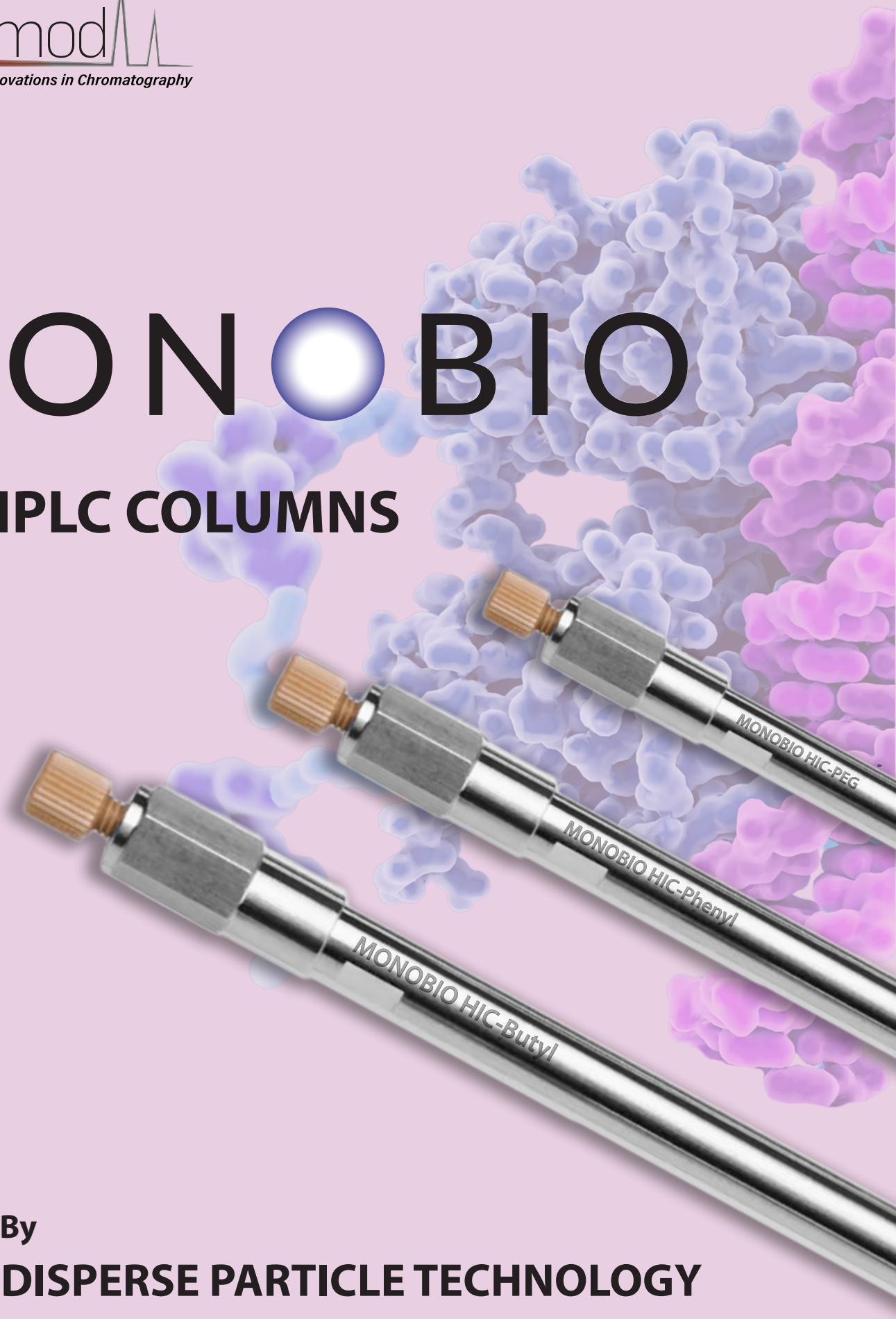
macmod

Innovations in Chromatography

MONOBIO

HIC HPLC COLUMNS



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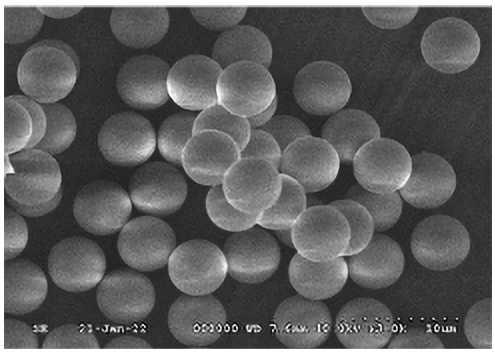
HIC Columns for HPLC Applications

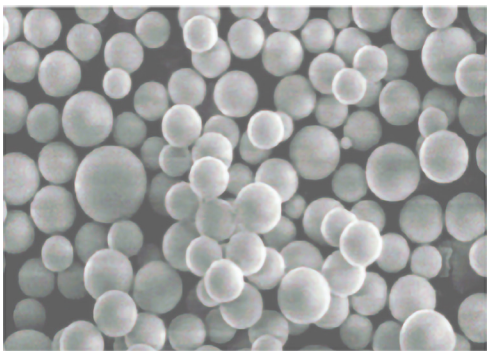
Table of Contents

What makes MONOBIO different?

