



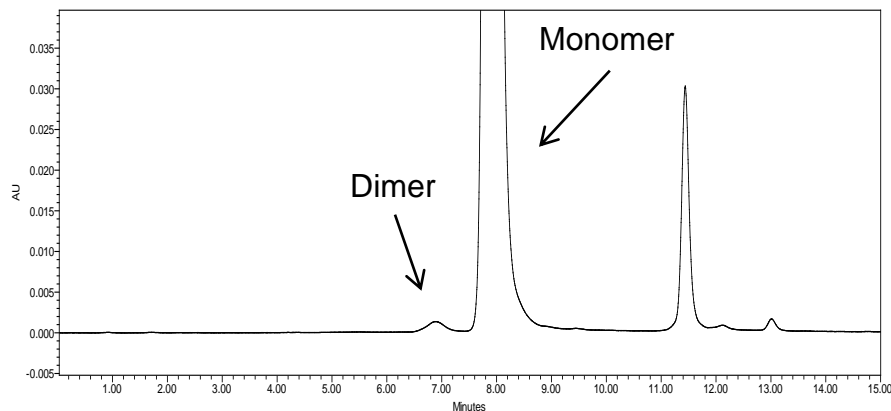
Chromatography-Based Characterization of Biologics

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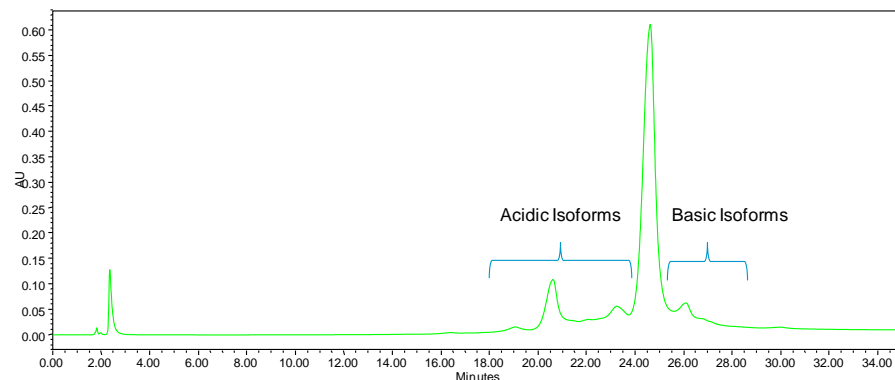
May 2010

Critical QA/QC Methods for Monoclonal Antibodies

Size Exclusion: Aggregation Analysis



Ion Exchange: Charge Isoform Analysis



Reversed Phase: Impurity Analysis & Disulfide Shifts

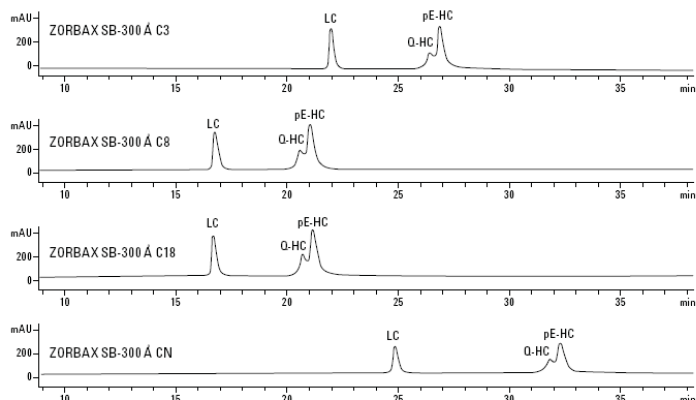
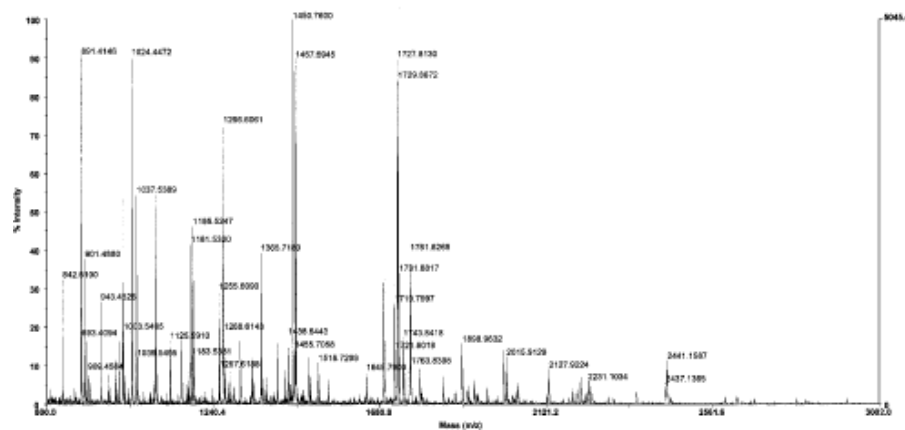


Figure 3. Reversed-phase chromatograms of reduced and alkylated IgG on ZORBAX StableBond SB-300 A, 4.6 mm × 150 mm columns packed with 3.5 µm particles, 300 Å pore size, C3, C8, C18, CN and Varian DiPhenyl 150 mm × 4.6 mm column. The separation was achieved using the same linear gradient from 23 to 35% B.

Peptide Mapping: Post-translational modifications



Agilent Columns for Biologic Characterization

Size Exclusion BioHPLC Columns

Agilent Bio SEC-5

High Resolution Size Exclusion Columns

Agilent Bio SEC-3

High Efficiency and High Resolution

Ion Exchange BioHPLC Columns

Agilent Bio MAb

High Resolution Separations of Monoclonal Antibodies

Agilent Bio IEX

High Resolution Ion Exchange Columns

Reversed Phase

Poroshell 300

High efficiency – fast protein and peptide separations

Agilent Poroshell 120

Advancing capabilities for high-performance HPLC



Size Exclusion BioHPLC Columns

Size Exclusion Columns

Agilent Bio SEC-5

High Resolution Size Exclusion Columns

- 5 μ m Particle
- Polymeric coated silica, minimizes non-specific binding
- Optimized for use at low salt concentrations (150mM Phosphate)
- High stability and long lifetime
- Great reproducibility
- Exclusion Ranges Available
 - 100 \AA , 0.1 – 100 kDa
 - 150 \AA , 0.5 – 150 kDa
 - 300 \AA , 5 – 1,250 kDa
 - 500 \AA , 15 – 5,000 kDa
 - 1000 \AA , 50 – 7,500 kDa
 - 2000 \AA , >10,000 kDa



When to choose the Bio SEC-5?

- Need higher resolution for mAb aggregates (300 \AA) and same particle size as Tosoh
- Require higher exclusion limits (500 \AA , 100 \AA , 2000 \AA)
- Want to separate proteins with lower salt concentrations, better for your instruments

Column Evaluation and Comparison

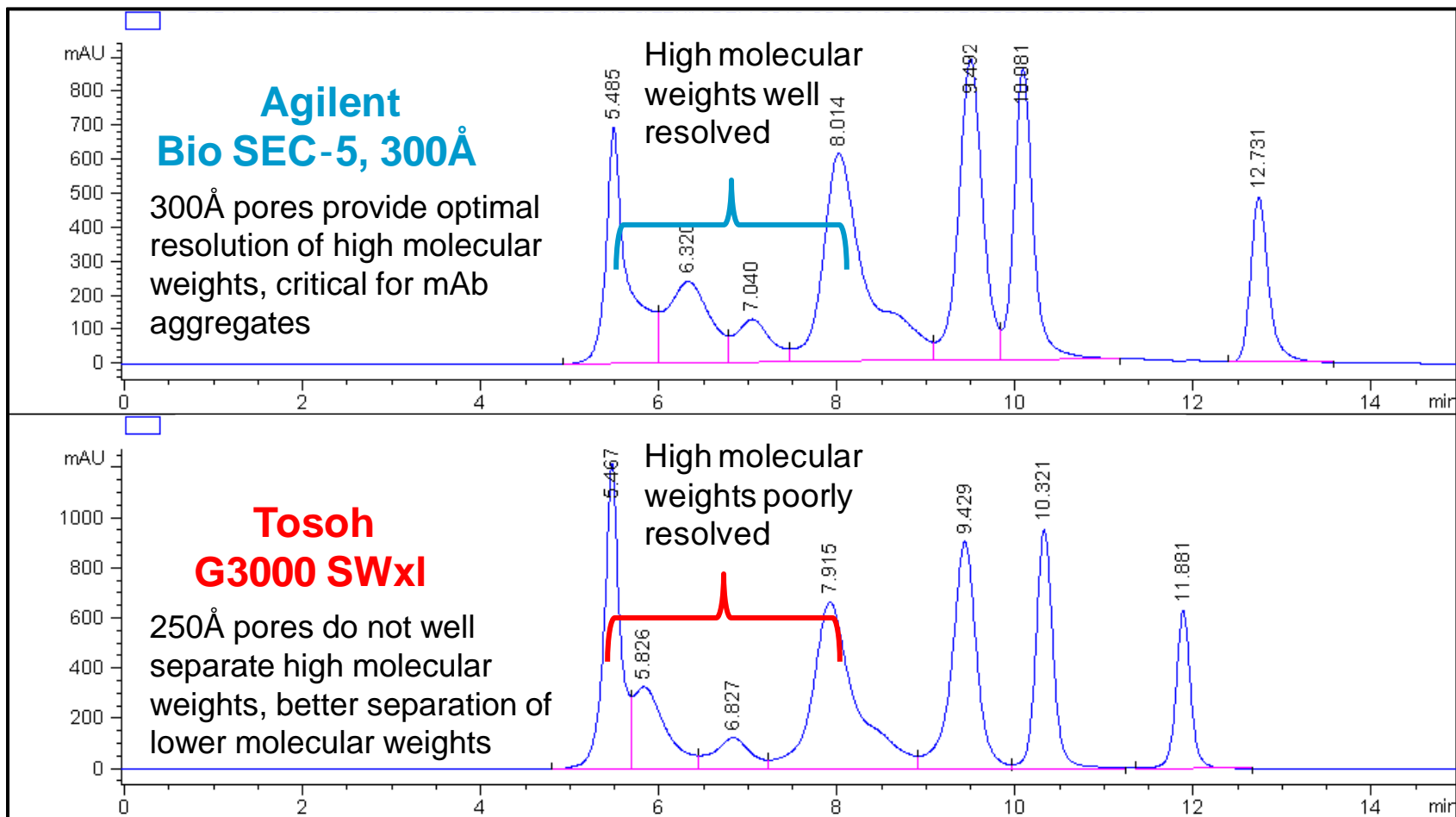
Bio-Rad Size Exclusion Standards

1. Thyroglobulin- 670 kDa
2. Gamma Globulin – 320 kDa and 150 kDa
3. Ovalbumin – 44.3 kDa
4. Myoglobin- 17 kDa
5. Vitamin B12 - 1,350 Da

