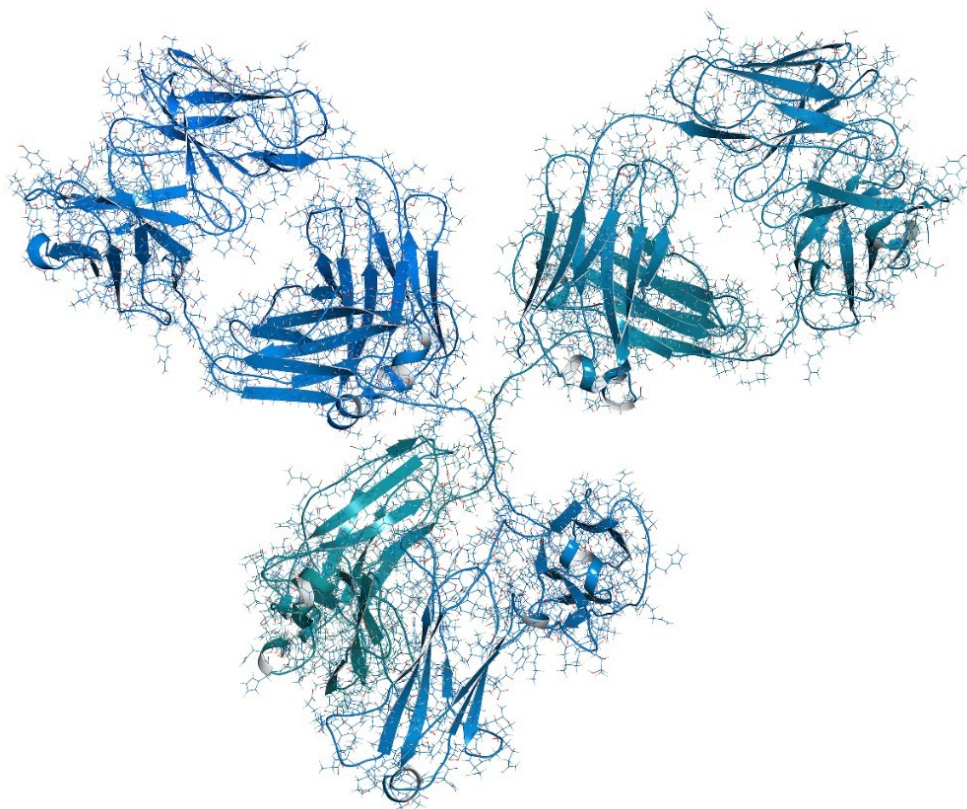


Application Note

Demonstrating a Fit-for-Purpose Solution for Biopharmaceutical Separations with the ACQUITY UPLC H-Class PLUS Bio Binary System

Brooke M. Koshel, David Dao, Robert E. Birdsall, Ying Qing Yu

Waters Corporation



Abstract

Many biopharmaceutical laboratories do not have the resources available to dedicate LC instrumentation to individual analytical methods. Although binary LC systems support more stringent method conditions such as slow flow rates and shallow gradients generally associated with peptide mapping methods, it is important that reproducible results can be achieved for all assays, regardless of LC platform. In this work, HILIC, HIC, and SEC methods are used to assess the performance of the ACQUITY UPLC H-Class PLUS Bio Binary System as a fit-for-purpose LC system for common biopharmaceutical methods.

Benefits

- Demonstrated method equivalency when comparing results between quaternary and binary LC systems without any method changes required
- Proven LC system robustness over a multiple-day study for a method requiring high salt mobile phase conditions
- Enables future-proofing of the QC lab through the combination of modern column and LC technologies

Introduction

Analytical methods that incorporate slow flow rates and shallow gradients to achieve optimal resolution of complex samples are some of the most difficult methods to reproduce. Peptide mapping methods are among such methods within the biopharmaceutical landscape, where retention time repeatability is critical for proper identification and quantitation of peaks. Among scientists conducting peptide mapping experiments, it is generally accepted that binary LC systems offer more accurate and precise gradient delivery compared to quaternary LC systems due to fundamental differences in how each of the pumps are designed. While the ACQUITY UPLC H-Class PLUS Bio Binary System has been shown to provide exceptional repeatability of peptide maps,^{1,2} non-denaturing analytical methods such as HIC, IEX, or SEC do not generally require the rigor of a binary pump to deliver reliable results. Quaternary LC systems are often considered a more affordable option that provides the added benefit of being more suitable for method development due to the extra in-line solvent reservoirs available. Because laboratories do not always have dedicated instrumentation for individual analytical methods, it becomes important to demonstrate that the desired results can be obtained regardless of the LC platform used. This work evaluates the performance of the ACQUITY UPLC H-Class PLUS Bio Binary System for methods not traditionally requiring the performance of a binary system to confirm that the LC system is fit-for-purpose for a host of biopharmaceutical assays.

