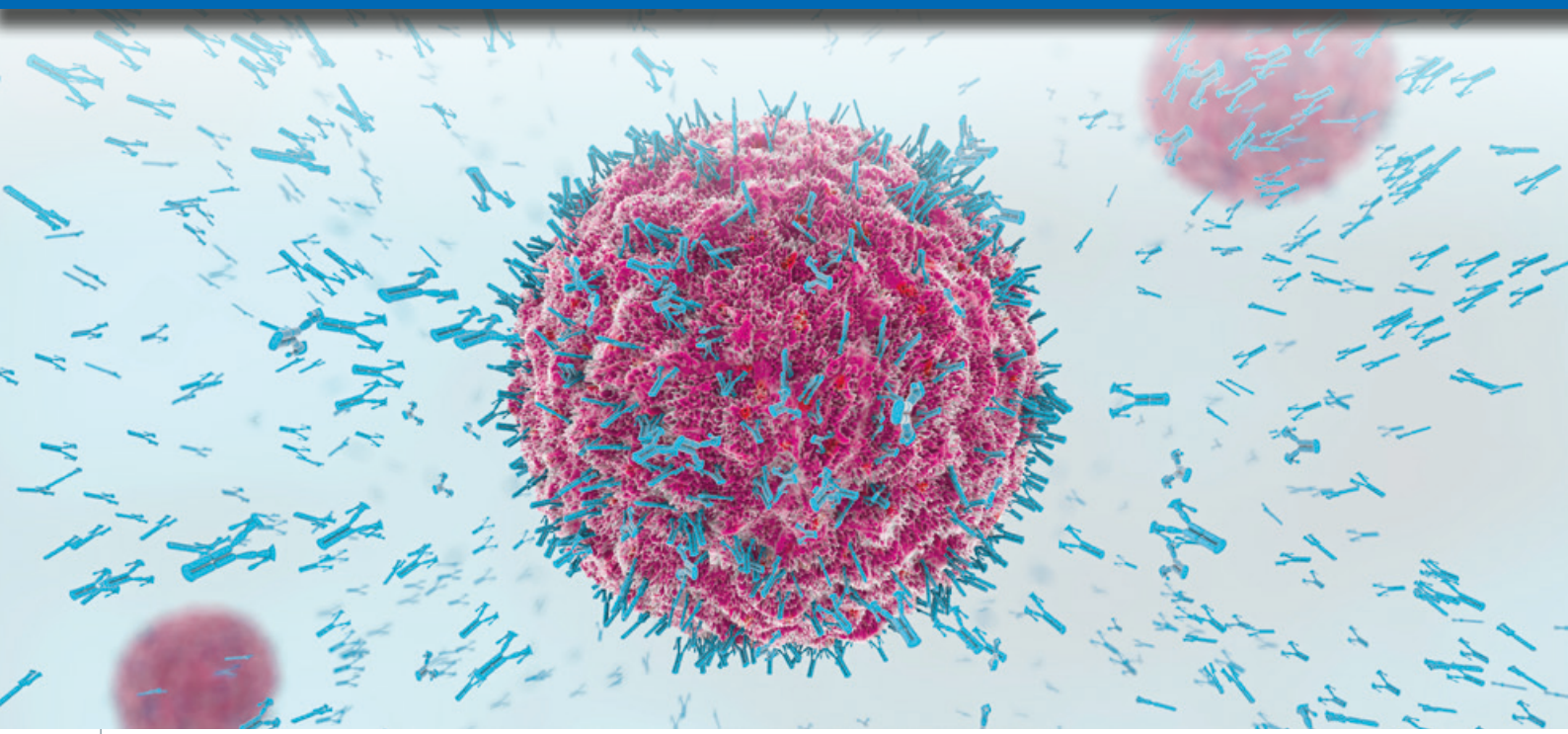

Routine analysis of drug to antibody ratio and drug distribution with maXis II

Antibody drug conjugates (ADC) are small molecule conjugated mAbs. These are rapidly emerging complex biological molecules in the biopharma space.

Abstract

Characterization of ADC's includes intact mass analysis and Drug to Antibody Ratio (DAR) estimation using advanced high-resolution LC-MS analytical tools. Intact mass confirms the amino acid sequence accuracy while DAR estimate provides an insight into the potency and efficacy. LC-MS offers a unique advantage in providing accurate quantification and in-depth characterization of ADC's along with their conjugated and unconjugated species. In our current work the DAR content of Kacyla (ado-trastuzumab emtansine, also known as T-DM1) was estimated using the Bruker maXis II ultrahigh resolution QTOF-MS. Our experimental DAR value of 3.54 corroborates already published data [1].

