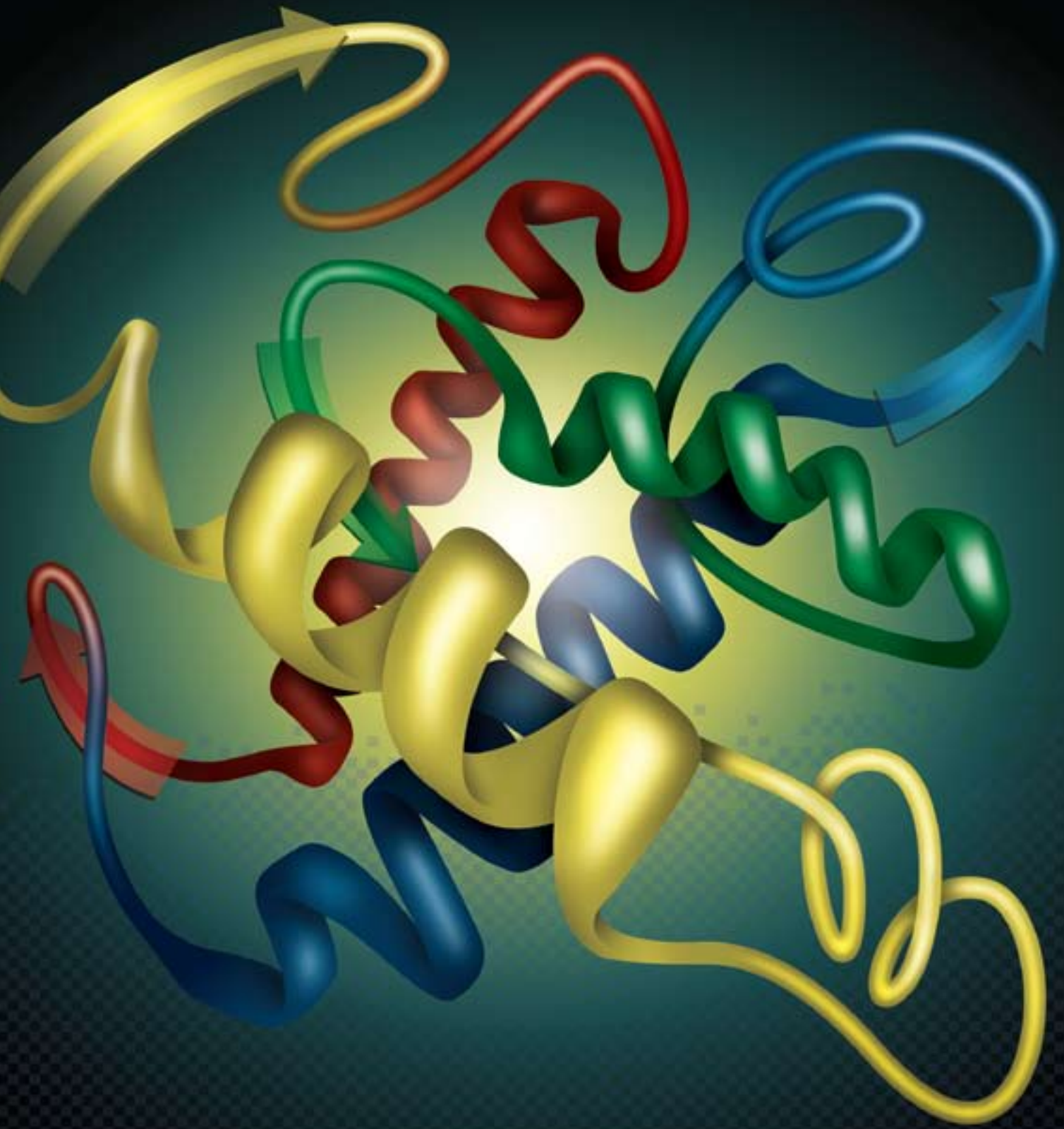
Protein Separation Technology



Waters

THE SCIENCE OF WHAT'S POSSIBLE.™

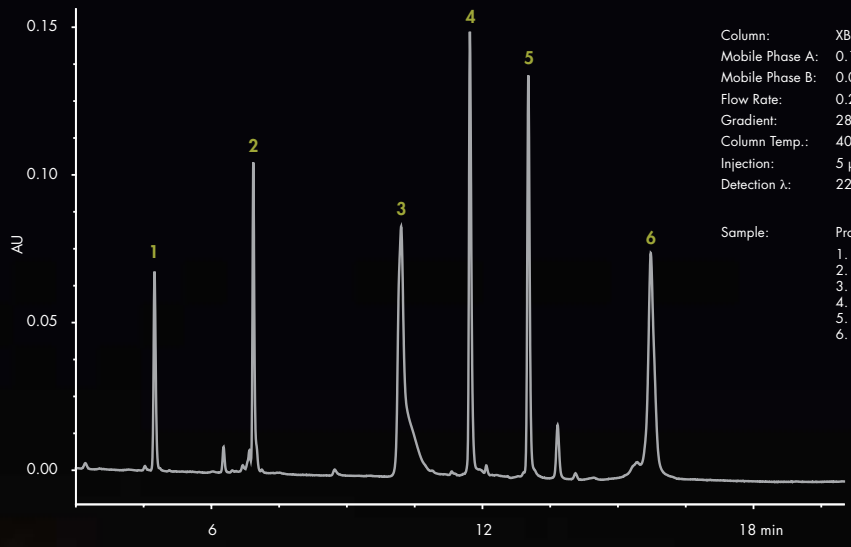
Protein Separation Technology

The analysis and characterization of protein samples requires the detection of small chemical differences between large molecules. Most often these analyses have employed an array of analytical techniques, each sensitive to a different property of the protein. Reversed-phase HPLC has not been fully exploited in these tests because the separation of proteins often yields relatively broad and asymmetrical peaks with poor recovery and significant carryover. Waters new reversed-phase, ethylene bridged hybrid (BEH) Protein Separation Technology columns are specifically designed for the high resolution analysis of proteins.

