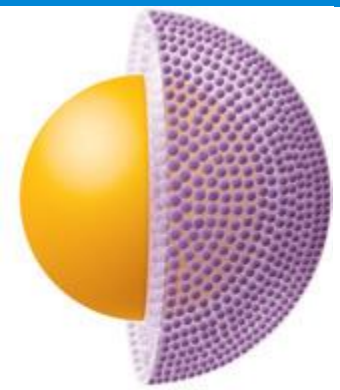
Comparison of Optimized Wide Pore Superficially Porous Particles (SPPs) Synthesized By One-Step Coating Process With Other Wide Pore SPPs For Fast And Efficient Separation Of Large Biomolecules



Wu Chen, Anne Mack, and Xiaoli Wang

Agilent Technologies, Inc.

Outline

1. Introduction of wide pore superficially porous particles (SPPs)
2. Performance of optimized SPPs for large molecule separation
3. Comparison of wide pore totally porous particles (TPPs)
4. Comparison of other wide pore SPPs
5. Conclusion

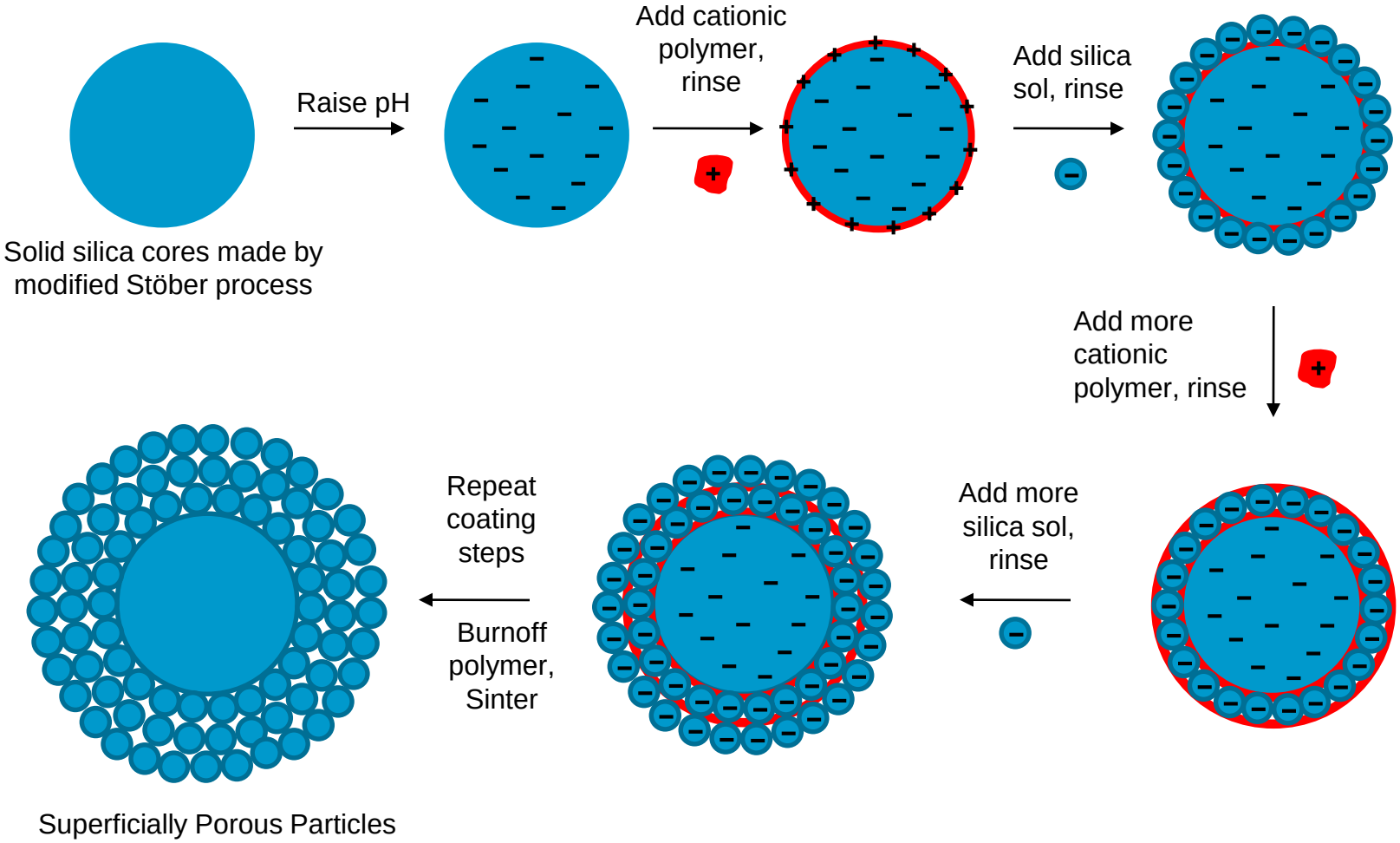
Current Status of Superficially Porous Particles

	Status in 2000	Status in 2010	Status in 2015			
	# of Vendors	# of Vendors	# of Vendors	Particle size range	Pore size range	# of Phase chemistries
Small molecules	0	3	16	1.3um to 5um	80Å to 120Å	>10 chemistries
Large molecules	1	1	9	2.6um to 5um	160Å to 450Å	>8 chemistries

- Ron Majors: “In my recent Pittcon 2014 article, in terms of new product introduction, it seems like the entire columns world has turned to SPP as the favored type of HPLC or UHPLC column, with SPP introductions exceeding sub-2-um columns by a 10:1 margin”.

Bell, LC-GC, 2015 June
Majors, LC-GC, 2014 Nov

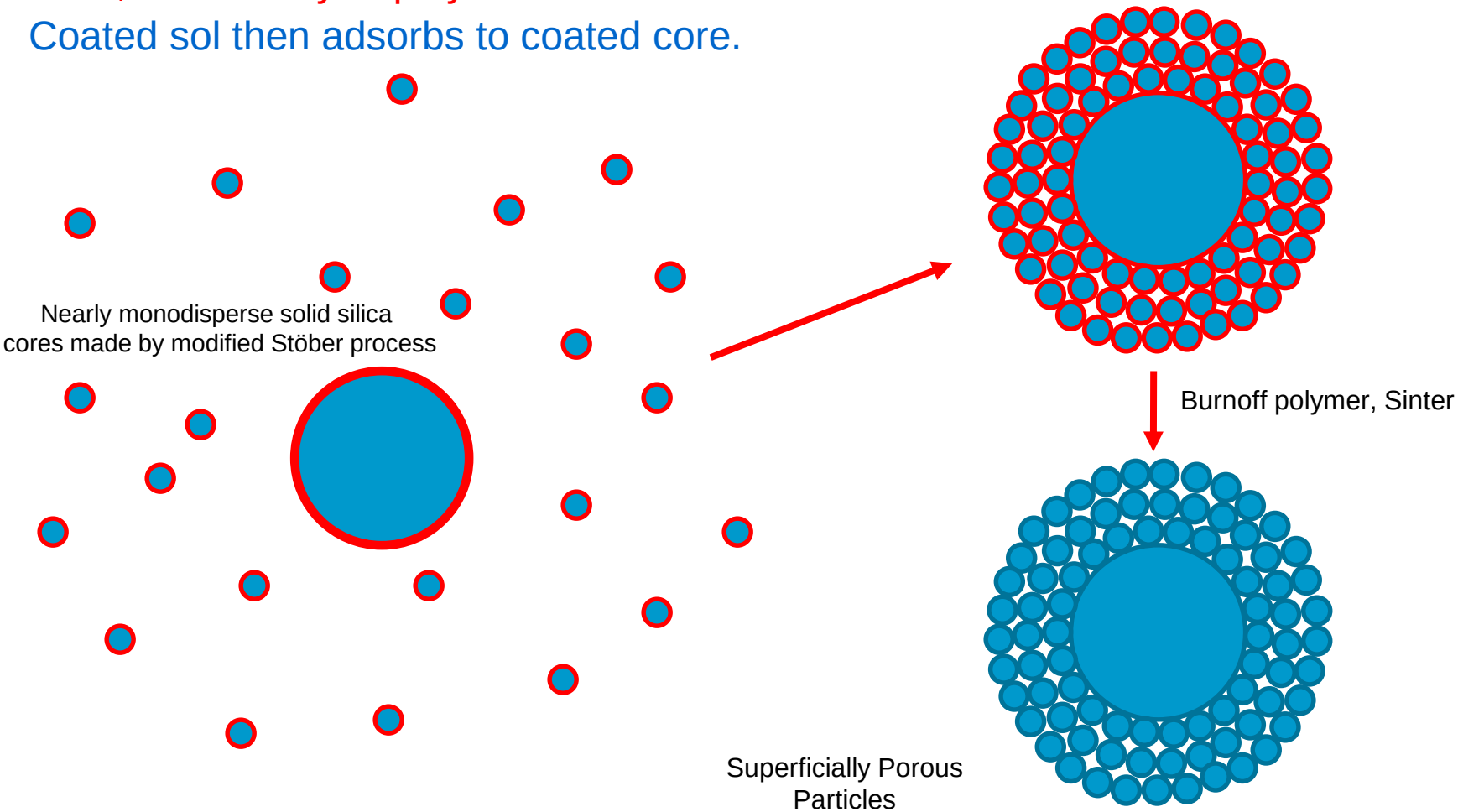
Layer-by-Layer Process for Producing Superficially Porous Particles



Coacervation Process for Producing Superficially Porous Particles

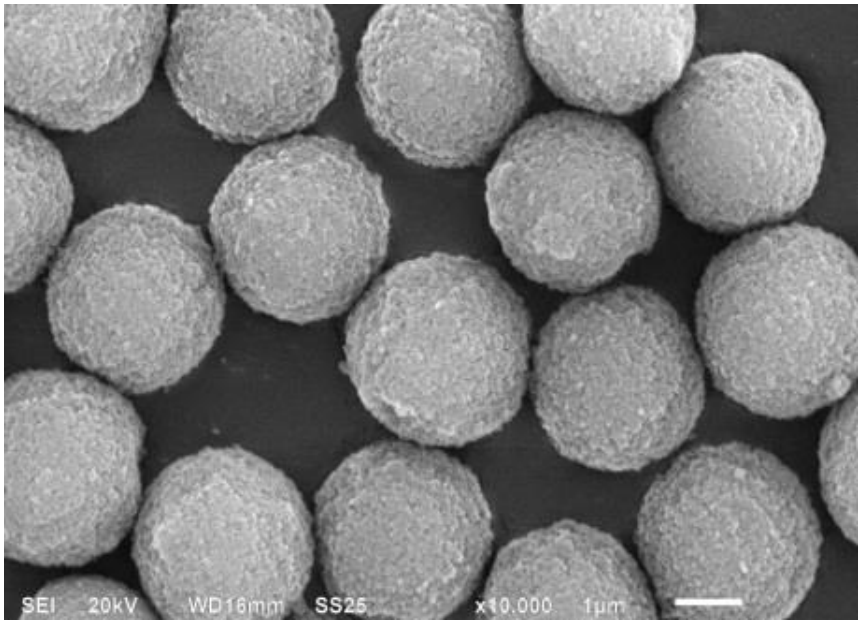
Urea, formaldehyde polymerization coats sol and core

Coated sol then adsorbs to coated core.

