Poster Reprint

ASMS 2021
Poster number ThP003

Characterization of Antibody-Drug Conjugates (ADC) using 2-Dimension Liquid Chromatography (2D-LC) and Native Mass Spectrometric Technologies

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Introduction

Antibody drug conjugates (ADCs), a fast-growing class of biomolecules, comprise a monoclonal antibody (mAb) conjugated to a small molecule drug through synthetic linkers[1]. The ratio of the conjugated drug to mAb (drug-to-antibody ratio or DAR) is one of the critical quality attributes for ADC development because it can affect efficacy and safety. To characterize the ADC molecules, a typical 2D-LC/MS approach where Hydrophobic Interaction Chromatography (HIC) and Reversed-phase column were used. However, many degraded ADC products were detected under the organic and acid solvent conditions.

In this presentation, we present a novel 2D-LC approach to overcome this obstacle, which utilizes HIC, Multiple Heart-cutting (MHC) and subsequent desalting/separation using Size Exclusion Chromatography (SEC) online with native MS analysis.

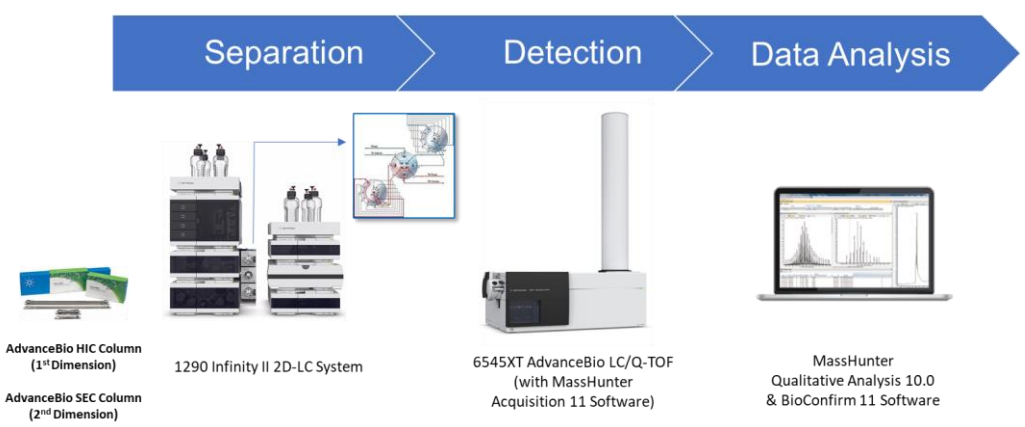


Figure 1. Analytical components of the 2D-LC and native MS protein analysis workflow.

This workflow features the Agilent 1290 Infinity II 2D-LC system utilizing various AdvanceBio columns (AdvanceBio HIC and AdvanceBio SEC) for sample separation, and the 6545XT AdvanceBio LC/Q-TOF system.

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

Experimental

Antibody-Drug Conjugates (ADC) sample

Lyophilized brentuximab vedotin was dissolved in deionized (DI) water to 50 mg/mL. Approximately 10 – 20 µL of samples were injected for each 2D-LC/MS analysis.

Instrumentation

- Agilent 1290 Infinity II 2D-LC
- Columns:
  - 1st dimension: Agilent AdvanceBio HIC column, 3.5 µm, 4.6 × 100 mm
  - 2nd dimension (Denatured condition): Agilent PLRP-S, 1000 Å, 2.1 × 50 mm, 5 µm
  - 2nd dimension (Native condition): AdvanceBio SEC (200 Å, 4.6 × 300 mm, 1.9 µm)

