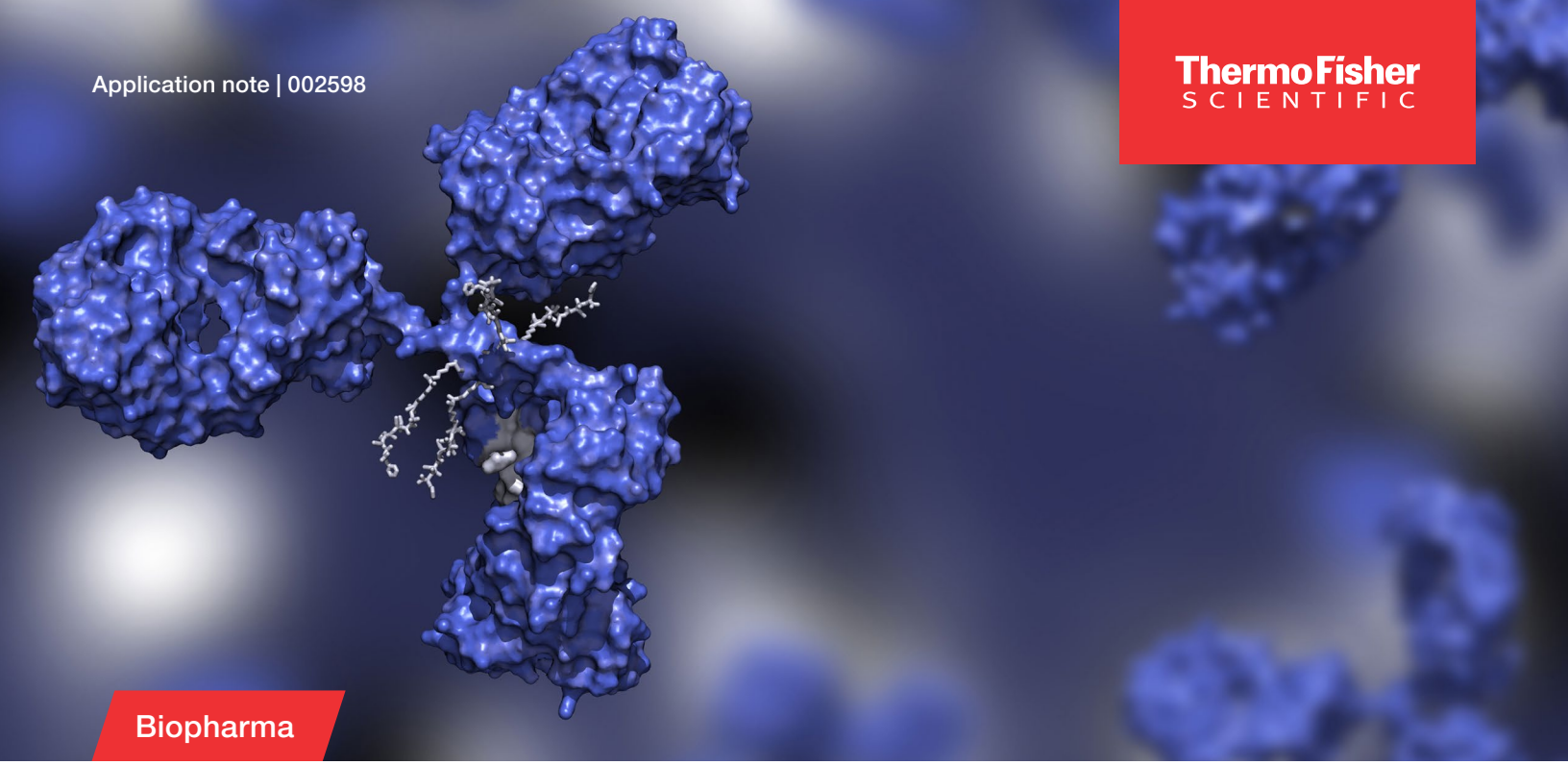

Application note | 002598

Biopharma

Automated method optimization for drug-to-antibody ratio determination using hydrophobic interaction chromatography

Authors

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Keywords

Drug-to-antibody ratio (DAR), antibody drug conjugates (ADCs), hydrophobic interaction chromatography (HIC), brentuximab vedotin, disitamab vedotin, polatuzumab vedotin, Vanquish Flex UHPLC, AQbD, MODR, robustness, ICH Q14, ICH Q2(R2), USP Chapter <1220>

Application benefits

- Robust and reproducible DAR determination methods for cysteine-linked ADCs, brentuximab vedotin, disitamab vedotin, and polatuzumab vedotin
- Achieving robust and reliable performance for the developed HIC method with high salt concentration in the eluent on a fully biocompatible Thermo Scientific™ Vanquish™ Flex UHPLC system
- Fusion QbD™ software provides an automated method optimization workflow and generates the method operable design region (MODR) for the final method

Goal

Demonstrate an automated method optimization procedure for the DAR test of ADCs using HIC on the Vanquish Flex UHPLC system

Introduction

Antibody-drug conjugates (ADCs) are a fast-growing class of biopharmaceutical drugs for the treatment of cancer. ADCs are typically composed of a monoclonal antibody (mAb) covalently attached to a cytotoxic drug via a chemical linker; this conjugation utilizes the exquisite selectivity of antibodies to achieve targeted delivery of cytotoxic drugs. Cysteine-linked ADCs make up the majority of the approved ADCs in the global market.[1] Cysteine-linked ADCs are generated by first reducing inter-chain disulfide bonds of mAbs, and then by coupling cytotoxic drugs with free thiols. This process yields a controlled but heterogeneous number of products with a distribution of 0, 2, 4, 6, and 8 cytotoxic drugs conjugated per antibody.[2] The average DAR is a critical quality attribute of ADCs, as it influences some key factors such as drug efficacy, pharmacokinetics, and toxicity, which should be determined and monitored during development and production.

