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Overcoming the Challenges of Bispecific Antibody Characterization Using New Column Chemistries to Detect Product Impurities

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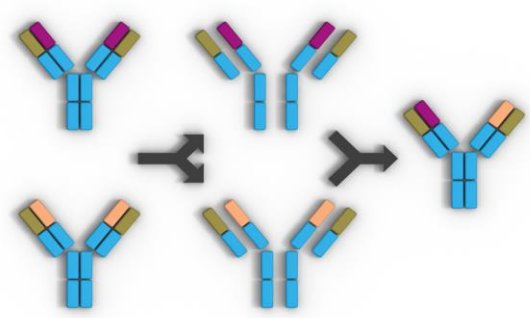
Introduction

As of today, three bispecific monoclonal antibodies (Bs-mAbs) are currently approved worldwide, Blinatumomab, Emicizumab and Amivantamab. These Bs-mAbs are representative of new engineered antibodies. Different Bs-mAbs have been studied containing various molecular architectures, for this reason the biomanufacturing of such molecules is a critical step. Keeping in mind critical parameters such as safety and efficacy, we provide an overview of the separation and characterization of Emicizumab using different biocolumns optimized for the separation of impurities such as aggregates and product related impurities. Intact and fragment analysis of the Bs-mAbs was obtained by reversed-phase chromatography (RP). Following RP chromatography, size exclusion chromatography (SEC), an ideal technique for the separation of protein aggregates, was used. Ion-exchange chromatography (IEX), a mild, nondenaturing technique often used for characterization and purification of proteins in their native form was performed. Finally, hydrophobic interaction chromatography (HIC) is presented.



Schematic overview showing the main steps for bispecific Ab production.

Experimental

Mobile phase & Sample Preparation

Emicizumab (mg/mL) was a gift from a customer. Monobasic and dibasic sodium hydrogen phosphate and sodium chloride were purchased from Millipore Sigma. All chemicals used were ≥99.5% pure. Mobile phases were prepared fresh daily and filtered through a 0.2 µm membrane filter before use.

Instrumentation

An Agilent 1260 Infinity II bio-inert LC System was used for SEC, IEX and HIC. LC/MS analysis were performed on a 1290 Infinity II LC coupled with a 6545XT AdvanceBio LC/Q-TOF system.

Experimental

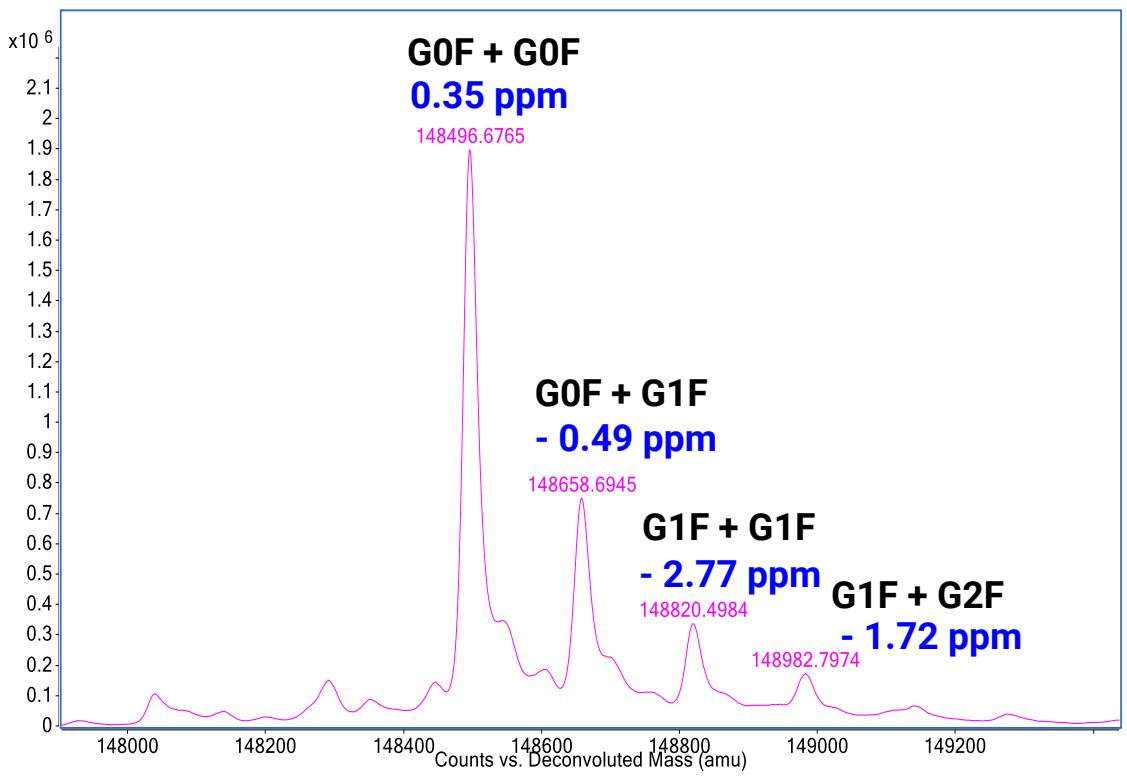
Agilent 1290 Infinity II LC		
Sample Type	Intact mAb	mAb Subunits (HC and LC)
Thermostat	4 °C	
Column	Agilent PLRP-S, 2.1 × 50 mm, 1000 Å, 5 µm (p/n: PL1912-1502)	
Column Temperature	80 °C	60 °C
Solvent A	0.1% Formic acid in DI water	
Solvent B	0.1% Formic acid in 100% acetonitrile	
Gradient	0–1 min, 0–20% B 1–3 min, 20–50% B 3–4 min, 50–70% B	0 min, 25% B 5 min, 55% B 6 min, 70% B 6–7 min, 70% B
Flow rate	0.5 mL/min	0.8 mL/min

