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Characterization of Antibody-Drug Conjugates Using 2D-LC and Native MS

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Abstract

Antibody drug conjugates (ADCs), which comprise a monoclonal antibody (mAb) conjugated to a small molecule drug through synthetic linkers, have been a fast-growing class of effective biotherapeutics in recent years. The ratio of the conjugated drug to mAb (drug-to-antibody ratio or DAR) is one of the critical quality attributes (CQAs) for ADC development because it can affect efficacy and safety. 2D-LC/MS is commonly used to characterize ADC molecules. This approach uses a hydrophobic interaction chromatography (HIC) column and a reversed-phase (RP) column coupled to a mass spectrometer. However, many ADC products degrade under the organic and acidic solvent conditions.

This application note demonstrates a novel 2D-LC approach to overcome this obstacle. The method uses HIC, multiple heart-cutting (MHC), and subsequent desalting and separation using size exclusion chromatography (SEC) online with native MS analysis. This workflow uses an Agilent 1290 Infinity II 2D-LC system, Agilent 6545XT AdvanceBio LC/Q-TOF, Agilent MassHunter Workstation 11.0 software, and Agilent MassHunter BioConfirm 11.0 software.

Introduction

ADCs are considered a new class of biotherapeutics that demonstrate promising results towards cancer treatment. Unlike the classical mAbs of human therapeutics, ADCs show excellent results in antigen-specific selectivity and efficiency because these ADCs are monoclonal antibodies to which a biologically active cytotoxic small molecule drug is chemically linked.[1] The conjugation types of the first two successful ADCs are through lysine side chains (Kadcyla, T-DM1) and cysteine residues (brentuximab vedotin, trade name Adcetris by Takeda) of mAbs. Adcetris comprises the partially reduced mAb conjugated through the free thiol groups of cysteine residues to the small drug molecule (monomethyl auristatin E, MMAE). A mixture of zero, two, four, six, and eight drugs per antibody is commonly observed (Figure 1). The average number of drugs conjugated to the mAb (DARs) is one of the most important critical quality attributes of an ADC because it can directly affect safety and efficacy.

Although the characterization of ADCs and determination of DAR values seem straightforward and analytically feasible, ADCs are substantially more complex than either "unconjugated" mAbs or small drug molecules. This complexity is due to their combined chemical linker and the heterogeneous distribution of conjugation sites. Therefore, ADC characterization and stability (or DAR value) monitoring can be challenging.
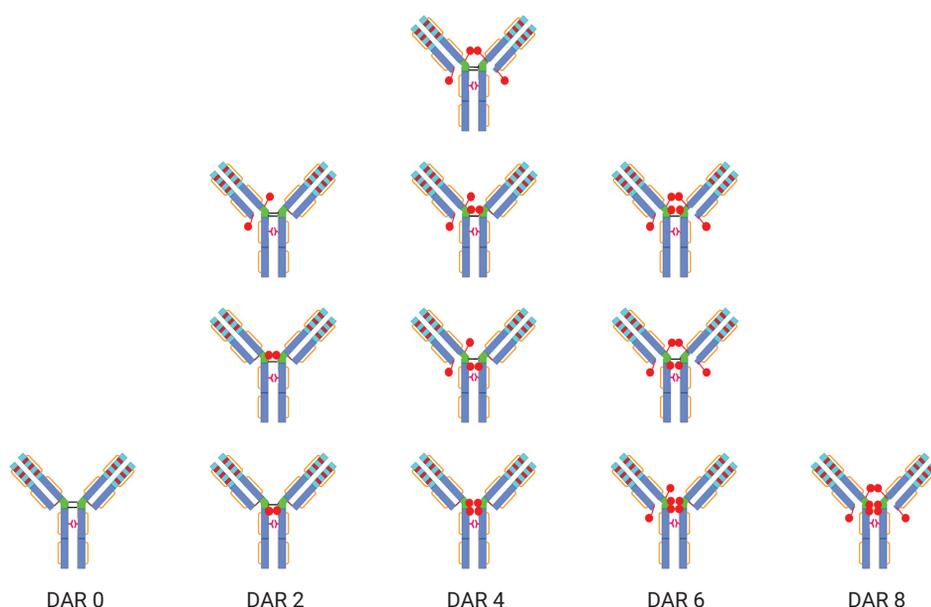
Figure 1. Various drug distributions in cysteine-linked ADCs (brentuximab vedotin).

Analysis of ADCs to characterize chemical stability issues and determine DAR values traditionally can be accomplished by various analytical techniques, such as UV-Vis, fluorescence, DSC, and SEC followed by MALS detection. Recently, HIC has become an essential technique for confirmation of DAR values for cysteine-conjugated ADCs.[2] The small drug molecules that chemically linked to the antibody are often hydrophobic. Therefore, the overall hydrophobicity of the ADC increases as its DAR value becomes larger, making HIC the perfect tool for DAR monitoring. A downside of HIC is that it separates proteins by running high concentration of a nonvolatile salt buffer, which is incompatible with the subsequent MS analysis. To overcome this analytical obstacle, a 2D-LC solution with an RP column in the second dimension was developed. In this online 2D-LC/MS approach, the protein peaks of interest (ADCs with various DAR values) can be selected from the first dimension HIC separation using either MHC or high-resolution mode. Subsequent desalting and separation occur by the second dimension RP column before MS analysis.

The new MassHunter Workstation 11.0 software now controls both the 2D-LC and MS in the same software. One comprehensive software makes multidimensional experiments, such as the analysis of ADCs with both

chromatographic separation and subsequent desalting before MS detection, easier to perform. Most of the 2D-LC acquisition capabilities from the Agilent ChemStation software are now possible in MassHunter including MHC and comprehensive 2D-LC, as well as both time- and peak-based 2D-LC. This software release also includes some new features such as a new multi-inject method with high-resolution sampling and post-acquisition file splitting for MHC workflows. The multi-inject method allows users to collect several fractions across a broad peak from the first dimension and, instead of injecting each fraction individually as with the high-resolution method, they can all be injected together for a single second dimension analysis. The multi-inject method increases the amount of sample that can be transferred from the first to second dimension without requiring

hardware changes and reduces the total run time considering the decreased number of needed second dimension runs. Now that the 2D-LC and MS are controlled by the same software, only one Agilent MassHunter .d data file is produced. For MHC experiments this data file strings together all the individual second dimension runs in the order they were acquired. A new file splitter utility splits this data file into individual second dimension LC/MS .d files and names them according to the order the cuts were made in the first LC dimension. The files are split immediately after the acquisition is complete, which streamlines the process for the user. Now that the 2D-LC data file has been split into essentially 1D-LC/MS data files, many data analysis workflows are possible.