HIC is the reference technique to determine the average DAR and drug distribution. A typical method for HIC is a linear salt gradient elution with decreasing salt concentration. High salt concentration generally reduces protein solubility and facilitates interactions between the protein's nonpolar surfaces and the stationary phase's hydrophobic functional ligands. With decreasing salt concentration in the gradient, proteins have a greater affinity to the highly aqueous mobile phase and are eluted from the column in their order of increasing molecular hydrophobicity.

Our previous application note demonstrated that the Thermo Scientific™ MAbPac™ HIC-Butyl column and the Thermo Scientific™ UltiMate™ 3000 BioRSLC system are a suitable combination for DAR determination of cysteine-conjugated ADC mimics.[2] However, optimal conditions are quite different for different ADCs. Given a constant column and salt type, organic modifiers, column temperature, and gradient time can change selectivity and retention. Therefore, it is necessary to perform a systematic method optimization study rather than just applying historical conditions or executing random trials for new ADCs. The analytical quality by design (AQbD) approach is a systematic approach to method development based on sound science and quality risk management. This approach is strongly advocated by regulatory agencies such as the U.S. Food and Drug Association and the European Medicines Agency. The recently published ICH Q14 guideline and USP Chapter <1220> introduced the AQbD approach in detail. It is expected that the AQbD approach to method development and validation will help define a proper control strategy for analytical procedures to control sources of variability and consistently provide credible results with constant quality.[3-5] Although developing methods that meet the AQbD criteria can be labor intensive as it involves design-of-experiment (DoE) studies and mathematical modeling, software tools, such as the Fusion QbD software, can be of great assistance.

In this application note, we demonstrate an AQbD-based generic method optimization approach for the DAR test using the MAbPac HIC-Butyl column and a Vanquish Flex UHPLC system. Fusion QbD software platform was used to perform DoE studies, data analysis, and mathematical modeling for an optimum and robust final method. The DAR determination methods for disitamab vedotin, polatuzumab vedotin, and brentuximab vedotin were successfully optimized using this approach.

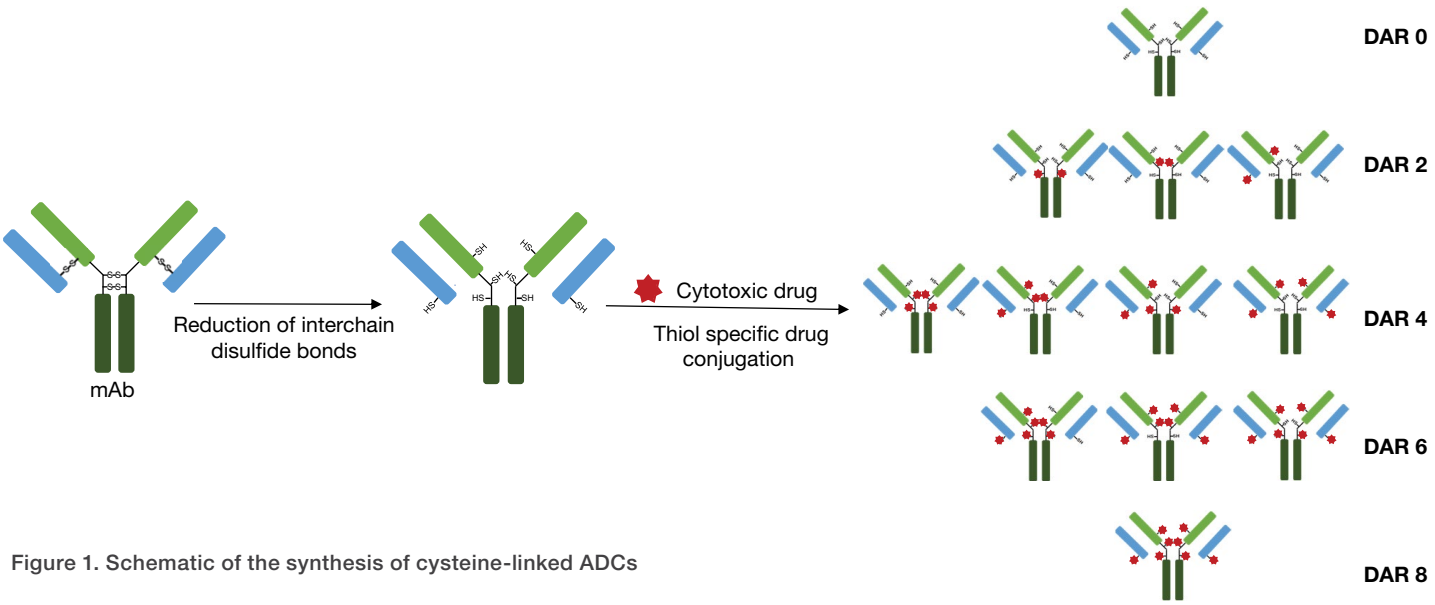


Figure 1. Schematic of the synthesis of cysteine-linked ADCs

Experimental

Instrumentation

Vanquish Flex UHPLC system consisting of:

- System Base Vanquish Flex (P/N VF-S01-A)
- Binary Pump F (P/N VF-P10-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Pre-column Heater 1 µL, biocompatible MP35N (P/N 6732.0174)
- Variable Wavelength Detector F (P/N VF-D40-A) with 11 µL standard biocompatible flow cell (6077.0200)

Thermo Scientific™ Orion Star™ A211 pH meter (P/N STARA2110)

Software

Thermo Scientific™ Chromeleon™ 7.3.1 Chromatography Data System (CDS) was used for data acquisition and basic data processing. Fusion QbD software, version 9.9.2, was used to perform DoE studies, data analysis, and mathematical modeling.

