Strategies to Improve Your HIC Analysis

You will learn insights about:

- **How to increase the retention by using elevated temperature and high initial salt concentrations**
- **The influence of salt concentration on sample precipitation**
- **Improving hydrophobic interactions by selecting the right pH**
- **How shallower gradients provide higher resolution**
- **The influence of organic modifiers on peak shape and retention**

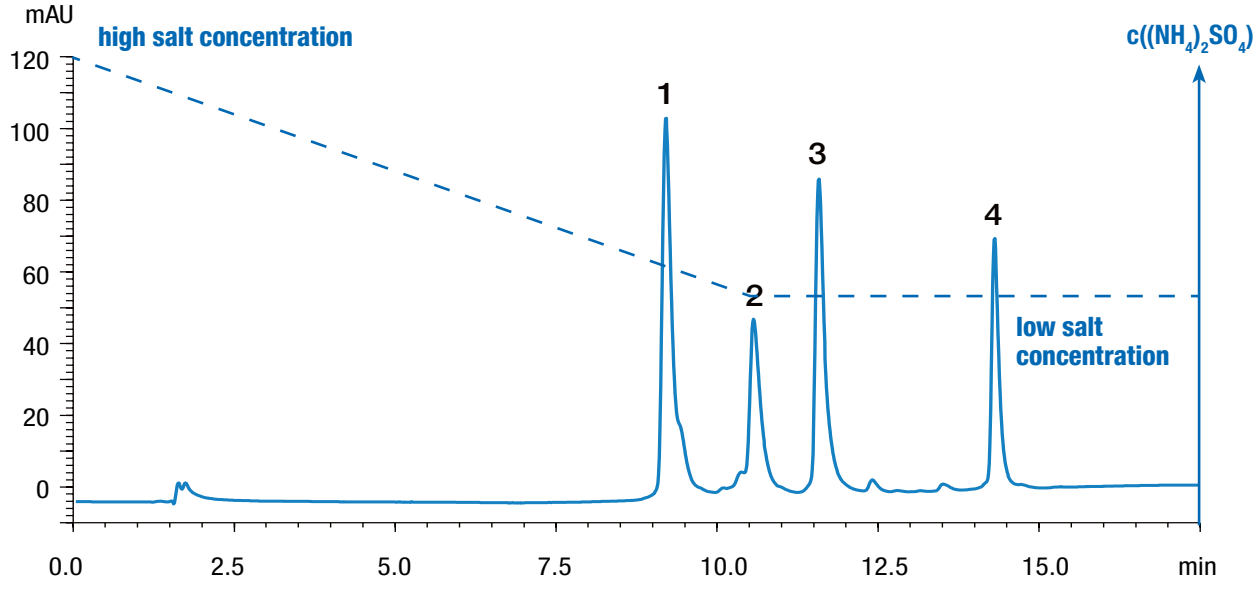
Hydrophobic interaction chromatography (HIC)

HIC is a separation mode used for the analysis of biomolecules like:

- **antibody-drug-conjugates (ADCs)**
- **monoclonal antibodies (mAbs)**
- **proteins in general.**

Analytes are separated based on the differences in their surface hydrophobicity similar to reversed phase (RP) chromatography. However, a big plus is that analytes maintain their original structure and therefore functionality, making HIC a useful separation mode for intact protein analysis or protein purification.

HIC utilises reversible interactions that occur between the analyte and hydrophobic stationary phase ligands attached to the particle surface by applying an inverse salt gradient.
A high salt concentration in the beginning of the gradient enhances the hydrophobic interactions between the target molecules and the stationary phase resulting in their retention. By decreasing the salt concentration, the strength of the interaction weakens and the analytes elute (see Fig. 1).



Column:	BioPro HIC BF (100 x 4.6 mm ID)
Part No.:	BHB00S04-1046WT
Eluent:	A) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0) containing 2.0 M $(NH_4)_2SO_4$ B) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)
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

Samples:
1. Myoglobin (0.73 mg/mL)
2. Ribonuclease A (0.75 mg/mL)
3. Lysozyme (0.25 mg/mL)
4. α-Chymotrypsinogen A (0.25 gm/mL)

Figure 1: HIC analysis of four proteins using a decreasing salt gradient on a BioPro HIC BF column.

