Getting the best out of solid core technology for small and large molecule analysis

Matt James
Matt.James@avantorsciences.com

avantor™

In Partnership with MAC-MOD, our authorized channel partner. MAC-MOD have been a trusted partner for over 20 years with expertise in HPLC & UHPLC

Introduction

01

– History of solid core

02

– Modelling of solid core

03

– Separating small molecules

04

– Separating big molecules

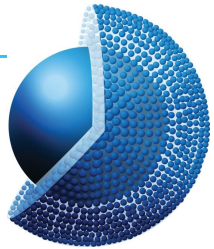
05

– Method development considerations

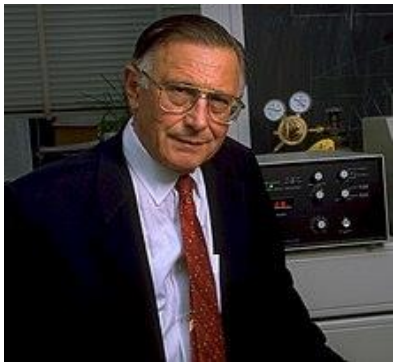
06

– Conclusions

History of Solid Core Particles



- 1960s
  - Horvath and Lipsky introduce the concept of pellicular/shell particles
- 1970s
  - Core-shell particles developed:-
  - Zipax (DuPont later Rockland Technologies and finally acquired by HP/ Agilent), Corasil I&II (Waters), Perisorb (Merck)
  - Improvement in the manufacturing of high-quality fully porous spherical particles inhibits success of the shell particles
  - 10 µm fully porous spherical particles
- 1980s
  - 5 µm porous particles
- 1990s
  - 3 µm porous particles
- 2000–Present
  - <2 µm porous particles
  - 1st solid core material

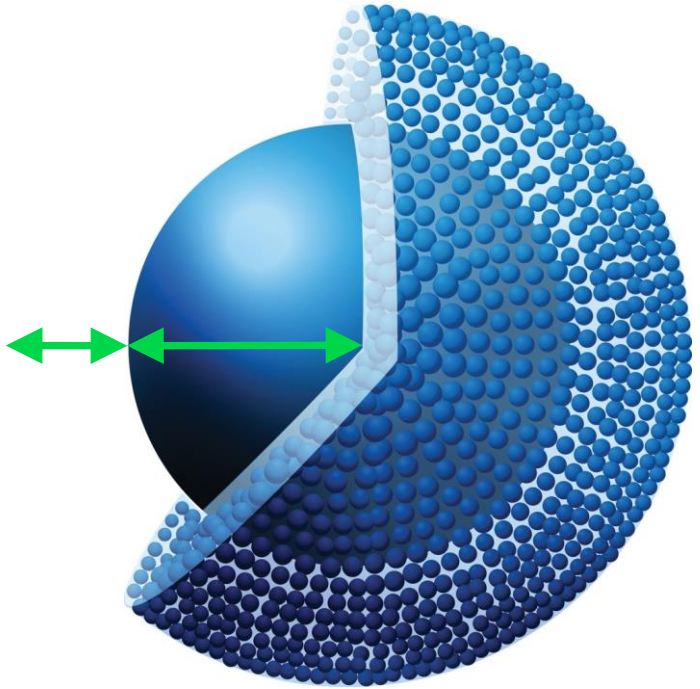


Reprinted with kind permission from Imre Molnár

Solid Core Particle architecture

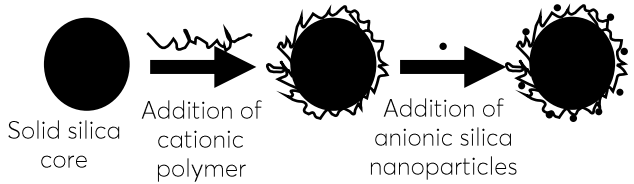
- 2.4-2.7 µm & 5 µm typical
- Smaller particles also available (1.3-1.7 µm)
  (with their own challenges!)

Rho (ρ) = solid core diameter : particle diameter ratio
Typically 0.6 – 0.75 for solid core particles

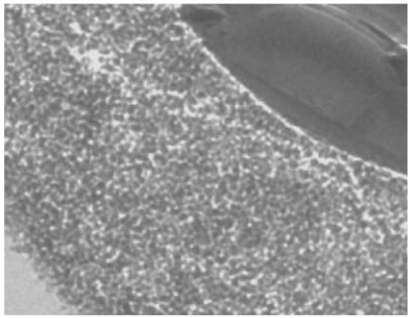


How to make a Solid Core particle

- **Layer-by-layer approach**
  - Most core–shell particles prepared by this approach
  - Electrostatic interaction between positively charged and negatively charged species assemble multiple layers.
  - XS polymer washed off after each layer of silica nano particles added
  - Process repeated, finally organic polyelectrolyte removed by heating.



- **Shell synthesis on a core**
  - Shell formed on a core particle by synthetic methods.
  - Silica microspheres, or even polymer microspheres can be used as the cores to prepare a wide range of core shell particles.
  - One pot mechanism is available.



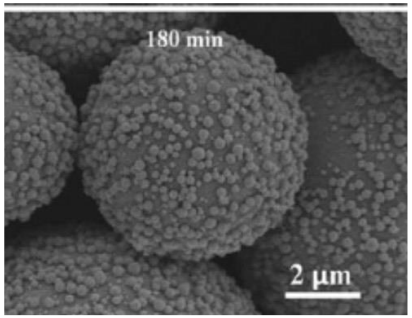
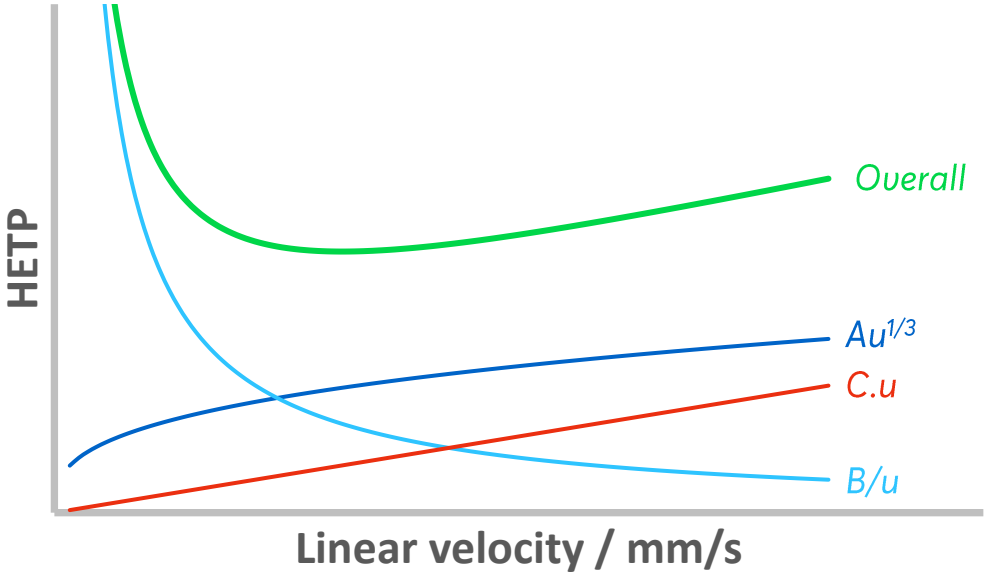


Image reprinted with kind permission from R. Hayes et al. / J. Chromatogr. A 1357 (2014) 36–52

Measuring Chromatographic Performance

Knox Curve

$$HETP = Au^{1/3} + \frac{B}{u} + Cu$$

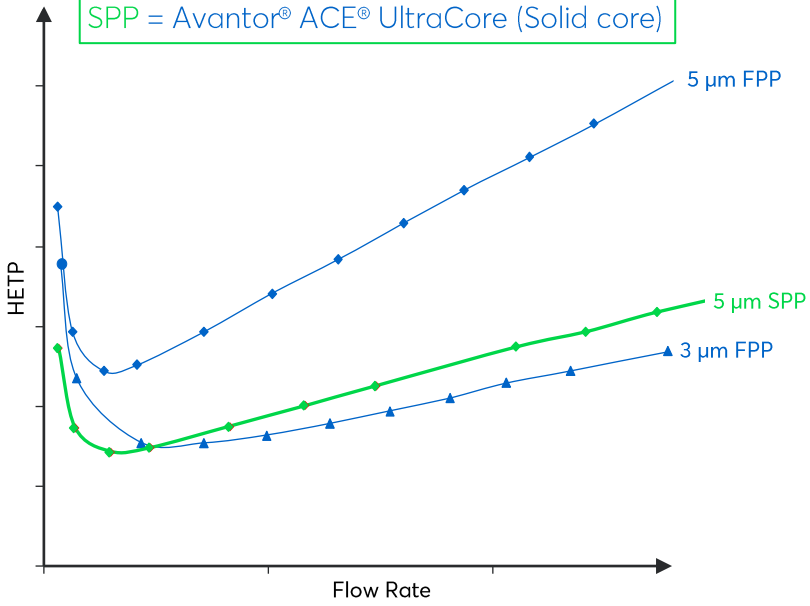


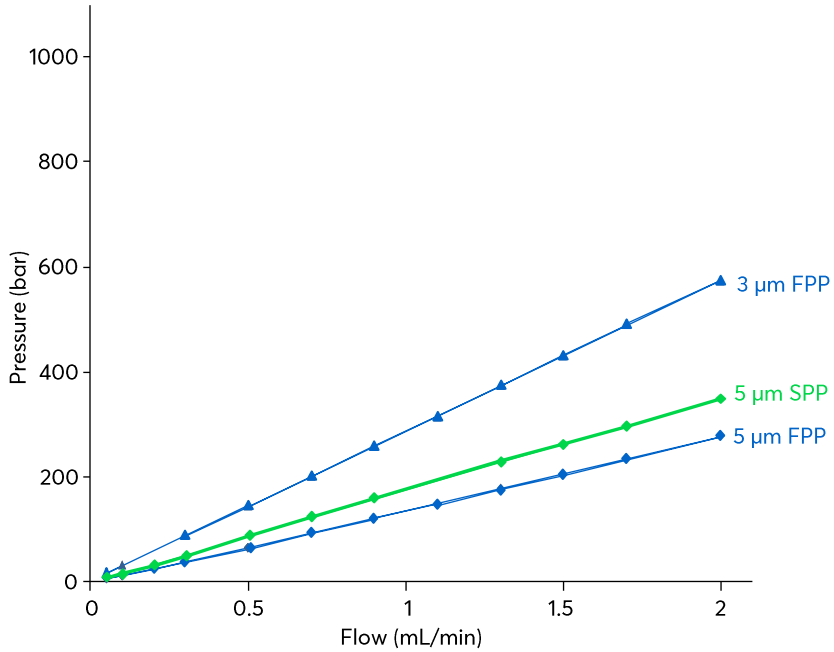
$A.u^{1/3}$	Eddy diffusion (analyte paths, packing, wall effects)
B/u	Analyte longitudinal / axial diffusion
C.u	Analyte mass transfer between stationary & mobile phases / radial transfer

Picture reprinted with kind permission from ChromCom 2020

Porous vs Solid Core

FPP = Avantor® ACE® Fully Porous Particles
SPP = Avantor® ACE® UltraCore (Solid core)

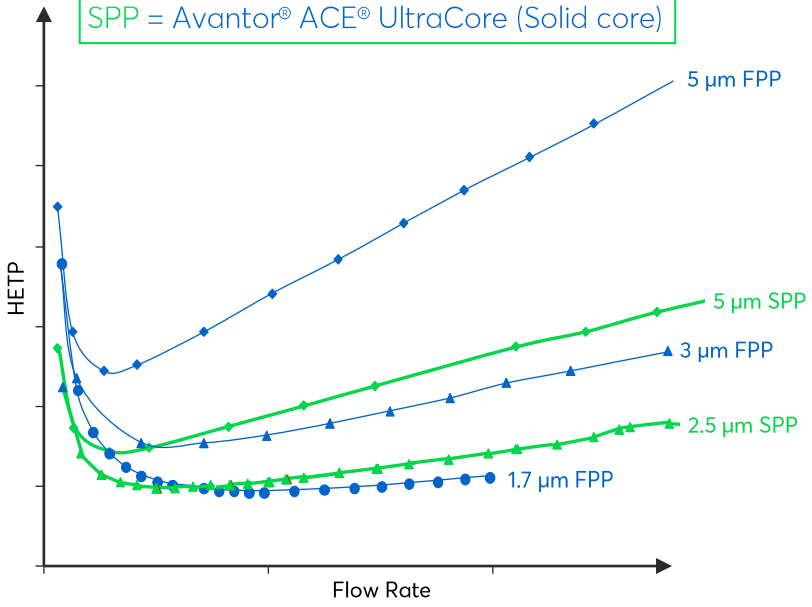


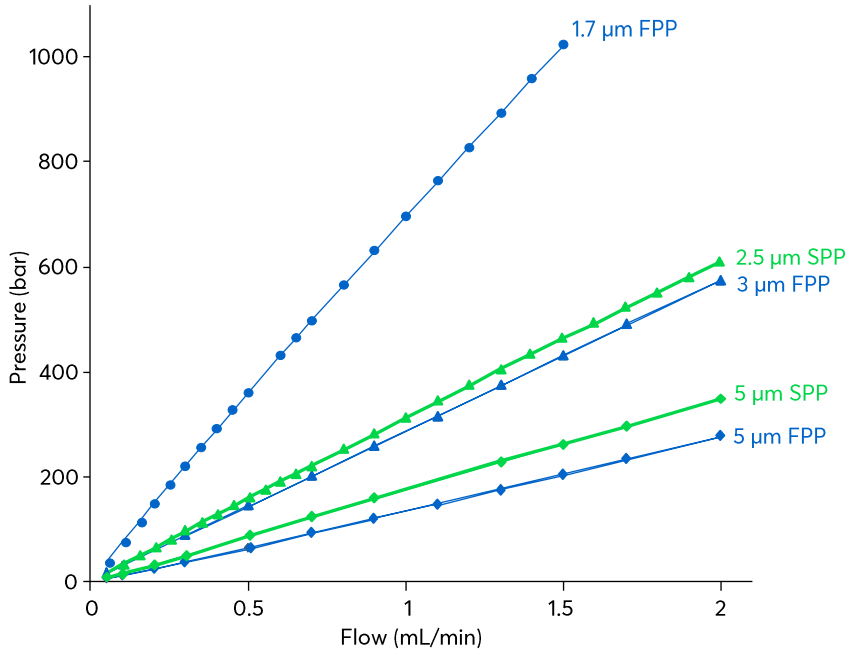


Isocratic analysis, 50x2.1 mm columns,
eluent = MeCN / water + 0.1% TFA, analyte = naproxen, constant k = 10, 40 °C, λ = 256 nm