Reagents and consumables

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Isopropanol (IPA), HPLC grade, Fisher Chemical™ (P/N A451-4)
- Ammonium sulfate, disodium hydrogen phosphate (anhydrous), sodium dihydrogen phosphate (anhydrous), and sodium hydroxide, AR grade, Sigma-Aldrich

Eluents and sample preparation

Eluents

- **50 mM phosphate buffer:** Added 5.076 g of NaH_2PO_4 and 8.191 g of Na_2HPO_4 to a 2.0 L bottle and filled up to 2.0 L using ultrapure water.
- **Eluent A:** Added 198.21 g of $(NH_4)_2SO_4$ to a 1.0 L bottle and filled up with 0.95 L prepared 50 mM phosphate buffer, adjusted the pH to 7.0 using NaOH solution, and then added 0.05 L IPA and mixed it well.
- **Eluent B1 to B4:** 100, 150, 200, 250 mL of IPA and 900, 850, 800, 750 mL of the prepared 50 mM phosphate buffer were mixed well and added to 1.0 L bottles as eluents B1 to B4, respectively.

Sample

Three commercially available ADC products were weighed and then dissolved in an amber vial with a mixed solvent of eluent A and water (1:1, v/v). The final concentration for brentuximab vedotin was 100.0 mg/mL, for disitamab vedotin was 40.0 mg/mL, and for polatuzumab vedotin was 30.0 mg/mL.

Results and discussion

Method optimization

Based on our previous experience, the MAbPac HIC-Butyl column is an optimal choice for DAR determination. For the eluent, it was reported that very similar retention and selectivity can be achieved with different salt systems when correcting the salt concentration for the same lyotropic strength.[6] Here, a typical eluent consisting of 1.5 M $(NH_4)_2SO_4$ with 50 mM phosphate buffer was used. This combination has been widely demonstrated to be effective for DAR determination.

ADCs with hydrophobic payloads usually cover a broader hydrophobicity range than bare mAbs; DAR 6 and DAR 8 are very hydrophobic and not able to be eluted with 100% aqueous phase on the butyl column, even without salt in the final phase. Therefore, an organic phase is usually added to elute DAR 6 and DAR 8 and sharpen the peaks. In preparation for this study, a range of 10%–25% of IPA in eluent B was tested for DAR determination. The conditions are listed in Table 1.

The results show that DAR 8 was not fully eluted when the IPA amount was less than 20%. Though 25% of IPA can elute all the DARs with good peak shape, the viscosity of IPA increases the column pressure, which is close to the recommended upper limit. This can negatively affect the lifetime of the column. Therefore, 20% IPA was used in this optimization study. Other optimized variables in the study are listed in Table 2.

Table 1. Initial HPLC conditions for IPA% optimization for DAR determination of brentuximab vedotin

Items	Value		
Column	MAbPac HIC-Butyl, 4.6 × 100 mm, 5 µm (P/N 088558)		
Eluent	A: 1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 / IPA (95:5 v/v) B1 to B4: 50 mM sodium phosphate, pH 7.0 / IPA (90:10,85:15,80:20,75:25, v/v)		
Gradient	Time (min)	%A	%B
	-5	100	0
	0	100	0
	2.0	100	0
	17.0	0	100
	22.0	100	0
	25.0	100	0
Flow rate	0.8 mL/min		
Injection volume	10 µL		
Column temperature	30 °C		
Detector	280 nm		

Table 2. DoE platform for HIC method optimization

Variable	DoE range
Column temperature	25–40 °C
Flow rate	0.7–0.9 mL/min
Gradient time	10–20 min
Initial B%	0–20%

Fusion QbD software was used for the following optimization study. After defining the variables and their ranges in the software, it automatically generated the most efficient experiment design to characterize the independent and interactive effects of the study parameters. The software's export routine then automatically constructed the design within the Chromeleon CDS as a ready-to-run sequence and methods; the workflow using Fusion QbD software and Chromeleon CDS for method optimization is shown in Figure 2. In this optimization, a 30-run statistical experimental design was generated, which required about 42 hours of instrument time (including the conditioning runs and blank injections).

After the optimization, the integrated data were imported into Fusion QbD software for data analysis and mathematical modeling. The best answer method predicted by Fusion QbD software and the experimental chromatogram generated using

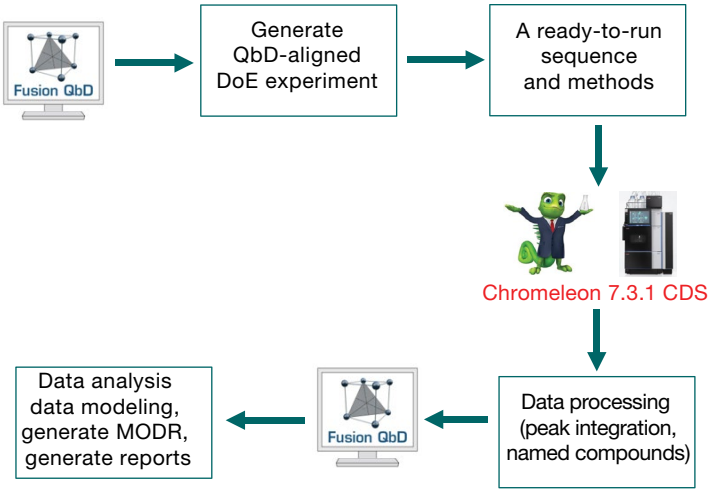


Figure 2. A QbD-based method optimization workflow using Fusion QbD software and Chromeleon CDS

this method are shown in Figure 3. The predicted retention times are in excellent agreement with the experimental ones, with an average error of ±0.22 min. Most importantly, the Fusion QbD software predictions of relative retention, which more directly relate to the software's resolution predictions, are in almost perfect agreement with the observed data. In this case, the average difference is only -0.10 min (<6.0 s). The tested resolution and peak asymmetry using this condition exceeded all the specified performance goals.