The least hydrophobic analytes are eluted at still high salt concentrations, whilst more hydrophobic molecules may require mobile phases with lower salt concentration or even free of salts for their elution (see Fig. 2). Often, also certain amounts of organic solvents such as isopropanol or ethanol are used for separating very hydrophobic compounds.

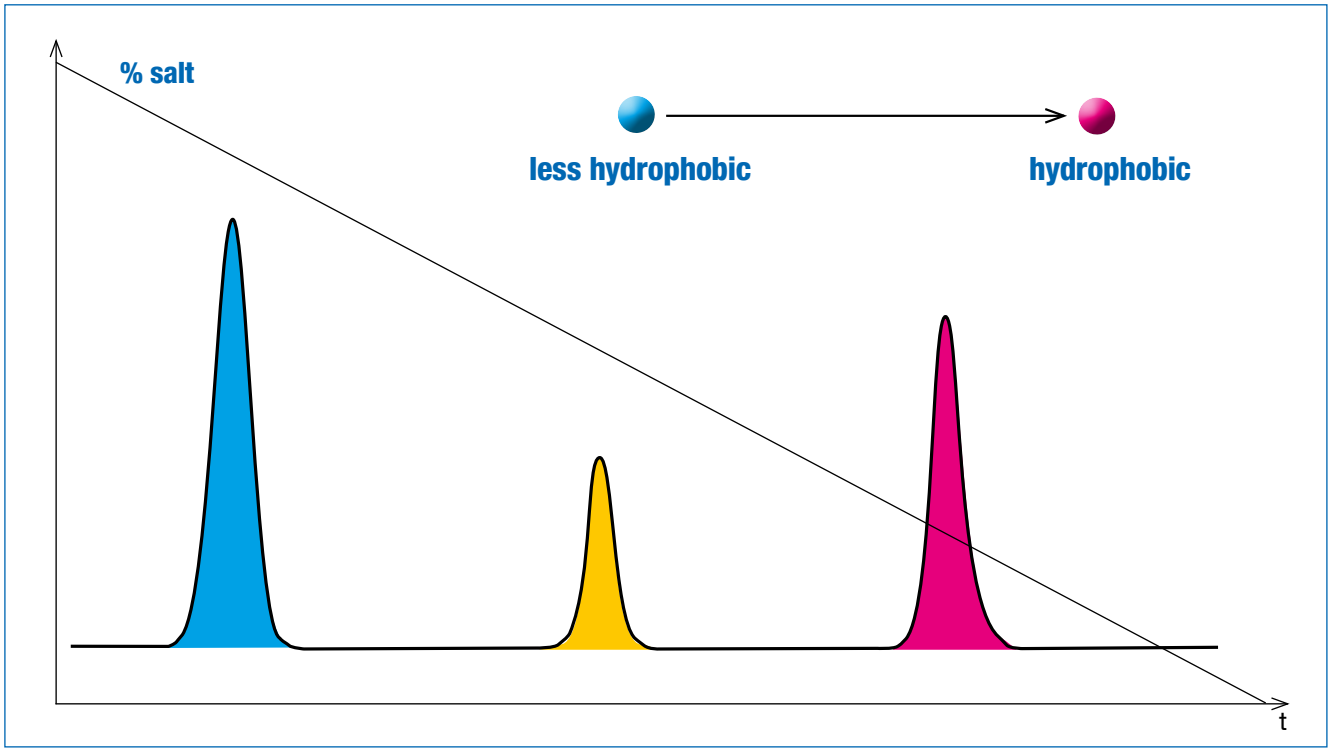


Figure 2: Elution order in HIC separations.

1. Type of Salt

The most commonly used salt in HIC analysis is ammonium sulphate, but it is often helpful to consider different non-denaturing salts during method development. As different types of salt provide different selectivities, this can be especially beneficial when separation is not sufficient.