While the 2D-LC (HIC + RP) experiments

were successful on various ADCs' separation and the subsequent desalting, major degradation from the drug payload (MMAD) and denaturation of mAbs were observed. It has been confirmed that the degradation of the ADC was caused by mainly using organic and acidic solvents in the second dimensional RP chromatography.

In this study, a novel native 2D-LC/MS analytical method for characterization of native intact brentuximab vedotin and the accurate determination of DAR values was developed. This workflow features the 1290 Infinity II 2D-LC system using various Agilent AdvanceBio columns (AdvanceBio HIC and AdvanceBio SEC) for sample separation, and the 6545XT AdvanceBio LC/Q-TOF with the large molecule SWARM autotune feature and extended mass range of up to *m/z* 30,000 for native MS analysis (Figure 2).

Separation > **Detection** > **Data analysis**

Agilent AdvanceBio HIC (first dimension)

Agilent AdvanceBio SEC (second dimension)

Agilent 1290 Infinity II 2D-LC system

Agilent 6545XT AdvanceBio LC/Q-TOF with Agilent MassHunter Acquisition 11.0 software

Agilent MassHunter Qualitative Analysis 10.0 and Agilent MassHunter BioConfirm 11.0 software
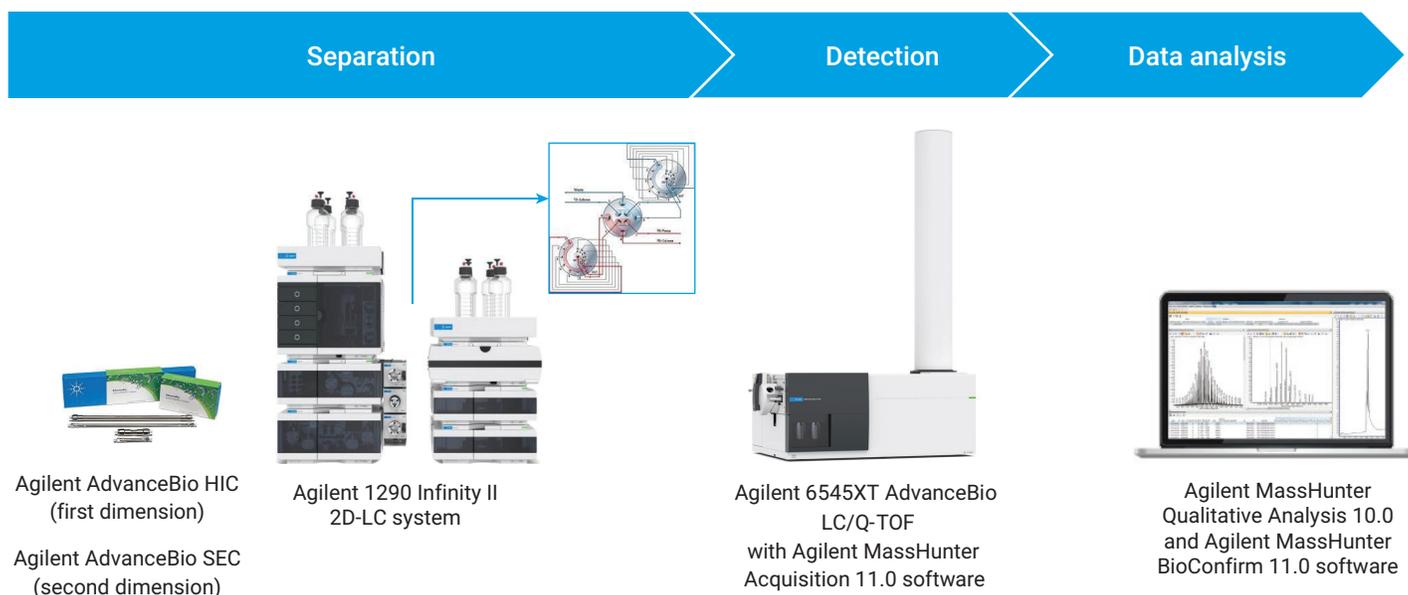
Figure 2. Analytical components of the 2D-LC and native MS protein analysis workflow.

Experimental

Materials and methods
Ammonium sulfate, sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, and ammonium acetate were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

ADC sample
Lyophilized brentuximab vedotin was dissolved in solvent A to 50 mg/mL. Approximately 10 to 20 µL of samples were injected for each 2D-LC/MS analysis.

Note: As the formulated Adcetris contains many adjuvants, this concentration is not purely the protein or ADC concentration. It rather represents the total concentration of all components in the ADC drug sample.

First dimension (HIC) buffer preparation
- **Buffer A:** 2 M ammonium sulfate in 100 mM phosphate buffer at pH 7
- **Buffer B:** 100 mM phosphate buffer at pH 7 + 25% isopropanol

To make 2 L of 100 mM phosphate buffer at pH 7, 11.68 g of sodium phosphate monobasic monohydrate and 30.94 g of sodium phosphate dibasic heptahydrate were used. DI water was added to a total volume of 2 L, and the solution was adjusted pH to 7. For buffer A, 264.28 g of ammonium sulfate was added to 1 L bottle and 100 mM of phosphate buffer was added for a total volume of 1 L. To make buffer B, 250 mL of isopropanol and 750 mL of phosphate buffer was used.