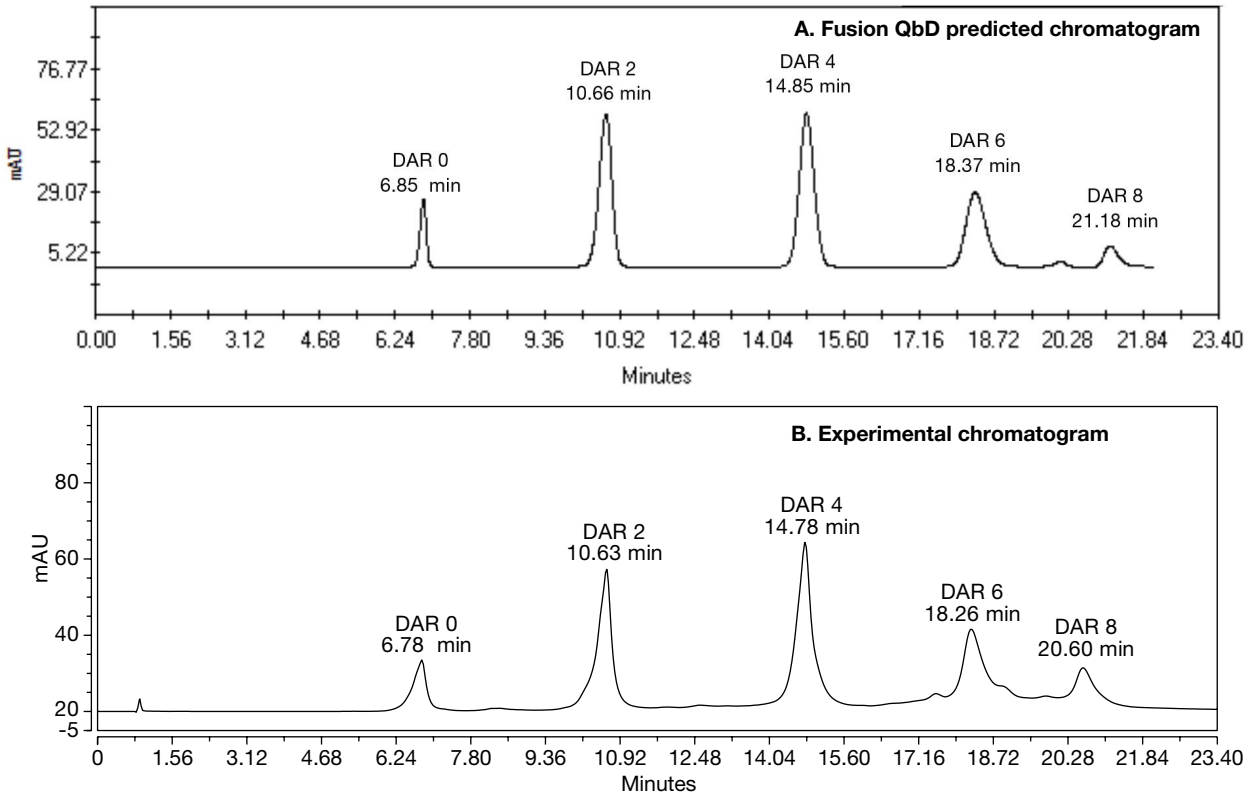


Figure 3. Comparison of predicted (A) and experimental (B) chromatograms of brentuximab vedotin. Fusion QbD software predicted the best conditions: Pump flow rate: 0.80 mL/min, Gradient time: 18.5 min, Initial % eluent B: 5.0%, Column temp.: 25.0 °C.

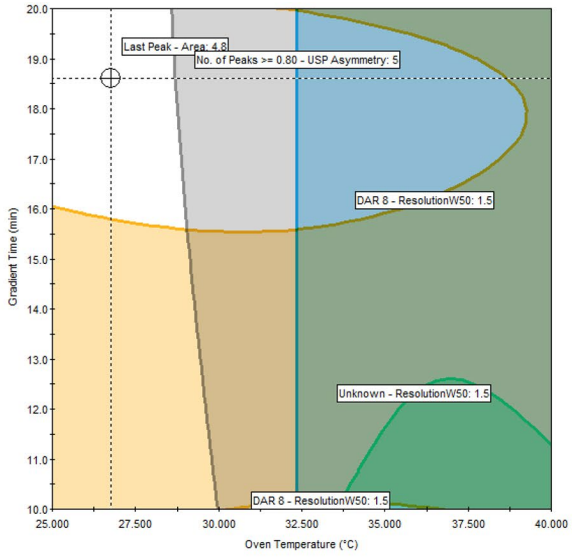
Using the following equation, the average DAR of brentuximab vedotin was calculated to be 3.90, which agrees with the results of high-resolution mass spectrometry (HRMS) analysis obtained in our lab, and with the previously reported average DAR value in literature.[7]

$$DAR = \sum_{n=0}^{8} \frac{LC\ peak\ area \times n_{drug}}{Total\ LC\ peak\ area}$$

The impact of various parameters on method performance can be evaluated by using the MODR, or analytical design space, generated in Fusion QbD software. Figure 4 presents graphs that display the variable effects and the MODR. To generate these graphs, the non-graphed variables flow rate and initial B% were set to their optimal levels. In these graphs, each critical performance characteristic is assigned a color. The graph region shaded with that color identifies the method conditions that fail to meet the specified performance requirements for that characteristic. For example, in Figure 4A, the orange-colored area designates methods that result in a resolution of DAR 8 and its previous peak of less than 1.5. The remaining unshaded region in the graph is the MODR—the region containing the methods that simultaneously meet or exceed all performance requirements specified for resolution, peak area, asymmetry, and peak number. Within the MODR, parameters can be varied independently or simultaneously without compromising any of the method performance requirements.

