Analysis of Monoclonal Antibody in Critical Quality Attributes by UHPLC/HPLC (under Non-Denatured Condition)

Tosoh Corporation

Content

- Characteristics of antibody
  - Risk analysis of quality, Critical quality attribute, USP regulation
  - Characteristics of antibody
  - Analytical tool, chromatographic technique
- Chromatographic technique
  - Non-denature chromatography (SEC)
  - Bridge from HPLC to UHPLC for SEC
  - Charge variants by Ion-exchange chromatography (IEC)
  - Chromatographic analysis by sugar moiety (AFC)
  - HIC analysis of antibody-drug conjugate (ADC)
  - Denature chromatographic technique (RPC, HILIC)

Risk Analysis of mAb Pharmaceutical Quality (A-Mab Case Study)

		Property	Impact (A)	uncertainty (B)	Risk score (A x B)	Risk judgement
Structure of mAb	Sugar	Galactose	16	3	48	High
		Fucose	20	3	60	High
		Sialic acid	12	5	60	High
		High mannose type	16	5	80	Very High
		Non-glycosyl	16	5	80	Very High
	Amino acid	Oxidation	4	3	12	Low
		Truncation of C terminal lysine	2	2	4	Very Low
		Deamidation	2	2	4	Very Low
	Aggregate	Aggregate	12	5	60	High
Impurities	Host cell	DNA	2	3	6	Very Low
		HCP	12	3	36	Middle
	Raw materials	Protein A	16	1	16	Low
		Methotrexate	16	1	16	Low

Original data: Report of CMC-biotech Working Group, A-Mab: a Case Study in Bioprocess Development was modified.

UHPLC/HPLC TSKgel® Columns for CQAs in mAb

Category	Quality attribute	Methods	TSKgel Column
Physicochemical characterization			
Primary structure	Amino acid sequence	Red. RP-HPLC-ESI-MS peptide mapping, intact mass of mAB, HC and LC by RP-HPLC-ESI-MS, Red. RPHPLC-UV peptide mapping	TSKgel Protein C4-300 TSKgel ODS-100V
Higher order structure	Disulfide bridging	Non-red. RP-HPLC-ESI-MS peptide mapping	TSKgel Protein C4-300
	Free thiols	Ellman's assay	
	Secondary and tertiary structure	CD, FTIR, HDX-MS, X-ray	
	Thermodynamic stability	DSC	
General charge heterogeneity and amino acid modifications	0K variant, acidic variants, basic variant, Gln-variant, Lys-variant, amidated proline	CEX digested/undigested	TSKgel CM-STAT (TSKgel Butyl-NPR/HIC)
	Glycation	Boronate affinity	TSKgel Boronate-5PW
	Oxidation/deamidation/C-terminal variants	RP-HPLC-UV/MS peptide mapping	TSKgel ODS-100V (HIC; TSKgel Butyl-NPR)
Glycosylation	Galactosylation, sialylation, mannosylation, afucosylation, bisecting GlcNAc, NGNA, a-galactose, qualitative glycosylation pattern	Normal Phase (NPC) Hydrophilicity (HILIC)	TSKgel Amide-80
Size heterogeneity	Monomer, low-molecular weight (LMW) and high molecular weight (HMW) variants (aggregates)	SEC, AFFFF(AF4; asymetry flow field flow fractionation)	TSKgel UP-SW3000 TSKgel UltraSW-Aggregate TSKgel G3000SWXL
	Heavy chain (HC), light chain (LC), aglycosylated HC, clipped variants	Red. CE-SDS	
	Monomer, LMW (e.g., half antibodies (HL) and HHL variant) and HMW variants	Non-red. CE-SDS	
	Subvisible particles	Light obscuration (PhEur, ≧10 um and > 25um)	
	Visible particles	Visual inspection (PhEur)	
Functional characterization			
Target and receptor binding	FcRn binding	SPR	TSKgel FcR-IIIA-NPR
	FccR binding (FccRIa, FccRIIa, FccRIIb, FccRIIIa(F158), FccRIIIa(V158), FccRIIIb)	SPR	
Bioactivity	CD20 target binding	Cell-based binding assay	
	CDC potency	Cell-based CDC assay	
	ADCC potency	Cell-based ADDC assay	TSKgel FcR-IIIA-NPR
	Apoptosis	Cell-based apoptosis assay	
Other characterization			
Titer	Concentration in culture media	Protein A affinity	TSKgel Protein A-5PW
Conjugate	Drug antibody Ratio (DAR)	HIC-HPLC	TSKgel Butyl-NPR
Bi-specific antibody	Variant AA/AB/BB	IEC-HPLC	TSKgel SP-STAT

* Table modified from J. Visser et al., BioDrug (2013) 27, 495-507

USP Standard for Characterization of Therapeutic Proteins

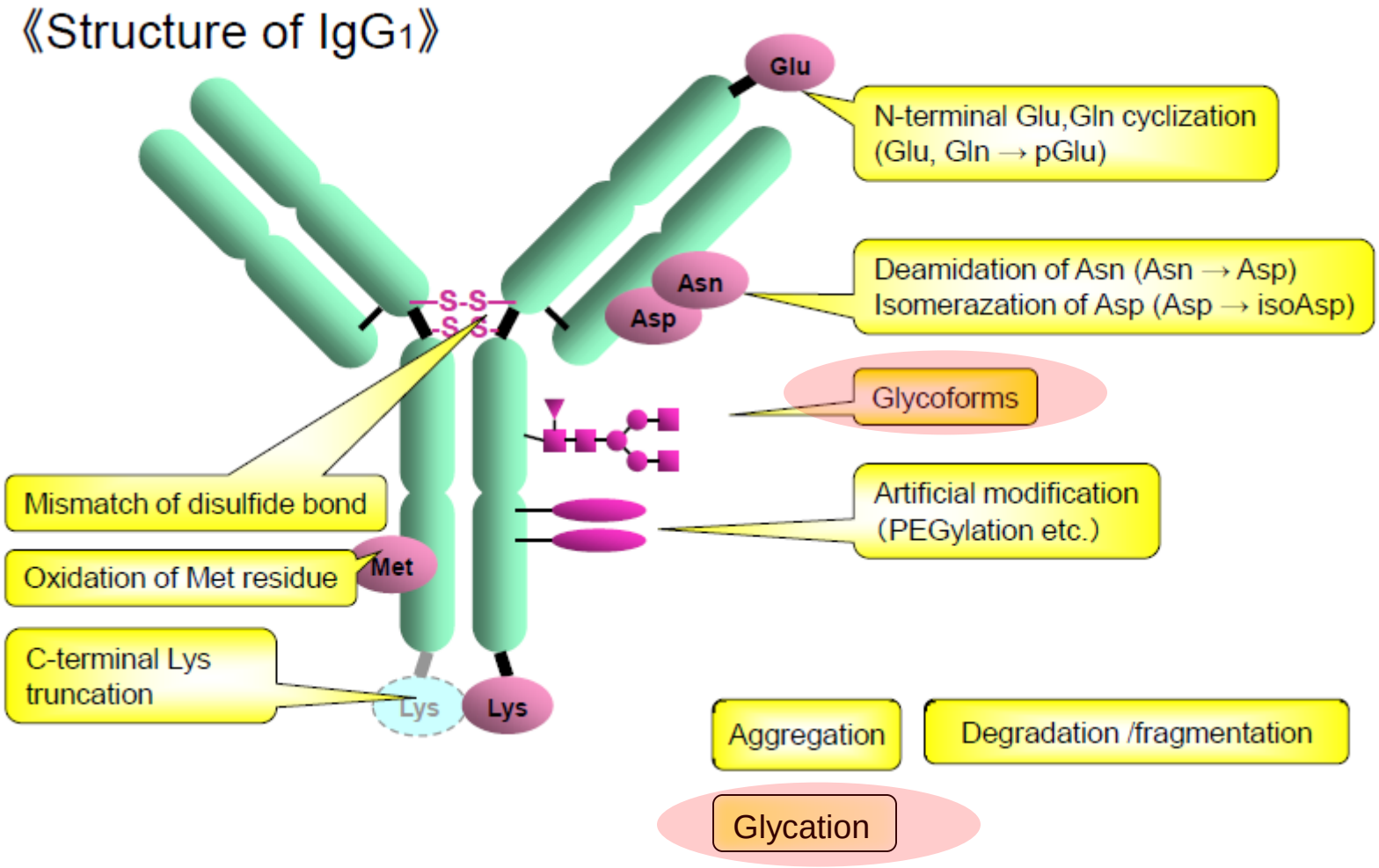
- USP General Chapter 129
  - Analytical procedures for recombinant therapeutic monoclonal antibody
- USP General Chapter 212
  - Oligsaccharide analysis
- USP General Chapter 507, 509
  - Protein determination procedures
  - Residual DNA testing
- Chapter 1132
  - Residual host cell protein measurement in biopharmaceuticals

Reference

- D. Schmidt et al., BioPharm International, 28 (3) 2016
- T. L. Mutlu, Quality Matters (2016), http://qualitymatters.usp.org/new-usp-standards-characterization-therapeutic-proteins
- J. Venema, USP presentation slides (2016)
- M. C. Kibbey, BioPharm International (2017)

Chromatographic Technique (SEC, IEC, HIC and AFC) under Non-Denatured Condition

Characteristics and Heterogeneity of Antibody



Chromatographic Techniques

- **Non-denatured condition techniques**
  - Size-exclusion chromatography (SEC); molecular size
  - Ion-exchange chromatography (IEC); charge
  - Hydrophobic interaction chromatography (HIC); hydrophobicity
  - Affinity chromatography (AFC); glycosylated or glycated moiety
    - Boronate affinity; glycated form
    - Fc receptor affinity; glycosylated (oligosaccharide) form related to ADCC
    - Protein A affinity; Fc region
- **Denatured condition techniques**
  - Reversed-phase chromatography (RPC); hydrophobicity
  - Normal phase (hydrophilic) chromatography (NPC/HILIC); glycosylated
    - Oligosacchride analysis after depletion from antibody molecule
- Other techniques
  - 2-Dimensional HPLC; SEC/RPC, IEC/RPC, HIC/RPC, HILIC/RPC
  - Detection; HPLC/MS, HPLC(SEC)/LS

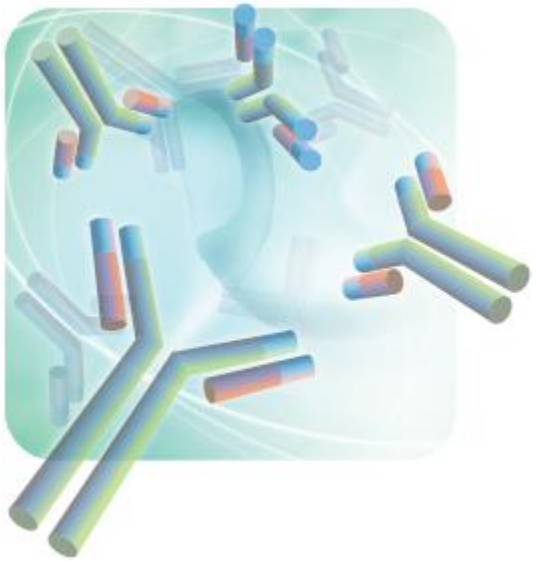
Typical Release Test Used for mAb of Therapeutic Products (Example)

Test	Purpose	Specification	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5
Protein concentration by UV absorbance at 280 nm (mg/mL)	Quantity	50–60	55	54	55	55	56
Percent purity by high-performance SEC	Purity (size)	≥98.0	99.5	99.1	99.7	99.8	99.5
Ion-exchange purity	Purity (charge)	≥95.0	98.0	97.5	98.5	100.0	98.9
Percent deamidation by percent IEC	Purity (charge)	≤5.0	2.0	2.2	1.5	1.0	1.1
Capillary zone electrophoresis	Identity	Conforms to standard	Yes	Yes	Yes	Yes	Yes
Peptide mapping	Identity	Conforms to standard	Yes	Yes	Yes	Yes	Yes
Antigen binding assay or other appropriate	Potency	10–120%	90	95	92	101	105
Host cell proteins (ng/mg)	Impurities	≤100	10	2	5	2	5
Residual DNA (pg/mg)	Impurities	≤20	2	2	3	2	2
Endotoxin (EU/mg)	Impurities	≤0.1	0.01	0.01	0.02	0.01	0.02
pH	General	6–6.5	6.2	6.2	6.2	6.2	6.2
Volume (mL)	General	≥15	15	15	15	15	15
Appearance	General	Colorless	Colorless	Colorless	Colorless	Colorless	Colorless

Ref. ; T. Y. Zhang et al., LCGC North Am, 32 (10) (2014) 796

Size-Exclusion Chromatography (SEC)

- Aggregates and fragments Analysis
- Ultra-High performance column
- Bridge from HPLC to UHPLC



USP 129 Monoclonal Antibody Guideline

Analytical procedures for checking common quality attributes of monoclonal antibody (mAbs) and subtypes (e.g. IgG_1, IgG_2)

- Chromatography separation of size variants (SEC)
- Capillary electrophoresis (CE-SDS)
- Analysis of oligosaccharides and sialic acid in mAb
- IgG system Suitability Reference Standard for SEC and CE-SDS.
- Chapter 129 appears in USP 39-NF 34 (2016)

- This chapter includes validated procedures and system suitability criteria for purity assessments.
- This chapter does not contain product- or class-specific acceptance criteria, and thus cannot replace a monograph or delicate requirements for an individual mAbs. Quality attributes of monoclonal mAbs that are highly product specific would be addressed at the monograph level.