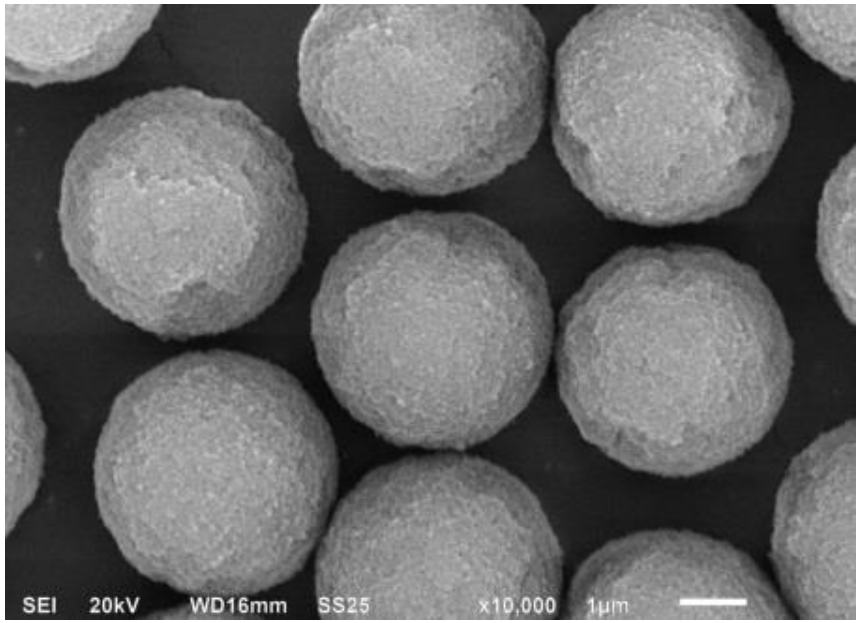


Wide Pore SPPs Made by Coacervation Process

Particle Size (µm)	Core Size (µm)	Shell Thickness (µm)	SA (m^2/g)	BET Pore Size (Å)	Pore Volume (cm^3/g)
2.72 - 3.77	1.69 - 2.95	0.20 - 0.51	10 - 31	253 - 460	0.07 - 0.19



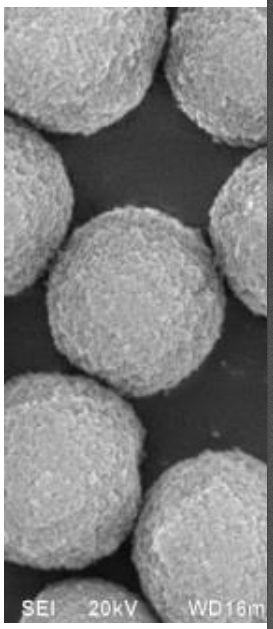
2.7 µm

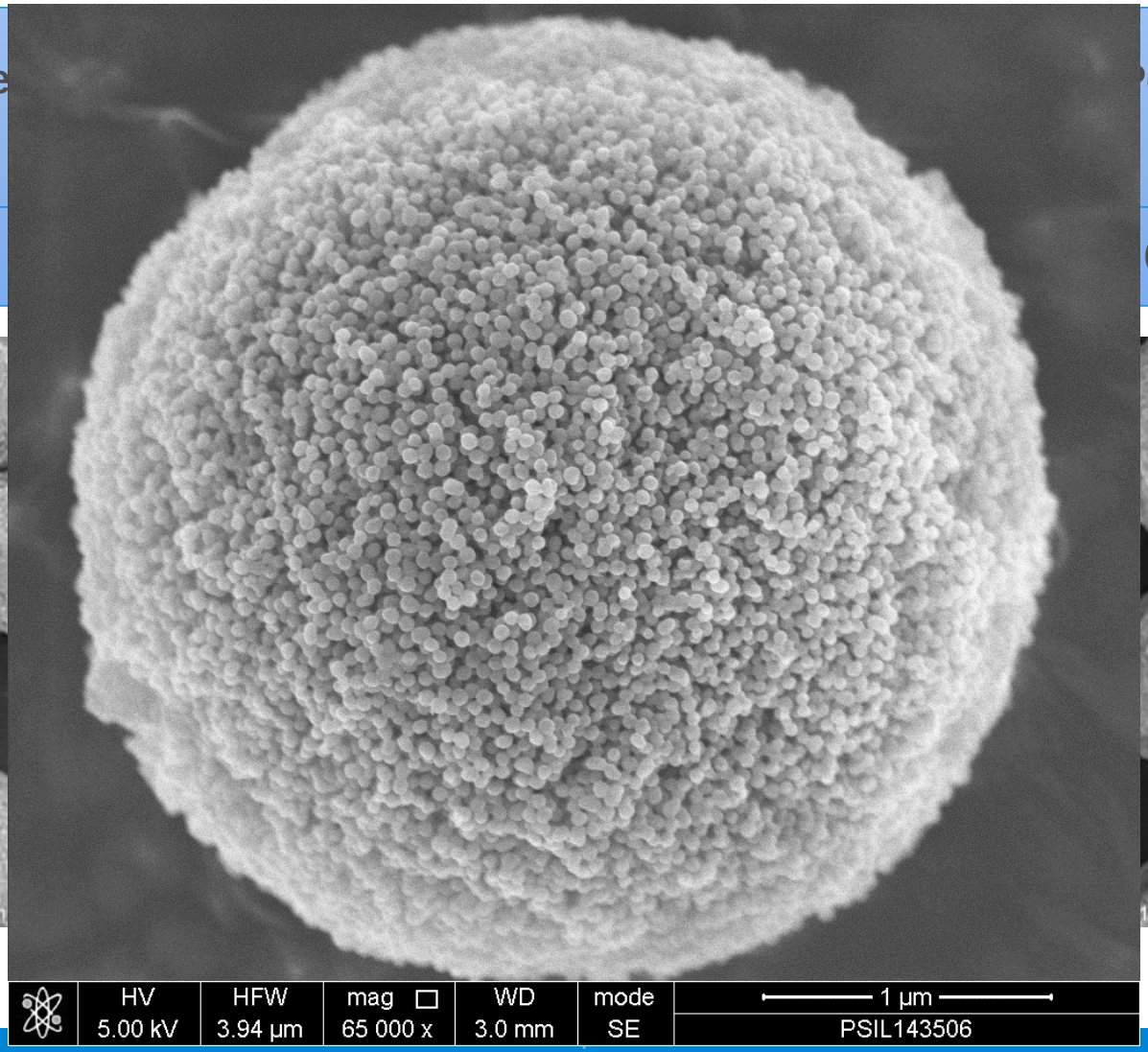


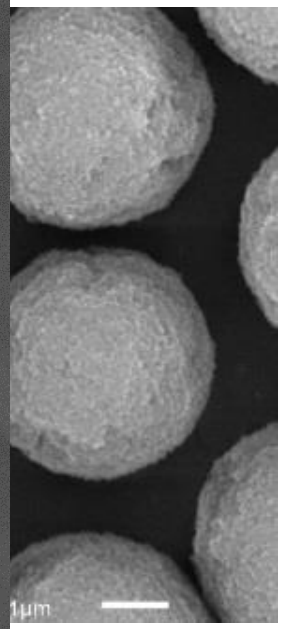
3.5 µm

Wide Pore SPPs Made by Coacervation Process

Particle Size (µm)	Pore Volume (cm^3/g)
2.72 - 3.77	0.07 - 0.19







Effect of Shell Thickness

Particle Size (µm)	Core Size (µm)	**Shell Thickness (µm)**	SA (m^2/g)	Pore Size (Å)	Pore Volume (cm^3/g)
2.72	1.69	**0.51**	31	253	0.19
2.70	2.25	**0.23**	10	281	0.07

Effect of Pore Size

Particle Size (µm)	Core Size (µm)	Shell Thickness (µm)	SA (m^2/g)	**Pore Size (Å)**	Pore Volume (cm^3/g)
3.55	2.95	0.30	13	**278**	0.10
3.46	2.95	0.26	13	**454**	0.15

Effect of Particle Size

Particle Size (µm)	Core Size (µm)	Shell Thickness (µm)	SA (m^2/g)	Pore Size (Å)	Pore Volume (cm^3/g)
2.67	2.25	0.21	10	452	0.11
3.46	2.95	0.26	13	454	0.15

W. Chen, etc. Synthesis and optimization of wide pore superficially porous particles by a one-step coating process for separation of proteins and monoclonal antibodies, J. Chromatogr. A, 1414 (2015) 147–157

Summary of Physical Property Study on Large Molecule Separation

Effect of physical property on large molecule separation

Pore size	>>	shell thickness	>	particle size
(450 Å > 300 Å)		(0.25 µm > 0.50 µm)		(3.5 µm > 2.7 µm)

Pore size has the biggest effect on large molecule separation. Large pores results in better resolution and narrow peak width.