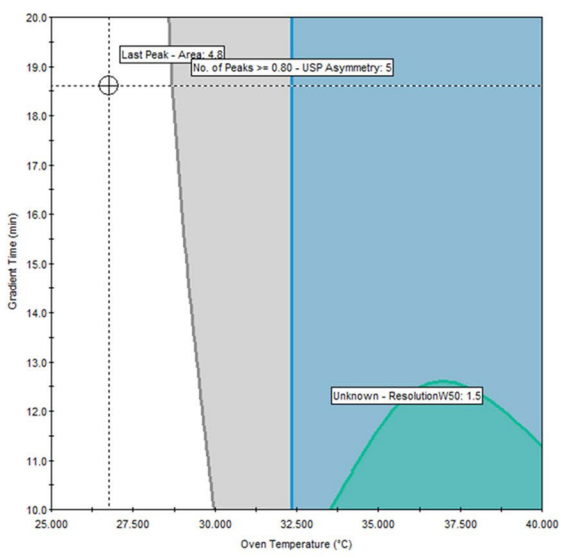
The peak area of the last peak in the chromatogram was added to the method performance goal set, as it was found that when the column temperature increased, the peak area decreased, especially for DAR 2 to DAR 8, as shown in Figure 5. A lower bound of 4.8 was defined in the goal, which is about 90% of the average area under the ambient temperature of 25.0 °C. The MODR in Figure 4A shows that the peak area gets below 4.8 when the column temperatures are above 28.8 °C. This result can be explained by the increased hydrophobic interaction between proteins and HIC-butyl ligand at the higher column temperatures; the protein can't be fully eluted. Higher temperatures also result in poor chromatographic recovery and peak shape. Another reason to control the column temperature is to maintain non-denaturing conditions and the native conformations of the proteins. Therefore, it is necessary to use column temperature control even in ambient conditions.

A



	Name	Units	Goal	Lower Bound	Upper Bound	Color	Crosshair Prediction	Contour Label
☐	Rs-Map Response	*	---	---	---	---	---	---
☑	DAR 2 - ResolutionW50	*	Maximize	1.500		Red	10.526	☑
☑	DAR 4 - ResolutionW50	*	Maximize	1.500		Blue	7.834	☑
☑	DAR 6 - ResolutionW50	*	Maximize	1.500		Green	4.962	☑
☑	DAR 8 - ResolutionW50	*	Maximize	1.500		Orange	2.328	☑
☑	Unknown - ResolutionW50	*	Maximize	1.500		Teal	2.655	☑
☑	No. of Peaks	*	Maximize	5.0		Purple	6.12	☑
☑	No. of Peaks >= 1.50 - Resolution (USP)		Maximize	4.0		Lime	4.35	☑
☑	No. of Peaks >= 0.80 - USP Asymmetry		Maximize	5.0		Sky	5.43	☑
☑	Last Peak - Area	n.a.	Maximize	4.80		Gray	5.101	☑

B



	Name	Units	Goal	Lower Bound	Upper Bound	Color	Crosshair Prediction	Contour Label
☐	Rs-Map Response	*	---	---	---	---	---	---
☑	DAR 2 - ResolutionW50	*	Maximize	1.500		Red	10.526	☑
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☑	DAR 6 - ResolutionW50	*	Maximize	1.500		Green	4.962	☑
☐	DAR 8 - ResolutionW50	*	---	---	---	---	---	---
☑	Unknown - ResolutionW50	*	Maximize	1.500		Teal	2.655	☑
☑	No. of Peaks	*	Maximize	5.0		Purple	6.12	☑
☑	No. of Peaks >= 1.50 - Resolution (USP)		Maximize	4.0		Lime	4.35	☑
☑	No. of Peaks >= 0.80 - USP Asymmetry		Maximize	5.0		Sky	5.43	☑
☑	Last Peak - Area	n.a.	Maximize	4.80		Gray	5.101	☑

Figure 4. The MODR at different oven temperatures and gradient times. Flow rate = 0.8 mL/min, initial % eluent B = 5.0%. The unshaded region in the graph represents the MODR. After deleting the requirement on resolution between DAR 8 and the previous unknown peak from the performance goal, the MODR was broadened (graph B).

It should be noted that the size and shape of the MODR depend on the goals that are defined. In Figure 4A, the resolutions of all integrated peaks (DAR 0 to DAR 8, and 1 unknown peak before DAR 8) should be greater than 1.50. This resulted in a MODR being restricted to the top-left quadrant of the graph. In this region, the operable range of column temperature is 25.0 °C to 28.8 °C, and the gradient time is 16–20 min. If the resolution between DAR 8 and its previous unknown peak is ignored, the MODR is broadened, and the gradient time is down to 10 min, as shown in Figure 4B. This demonstrates that a gradient time of 16 min provides a better separation for some small unknown peaks, as can be also seen in Figure 6. The better separation of the small peak facilitates peak identification when combined with other techniques, such as two-dimensional LC or fraction collection with subsequent HRMS analysis. However, the resolution >1.50 is not a general rule for protein separation, because the isomers and conformations of protein make it difficult to produce baseline resolved separation for all small peaks, this differs from traditional applications for small molecule in pharma analysis.

