Addressing Analytical Challenges in Biopharmaceuticals Analysis & QC

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Santa Clara, California, USA

Outline

The big switch in Pharma

Biopharma customers have different challenges

The best kept secrets in Agilent

- Bioanalyzer – The Lab-on-a-Chip
- TapeStation – The Next-gen electrophoresis platform
- OFFGEL Fractionator – A novel sample prep tool
- CE and CE/MS in biopharmaceutical analysis
- HPLC-Chip Technology

Biosimilars

- Definitions & Regulations
- How similar is similar enough
- Case studies: Comparability data between a biosimilar and its innovator reference

Summary

Traditional Pharma business model is changing

It's a mAb!

It's a large Pharma!

HEALTH

New melanoma drug boosts survival time

By Marilynn Marchione
ASSOCIATED PRESS

CHICAGO — Researchers have scored the first big win against melanoma, the deadliest form of skin cancer. An experimental drug significantly improved survival in a major study of people with very advanced disease.

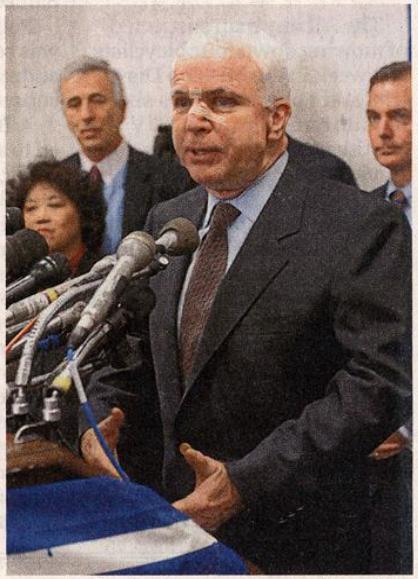
The results, reported Saturday at a cancer conference, left doctors elated.

"We have not had any therapy that has prolonged survival" until now, said Dr. Lynn Schuchter of the Abramson Cancer Center at the University of Pennsylvania, a skin cancer specialist with no role in the study or ties to the drug's maker.

The drug, ipilimumab, works by helping the immune system fight tumors. The federal Food and Drug Administration has pledged a quick review, and doctors think the drug could be available by the end of this year.

"People are going to have a lot of hope and want this drug, and it's not on their doctors' shelves," although some may be able to get it through special programs directly from its maker, Bristol-Myers Squibb Co., Schuchter said.

Melanoma is the most serious form of skin cancer. Last year in the United States, there were about 68,720



Stephen J. Boitano / Associated Press 2002

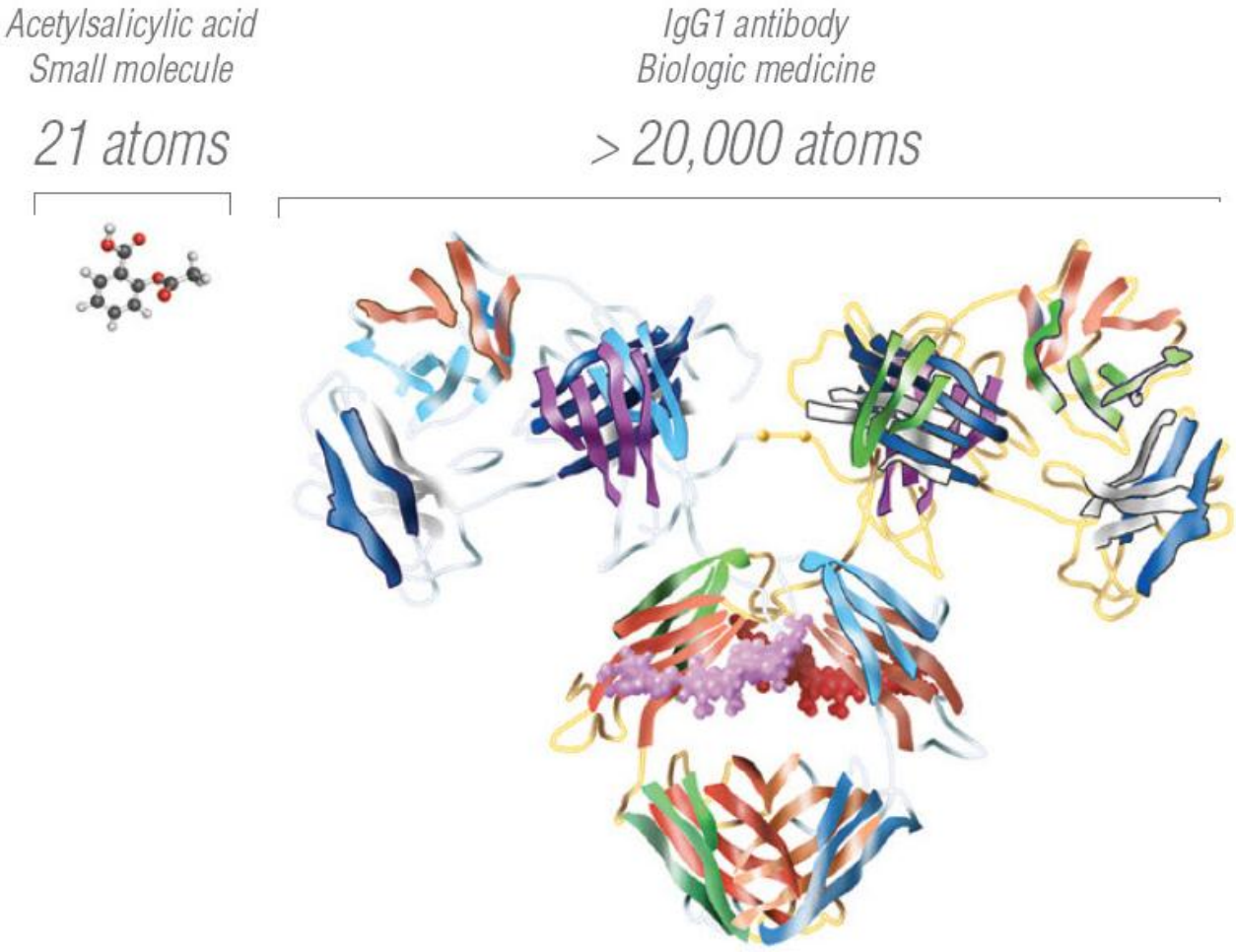
Sen. John McCain, R-Ariz., had a melanoma removed from his nose.

immune-stimulating treatment, or the immune-stimulating treatment alone.

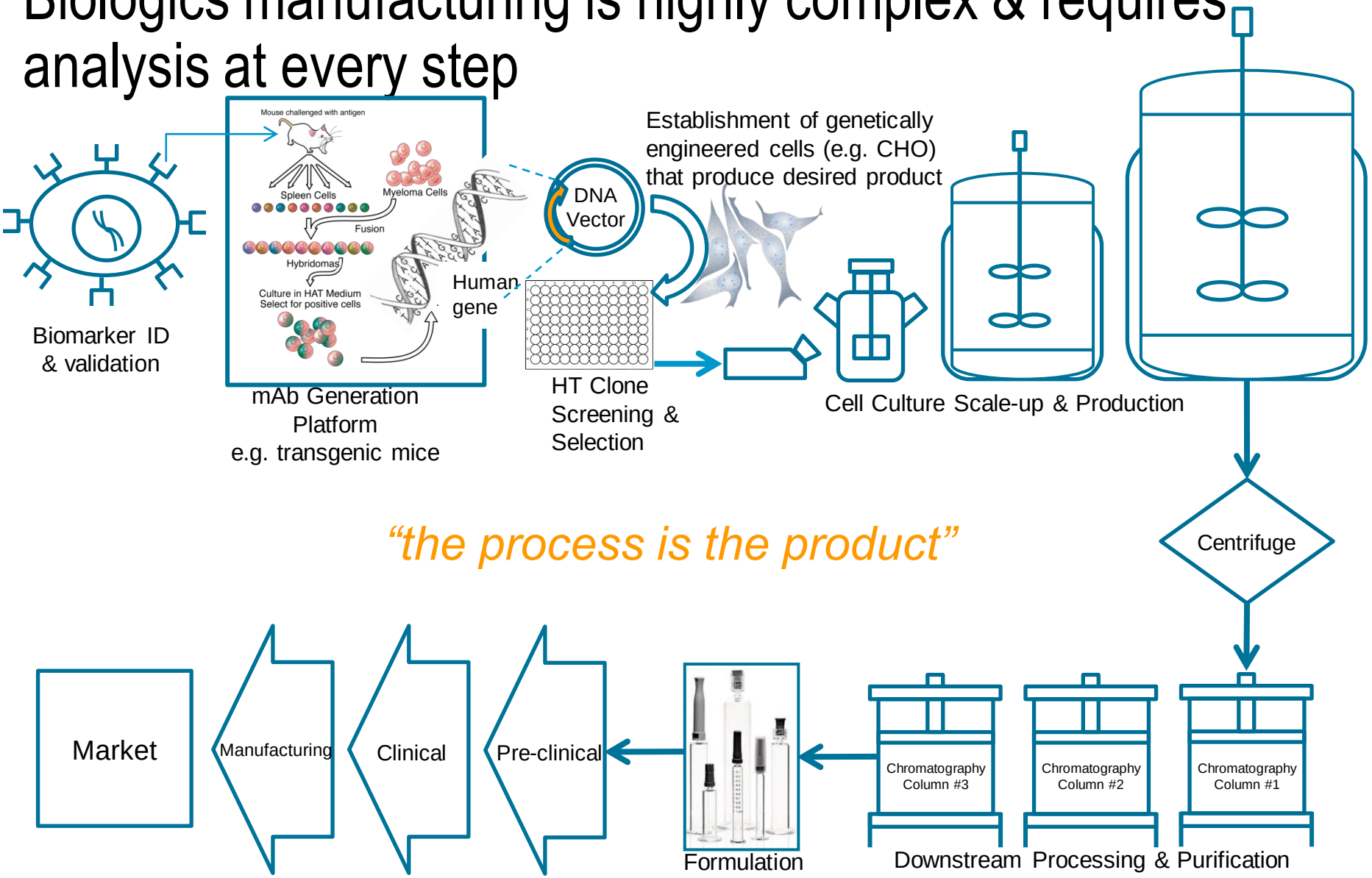
After two years, 24 percent of those given the drug alone or in combination were alive, versus 14 percent of those given just the immune-stimulating treatment.

Average survival was 10 months with ipilimumab versus just over 6

Antibodies are unlike Aspirins

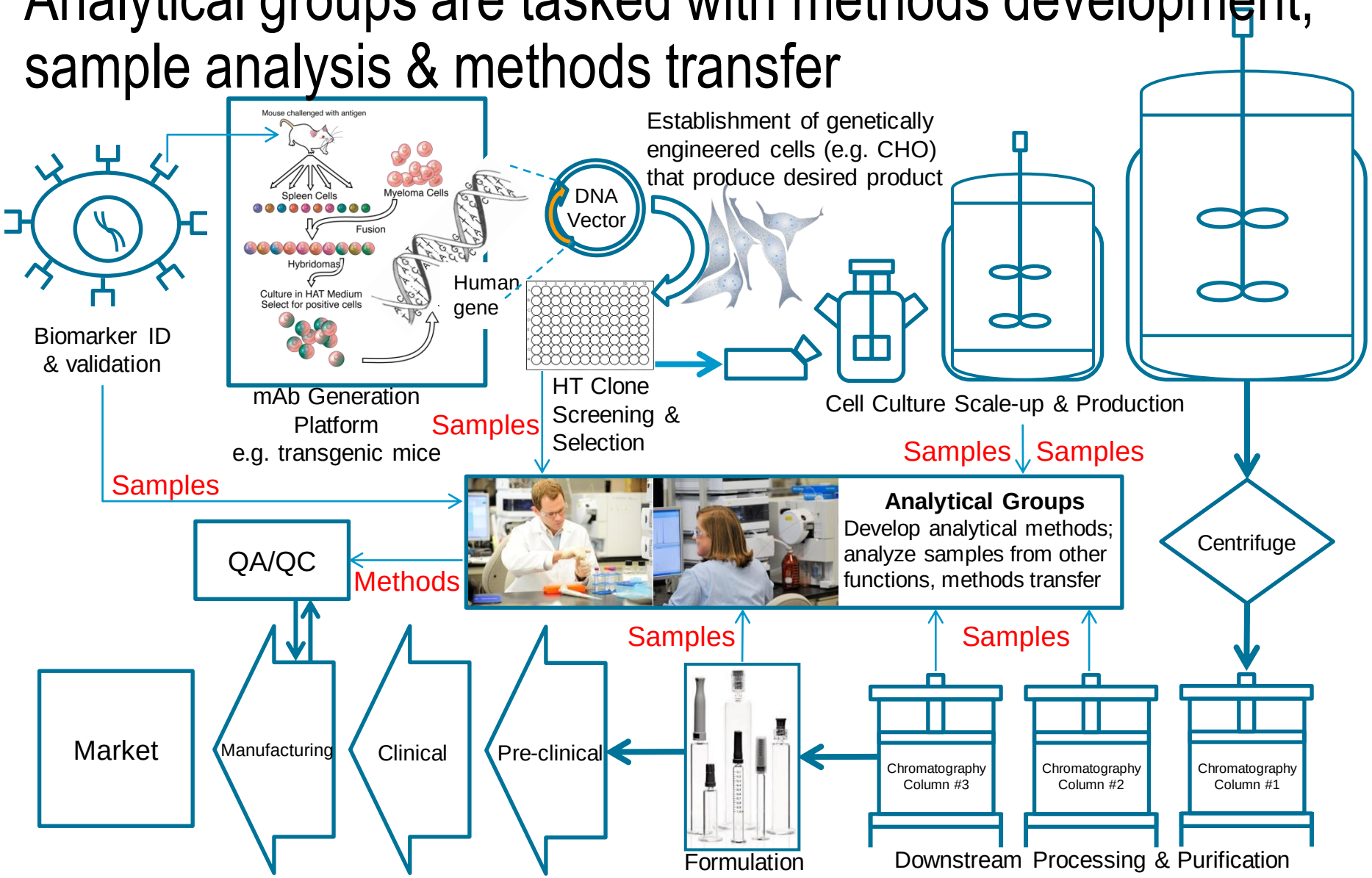


Biologics manufacturing is highly complex & requires analysis at every step



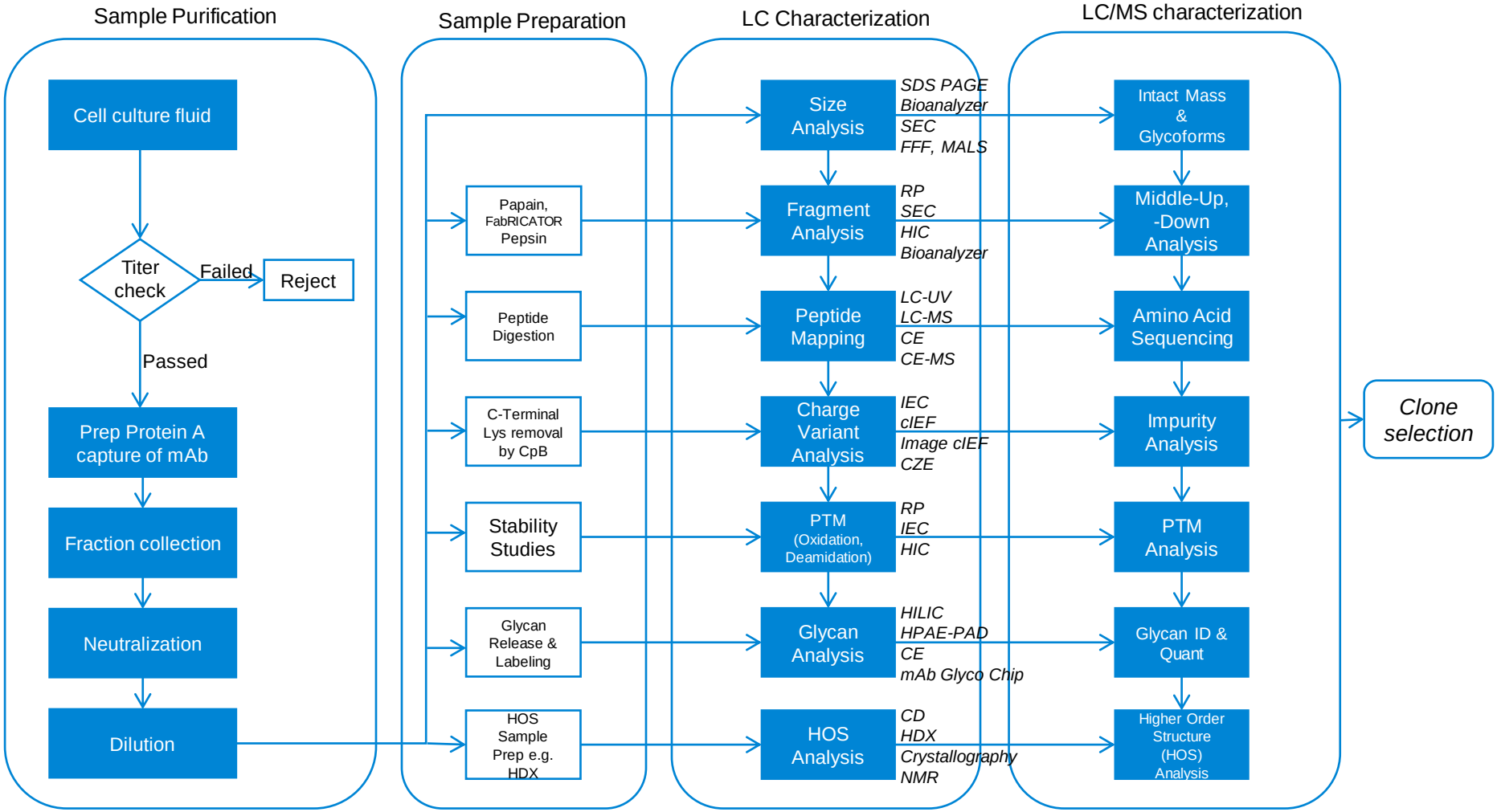
"the process is the product"

Analytical groups are tasked with methods development, sample analysis & methods transfer



Typical workflows in biopharma analytical labs — from cell culture harvest to clone selection

Samples from cell culture & purification process development, formulation, stability studies, QA/QC



Biopharma customers have different challenges

- Biopharma sample prep is complex
  - Too many timed-steps that tie scientist to bench
- Bioseparation methods are longer, product specific & require long method development time
- There are many steps & methods within workflows
  - Need to automate and streamline workflows and methods to meet the demand without impacting quality
- Bioseparation methods uses corrosive buffers not amenable to LC/MS analysis
- MS are becoming important and the need to make them biologist friendly

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  - TapeStation – The Next-gen electrophoresis platform
  - OFFGEL Fractionator – A novel sample prep tool
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- Biosimilars
  - Definitions & Regulations
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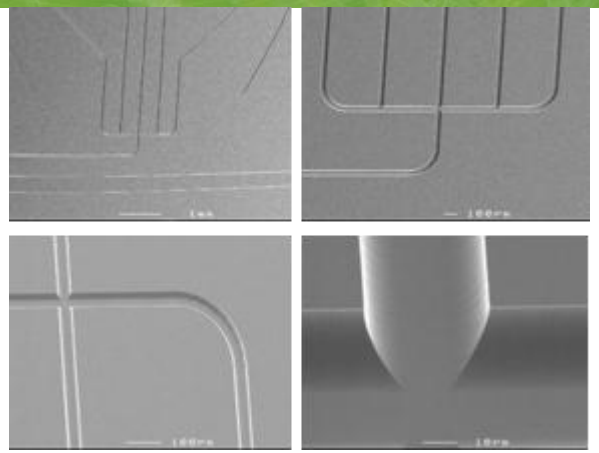
Traditional Slab Gel Analysis

The bottleneck in Protein, DNA and RNA analysis

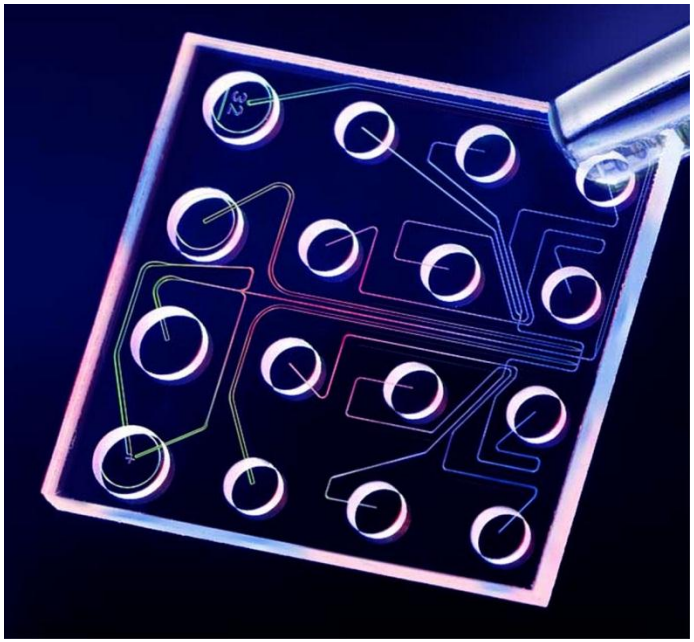
- Manual process
- Difficult to automate
- Slow
- Not accurate enough
- Bad reproducibility
- No direct comparison

Agilent solutions offer the potential to address this

2100 Bioanalyzer:
The Lab-on-a-Chip Approach



Increasing quality and speed of gel electrophoresis



- Highly reproducible results
- Qualitative & Quantitative in a single step
- Sample volumes 1 -5 µl
- Up to 12 samples depending on Assay
- Digital results in 30 minutes available
- No extra waste removal needed
- Disposable Chip, no crosscontamination

Bioanalyzer Protein Kit portfolio

Agilent Protein 80 kit	Prod Number	5067-1515
Agilent Protein 230 kit	Prod Number	5067-1517
Agilent High Sensitivity Protein 250 kit	Prod Number	5067-1575

P 80

Range 5 - 80 kDa
Sensitivity: Coomassie
Samples: 10

Samples
-Antibodies (reduced)
-Small Proteins

P 230

Range 14 - 230 kDa
Sensitivity: Coomassie
Samples: 10

Samples
-Antibodies (all types)
-Standard Proteins

Coomassie Range (5 ng/µL BSA)

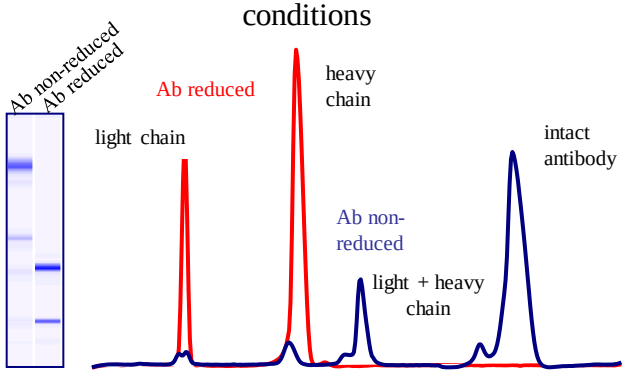
HSP 250

Range: 10 - 250 kDa
Sensitivity: 1 pg/µl BSA on Chip
Samples #: 10 per Chip
Chips #: 10 per Kit
Labeling Conc: 1 ng – 1 µg /µl