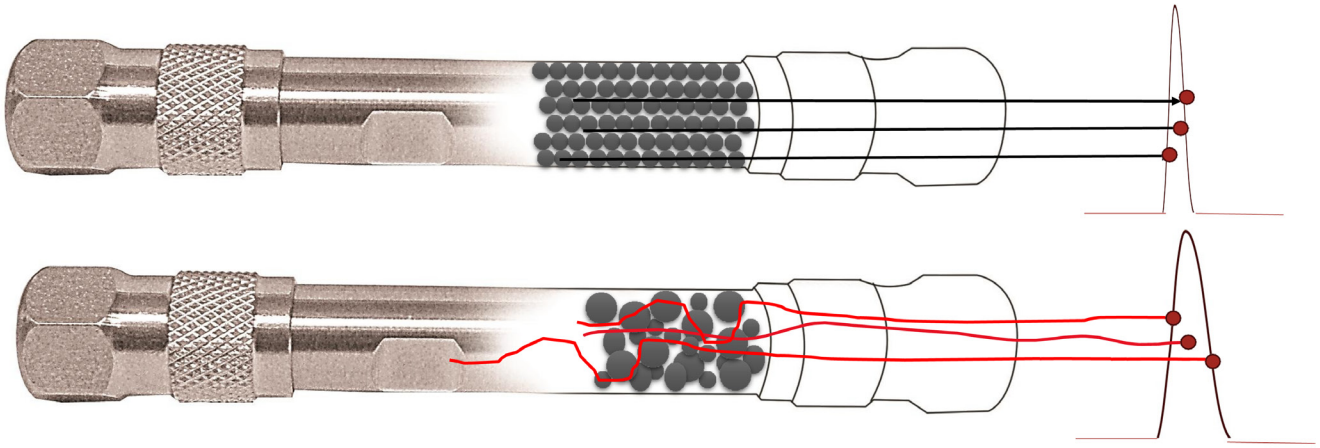
MONOBIO HIC HPLC Columns are based solely on monodisperse fully porous spherical silica particles. The monodispersity of the silica packed bed allows the column to minimize Eddy diffusion of analytes traveling through the packed bed, which inherently increases their efficiency and gives exciting resolution as compared to traditional polydisperse HIC columns.



Monodisperse Silica



Traditional Polydisperse Silica



Note: How monodispersity of MONOBIO HPLC columns impact the efficiency of analytes as depicted in the image above.

What is HIC (Hydrophobic Interaction Chromatography)?

1. **What is HIC Chromatography?**

   Hydrophobic Interaction Chromatography (HIC) is an analytical technique used to **characterize proteins, antibodies, and ADCs** based on differences in **surface hydrophobicity** under non-denaturing (native aqueous salt) conditions.

2. **What is the main governing principle of HIC?**

   Proteins have both **hydrophilic (water-loving)** and **hydrophobic (water-repelling)** region(s) depending on the protein species. As the protein is placed in a **high-salt environment**, the protein(s) expose more of their hydrophobic surfaces. The surface then interacts with a **hydrophobic stationary phase** that is applied to the surface (in the case of MONOBIO a silica surface).

   The **more hydrophobic** a protein is, the **more attracted** it is to the column (i.e. more retention), and by that approach the **less hydrophobic** it is, the **less attracted** the protein is to the column and elutes earlier (i.e. less retention).

3. **Most Commonly used Applications of HIC?**
   - **Protein purification**
     - Antibodies, enzymes, and other biomolecules
   - **Polishing step in mAb production**
     - **HIC** is often used after the ion exchange chromatography step to remove aggregates or variants
   - **Analytical Protein characterization**
     - Bioanalytical team assessment of surface hydrophobicity
   - **Monoclonal antibody (mAb) variant profiling**
     - Detect and quantify oxidized, deamidated, or aggregated forms, which alter surface hydrophobicity
   - **Stability studies**
     - Assess structural changes or degradation that modify hydrophobic surface exposure
   - **DAR Analysis**
     - Monitor drug-to-antibody ratio of variant(s) of multi-linker/killer molecules associated with ADC (antibody-drug-conjugate platform)

How is HIC applied to DAR Analysis?

1. **How is HIC applied to DAR analysis?**

   HIC, applied to ADCs, allows the chromatographer to gauge hydrophobicity in proportion to drug loading. An **ADC** is a monoclonal antibody linked to a **cytotoxic drug molecule(s)** via a chemical linker on the ADC. The **Drug-to-Antibody Ratio (DAR)** provides the average number of drug molecules attached via the chemical linker per antibody. The DAR value of an ADC is considered a key quality attribute.

   DAR may indicate the following:

   - **Potency**
   - **Stability**
   - **Pharmacokinetics**
   - **Safety and efficacy**

   Typical DAR values range from **~0 to 8**, depending on the conjugation chemistry. Each additional drug increases retention, allowing separation and quantification of individual DAR species under mild, non-denaturing conditions.

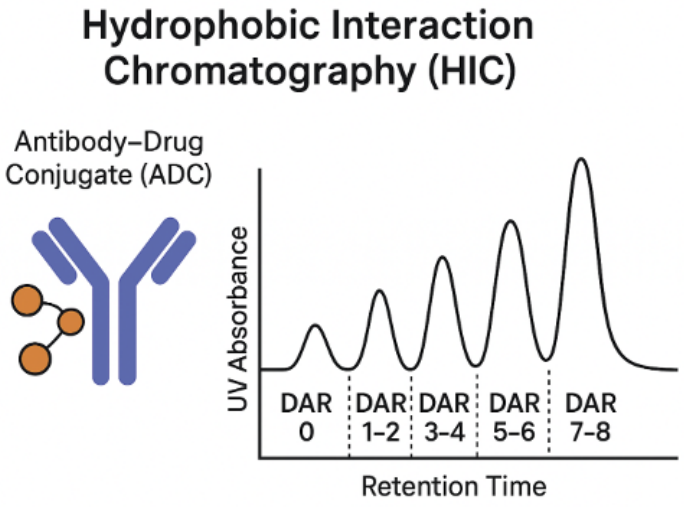
2. **Why HIC is used for DAR analysis in ADCs?**

   HIC separates ADC species **based on increasing hydrophobicity**, which correlates directly with the **number of attached hydrophobic cytotoxic drug molecules**. Each conjugated drug adds **hydrophobic surface area** to the antibody:

   Therefore:

   - **DAR 0 (unconjugated)** is considered the least hydrophobic (ie elutes first)
   - **DAR 1–8** as the DAR value increases the ADC becomes progressively more hydrophobic (i.e. elute later)