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

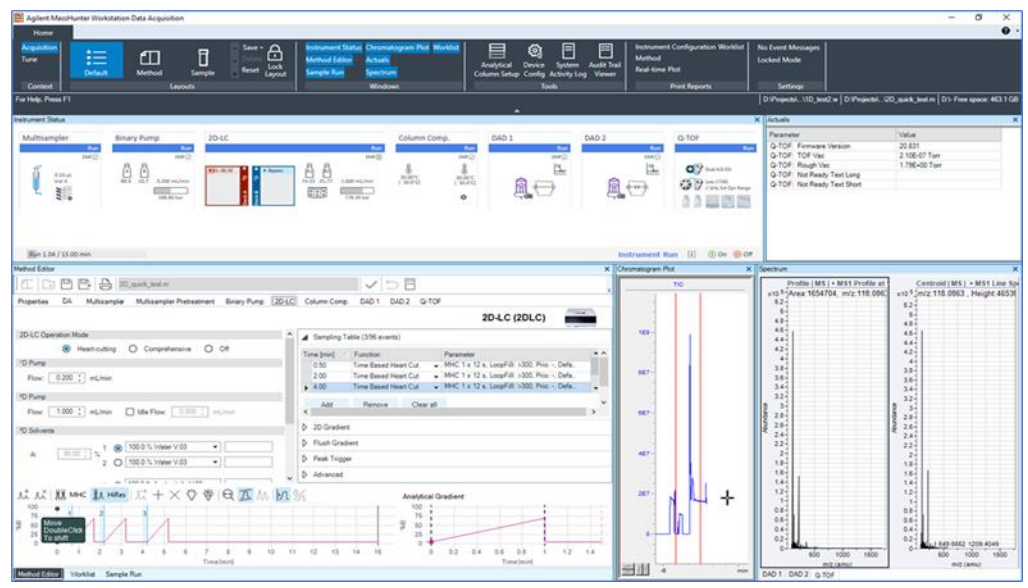


Figure 2. Screen capture of MassHunter Data Acquisition 11.0 showing the direct control of 2D-LC system coupled to the 6545XT AdvanceBio LC/Q-TOF MS system.

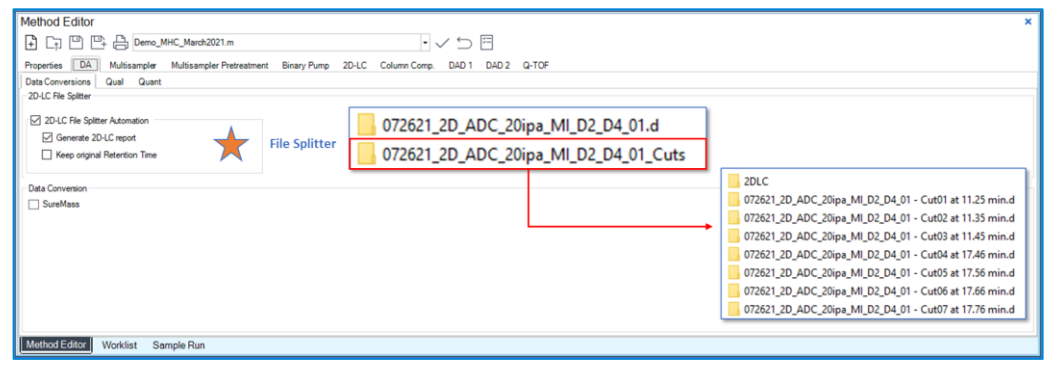


Figure 3. File Splitter is one of the unique features in MassHunter Data Acquisition 11.0. The 2D-LC data file is split into essentially 1D LC-MS data files which make many data analysis workflows possible.

2D-LC/MS Analysis of intact antibody-drug conjugates

Brentuximab vedotin 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.