Keywords: mAb, ADC, antibody drug conjugate, monoclonal antibody, intact mass analysis, average DAR, drug to antibody ratio, drug distribution, maXis, QTOF, biopharma, characterization

Centre for Advanced Protein Studies

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Introduction

Antibody drug conjugates rely on combining the target specificity of monoclonal antibodies (mAb) with the efficacy of small molecule drugs too toxic for systemic use. These molecules are designed to deliver their payload in proximity of the target tissue through the cleavage of the linker in between the mAb and the drug, allowing the free drug to interact with its target. For example, Trastuzumab is specific for HER2 markers overexpressed by tumor cells, giving the ADC Kadcyla the potential to preferentially deliver its cytotoxic payload to these cells.

Since the potency of the ADC is directly linked to the amount of free drug it can deliver to the target, it is necessary to know how much drug is linked to each mAb molecule, which is characterized by the average DAR. In addition, the drugs often strongly impact the hydrophobicity of the mAb and can impact the stability and pharmacokinetics. Understanding and characterizing the influence of the number of drug-linker entities on the ADC requires a tool to measure the distribution of DAR species in the drug substance.

Average Drug-to-Antibody ratio (DAR) is a key attribute of ADCs and refers to the average number of small molecule drugs conjugated to the antibodies. The average DAR value has a direct impact on the overall efficacy, as lower drug loading reduces the potency and higher drug loading can results in toxicity or stability related issues. Lysine side chain amidation or cysteine interchain disulfide bond reduction based conjugation mechanisms result in average DAR value ranging from D0 - D8 (where D represents the number of molecules per antibody). Kadcyla is a commercially available antibody–drug conjugate (ADC) that contains the humanized anti-HER2 IgG1 antibody trastuzumab, and DM1, a microtubule inhibitory maytansinoid, linked through a thioether bond. Trastuzumab emtansine retains the mechanisms of action of both trastuzumab and DM1.

Kadcyla is a lysine-conjugated ADC, it utilizes the solvent-exposed e-amino groups of lysine residues to attach drugs.

The analysis of intact ADCs by LC-MS provides a reliable way to establish these parameters. This measurement can be carried out ahead of the development of e.g. hydrophobic interaction chromatography methods, providing a tool to accelerate process development in parallel with analytical development. Furthermore, it provides an orthogonal assay to validate HPLC methods and avoid peak assignment errors due to shifts in hydrophobicity induced by glycans or other hydrophilic moieties.

Challenges of ADC characterization

The drug-linker assembly can be conjugated to various amino acids of the protein backbone, for example lysine, interchain cysteine (after partial reduction) or specifically engineered non-natural residues.

