YMC's HIC Phase Especially Designed for Antibodies, Antibody-Drug Conjugates and Proteins

A demanding aim in pharmaceutical -, food- and clinical research is to perform high-performance LC separations on natural products. In order to maintain the 3D structures of protein conformations and therefore their biological or enzymatic activity during analysis there is a need for:

- **Physiological conditions / aqueous environment**
- **Reduction of organic modifiers**

The most successful chromatographic technique for this purpose is the use of hydrophobic interaction chromatography (HIC), which provides an alternative separation mechanism to ion exchange chromatography (IEX) and size exclusion chromatography (SEC).

What is HIC?

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-sulfat or sodium chloride and a buffer to control pH (usually phosphate, 6 ≤ pH ≤ 7). The Hofmeister series of lyotropic and chaotropic ions in Fig. 1 provides a template for salt selection. High concentrations of salt, in general 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 in the charge on the protein due to the ionisation of acidic groups.

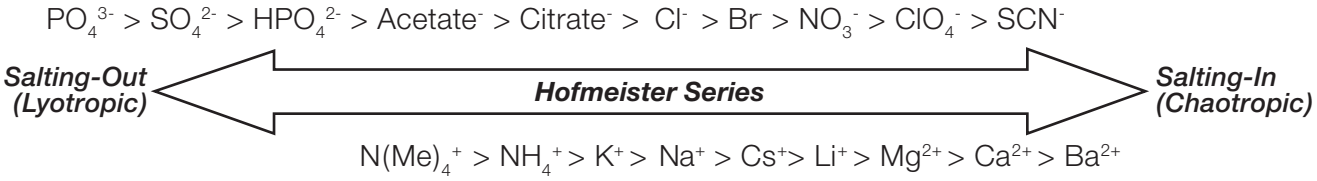


Fig. 1: The Hofmeister Series of lyotropic and chaotropic ions.

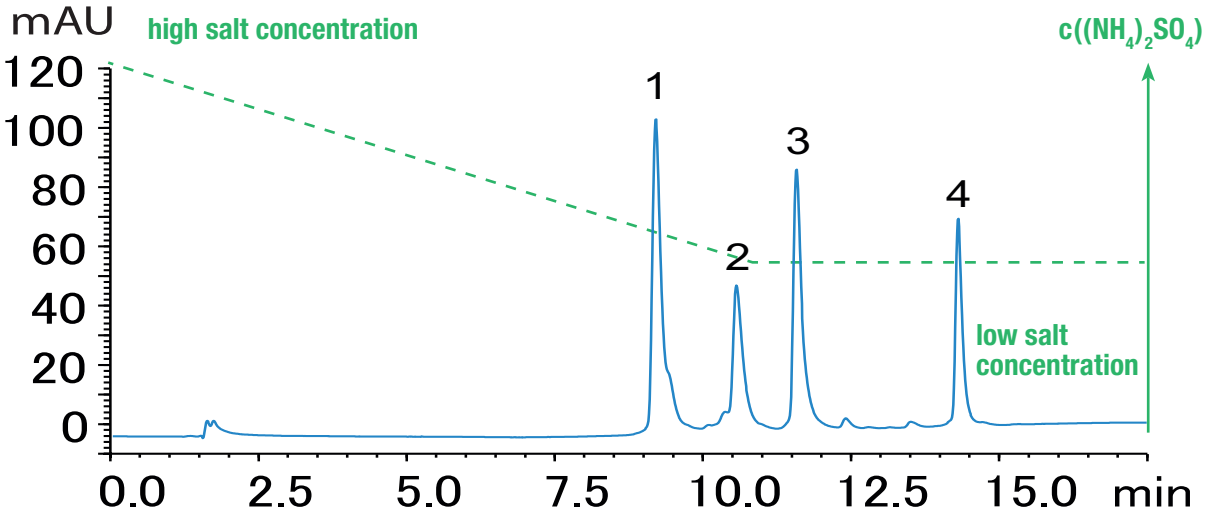


Fig. 2: Method with decreasing salt gradient.
Samples: 1. Myoglobin (0.73 mg/mL), 2. Ribonuclease A (0.75 mg/mL), 3. Lysozyme (0.25 mg/mL), 4. α-Chymotrypsinogen A (0.25 gm/mL). Column: BioPro HIC BF (100 x 4.6 mm ID).
Eluent: A) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0) containing 2.0 M $(NH_4)_2SO_4$; B) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0).
Flow: 0.5 mL/min. Gradient: 0-100% B (0-11 min) 100% B (11-15 min). Temp.: 25 °C. Detection: UV at 280 nm.
Injection: 15 µL.