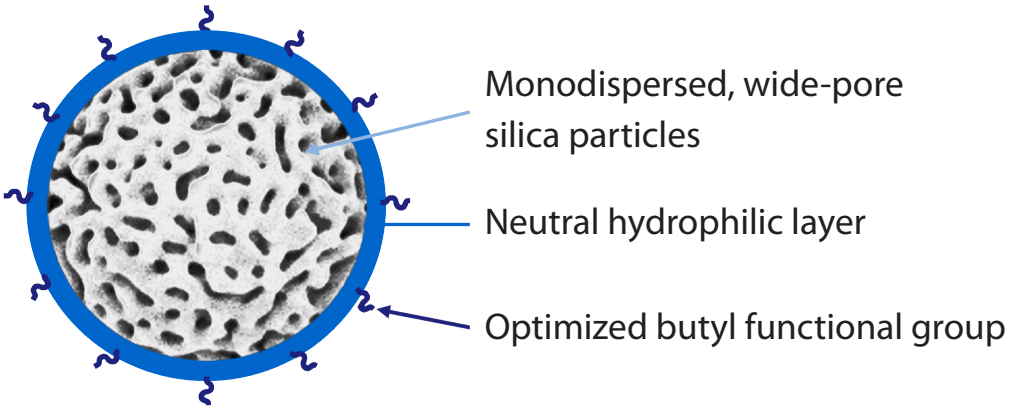
   This separation occurs under **mild, non-denaturing conditions**, preserving the antibody's structure — ideal for accurate DAR characterization.



MONOBIO HIC Specifications Table

Bonded Phase	Pore Size (Å)	Particle Size (µm)	Particle Composition	pH Range	Application Use:
MONOBIO HIC-Butyl	1000	3 5	Monodisperse Fully Porous Silica Particles	2-8	Hydrophobicity/ aggregation determination in mAbs, bispecific antibodies, ADCs and general protein mixtures
MONOBIO HIC-Phenyl	1000	3 5	Monodisperse Fully Porous Silica Particles	2-8	Hydrophobicity/ aggregation determination in mAbs, bispecific antibodies, ADCs and general protein mixtures
MONOBIO HIC-PEG	1000	3 5	Monodisperse Fully Porous Silica Particles	2-8	Hydrophobicity/ aggregation determination in mAbs, bispecific antibodies, ADCs and general protein mixtures

MONOBIO HIC-Butyl



mAbs (IgG1, IgG2, IgG4)

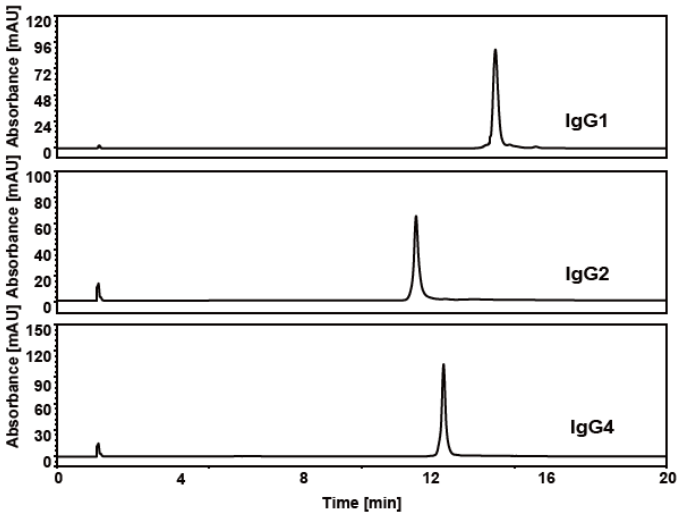


Figure 1

Part Number:	MBHICHBU424610		
Column:	**MONOBIO HIC-Butyl**, 5 µm		
Dimension:	4.6×100 mm		
Mobile Phase:	A) 2.0 M $(NH_4)_2SO_4$ in 100 mM Phosphate Buffer, pH 7.0		
	B) 100 mM Phosphate Buffer, pH 7.0		
Gradient:	t (min)	%A	%B
	-10	100	0
	0	100	0
	1	100	0
	15	0	100
	20	0	100
Flow Rate:	1.0 mL/min		
Temperature:	30 ℃		
Injection:	10 µL		
Detection:	UV 280 nm		
Sample:	IgG1, IgG2 and IgG4 (~1 mg/mL each in mobile phase A)		

In Figure 1, the MONOBIO HIC-Butyl column separates each IgG variant.

Bispecific

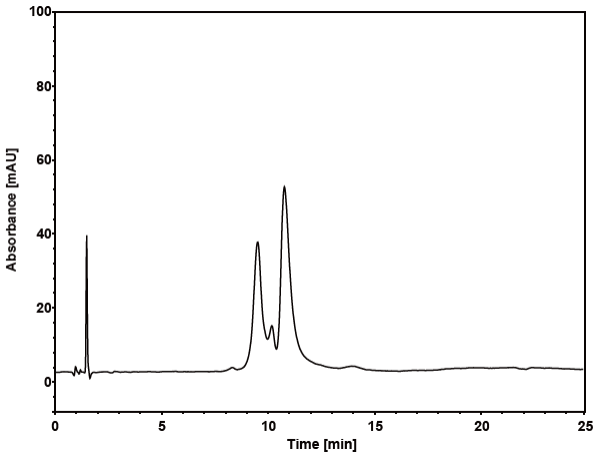


Figure 2

Part Number:	MBHICHBU424610			
Column:	**MONOBIO HIC-Butyl**, 5 µm			
Dimension:	4.6×100 mm			
Mobile Phase:	A) 2.0 M $(NH_4)_2SO_4$ in 100 mM Phosphate Buffer, pH 7.0			
	B) 100 mM Phosphate Buffer, pH 7.0			
	C) IPA			
Gradient:	t (min)	%A	%B	%C
	0	60	40	0
	20	0	80	20
	25	0	80	20
Flow Rate:	1.0 mL/min			
Temperature:	30 ℃			
Injection:	10 µL			
Detection:	UV 214 nm			
Sample:	Bispecific Antibody			

In Figure 2, the Biospecific Anitbody variants are well separated from each other using MONOBIO HIC-Butyl.

