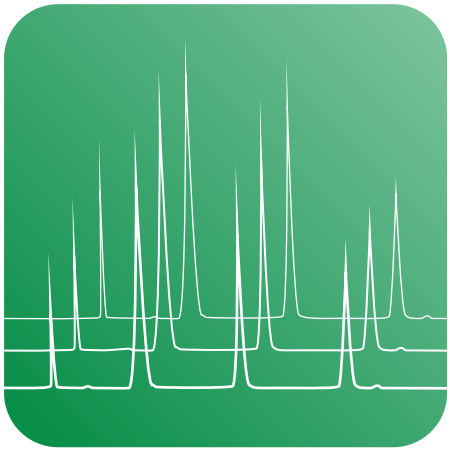
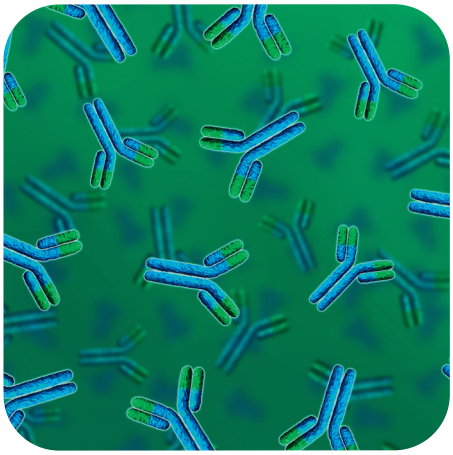



HIC



HIC – HPLC selectivities

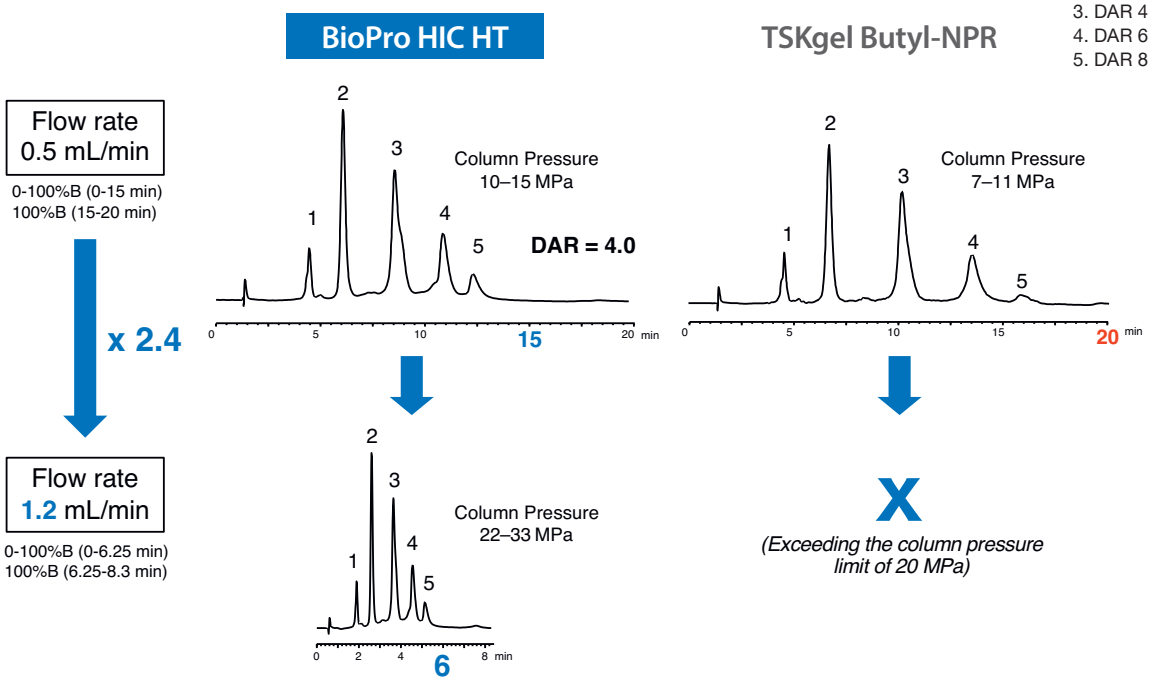
- **Specifically designed for drug-to-antibody conjugates (ADCs) and antibodies**
- **Ideal drug-to-antibody ratio (DAR) analysis**
- **High throughput by reducing analysis time**
- **Excellent batch-to-batch reproducibility**
- **Long term stability**

	BioPro HIC HT	BioPro HIC BF
Base particle	hydrophilic polymer (polymethacrylate)	hydrophilic polymer (polymethacrylate)
Particle size / µm	2.3	4
Pore	non-porous	non-porous
Functional group	butyl	butyl
pH range	2–12	2–12
Pressure limit (for 100 mm)	40 MPa (5,800 psi)	20 MPa (2,900 psi)
Temperature range	10–60 °C	10–60 °C