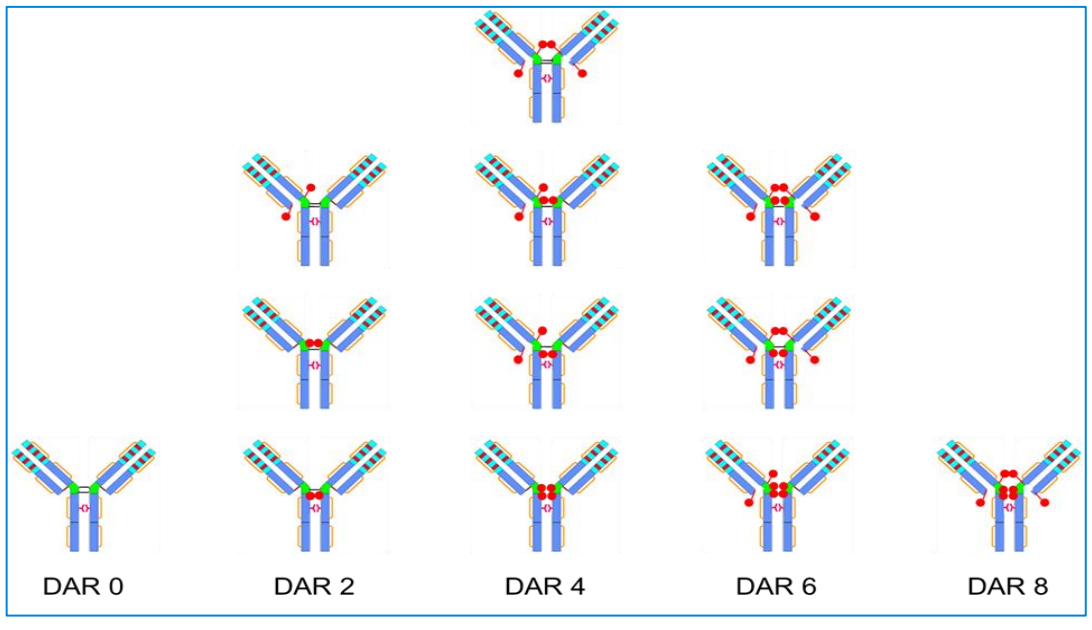
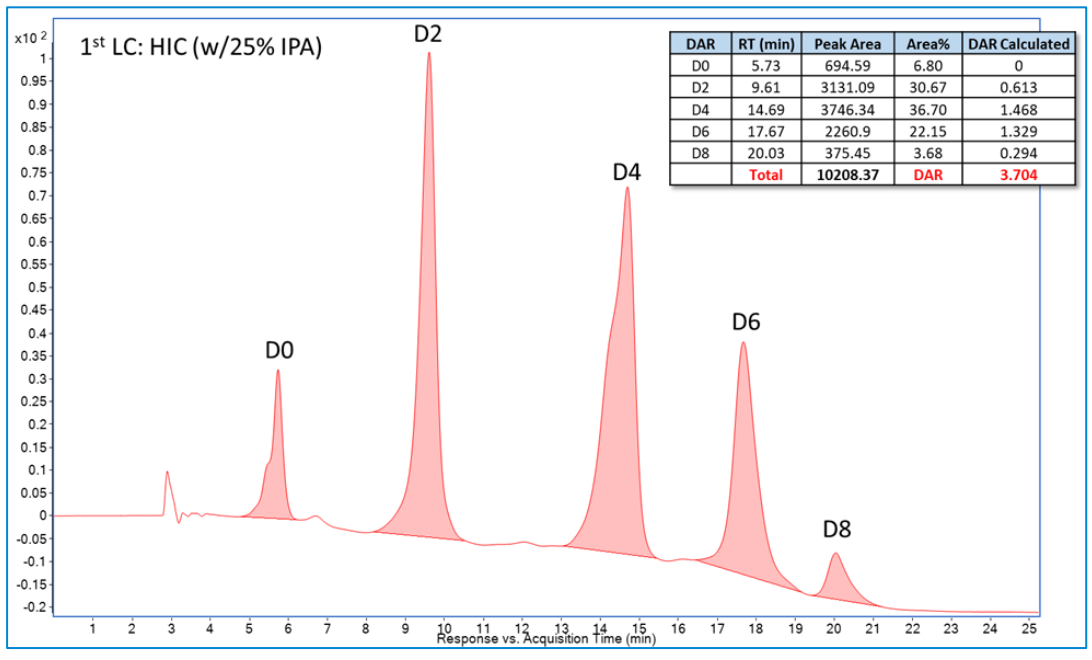


Figure 4. Various drug distribution in cysteine-linked ADCs (Brentuximab vedotin).