The particles with thinner shell have narrow peak width at high flow rate.

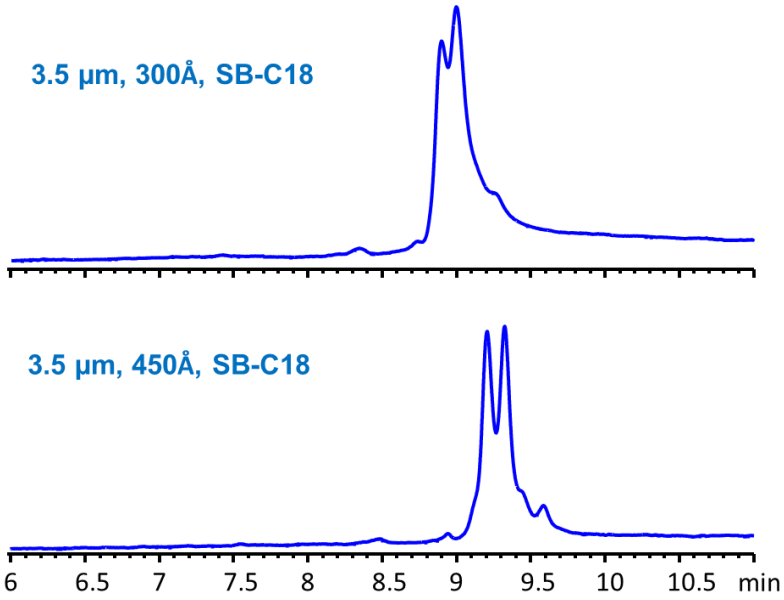
The particle size seems not affect peak width, but large particles have lower back pressure.

W. Chen, etc. Synthesis and optimization of wide pore superficially porous particles by a one-step coating process for separation of proteins and monoclonal antibodies, J. Chromatogr. A, 1414 (2015) 147–157

Effect of Pore Size-Large Molecule Separation

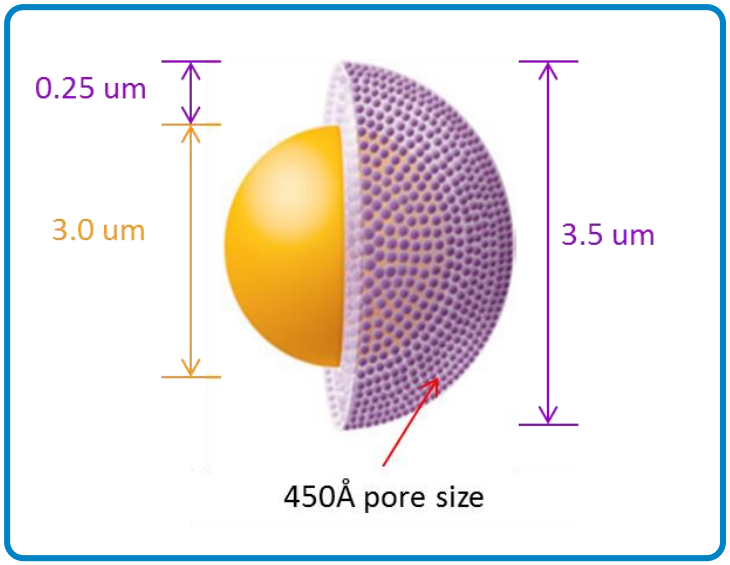
Particle Size (µm)	Core Size (µm)	Shell Thickness (µm)	SA (m^2/g)	**Pore Size (Å)**	Pore Volume (cm^3/g)
3.55	2.95	0.30	13	**278**	0.10
3.46	2.95	0.26	13	**454**	0.15

Separation of intact IgG2 lambda



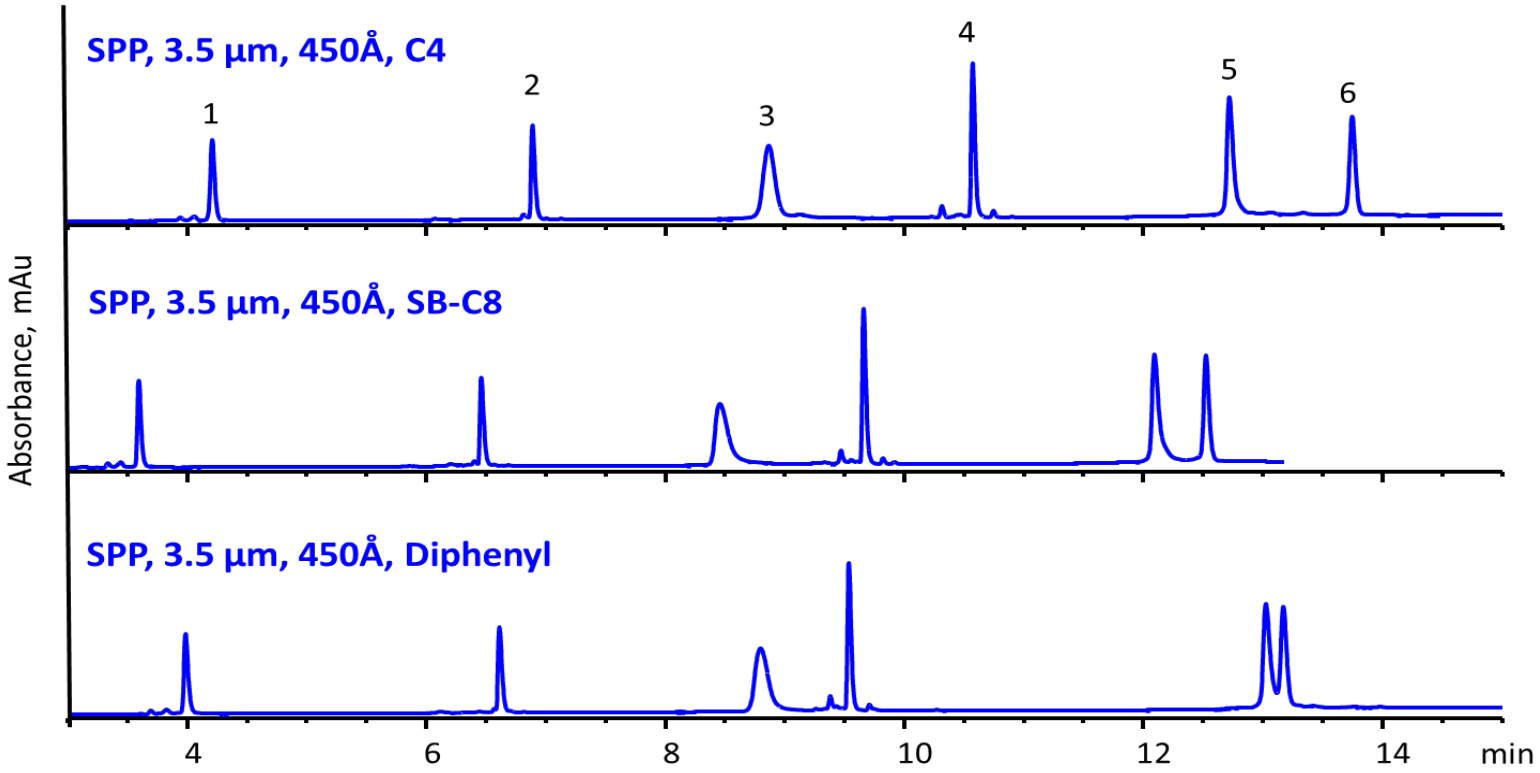
Column: 3.5 µm, SB-C18, 2.1 x 100 mm; flow rate: 1.5 mL/min; gradient: mobile Phase: A: 90% water/10% ACN, 0.1% TFA, 0.3% Polyethylene glycol, B: 90% ACN/10% water, 0.1% TFA, 0.3% PEG; hold 19% B for 0.5 min and then 19%-41% B in 11.5 min; injection: 1 µL; detector: 220 nm; temperature: 80 °C; sample: intact IgG2 lambda.