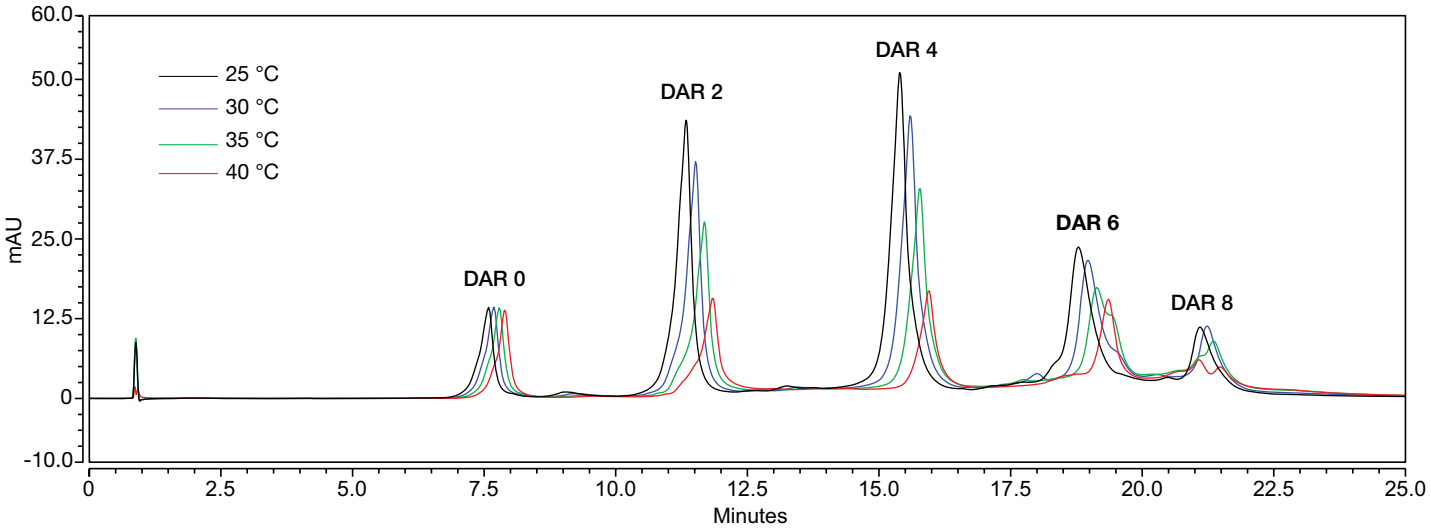


Figure 5. Brentuximab vedotin chromatograms under different column temperature conditions

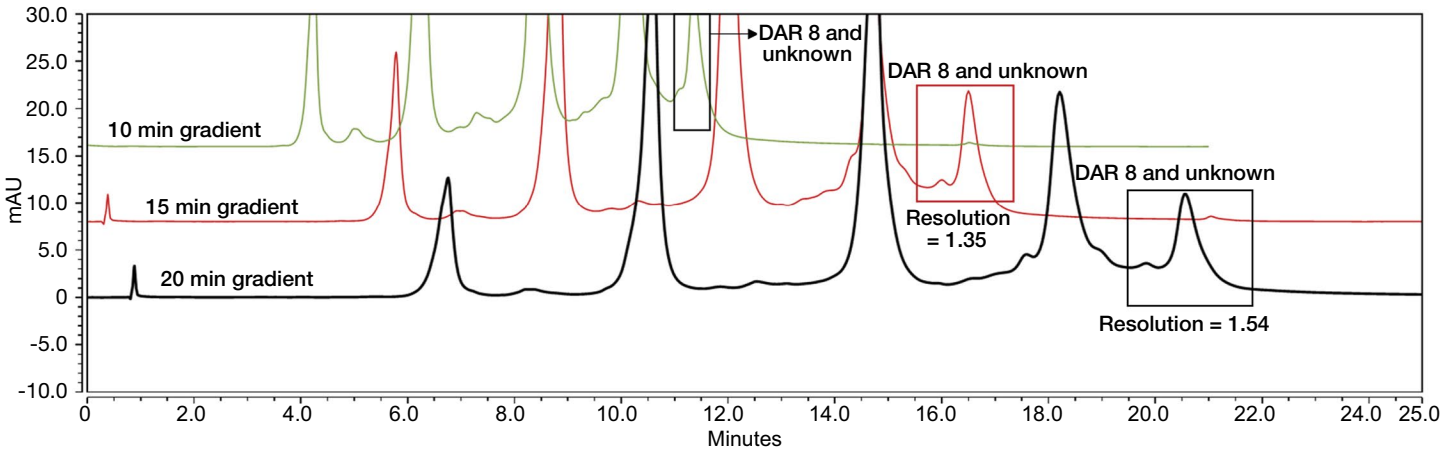


Figure 6. Separation for small unknown peaks in brentuximab vedotin chromatograms with different gradient times

Baseline subtraction

The decreasing salt concentration during the gradient leads to a baseline drop in the obtained chromatogram. Reagents of higher purity may result in lower fluctuations but are also associated with higher operational costs for the laboratory, as the concentration of ammonium sulfate in eluent A is 198.2 g/L. Figure 7 shows the chromatogram of a solvent (eluent A: Water = 1:1, v/v) injection using ammonium sulfate from different vendors; even different batches of ammonium sulfate from the same vendor can produce different baselines. The baseline subtraction functionality in Chromeleon CDS can be used to eliminate this effect by automatically subtracting the solvent injection from the sample chromatogram.

Robustness of the instrument and column

The eluent used in HIC contains high concentrations of salts and organic phase, which makes it challenging for the LC system and the column. The robustness of the method and the instrument were further tested by performing a replicate injection of solvent (eluent A: Water = 1:1, v/v) and sample using the final optimized method over a period of one week. The run-to-run reproducibility is shown in Figure 8. The RSD% of retention time was found to be less than 0.9%, and the RSD% of peak area was found to be less than 2.0%, which demonstrates the robustness of the method and instrument. To reduce the risk of salt precipitation and clogging, it is recommended to regularly rinse and purge the system and column with 20% methanol in water.