HIC is particularly effective when used to separate proteins and monoconal antibodies. The detection of monoclonal antibodies (MAb), MAb aggregates and glycosylated MAbs can be achieved due to their specific hydrophobic natures. It is a very useful method for determination of drug-to-antibody ratios (DAR) in antibody-drug conjugates (ADCs).

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

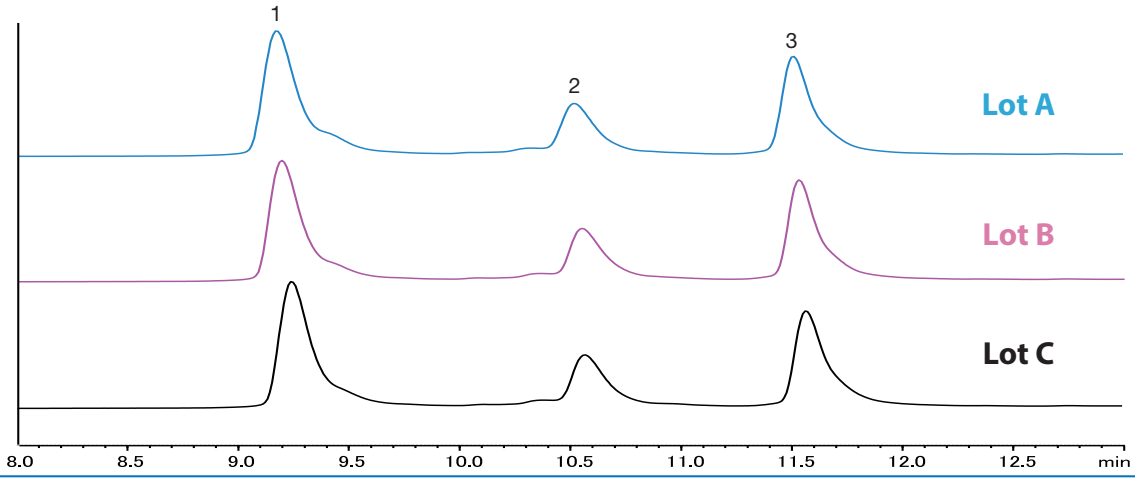
Column Technology

BioPro HIC BF from YMC is a newly-developed hydrophilic polymer stationary phase bonded with C4 (butyl) ligands. The 4 µm, non-porous particles provide high mechanical stability together with high column efficiency which results in fast analyses at higher flow rates. The butyl bonded polymer phase provides long-term stability and ensures excellent lot-to-lot reproducibility.
BioPro HIC BF improves analysis throughput with virtually no carryover effects.

Specifications	
Base particle	hydrophilic polymer (polymethacrylate)
Particle sizes	4 µm
Pore	non-porous
Functional group	butyl
pH range	2 – 12
Temperature range	10 – 60°C
Pressure limit	20 MPa (200 bar)

Excellent Reproducibility & Stability

In contrast to most other columns on the market, BioPro HIC BF columns provide an excellent lot-to-lot reproducibility to provide reproducible and reliable results (see Fig. 3) and therefore overcome the most important demands by users!

