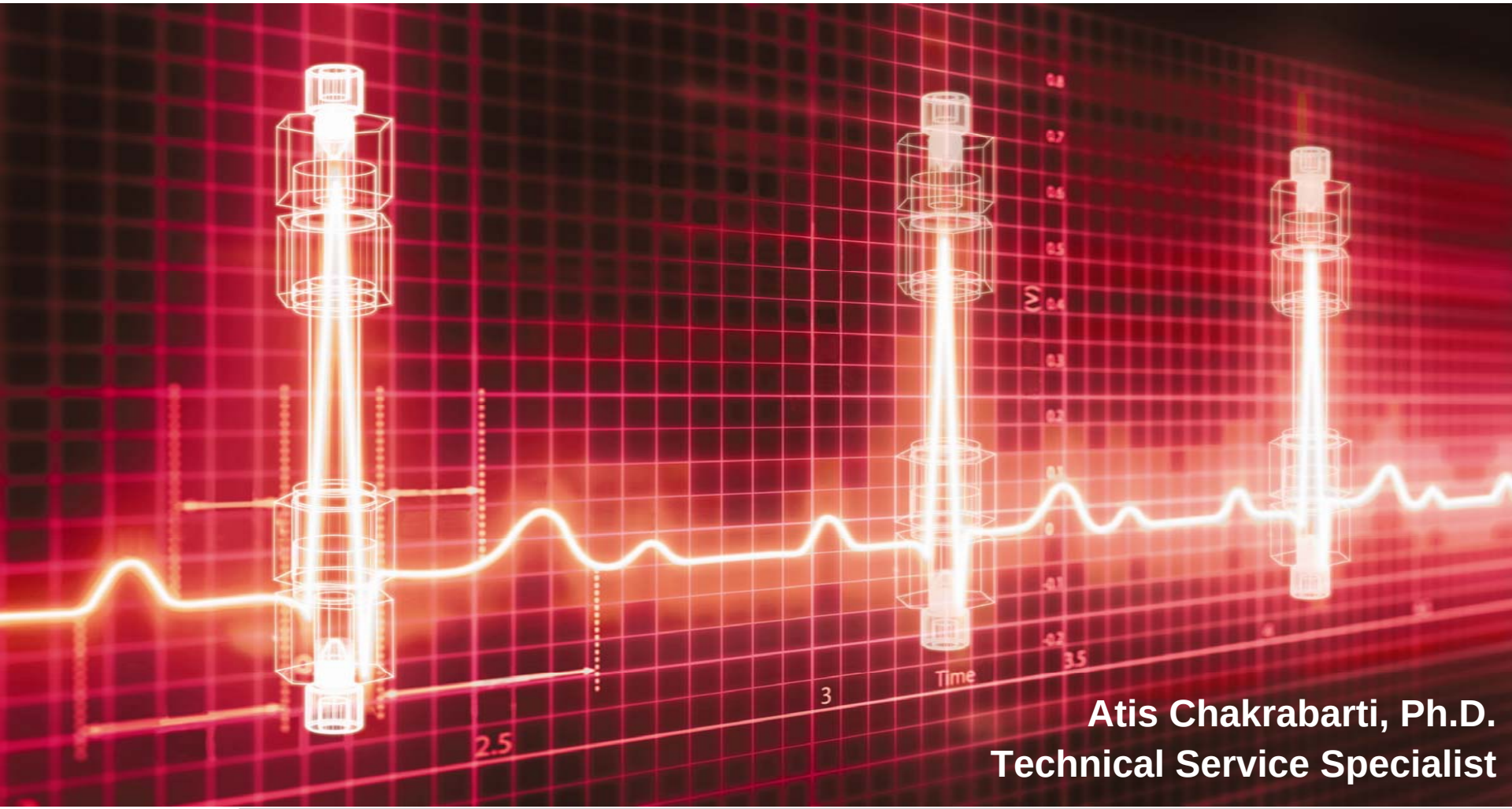
Protein and Peptide Analysis: Gel Filtration Chromatography (GFC) Applications and Recent Developments



Atis Chakrabarti, Ph.D.
Technical Service Specialist

Overview

- Brief review of Tosoh Corporation
- Brand names of Tosoh
- Size exclusion chromatography
- Applications useful for protein and peptide analysis by size exclusion chromatography
- I will take your questions

Tosoh Logo and Corporate History

Curved lines “the realization of happiness”

The right-angle cut at the top portrays an image of contributing to society.

Red corporate color – the “Tosoh Spirit”

Tosoh Corporation:	**1937**
Tosoh SID:	**1971**
TosoHaas:	**1987**
Tosoh Biosep:	**2000**
Tosoh Bioscience:	**2001**

Offices

- **Head Office in Japan with divisions all around the globe**
- Main office: (USA) King of Prussia, PA Supply Chain: Grove City, OH

Function

- Sales, marketing and support organization for chromatographic columns, media edia and GPC instrument manufactured by Tosoh Corporation
  - Territory; North and South America

Brand names

TSK- Toyo Soda Kogyo

TSKgel® chromatographic media

columns for the following modes of LC:

- Size exclusion
- HILIC
- Ion exchange
- Reversed phase
- HIC
- Affinity

bulk media for the following modes of LC:

- Size exclusion
- Ion exchange
- HIC

Toyopearl® chromatographic bulk media

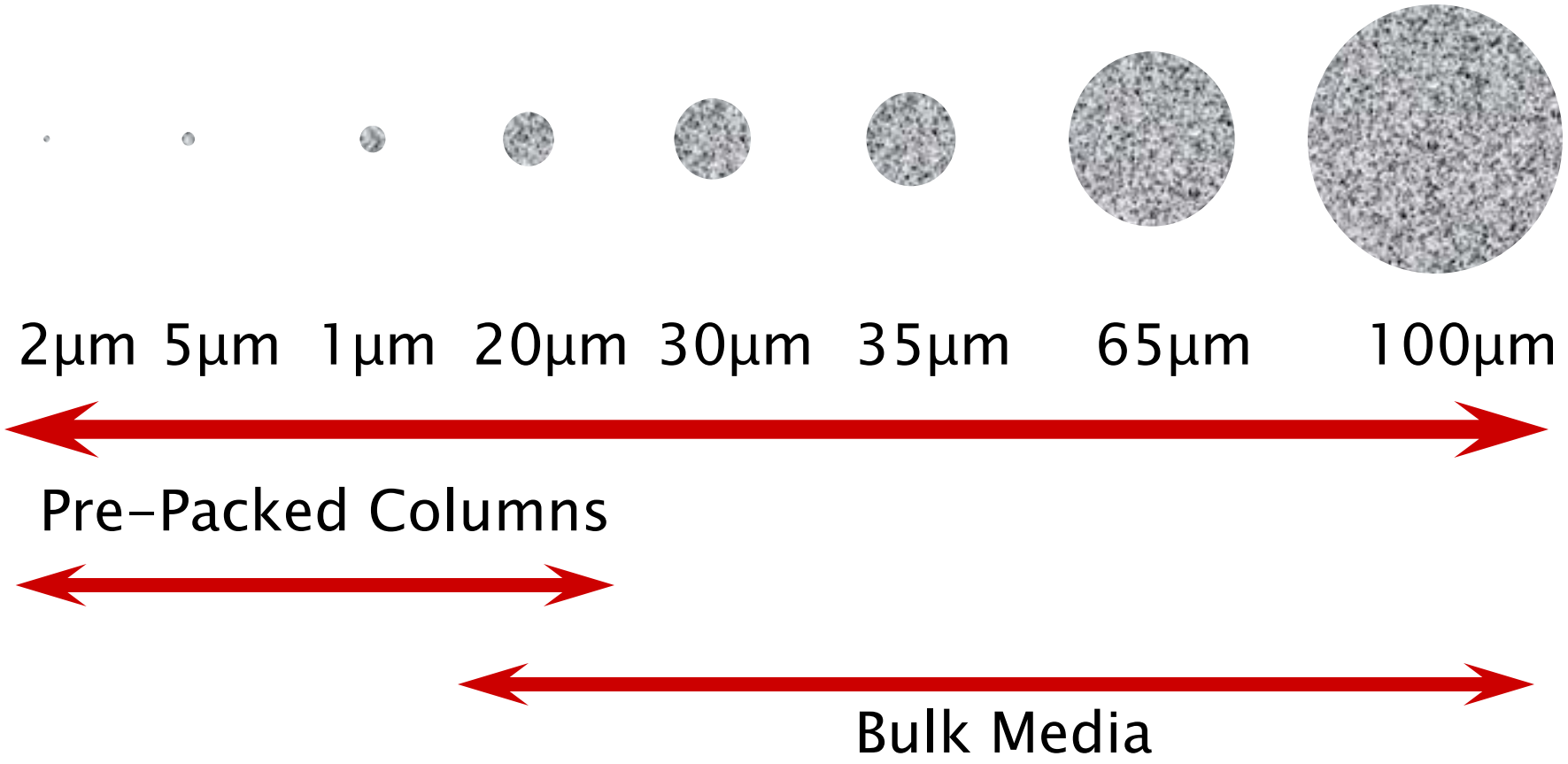
for the following modes of LC:

- Size exclusion
- Ion exchange
- HIC

ToyoScreen® process development columns

pre-packed with our most popular Toyopearl resins

TSKgel and Toyopearl resins:
Seamless scale-up and scale-down



TOSOH

Can I see the protein aggregates?

Can I use your column for higher pH and temperature?

Which SEC column I should select?

Can I use surfactants?

How much protein I can load?

I am having a column shedding issue – what's your suggestion?

Expected SEC column features for the separation of proteins and peptides by SEC

- Rigid silica support → excellent mechanical strength
- High resolution and sharp peak
- Fast separation
- Reproducibility
- Column stability
- Analytical and preparative size columns
- High recovery
- Maintaining biological activity

Factors affecting the protein and peptide analysis

- Complex mixture
- Low sample concentration
- Aggregation
- Secondary interaction
- Chromatographic conditions
  - Type of salt
  - Salt concentration
  - pH
  - Surfactant
  - Other additives
  - Denaturant
  - Heat

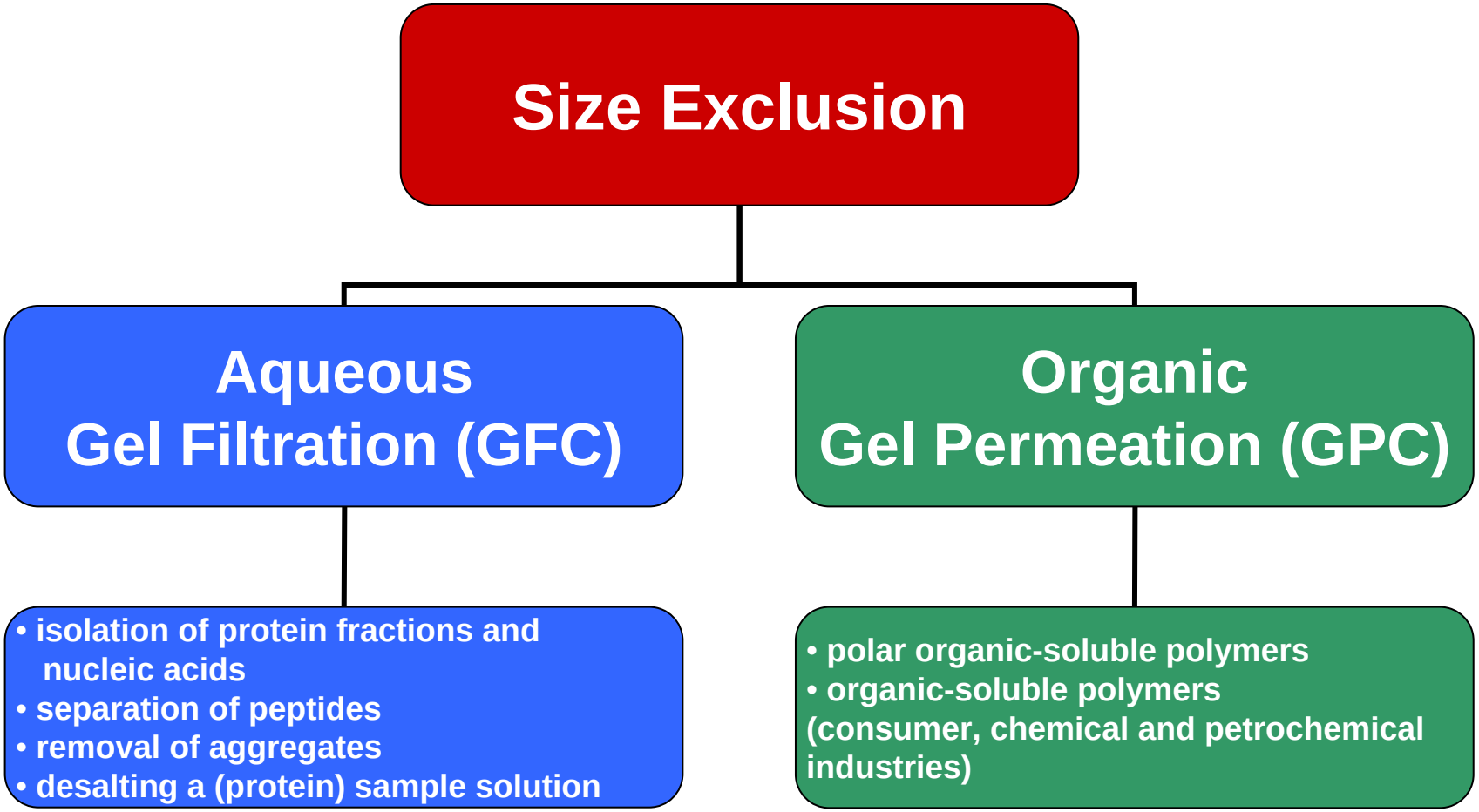
Protein and peptide analysis

- High-performance size-exclusion liquid chromatography on silica-based columns is the industry's workhorse for separating and quantifying soluble protein aggregates.
- Same is true for peptide analysis.
- Aggregation analysis of therapeutic proteins using SEC is almost always required for regulatory approval.

Size exclusion chromatography

- Dominant mode of separation for **polymers.**
- Separation is based on **hydrodynamic radii** of the sample molecule.
- Large sample molecules cannot or can only partially **penetrate the pores**, whereas smaller molecules can access most or all pores.
- Thus, large molecules elute first, smaller molecules elute later, while molecules that can **access** all the pores elute last from the column.