Optimized Wide Pore SPPs for Large Molecule Separation- AdvanceBio RP-mAb



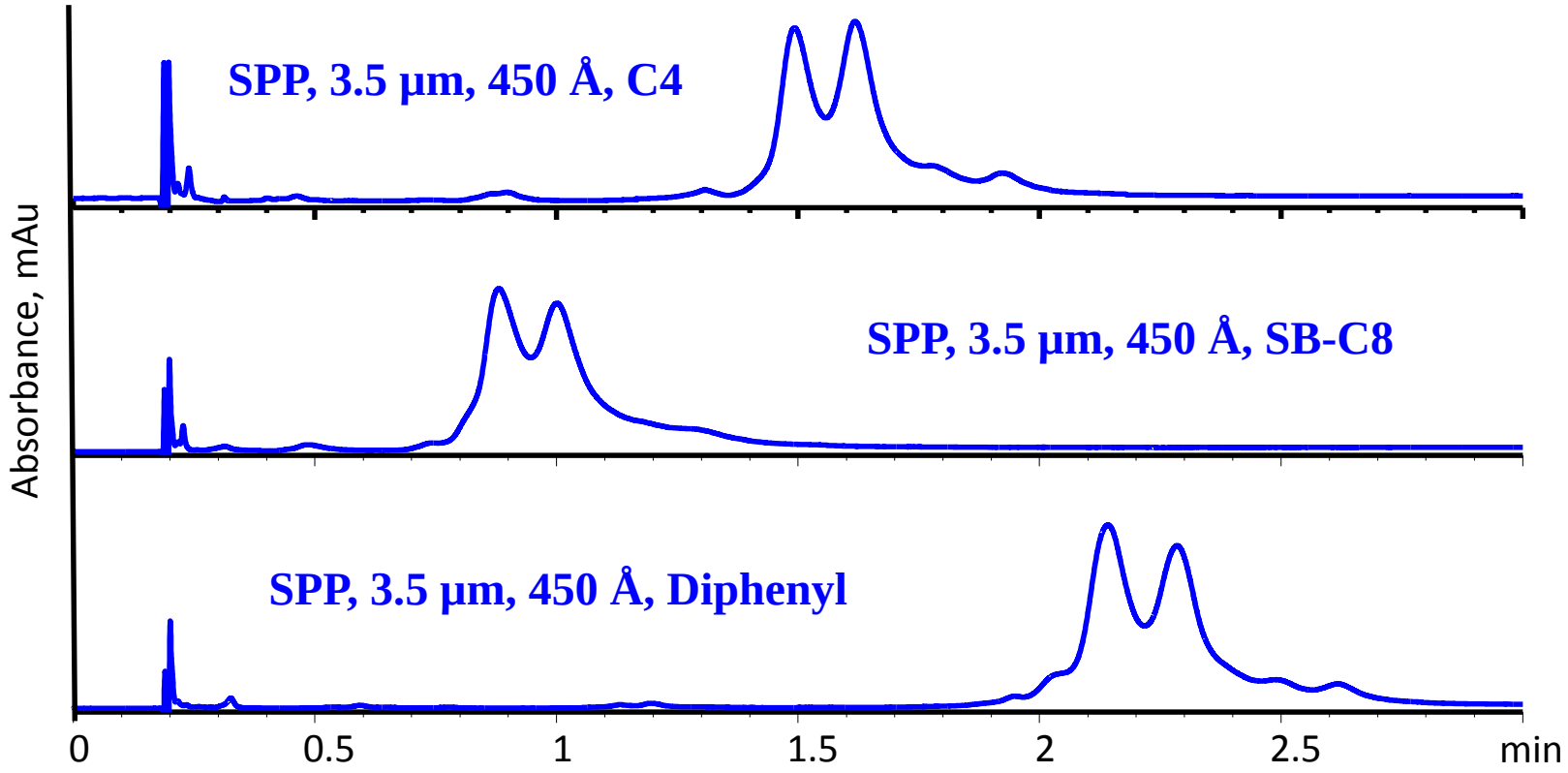
Stationary phase	Si ligand type	End-capping
C4	n-butyldimethyl	Yes
SB-C8	n-octyldiisopropyl	No
Diphenyl	diphenylmethyl	Yes

Difference Selectivity of A Protein Standard



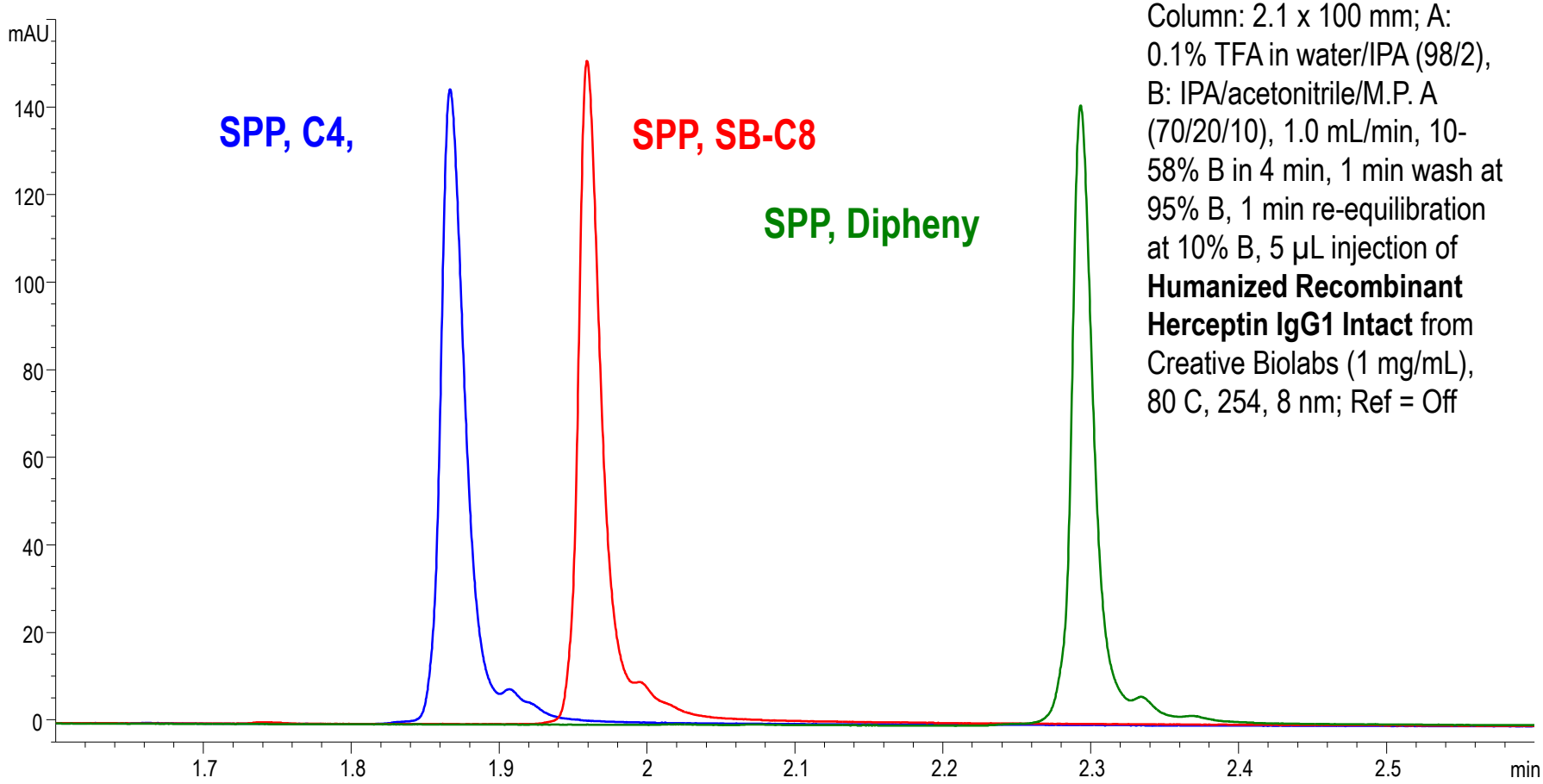
Columns: 2.1 x 100 mm; gradient: A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile, 20-50% B in 15 min, 3 min wash at 95% B, 2 min re-equilibration at 20% B; flow rate: 0.3 mL/min; temperature: 60 C; detector: 220, 8 nm; Ref = Off; injection: 5 µL injection of Protein Standard; samples: 1. ribonuclease A (14 kDa), 2. cytochrome C (12 kDa), 3. holo-transferrin (80 kDa), 4. α-lactalbumin (14 kDa), 5. catalase (240 kDa), 6. carbonic anhydrase (30 kDa)

Difference Selectivity of Intact IgG2



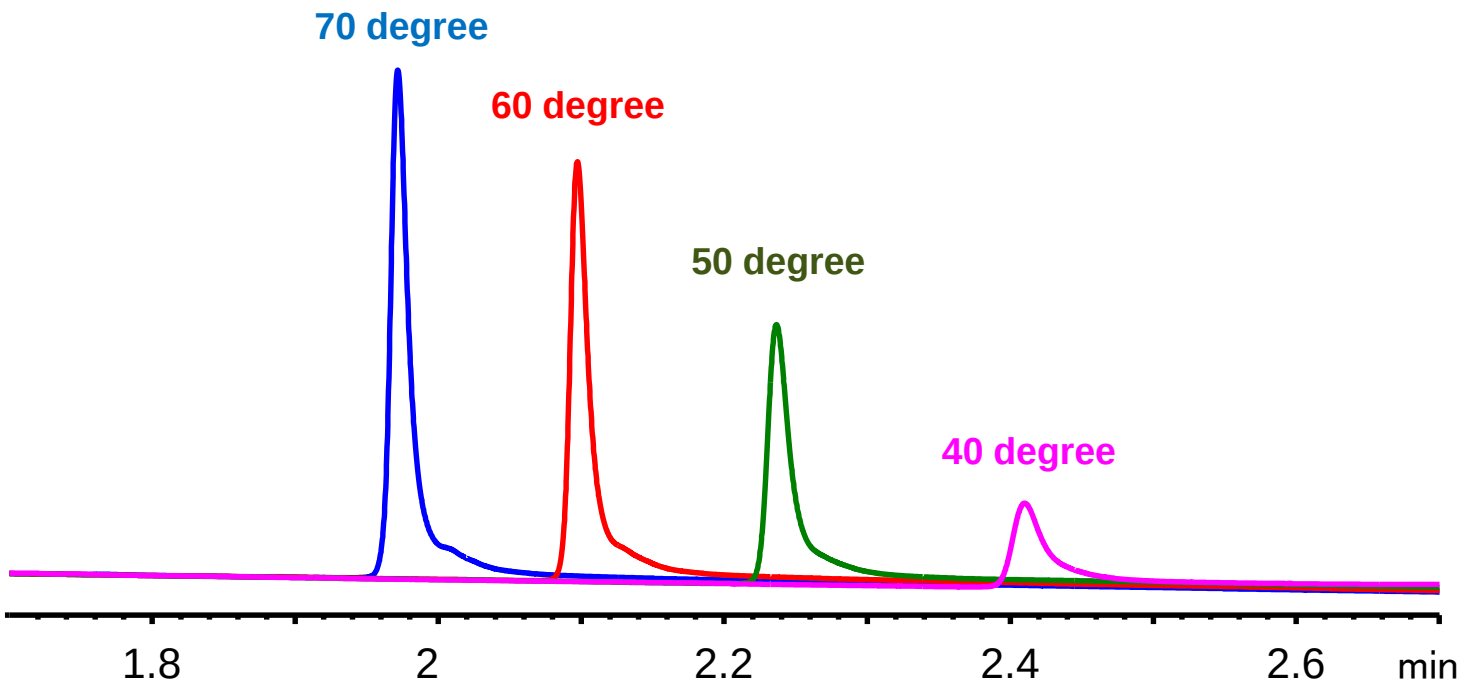
Columns: RP-mAb, 2.1 x 100 mm; mobile phase A: 0.1% TFA in water, mobile phase B: 0.1% TFA in acetonitrile; gradient: 34-38% B in 3 min, 3 min wash at 95% B, 2 min re-equilibration at 34% B; flow rate: 1.0 mL/min; injection: 5 µL; temperature: 85 °C; detector: 215 nm; sample: intact IgG2 lambda from human myeloma plasma.