Agilent 6545XT AdvanceBio LC/Q-TOF		
Sample Type	Intact mAb	mAb Subunits (HC and LC)
Source	Dual Agilent Jet Stream	Dual Agilent Jet Stream
Gas Temp	350 °C	350 °C
Gas Flow	12 L/min	12 L/min
Nebulizer	60 psig	35 psig
Sheath Gas Temp	400 °C	350 °C
Sheath Gas Flow	11 L/min	11 L/min
VCap	5500 V	4000 V
Nozzle Voltage	2000 V	500 V
Fragmentor	380 V	180 V
Skimmer	140 V	65 V
Quad amu	1000	300
Mass Range	500-8000 *m/z*	100-3200 *m/z*
Acquisition Rate	1.0 spectra/s	1.0 spectra/s
Reference Mass	922.0098	922.0098
Acquisition Mode	Positive, Extended (10,000 *m/z*) Mass Range	Positive, Standard (3200 *m/z*) Mass Range, HiRes (4 GHz)

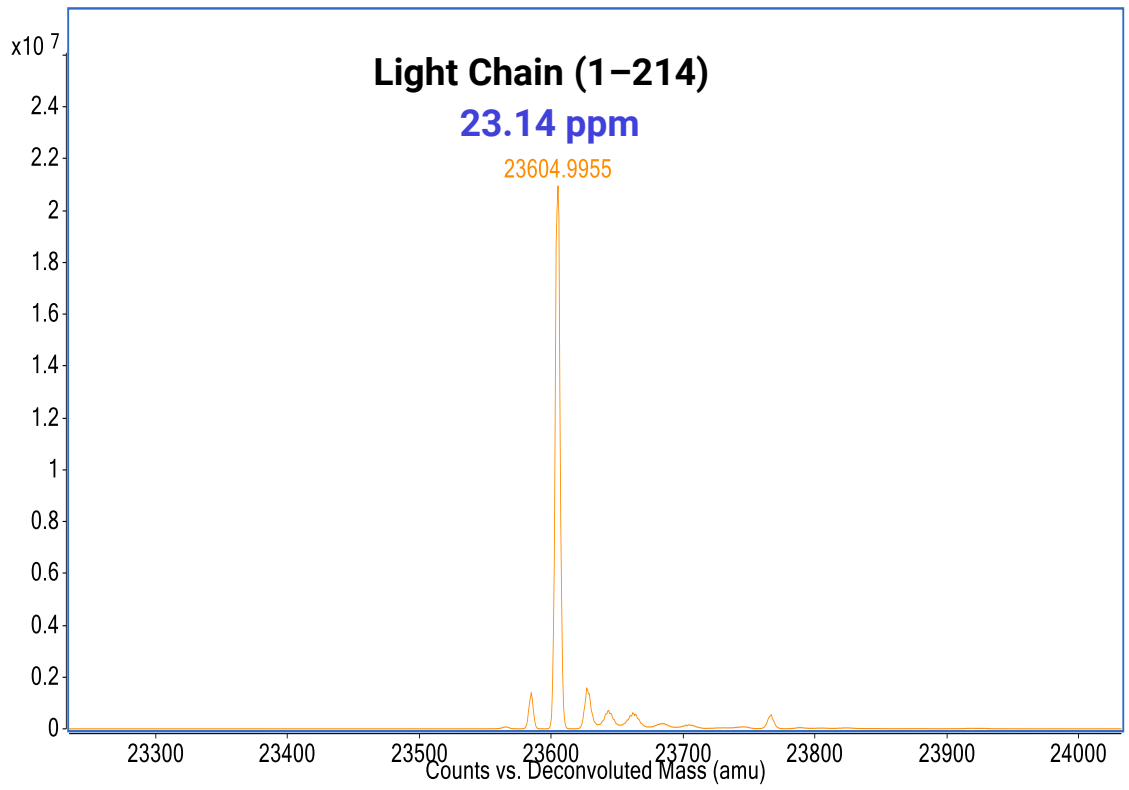
Intact and fragment analysis by reversed-phase chromatography (RP)