Comparison of Agilent And Tosoh

Agilent Bio SEC-5 in 150 mM NaPO₄ pH 7.0

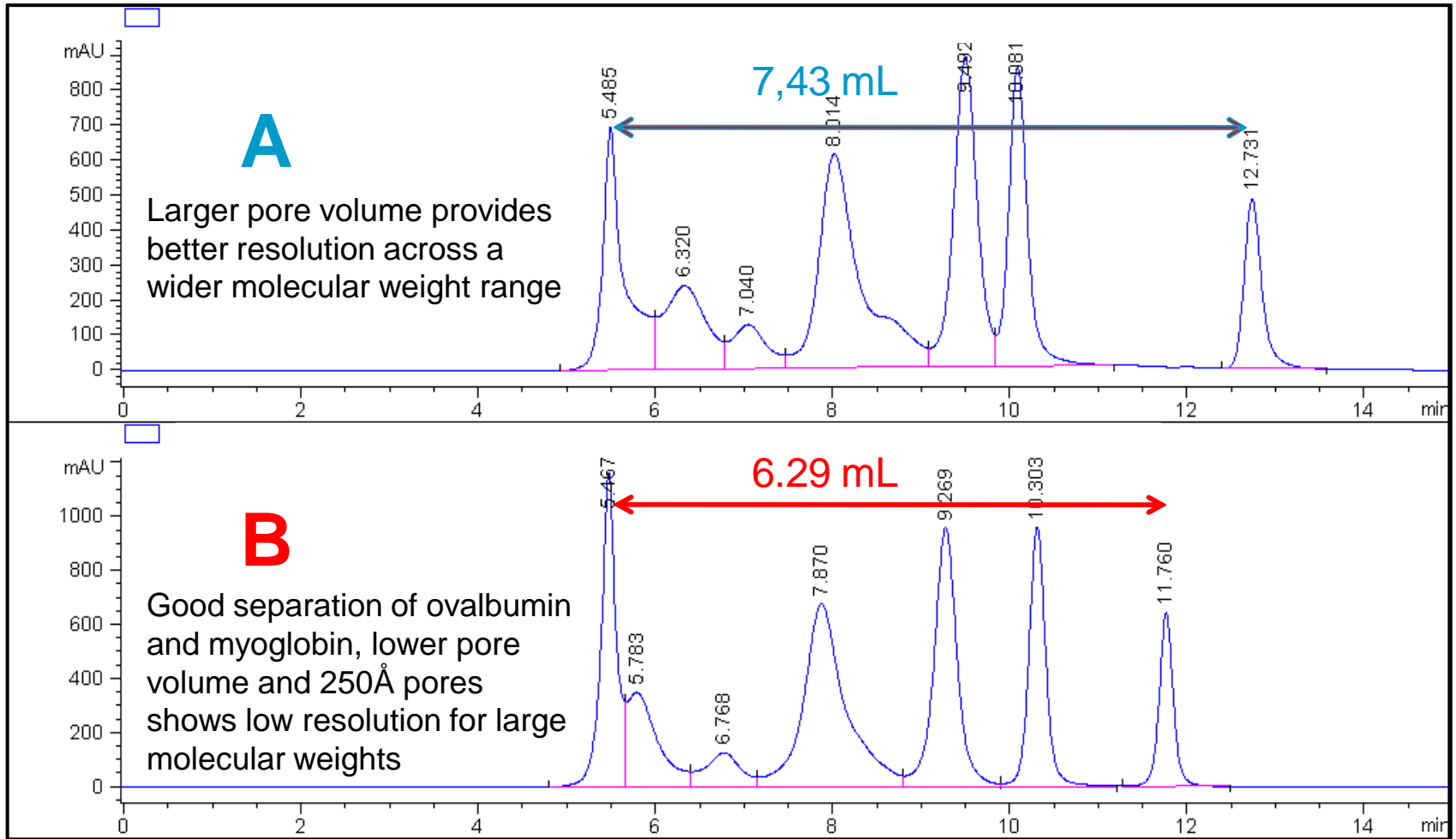
Tosoh TSKgel G3000 SWxl in 100 mM NaPO₄ + 100 mM Na₂SO₄, pH 7.0



Total Column Pore Volume Comparison

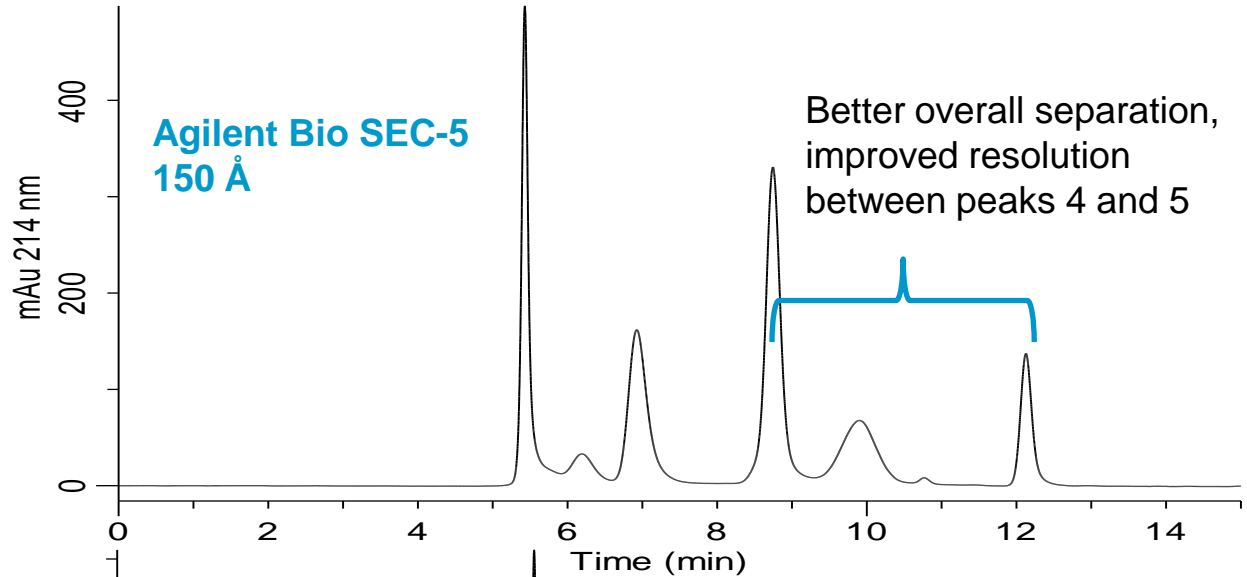
(A) Agilent Bio SEC-5 in 100 mM NaPO₄+100 mM NaSO₂ buffer, pH 7.0