Selectivity Comparison of Intact IgG1



Column: 2.1 x 100 mm; A: 0.1% TFA in water/IPA (98/2), B: IPA/acetonitrile/M.P. A (70/20/10), 1.0 mL/min, 10-58% B in 4 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B, 5 µL injection of **Humanized Recombinant Herceptin IgG1 Intact** from Creative Biolabs (1 mg/mL), 80 C, 254, 8 nm; Ref = Off

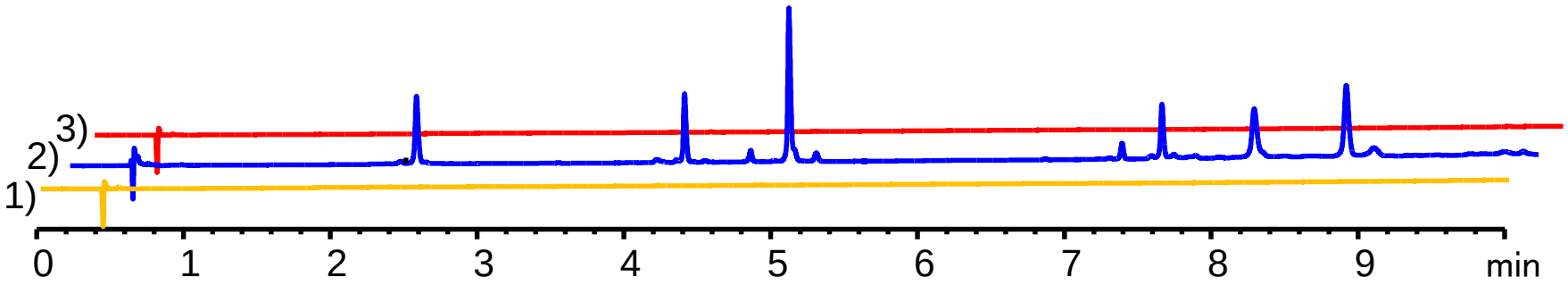
Effect of Temperature on Separation of Intact IgG1



Column: SPP, C4, 3.5 µm, 450 Å, 2.1 x 100 mm; mobile phase A: 0.1% TFA in water:IPA (98:2), mobile phase B: IPA:acetonitrile:mobile phase A (70:20:10); flow rate: 1.0 mL/min; gradient: 10-70% B in 5 min; detection: UV at 254 nm; sample: 5 µL humanized recombinant Herceptin IgG1 (1 mg/mL).

Carryover Study of A Protein Standard

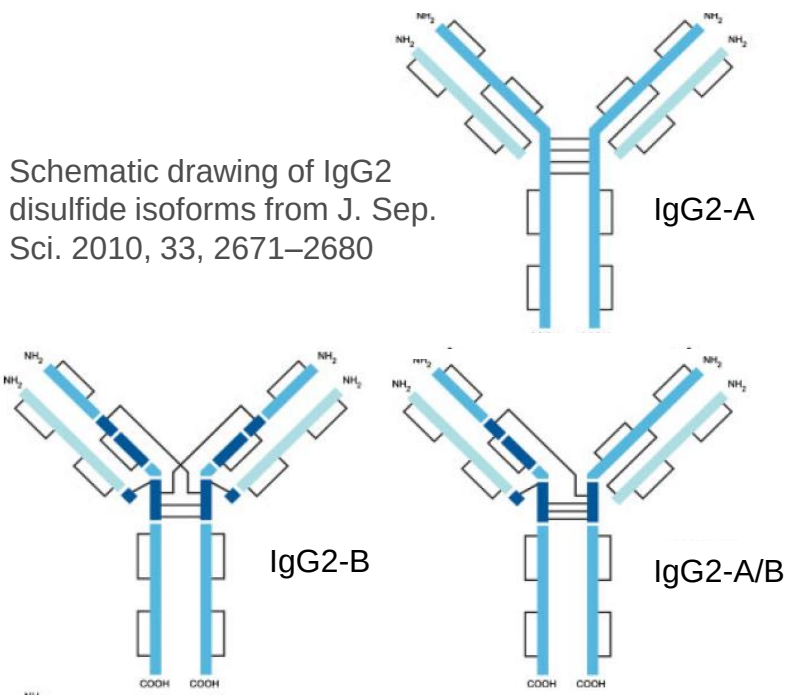
1) (yellow): blank injection before real sample injection;
2) (blue): real sample injection;
3) (red): blank sample injection after real sample injection.

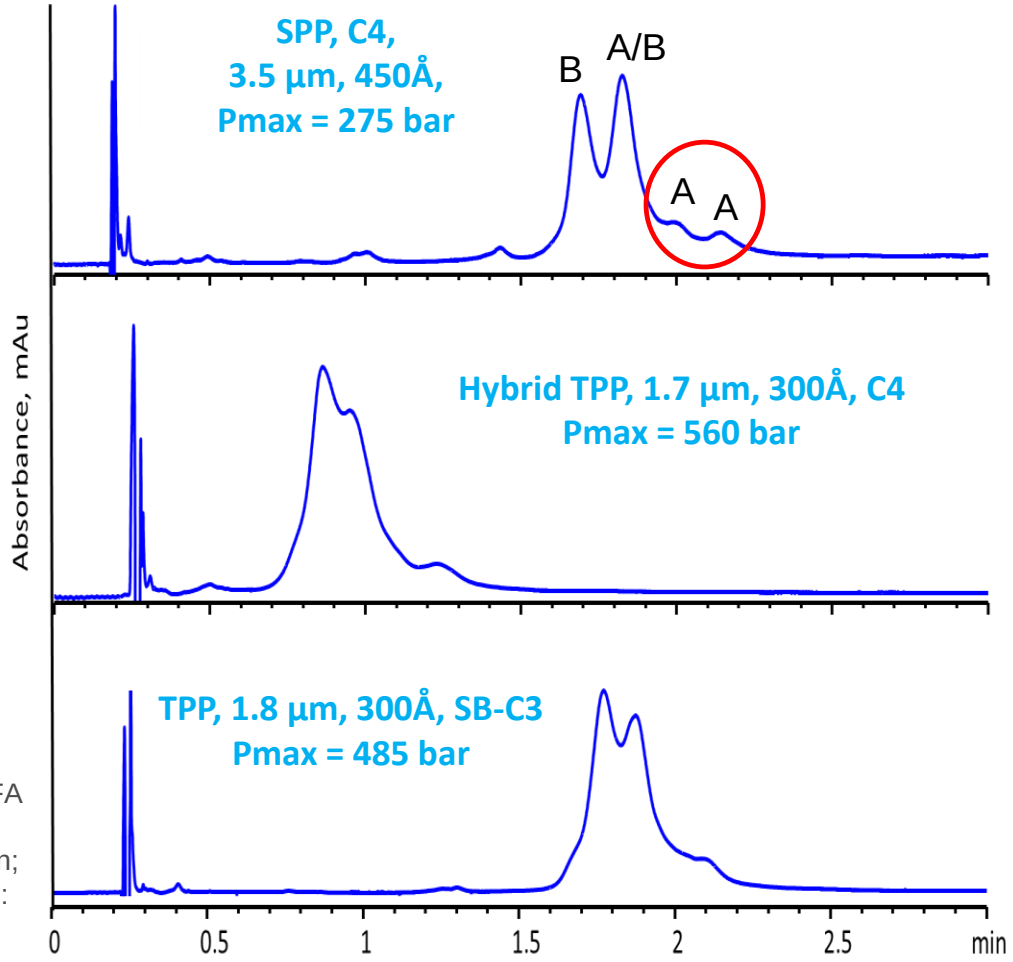


Column: SPP, C4, 3.5 µm, 450Å, 2.1 x 100 mm; mobile phase A: 0.1% TFA in water, mobile phase B: 0.1% TFA in acetonitrile; gradient: 20-50% B in 10 min, 3 min wash at 95% B, 2 min re-equilibration at 20% B; flow rate: 0.5 mL/min; temperature: 60 °C; detector: 220, 8 nm; Ref = off; injection: 2 µL. 1. Ribonuclease A (14 kDa), 2. Lysozyme (14.3 kDa), 3. Cytochrome c (12 kDa), 4. α-Lactalbumin (14 kDa), 5. Catalase (240 kDa), 6. Carbonic anhydrase (30 kDa);

