Analysis of Monoclonal Antibodies

Using Multiple Heart-cutting Hydrophobic Interaction/Reversed Phase 2D-LC/MS

Application Note

Biotherapeutics & Biologics

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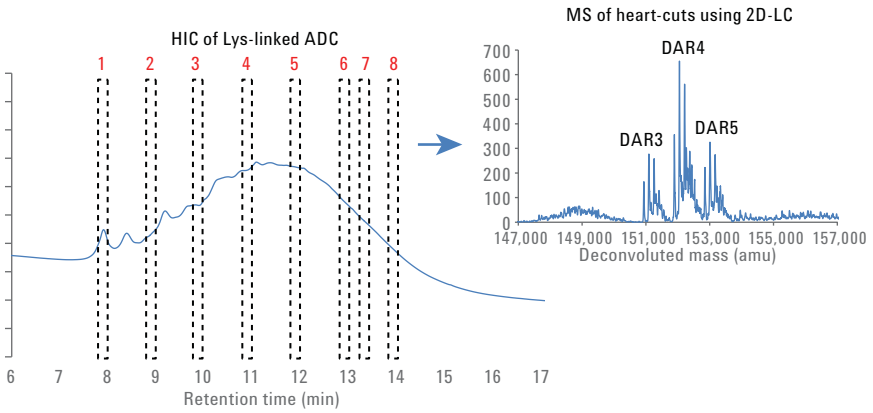
Abstract

Hydrophobic interaction chromatography (HIC) is a popular approach for both downstream processing and analytical scale analysis of monoclonal antibodies (mAbs). In the latter case, HIC is a useful characterization tool because it can often separate mAb variants, for example certain isomers or oxidized species. HIC separations are also gaining popularity for the characterization of antibody-drug conjugates (ADCs). Such investigations often benefit from mass measurement using MS, but the high salt conditions used for HIC separations are completely incompatible with ESI. In this work, we overcome this obstacle using a 2D-LC system, which affords multiple heart-cutting and subsequent desalting/separation using reversed-phase chromatography on-line with TOF MS.



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Introduction

Proteins are widely recognized for their potential as therapeutic agents, evidenced by the growing number of molecules approved in various countries for treating a diverse group of diseases. Most therapeutic proteins are produced in mammalian cell culture. As such, the final products are populations of molecules with heterogeneity reflective of the biosynthetic process. Further heterogeneity can be imparted by the formulation and storage process of the drug products. Combined, all of these factors contribute to make the physicochemical characterization of protein therapeutics a great challenge.

Monoclonal antibodies (mAbs) represent the majority of approved therapeutic proteins. Many of the performance aspects of mAbs, such as efficacy and immunogenicity, are affected by the physicochemical properties of the molecule. These features are known as critical quality attributes (CQAs), and must be measured carefully to ensure product quality. mAb CQAs can include N- and C-terminal features, disulfide bonding, glycosylation, deamidation, oxidation, and glycation. The emerging class of ADCs, consisting of small molecular weight drugs conjugated to mAbs, represents an additional layer of structural complexity that further complicates characterization efforts.

Among the many tools available to the research analyst for mAb characterization is HIC. In HIC separations, proteins interact with a weakly hydrophobic stationary phase in the presence of a high initial concentration of lyotropic or cosmotropic salts, most commonly ammonium sulfate. The concentration of ammonium sulfate is lowered as the gradient proceeds, resulting in elution of the protein from the stationary phase.

Generally, it is believed that proteins retain their native structure during HIC separation. Thus, HIC employs a much different separation mechanism than reversed-phase (RP) separations. HIC is capable of resolving protein populations that differ in their surface hydrophobicity, which can arise from common modifications such as oxidation.

A particular downside of HIC is that the high proportion of nonvolatile salt used to drive the separation makes it completely incompatible with LC/MS. This is unfortunate, as mass information for a given chromatographic peak is a very useful first step in identification. This issue can be solved using 2D-LC with a RP column in the second dimension (^{2}D). In this approach, peaks of interest can be selected from the first dimension (^{1}D) HIC separation and subsequently desalted, separated, and introduced into the LC/MS system.

