS method conditions.

Q-TOF Parameters	
Ionization Mode	Positive
Gas Temperature	350 °C
Drying Gas	12 L/min
Nebulizer	60 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	5,500 V
Nozzle	2,000 V
Fragmentor	270 V
Skimmer	140 V
Oct 1 RF Vpp	750 V

Results and discussion

Altura PLRP-S 1000 Å minimizes passivation time and enhances recovery of a broad range of biomolecules

As proteins and peptides are composed of amphoteric amino acids, they have both positive and negatively charged sites which can interact non-specifically with charged sites on metal surfaces. Figure 1 demonstrates the benefit of the Altura PLRP-S 1000 Å column hardware with respect to passivation (Figure 1A) of the ADC sacituzumab govitecan, where a stable analyte signal is reached by the third injection. While the stainless-steel hardware showed no signal until injection 3, it took almost 20 injections to reach a similar signal intensity as the Altura column. Additionally, when injecting a larger amount of sacituzumab govitecan to see if higher mass loading overcame the difference in recovery, we still observed a roughly 8% higher recovery on the Altura hardware following several injections (Figure 1B).

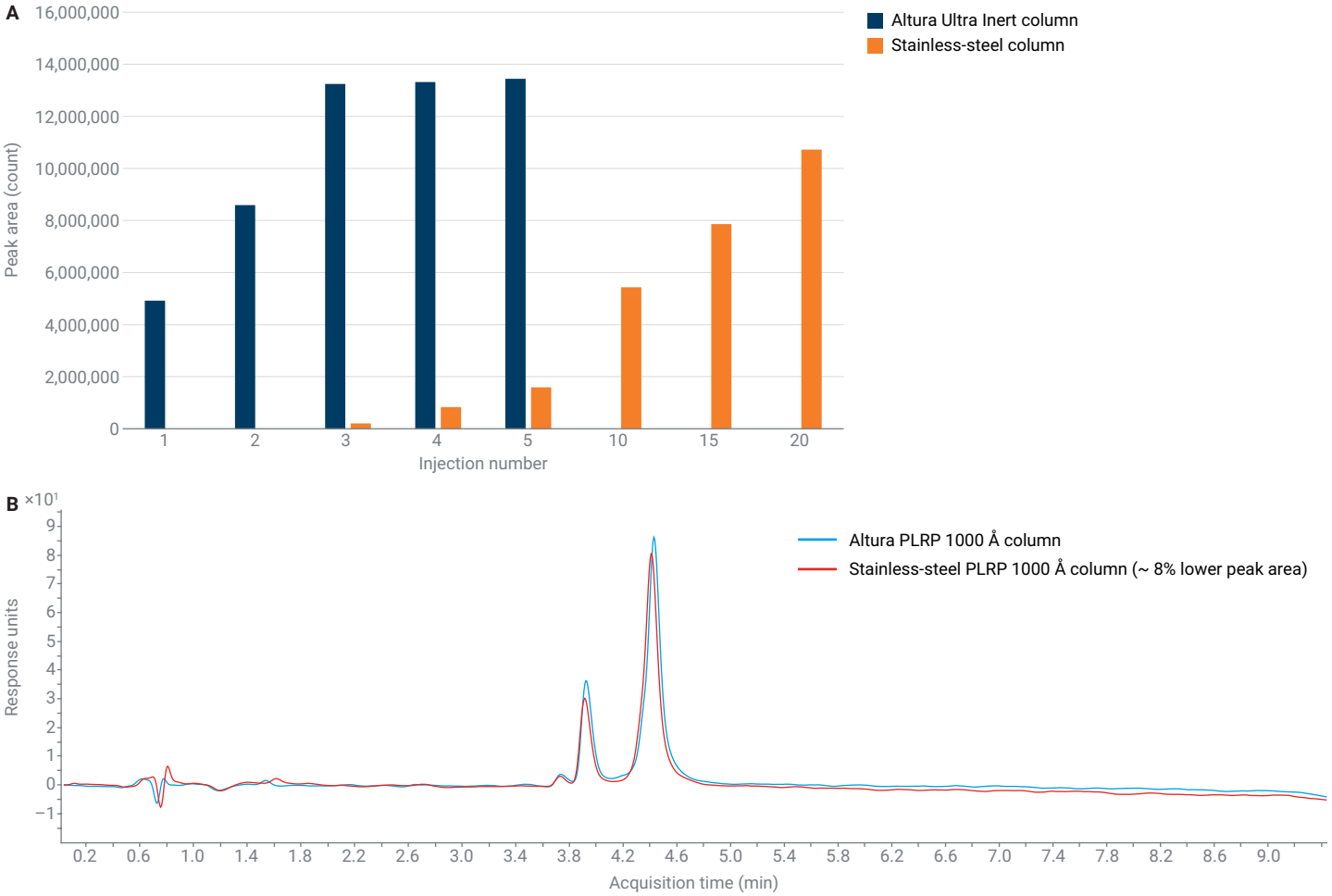

Figure 1. (A) Passivation of the Altura PLRP-S 1000 Å column (red) and the stainless-steel PLRP-S 1000 Å column (blue) when injecting 1 µL at 0.05 mg/mL. (B) Difference in peak recovery for with the Altura PLRP-S 1000 Å column (blue chromatograms) versus stainless-steel (red chromatograms) of 1 µL sacituzumab govitecan at 0.1 mg/mL.

Additionally, the recovery of the intact aglycosylated mAb atezolizumab also demonstrated improved recovery with the Altura PLRP-S 1000 Å column with a gain of 5% (Figure 2A) which resulted in a higher quality of deconvoluted MS spectra (Figure 2B) which identified the loss of either two lysine residues (−256 Da) or one (−128 Da).

While the gain in recovery was moderate for intact mAbs under low pH conditions, for smaller biotherapeutics such as the GLP-1 exenatide, the gain in recovery was significantly greater at almost double the recovery rate with the Altura column (Figure 3A). Small globular proteins like lysozyme showed about 85% recovery on the stainless-steel column versus the Altura (Figure 3B). This suggests that the Altura PLRP-S 1000 Å would be a good option for a wide variety of biotherapeutic molecules to ensure maximal sensitivity in comparison to the current stainless-steel columns.