DAR determination of disitamab vedotin and polatuzumab vedotin

Using this approach, DAR determination methods for disitamab vedotin and polatuzumab vedotin were further optimized, and the best HPLC conditions and average DAR values are shown in Figure 9. The results are consistent with HRMS results obtained in our lab, and with previously reported DAR values in literature.[8,9]

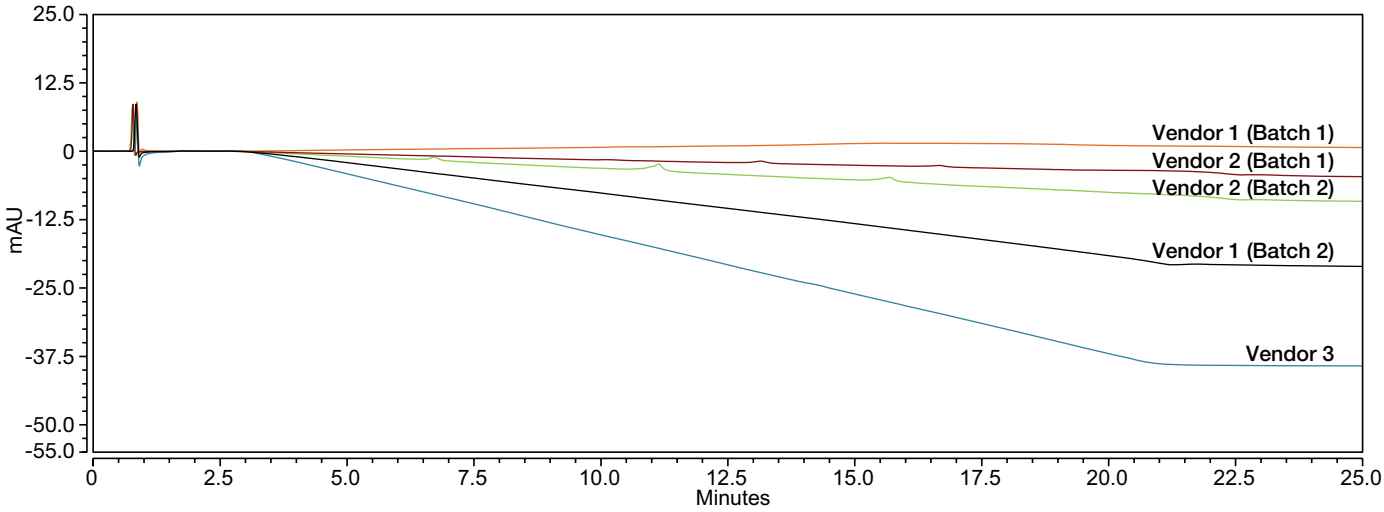


Figure 7. Chromatogram of a mixed solvent of eluent A and water (1:1, v/v) injections using ammonium sulfate from different vendors

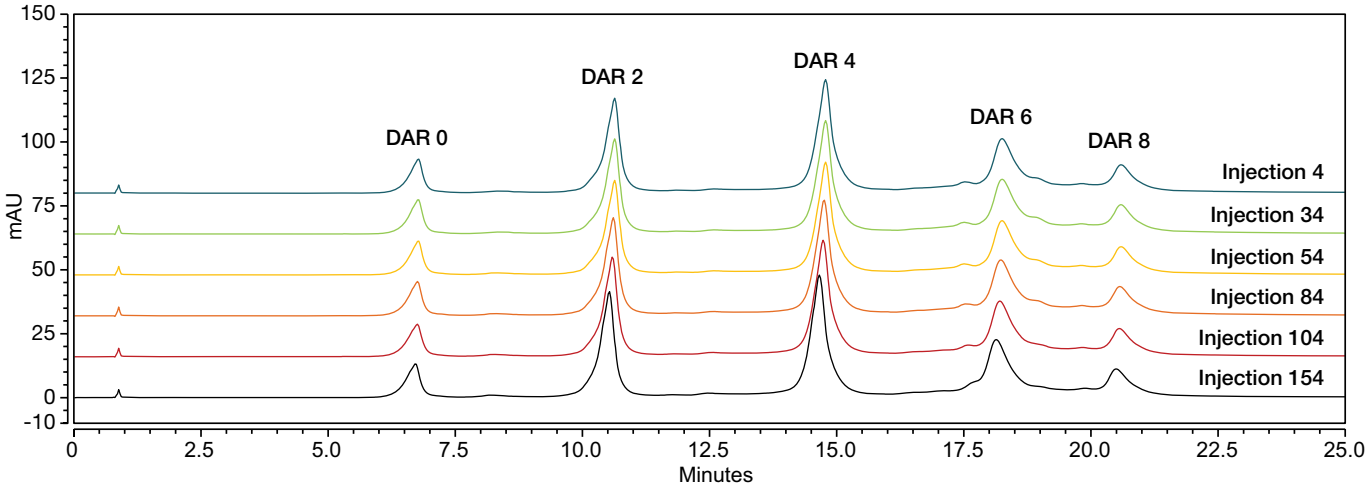
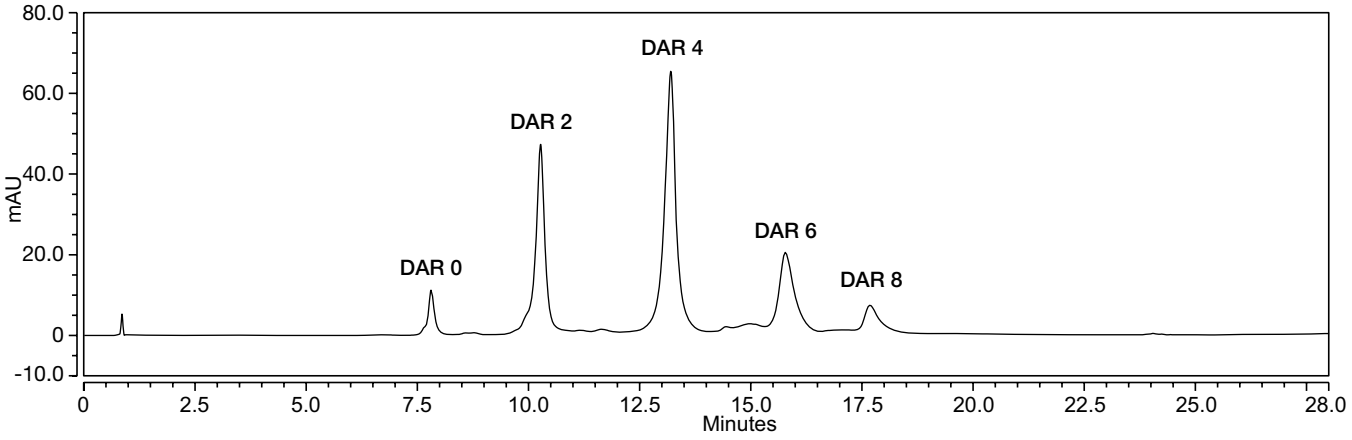