Introducing a New Family of BEH300 C_4 Columns for Protein Separations

- Separates proteins of various sizes, hydrophobicities, and isoelectric points
- Available in 3.5 µm particles for HPLC and 1.7 µm material for UPLC® and nano UPLC applications
- Maximizes recovery and minimizes protein carryover
- Tolerates extreme pH and temperature
- Quality-control tested with protein mixture
- Couples directly to ESI-MS for protein identification

300Å C_4 Columns Developed for Protein Chromatography



Column:	XBridge™ BEH300 C_4, 3.5 µm, 2.1 × 50 mm
Mobile Phase A:	0.1% TFA in water
Mobile Phase B:	0.075% TFA in 71.4% acetonitrile
Flow Rate:	0.2 mL/min
Gradient:	28-100% B in 25 min
Column Temp.:	40 °C
Injection:	5 µL
Detection λ:	220 nm

Sample: Protein mixture prepared in 0.1% TFA in 5% acetonitrile

1. Ribonuclease A (0.04 mg/mL)
2. Cytochrome *c* (0.06 mg/mL)
3. Bovine Serum Albumin (0.20 mg/mL)
4. Myoglobin (0.13 mg/mL)
5. Enolase (0.22 mg/mL)
6. Phosphorylase b (0.59 mg/mL)

BEH300 C_4 columns can be used with proteins that have a wide range of properties. This protein mix was chosen to represent a range of isoelectric points, molecular weights, and hydrophobicities.

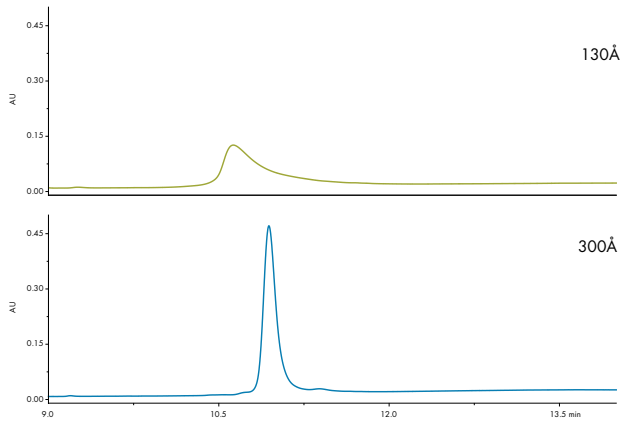
COLUMNS DESIGNED FOR PROTEIN SEPARATIONS

The new Protein Separation Technology material is based on Waters patented BEH Technology™ hybrid material. The combination of the hybrid particle, short chain bonded phase, and wide pore overcomes the performance limitations of traditional silica-based column packings.

- 300Å pores
- C_4 ligand
- Minimal secondary interactions
- Long column lifetime at elevated temperature
- Minimal carryover and maximum recovery

Effect of Pore Size on Separation of a Monoclonal Antibody

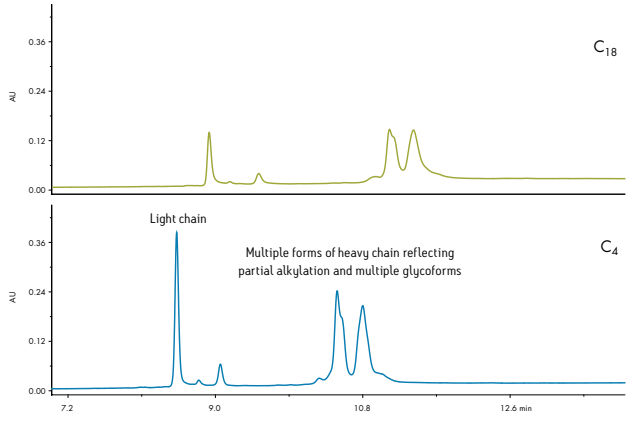
Sample:	Intact mouse monoclonal antibody (IgG) – 0.5 mg/mL prepared in 0.1% TFA in water
Column:	XBridge BEH300 C_{18} (bottom) and XBridge BEH130 C_{18} (top), 3.5 µm, 2.1 × 50 mm
Mobile Phase A:	0.1% TFA in water
Mobile Phase B:	0.075% TFA in 100% acetonitrile
Flow Rate:	0.2 mL/min
Gradient:	20-71.4% B in 25 min
Column Temp.:	80 °C
Injection:	3.3 µL
Detection λ:	220 nm



A murine monoclonal IgG shows a small, badly tailing peak on a C_{18} column with 130Å pores and a better recovered, symmetrical peak on the larger pore size.