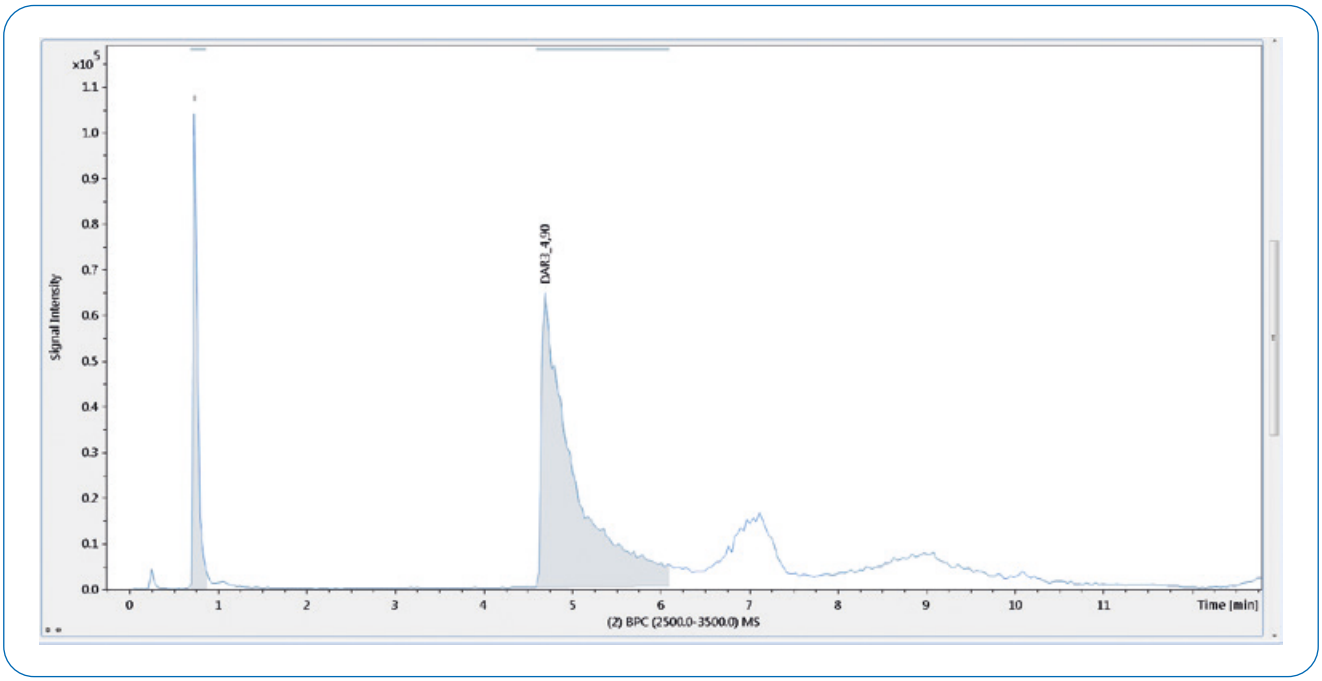


Figure 1: Base peak chromatogram of TDM-1 reverse phase separation (2500-3500 m/z)

