Size Exclusion Chromatography/ Mass Spectrometry Analysis of Antibody Drug Conjugates Using the Agilent 1260 Infinity II Bio-Inert LC

Suitable for Agilent
1260 Infinity III LC

Application Note

Biologics & Biosimilars

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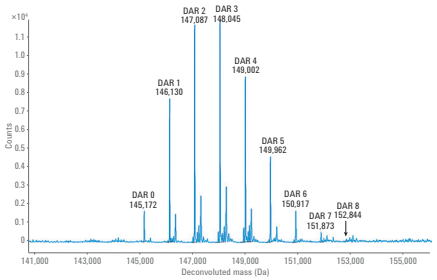
Abstract

Recombinant monoclonal antibodies (mAbs) have emerged as important therapeutic agents for the treatment of different diseases[1]. These biotherapeutics have been extended by the development of antibody drug conjugates (ADCs). ADCs are mAbs to which cytotoxic payloads are covalently attached, enabling delivery of potent drugs to a specific target. This Application Note describes the analysis by size exclusion chromatography with online mass spectrometry (SEC/MS) of an mAb (Trastuzumab) and an ADC (Trastuzumab emtansine) using the Agilent 1260 Infinity II Bio-inert LC with quadrupole time-of-flight (Q-TOF) mass spectrometry (MS) detection. SEC is a straightforward technique of introducing mAbs and ADCs into the LC/MS system, requiring little method optimization. From the MS data collected for the ADC, it was possible to calculate a drug-to-antibody ratio (DAR) of 3.1 using the Agilent DAR calculation software. We demonstrate that the 1260 Infinity II Bio-inert LC is an optimal front-end for LC/MS analysis of biopharmaceuticals.



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Introduction

Antibody drug conjugates (ADCs) are recombinant monoclonal antibodies (mAbs) conjugated to cytotoxic drug molecules using various available linker chemistries[2]. The combination of highly potent drugs with antibodies targeted to specific antigens is expected to enhance the therapeutic action by the sensitive discrimination between healthy and diseased tissue[3]. The advantage of ADCs as therapeutic agents is the increased control of the pharmacokinetics of the drug[4]. The delivery to the target diseased tissue is improved, while the exposure of healthy tissues to toxic drugs is minimized, leading to selective elimination of tumor cells expressing the target antigen[2].

Figure 1 shows the structure of the ADC Trastuzumab emtansine (T-DM1, Kadcyla). It consists of three components:

- mAb (Trastuzumab)
- A bifunctional linker ([N-maleimidomethyl]cyclohexane-1-carboxylate, MCC)
- Small molecule drug (DM1, derivative of maytansine)

T-DM1 is typically administered for the treatment of human epidermal growth factor receptor 2 (HER2)-positive breast cancer[5]. The mAb part of T-DM1 binds selectively to HER2-positive cells, leading to internalization of the ADC-antigen complex into the cell. Inside the cell, the complex is degraded, releasing the cytotoxic drug, which destroys the cancer cell[6]. Emtansine is the combination of the cytotoxic maytansinoid DM1 (which inhibits tubulin polymerization to induce cell-cycle arrest and cell death) and a bifunctional MCC linker. MCC is chemically linked to the antibody through lysine residues on the one side and bound to DM1 by nonreducible thioether linkages on the other side[7]. The resulting ADC is typically highly heterogeneous regarding both the distribution and the loading of the cytotoxic drug species on the antibody. Therefore, physicochemical characterization of ADCs is more complex, compared to the analysis of the corresponding mAb[8].

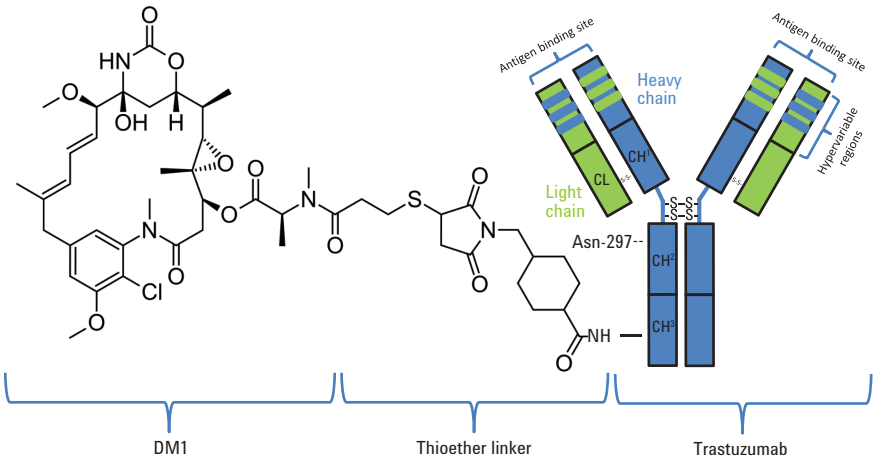

Figure 1 - Structure of Trastuzumab emtansine.