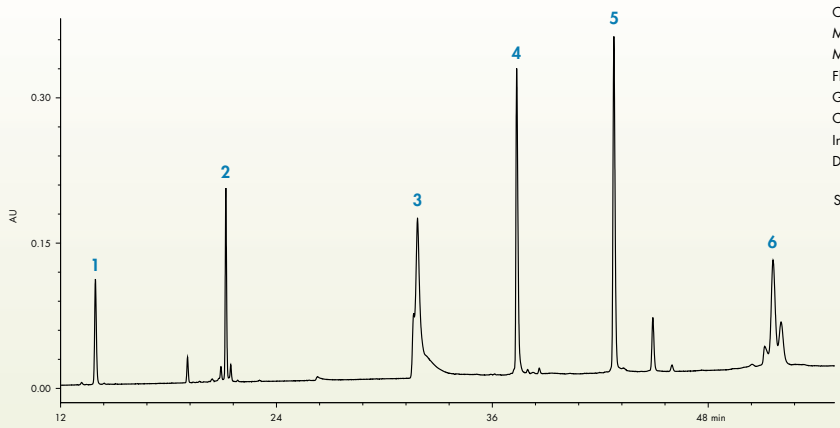
Effect of Bonded-Phase Chain Length on Separation of IgG Heavy and Light Chains

Sample:	Reduced and alkylated mouse monoclonal antibody (IgG) – 0.5 mg/mL prepared in 0.1% TFA in water
Column:	XBridge BEH300 C_4 (bottom) and XBridge BEH300 C_{18} (top), 3.5 µm, 2.1 × 50 mm
Mobile Phase A:	0.1% TFA in water
Mobile Phase B:	0.075% TFA in 100% acetonitrile
Flow Rate:	0.2 mL/min
Gradient:	20-71.4% B in 25 min
Column Temp.:	80 °C
Injection:	3.3 µL
Detection λ:	220 nm



Generally, proteins are expected to give better reversed-phase chromatography on short chain bonded phases. For testing this effect, a murine monoclonal antibody was reduced and partially alkylated to create a sample that includes several large protein subunits that are distinguished by very small differences in structure. The light and heavy chain components of the mouse monoclonal antibody offer improved peak shape, resolution, and recovery on the XBridge BEH300, C_4 relative to the same particle and pore size with the longer chain C_{18}.

Minimal Secondary Interactions

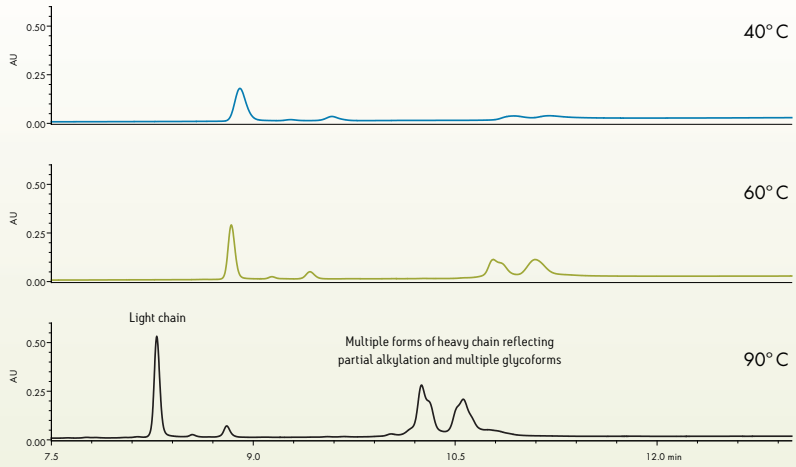


Column:	ACQUITY UPLC® BEH300 C_4, 1.7 µm, 2.1 × 150 mm
Mobile Phase A:	0.1% TFA in water
Mobile Phase B:	0.1% TFA in 100% acetonitrile
Flow Rate:	0.2 mL/min
Gradient:	20-55% B in 60 min
Column Temp.:	40 °C
Injection:	3.3 µL
Detection λ:	220 nm
Sample:	Protein mixture prepared in 0.1% TFA in 5% acetonitrile

1. Ribonuclease A (0.04 mg/mL)
2. Cytochrome *c* (0.06 mg/mL)
3. Bovine Serum Albumin (0.20 mg/mL)
4. Myoglobin (0.13 mg/mL)
5. Enolase (0.22 mg/mL)
6. Phosphorylase b (0.59 mg/mL)

The surface chemistry of the BEH300 C_4 particles gives symmetrical peak shapes for a variety of protein types. For example, the sharp peak for the basic protein cytochrome c supports the lack of non-desired secondary interactions with the BEH particle surface.