Experimental

LC Conditions

LC systems:	ACQUITY UPLC H-Class PLUS Bio Binary
	System with CH-A (Binary)
	ACQUITY UPLC H-Class PLUS Bio System with CH-A (Quaternary)

HILIC

Sample Description

Waters *RapiFluor*-MS (RFMS) Glycan Performance Test Standard (p/n: [186007983 < https://www.waters.com/nextgen/us/en/shop/standards--reagents/186007983-rapifluor-ms-glycan-performance-test-standard.html>](https://www.waters.com/nextgen/us/en/shop/standards--reagents/186007983-rapifluor-ms-glycan-performance-test-standard.html)), a labeled standard containing N-released glycans from pooled human IgG, was reconstituted in 9 μ L water, 10 μ L DMF, and 21 μ L ACN for a final concentration of 400 pmol. Glucose unit (GU) values were determined using the *RapiFluor*-MS Dextran Calibration Ladder (p/n: [186007982 < https://www.waters.com/nextgen/us/en/shop/standards--reagents/186007982-rapifluor-ms-dextran-calibration-ladder.html>](https://www.waters.com/nextgen/us/en/shop/standards--reagents/186007982-rapifluor-ms-dextran-calibration-ladder.html)), a calibration ladder composed of glucose multimers from 2 to 30 units long. The dextran standard was reconstituted in 100 μ L water.

Column:	ACQUITY UPLC
	Glycan BEH Amide
	Column, 130 Å, 1.7 μ m,
	2.1 mm x 150 mm
	(p/n: 186004742)

Wavelength:	FLR detection at λ Ex.
	265 nm and λ Em. 425
	nm

Injection volume:	6 μ L (RFMS glycan
	standard), 1 μ L (dextran

ladder)

Column temp.: 60 °C

Mobile phase A: 50 mM NH_4HCO_2 , pH
4.4

Mobile phase B: Acetonitrile

Gradient

Time (min)	Flow (mL/min)	%A	%B
Initial	0.400	25.0	75.0
35.00	0.400	46.0	54.0
36.50	0.200	100.0	0.0
39.50	0.200	100.0	0.0
43.10	0.200	25.0	75.0
47.60	0.400	25.0	75.0
55.00	0.400	25.0	75.0

HIC

Sample Description

A cysteine-conjugated antibody drug conjugate (ADC), was provided by a collaborator at 10 mg/mL and diluted to 2 mg/mL in 1 M $(\text{NH}_4)_2\text{SO}_4$ (mobile phase A).

Column: Protein-Pak Hi Res
HIC Column, 2.5 μm ,
4.6 mm x 100 mm
(p/n:186007583)

Wavelength: TUV detection at 280

nm

Injection volume: 10 μ

Column temp.: 25 °C

Flow rate: 0.500 mL/min

Mobile phase A: 1 M (NH₄)₂SO₄ in 50 mM NaH₂PO₄/Na₂HPO₄, pH 6.8

Mobile phase B: 50 mM NaH₂PO₄/Na₂HPO₄, pH 6.8 + 10% IPA

Method: 0–100% B in 10 minutes (30 minute total run time)

SEC

Sample Description

Waters mAb Size Variant Standard (p/n: [186009429 <https://www.waters.com/nextgen/us/en/shop/standards--reagents/186009429-mab-size-variant-standard.html>](https://www.waters.com/nextgen/us/en/shop/standards--reagents/186009429-mab-size-variant-standard.html)), a mixture of NIST mAb and non-reduced IdeS digested NIST mAb fragments, was reconstituted in 120 μ water for a final concentration of 1.5 mg/mL.

Column: BioResolve SEC mAb
Column 200 Å, 2.5 μ ,
4.6 mm x 150 mm
(p/n: 176004592)

Wavelength: TUV detection at 280 nm

Injection volume:	5 μ
Column temp.:	35 °C
Flow rate:	0.200 mL/min
Mobile phase:	50 mM Na ₃ PO ₄ pH 7.0, 200 mM KCl
Method:	8.5 min isocratic run time

MS Conditions (HILIC Only)

MS system:	ACQUITY QDa Detector
Ionization mode:	ESI+
Acquisition range:	350–1250 <i>m/z</i>
Capillary voltage:	1.5 kV
Cone voltage:	15 V
Probe temp.:	400 °C

Data Management

Empower 3 Chromatography Data Software FR4

Results and Discussion

HILIC: Evaluating a Quaternary-Based LC Method Using a Binary LC System

Characterization and monitoring of released glycans is routinely used in the development of biopharmaceuticals. Well established workflows are available to support sample preparation, fluorescence, and MS detection, as well as informatics for data processing and reporting. Historically, the LC system of choice for analysis has been a quaternary LC system, as methods used for analysis have not demanded the performance afforded by a binary LC system.

To evaluate the ACQUITY UPLC H-Class PLUS Bio Binary System for glycan analysis, the *RapiFluor*-MS Glycan Performance Test Standard was used to compare results to those obtained on a quaternary LC system. Figure 1 shows representative chromatograms from an injection series on both the binary and quaternary LC systems. The standard contains 19 identified N-glycans which are labelled in the chromatograms.³ Results generated from the quaternary LC and binary LC have similar chromatographic profiles and relative abundance for each of the 19 peaks. An ACQUITY QDa Detector was placed in-line after the FLR detector on the binary LC system to confirm that the pressure on the FLR flow cell would not exceed specification due to the increased pressure from the MS detector. The pressure across the FLR flow cell was approximately 60 psi, which is well below the instrument specification. The addition of the ACQUITY QDa Detector allows for the acquisition of complementary mass data as shown in the corresponding TIC (bottom panel) of Figure 1 for increased flexibility in glycan analysis.