The Agilent 1290 Infinity 2D-LC Solution for Multiple Heart-cutting (MHC) is an enabling tool for the situation described above. Figure 1 shows a diagram of this system. In this configuration, the ^{1}D and ^{2}D are interfaced using the 2-position/4-port-duo valve. The duo valve itself is interfaced to two 6-position/14-port valves, each plumbed with six 40-µL loops. This arrangement permits the storage of peaks from the ^{1}D for later injection onto the ^{2}D. Effectively, MHC decouples the ^{1}D and ^{2}D. This provides the user with time to perform optimal ^{2}D separation. This is advantageous for protein separations, where separation cycle times can often be longer than those used for small molecules. A detailed description of the operation of the MHC 2D-LC system, including software features, can be found in Agilent Technologies Technical Overview publication number 5991-5615EN[1].

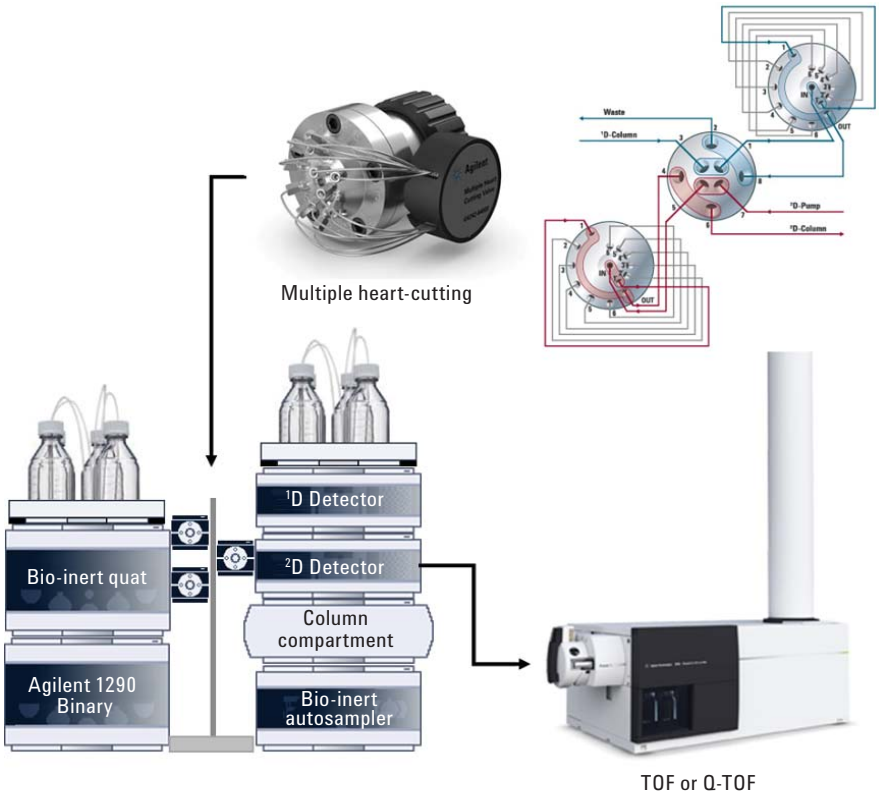

Figure 1. Diagram of the MHC 2D-LC/MS system used in the current work.

Experimental

The Agilent 1290 Infinity 2D-LC Solution was comprised of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary Pump (G5611A) in ^{1}D
- Agilent 1290 Infinity Binary Pump (G4220A) in ^{2}D
- Agilent 1260 Infinity Bio-inert High Performance Autosampler (G5667A) with an Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2-Position/4-Port Duo valve (G4236A) equipped with Multiple Heart-Cutting Valve Upgrade Kit (G4242A)
- Agilent 1290 Infinity Diode Array Detector (G4212A)