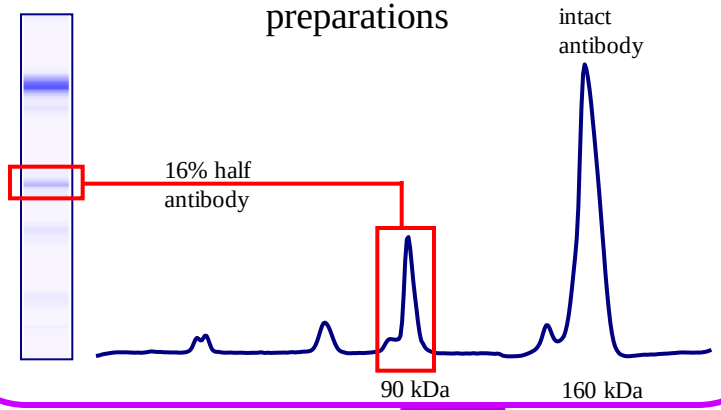
Silver stain Range (200 pg/µL BSA)

Quality Control of Antibodies

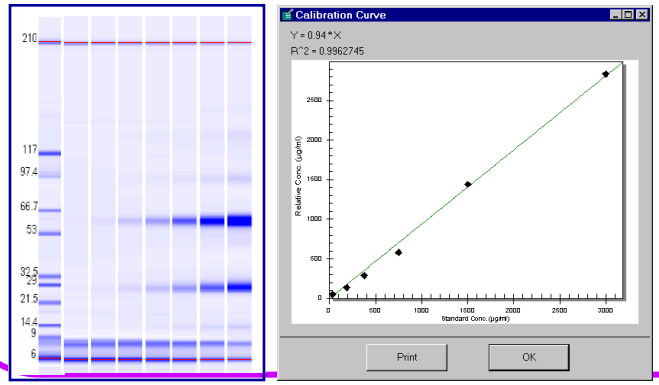
Antibody analysis under reducing and non-reducing conditions



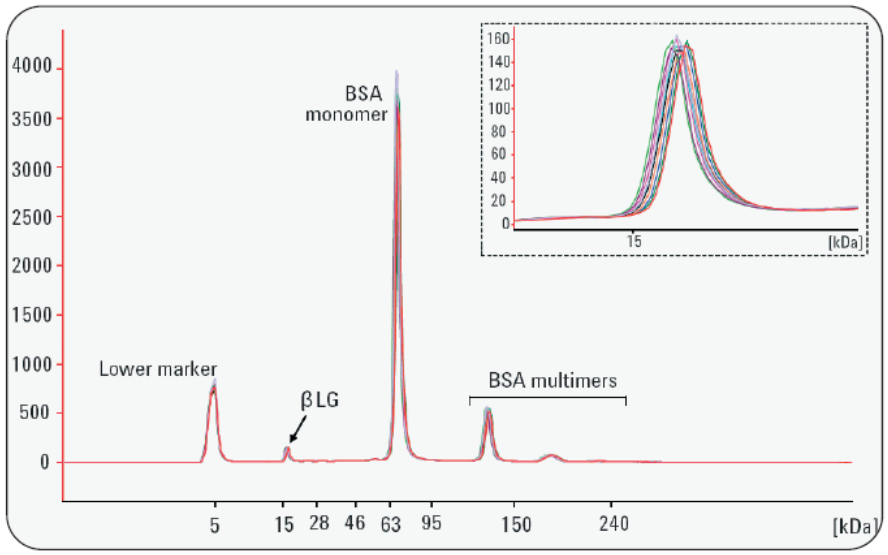
Determine the half antibody content in IgG preparations



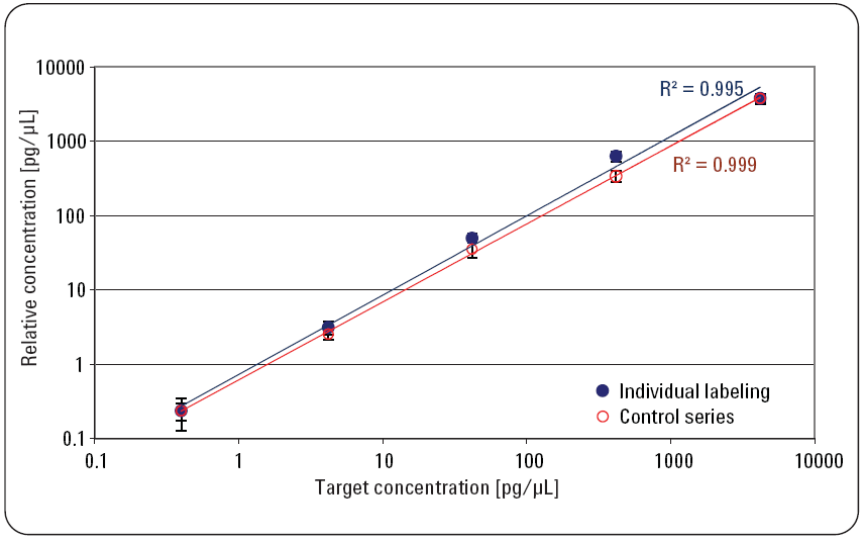
Absolute Quantitation of IgG samples



Quantification and Purity Analysis



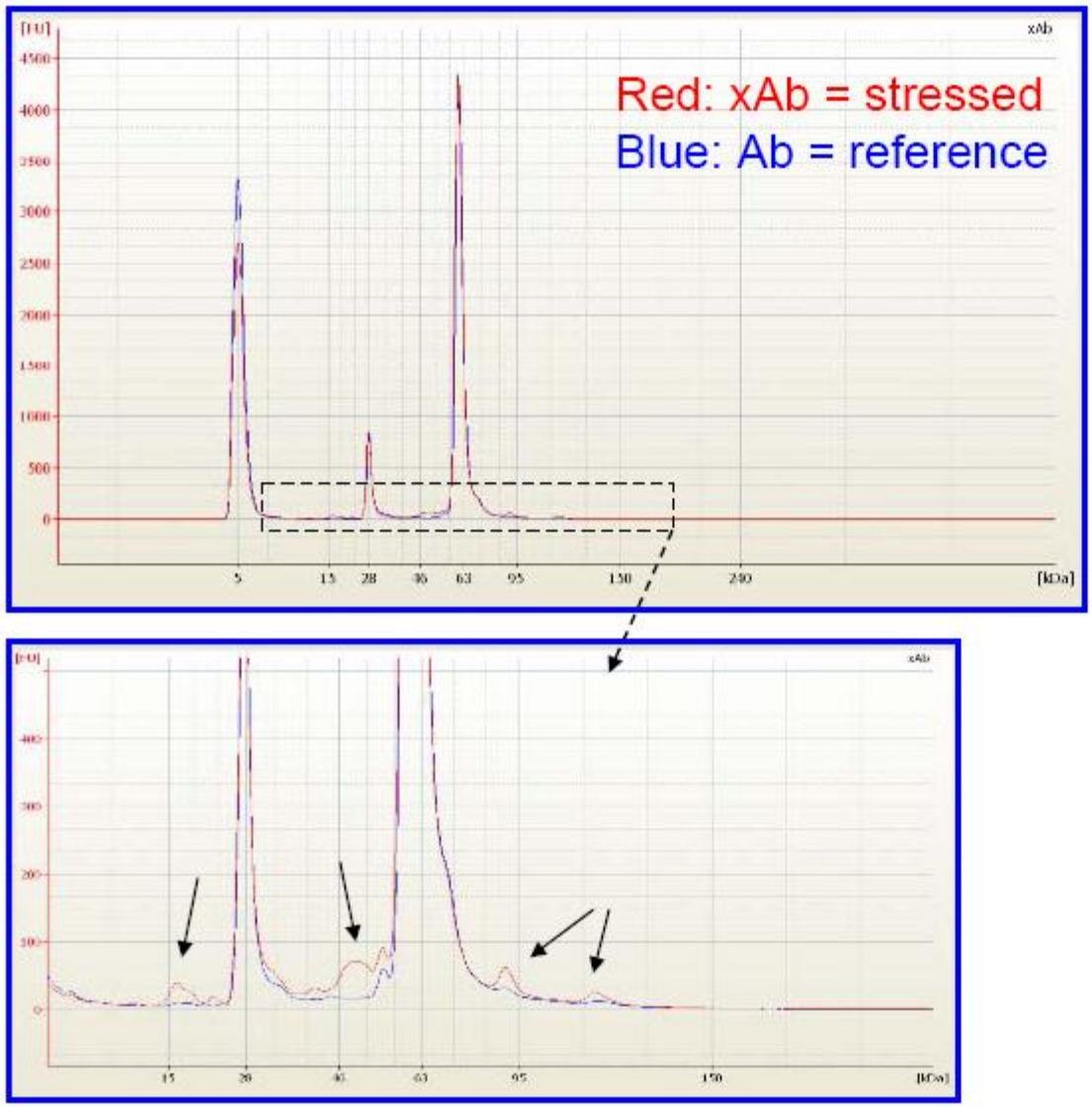
Protein		Rel. Conc. [pg/µL]	% Total
βLG	Average	184	3.3
	% CV	3.3	1.5
BSA monomer	Average	4191	75.7
	% CV	3.1	0.3

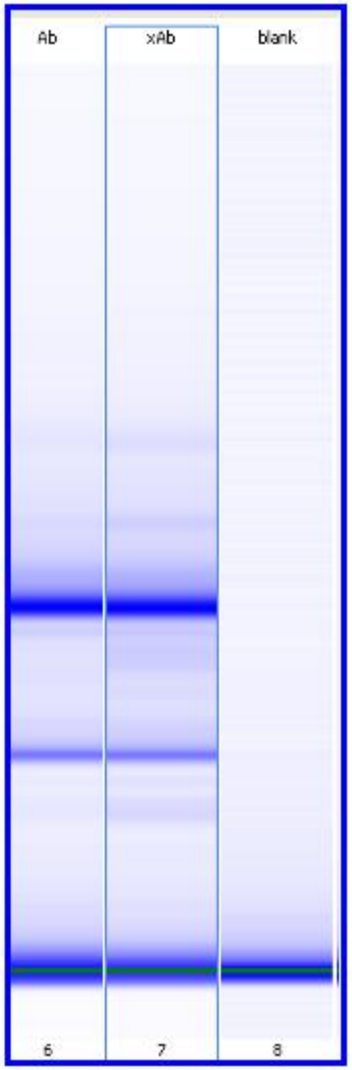


Impurity analysis

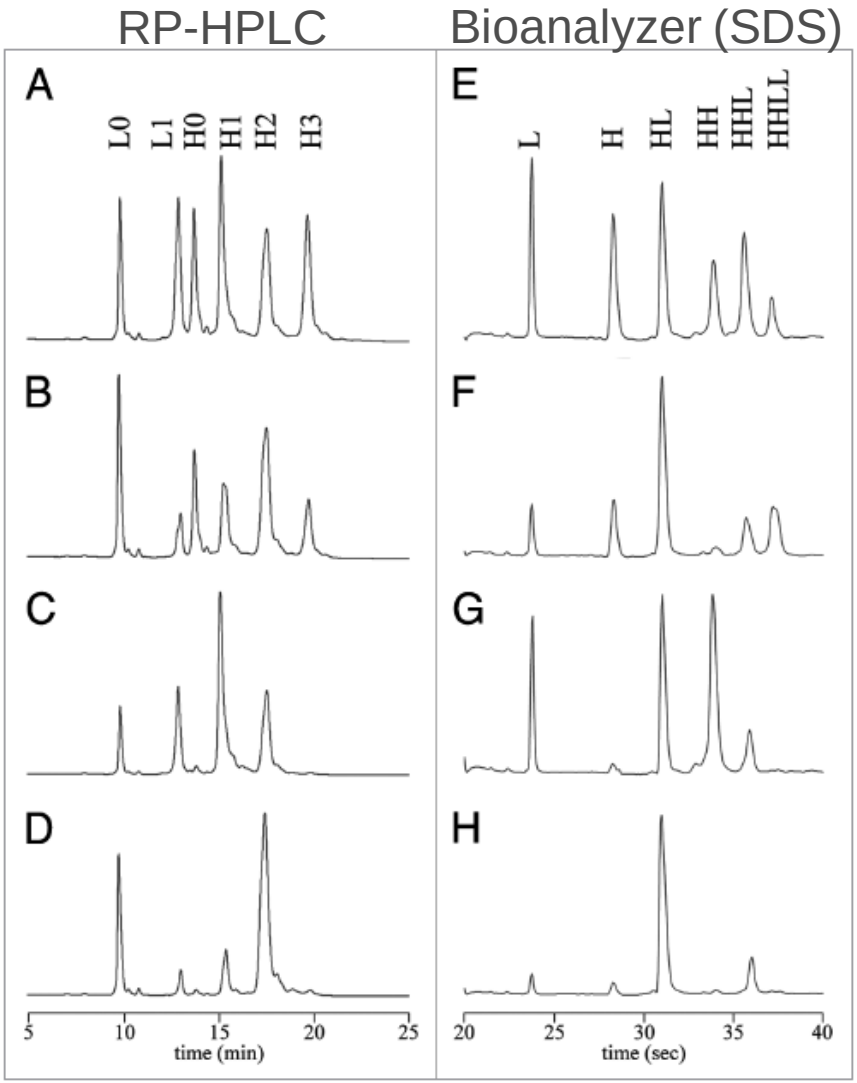
The High Sensitivity Protein 250 kit is perfectly suited to measure minor impurities besides dominant main compounds. With highest sensitivity and a 4 orders of magnitude range for linear quantification the 2100 can reliably analyze up to 10 samples in 40 minutes

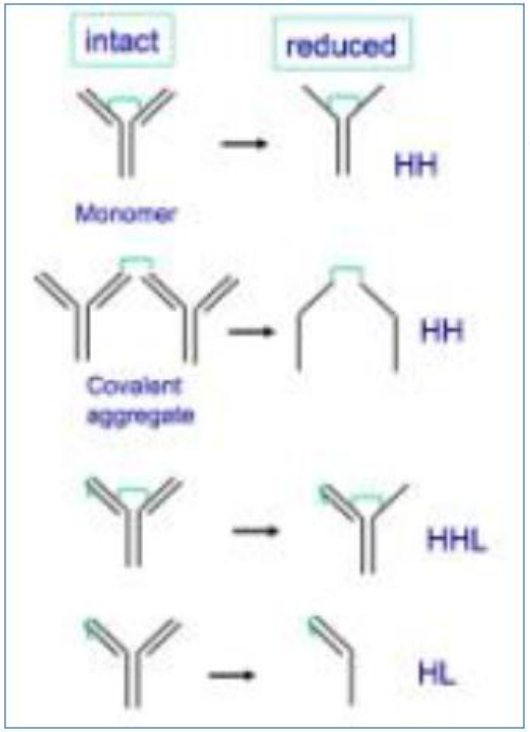
IgG Stress Tests





Analysis of Reduced ADC fragments





Bioanalyzer (SDS)

Figure 4. (A–D) Reversed-phase HPLC analysis of DTT-reduced conjugates produced using different reduction/reoxidation protocols. (E–H) Analysis of the same conjugate samples in (A–D), under non-reducing conditions, using the Agilent Bioanalyzer™, a silicon chip based system for capillary electrophoresis in the presence of SDS (CE-SDS).[12] Adapted with permission from Sun MM, Beam KS, Cerveny CG, Hamblett KJ, Blackmore RS, Torgov MY, et al. Reduction-alkylation strategies for the modification of specific monoclonal antibody disulfides.[12]

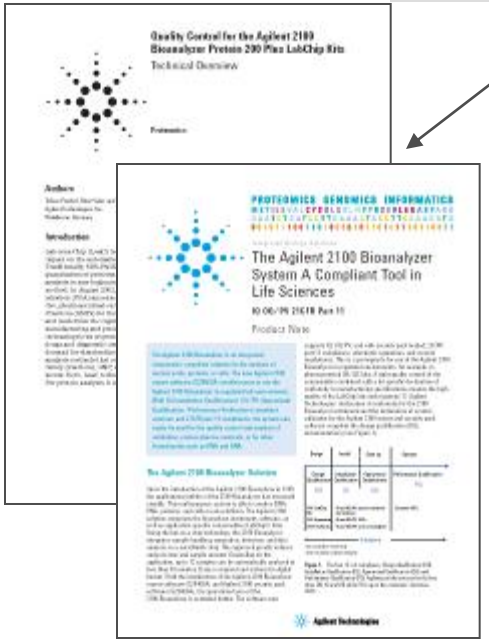
A Wakankar, Y Chen, Y Gokarn & F Jacobson (Genentech)

2100 Bioanalyzer – 21 CFR Part 11 Compliance

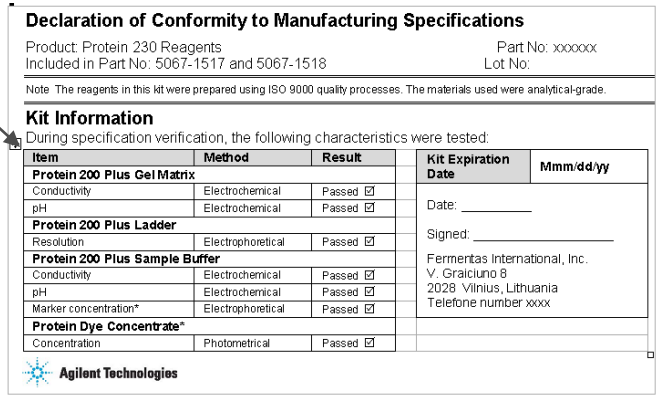
Compliance Services

21 CFR part 11 compliant Security Pack Software

Application Notes



Declaration of Conformity (consumables)



Declaration of Conformity to Manufacturing Specifications

Product: Protein 230 Reagents — Part No: xxxxxx
Included in Part No: 5067-1517 and 5067-1518 — Lot No:

Note The reagents in this kit were prepared using ISO 9000 quality processes. The materials used were analytical-grade.

Kit Information

During specification verification, the following characteristics were tested:

Item	Method	Result
Protein 200 Plus Gel Matrix		
Conductivity	Electrochemical	Passed ☑
pH	Electrochemical	Passed ☑
Protein 200 Plus Ladder		
Resolution	Electrophoretical	Passed ☑
Protein 200 Plus Sample Buffer		
Conductivity	Electrochemical	Passed ☑
pH	Electrochemical	Passed ☑
Marker concentration*	Electrophoretical	Passed ☑
Protein Dye Concentrate*		
Concentration	Photometrical	Passed ☑

Kit Expiration Date	Mmm/dd/yy

Date: _____

Signed: _____

Fermentas International, Inc.
V. Graiciuno 8
2028 Vilnius, Lithuania
Telefone number xxxx

Agilent Technologies

Agilent 2200 TapeStation – Extension of Agilent's Electrophoresis Platforms

Ease of use

2100 Bioanalyzer

Gold Standard for NGS sample and library QC

2200 TapeStation

- 96-well compatible
- 1min/sample run time
- Fully scalable throughput
- Ready-to-use ScreenTape
- 1µl - 2µl of sample

Automation & Throughput

ScreenTapes

Co-extruded & formed polymer

16 individual acrylamide gel & buffer filled channels

Pre-packaged (no reagent or chip priming)

Barcoded to trace batch and usage

Run 2 to 16 samples

Unused lanes can used within 2 weeks

Only 1-2ul sample required

4 month shelf life at 4ºC

Various ScreenTape types available

2200 TapeStation

Monitoring mAb Stability

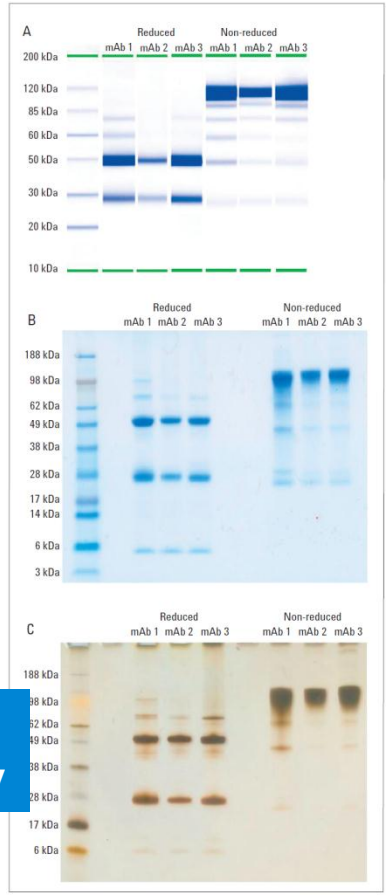


Figure 1
Separation of three different mAb preparations with the P200 ScreenTape (A), or 4-12% SDS-PAGE gel stained with Coomassie blue (B) or with silver stain (C) in reducing and non-reducing conditions. Lane 1 contains the P200 ladder (A) or a SDS-PAGE ladder (B and C). Green bands in each lane are internal P200 markers.

Monitoring Purification Schemes

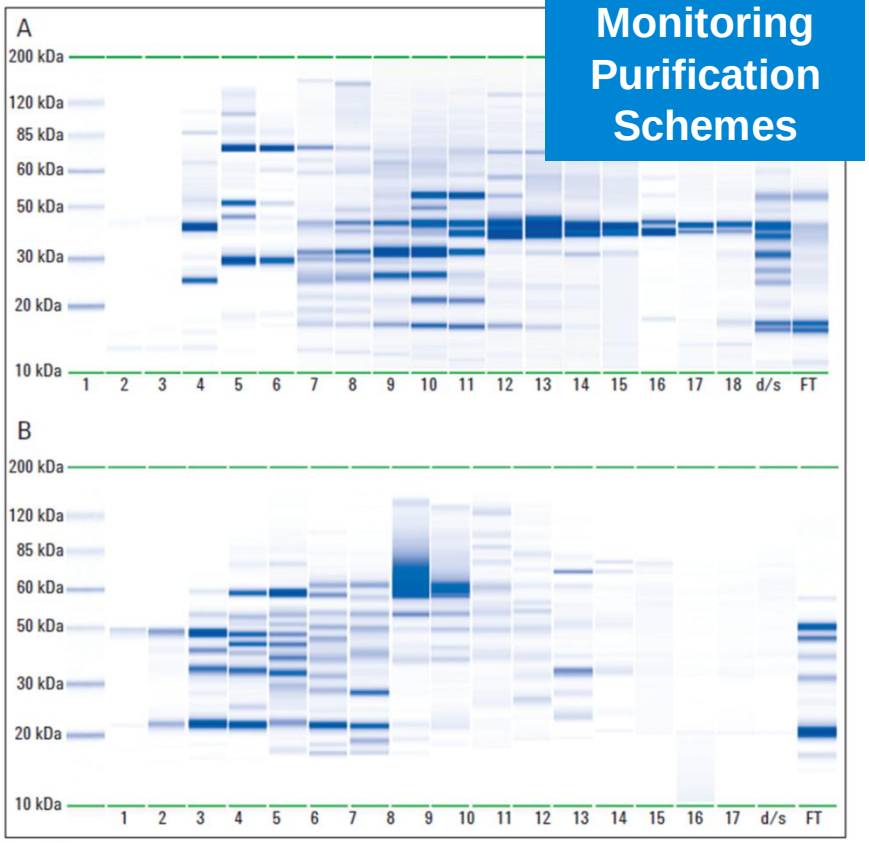


Figure 1
P200 ScreenTape gel images from anion (A) and cation (B) exchanger fractions. Cation exchanger samples were diluted 1:5 prior to analysis. Column flow through (FT) and low salt wash (d/s) from the anion exchanger were combined to form the starting material for the cation exchange purification. The P200 ladder is shown in the first lane, internal markers are highlighted in green.