Two types of ADC are currently available on the market; lysine-linked ADCs (such as T-DM1) and cysteine-linked ADCs[9]. Some cysteine conjugates can be analyzed using hydrophobic interaction chromatography (HIC) to determine the drug-to-antibody ratio (DAR). The analysis of lysine conjugates, however, is not feasible using HIC. Due to the higher heterogeneity, UV-Vis spectroscopy and MS are generally the methods of choice to determine the DAR. In contrast to cysteine conjugates, lysine-conjugated ADCs do not dissociate under acidic or high organic conditions (such as those commonly used in MS) due to their intact interchain disulfides. The analysis of ADCs using reversed-phase (RP) liquid chromatography (LC) coupled to MS detection can be problematic due to irreversible binding of the proteins to the stationary phase[8,10]. Elevated column temperatures (60 to 80 °C) and propanol-containing buffers can reduce these effects. However, these conditions might lead to problems regarding temperature-sensitive proteins. SEC is usually carried out at room temperature or slightly above without the need for high temperatures.

This Application Note describes the SEC/MS analysis of T-DM1 as well as Trastuzumab using an Agilent 1260 Infinity II Bio-inert LC coupled to quadrupole time-of-flight (Q-TOF) mass spectrometry (MS).

Experimental

Instrumentation

The SEC-MS experiments were carried out on a 1260 Infinity II Bio-inert LC with online Q-TOF detection comprising the following modules:

Agilent 1260 Infinity II Bio-inert LC

- Agilent 1260 Infinity II Bio-inert Pump (G5654A)
- Agilent 1260 Infinity II Bio-inert Multisampler (G5668A)
- Agilent 1260 Infinity II Multi Column Thermostat (G7116A) with bio-inert heat exchanger (Option #019)
- Agilent 1260 Infinity II Diode Array Detector WR (G7115A) with bio-inert flow cell (Option #028)

MS System

Agilent 6550 iFunnel quadrupole time-of-flight LC/MS

Column

Agilent Bio SEC-3, 300 Å,
7.8 × 300 mm, 3 µm (p/n 5190-2511)

Software

Agilent MassHunter Workstation software with Bioconfirm software, version B.08.00. The DAR was calculated using Agilent DAR Calculator software, version B.01.00, build 1.15.2.0.

Samples

T-DM1 (Kadcyla) and Trastuzumab (Herceptin)

Chemicals

All solvents used were LC/MS grade. LC/MS grade acetonitrile and formic acid (FA) were purchased from Sigma-Aldrich, St. Louis, Missouri, US. N-glycanase and MS-friendly deglycosylation (Tris) Buffer was purchased from Prozyme, Hayward, CA, USA). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22 µm membrane point-of-use cartridge (Millipak).

Sample preparation

Both samples (mAb and ADC) were deglycosylated by adding 25 mU of N-glycanase to 100 µL of 1 mg/mL protein solution (in Tris), and incubated overnight at 37 °C.

Column preparation

Agilent Bio SEC-3 columns are shipped in 0.1 M sodium phosphate buffer, pH 7.0. Sodium phosphate buffer is not compatible with MS analyses as it is a nonvolatile salt. The column was extensively flushed with mobile phase for approximately 48 hours before LC/MS analysis.

Table 1. Chromatographic and MS conditions.

Parameter	Condition
Mobile phase	49 % Water, 50 % ACN, 1 % FA
Flow rate	0.5 mL/min
Stop time	25 minutes
Needle wash mode	Standard wash
Injection volume	5 µL
Column temperature	30 °C
Diode array detection	280 nm/ 4 nm, Ref.: OFF > 0.025 minutes (0.5 seconds response time) (10 Hz)
MS detection	
Acquisition mode MS1	
Minimum range	100 *m/z*
Maximum range	7,000 *m/z*
Ion polarity	Positive
Source parameters	
Gas temperature	290 °C
Gas flow	14 L/min
Nebulizer	20 psig
Sheath gas temperature	400 °C
Sheath gas flow	12 L/min
Scan source parameters	
Vcap	5,000 V
Nozzle voltage	2,000 V
Fragmentor	500 V
Skimmer 1	0
Octopole RF peak	750
Reference masses	922.0098, 1,821.9523