High column stability

High throughput by shortening analysis time using high flow rates

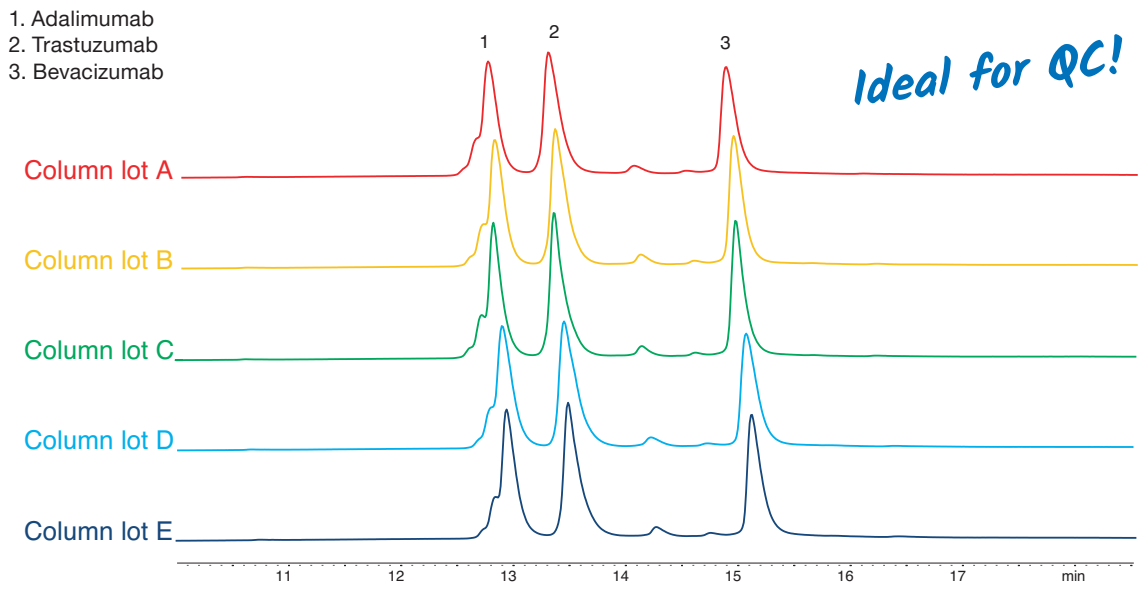
1. DAR 0
2. DAR 2
3. DAR 4
4. DAR 6
5. DAR 8



Column:	100 x 4.6 mm ID	Temperature:	25 °C
Part No.:	BHH00SQ3-1046PTH	Detection:	UV at 280 nm
Eluent:	A) 20 mM NaH_2PO_4-Na_2HPO_4 containing 1.0 M $(NH_4)_2SO_4$ (pH 7.0)	Injection:	10 µL
	B) 20 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)/2-propanol (85/15)	Sample:	Brentuximab vedotin (2.5 mg/mL)

BioPro HIC HT improves analysis throughput of ADCs by 2–3 times with an excellent Drug-to-Antibody Ratio (DAR). The rapid analysis is possible without loss of resolution. Competitor HIC columns fail under these conditions.

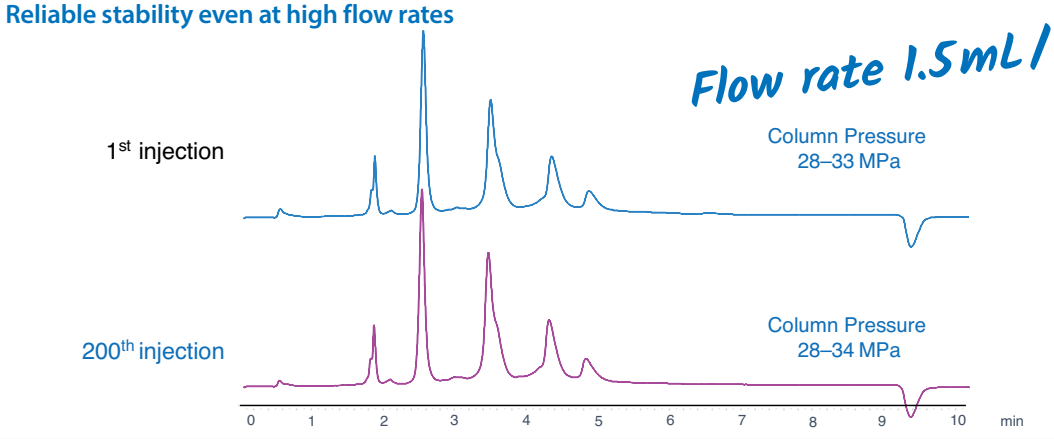
Excellent batch-to-batch reproducibility



Column:	BioPro HIC HT (2.3 µm) 100 x 4.6 mm ID	Flow rate:	0.5 mL/min
Part No.:	BHH00SQ3-1046PTH	Temperature:	25°C
Eluent:	A) 100 mM NaH_2PO_4-Na_2HPO_4 containing 2.0 M $(NH_4)_2SO_4$ (pH 7.0)	Detection:	UV at 280 nm
	B) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)	Injection:	15 µL
Gradient:	0%B (0–1 min), 0–100%B (1–11 min), 100%B (11–15 min)		

BioPro HIC HT exhibits an excellent batch-to-batch reproducibility making it the ideal choice for quality control analysis of biopharmaceuticals such as mAbs.