Drug-mAb Ratio (DAR) determination

HIC is a commonly used analysis technique to separate proteins using a high to low salt gradient. As it has very mild running condition (neutral pH), the native intact ADC structures can be preserved[2].



DAR	RT (min)	Peak Area	Area%	DAR Calculated
D0	5.73	694.59	6.80	0
D2	9.61	3131.09	30.67	0.613
D4	14.69	3746.34	36.70	1.468
D6	17.67	2260.9	22.15	1.329
D8	20.03	375.45	3.68	0.294
	Total	10208.37	DAR	3.704

Figure 5. HIC separation and DAR determination of brentuximab vedotin on Agilent 1290 Infinity II 2D-LC system. D0 to D8 refers to the number of drugs bound to mAb.

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

To further investigate and characterize the intact ADCs, each DAR variant peaks from the 1st dimension HIC run were collected either by MHC or High-Resolution mode. These fractions were subsequently analyzed by the 2nd dimension reversed-phase HPLC and mass spectrometric analysis.

Under the reversed-phase condition (organic and acidic solvents), the intact ADC became denatured with multiple peaks shown in the UV chromatogram (Figure 6, left). The MS deconvolution results indicated that many degraded/reduced mAb or ADCs were detected and identified, as shown in Figure 6 (right).

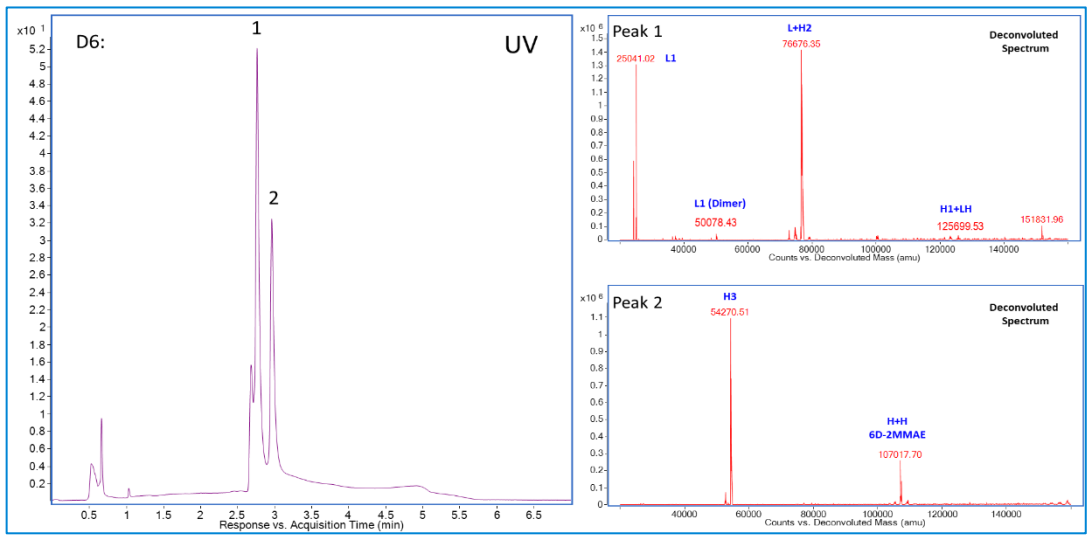


Figure 6. 2nd dimension liquid chromatogram (PLRP-S, UV) and MS deconvoluted spectrum of HIC separated ADC sample (DAR6). LC/MS analysis was performed under denaturing MS conditions. Various degraded ADC molecules were detected, L1: mAb light chain with 1 drug, L+H2: half mAb with 2 drugs, H1+LH: half mAb + light chain +1 drug, H3: mAb heavy chain with 3 drugs, and H+H+6D-2MMAE: 2 heavy chains with 6 drugs but lost of 2 MMAE molecules.