Instrumentation
Agilent 1290 Infinity II 2D-LC, including:

- Two Agilent 1290 Infinity II High-Speed Pumps (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) with Agilent Infinity II Sample Cooler (Option #100)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 1290 Infinity II DAD (G7117A)
- Agilent 1290 Infinity II DAD (G7117B)
- Agilent 1290 Infinity Valve Drive (G1170A) with Agilent InfinityLab 2D-LC ASM Valve, 1300 bar (part number 5067-4266)
- Two Agilent 1290 Infinity Valve Drives (G1170A) with Agilent Multiple Heart-Cutting Valves (G4242-64000) equipped with 40 µL loops
- Agilent 1290 Infinity Valve Drive (G1170A) with diverter valve 2 position/6 port ultrahigh pressure valve head, 1200 bar (part number 5067-4241)
- Agilent 6545XT AdvanceBio LC/QTOF system with Agilent Dual Jet Stream ESI source

Columns
- First dimension: Agilent AdvanceBio HIC, 4.6 × 100 mm, 3.5 µm (part number 685975-908)
- Second dimension (denatured condition): Agilent PLRP-S 1000Å, 2.1 × 50 mm, 5 µm (part number PL1912-1502)
- Second dimension (native condition): Agilent AdvanceBio SEC 200Å, 4.6 × 300 mm, 1.9 µm (part number PL1580-5201)

2D-LC/MS analysis

2D-LC/MS analyses were conducted on a 1290 Infinity II 2D-LC system coupled with a 6545XT AdvanceBio LC/Q-TOF that was equipped with a Jet Stream ESI source. MassHunter Workstation for LC/TOF and LC/Q-TOF version 11.0 software was used with new features in 2D-LC (i.e., multi-injection method and file splitter utility) and the large molecule SWARM autotune feature.

The collected HIC peak fractions from the first LC dimension were injected and analyzed by the second dimension LC/MS analysis. Traditionally, the LC/MS analysis of the intact mAb or intact ADC under denaturing conditions is carried out using a traditional RP HPLC method. A PLRP-S column was used with acetonitrile as the organic solvent and 0.1% formic acid.

ADC sample characterization under the native LC/MS condition was obtained with an AdvanceBio SEC column in the second dimension and a 14-minute isocratic run using 100 mM ammonium acetate solvent.

For the second dimension AdvanceBio SEC column, which elutes the salt at the end of the run instead of at the beginning, the diverter valve was plumbed backwards. This allows the first seven minutes of the second LC dimension run to go to the MS, and the end of the run (with the salt) to go to waste. This configuration results in less salt transfer to the MS than if the diverter valve is not used.

Tables 1 and 2 list the detailed LC/MS parameters used.

Data processing

All LC/MS data files of the denatured and intact ADC were processed using MassHunter Qualitative Analysis 10.0 and BioConfirm 11.0 software.

Table 1. Liquid chromatography parameters.

First Dimension	
Column	Agilent AdvanceBio HIC, 4.6 × 100 mm, 3.5 μm (p/n 685975-908)
Thermostat	4 °C
Solvent A	2 M ammonium sulfate in 100 mM sodium phosphate buffer pH 7.0
Solvent B	100 mM sodium phosphate buffer pH 7.0 + 25% IPA
Gradient	0 to 17 min, 30 to 100% B 17 to 27 min, 100% B
Column Temperature	Room temperature
Flow Rate	0.4 mL/min
Injection Volume	20 μL of 20 to 50 mg/mL brentuximab vedotin
UV Detection	280 nm at 10 Hz
Second Dimension with Denatured Condition	
Column	Agilent PLRP-S 1000Å, 2.1 × 50 mm, 5 μm (p/n PL1912-1502)
Thermostat	4 °C
Solvent A	0.1% formic acid in DI water
Solvent B	0.1% formic acid in 100% acetonitrile
Gradient	0 to 1 min, 0 to 20% B 1 to 3 min, 20 to 50% B 3 to 4 min, 50 to 70% B
Column Temperature	60 °C
Flow Rate	0.5 mL/min
Second Dimension with Native Condition	
Column	Agilent AdvanceBio SEC 200Å, 4.6 × 300 mm, 1.9 μm (p/n PL1580-5201)
Thermostat	4 °C
Solvent A	100 mM ammonium acetate buffer pH 7.0
Isocratic Elution	0 to 14 min, 100% A
Column Temperature	Room temperature
Flow Rate	0.4 mL/min

Table 2. Denatured and native MS data acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF System		
Sample Type	ADC (Denatured)	ADC (Native MS)
Source	Agilent Jet Stream ESI	Agilent Jet Stream ESI
Drying Gas Temperature	350 °C	365 °C
Drying Gas Flow	12 L/min	12 L/min
Nebulizer	60 psig	35 psig
Sheath Gas Temperature	400 °C	300 °C
Sheath Gas Flow	11 L/min	12 L/min
Capillary Voltage	5,500 V	5,500 V
Nozzle Voltage	2,000 V	2,000 V
Fragmentor	380 V	300 V
Skimmer	140 V	220 V
Quad amu	*m/z* 500	*m/z* 3,000
Mass Range	*m/z* 100 to 10,000	*m/z* 3,000 to 10,000
Acquisition Rate	1.0 spectra/s	1.0 spectra/s
Acquisition Mode	Positive, extended (*m/z* 10,000) mass range	Positive, extended (*m/z* 10,000) mass range

Results and discussion

ADCs

This study used brentuximab vedotin as a targeted ADC for its physical and chemical stability investigation. This ADC is a cysteine-based through maleimide-thiol conjugation, which is widely used for ligand-mediated drug delivery systems. Its DAR values determination and specific degradation have also been examined in detail. In the brentuximab vedotin conjugation, various small drug molecules (monomethyl auristatin E, MMAE) are site-specifically conjugated via a protease-cleavable linker to the free thiol group of the cysteine residues in an IgG1 mAb. The overall drug distribution ranges from 0, 2, 4, 6, or 8 drug molecules per mAb.