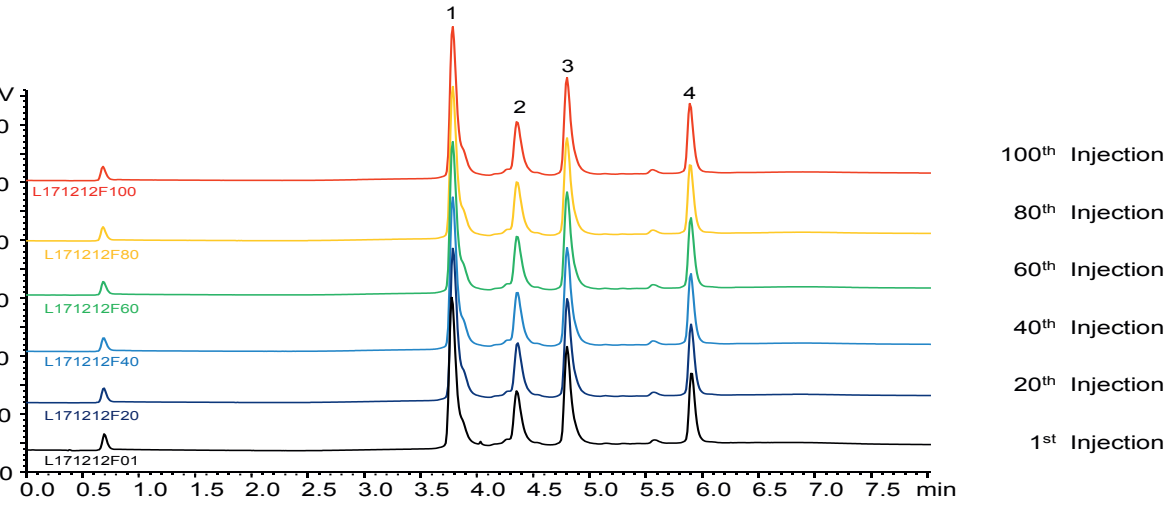


Column: BioPro HIC BF (4 µm) 100 x 4.6 mm ID
Eluent: A) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0) containing 2.0 M $(NH_4)_2SO_4$
B) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)
Gradient: 0-100%B (0-11 min), 100%B (11-15 min)
Flow rate: 0.5 mL/min
Temperature: 25°C

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)

Fig. 3: Reproducibility when using different batches of BioPro HIC BF phase.

Additionally, the high robustness of BioPro HIC BF columns provides long-term