Exceptional stability



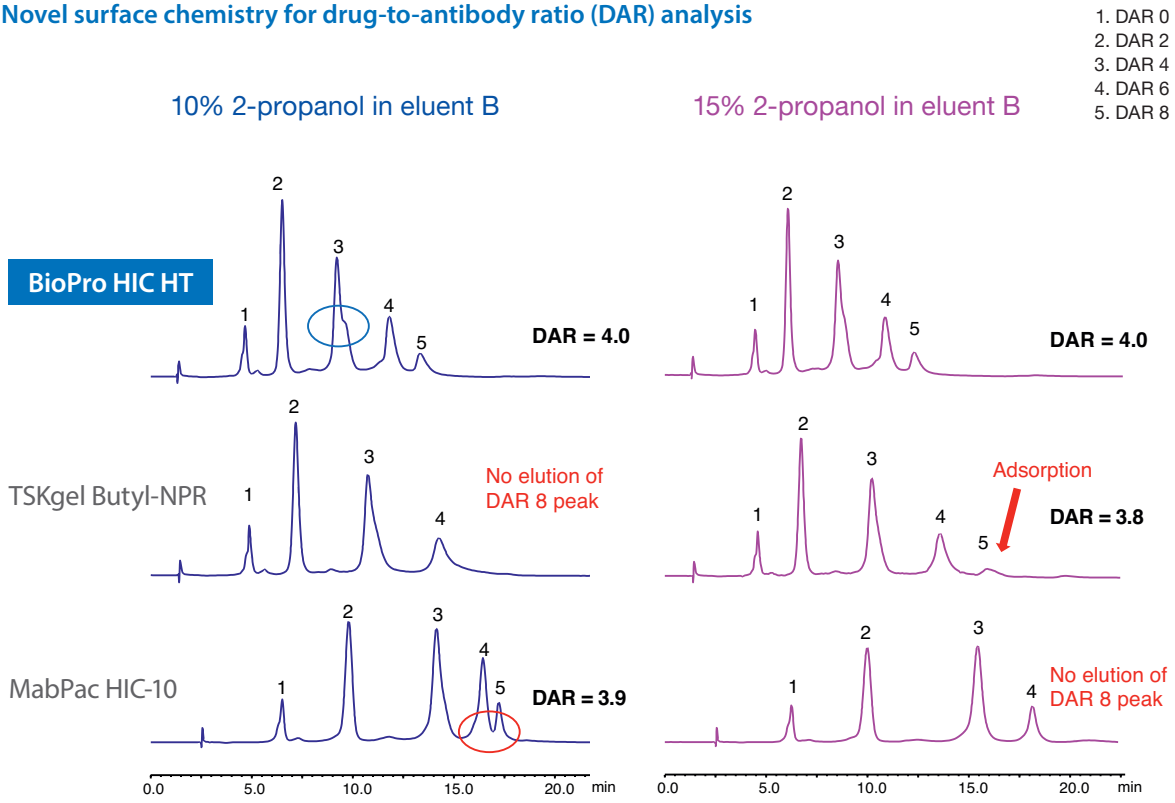
Column:	BioPro HIC HT (2.3 µm) 100 x 4.6 mm ID	Flow rate:	1.5 mL/min
Part No.:	BHH00SQ3-1046PTH	Temperature:	25°C
Eluent:	A) 20 mM NaH_2PO_4-Na_2HPO_4 containing 1.0 M $(NH_4)_2SO_4$ (pH 7.0)	Detection:	UV at 280 nm
	B) 20 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)/2-propanol (90/10)	Injection:	10 µL
Gradient:	0–100%B (0–5 min), 100%B (5–8.33 min), 0%B (8.33–12 min)	Sample:	Brentuximab vedotin (2.5 mg/mL)

BioPro HIC HT offers excellent stability under high flow rates / high pressure conditions due to its unique rigid particle and optimised column packing technology.

HIC – BioPro HIC: ADC analysis

Designed for analysis of ADCs

Novel surface chemistry for drug-to-antibody ratio (DAR) analysis



Column:	100 x 4.6 mm ID
Part No.:	BHH00SQ3-1046PTH
Eluent:	A) 20 mM NaH_2PO_4-Na_2HPO_4 containing 1.0 M $(NH_4)_2SO_4$ (pH 7.0) B) 20 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)/2-propanol (90/10) or (85/15)
Gradient:	0–100%B (0–15 min), 100%B (15–20 min), 0%B (20–35 min)
Flow rate:	0.5 mL/min
Temperature:	25°C
Detection:	UV at 280 nm
Injection:	10 µL
Sample:	Brentuximab vedotin (2.5 mg/mL)