Agilent 3100 OFFGEL Fractionator

IEF of Peptides and Proteins

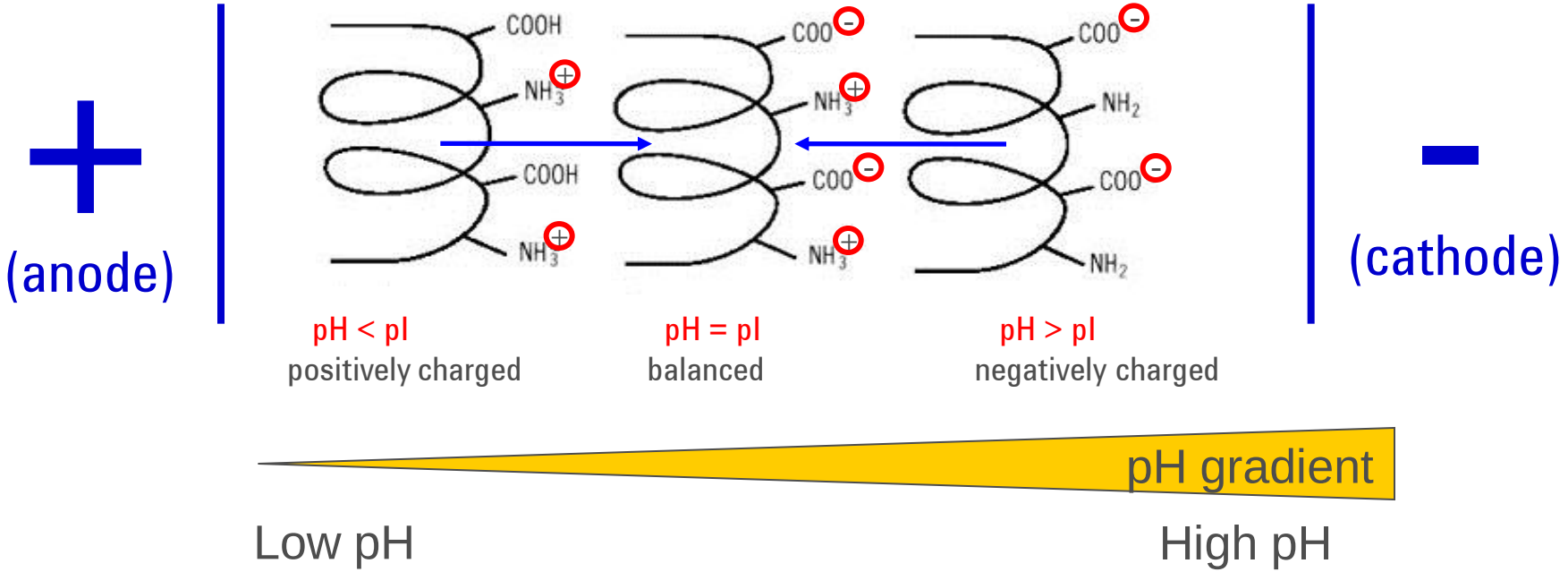
Agilent Technologies

Isoelectric Focusing IEF

What does the instrument do?

It sorts proteins or peptides in a pH gradient (isoelectric focusing, IEF) = It separates proteins or peptides according to their isoelectric points (pI)

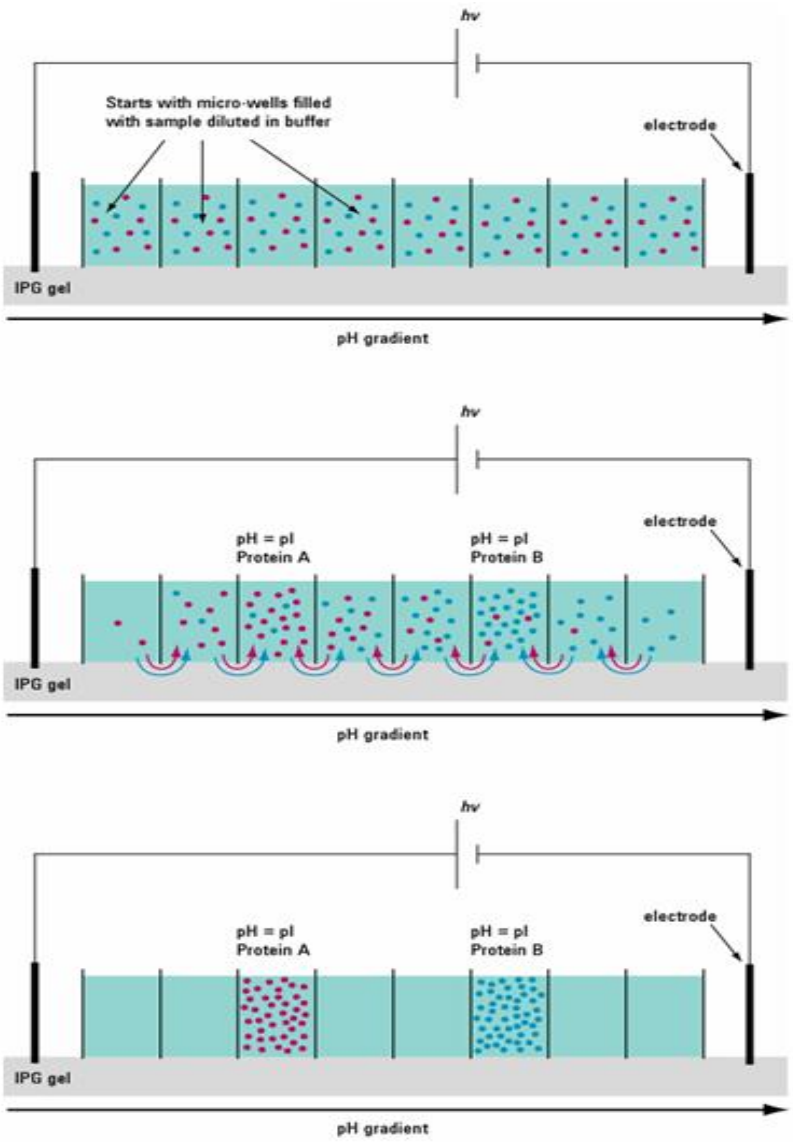
Isoelectric point: pH at which the net charge of the protein is zero (can be calculated from the number of the basic and acidic side chains)



OFFGEL Principle
pI-Based Fractionation

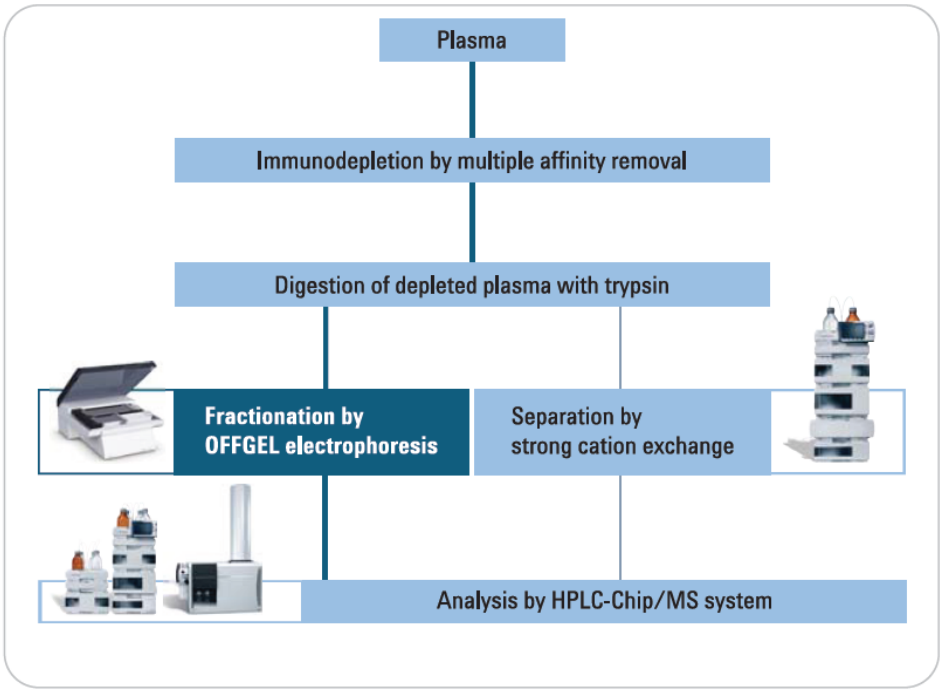
- after rehydration the IPG gel seals tightly against the compartment frame
- the diluted sample is distributed across all wells in the strip
- after fractionation the liquid fractions containing can be removed with a pipette

Number of fractions	**12 or 24**
Samples	**proteins or peptides**
Fraction volume	**150 ul**
Resolution	**0.1/0.6 pH**
Loading capacity	**50 µg – 5 mg per sample**
Fractionation time	**8 - 24 h**
Typical recovery	**70% for proteins, 90% for peptides**

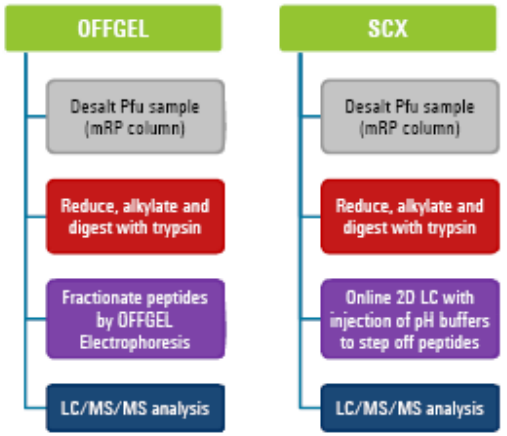


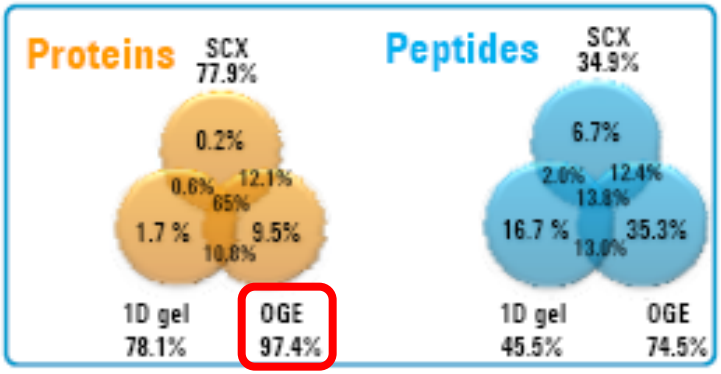
OFFGEL Increases MS Sensitivity

Comparison to SCX (MudPIT) with Stratagene Pfu Standard



Workflow: orthogonal methods!





OFFGEL Prefractionation lead to the highest number of peptides and proteins identified: > **97%** of all proteins in the PFU standard!

Miller, C., *et. al.*, HUPO 2010

OFFGEL Used to Isolate Antibody Charge Heterogeneity Observed by Capillary IEF

Anal. Chem. **2010**, *82*, 3510–3518

Characterization of Antibody Charge Heterogeneity Resolved by Preparative Immobilized pH Gradients

Charlie D. Meert, Lowell J. Brady, Amy Guo, and Alain Balland*

Amgen Inc., Analytical and Formulation Sciences, 1201 Amgen Court West, Seattle, Washington 98119

Workflow

Protein purification

***Native* protein OFFGEL fractionation**

Fraction clean-up

Fraction analysis:

- LC/MS-MS (TOF), native & reduced mass
- Peptide fingerprinting
- cIEF (control)

Charge Variant Identification of Protein Drugs

Workflow

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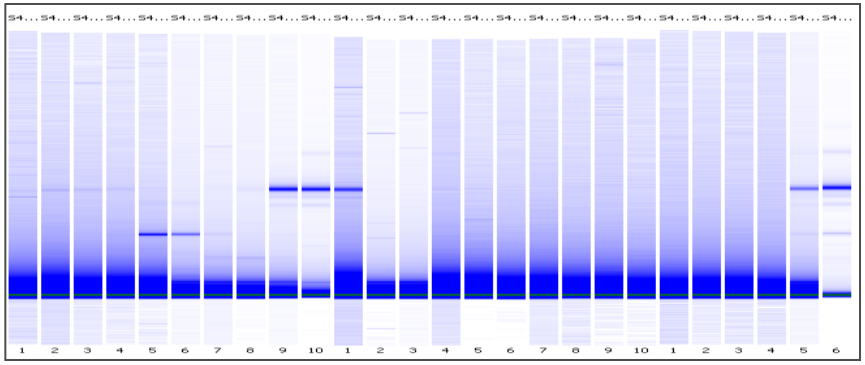


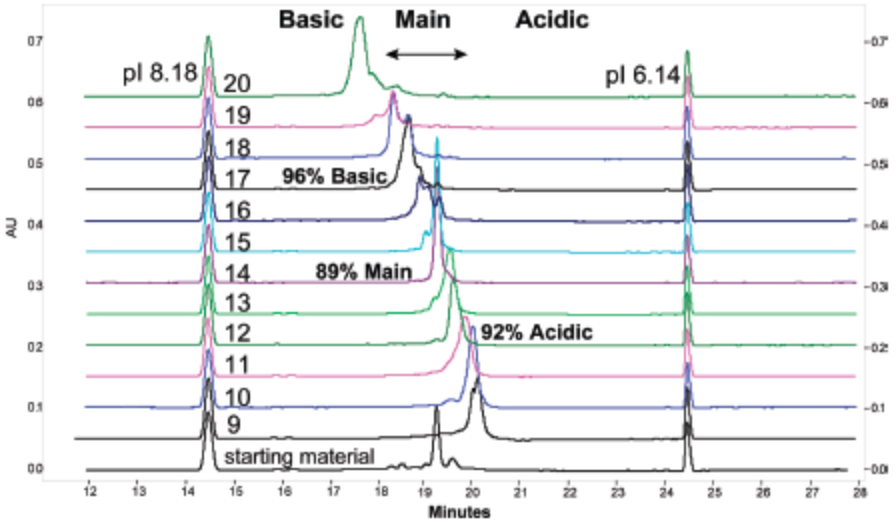
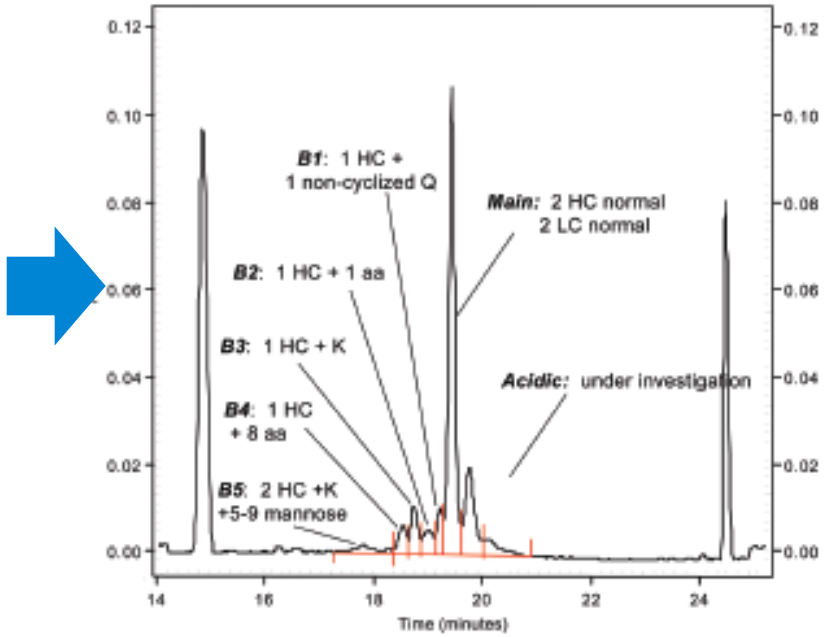


Figure 2. cIEF profiles of fractions obtained from an optimized OFFGEL preparative IEF fractionation of the antibody show isolation of the basic, main, and acidic peaks across 12 fractions compared to the starting material. The composition of the isoform present, expressed in percentage, was determined using cIEF peak area and is indicated for fractions 12, 14, and 17.



Meert, C,. *et. al.*, Anal. Chem. 2010
5990-6521EN

Sample Preparation for Charge Variant Identification of Protein Drugs by OFFGEL

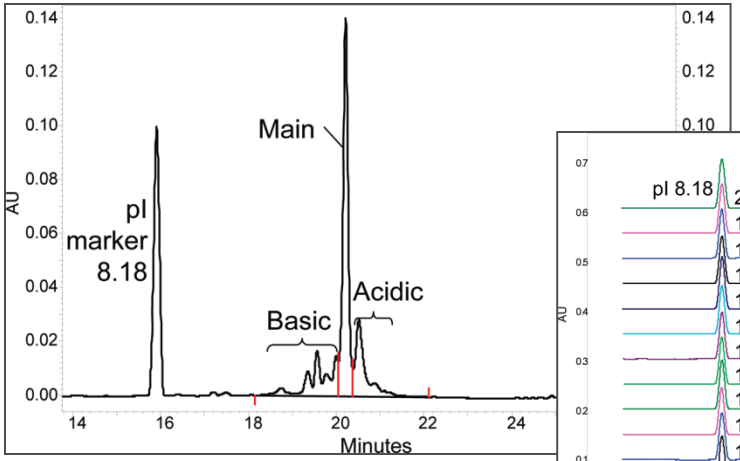
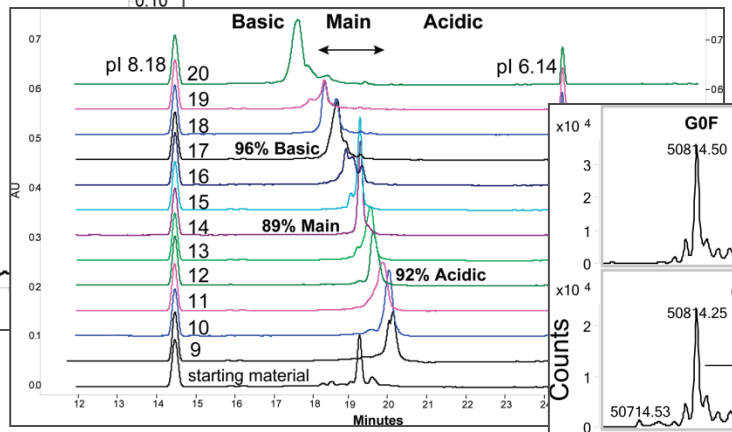


Figure 1. Representative cIEF profile of the antibody of interest for characterization using preparative IEF

Balland, A., et al, 2010, Characterization of Antibody Charge Heterogeneity Resolved by Preparative Immobilized pH Gradients, Anal. Chem. 2010, 82, 3510–3518



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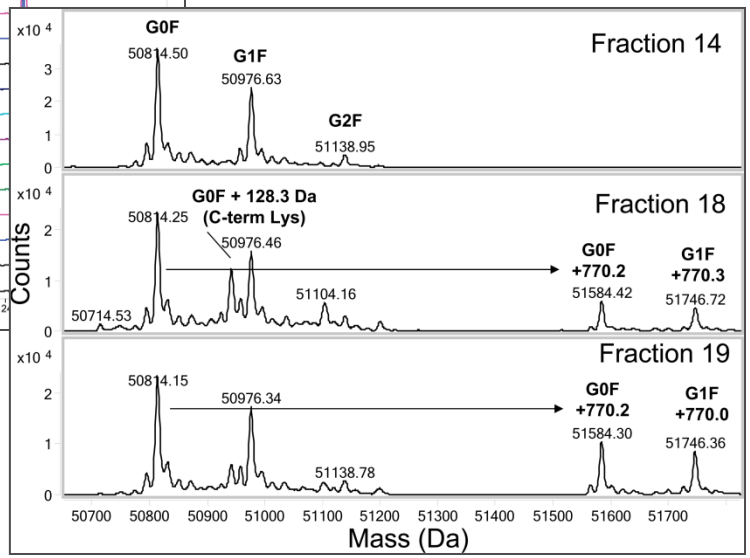
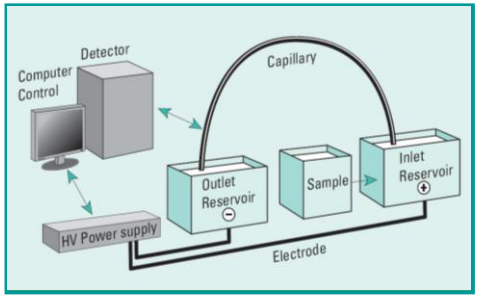


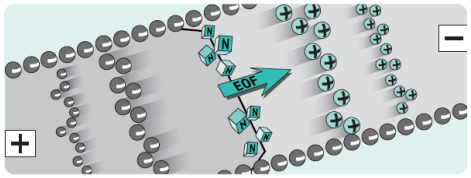
Figure 4. Detailed view of the deconvoluted heavy chain spectra for fractions 14, 18, and 19 from the reduced mass analysis shows the enrichment of a +770 Da species in fractions 18 and 19 compared to the main peak in fraction 14.

CE and CE/MS in Biopharmaceutical analysis

Capillary Electrophoresis Applications

Separation Principles of Capillary Electrophoresis (CE)





- Separation based on compound mobility (mass/charge) in an electrical field
- High resolution separations (usually >> 40,000 plates)
- Fast separation (few minutes)
- Smallest sample volumes (few µL)
- Less sample prep required (no stationary phase, just an open glass tube)
- Low consumption of sample and buffer (green method)

- Orthogonal technique complementing HPLC

Sample Capacity
Detector Options
Sensitivity
Reproducibility
Green Methods
Neutral Compounds
Mainstream Technique
Cost of Ownership
Biocompatibility

Ideal

Large Molecules
Biocompatibility
Small sample Volume
Green Methods
Cost of Ownership
Sample Capacity
Reduced Sample Prep
High Resolution
Charged Compounds

Ideal

CE in Biopharma Application

Abbreviations: CZE-Capillary Zone Electrophoresis; MECC-Micellar ElectroKinetic Chromatography; CEC-Capillary ElectroChromatography; cIEF-CapillaryIsoeElectric Focussing; cGE-Capillary GelElectrophoresis

- Different resolution and separation than reversed phase LC
- Avoids retention problems of Ions or polar compounds with RP-columns
- No column packing materials – less adsorption, easy cleaning by capillary flush
- Offers non-denaturing separations of bio molecules (e.g. Proteins)
- Many separation modes on one instrument CZE, MECC, CEC, cIEF, cGE,...

