Analysis of Biomacromolecules by LC-MS Utilizing Novel, Narrow-bore, Wide Pore Monolithic and Superficially Porous Stationary Phases

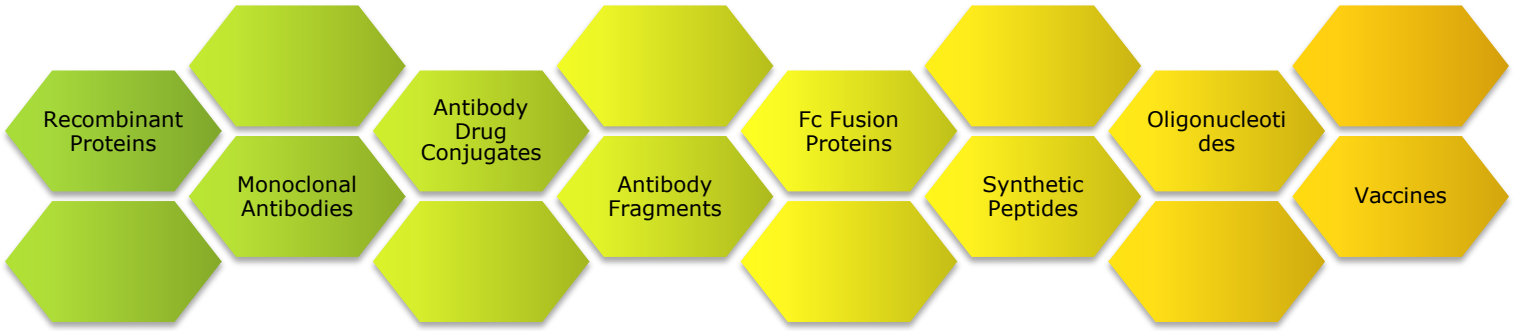
Dr. Egidijus Machtejevas
Sr. Technical Advisor,
Analytical Chromatography Workflows
Advanced Analytical, Chemistry,
Science and Lab Solutions

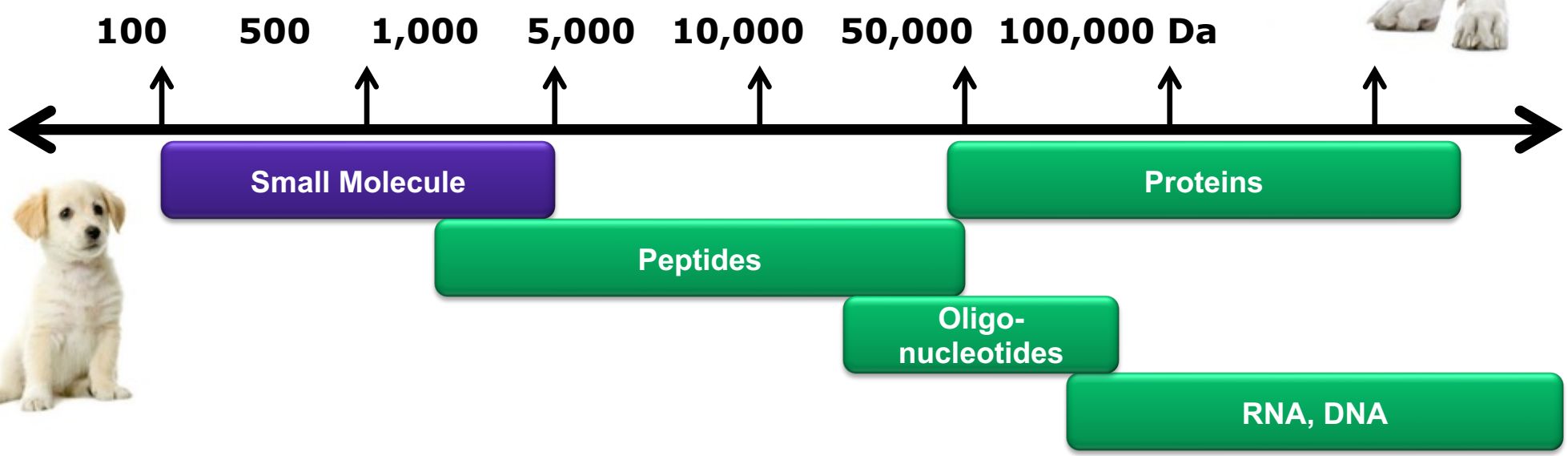
Supelco®
Analytical Products

Small molecules – Large molecules

Biomolecules are compounds created by living organisms.

They can range in size from amino acids and small lipids to large polynucleotides such as DNA or RNA.





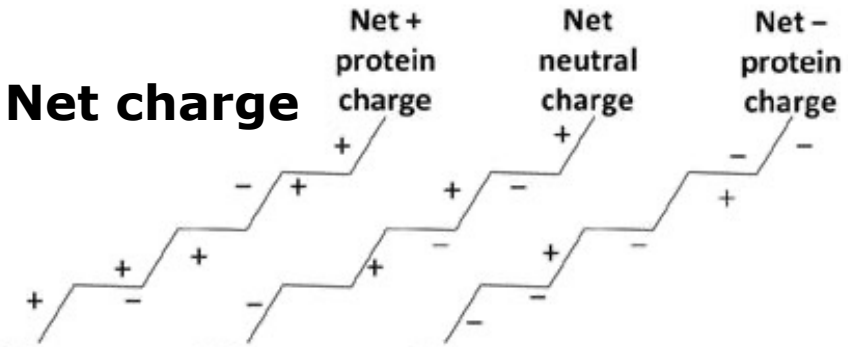
Supelco®

Chemical Properties of Proteins and peptides

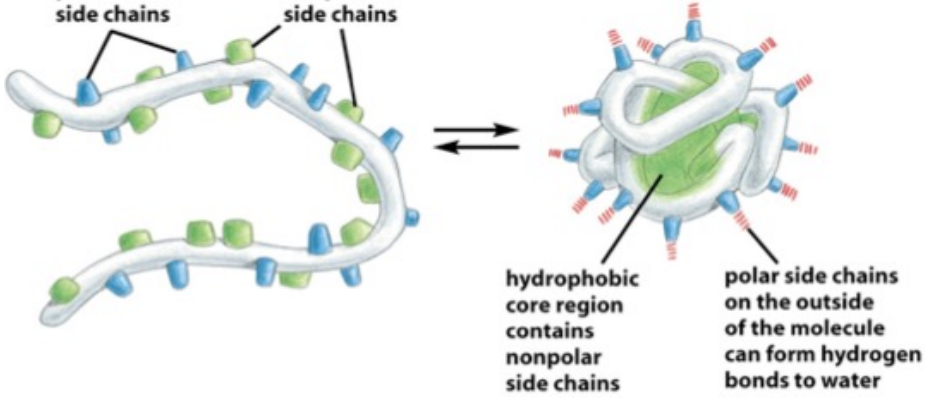
Proteins and peptides consist of amino acids (20 naturally occuring)

The composition determines:

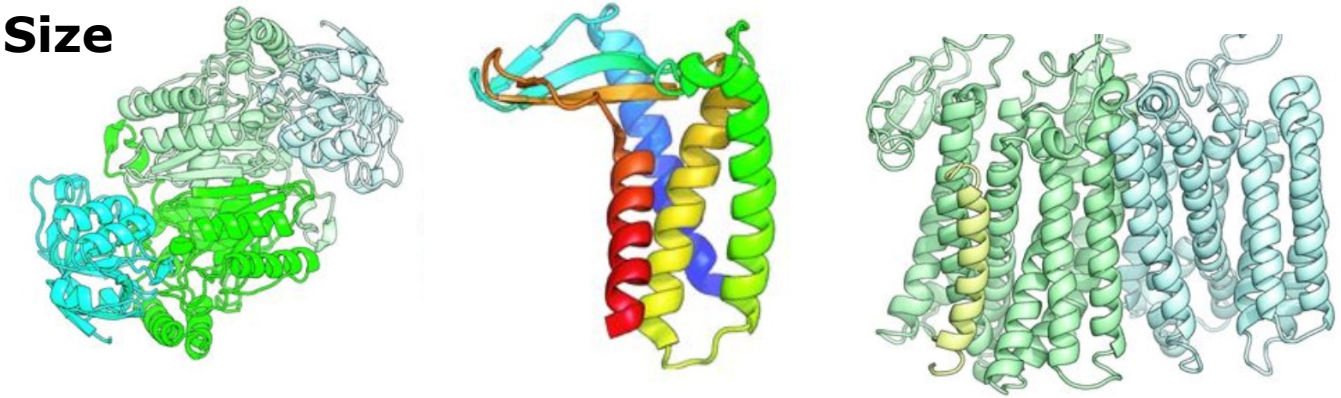
Net charge



Hydrophobicity



Size



Techniques used to separate & analyse proteins

SDS-PAGE (Sodium dodecyl polyacrylamide electrophoresis)

IEF (Isoelectric focusing)

CE (Capillary Electrophoresis)

Immunoassay/ELISA

- Monoclonal antibodies (MAbs) typically analysed using this but they could also be done as an intact protein by MALDI MS, or as a digest, using LC/MS/MS

Immunohistochemistry (IHC)

HPLC – various techniques

HPLC Separation Techniques for Proteins/Peptides

SEC (Size Exclusion Chromatography)

- For proteins: GFC (gel filtration chromatography)
- Proteins separates according to their relative size

Ion exchange

- Protein is displaced by salt concentration or pH changes from ionic surface

Reversed phase

- Simple adsorption and desorption, protein is displaced by organic solvent.
- Usually uses wide-pore silica (pore size 300 Å or larger) with C18, C8, C5/C4, CN

Affinity chromatography

- Protein is displaced by salt concentration or pH changes from specific adsorption

Hydrophobic interaction

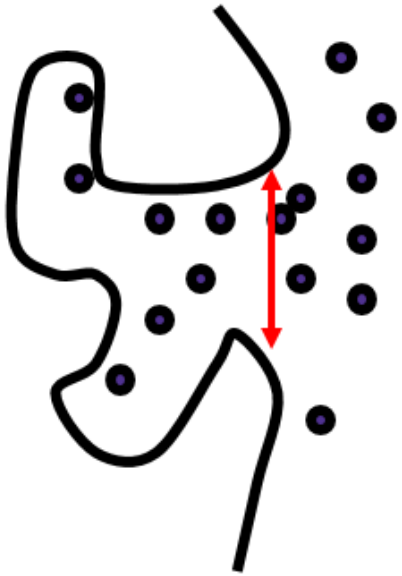
- Separates by dispersion interactions, protein is displaced by water (decreasing salt concentration)

HILIC

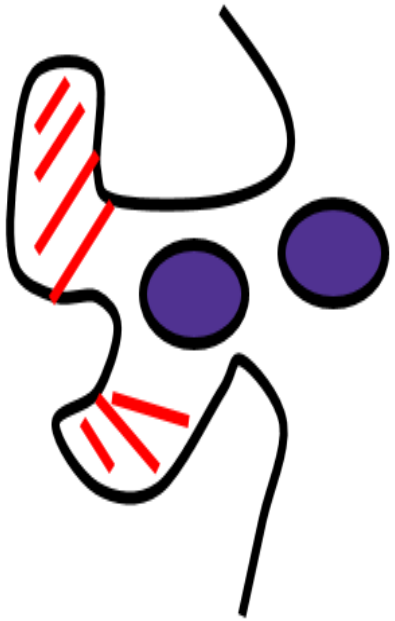
- For highly proteins, but more often used in Glycan Analysis

Sometimes multiple LC techniques are combined: orthogonal separation techniques confirm purity, orthogonal techniques may be required for difficult applications

Large molecules require larger pores

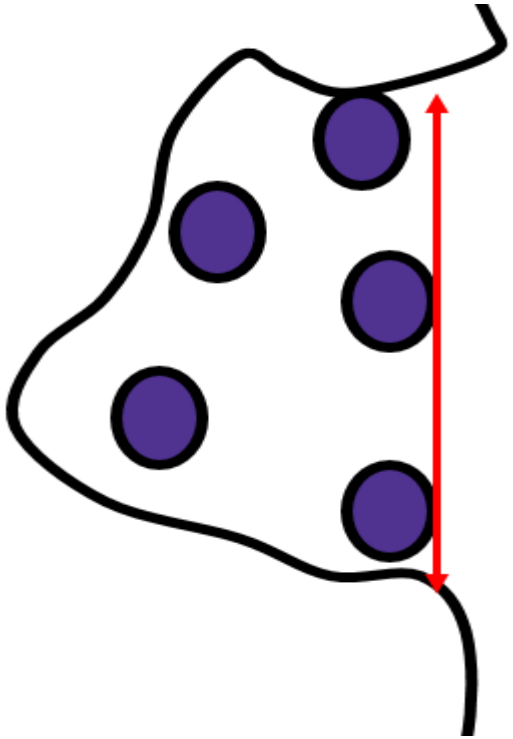


Small Pores



Small Pores

The surface area in the pores is not fully accessible for large molecules.