DAR determination

HIC is a common analysis technique to separate proteins using a high to low salt gradient. As it has mild running conditions (neutral pH), the native intact ADC structures can be preserved. The relative hydrophobicity of the ADC molecule increases with more drug molecules attached to the antibody. Therefore, the HIC technique is the perfect tool to separate various ADCs and determine the DAR value.

Figure 3 shows the excellent chromatographic separation of various ADCs (D0 to D8) by HIC (first dimension) using a 25-minute LC gradient. A small portion of organic solvent (25% isopropanol) was added to solvent B to elute the D8 molecule efficiently. The HIC result enabled the direct determination of the DAR value. By integrating the peak areas of the different DAR variants, the overall DAR was calculated to be approximately 3.7 (Figure 3, inserted table), which is consistent with the expected value for brentuximab vedotin.[3]

New 2D-LC/MS system for mAb-drug conjugate characterization

Key 2D-LC/MS features:

- MassHunter Workstation 11.0 software controls both the 2D-LC and MS systems (Figure 4).

- 2D-LC acquisition program includes both multiple heart-cutting and comprehensive modes.

- The multi-inject method allows users to collect several fractions across a broad peak from the first dimension and to inject them all together for a single second dimension analysis.

- The new file splitter utility splits the single data file string into individual second dimension LC/MS .d files and names them based on where the cuts were made in the first dimension (Figure 5).