Generic SEC Conditions in mAb Analysis

• Sample concentration;	1.0 g/L (0.5 g/L for FL* detection)
• Sample Injection volume;	10 uL (0.5 uL for FL* detection)
• Column;	TSKgel UP-SW3000 (2.0 um, 4.6 mm I.D. x 30 cm)
• Mobile phase;	100 mM potassium phosphate and 300 mM NaCl (pH 6.8)
• Column Temperature;	25 degrees C
• Flow rate;	0.35 mL/min
• First equilibration;	60 min
• Autosampler;	water/methanol (85:15, v/v), 6 sec
• Autosampler temperature;	8 degrees C
• Detection;	UV (280 nm) Fluorescence (FL*) Excitation/emission (280 nm/340 nm)

- Ref.; Data modified from A. Goyon et al., J. Chromatogr., B 1058 (2017) 73-84

SEC Column Line-up on TSKgel

P/N	Product Name	Particle size (μm)	Column size	Efficiency TP/column	Remark
0023449	TSKgel UP-SW3000	2	4.6 mm I.D. x 15 cm	25,000	Rapid analysis
0023448	TSKgel UP-SW3000	2	4.6 mm I.D. x 30 cm	45,000	High resolution
0023515	TSKgel UP-SW2000	2	4.6 mm I.D. x 15 cm	25,000	Rapid analysis
0023514	TSKgel UP-SW2000	2	4.6 mm I.D. x 30 cm	45,000	High resolution
0023450	TSKgel guardcolumn UP-SW	–	4.6 mm I.D. x 2 cm	–	Guardcolumn
0023451	TSKgel guardcolumn UP-SW DC	–	4.6 mm I.D. x 2 cm	–	Guardcolumn (direct)
0023516	TSKgel guardcolumn UP-SW2	–	4.6 mm I.D. x 2 cm	–	Guardcolumn
0023517	TSKgel guardcolumn UP-SW2 DC	–	4.6 mm I.D. x 2 cm	–	Guardcolumn (direct)
0022854	TSKgel SuperSW mAb HR	4	7.8 mm I.D. x 30 cm	30,000	High resolution
0022855	TSKgel SuperSW mAb HTP	4	4.6 mm I.D. x 15 cm	15,000	Rapid analysis
0022856	TSKgel UltraSW Aggregate	3	7.8 mm I.D. x 30 cm	35,000	Aggregate analysis
0022857	TSKgel guardcolumn SuperSW mAb	–	6.0 mm I.D. x 4 cm	–	Guardcolumn (for 7.8 mm I.D.)
0022858	TSKgel guardcolumn SuperSW mAb	–	3.0 mm I.D. x 2 cm	–	Guardcolumn (for 4.6 mm I.D.)
0022859	TSKgel guardcolumn UltraSW	–	6.0 mm I.D. x 4 cm	–	Guardcolumn
0018675	TSKgel SuperSW3000	4	4.6 mm I.D. x 30 cm	30,000	High resolution
0021485	TSKgel SuperSW3000	4	2.0 mm I.D. x 30 cm	25,000	High resolution, for capillary LC
0021845	TSKgel SuperSW3000	4	1.0 mm I.D. x 30 cm	18,000	High resolution, for capillary LC
0018674	TSKgel SuperSW2000	4	4.6 mm I.D. x 30 cm	30,000	High resolution
0018762	TSKgel guardcolumn SuperSW	–	4.6 mm I.D. x 3.5 cm	–	Guardcolumn
0008541	TSKgel G3000SWXL	5	7.8 mm I.D. x 30 cm	20,000	General analysis
0008540	TSKgel G2000SWXL	5	7.8 mm I.D. x 30 cm	20,000	General analysis
0008543	TSKgel guardcolumn SWXL	–	6.0 mm I.D. x 4 cm	–	Guardcolumn

Bridge from 5 um TSKgel G3000SWXL to 2 um TSKgel UP-SW3000

- Transition stage HPLC to UHPLC
- TSKgel UP-SW3000 (2 μm) has the function of bridging from HPLC to UPLC
  - There are some application to TSKgel UP-SW3000 on HPLC system.
  - SEC market moves to 2 - 3 μm columns in future.
- TSKgel UP-SW3000 is applicable to both HPLC and UHPLC system and higher performance than SWXL/HPLC system.
- Great success in USA; Screening of new mAb in R&D Lab.
  - Presentation by major pharmaceutical companies about higher performance on TSKgel UP-SW3000.
  - Related poster by Genentech
  - Related poster by Boehringer Ingelheim

For Usage of TSKgel UP-SW3000 on Regular HPLC System

- Operation as semi-micro HPLC system required*.
  - Tubing; inner diameter 0.13 mm, length within 20 cm (connection before and after column)
  - Sample injector; Reodyne® 8125 or equivalent
  - UV flow cell; micro-flow cell type (cell volume; about 2 µL)

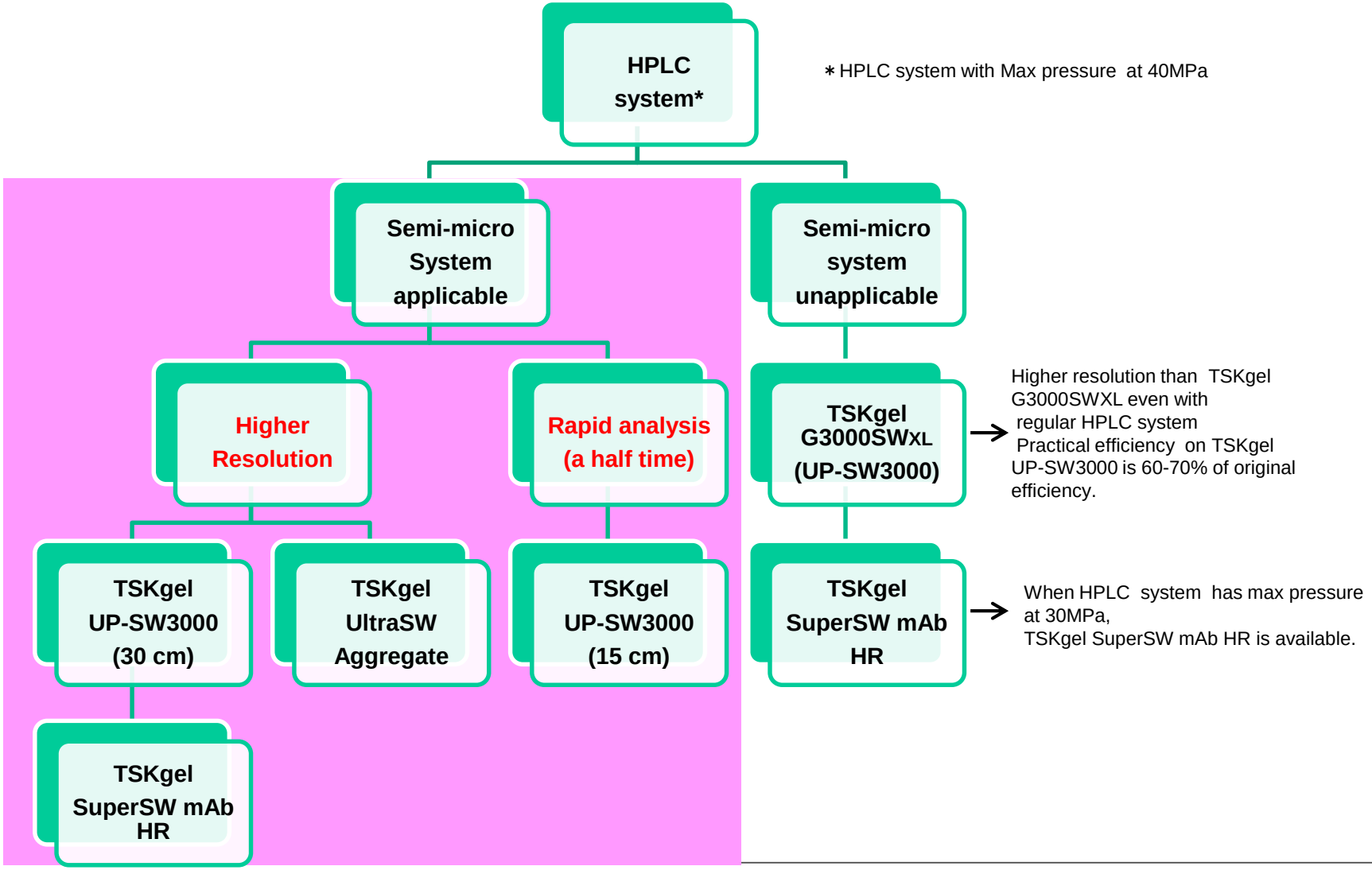
*1

When applied regular HPLC system, column efficiency of TSKgel UP-SW3000 is about 70 % of the original performance.

*2

Even after tune-up of HPLC system, the identical performance of the column may not be achieved as that on Inspection Data Sheet.

Selection of TSKgel SEC Columns on HPLC System



Comparison of mAb Separation on UHPLC Columns by SEC as USP 129 Application

Parameter	Column		
Column	**TSKgel UP-SW3000**	**TSKgel UP-SW3000**	**USP SEC column (L59)**
Particle size (um)	2	2	5
Coumn size	4.6 mm I.D. x 30 cm	4.6 mm I.D. x 15 cm	7.8 mm I.D. x 30 cm
Elution	0.14 mol/L monobasic potassium phosphate, 0.06 mol/L dibasic potassium phosphate, 0.25 mol/L KCl (pH 6.2)		
Flow rate (mL/min)	0.4	0.4	0.5
Temperature (degrees C)	25	25	25
Detection	UV (280 nm)	UV (280 nm)	UV (280 nm)
Sample	Standard Monoclonal IgG System Suitability (USP No. 1445550)		
Sample concentration and injection	5 uL (1 g/L)	5 uL (1 g/L)	20 uL (10 g/L)
Retention time (min)	6.61	3.59	15.48
Peak height (mAU)	80	124	650
Response (mAU/ug sample)	16.0	24.8	3.3
Run time (min)	12	7	30

Ref.; Data modified from the report by Knauer web site
https://www.knauer.net/en/fast-and-sensitive-size-exclusion-chromatography-of-igg-antibody/a440

- UHPLC column performs small sample loading, rapid analysis and high sensitivity detection.

Evaluation for Sub 3 micron SEC Columns

- Size variants analysis; aggregates and fragments
- Higher resolution and fast analysis
- SEC column from 5 micron to sub 3 micron silica

J. Chromatogr. A, 1498 (2017) 80-89

- Evaluation by Geneva Univ and other academic.

- Evaluation of UHPLC and HPLC (sub 3 micron) columns
  - Tosoh; TSKgel® UP-SW3000 (2.0 um)
  - Agilent; AdvanceBio SEC (2.7 um)
  - Phenomenex; Yarra SEC X-150 (1.8 um)
  - Waters; Acquity® BEH2000 (1.7 um)
- Evaluation category
  - Pore volume
  - Non-specific interaction (electrostatic and hydrophobic interaction)
  - Resolution of various monoclonal antibodies (dimer and monomer)
  - Separation of antibody-drug-conjugates (ADC)

Comparison of Sub 3 um SEC Columns for mAb

Waters Acquity UPLC® Protein BEH200 (UPLC)

- Lot-to-Lot variation was reported
- Narrower separation range
- High non-specific adsorption (ionic, hydrophobic)
- Higher column back pressure

Phenomenex Yarra SEC X-150 (UHPLC)

- Narrower separation range (lower resolution between dimer and monomer)
- High non-specific adsorption (ionic, hydrophobic)
- Higher column back pressure
- Lot-to-Lot variation unknown, but occurred on 3 um type column)

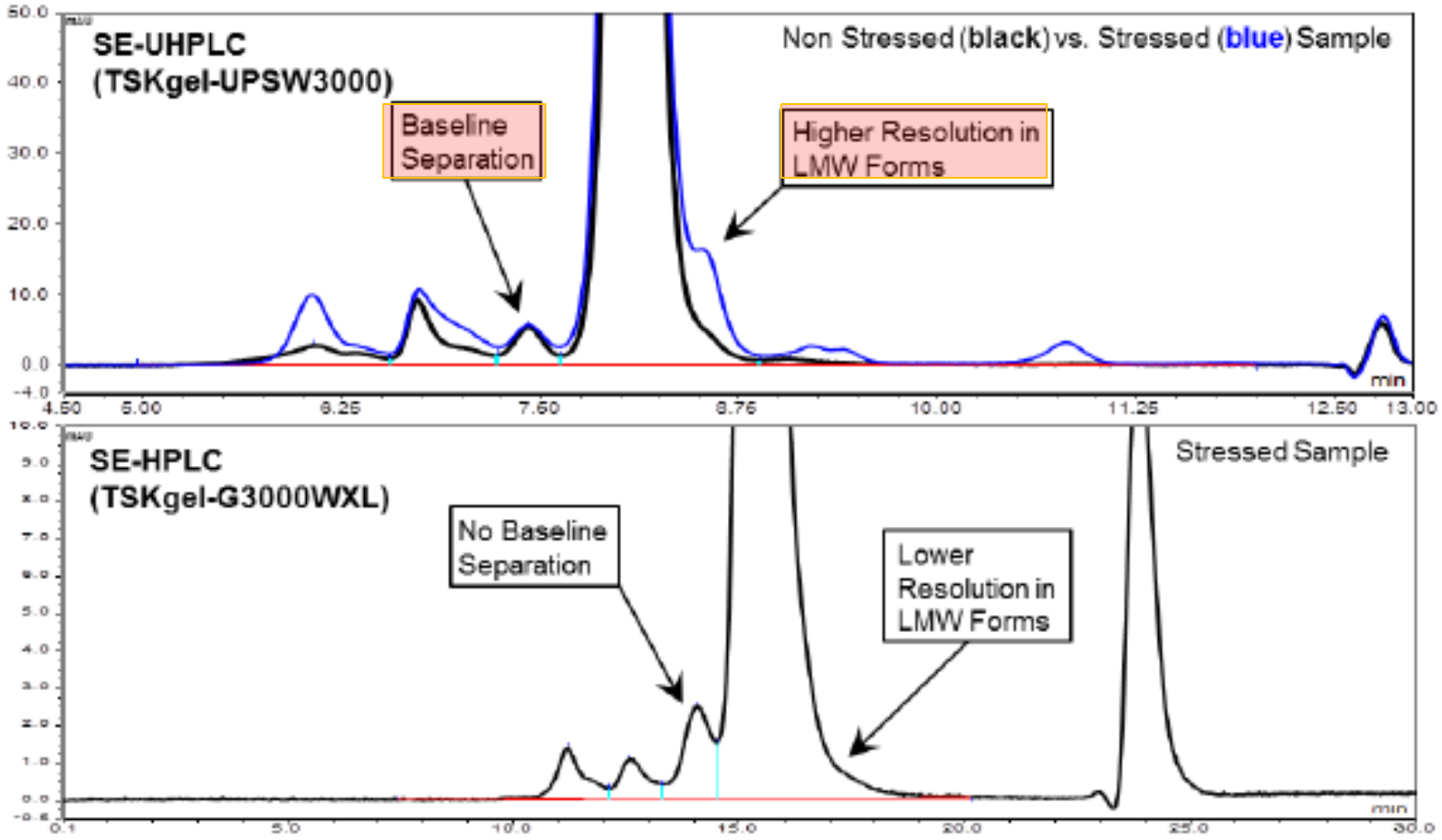
Agilent AdvanceBio SEC (sub 3 micron)