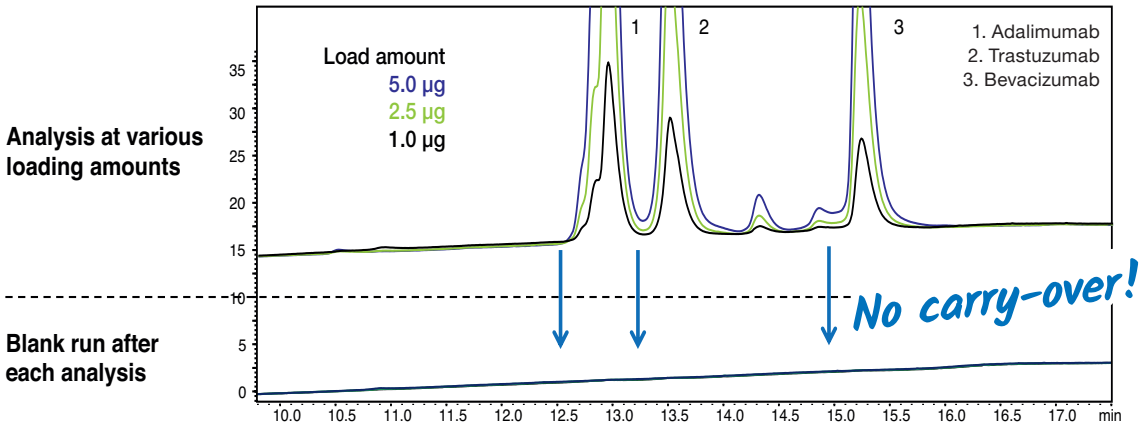
BioPro HIC HT offers higher resolution than conventional HIC columns. Its surface modification suppresses excessive or too strong adsorption of ADCs and results in highly reliable quantification. With varying 2-propanol content, all peaks are completely eluted from the BioPro HIC HT column with high resolution. Another peak is partially separated from peak 3. Additionally, the same DAR values are observed at any content of 2-propanol.

BioPro HIC HT offers:

- **Higher resolution than conventional HIC columns**
- **Highly reliable quantification**
- **Flexible method development**

Excellent recovery and virtually no carry-over

Highly accurate quantification of ADCs and antibodies

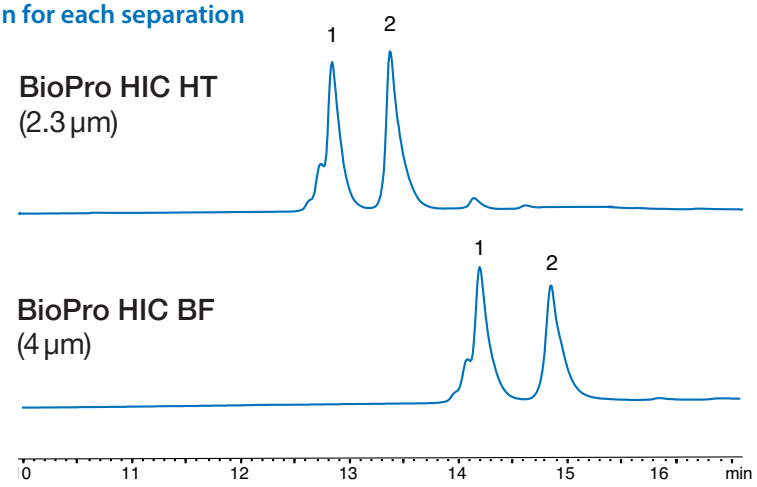


Column:	BioPro HIC HT (2.3 µm) 100 x 4.6 mm ID	Gradient:	0%B (0–1 min), 0–100%B (1–11 min), 100%B (11–15 min)
Part No.:	BHH00SQ3-1046PTH	Flow rate:	0.5 mL/min
Eluent:	A) 100 mM NaH_2PO_4-Na_2HPO_4 containing 2.0 M $(NH_4)_2SO_4$ (pH 7.0)	Temperature:	25 °C
	B) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)	Detection:	UV at 280 nm

BioPro HIC HT offers higher linearity over wide loading and virtually no carry-over. This contributes to highly accurate quantitation of ADCs and antibodies.

Different hydrophobicity

The right column for each separation



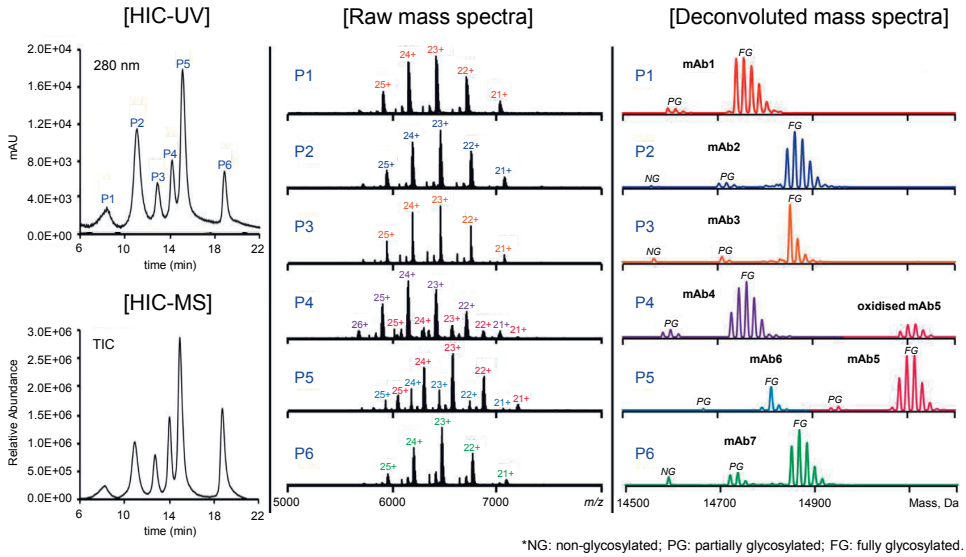
Column:	100 x 4.6 mm ID	Flow rate:	0.5 mL/min
Part Nos.:	BHH00SQ3-1046PTH	Temperature:	25 °C
	BHB00S04-1046WT	Detection:	UV at 280 nm
Eluent:	A) 100 mM NaH_2PO_4-Na_2HPO_4 containing 2.0 M $(NH_4)_2SO_4$ (pH 7.0)	Injection:	15 µL
	B) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)	Sample:	1. Adalimumab (Humira®; 0.5 mg/mL)
Gradient:	0%B (0–1 min), 0–100%B (1–11 min), 100%B (11–15 min)		2. Trastuzumab (Herceptin®; 0.5 mg/mL)