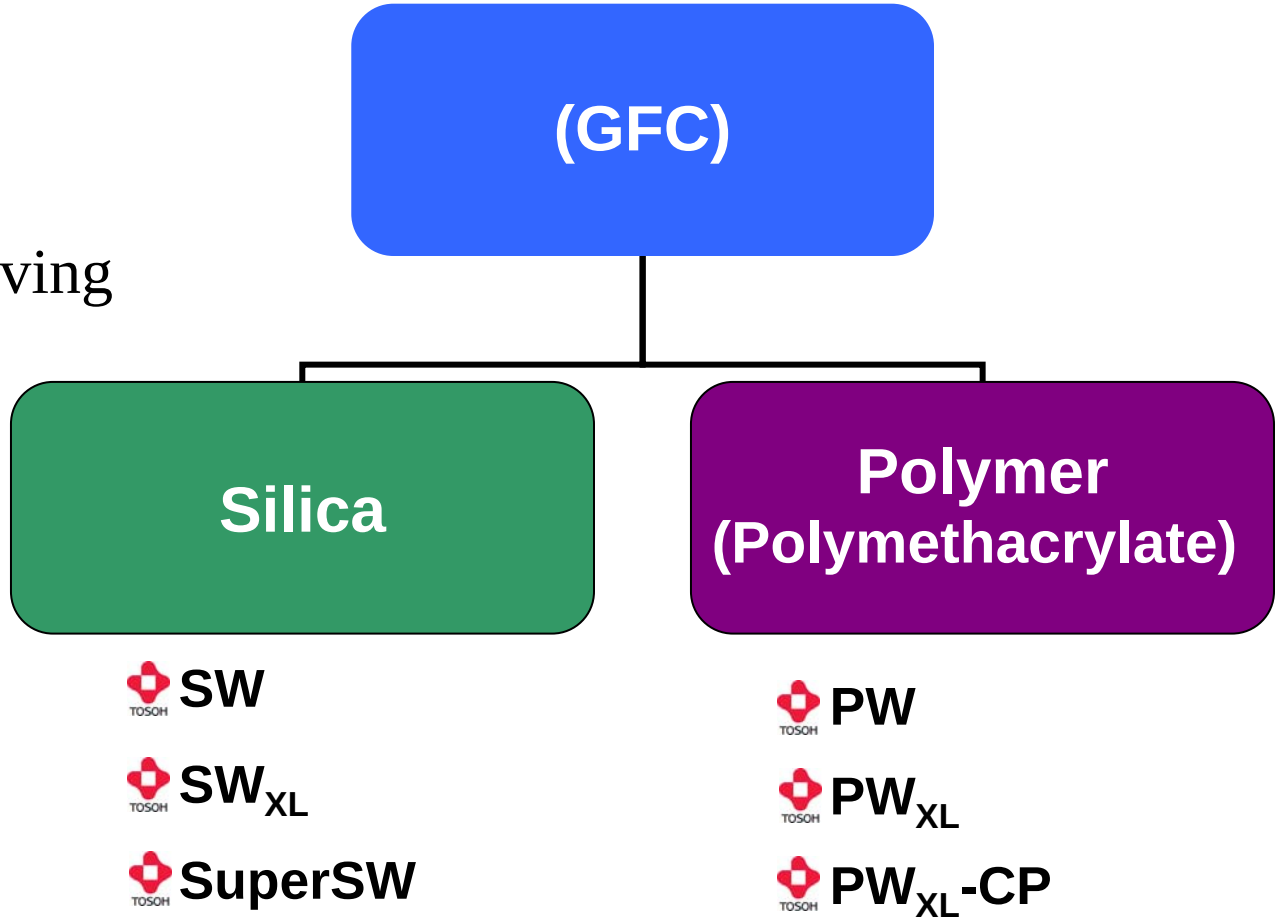
TSKgel size exclusion columns



TSKgel size exclusion columns

Gel Filtration Chromatography

S = Silica
P = Polymer
W= water loving



TSKgel SW column line

- **Silica-based**
  - **Rigid**
  - **Hydrophilic diol-type** bonded phase
    - shields the silica surface from protein samples
- **Pore sizes:**
  - 125Å – small proteins and peptides
  - 250Å – most protein samples
  - 450Å – very large proteins and nucleic acids

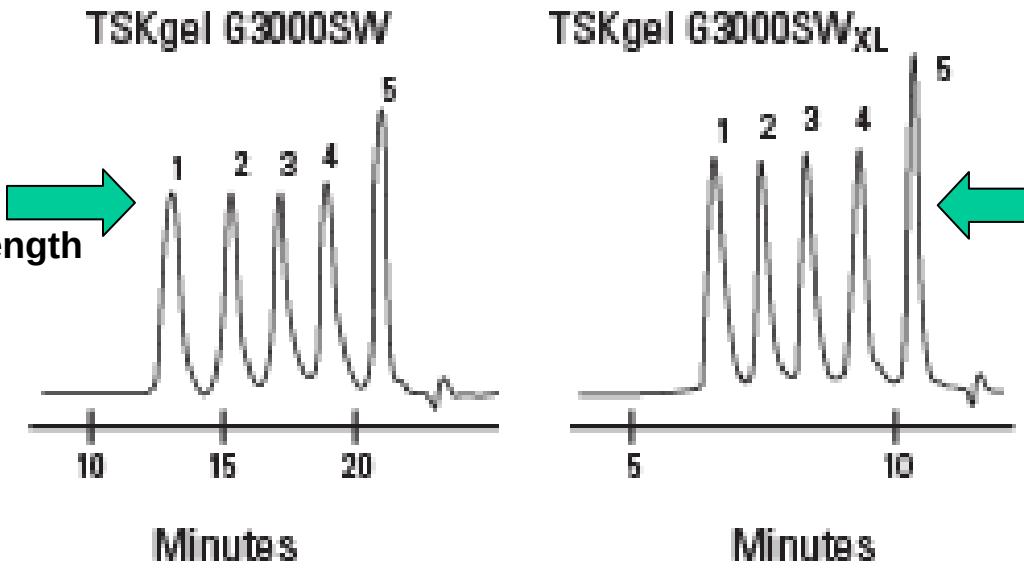
Pore volume per unit column volume:

- **SW-type columns >> competitive HPLC columns**
  - **Higher MW selectivity or better resolution**

- **Particle sizes:**
  - SW: 10-13µm – very large proteins
  - SW_{XL}: 5-8µm – QC of mAb, most proteins
  - SuperSW: 4µm – small protein and peptide

TSKgel SW column vs. TSKgel SW_{XL} column

Two 30 cm columns
60cm total column length

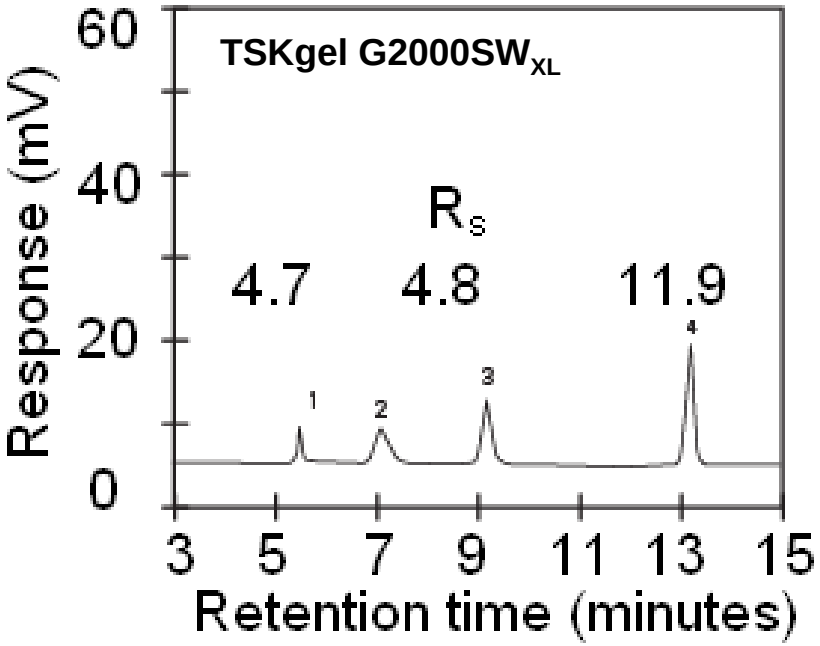


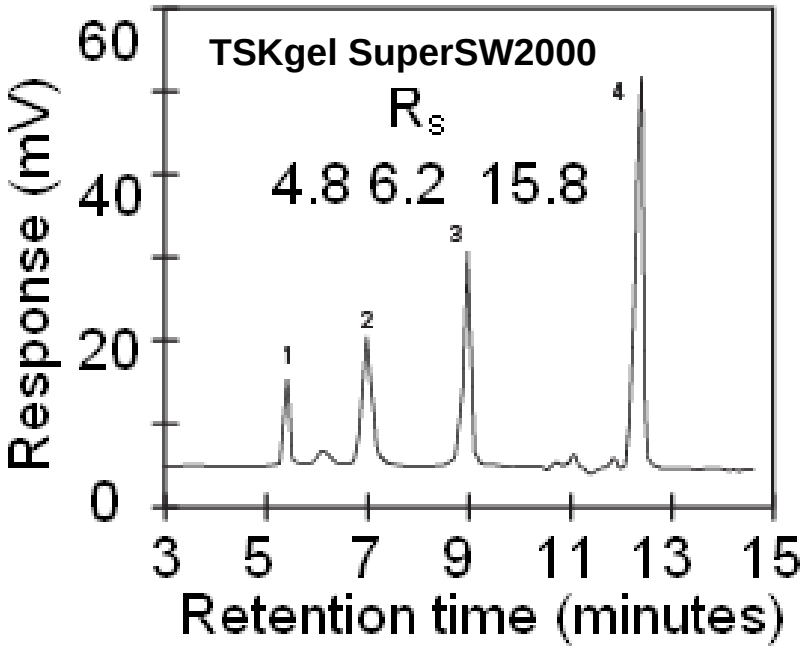
One 30cm column
30cm total column length

Shorter run time and higher resolution obtained with one, 5µm TSKgel SW_{XL} 30cm column when compared with two, 10µm TSKgel SW 30cm columns

Column:	Left: TSK-GEL SW, two 10µm 7.5mm x 30cm columns in series Right: TSK-GEL SW_{XL}, one 5µm, 7.8mm x 30cm column
Sample:	1. glutamate dehydrogenase (55,000Da) 2. lactate dehydrogenase (36,500Da) 3. enolase (67,000Da) 4. adenylate kinase (6,000Da) 5. cytochrome C (12,400Da)
Elution:	0.3M NaCl in 0.05M phosphate buffer, pH 6.9
Flow Rate:	1.0mL/min
Detection:	UV @ 220nm

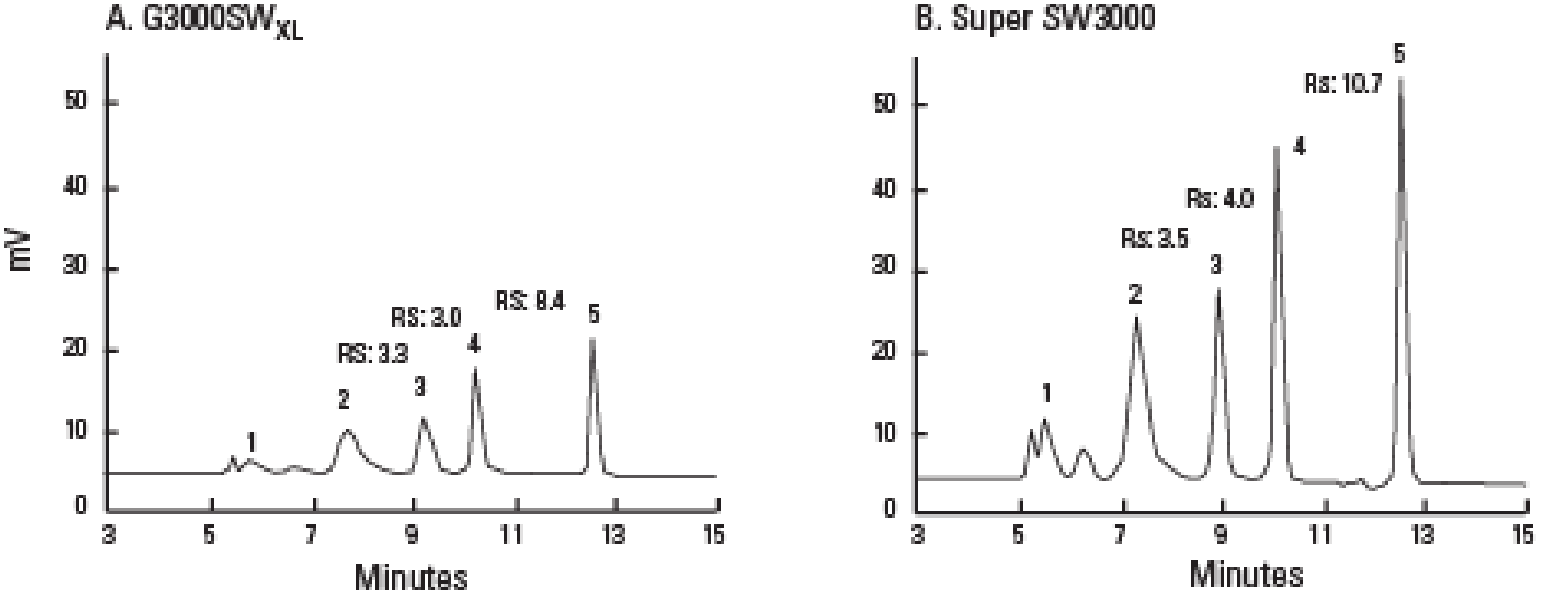
TSKgel G2000SW$_{XL}$ column vs. TSKgel SuperSW2000 column





Column:	A. TSKgel G2000SW$_{XL}$, 7.8mm x 30cm; B. TSKgel Super SW2000, 4.6mm x 30cm
Sample:	1. thyroglobulin (0.2mg/mL); 2. albumin (1.0mg/mL); 3. ribonuclease A (1.0mg/mL); 4. *p*-aminobenzoic acid (0.01mg/mL)
Injection Volume:	5µL
Elution:	0.1M phosphate buffer + 0.1M Na_2SO_4 + 0.05% NaN_3 (pH 6.7)
Flow Rate:	0.35mL/min for Super SW2000; 1.0mL/min for G2000SW$_{XL}$
Temperature:	25°C
Detection:	UV @ 280nm

TSKgel G3000SW$_{XL}$ column vs. TSKgel SuperSW3000 column



Column:	A.TSKgel G3000SW$_{XL}$, 7.8mm x 30cm; B. TSKgel Super SW3000, 4.6mm x 30cm
Sample:	5µL of a mixture of 1. thyroglobulin, 0.5mg/mL (660,000Da); 2. γ-globulin, 1.0mg/mL; (150,000 Da); 3. ovalbumin, 1.0mg/mL (43,000Da); 4. ribonuclease A, 1.5mg/mL (12,600Da); 5. *p*-aminobenzoic acid, 0.01mg/mL (137Da)
Elution:	0.1M Na_2SO_4 in 0.1M phosphate buffer with 0.05% NaN_3, pH 6.7
Flow Rate:	1.0mL/min for G3000SW$_{XL}$; 0.35mL/min for Super SW3000
Temperature:	25°C
Detection:	UV @ 220nm

Characteristics of SW and PW column lines

Column line	TSKgel SW / SW_{XL} / SuperSW	TSKgel PW / PW_{XL}
Resin type	Silica	Methacrylate
No. of available pore sizes	3/2	7
PH stability	2.5 - 7.5	2.0 - 12.0
Solvent compatability	100% polar	50% polar
Max. temp.	30°C	80°C*
Max. flow rate (mL/min)	1.2 (SW, SW_{XL}) 0.4 (SuperSW)	1.2 (PW) 1.0 (PW_{XL})
Pressure**(MPa)	0.8 - 1.2	1.0 - 4.0
Application focus	Proteins	Water-soluble polymers

* Except for the TSKgel G-DNA-PW, which can be operated up to 50°C and the 55 mm ID TSK-GEL PW-type columns, which can be operated up to 60°C. When operating below 10°C, reduce the flow rate to ensure that the maximum pressure is not exceeded.