- Narrower separation range (lower resolution between dimer and monomer)
- Lot-to-Lot variation unknown

Transfer from TSKgel G3000SW$_{XL}$ to TSKgel UP-SW3000 in Roche, Germany

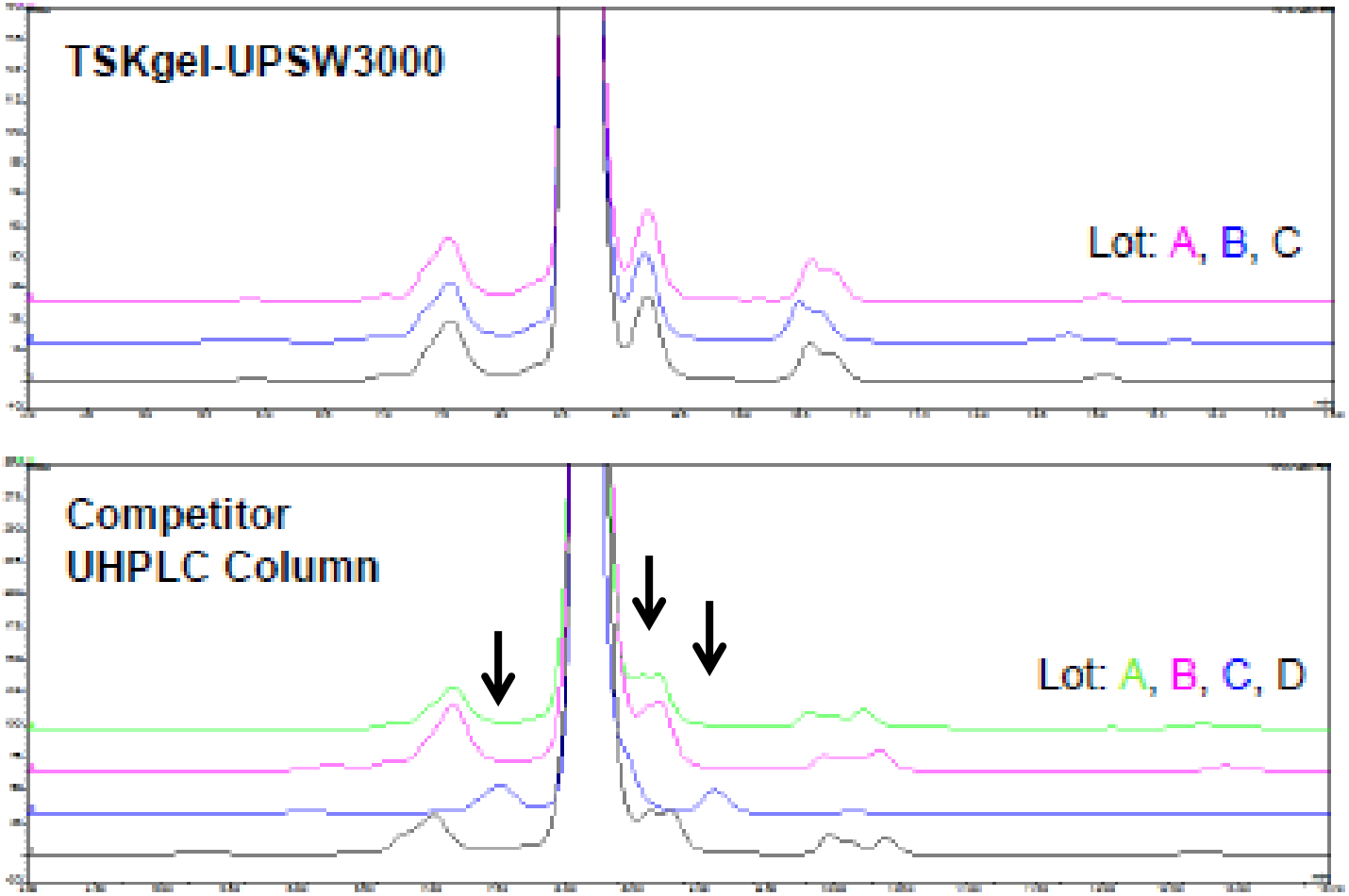
- Poster presentation by Roche at CSSS 2017
- For QC for bi-specific antibody, evaluation of transition from TSKgel G3000SW$_{XL}$ to TSKgel UP-SW3000
- Transition from HPLC to UHPLC system
  - There is no confusion for understanding of sample loading, tubing, cell volume on detector for UHPLC operation.
- Less Lot to Lot difference
  - Other UHPLC columns showed large column to column difference.
- Advantage on TSKgel UP-SW3000
  - Better (base-line) resolution for aggregates (dimer), and improvement of low molecule IgG.
  - Higher reproducibility and robustness
  - Shorter analysis time
- Similar reports for TSKgel UP-SW3000
  - By **Genentech** (HPLC 2016, poster)
  - By **Boehringer Ingelheim** (HPLC 2016 Conference, poster)

Comparison of SEC-HPLC and SEC-UHPLC for Size Variants in Bispecific Antibody



Ref.; R. Ruppert et al., Roche Diagnostics, GmbH, poster (2017)

Lot to Lot Difference on TSKgel UP-SW3000 and UHPLC Clumn for Separation of Bispecific Antibody



Ref.; R. Ruppert et al., Roche Diagnostics, GmbH, poster (2017)

Summary of Sub 3 Micron SEC Columns

TSKgel UP-SW3000 showed higher performance in general aspects.

	TSKgel UP-SW3000	Agilent AdvanceBioSEC	Waters BEH200	Phenomenex Yarra SEC X-150
Particle size	2 µm	2.7 µm	1.7 µm	1.8 µm
Separation range	AAA	A	A	A
Resolution				
Dimer/monomer	AAA	AA	AAA	AA
Fragment	AA	n/a	AAA	n/a
Non-specific interaction				
Ionic interaction	AAA	AA	A	A
Hydrophobic interaction	AAA	AAA	A	A
Lot-to-lot variation	AAA	n/a	A	n/a
Operational back pressure	AA	AAA	A	A
Total score	**AAA**	**AA**	**A**	**A**

*References;

R. Ruppert et al., CASS, poster (2017)

D. Fulchiron et al., HPLC 2016, poster

X. Chen et al., HPLC 2016, poster

A. Goyon et al., J. Chromatogr. A, 1498 (2017) 80-89

Summary;
High Reputation on TSKgel UP-SW3000

- Larger pore volume and pore size
  →wider separation range
  - Tosoh original high technology for silica matrix!
- Higher resolution between mAb dimer and monomer
  - Best in average resolution for 10 mAbs on TSKgel UP-SW3000
- Less non-specific adsorption (ionic, hydrophobic)
  - TSKgel UP-SW3000 and AdvanceBioSEC showed better results.
- Lower column pressure on sub 3 micron column
  - UHPLC column; sub 2 micron column can achieve high-throughput analysis
  - Operation of UHPLC column at 50 - 60° C may not be appropriate for protein separation.
- Lower Lot-to-Lot variation
  - Some difficulty on UPLC column manufacturing and quality control?
- Big pharma companies reported better results on TSKgel UP-SW3000
  - Report from Roche, Genentech, Boehringer Ingelheim on TSKgel UP-SW3000

New SEC Conditions by FDA (2017)

Journal of Pharmaceutical and Biomedical Analysis 138 (2017) 330–343

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An improved size exclusion-HPLC method for molecular size distribution analysis of immunoglobulin G using sodium perchlorate in the eluent

Hsiaoling Wang, Mark S. Levi, Alfred V. Del Grosso, William M. McCormick, Lokesh Bhattacharyya*

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- Evaluation of TSKgel SuperSW3000 and TSKgel G3000SW$_{XL}$
- Comparion of elution buffer between EP method and FDA method
- Faster analysis at 4.6 mm I.D. column
- Improvement (increase) of aggregate contents, peak sharpness, resolution and analysis time

Conditions (Trace Test by Tosoh)

Elution buffer;

FDA method; 40 mmol/L sodium phosphate (pH 6.8) containing 0.4 mol/L $NaClO_4$

EP method; 40 mmol/L sodium phosphate (pH 7.0) containing 0.2 mol/L NaCl, 0.05 % NaN_3

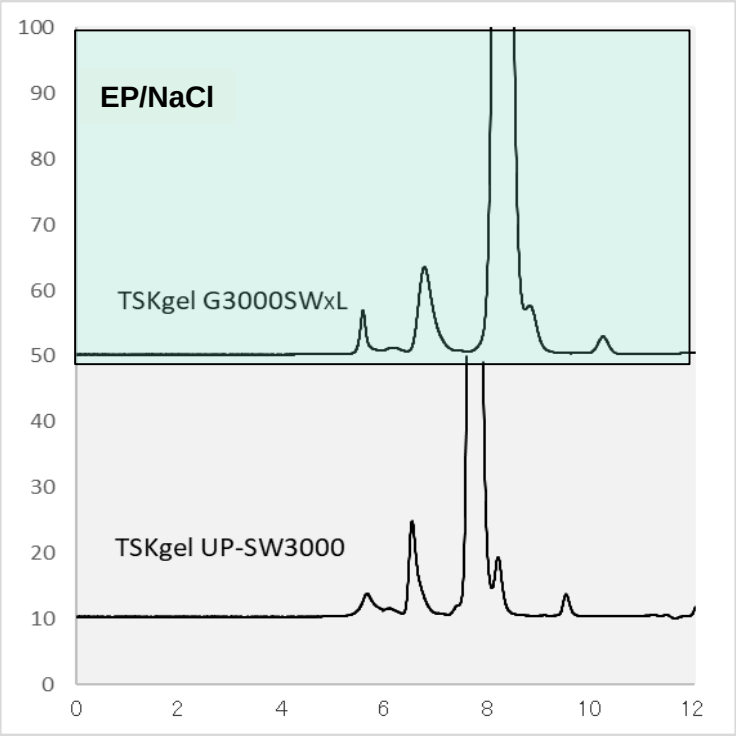
Column; TSKgel UP-SW3000, 4.6 mm I.D. x 30 cm
Flow rate; 0.35 mL/min
Temperature; 25 degrees C, Detection; UV (280 nm)
Sample; monoclonal IgG or fragment, 4 uL

Column; TSKgel G3000SW_{XL}, 7.8 mm I.D. x 30 cm
Flow rate; 1.0 mL/min
Temperature; 25 degrees C, Detection; UV (280 nm)
Sample ; IgG or fragment, 12 uL

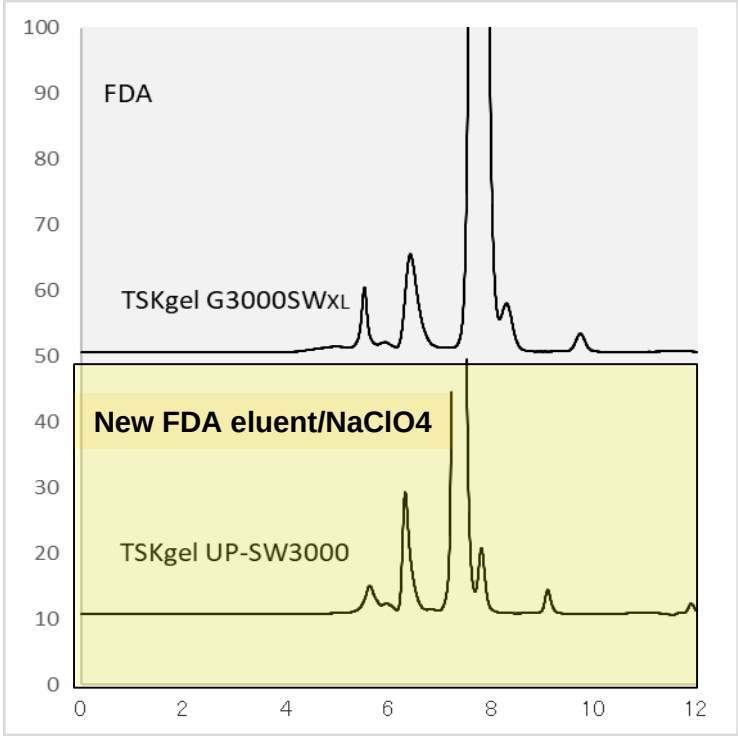
Sample; Rituxan®, Remicade®, Herceptin®, Avastin®, mouse IgG_1
Sample concentration; 5 – 100 mg/mL

Separation of mAb by European Pharmacopeia and FDA Elution Conditions by HPLC/UHPLC

TSKgel G3000SWXL



TSKgel UP-SW3000

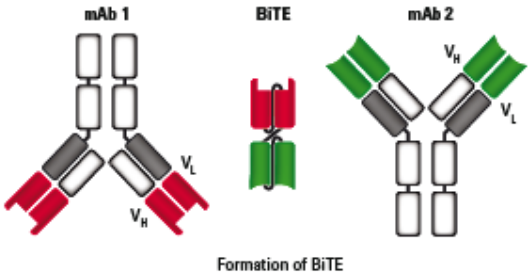


- EP conditions; 40 mmol/L sodium phosphate (pH 7.0) containing 0.2 mol/L NaCl, 0.05 % NaN_3
- New FDA conditions; 40 mmol/L sodium phosphate (pH 6.8) containing 0.4 mol/L $NaClO_4$
- New buffer proposed by FDA, some monoclonal antibodies improved aggregates contents compared with buffer described in EP.
- Higher performance TSKgel UP-SW3000 gave also smaller amounts of injection of precious sample, which would be favorable of cost of analysis.