BioPro HIC HT is the first choice for ADCs or mAbs. BioPro HIC BF columns show a stronger retention and can therefore be used for the separation of low hydrophobic proteins or especially for the analysis of oxidised mAbs.

HIC – BioPro HIC: Direct HIC-MS coupling

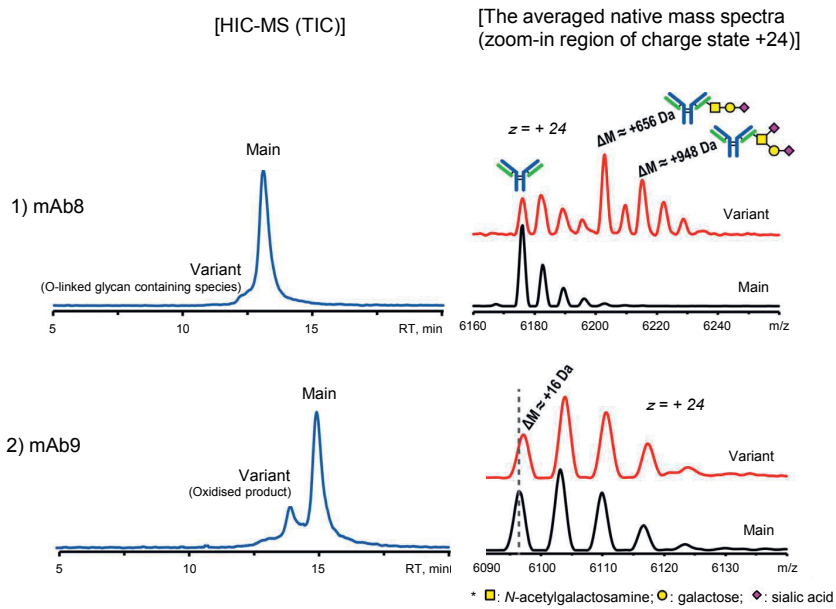
Online native HIC-MS analysis of mAbs and their molecular variants

Separation of an antibody mixture of seven different mAbs



*NG: non-glycosylated; PG: partially glycosylated; FG: fully glycosylated.

Separation of two mAbs from their molecular variants



* □: *N*-acetylgalactosamine; ○: galactose; ◆: sialic acid

Column:	BioPro HIC BF (4 µm) 100 x 4.6 mm ID	Injection:	mAb mixture: 3 µL (3–6 µg) mAb 8 and mAb 9: 10 µg each
Part No.:	BHB00S04-1046WT	Sample:	Mixture of 7 in-house mAbs at 1–2 mg/mL each 2 in-house mAbs with molecular variants
Eluent:	A) 3 M ammonium acetate in water B) 100 % water	Setup:	Post-column makeup flow: 100 % water at 1.5 mL/min (reducing salt conc. 6-fold) Splitter to reduce the flow rate to 1–5 µL/min
Gradient:	0 %B (0–2 min), 0–90 %B (2–18 min), 90 %B (18–22 min)		
Flow rate:	0.3 mL/min		
Temperature:	ambient		
Detection:	UV at 280 nm, NSI-MS		

Courtesy by S. Wang, Regeneron Pharmaceuticals Inc.

To enable simultaneous UV and MS detection a post-column makeup flow and a splitter were used. The make-up flow decreases the salt concentration while the splitter reduces the flow rate to enable the coupling to MS. A nanospray ionisation (NSI) was chosen because of its high sensitivity and salt tolerance.

Reference: Y. Yan, T. Xing, S. Wang, T. J. Daly, N. Li, Online coupling of analytical hydrophobic interaction chromatography with native mass spectrometry fo the characterization of monoclonal antibodies and related products, J. Pharm. Biomed. Anal. 186 (2020) 113313.

The influence of salts in HIC separations

The technique known as hydrophobic interaction chromatography is a mode of chromatography that separates proteins by differences in surface hydrophobicity. [1] This method utilises reversible interactions that occur between protein molecules and hydrophobic stationary phase ligands attached to the particle surface.

Certain non-denaturing salts are used to improve the hydrophobic interactions between proteins and the stationary phase. The mobile phase is typically an aqueous solution of salts such as ammonium sulfate or sodium chloride and a buffer to control pH (usually phosphate buffer between pH 6 and 7). The Hofmeister series of lyotropic and chaotropic ions shown below in Fig. 1 provides a template for salt selection. High concentrations of salt, particularly ammonium sulfate, may precipitate proteins; therefore, solubility should be checked under the initial gradient (binding) conditions. The strength of the interaction between the protein and stationary phase decreases with decreasing salt gradient (see Fig. 2). Another option is a change of pH which results in an increase or decrease in the charge on the protein due to the ionisation of acidic or basic groups.