stability. No change can be found in the separation parameters including retention times, even after 100 injections (see Fig. 4).



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 $(NH4)_2SO_4$
B) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)
0-100%B (0-4.58 min), 100%B (4.58-6.25 min)
Flow rate: 1.2 mL/min
Temperature: 25 °C

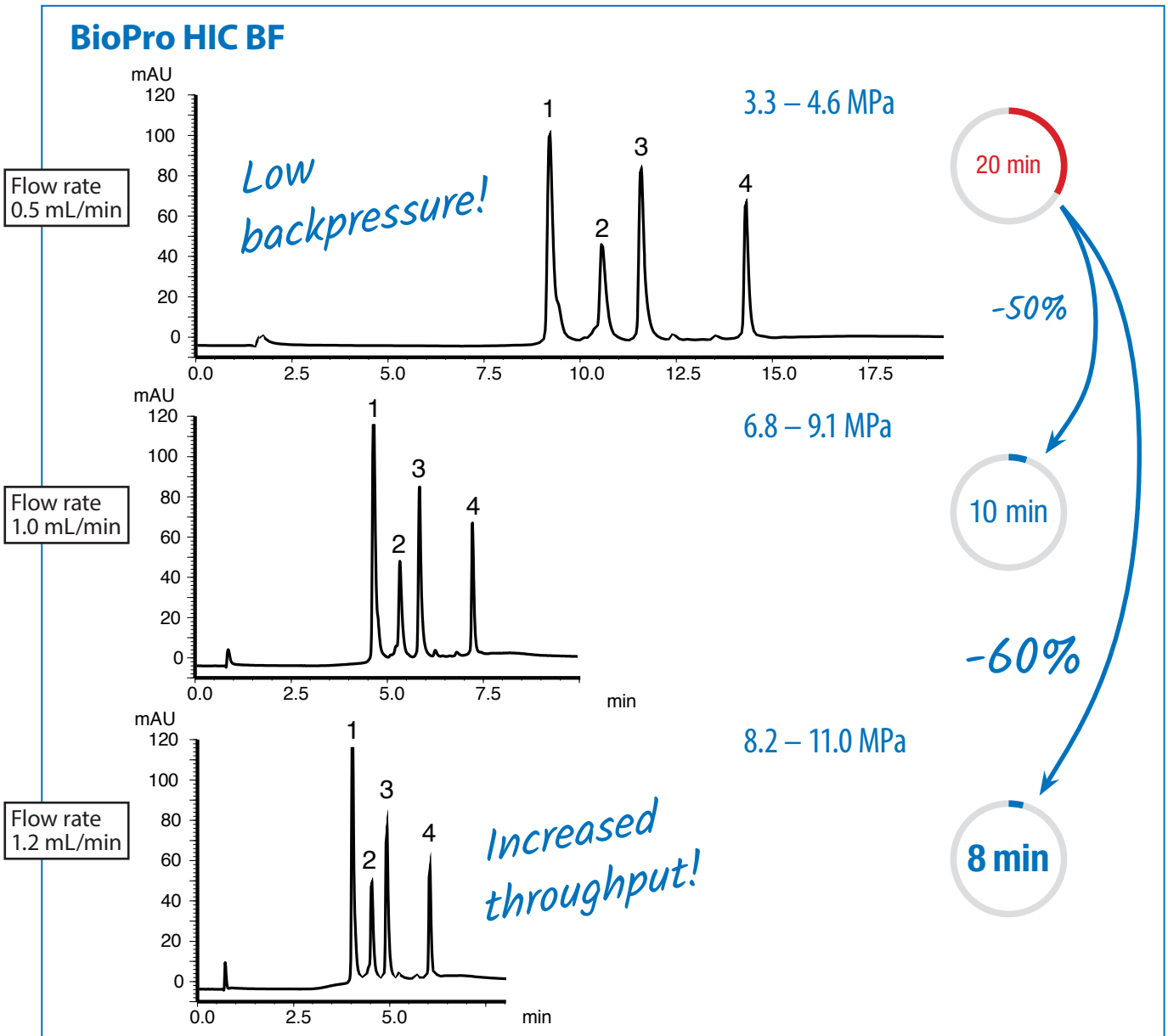
Detection: UV at 280 nm
Injection: 15 µL
Samples:
1. Myoglobin (0.73 mg/mL)
2. Ribonuclease A (0.75 mg/mL)
3. Lysozyme (0.25 mg/mL)
4. α-Chymotrypsinogen A (0.25 mg/mL)