Ions	Anion and Cation analysis. Quantitation and ID of ions	Modes: CZE Detectors: UV-DAD, CCD
Small polar compounds	ID and quantification of polar or charged compounds	Modes: CZE Detectors: UV-DAD, CCD
Metabolites	ID and quantification of Amines, Organic Acids, Nucleosides, ...	Modes: CZE Detectors: UV-DAD, MS
Proteins & Peptides	Native or denatured protein analysis, charge states, sizing	Modes: CZE, cIEF, cGE Detectors: UV-DAD, LIF, MS
Glycans	Profiling of complex and linked Glycans	Modes: CZE Detectors: LIF, MS
Nucleic Acids	Sizing and quantification of Oligonuclotides or dsDNA	Modes: CZE, cGE Detectors: LIF, MS

Agilent 7100 CE

Performance	Highest sensitivity for UV
Handling	Quick, direct and easy
Automation	Agilent replenishment system
Flexibility	All modes, open to external detectors
CE/MS	Complete single vendor solution
Economic	Reducing cost of ownership

Slim housing

Sensitive UV diode array detector based on the Agilent 1200 Series LC

Quick access cassette type

Vial sensor for sample tray

Fast Agilent 1200 electronics

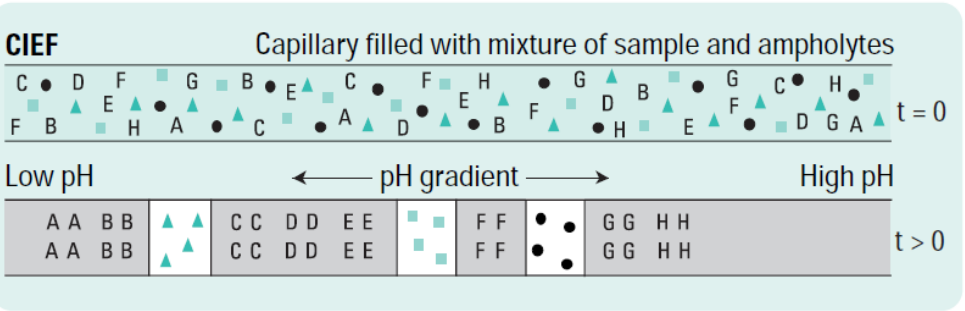
Low volume replenishment system

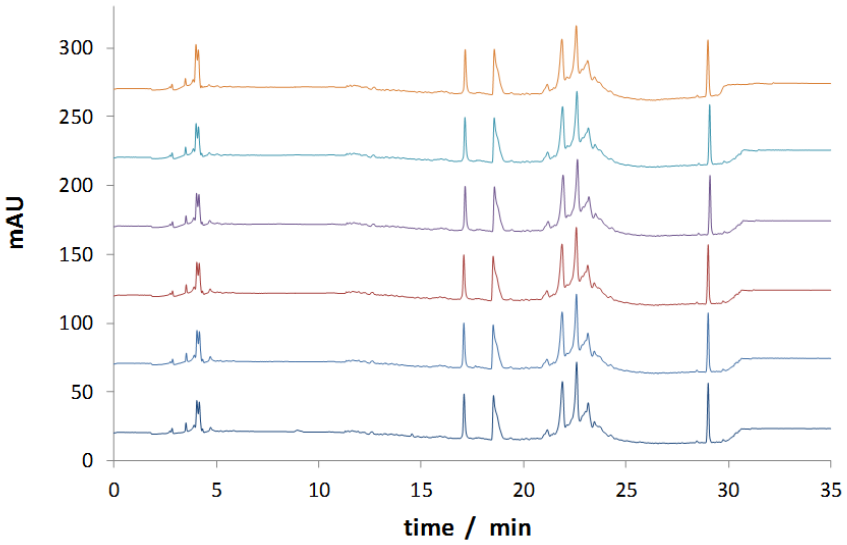
Improved pressure/vacuum pumps

Agilent Open LAB CDS software
LabAdvisor Diagnostic tools

Application of cIEF
Separate Peptides & Proteins Based on Their Isoelectric Point (pI)

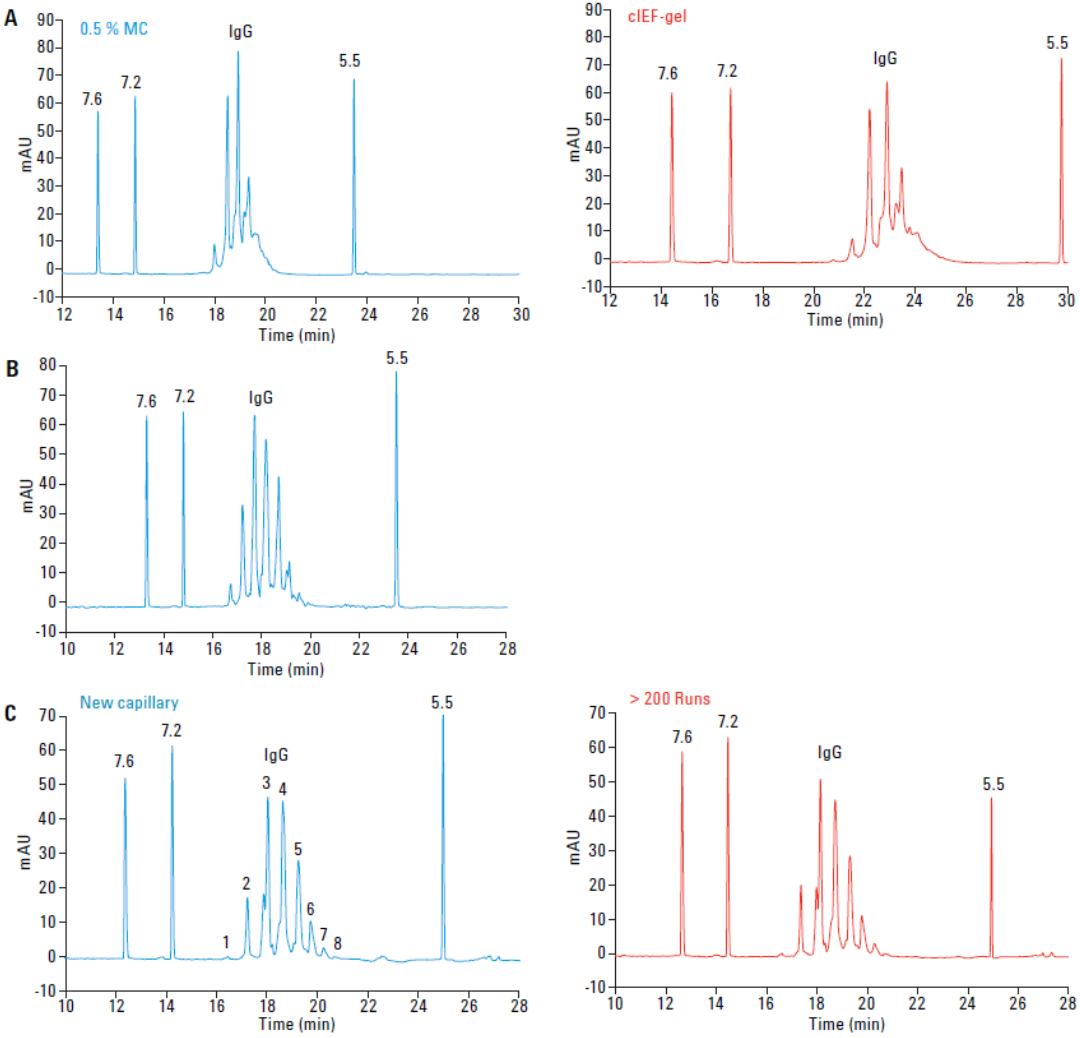
1) The whole capillary is filled by a mix of sample and ampholytes.
2) Ampholytes will create a pH gradient after an electrical field is applied.
3) Focusing step all sample compounds will migrate to reach their distinct pI value were they get uncharged and stop moving.
4) Detection, moving content of the capillary via pressure or chemical mobilization through the detection window





Agilent Application Notes
(Publication 5991-1142EN)
(Publication 5991-2885EN)

cIEF Analysis using Agilent µSIL FC capillaries



cIEF analysis of commercially available mAb samples on fluorocarbon coated capillaries

Electropherograms of samples containing mouse IgG1-k and either cIEF gel (red) or 0.5 % MC (blue) are shown in **(A)**

The resolution of main mAb isoform peaks was 2.74 ± 0.05 with the cIEF gel and 2.66 ± 0.09 with 0.5 % MC (n = 5). Samples containing an mouse anti-a1-antitrypsin mAb **(B)** or a rat anti-DYKDDDDK mAb **(C)** were analyzed in presence of 0.5 % MC.

Rat mAb, electropherograms and peaks labeled from 1 to 8 obtained on:

new capillary	--- blue line
after more than 200 injections	--- red line

Agilent Application Note (Publication 5991-2885EN)

The Potential of CZE for Charge Variant Analysis!

Journal of Chromatography B, 983–984 (2015) 101–110

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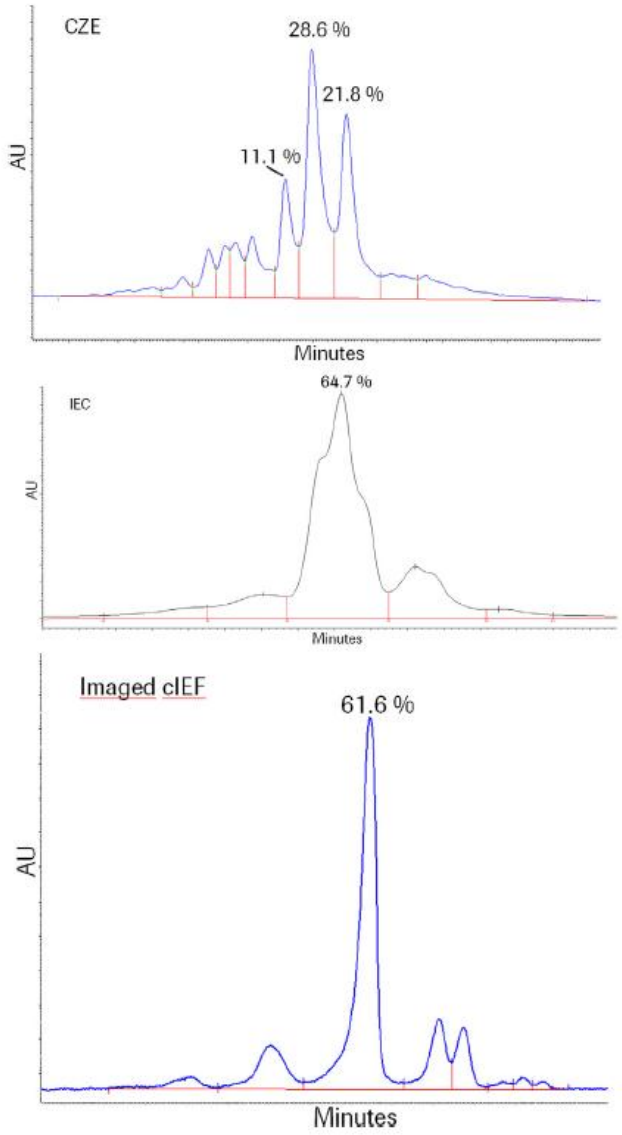
journal homepage: www.elsevier.com/locate/chromb

Evaluation of capillary zone electrophoresis for charge heterogeneity testing of monoclonal antibodies

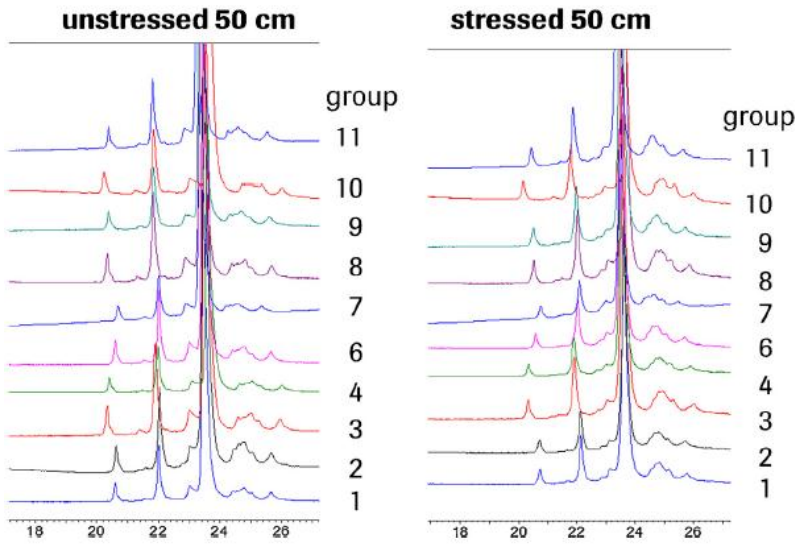
Bernd Moritz[a,*], Volker Schnaible[a], Steffen Kiessig[a], Andrea Heyne[a], Markus Wild[a], Christof Finkler[a], Stefan Christians[b], Kerstin Mueller[c], Li Zhang[d], Kenji Furuya[d], Marc Hassel[e], Melissa Hamm[f], Richard Rustandi[f], Yan He[g], Oscar Salas Solano[h], Colin Whitmore[h], Sung Ae Park[i], Dietmar Hansen[j], Marcia Santos[k], Mark Lies[k]

[a] F Hoffmann-La Roche Ltd, Grenzacher Str. 124, 4070 Basel, Switzerland
[b] Paul-Ehrlich Institut, Paul-Ehrlich-Str. 51-59, 63225 Langen, Germany
[c] Boehringer Ingelheim, Birkendorfer Straße 65, 88397 Biberach/Riß, Germany
[d] Boehringer Ingelheim, 6701 Kaiser Dr., Fremont, CA 94555 USA
[e] Novartis, Fabrikstrasse 2, 4056 Basel, Switzerland
[f] Merck, 770 Sumneytown Pike, West Pt, PA, USA
[g] Pfizer, 700 Chesterfield Pkwy, St Louis, MO, USA
[h] Seattle Genetics, 21823 30th Dr SE, Bothell, WA 98021, USA
[i] Amgen, One Amgen Center Drive Thousand Oaks, CA 91320-1799, USA

Charge Variant Analysis – CZE vs. IEC vs. cIEF



B. Moritz et al. / J. Chromatogr. B 983–984 (2015) 101–110



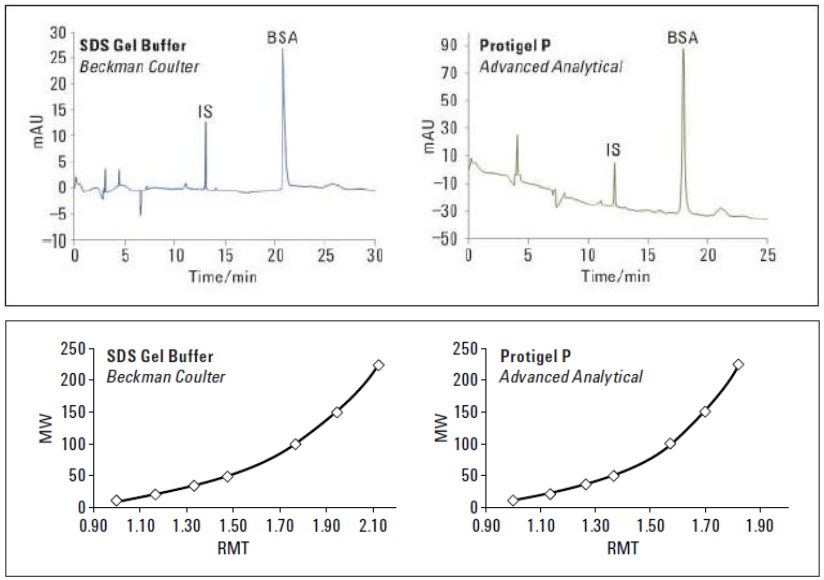
It was shown that CZE is applicable across a broad p*I* range between 7.4 and 9.5. The coefficient of correlation was above 0.99 which demonstrated linearity. Precision by repeatability was around 1% (maximum relative standard deviation per level) and accuracy by recovery was around 100% (mean recovery per level). Accuracy was further verified by direct comparison of IEC, IEF and CZE, which in this case showed comparable %CPA results for all three methods. However, best resolution for the investigated MAb was obtained with CZE. In dependence on sample concentration the detection limit was between 1 and 3%.

4. Conclusions

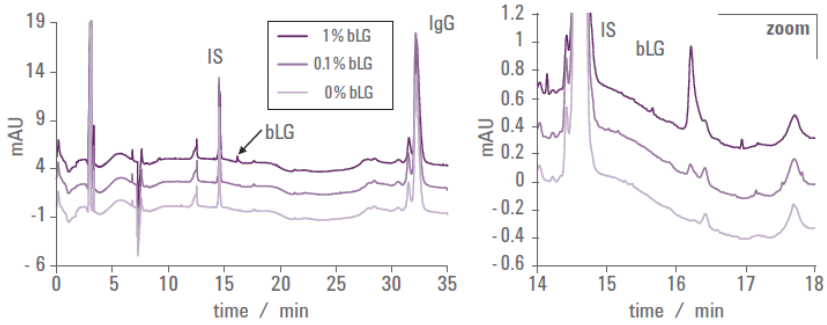
CZE is a very efficient and robust platform method for charge heterogeneity testing of biopharmaceuticals. The implementation of CZE for different products is very easy. Sample preparation and separation are fast and allow high throughput applications. The intercompany study performed and described here delivered precise and robust results without the need for prior method training. CZE for charge heterogeneity profiling of MAbs is stability indicating, precise, accurate, robust, linear and sensitive. This

Application of cGE

Protein Sizing by Capillary Gel Electrophoresis
Agilent 7100 CE UV-DAD System



Performance of commercially available gels for protein characterization by capillary gel electrophoresis cGE with UV detection on the Agilent 7100 CE System

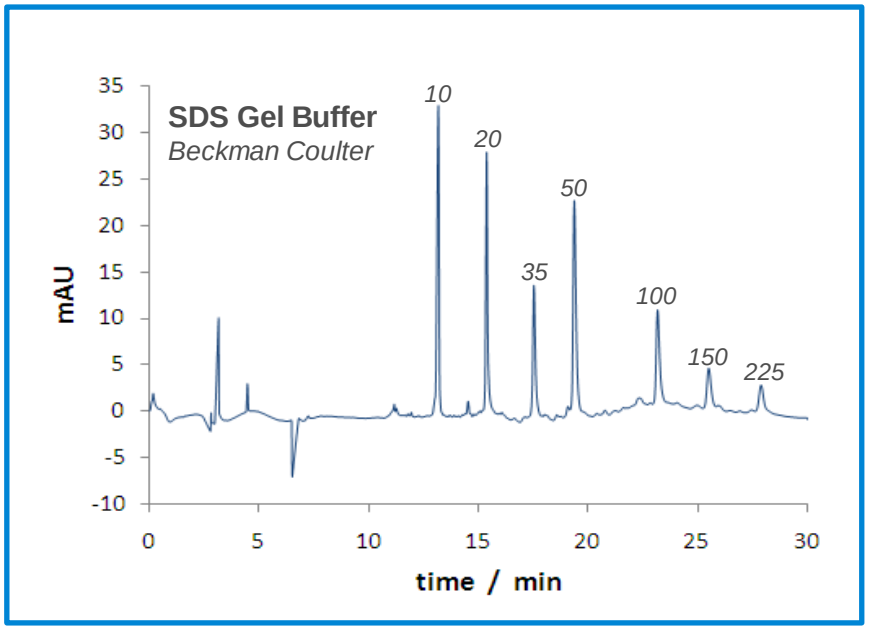


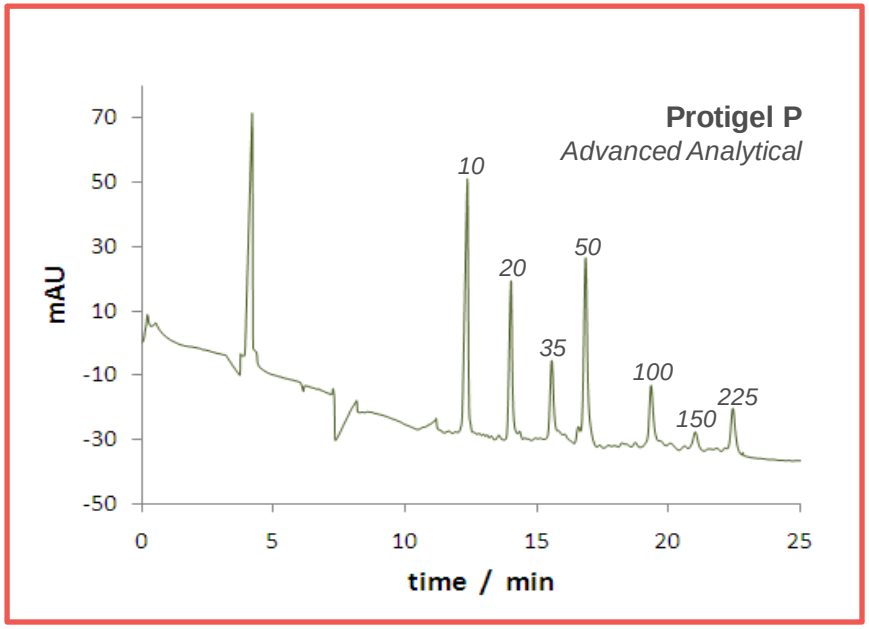
Low-level impurity detection with IgG samples

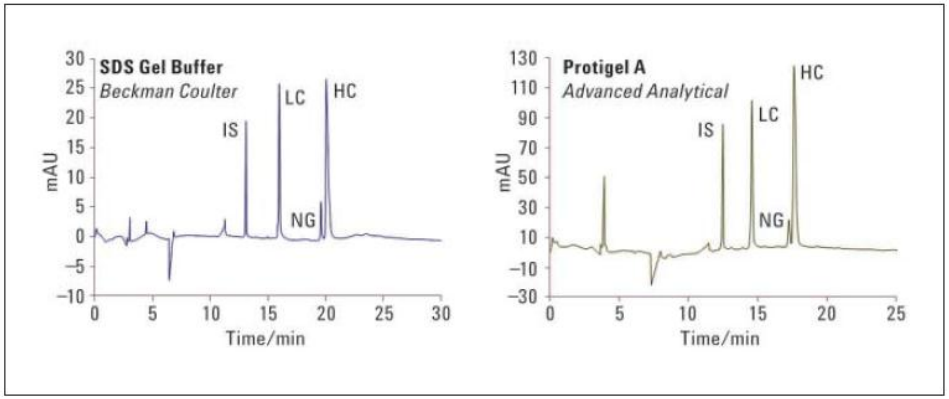
Agilent Application Note (Publication 5990-7976EN)

Protein Sizing by cGE

Use of Commercial Capillary Gel-Electrophoresis Kits







Agilent Application Note (Publication 5990-7976EN)

Combined CE-LIF Solution
with Picometrics Technologies Zetalif LED

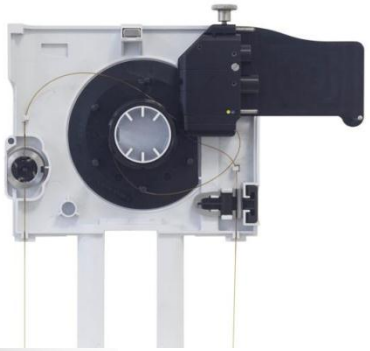
Laser-induced fluorescence detection (LIF) offers one way to achieve very high sensitivity and compound specificity in capillary electrophoresis. A tailored and seamless solution for CE-LIF is possible by combining Agilent 7100 CE instruments with LIF detectors from Picometrics*.