LC/MS was performed on both the intact and reduced sample using a PLRP-S reversed-phase column, the deconvoluted intact mass clearly shows the different glycoforms corresponding to the different combinations of G0F, G1F, and G2F on both heavy chains. Analysis of the reduced sample gave a single light chain, and two nonidentical heavy chains corresponding to the expected 1 to 444 and 1 to 448 sequences.

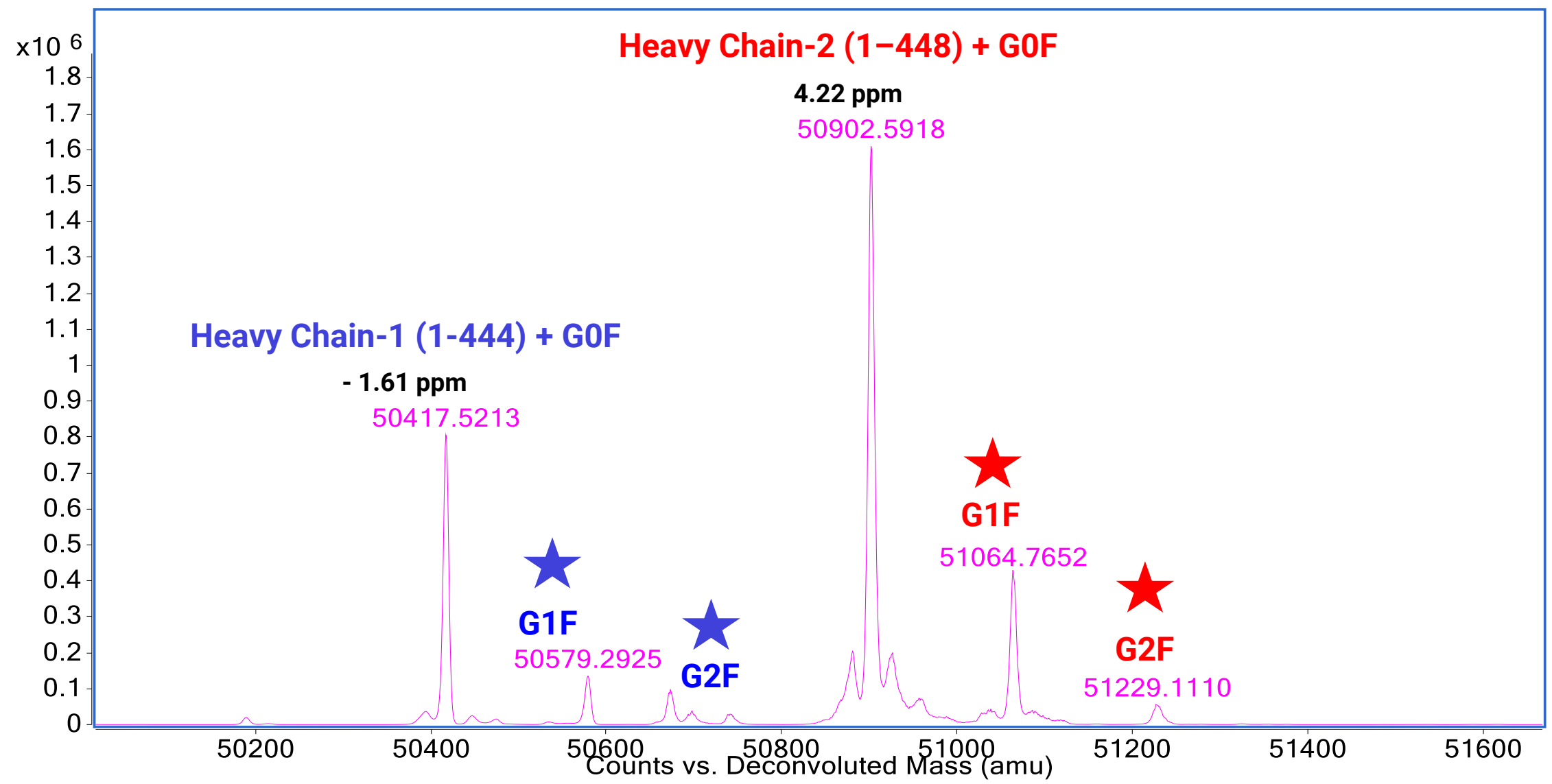
MS deconvolution spectra of intact Emicizumab (0.25 µL injection).