Fig. 4: Long term stability of BioPro HIC BF, even after 100 injections.

High Resolution with Greater Throughput

Pore diffusion often represents the rate-limiting step in the mass transport of large biomolecules through a porous phase. Eliminating the pores by using non-porous particles provides higher resolution at higher flow rates.

YMC's HIC column provides a non-porous base particle with a size of 4 µm, which allows a greater throughput at high flow rates (see Fig. 5). The high flow rate is possible due to the high mechanical stability of the polymer beads, which cannot be achieved with other media. This saves up until 60 % of method time.

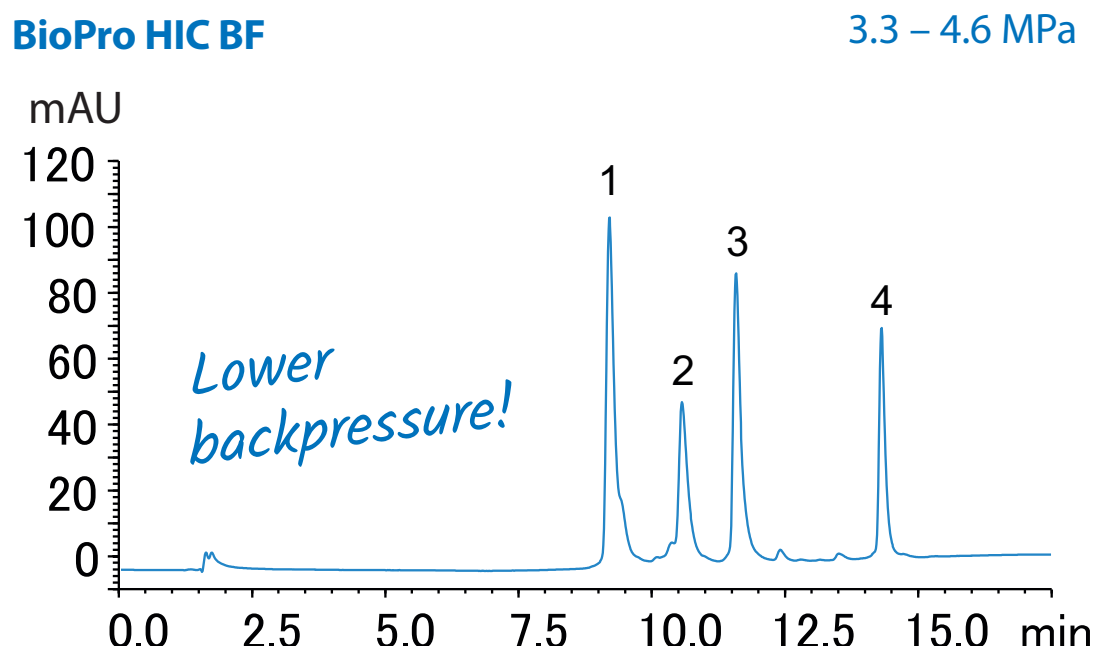


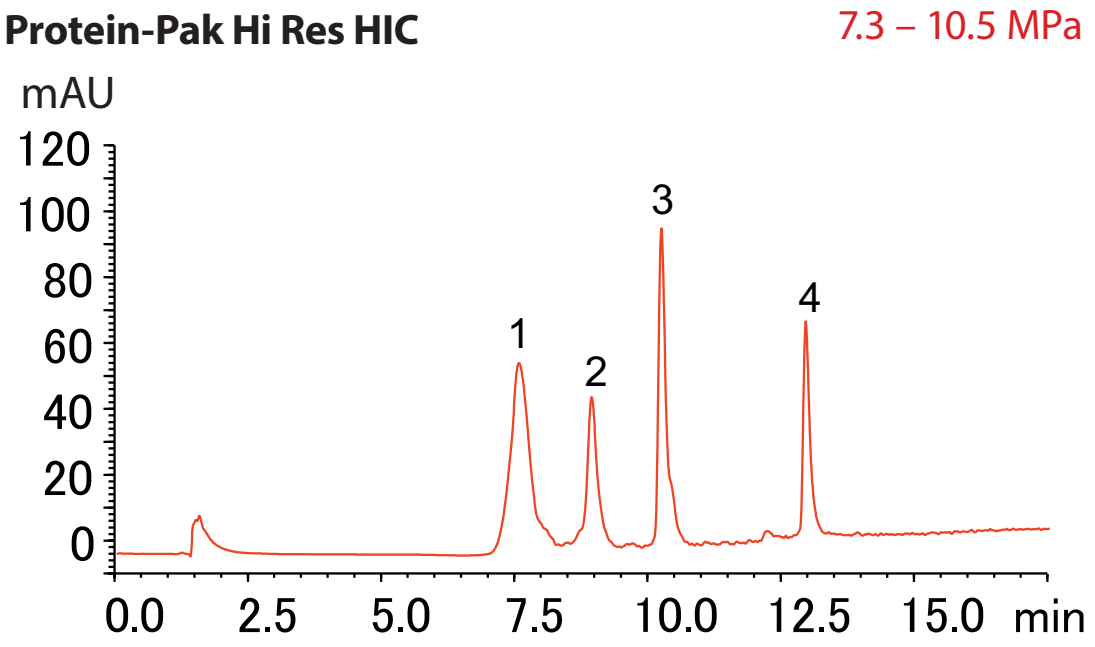
Column:	BioPro HIC BF (4 µm) 100 × 4.6 mm ID	
Eluent:	A) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0) containing 2.0 M $(NH_4)_2SO_4$	
	B) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)	
Gradient 0.5 mL/min:	0-100% B (0-11 min) 100% B (11-15 min)	
1.0 mL/min:	0-100% B (0-5.5 min) 100% B (5.5-5.8 min)	
1.2 mL/min:	0-100% B (0-4.58 min) 100% B (4.58-6.58 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 mg/mL)

Fig. 5: Effect of increasing flow rate on the resolution achieved using BioPro HIC BF.

The performance provides a high resolution comparable to sub 3 µm particle phases while additionally allowing method flexibility due to lower backpressures (see Fig. 6).