Cysteine Conjugated ADC

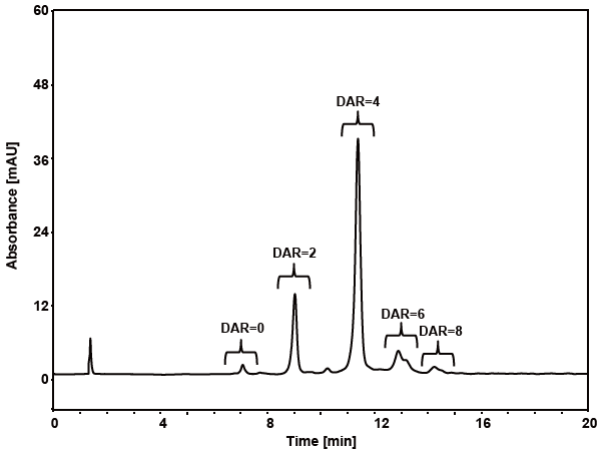


Figure 3

Part Number:	MBHICHBU424610			
Column:	**MONOBIO HIC-Butyl**, 5 µm			
Dimension:	4.6×100 mm			
Mobile Phase:	A) 2.0 M $(NH_4)_2SO_4$ in 100 mM Phosphate Buffer, pH 7.0			
	B) 100 mM Phosphate Buffer, pH 7.0			
	C) IPA			
Gradient:	t (min)	%A	%B	%C
	-10	75	25	0
	0	75	25	0
	1	75	25	0
	15	0	75	25
	20	0	75	25
Flow Rate:	1.0 mL/min			
Temperature:	30 ℃			
Injection:	10 µL			
Detection:	UV 280 nm			
Sample:	Cysteine Conjugated ADC (~1 mg/mL in mobile phase A)			

In Figure 3, seven Cysteine Conjugated ADC DAR species can be easily separated including two DAR-6 species and two DAR-8 species. The other minor peak between DAR-2 and DAR-4 is considered and impurity peak.

ADC and Pro-mAb

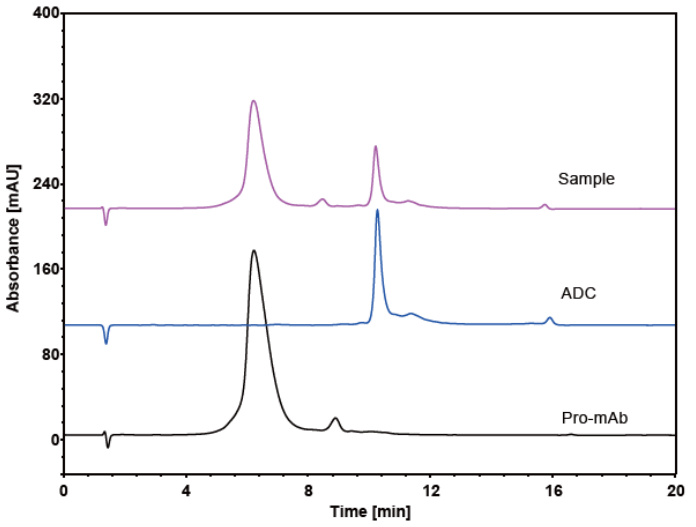
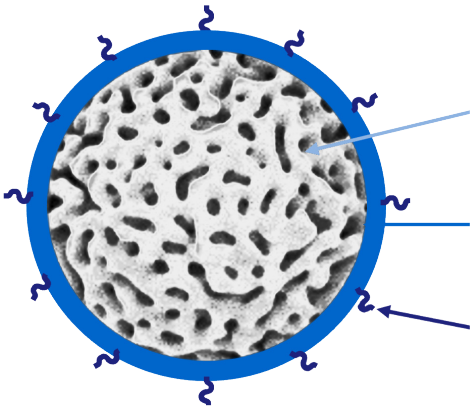


Figure 4

Part Number:	MBHICHBU424610			
Column:	**MONOBIO HIC-Butyl**, 5 µm			
Dimension:	4.6×100 mm			
Mobile Phase:	A) 2.0 M $(NH_4)_2SO_4$ in 100 mM Phosphate Buffer, pH 7.0			
	B) 100 mM Phosphate Buffer, pH 7.0			
	C) IPA			
Gradient:	t (min)	%A	%B	%C
	0	50	50	0
	1	50	50	0
	15	0	75	25
	20	0	75	25
	20.1	50	50	0
	35	50	50	0
Flow Rate:	1.0 mL/min			
Temperature:	30 ℃			
Injection:	20 µL			
Detection:	UV 220 nm			
Sample:	ADC (0.46 mg/mL)			
	Pro-mAb (1.8 mg/mL)			

In Figure 4, the ADC and Pro-mAb are easily separated showing the increase in hydrophobicity once the drug conjugate is added to the antibody candidate.