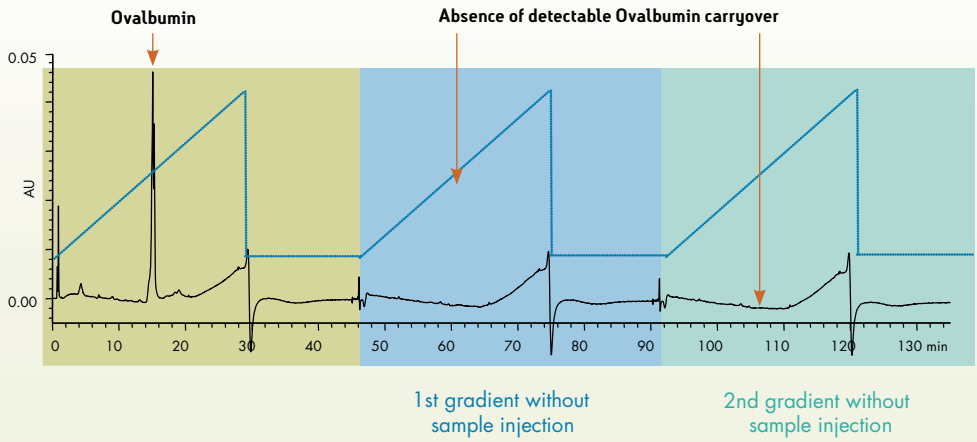
Separations at Elevated Temperature



Sample:	Reduced and alkylated mouse monoclonal antibody (IgG) – 0.5 mg/mL prepared in 0.1% TFA in water
Column:	XBridge BEH300 C_4, 3.5 µm, 2.1 × 50 mm
Mobile Phase A:	0.1% TFA in water
Mobile Phase B:	0.075% TFA in 100% acetonitrile
Flow Rate:	0.2 mL/min
Gradient:	20-71.4% B in 25 min
Column Temp.:	40 °C (top), 60 °C (middle), 90 °C (bottom)
Injection:	3.3 µL
Detection λ:	220 nm

Elevated temperature is routinely used in protein separations. For testing this effect, a murine monoclonal antibody was reduced and partially alkylated to create a sample that includes several large protein subunits that are distinguished by very small differences in structure. The yield of the heavy and light chains improves the temperature is increased from 40-90 °C. BEH Technology columns were developed for use at extreme temperatures.

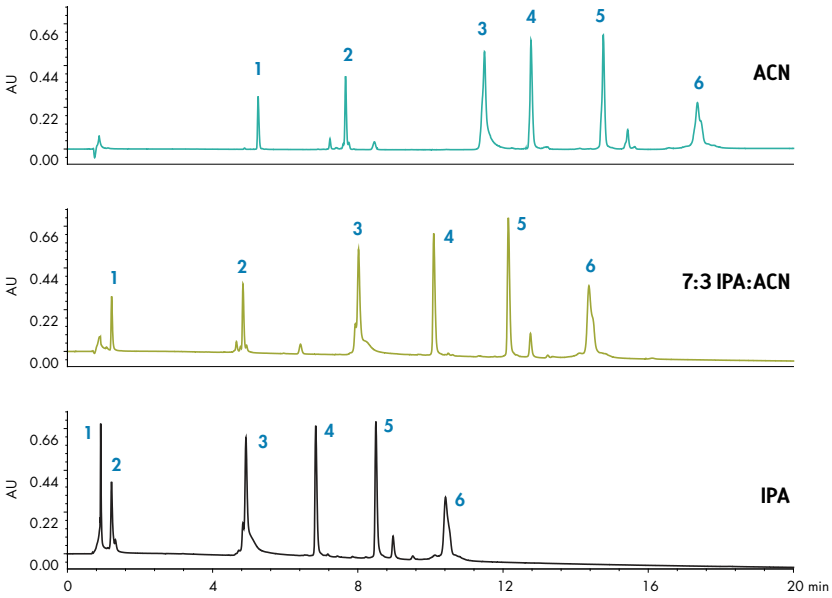
Minimal Protein Carryover



Sample:	1.3 µg Ovalbumin injected
Columns:	ACQUITY UPLC BEH300 C_4, 1.7 µm, 2.1 mm x 50 mm
Eluent A:	0.1% TFA in 100% HPLC grade water
Eluent B:	0.075% TFA in 71.4% acetonitrile
Flow Rate:	0.2 mL/min
Gradient:	28-100% B in 25 min Hold at 100 %B for 2 min then re-equilibrate column at 28% B for 18 min Gradient restarted at 45 min and at 90 min
Column Temp.:	40 °C
Detection λ:	220 nm

Column carryover was tested by running multiple gradients following a single injection. Protein peaks observed during the first gradient are not found in subsequent gradients.

Effective Alternatives to Use of 100% Acetonitrile



Sample:	Protein Mixture prepared in 0.1% TFA in 5% MeCN: 1. Ribonuclease A (0.08 mg/mL), 2. Cytochrome c (0.11 mg/mL), 3. Bovine Serum Albumin (0.40 mg/mL), 4. Myoglobin (0.25 mg/mL), 5. Enolase (0.43 mg/mL), 6. Phosphorylase b (1.18 mg/mL)
Column:	BEH300 C_4, 1.7 µm, 2.1 x 50 mm
Mobile Phase A:	0.1% TFA in water
Mobile Phase B:	0.1% TFA in MeCN; 0.1% TFA in 7:3 IPA:MeCN; 0.1% TFA in IPA;
Flow Rate:	0.2 mL/min
Column Temperature:	80 °C
Injection:	3.3 µL
Detection λ:	220 nm

Gradient Table:

Time (min)	%A	%B	Curve	Column Volumes (CV)	% Change (CV)
Initial	80	20	–	–	–
29.6	28.6	71.4	6	23	1.5
31.6	28.6	71.4	6	3	*
49.6	80	20	1	10	–