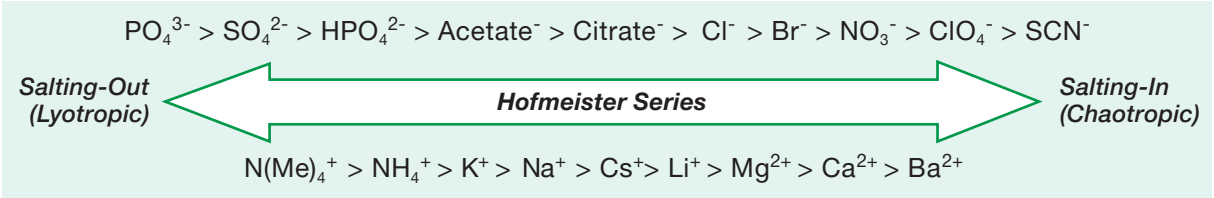


Fig. 1: The Hofmeister Series of lyotropic and chaotropic ions.

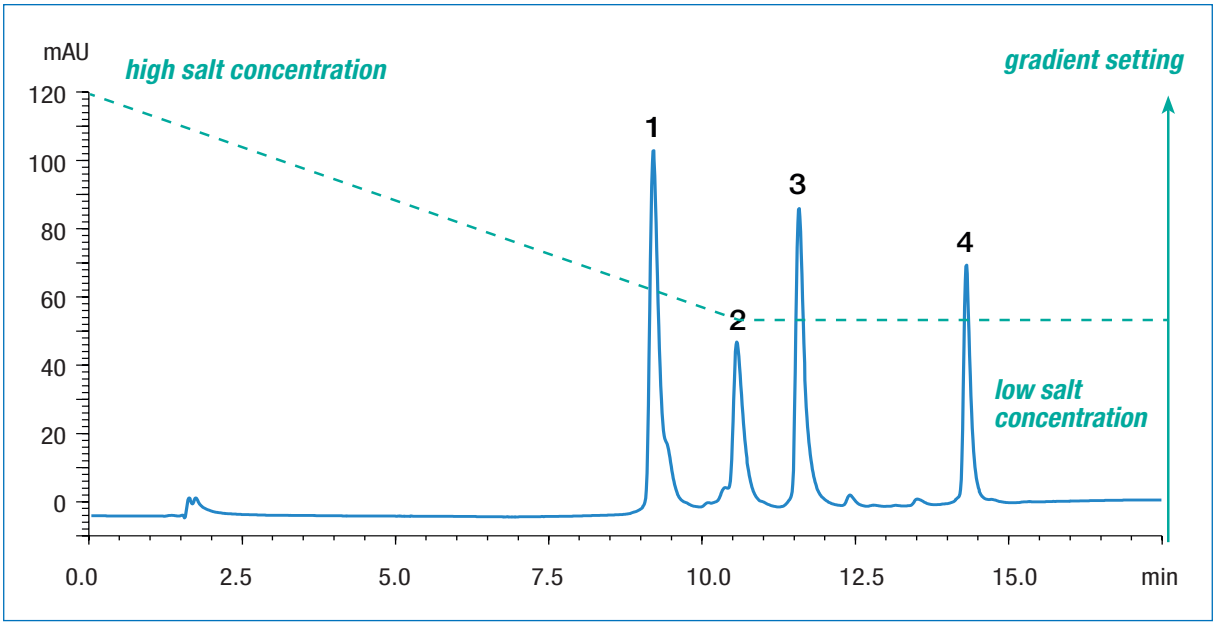


Fig. 2: Method with decreasing salt gradient.

Column:	BioPro HIC BF (100 x 4.6 mm ID)	Samples:	1. Myoglobin (0.73 mg/mL)
Part No.:	BHB00S04-1046WT		2. Ribonuclease A (0.75 mg/mL)
Eluent:	A) 100 mM NaH_2PO_4-Na_2HPO_4 containing 2.0 M $(NH_4)_2SO_4$ (pH 7.0)		3. Lysozyme (0.25 mg/mL)
	B) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)		4. α-Chymotrypsinogen A (0.25 gm/mL)
Flow rate:	0.5 mL/min		
Gradient:	0–100%B (0–11 min), 100%B (11–15 min)		
Temperature:	25°C		
Detection:	UV at 280 nm		
Injection:	15 µL		

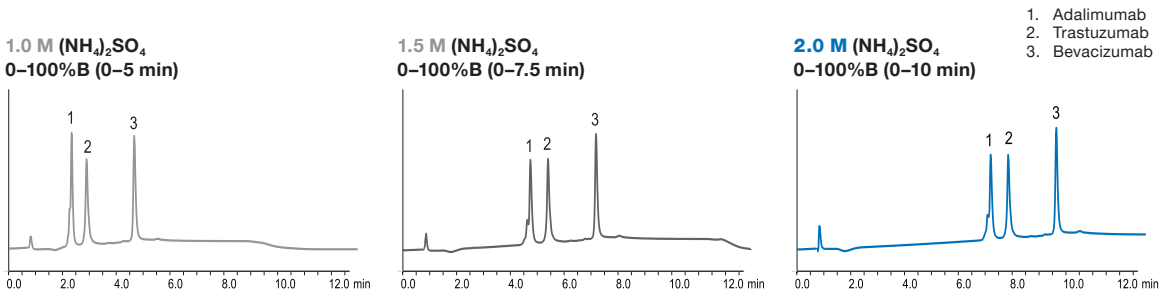
HIC is particularly effective when used to separate proteins and monoconal antibodies. The separation of monoclonal antibodies, mAb aggregates and glycosylated mAbs can be achieved due to their specific hydrophobic properties. It also provides an excellent method for determination of drug-to-antibody ratios in antibody-drug conjugates.