Reversed-phase chromatography of Emicizumab subunit – light chain (0.5 µL injection).



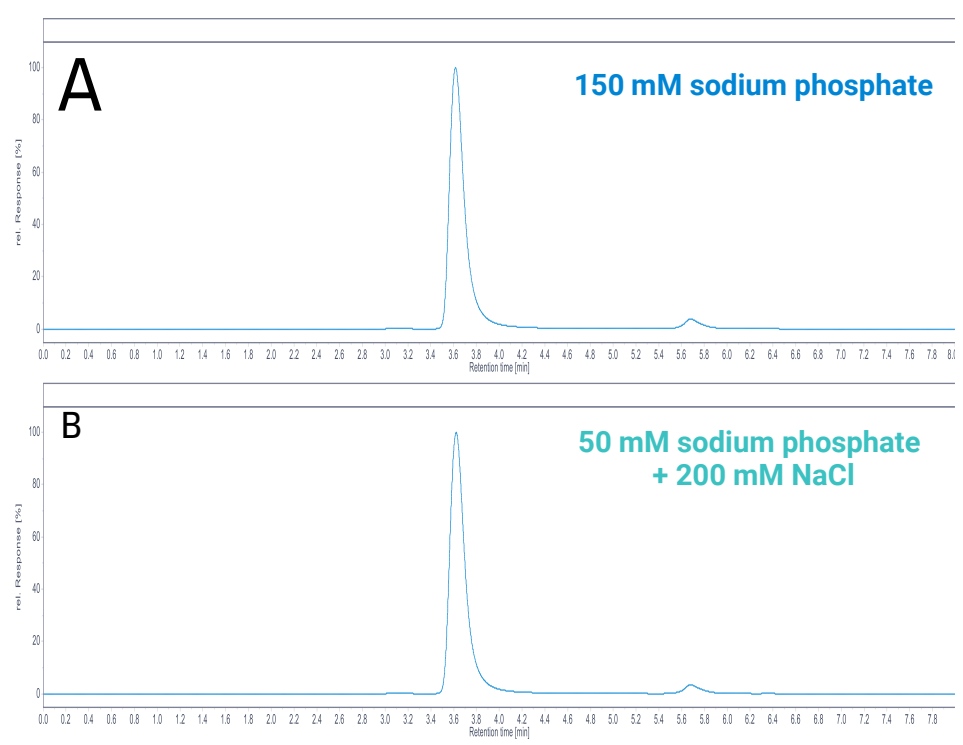
Reversed phase chromatography of Emicizumab of Emicizumab subunit – heavy chains (0.5 µg injection).