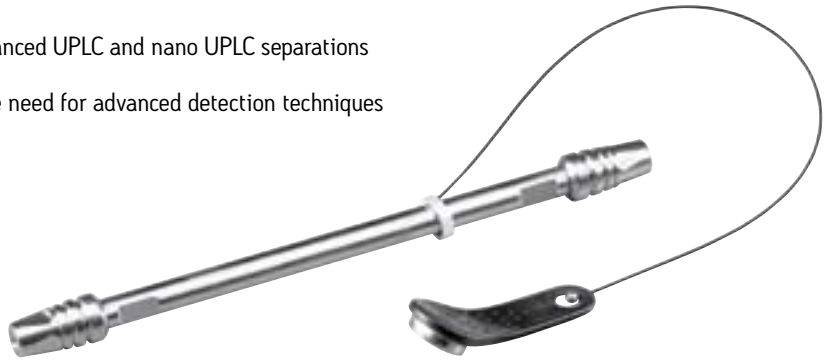
Acetonitrile is the preferred solvent for reversed-phase protein separations. Altered selectivity and improved recovery in reversed-phase protein separations can be achieved with different solvents. It is important to consider alternatives to acetonitrile during the recent worldwide shortage of this solvent. A protein standard mixture was analyzed on BEH300 C_4 with 100% acetonitrile (top), pure isopropanol (bottom) and a 70:30 blend (middle). All three solvents give useful separations with similar selectivity.

WATERS UPLC TECHNOLOGY AND ADVANCED DETECTION FOR PROTEIN CHARACTERIZATION

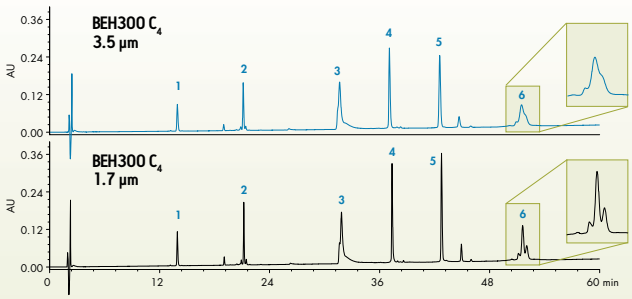
Waters introduced UPLC Technology in 2004. This new class of separation technology brings improved resolution, sensitivity, and speed to a wide range of chromatographic analyses. With the BEH300 C_4 packing material in 1.7 µm particles, these enhancements can now be realized with protein separations.

- Easily scaled 3.5 µm for HPLC and 1.7 µm for enhanced UPLC and nano UPLC separations
- Effective with MS compatible eluents to address the need for advanced detection techniques

Acquity
UltraPerformance LC®



Improved Protein Resolution with UPLC Technology

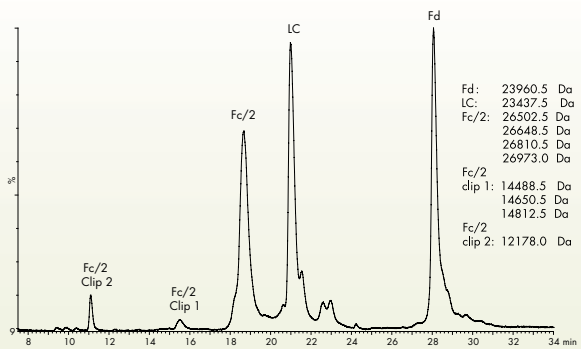


Column:	ACQUITY UPLC BEH300 C_4, 2.1 ×150 mm, 1.7 µm (top), 3.5 µm (bottom)
Mobile Phase A:	0.1% TFA in water
Mobile Phase B:	0.1% TFA in 100% acetonitrile
Flow Rate:	0.2 mL/min
Gradient:	20-55% B in 60 min
Column Temp.:	80 °C
Injection:	3.3 µL
Detection λ:	220 nm

Sample: Protein mixture prepared in 0.1% TFA in 5% acetonitrile
1. Ribonuclease A (0.04 mg/mL)
2. Cytochrome c (0.06 mg/mL)
3. Bovine Serum Albumin (0.20 mg/mL)
4. Myoglobin (0.13 mg/mL)
5. Enolase (0.22 mg/mL)
6. Phosphorylase b (0.59 mg/mL)

The protein test mixture was separated on two BEH300 C_4 columns, one with 3.5 µm particles and the other with 1.7 µm particles. The UPLC separation provides sharper peaks for all the proteins in the test mixture. This translates into better resolution as shown by the multiple peaks around phosphorylase, at approximately 50 min. This comparison, with both columns, was performed on a UPLC system to preserve the minimized band-broadening. The benefits of the small particle UPLC BEH300 C_4 column would be lost without the optimized ACQUITY UPLC System.

BEH300 C_4 Columns for Protein Analysis with UPLC/MS



Sample:	Lys-C digested (limited) reduced monoclonal antibody
Column:	ACQUITY UPLC BEH300 C_4, 1.7 µm, 2.1 mm × 50 mm
Eluent A:	0.1% FA in 100% HPLC grade water
Eluent B:	0.1% FA in 100% acetonitrile
Flow Rate:	0.2 mL/min
Gradient:	5% B for 5 min Then 5-21% B in 0.1 min. Then 21-29% B in 30 min.
Column Temp.:	80 °C
Waters LCT Premier™ XE MS Detection	
Capillary Voltage:	3200 V
Cone Voltage:	45 V
Source Temp.:	150 °C
Desolvation Temp.:	350 °C
Desolvation Gas:	1000 L/h