The Hofmeister series of lyotropic and chaotropic ions shown in Fig. 3 provides a template for salt selection. The more lyotropic salts, such as ammonium sulphate, have a high salting-out capacity which means that they are promoting the hydrophobic interactions between analyte and stationary phase.

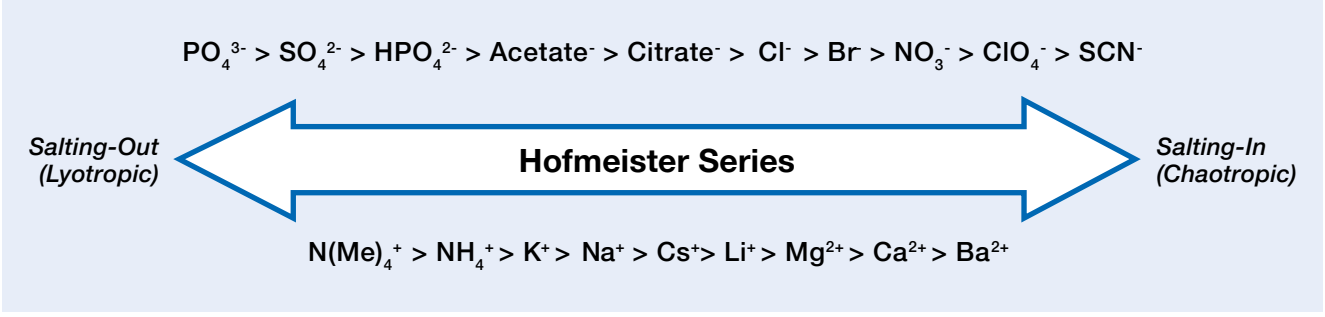
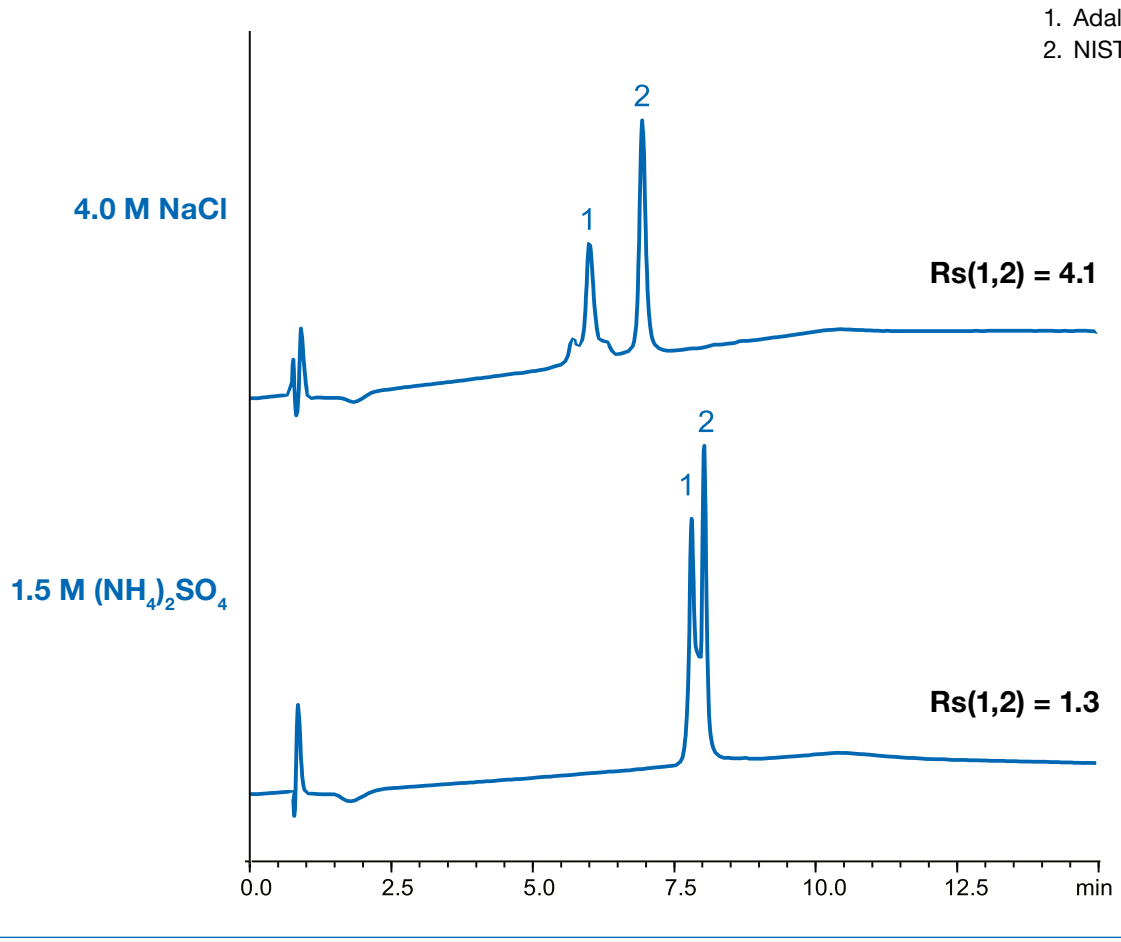