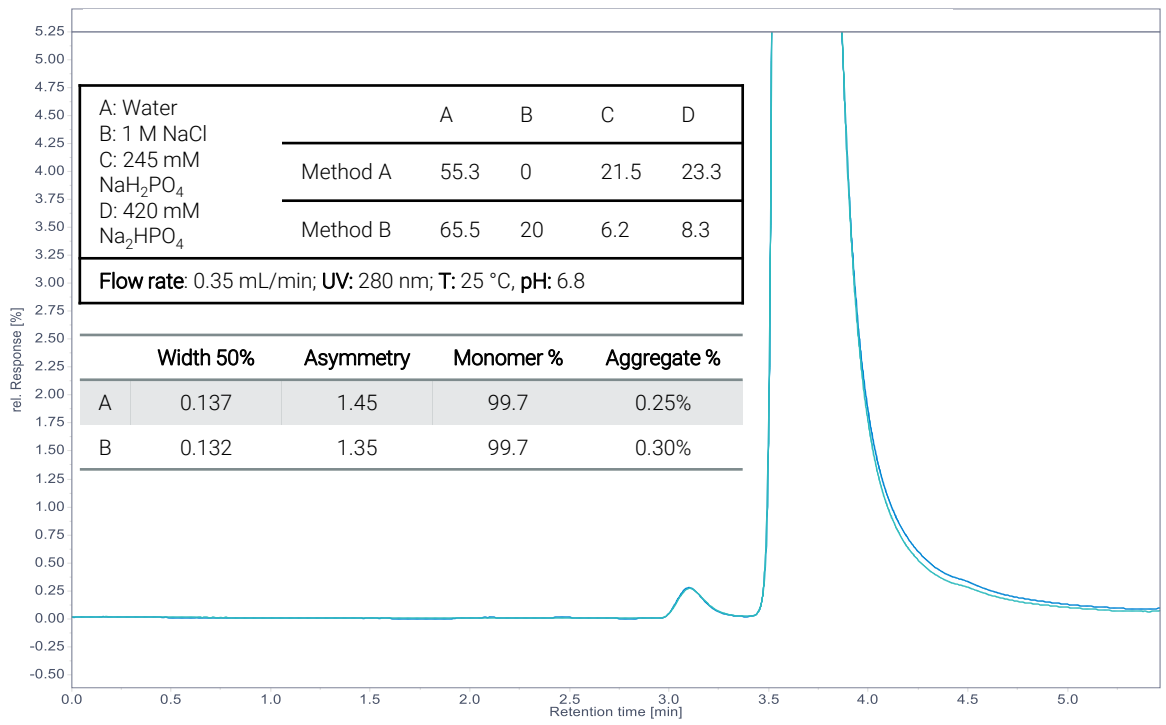


Results and Discussion

Intact and aggregate analysis by size exclusion chromatography (SEC)

SEC was used to characterize size variants. The separation was performed with an AdvanceBio SEC 200 Å 1.9 µm column. Two mobile phases, 150 mM phosphate buffer at pH 6.8 and 50 mM phosphate buffer with 200 mM NaCl at pH 6.8 were investigated for SEC analysis.