MS system

Agilent 6224 TOF LC/MS system with dual-nebulizer ESI source

Columns

- Silica-based HIC column, 4.6 × 100 mm (for 1st dimension HIC separation of mAb A and mAb B)
- Polymer-based HIC column, 4.6 × 100 mm (for 1st dimension HIC separation of mAb C)
- Agilent AdvanceBio RP-mAb C4 column, 2.1 × 50 mm (p/n 799775-904) (for 2nd dimension RP of all mAb samples)

Chromatographic conditions

^{1}D Separation				
For mAbs A and B				
Mobile phase A	50 mM sodium phosphate pH 7.0			
Mobile phase B	2 M ammonium tartrate in 50 mM sodium phosphate pH 7.0			
Gradient	Time (min)	%A	%B	
	2	25	75	
	17	100	0	
	20	100	0	
	22	25	75	
	25	25	75	
For mAb C				
Mobile phase A	50 mM sodium phosphate pH 7.0			
Mobile phase B	2 M ammonium tartrate in 50 mM sodium phosphate pH 7.0			
Mobile phase C	Isopropanol			
Gradient	Time (min)	%A	%B	%C
	0	25	75	0
	15	75	0	25
	20	75	0	25
	21	25	75	0
	25	25	75	0
^{2}D Separation				
For all mAbs				
Mobile phase A	0.5 % AcOH 0.05 % TFA in H_2O			
Mobile phase B	0.5 % AcOH 0.05 % TFA in 80:10:10 ACN:1-propanol:H_2O			
Valve and loop configuration	2-position/4-port-duo 2×6 loops (concurrent) Loop size: 40 µL			

Method parameter	Value
Autosampler temperature	4 °C
DAD	280 nm
Injection volume	1–10 µL
^{1}D Parameters	
Column temperature	25 °C
^{1}D flow rate	0.4 mL/min
^{2}D parameters	
Column temperature	80 °C
^{2}D flow rate	0.2 mL/min
2D-LC mode	Multiple heart-cutting
^{2}D gradient stop time	5.0 minutes
^{2}D cycle time	7.0 minutes
Idle flow	n/a
^{2}D gradient	Time (min) %B 2.00 28 4.00 42 4.60 50 4.61 28
Loop filling	100 %

Software

- Agilent OpenLAB CDS ChemStation Edition Rev. C.01.07 [27] with 1290 Infinity 2D-LC acquisition software product version A.01.02 [24]
- Agilent MassHunter Workstation Software, Version B.05.01, Build 5.01.5125.1

Solvents and samples

All reagents and solvent used were of the highest purity available.

Results and Discussion

An analytical platform based on the Agilent 1290 Infinity 2D-LC Solution was assembled with the goal of making mass measurements from HIC separations of therapeutic proteins. The previously described MHC kit was used to transfer any ^{1}D fraction of interest to the ^{2}D for desalting and introduction into the LC/MS system. While initial experiments were successful, MS spectra were dominated by +98 Da adducts (data not shown), which were resultant from the use of ammonium sulfate in the ^{1}D HIC separation. To mitigate this problem, ammonium tartrate was substituted for ammonium sulfate, as recently described by Xiu[2].

The performance of the HIC/RP 2D-LC/MS system was then investigated using three test cases: separation of an intact mAb, separation of a force-oxidized mAb, and separation of a Lys-linked ADC.

HIC/RP-MS of mAb A

Figure 2A shows the HIC separation of mAb A, where the main peak eluted at RT 17.5 minutes. While this peak represented the majority of the UV signal, three other peaks eluting at RTs 16.4, 16.8, and 18.9 minutes were apparent when the baseline of the chromatogram was examined more closely. Heart cuts of each of these peaks along with the main peak were taken from the ^{1}D HIC, and sent onward to the ^{2}D RP column interfaced to a TOF LC/MS system.