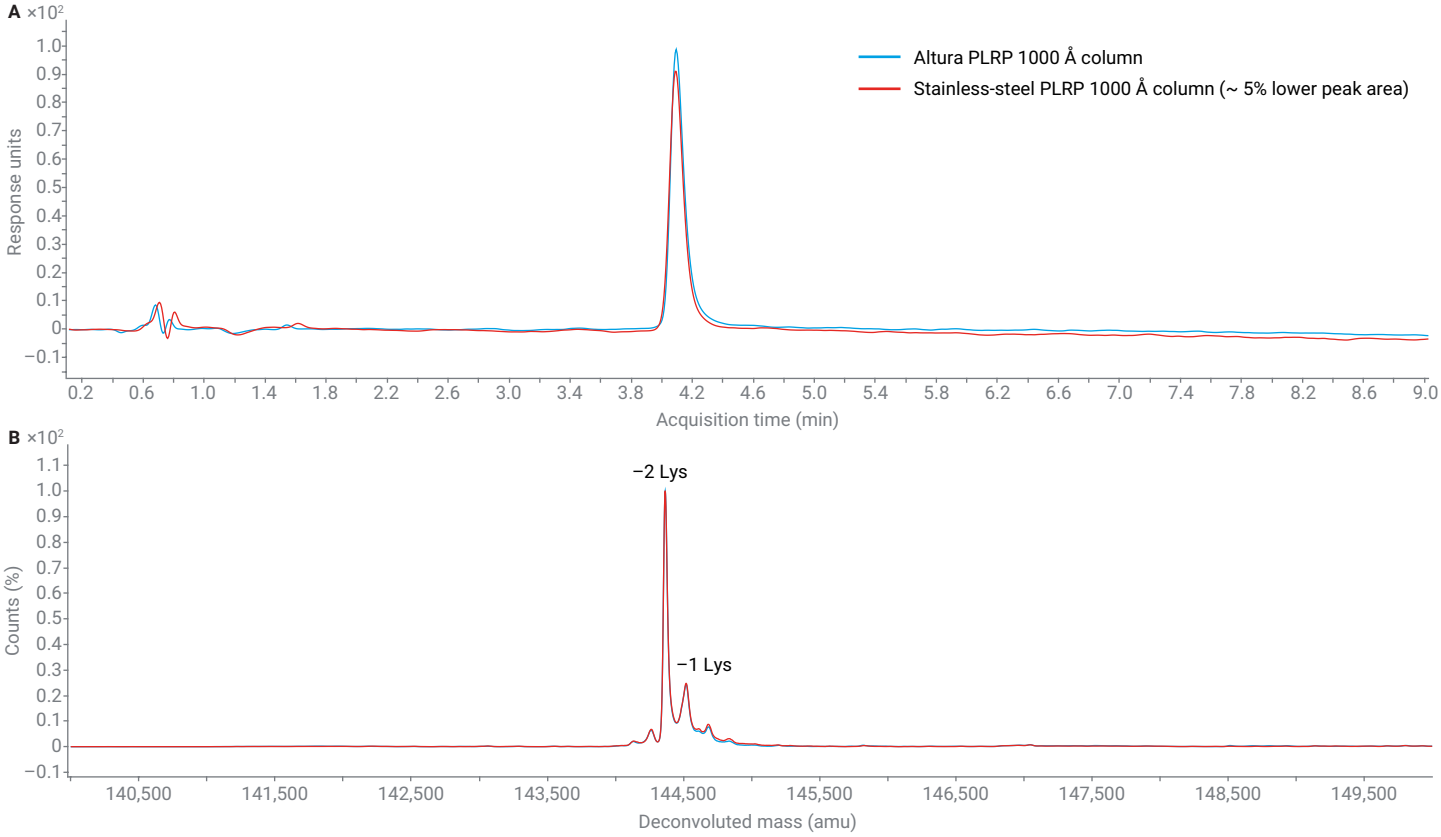

Figure 2. (A) Difference in peak recovery with the Altura PLRP-S 1000 Å column (blue chromatograms) versus stainless-steel (red chromatograms) of 1 µL Atezolizumab at 0.1 mg/mL. (B) deconvoluted spectrum of Atezolizumab.