Figure 3: Hofmeister series of lyotropic and chaotropic ions.

In order to gain comparable retention with less lyotropic salts such as sodium chloride very high concentrations are required. The concentration of sodium chloride needs to be more than double compared to ammonium sulphate when trying to achieve similar retention for Adalimumab and NIST mAb (see Fig. 4).



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 (pH 7.0) containing salt B) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)	Detection:	UV at 280 nm
		Injection:	10 µL (0.25 mg/mL)
Gradient:	0–100%B (0–10 min), 100%B (10–15 min)		

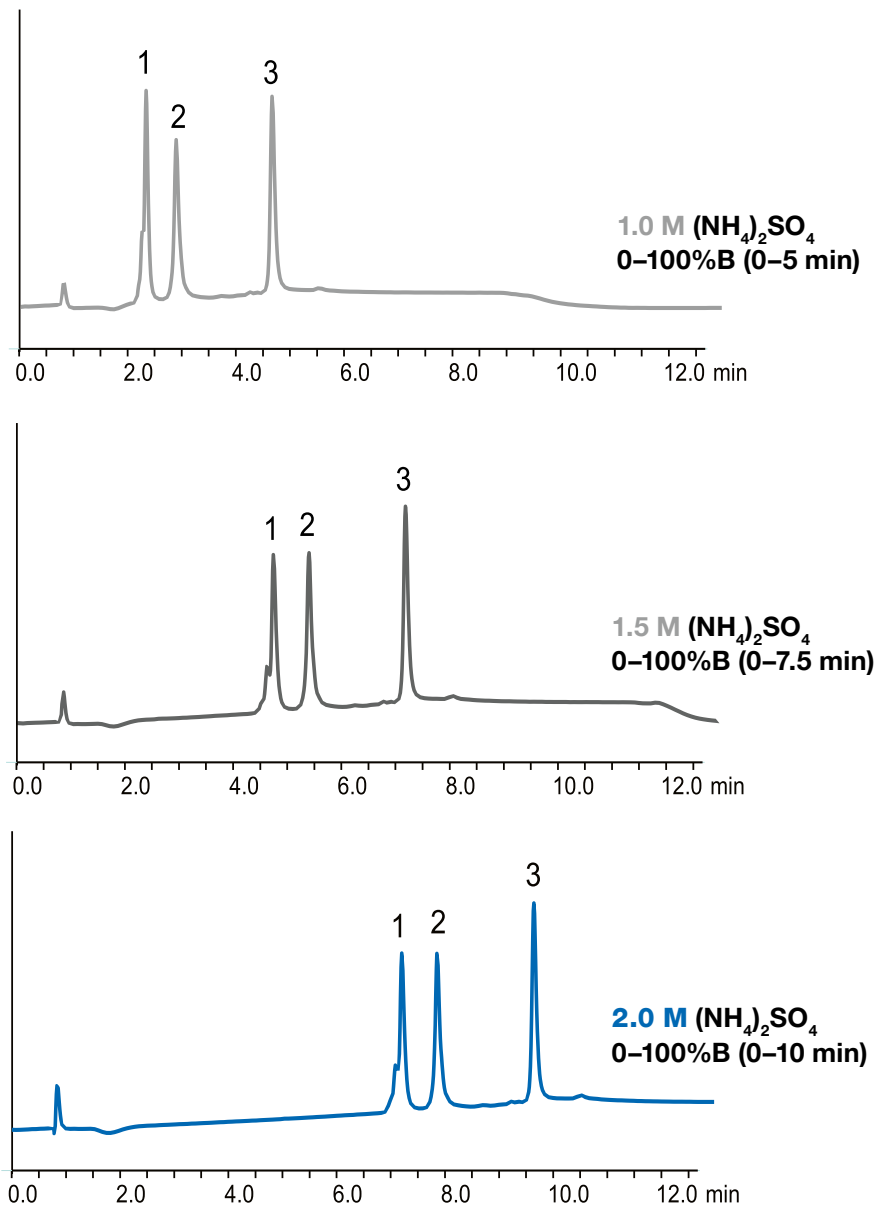
Figure 4: Separation of Adalimumab and NIST mAb using different type of salts.