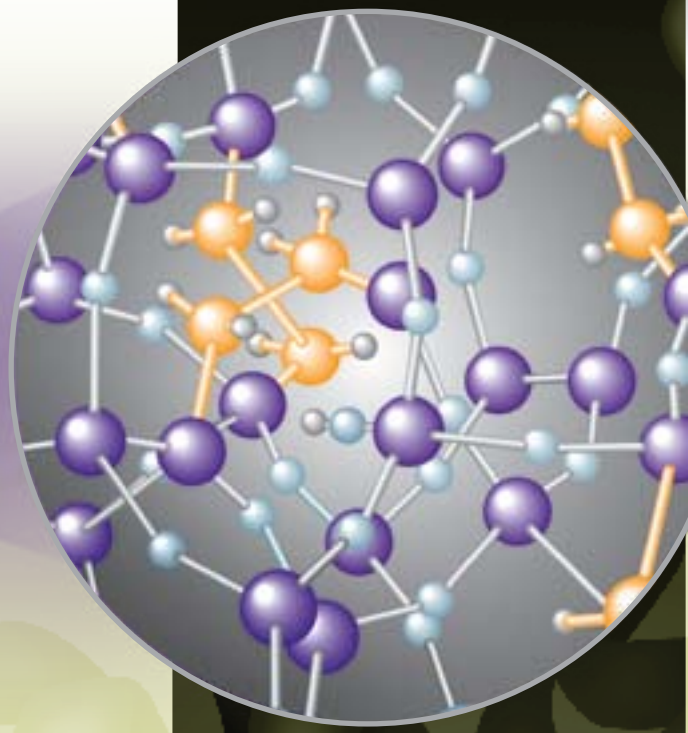
The large fragments obtained through LysC digestion of a monoclonal antibody can be separated on the UPLC BEH300 C_4 column coupled directly to ESI/Tof MS for identification of the individual peptide products.

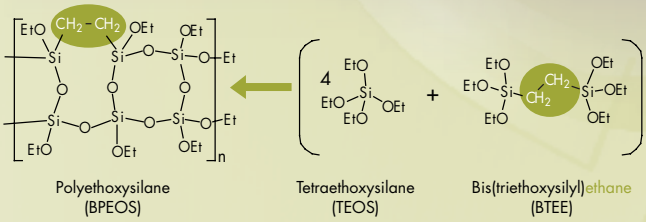
BEH PARTICLE SYNTHESIS AND BONDING TECHNOLOGY FOR STABLE AND REPRODUCIBLE PROTEIN SEPARATIONS

Waters has developed a second generation hybrid particle synthesis. The patented process ensures the highest purity and most consistent column properties. The material can be prepared in particle sizes for HPLC and UPLC. The pores can be enlarged as appropriate for protein separations.

- Well characterized, state-of-the-art bonding procedures for short-chain ligand
- Particle structure and bonding chemistry stable at low pH and at elevated temperature
- Quality-control tested with a diverse protein mixture
- Consistent protein separations from batch-to-batch

BEH Technology™



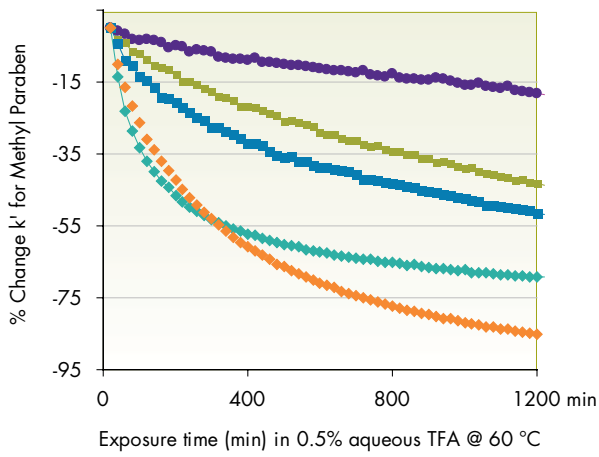


Anal. Chem. 2003, 75, 6781-6788, U.S. Patent No. 6,686,035 B2

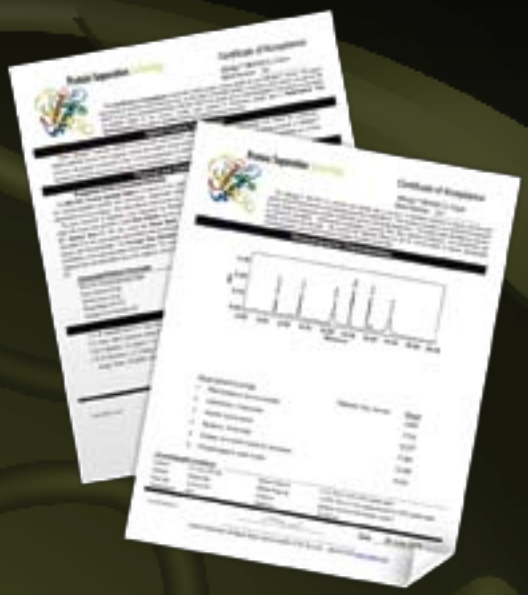
XBridge™ COLUMNS

Waters innovative BEH300 C_4 particle synthesis and bonding process yields HPLC and UPLC columns that tolerate separation conditions of low pH and elevated temperature. Shown above are our available XBridge BEH300 C_4 Columns. ACQUITY UPLC BEH300 C_4 Columns are also available.

Accelerated Low pH Stability Testing on BEH300 C_4 vs. Competitive Columns



Quality Control Document for BEH300 C_4 Columns



Each batch of BEH300 C_4 material is rigorously tested to ensure consistent performance. The Certificate of Analysis included with each column reports physical, chemical, and chromatographic tests.