Standard Agilent 7100 CE instrument

Detection inside of Agilent capillary cassette (full temperature control)

Picometrics Zetalif LED using fiber optical connection to the CE

Agilent OpenLAB CDS ChemStation Edition software



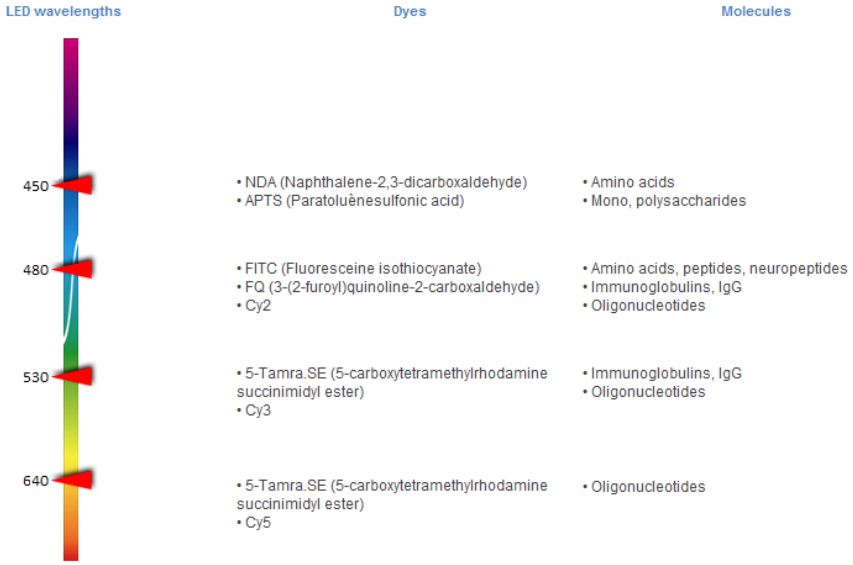
*** Picometrics Technologies SAS (Toulouse, France)**

Advantage of Agilent-Picometrics CE-LIF

The **Agilent 7100 CE** is the most flexible CE instrument to host external detectors.

The **Zetalif LED** detector is a sensitive solution and offers a range of wavelengths: 450, 480, 530 or 640 nm.

- Easy-to-access cassette type (no liquids or sealants)
- Multiple detectors at a time (e.g., direct LIF-MS)
- Full software control of LIF through RC.net driver
- Signal transfer into Agilent OpenLAB CDSChemStation

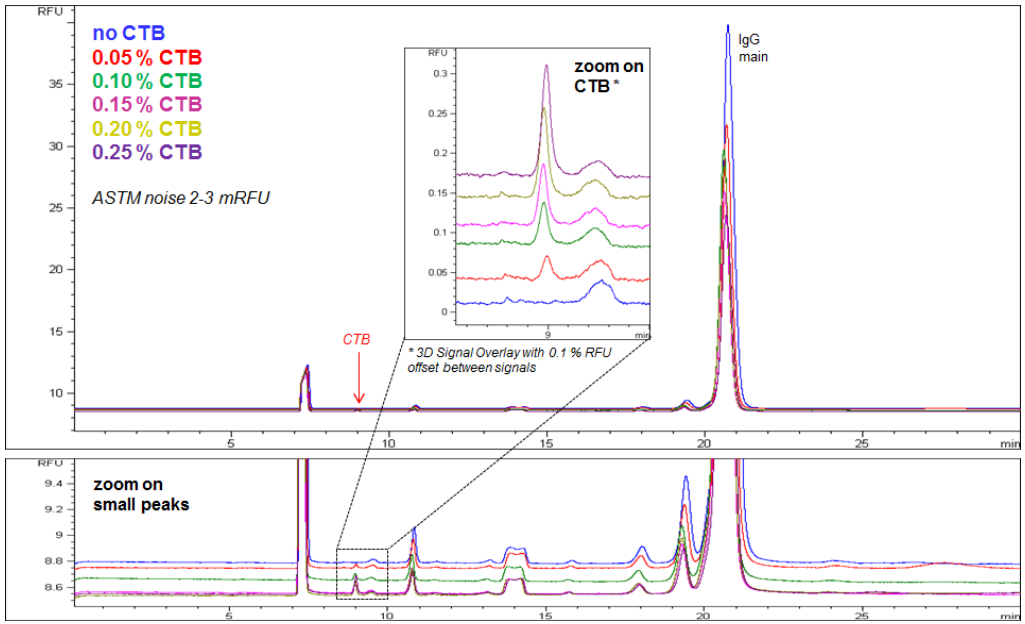


- **Complete solution with easy setup**
- **Small footprint of solution + flexible combinations**
- **Reduced capital cost for LIF detection**

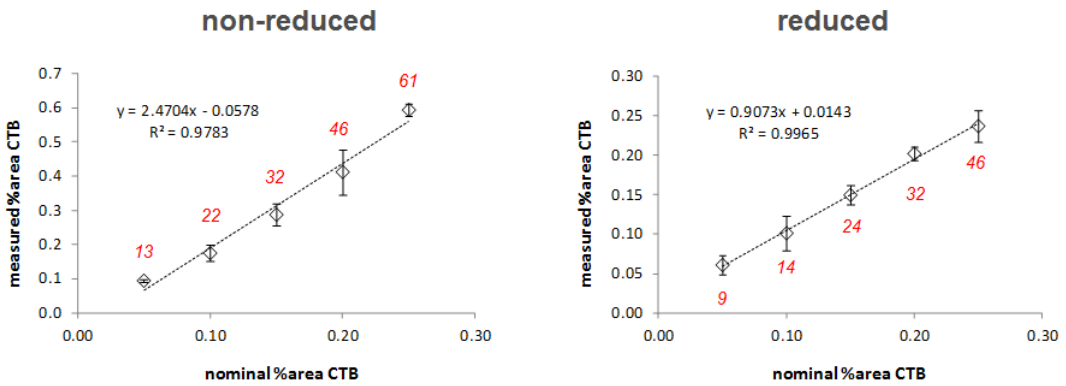
► CE-LIF, CE-LIF-MS, HPLC-LIF, ...
► detection inside or outside of CE, A/D converter included
► long-life LED based LIF, flexible use of modular devices

Application of CE-LIF
Impurity Analysis

Separation of non-reduced mAb spiked with cholera toxin B (CTB)



Quantification of cholera toxin B (CTB)



CE/MS
A Fully Integrated Solution

Whole solution
- CE hardware
- MS hardware
- CE/MS Interface
- Integrated Software for LC/MS & CE/MS

Typical Applications
- Small Molecules / Metabolites
- Peptide mapping
- Protein ID and characterization
- mAb and mAb conjugates

Target Analysis

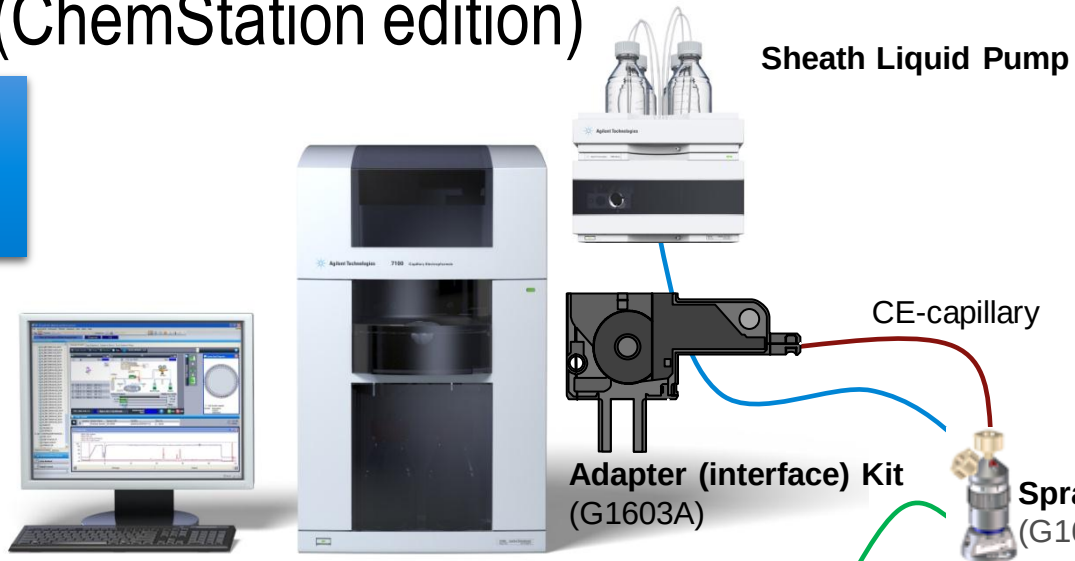
MS choice: MSD/QQQ

Screening

MS choice: TOF/QTOF

Agilent CE/MSD Setup
OpenLAB CDS (ChemStation edition)

Single point SW control
- 7100 CE instrument
- MSD Single quadrupole
- LC make-up flow



Sheath Liquid Pump

CE-capillary

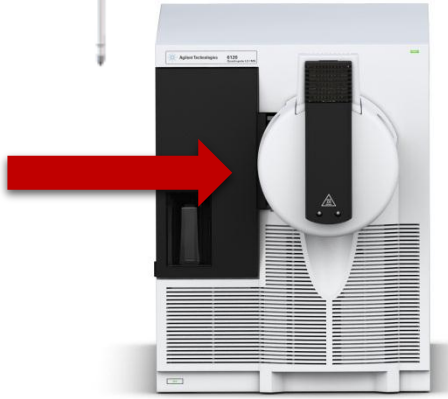
Adapter (interface) Kit
(G1603A)

Sprayer Kit
(G1607B)

Nitrogen
nebulizing gas

PC-system controlling
- 7100 CE system
- MS system
- Sheath liquid pump

MS source
JetStream or ESI
On request also APPI / APCI



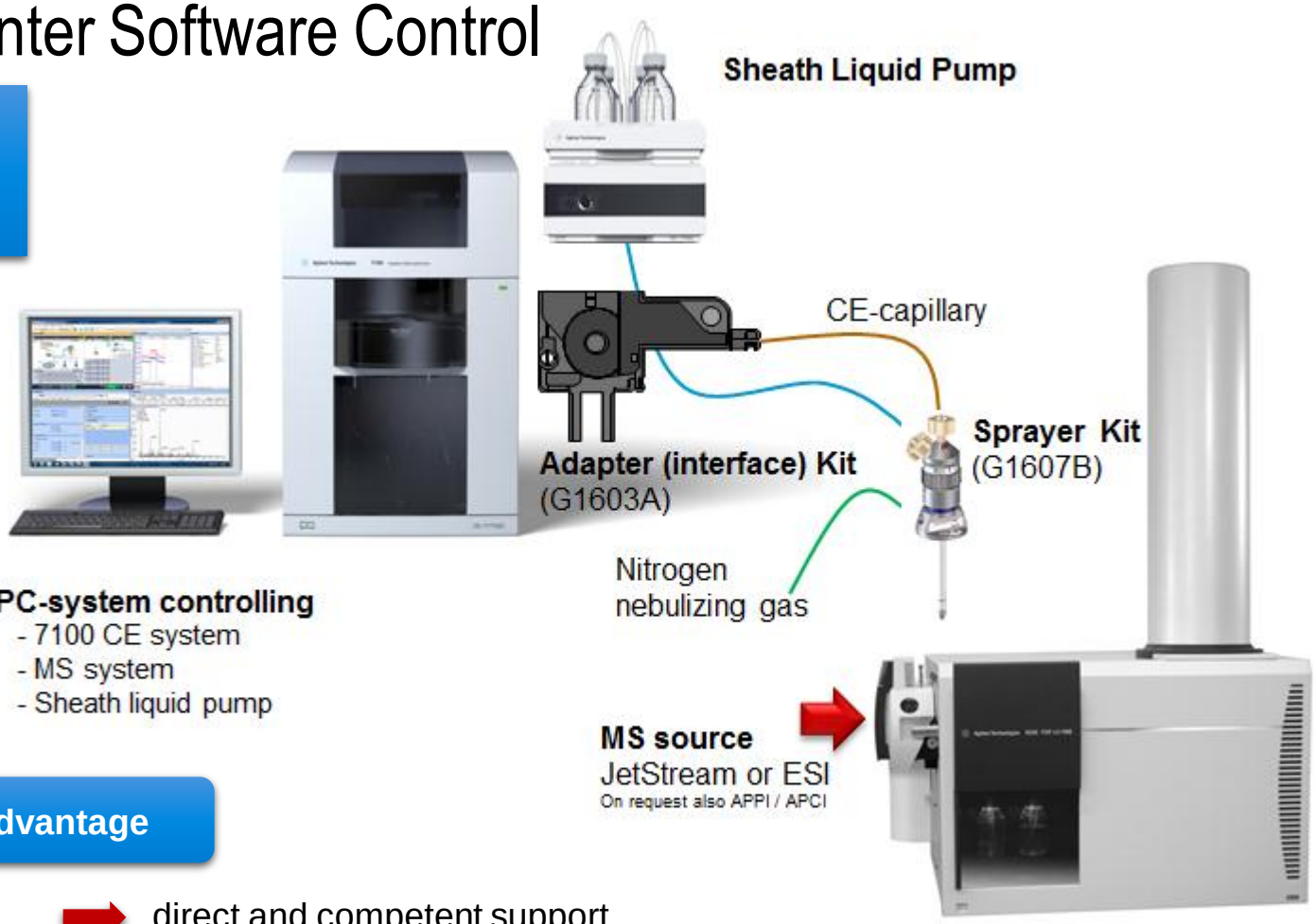
The Agilent CE/MS Advantage

Single vendor solution: ➡ direct and competent support
Sheath Liquid Interface: ➡ robust and reliable, offering efficient control on chemistry
Capillary outlet on ground: ➡ no compromises on voltages for CE or ESI-MS

Agilent CE/MS Setup

Agilent MassHunter Software Control

Single point SW control
- 7100 CE instrument
- TOF,QTOF,QQQ
- LC make-up flow



The Agilent CE/MS Advantage

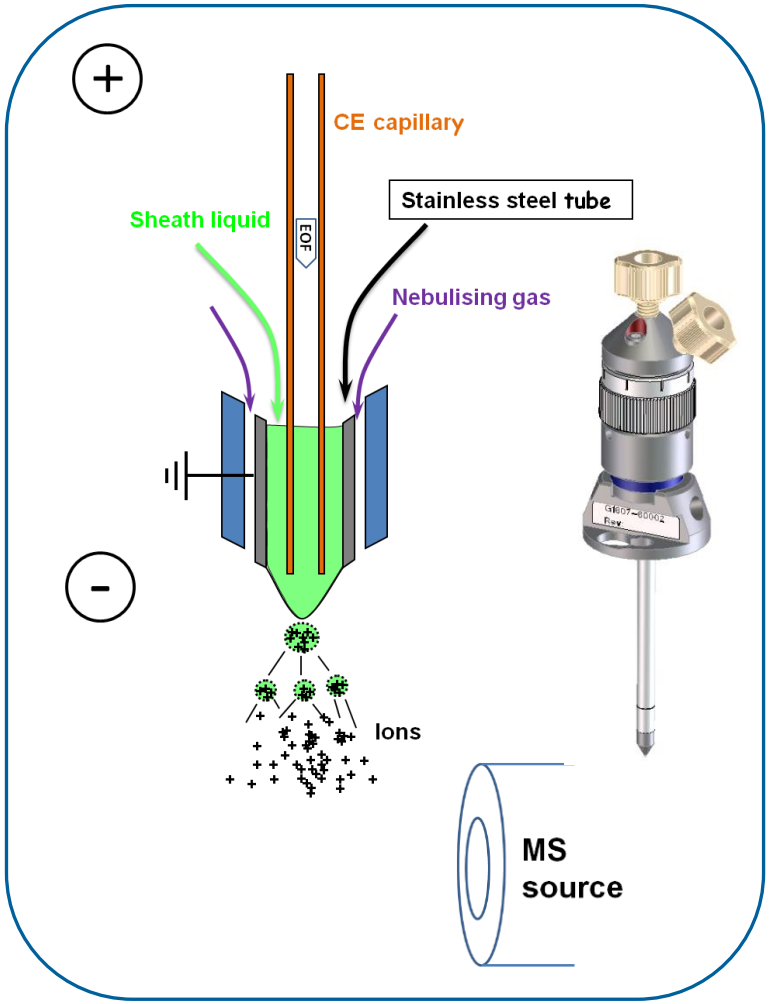
Single vendor solution: ➡ direct and competent support
Sheath Liquid Interface: ➡ robust and reliable, offering efficient control on chemistry
Capillary outlet on ground: ➡ no compromises on voltages for CE or ESI-MS

Agilent interface for CE/MS
Sheath-Liquid Type

Sheath liquid is added to the CE eluent by a software controlled LC pump at a rate of typically 1 - 5 µL/min.

It often consists of a mix of water, methanol or isopropanol, adjusted for desired pH range by volatile acids or bases)

Besides controlling flow rate and chemical conditions for ESI ionization of molecules it allows grounding of the non-conductive fused silica capillary to the metal tube of the spray needle



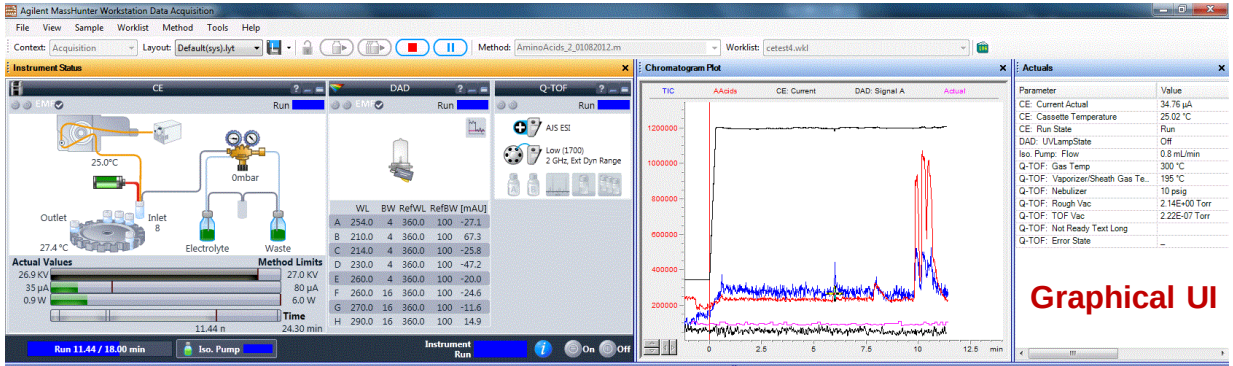
Advantages of Sheath Liquid interface:

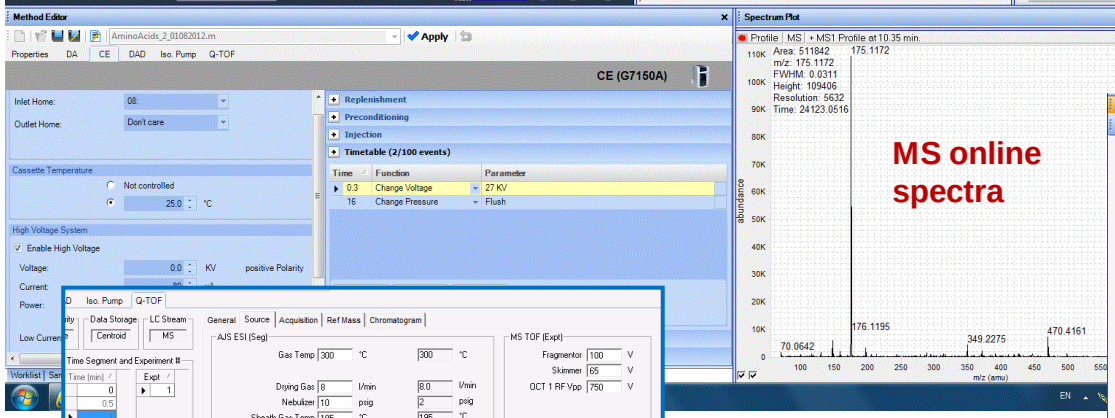
- **High stability & reproducibility for routine analysis**
- **Decoupling chemistry (CE separation / MS ionization)**
- **Constant flow rates during runs and sequences**
- **No modification of capillary / columns required**

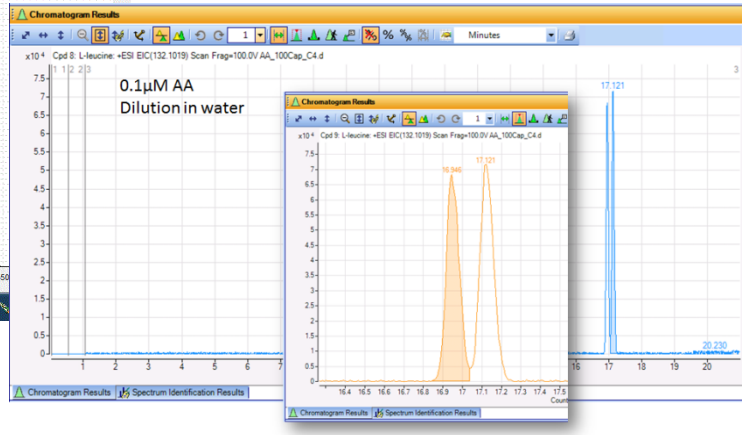
Agilent interface for CE/MS

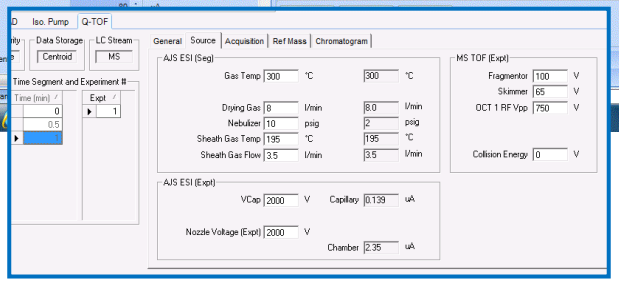
Agilent MassHunter software for LC-MS & CE/MS

MassHunter versions B.05.01 and higher are integrating and controlling Capillary Electrophoresis for CE/MS analysis as a single software package under Windows 7 (64 bit)







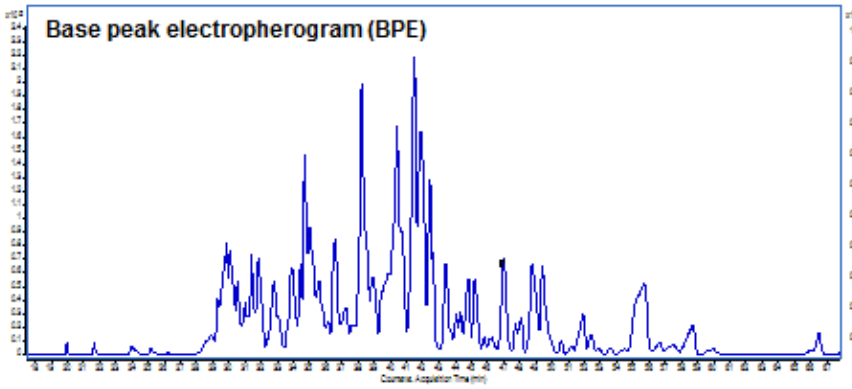


Agilent Technologies

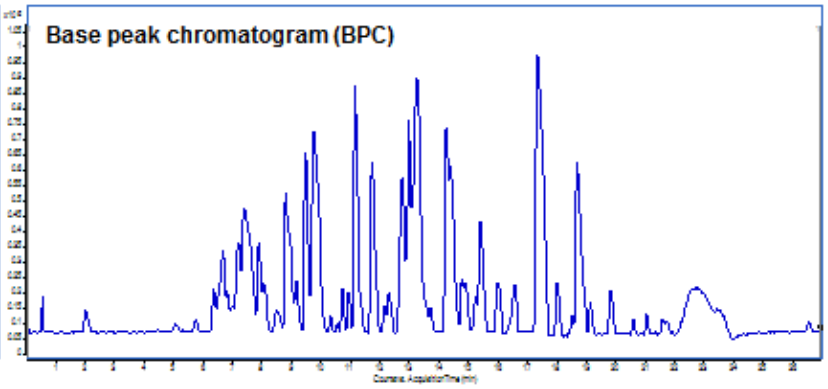
Instrument type :
Agilent Q-TOF

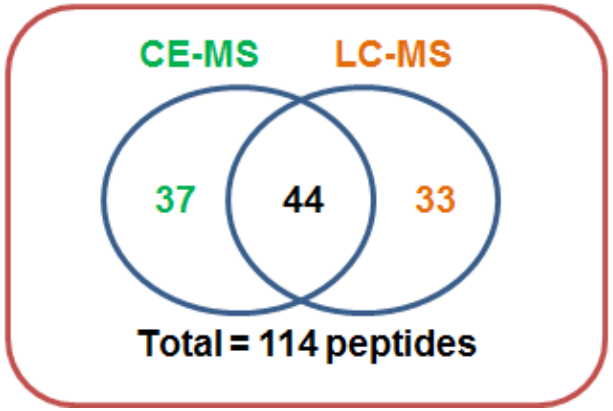
Peptide Mapping: CE/MS vs. LC/MS
Comparison of Tryptic Digests

CE-QTOF MS



LC-QTOF MS





CE/MS and LC/MS peptide map of BSA

Among the total number of peptides identified (114), 37 peptides are unique to CE/MS and 33 peptides are unique to LC/MS.
Each of the techniques contained 44 common peptides.