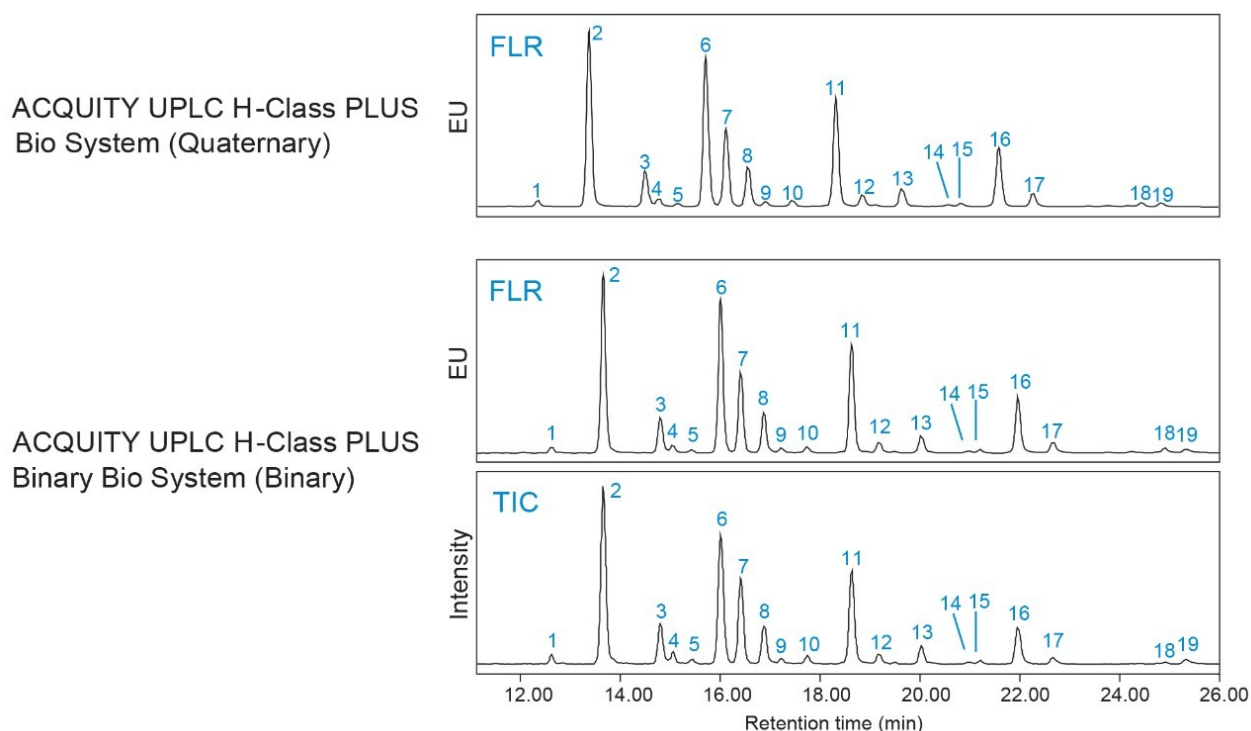


Figure 1. HILIC of released glycans on an ACQUITY UPLC Glycan BEH Amide Column (1.7 μ m, 2.1 mm x 150 mm). The Waters RapiFluor-MS Glycan Performance Test Standard contains 19 identified N-released glycans.³ The corresponding TIC is from an ACQUITY QDa Detector placed in-line post optical detection on the ACQUITY UPLC H-Class PLUS Binary Bio System. The slight shift in retention time between the quaternary and binary LC systems is due to the difference in dwell volume between the two systems.

Identification of glycans according to glucose unit (GU) values reduces differences in retention time due to different LC systems. GU values are determined through a dextran calibration ladder which correlates elution times of glucose multimers to glycan retention time. To compare results more systematically, GU values were determined for each of the 19 standard peaks and averaged over five injections (Table 1). The difference in GU values between the LC systems is also reported in Table 1, where the average difference is less than 0.1 GU values (3.5 s) between systems. This difference is well within the expectations of GU repeatability and would allow a user to refer to the Waters GU scientific library if desired. The average GU values from each system were also plotted against one another to observe any differences in selectivity (Figure 2). The R^2 value is 0.99996, indicating that results between the two LC platforms are highly comparable. Peak area percent was also determined to be highly similar across both systems (Figure 3). The difference in peak area was negligible in most cases (<0.06% for 16 of 19 peaks). The largest observed difference in peak area was 0.6% for Peak 11, which is again considered negligible as Peak 11 is present at approximately 15% and is more highly abundant. This data suggests that the ACQUITY UPLC H-Class PLUS Binary Bio System can be used for released glycan analysis and generate results comparable to results

obtained on a quaternary LC platform.

	ACQUITY UPLC H-Class PLUS Bio System (Quaternary)	ACQUITY UPLC H-Class PLUS Binary Bio System (Binary)	Quaternary – Binary
Peak	Average GU value (min)	Average GU value (min)	Δ GU Value
1	3.432	3.370	0.062
2	3.752	3.690	0.062
3	4.102	4.040	0.062
4	4.184	4.118	0.066
5	4.304	4.230	0.074
6	4.482	4.416	0.066
7	4.610	4.540	0.070
8	4.752	4.690	0.062
9	4.862	4.800	0.062
10	5.036	4.970	0.066
11	5.326	5.260	0.066
12	5.506	5.442	0.064
13	5.782	5.734	0.048
14	6.108	6.068	0.040
15	6.204	6.152	0.052
16	6.486	6.440	0.046
17	6.748	6.706	0.042
18	7.636	7.614	0.022
19	7.806	7.798	0.008

Table 1. Comparison of average GU values between the ACQUITY UPLC H-Class PLUS Binary Bio System (binary) and the ACQUITY UPLC H-Class PLUS Bio System (quaternary) (N=5).