Results and Discussion

Typically, SEC of mAbs is carried out in a physiological buffer such as phosphate buffered saline (PBS) with a pH of approximately 7.4. The buffer maintains the native structure of the protein and reduces undesired binding of the proteins to the column. However, PBS is incompatible with MS analysis. Instead, for the SEC/MS analysis, a 1260 Infinity II Bio-inert LC was used together with a solvent mixture of 50 % ACN, 49 % water, and 1 % FA. Although a denaturing solvent was used for the analysis, a highly reproducible analysis was possible, resulting in minimal relative standard deviations of 0.012 % for the retention time and 0.486 % for area precision (Figure 2).

The SEC/MS analyses of intact and deglycosylated Trastuzumab are displayed as deconvoluted spectra in Figure 3. The insets show the Gaussian distribution of the charge state envelope for the mAbs. The spectra were deconvoluted using the maximum entropy deconvolution algorithm in Agilent MassHunter BioConfirm software. The deconvoluted spectrum of the intact Trastuzumab (Figure 3A) shows multiple mass peaks corresponding to different glycoforms of the mAb. As a comparison, Figure 3B shows the deconvoluted spectrum of the deglycosylated Trastuzumab, observed as a single main peak with a mass of 145,173 Da. Using SEC before MS analysis, adduct peaks (which would arise from buffer salts when performing static infusion experiments) are minimized[7].

Mass spectra of ADCs are even more complex due to the combination of both glycosylation and drug loading heterogeneities, which pose a challenge for interpretation (Figure 4). Therefore, the ADC was deglycosylated prior to further SEC/MS analysis to reduce the spectrum complexity.

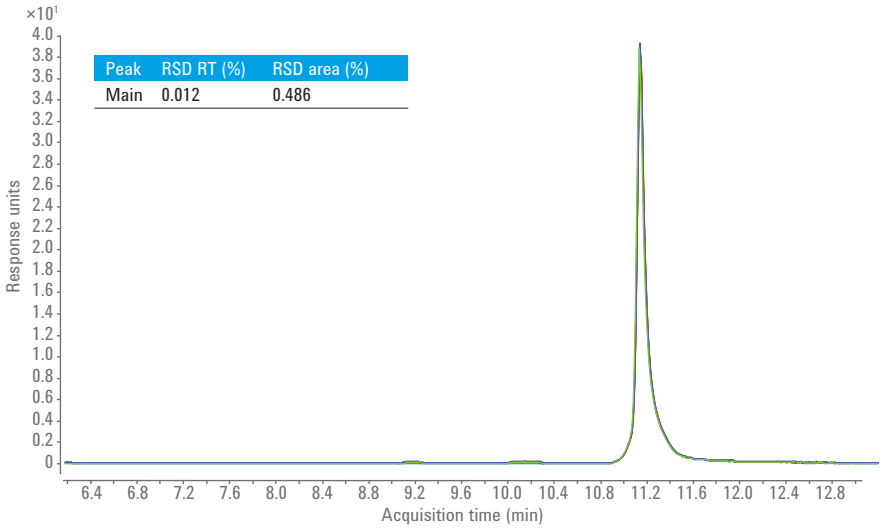

Figure 2. Overlay of seven consecutive SEC/UV runs of T-DM1 at 280 nm.