(B) Tosoh TSKgel G3000SWxl in 100 mM NaPO₄+100 mM NaSO₂ buffer, pH 7.0

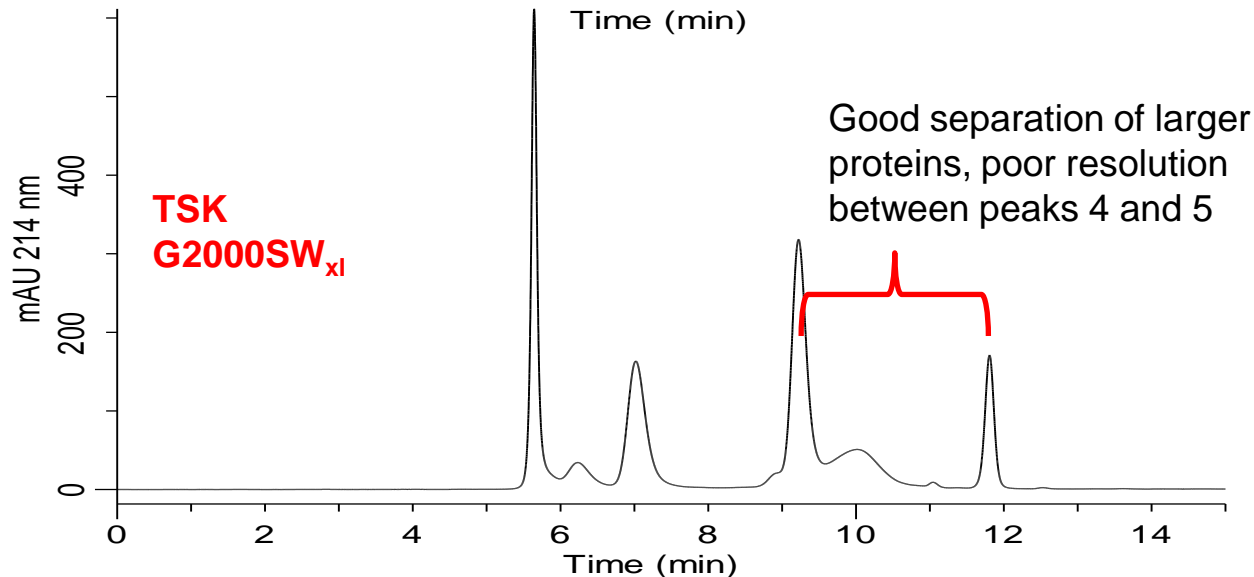


Comparison of Agilent and Tosoh

Larger Pore Volume Provides Higher Resolution



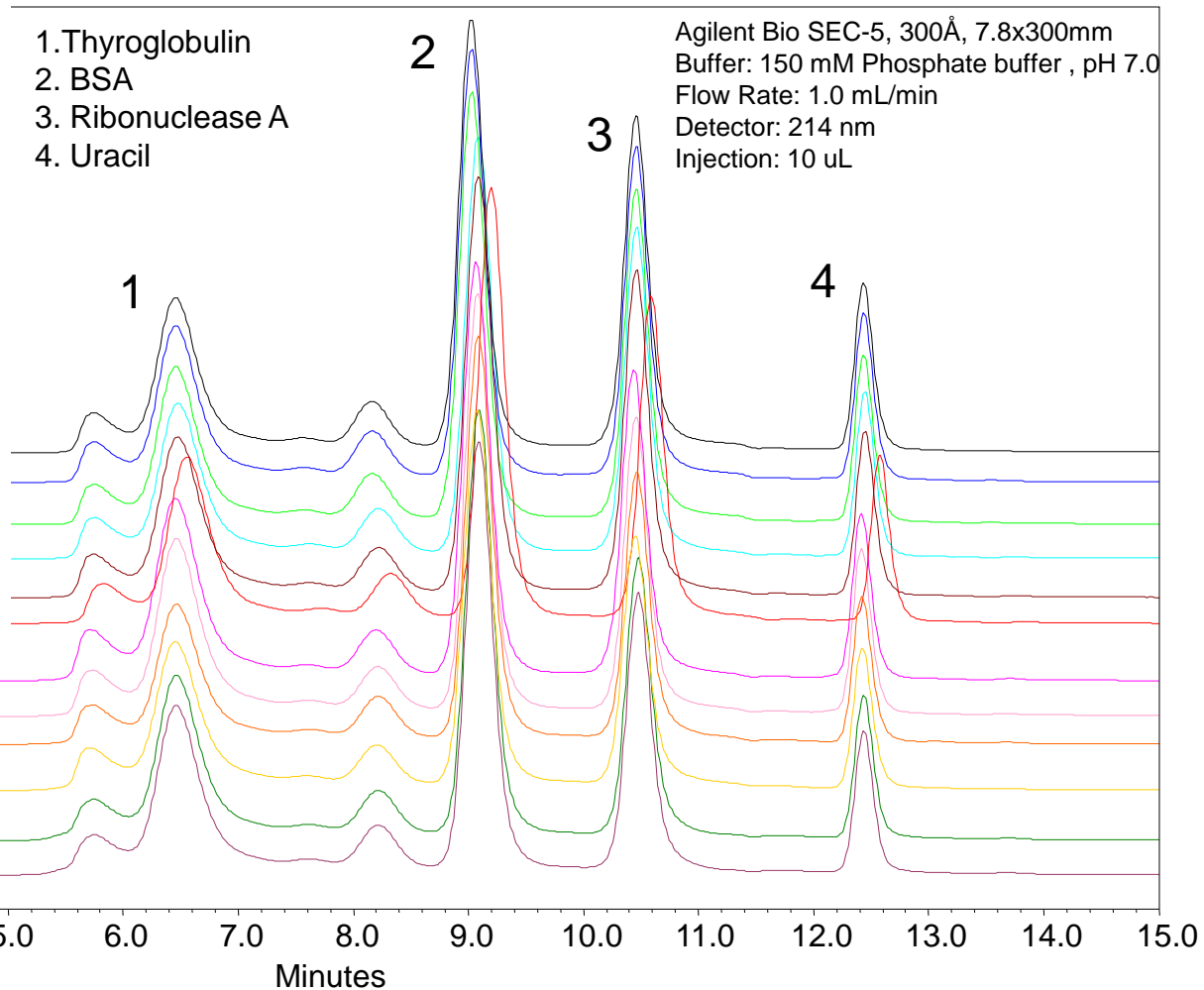
1. thyroglobulin, 5.64 min
2. BSA dimer, 6.23 min
3. BSA monomer, 7.02 min
4. ribonuclease A, 9.22 min
5. poly-DL-alanine (1-5 kDa), 10.02 min
6. uracil, 11.81 min



Agilent Bio SEC-5

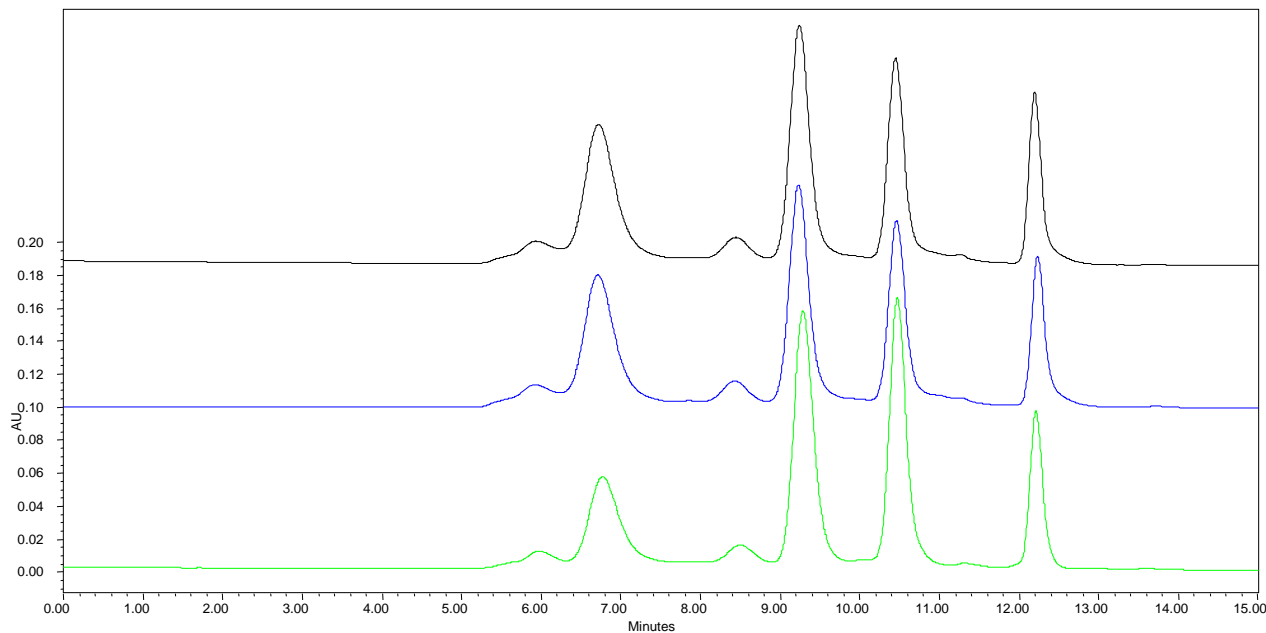
Reproducibility and Lifetime

Injection No.	Efficiency	Tailing	Line
1	26512	1.192	—
2	25959	1.183	—
3	26182	1.180	—
10	25682	1.190	—
20	25872	1.194	—
64	25811	1.187	—
102	25776	1.170	—
140	25910	1.174	—
175	25177	1.208	—
192	24300	1.196	—
250	25707	1.172	—
300	25720	1.166	—



Agilent Bio SEC-5

Lot to Lot Reproducibility, 300Å, 7.8x300mm

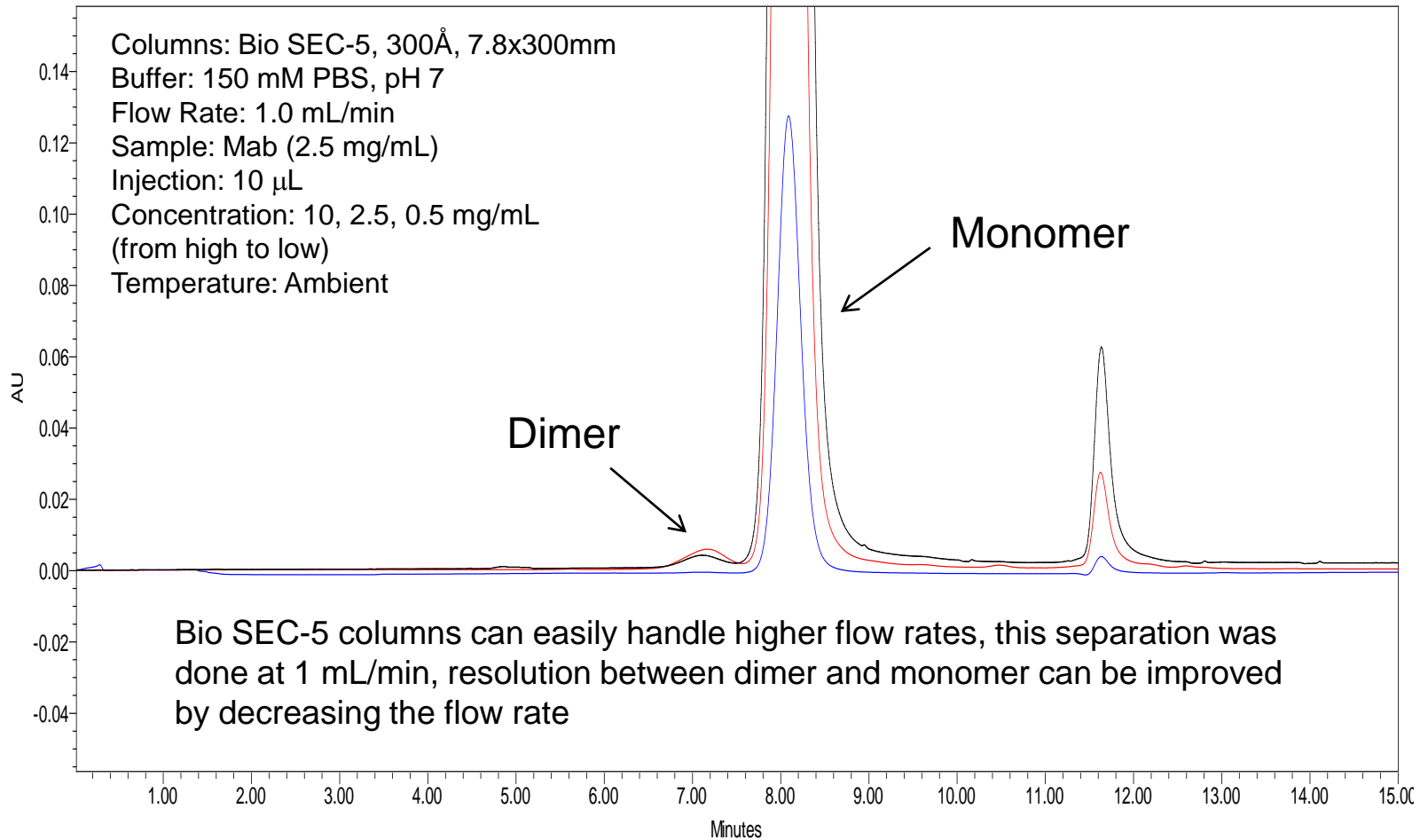


Black: Lot #1
Blue: Lot #2
Green: Lot #3

Lot	Thyroglobulin		BSA		Ribonuclease A		Uracil	
	RT	Eff.	RT	Eff.	RT	Eff.	RT	Eff.
#1	6.72	1536	9.24	6781	10.45	12372	12.20	28733
#2	6.71	1572	9.23	6811	10.46	12016	12.23	26429
#3	6.77	1603	9.29	6515	10.47	11812	12.21	26709

Agilent Bio SEC-5

Monoclonal Antibody Aggregation Monitoring



Size Exclusion Columns

Agilent Bio SEC-3

High Efficiency and High Resolution

- 3 μ m Particle
- Polymeric coated silica, minimizes non-specific binding
- Optimized for use at low salt concentrations (150mM Phosphate)
- Highest resolution
- Faster SEC separations
- Exclusion Ranges Available
 - 100Å, 0.1 – 100 kDa
 - 150Å, 0.5 – 150 kDa
 - 300Å, 5 – 1,250 kDa