Comparison of Totally Porous Particles-Separation of Intact IgG2 Isomers

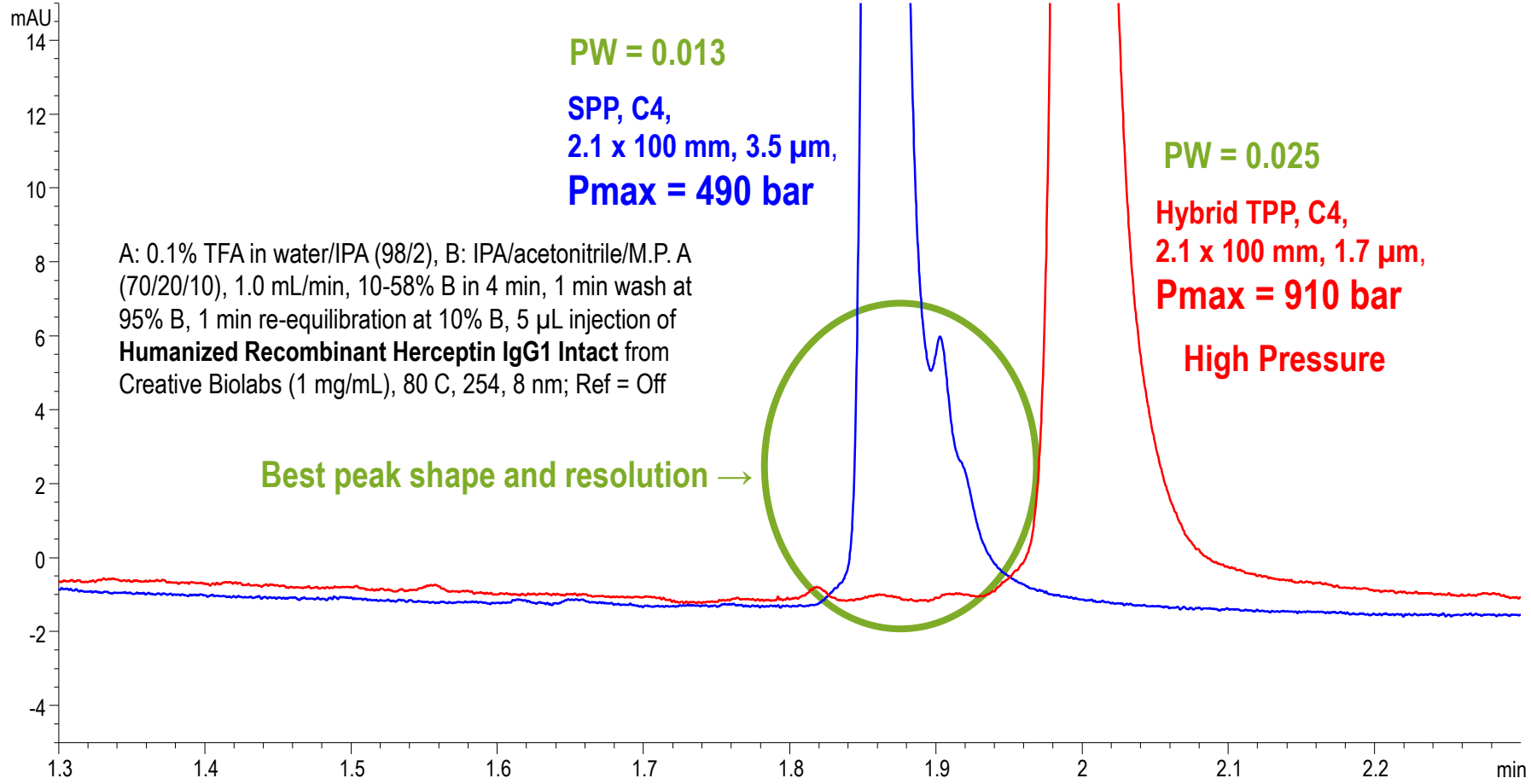
Schematic drawing of IgG2 disulfide isoforms from J. Sep. Sci. 2010, 33, 2671–2680





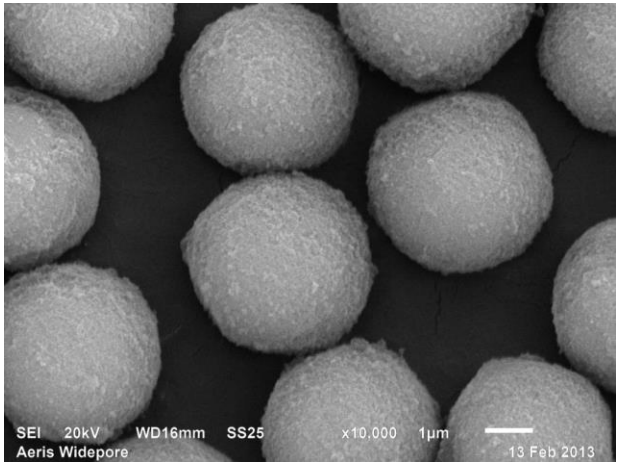
Column: 2.1 x 100 mm; gradient: A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile, 34-38%B in 8 min, 3 min wash at 95%B, 2 min re-equilibration at 34%B; flow rate: 1.0 mL/min; injection: 5 µL injection; temperature: 85 degree; detector: 215 nm, 8 nm, Ref = Off; sample: intact IgG2 Lambda from human myeloma plasma

Comparison of Totally Porous Particles-Separation of Intact IgG1

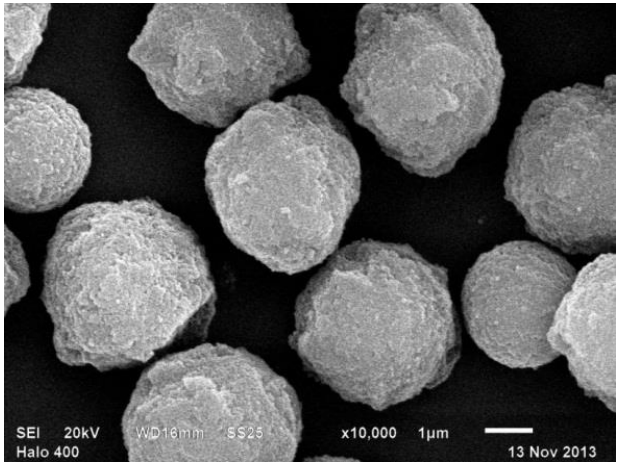


Comparison of Other Wide Pore Superficially Porous Particles-Physical Properties

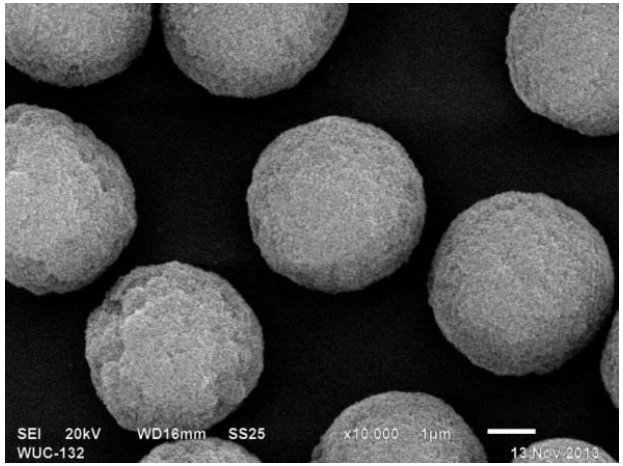
	Particle Size (µm)	Average Pore Size (Å)	Surface Area (m^2/g)	Shell Thickness (µm)	Pore Volume (cm^3/g)
Other SPP	3.6	200	25	0.20	0.12
Other SPP	3.4	400	15	0.20	0.11
SPP	**3.5**	**450**	**12**	**0.25**	**0.12**