Ref.; H. Wang et al, J. Pharm. Biomed. Analysis, 138 (2017) 330

Separation of Bispecific scFv by UP-SEC/MS

Figure 1. Formation of Bispecific T Cell Engager (BiTE)

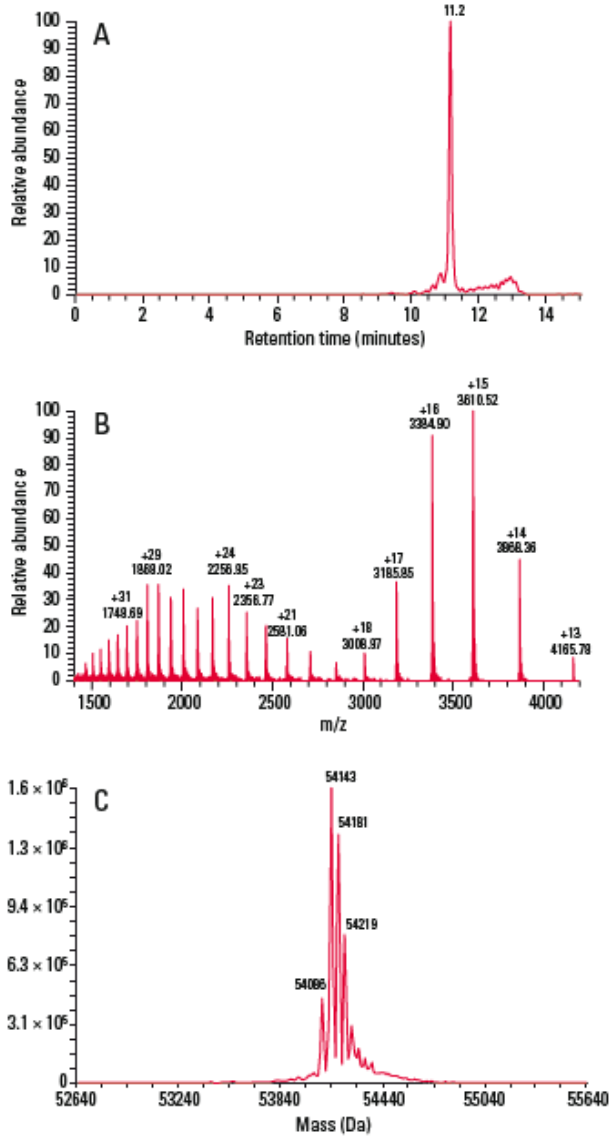


Experimental HPLC Conditions

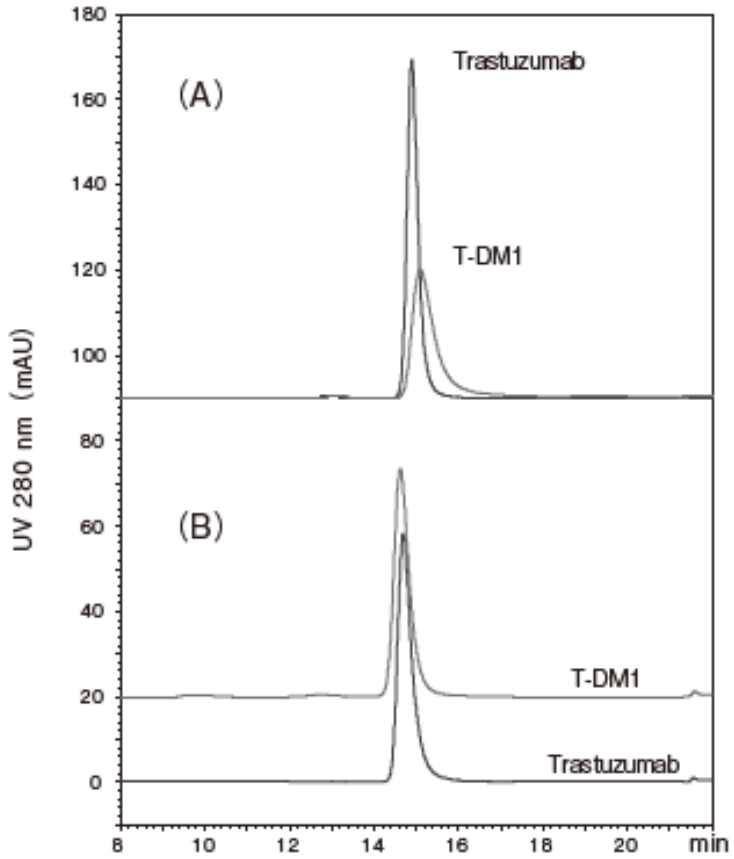
Column:	TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm
HPLC Instrument:	Nexera® XR UHPLC system
MS Instrument:	Q Exactive™ Plus
Mobile phase:	20 mmol/L ammonium acetate, 10 mmol/L ammonium bicarbonate; pH 7.2
Gradient:	isocratic
Flow rate:	0.35 mL/min
Detection:	UV @ 280 nm
Temperature:	30 °C
Injection vol.:	5.0 µL
Samples:	BiTE, 0.3 mg/mL *(Creative Biolabs)* parent mAb shown, 0.5 mg/mL *(Creative Biolabs)*
Ionization mode:	Electrospray ionization, positive mode
MS mode:	Scanning, m/z 800-6000

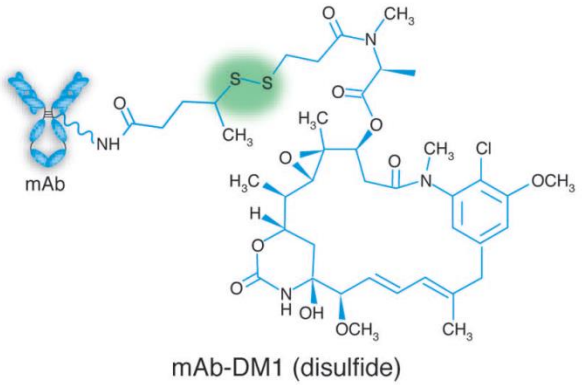
*SEC/MS analysis was performed by the Wistar Proteomics and Metabolomics Facility *(Philadelphia, PA)*

Figure 2. SEC/MS analysis of the BiTE. Accurate molar mass of the BiTE was identified as 54.1 kDa via SEC/MS.



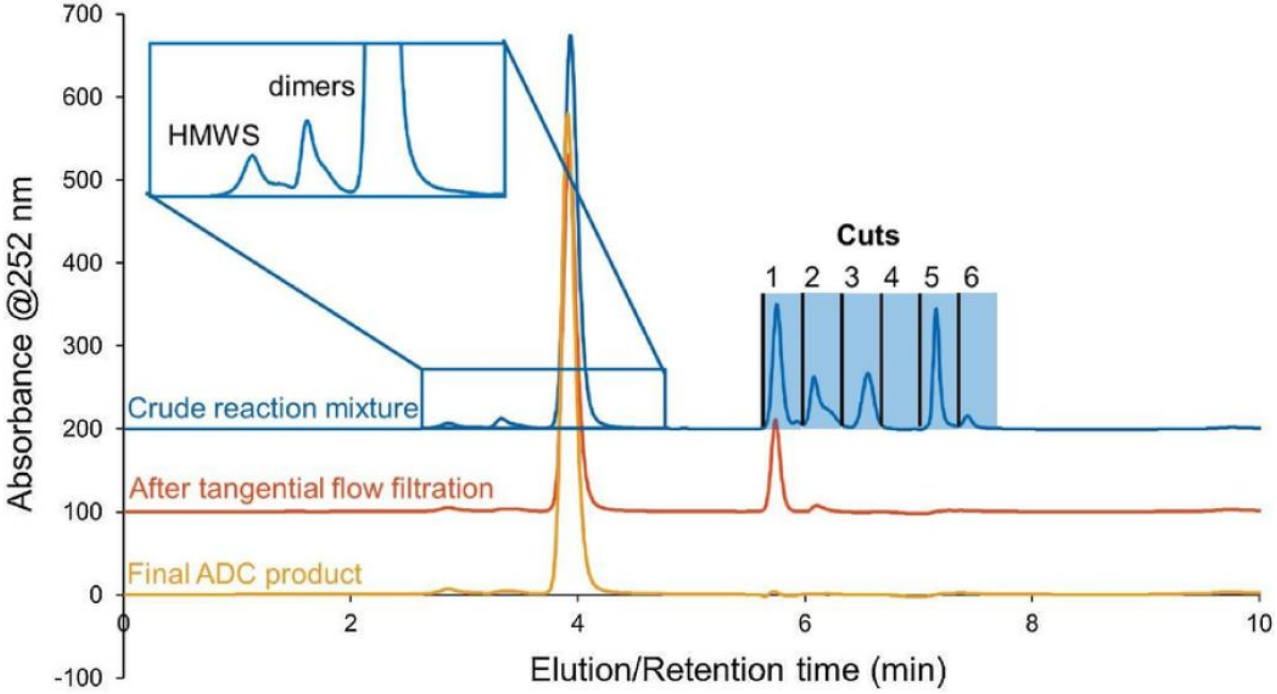
Analysis of Antibody-Drug-Conjugate (ADC) by SEC on TSKgel UP-SW3000





Column; TSKgel UP-SW3000 (4.6 mm I.D. x 30 cm)
Eluent; Buffer A: 20 mmol/L phosphate buffer (pH7.0) + 0.3 mol/L NaCl
Buffer B: 2-propanol / Buffer A = 15/85
Flow rate: 0.175 mL/min, Detection; UV(280 nm), Temperature: 25 degrees C
Sample; Trastuzumab/T-DM1 (5 uL)

Separation of Crude ADC Reaction Mixture on TSKgel UP-SW3000 by SEC with Step Gradient Mode



Conditions

Column; TSKgel UP-SW3000 (4.6 mm I.D. x 15 cm)

Elution: Buffer A: 0.05 M potassium phosphate buffer + 0.25 M KCl (pH 6.8), Buffer B: 100% acetonitrile

Gradient; 0 min (Buffer B 3 min), 0.5 min (Buffer B 3 %), 1 min (Buffer B 20%), 3 min (Buffer B 20%), 3.5 min (Buffer B 3 %)

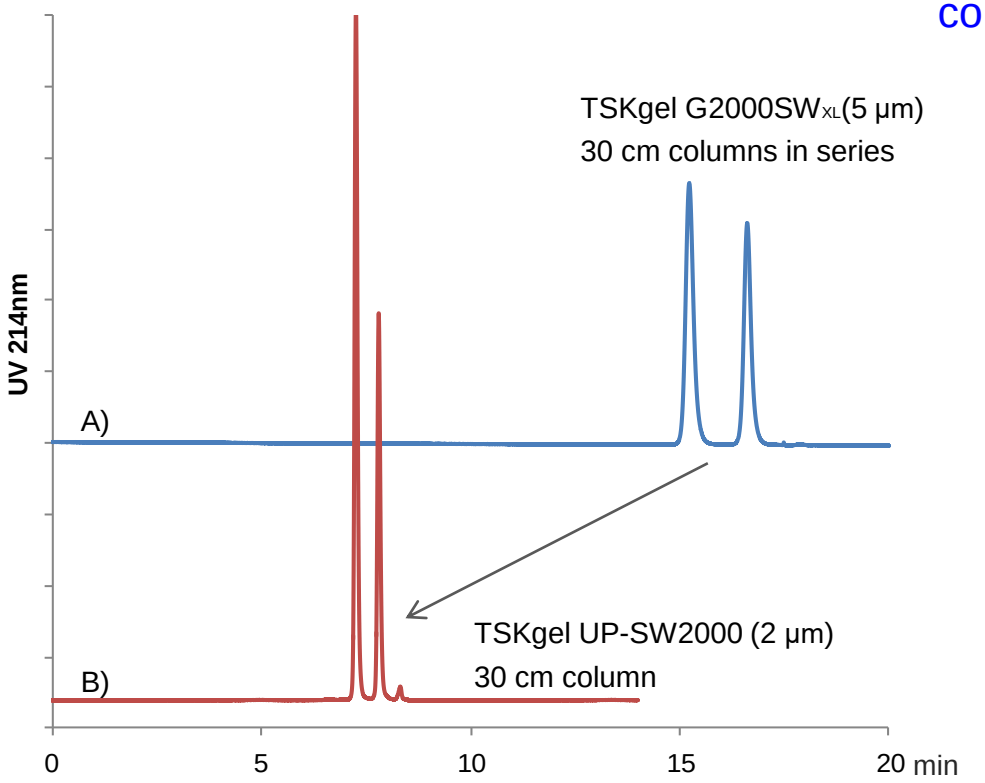
Flow rate; 0.35 mL/min, Detection; UV (280 nm), Sample; crude ADC, 2.0 uL

Ref.; A. Goyon et al., J. Chromatogr. A, 1539 (2018) 19-29

Comparison of TSKgel UP-SW2000 and TSKgel G2000SW$_{XL}$ (in Two Column Series) for Separation of Peptides

- TSKgel UP-SW2000

Higher resolution and fast analysis with high sensitivity on UHPLC-SEC compared with traditional columns



Column	Rs (1/2)
A) TSKgel G2000SW$_{XL}$ x 2	4.27
B) TSKgel UP-SW2000	4.30

Condition

Column:	A) TSKgel G2000SW$_{XL}$ 5 µm, 7.8 mm I.D. x 30 cm x 2 B) TSKgel UP-SW2000 2 µm, 4.6 mm I.D. x 30 cm
Eluent:	30 % acetonitrile + 0.1 % trifluoroacetic acid
Flow rate:	A) 1.0 mL/min, B) 0.35 mL/min
Temperature:	25 °C
Detection:	UV (214 nm)
Injection vol.:	10 µL
Sample:	Big gastrin (MW 3,849 Da), Bombesin (MW 1,620 Da)

Other HPLC Modes (IEC, AFC, HIC) for mAb Characterization

Generic IEC Conditions in mAb Analysis

• Sample concentration;	0.5 g/L
• Sample Injection volume;	20 uL
• Column;	TSKgel CM-STAT (5 um, 4.6 mm I.D. x 10 cm)
• Mobile phase;	Buffer A; 20 mM MES, (pH 6.8) Buffer B; Buffer A, 0.5 M NaCl
• Linear gradient;	0 min (10% B), 15 min (30% B), 15 min (100% B), 18 min (100% B)
• Column Temperature;	25 degrees C
• Flow rate;	1.0 mL/min
• First equilibration;	60 min
• Detection;	UV (280 nm)