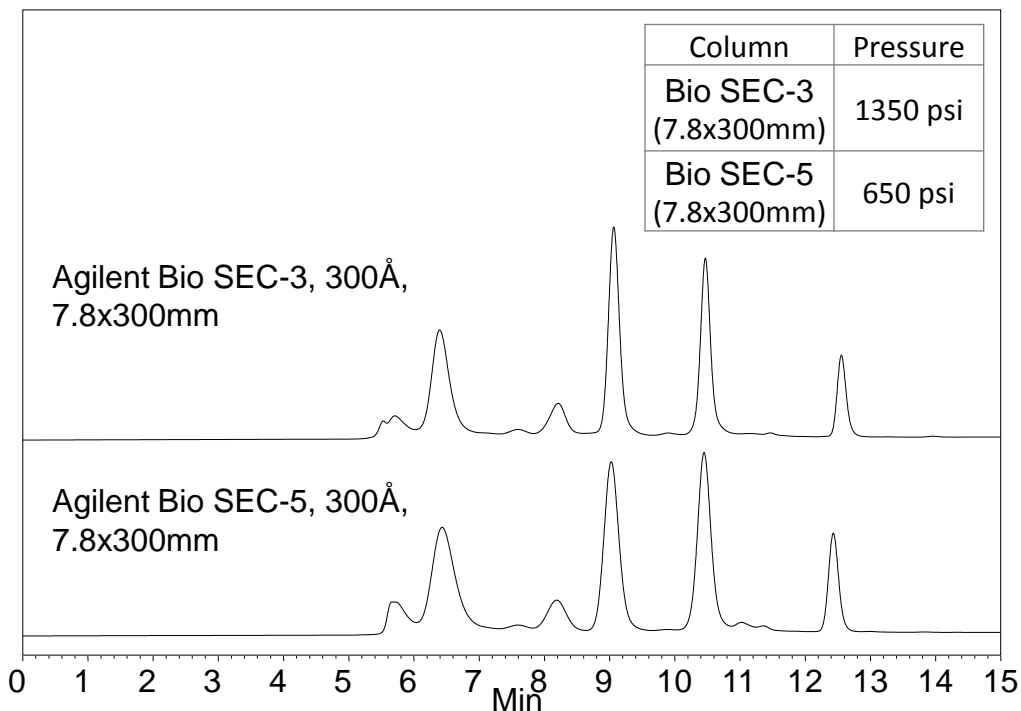


When to choose the Bio SEC-3?

- Need the maximum resolution for mAb aggregates (300Å) and same particle size as Tosoh
- Want to speed up your SEC separations without losing resolution
- Want to separate proteins with lower salt concentrations, better for your instruments

Agilent Bio SEC-3

Improved Efficiency With Smaller Particles



Peak	Protein	SEC-3, 300Å (7.8x300mm)	SEC-5, 300Å (7.8x300mm)
1	Thyroglobulin	2460	1120
2	BSA Dimer	5100	2720
3	BSA	13090	6590
4	Ribonuclease A	22000	11160
5	Uracil	38500	27860

Column: Bio SEC-3 300Å and Bio SEC-5 300Å

Buffer: 150 mM Phosphate buffer, pH 7

Flow rate: 1.0 mL/min for 7.8x300 mm

Temperature: Ambient (~23° C)

Detection: UV 214nm

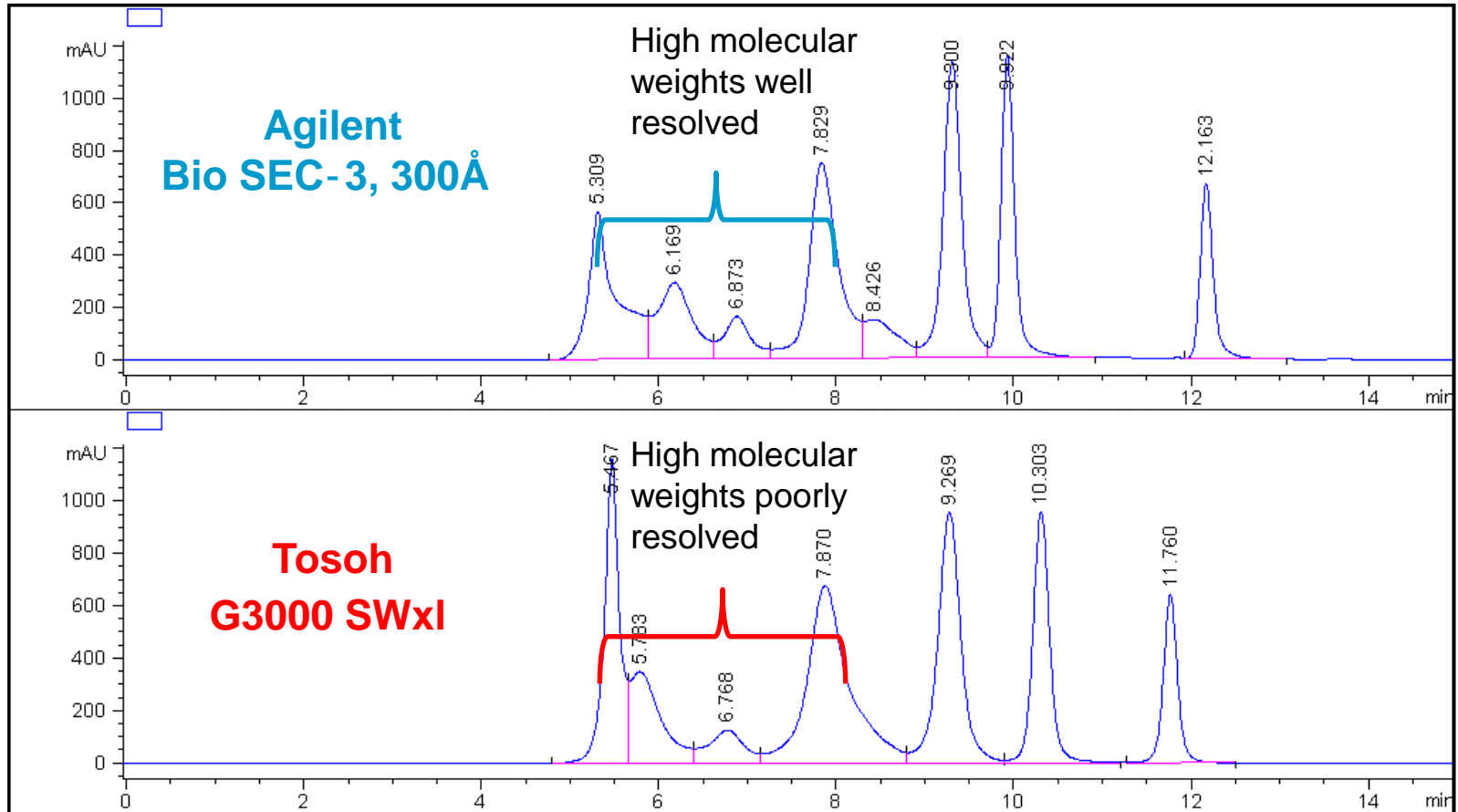
Injection: 10 µL (3 µL for 4.6x300 mm)

Sample: 1) Thyroglobulin (1.0 mg/mL), 670 kD; 2) BSA dimer, 132 kD; 3) BSA (1.0 mg/mL), 66 kD; 4) Ribonuclease A (1.0 mg/mL), 13.7 kD, and 5) Uracil (2.5 µg/mL), 120D.

Comparison of Agilent and Tosoh

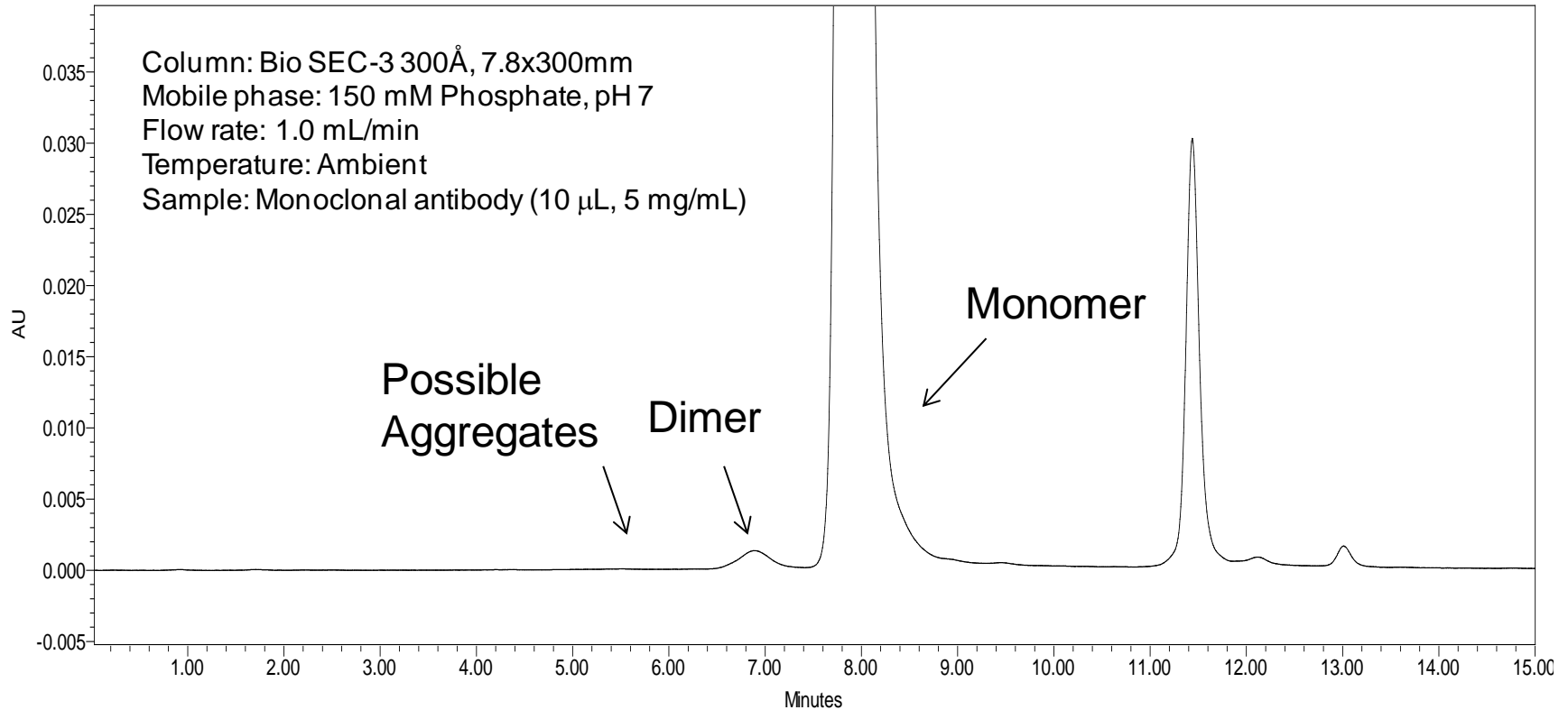
Agilent Bio SEC-3 in 150 mM NaPO₄ pH 7.0

Tosoh TSKgel G3000 SWxl in 100 mM NaPO₄+100 mM Na₂SO₄, pH 7.0



Agilent Bio SEC-3

Monoclonal Antibody Aggregation Monitoring



Baseline separation of aggregates, dimer and monomer in 15 minutes.
Separation run at 1.0 mL/min, using 150mM phosphate, pH 7.0