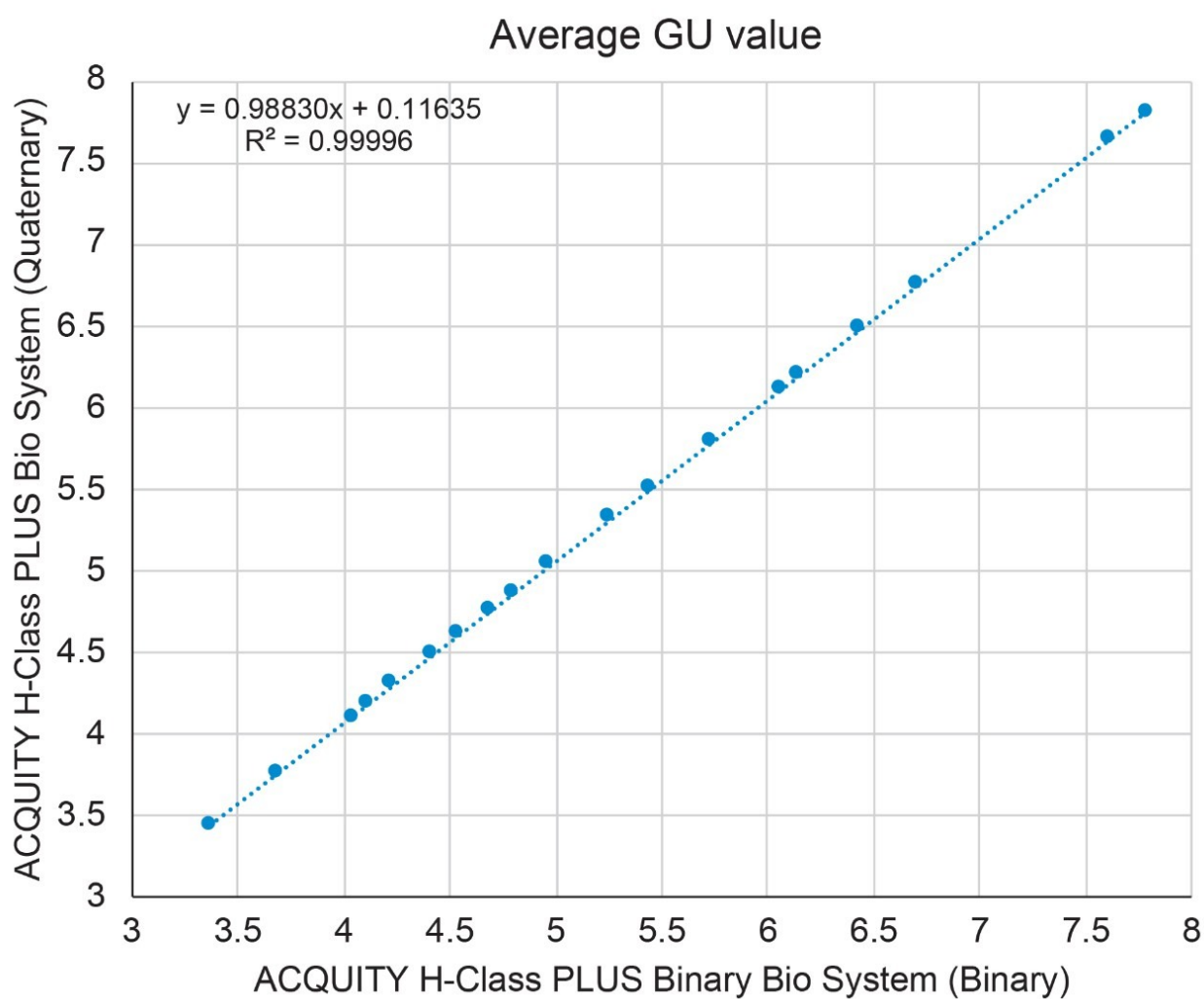


Figure 2. Comparison of GU values of the ACQUITY UPLC H-Class PLUS Binary Bio System (binary) and the ACQUITY UPLC H-Class PLUS Bio System (quaternary) ($N=5$). A high R^2 value indicates selectivity is maintained between systems.