Separation of Charge Variants of Biosimilar Herceptin by IEC on TSKgel CM-STAT

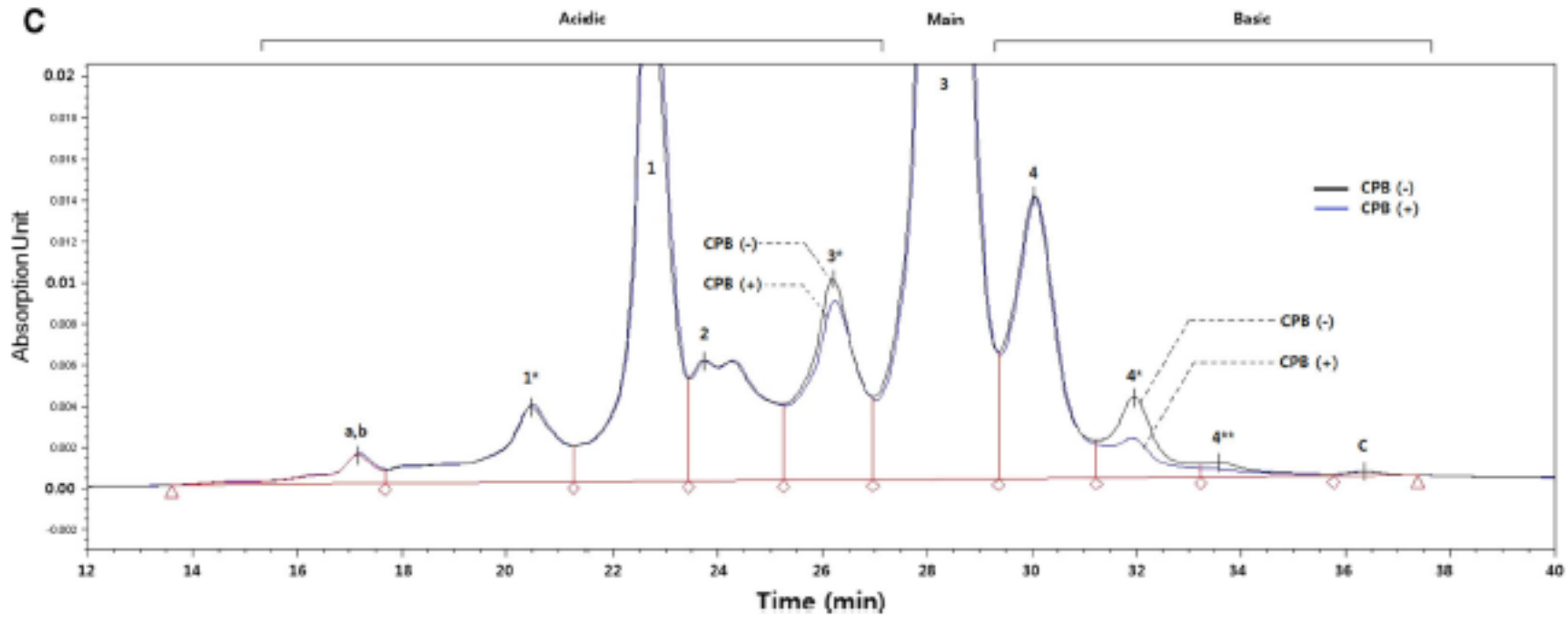
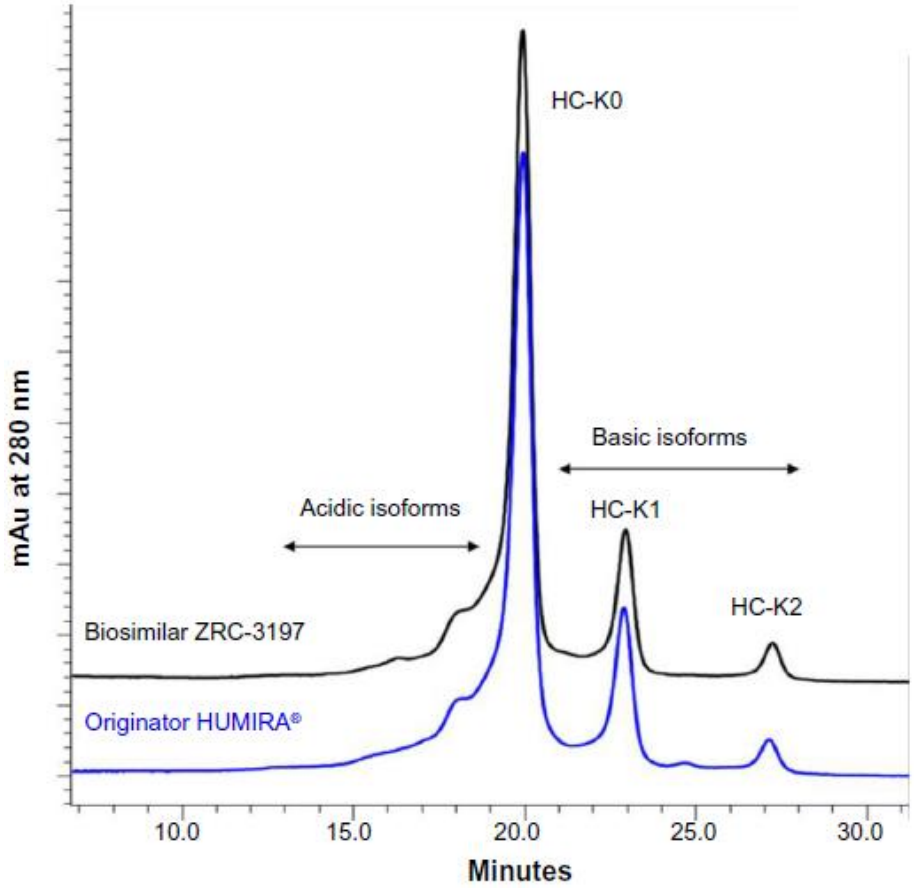


Fig. 1 Cation-exchange chromatography profiles of control and CPB-treated Trastuzumab. **a**, **b** Profiles of control and CPB-treated samples, respectively. Eluted charge variants were monitored at 280 nm and acquired for 55 min, as described in Table 2. Variants a,b, 1*, 1, 2 and 3* are acidic species present in both control and CPB-treated Mab. The basic species in the control Mab are variants 4, 4*, 4** and C while those in the CPB-treated Mab are 4, 4* and C. **c** The comparative elution profiles of acidic variants in control and CPB-treated Mabs. *CPB (−)* control Mab, *CPB (+)* CPB-treated Mab

IEC; Shallower NaCl gradient, Temperature at 40 degrees C

Reference; D. G. Kim et al., Arch. Pharm., Res., August 2016

Analysis of Exemptia™ (Biosimilar Humira®) by IEC on TSKgel CM-STAT



Shallower NaCl gradient elution

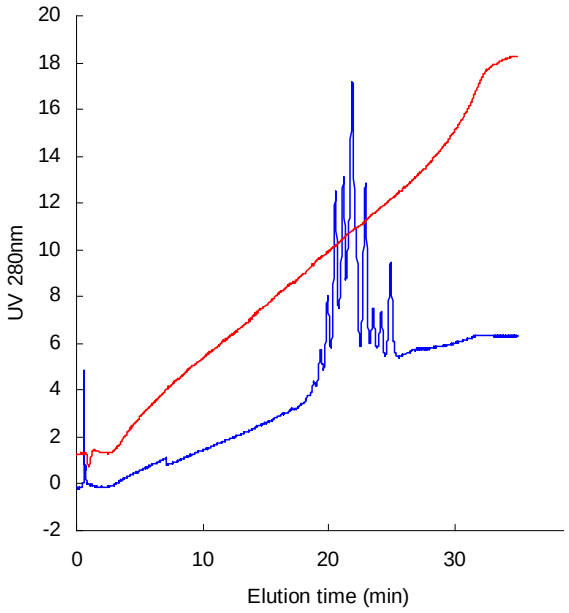
Temperature at 40 degrees C

Figure 5 Comparison of the charge heterogeneity profile of the biosimilar ZRC-3197 with respect to the originator HUMIRA® by HP-IEC. Samples containing 50 µg adalimumab from originator HUMIRA® or ZRC-3197 preparation were injected onto a TSK-Gel CM-STAT weak cation-exchange column equilibrated with phosphate buffer of pH 6.9 containing NaCl. Separation of the charged species variants was performed with a linear salt gradient at a flow rate of 1 mL/min, and elution was monitored at 280 nm with UV detection.

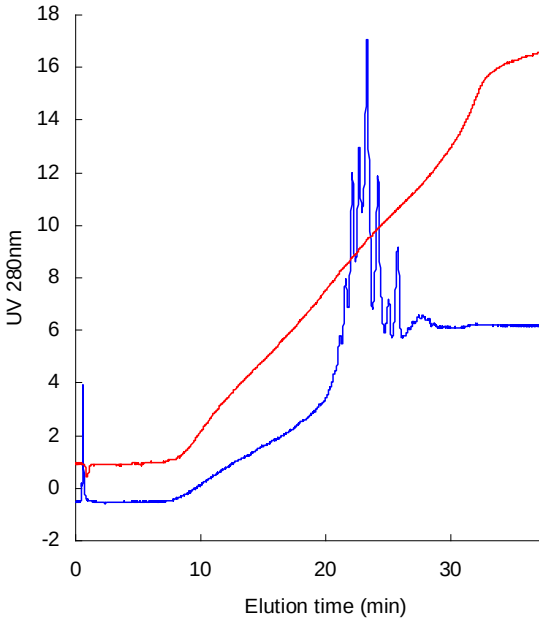
Reference; S. Bandyopadhyay et al., Biosimilar 2015:5, 1-18

Separation of Charge Isomer of mAb Therapeutic by CEX with pH Gradient System

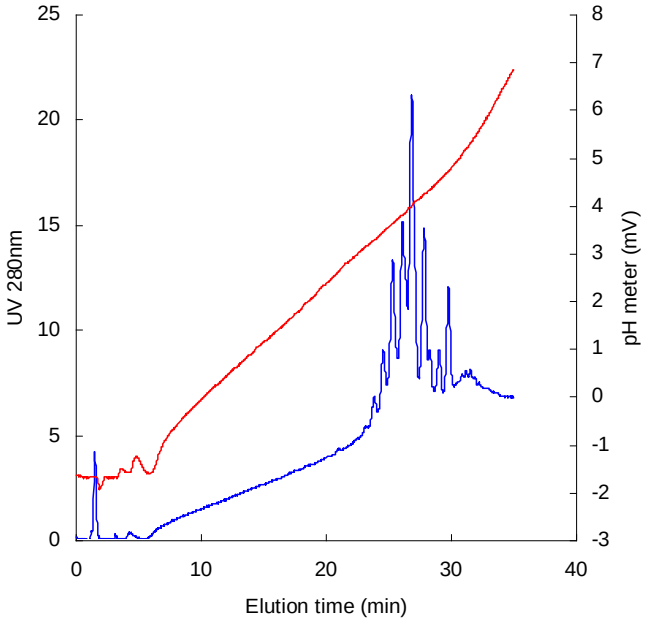
TSKgel SP-STAT



TSKgel CM-STAT

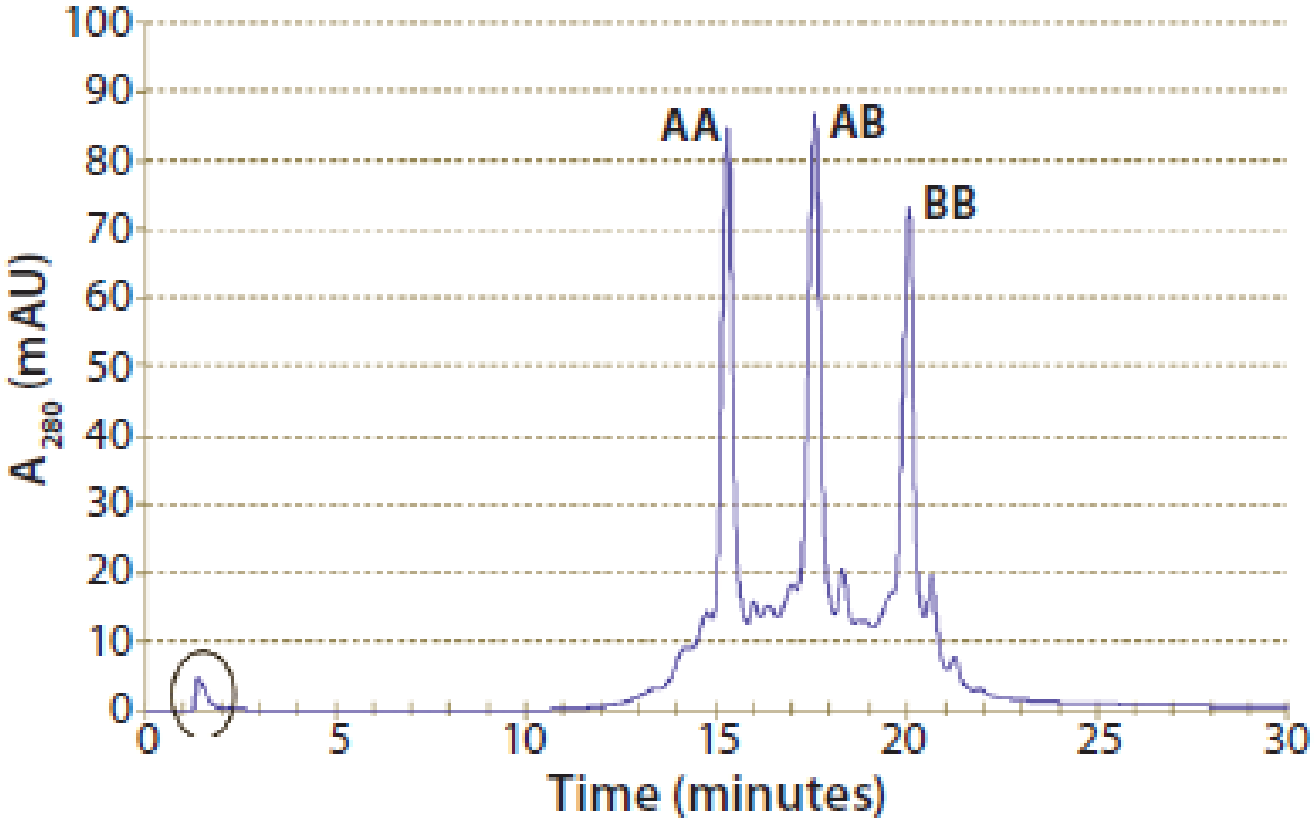


ProPac ™ WCX



Column: TSKgel SP-STAT (4.6 mm I.D. x 10 cm), TSKgel CM-STAT (4.6 mm I.D. x 10 cm),
ProPac WCX (4.0 mmI.D. x 25 cm)
Eluent A: 10 mM NaCl in 20 mM MES + 20 mM HEPES (pH5.4)
Eluent B: 10 mM NaCl in 20 mM MES + 20 mM HEPES (pH9.0)
Gradient: A →B 30min linear
Flow rate: 1.0 mL/min, 0.76 mL/min (ProPac WCX)
Sample: mAb C