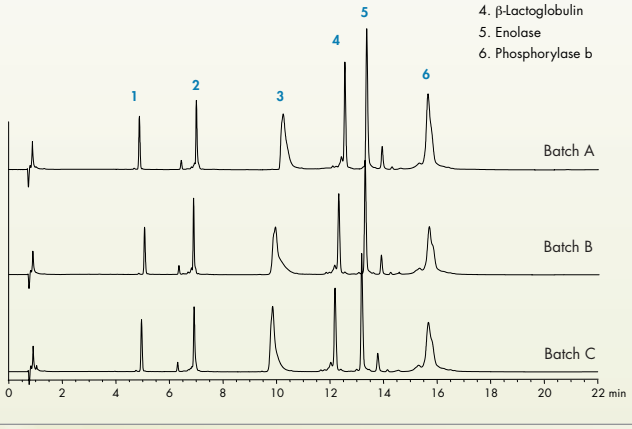
Batch-to-Batch Reproducibility

Column:	ACQUITY UPLC BEH300 C_4, 1.7 µm, 2.1 mm × 50 mm
Eluent A:	0.1% TFA in 100% HPLC grade water
Eluent B:	0.075% TFA in 71.4% acetonitrile
Flow Rate:	0.2 mL/min
Gradient:	28-100% B in 25 min
Column Temp.:	40 °C
Detection λ:	220 nm

Sample:

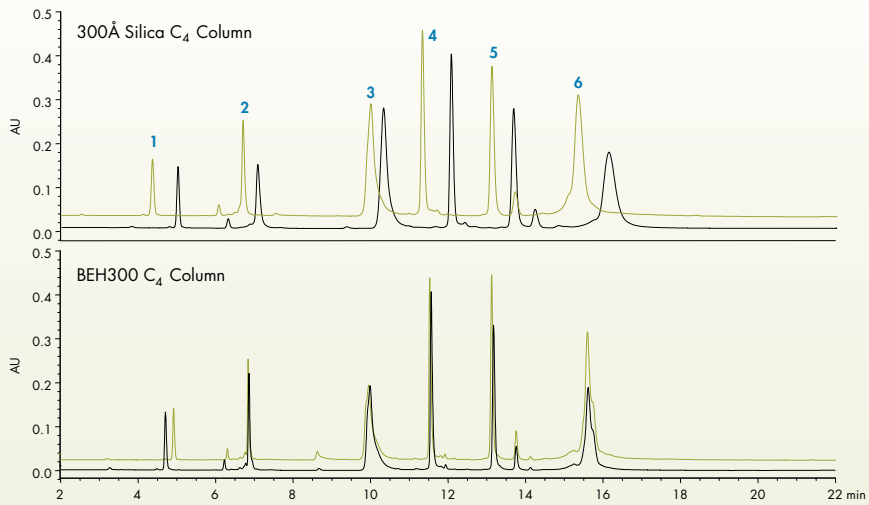
Protein mixture prepared in 0.1% TFA in 5% acetonitrile

1. Ribonuclease
2. Cytochrome *c*
3. BSA
4. β-Lactoglobulin
5. Enolase
6. Phosphorylase b



This comparison shows the consistent batch-to-batch performance with a protein separation.

Protein Separation Stability Comparison Between BEH300 C_4 and Competitive Column



Columns:	XBridge BEH300 C_4, 3.5 µm (top), 100% silica-based, 300Å C_4, 3.5 µm (bottom), 2.1 mm × 50 mm
Eluent A:	0.1% TFA in 100% HPLC grade water
Eluent B:	0.075% TFA in 71.4% acetonitrile
Flow Rate:	0.2 mL/min
Gradient:	28-100% B in 25 min
Column Temp.:	40 °C
Detection:	220 nm
Sample:	Protein mixture prepared in 0.1% TFA in 5% acetonitrile

1. Ribonuclease
2. Cytochrome *c*
3. BSA
4. myoglobin
5. Enolase
6. Phosphorylase b

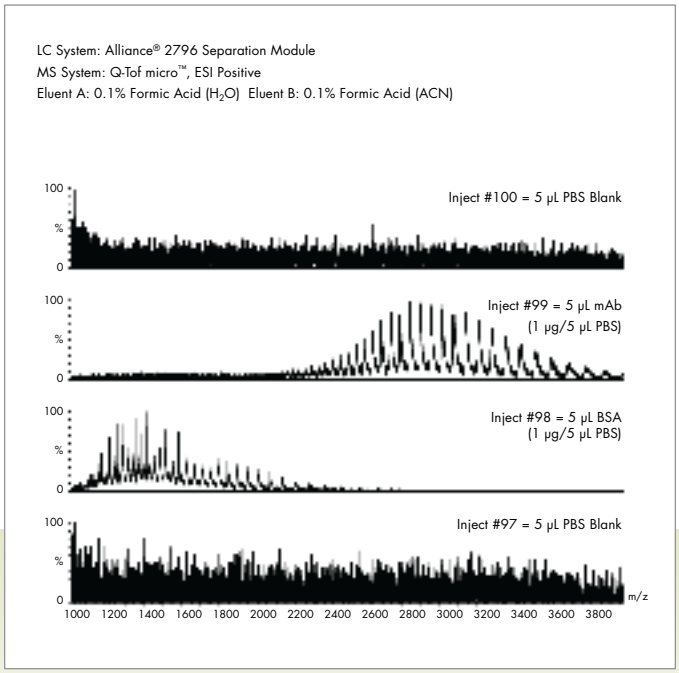
The two columns were tested with an initial analysis of the protein standard mixture (black line). Each column was operated continuously for one week with the same gradient at 90 °C. The protein separation test at 40 °C was then repeated (green line). Note that the retention shifts earlier on the silica-based column while the separation is stable on the BEH300 C_4.