** Depends on column dimensions and particle size

Note: The operating conditions and specifications for each column are listed on the Operating Conditions and Specifications sheet (OCS) shipped with the column.

TSKgel SW Series – Column Selection

Proteins (general)

Select appropriate pore size based on knowledge or estimate of protein MW

Protein of unknown molecular weight

TSKgel G3000SW$_{XL}$

Ideal investigational column (Scouting column)

If peak elutes near the exclusion volume

Switch to TSKgel G4000SW$_{XL}$

If peak elutes near the end of the chromatogram

Switch to TSKgel G2000SW$_{XL}$

TSKgel SW Series – Column Selection

Monoclonal antibodies

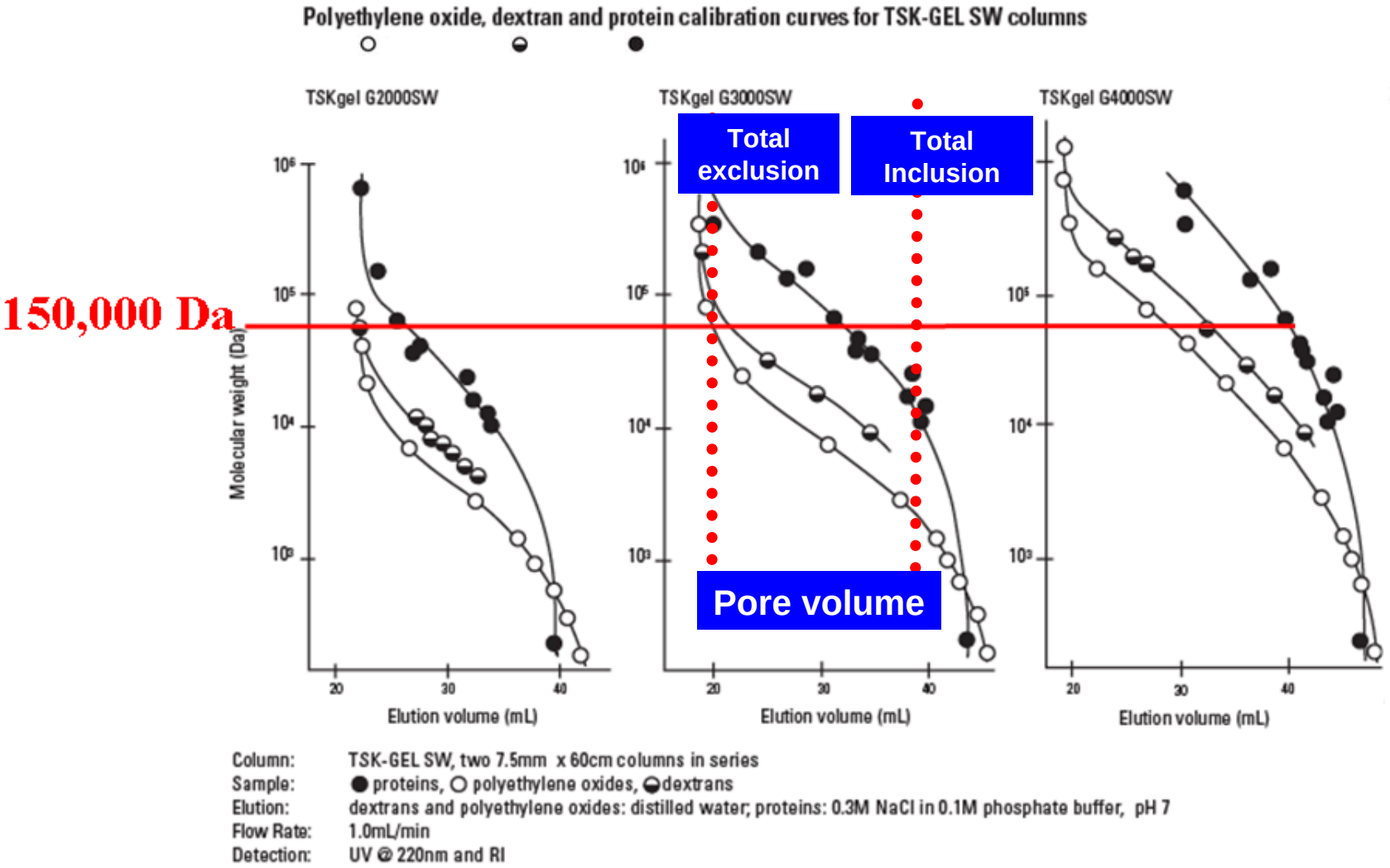
TSKgel G3000SW$_{XL}$ columns

Widely used for quality control

TSKgel SuperSW3000 columns

When sample is limited or very low concentration

TSKgel SW Series – Column Selection



Monoclonal antibodies (mAbs) have a MW of about 150,000 and would fall in the area indicated on the calibration curves. Most of our customers use TSKgel G3000SW and TSKgel G3000SW$_{XL}$ columns for their mAb analysis.

TSKgel SW_{XL} – Column Selection

Molecular mass separation range (Da) of TSKgel SW_{XL} Columns			
Column	**Polyethylene glycol (Straight Chain)**	**Dextran (Branch)**	**Protein (Globular)**
G2000SW_{XL}	**500~15,000**	**1,000~30,000**	**5,000~100,000**
G3000SW_{XL}	**1,000~35,000**	**2,000~70,000**	**10,000~500,000**
G4000SW_{XL}	**2,000~250,000**	**4,000~500,000**	**20,000~7,000,000**

TSKgel SW Series – Column Selection

Peptides

TSKgel G2000SW$_{XL}$

first choice

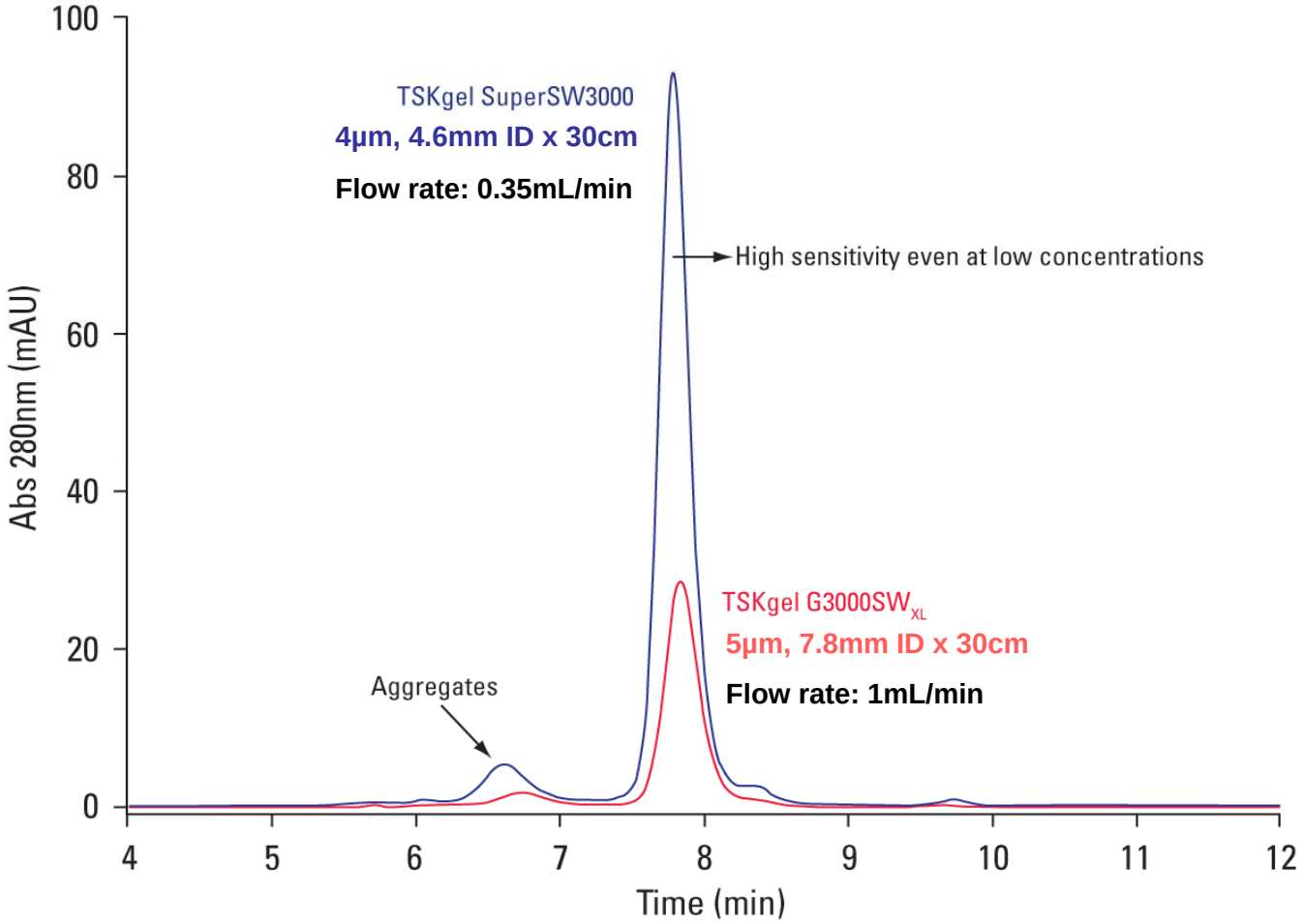
TSKgel SuperSW2000 columns

when sample is limited or very low concentration, also for 2.6 fold reduction in solvent consumption

Other

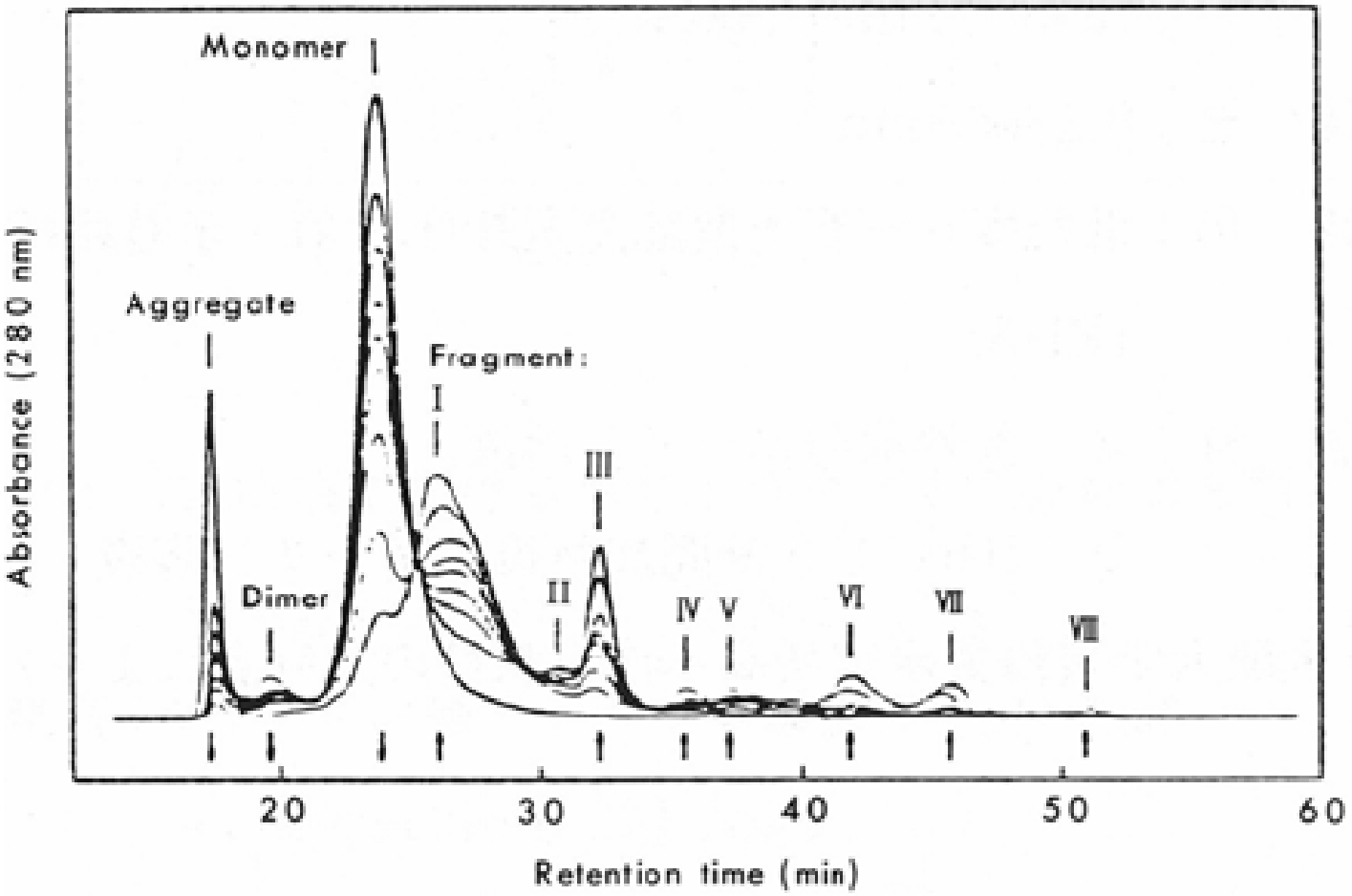
TSKgel SW columns for (preparative) product isolation

Analysis of purified mAb



Mobile phase: 0.1mol/L phosphate buffer (mono/dibasic), 0.1mol/L NaCl, 0.05% NaN_3, pH 6.8;
Flow rate: 1mL/min; Detection: UV@280nm (micro flow cell); Injection volume: 5µL