Column:	BioPro HIC BF (4 µm) 100 × 4.6 mm ID ProteinPak Hi Res HIC (2.5 µm) 100 × 4.6 mm ID
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

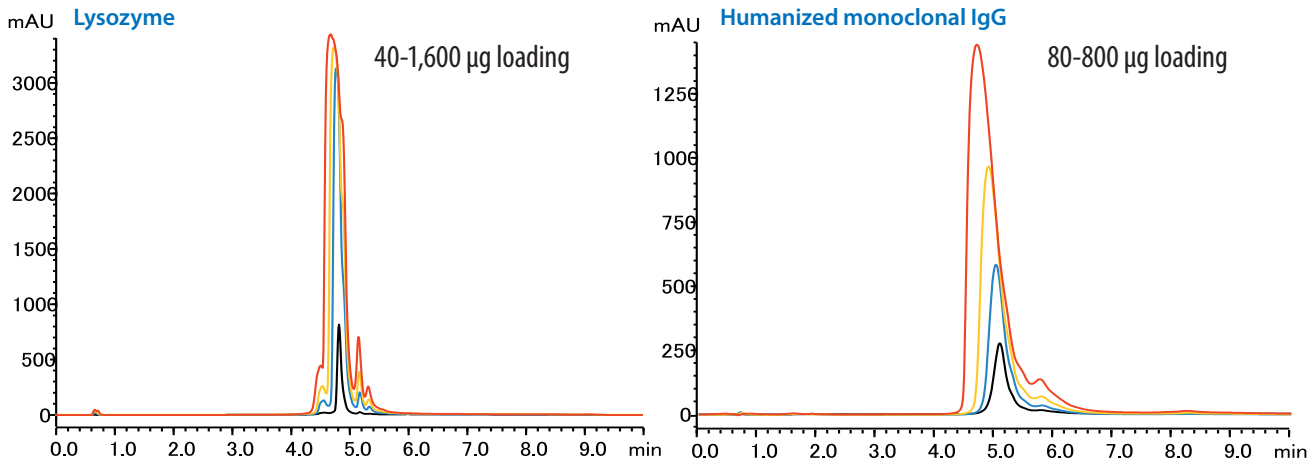
Samples:
1. Myoglobin (0.73 mg/mL)
2. Ribonuclease A (0.75 mg/mL)
3. Lysozyme (0.25 mg/mL)
4. α-Chymotrypsinogen A (0.25 mg/mL)

Fig. 6: Comparison of separations using BioPro HIC BF (above) and Protein-Pak Hi Res HIC (below).

Sharp Peaks & High Loadability

For the detection of variants and impurities of proteins or antibodies in low concentrations, high resolution of sharp peaks is necessary. Fig. 7 shows that extraordinary sharp peak shapes can be achieved with BioPro HIC BF even at high loading conditions. This characteristic provides the option to use this material for lab-scale purification with high recovery rates.

The columns can be used for the isolation of variants in trace amounts and in quantitation analysis.