3.6 µm, 200 Å, SPP

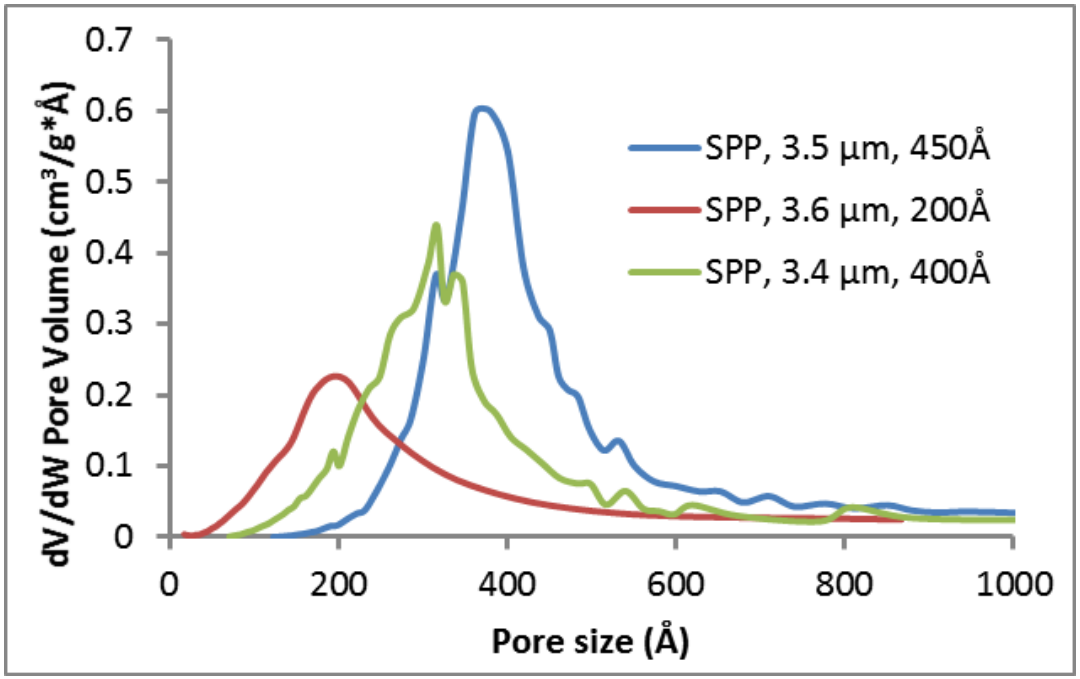


3.4 µm, 400 Å, SPP



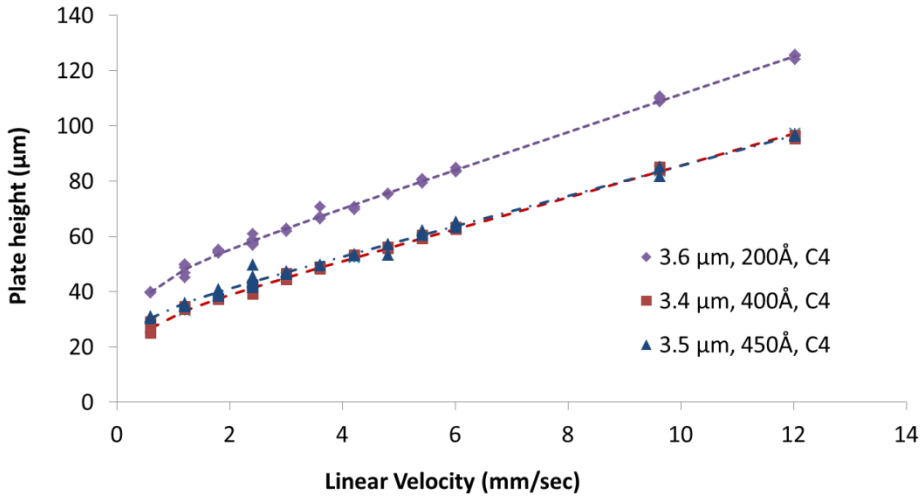
3.5 µm, 450 Å, SPP

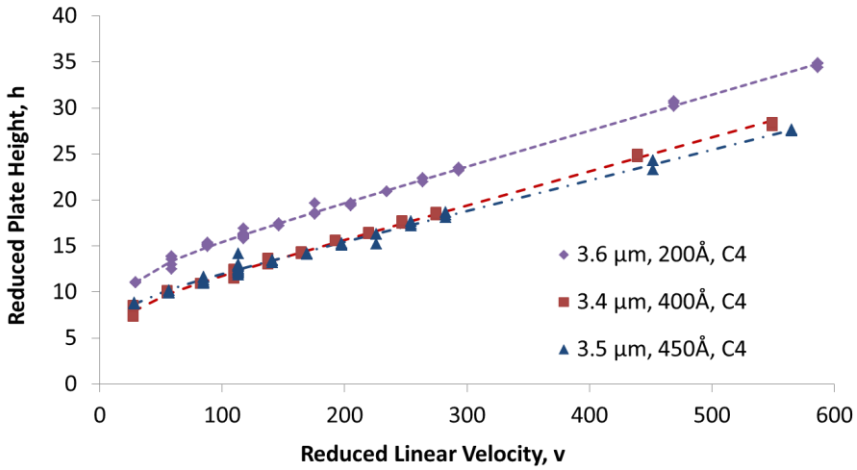
Comparison of Other Wide Pore Superficially Porous Particles-Pore Size Distribution



- The volume percentage of pores smaller than 200 Å is 0.8% for the 450 Å SPP, 12% for the 400 Å SPP, and 66% for the 200 Å SPP.

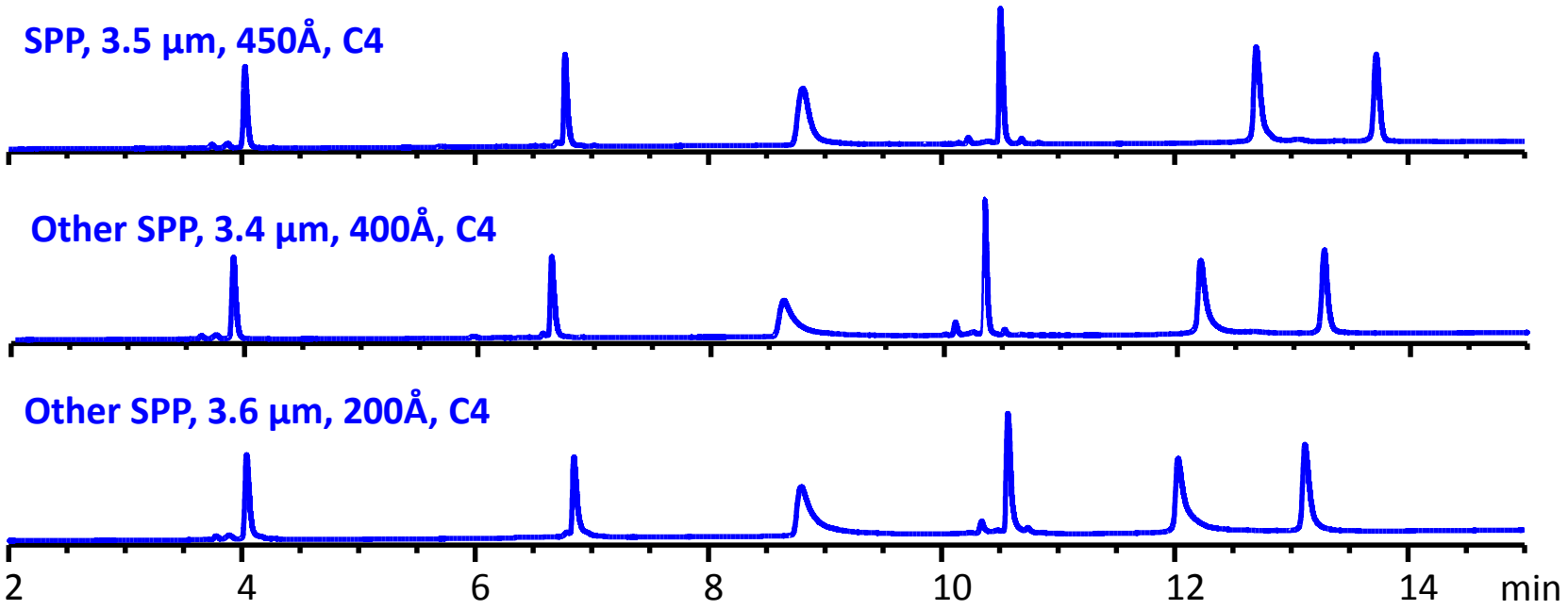
Comparison of van Deemter Plot





Columns: 2.1 x 100 mm; mobile phase A: 0.1% TFA in water; mobile phase B: 0.1% TFA in ACN; 43.7% mobile phase B; 41.6% B for the 200Å, 43.3% B for the 400Å and the 450Å SPPs; injection: 1 µL; detector: 220 nm; temperature: 60 ^{o}C; sample: carbonic anhydrase (30 kDa).

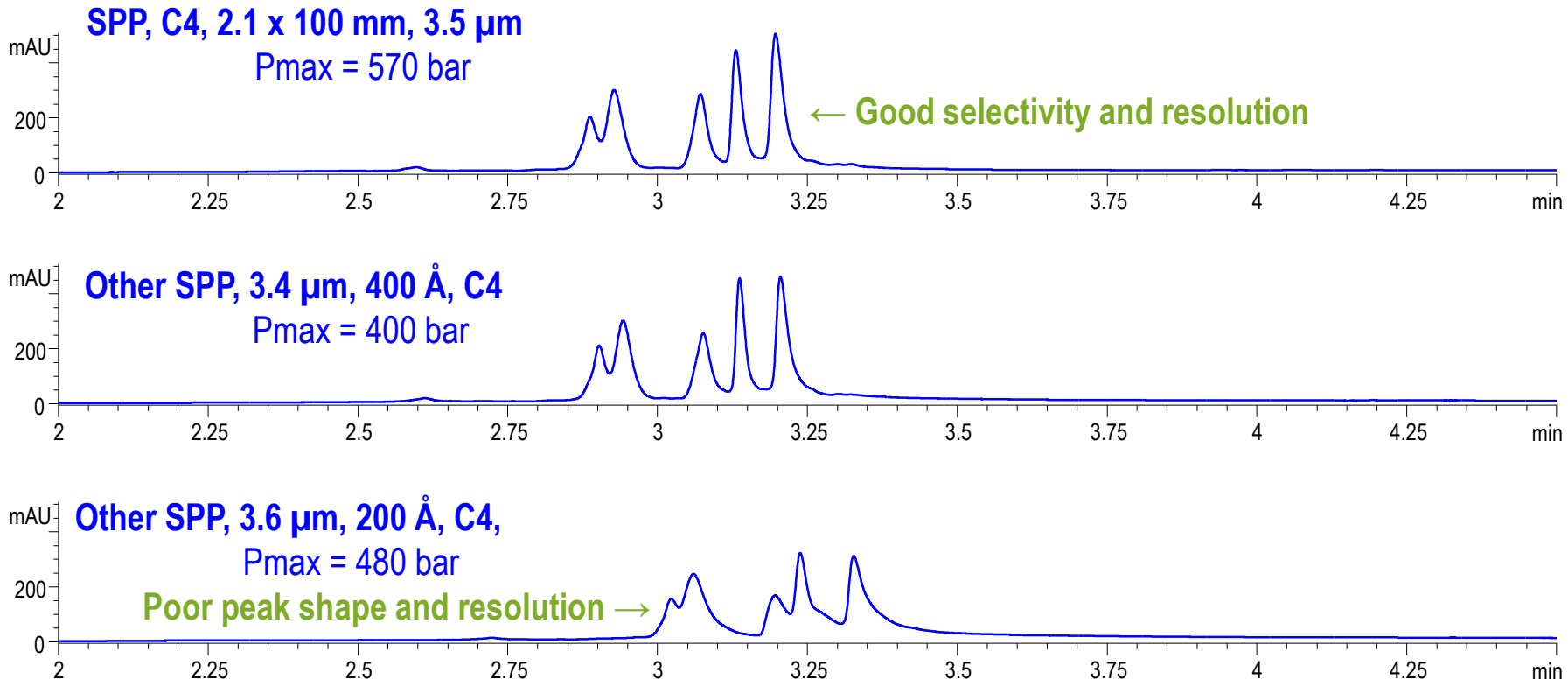
Comparison of Protein Separation



Peak Widths (min)	RP-mAb, 3.5 µ, 450 Å, C4	3.4 µm, 400 Å, C4	3.6 µm, 200 Å, C4
Ribonuclease A (14 kDa)	0.035	0.036	0.040
Cytochrome C (12 kDa)	0.035	0.038	0.041
Holo-transferrin (80 kDa)	0.106	0.126	0.121
α-Lactalbumin (14 kDa)	0.035	0.034	0.041
Catalase (240 kDa)	0.059	0.068	0.074
Carbonic Anhydrase (30 kDa)	0.052	0.054	0.060