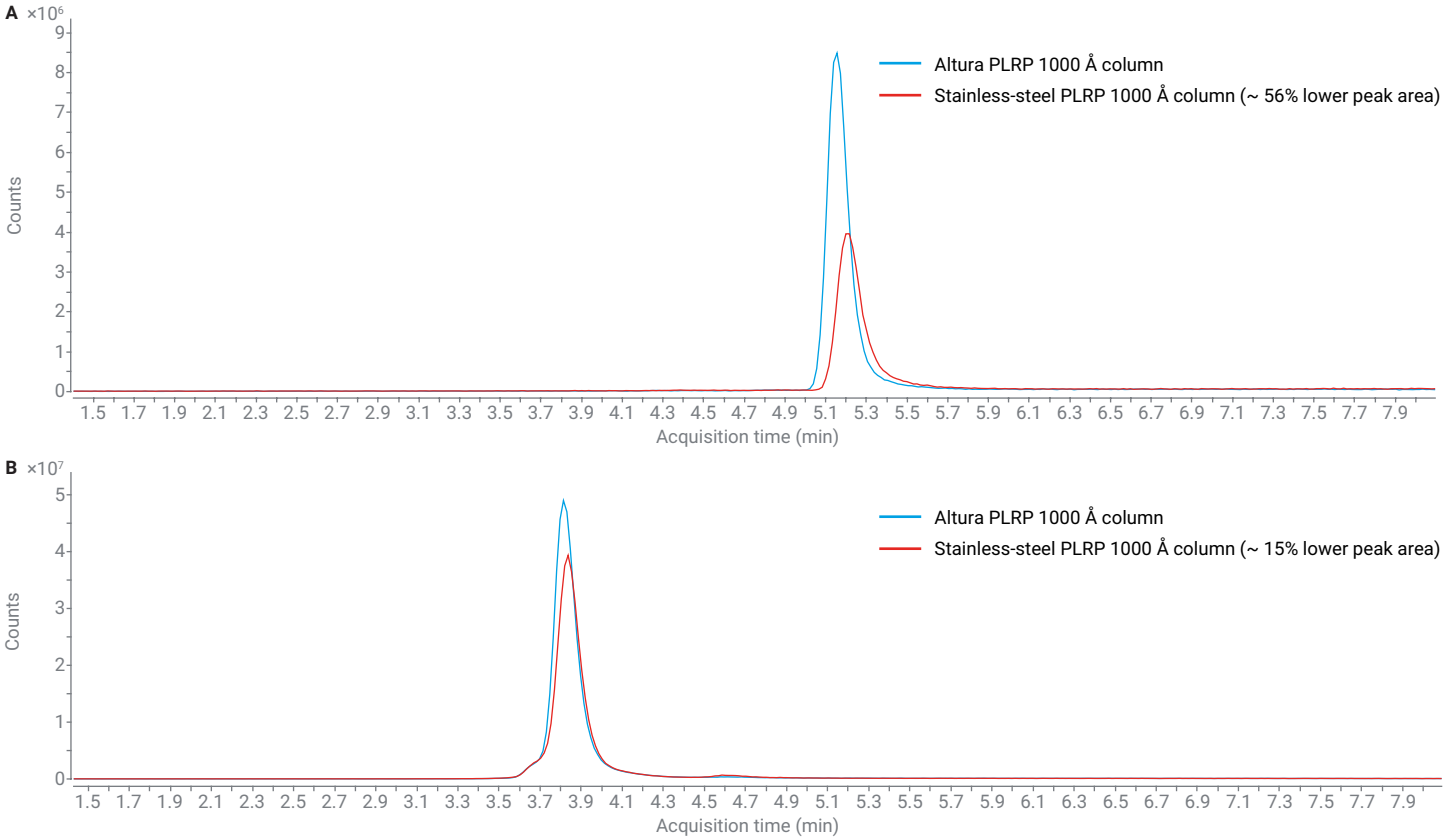

Figure 3. Difference in peak recovery with the Altura PLRP-S 1000 Å column (blue chromatograms) versus stainless-steel (red chromatograms) for 1 µL of exenatide at 0.1 mg/mL (A) and 1 µL of Lysozyme at 0.1 mg/mL (B).

The Altura PLRP-S 1000 Å enabled improved PTM identification and accurate determination of ADC DAR

The improvements in peak recovery on the Altura PLRP-S 1000 Å column resulted in improved MS spectral quality which is beneficial to the analysis of accurate mass. Sacituzumab govitecan is a cysteine-linked ADC which under reverse phase conditions dissociated into the heavy and light chains during the separation, requiring high separation power to quantify both (Figure 4). Curiously, the mass spectra of both the heavy and light chain showed a loss of 418 Da which was the loss of the SN-38 drug payload from the ADC. This could be due to leaving the sample at room temperature for an extended period.

One of the most important aspects of intact mass analysis via RP-LC is the accurate quantitation of the drug antibody ratio (DAR), to determine if the loading of small molecule onto the mAb was successful. In the case of sacituzumab govitecan, the validated DAR is 7.6.[3] The Altura PLRP-S 1000 Å column provided sufficient separation power, recovery, and spectral quality for both heavy and light chains to accurately quantify the DAR (Figure 5).