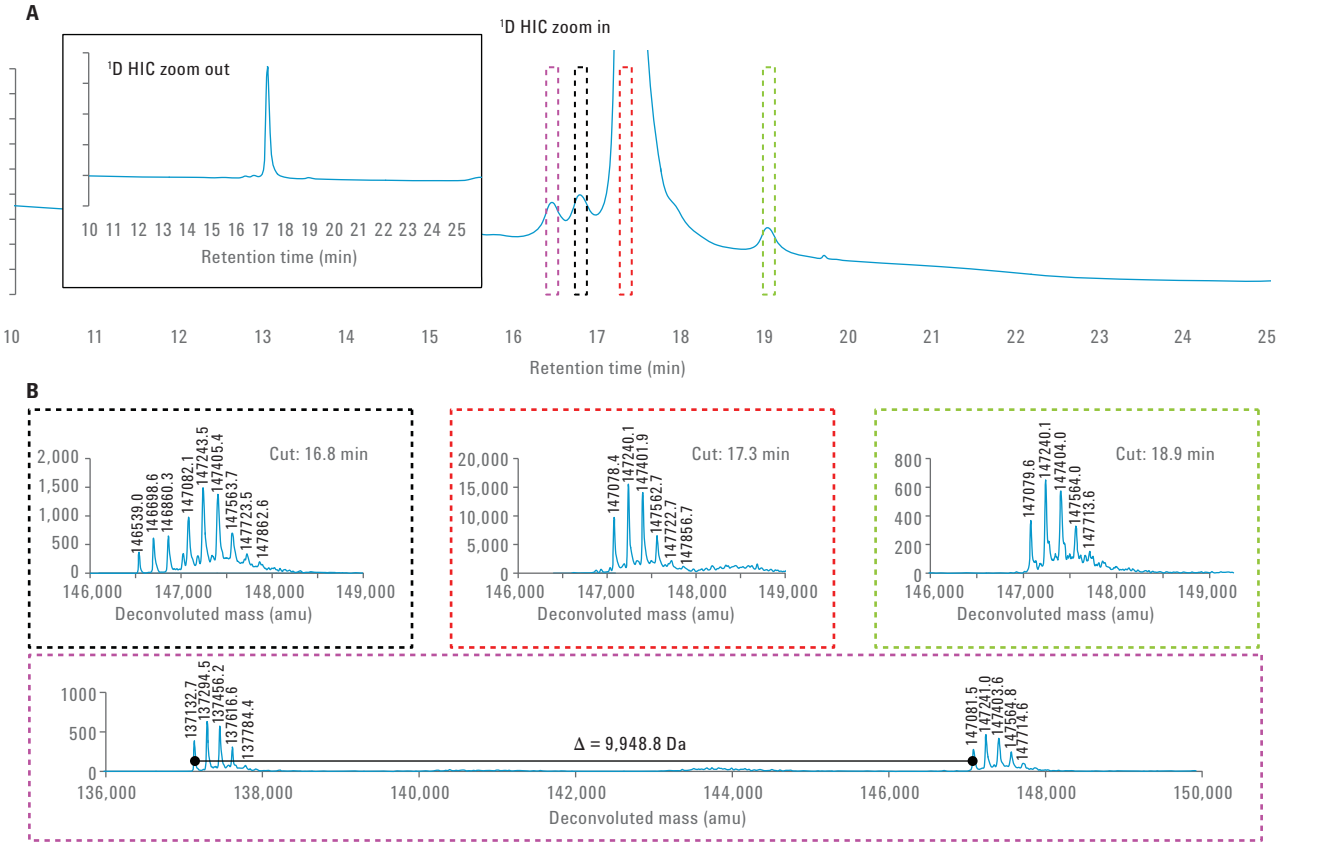

Figure 2. Analysis of mAb A using the HIC/RP 2D-LC/MS system. (A) HIC chromatogram of mAb A revealing multiple satellite peaks (see zoom in) as well as one major peak (see zoom out). (B) Heart-cuts from the first dimension at 16.4, 16.8, 17.3, and 18.9 minutes were stored, then separated on the second dimension followed by MS detection. Various impurities were detected that ranged in mass difference from the main peak.

Figure 2B shows the resultant mass spectra. The peak at RT 16.4 minutes was 9948.8 Da lower in mass as compared to the main peak. The peak at RT 16.8 minutes was also lower in mass, in this case by 539.4 Da. Finally, the peak at RT 18.9 minutes showed no mass difference from the main peak. It appears from the data that the earliest eluting peaks represent truncated variants of the mAb, while the latest eluting peak may represent an isomer having significant differences in surface hydrophobicity from the main mAb peak.