Agilent Application Note (Publication 5991-2583EN**)**

CE/MS vs. LC/MS

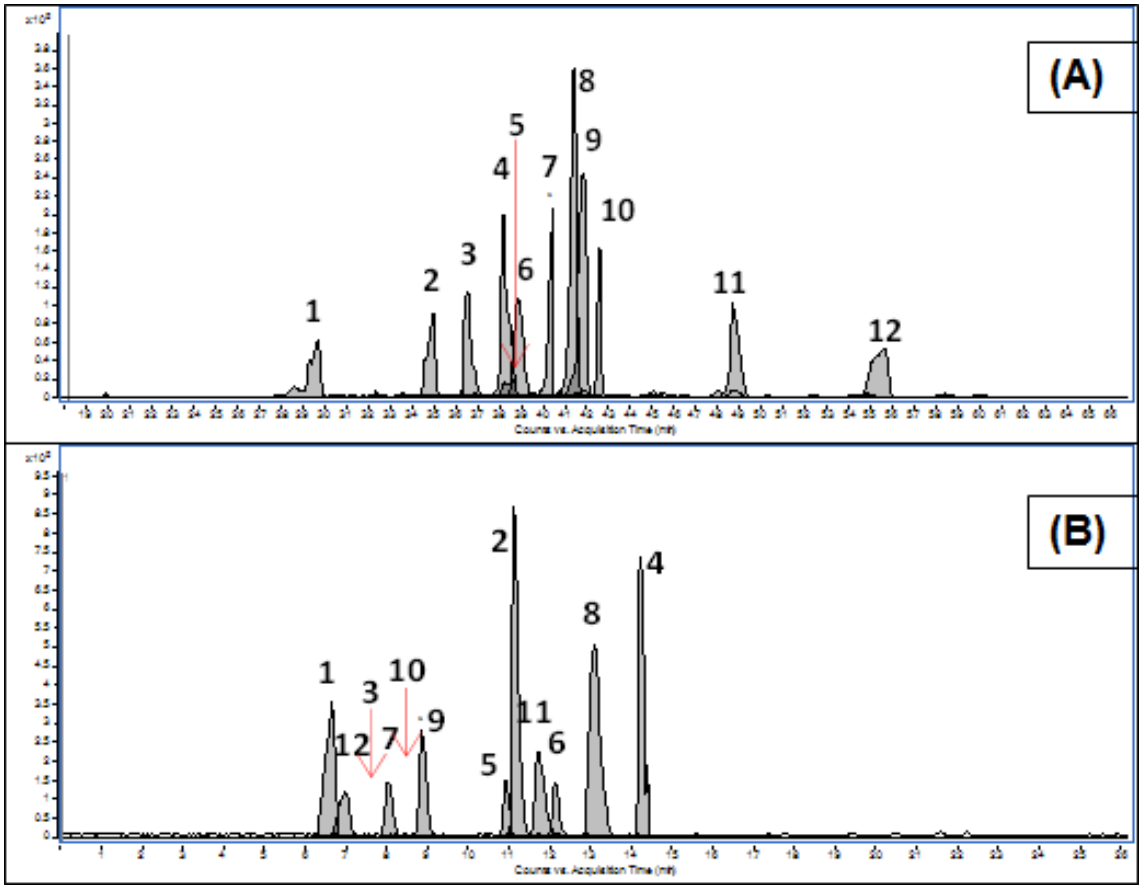
Comparison of Tryptic Peptide Maps

Separation comparison of same set of BSA tryptic peptides

(A) CE-MS
(B) LC-MS

List of peptides:

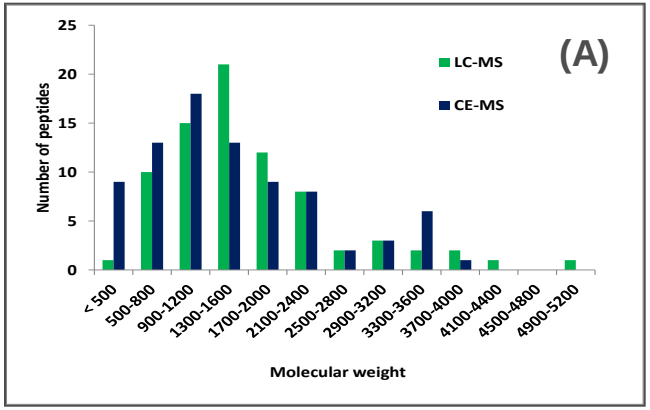
1 . LCVLHEK,
2. HLVDEPQNLIK,
3. NYQEAK,
4. QTALVELLK
5. YLYEIAR,
6. ECCHGDLLECADDR
7. LVTDLTK
8. LVNELTEFAK
9. AEFVEVTK
10. LCVLHEKTPVSEK
11. LVVSTQTA
12. DDSPDLPK

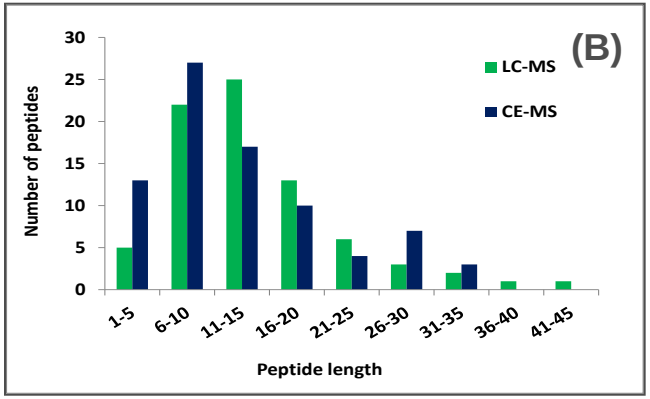


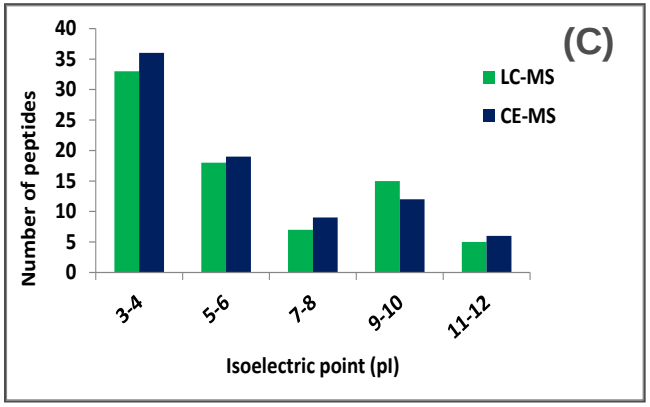
Orthogonal techniques: CE and LC

Due to different physical separation principles the elution order is totally different, reducing the risk of overlapping peaks if both methods are applied

CE/MS vs. LC/MS
Comparison of Tryptic Peptide Maps







CE-MS and LC-MS comparison of peptide distributions.

(A) Molecular weight plot
(B) Peptide length plot
(C) Isoelectric point plot

CE/MS vs. LC/MS
Comparison of Tryptic Peptide Maps

	CE-QTO MS (6520)	LC-QTOF MS (Chipcube-6540)
Sample injected	44nl (0.34pmole)	2ul (15pmole)
Peptide elution window	30 min	16 min
Sequence coverage	80%	81%
Total peptides identified	82	78
Distinct peptides ID'ed	37	33
Selectivity & resolution	Change in elution order of few peptide – shows the complementary value of two techniques	
Selectivity	CE-MS is shows the best separation/ionization for hydrophilic peptides	
Peptide distribution	• Shorter peptides are represented (1-5 amino acid peptide length) • Identified peptides starting with 3 amino acid length • Low MW peptides are well presented (<500Da) • Acidic peptides (pI 3-4) are well represented	• Shorter peptides are less represented (1-5 amino acid peptide length) and also cover wide range of peptide length identified • Identified peptides starting with 4 amino acid length • Low MW peptides are less represented (<500Da)

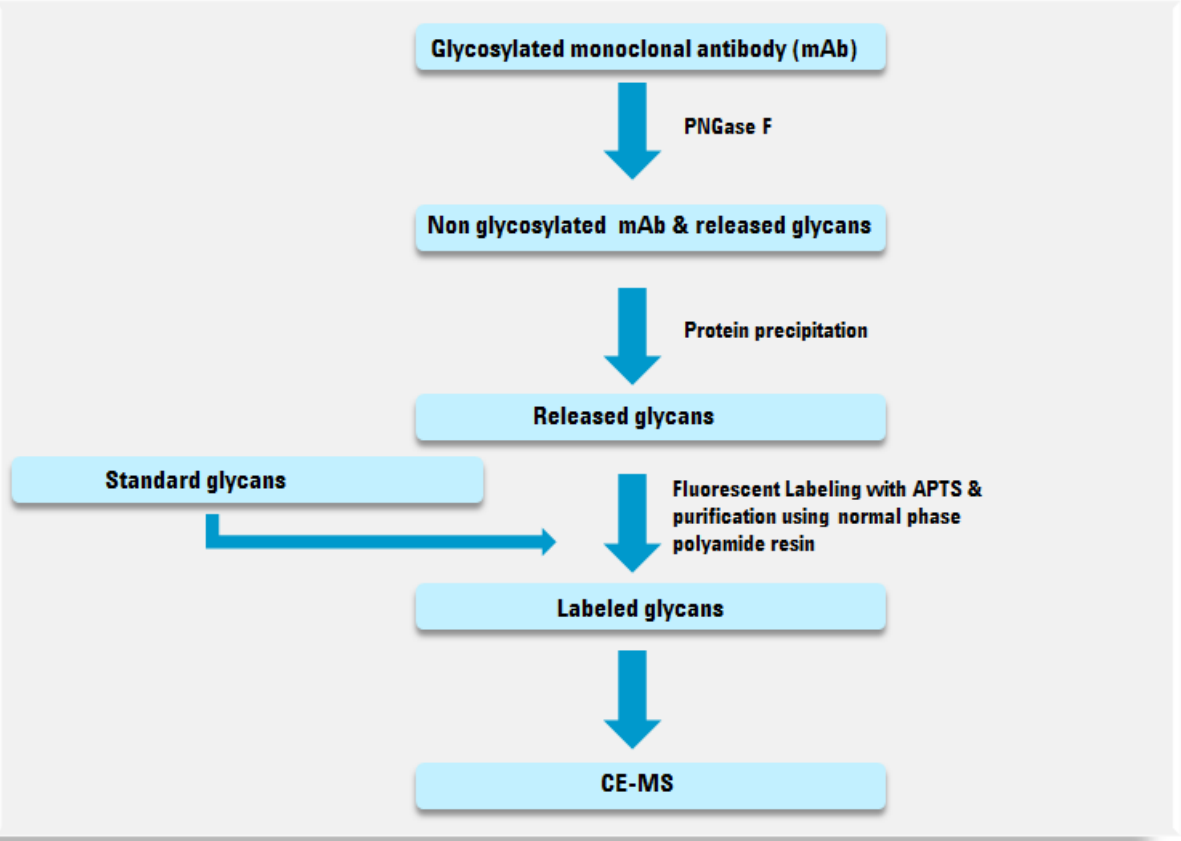
Analysis of N-Glycans of a Monoclonal Antibody by CE/MS

Capillary Electrophoresis (CE)

CE:	Agilent 7100 Capillary Electrophoresis System
Sample:	Standard glycans and released glycans from mAb
Injection:	40s @ 30 mbar
Capillary:	PVA, total length 60 cm, 50 µm id
Buffer:	40 mM ε-aminocaproic acid, pH 4.5
Voltage:	- 25 kV
Pressure:	10 mbar
Temp:	20° C

Mass Spectrometry (MS)

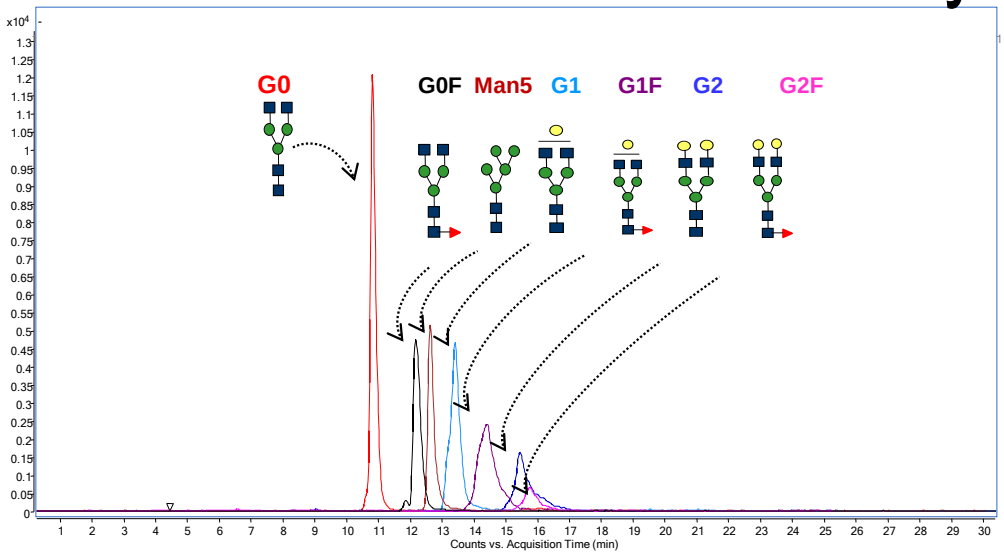
MS:	Agilent 6520 Accurate-Mass Q-TOF LC/MS
Ionization mode:	ESI (negative mode)
Acquisition mode:	MS (mass range 400-3200 m/z)
Sheath liquid:	1:1 isopropanol:water+0.2% NH_3, 5 µL/min
Drying gas flow:	5 L/min
Nebulizer:	8 psi
Drying gas temp:	250 °C
Fragmentor:	175 V
Vcap:	3200 V



Schematic overview of the glycoprofiling of mAb using CE/MS

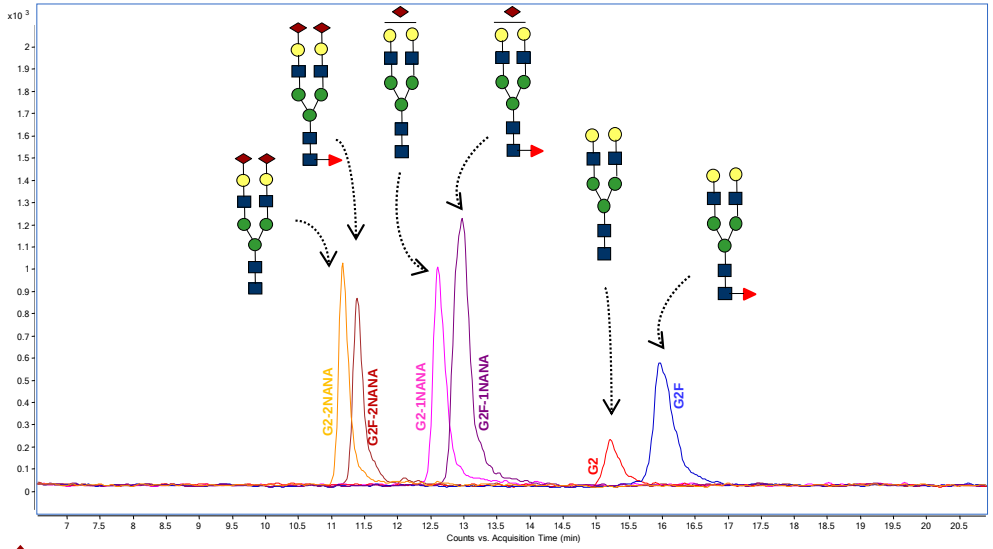
Agilent Application Note (Publication 5991-1020EN)

CE/MS of APTS Labeled Glycan Standards



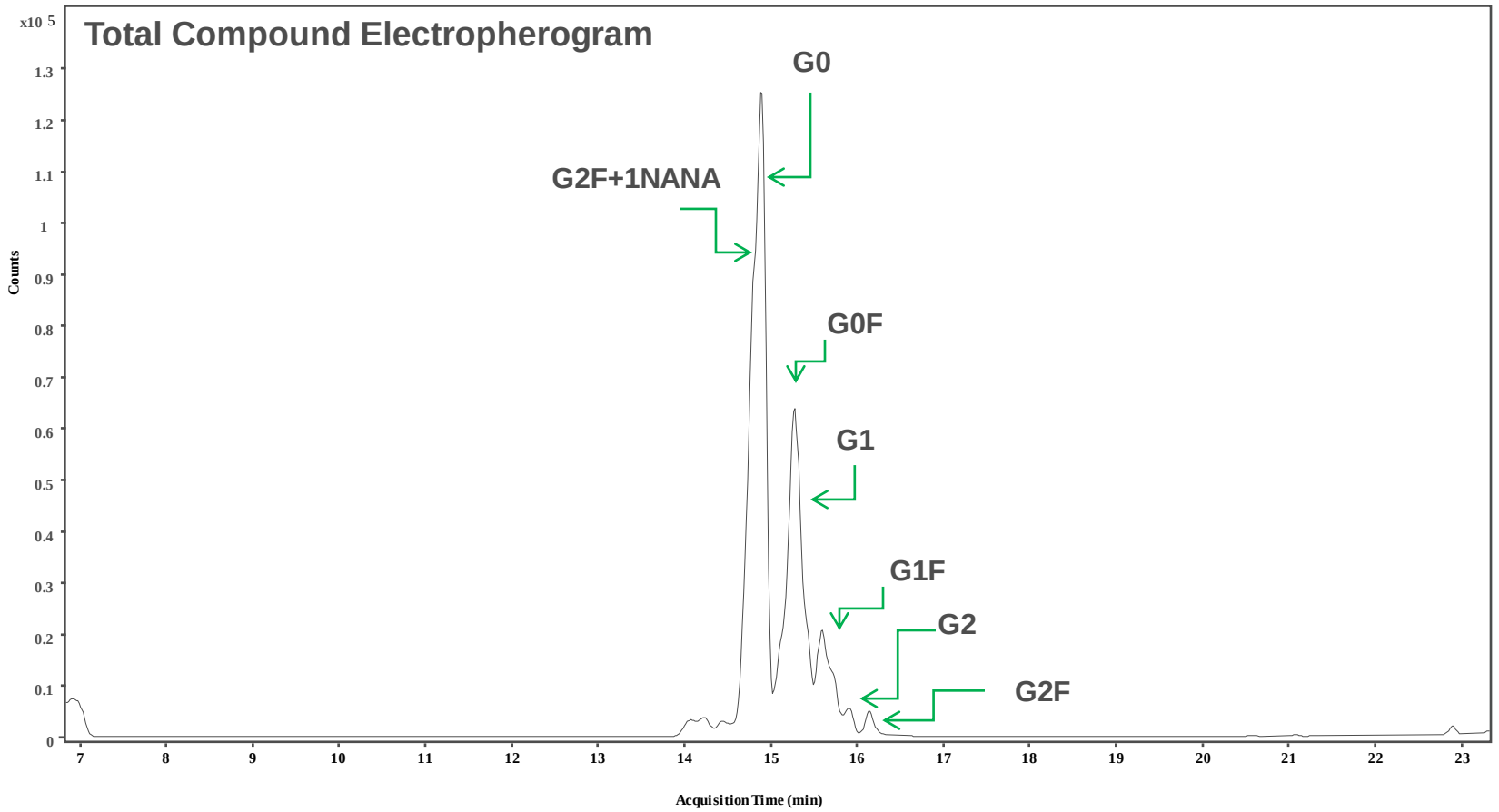
Mixture of neutral glycan

Mixture of neutral glycan & sialylated glycans



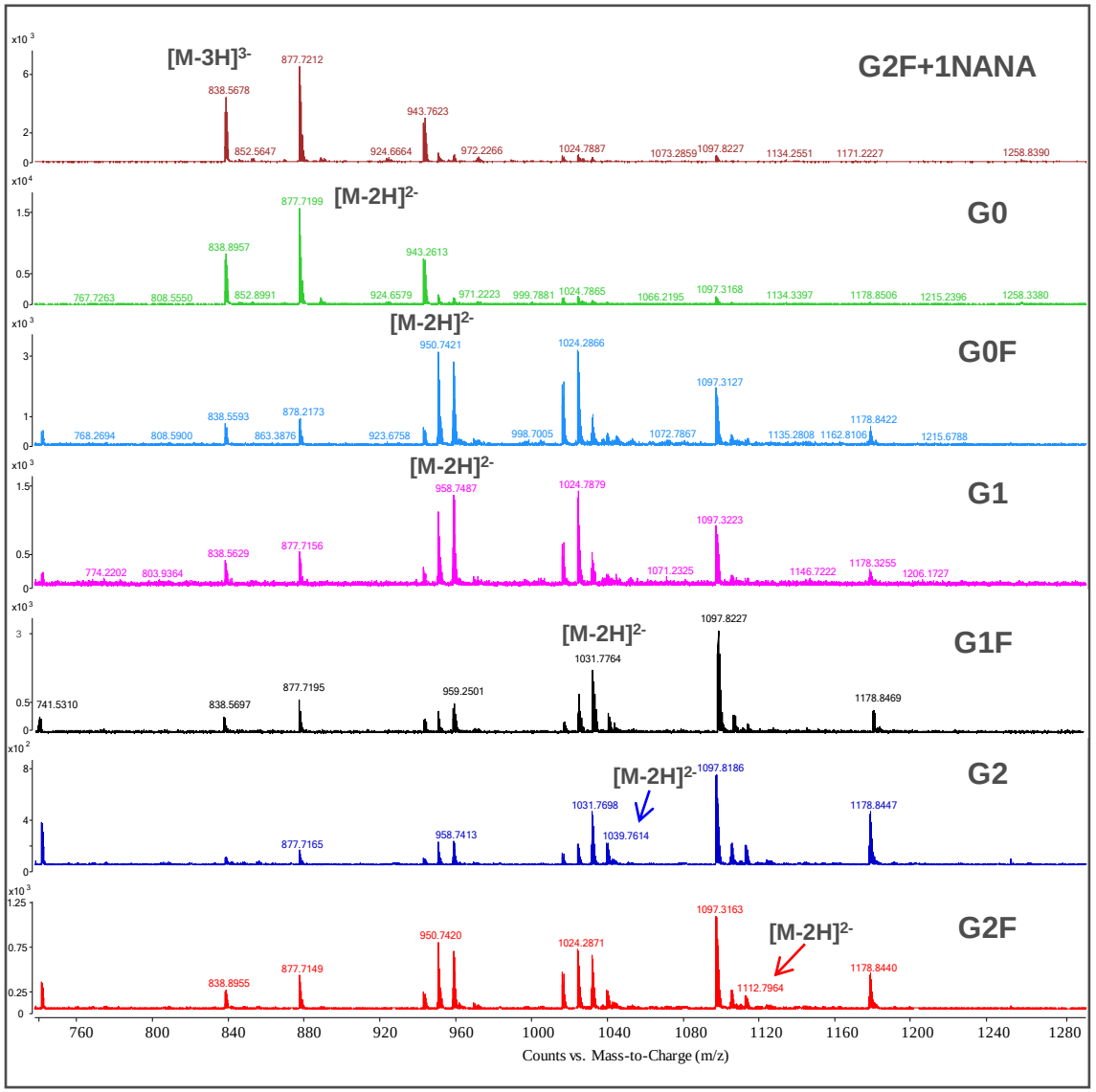
Galactose Mannose Fucose N-acetylglucosamine sialic acid