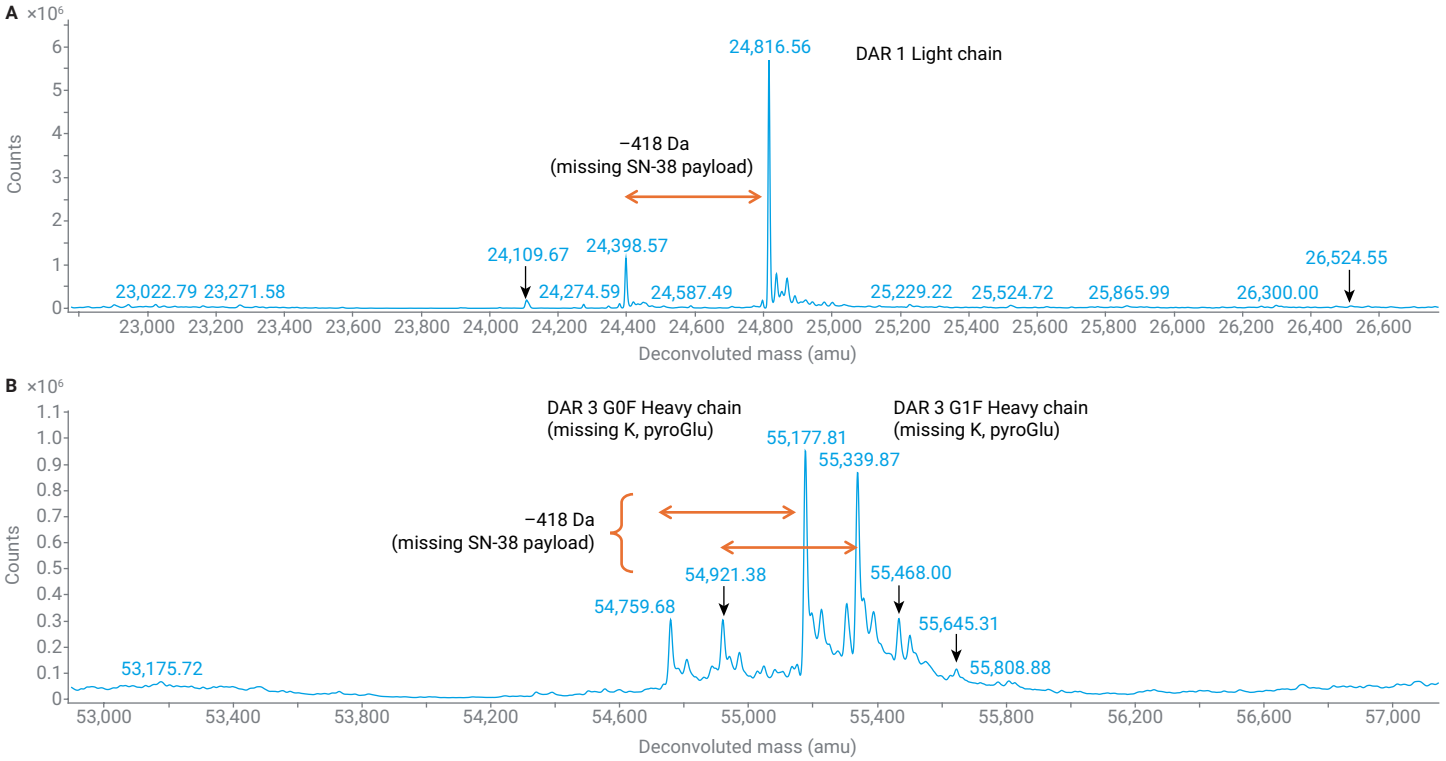

Figure 4. Deconvoluted mass spectra of Sacituzumab govitecan light and heavy chains demonstrating the loss of the SN-38 payload (−418Da) and the glycosylation patterns on both chains (mass accuracy of 28 ppm for both chains).