Tracking changes over time in degradation products of commercial IgG by pepsin



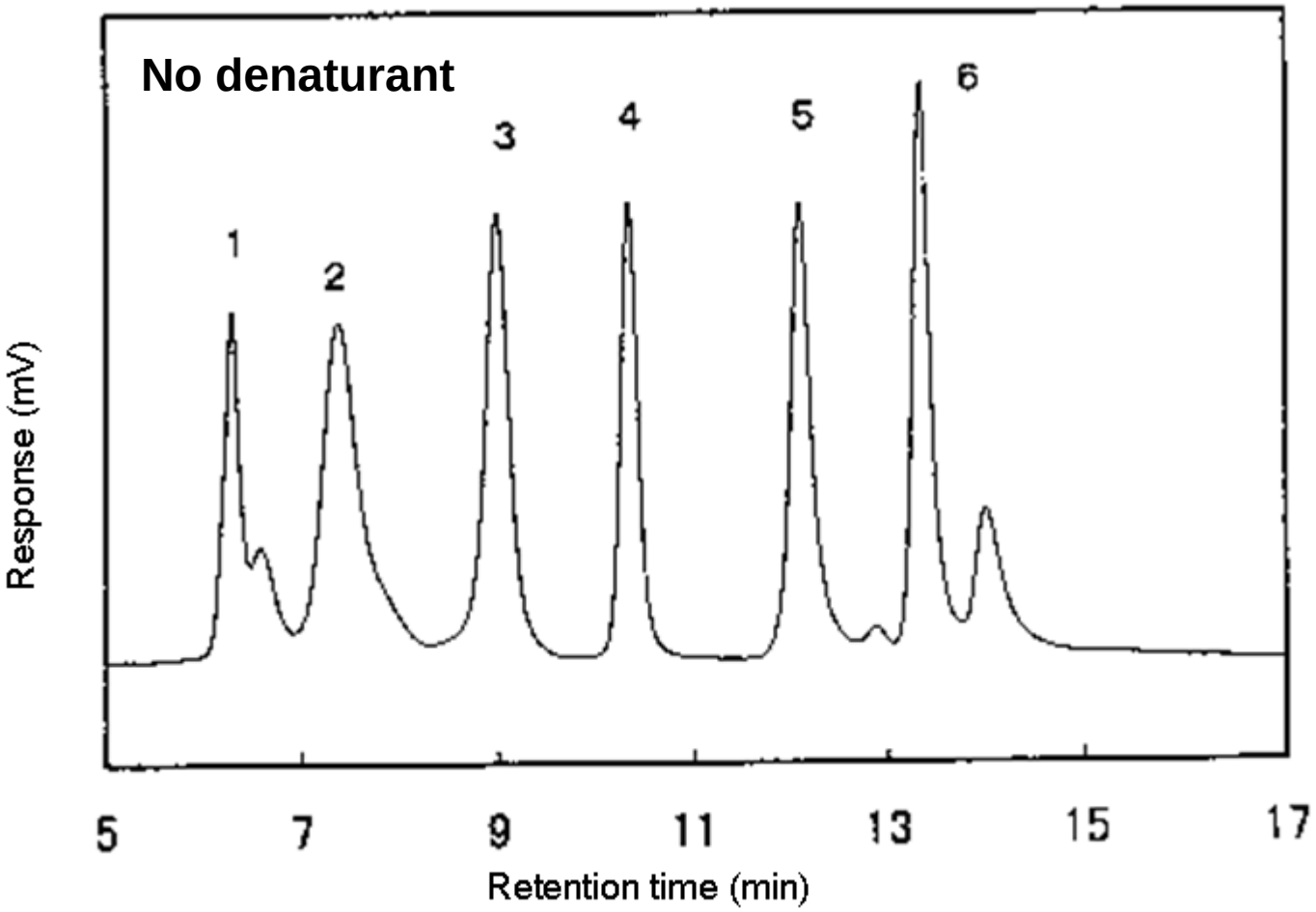
Column: TSKgel G3000SW, 7.5mm ID x 60cm x 2 Solvent: 0.05 mol/L acetate buffer (pH 5.0) + 0.1 mol/L sodium sulfate
Flow rate: 1.0mL/min Samples*: 100µL solutions produced by digestion of IgG (20g/L) by pepsin after 0, 2, 4, 6, 8, 10, 15, 30, and 60min

Different buffer conditions for the separation of proteins and peptides

Characteristic	Ordinary buffer	SDS	Guanidine-HCl	Urea
1. Native or denaturated	□ native	denaturated	denaturated	denaturated
2. Separation range	○ wide	narrow	medium	medium
3. Linearity I) MW > 10,000	good	○ very good	□ excellent	good
2) MW < 10,000	not good	× bad	○ very good	good
4. Sensitivity to ionic strength	sensitive	× very sensitive	○ not sensitive	sensitive
5. Corrosion concern	not severe	not severe	× severe	not severe
6. Operating cost	○ low	○ low	× high	low
Typical	sodium	0.2% SDS 0.2mol/L SP	6mol/L Guanidine-HCl	8mol/L urea

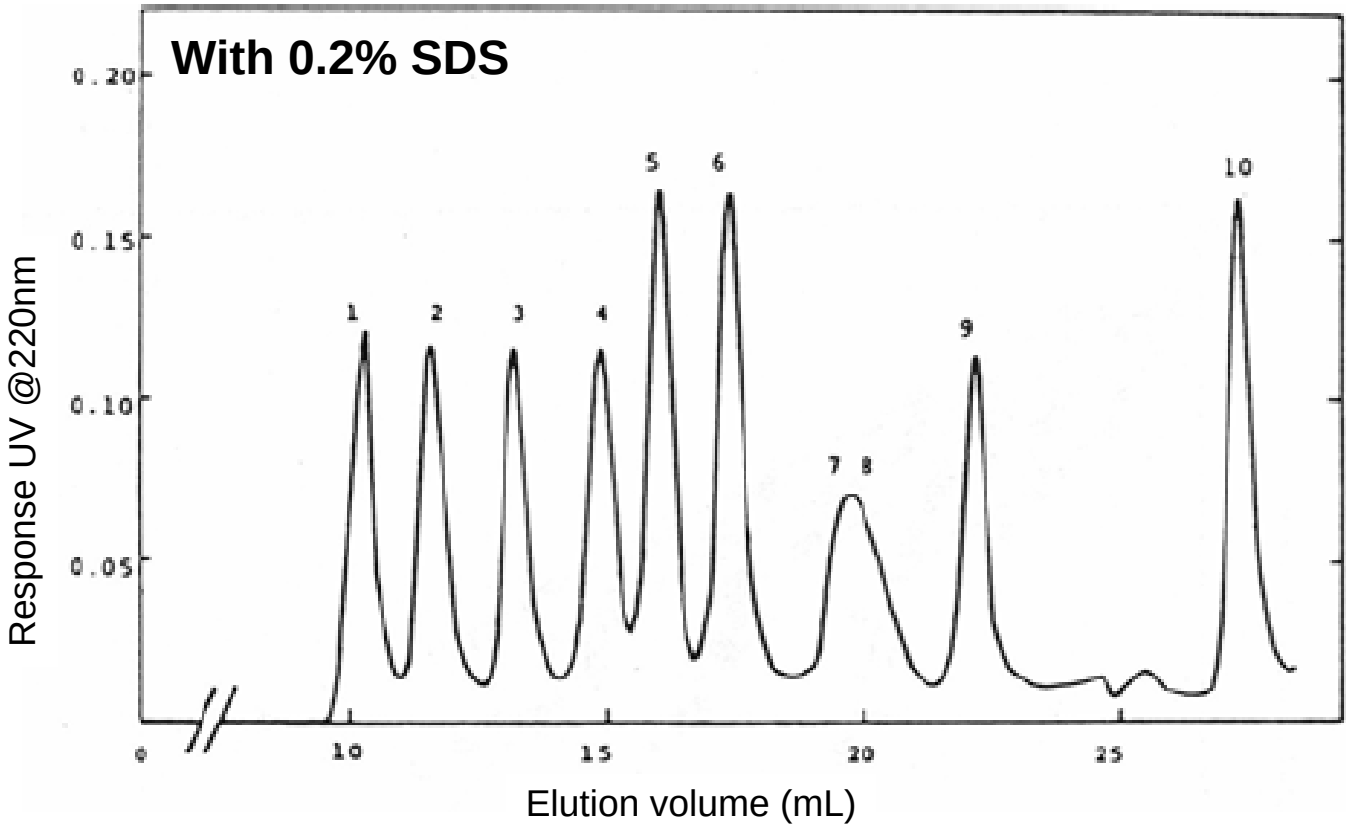
But surfactants change the bonding phase permanently so you need to dedicate the column for the particular application.

Separation of polypeptides by TSKgel G3000SW column



Column: TSKgel SuperSW2000, 4.6mm ID x 30cm, Mobile phase: 0.2mol/L phosphate buffer, pH 6.7, Flow rate: 0.35mL/min, Detection: UV@220nm, Injection vol.: 5µL, Sample load: 0.10mg/mL

Separation of mixture of peptides in SDS system

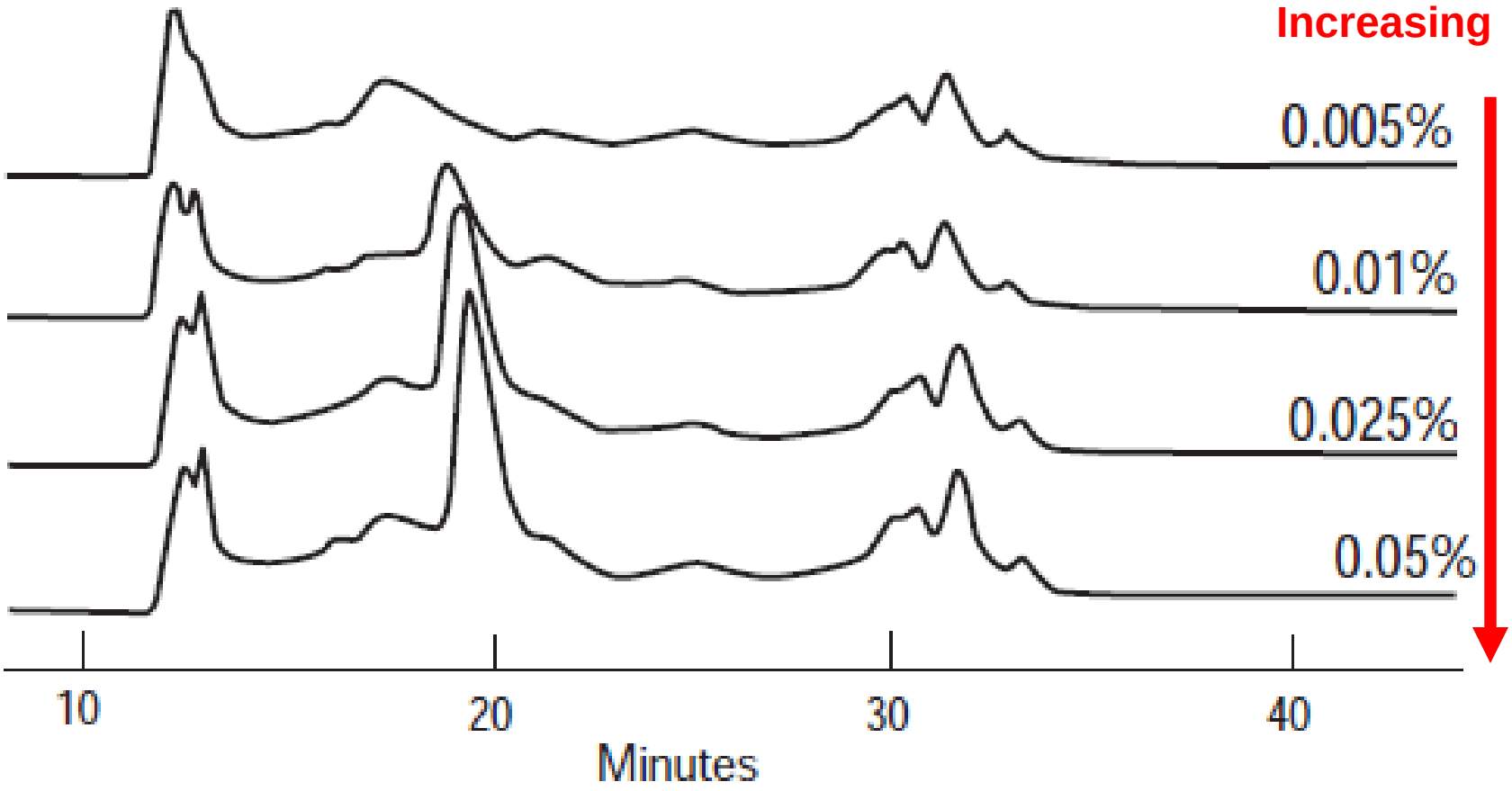


1. blue dextran
2. serum albumin
3. pepsin
4. tripsinogen
5. myoglobin
6. cytochrome C
7. aprotinin
8. insulin B chain
9. insulin A chain
10. 2-mercaptoethanol

Column: TSKgel G3000SW, 7.5mm ID x 60cm Solvent: 0.2mol/L sodium phosphate buffer (pH 7.0) + 0.2% SDS + 0.2mol/L NaCl Flow rate: 0.5mL/min Detection: UV@280 nm