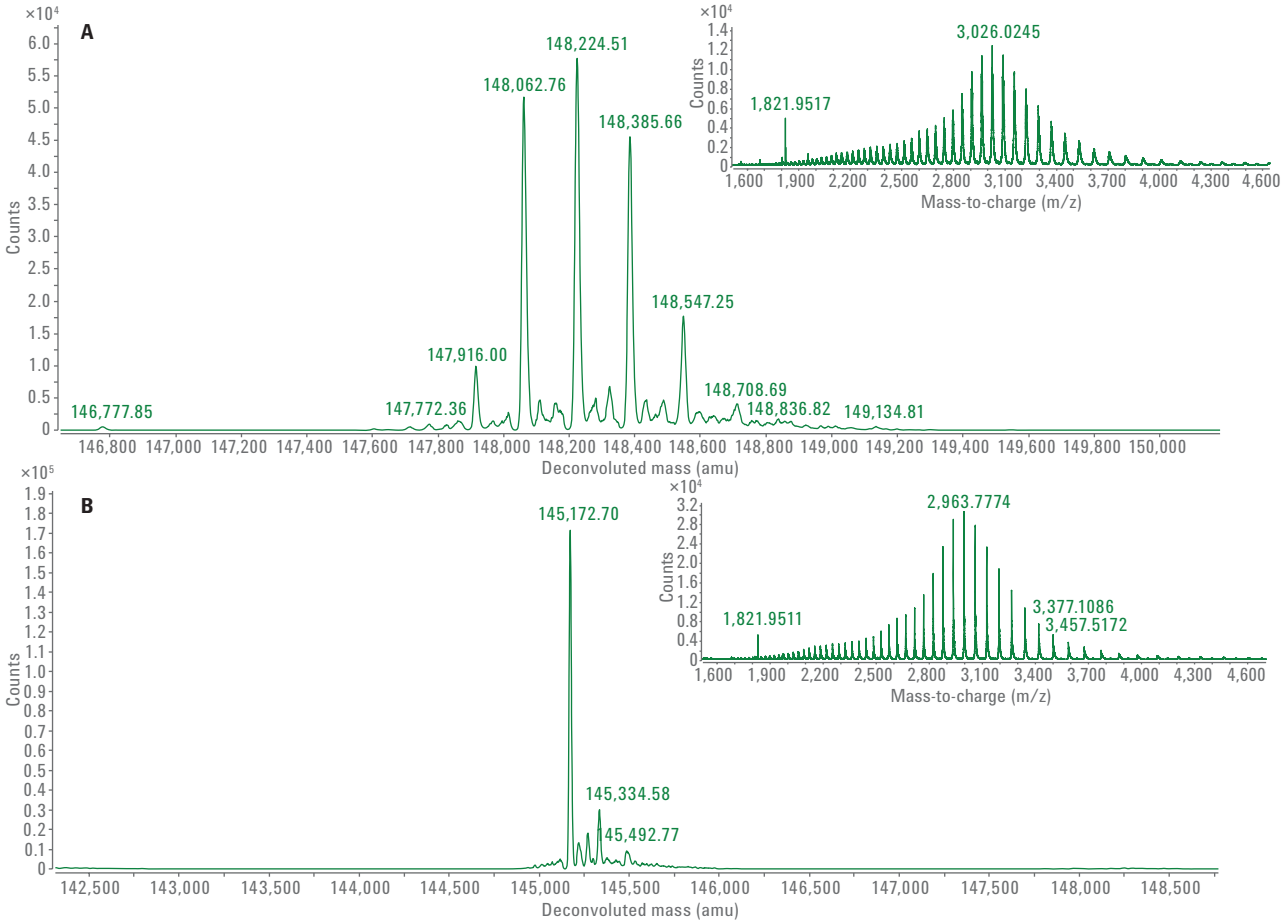

Figure 3. A) Deconvoluted spectrum of intact Trastuzumab. B) Deconvoluted spectrum of deglycosylated Trastuzumab. The insets show the raw mass spectra of the analyte.