Porous vs Solid Core

FPP = Avantor® ACE® Fully Porous Particles
SPP = Avantor® ACE® UltraCore (Solid core)





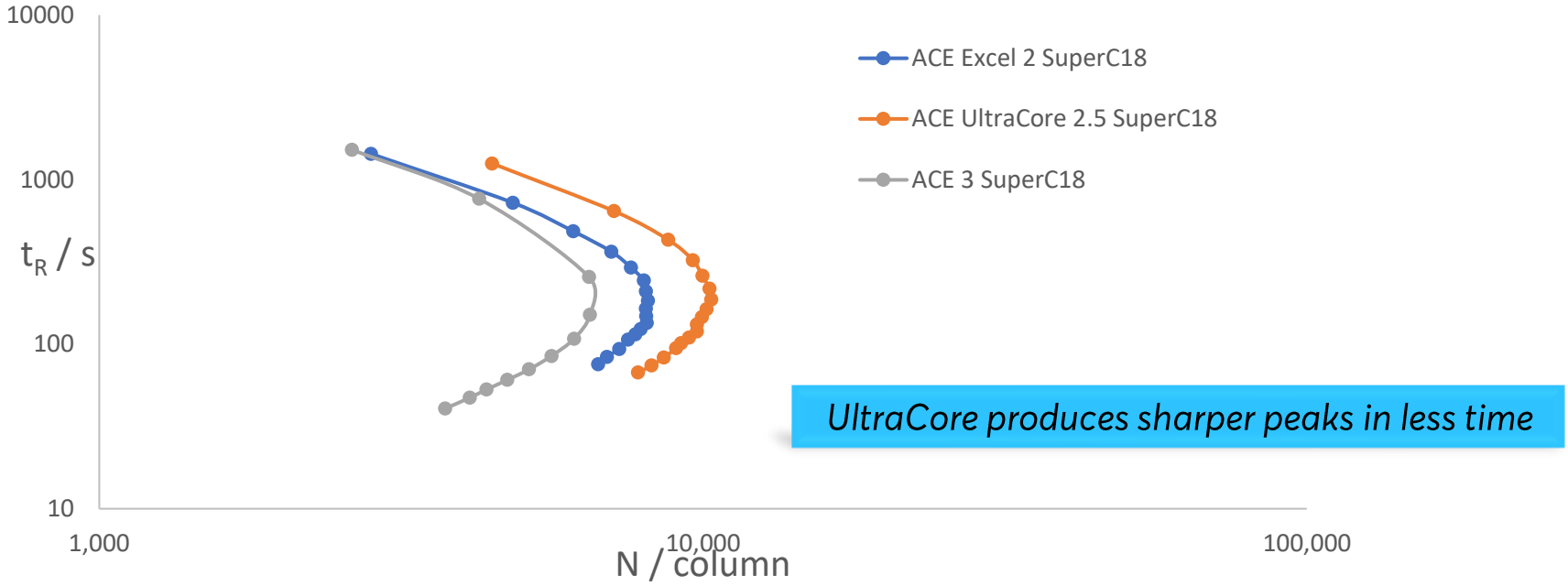
Isocratic analysis, 50x2.1 mm columns,
eluent = MeCN / water + 0.1% TFA, analyte = naproxen, constant k = 10, 40 °C, λ = 256 nm

Limitations of Knox Approach

- 3 parameters
  - A – Eddy diffusion
  - B – Longitudinal diffusion
  - C – Resistance to mass transfer
- Optimization of these parameter will give the best peak shape/efficiency

- However it does not take into account;
  - Analysis time
  - Pressure restrictions on a system

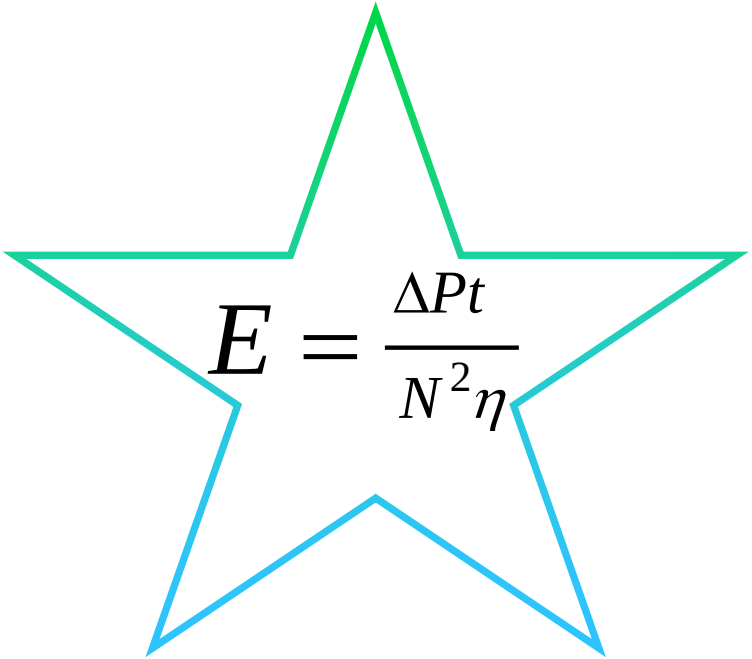
Kinetic Plots – Retention Time



UltraCore produces sharper peaks in less time

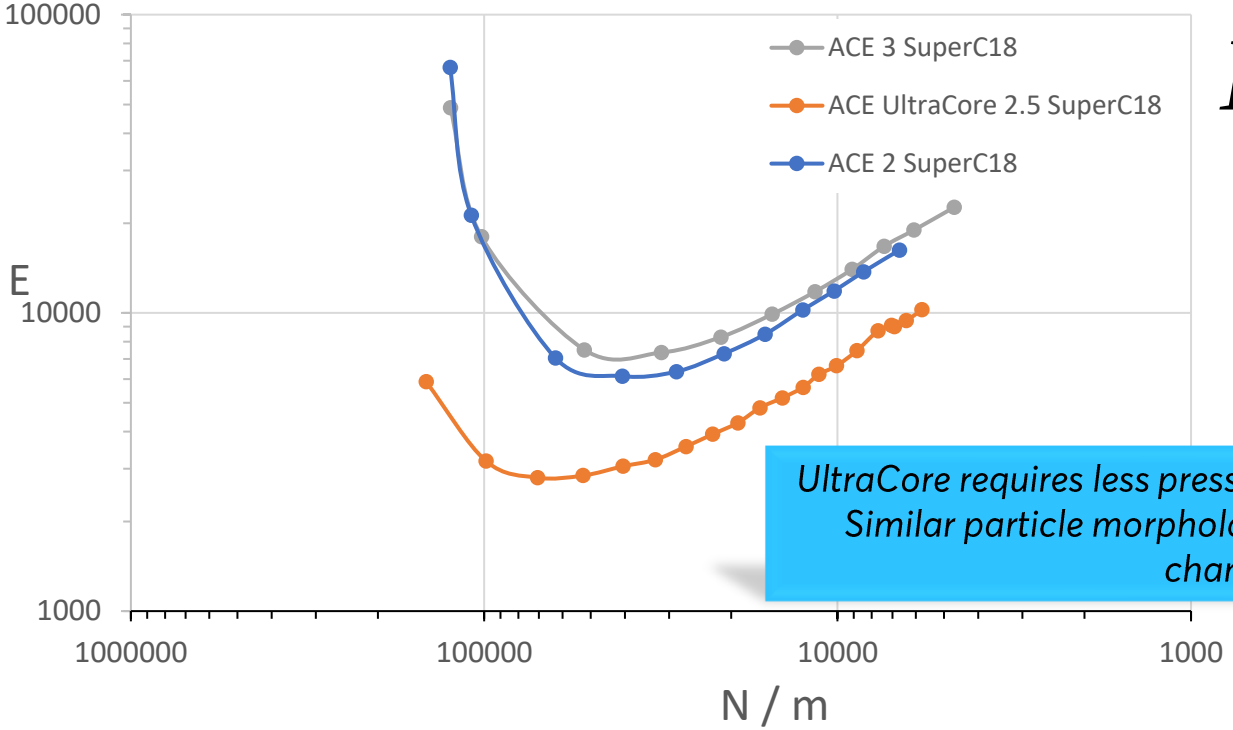
Impedance

- Devised by John Knox (Edinburgh Uni) and Paul Bristow (ICI) in 1977
  - Defines the resistance a compound has to moving down a column relative to the performance of that column
  - Pressure is now considered



- Plotted with a reverse axis
  - Mimics van Deemter plot
  - Minimum value optimum conditions

Kinetic Plots – Impedance

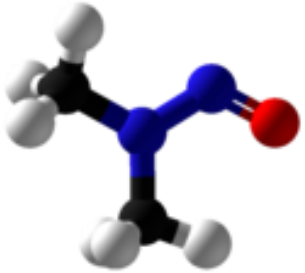


$$E = \frac{\Delta P t_0}{N^2 \eta}$$

UltraCore requires less pressure to obtain < 2 µm performance
Similar particle morphologies have similar performance characteristics

Method Development on UltraCore for Small Molecules

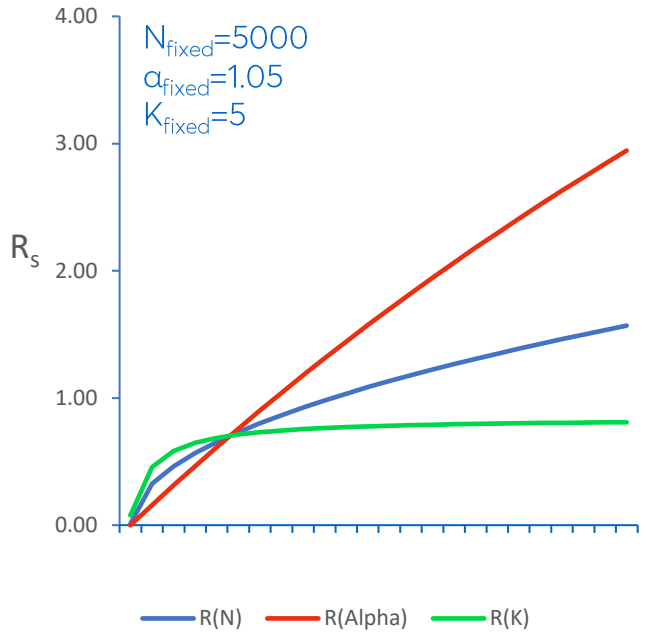
- Pressure
- Column Dimensions / Particle Size / Pore size
- Column Chemistry
- Solvents (type, gradient, modifier etc.)
- Temperature
- pH
- Buffer strength



Resolution, Selectivity, Efficiency & Retention

$$R_s = \frac{\sqrt{N_2}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k_2}{1 + k_2}$$

Selectivity is the key to resolution and efficiency boosts performance



Zhao, J.H. and P.W. Carr. Analytical Chemistry, (1999) 71, 2623-2632

Which Factors[1] Affect Selectivity?

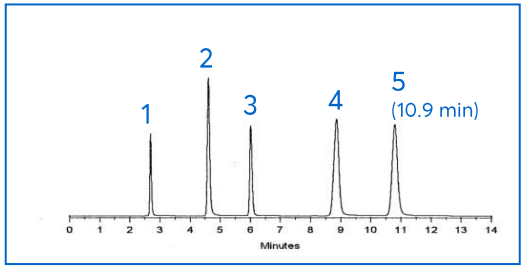
- Strongly influenced by physicochemical properties of the analyte, stationary phase, eluent etc.
- From a practical perspective:

Isocratic Separations	MOST Influence ↑ LEAST Influence	Gradient Separations
– Column stationary phase type – pH (ionisable analytes only) – Organic modifier type – % Organic modifier – Buffer selection – Column temperature – Buffer concentration		– All parameters for isocratic PLUS – Gradient steepness, – k* (t_G, F, V_m, $\Delta\Phi$, M), $$k^* = \frac{t_G F}{\Delta\Phi V_m M}$$ – Dwell volume, – Column dimensions.

1 Adapted from 'Introduction to Modern Liquid Chromatography", 3rd Edition, Snyder, Kirkland, Dolan, 2010, p.29, Wiley & sons

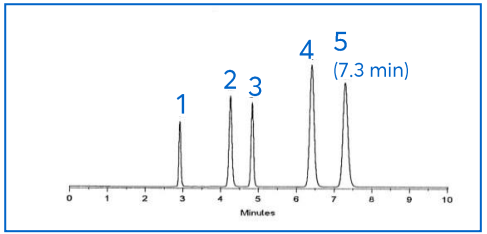
Exploring Selectivity: Porous Silica Bonded Phase Effects

ACE C18 – Increase Retention

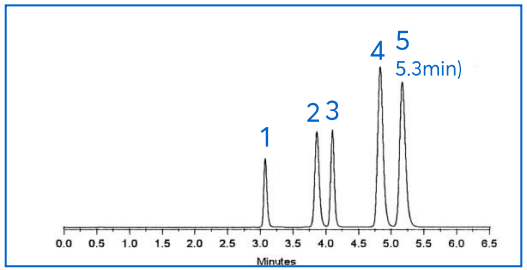


Hydrophobicity Differences

ACE C8 (start point)



ACE C4 – Decrease Retention



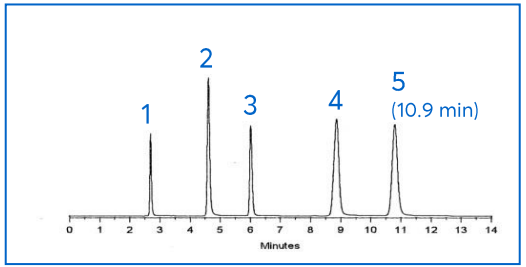
Sample: 1. Norephedrine 2. Nortriptyline 3. Toluene 4. Imipramine 5.Amitriptyline

Column: 250 x 4.6mm 5µm Mobile phase: 80:20 v/v MeOH/25mM KH_2PO_4 (pH6.0) Flow: 1.0mL/min, Wavelength: 215nm

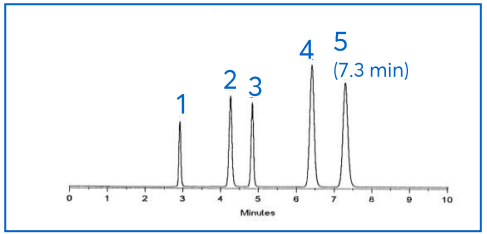
Exploring Selectivity: Porous Silica Bonded Phase Effects