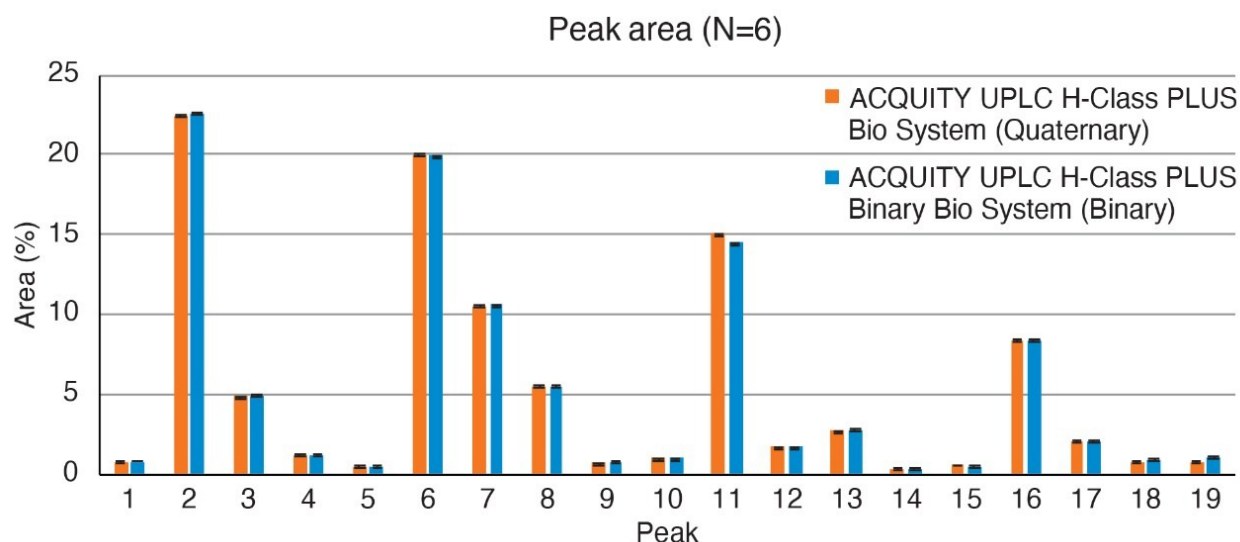


Figure 3. Comparison of peak area percent between the ACQUITY UPLC H-Class PLUS Binary Bio System (binary) and the ACQUITY UPLC H-Class PLUS Bio System (quaternary) (N=5).

HIC: Confirming LC Robustness for High Salt Separations

Native or non-denaturing protein separations often require mobile phases containing high salt. Although low dispersion LC systems are often desirable for achieving optimal performance and resolution, the narrow tubing diameter can be more susceptible to clogging and precipitation than the wider diameter tubing associated with conventional HPLC systems. To evaluate the ACQUITY UPLC H-Class PLUS Bio Binary System for clogging and precipitation after prolonged periods of exposure to high salt, a HIC method for ADCs was used for a multi-day study. A cysteine-conjugated ADC with a non-toxic payload was provided by a collaborator and injected at 2 mg/mL on a Protein-Pak Hi Res HIC Column (2.5 μ m, 4.6 mm x 100 mm). Experiments were conducted so that four blank injections (mobile phase A) were followed by a sample injection. This injection series was repeated ten times so that total run time of the sample set was 25 hours. Fresh mobile phase and sample were prepared daily, and experiments were run over three consecutive days. Figure 4 shows chromatographic overlays of every fifth ADC injection over the 25-hour run time for each of the three days. The chromatograms visually overlay well within each data set but also align nicely across each of the subsequent days. Ten peaks were identified in the chromatogram and their average retention times are reported in Table 2. The standard deviation of retention time for each peak within a data set is negligible, where the average standard deviation is less than one second on each of the three days. Small differences in retention time can be noted between days which is expected due to different mobile phase preparations.