[1] Queiroza, J.A.; Tomaza, C.T.; Cabral, J.M.: Hydrophobic interaction chromatography of proteins, J Biotechnol. 2001, 87, 143-159.

HIC – Expert Tips: Separation factors

Effect of initial salt concentration

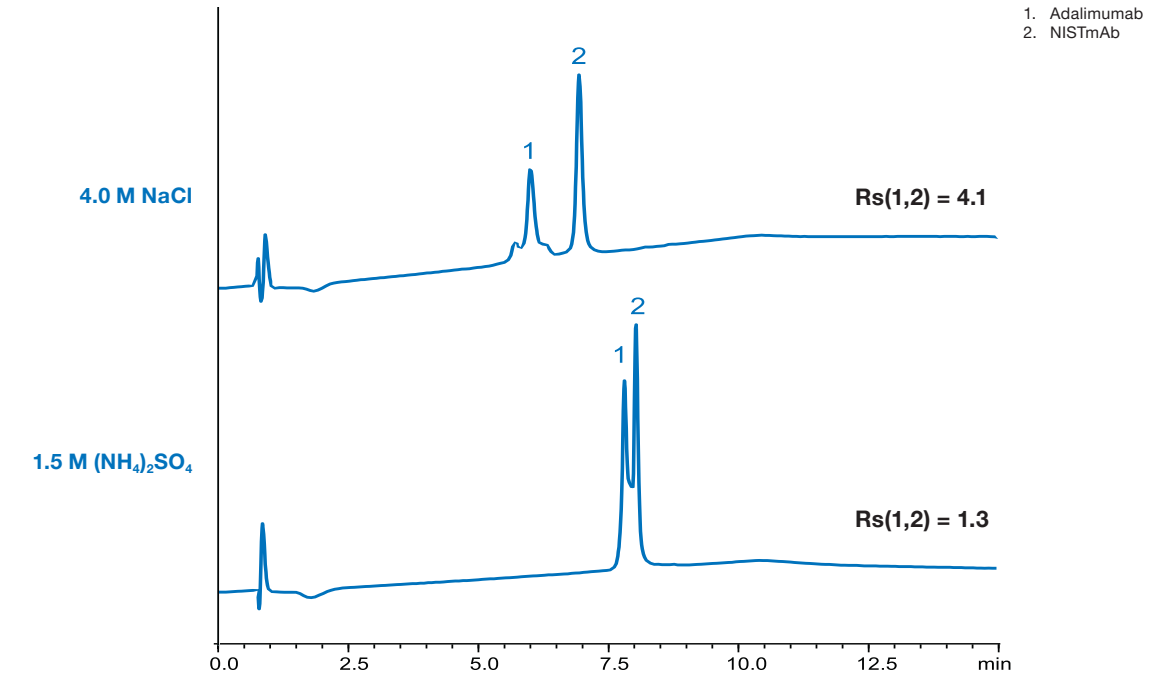
Buffers containing $(NH_4)_2SO_4$ are often used as a mobile phase in HIC mode because $(NH_4)_2SO_4$ has a strong salting-out effect. The higher the initial concentration of $(NH_4)_2SO_4$; the stronger will be the retention of proteins. Therefore, a buffer with a high salt concentration is more suitable for the separation of low hydrophobic proteins with weak retention.



Column:	BioPro HIC BF (4 µm) 100 x 4.6 mm ID	Flow rate:	1.0 mL/min
Part No.:	BHB00S04-1046WT	Temperature:	25 °C
Eluent:	A) 100 mM NaH_2PO_4-Na_2HPO_4 containing salt (pH 7.0)	Detection:	UV at 280 nm
	B) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)	Injection:	5 µL (0.5 mg/mL)
Gradient:	0.2 M/min (gradient slope is the same)		