MONOBIO HIC-Phenyl



Bispecific Antibody

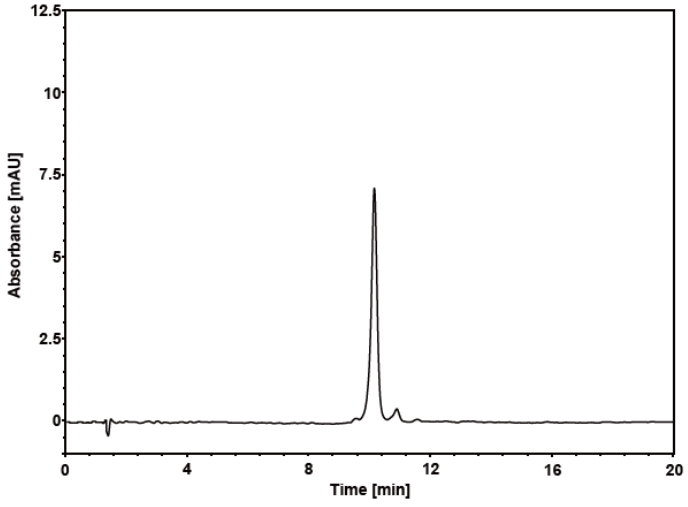


Figure 5

Part Number:	MBHICHPH424610		
Column:	**MONOBIO HIC-Phenyl**, 5 µm		
Dimension:	4.6×100 mm		
Mobile Phase:	A) 7.5/92.5 v/v IPA/2.0 M $(NH_4)_2SO_4$ in 100 mM Phosphate Buffer, pH 7.0		
	B) 7.5/92.5 v/v IPA/100 mM Phosphate Buffer, pH 7.0		
Gradient:	t (min)	%A	%B
	0	100	0
	1	100	0
	20	0	100
	20.1	100	0
	26	100	0
Flow Rate:	1.0 mL/min		
Temperature:	30 ℃		
Injection:	2 µL		
Detection:	UV 280 nm		
Sample:	Bispecific Antibody (1.72 mg/mL)		

Antibody Drug Conjugates (ADC)

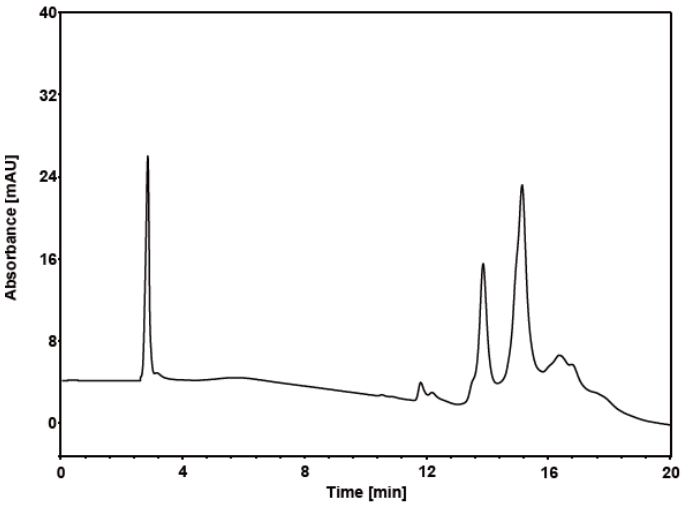
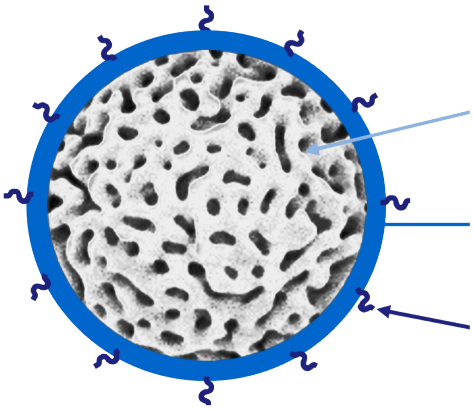


Figure 6

Part Number:	MBHICHPH424610		
Column:	**MONOBIO HIC-Phenyl**, 5 µm		
Dimension:	4.6×100 mm		
Mobile Phase:	A) 1.5 M $(NH_4)_2SO_4$ in 20 mM Phosphate Buffer, pH 7.0		
	B) 25/75 v/v IPA/20 mM Phosphate Buffer, pH 7.0		
Gradient:	t (min)	%A	%B
	0	100	0
	20	0	100
	21	100	0
	30	100	0
Flow Rate:	0.5 mL/min		
Temperature:	30 ℃		
Injection:	20 µL		
Detection:	UV 280 nm		
Sample:	ADC-DAR4 (0.5 mg/mL)		

MONOBIO HIC-PEG



Monodispersed, wide-pore silica particles

Neutral hydrophilic layer

Optimized PEG (polyethylene-glycol)

ADC

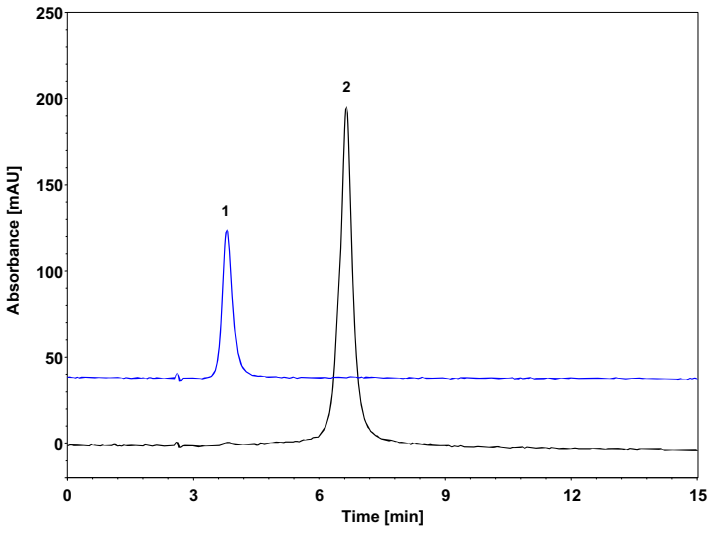


Figure 7

Part Number:	MBHICPEG424610		
Column:	**MONOBIO HIC-PEG**, 5 µm		
Dimension:	4.6×100 mm		
Mobile Phase:	A) 1 M $(NH_4)_2SO_4$ in 100 mM PB, pH 7.0		
	B) 20/80 v/v Isopropanol/100 mM PB, pH 7.0		
Gradient:	t(min)	%A	%B
	0	100	0
	20	0	100
	25	0	100
	25.1	100	0
	35	100	0
Flow Rate:	0.5 mL/min		
Temperature:	30 ℃		
Injection:	3 µL		
Detection:	UV 280 nm		
Sample:	ADC1		
Peaks:	1. mAb (3.3 mg/mL)		
	2. DAR6 (10 mg/mL)		