Large Pores

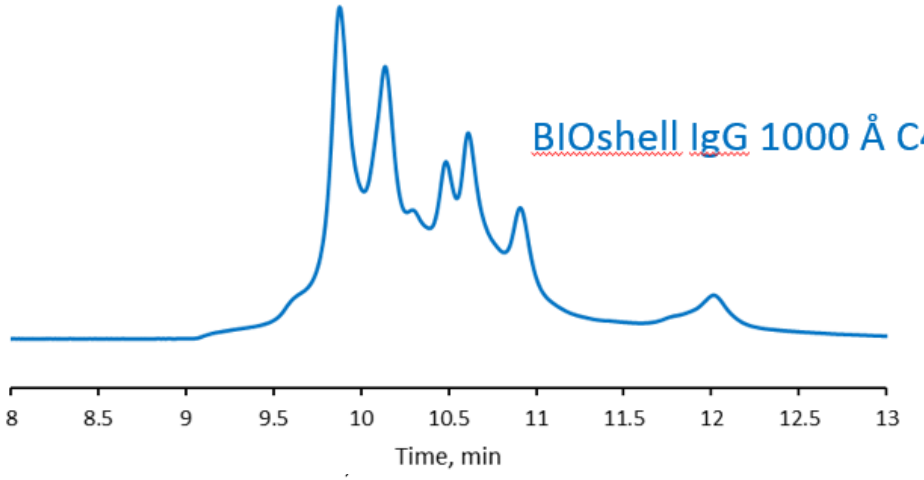
Small Molecule MW <0.5kD

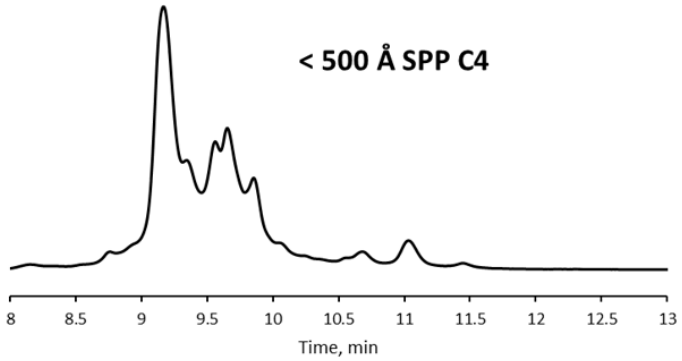
Large Molecule / MW > 2.0kD

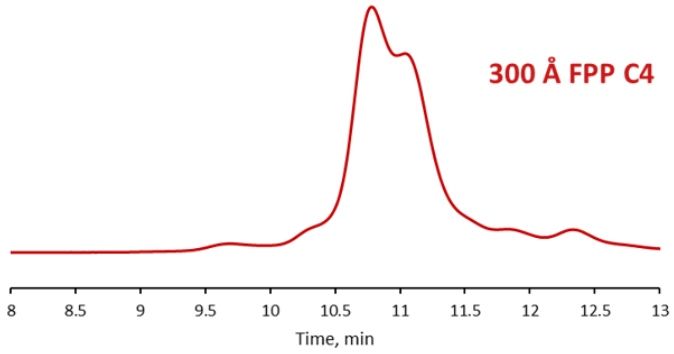
Pore Size Matters!

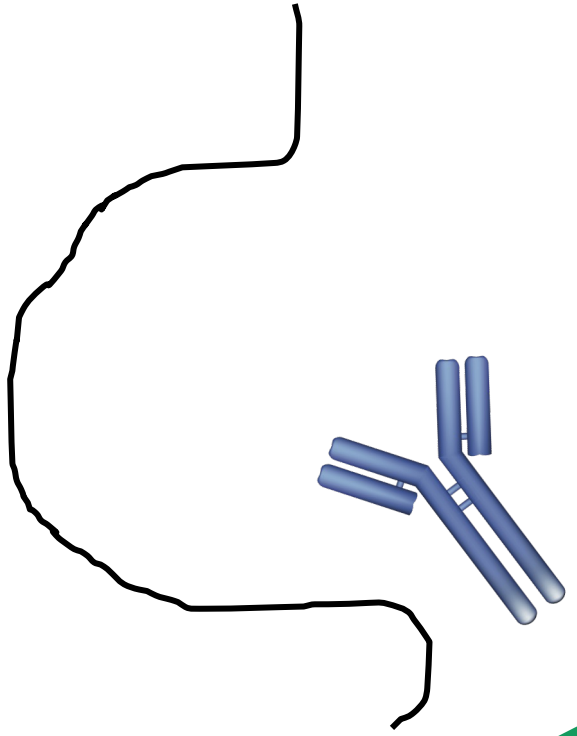
Trying to analyze intact proteins, mAbs, bsAbs or other large molecules >150kDa?
BIOshell IgG 1000Å pore can offer high resolution separations!

Separation of IgG2 variants

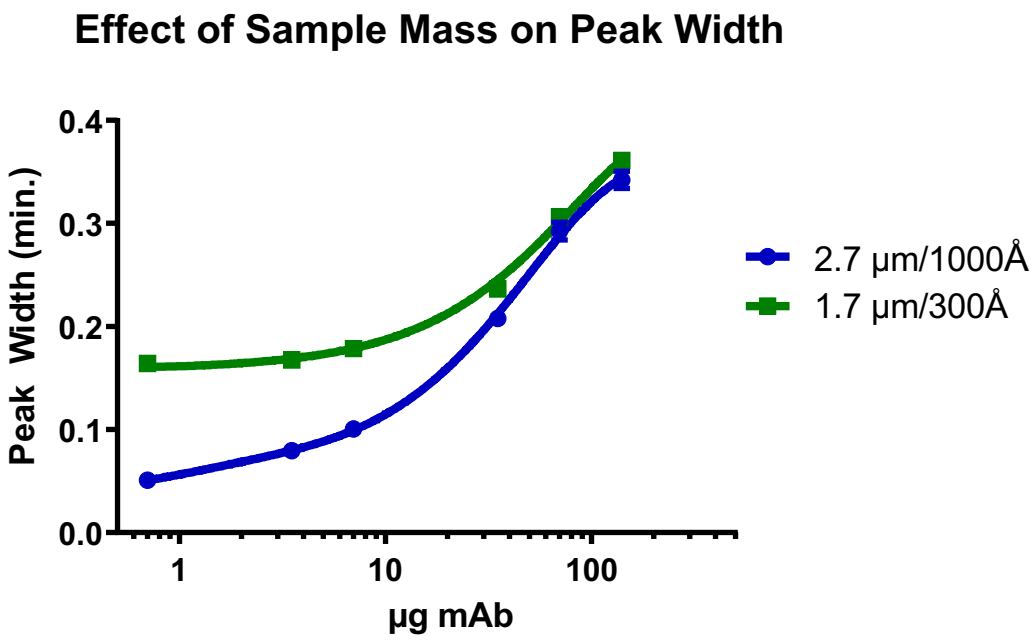








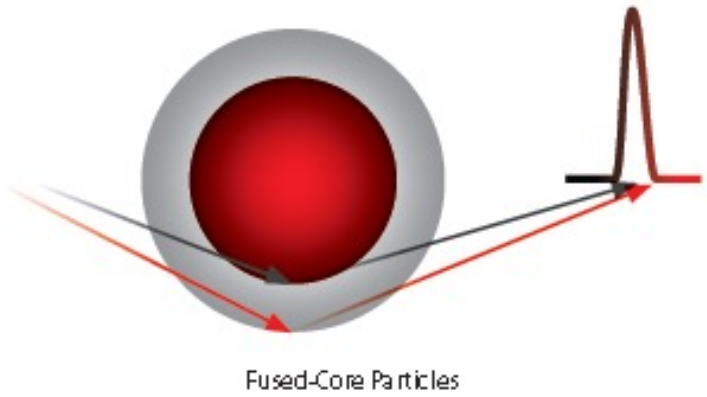
Pore Size Matters!

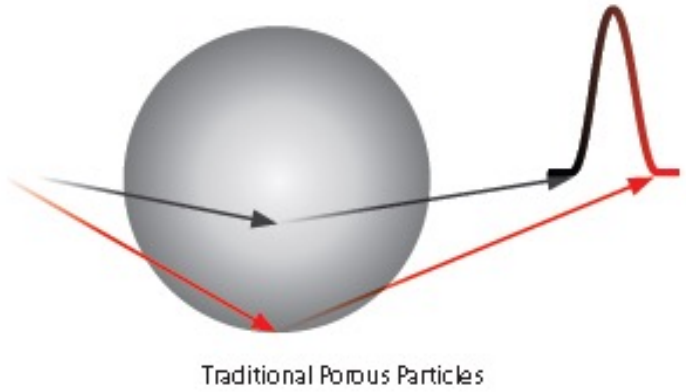


Conditions
Column: As indicated; 15 cm x 2.1 mm I.D., C4
Mobile Phase: [A] Water (0.1% DFA); [B] Acetonitrile (0.1% DFA)
Gradient: 27% B to 37% B in 10 min
Flow Rate: 0.5 mL/min
Column Temp.: 80 °C
Detector: UV, 280 nm
Injection: 0.1, 0.5, 1, 5, 10, and 20 µL
Sample: mAb, 7 mg/mL, water

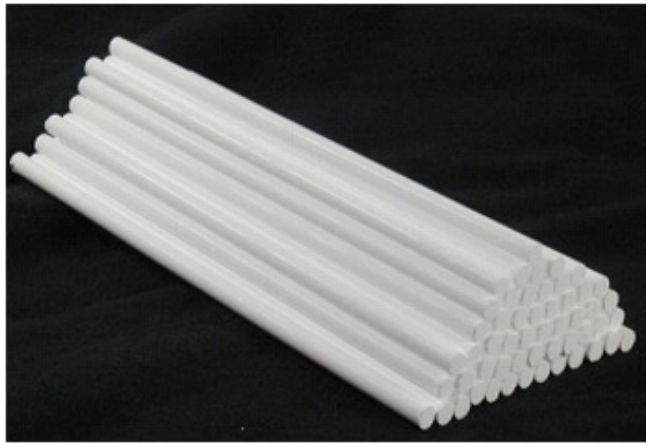
Alternatives to smaller, fully porous particles

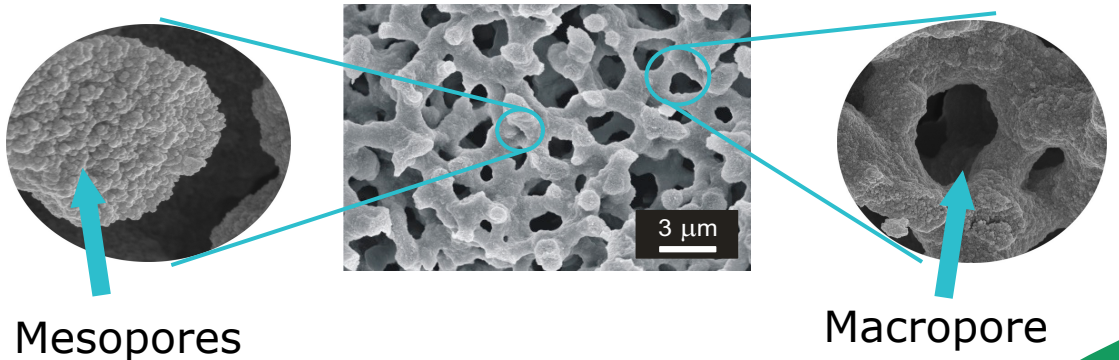
Ascentis® Express – Core-shell technology