Influence of the type of salt

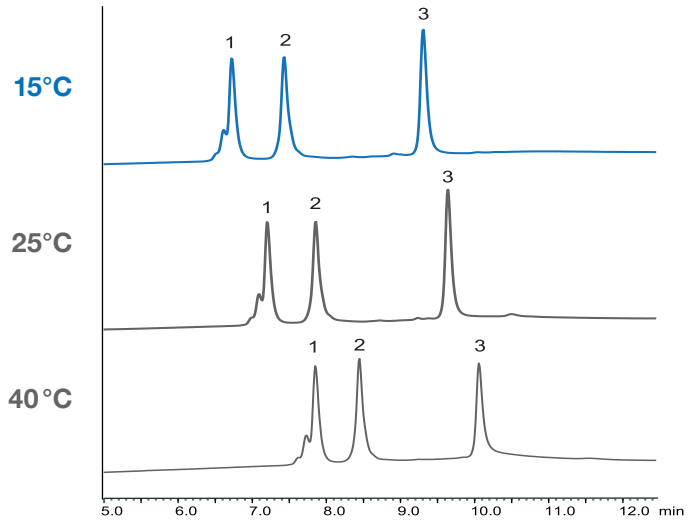
NaCl and CH_3COONH_4 are also used as buffer salts. The separation selectivity varies with the type of salt used in some cases, so changing the type of salt can also be effective when the separation is not sufficient. However, these salts have to be used at very high concentrations to gain retentions comparable to $(NH_4)_2SO_4$. Attention needs to be paid to the prevention of precipitation of salts in the buffer and damage of the LC system.



Column:	BioPro HIC BF (4 µm) 100 x 4.6 mm ID	Flow rate:	1.0 mL/min
Part No.:	BHB00S04-1046WT	Temperature:	25 °C
Eluent:	A) 100 mM NaH_2PO_4-Na_2HPO_4 containing salt (pH 7.0)	Detection:	UV at 280 nm
	B) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)	Injection:	10 µL (0.25 mg/mL)
Gradient:	0–100%B (0–10 min), 100%B (10–15 min)		

Temperature influence

In HIC mode, higher temperatures result in longer retention times of proteins. This assumes that the hydrophobic area interacting with the stationary phase becomes larger due to a change in the structure of proteins with increasing temperature so that the hydrophobic interactions become stronger.

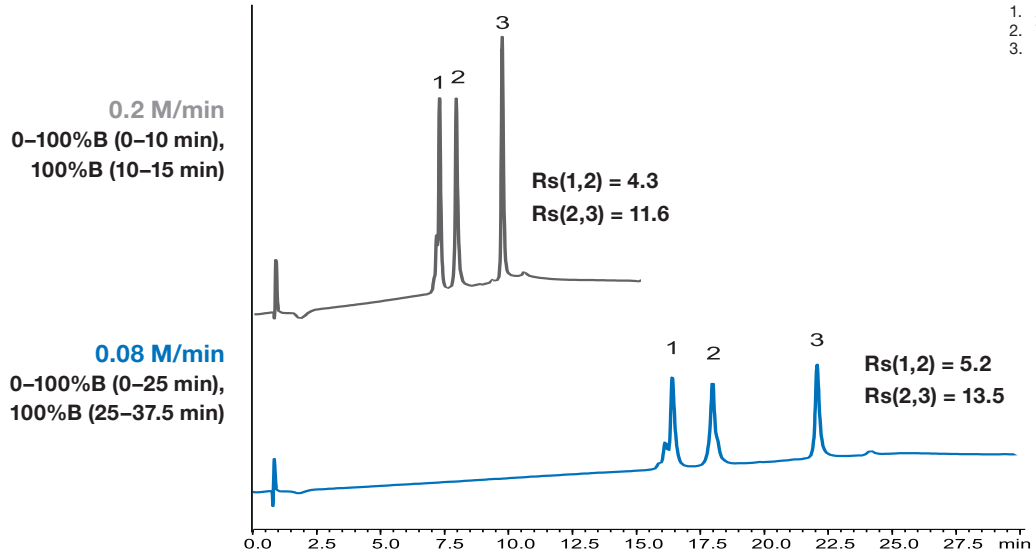


1. Adalimumab
2. Trastuzumab
3. Bevacizumab

Column:	BioPro HIC BF (4 µm) 100 x 4.6 mm ID	Gradient:	0–100%B (0–10 min), 100%B (10–15 min)
Part No.:	BHB00S04-1046WT	Flow rate:	1.0 mL/min
Eluent:	A) 100 mM NaH_2PO_4-Na_2HPO_4 containing 2.0 M $(NH_4)_2SO_4$ (pH 7.0)	Detection:	UV at 280 nm
	B) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)	Injection:	5 µL (0.5 mg/mL)

Variation of gradient slope

In general, shallower gradients improve the separation and the resulting resolution.



1. Adalimumab
2. Trastuzumab
3. Bevacizumab

Column:	BioPro HIC BF (4 µm) 100 x 4.6 mm ID	Flow rate:	1.0 mL/min
Part No.:	BHB00S04-1046WT	Temperature:	25 °C
Eluent:	A) 100 mM NaH_2PO_4-Na_2HPO_4 containing 2.0 M $(NH_4)_2SO_4$ (pH 7.0)	Detection:	UV at 280 nm
	B) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)	Injection:	10 µL (0.5 mg/mL)

HIC – Ordering information

2.3 and 4 µm non-porous analytical columns (max. pressure 20–40 MPa)

Phase	Particle size [µm]	Column ID [mm]	Column length [mm]	Part number	Precolumn filter 2 µm*
					(pack of 5)
BioPro HIC HT	2.3	4.6	100	BHH00SQ3-1046PTH	XRPRCS35
		4.6	33	BHH00SQ3-H346PTH	XRPRCS35
BioPro HIC BF	4	4.6	100	BHB00S04-1046WT	XRPRCS35

*Holder required, part no XRPRCS03
Other dimensions on demand