DAR	RT (min)	Peak Area	Area %	DAR Calculated
D0	5.73	694.59	6.80	0
D2	9.61	3,131.09	30.67	0.613
D4	14.69	3,746.34	36.70	1.468
D6	17.67	2,260.90	22.15	1.329
D8	20.03	375.45	3.68	0.294
	Total	10,208.37	DAR	3.704
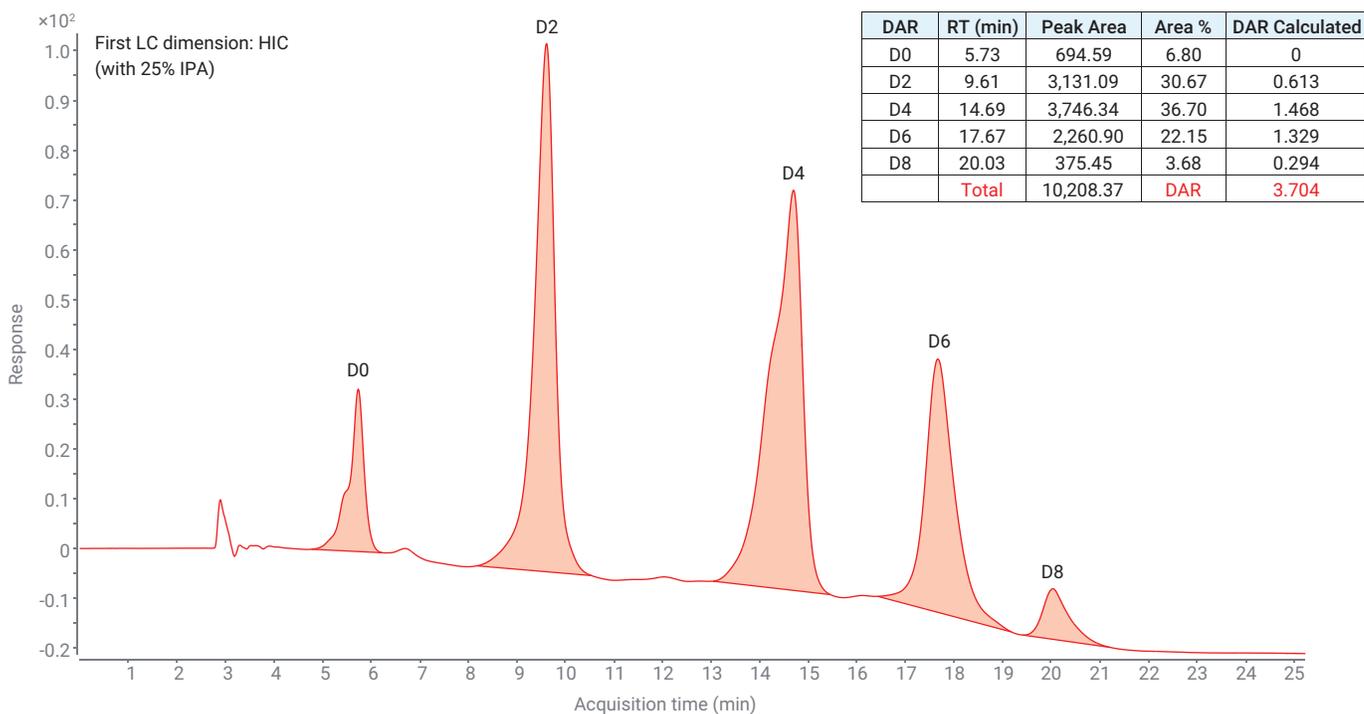
Figure 3. HIC separation and DAR determination of brentuximab vedotin on an Agilent 1290 Infinity II 2D-LC system. D0 to D8 refers to the number of drugs bound to the mAb.
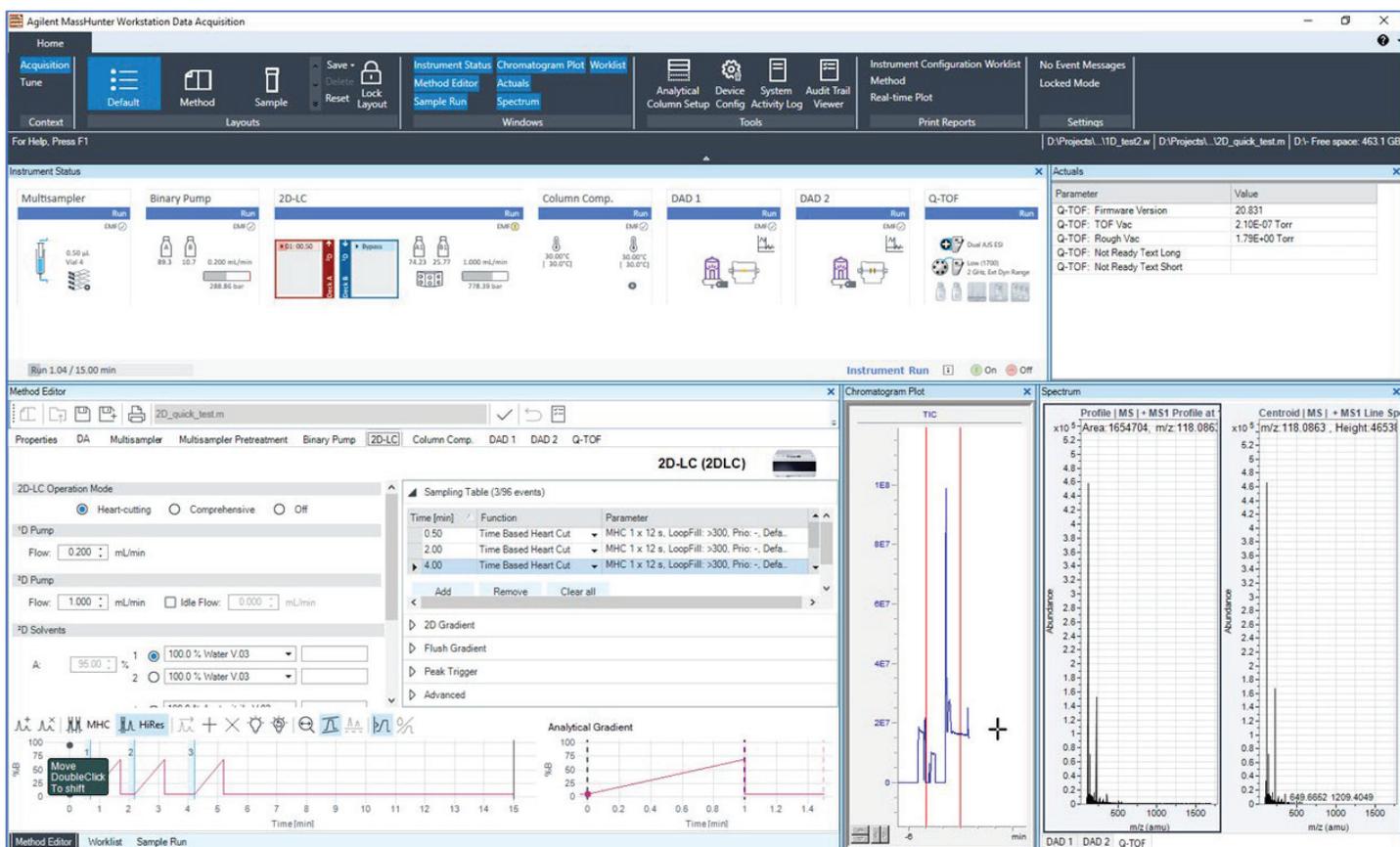
Figure 4. Screen capture of Agilent MassHunter Workstation 11.0 software demonstrates the single software platform controlling both the 2D-LC and the MS systems during sample analysis.
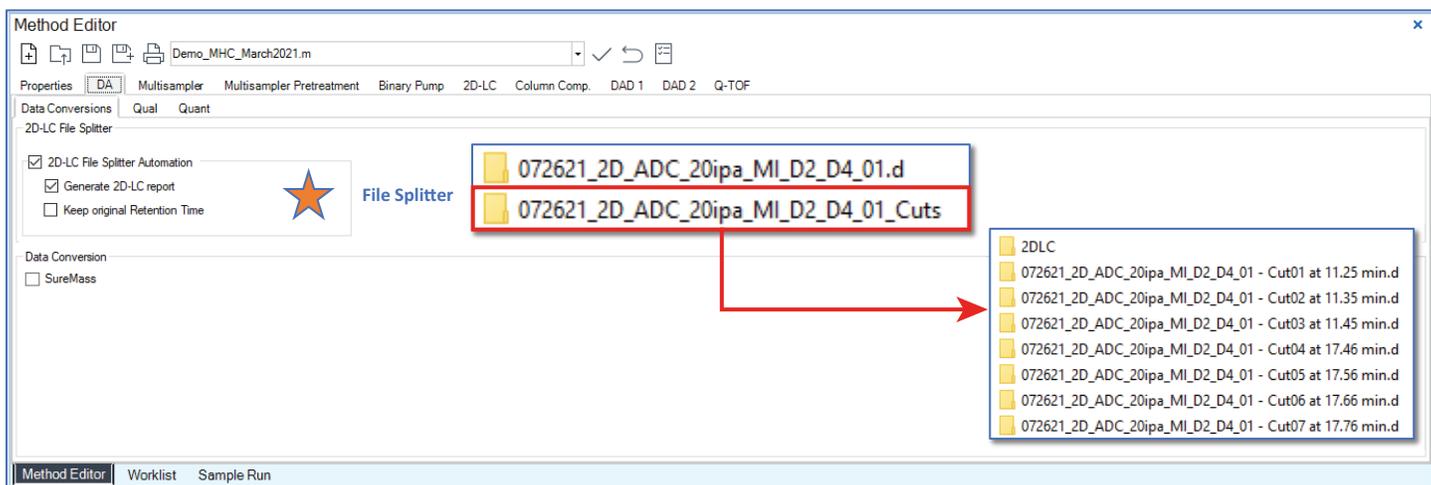
Figure 5. The file splitter utility divides the single full data file into individual second dimension LC/MS data files that are named based on when the first dimension LC cuts were made. The split first dimension LC/MS data files enable many data analysis workflows.

2D-LC/MS analysis of intact mAb-drug conjugates under denaturing conditions

To further investigate and characterize the intact ADCs, each DAR variant peak from the first dimension HIC run was collected either by MHC or high-resolution mode. These fractions were later analyzed by the second dimension RP LC/MS analysis. A PLRP-S column was used with an 8-minute gradient at a flow rate of 0.5 mL/min.