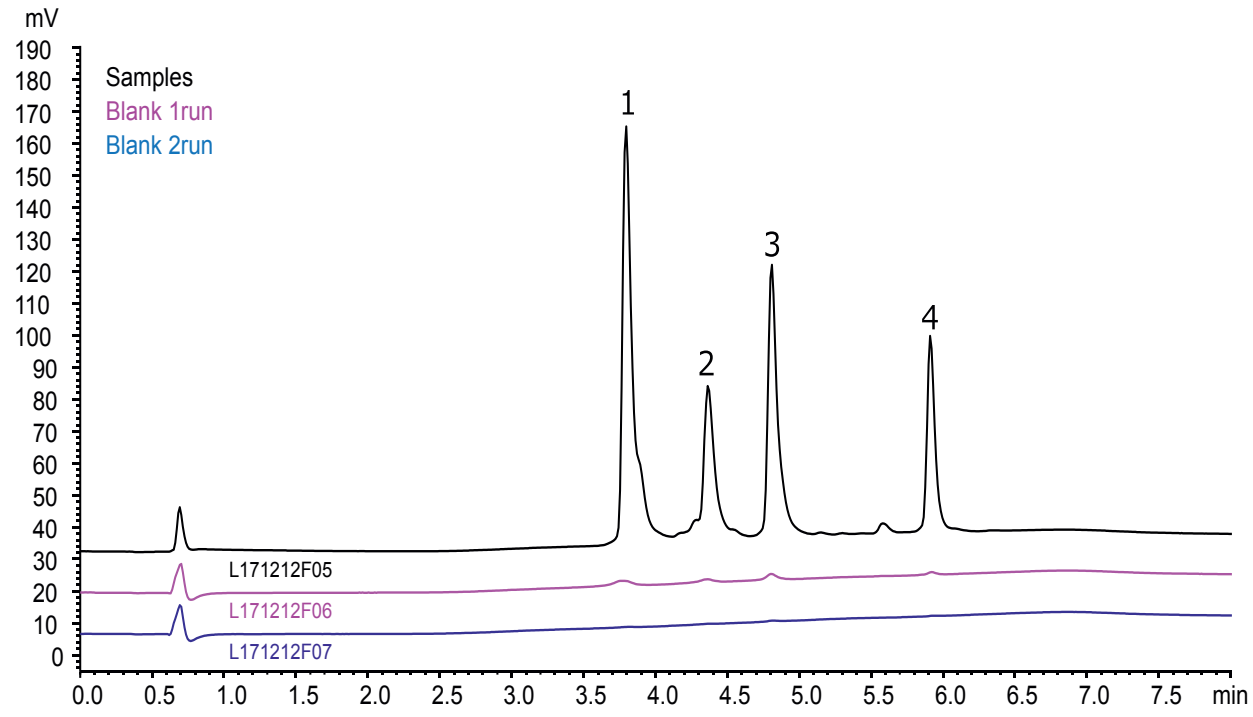


Column:	BioPro HIC BF (4 µm) 100x4.6 mm ID	Temperature:	25°C for Lysozyme 30°C for IgG
Part No.:	BHB00S04-1046WT	Detection:	UV at 280 nm
Eluent:	A) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0) containing 2.0 M $(NH_4)_2SO_4$ B) 100 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)	Injection:	4, 40, 80, 160 µL for Lysozyme 32, 80, 160, 320 µL for IgG
Gradient:	0-100%B (0-4.58 min), 100%B (4.58-6.58 min) for Lysozyme 60%B(0-0.5 min), 60-100%B (0.5-7.5 min), 100%B (7.5-10 min) for IgG	Sample:	Lysozyme (10 mg/mL) Humanized monoclonal IgG (2.5 mg/mL)
Flow rate:	1.2 mL/min		

Fig. 7: Loadability study for lysozyme and IgG using BioPro HIC BF.

Virtually No Carryover Effects

Carryover is a term used to describe contamination of an analysis caused by sample peaks reappearing in a later separation which does not actually contain the sample (e.g. blank runs).
With BioPro HIC BF this effect can be minimized to virtually zero which greatly contributes to analysis reliability and reproducibility.