Screening Multiple HIC Phase Chemistries for Optimal Method Development

Protein Mixture

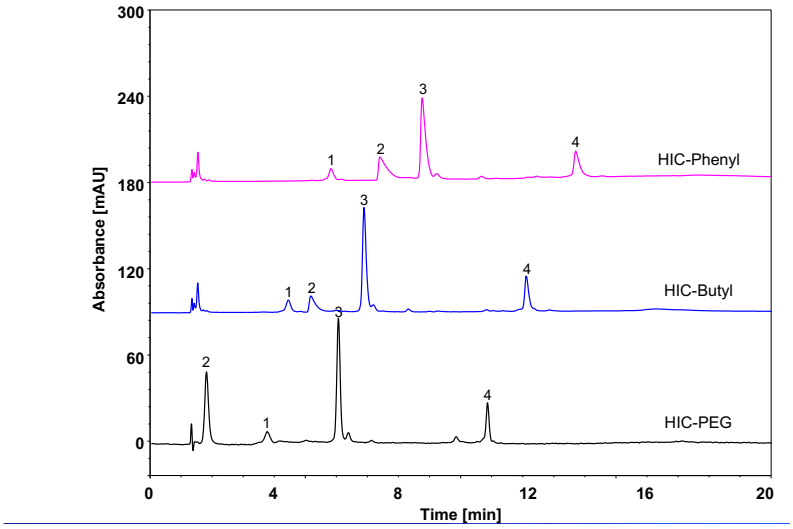


Figure 8

Part Number:
- Pink: MBHICHPH424610
- Blue: MBHICHBU424610
- Black: MBHICPEG424610

Column:
- Pink: **MONOBIO HIC-Phenyl**, 5 µm
- Blue: **MONOBIO HIC-Butyl**, 5 µm
- Black: **MONOBIO HIC-PEG**, 5 µm

Dimension: 4.6×100 mm

Mobile Phase: A) 2 M $(NH_4)_2SO_4$ in 100 mM PB, pH 7.0
B) 100 mM PB, pH 7.0

Gradient:

t (min)	%A	%B
0	100	0
1	100	0
15	0	100
20	0	100
20.1	100	0
30	100	0

Flow Rate: 1.0 mL/min

Temperature: 30 ℃

Injection: 5 µL

Detection: UV 280 nm

Peaks:
1. RNase A
2. Myoglobin
3. Lysozyme
4. α-Chymotrypsin

In Figure 8, a protein mixture was screened against the three MONOBIO phase chemistries to show the power of selectivity when choosing different column bonded phases.

mAbs Sample

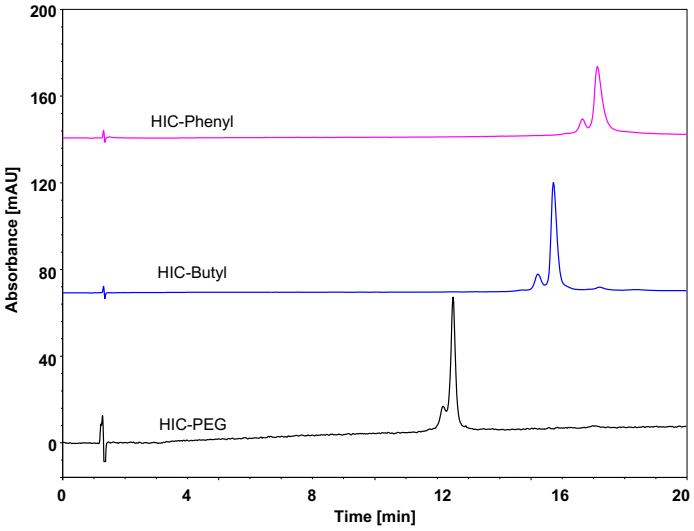


Figure 9

Part Number:
- Pink: MBHICHPH424610
- Blue: MBHICHBU424610
- Black: MBHICPEG424610

Column:
- Pink: **MONOBIO HIC-Phenyl**, 5 µm
- Blue: **MONOBIO HIC-Butyl**, 5 µm
- Black: **MONOBIO HIC-PEG**, 5 µm

Dimension: 4.6×100 mm

Mobile Phase: A) 2 M $(NH_4)_2SO_4$ in 100 mM PB, pH 7.0
B) 100 mM PB, pH 7.0

Gradient:

t (min)	%A	%B
0	100	0
1	100	0
15	0	100
20	0	100
20.1	100	0
30	100	0

Flow Rate: 1.0 mL/min

Temperature: 30 ℃

Injection: 10 µL

Detection: UV 280 nm

Sample: mAb1 (1 mg/mL)

In Figure 9, using a standard mAb, it is easy to see the hydrophobicity differences between phase chemistries.