Ion Exchange BioHPLC Columns

NEW Ion Exchange Columns

Agilent Bio MAb

High Resolution Separations of Monoclonal Antibodies

- Non-porous PS/DVB particles with a uniform polymeric coating
- Particle has a dense, weak cation exchange (WCX) mono-layer with excellent selectivity for basic monoclonal antibodies
- Available in 10 μm , 5 μm , 3 μm , 1.7 μm particle sizes
- Small particles offering higher resolution than the commonly used 10 μm particles

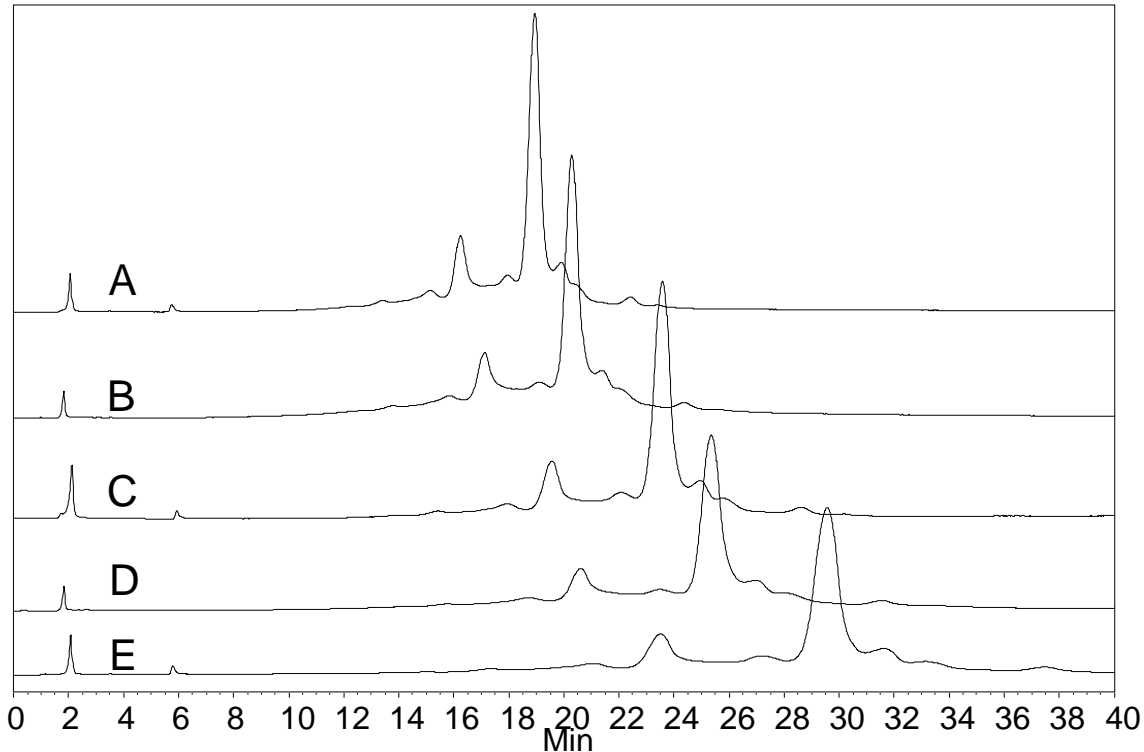


When to choose the Bio Mab Column?

- **Want to separate charge isoforms of monoclonal antibodies**
- **In need of higher resolution separations than current WCX column can offer**
- **Want a more reproducible selectivity from batch to batch and column to column**

Monoclonal Antibody Characterization

Charge-Based Separations on Agilent Bio MAb Column (WCX)



Columns: Agilent Bio MAb, NP10, 4.6x250 mm

Mobile phase: A, 10 mM phosphate, pH 7.5

B, A + 0.1M NaCl

Gradient: A) 15-75%B in 30 min

B) 15-65%B in 30 min

C) 15-55%B in 30 min

D) 15-47.5%B in 30 min

E) 15-40%B in 30 min

Flow rate: 0.8 mL/min

Sample: Monoclonal Antibody

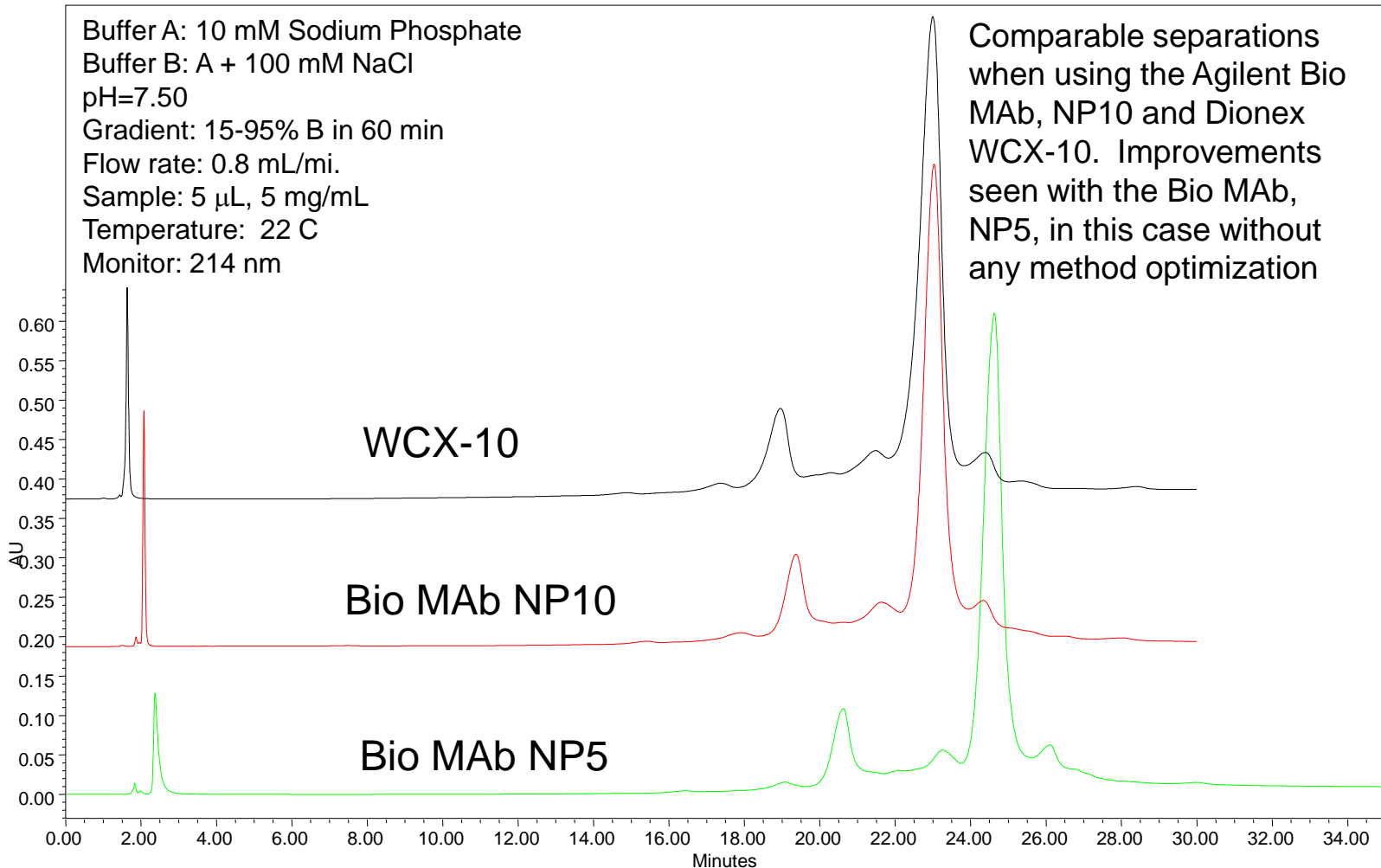
Injection: 10 μ L (1.5 mg/mL)

Temperature: 25 $^{\circ}$ C

Detection: UV 214 nm

Optimization of method conditions for the isoform characterization of a monoclonal antibody. Changes in the buffer conditions, pH and gradient conditions sharpen peaks and increase resolution of acidic and basic isoforms.

Agilent Bio MAb NP10, NP5, Dionex WCX-10



NEW Ion Exchange Columns

Agilent Bio IEX

High Resolution Ion Exchange Columns

- Non-porous PS/DVB particles with a uniform polymeric coating
- Available in SCX, WCX, SAX, WAX phases, designed for protein and peptide separations
- Available in 10 μm , 5 μm , 3 μm , 1.7 μm particle sizes
- Small particles offering higher resolution than the commonly used 10 μm particles
- Multiple ion exchange functional groups are bound at each site providing higher surface area and high capacity for a non-porous particle column
- Can be used to separate, proteins, antibodies, peptides, glycans (glycosylated bio-molecules, not sugar analysis), polynucleotides, cell lysates and in 2-D separations



When to choose the Bio IEX Column?

- **Want to separate proteins, peptides and a variety of other bio-molecules**
- **In need of higher resolution separations than current IEX columns can offer**
- **Want a more reproducible selectivity from batch to batch and column to column**