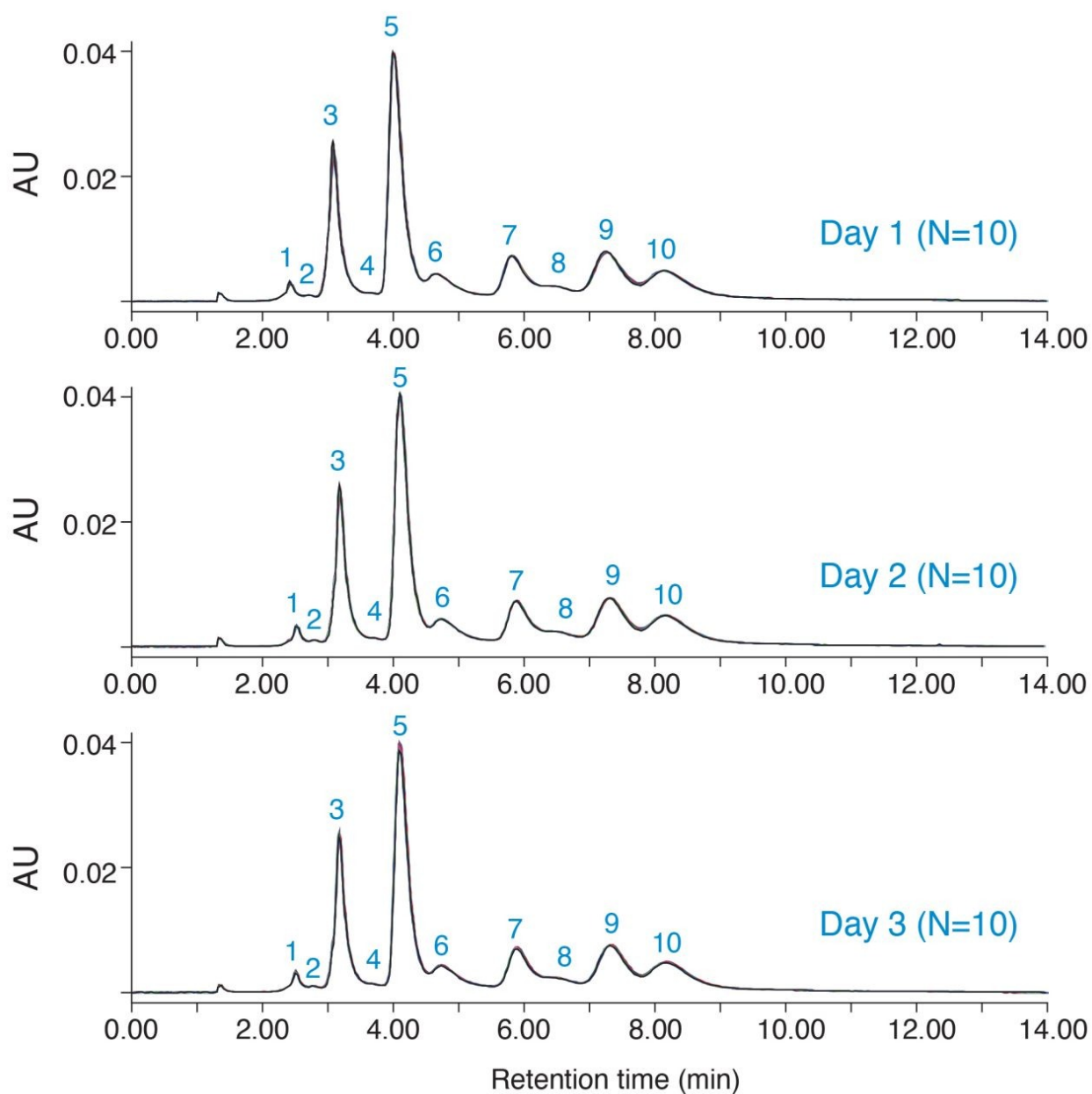


Figure 4. HIC of a cysteine-conjugated ADC on a Protein-Pak Hi Res HIC Column (2.5 μ m, 4.6 mm x 100 mm) over a three-day study. Sample sets contained four blank injections (mobile phase A) followed by an ADC injection. Overlays represent every fifth injection over a 25-hour sample set (N=10). Sample and mobile phase were prepared fresh daily.

Peak	Day 1 (N=10)		Day 2 (N=10)		Day 3 (N=10)	
	Average RT (min)	Std dev (min)	Average RT (min)	Std dev (min)	Average RT (min)	Std dev (min)
1	2.423	0.006	2.526	0.005	2.502	0.005
2	2.694	0.006	2.789	0.007	2.766	0.005
3	3.079	0.008	3.185	0.006	3.166	0.006
4	3.637	0.012	3.704	0.017	3.671	0.018
5	4.002	0.006	4.105	0.005	4.090	0.006
6	4.641	0.003	4.730	0.006	4.713	0.005
7	5.805	0.007	5.887	0.006	5.873	0.007
8	6.393	0.049	6.464	0.028	6.442	0.025
9	7.250	0.011	7.318	0.007	7.308	0.010
10	8.127	0.006	8.170	0.006	8.150	0.012

Table 2. HIC comparison of average retention time (min) across a three-day study (N=10).

Peak area was evaluated for this same data set in terms of the drug-to-antibody ratio (DAR). DAR refers to the average number of drugs conjugated to the antibody and is an important quality attribute of an ADC. Because payload is conjugated through disulfide bonds, a DAR of 0, 2, 4, 6, and 8 is possible. Figure 5 shows the chromatographic DAR distribution and the corresponding bar plots of peak area percent. Low standard deviations are observed for each data set. Furthermore, results are within error of each other across each of the three days.