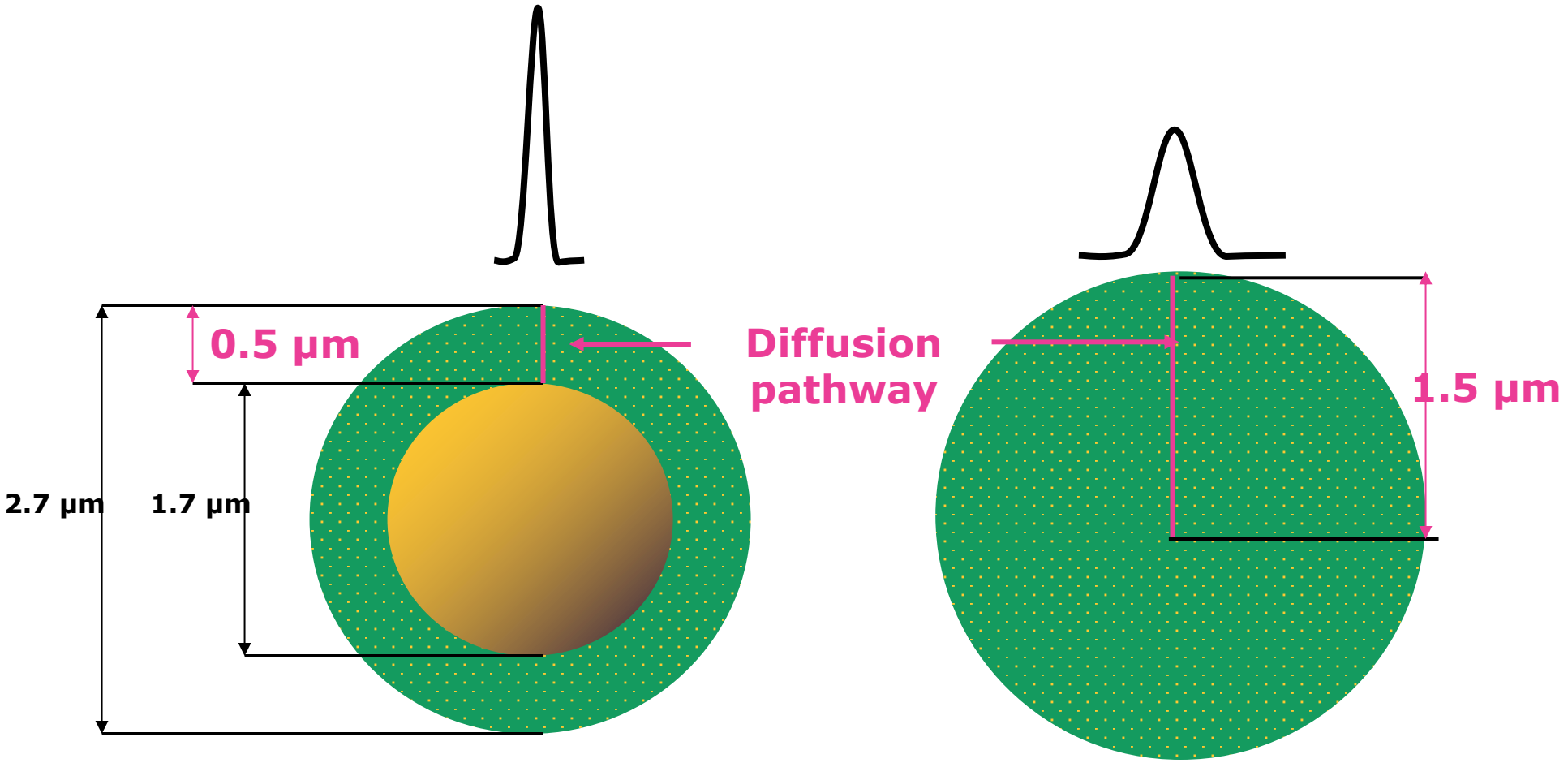


Chromolith® – monolithic silica technology





Fused-Core provides higher efficiency

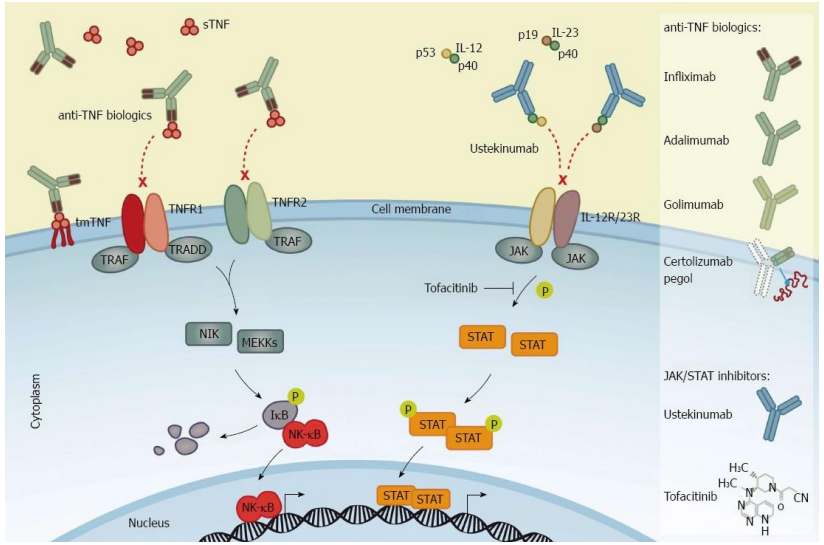


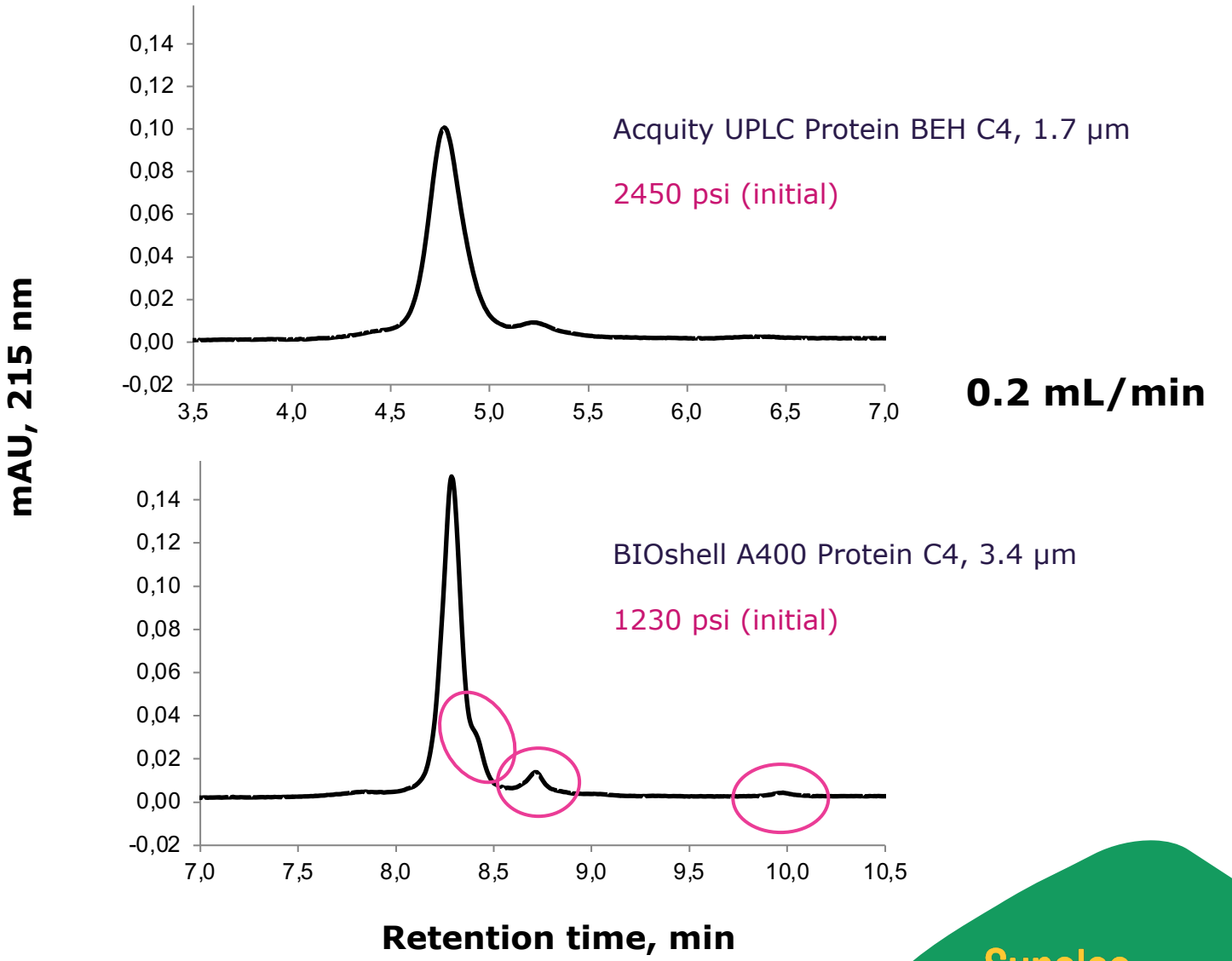
The shorter diffusion pathway facilitates the mass transfer (C term)!

FPP vs. SPP

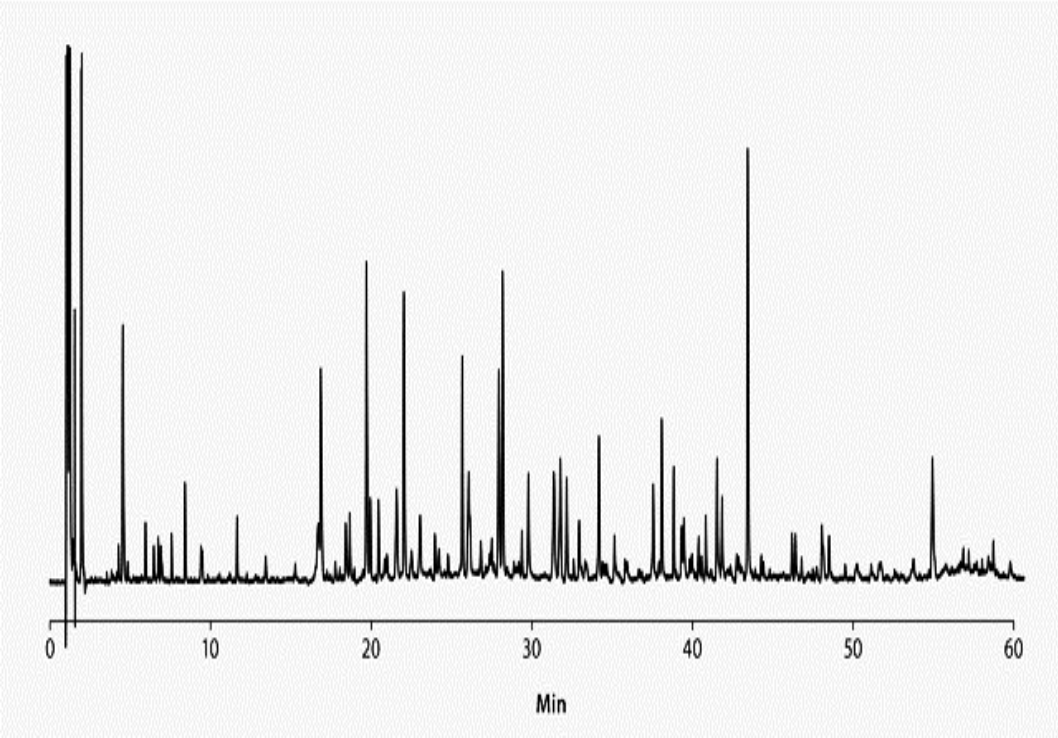
Humira (adalimumab)

Adalimumab, sold under the trade name **Humira** among others, is a medication used to treat rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, Crohn's disease, ulcerative colitis, chronic psoriasis, hidradenitis suppurativa, juvenile idiopathic arthritis, and uveitis.
In rheumatoid arthritis, adalimumab has a response rate similar to methotrexate, and in combination, it nearly doubles the response rate of methotrexate alone. Adalimumab is a TNF-inhibiting, anti-inflammatory, biologic medication. It binds to tumor necrosis factor-alpha (TNFα), which reduces the immune response. There is strong evidence that adalimumab increases the risk of life-threatening infections and cancers, particularly lymphoma.





Example of Fast Digestion Workflow:
Peptide Map Results with Small Protein



column: BIOshell™ A160 Peptide C18; 15 cm x 2.1 mm I.D., 2.0 µm

mobile phase: [A] 98:2 water:acetonitrile (both 0.1% TFA); [B] 50:50 water:acetonitrile (both 0.1% TFA)

gradient: 0% B to 63% B in 60 min

flow rate: 0.3 mL/min

column temp.: 35 °C

detector: UV, 215 nm

injection: 20 µL

sample: Lysozyme tryptic digest, 10 µg/mL, water (0.1% TFA)

Improving Peptide Separations: Peak Capacity

Peak Capacity Pc

- Pc (Gradient) = Duration of Gradient/mean Peak Width

Extraction of Peak Widths from 20 Peaks covering the whole range of the c-gram

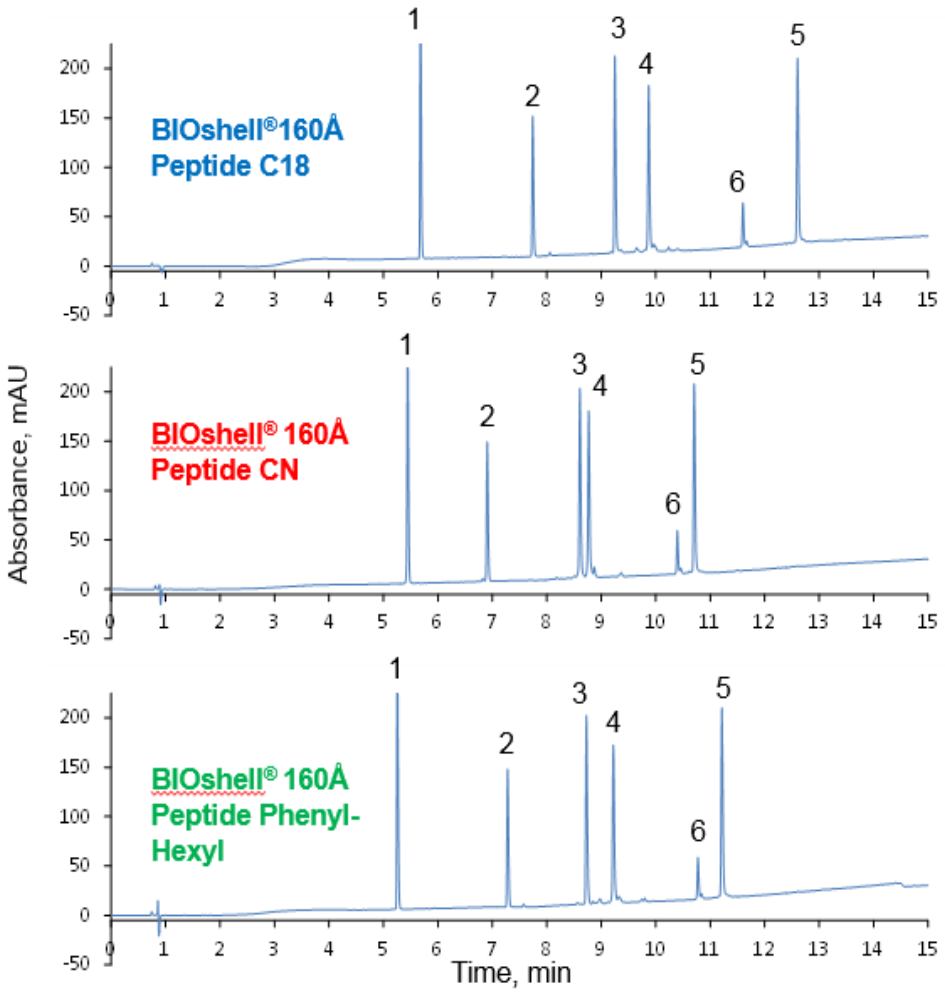
Column	t_g *	w_{ave}	P_c
BIOshell Peptide C18	41.3	0.1213	340
Standard 5µ C18	47.2	0.1951	242

t_g

* Gradient in column volumes (CV); while Peak Width in Minutes

Mean Peak Width on Fused-Core column is less than 40% compared to fully porous columns