Figure 8. Run-to-run reproducibility of brentuximab vedotin on a Vanquish Flex UHPLC system

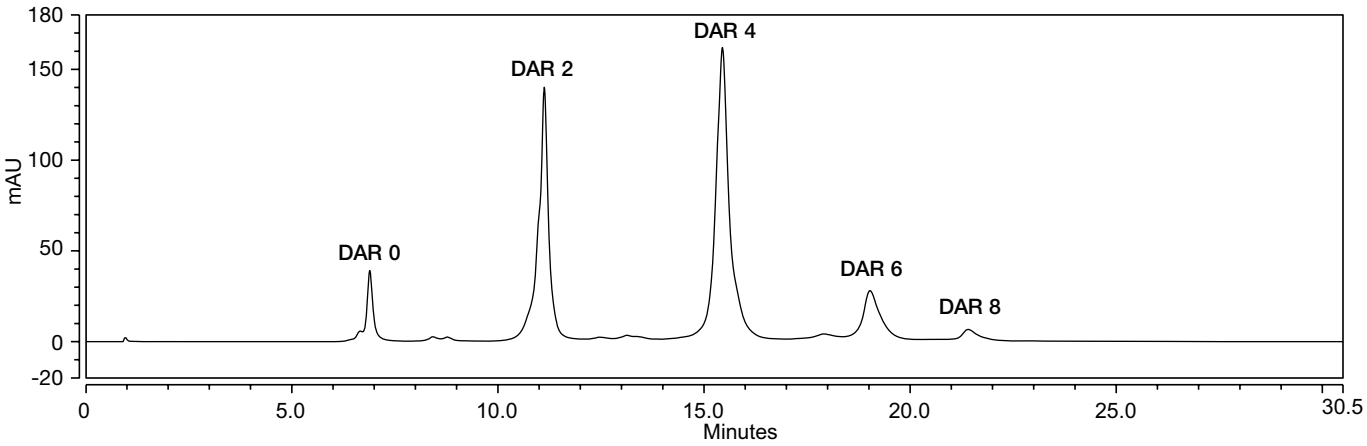
A. Disitamab vedotin



HPLC conditions			
Column temperature	25 °C		
Injection volume	10 µL		
Detector	280 nm		
Gradient	Time (min)	Flow rate (mL/min)	%B3
	-6	0.8	10
	2	0.8	10
	17.5	0.8	100
	22.5	0.8	100
	27.5	0.8	10
	28.5	0.8	10

Peak name	Peak area	Area%	DAR
DAR 0	1.976	4.3	0
DAR 2	12.188	26.51	2
DAR 4	20.176	43.88	4
DAR 6	9.255	20.13	6
DAR 8	2.385	5.19	8
		Average DAR	3.91

B. Polatuzumab vedotin



HPLC conditions			
Column temperature	25 °C		
Injection volume	10 µL		
Detector	280 nm		
Gradient	Time (min)	Flow rate (mL/min)	%B3
	-6	0.7	0
	2	0.7	0
	21.5	0.7	100
	25.5	0.7	100
	27.5	0.7	0
	30.5	0.7	0

Peak name	Peak area	Area%	DAR
DAR 0	7.964	6.07	0
DAR 2	41.318	31.49	2
DAR 4	64.650	49.27	4
DAR 6	14.889	11.35	6
DAR 8	2.396	1.83	8
		Average DAR	3.43

Figure 9. The DAR determination of disitamab vedotin (A) and polatuzumab vedotin (B)

Conclusion

In this study, an automated HIC method optimization approach was developed for the determination of the DAR using Fusion QbD software. The average DAR values of brentuximab vedotin, disitamab vedotin, and polatuzumab vedotin were determined, and the DAR values are consistent with HRMS results. This application note provides the following benefits:

- The chromatogram subtraction functionality in Chromeleon CDS easily eliminates the baseline fluctuation.
- Fusion QbD software facilitates the method optimization process. The DoE studies and the mathematical modeling were used to investigate the interaction of different method parameters and generate the MODR, which is in accordance with the AQbD principle.
- The column temperature, flow rate, and gradient were optimized in this method. The final method provides good separation and reproducible results for DAR determination of brentuximab vedotin, disitamab vedotin, and polatuzumab vedotin.

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