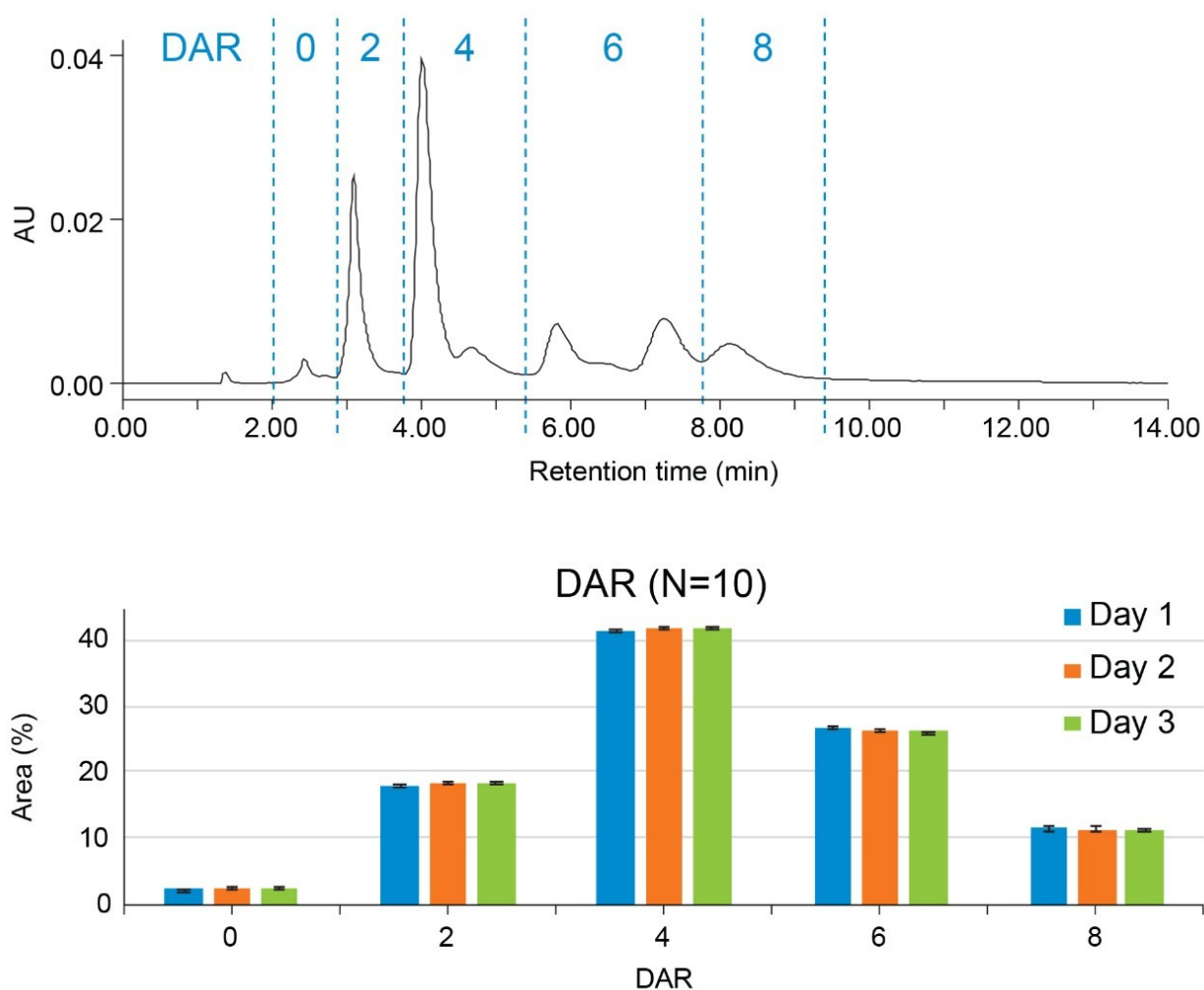


Figure 5. Drug-to-antibody (DAR) of a cysteine-conjugated ADC. Bar plot shows DAR peak area percent across three days ($N=10$).

From the retention time and peak area reproducibility assessment, there is no evidence to suggest that the column or LC system experienced clogging or salt precipitation. The reported standard deviation meets system specification and there were no leaks visibly observed during data collection. To further investigate the likelihood of these pitfalls, pressure traces were studied over the course of each injection series. Salt precipitation can manifest as spikes in the pressure (and optical) trace, however, a repeatable baseline free of noise was observed for all blank injections (data not shown). By plotting the maximum pressure observed for each of the ADC runs, the pressure is stable over the three-day study. The change in delta pressure is approximately 50 psi for each sample set. If clogging of the column or LC system were observed, the expectation would be that pressure would steadily increase over the course of an injection series as well as over multiple days as the column saw more injections, but this did not occur. The ACQUITY UPLC H-Class PLUS Bio Binary System offers a fit-for-purpose solution for high salt

separations using the conditions tested.

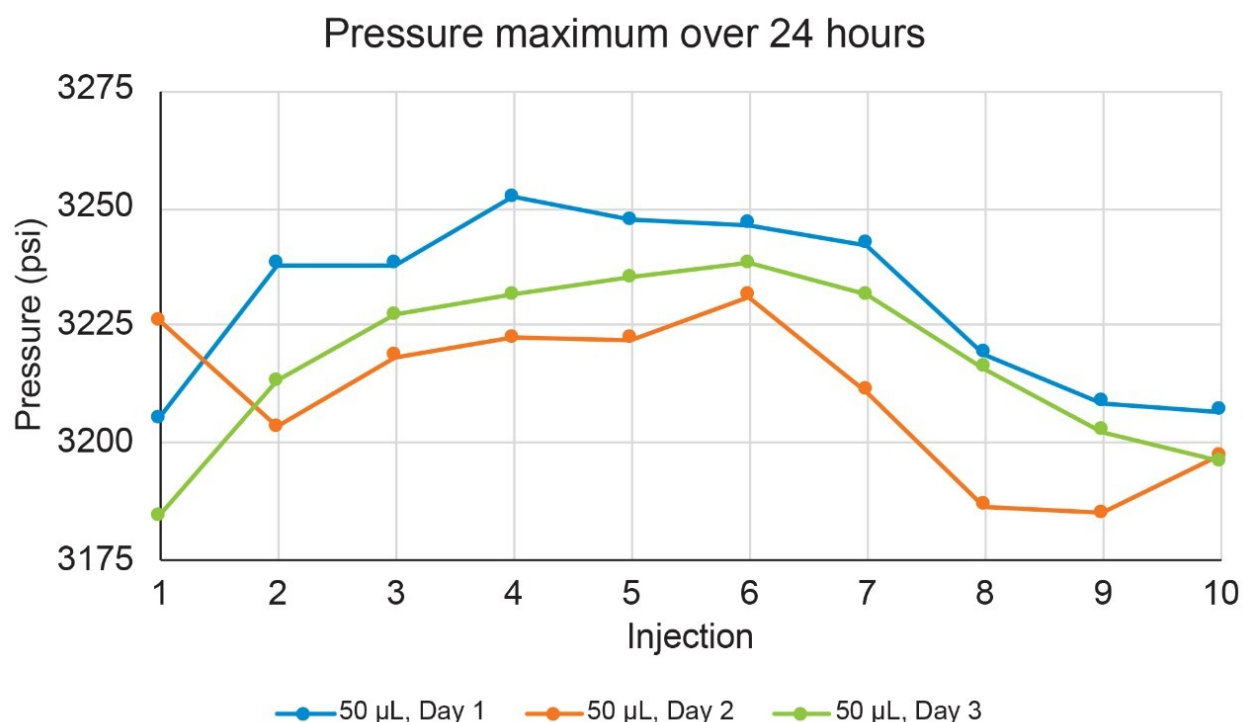


Figure 6. Maximum pressure for each of the ADC injections ($N=10$). A steady pressure trace over multiple days is indicative that clogging or precipitation is not a concern (Δ pressure ~ 50 psi for each sample set).