Mechanism Differences

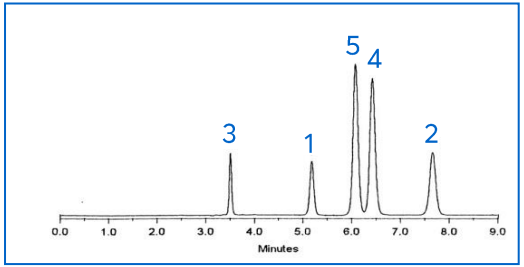
ACE C18 – Increase Retention



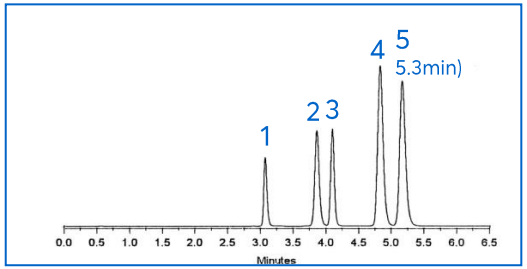
ACE C8 (start point)



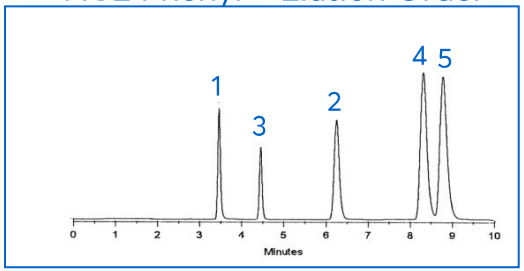
ACE CN – Elution Order



ACE C4 – Decrease Retention



ACE Phenyl – Elution Order



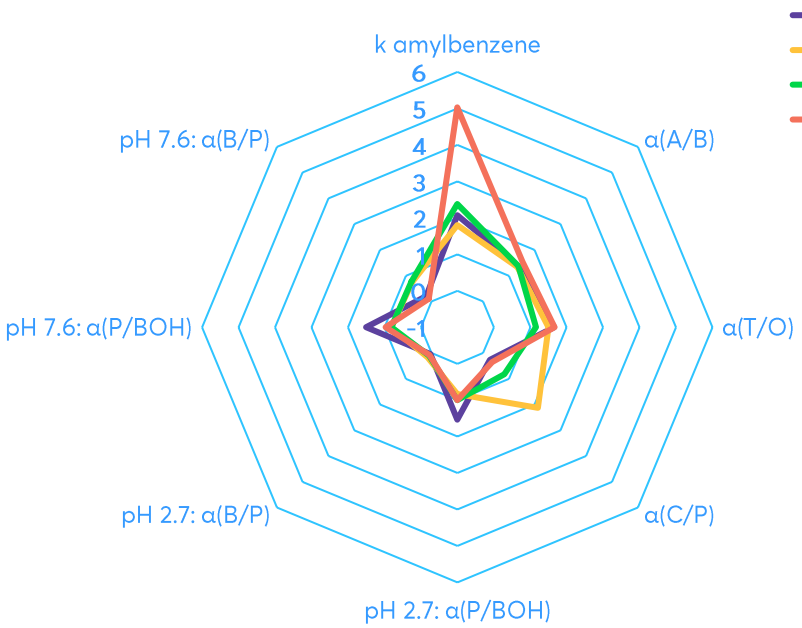
Stationary Phase Is Powerful With Selectivity & Retention

Sample: 1. Norephedrine 2. Nortriptyline 3. Toluene 4. Imipramine 5.Amitriptyline

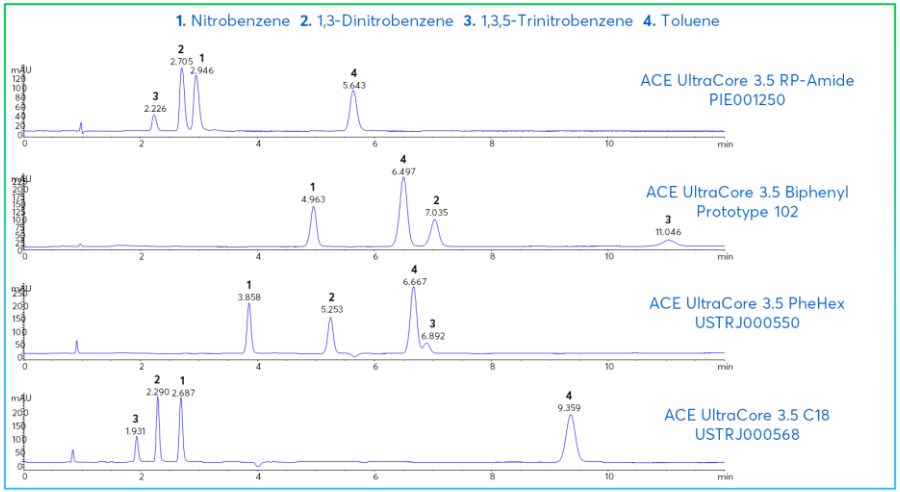
Column: 250 x 4.6mm 5µm Mobile phase: 80:20 v/v MeOH/25mM KH_2PO_4 (pH6.0) Flow: 1.0mL/min, Wavelength: 215nm

Tanaka Characterisation – UltraCore

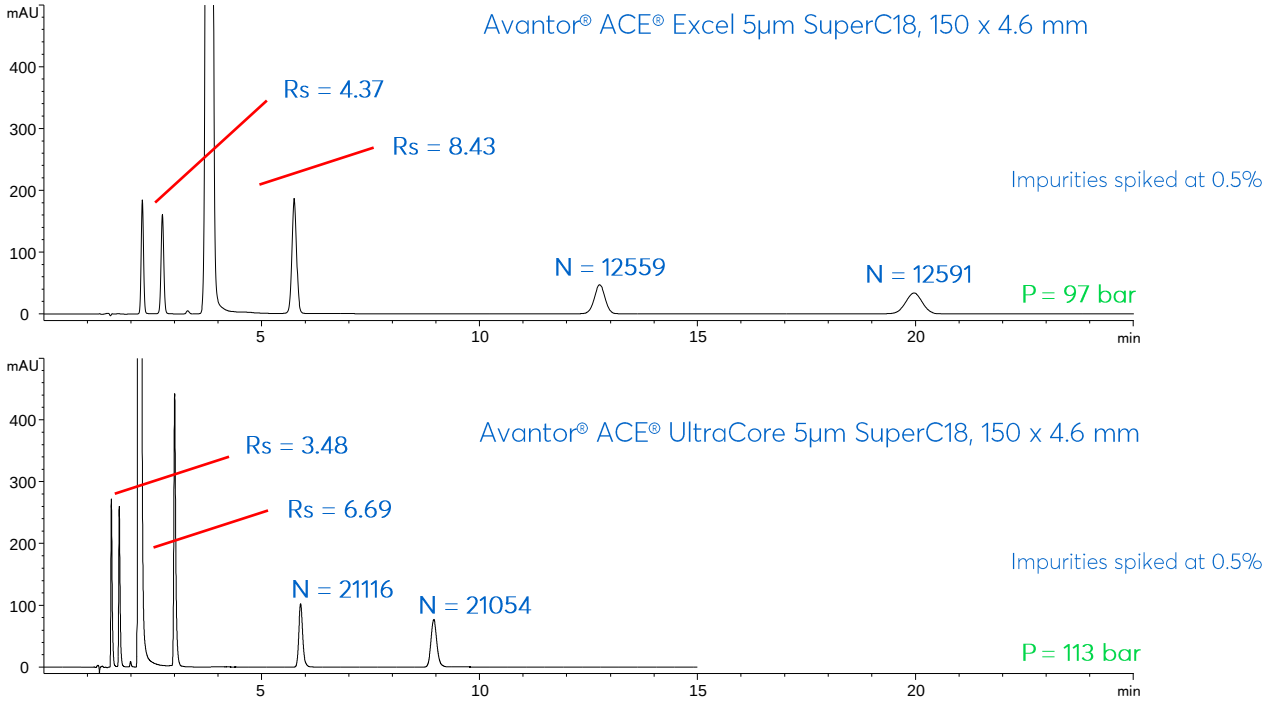
- Performed characterisation work to demonstrate differences in selectivity between the phases
  - Ideal for method development



ACE UltraCore 3.5 RP-Amide
ACE Ultracore 3.5 Biphenyl
ACE UltraCore 3.5 Phenyl-Hexyl
ACE UltraCore 3.5 C18



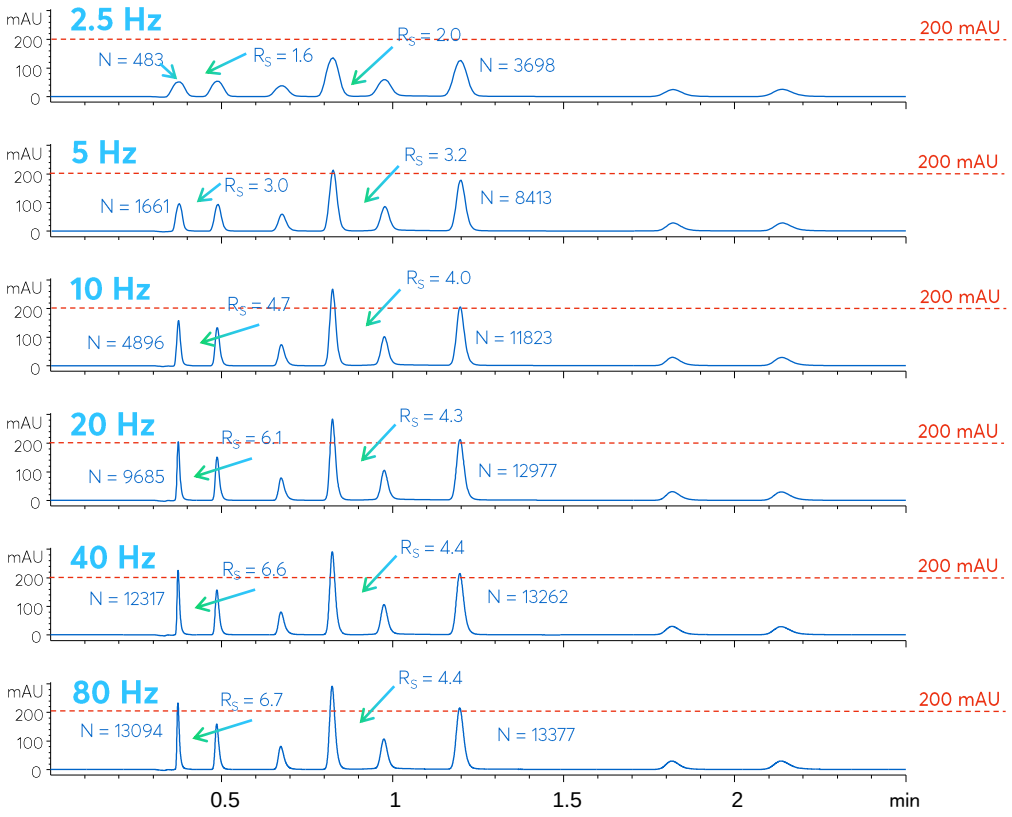
Isocratic Aspirin Analysis: Porous and UltraCore Columns

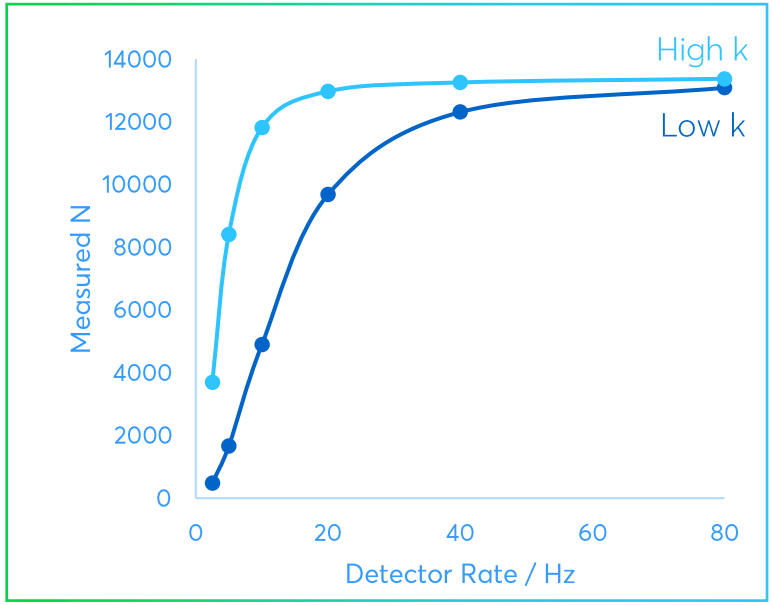


Reduced hydrophobicity of solid core particles leads to 'faster' analysis

Conditions: (Top): 60:35:5:0.2 v/v/v/v water:acetonitrile:methanol:85% phosphoric acid, 237 nm (2.5 Hz), 25°C, 1 mL/min, 5 µL injection
(Bottom): 60:35:5:0.2 v/v water:acetonitrile:methanol:85% phosphoric acid, 237 nm (20 Hz), 25°C, 1 mL/min, 3.9 µL injection

Impact of Detector Rate

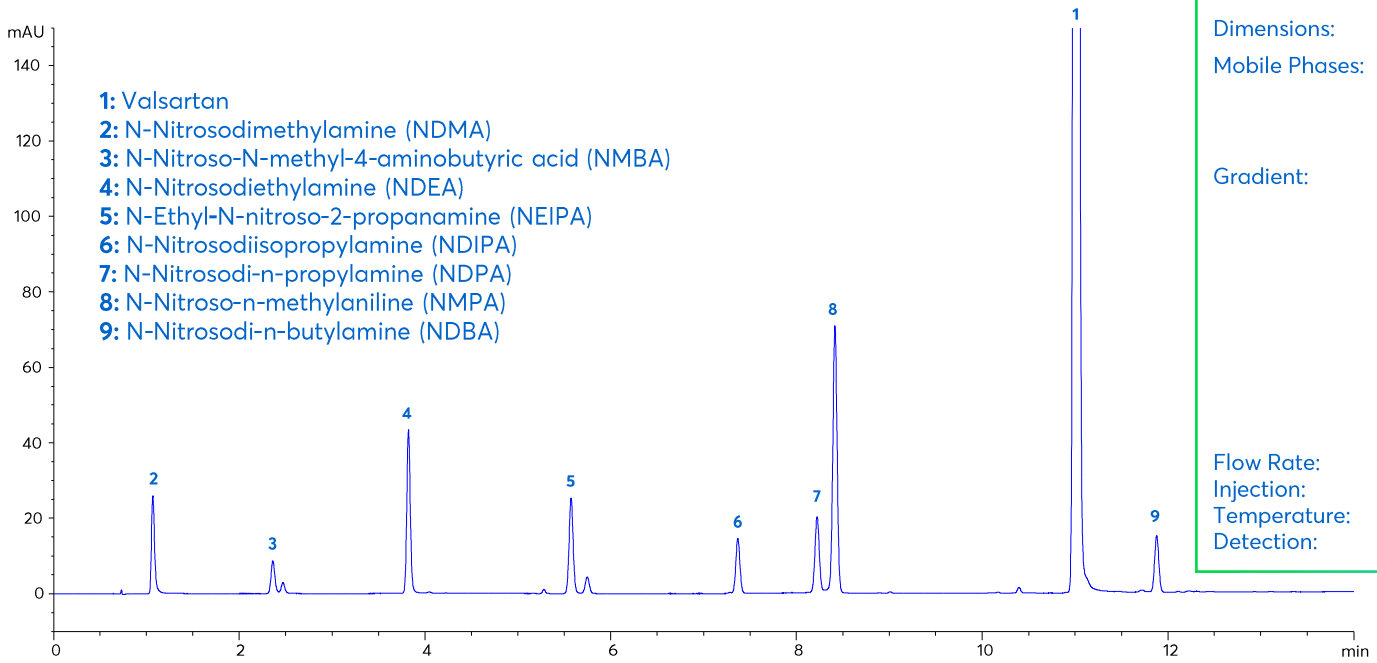




Column: Avantor® ACE® UltraCore 2.5 SuperC18 (75 x 3.0 mm).