Results and Discussion

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

Native MS has become a widely used technique for a variety of protein-based applications, such as protein-protein interaction, non-covalent protein complex, protein-ligand binding, protein folding and mAb-drug conjugates[3]. As no organic solvent and acid is used during the LC/MS analysis, less protein degradation is observed, and the integrity of non-covalent protein-protein complexes can be preserved. In this study, we have developed a highly sensitive native mass spectrometric methodology for the analysis of various ADC samples collected from the 1st dimension HIC separation with Multiple-Injection Method. This workflow utilizes the AdvanceBio size exclusion chromatography (SEC) column for the 2nd-dimension online sample separation.

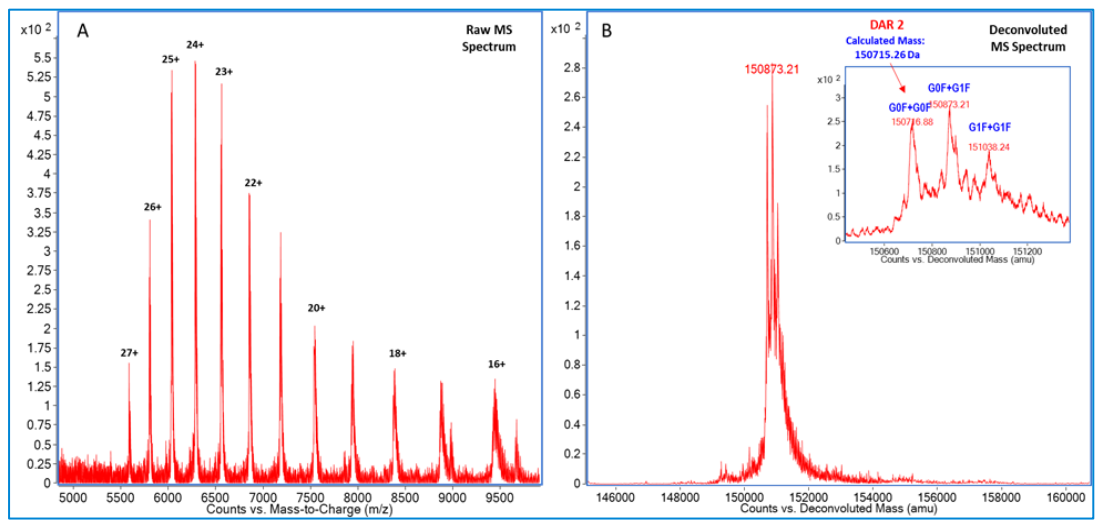


Figure 7. Native LC/MS Analysis of ADC (DAR2). A) Raw MS spectrum of intact ADC DAR2 under native MS condition. B) The deconvoluted MS spectrum of intact ADC with DAR=2.