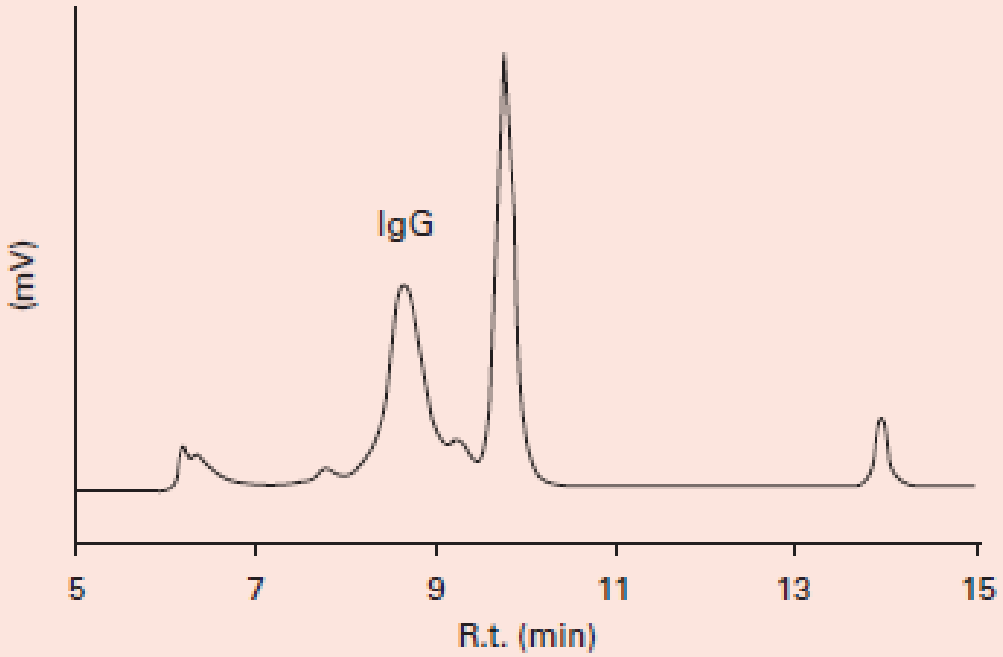
Effect of surfactants on SEC

Octa ethylene glycol dodecyl ether – nonionic surfactant



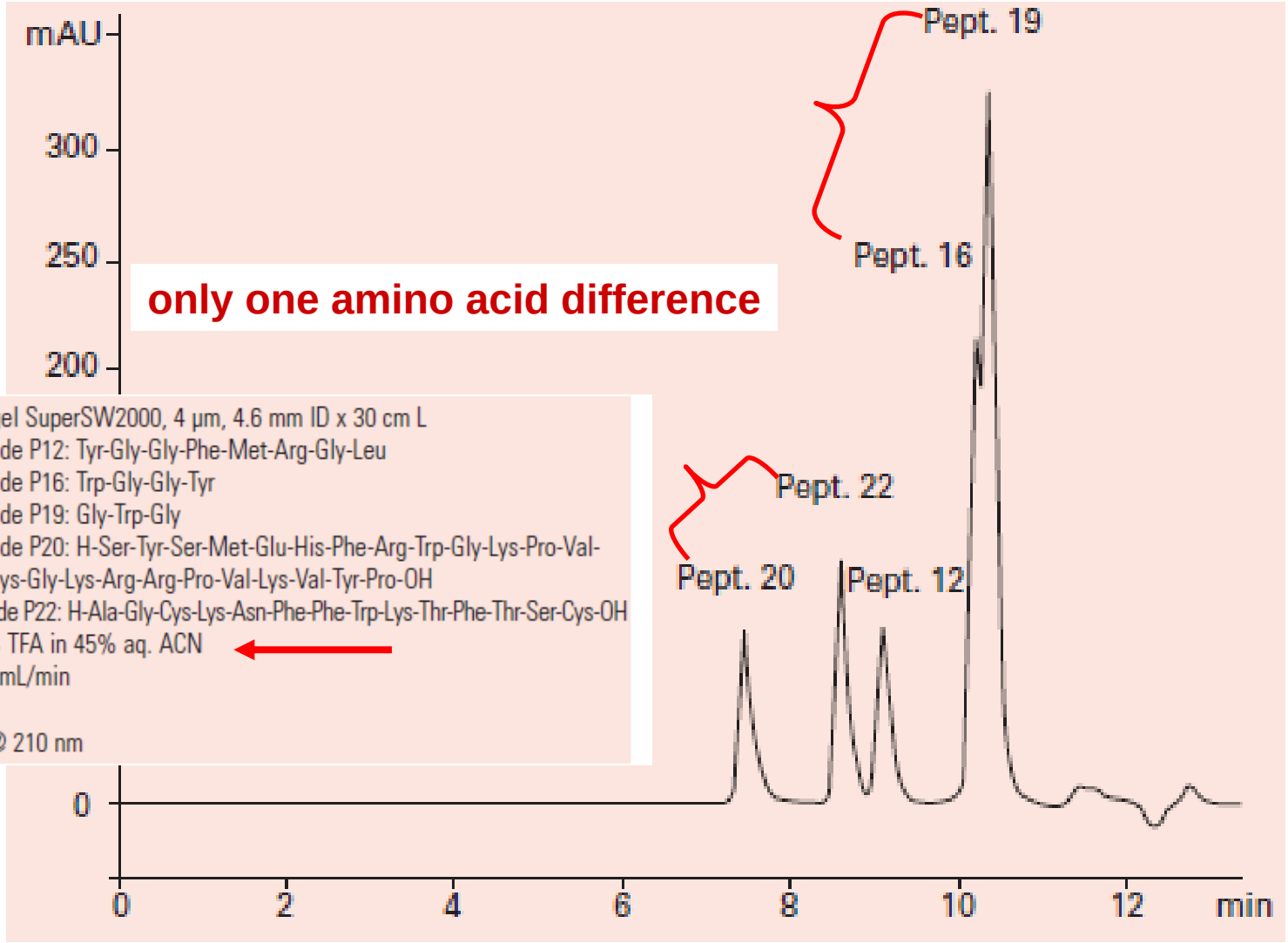
As the concentration of the surfactant increases from 0.005% to 0.05% the main peak becomes sharper and recovery increases.

Separation of IgG from human serum albumin



Column:	TSKgel SuperSW3000, 4 µm, 4.6 mm ID x 30 cm L
Sample:	Human serum
Elution:	0.2 M phosphate buffer (pH 6.7)
Flow rate:	0.35 mL/min
Injection vol:	5 µL
Detection:	UV @ 280 nm

Separation of peptides using TSKgel SuperSW2000 column



Column: TSKgel SuperSW2000, 4 µm, 4.6 mm ID x 30 cm L
Sample: Peptide P12: Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu
Peptide P16: Trp-Gly-Gly-Tyr
Peptide P19: Gly-Trp-Gly
Peptide P20: H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Gly-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH
Peptide P22: H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH
Elution: 0.1% TFA in 45% aq. ACN
Flow rate: 0.35 mL/min
Injection vol: 3 µL
Detection: UV @ 210 nm

Sample load in SEC

In SEC, sample load on the column is limited due to the absence of a stationary phase that participates in the retention process.

High sample loads

column overload

distort peak shapes

cause an overall decrease in efficiency

Optimal sample load highly depends on

the sample properties (sample matrix)

the separation task

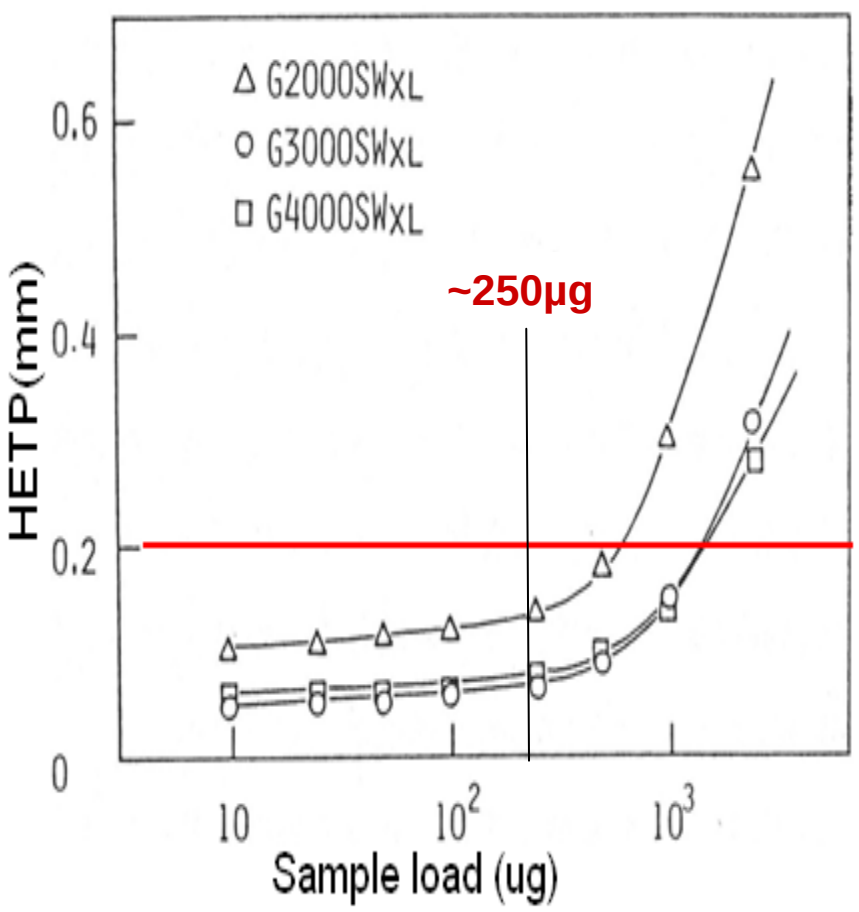
For analytical columns – recommended load is 1-20 mg/mL
Proteins can be loaded at higher concentrations and higher total loads than synthetic macro-molecules.

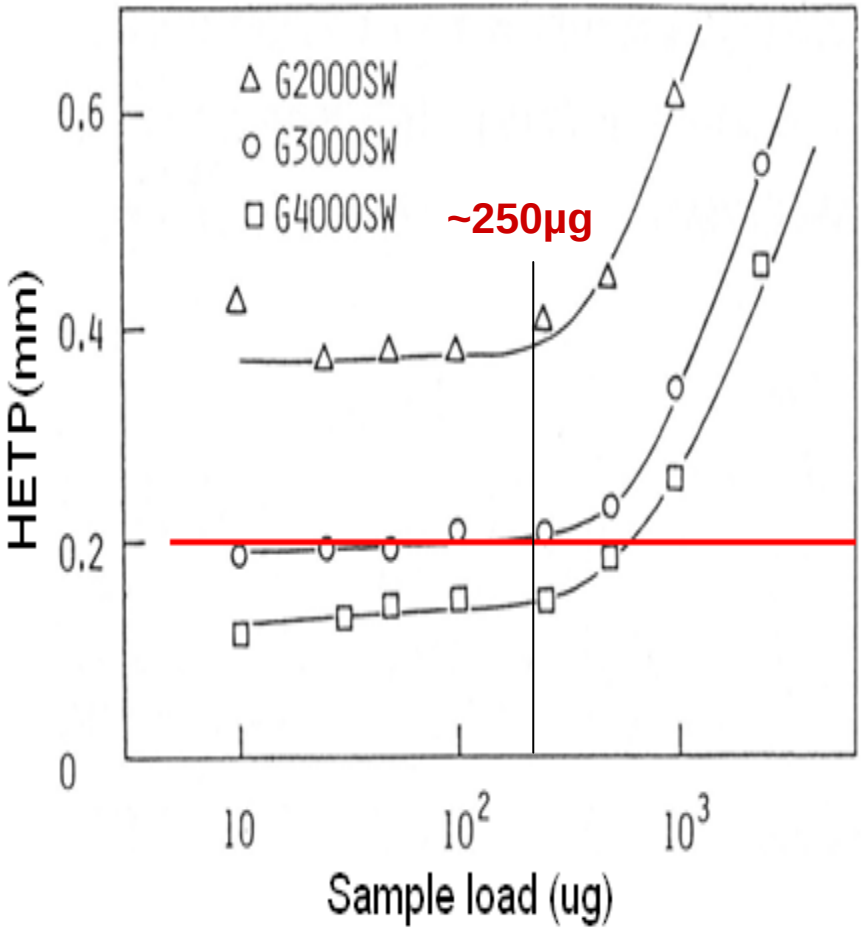
For preparative purposes

100 mg of BSA can be loaded on two 21.5mm ID x 60cm TSKgel G3000SW columns, but only 20 mg of PEG 7500.

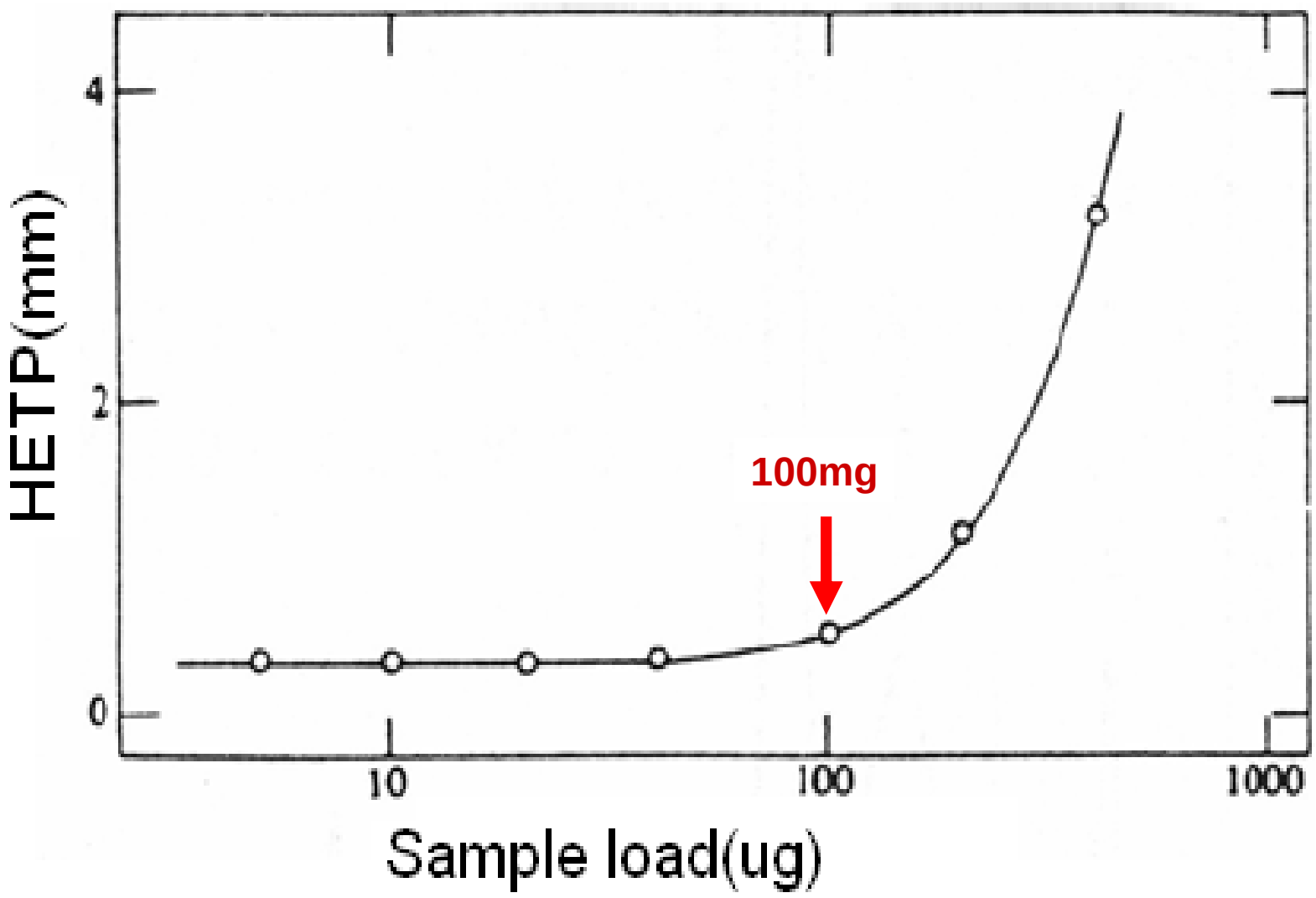
Dependence of HETP on sample load

Sample: Bovine Serum Albumin (BSA)