Conditions: Isocratic 30 mM KH_2PO_4 pH 2.7 in MeOH:H_2O 40:60 v/v, flow rate: 0.85 mL/min, injection volume: 0.9 µL, column temperature: 30 °C, detector: UV, 214 nm. Sample (in order of elution): 1. maleic acid, 2. norephedrine, 3. doxylamine, 4. salicylamide, 5. guaifenesin, 6. guaiacol, 7. chlorpheniramine, 8. triprolidine.

Nitrosamine Contaminants in Valsartan API



1: Valsartan
2: N-Nitrosodimethylamine (NDMA)
3: N-Nitroso-N-methyl-4-aminobutyric acid (NMBA)
4: N-Nitrosodiethylamine (NDEA)
5: N-Ethyl-N-nitroso-2-propanamine (NEIPA)
6: N-Nitrosodiisopropylamine (NDIPA)
7: N-Nitrosodi-n-propylamine (NDPA)
8: N-Nitroso-n-methylaniline (NMPA)
9: N-Nitrosodi-n-butylamine (NDBA)

CONDITIONS

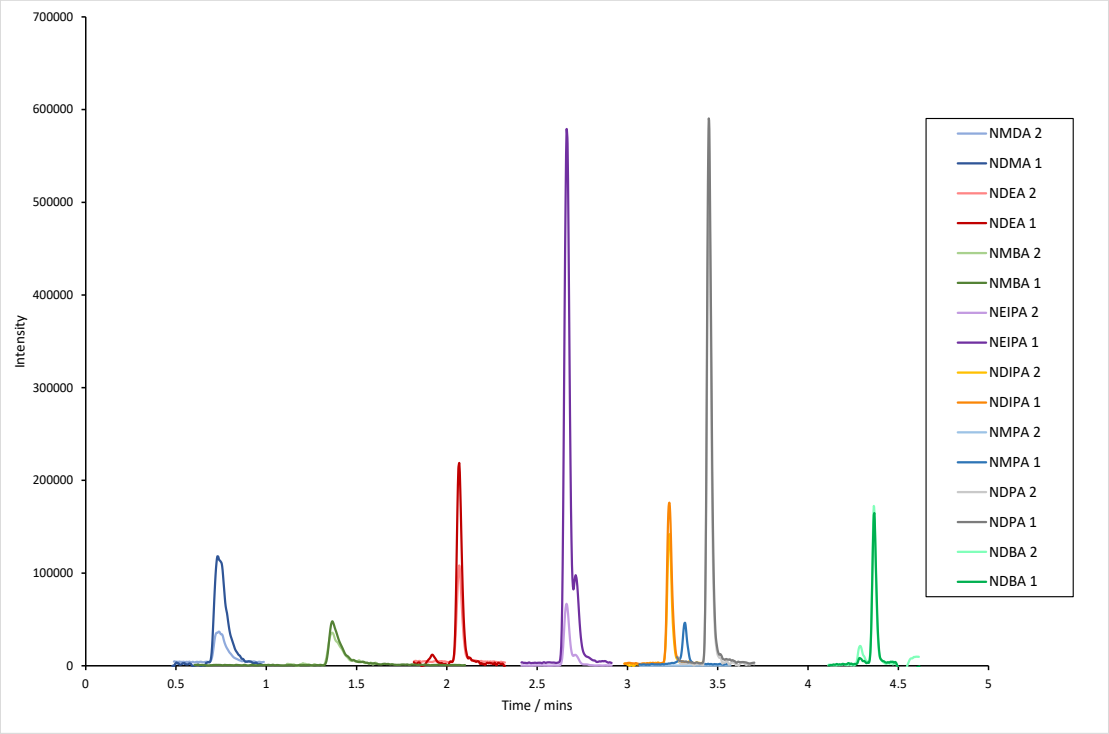
Column:	Avantor® ACE® UltraCore C18
Dimensions:	100 x 3.0 mm
Mobile Phases:	A: 20 mM KH_2PO_4 pH 2.7 in H_2O
	B: 20 mM KH_2PO_4 pH 2.7 in ACN/H_2O 70:30 v/v

Gradient:

Time (mins)	%B
0	4
1	4
15	95
17	95
17.5	4
25	4

Flow Rate:	0.6 mL/min
Injection:	1 µL
Temperature:	20 °C
Detection:	UV, 254 nm

Nitrosamine Contaminants in Valsartan API



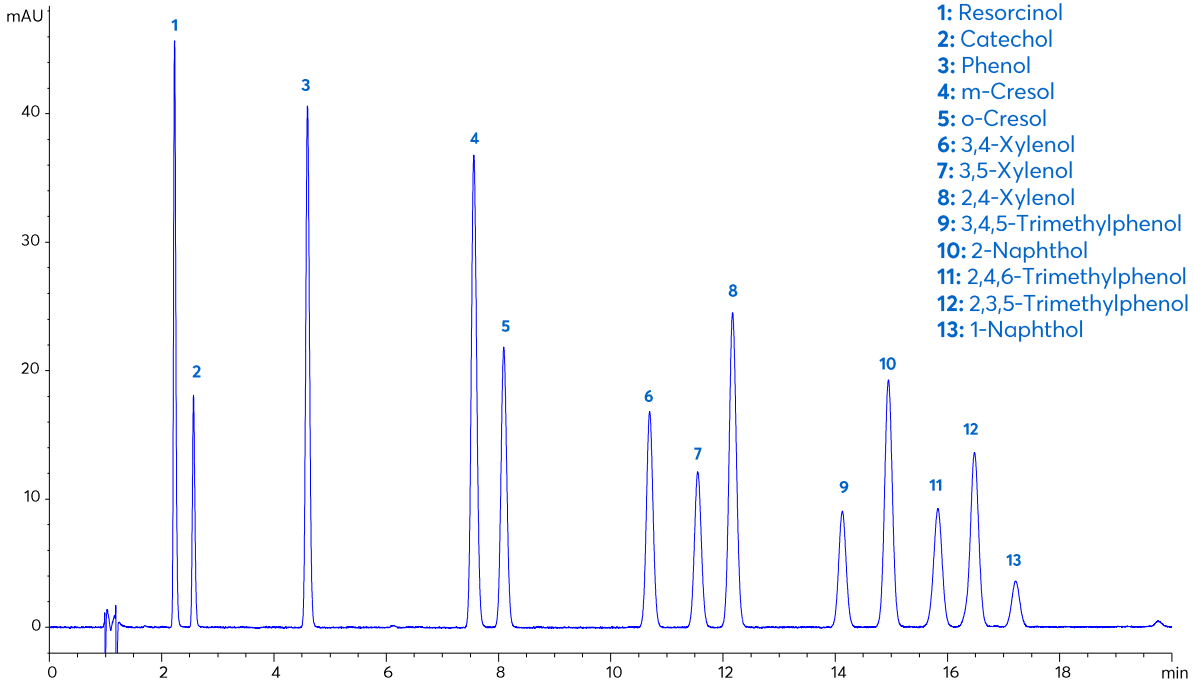
Column: Avantor® ACE® UltraCore C18
Dimensions: 100 x 2.1 mm, 3.5 µm,
Mobile phase: A: 0.1% formic acid in water,
B: 0.1% formic acid in methanol

Gradient:

Time (mins)	%B
0	2.5
0.2	2.5
4.2	80
4.5	80
4.6	2.5
7	2.5

Flow rate: 0.5 mL/min
Temperature: 40 °C
Injection volume: 40 µL

Separation of Phenolic Compounds



1: Resorcinol
2: Catechol
3: Phenol
4: m-Cresol
5: o-Cresol
6: 3,4-Xylenol
7: 3,5-Xylenol
8: 2,4-Xylenol
9: 3,4,5-Trimethylphenol
10: 2-Naphthol
11: 2,4,6-Trimethylphenol
12: 2,3,5-Trimethylphenol
13: 1-Naphthol

CONDITIONS

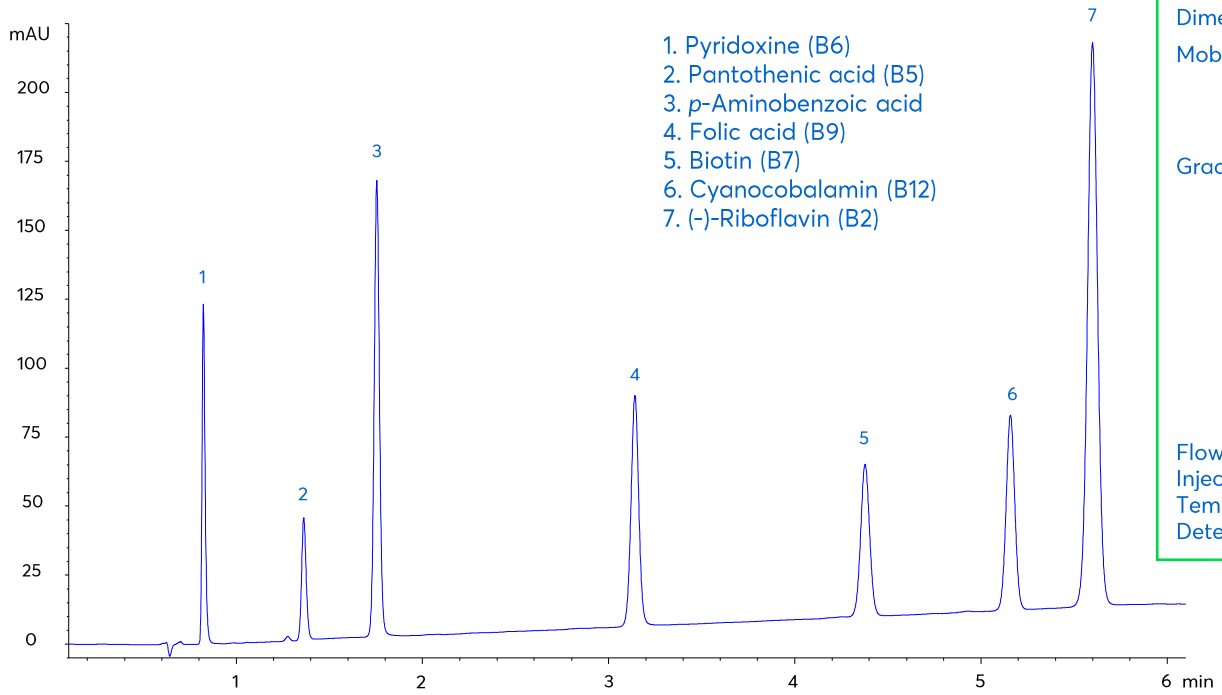
Column:	Avantor® ACE® UltraCore C18-Amide
Dimensions:	100 x 3.0 mm
Mobile Phases:	A: 0.1% Formic acid in H_2O
	B: 0.1% Formic acid in ACN

Gradient:

Time (mins)	%B
0	20
20	40
22	95
24	95
25	20
35	20

Flow Rate:	0.43 mL/min
Injection:	5 µL
Temperature:	40 °C
Detection:	UV, 274 nm

Separation of Water-soluble Vitamins



CONDITIONS

Column:	Avantor® ACE® UltraCore PhenylHexyl
Dimensions:	100 x 3.0 mm
Mobile Phase:	A: 20 mM KH_2PO_4 pH 2.7 in H_2O
	B: 20 mM KH_2PO_4 pH 2.7 in MeCN/H_2O 50:50 v/v

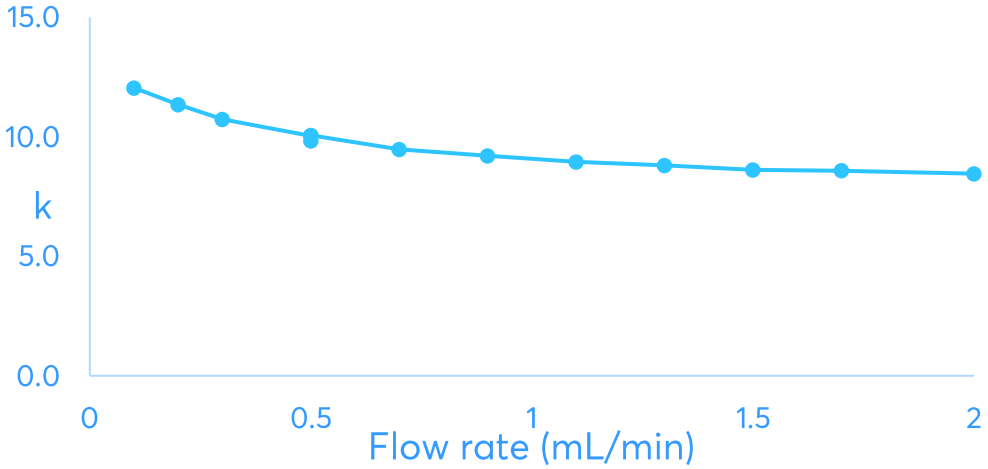
Gradient:

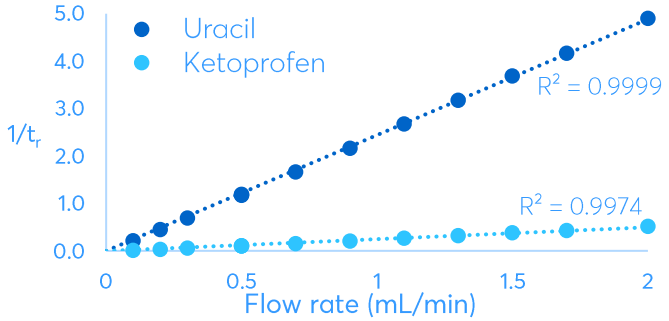
Time (mins)	%B
0	30
5	60
6	60
6.1	30
15	30

Flow Rate:	0.6 mL/min
Injection:	2.8 µL
Temperature:	40 °C
Detection:	UV, 214 nm

Effect of pH on Retention Time

– TFA replaced with formic acid to reduce impact on LC-MS work

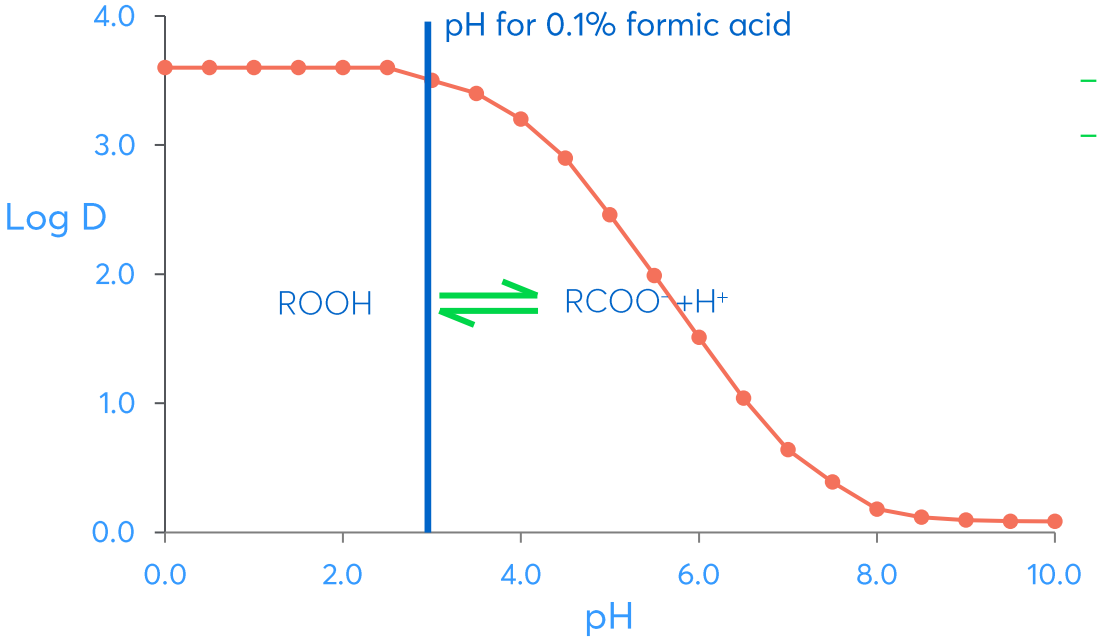




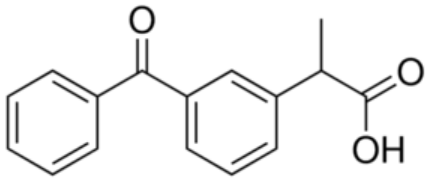
Flow rate mL/min	Accuracy $1/t_0$	Accuracy $1/t_r$
0.1	93%	29%
0.2	99%	86%
0.3	100%	98%
0.4	101%	105%
0.5	99%	101%
0.5	100%	104%
0.7	101%	104%
0.9	101%	103%
1.1	100%	101%
1.3	100%	101%

Isocratic analysis, 50 x 2.1 mm columns, eluent = MeCN / water + 0.1% FA, analyte = ketoprofen, 40°C, λ=256 nm

Effect of pH on Retention Time



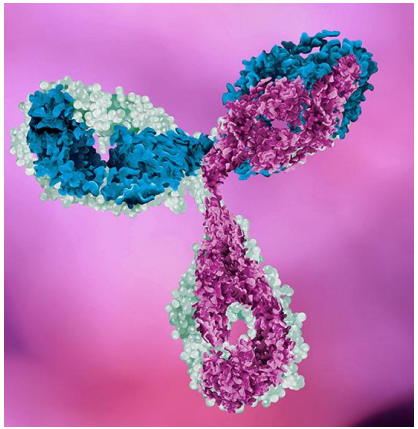
- Flow changed pressure
- Resulted in a shift in the equilibria
  - pK_a for ketoprofen is 3.9
  - Higher flows/pressure produces more ionised form of ketoprofen



Reproduced with kind permission from Chromatography Today March/April 2021

Method Development on UltraCore for Large Molecules

- Pressure
- Column Dimensions / Particle Size / Pore size
- Column Chemistry
- Solvents (type, gradient, modifier etc.)
- Temperature
- pH
- Buffer strength

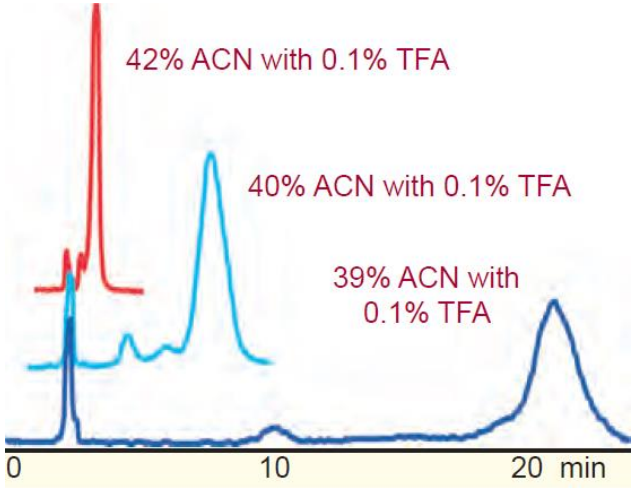


Pore size, column loading and adsorption kinetics are important considerations for large molecule separations

Image with kind permission from LC-GC "U/HPLC and LC-MS workflow solutions for biopharmaceutical analysis by T. Edge"

Challenges with RPLC of Large Molecules

- Isocratic conditions rarely used
  - Proteins and degradants can have large spans of *k*
- However - RPLC gradients can be problematic
  - Potential for denaturing of the proteins causing irreversible adsorption
- Lack of understanding on the retention mechanism due to multiple points of interaction and synergistic effects
- Longer chain lengths (C8, C18) often give lower recovery than shorter ones (C4)
  - Carryover



Separation of lysozyme with small changes in acetonitrile concentration under isocratic conditions – David Carr *A guide to the analysis and purification of proteins and peptides by reversed phase HPLC* – ACE HPLC primer

General Approach to RPLC Large Molecule Analysis

- Organic solvent: Acetonitrile or isopropanol (higher elution strength)

- Column length:
  - Protein elution largely governed by strength of 'hydrophobic foot' and amount of organic required to initiate elution
  - Column length less important with proteins than peptides

- Temperature:
  - ≥60 °C to improve chromatographic efficiency
  - Proteins can start to denature at these temps. (eluting in organic solvent also causes denaturing)

Considerations with RPLC Large Molecule Analysis

- Biomolecules can stick to stationary phases and column frits
  - Decreased recovery, changes in selectivity and potentially increases in column backpressure
- Column conditioning is often required to stabilise surface
- Occasional cleaning is prudent
  - If strong ionic interactions suspected – clean with a denaturing solution e.g. 6 M guanidine hydrochloride or 10% aqueous DMSO
  - For hydrophobic proteins, flush buffer from column (95-100% water) followed by 5-95% aqueous acetonitrile gradients
  - Backflushing column can help remove debris from frits, but beware of impact that this will have on bed of column and baseline profile

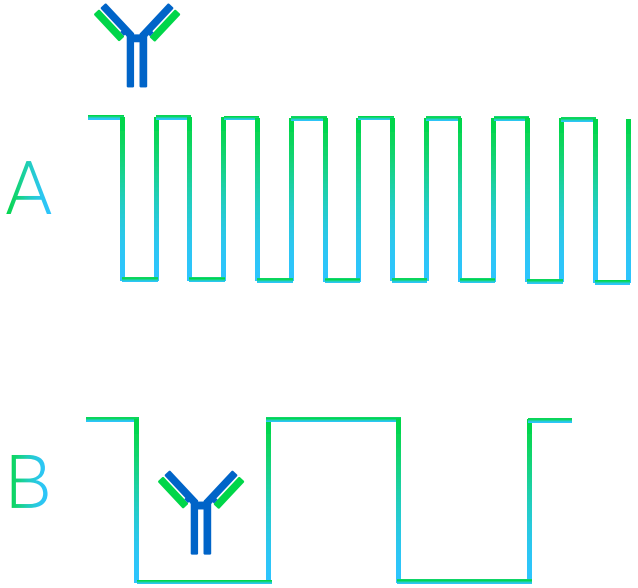
Stationary Phase Properties - Pore Size

Pore size is critical for protein analysis

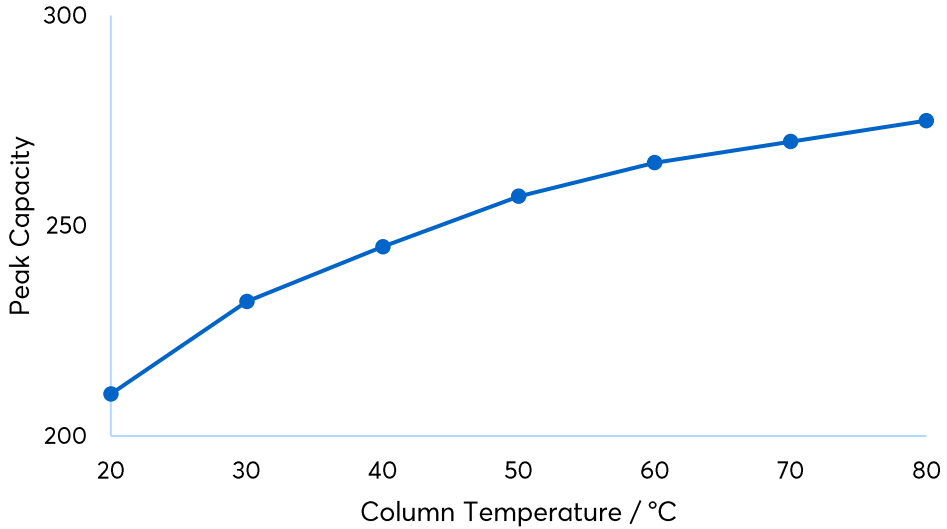
Use wide pore stationary phases (300, 500 Å)

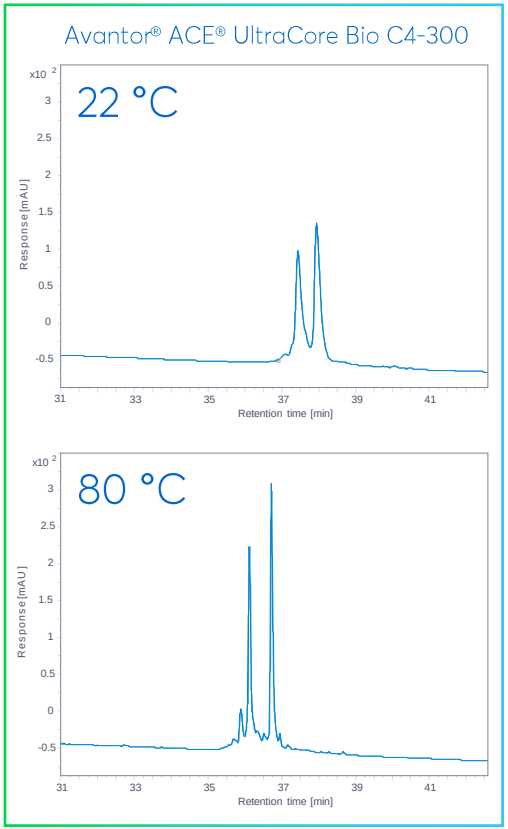
Pore diameter:

- Defines the average diameter of pore
- Affects ability of analyte molecules to penetrate inside the particle and interact with its inner surface
  - Affects the surface area
  - Greater surface area gives higher retention due to increased number of potential retention sites



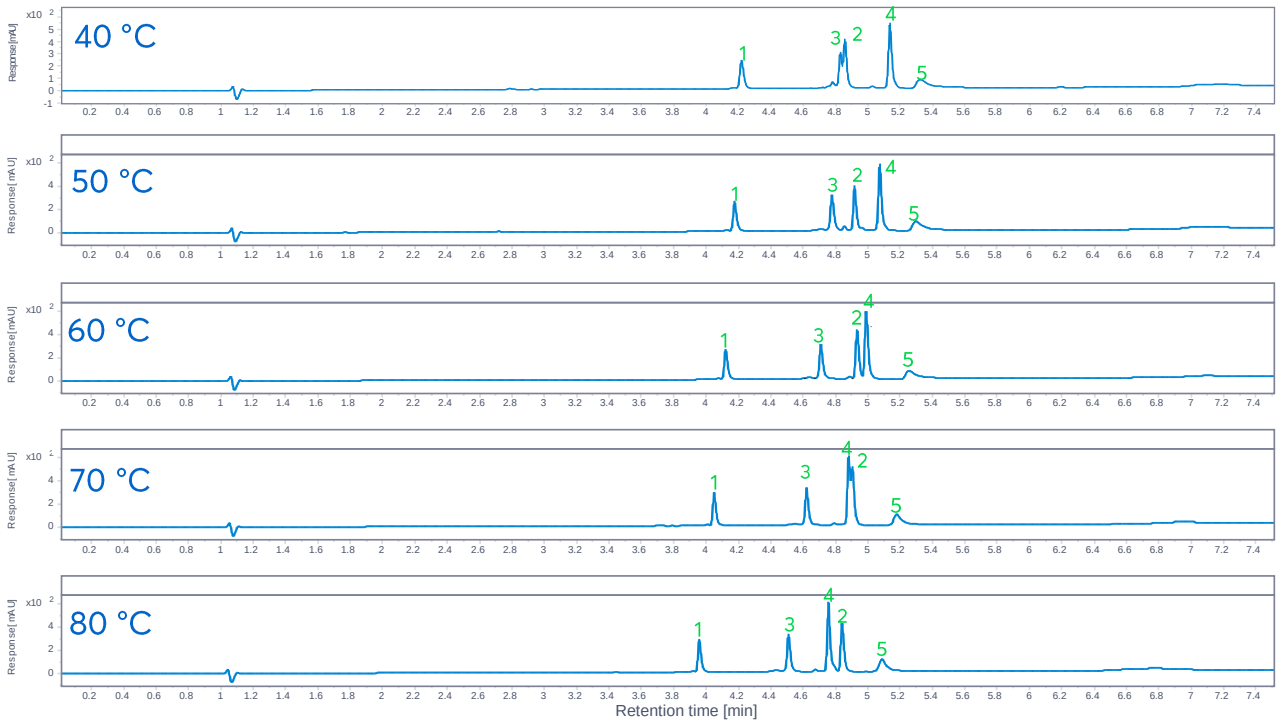
Effect of Column Temperature on Peak Capacity