Separation of Bispecific Monoclonal Antibody by IEC on TSKgel SP-STAT

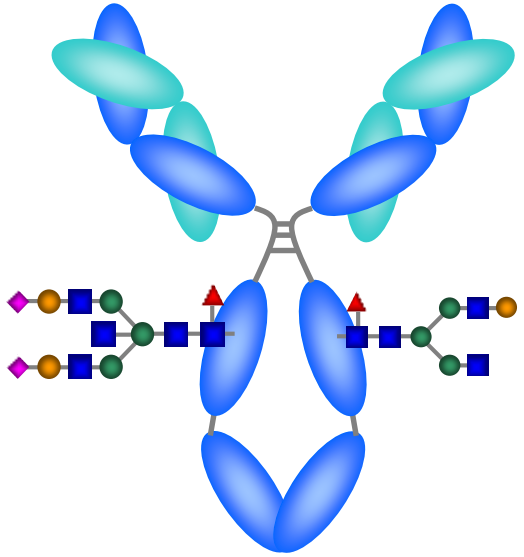


Reference; T. Mulelr-Spath et all, BioProcess International, (2013) May, 36-44

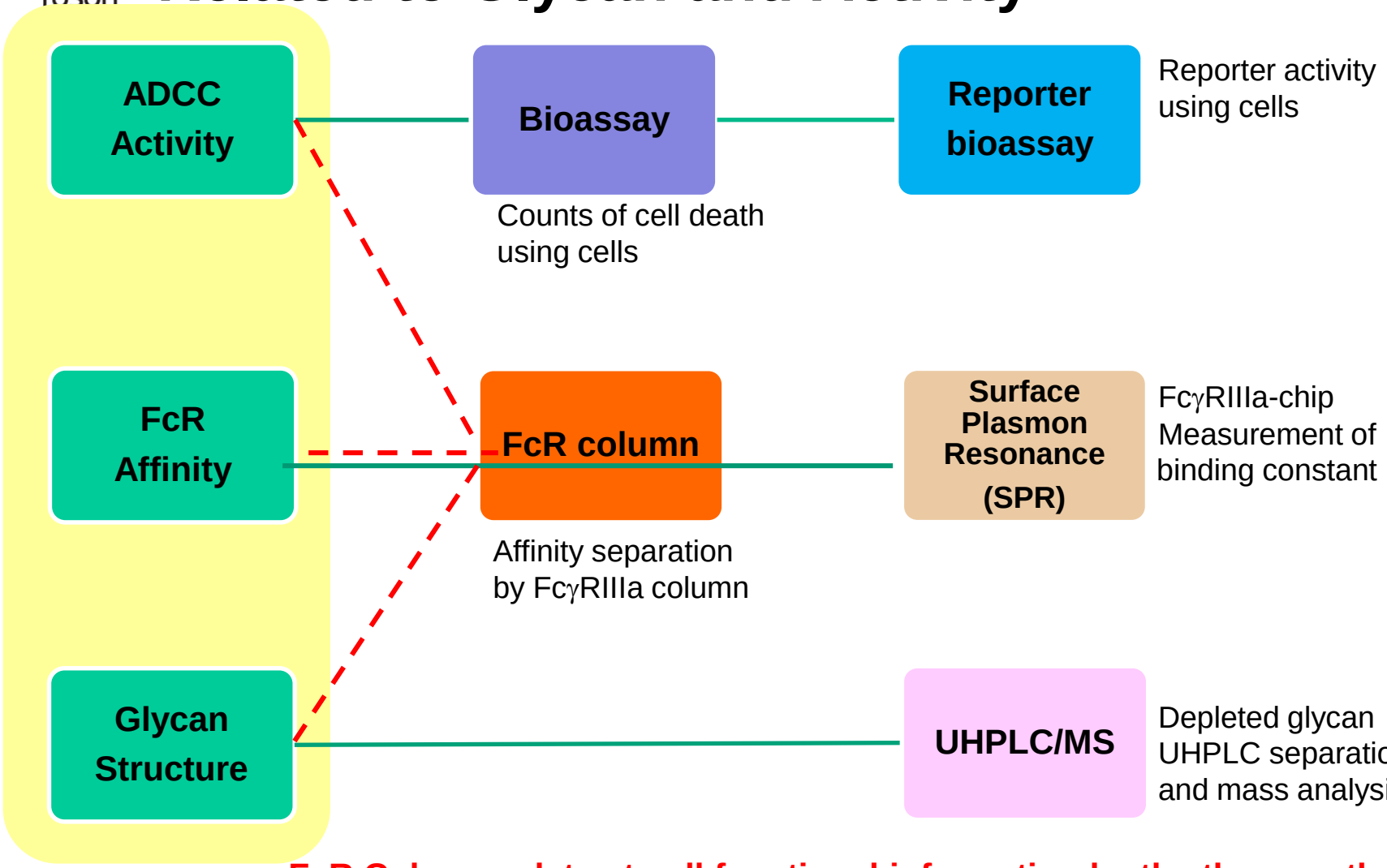
TOSOH

AFC Analysis of Sugar Related Moiety of Antibody

- Glycosylation
- Glycation
- ADCC Activity



Comparison of Analytical Methods for mAb Related to Glycan and Activity

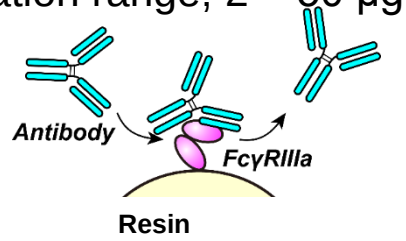


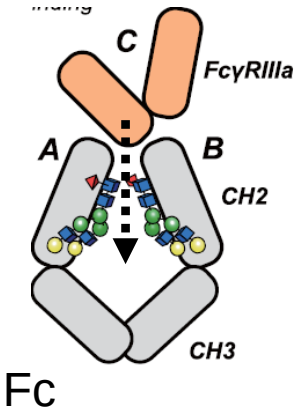
FcR Column relates to all functional information by the three methods.

Principle of TSKgel FcR-IIIA-NPR

- Binding to column depends on number of galactose in Fc glycan part. More galactose, higher binding to the column.
  - (Afucosylated and syalylated enhances retention)
- Antibody is separated to roughly three peaks.
- Quantitation range; 2 – 50 µg

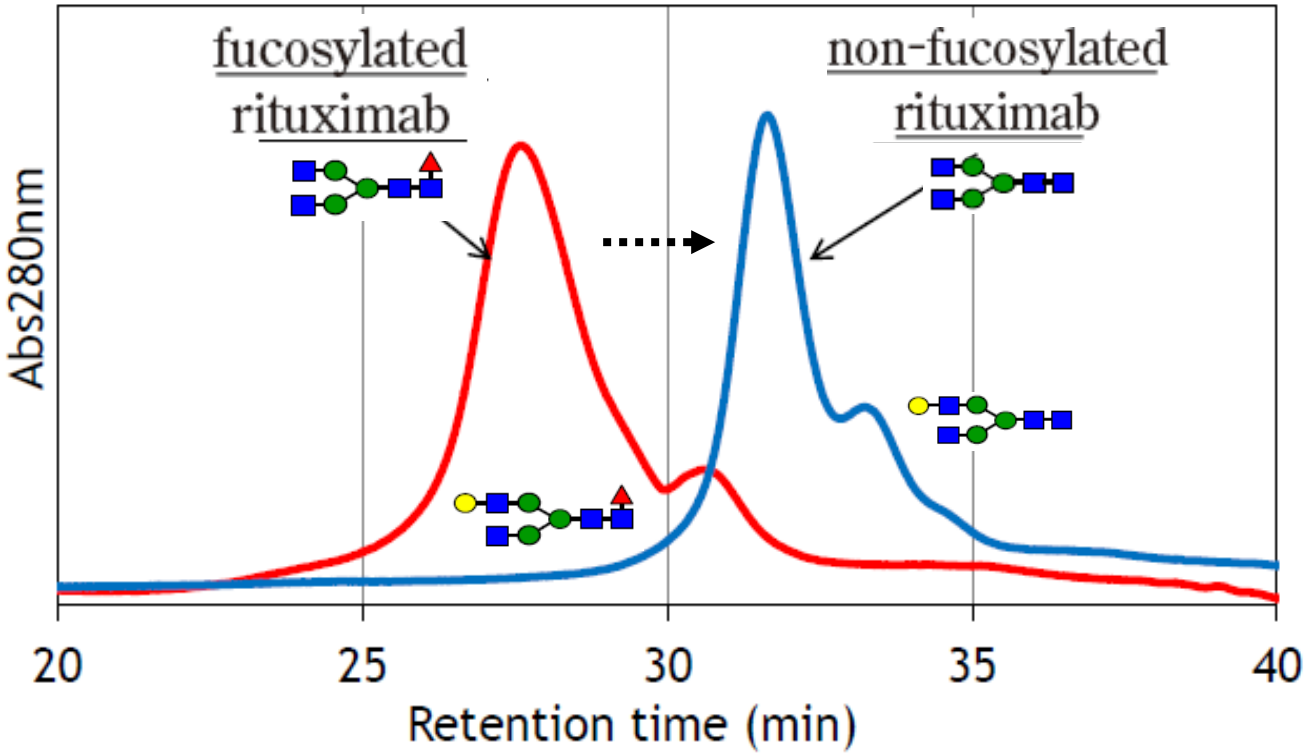
Peak 1	G0F G0F
Peak 2	G0F G1F
Peak 3	G1F G1F or G2F





Average Mixture activity vs. individual activity (Peak 1, 2, 3)

Separation of Fucosylated and Non-Fucocylated mAb (Rituximab) by AFC on TSKgel FcR-IIIA-NPR

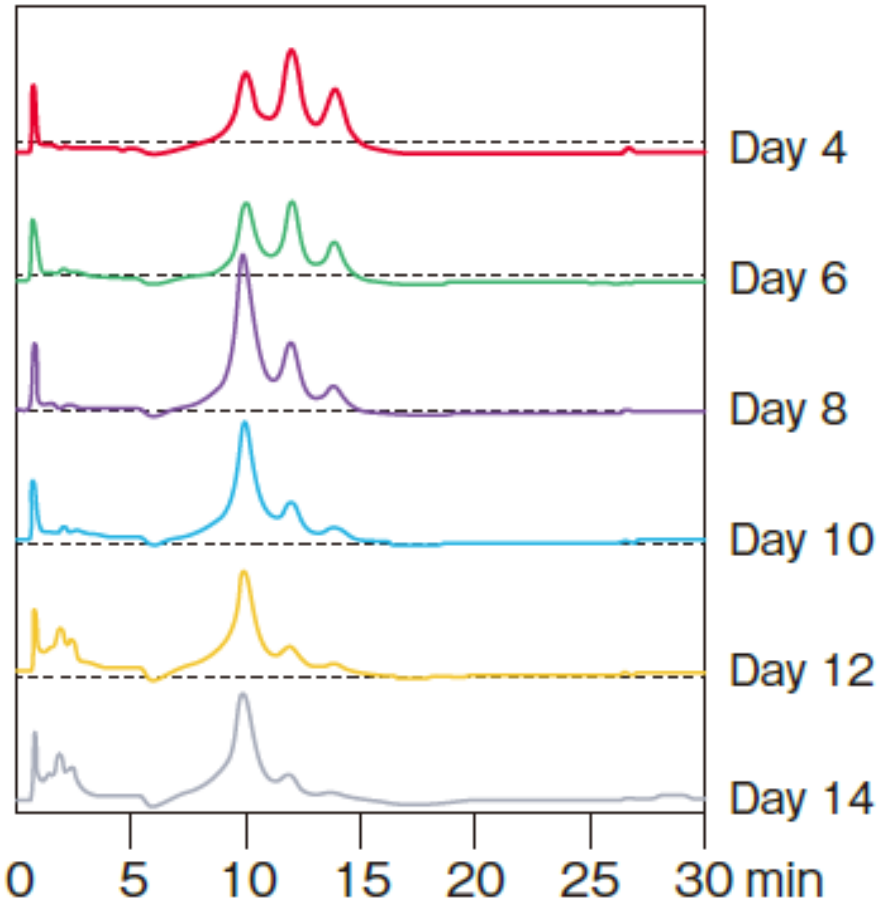


uipment	HPLC system
olumn	FcR column (4.6 mmI.D.×75 mm)
uffer A	20 mM sodium acetate + 50 mM NaCl pH 5.0
uffer B	10 mM glycine-HCl pH 3.0
radient	0-2 min 0%B
	2-30 min 0%B to 100%B linear
	30-40 min 100%B
	40-50 min 0%B (re-equilibration)
emperature	25 ℃
ow Rate	0.6 mL/ min
mple	antibody 5 μg
etection	UV absorbance at 280 nm

- **Afucosylated (non-fucosylated) monoclonal antibody showed stronger retention and more retention with galactose in glycan**

Ref.; Y. Terao et al., Prep 2016, poster

Analysis of Herceptin® Biosimilar Antibody during Fermentation (In-process Monitoring)