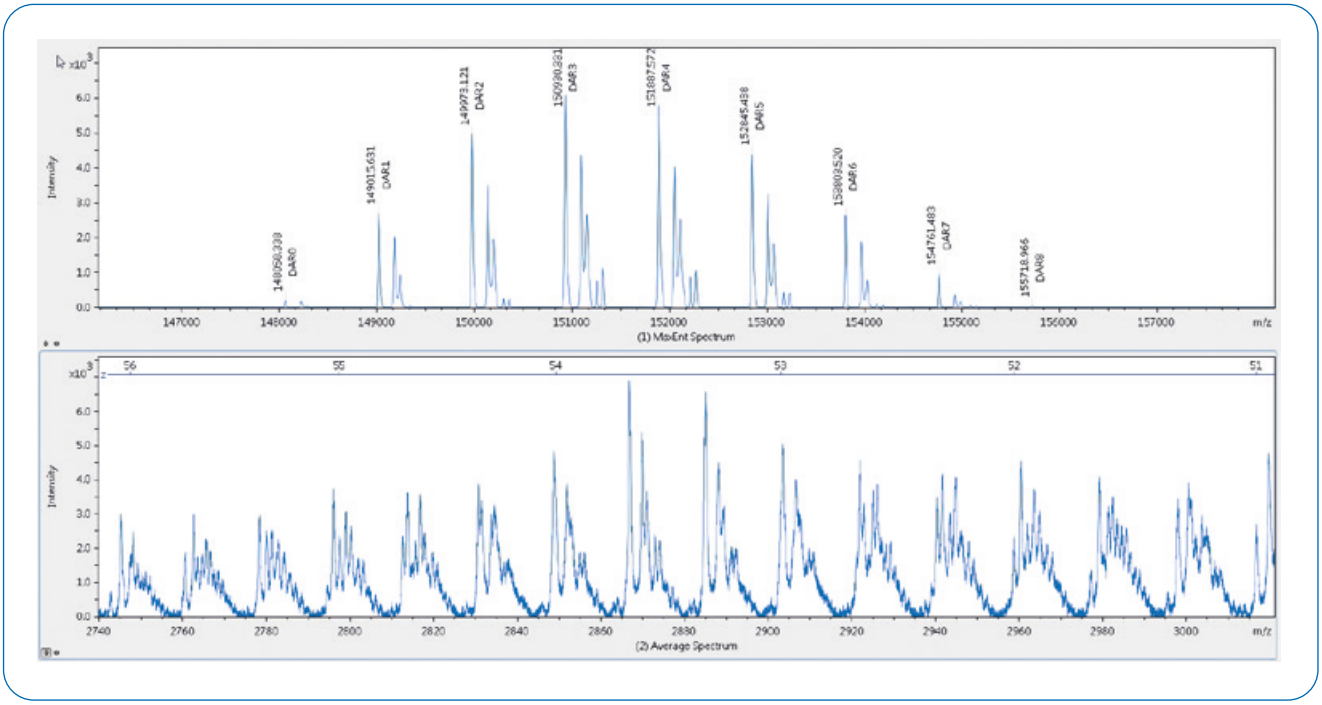


Figure 2: Deconvoluted spectrum (top) and raw spectrum (bottom) for TDM-1

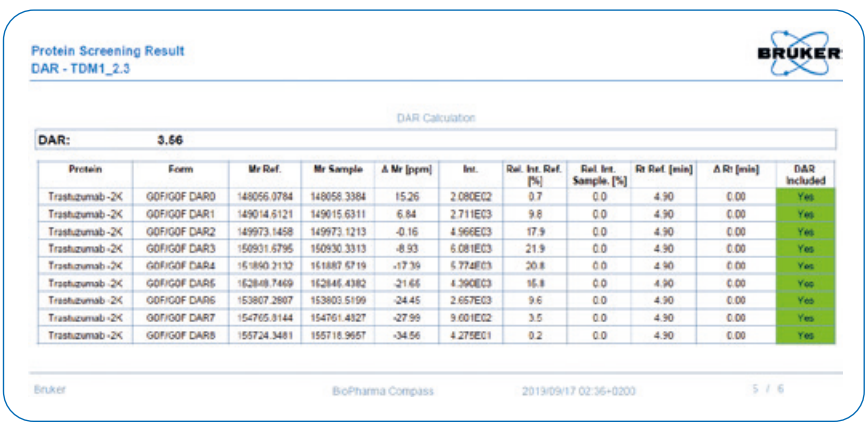
In the case of conjugation to cysteine residues, the ADC must be analyzed under non-denaturing conditions to conserve the integrity of the protein in absence of interchain disulfide linkages. This requires an instrument capable of efficient desolvation in native MS conditions without disrupting the protein stoichiometry.

ADC formed using lysine conjugation can be analyzed under denaturing conditions, however high resolution is required to separate the various heterogeneity peaks which have reduced *m/z* distances in the higher charge states typically obtained with fully denatured proteins.

Bruker maXis II offers a resolution up to 80,000 providing high quality intact mass data for mAbs based therapeutics such as ADCs. In addition, the low heat (<250 °C) in the ESI source, soft transfer optics and tunable pressure in the dual funnel inlet (High mass option) make it ideally suited for the analysis of ADCs, and retain the intact molecular structure during the initial ionization process and subsequent analysis. In addition, the very high intra scan dynamic range makes it possible to observe the desired ions with high S/N ratio. This application note focuses on the lysine based ADC being analyzed under denaturing conditions.

Data acquisition

Trastuzumab conjugated with emtansine (TDM-1) samples were diluted to a final concentration of 1 mg/mL. 1 µL of the sample was analyzed by LC-MS (Fig.1). The HPLC separation was performed on a Bruker Elute UHPLC with a BEH 300 C4 2.1X100 mm (Waters) at 200 µL/min, at 60 °C separated on a gradient from 5% to


Protein Screening Result
DAR - TDM1_2.3

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DAR Calculation

DAR: 3.56

Protein	Form	Mr Ref.	Mr Sample	Δ Mr [ppm]	Int.	Rel. Int. Ref. [%]	Rel. Int. Sample. [%]	Rt Ref. [min]	Δ Rt [min]	DAR Included
Trastuzumab -2K	G0F/G0F DAR0	148056.0784	148056.3384	15.26	2.080E02	0.7	0.0	4.90	0.00	Yes
Trastuzumab -2K	G0F/G0F DAR1	149014.6121	149015.6311	6.84	2.711E03	9.8	0.0	4.90	0.00	Yes
Trastuzumab -2K	G0F/G0F DAR2	149973.1458	149973.1213	-0.16	4.966E03	17.9	0.0	4.90	0.00	Yes
Trastuzumab -2K	G0F/G0F DAR3	150931.6795	150930.3313	-8.93	6.081E03	21.9	0.0	4.90	0.00	Yes
Trastuzumab -2K	G0F/G0F DAR4	151890.2132	151887.6719	-17.39	5.774E03	20.8	0.0	4.90	0.00	Yes
Trastuzumab -2K	G0F/G0F DAR5	152848.7469	152846.4382	-21.66	4.390E03	15.8	0.0	4.90	0.00	Yes
Trastuzumab -2K	G0F/G0F DAR6	153807.2807	153803.5199	-24.45	2.657E03	9.6	0.0	4.90	0.00	Yes
Trastuzumab -2K	G0F/G0F DAR7	154765.8144	154761.4327	-27.99	9.601E02	3.5	0.0	4.90	0.00	Yes
Trastuzumab -2K	G0F/G0F DAR8	155724.3481	155718.9657	-34.56	4.275E01	0.2	0.0	4.90	0.00	Yes