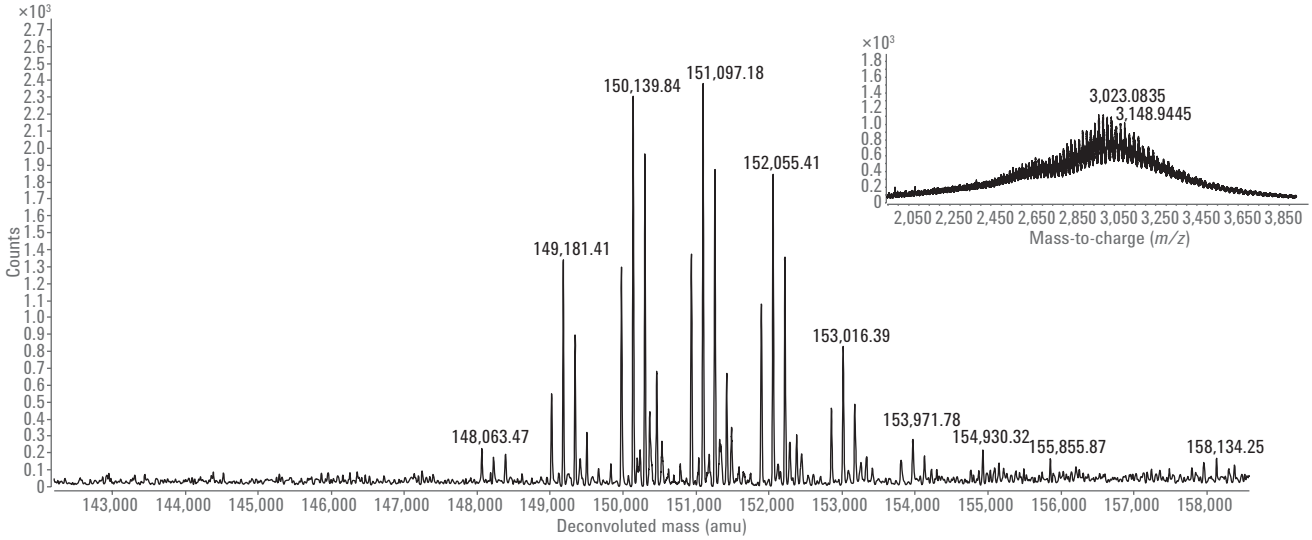

Figure 4. Deconvoluted spectrum of T-DM1. The inset shows the raw mass spectra of the analyte.

The SEC/MS analysis of the deglycosylated T-DM1 is shown in Figure 5A (raw mass spectrum) and Figure 5B (deconvoluted spectrum). The spectrum was also deconvoluted using the maximum entropy algorithm in MassHunter BioConfirm software. The spectrum of the deglycosylated ADC is much easier to interpret, and the resulting deconvolution (Figure 5B) is considerably simpler. The ADC spectrum is composed of nine equally spaced groups of peaks, where the masses of the most intense peaks are separated by 958 Da, on average. This mass corresponds to the mass of one DM1 drug (738.3 Da) and one MCC linker (219.7 Da), representing the drug load species[11]. The first peak in the deconvoluted ADC spectrum matches the mass of the deglycosylated Trastuzamab in Figure 3B (145173 Da). Therefore, D0 (Trastuzumab with zero drug load) can be assigned to the first peak of the spectrum.

The following peaks represent species with DARs ranging from 0 to 8. A second minor series of peaks, marked with an asterisk, is separated from the main peaks with a mass approximately 221.7 Da higher. These peaks can be assigned to species with an attached linker but without conjugated DM1, as shown by Marcoux *et al.*[9].

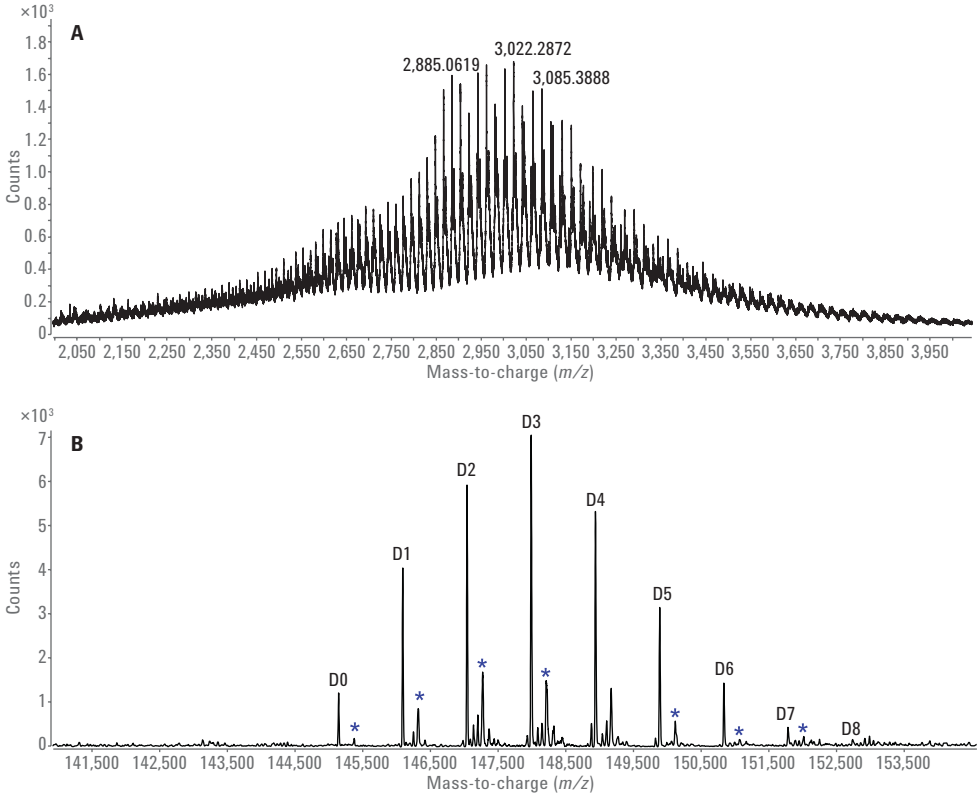

Figure 5. SEC-MS analysis of the deglycosylated T-DM1 A) raw mass spectrum and B) deconvoluted spectrum representing DAR species D0 to D8 plus satellite peaks (marked with an asterisk) representing Trastuzumab + linker signals.