HIC/RP-MS of force-oxidized mAb B

mAb B was subject to forced oxidation using 1 % TBHP for 72 hours at 37 °C. Treatment with TBHP produced a peak that eluted 0.5 minutes earlier than the main mAb peak at 12.5 minutes, as shown in Figure 3A. Each of the two peaks was selected for mass measurement using the MHC 2D-LC/MS approach.

As expected, the peak resultant from TBHP treatment was higher in mass than the main mAb peak, as shown in Figures 3B and 3C. In this case, the mass was 64 Da higher, which is consistent with four oxidation reactions. Based on prior knowledge of the molecule, these oxidation events are likely to occur at the four methionine residues present in the mAb, which are susceptible to this modification.

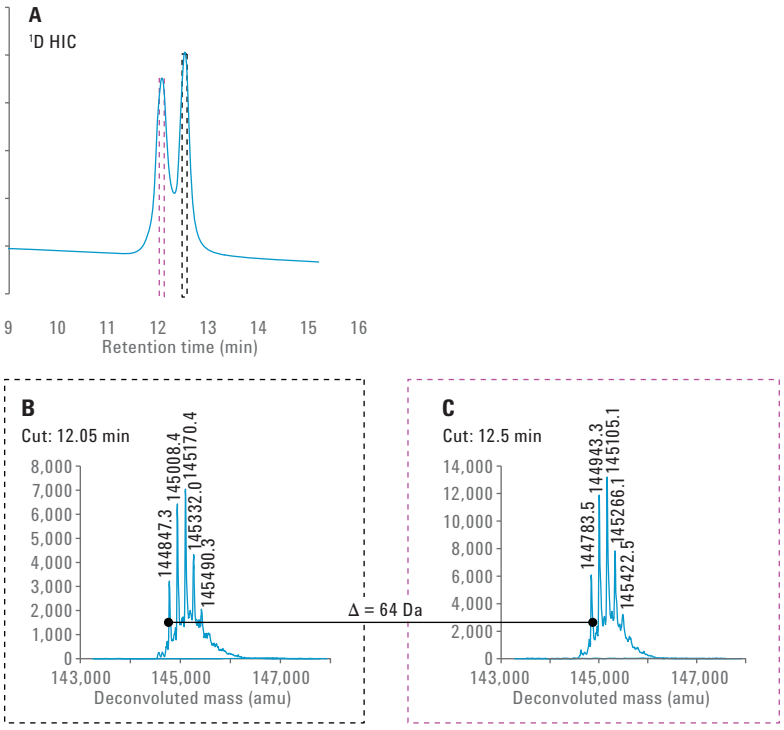

Figure 3. Analysis of mAb B using the HIC/RP 2D-LC/MS system. (A) HIC chromatogram of mAb B treated with 1 % TBHP. TBHP treatment resulted in a new peak at RT 12.05 minutes. (B) Mass measurement of the peak at 12.05 minutes. (C) Mass measurement of the peak at 12.5 minutes. The mass of the oxidized mAb was 64 Da higher than the untreated mAb, indicating four potential sites of oxidation.

HIC/RP-MS of mAb C, a Lys-linked ADC

Finally, we performed a series of mass measurements from the HIC separation of a Lys-linked ADC (mAb C). As shown in Figure 4, the HIC separation of mAb C lacks the resolution required for detailed characterization. This is typical for HIC separations of Lys-linked ADCs where small molecule drugs are linked to the dozens of Lys residues accessible on the surface of the mAb. Such ADC preparations consist of a very complex mixture of positional isomers. While HIC is often useful for determining the drug to antibody ratio (DAR) of an ADC, the present case is a clear exception.