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Figure 5: DAR calculation windows and drug distribution report

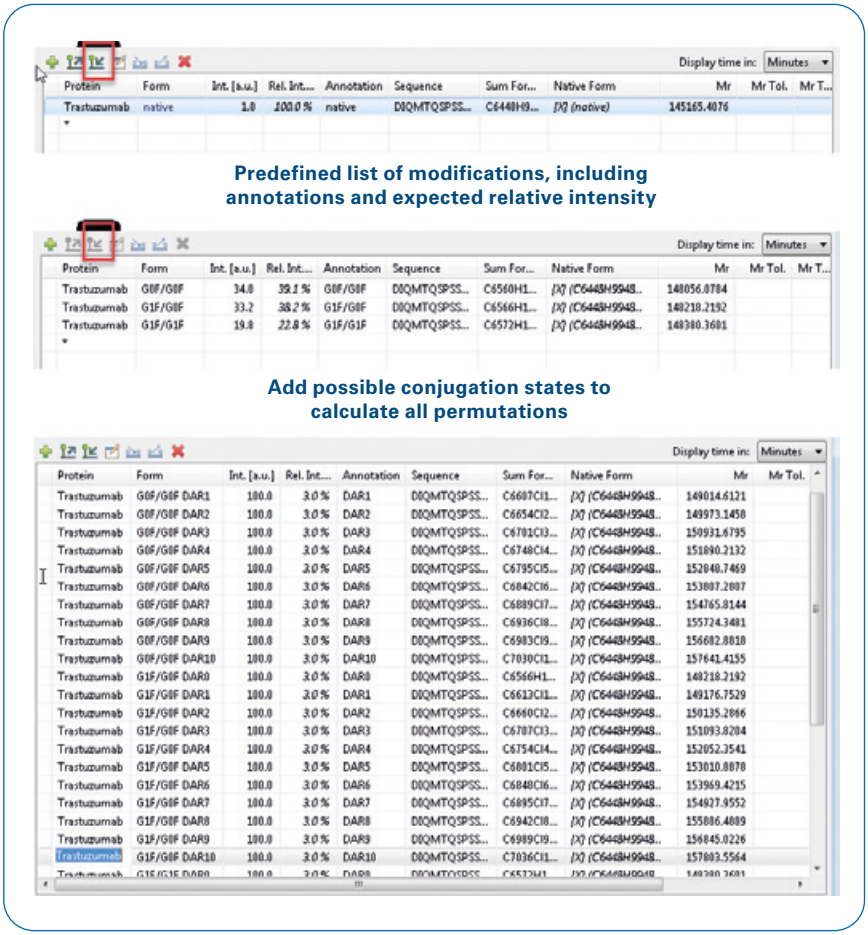

Figure 3: Iterative process for easy reference profile creation

95% mobile phase B in 12 min (A: 0.1% formic acid, B: 0.1% acetonitrile).

MS detection was carried out with a Bruker maXis II ETD using a high mass method. The following acquisition parameters were adopted (Source voltage - 4500 V, Nebulizer gas, Dry gas - 1.5 bar 8 L/min and Source temperature 200°C, 120 eV ISCID).

Data processing

The data were processed in BioPharma Compass 2021 for automated deconvolution and average DAR calculation. The average spectrum was determined based on the integration of the TIC in the 4 to 8 min range. Maximum entropy deconvolution was performed with 2500-3500 *m/z* input range, 140,000 to 180,000 Mr range, baseline subtraction on and a sensitivity of 0.2

Rapid inspection of the deconvoluted data (Fig.2) reveals the presence of a low intensity peak consistent with trastuzumab G0F/G0F indicating that some unconjugated material is present. Peaks of higher intensity with a spacing of 958.5 Da can be observed, consistent with the mass for SMCC-Entamsine, the expected linker drug.

In addition, the more abundant glycoforms of tratuzumab can be observed (galactosylation) as well as a peak shifted by 219.2 Da, consistent with free MMC linker.