Highest peak capacity at high column temperature (higher D_m and D_s)
Reduced viscosity leads to lower column pressures

Effect of Temperature



CONDITIONS

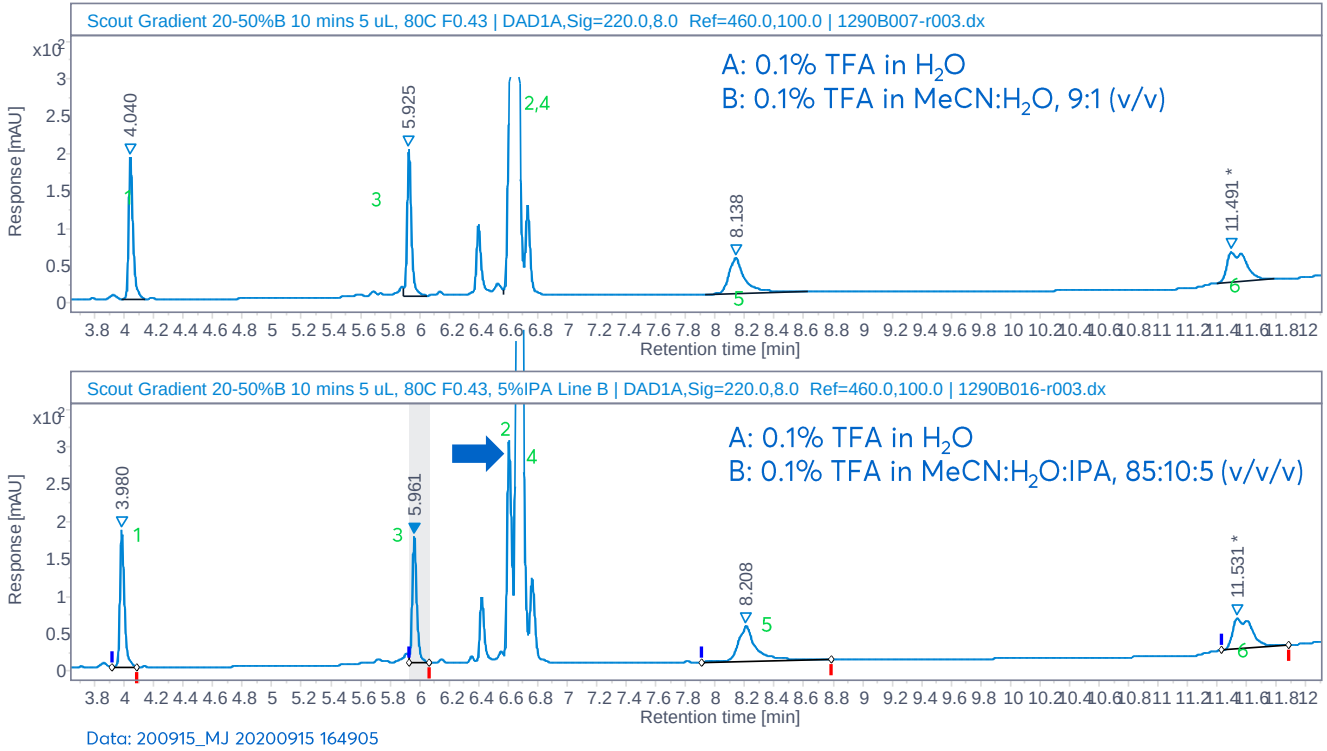
Column: Avantor® ACE® UltraCore Bio C4-500
Dimensions 100 x 3.0 mm
Mobile phase:
A1: 0.1% TFA in H_2O
B1: 0.1% TFA in ACN/H_2O 90:10)

Time (mins)	%B
0	5
10	100
12	100
12.5	5
32.5	5

Flow rate: 0.43 mL/min
Temperature: 40, 50, 60, 70, 80 °C
Injection volume: 5 µL

1 Ribonuclease A (13.7 kDa)
2 Insulin (human - 5.7 kDa)
3 Cytochrome C (12.3 kDa)
4 Lysozyme (14.3 kDa)
5 BSA (66.5 kDa)

Effect of adding 5% IPA to line B



Data: 200915_MJ 20200915 164905

CONDITIONS

Column: Avantor® ACE® UltraCore Bio C4-500
Dimensions 100 x 3.0 mm

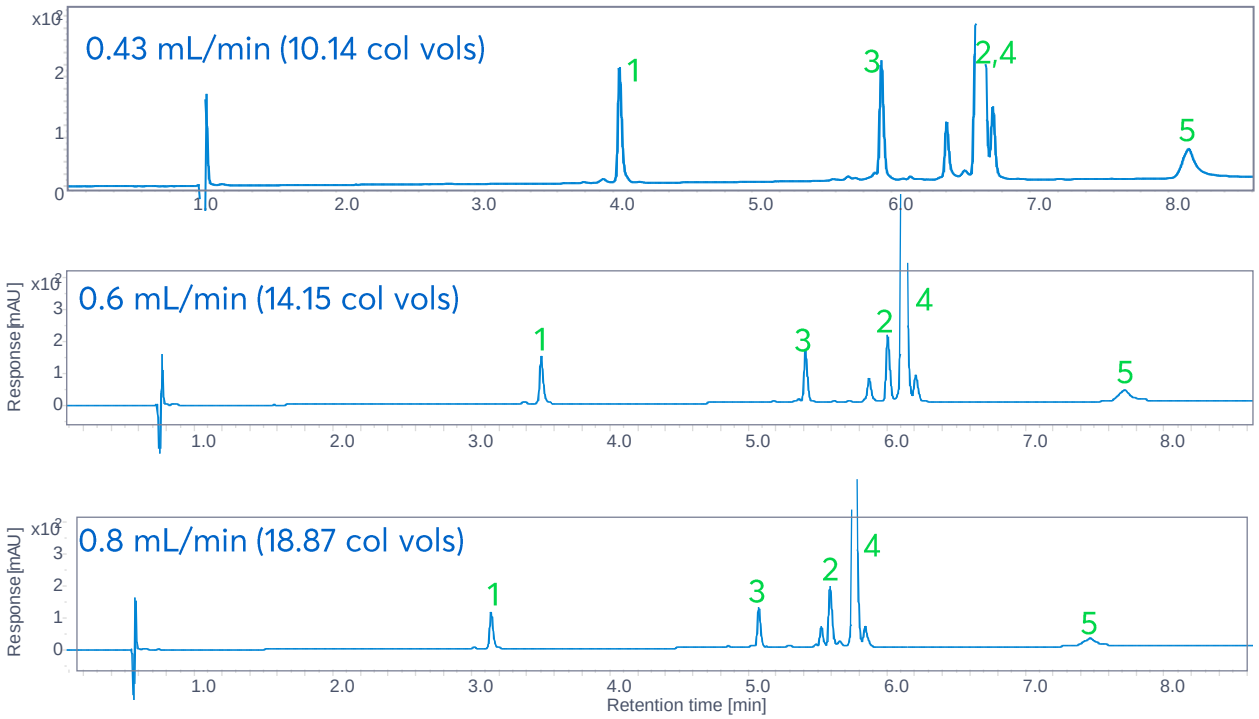
Time (mins)	%B
0	20
10	50
12	100
12.5	20
23	20

Flow rate: 0.43 mL/min
Temperature: 80 °C
Injection volume: 5 µL

1 Ribonuclease A (13.7 kDa)
2 Insulin (human - 5.7 kDa)
3 Cytochrome C (12.3 kDa)
4 Lysozyme (14.3 kDa)
5 BSA (66.5 kDa)
6 Thyroglobulin (660 kDa)

Slightly longer retention, small selectivity change

Shallow gradient @80 °C – Effect of flow



CONDITIONS

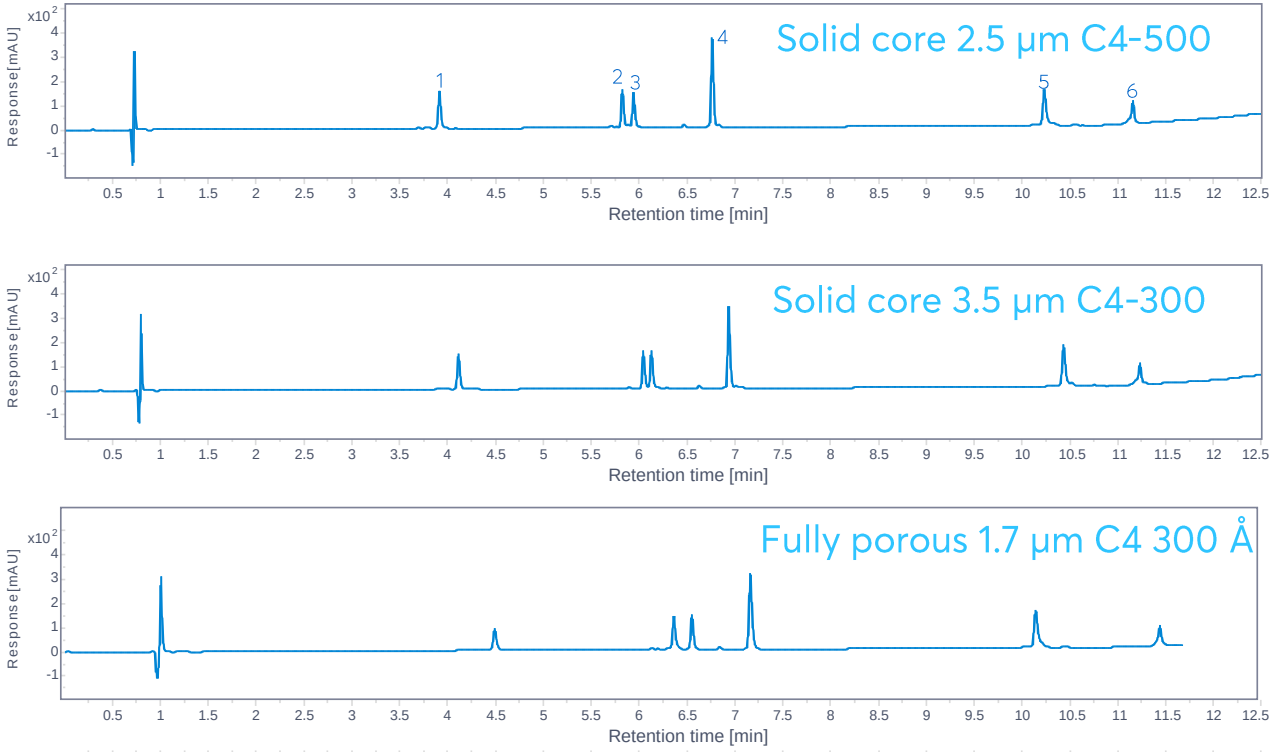
Column: Avantor® ACE® UltraCore Bio C4-500
Dimensions 100 x 3.0 mm
Mobile phase:
A1: 0.1% TFA in H_2O
B1: 0.1% TFA in ACN/H_2O 90:10)

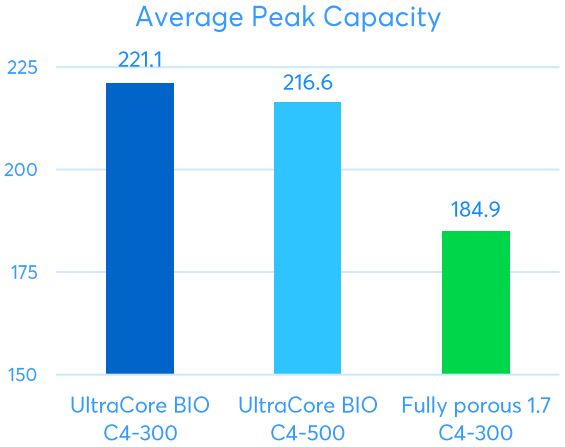
Time (mins)	%B
0	20
10	50
12	50
12.5	20
13.5	20

Temperature: 80 °C
Injection volume: 5 µL

1 Ribonuclease A (13.7 kDa)
2 Insulin (human - 5.7 kDa)
3 Cytochrome C (12.3 kDa)
4 Lysozyme (14.3 kDa)
5 BSA (66.5 kDa)
6 Thyroglobulin (660 kDa)

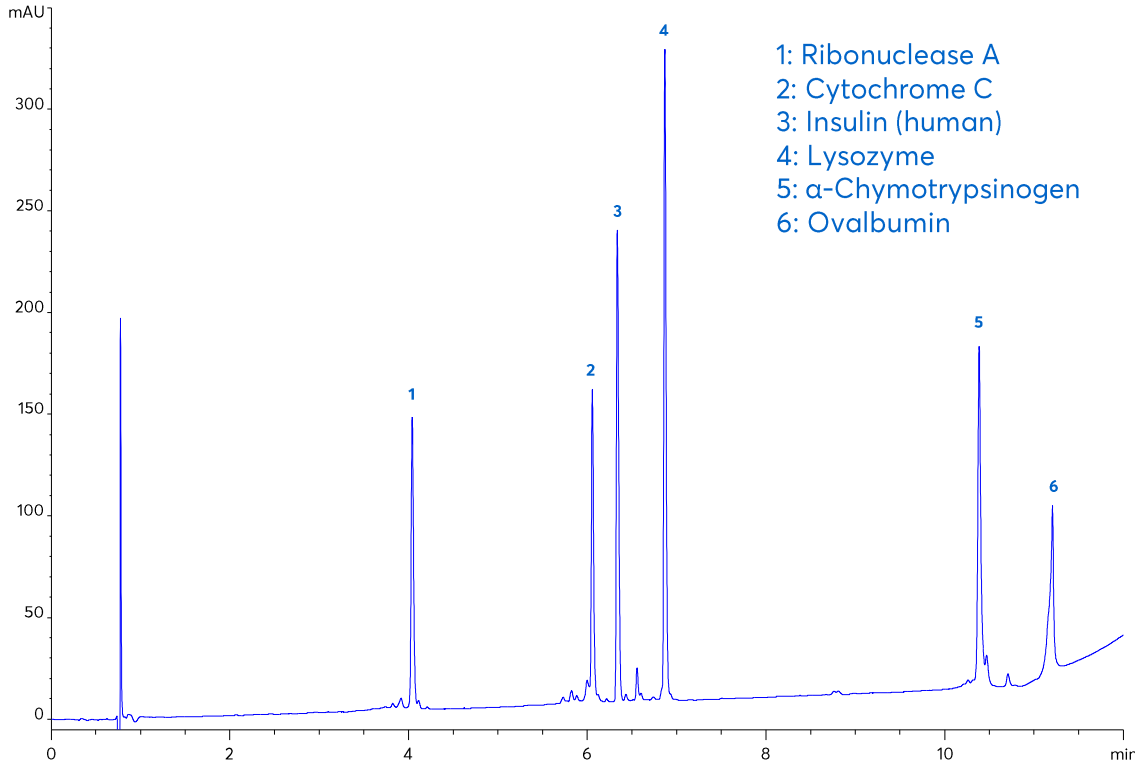
Do Solid Core Particles Provide Improved Peak Capacity?