Figure 6 illustrates the LC/MS profile of the intact ADC with a DAR value of 6 (D6). Under the RP condition (organic and acidic solvents), this intact ADC became denatured with multiple peaks shown in the UV chromatogram (Figure 6, left). MS data deconvolution and mass matching to the mAb plus small drug molecules were performed on peak 1 and peak 2 using MS deconvoluted mass range from 20 to 160 kDa. This broad mass range should cover from mAb light chain to intact ADC with DAR of 8. The MS deconvolution results indicated that many degraded/reduced mAbs or ADCs were detected and identified, as shown in Figure 6 (right). These degraded molecules are: mAb light chain with one drug, half mAb with two drugs, half mAb + heavy chain + 1 drug, mAb heavy chain with three drugs, and more. Similarly, many degraded ADCs were detected in other RP peaks. These results suggested that most of the ADCs were dissociated under the denaturing LC/MS condition. In the cysteine-conjugated ADCs, the small drugs are conjugated to the mAb through its partially reduced disulfide bonds. Therefore, those previously strong disulfide bond linkages are broken, and the new ADC structures that are held together through weak electrostatic interactions become unstable, more organic/acid labile molecules. Thus, the native LC/MS analysis is needed to characterize these intact ADC molecules.
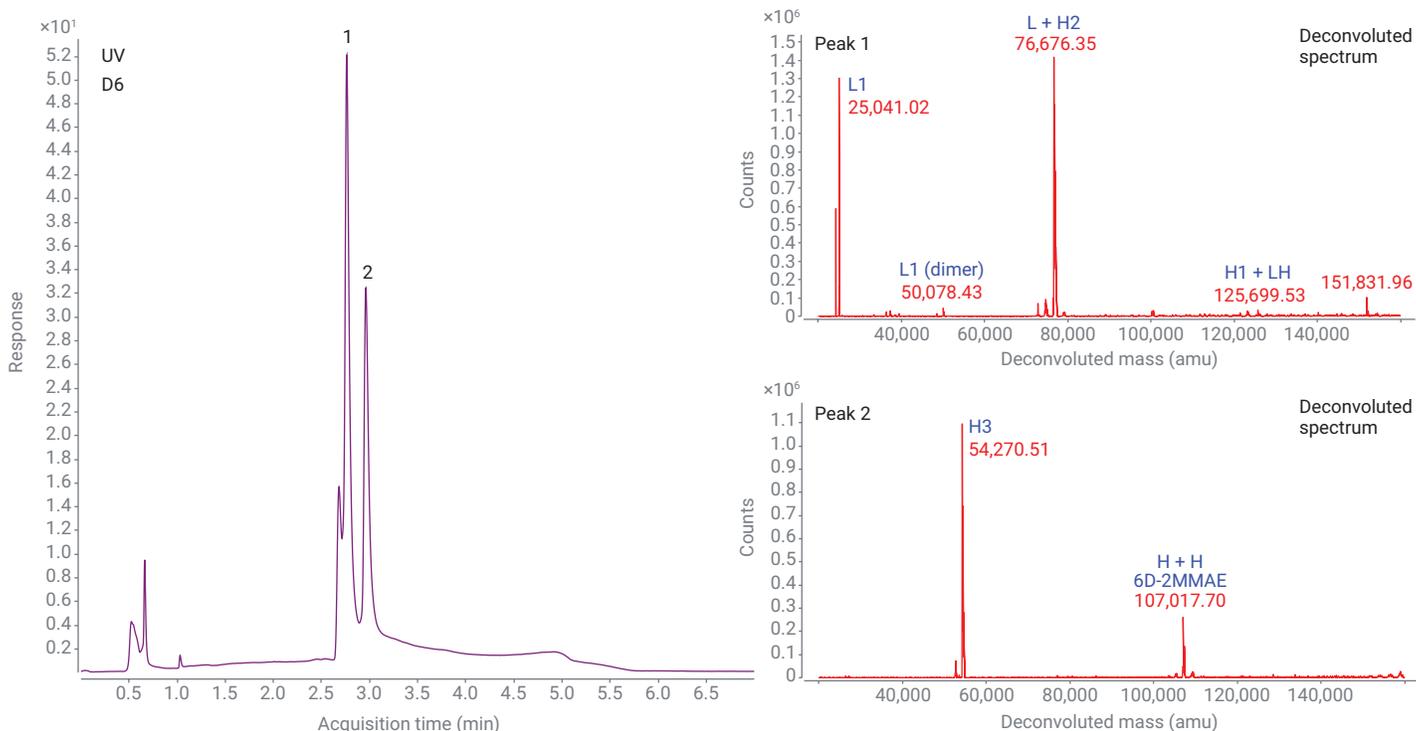
Figure 6. Second dimension liquid chromatogram (Agilent PLRP-S, UV) and deconvoluted MS spectrum of HIC separated ADC sample (D6). LC/MS analysis was performed under denaturing MS conditions. Various degraded ADC molecules were detected. L1: mAb light chain with 1 drug; L + H2: half mAb with 2 drugs; H1 + LH: half mAb + heavy chain + 1 drug; H3: mAb heavy chain with 3 drugs; H + H + 6D-2MMAE: 2 heavy chains with 6 drugs but loss of 2 MMAE molecules.

Native 2D-LC/MS analysis of intact mAb-drug conjugates

Native MS has become a widely used technique for many protein-based applications, such as protein-protein interaction, noncovalent protein complex structures, protein-ligand binding, protein folding, and antibody-drug conjugates. As no organic solvent and acid is used during the LC/MS analysis, less protein degradation is observed, and the integrity of noncovalent protein-protein complexes can be preserved.[4,5] However, major MS analytical challenges, such as lower abundance MS signals at higher *m/z* ranges and fewer charges per molecule, have been observed mainly due to the use of neutral pH solvent (100 mM ammonium acetate, pH 7) in LC/MS analysis.

This study has developed a highly sensitive native MS methodology for the analysis of various ADC samples collected from the first dimension HIC separation. This workflow uses the AdvanceBio SEC column for the second dimension online sample separation.