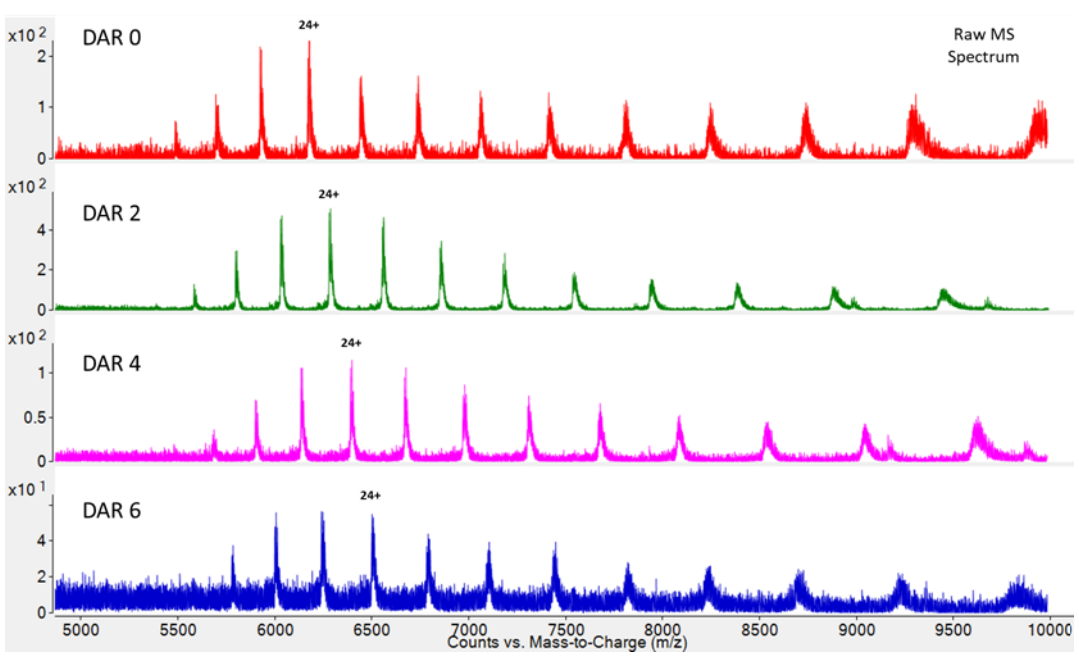


Figure 8. Raw MS spectrum of native SEC LC/MS analysis of various ADC DARs (DAR 0 to DAR 6).

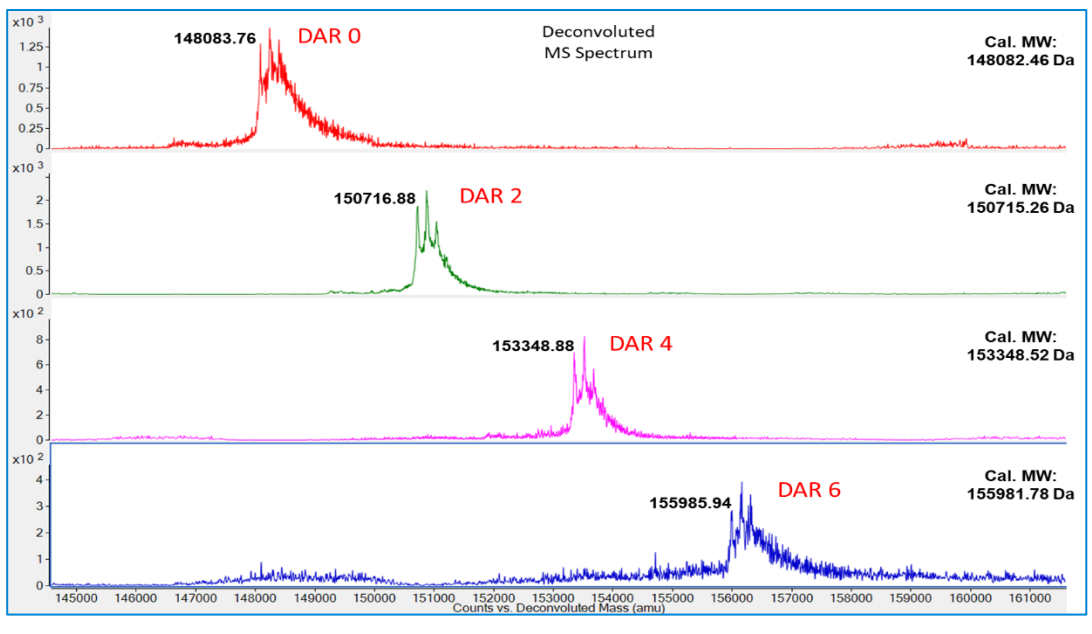


Figure 9. The deconvoluted MS spectrum of native SEC LC/MS analysis of various ADC DARs (DAR 0 to DAR 6).

Conclusions

- We have 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 1290 Infinity II 2D-LC with the AdvanceBio HIC column, the AdvanceBio SEC column, the 6545XT AdvanceBio LC/Q-TOF with extended mass range up to *m/z* 30,000, MassHunter LC/MS Data Acquisition 11.0, and MassHunter BioConfirm 11.0 software.
- This 2D-LC/native MS analysis method not only provides accurate average DAR value for the ADC sample, but also enables excellent chromatographic separation, preserves the intact native structures and performs the accurate intact mass determination for all ADCs with various DARs.

References

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Published in USA, October 20, 2021

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