Using high salt concentrations, attention needs to be paid to the prevention of salt precipitation. In general, it is recommended to keep the salt concentration as low as possible to prevent precipitation causing blockages within the LC system. Flushing the system with water during the daily routine helps to prevent from further damage.

2. Initial Salt Concentration

In addition to the type of salt, its initial concentration can greatly influence the retention behaviour. Three different mAbs were analysed using initial concentrations of 1.0 M, 1.5 M and 2.0 M of ammonium sulphate using the same gradient slope (see Fig. 5). By increasing the initial salt concentration, retention gets stronger, making this a beneficial tool for method improvement of low hydrophobic biomolecules with weak retention.

A similar example with intact proteins using different salt concentrations can be found in a **further technical note** about HIC columns.

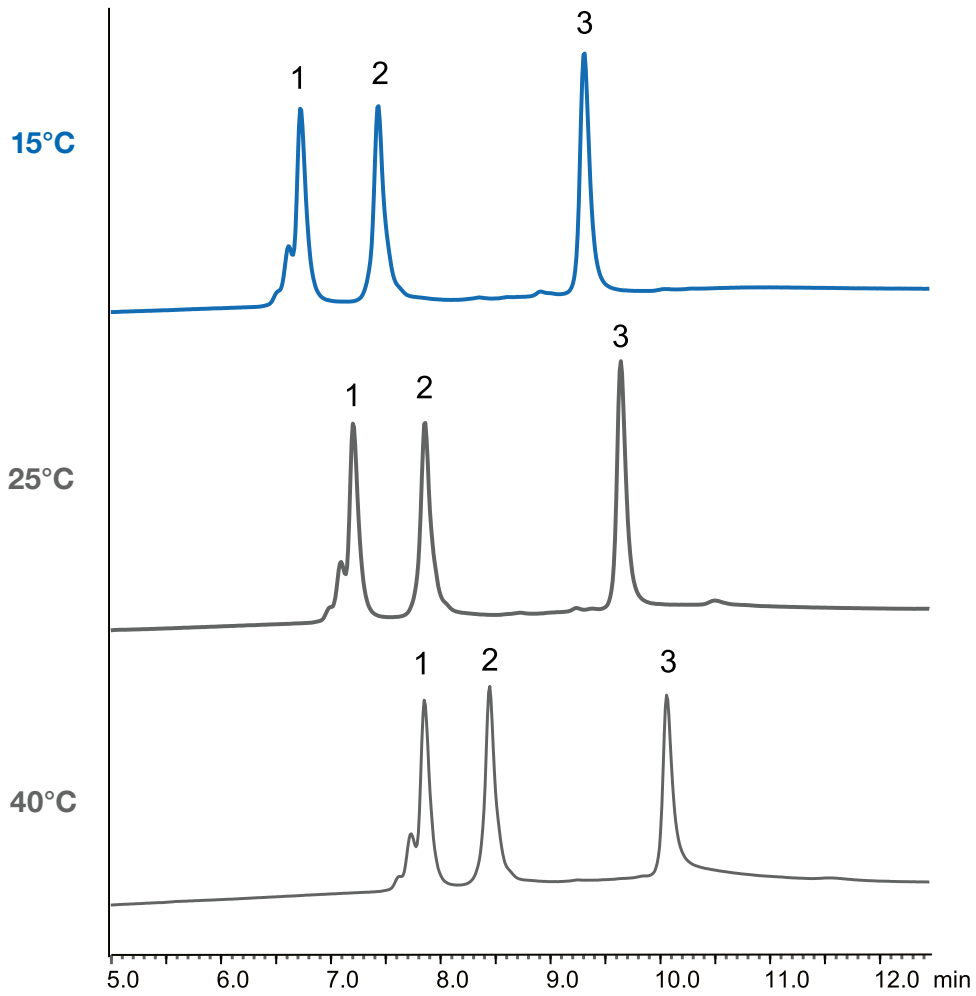


Column:	BioPro HIC BF (4 µm) 100 x 4.6 mm ID	Flow rate:	1.0 mL/min
Part No.:	BHB00S04-1046WT	Temperature:	25°C
Eluent:	A) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0) containing salt B) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)	Detection:	UV at 280 nm
		Injection:	5 µL (0.5 mg/mL)
Gradient:	0.2 M/min (gradient slope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

Figure 5: Separation of three mAbs using different initial concentrations of ammonium sulphate.

3. Temperature

Further improvement can be achieved by considering different temperatures. In HIC mode, higher temperatures result in longer retention times. This assumes that the hydrophobic area interacting with the stationary phase becomes larger due to a change in the analyte's structure with increasing temperature, so that the hydrophobic interactions become stronger. Fig. 6 shows the increased retention for three different mAbs applying 15°C, 25°C and 40°C. For intact proteins, the temperature effect is also shown in the **technical note about intact protein HIC analyses**.



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

Figure 6: Separation of three mAbs using different temperatures.

4. pH

As the mobile phase pH influences the analyte's net surface charge, it plays an important role in HIC analyses. When working close to the isoelectric point (pI) of the target molecule, electrostatic repulsion between the analytes is decreased. This results in increased hydrophobic interactions as the analytes are allowed for a closer arrangement on the stationary phase.