To further investigate the HIC separation of mAb C, a total of eight heart-cuts were taken and sent to the ^{2}D for mass measurement. These heart-cuts are represented by the dashed rectangles in Figure 4 labeled 1–8. The MS results from each of these heart-cuts are shown in Figure 5. The deconvoluted mass spectra revealed that RT increases as a function of DAR, as expected. It is also evident, based on the fact that certain DAR species are present in multiple heart-cuts, that there is some selectivity based on the positional isomers in the mixture. Species containing between 0 (unconjugated mAb) and a maximum of 8 small molecule drugs were detected. In addition, species containing linker molecules without any small molecule drug were also present in the mixture. This is one factor that confounds the sensitive mass measurement of molecules having the complexity of mAb C. Additional confounding factors include the fact that only 20 µg of mAb C were loaded onto the HIC dimension, and only a portion of the total mass was transferred to the second dimension. Also, we purposefully chose not to deglycosylate the sample to simplify the sample preparation. The results are unambiguous despite the sum of each of these factors that serve to dilute the overall MS signal. This becomes an advantage in that a large amount of sample is not required for this type of analysis. Overall, the obtained data indicate that the desalting efficiency of the MHC 2D-LC HIC/RP platform is extremely high.

Conclusion

HIC is an incredibly useful tool for characterizing mAbs, even when modified components are present as minor species. We have illustrated the utility of HIC by using the separation of mAbs and ADCs as examples. The unique selectivity of HIC makes it an excellent addition to the bioanalytical toolbox. However, mass measurement of protein variants, often a first step in the determination of an unknown peak, is not straightforward when using HIC due to the high concentration of nonvolatile salts used in the separation. These difficulties can be overcome when using a 2D-LC/MS platform combining HIC and RP. Especially when dealing with unknown peaks, as shown for mAb A, the MS measurements obtained in this work are useful first steps toward determining identity. For the intact mAbs examined, the RP column works principally as a desalting tool. For other samples, for example those that have been reduced, those that have been digested into fragments, or those that originate from interchain Cys-linked ADCs, the ^{2}D RP column would provide additional separation useful for product characterization.

MHC allows the investigator to store peaks of interest from the ^{1}D, permitting the allocation of whatever time is necessary to conduct optimized ^{2}D separations. In the current work, this was advantageous because adequate time could be allotted to sample desalting and sensitive introduction into the MS at a relatively low flow rate (0.2 mL/min). Even when desalting for MS analysis is not the goal, MHC style 2D-LC allows the flexibility to perform longer ^{2}D gradients, which are often needed when working with protein samples.

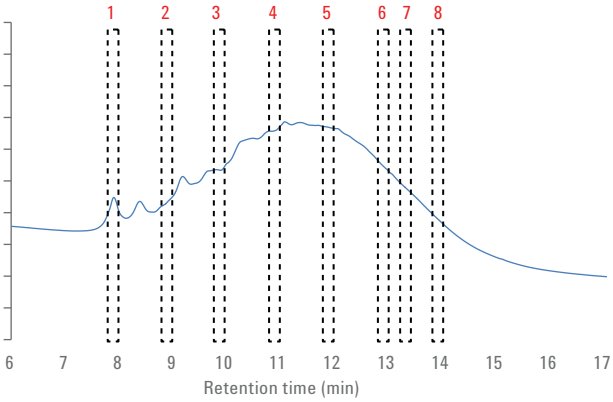

Figure 4. mAb C, a Lys-linked ADC, was analyzed using the HIC/RP 2D-LC/MS system. The resulting HIC chromatogram consisted of a very poorly resolved group of peaks eluting over the course of ~7 minutes, reflective of the multiple positional isomers present in the preparation. Eight heart-cuts at RTs 7.9, 8.9, 9.9, 10.9, 11.9, 12.9, 13.4, and 13.9 minutes were selected and sent to the ^{2}D.