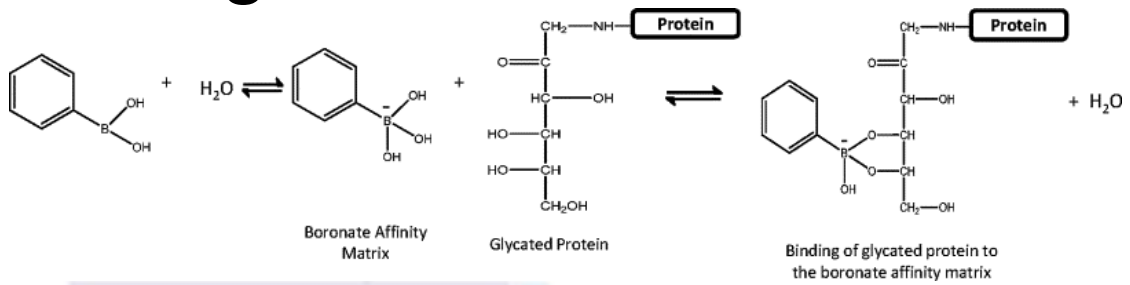


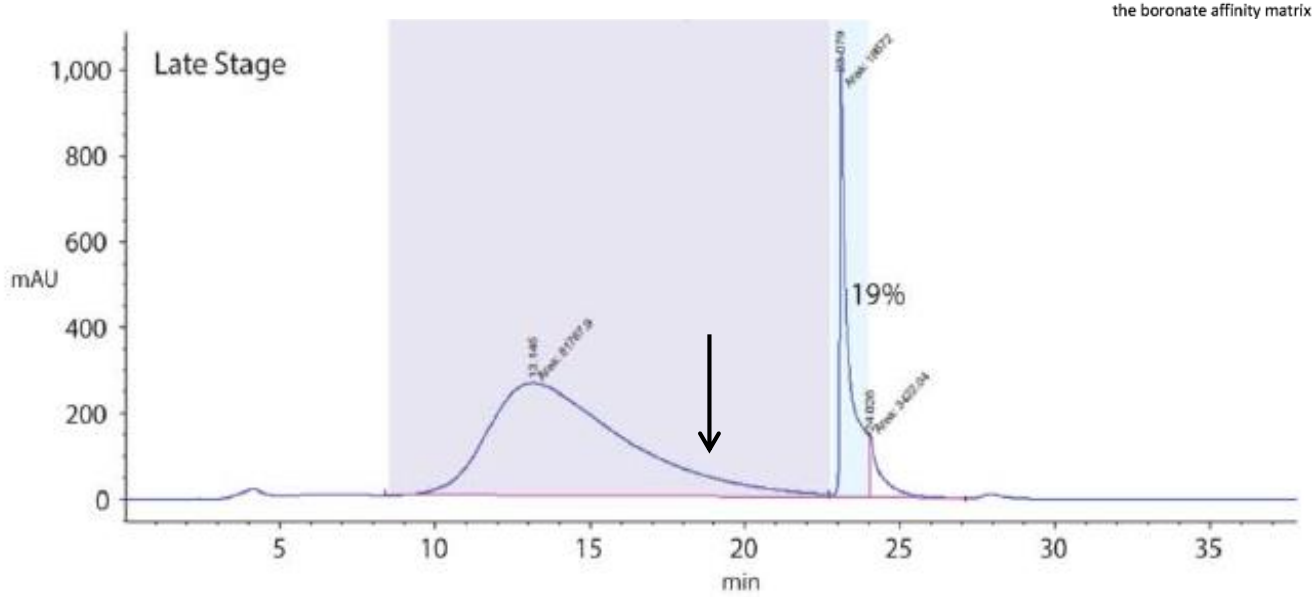
Conditions
Column; TSKgel FcR-IIIA-NPR (4.6 mm I.D. x 7.5 cm)
Elution; Buffer A: 50 mmol/L sodium citrate buffer (pH 6.5)
Buffer B: 50 mmol/L sodium citrate buffer (pH 4.5)
Linear gradient (20 min, Buffer B 2-20 min)
Flow rate; 1.0 mL/min
Detection; UV (280 nm)
Temperature; 25 degrees C
Sample; mAb, 5 ug
CHO cell culture media* was filtrated and purified by Protein A affinity column.

*CHO cell culture media was by the courtesy of MAB.

- Antibody peaks shifted elution volume toward to earlier, which suggested number of galactose in Fc glycan decreased with increase of days of fermentation and suggested lower ADCC activity.

Quantitation of Glycation of Antibody by AFC on TSKgel Boronate-5PW

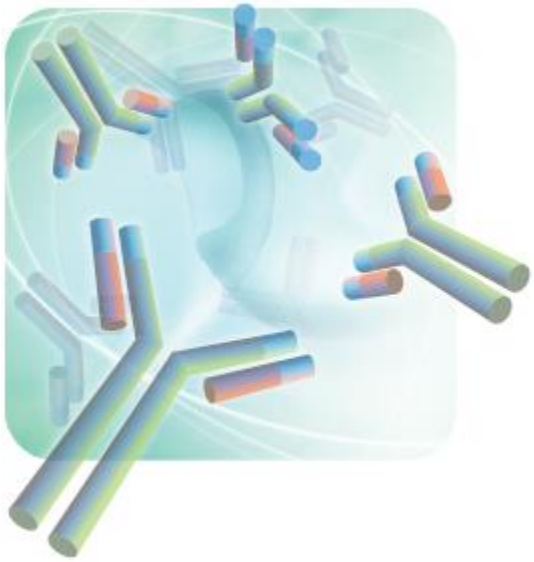




Column; TSKgel Boronate-5PW (7.5 mm ID x 7.5 cm)
Elution; A: 100 mM HEPES, 25 mM Tris (pH 8.5) + 200 mM NaCl
B: Buffer A containing 500 mM sorbitol
Buffer A: 0-19 min, Buffer B from 19 min
Sample; monoclonal antibody IgG1

Ref. R. A. Saleen et al., mAbs 7:4, 719-731 (2015)

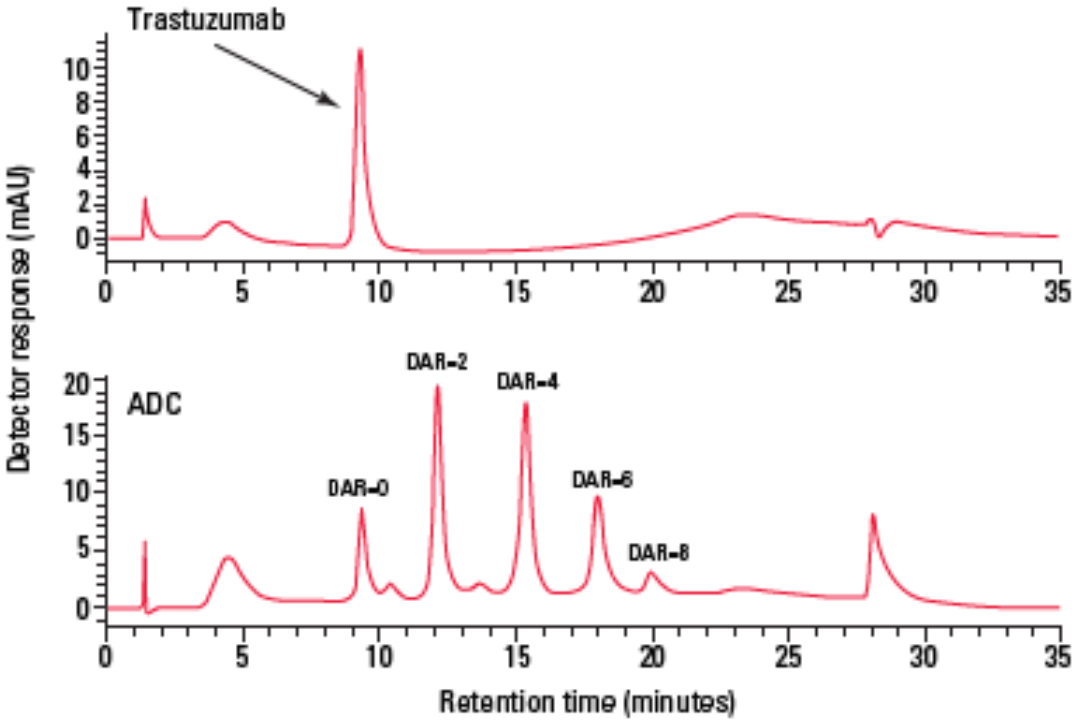
HIC Analysis of Antibody Drug Conjugate (ADC)

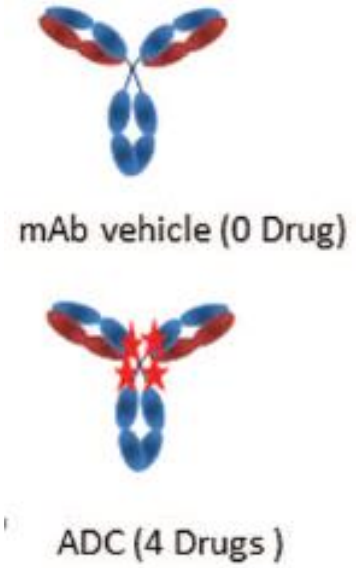


Application to Antibody-Drug Conjugate (ADC)

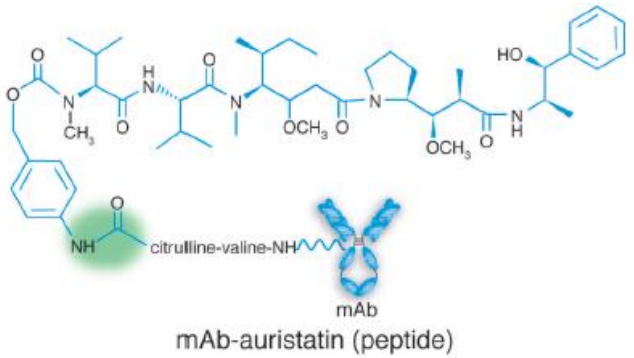
- 3 ADC approved, over 60 products in clinical trial.
- ADC increases its hydrophobicity to become more aggregation.
- Effective drug-to-antibody ratio (DAR) is preferably between 2 and 4. →Separation by SEC and/or HIC
  - TSKgel UP-SW3000, TSKgel Butyl-NPR, TOYOPEARL PPG-600M
  - Recently Daiichi Sankyo reported DAR 7-8 was effective in clinical trial (2018)
- New ADC development as peptide-drug conjugate (PDC), antibody-drug conjugate micelle (ADCM)

Drug-Antibody-Ratio (DAR) on Antibody-Drug Conjugate (ADC) by HIC on TSKgel Butyl-NPR

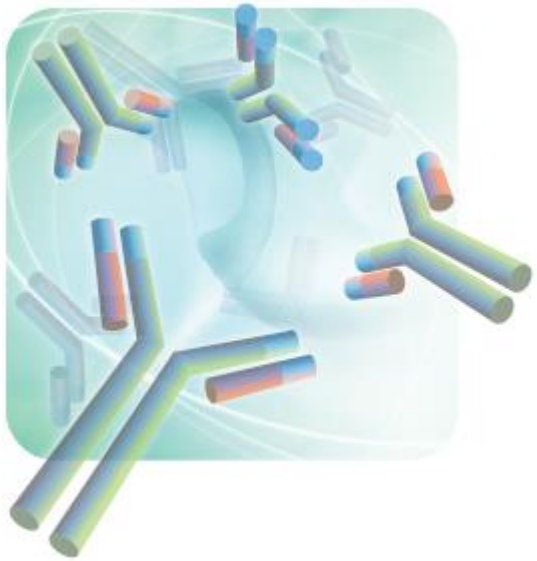




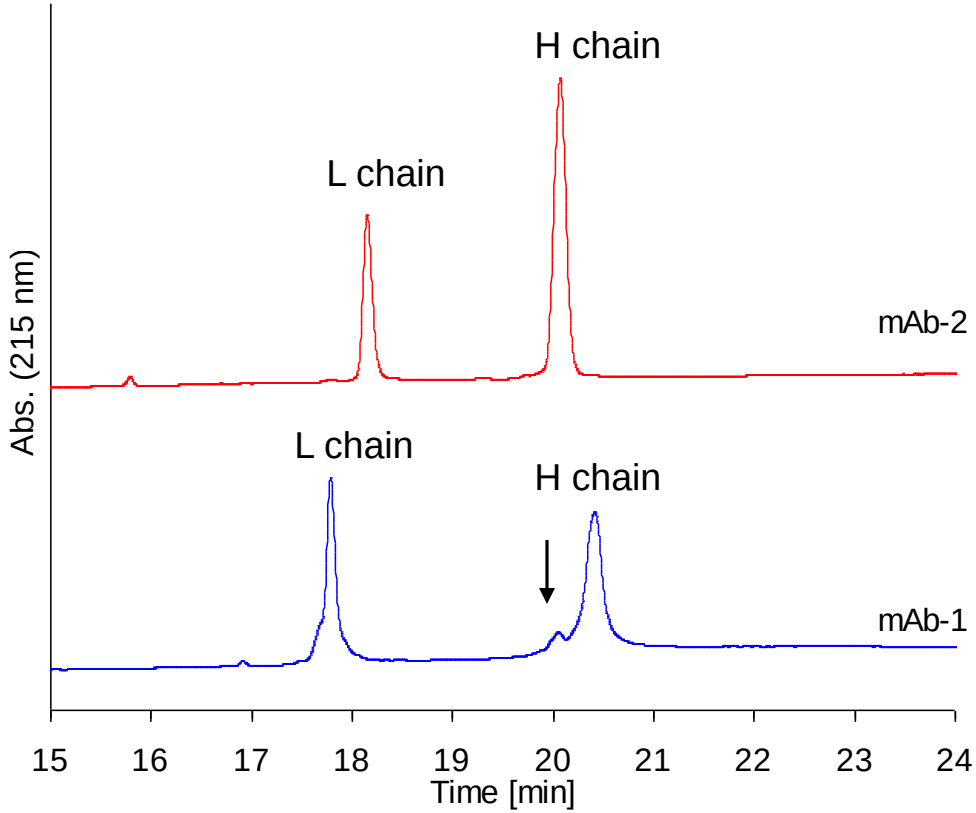
Column; TSKgel Butyl-NPR, 2.5 µm, 4.6 mm I.D. x 10 cm
Elution; A: 25 mmol/L sodium phosphate buffer (pH 7.0)
+ 1.5 mol/L ammonium sulfate
B: Buffer A/2-propanol (80/20)
20 min linear gradient from Buffer A to B
Flow rate; 0.5 mL/min
Detection; UV (280 nm)
Sample; Trastuzumab (0.2 g/L), 10 µL
Trastuzumab-vcMMAE (2.2 g/L), 10 µL



Chromatographic Techniques under Denatured Conditions (RPC, HILIC)