CE/MS Analysis of N-Glycans Released from mAb



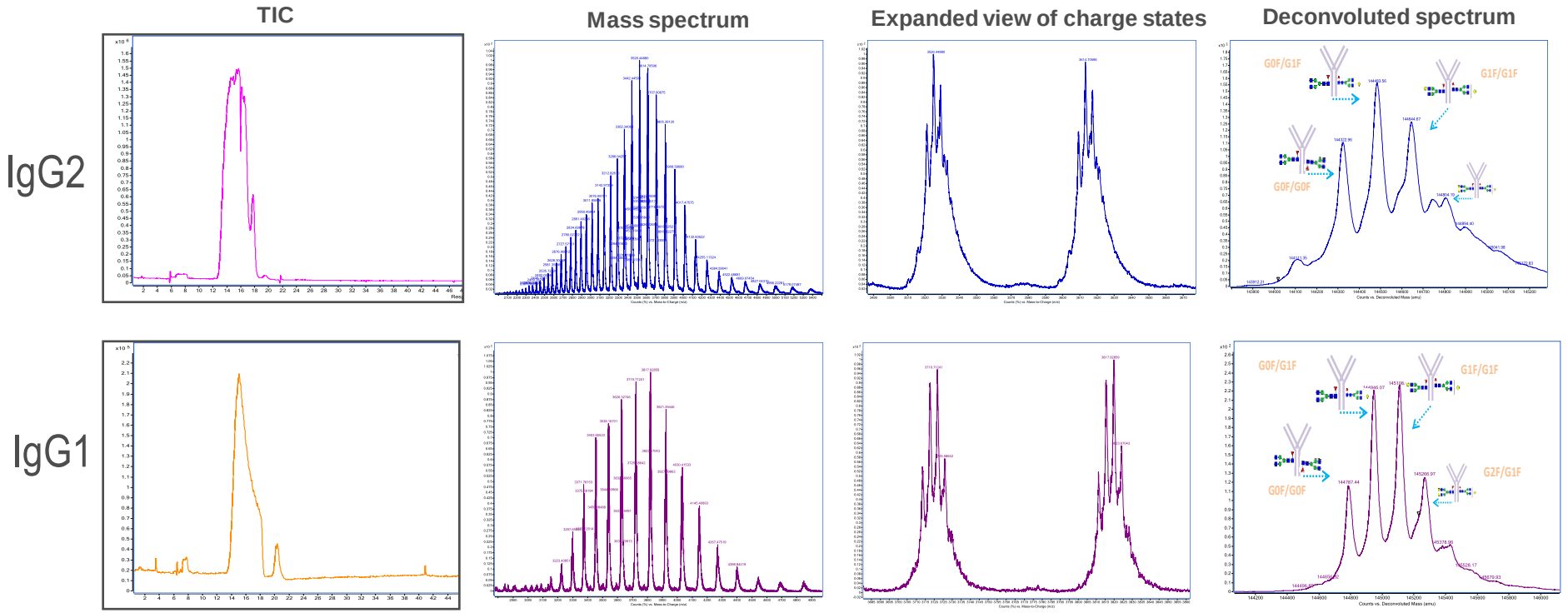
CE/MS of APTS labeled N-glycans released from mAb

CE/MS Analysis of mAb



Mass spectra of APTS labeled mAb N-glycans

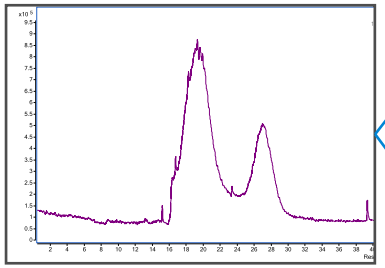
CE/MS of Intact mAb

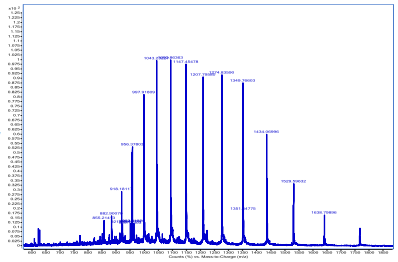


CE/MS of Reduced mAb

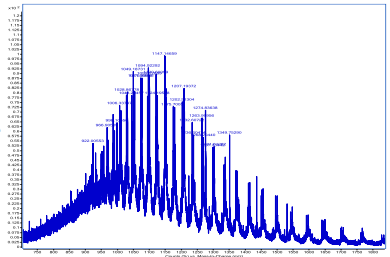
IgG2

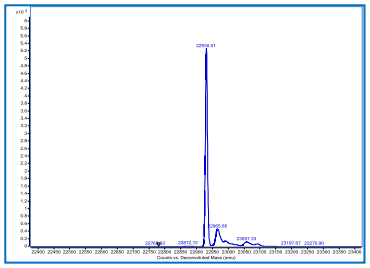
TIC



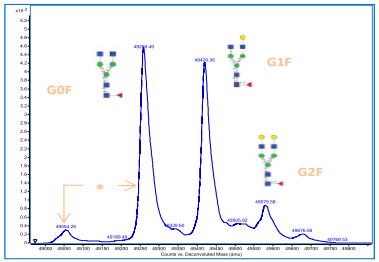


Mass spectrum





Deconvoluted spectrum

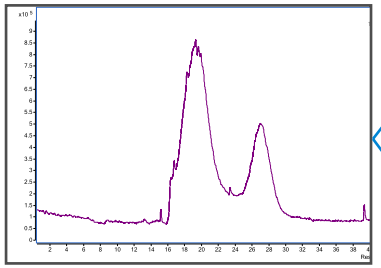


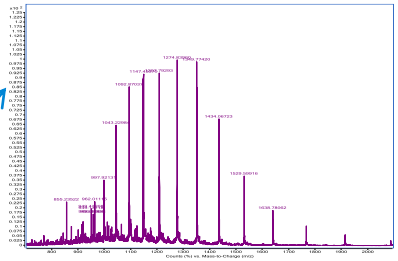
Light Chain

Heavy Chain

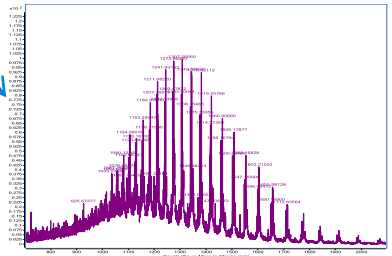
IgG1

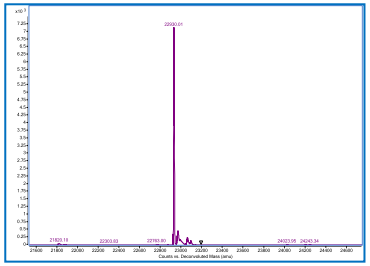
TIC



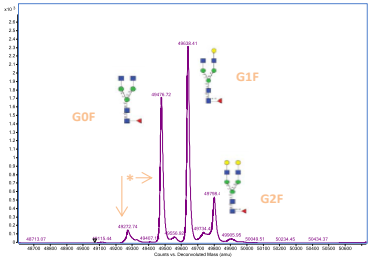


Mass spectrum





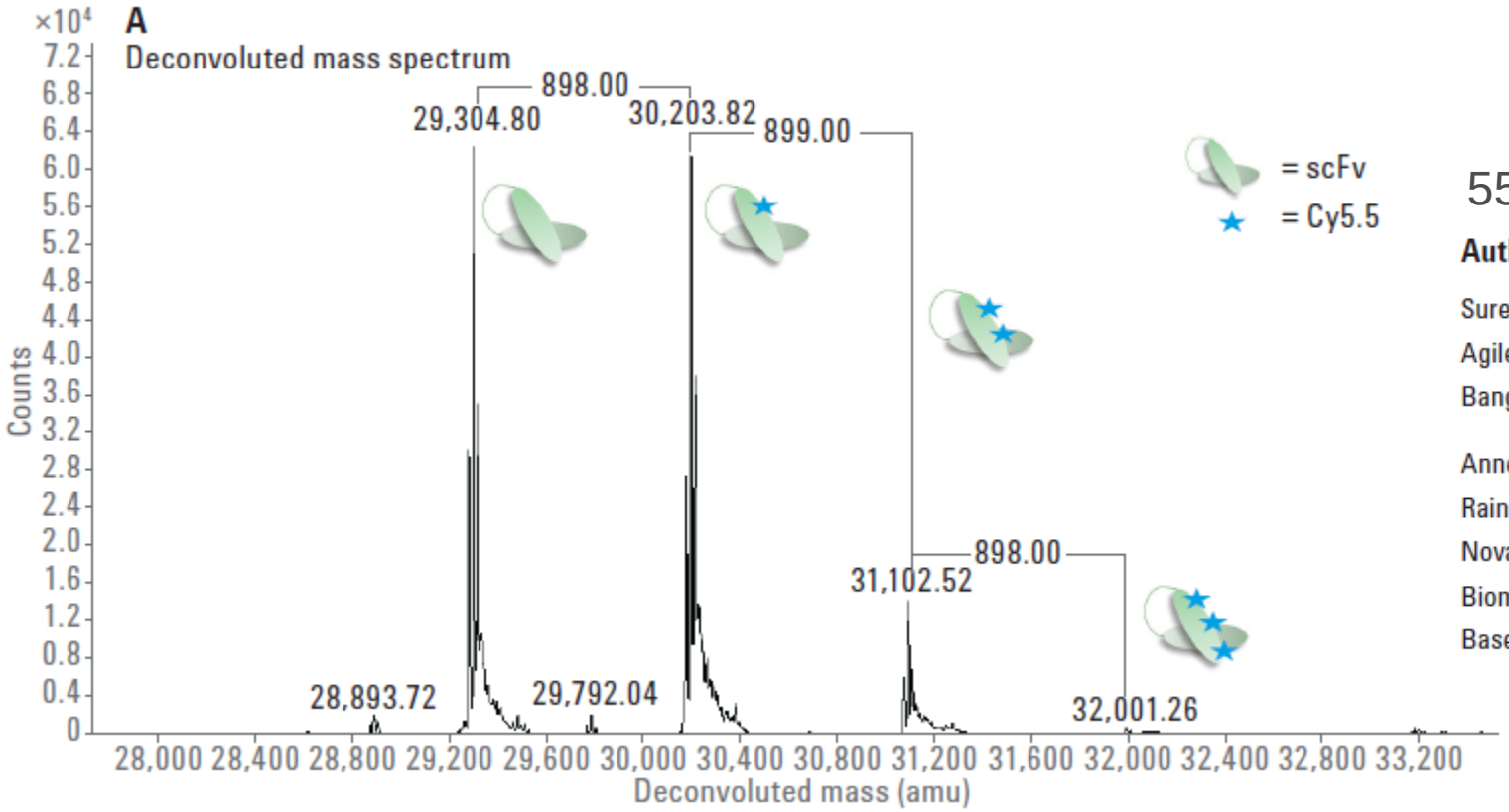
Deconvoluted spectrum



Light Chain

Heavy Chain

Characterization of Small Immunoconjugates (< 40 kDa) Using CE-MS



5591-4433EN

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Anne Basler, Sina Bunzendahl, and
Rainer Kneuer
Novartis Institutes for
Biomed. Research
Basel, Switzerland

Summary
7100 CE/MS Systems

Agilent is the only sole vendor to provide a completely integrated robust and sensitive CE/MS solution for research and for routine analysis
Full Agilent series 6000 MS portfolio available – single quad, QQQ, TOF, and QTOF
Triple-tube interface to optimize individually separation and MS ionization – no compromises
Range of ion sources available - standard ESI and Agilent JetStream (APPI and APCI on demand)
Flexibility on additional detectors – UV-DAD, LIF, and CCD in parallel to MS
iFunnel-Sensitivity for small molecules down to the ppt range
Agilent MassHunter software control – one software, one workstation
Single-vendor solution – integrated system and single-source support
More information at: **www.agilent.com/chem/cems**

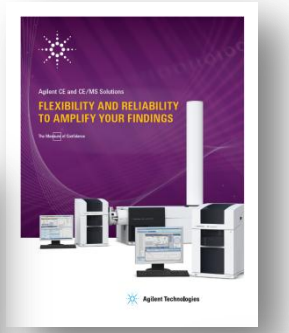
Information on Agilent 7100 CE

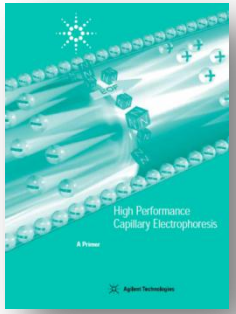
Visit our webpage at
to find helpful documentation on **http://www.agilent.com/chem/ce**

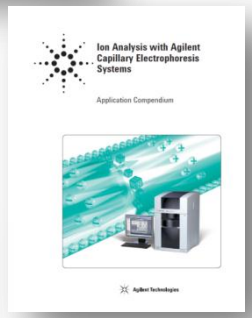
http://www.agilent.com/chem/cems

Product

- 5991-1511EN Brochure
- 5990-3962EN Data Sheet
- 5990-3822EN CE consumables catalog
- 5990-3980EN CE Partner CD







Basics on CE methodology and Application data

- 5990-3777EN Primer tutorial
- 5990-5244EN Ion Analysis Compendium
- WebPage go: Applications

Videos on CE/MS

Agilent 7100 CE/MS

Metabolomics by CE/MS (Keio University, Japan)

New trends in Biopharma presents new analytical challenges

- Biosimilars
  - Need to demonstrate similarity/comparability between biosimilar to its innovator molecule
- Antibody Drug Conjugates (ADCs)
  - Increased analytical complexity due conjugate, linker & conjugation chemistries

BIOSIMILARS

Definitions

- Innovator biologic
  - Novel clinically-validated biologic on which biosimilars or biobetters are designed
- **Biosimilar**
  - **Biologic molecule with identical primary amino acid sequence as innovator biologic and developed with intention to be as close to the innovator product as possible**
- Biobetter
  - Biologic molecule based on the innovator molecule but with improvements intended to increase efficacy, potency, marketability, safety, or patient compliance
- Next-generation
  - Biologic molecule based on same validated target as innovator biologic, but with novel VH/VL chains and (typically) different epitope, with intent of making an improved biologic against the validated target

Biosimilars—*The Race is "ON"*

GEN Genetic Engineering & Biotechnology News

SEND TO PRINTER

Insight & Intelligence™ : Dec 19, 2011

Firms Are Upping the Stakes on mAb Biosimilar Development

As originators try to defend their patents, companies make larger investments in biosimilars.

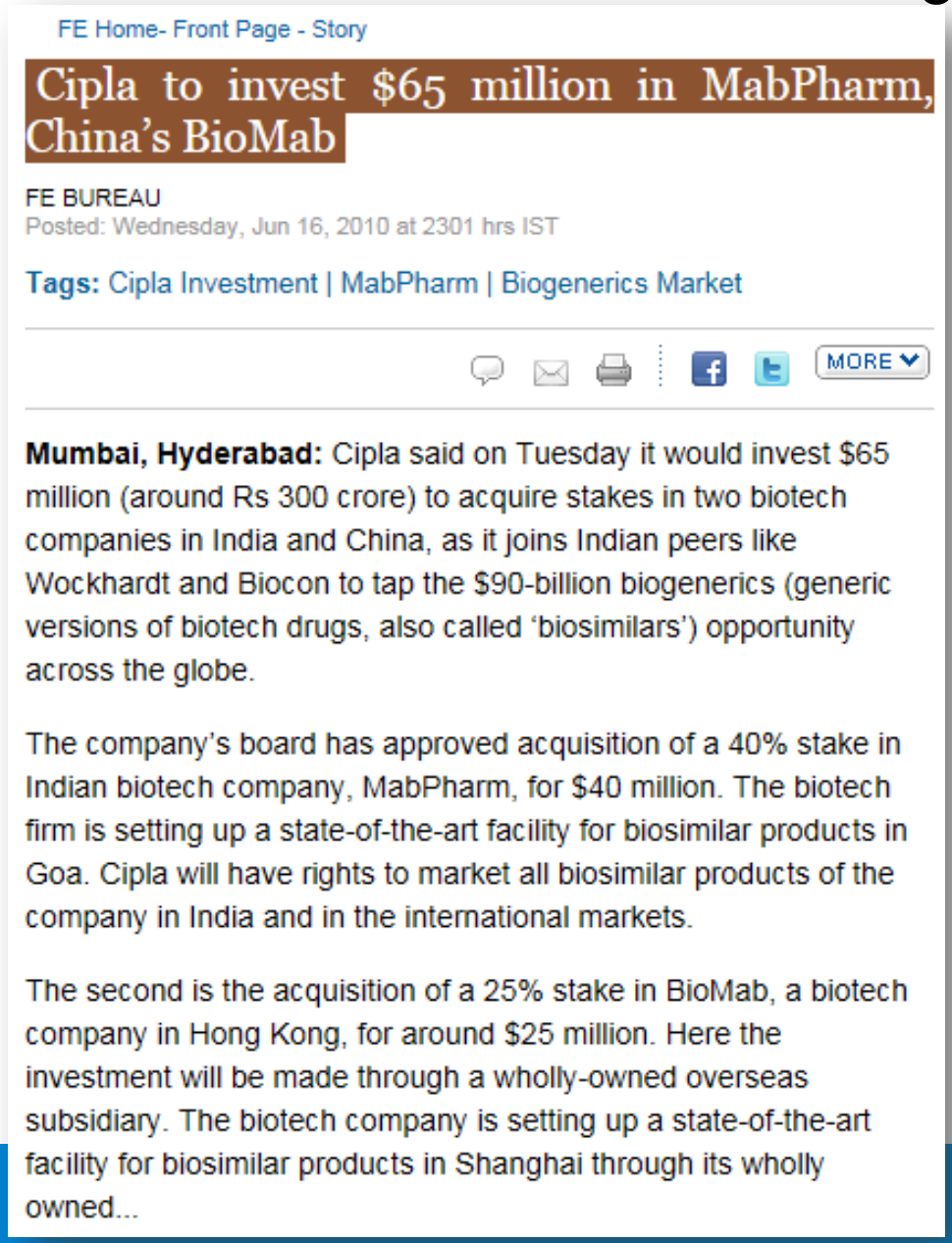
Patricia F. Dimond, Ph.D.

Despite delays by the FDA and some opposition from originator companies, biosimilars now represent one of the most rapidly evolving areas of product development in the biopharmaceutical industry. The EU already has legislation in place for the approval of biosimilars, and the FDA has publicly committed to publishing biosimilar guidelines by the end of this year.

Judging from the feverish activity among potential biosimilar marketers, mAb follow-on proteins will be the hottest competitive area. At $6.6 billion in 2010 sales, Rituxan is the largest revenue-producing biologic yet to be targeted by biosimilar developers. This anti-CD20 chimeric mAb is approved for chronic lymphocytic leukemia, non-Hodgkin's lymphoma, and RA and is due to come off patent in 2015.

South Korea's Celltrion has initiated clinical trials of CT-P13, its Rituxan biosimilar. Sandoz, Novartis' genetics arm, has a Phase II RA trial with its own version of Rituxan. Teva Pharmaceuticals and Spectrum Pharmaceuticals are also working on Rituxan biosimilars; Teva obtained therapeutic protein production capacity and expertise through its 2009 joint venture agreement with Lonza focused on biosimilars.

Biosimilars—*"Foot-in-the-Door"* for Emerging Markets



FE Home- Front Page - Story

Cipla to invest $65 million in MabPharm, China's BioMab

FE BUREAU
Posted: Wednesday, Jun 16, 2010 at 2301 hrs IST

Tags: Cipla Investment | MabPharm | Biogenerics Market

MORE

Mumbai, Hyderabad: Cipla said on Tuesday it would invest $65 million (around Rs 300 crore) to acquire stakes in two biotech companies in India and China, as it joins Indian peers like Wockhardt and Biocon to tap the $90-billion biogenerics (generic versions of biotech drugs, also called 'biosimilars') opportunity across the globe.