Modification impact on separation



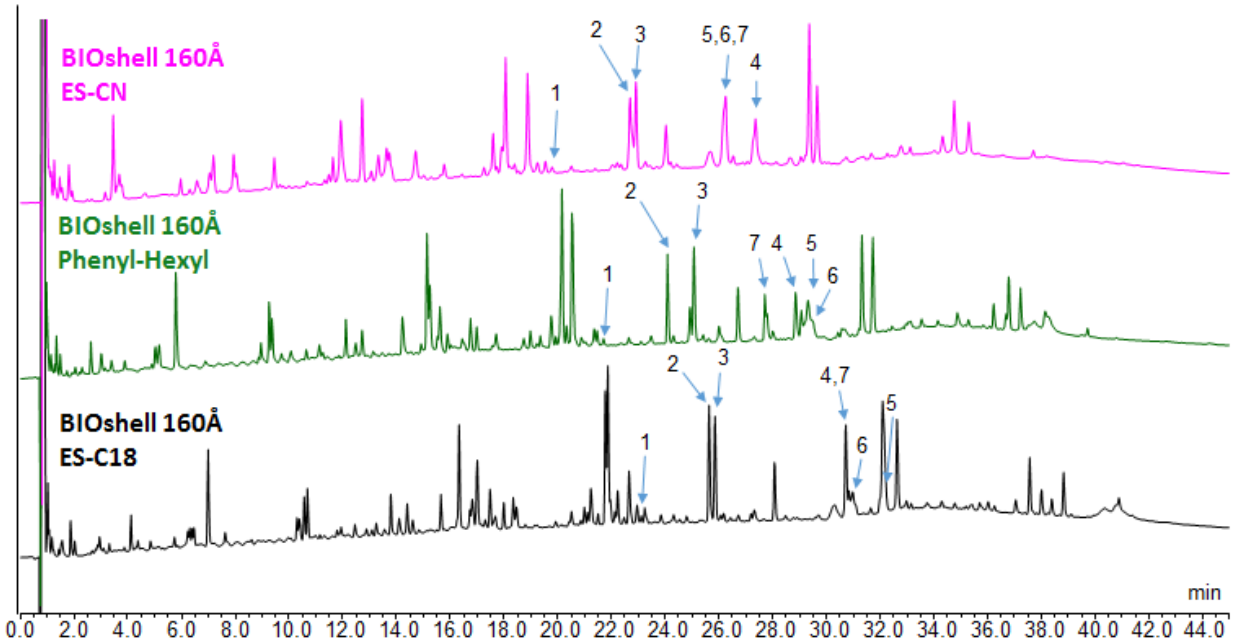
Chromatographic Conditions:

Columns: BIOshell® A160 Peptide 2.1 x 150 mm
C18, 2.7 µm
CN, 2.7 µm
Phenyl-Hexyl, 2.7 µm
Mobile Phase A: water/0.1% TFA
Mobile Phase B: ACN/0.1% TFA
Gradient: 0-60% B in 15 min
Flow Rate: 0.4 mL/min
Temperature: 60 °C
Detection: 220 nm
Injection: 2 µL

Peak Identities:

1. Tyr-Tyr-Tyr
2. Angiotensin II
3. Angiotensin 1-12
4. Melittin
5. Sauvagine
6. β-Endorphin

Trastuzumab Tryptic Digest:
Effect of Phase Chemistry



Trastuzumab Tryptic Peptides		
1.FTISADTSKNTAYLQMNSLR	4. LLIYSASFLYSGVPSR	7. VVSVLTVLHQDWLNGKEYK
2. LScAASGFNIKDTYIHWVR	5. SGTASVVcLLNNFYPR	
3. GFYPSDIAVEWESNGQPENNYK	6. ScDKTHTcPPcPAPELLGGPSVFLFPPKPK	

column: As indicated; 10 cm x 2.1 mm I.D., 2.7 µm

mobile phase: [A] Water (0.1% DFA); [B] Acetonitrile (0.1% DFA)

gradient: Hold at 2% B for 2 min; 2% B to 50% B in 60 min

flow rate: 0.3 mL/min

column temp.: 60 °C

detector: MSD

injection: 5 µL

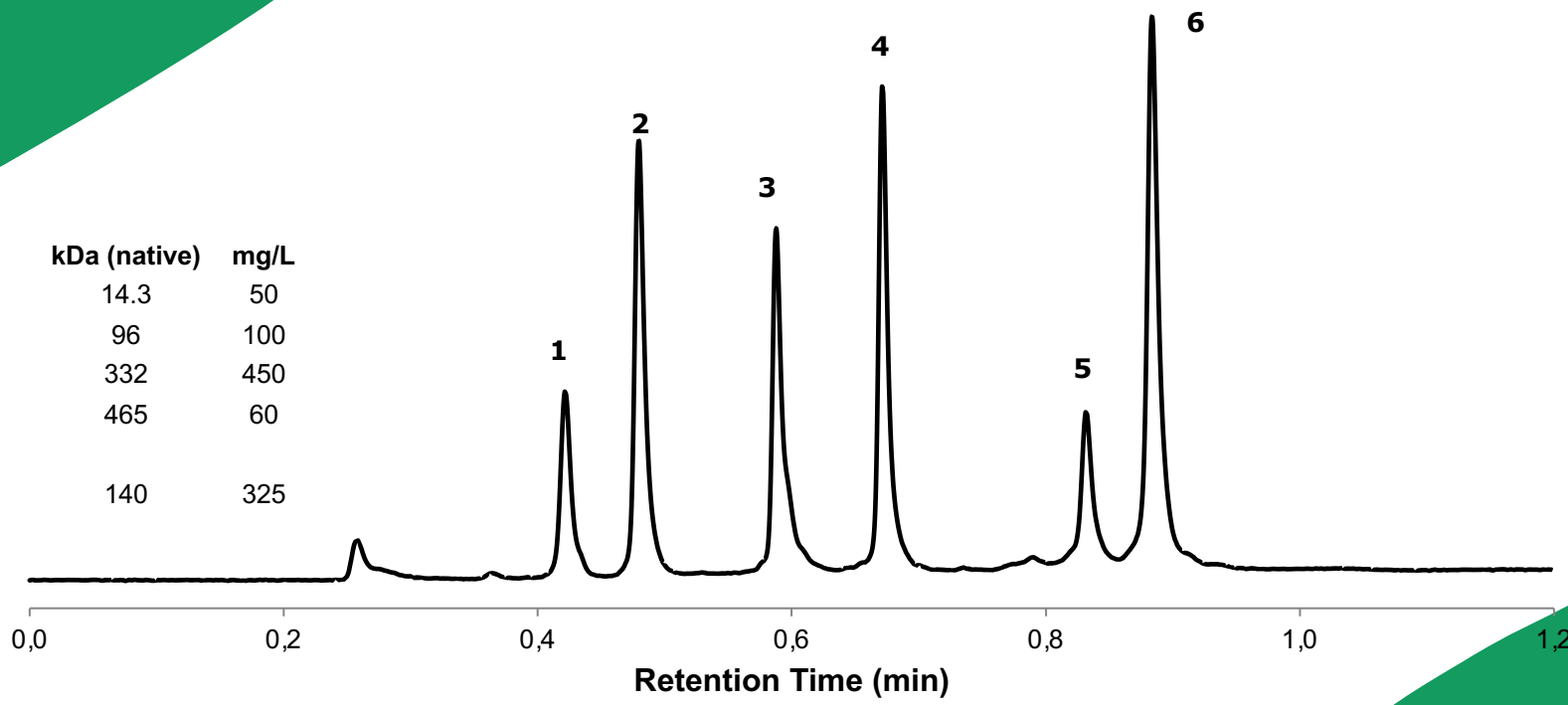
sample: Trastuzumab tryptic digest, 0.2 µg/mL, water

Muraco, C. E. "Breaking Up is Not So Hard to Do: Recent Advances in Peptide Mapping of Biotherapeutics." Oral Seminar given at HPLC 2018; July 31, 2018.

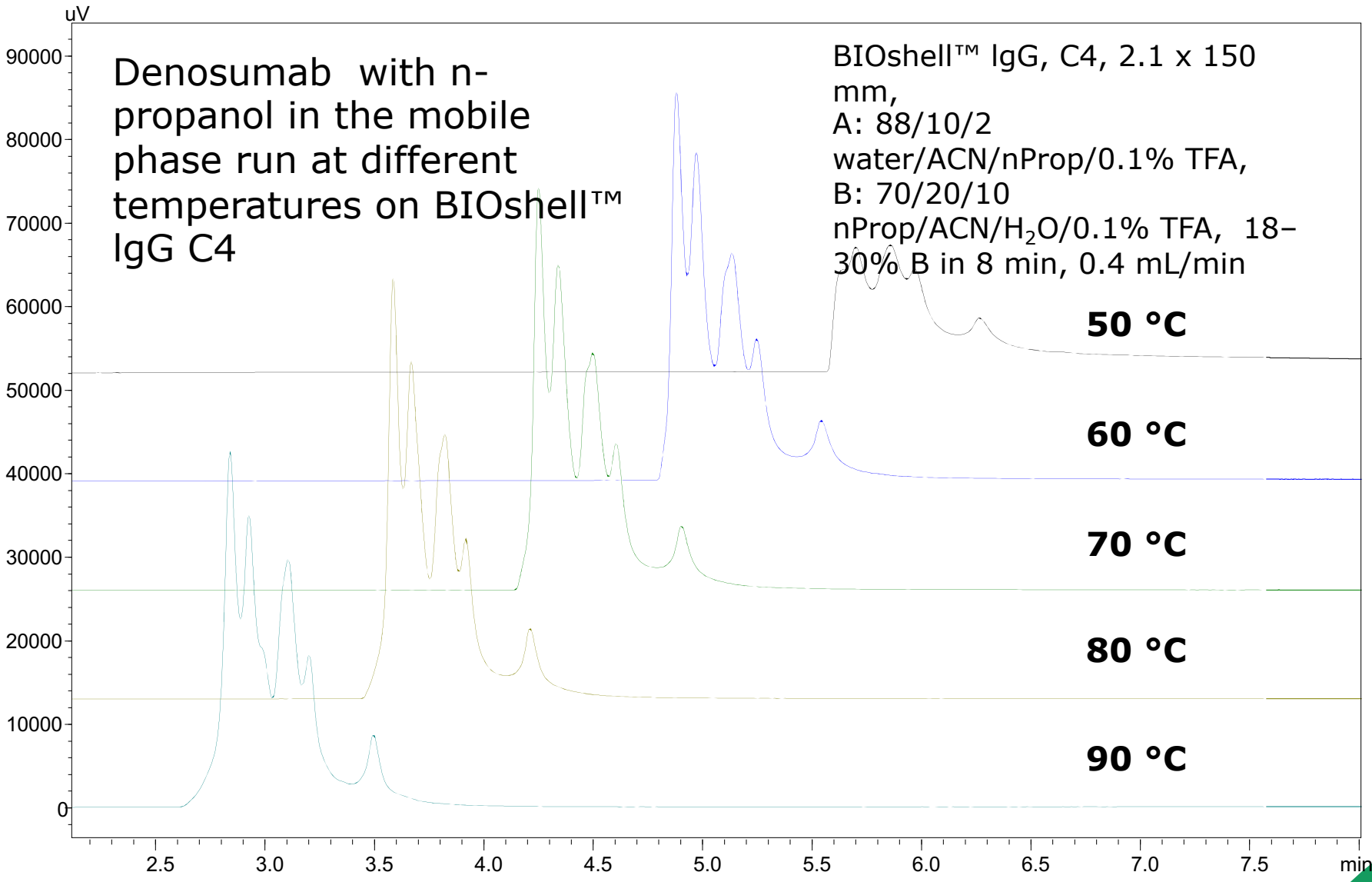
Fast Protein Separation at High Temperature

column:	BIOshell A400 Protein C4, 5 cm x 2.1 mm I.D., 3.4 µm
mobile phase A:	75:25, (water, 0.1% TFA) : (acetonitrile, 0.1% TFA)
mobile phase B:	25:75, (water, 0.1% TFA) : (acetonitrile, 0.1% TFA)
gradient:	12 to 100% B in 1 min
flow:	0.4 mL/min
pressure:	900 psi initial
column temp:	**90° C**
detector:	UV, 215 nm
injection:	1 µL
sample:	protein mix in 0.05% TFA

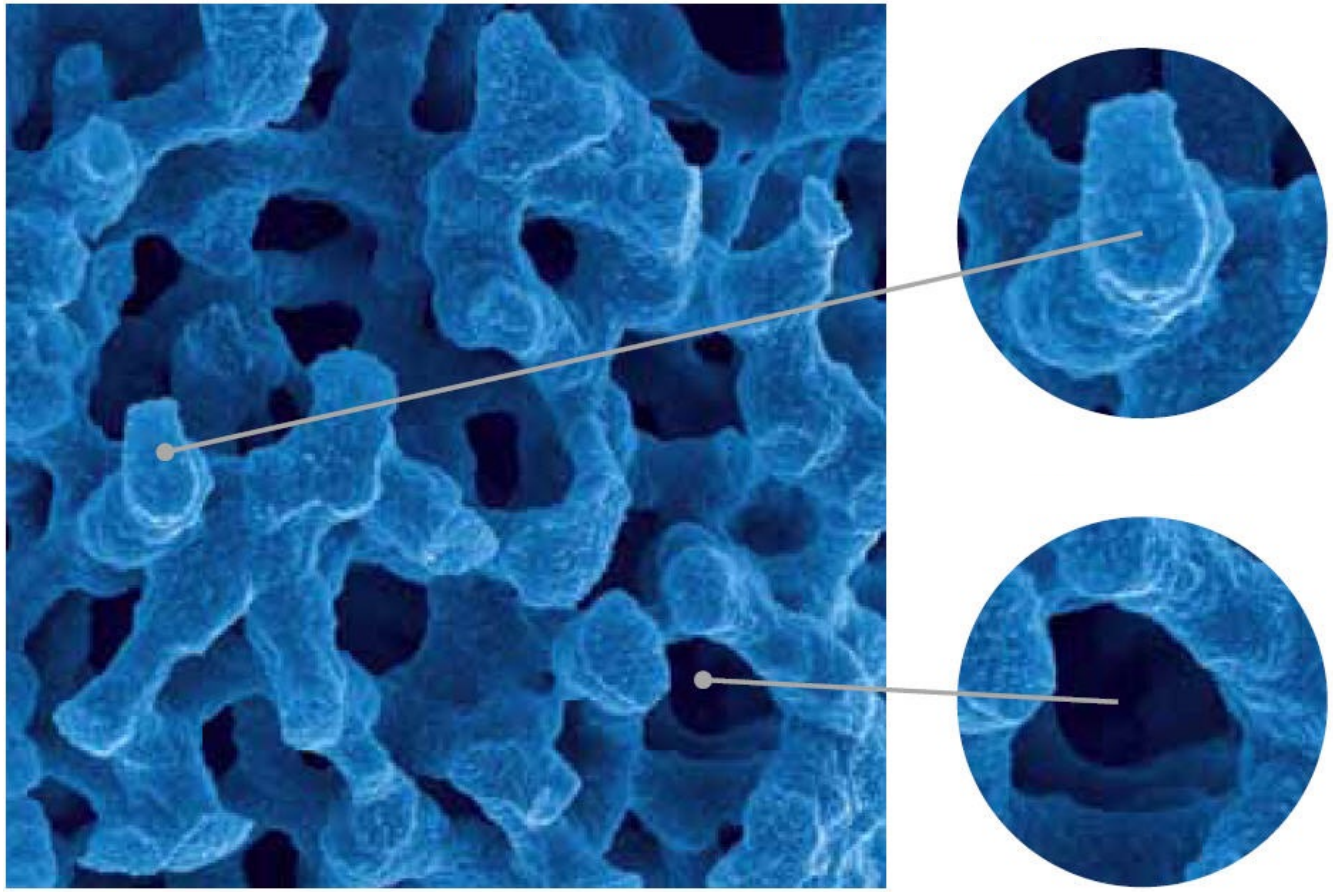
Peak	Name	kDa (native)	mg/L
1	lysozyme; chicken	14.3	50
2	haptoglobulin, phenotype 1-1; human	96	100
3	glutamate dehydrogenase; bovine	332	450
4	ß-galactosidase; *E. coli*	465	60
5	lactate dehydrogenase contaminant		
6	lactate dehydrogenase; rabbit	140	325