SEC: Combining Modern Chemistry and LC Technologies

SEC is a routine assay used in biopharmaceutical development that is traditionally used to assess protein aggregation, or high molecular weight species (HMWS). Legacy methods using columns having large particle size and high dispersion LC systems cannot achieve optimal resolution of low molecular weight species (LMWS) such as clips or fragments. With the advent of new column technologies and lower dispersion LC systems, better separation of these LMWS allows for more accurate quantitation for greater confidence and a deeper understanding of results.

The BioResolve SEC mAb Column (2.5 μ m, 4.6 mm x 150 mm) was used in combination with the ACQUITY UPLC H-Class PLUS Bio Binary System, a low dispersion LC system, to demonstrate that adequate resolution of the Waters mAb Size Variant Standard monomer and LMWS could be achieved (Figure 7). From the chromatogram, the HMWS are well separated from the monomer as expected due to a large difference in molecular weight. The LMWS 1&2 which would typically co-elute with the monomer peak when using traditional columns and LC systems is also adequately resolved, even when using a short column. To further evaluate results, peak area and

retention time are reported (Table 3). Results are shown to be highly reproducible for all species, regardless of relative peak area percentages. By incorporating more modern technologies into development and QC environments, routine assays can provide additional information compared to legacy methods.

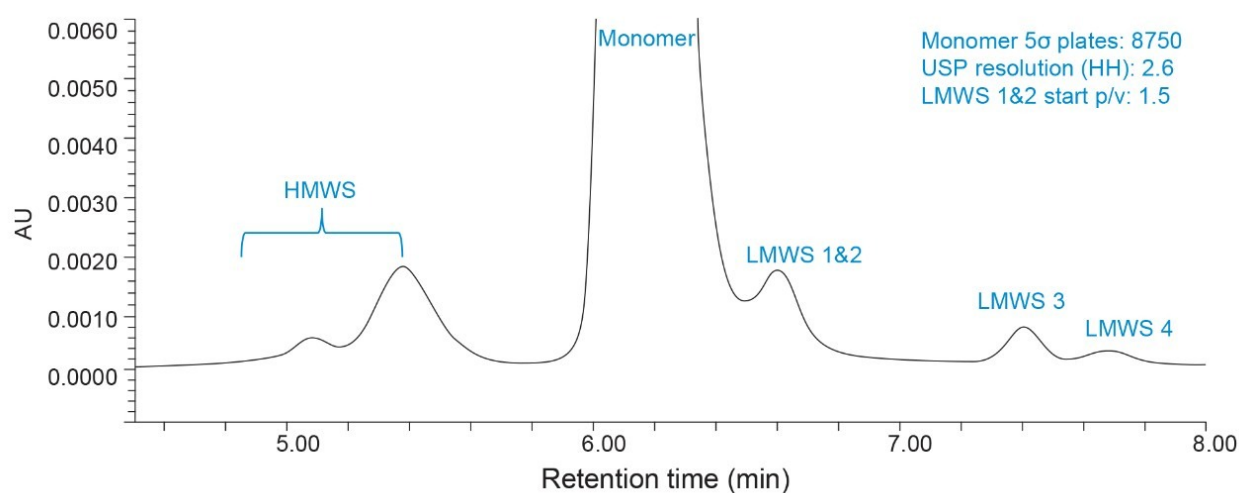


Figure 7. SEC of Waters mAb Size Variant Standard on a BioResolve SEC mAb Column (2.5 μ m, 4.6 mm x 150 mm) on a low dispersion LC system. Separation of both high molecular weight species (HMWS) and low molecular weight species (LMWS) from the monomer can be achieved. It should be noted that if greater resolution is desired, a 300 mm length column can be used.

	Retention time (N=4)		Peak area (N=4)	
	Average (min)	Std dev (min)	Average (%)	Std dev (%)
HMWS	5.370	0.002	2.200	0.033
Monomer	6.145	0.001	96.816	0.313
LMWS 1&2	6.602	0.003	0.458	0.005
LMWS 3	7.395	0.004	0.374	0.006
LMWS 4	7.681	0.005	0.150	0.000

Table 3. SEC comparison of average retention time (min) and peak area (%) (N=4).

Conclusion

Our previous work has demonstrated that the ACQUITY UPLC H-Class PLUS Bio Binary System provides superior performance for methods requiring slow flow rates and shallow gradients. Because many laboratories do not have the resources available to dedicate LC systems to individual assays, it is critical that reliable results can be generated as analytical methods are migrated among different LC platforms. HILIC, HIC, and SEC methods were used to assess performance of the ACQUITY UPLC H-Class PLUS Bio Binary System for methods more frequently run on quaternary LC systems. All the methods evaluated in this study could be used without making any changes or special considerations to traditionally used method conditions, demonstrating that the ACQUITY UPLC H-Class PLUS Bio Binary System is fit-for-purpose for common biopharmaceutical chromatographic methods.

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720007304, July 2021

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