ADC Sample – DAR Analysis

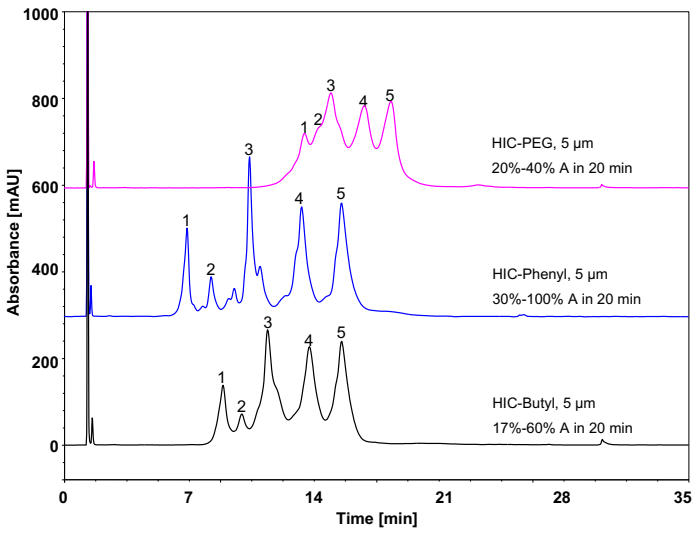


Figure 10

Part Number:
- Pink: MBHICPEG424610
- Blue: MBHICHPH424610
- Black: MBHICHBU424610

Column:
- Pink: **MONOBIO HIC-PEG**, 5 µm
- Blue: **MONOBIO HIC-Phenyl**, 5 µm
- Black: **MONOBIO HIC-Butyl**, 5 µm

Dimension: 4.6×100 mm

Mobile Phase: A) 1.8 M $(NH_4)_2SO_4$ in 50 mM PB, pH 6.0
B) 20% IPA in 50 mM PB, pH 6.0

Gradient:

t (min)	%A	%B
0	83/70/80	17/30/20
20	40/0/60	60/100/40
25	20/0/20	80/100/80
25.1	0	100
28	0	100
28.1	83/70/80	17/30/20
40	83/70/80	17/30/20

Flow Rate: 1.0 mL/min

Temperature: 25 ℃

Injection: 20 µL

Detection: UV 214 nm

Sample: ADC2 (1 mg/mL, 158 kDa)

Peak: 1. DAR=0 2. DAR=2 3. DAR=4 4. DAR=6 5. DAR=8

In Figure 10, the same ADC sample was screened against the three MONOBIO chemistries, and HIC-Phenyl was selected to be taken for further method development and validation.

ADC Sample 2 – DAR Analysis

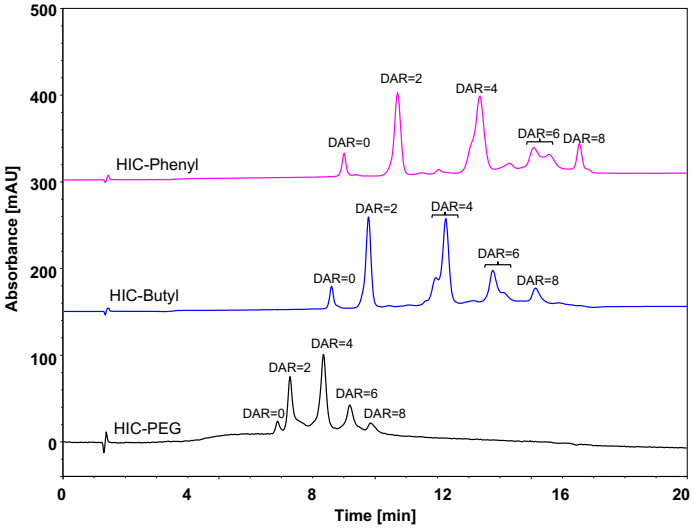


Figure 11

Part Number:
- Pink: MBHICHPH424610
- Blue: MBHICHBU424610
- Black: MBHICPEG424610

Column:
- Pink: **MONOBIO HIC-Phenyl**, 5 µm
- Blue: **MONOBIO HIC-Butyl**, 5 µm
- Black: **MONOBIO HIC-PEG**, 5 µm

Dimension: 4.6×100 mm

Mobile Phase: A) 2 M $(NH_4)_2SO_4$ in 100 mM PB, pH 7.0
B) 100 mM PB, pH 7.0
C) Isopropanol

Gradient:

t (min)	%A	%B	%C
0	75	25	0
1	75	25	0
15	0	75	25
20	0	75	25
20.1	75	25	0
30	75	25	0

Flow Rate: 1.0 mL/min
Temperature: 30 ℃
Injection: 10 µL
Detection: UV 280 nm
Sample: ADC4 (5 mg/mL)

In Figure 11, MONOBIO HIC-Butyl would be chosen, as this column exhibits some selectivity improvements for DAR04 and DAR-6 which impact resolution.

Bispecific Antibody Screening

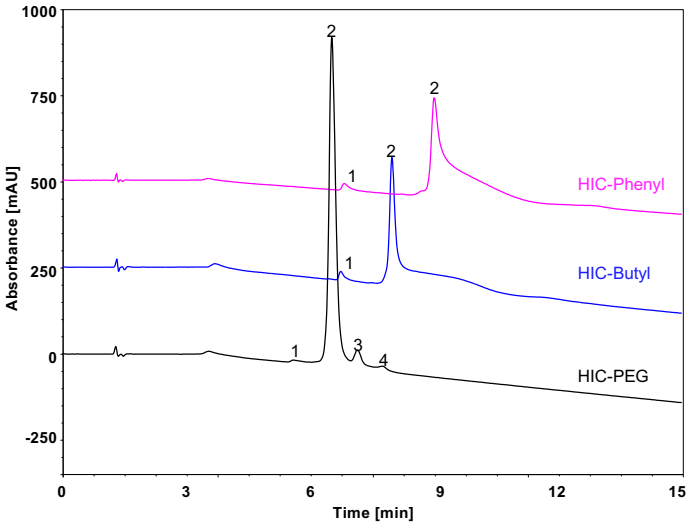


Figure 12

Part Number:
- Pink: MBHICHPH424610
- Blue: MBHICHBU424610
- Black: MBHICPEG424610

Column:
- Pink: **MONOBIO HIC-Phenyl**, 5 µm
- Blue: **MONOBIO HIC-Butyl**, 5 µm
- Black: **MONOBIO HIC-PEG**, 5 µm

Dimension: 4.6×100 mm

Mobile Phase: A) 2 M $(NH_4)_2SO_4$ in 100 mM PB, pH 7.0
B) 100 mM PB, pH7.0
C) IPA

Gradient:
Red: 50%-75% B, 0%-25% C in 1-15 min, 75% B, 25 % C in 15-20 min, 50% B, 0% C in 20.1-30 min

Blue and Black: 25%-75% B, 0%-25% C in 1-15 min, 75% B, 25 % C in 15-20 min, 25% B, 0% C in 20.1-30 min

Flow Rate: 1.0 mL/min
Temperature: 30 ℃
Injection: 5 µL
Detection: UV 214 nm
Sample: Bispecific Antibody (2 mg/mL)

In Figure 12, the same Bispecific Antibody sample was screened against the three MONOBIO chemistries, and HIC-PEG was selected to be taken for further method development and validation. It provided improved resolution for the variant peaks.