Effect of Temperature on IgG2



Monolithic silica columns
Bi-modual pore structure



Mesopores:

Chromolith Performance: 130 Å

Chromolith 2mm ID: 130 Å

Chromolith HR: 150 Å

Macropores:

Chromolith Prep 3µm

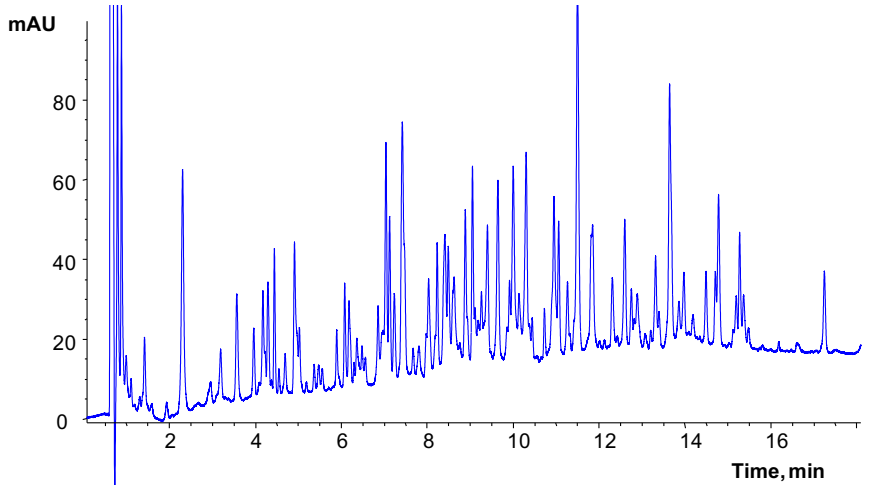
Chromolith Performance (3, 4.6, 10, 25 mm ID): 2 µm

Chromolith 2mm ID: 1.5 µm

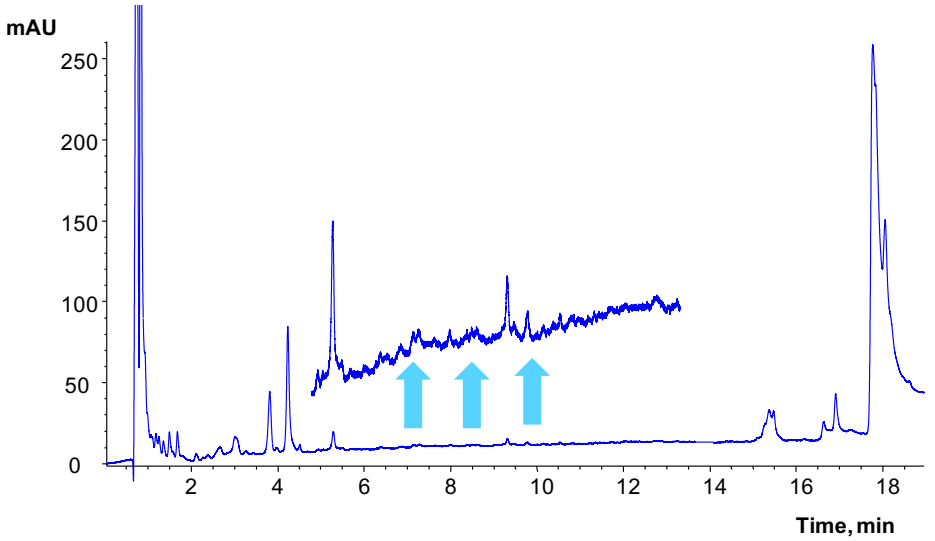
Chromolith HR: 1.15 µm

Pore volume	1.0 mL/g
Total porosity	>80 %
Surface area	300 m2/g

Application: Proteomics
Monolithic silica column RP-18e 100-2mm



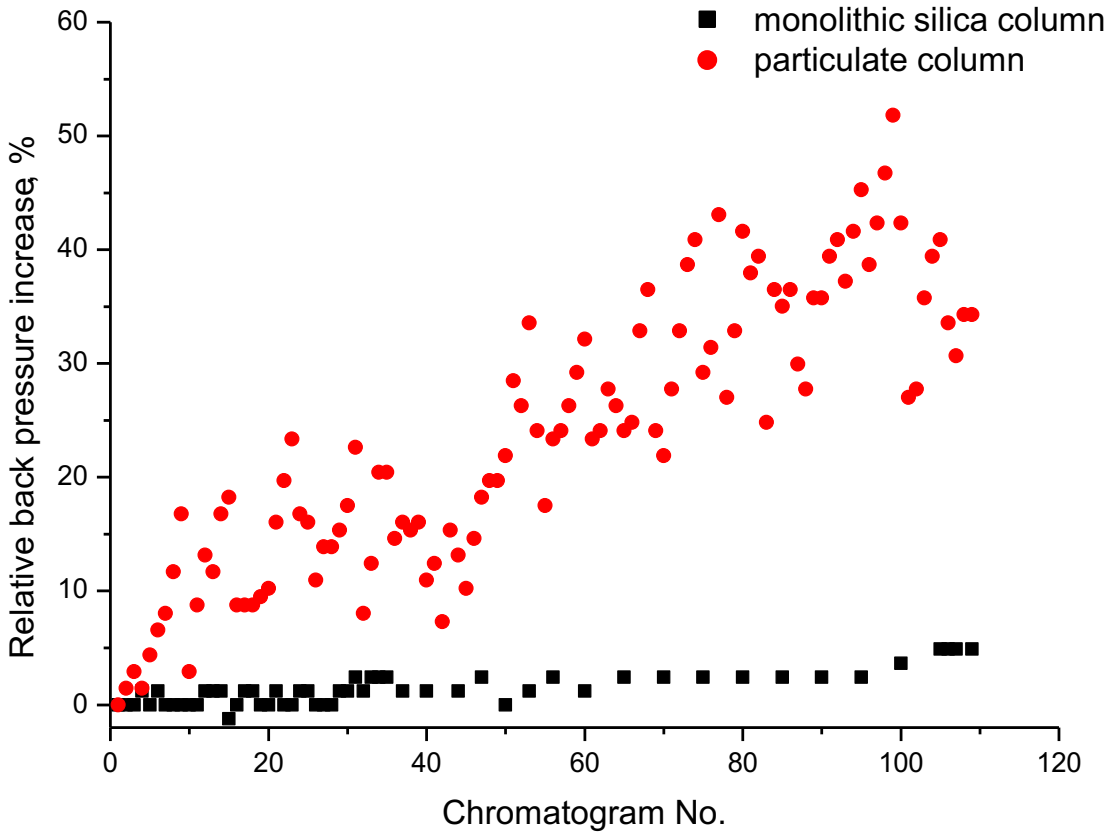
1 µl BSA digest (1 mg/ml)



8 µl of filtered amniotic fluid

Eluents A: 95% H_2O/5% ACN/0.1% TFA (v/v/v),
B: 5% H_2O/95% ACN/0.085% TFA (v/v/v);
Gradient from 5% B to 50% B in 20 min
Flow Rate 0.3 ml/min
Detection UV 214 nm

Direct injection
Human plasma



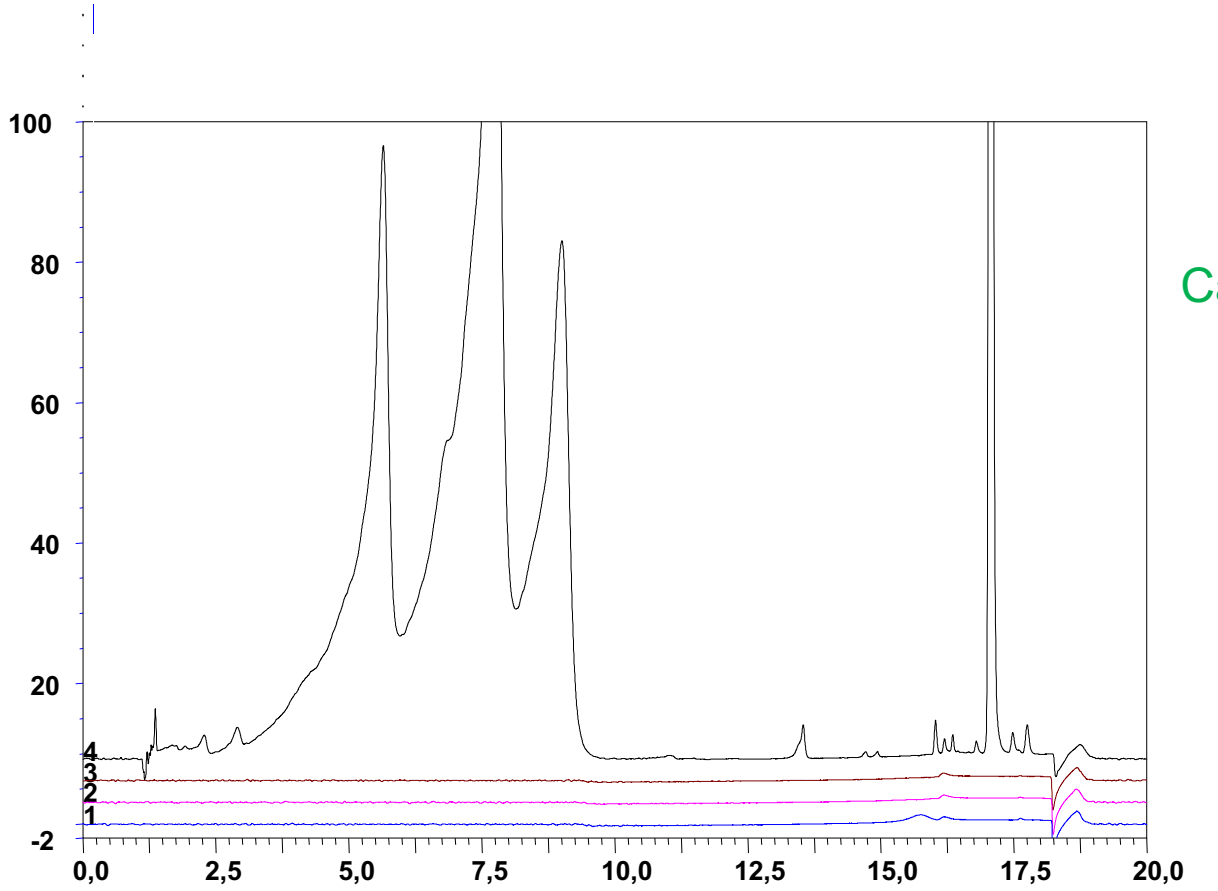
Changes in back pressure of the particulate (25-µm particles) and the monolithic silica columns injecting filtrated human plasma sample.

- **Particulate column:** 25 x 4 mm I.D. cm, 25µm RAM-SCX particles.
- **Monolithic silica column:** 5 x 4.6 mm I.D., Chromolith guard column.

Sample human plasma, 50 µl per injection.

Applications

UV-Filters in Sun-Lotion – Colipa S (QC-method)



Carry over with cream sample

100µL inj. vol.

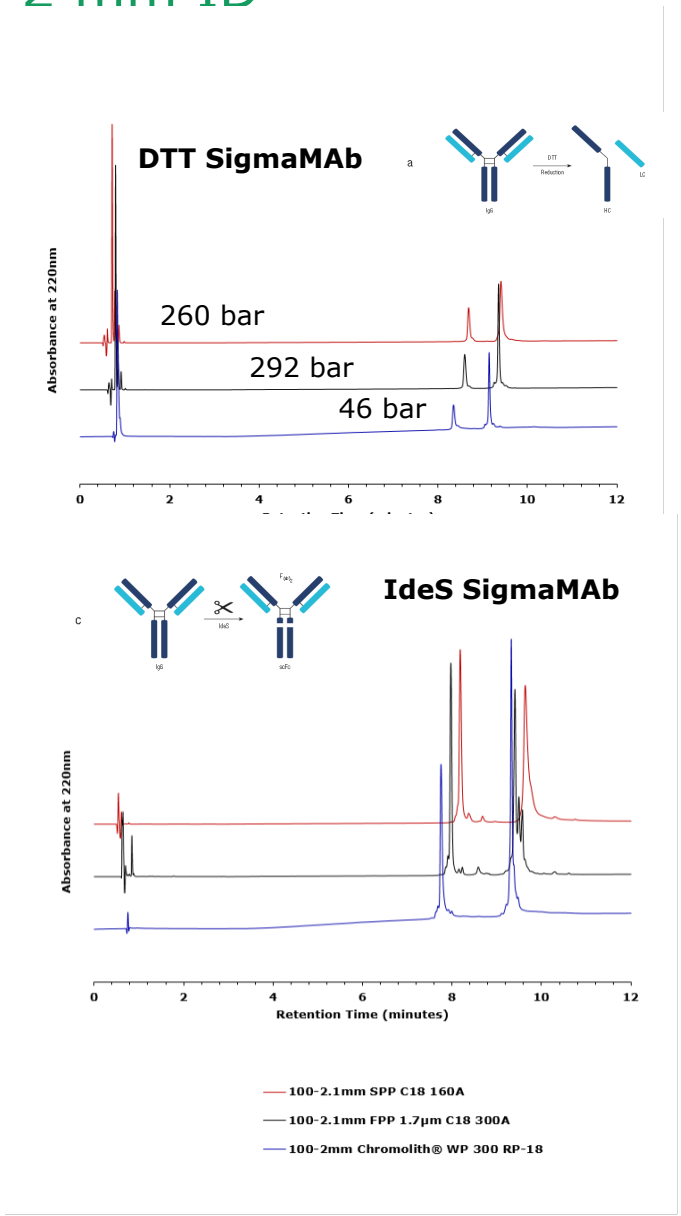
0% Carry Over of UV-Filters!!!