Dependence of HETP on sample load

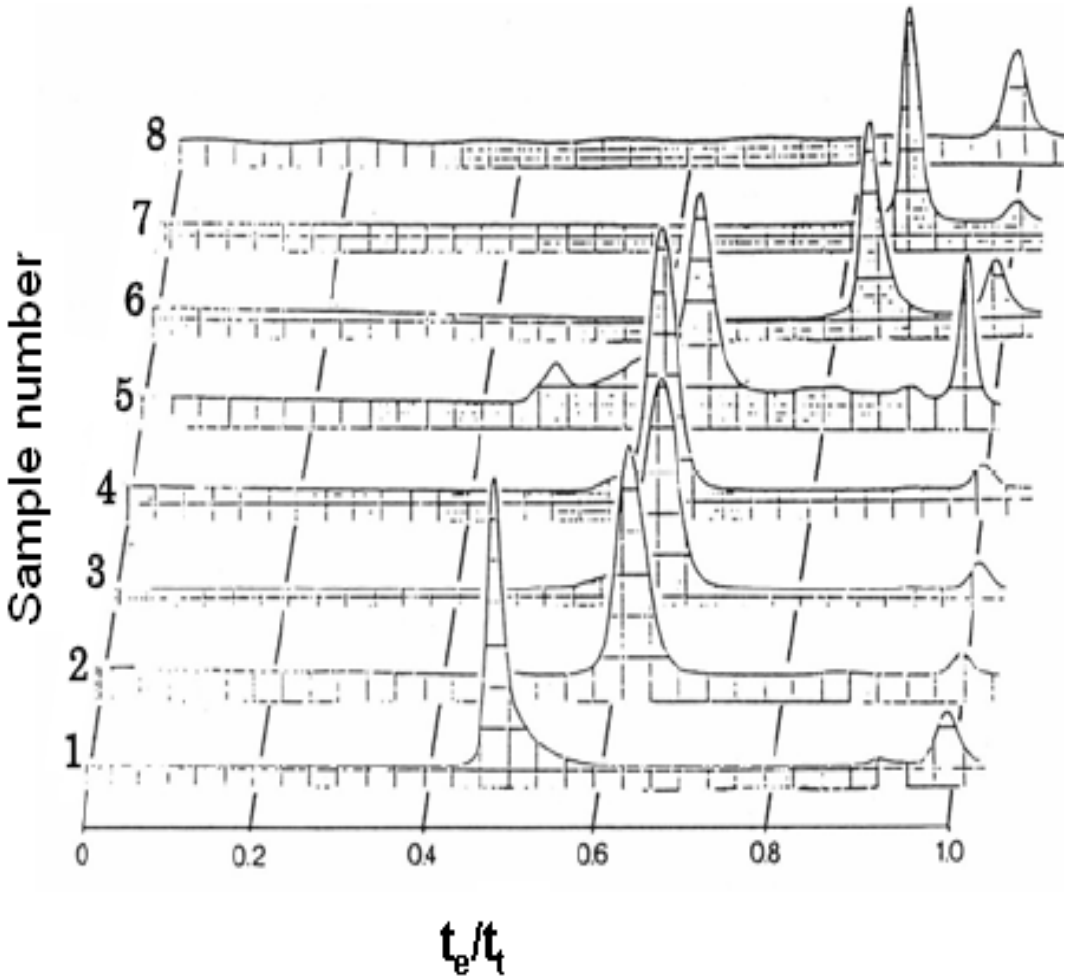


Protein recovery %

	Sample load				
	1 µg	5 µg	10 µg	50 µg	100 µg
G2000SW$_{XL}$					
Ribonuclease A	95	83	96	98	94
Thyroglobulin	107	92	101	-	-
γ-globulin	103	109	116	98	107
G3000SW$_{XL}$					
Ribonuclease A	96	97	97	95	94
Thyroglobulin	92	97	101	99	91
γ-globulin	106	103	97	97	108
G4000SW$_{XL}$					
Ribonuclease A	104	106	103	103	94
Thyroglobulin	78	90	91	102	101
γ-globulin	91	90	107	97	104

The recovery was virtually quantitative, **regardless of the sample load.**

Separation of virus and protein by TSKgel G5000PW



Column: TSKgel G5000PW, 21.5mm ID x 60cm

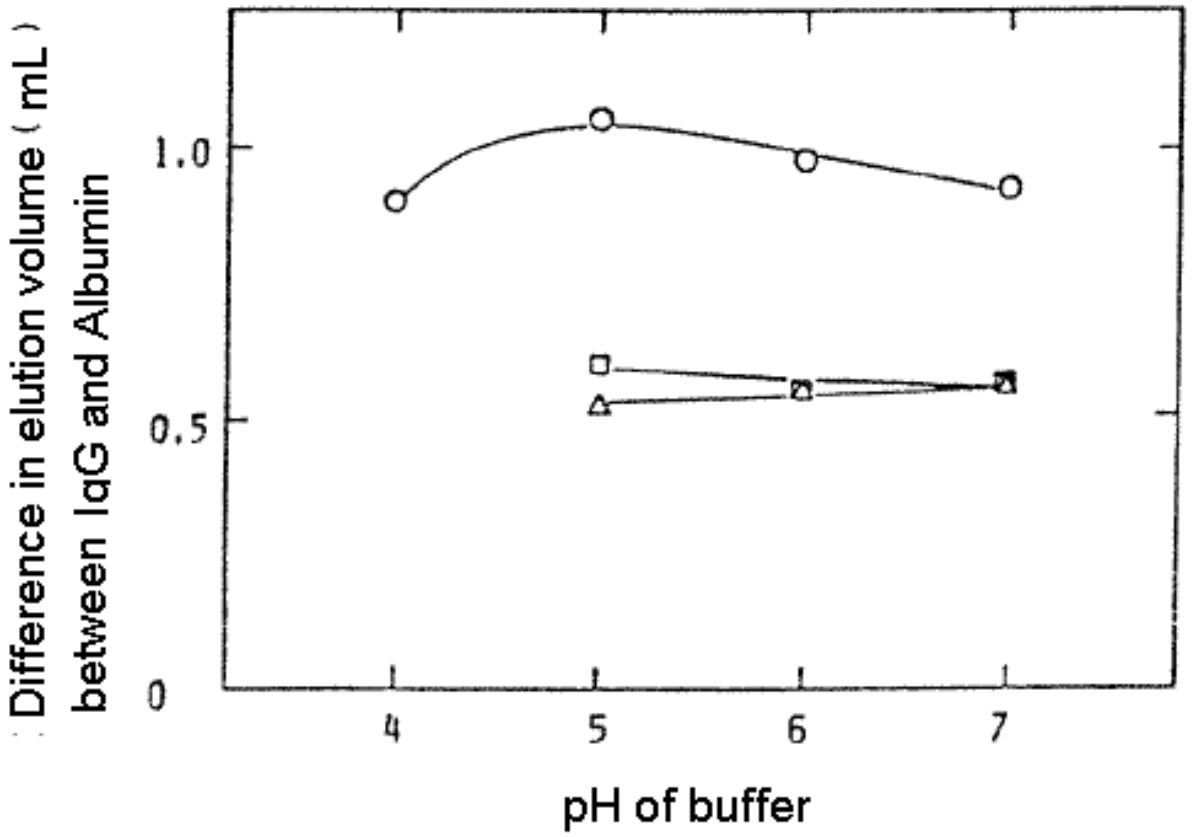
Solvent: 0.01mol/L phosphate buffer (pH 7.0) + 0.1mol/L potassium chloride

Flow rate: 0.96mL/min

Detection: UV@280 nm

Samples:
1. TMV (tobacco mosaic virus)
2. TBSV (tomato bushy stunt virus)
3. SBMV (southern bean mosaic virus)
4. TYMV (turnip yellow mosaic virus)
5. apoferritin dimer
6. hemoglobin
7. myoglobin
8. cytochrome C

Effect of buffer composition on separation



50mmol/L sodium phosphate at various pH containing

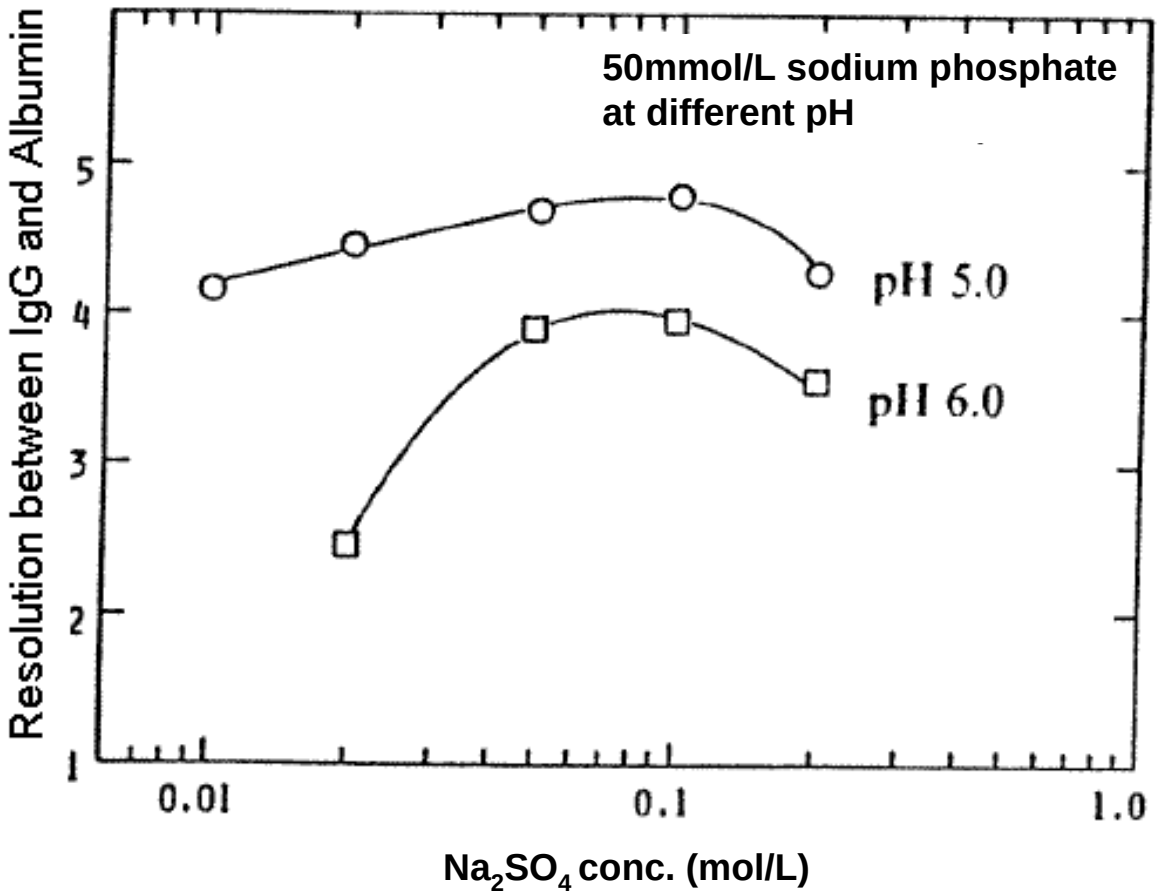
(○) **0.1mol/L Na_2SO_4**

(□) **0.3mol/L NaCl**

(△) **0.3mol/L $NaClO_4$**

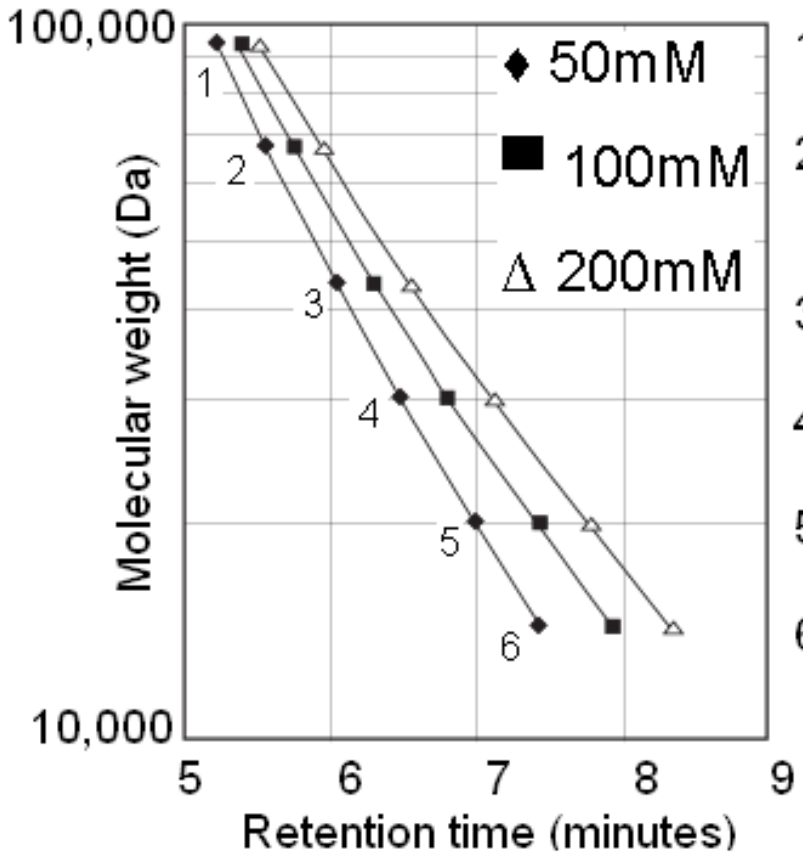
Column: TSKgel G3000SW$_{XL}$

Effect of buffer composition on separation



Column: TSKgel G3000SW$_{XL}$

Effect of buffer composition on calibration curves



1. Phosphorylase (94,000Da)
2. BSA (67,000Da)
3. Ovalbumin (43,000Da)
4. Carbonic anhydrase (30,000Da)
5. Soybin trypsin inhibitor (20,100Da)
6. alpha-lactalbumin (14,000da)

Sample: proteins Proteins are denatured in phosphate buffer containing SDS and DTT at 40°C during 15 min.
Eluent: 0.1M phosphate buffer (pH 6.8) containing 0.1% SDS Flow Rate: 0.35mL/min
Temperature: 25°C Detection: UV @ 280nm

Column: TSKgel SuperSW

Influence of Buffer on Recoveries

Protein	Recovery%		
	Sodium Phosphate	Potassium Phosphate	Tris-HCl
Cytochrome C	98	101	92
Lysozyme	92	96	75
α-Chymotrypsinogen	95	98	90
IgG	95	98	88
Thyroglobulin	94	94	85
Ovalbumin	96	92	66

Column: TSKgel G3000SW, 7.5mm × 60cm,
Mobile phase: A: 0.2M NaH_2PO4 and 0.2M Na_2HPO4, pH6.9
B: 0.2M KH_2PO4 and 0.2M K_2HPO4, pH6.9
C:0.02M NaCl and 0.05M Tris-HCl, pH7.8
Flow rate: 1.0mL/min
Detection: UV@220nm