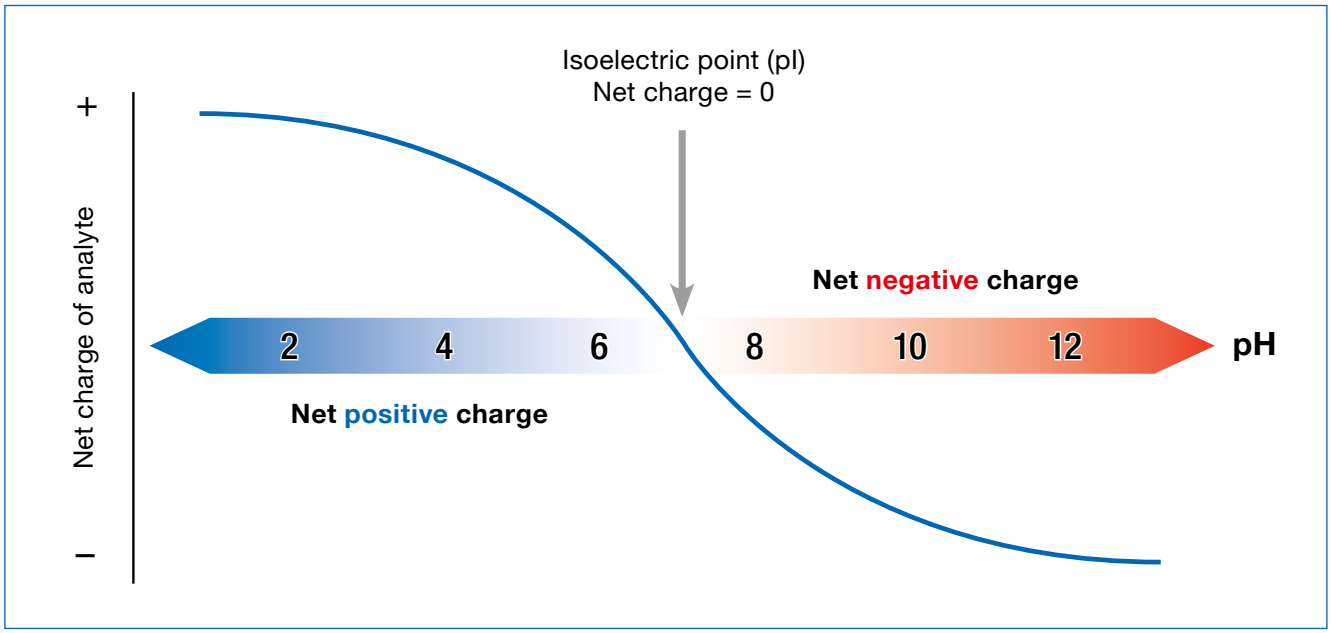
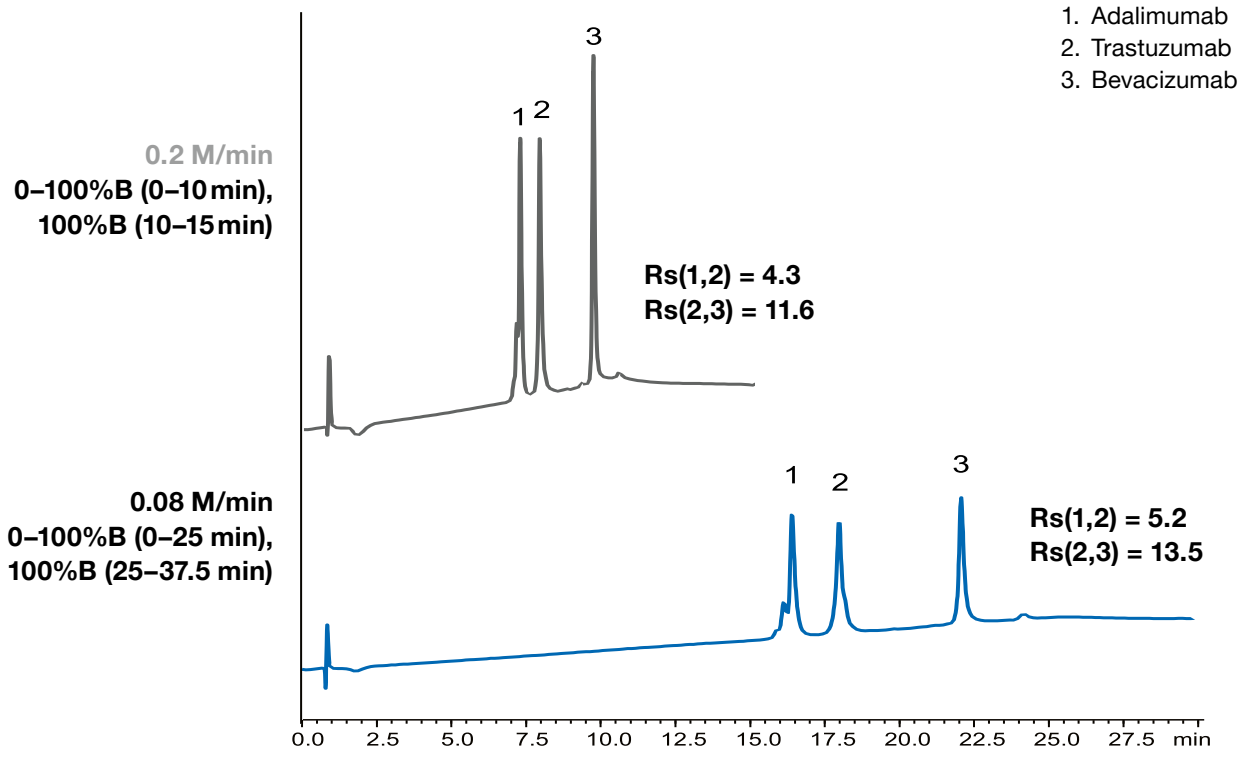


Figure 7: Net charge of biomolecules including the isoelectric point.