Antibody Fragment Analysis – vs. FPP and SPP

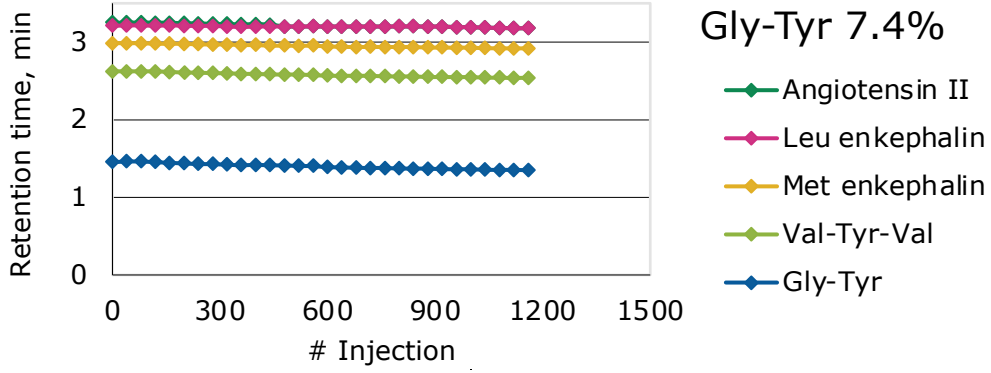
Monolithic silica WP 300 RP-18 2 mm ID

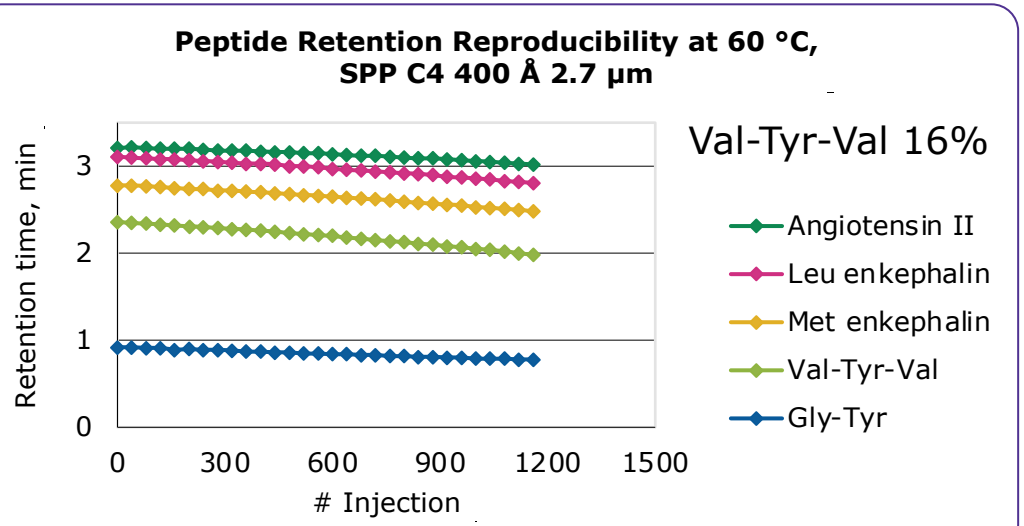
Chromatographic conditions

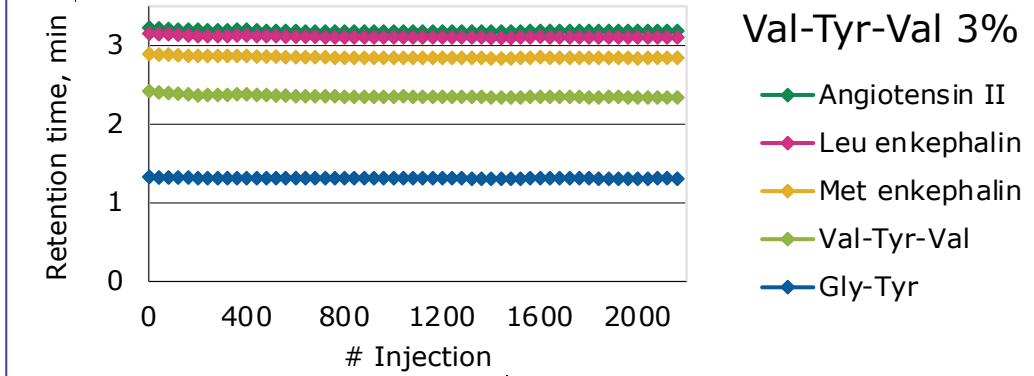
Columns:	Chromolith® WP 300 RP-18, 2 mm I.D. SPP, C18, 160 Å, 2.0 µm, 100-2.1 mm FPP, C18, 300 Å, 1.7 µm, 100-2.1 mm
Mobile Phase:	A: Water (0.1% (v/v) TFA) B: Acetonitrile (0.08% (v/v) TFA)
Gradient:	0 min 20% B 1 min 20% B 9 min 45% B
Detection:	UV, 220 nm
Temperature:	80 °C
Injection Volume:	1.0 µL
Flow rate:	380 µL/min
Sample:	SigmaMAb, 2 mg/mL (SiLu™ Lite Universal Antibody) NISTmAb, NIST® RM 8671
Sample preparation:	DTT digest: 60 µL of 40 mM Dithiothreitol (DTT) solution was added in a PCR vial, 40 µL mAb was added and incubated at 37 °C for 30 minutes creating LC and HC parts of the antibody. IdeS digest: 4 µL IdeS-Protease and 56 µL water were added in a PCR vial, 40 µL mAb was added and incubated at 37 °C for 30 minutes creating $F(ab')_2$ and scFc parts of the antibody.



Stability of short chain columns





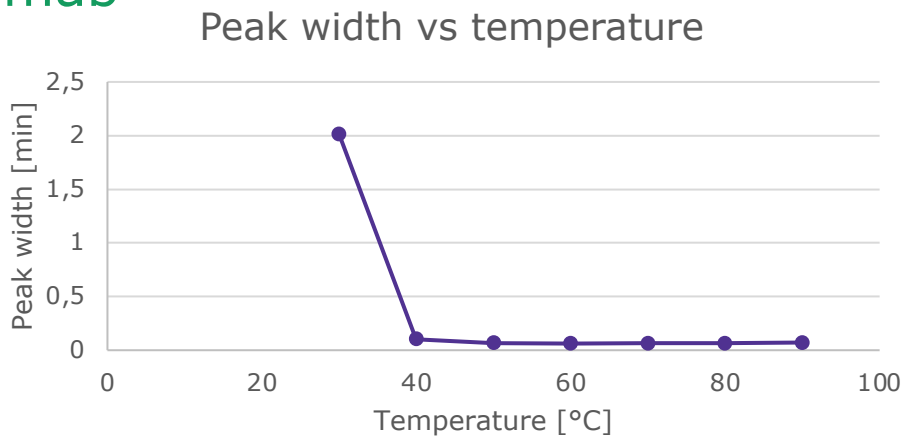


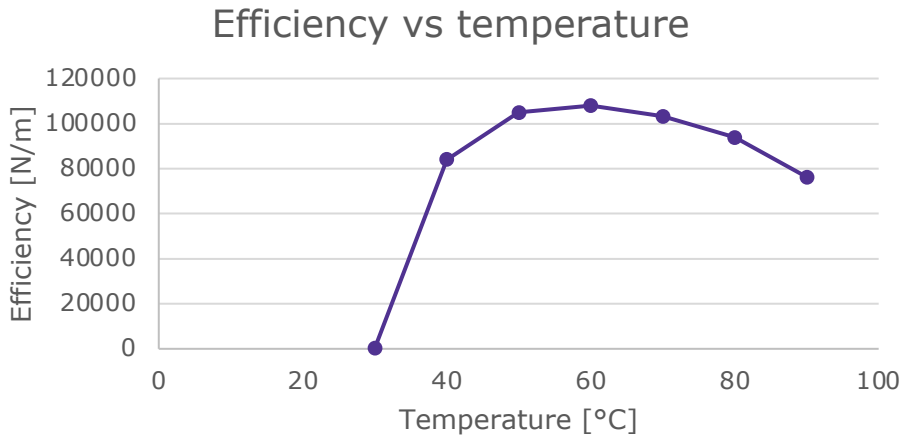
Mobile phase:	[A] Acetonitrile (0.08% TFA)
	[B] Water (0.1% TFA)
Gradient:	98% B to 55% B in 3 min
Flow rate:	1.5 mL/min
Detection:	UV, 220 nm
Temperature:	60 °C
Injection vol:	2 µL
Sample:	peptide standard mixture (H2016)
Column:	as indicated, 100 x 4.6 mm

Monolithic silica WP 300 RP4

Influencing peak width and efficiency of cetuximab

Temperature [°C]	Peak width 10% [min]	Separation efficiency [N/m]
30	2,013	207
40	0,103	84090
50	0,067	104918
60	0,062	107973
70	0,063	103188
80	0,064	93865
90	0,069	76228





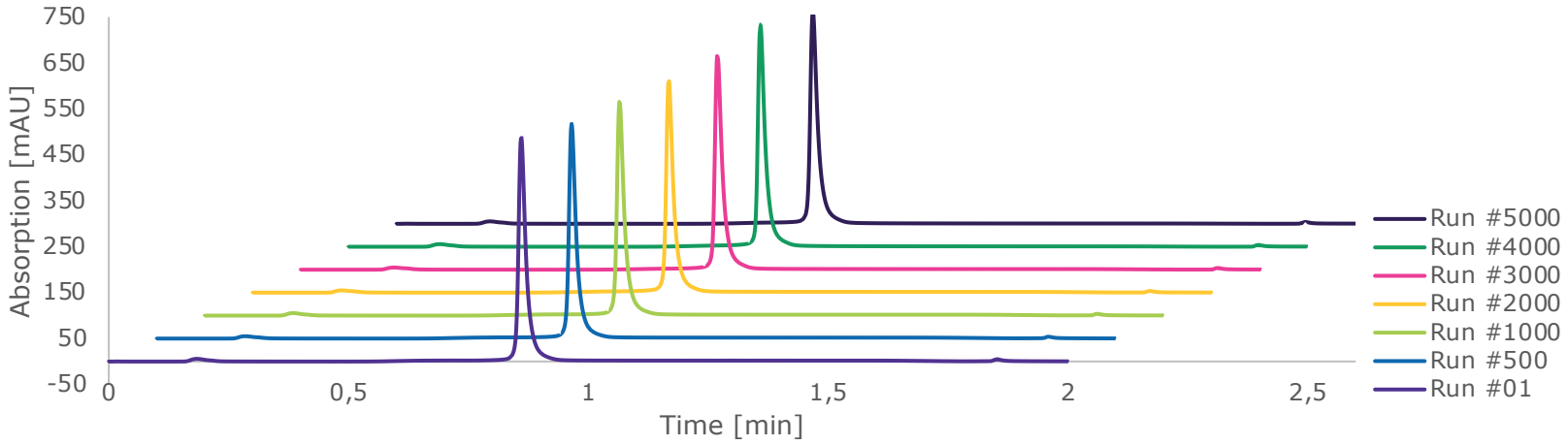
Conditions: ACN 0,08%TFA/ Wasser 0,1%TFA 4/96 in 5min to 60/40, 2,2mL/min, UV220nm, 60°C, 0,5µL Inj.vol.

Column: Chromolith WP 300 RP4, 100x4.6mm (1.52260.0001)

Peak shape of biomolecules can be improved with increasing temperature. However for Chromolith WP300 RP4 and the separation of cetuximab a temperature of 60°C leads to the best results.

Nevertheless this is strongly depending on the protein.