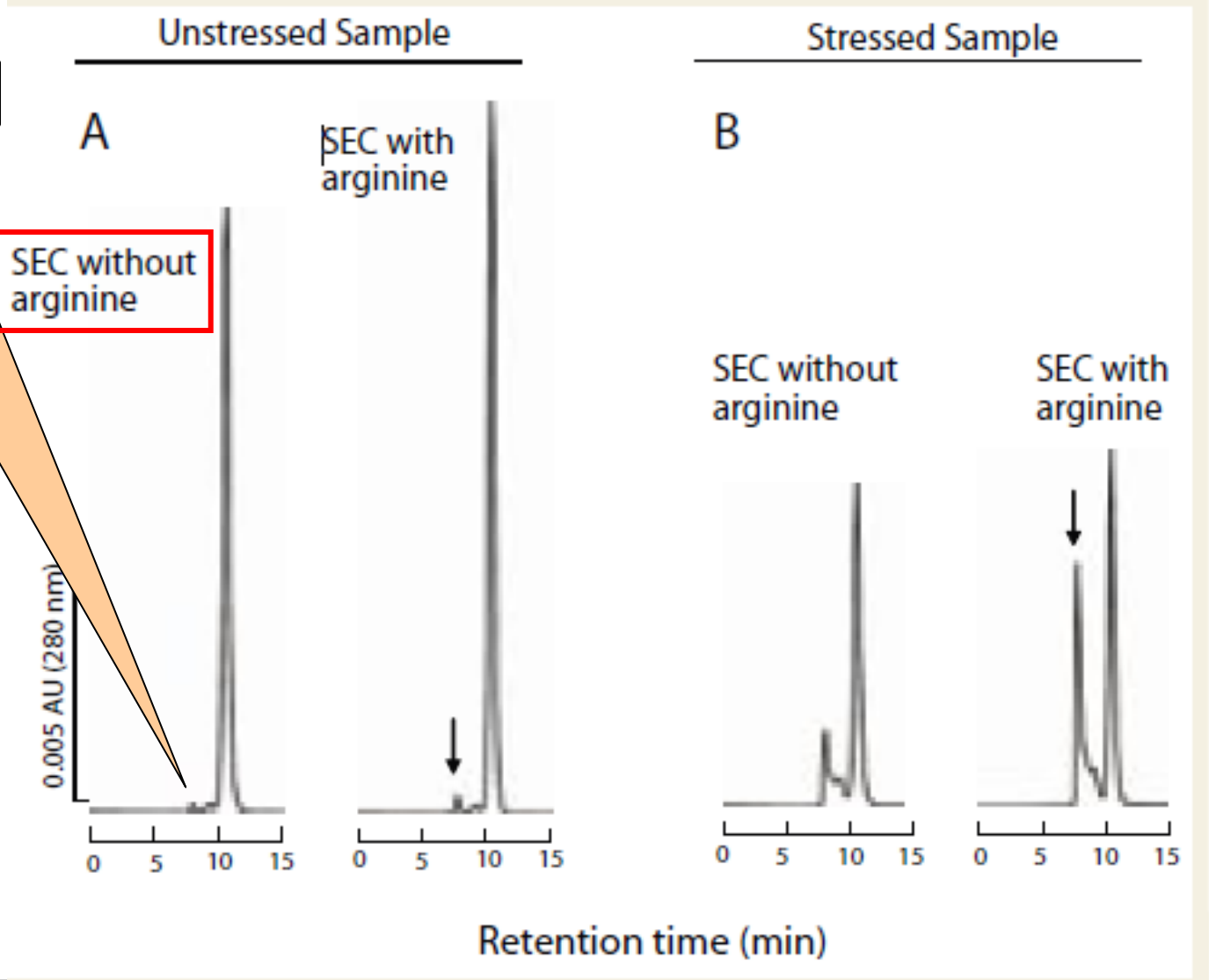
Effect of arginine on recovery of aggregates

Analytical SEC for MAb-a using a TSKgel G3000SW$_{XL}$ column

2% total aggregates

Elution solvent 0.1mol/L phosphate, pH 6.8, in the absence and presence of 0.2mol/L arginine.

To generate aggregates, the MAb sample was subjected to low pH and heat treatment (in the absence of arginine)



Ref: Arisaka F. et al., Bioprocess International, 4(10) 42-43 (November 2006)

Effect of arginine on recovery of aggregates

Analytical SEC for MAb-a using a TSKgel G3000SW$_{XL}$ column

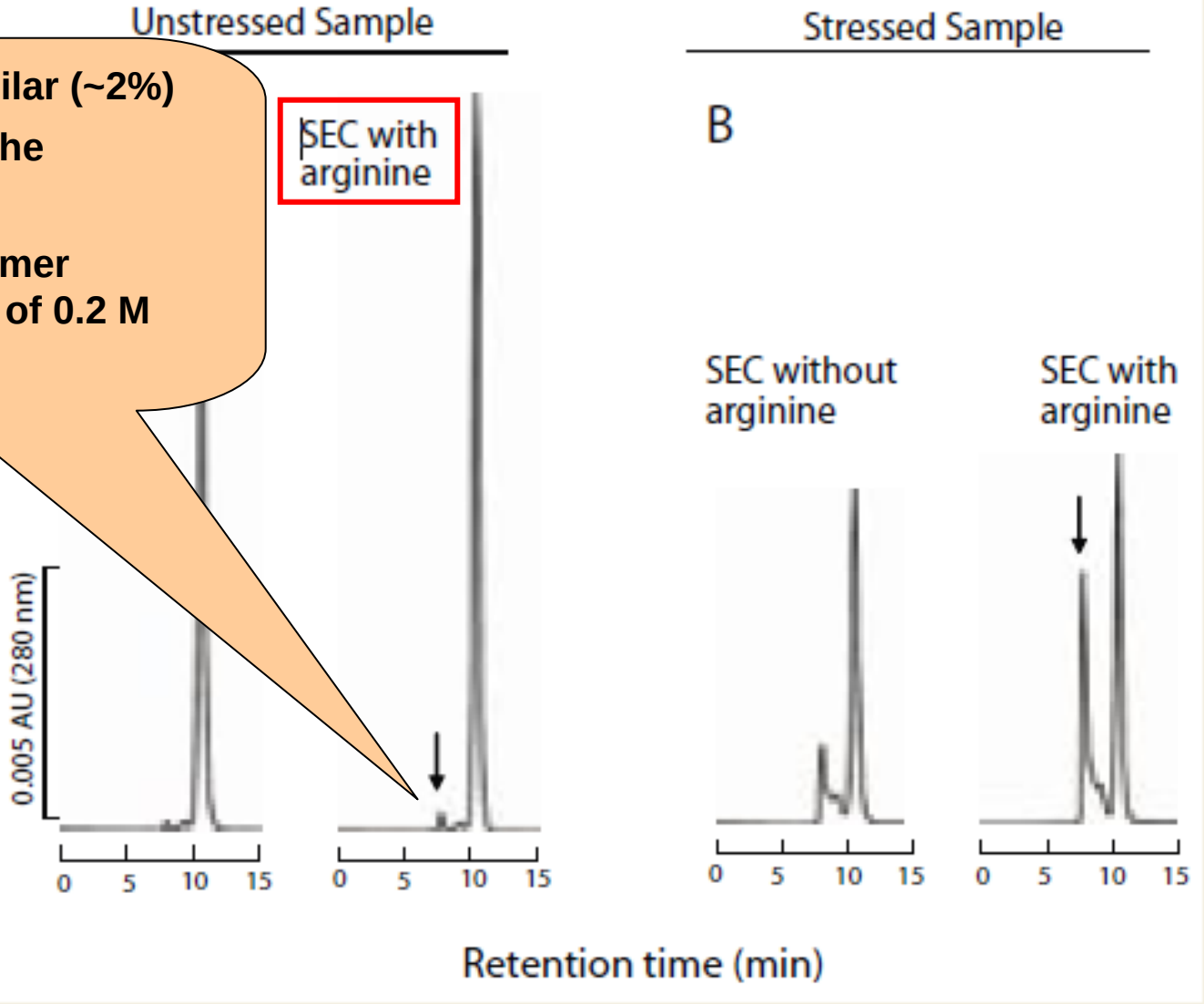
Aggregate content is similar (~2%)

Clear increase in one of the aggregate species

No decrease in the monomer recovery in the presence of 0.2 M arginine

Elution solvent 0.1mol/L phosphate, pH 6.8, in the absence and presence of 0.2mol/L arginine.

To generate aggregates, the MAb sample was subjected to low pH and heat treatment (in the absence of arginine)



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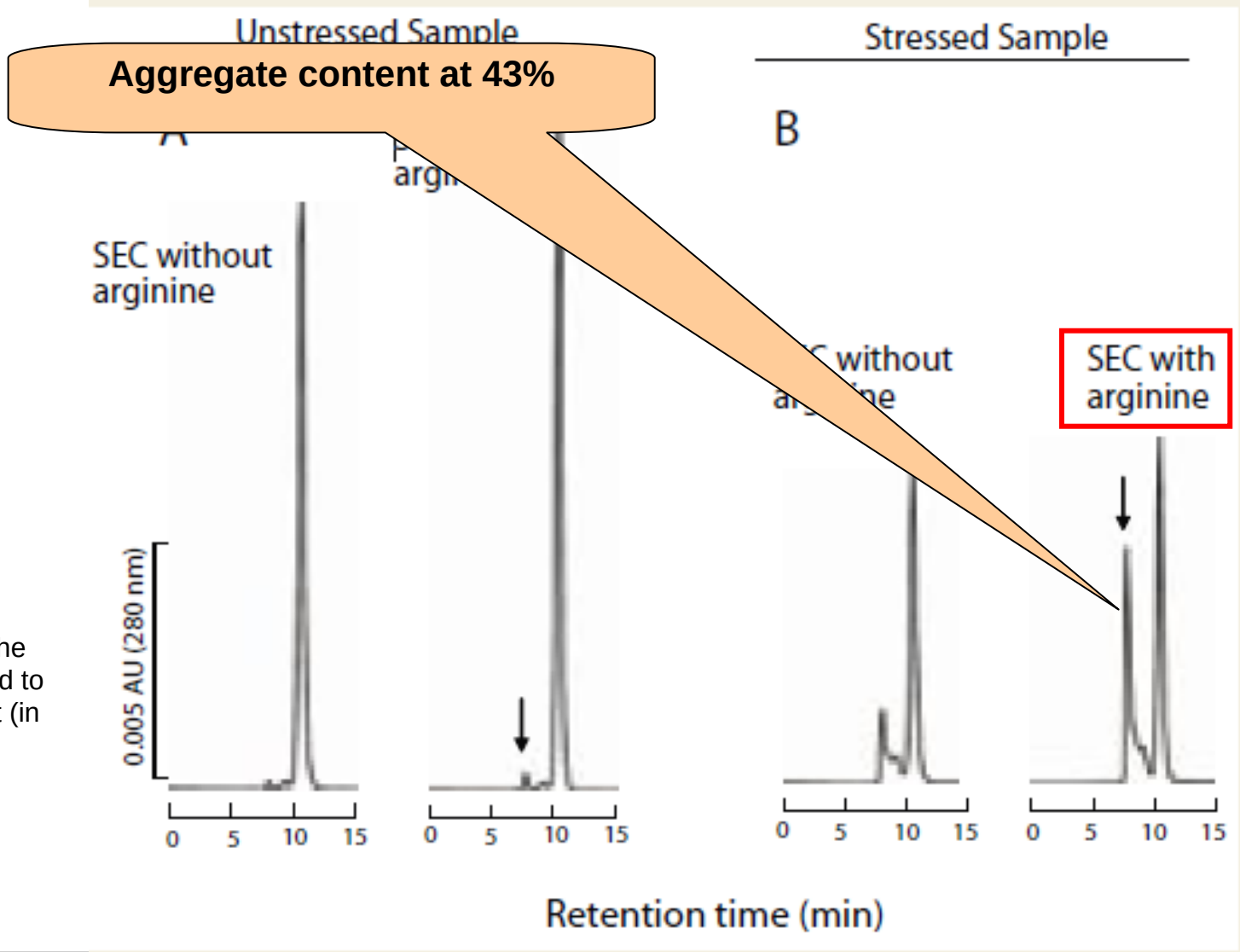