5. Variation of Gradient Slope

The gradient slope has a large effect on the chromatographic results. On one hand, shallower gradients improve the separation by increasing the resolution.

On the other hand, sensitivity decreases and peaks get broader with shallower gradients. Therefore, different gradient slopes should be tested during method development to find a good compromise.

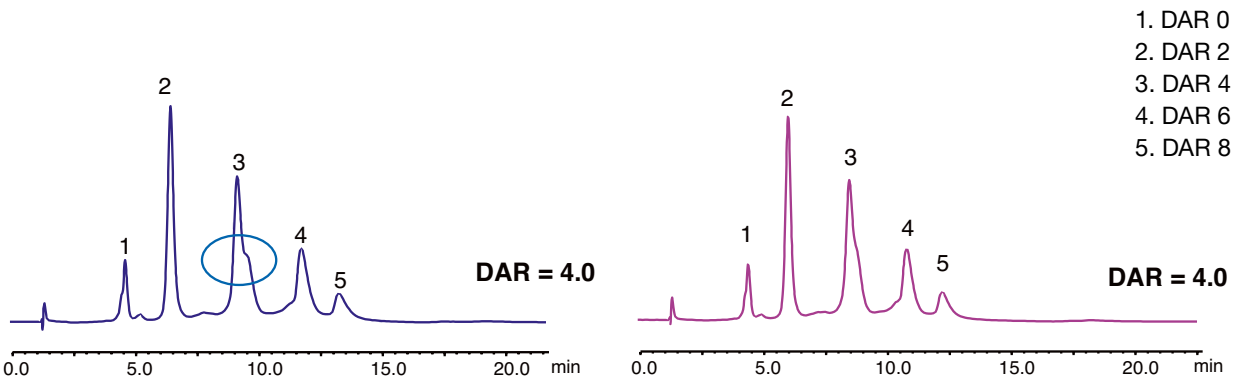


Column:	BioPro HIC BF (4 µm) 100 x 4.6 mm ID
Part No.:	BHB00S04-1046WT
Eluent:	A) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0) containing 2.0 M $(NH_4)_2SO_4$ B) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)
Flow rate:	1.0 mL/min
Temperature:	25 °C
Detection:	UV at 280 nm
Injection:	10 µL (0.5 mg/mL)

Figure 8: Separation of three mAbs using different gradient slopes.

6. Organic Modifier

The addition of an organic modifier such as isopropanol or ethanol is a conventional strategy to improve HIC analyses, but is not mandatory. It influences the interaction between the target molecule and the stationary phase, resulting in improved peak shapes and a decreased retention.
However, organic modifiers can also have an effect on the biomolecules' structure which is why they should be used carefully. Additionally, special caution is required when elevated temperatures are also applied as the risk of denaturation is even higher. For this reason, only low amounts of organic modifiers (such as 10-15%) are commonly used. Fig. 9 shows the drug-to-antibody (DAR) analysis of the ADC brentuximab vedotin using two different concentrations of isopropanol. Good peak shapes are obtained and the lower concentration allows for a partially separated peak close to peak 3.



Column:	BioPro HIC HT (2.3 µm) 100 x 4.6 mm ID
Part No.:	BHH00SQ3-1046PTH
Eluent:	A) 20 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0) containing 1.0 M $(NH_4)_2SO_4$ 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)

Figure 9: DAR analysis of brentuximab vedotin using 10 and 15% isopropanol as organic modifier.

Strategies for the improvement of HIC analyses:

- **Considering various salts with different selectivities**
- **Higher initial salt concentrations provide increased retention**
- **Retention increases with increasing temperature**
- **Working near the pI improves hydrophobic interactions**
- **Shallower gradients provide higher resolution**
- **Organic modifiers are able to improve peak shape and decrease retention**