MONOBIO Part Numbers

HIC-Butyl

Pore Size (Å)	Particle Size (µm)	Column ID (mm)	Column Length (mm)	Part Number
1000	3	4.6	100	MBHICHBU414610
1000	5	4.6	50	MBHICHBU424605
1000	5	4.6	100	MBHICHBU424610
1000	5	4.6	250	MBHICHBU424625

HIC-Phenyl

Pore Size (Å)	Particle Size (µm)	Column ID (mm)	Column Length (mm)	Part Number
1000	3	4.6	100	MBHICHPH414610
1000	5	4.6	50	MBHICHPH424605
1000	5	4.6	100	MBHICHPH424610
1000	5	4.6	250	MBHICHPH424625

HIC-PEG

Pore Size (Å)	Particle Size (µm)	Column ID (mm)	Column Length (mm)	Part Number
1000	3	4.6	100	MBHICPEG414610
1000	5	4.6	50	MBHICPEG424605
1000	5	4.6	100	MBHICPEG424610
1000	5	4.6	250	MBHICPEG424625

NOTES

MONOBIO

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MONODISPERSE PARTICLE TECHNOLOGY

HIC Columns for HPLC Applications

CONTACT US

Ordering: info@mac-mod.com

Technical Support: technical@mac-mod.com

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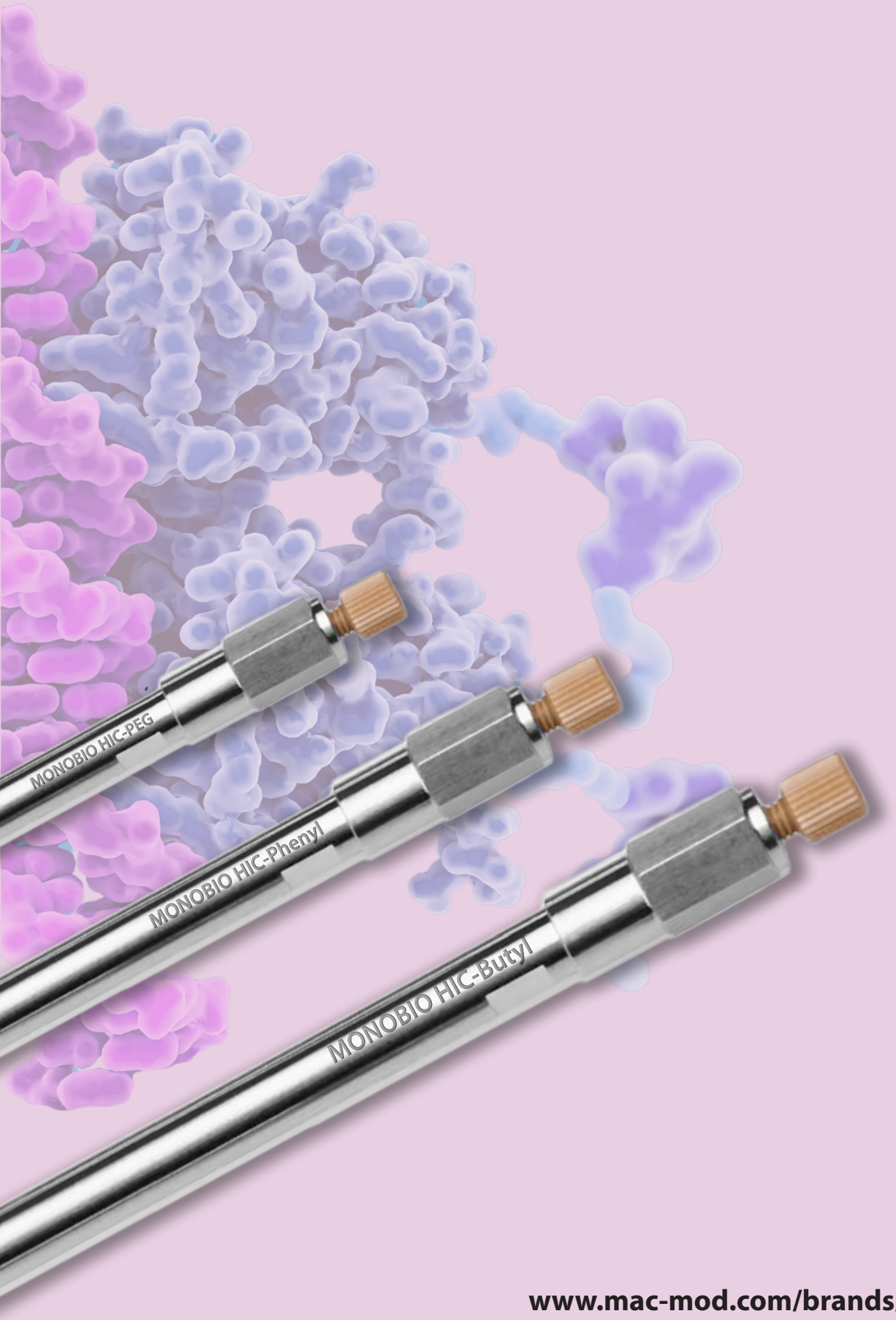

www.mac-mod.com/brands/monobio/hic/