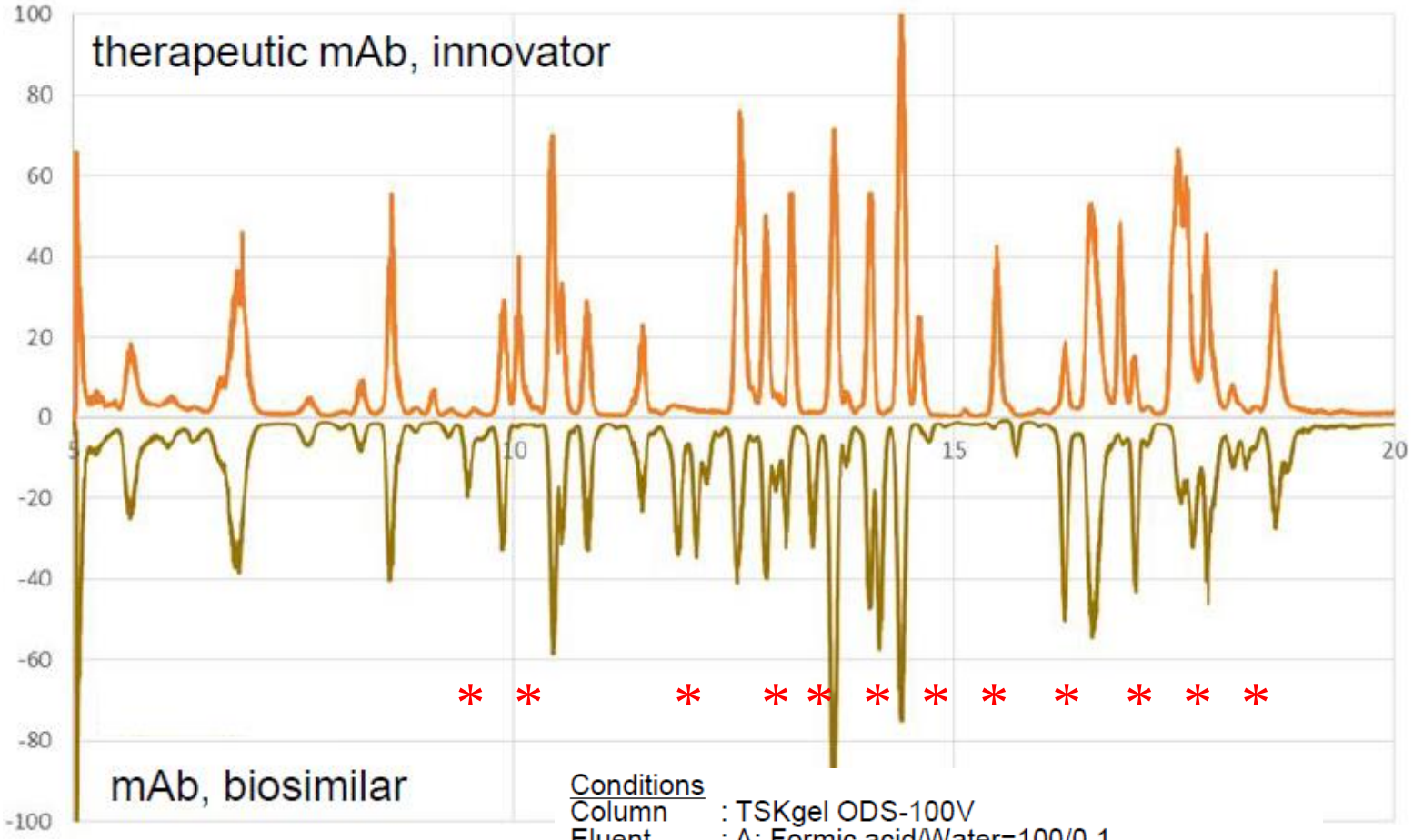


Intact IgG (reduced form) Separation by RPC on Large Pore (300 A) C_4 Column



Column; TSKgel Protein C_4-300 (4.6 mmI.D. ×15 cm)
Eluent A; H_2O/ACN/TFA = 90/10/0.05 (v/v/v)
Eluent B; H_2O/ACN/TFA = 20/80/0.05 (v/v/v)
Eluent; B 0% (0 min) — 100% (45 min)
Flow rate; 1.00 mL/min, Detection; UV 215nm, Temperature; 50℃ (100 uL)
Sample; mAb-1, mAb-2, each reduced with dithiothreitol

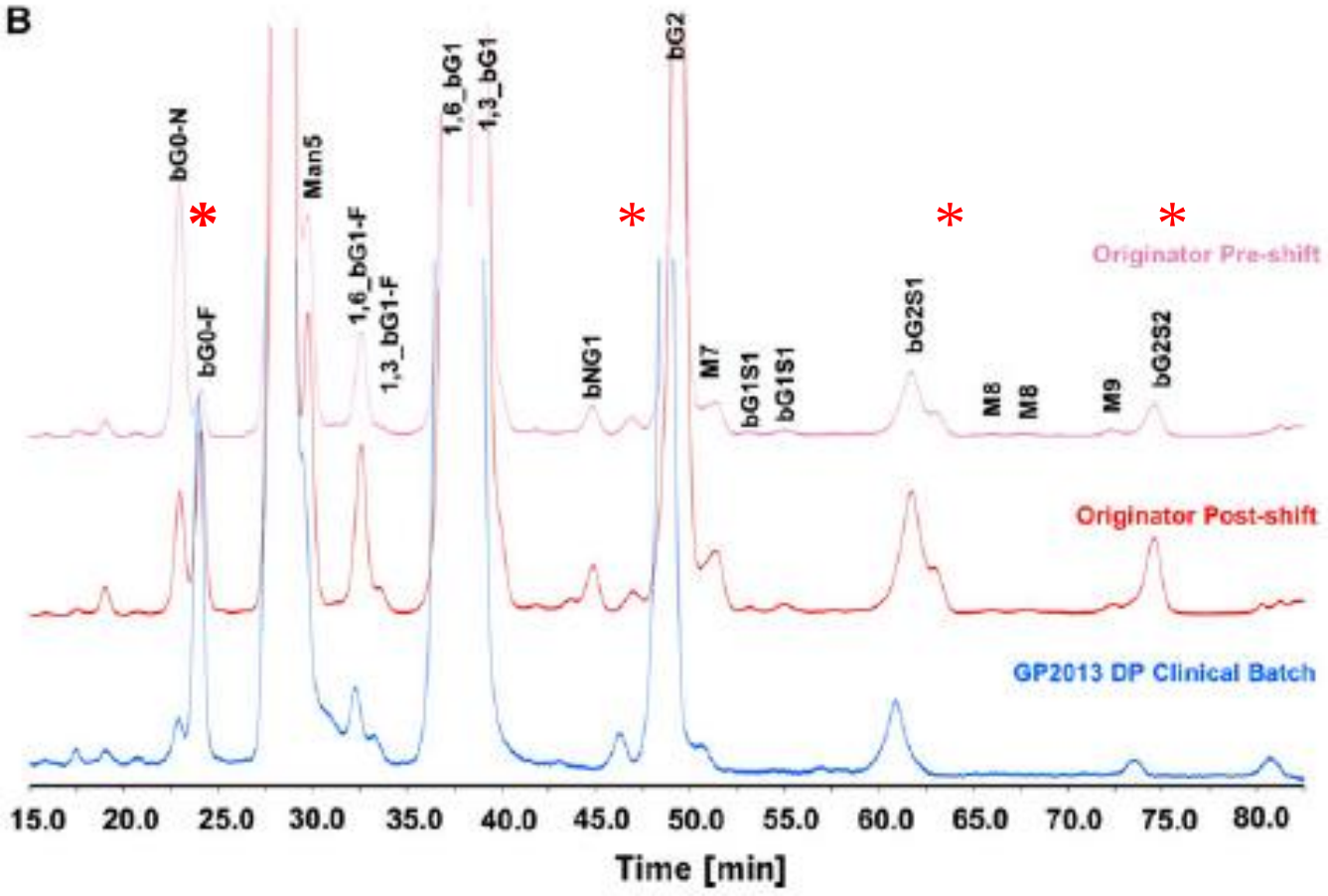
RPC Peptide Mapping Analysis of Biosimilar mAb on TSKgel ODS-100V



Conditions

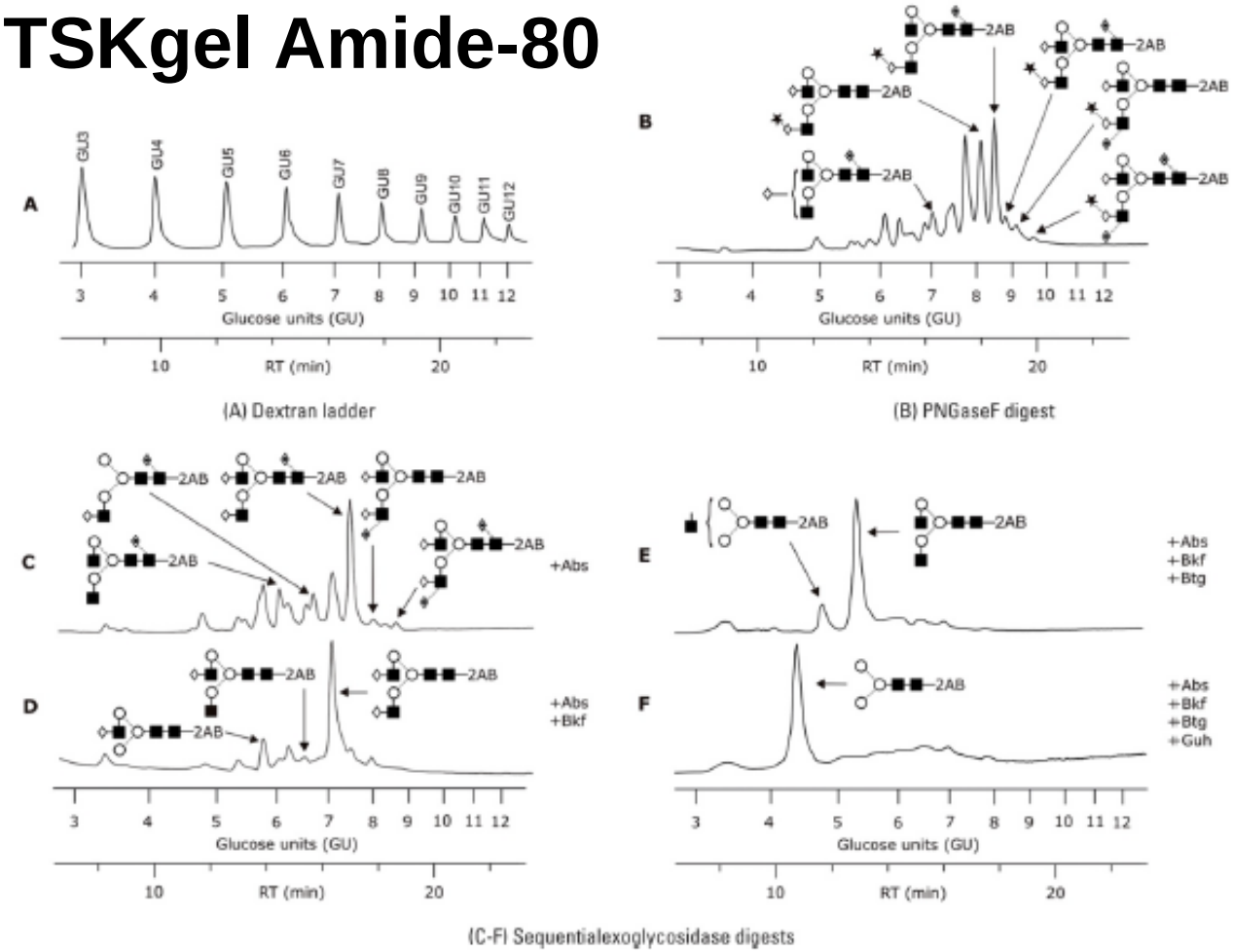
Column	: TSKgel ODS-100V
Eluent	: A; Formic acid/Water=100/0.1 B; Aetonitlile/FA=100/0.1
Gradient	: 10 % B(0 min)-10 % B(2 min)-50 % B(24 min)
Flow rate	: 0.5 mL/min
Detection	: MS(positive ion mode)
Temp.	: 40 °C
Inj. volume	: 10 µL
Sample	: trypsin digested mAb

Analysis of Glycosylation in Biosimilar Rituximab GP2013 (Rituxan®) by HILIC on TSKgel Amide-80



Ref.; J. Visser et al., BioDrugs (2013) 27, 495-507

Separation of 2-AB N-Glycan for Transformer Growth Factor by HILIC/MS on TSKgel Amide-80



Used exoglycosidase:

- Sialidase A (Abs)
- α-Fucosidase (Bkf)
- β-Galactosidase (Btg)
- β-N-Acetylhexoamidase (Guh)

Reference; A. Chakrabarti et al., AMS 2011, poster

HPLC Columns for Antibody Analysis

Target Form, Function	Principle	chromatographic Mode	Chromatography System		Features
			UHPLC	HPLC	
Aggregate Dimer Monomer Fragment	Molecular size	Size-Exclusion Chromatography (SEC)	TSKgel UP-SW3000		Dimer, monomer, fragment
				TSKgel SuperSW-mAb HR/HTP	Dimer, monomer, fragment
				TSKgel UltraSW Aggregate	High molecular aggregate
				TSKgel SuperSW3000	1.0, 2.0 4.6 mm I.D. columns
				TSKgel G3000SW$_{XL}$	Dimer, monomer, fragment
Charge Variants	Ionic interaction	Ion-Eschange Chromatography (IEC)		TSKgel CM-STAT	by salt gradient
				TSKgel SP-STAT	by pH gradient
Aggregate Met-oxidant	Hydrophobicity	Hydrophobic Interaction Chromatography (HIC)		TSKgel Butyl-NPR	Methionine-oxidation ADC analysis
				TSKgel Phenyl-5PW	
mAb with different ADCC Glycosylation, Glycation, Titer	Affinity	Affinity Chromatography (AFC)		TSKgel FcR-IIIA-NPR	ADCC activity
				TSKgel Boronate-5PW	Glycation
				TSKgel Protein A-5PW	Titer determination
Peptide mapping Fragment	Hydrophobicity	Reversed-Phase Chromatography (RPC)		TSKgel ODS-120H	Peptide mapping
				TSKgel SuperODS	Teptide mapping
				TSKgel ODS-100V 3 um	Peptide mapping
				TSKgel Protein C$_4$-300	Protein, fragment
Glycosylation	Hydrophilicity	Normal Phase Chromatography (HILIC) (NPC/HILIC)		TSKgel Amide-80 2 um, 3 um	Glyco-form analysis
				TSKgel NH$_2$-100 3 um	Glyco-form analysis

Thank you for your attention.

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