Figure 7 illustrates the SEC column separation of the native LC/MS analysis of an ADC (DAR 2) sample. This sample was separated from the first dimension HIC. Therefore, the sample had only a single LC peak on the second dimension AdvanceBio SEC column. Figure 7A shows the raw MS spectrum of the intact DAR 2 sample with MS charge envelope ranging from *m/z* 5,500 to 10,000 (16+ to 27+). The deconvoluted spectrum (Figure 7B) indicates that there was only ADC DAR 2 in this sample. The

zoomed-in spectrum (Figure 7B, inset) demonstrated that the 6545XT system had excellent MS resolution with all three major glycoforms separated and great MS accuracy (~10 ppm, Δm = 1.6 Da) under the native LC/MS conditions.

Similarly, excellent MS spectra quality and detection sensitivity were obtained for all DAR 0 to DAR 6 samples as shown in Figure 8. The MS deconvolution analysis on these MS data confirmed their correct DAR value assignments with excellent MS accuracies (Figure 9).

Due to the low abundance and sample degradation under higher IPA content, MS data for the DAR 8 sample could not be collected under the native LC/MS analysis.
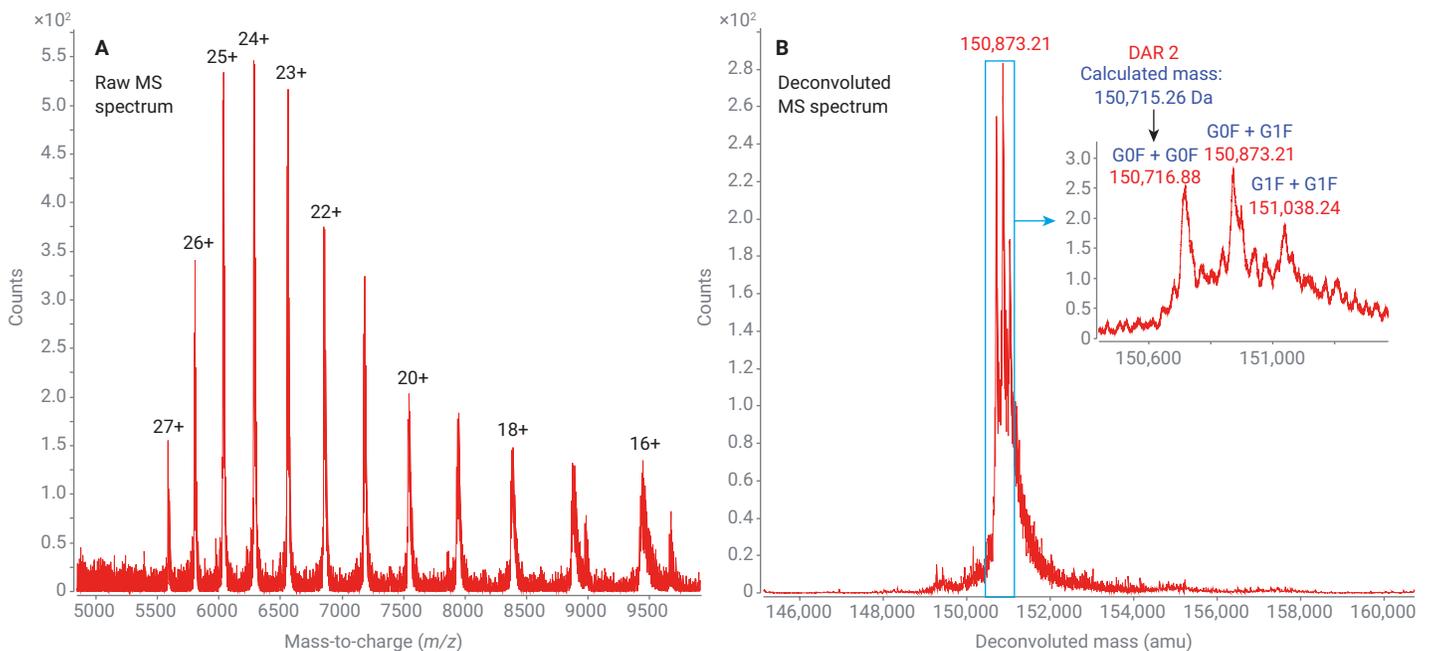