Agilent Bio IEX Columns

Dynamic Binding Capacity

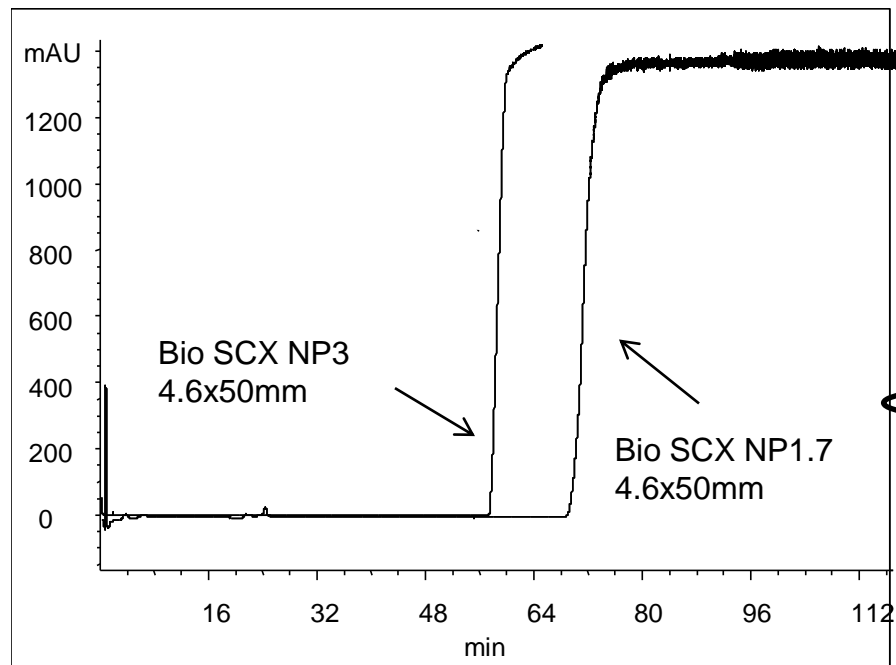
Test Conditions

Buffer: 10 mM Phosphate, pH 6.0

Sample: 3 mg/mL Lysozyme

Flow rate: 0.25 mL/min

Detector: 254 nm



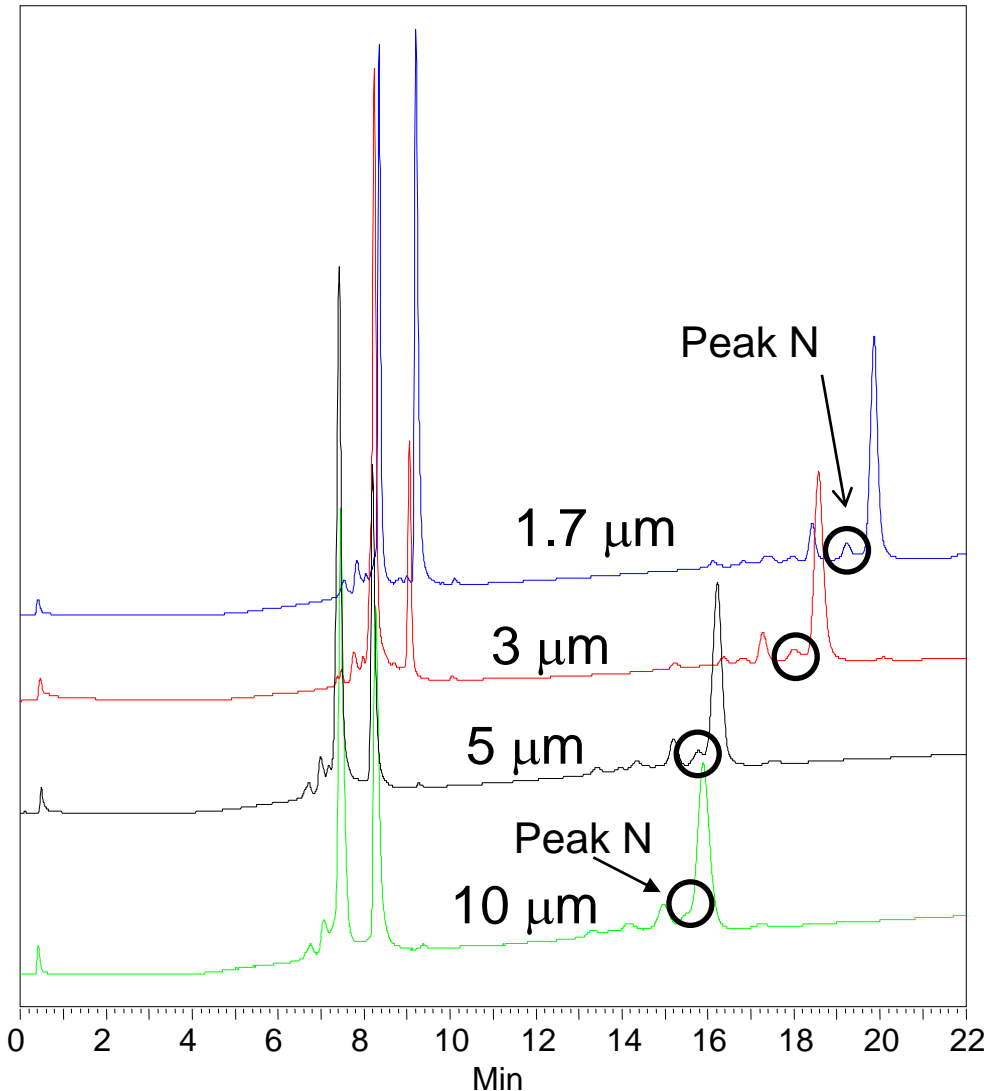
Agilent 10 μ m non-porous particles have higher capacity than other 10 μ m non-porous particles, even higher with smaller particles

Column Packing	Particle (μ m)	Surface Area (m^2)	Capacity (mg/mL)
Bio SCX, NP1.7	1.7	NP	65
Bio SCX, NP3	3	NP	53.5
Bio SCX, NP5	5	NP	38
Bio SCX, NP10	10	NP	20
Bio WCX, NP1.7	1.7	NP	25
Bio WCX, NP3	3	NP	19

Column Packing	Particle (μ m)	Surface Area (m^2)	Capacity (mg/mL)
SP-NPR (TSK)	2.5	16	5
SP-5PW (TSK)	10	50	40
Mini S (GE)	3	NP	<2
ProPac SCX	10	NP	3
ProPac WCX	10	NP	6

Agilent Bio IEX Columns

Comparing Separations on Each Particle Size



Column: Bio WCX-NP, 4.6x50mm

Buffer A: 20 mM PBS

Buffer B: A+1.0 M NaCl

Gradient: 0-100%B (20 min)

Flow rate: 1.0 mL/min for NP10, NP5, NP3

0.75 mL/min for NP1.7

Sample

1) Ribonuclease A

2) Cytochrome C

3) Lysozyme

Concentration: 1.0 mg/mL

Detector: 280 nm

Average N~80,000 for WCX-NP1.7

Small (5 μ m, 3 μ m, 1.7 μ m) non-porous particles provide higher resolution, resolving peaks not seen with traditional larger particles (10 μ m)



Reversed Phase BioHPLC Columns

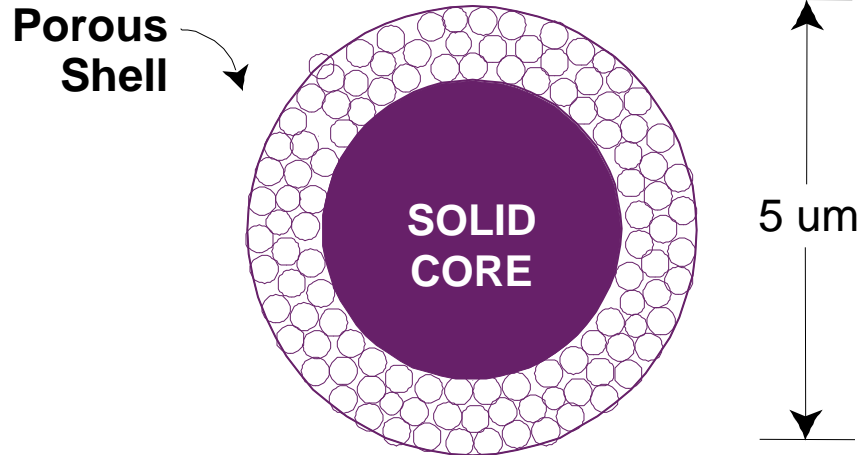
Reversed Phase Columns

Poroshell 120 and Poroshell 300

Poroshell 300 – Agilent's First Poroshell Columns

Poroshell 300

High efficiency – fast protein and peptide separations



Poroshell 300 columns are for the separation of large peptides, proteins and antibodies.

Poroshell 300 particles have a solid silica core with a superficially porous surface with a 300Å pore size

This results in more efficient mass transfer, sharper peaks and fast, efficient separations with large proteins and peptides

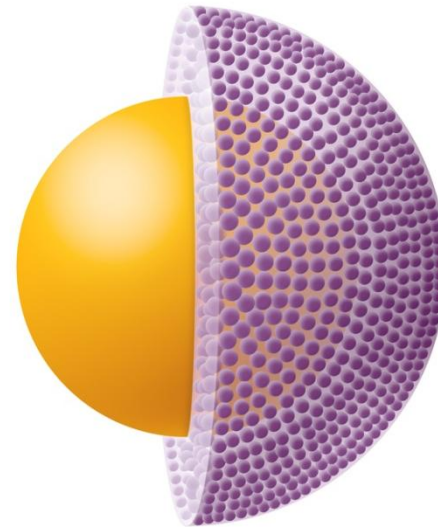
Reversed Phase Columns

Poroshell 120 and Poroshell 300

Agilent Poroshell 120

Advancing capabilities for high-performance HPLC

- 80-90% efficiency of sub 2 μ m
- At ~40-50% lower pressure
- 2.7 μ m particle size
- 2 μ m frit to reduce clogging
- 600 bar pressure limit
- The particle has a solid core (1.7 μ m) and porous outer layer with a 0.5 μ m diffusion path for a 2.7 μ m total particle size.



Poroshell 120 vs. Poroshell 300



Use Poroshell 120 for **peptide mapping**

- Peptide digests of proteins or monoclonal antibodies
- Use Poroshell 120 EC-C18 for LC/MS applications with formic acid

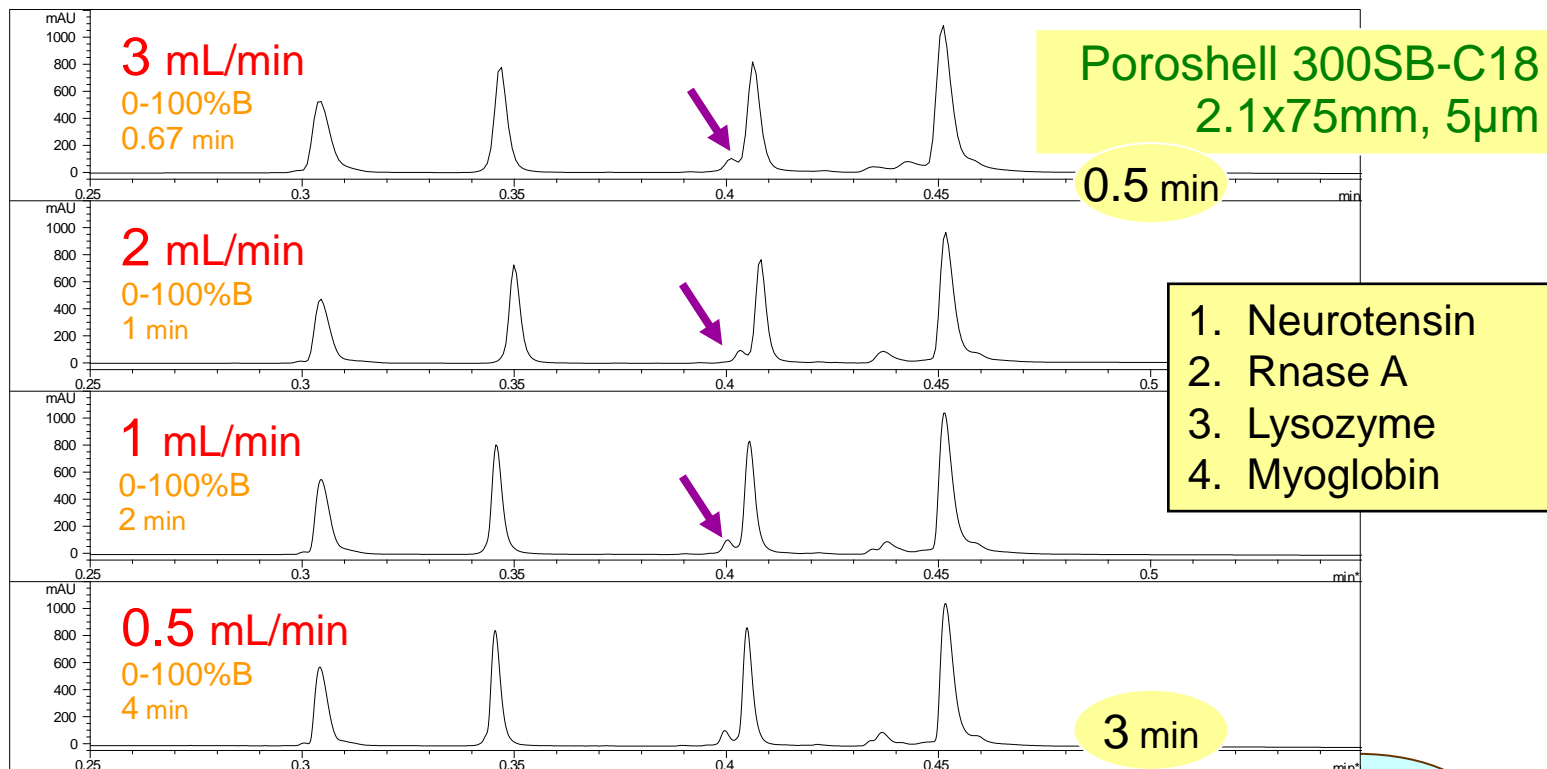
Use Poroshell 300 for reversed phase separations of **large biomolecules**