Monolithic silica WP 300 Protein A: Stability test



Stability is given for 10.000 pH shifts

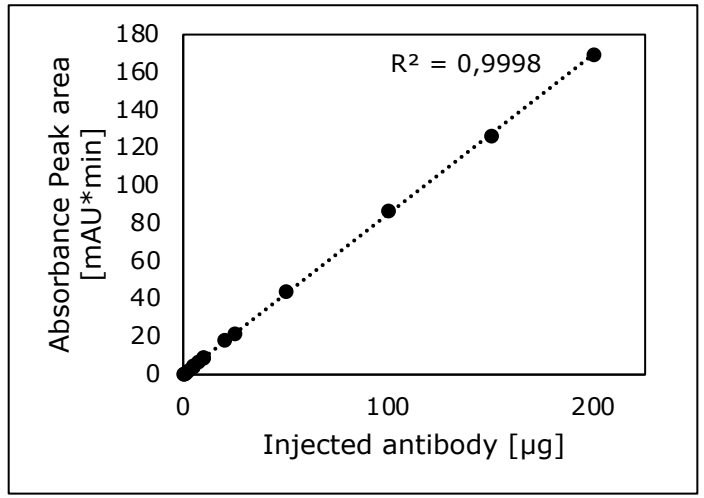
- Performance was checked every 10th run with IgG
- RSD elution time IgG < 0.5%
- RSD peak area IgG < 1.1%

Reproducibility of 50 injections

RSD elution time IgG	< 0.1%
RSD peak area IgG	< 0.5%

Linearity

- 0.125 mg/mL – 10 mg/mL

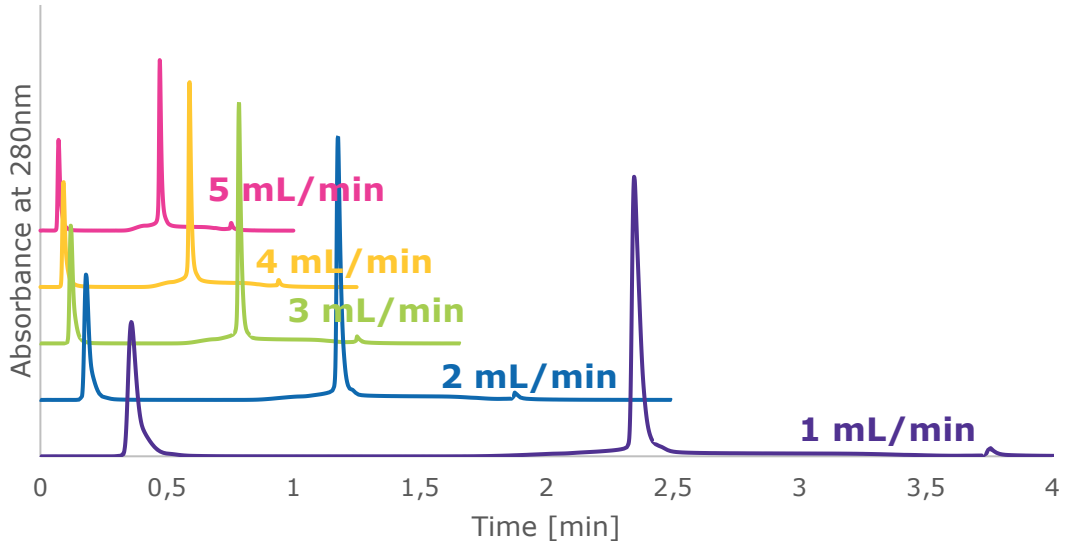


Stability test

- 2.0 mL/min
- 280 nm
- 25°C
- 10 µl IgG (1 mg/mL)
- Buffer A: 100 mM sodium phosphate pH7.4
- Buffer B: 100 mM sodium phosphate pH2.5

Monolithic silica WP 300 Protein A: Flow rate

Antibody binding is not affected by flow rate



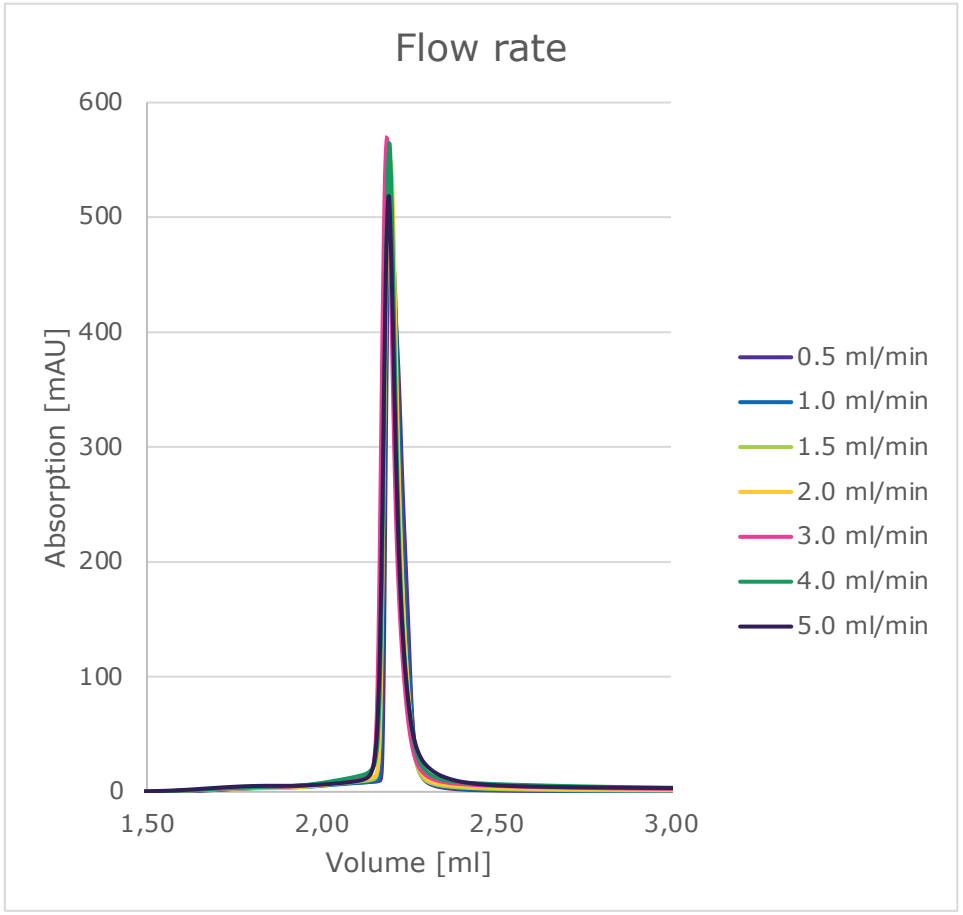
- high-speed separation at high flow rate due to excellent mass transfer properties of the monolithic skeleton
- separation of IgG demonstrates the extreme time savings and high separation efficiency made possible with Chromolith® protein A columns.

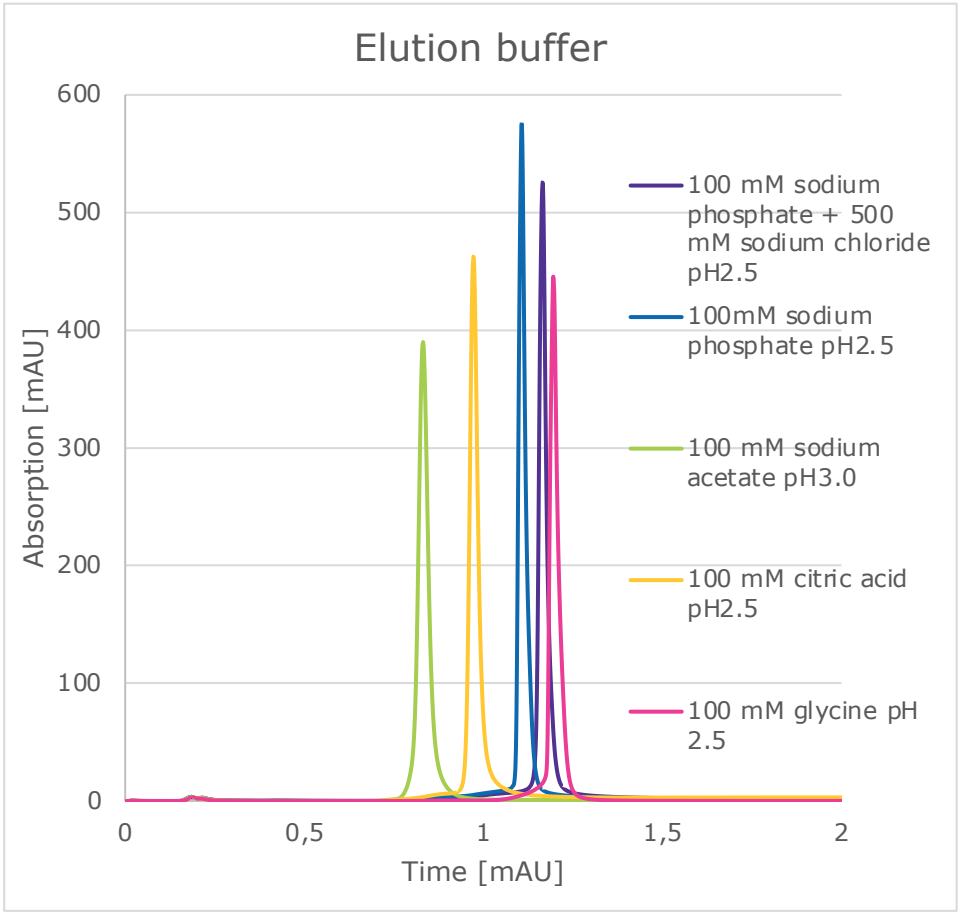
Monolithic column provides low column backpressure at high flow rates

Flow rate	Unbound area	IgG area	Pressure [bar]
1 mL/min	39%	61%	**3**
2 mL/min	39%	61%	**6**
3 mL/min	39%	61%	**10**
4 mL/min	39%	61%	**13**
5 mL/min	39%	61%	**21**

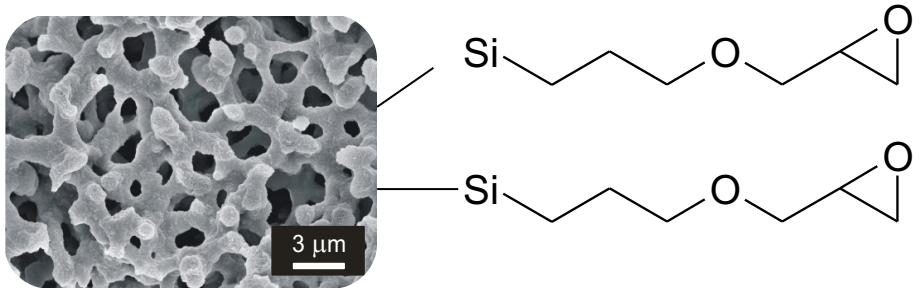
- IgG was well separated with excellent peak symmetry
- At 5 ml/min the total analysis time is less than 1 minute and the net column backpressure is only 21 bar
- Antibody binding is not affected by flow rate

Monolithic silica WP 300 Protein A: Flow rate





Monolithic silica WP 300 Epoxy

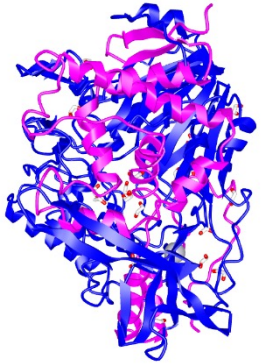


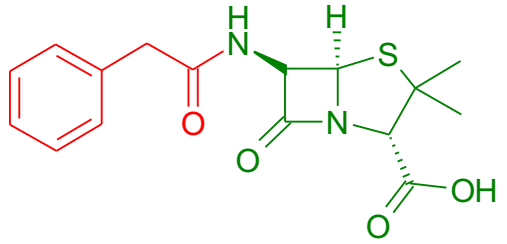
Product description

Silica type	High purity
Particle size	monolithic
Macropore size	~ 2 µm
Mesopore size	~ 30 nm (300 Å)
Surface area	~ 120 m^2/g
Epoxide concentration	~ 3,2 $\mu mol/m^2$
Pressure limit	200 bar
pH stability	1.5 – 7.5
immobilization	8.0 (up to 24 hours)
Operating temperature	2 – 45°C
Shipping solution	2-Propanol
Storage temperature	2 – 8°C

Immobilization of penicillin acylase

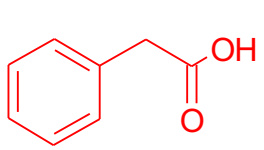
- According to Epoxy method
- Chromolith® WP 300 Epoxy 100-4.6mm
- 80 mg penicillin acylase dissolved in 25 ml 50mM sodium phosphate + 1.9M ammonium sulfate pH8.0
- Immobilization for 24 hours at 0.2 ml/min
- Quenching of remaining epoxide functions with glycine