Figure 7. Native LC/MS analysis of ADC (DAR 2). (A) Raw MS spectrum of intact ADC DAR 2 under native MS condition. (B) The deconvoluted MS spectrum of intact ADC with DAR 2.
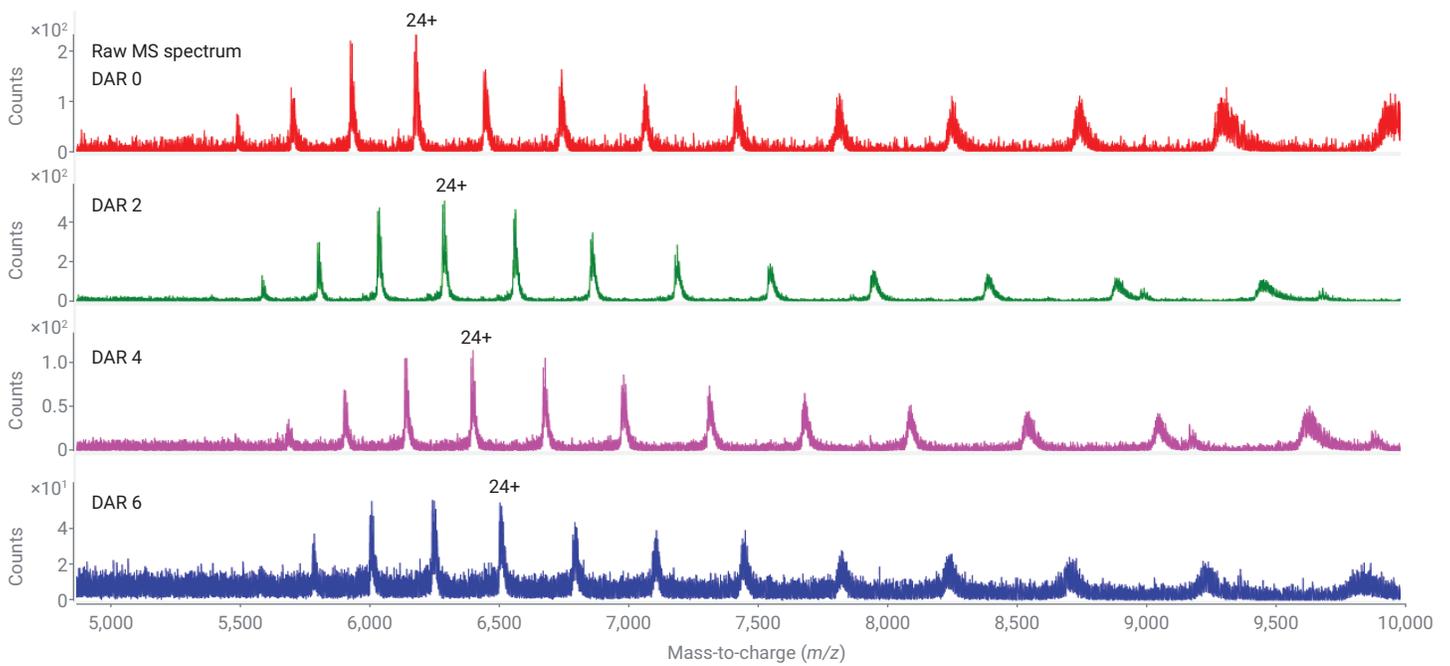
Figure 8. Raw MS spectrum of native SEC LC/MS analysis of various ADC DARs (DAR 0 to DAR 6).
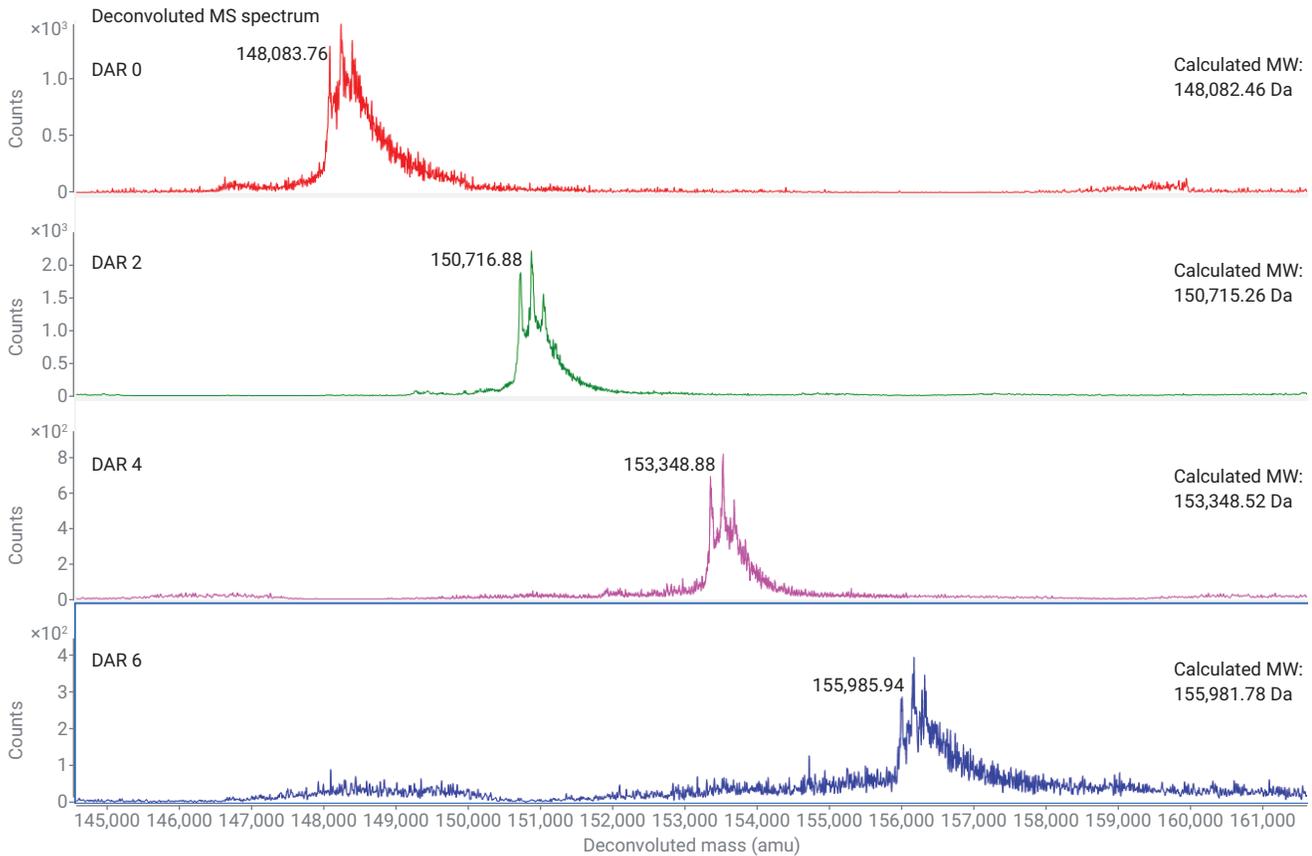
Figure 9. The deconvoluted MS spectrum of native SEC LC/MS analysis of various ADC DARs (DAR 0 to DAR 6).

Conclusion

Agilent has developed a novel 2D-LC/MS method for the characterization of various intact DARs under their native LC/MS conditions. This optimized workflow uses the Agilent 1290 Infinity II 2D-LC with the Agilent AdvanceBio HIC column, the Agilent AdvanceBio SEC column, and the Agilent 6545XT AdvanceBio LC/Q-TOF with extended mass range up to m/z 30,000. Agilent MassHunter Workstation for LC/TOF and LC/Q-TOF 11.0 and Agilent MassHunter BioConfirm 11.0 software were used for data acquisition and analysis.

This native MS analysis method not only provides the accurate average DAR value for ADC samples, but also enables excellent chromatographic separation, preserves the intact native structures, and performs the accurate intact mass determination for all ADCs with various DARs.

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