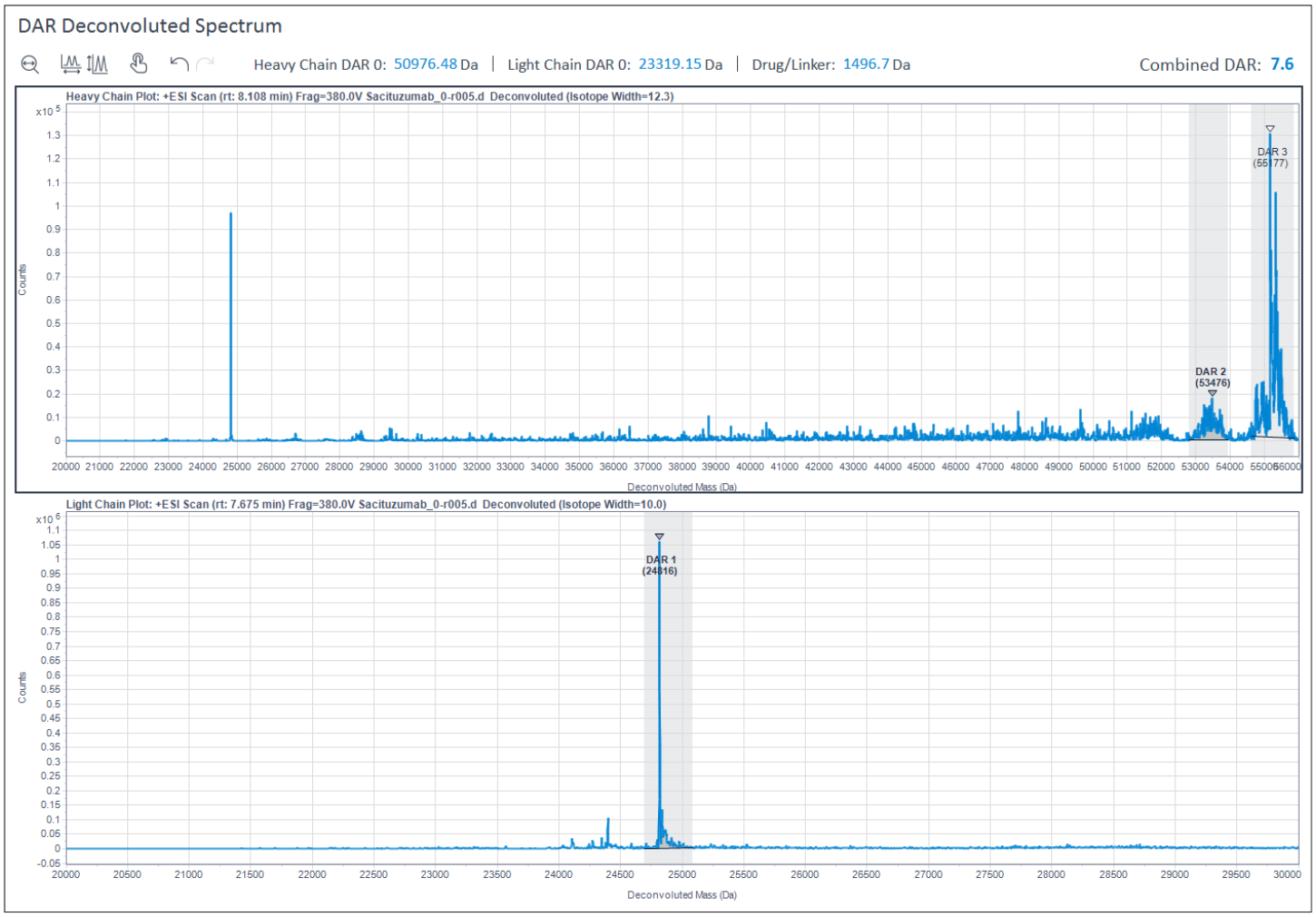

Figure 5. Quantification of the DAR of the ADC sacituzumab govitecan using the Agilent DAR calculator software.

Conclusion

Intact mass analysis is a critical methodology to determine the purity of biotherapeutics, but due to the chemical complexity of these analytes, nonspecific adsorption from both silica stationary phase and metal column housing can be highly deleterious to separation performance.

The Agilent Altura PLRP-S 1000 Å provides a highly robust solution combining the chemical inertness of polystyrene/divinyl benzene stationary phase with the ultra-inert Altura hardware to dramatically reduce deleterious peak tailing and increase recovery for biotherapeutics. Herein, we see that analyte recovery for both mAbs, ADCs, GLP-1s, and globular proteins are improved versus stainless-steel, while the Altura PLRP-S column is capable of monitoring both quality critical PTMs such as loss of drug payload as well as accurate quantification of ADC DAR.

References

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3. Kopp, A.; *et al.* Antibody–Drug Conjugate Sacituzumab Govitecan Drives Efficient Tissue Penetration and Rapid Intracellular Drug Release. *Mol. Cancer Ther.* **2022**, *22(1)*, 102–111. doi:10.1158/1535-7163.mct-22-0375.

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