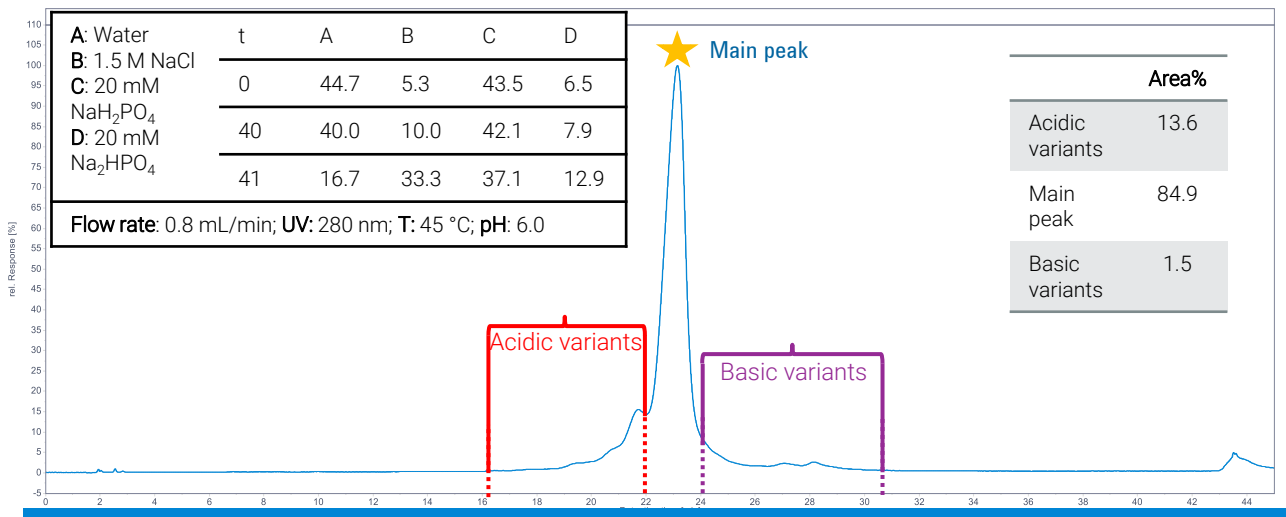


	A	B	C	D
Method A	55.3	0	21.5	23.3
Method B	65.5	20	6.2	8.3

	Width 50%	Asymmetry	Monomer %	Aggregate %
A	0.137	1.45	99.7	0.25%
B	0.132	1.35	99.7	0.30%

Charge variants analysis by ion exchange chromatography (IEX)

The charge heterogeneity could indicate different PTMs including glycosylation and loss of C-terminal lysine among others, which alter the surface charge of the protein. These modifications need to be monitored and quantified throughout drug manufacturing.

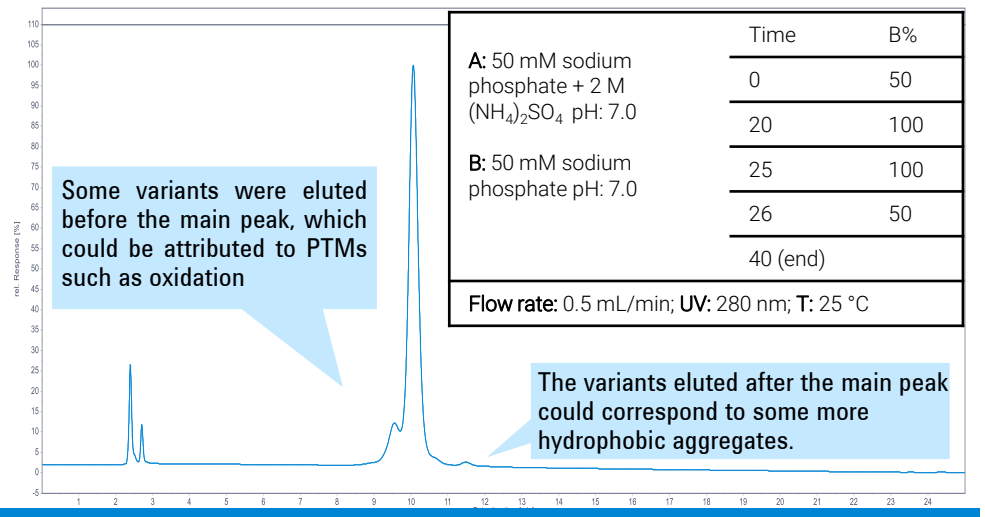


t	A	B	C	D
0	44.7	5.3	43.5	6.5
40	40.0	10.0	42.1	7.9
41	16.7	33.3	37.1	12.9

	Area%
Acidic variants	13.6
Main peak	84.9
Basic variants	1.5

Intact analysis by Hydrophobic interaction chromatography (HIC)

The AdvanceBio HIC columns with finely tuned hydrophobicity are suitable for a wide range of modalities including IgG like Bs-MAbs.



Time	B%
0	50
20	100
25	100
26	50
40 (end)	

Conclusions

This study demonstrates that different chromatographic techniques can be used to overcome the drawbacks associated with the analysis of difficult molecules such as bispecific antibodies.

- Reversed phase RP was used to confirm the correct structure with both intact mass and verification of identical light chains and nonidentical heavy chains through fragment analysis of the reduced molecule.
- SEC, IEX, and HIC were also successfully used to determine size variants, charge variants, and PTMs, respectively, supporting the typical CQAs of such complex molecules.

References

- Surmounting the Challenges of Bispecific Antibody Characterization (5994-2997EN)

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