The deconvoluted spectra were then exported as a .csv file, and imported into the Agilent DAR calculator. After inputting/selecting D0 mass and drug/linker mass, the DAR calculator automatically selects, annotates, and integrates mass peak groups of ADCs with various drug loadings. It then calculates the average DAR, and generates a peak list table[11]. The average DAR was calculated as approximately 3.1, matching previously determined values for this molecule[12]. Figure 6 shows a representative graph of the DAR calculation of the deglycosylated ADC. Figure 7 displays the DAR peak list ranging from DAR 0 to DAR 8 together with the masses, area, and area % from which the average DAR of 3.1 was calculated.

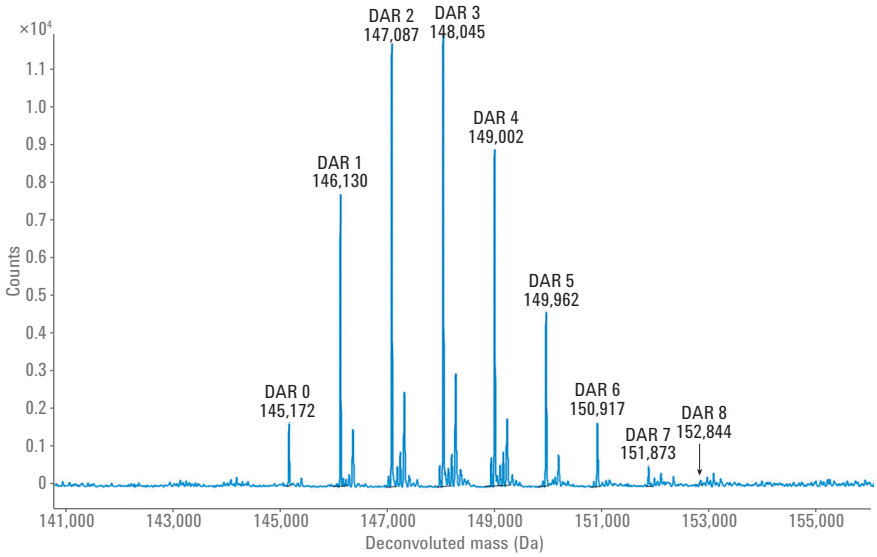

Figure 6. DAR calculation of the deglycosylated T-DM1 with the Agilent DAR calculator, resulting in an average DAR of 3.1.

Conclusions

SEC/MS using MS-compatible buffer (solvent mix of 50 % ACN, 49 % water, and 1 % FA) was performed for the analysis of Trastuzumab and T-DM1 on the Agilent 1260 Infinity II Bio-inert LC with Q-TOF detection. SEC prior to MS was useful because mAbs and ADCs can easily be desalted before introduction into the MS ion source. The spectrum of Trastuzumab revealed multiple mass peaks corresponding to different glycoforms of the mAb. The spectrum of T-DM1 revealed a highly complex peak pattern due to both glycosylation and drug loading heterogeneities. Deglycosylation of Trastuzumab and T-DM1 permitted a more facile interpretation of the data. The deconvolution of the deglycosylated T-DM1 revealed nine equally spaced peaks that were assigned to nine versions of Trastuzumab with zero to eight molecules of DM1 attached. The average DAR of 3.1 was calculated using the Agilent DAR calculator.

DAR Peak List

	DAR Peak	Start Mass (Da)	End Mass (Da)	Area	% Area
☑	0	145135	145212	2.87E+004	2.88
☑	1	145996	146267	1.21E+005	12.09
☑	2	146984	147165	2.07E+005	20.69
☑	3	147926	148180	2.34E+005	23.43
☑	4	148831	149295	2.59E+005	25.93
☑	5	149807	150005	9.20E+004	9.21
☑	6	150787	150969	4.06E+004	4.07
☑	7	151831	151952	1.37E+004	1.37
☑	8	152818	152869	3.31E+003	0.33

Figure 7. DAR Peak List, screenshot from the Agilent DAR calculator.

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