- Does the sample contain all polypeptides or larger?
- Poroshell 300 can be used with very large proteins/monoclonal antibodies
- Use at a high flow rate for optimum efficiency (i.e. 1.0 mL/min on 2.1 mm ID columns)

Poroshell 300 Reverse Phase

Effect of Increasing Flow Rate in Protein Analysis with Poroshell 300

Sustained Efficiency and Resolution



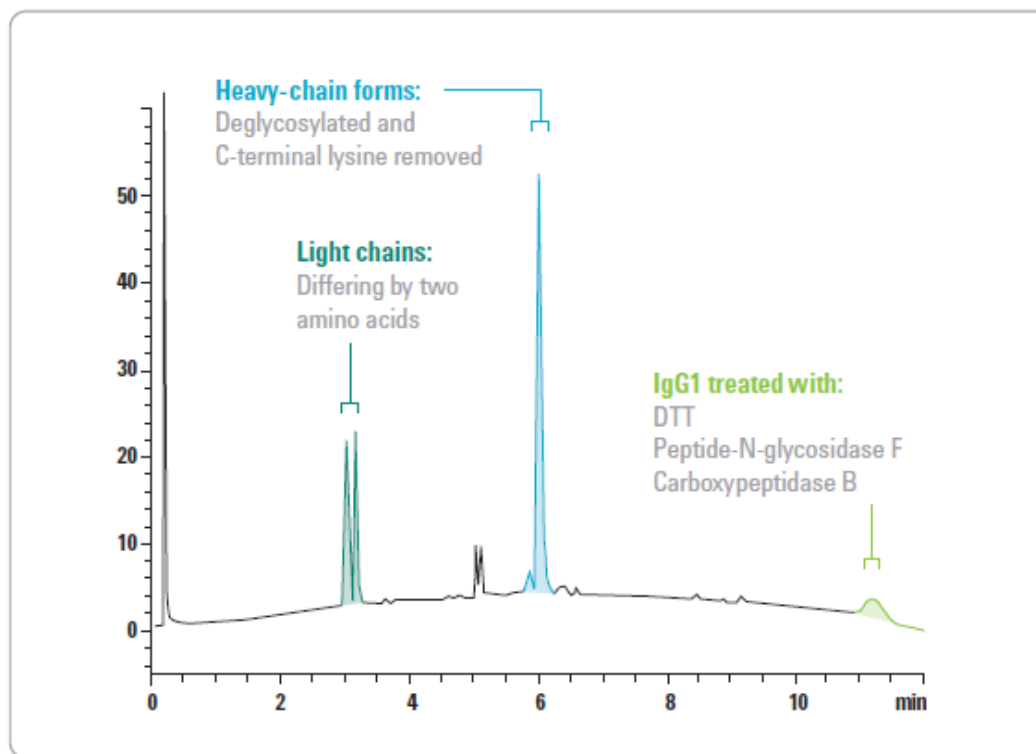
Aligned

Agilent 1100 WPS with AutoBypass; Piston Stroke: 20 μ L, Temp.: 70 $^{\circ}$ C, Det.: 215 nm
Mobile Phase: A= 95% H₂O, 5% AcN with 0.1%TFA; B= 5% H₂O, 95% AcN, with 0.07%TFA

As flow rate increases, peak width and resolution are maintained when using superficially porous particles!

Poroshell 300

Heavy and Light Chain Characterization

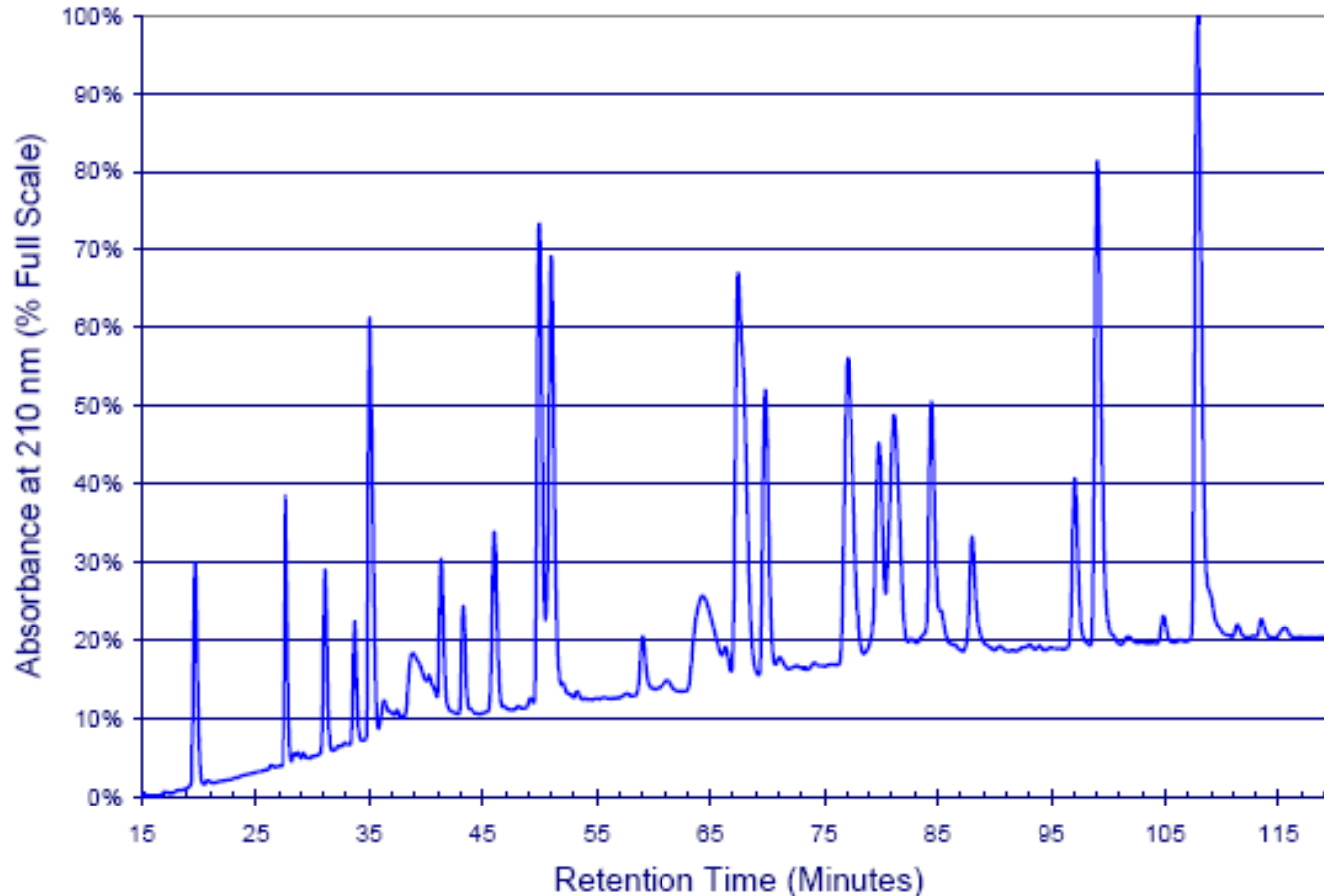


High resolution separations of antibodies. The Agilent Poroshell 300SB-C8 columns enhance peak shape and recovery through simplified interaction with the bonded phase. The Poroshell 300 column provides clear separation between heavy and light antibody chains of a monoclonal antibody treated with DTT, peptide-N-glycosidase F and carboxypeptidase B.