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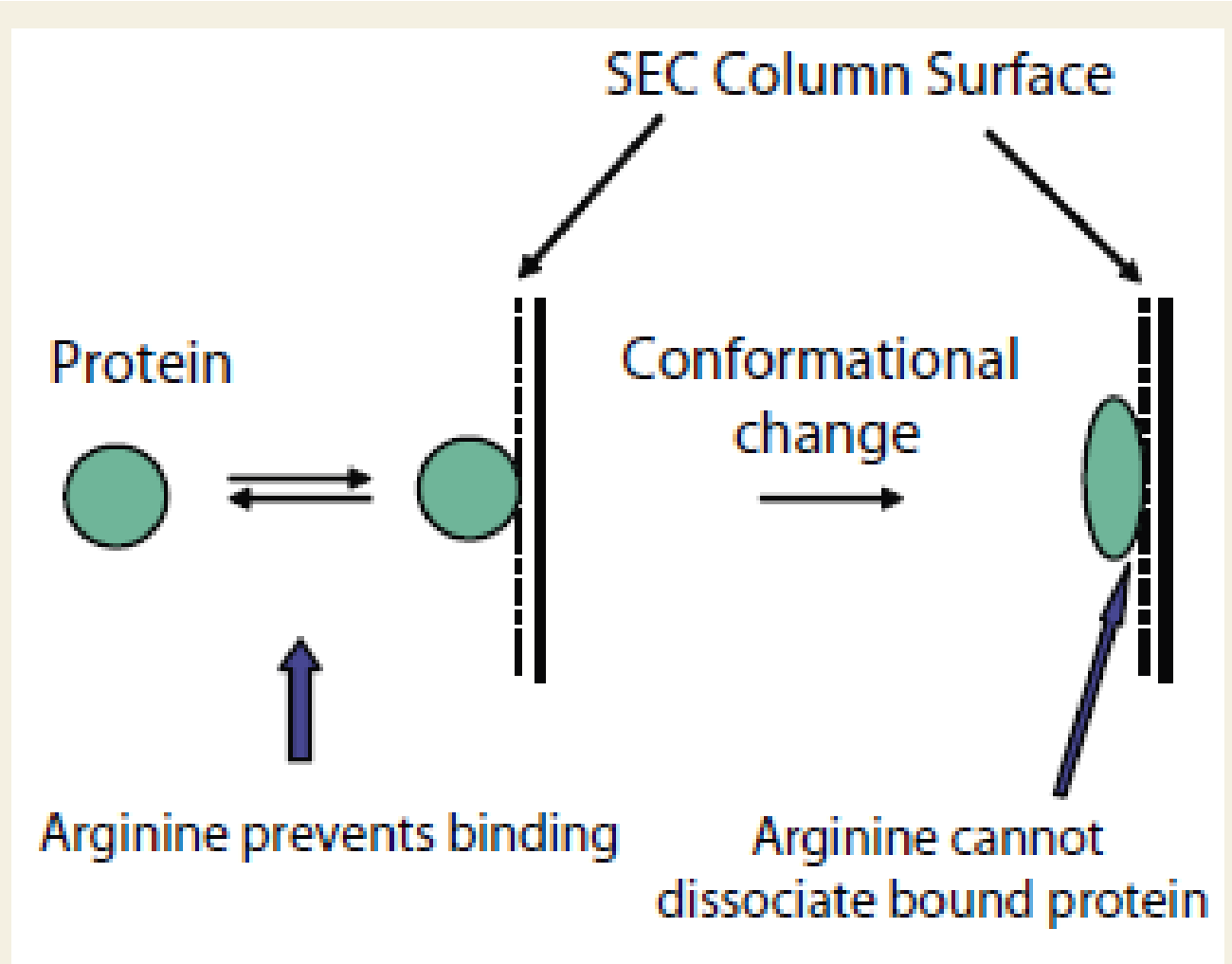
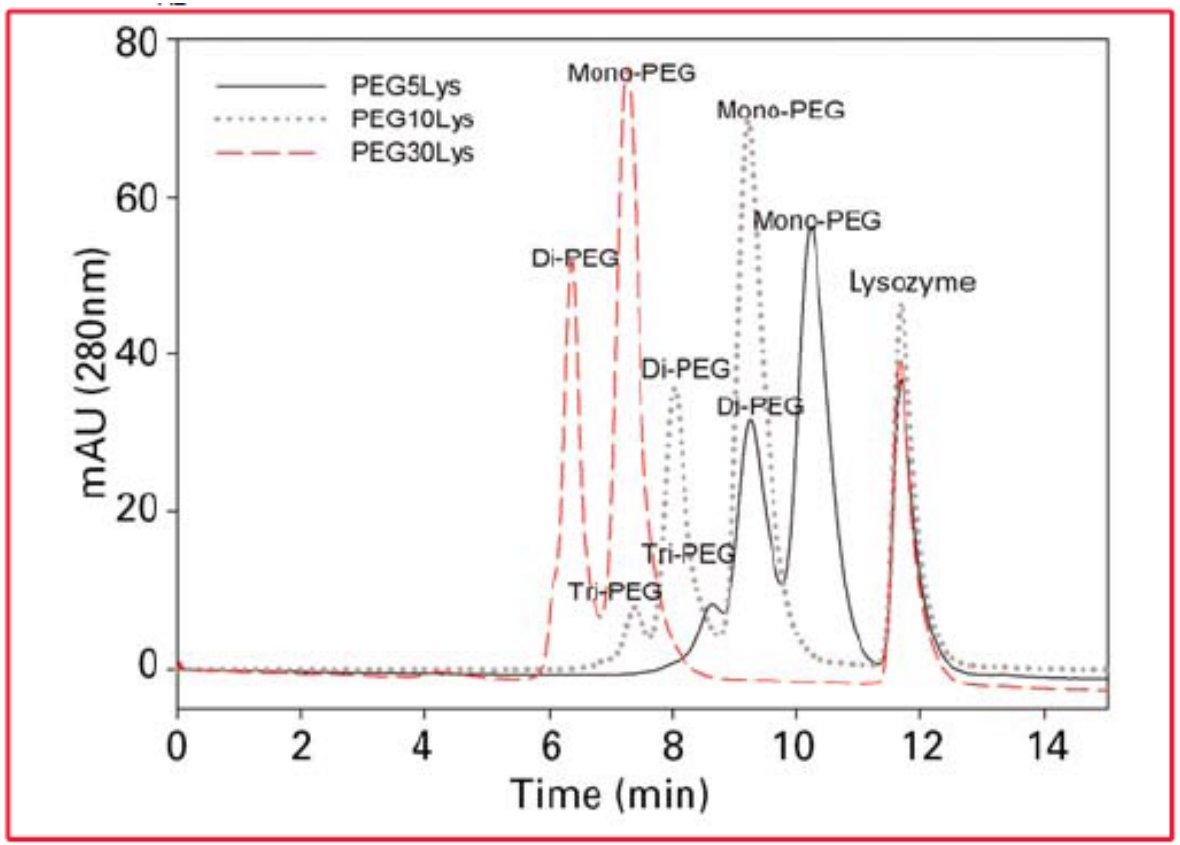
Ref: Arisaka F. et al., Bioprocess International, 4(10) 42-43 (November 2006)

Mechanism



Ref: Arisaka F. et al., Bioprocess International, 4(10) 42-43 (November 2006)

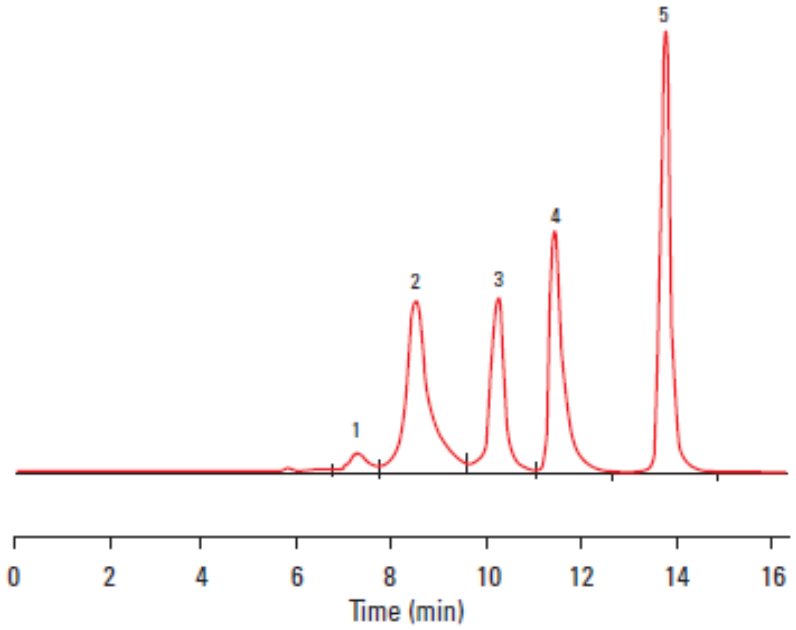
Separation of PEGylated proteins



Column: TSKgel G3000SW$_{XL}$, 5µm, 7.8mm ID x 30cm
Mobile phase: 0.1mol/L phosphate buffer, 0.1mol/L Na_2SO_4, pH 6.7
Flow rate: 1.0mL/min
Detection: UV@280nm
Injection vol.: 20µL

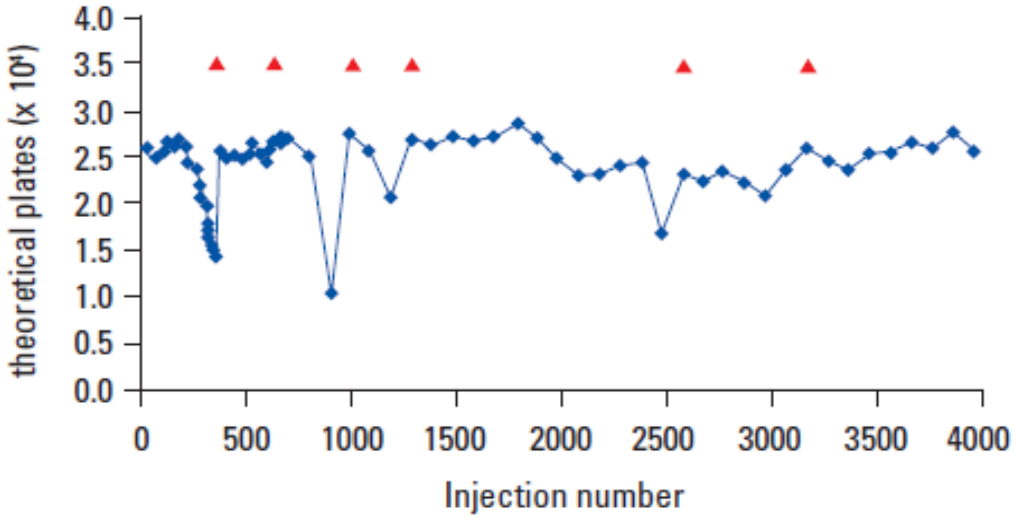
A. Moosmann et al.; J. Chromatogr. A (2010) 1217 (2): 209-215

Stability of the SEC column



Column:	TSKgel SuperSW3000, 4.6mm ID x 30cm
Guard column:	TSKgel SuperSW3000, 4.6mm ID x 3.5cm
Injection:	5µL
Eluent:	0.1M phosphate buffer, 0.1M Na_2SO_4, 0.05% NaN_3
Flow:	0.35mL/min
Temperature:	ambient
Detection:	280nm, 1µL cell volume, 0.2 second response time
Peaks:	1 = impurity 2 = g-globulin (156 kDa, 1mg/mL) 3 = ovalbumin (43 kDa,1mg/mL) 4 = cytochrome C (12.4 kDa, 0.5mg/mL) 5 = PABA (137 Da, 0.01mg/mL) in mobile phase

Stability of the SEC column



Guard column:	TSKgel SuperSW3000, 4.6mm ID x 3.5cm
Injection:	5µL
Eluent:	0.1M phosphate buffer, 0.1M Na_2SO_4, 0.05% NaN_3
Flow:	0.35mL/min
Temperature:	ambient
Detection:	280nm, 1µL cell volume, 0.2 second response time
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Mobile phase and secondary interaction

What is secondary interaction?

Ionic and hydrophobic interaction between the sample and the column packing material

- Low ionic strength (< 0.1 M) – ionic interactions
- high ionic strength (>1.0 M) – hydrophobic interactions

How to prevent?

Maximize molecular sieving mechanism

What to do?

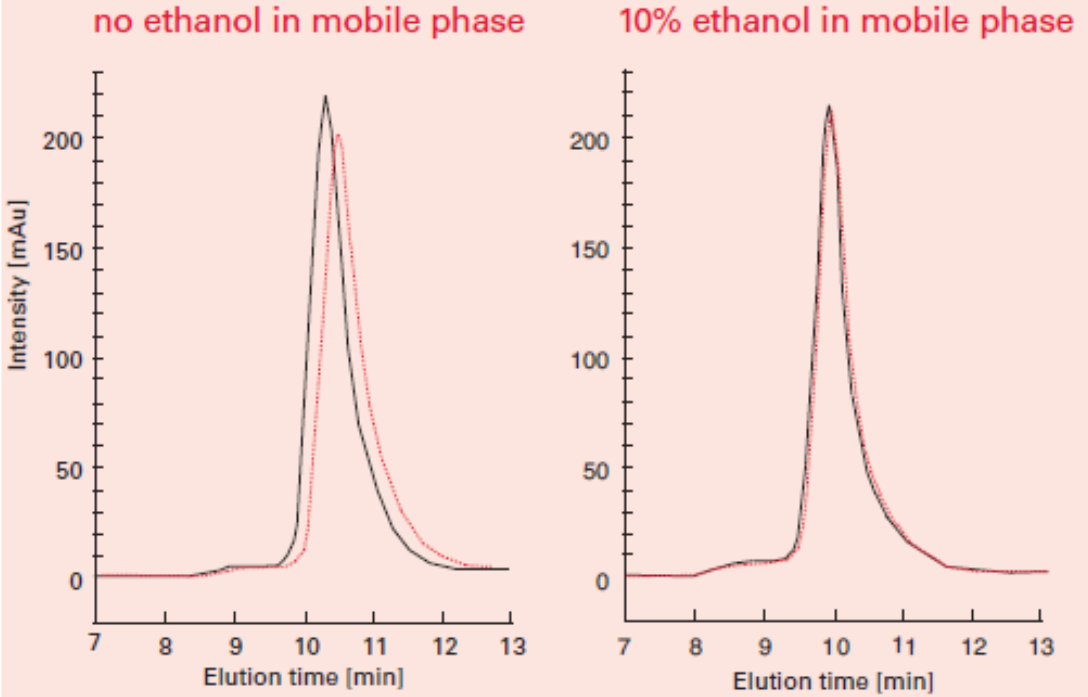
Select proper mobile phase

For each sample there will be an optimum buffer type and concentration that results in the highest resolution and recovery.

You must find it out by trial and error approach.

Mobile phase and secondary interaction

SEC of pegylated proteins



Column:	G3000SW$_{XL}$, 5 µm, 7.8 mm ID x 30 cm L
Sample:	10 mL of PEG-r-HuMGDF
	——— initial Injection
	····· after 150 injections
Mobile phase:	0.1 M sodium phosphate, pH 6.9, with 0.5 M NaCl
Flow rate:	0.7 mL/min
Detection:	UV @ 215 nm

(courtesy : J.J. Ratto et al. Amgen Inc., 1996)

Mobile phase and secondary interaction

Overlay plot of the Protein A sample pre and post-treatment with BSA

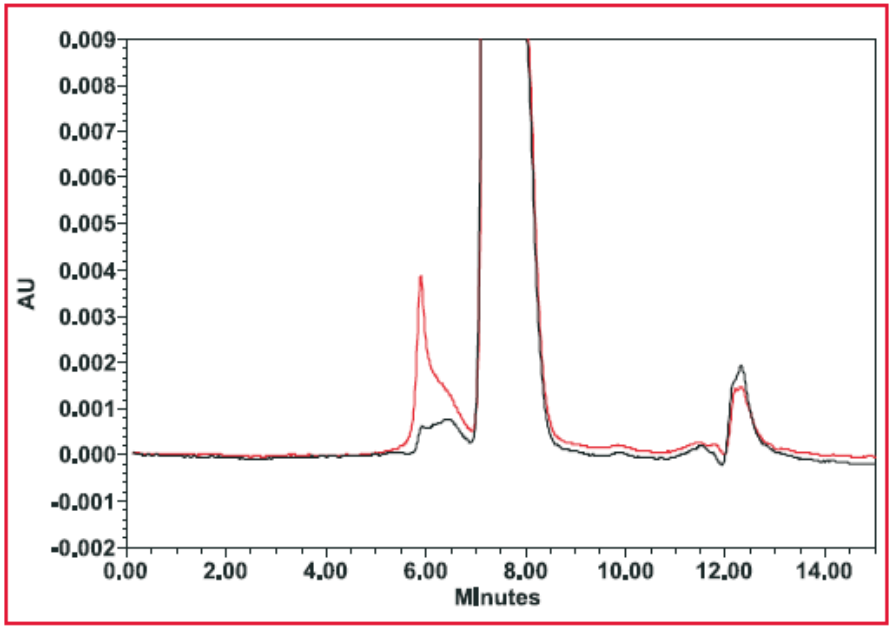


Table 1: Recoveries of all three types of samples pre and post-treatment with BSA

Sample	% HMW pre treatment	% HMW post treatment
Post Protein A	1.1	3.4
Post Polishing chromatographic step1	1.5	1.5
Post Polishing chromatographic step 2 (final product)	0.2	0.2

Mobile phase and secondary interaction

Recommendation for TSKgel SW columns:

- Buffer concentration: 0.1 – 0.5mol/L
- Add neutral salt e.g. sodium sulphate to increase buffer ionic strength.
- Using polymeric TSKgel PW and Alpha-type columns? Use low salt in the mobile phase.
- Non-ionic, non-polar compounds – just use water.
- Ionic polymeric compounds, add neutral salt such as sodium nitrate (0.1 – 0.2mol/L) is sufficient to overcome undesirable ionic interactions.
- Hydrophobic interaction – use water soluble organic solvent in the mobile phase.