MASSPREP ON-LINE PROTEIN DESALTING DEVICES

MassPREP™ on-line desalting columns can effectively desalt proteins prior to LC/MS analyses. Since non-volatile salts (e.g., NaCl) can suppress ionization of intact proteins leading to poor detection sensitivity, it is important to remove, or significantly minimize, the introduction of these compounds into the mass analyzer. The reversed-phase, phenyl material contained in MassPREP on-line columns successfully "traps" proteins, allowing the salts to be washed to waste prior to protein elution into the mass spectrometer. With an optimized LC/MS method, cycle times as low as 4 minutes (for intact antibody) and 10 minutes (for reduced antibody) are achievable.

- Effectively desalts proteins yielding improved LC/MS results
- Fast on-line method for high throughput applications
- Excellent protein recoveries and no detectable carryover
- 100 injections from a single cartridge

Waters MassPREP On-Line Desalting Cartridge (2.1 x 10 mm)

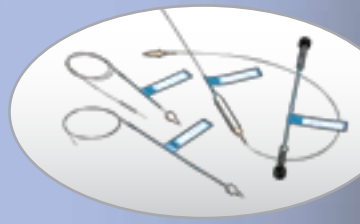


Over a series of 100 injections, satisfactory results were obtained for BSA and a mAb, as shown for injections #97-100 on a MassPREP on-line desalting cartridge.

Reference Desalting of Proteins Using MassPREP On-line Desalting Cartridges Prior to Mass Spectrometry. 2005 Waters Application Note 720001077EN

Waters ACQUITY UPLC System and Xevo™ Mass Spectrometer with Protein Separation Technology BEH 300 C_4 columns and MassPREP On-Line Desalting Devices

Waters nanoACQUITY UPLC® System with nanoACQUITY® BEH 300 C_4 columns



ORDERING INFORMATION

Ordering information for Waters BEH300 C_4 offerings for traditional HPLC and advanced UPLC protein separations are shown below.

UPLC Columns	Part Number
ACQUITY UPLC BEH300 C_4, 1.7 µm, 2.1 x 50 mm	186004495
ACQUITY UPLC BEH300 C_4, 1.7 µm, 2.1 x 100 mm	186004496
ACQUITY UPLC BEH300 C_4, 1.7 µm, 2.1 x 150 mm	186004497
ACQUITY UPLC BEH300 C_4, 1.7 µm VanGuard™ Pre-Column	186004623

Note: ACQUITY UPLC BEH300 C_4, 1.7 µm columns are designed for use with the ACQUITY UPLC system. The benefits of the small particle packing in ACQUITY UPLC BEH300 C_4, 1.7 µm columns are only realized with the low system volume and low detector dispersion of an ACQUITY UPLC system.

nanoACQUITY UPLC	Part Number
nanoACQUITY UPLC BEH300 C_4, 1.7 µm, 75 µm x 100 mm	186004639
nanoACQUITY UPLC BEH300 C_4, 1.7 µm, 100 µm x 100 mm	186004640
nanoACQUITY UPLC BEH300 C_4, 1.7 µm, 150 µm x 100 mm	186004641

For use with nanoACQUITY UPLC systems rated to 10,000 psi only. Not for use with nanoACQUITY UPLC systems rated to 5,000 psi.

HPLC Columns	Part Number
XBridge BEH300 C_4, 3.5 µm, 2.1 x 50 mm	186004498
XBridge BEH300 C_4, 3.5 µm, 2.1 x 100 mm	186004499
XBridge BEH300 C_4, 3.5 µm, 2.1 x 150 mm	186004500
XBridge BEH300 C_4, 3.5 µm, 2.1 x 250 mm	186004501
XBridge BEH300 C_4, 3.5 µm, 4.6 x 50 mm	186004502
XBridge BEH300 C_4, 3.5 µm, 4.6 x 100 mm	186004503
XBridge BEH300 C_4, 3.5 µm, 4.6 x 150 mm	186004504
XBridge BEH300 C_4, 3.5 µm, 4.6 x 250 mm	186004505
Custom BEH300 C_4	186004506

MassPREP On-Line Protein Desalting Devices	Quantity	Part Number
MassPREP Micro Desalting Column	1/pk	186004032
MassPREP On-line Desalting Cartridge (2.1 x 10 mm)	2/pk	186002785
UPLC Intact Mass Analysis Application Kit* (Includes MassPREP Micro Desalting Column and ACQUITY Tubing Kit)	1/pk	176001519
Sentry™ 2.1 x 10 mm Guard Cartridge Holder. Required for use of MassPREP On-line Desalting Cartridge	1/pk	WAT097958

** See: UPLC Intact Mass Analysis Application Kit Manual (715001664)*

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720002577EN April 2009 SC-AC

The quality management system of Waters' manufacturing facilities in Taunton, Massachusetts and Wexford, Ireland complies with the International Standard ISO 9001:2000 Quality Management and Quality Assurance Standards. Waters' quality management system is periodically audited by the registering body to ensure compliance.