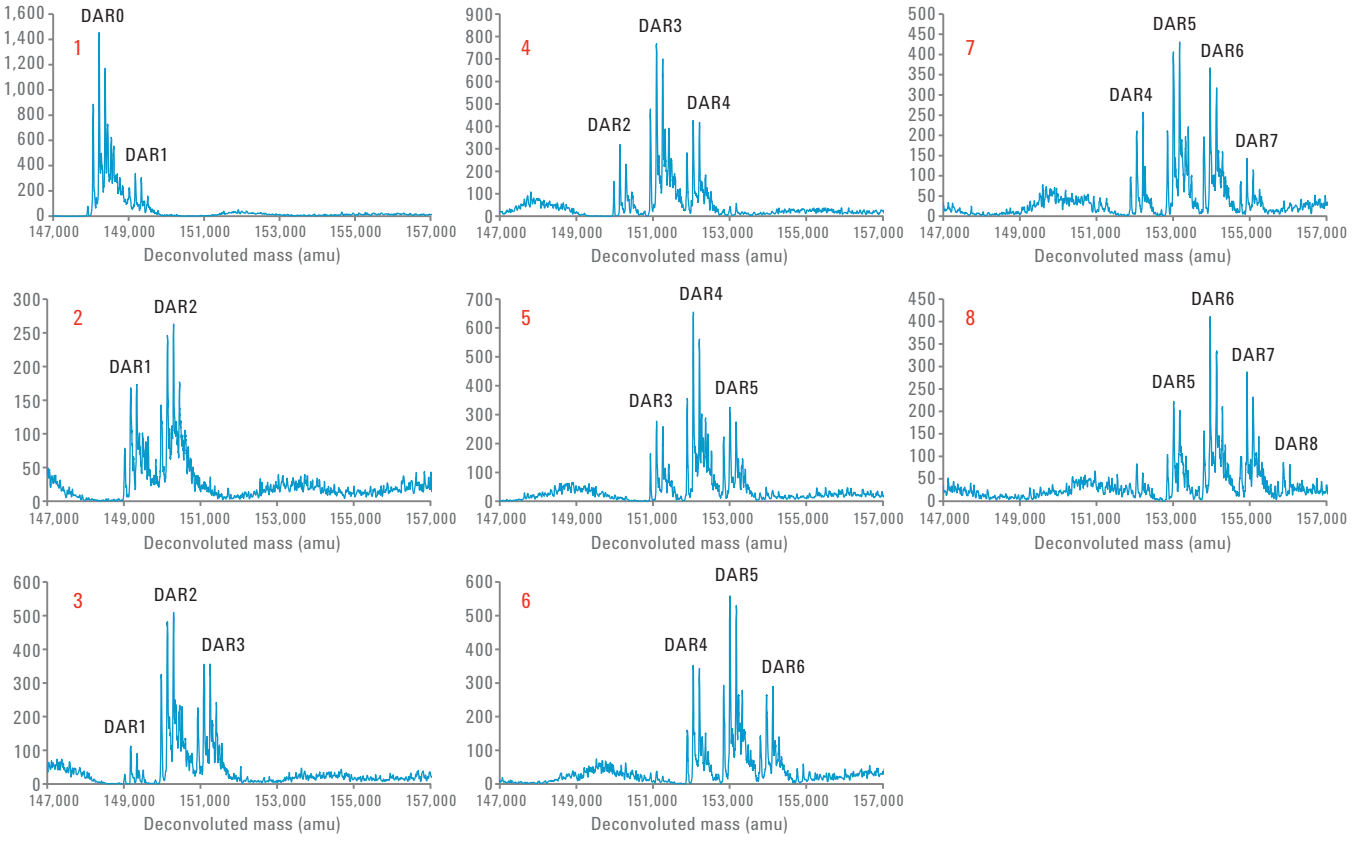

Figure 5. MS spectra corresponding to the heart-cuts from Figure 4. mAbs with DAR ranging from 0–8 were observed. The spectra also reflect the glycoform distribution of the mAb in addition to a subpopulation of molecules modified by linker without payload.

References

1. Buckenmaier, S. Agilent 1290 Infinity 2D-LC Solution for Multiple Heart-Cutting, *Agilent Technologies Technical Overview*, publication number 5991-5615EN, **2015**.

2. Xiu, L. *et al.* Effective Protein Separation by Coupling HIC and RP for Top-down Proteomics. *Anal. Chem.* **2014**, *86*, pp 7899-7906.

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Published in the USA, February 1, 2016
5991-6376EN

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