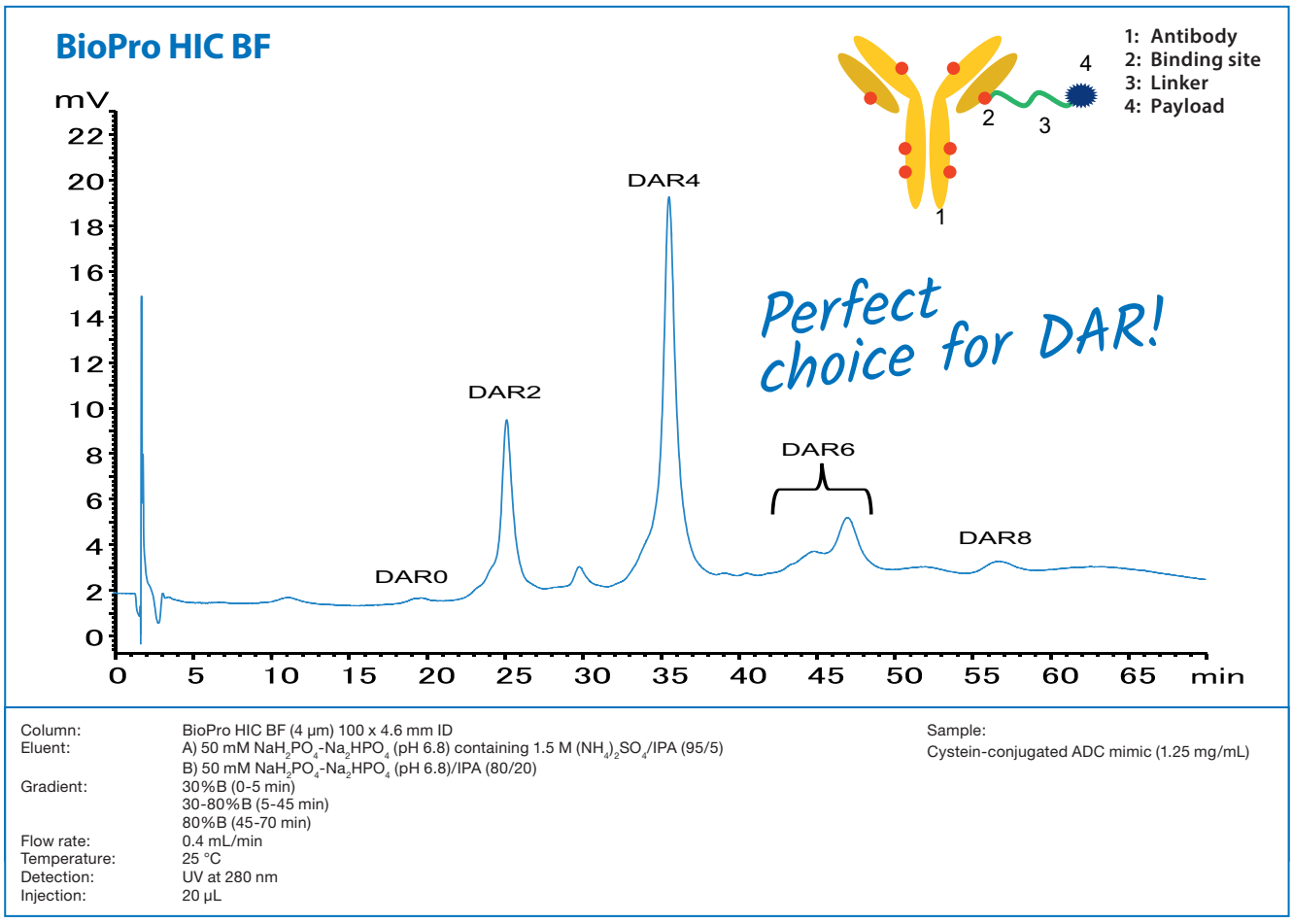
Column:	BioPro HIC BF (4 µm) 100x4.6 mm ID
Part No.:	BHB00S04-1046WT
Eluent:	A) 100 mM NaH2PO4-Na2HPO4 (pH 7.0) containing 2.0 M (NH4)2SO4
	B) 100 mM NaH2PO4-Na2HPO4 (pH 7.0)
Gradient:	0-100%B (0-4.58 min), 100%B (4.58-6.25 min)
Flow rate:	1.2 mL/min
Temperature:	25
Detection:	UV at 280 nm
Injection:	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 mg/mL)

Fig. 8: Virtually no effect of sample carryover using BioPro HIC BF.

Drug-to-Antibody Ratio (DAR) Analysis of ADCs

Antibody-drug conjugates (ADCs) are a new class of bio-therapeutics. ADCs are monoclonal antibodies chemically linked to biologically active small molecule drugs. Drug-to-antibody ratio (DAR), which is an important property of ADCs, is the average number of drug molecules bound to the antibody. The DAR value affects the efficiency of the drug, as low drug loading reduces the potency, while high drug loading can negatively affect pharmacokinetics and toxicity. The unmodified MAb and ADCs with DAR values ranging from 2 to 8 are well resolved on the BioPro HIC BF column (see Fig. 9).



Column:	BioPro HIC BF (4 µm) 100 x 4.6 mm ID
Eluent:	A) 50 mM NaH_2PO_4-Na_2HPO_4 (pH 6.8) containing 1.5 M $(NH_4)_2SO_4$/IPA (95/5)
	B) 50 mM NaH_2PO_4-Na_2HPO_4 (pH 6.8)/IPA (80/20)
Gradient:	30%B (0-5 min)
	30-80%B (5-45 min)
	80%B (45-70 min)
Flow rate:	0.4 mL/min
Temperature:	25 °C
Detection:	UV at 280 nm
Injection:	20 µL

Sample:
Cystein-conjugated ADC mimic (1.25 mg/mL)

Fig. 9: Analysis of the drug-to-antibody ratio from DAR0 to DAR8 with BioPro HIC BF.

Benefits for HIC applications:

- **Especially designed for antibodies, antibody-drug conjugates (ADCs), proteins**
- **High separation performance**
- **High-throughput analyses**
- **Excellent lot-to-lot reproducibility**
- **Long term stability**
- **Virtually no carryover effects**

To adopt YMC supports for your analytical HIC method please contact:

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