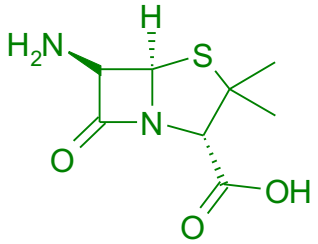


Penicillin G

Penicillin G Acylase



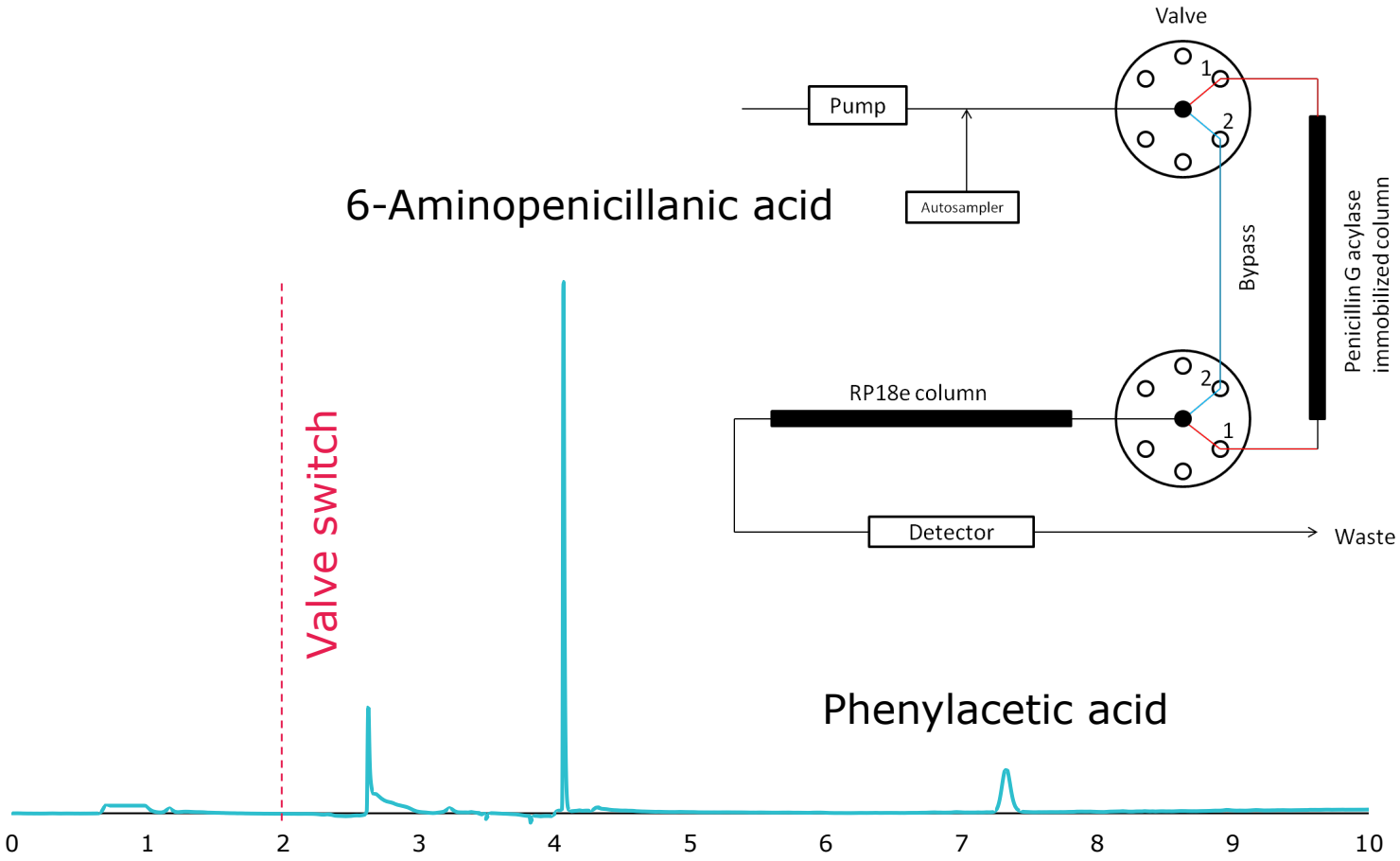
+



Phenylacetic acid

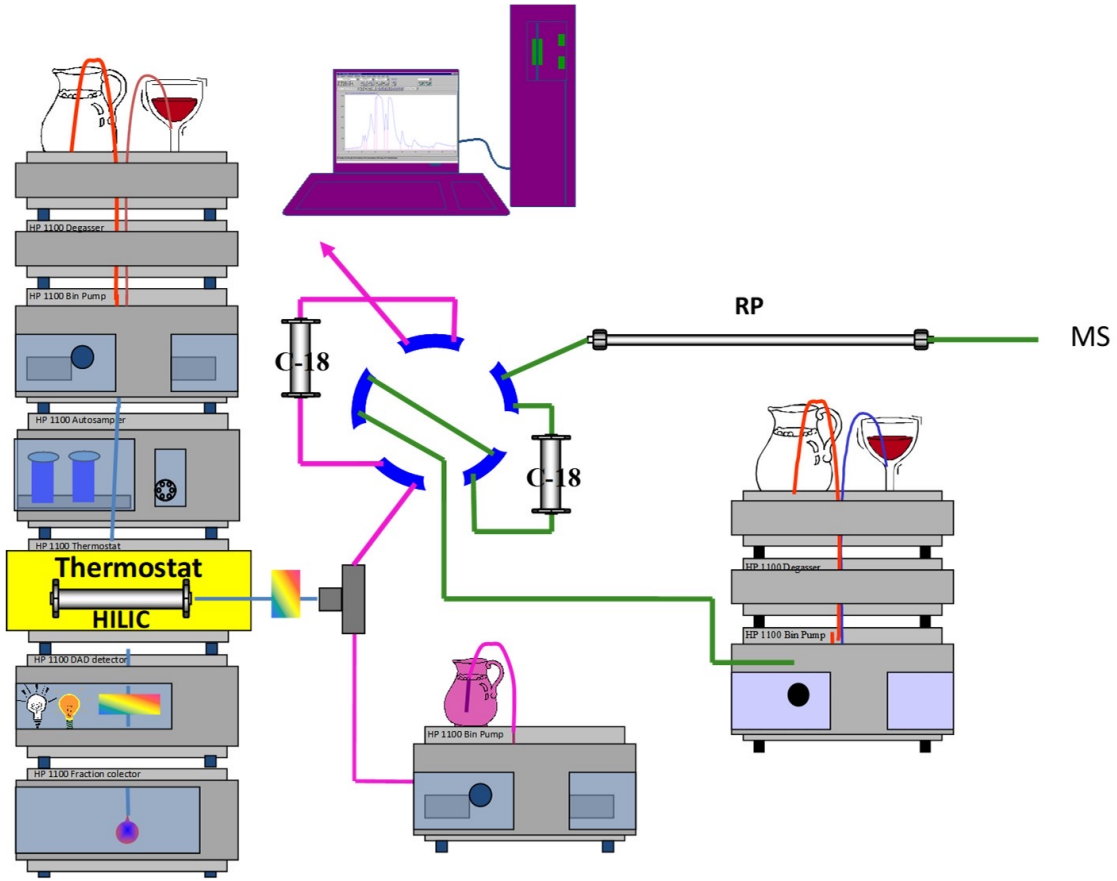
6-Aminopenicillanic acid

Immobilization of penicillin acylase – Enzymatic bioreactor

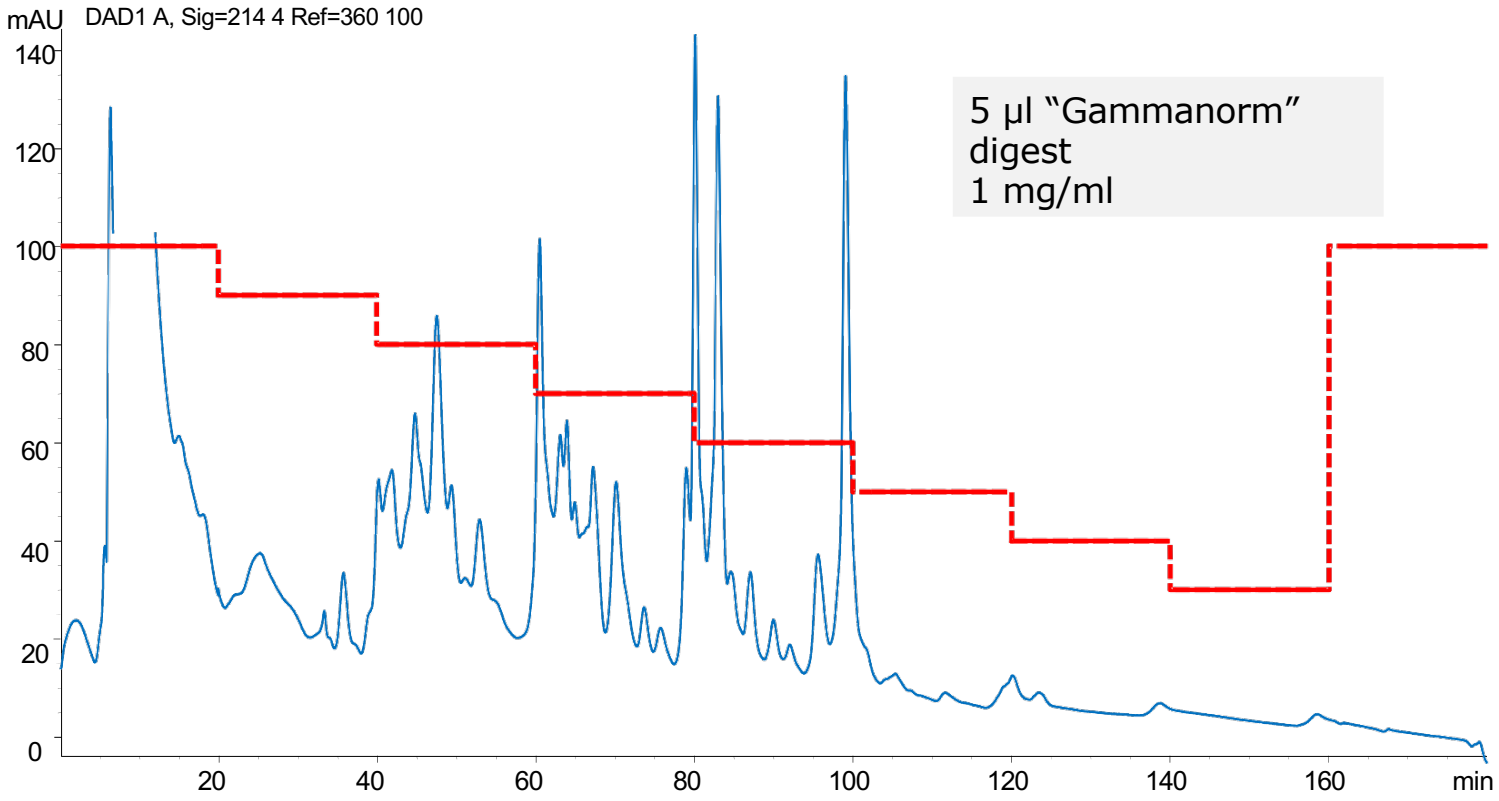


Chromatographic conditions				
Flow rate	1.0 ml/min			
Temperature	23°C			
Detection	UV 225nm			
Eluent A	10mM sodium phosphate pH7,0			
Eluent B	10mM sodium phosphate pH3,0			
Eluent C	Acetonitrile			
Sample	1.0 µl Penicillin G (3.5 mg/ml)			
Gradient				
Time	Valve	A	B	C
0.00	1	100	0	0
2.00	1	100	0	0
2.00	2	0	80	20
4.00	2	0	80	20
9.00	2	0	50	50
9.50	2	0	50	50
9.60	2	0	80	20
15.00	2	0	80	20

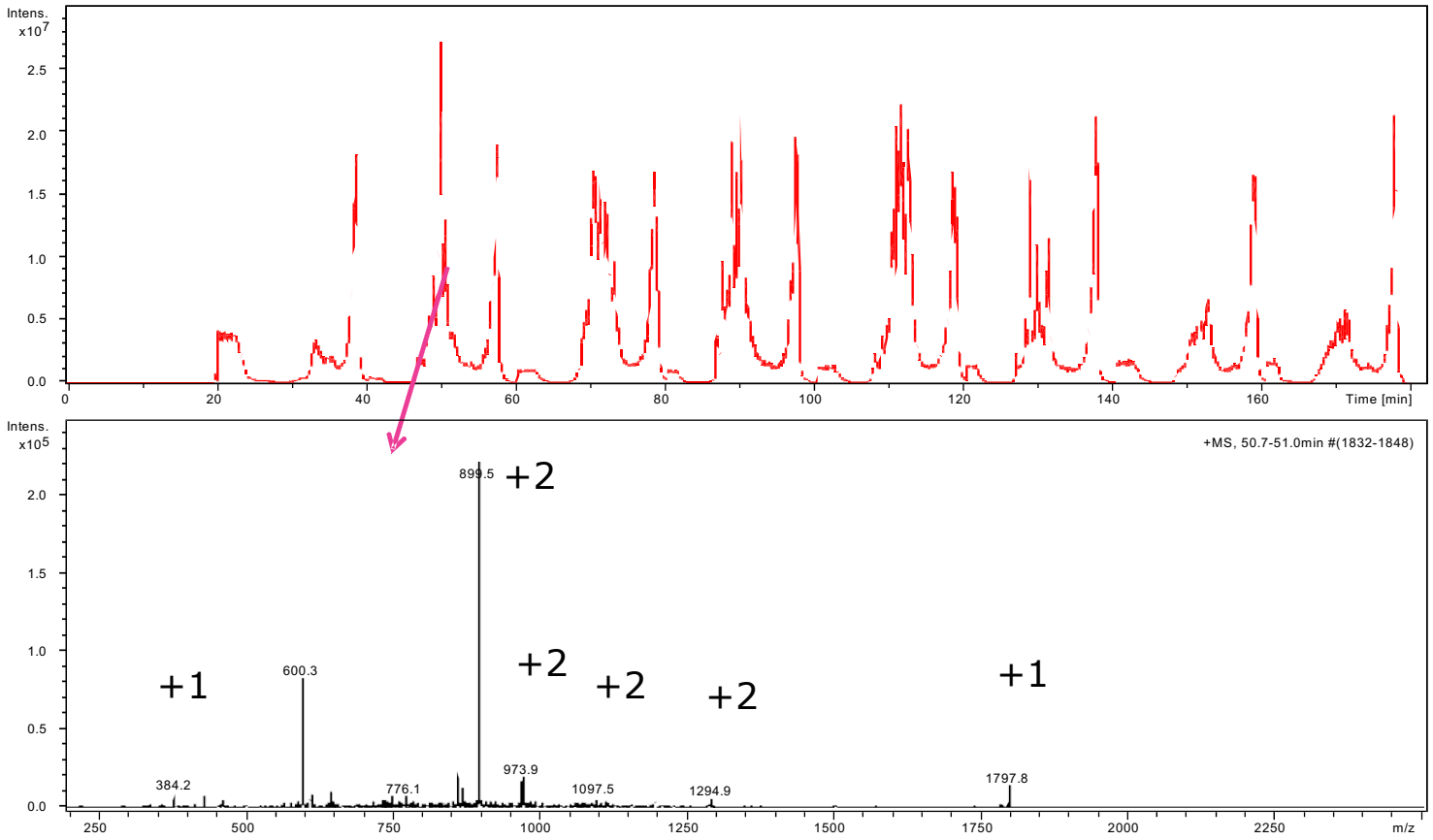
2D separation: HILIC & RP MS



2D separation: 1st dimension - HILIC



2D separation: 2nd dimension RP MS



Conclusions

- Pore size matters
- Superficially porous particles provides more efficiency, resolution and peak capacity
- Choosing right modification helps to achieve separation of coeluting substances

- Silica monoliths consist of macro- and mesopores, offering high permeability
  - SPP show highest separation efficiency
  - Silica monoliths show best column stability for more than 2000 injections
- Protein A immobilized silica monoliths enables robust and fast titer determination of immunoglobulines
  - Antibody binding is not affected by flow rate
  - Linearity up to 200 µg of injected antibody
- Immobilization of ligands onto epoxy-carrying monoliths leads to new degrees of freedom
  - Ligand binding via amino-groups using a simple, straightforward protocol
  - Various modes of chromatography are possible as well as the design of bioreactors or similar systems

Many thanks to our Chromatography R&D Groups in Darmstadt and Bellefonte and the Application labs in the different counties for providing the chromatographic data.

Find out more:
Sigmaaldrich.com