1: Ribonuclease A
2: Cytochrome C
3: Insulin (bovine)
4: Lysozyme
5: α-Chymotrypsinogen
6: Ovalbumin

Separation of Peptides and Proteins



1: Ribonuclease A
2: Cytochrome C
3: Insulin (human)
4: Lysozyme
5: α-Chymotrypsinogen
6: Ovalbumin

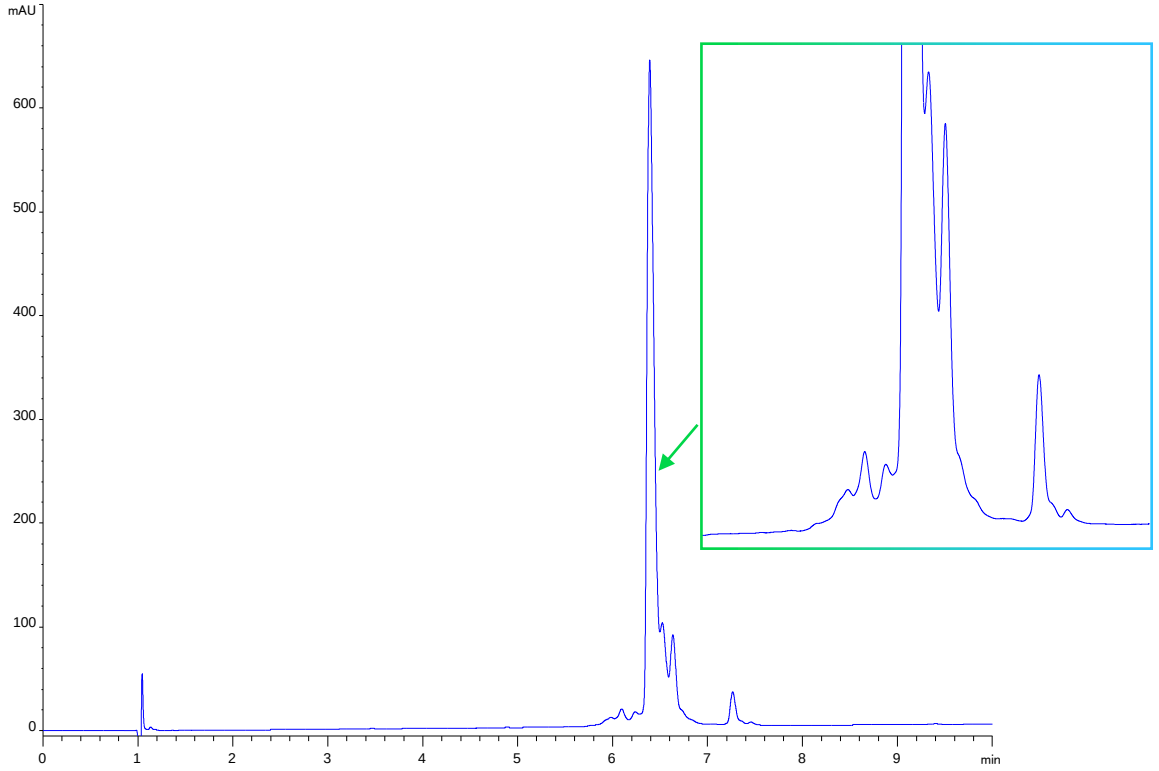
CONDITIONS

Column:	Avantor® ACE® UltraCore Bio C4-500
Dimensions:	100 x 3.0 mm
Mobile Phases:	A: 0.1% TFA in H_2O B: 0.1% TFA in ACN/H_2O 90:10 v/v
Gradient:	

Time (mins)	%B
0	20
10	50
12	100
12.5	20

Flow Rate:	0.6 mL/min
Injection:	5 µL
Temperature:	60 °C
Detection:	UV, 220 nm

Analysis of α-Chymotrypsinogen



CONDITIONS

Column: Avantor® ACE® UltraCore Bio C4-500

Dimensions: 100 x 3.0 mm

Mobile Phases: A: 0.1% TFA in H_2O
B: 0.1% TFA in ACN/H_2O 90:10 v/v

Gradient:

Time (mins)	%B
0	40
10	50
12	100
13	100
13.5	40

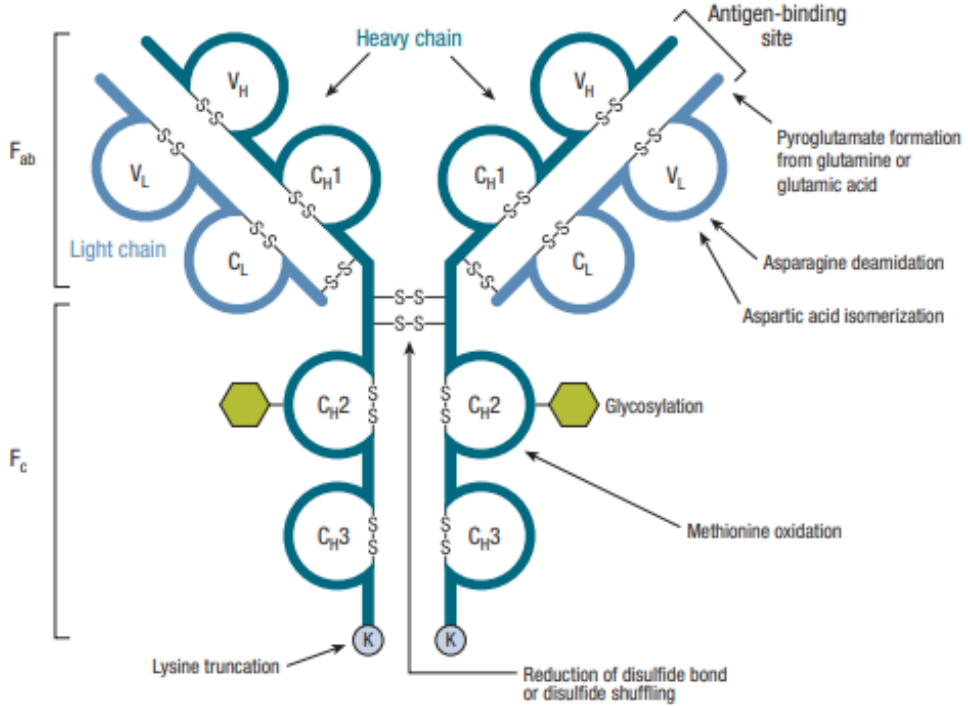
Flow Rate: 0.43 mL/min

Injection: 5 µL

Temperature: 80 °C

Detection: UV, 220 nm

Structure of mAb



V - Variable region

C - Constant region

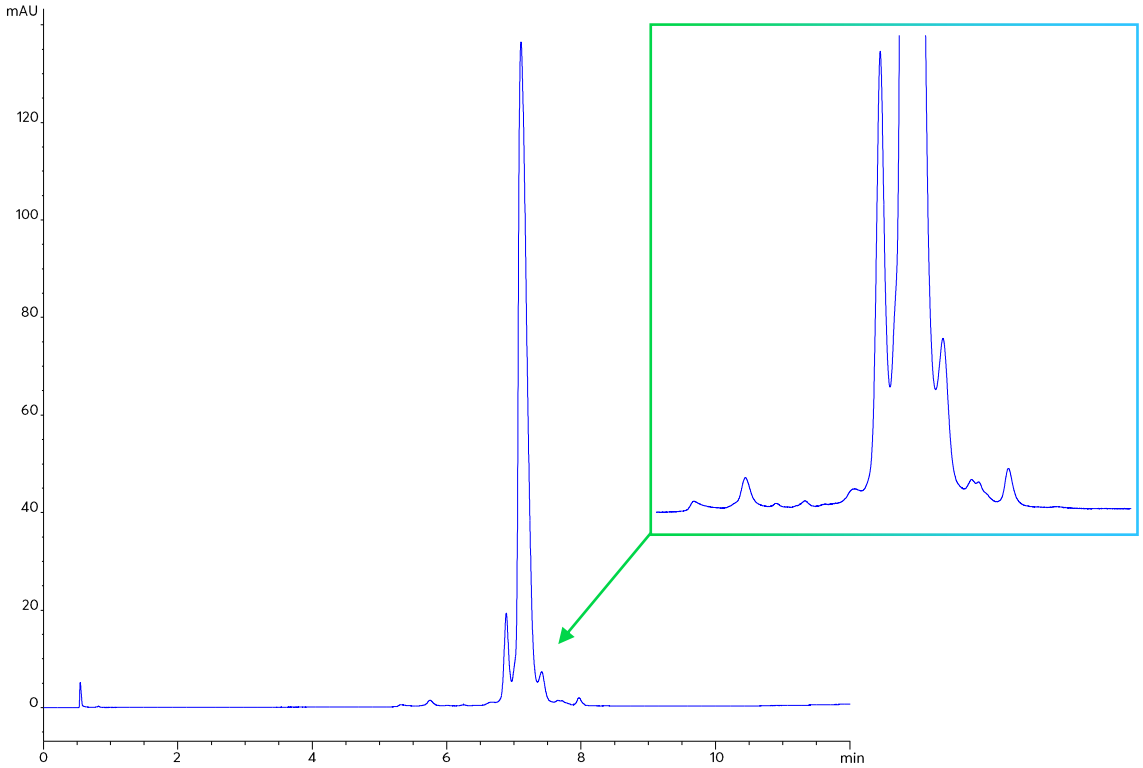
S-S – Disulfide bridges

Fab – Antigen binding fragment

Fc – Crystalline fraction

With kind permission from LC-GC "U/HPLC and LC-MS workflow solutions for biopharmaceutical analysis by T. Edge"

NIST mAb



CONDITIONS

Column: Avantor® ACE® UltraCore BIO C4-500

Dimensions: 100 x 3.0 mm

Mobile Phases: A: 0.1% TFA in H_2O

B: 0.1% TFA in ACN/H_2O 9:1 v/v

Gradient:

Time (mins)	%B
0	36
10	45
12	80
14	80
14.5	36

Flow Rate: 0.8 mL/min

Injection: 1 µL

Temperature: 80 °C

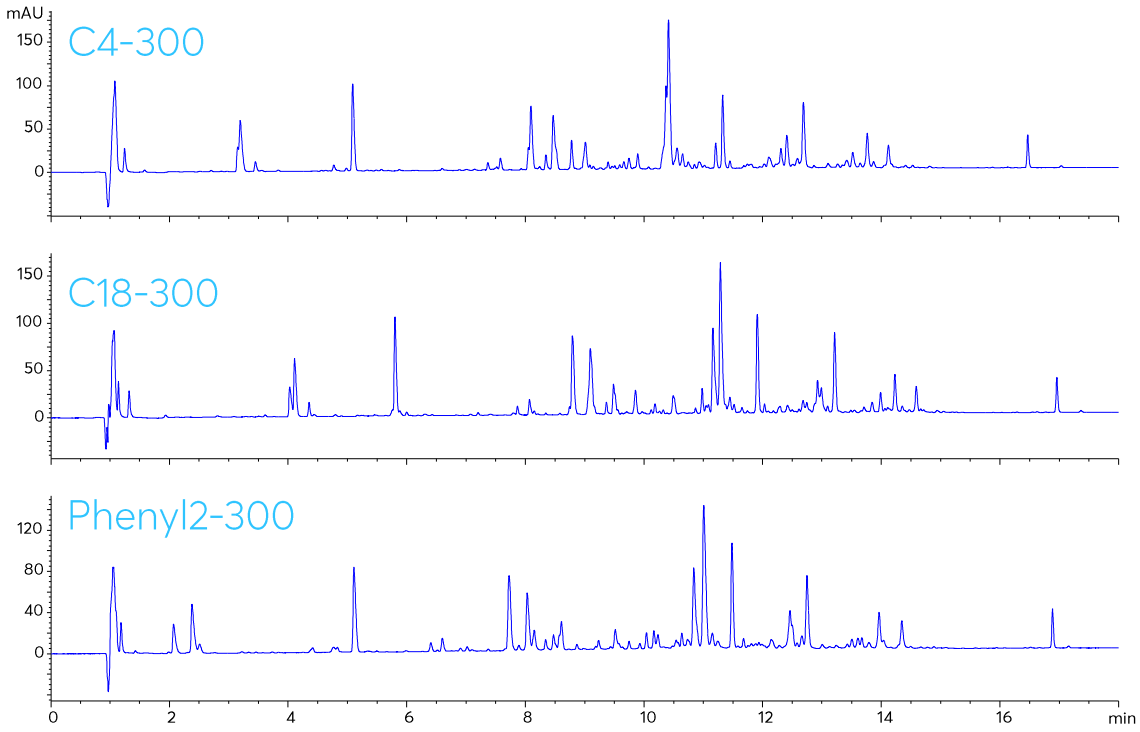
Detection: UV, 280 nm

Bottom Up Approach – Sample Pre-treatment

5 well defined stages to ensure optimal peptides produced

- Denaturing the protein
  - Addition of a chaotropic agents or rise in temperature.
- Reduction
  - Dithiothreitol (DTT) is commonly used to reduce disulfide bonds between cysteine residues.
- Alkylation
  - Alkylating cysteine residues prevents formation of disulfide bonds, ensures protein remains unfolded.
- Desalting
  - Salts and other reagents that may denature the enzyme need to be removed or diluted to ensure successful digestion.
- Digestion
  - Typically trypsin is added, however other reagents are also used

Tryptic Digest of Lysozyme



CONDITIONS

Columns: Avantor® ACE® UltraCore Bio C4-300
Avantor® ACE® UltraCore Bio C18-300
Avantor® ACE® UltraCore Bio Phenyl2-300

Dimensions: 100 x 3.0 mm

Mobile Phases:

A: 0.1% TFA in H_2O
B: 0.1% TFA in ACN

Gradient:

Time (mins)	%B
0	5
20	45
21	95
23	95
23.5	5
33.5	5

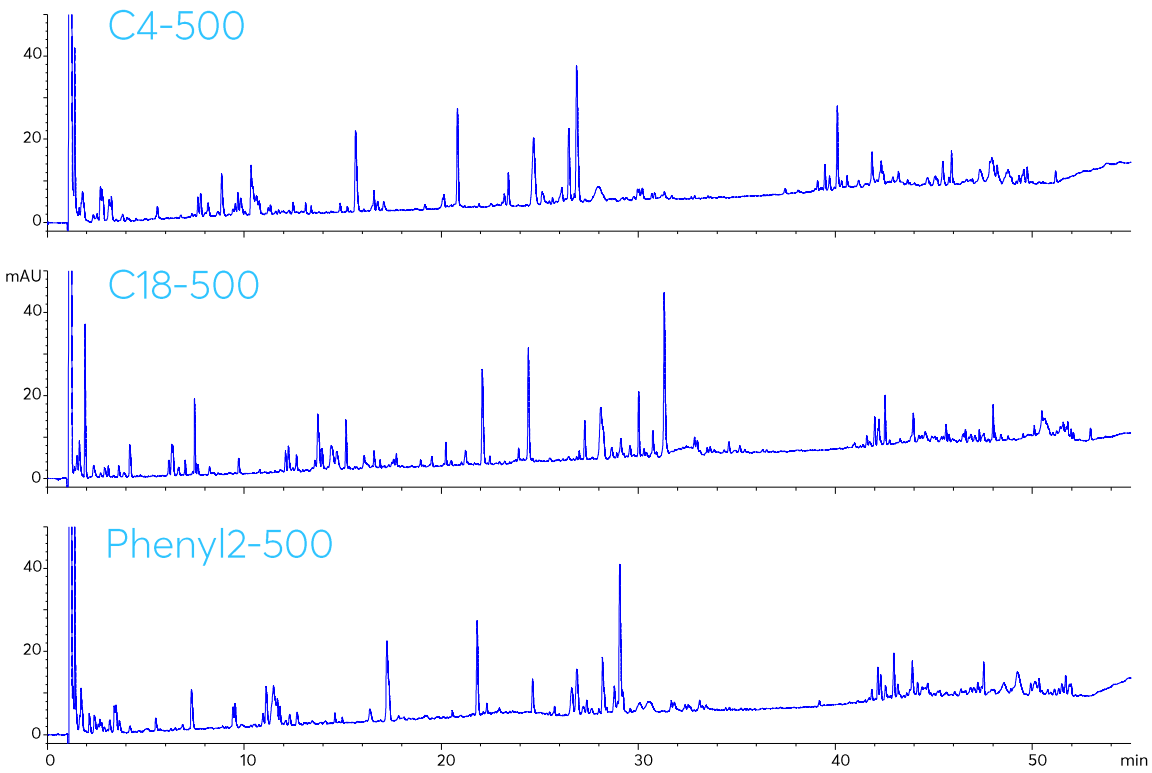
Flow Rate: 0.43 mL/min

Injection: 10 µL

Temperature: 60 °C

Detection: UV, 214 nm

Tryptic Digest of IgG



CONDITIONS

Columns: Avantor® ACE® UltraCore Bio C18-500
Avantor® ACE® UltraCore Bio C4-500
Avantor® ACE® UltraCore Bio Phenyl2-500

Dimensions: 100 x 3.0 mm

Mobile Phases:

A: 0.1% TFA in H_2O
B: 0.1% TFA in ACN

Gradient:

Time (mins)	%B
0	2
60	40
61	95
64	95
65	2
75	2

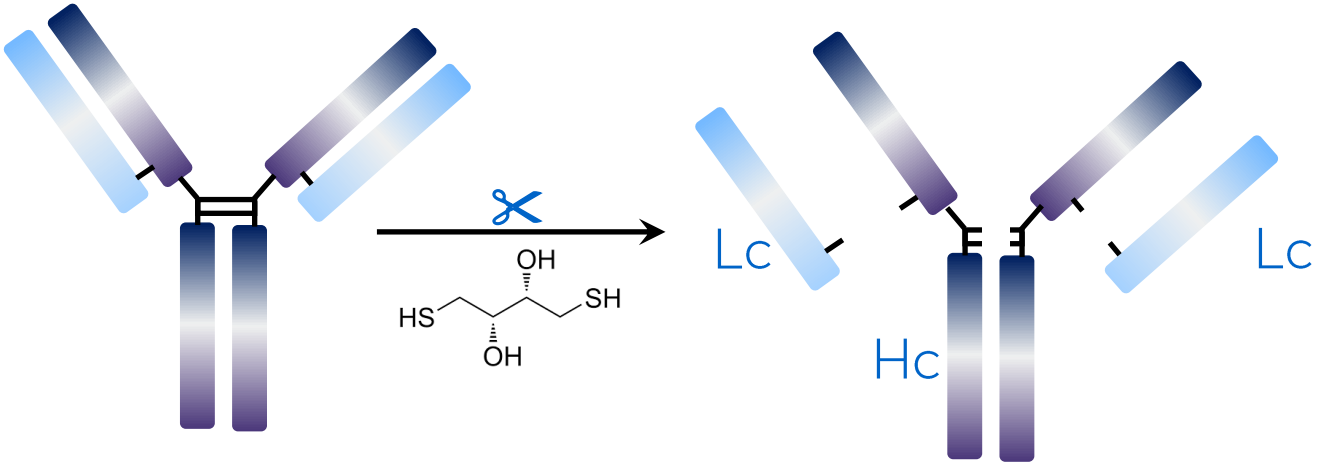
Flow Rate: 0.43 mL/min

Injection: 20 µL

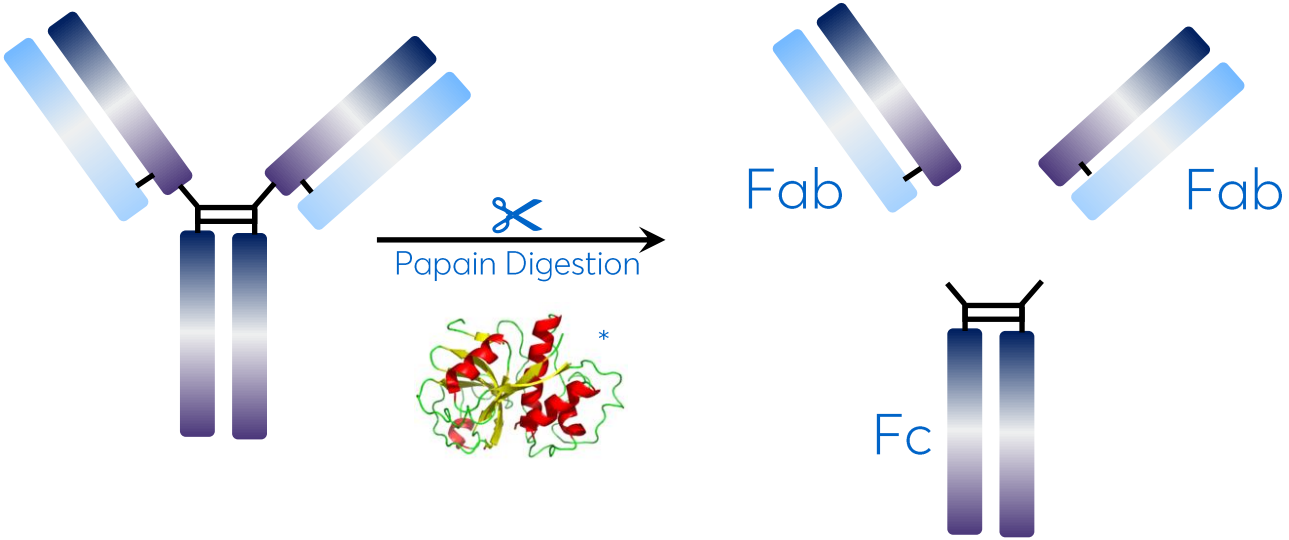
Temperature: 60 °C

Detection: UV, 214 nm

mAb Fragments – Reduction of Disulfide Bridges by DTT (Dithiothreitol)

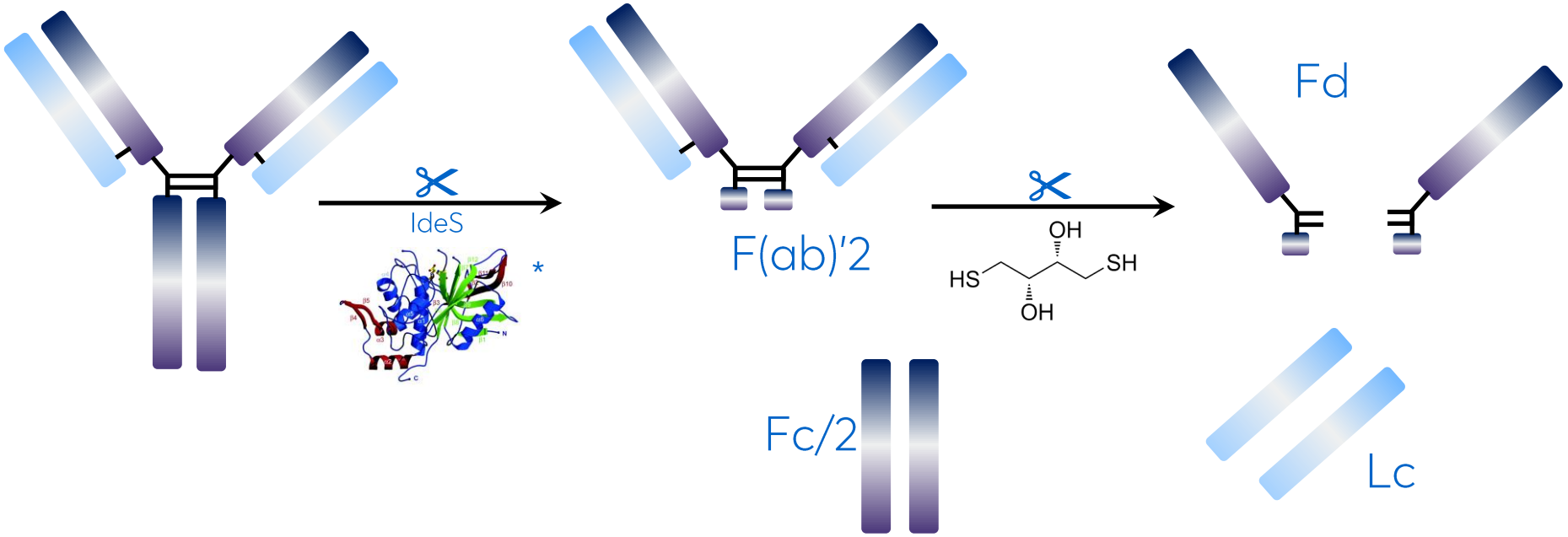


mAb Fragments – Papain Digestion



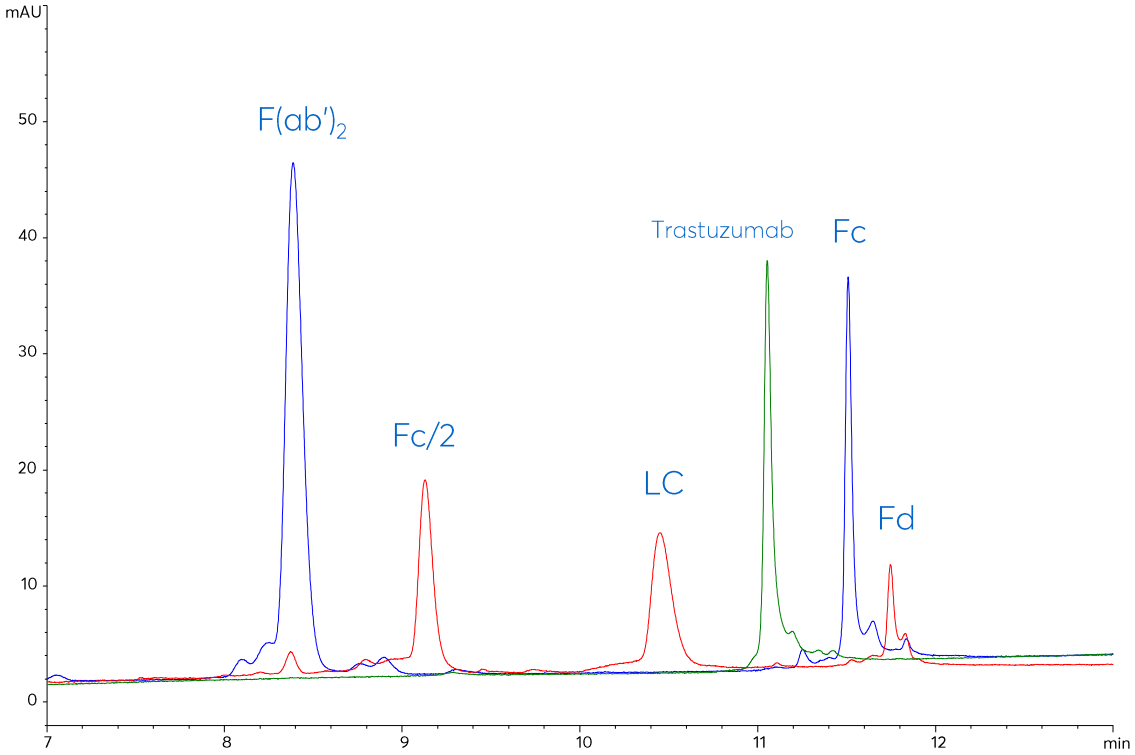
* - with kind thanks from Wikipedia, Roadnotaken, created it from PDB: 1PPP using PyMol and Photoshop

mAb Fragments – IdeS Digestion
(IgG-degrading Enzyme of Streptococcus Pyogenes)



* With kind permission from Katja Wenig et al. PNAS 2004;101:50:17371-17376

Analysis of Trastuzumab and Large Molecular Mass Fractions



CONDITIONS

Columns: Avantor® ACE® UltraCore Bio C18-300

Dimensions: 100 x 3.0 mm

Mobile Phases:

A: 0.1% TFA in H_2O
B: 0.1% TFA in ACN

Gradient:

Time (mins)	%B
0	25
20	50
21	95
23	95
24	25
34	25

Flow Rate: 0.43 mL/min

Injection: 20 µL

Temperature: 60 °C

Detection: UV, 214 nm

Conclusions

01

– History of solid core

02

– Modelling of solid core

03

– Separating small molecules

04

– Separating big molecules

05

– Method development considerations

06

– Conclusions

Thank you

info@mac-mod.com