A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile, 0.3 mL/min, 20-50% B in 15 min, 3 min wash at 95% B, 2 min re-equilibration at 20% B, 5 µL injection of **Protein Standard: Ribonuclease A (14 kDa), Cytochrome C (12 kDa), Holo-transferrin (80 kDa), α-Lactalbumin (14 kDa), Catalase (240 kDa), Carbonic Anhydrase (30 kDa)**, 60 C, 220, 8 nm; Ref = Off

Comparison of Digested IgG1 Separation

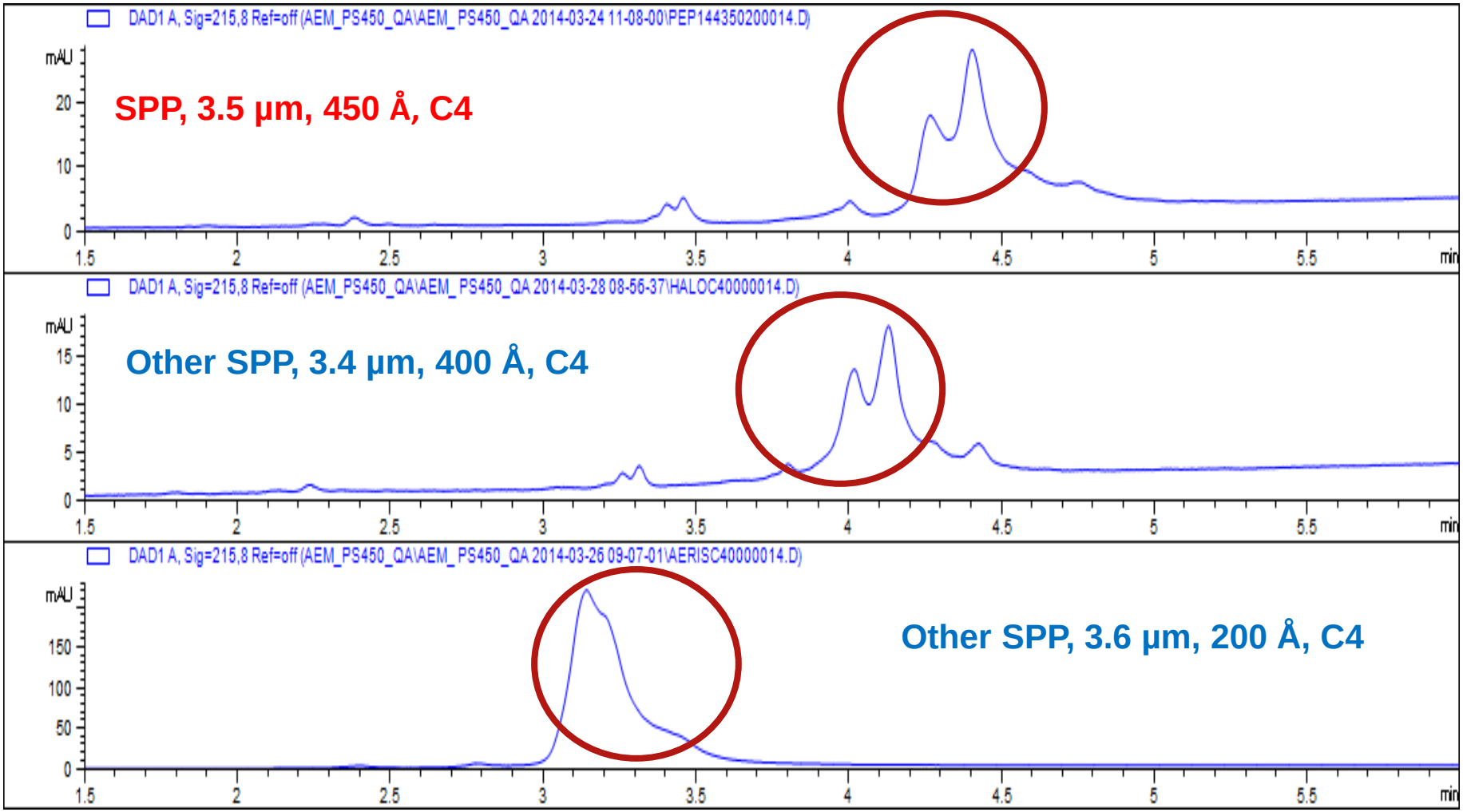


Column: 2.1 x 100 mm; A: 0.1% TFA in water, B: n-propanol/acetonitrile/M.P. A (80/10/10), 0.8 mL/min, 5-40% B in 5 min, 1 min wash at 95% B, 1 min re-equilibration at 5% B, 1 µL injection of **Fc/Fab, Papain Digested Humanized Recombinant Herceptin IgG1** from Creative Biolabs (2 mg/mL), 60 C, 220, 8 nm; Ref = Off

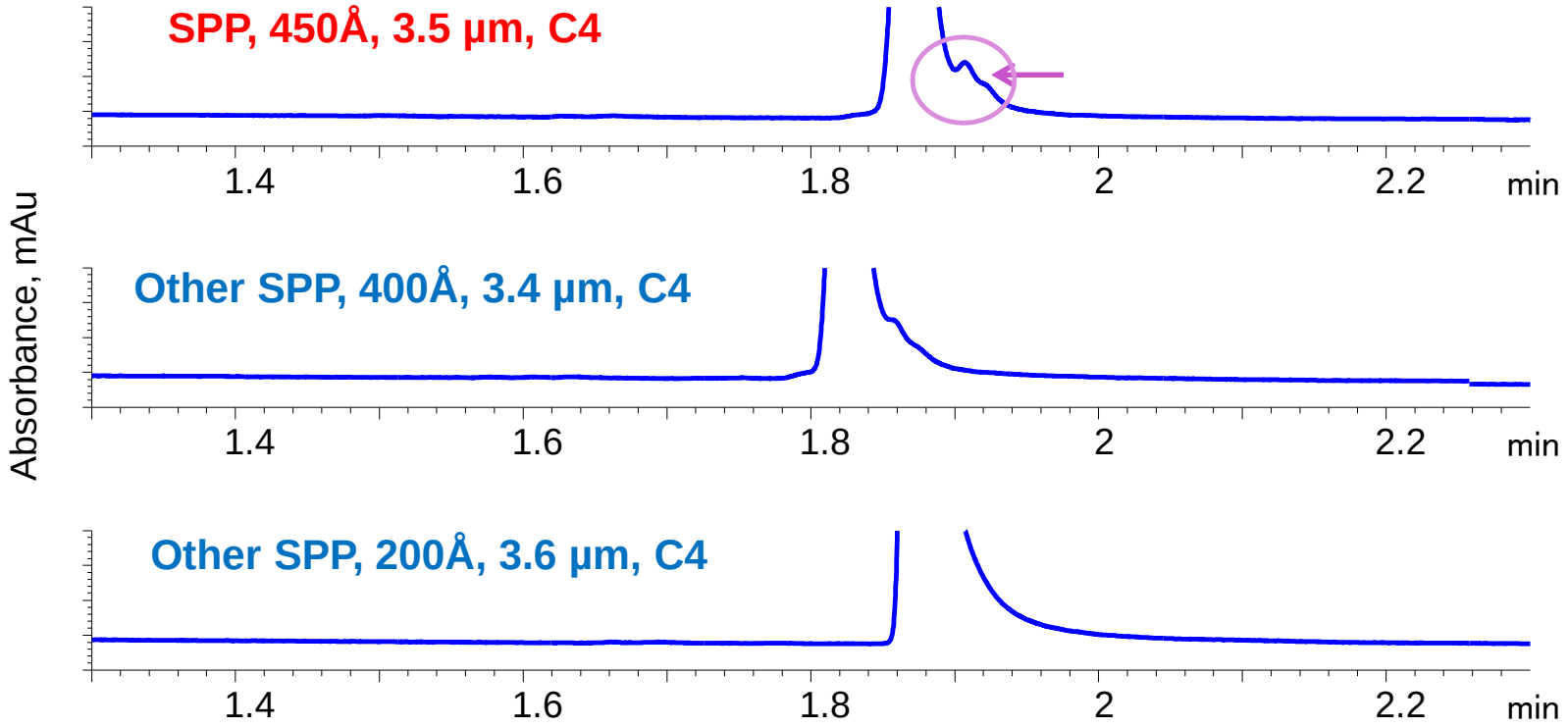
Intact IgG2

High resolution for intact mAb

A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile, 1.0 mL/min, 30-40%B in 8 min, 85 C, 215 nm
5 uL injection of IgG2 Lambda (Sigma I5279-1MG)
2.1 x 100 mm,



Comparison of Other Wide Pore SPPs-Fast Separation of Intact IgG1



Method Parameters
Column dimensions: 2.1 x 100 mm
Mobile phase A: 0.1% TFA in water/IPA (98/2)
Mobile phase B: IPA/acetonitrile/MPA (70/20/10)
Flow rate: 1.0 mL/min

Gradient: 10-58% B in 4 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B
Sample: 5 µL injection of Humanized Recombinant Herceptin Variant IgG1 Intact from Creative Biolabs (1 mg/mL)
Temperature: 80 °C
Detection: UV @ 254nm

Conclusion

3.5 µm SPPs with a 0.25 µm shell thickness and a 450 Å pore size were chosen as optimized base particles for large biomolecule separation.

The three phases (C4, SB-C8 and Diphenyl) of SPPs were evaluated with no carryover.

3.5 µm, 450 Å SPPs were compared with other commercially available wide pore SPPs with similar particle sizes and sub-two micron wide pore TPPs, and showed better performance for proteins and large biomolecules such as IgG1 and IgG2.