Use of 1200 LC, Poroshell 300SB columns and UV to characterize antibodies

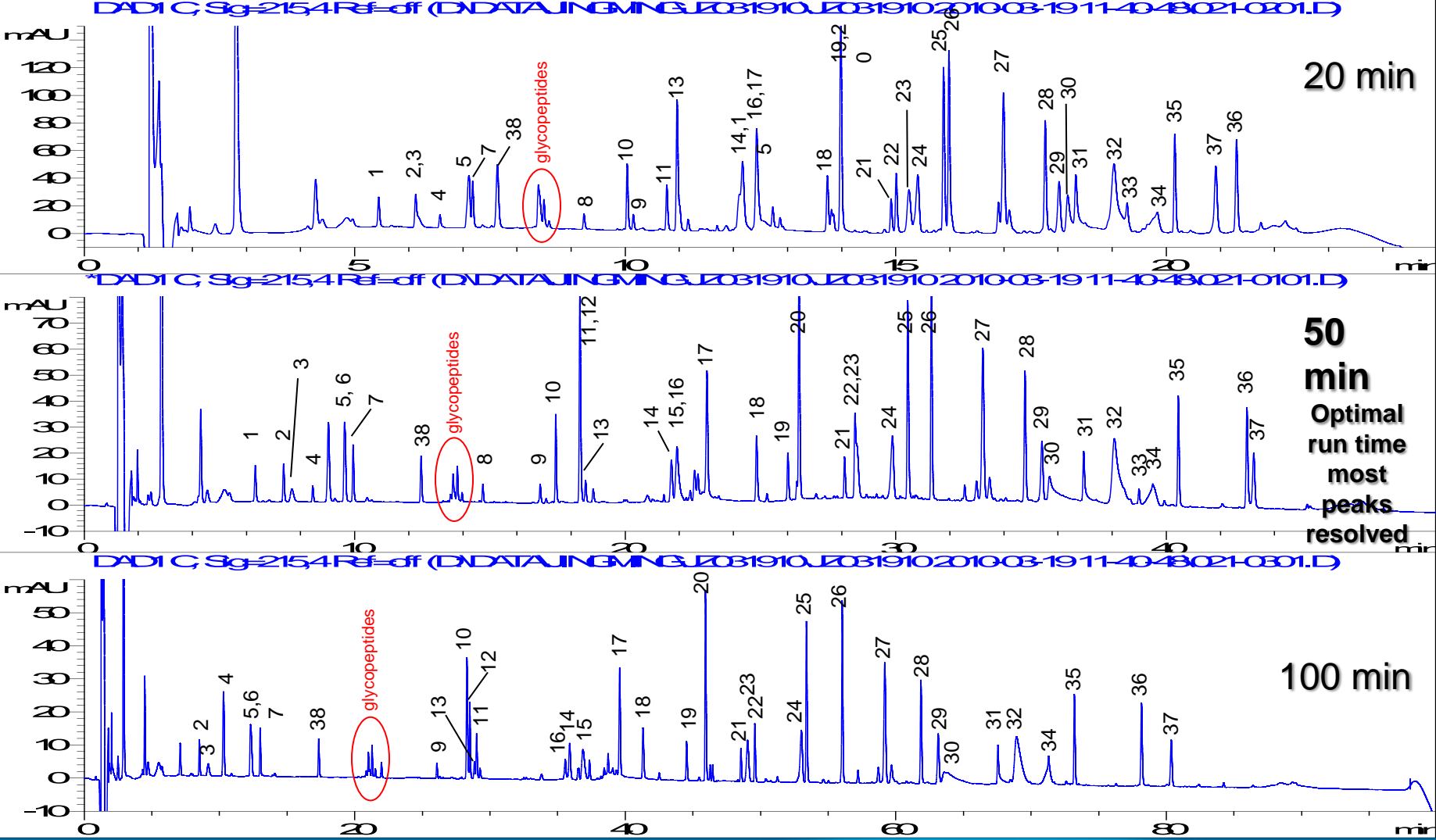
“Traditional” Peptide Map

Column: “C18” 4.6 X 250mm, 5um Mobile Phase: A= 0.1%TFA in water, B=0.1%TFA in ACN
Gradient: 10% B to 90 B in 100 min **Runtime: 115 minutes** Re-equilibration time: 20 min

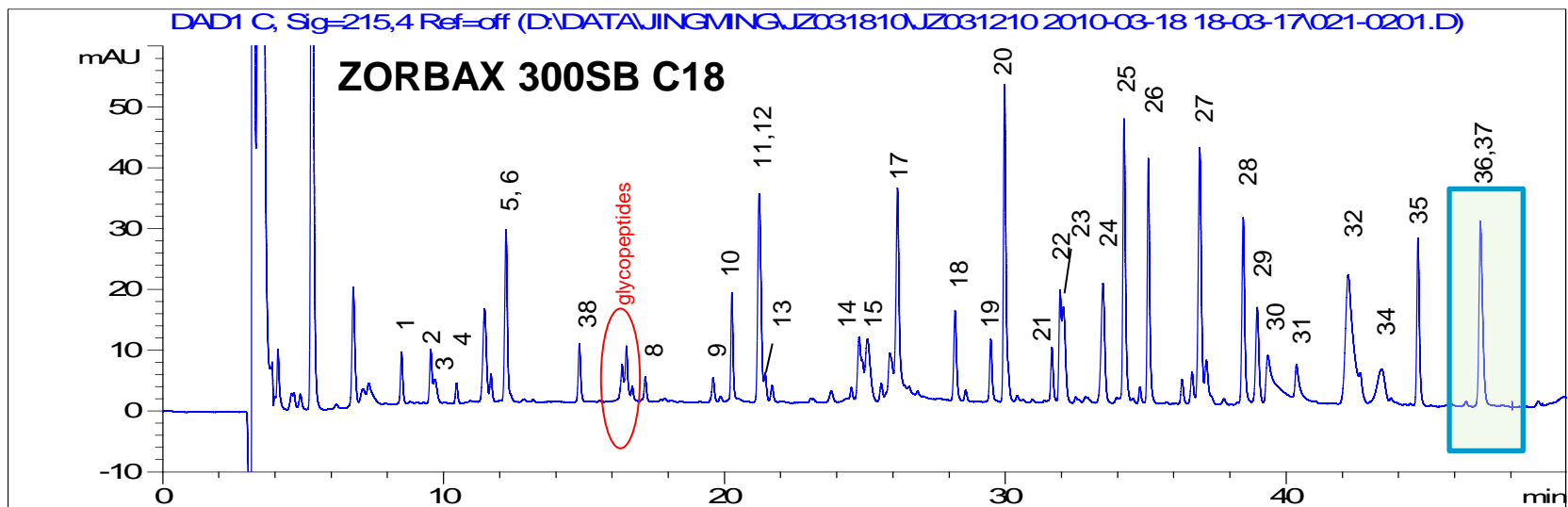
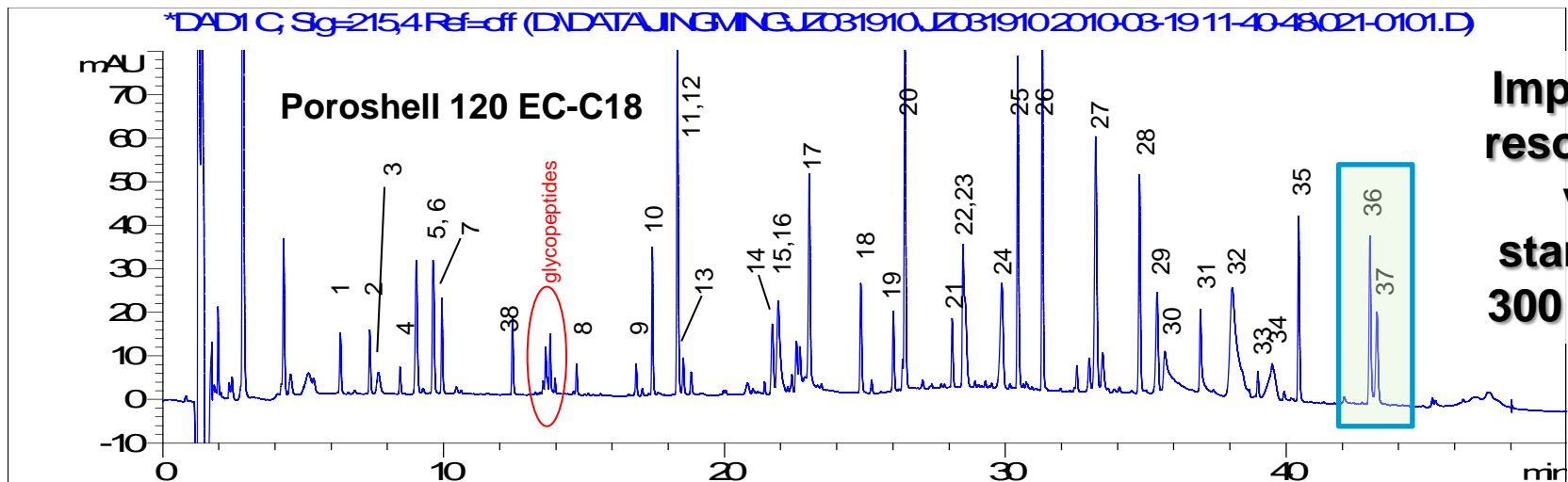


The very long runtimes of traditional peptide maps limit the utility of this approach.

Peptide Map Comparison of Run Times - Poroshell 120 EC-C18, 3.0 x 150mm 2.7um



Poroshell 120 and ZORBAX 300SB Comparison



New BioHPLC Columns Brochure

Confidently separate and characterize bio-molecules with Agilent BioHPLC columns

Size Exclusion BioHPLC columns

Ion Exchange BioHPLC columns

Our measure is your success.

products | applications | software | services

5990-5195EN

Agilent Bio MAb HPLC columns
High resolution ion exchange separations of monoclonal antibodies

Agilent Bio IEX HPLC columns
High resolution charge-based analytical separations of proteins, peptides, and other biological molecules

Agilent Bio SEC-3 HPLC columns (3 µm particle size)
High efficiency and high resolution size-based separations for bio-molecules

Agilent Bio SEC-3 HPLC columns are a unique technology for size

Agilent superior performance

- Particles, coated to high pressure resolution and
- Hydrophilic, non-specific
- A high cationic bond

Agilent Bio superior performance

- Particles, coated to high pressure resolution and
- Hydrophilic, non-specific
- Multiple ion on one anchor

Column Chart

Now Available

Agilent Poroshell 120
Advancing capabilities for high-performance HPLC

Make every HPLC in your lab work harder.

Now you can get the benefits of exceptional efficiency—more speed and more resolution—on your conventional HPLC instruments.

Poroshell 120 reinvents the technology introduced with Poroshell 200 to deliver the higher resolutions and faster separations that you have seen for proteins and polypeptides, but now for a complete range of small molecules. Poroshell 120 columns give you speed and resolution like a sub-2 µm column with up to 50% less back pressure—usually less than 400 bar.

Poroshell 120 highlights

- Up to ten times the number of theoretical plates vs conventional 3.5 µm particles
- Improved resolving power, even as you take advantage of speed gains
- Lower back pressure than 2 µm, even at higher flow rates
- More forgiving for dirty samples, due to 2 µm frits
- Manufactured by Agilent, to Agilent's typical strict quality standards, and backed by Agilent worldwide technical support

> 90% efficiency of sub-2 µm, 2X efficiency of 3.5 µm HPLC Pressure (< 400 bar)

Sample: 1. Saccharin, 2. Caffeine, 3. Phosphorylcholine acid, 4. Aspartame, 5. Dehydroacetic acid, 6. Benzoic acid

Poroshell 120 EC C18
2.0 x 100 mm, 2.2 µm
N = 23,280, P = 314 bar

Rapid Resolution High Throughput EdgePlus Plus C18
2.0 x 100 mm, 1.8 µm
N = 25,304, P = 450 bar

Rapid Resolution EdgePlus Plus C18
3.0 x 100 mm, 3.0 µm
N = 11,500, P = 150 bar

Distillates
C18max: 30% 100 ppm, Methylphen: 95%, A: 0.2%, Parathion acid: 25%
B: Methylphenol: 100 ppm, 0.5 mL/min, 1 µg injection 20°C, 0.5 µL, 200, 4 min, 100 - 0%

Our measure is your success.

products | applications | software | services

BioHPLC Columns on the Agilent Website



Visit www.agilent.com/chem/BioHPLC to learn more, and order online.