DAR measurement

The average DAR could be determined based on only the intensity of the most abundant glycoform, the intensity of the main glycoforms or even taking in account glycoforms and peaks with free linker. The result for these different scenarios can easily be determined in BioPharma Compass by generating reference profiles that consider various amounts of heterogeneity. The software includes a powerful editor to generate such lists even when they include complex modification profiles or when several drug-linkers need to be evaluated.

The starting mass for the project can be imported from the sequence editor or directly input in the protein reference list. The modification profile tool then allows users to automatically apply sets of modifications in an iterative manner, including customized annotations. For example, Fig.3 illustrates how this process allows creating a list of target masses for the 3 main glycoforms of trastuzumab and states in between DAR0 and DAR10. Fig.4 shows the full annotation for the DAR2 species, including the free linkers.

Once the list is ready the protein screening workflow can automatically process the data and assign the masses from the list to the deconvoluted peaks. Reprocessing a result allows automatically updating the reference profile with the measured relative intensities and retention time, giving users a tool to generate a target reference profile to compare against future processes or batches. With the predefined methods and reference profile, new data can be screened in less than 1 min per dataset.

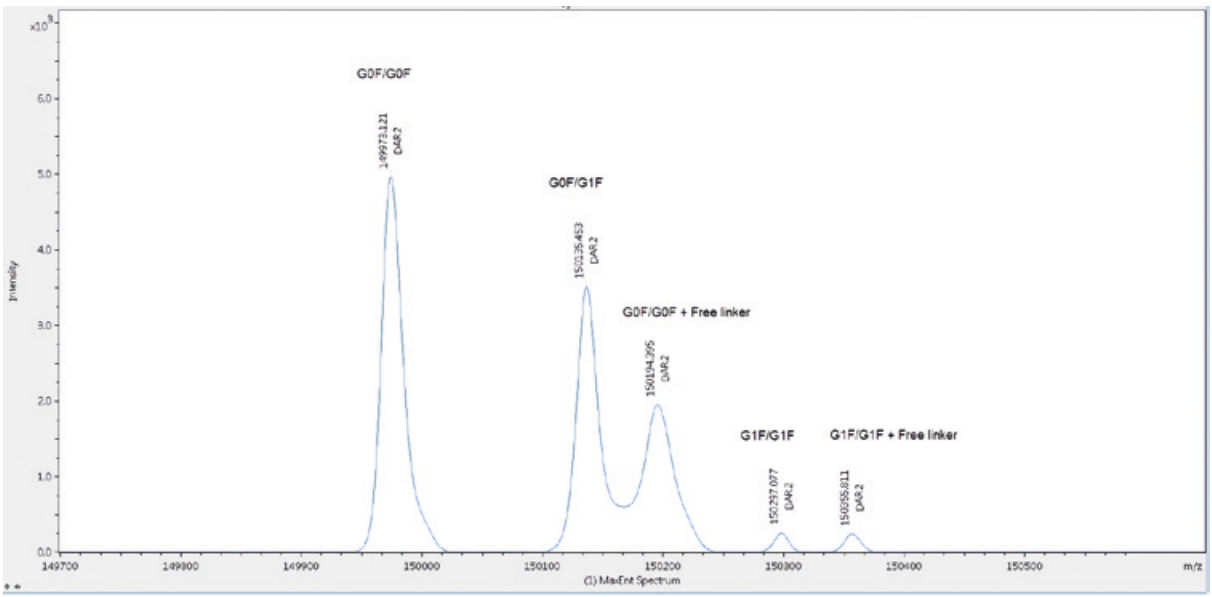

Figure 4: Annotated DAR2 species

The average DAR analysis considering only the main G0F/G0F peaks yielded an average DAR of 3.56 (Figure 5). The analysis with 3 main glycoforms and 3 glycoforms plus free linker resulted in an average DAR of 3.54. These values are comparable to the one reported in the literature [1]. The ability to perform these measurements on a glycosylated molecule is essential as some ADC products may be less stable after deglycosylation.

Conclusion

- Average DAR calculated from the MS data obtained from the glycosylated sample was found to be 3.5, in addition the deconvoluted spectra provide a direct measurement of the average DAR distribution from 0 to 8.
- The Bruker maXis II resolution and intra-scan dynamic range make it excellently suited for the analysis of ADC such as the determination of average DAR and drug distribution.
- The protein screening workflow of BioPharma Compass 2021 now simplifies average DAR studies by providing a simple mechanism to establish a complex target reference profile.
- Moreover, the capabilities of BioPharma Compass to fully automate data acquisition, data security, data processing and report provide an easy to learn platform for the routine analysis of mAb and mAb derived products.

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