The company's board has approved acquisition of a 40% stake in Indian biotech company, MabPharm, for $40 million. The biotech firm is setting up a state-of-the-art facility for biosimilar products in Goa. Cipla will have rights to market all biosimilar products of the company in India and in the international markets.

The second is the acquisition of a 25% stake in BioMab, a biotech company in Hong Kong, for around $25 million. Here the investment will be made through a wholly-owned overseas subsidiary. The biotech company is setting up a state-of-the-art facility for biosimilar products in Shanghai through its wholly owned...

…and large corporations like Samsung

GEN News Highlights: Dec 6, 2011

Biogen Idec, Samsung Establish $300M Biosimilar Joint Venture

UPDATED: Samsung will make some Roche biologics

Bristol-Myers extends manufacturing pact with Samsung

Samsung BioLogics to expand new Songdo plant

South Korean company anticipating big growth in biologics

February 17, 2015 | By Eric Palmer

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8

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South Korea's Samsung BioLogics has pledged to be a big deal in biosimilars, but so far its biologics subsidiary has produced mostly operating losses for its parent. In anticipation of turning that around, the company will undertake a significant expansion of the biologics plant in Songdo, Incheon, that it opened in 2013.

The division of Samsung Group will invest about $700 million in an expansion of the facility, the *Korea Herald* reports. The project is slated to be completed in

Amgen’s biosimilars push

Tuesday, February 19, 2013

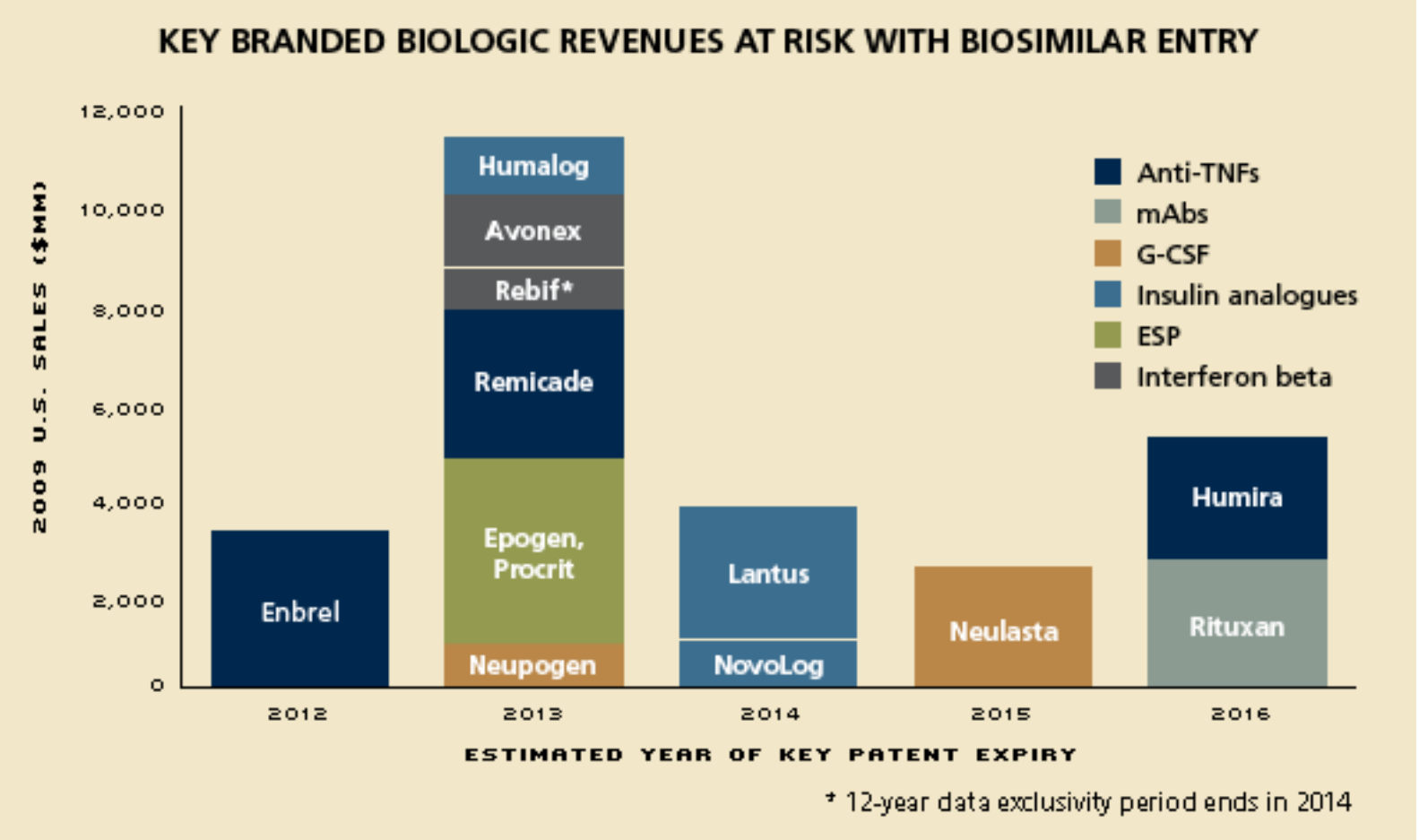
Amgen's Biosimilars Gambit

About a week ago, Amgen rocked the biotech industry's proverbial boat with their announcement that they'd be entering the biosimilars market. Multiple news outlets like Yahoo!, Forbes, and CNBC report that Amgen, starting in 2017, will be making six generic versions of blockbuster biologics:

- Abbvie's Humira
- Janssen's[OLY 02/19/13] Remicade
- Roche's Avastin, Herceptin and Rituxan
- Eli Lilly's Erbitux

This comes as a surprise to many, because for years, Amgen has been saying that biologics really can't be copied.

Biologics are falling off the patent cliff too!



BPCI* Act defines Biosimilar or Biosimilarity

- Biosimilar or Biosimilarity means:
  - that the biological product is highly similar to the reference product notwithstanding minor differences in clinically inactive components; and
  - there are no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity, and potency of the product

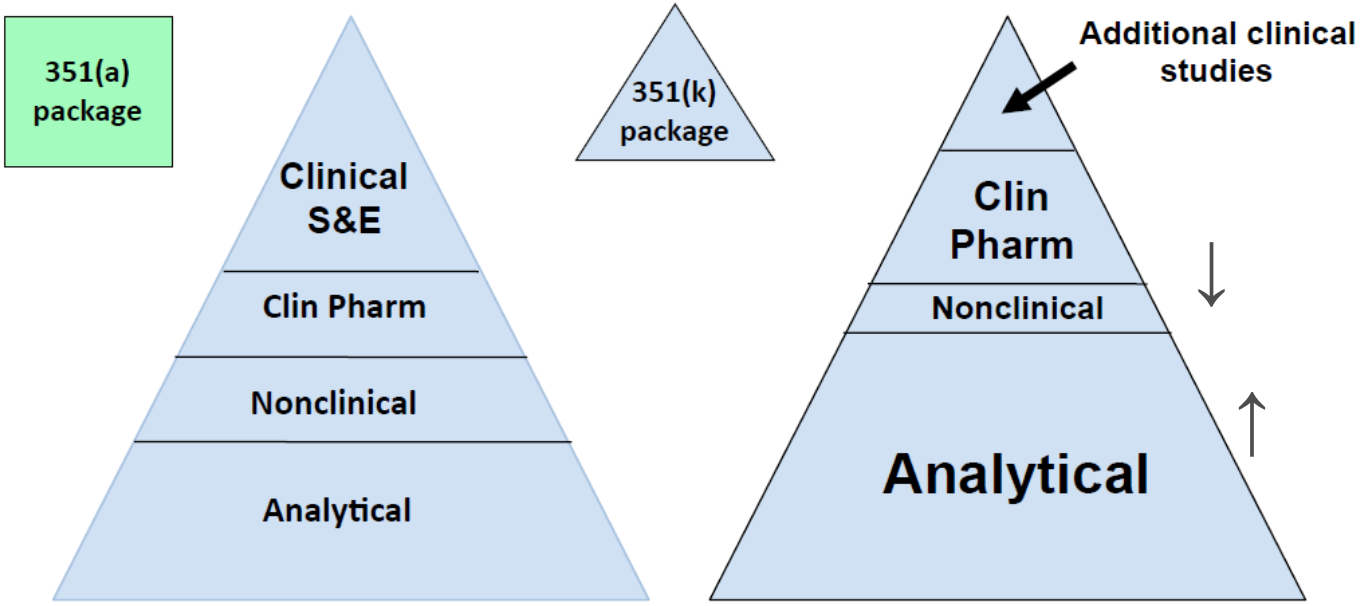
- FDA Biosimilars Guidance Outlines 'Stepwise' Development Approach
  - The FDA has issued three long-awaited biosimilars guidance documents, recommending a stepwise approach to showing biosimilarity that could allow eased trial requirements if a sponsor can demonstrate biosimilarity in earlier steps

*Biologics Price Competition and Innovation Act of 2009

New paradigm for biosimilar development

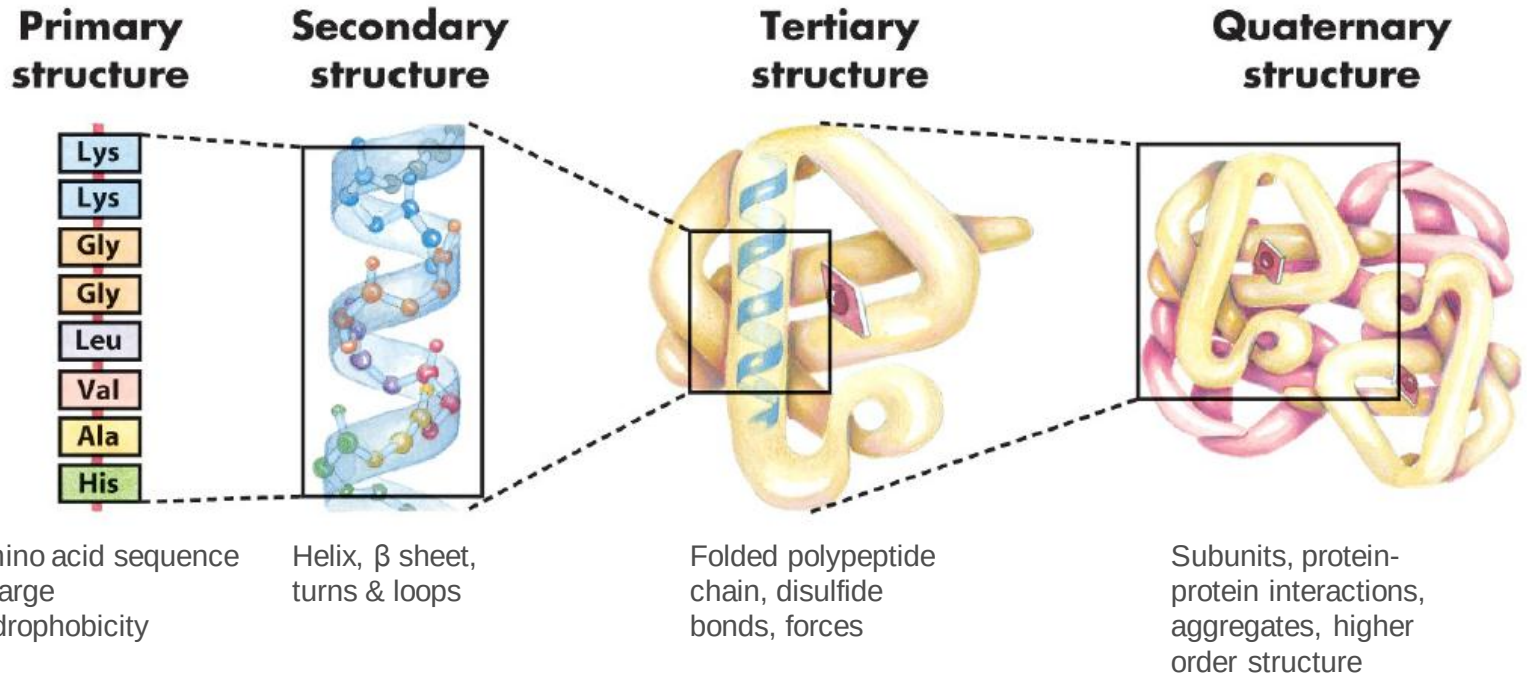
Scientific Considerations Draft Guidance

- The stepwise approach should start with extensive structural and functional characterization of both the proposed product and the reference product, which serves as the foundation of a biosimilar development program



- Highly similar analytical & PK/PD data = ↓ Risk of clinical differences
  - Reduce requirements for clinical studies

What does extensive structural and functional characterization means?



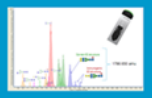
- All need to be evaluated as part of analytical similarity studies

Analytical tools to evaluate biosimilarity are the same but focus on comparability features

Attributes	Analytical tools
Amino acid sequence and modifications	Mass spectrometry (MS), peptide mapping, chromatographic separations
Folding	S-S bonding, calorimetry, HDX and IM-MS, NMR, circular dichroism, Fourier transform & Raman spectroscopy, fluorescence, interaction chromatographies
Subunit interactions	Chromatography, IM-MS
Heterogeneity (size, charge, hydrophobicity)	Chromatography resins; gel & capillary electrophoresis, light Scattering, IM-MS
Glycosylation	Anion exchange, enzymatic digestion, peptide mapping, CE, MS
PEGylation & isomers	Chromatography, peptide mapping
Bioactivity	Cellular and animal bioassays; ligand & receptor binding (ELISA, surface plasmon resonance), signal transduction
Aggregation	Analytical ultracentrifugation, size-exclusion chromatography, field flow fractionation, light scatter, microscopy
Proteolysis	Electrophoresis, chromatography, MS
Impurities (HCP, DNA)	LC, LC/MS, LBAs, PCR, metal (ICP-MS) & solvent analysis

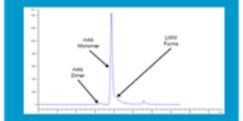
We have the most comprehensive portfolio of analytical instrumentation & solutions for biosimilars

Glycan Analysis



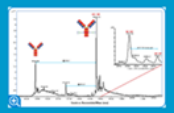
CE-LIF, CE-MS
LC-FLD, LC-MS
LC-Chip/MS mAb-Glyco kit

Aggregation



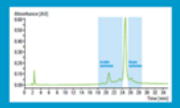
LC-UV Size Exclusion
CE-UV
Field-flow fractionation

Molecular Weight Determination



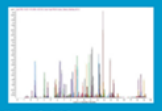
CE SDS-PAGE, CE-MS
Microfluidic SDS-PAGE
LC-UV or LC-MS

Charge Variants



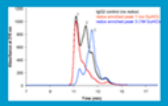
IEF analyzers (iCE 280)
CE (cIEF), CE-MS
Bio-LC-UV Ion Exchange

Peptide Characterization/ Mapping



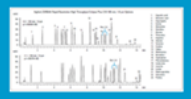
LC-UV, LC-MS
CE-UV, CE-MS

Oxidation



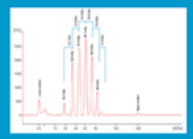
CE-MS
LC-UV , LC-MS
HIC and Reversed Phase

Amino Acids



LC-UV , LC-MS
CE, CE-MS

Protein PEGylation



LC-UV SEC / Cation Exchange
Microfluidic SDS-PAGE

Comparison of follow on biologics to an innovator mAb by HPLC, SEC and Peptide Mapping

Samples:

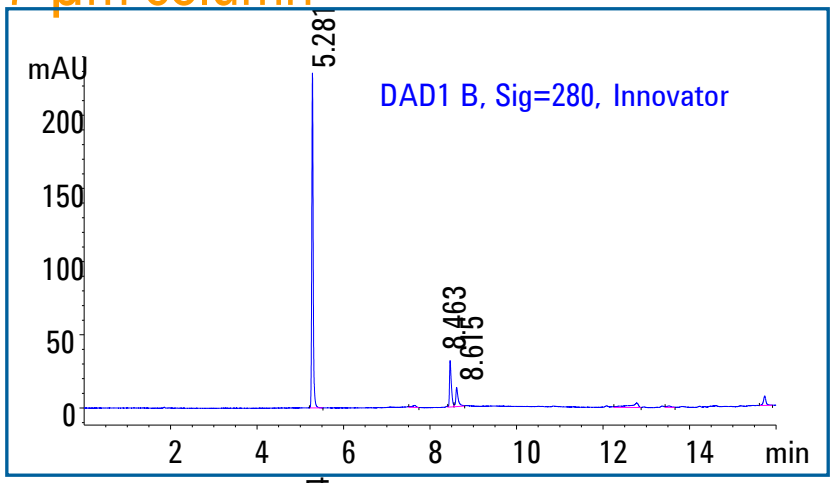
- Innovator – **Ristova** (Rituximab/Roche)
- Biosimilar – _____ (Rituximab/Indian Manufacturer)
  - Samples purchased from local Pharmacy in Bangalore, India

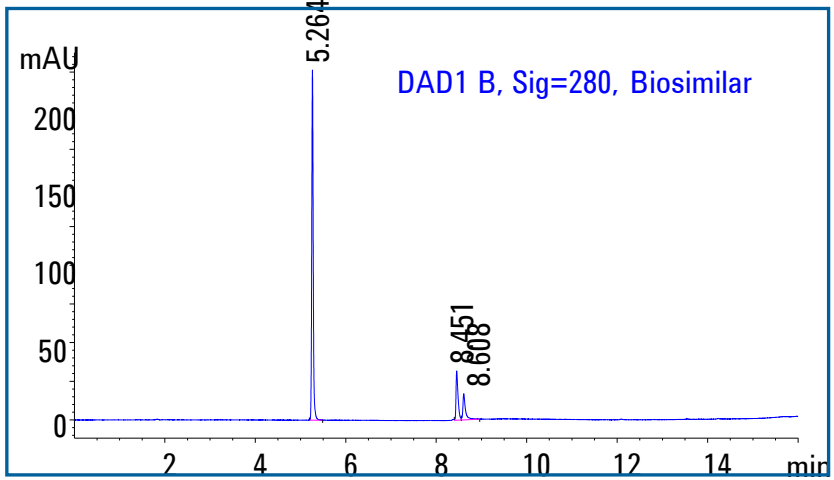
Analytical tools:

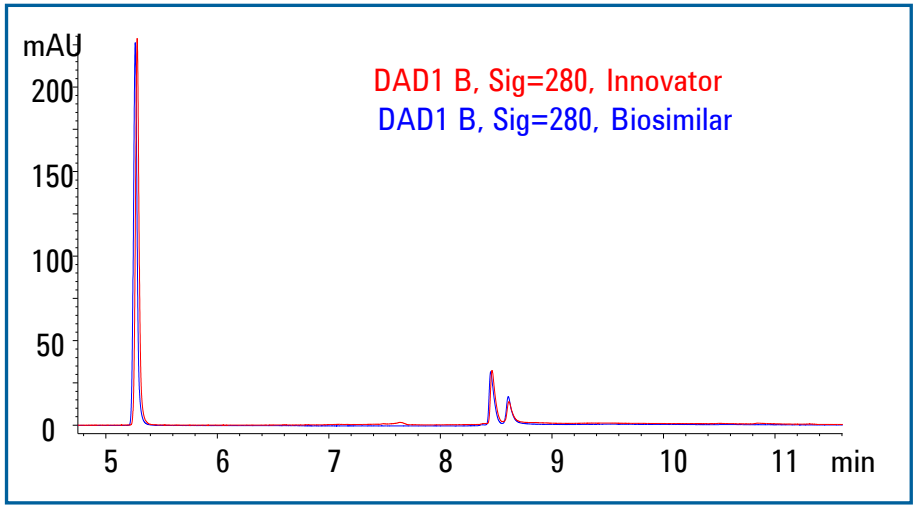
- The Agilent 1260 Bio-inert LC
- Biocolumns
- Match Compare Software

RP HPLC of Biosimilar and Innovator mAb

Agilent 1260 Infinity Bio-inert LC using Poroshell 120 SB C18 4.6x150 mm, 2.7 µm column



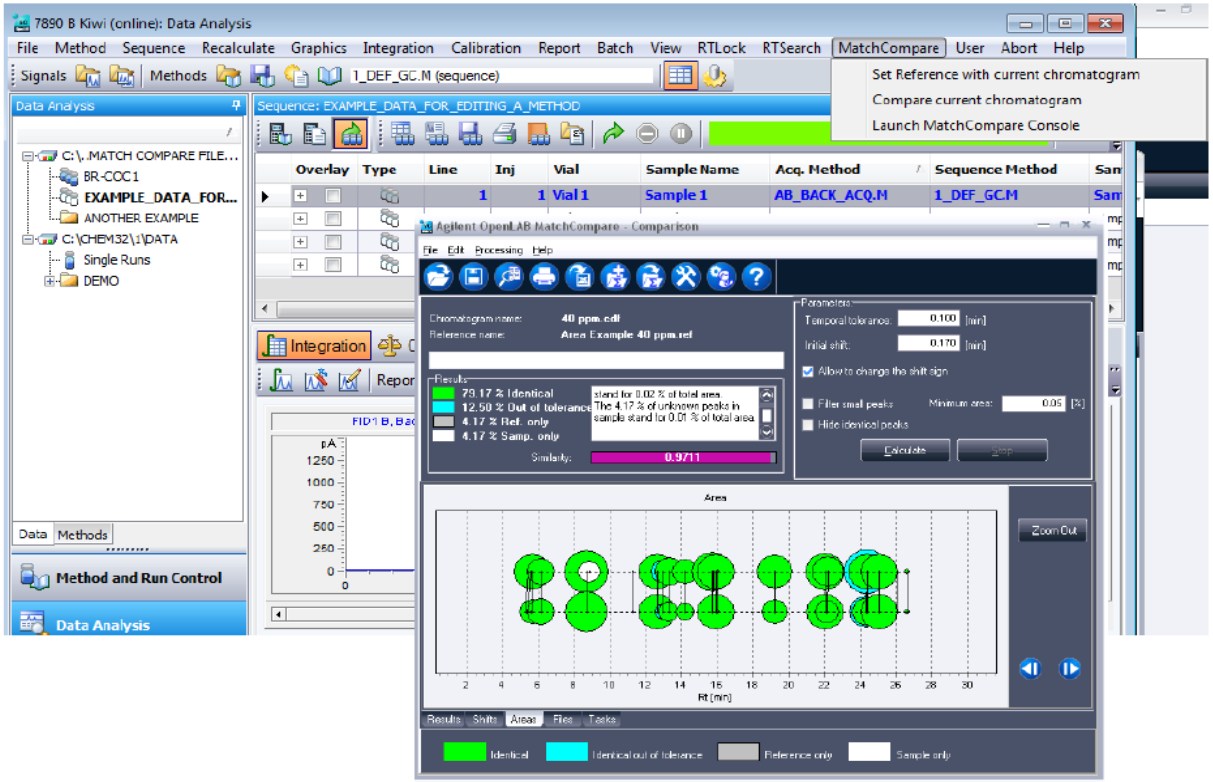




Agilent Match Compare tool for comparison

Compare an unknown sample, by selecting the sample chromatogram, in data analysis within OpenLAB CDS.

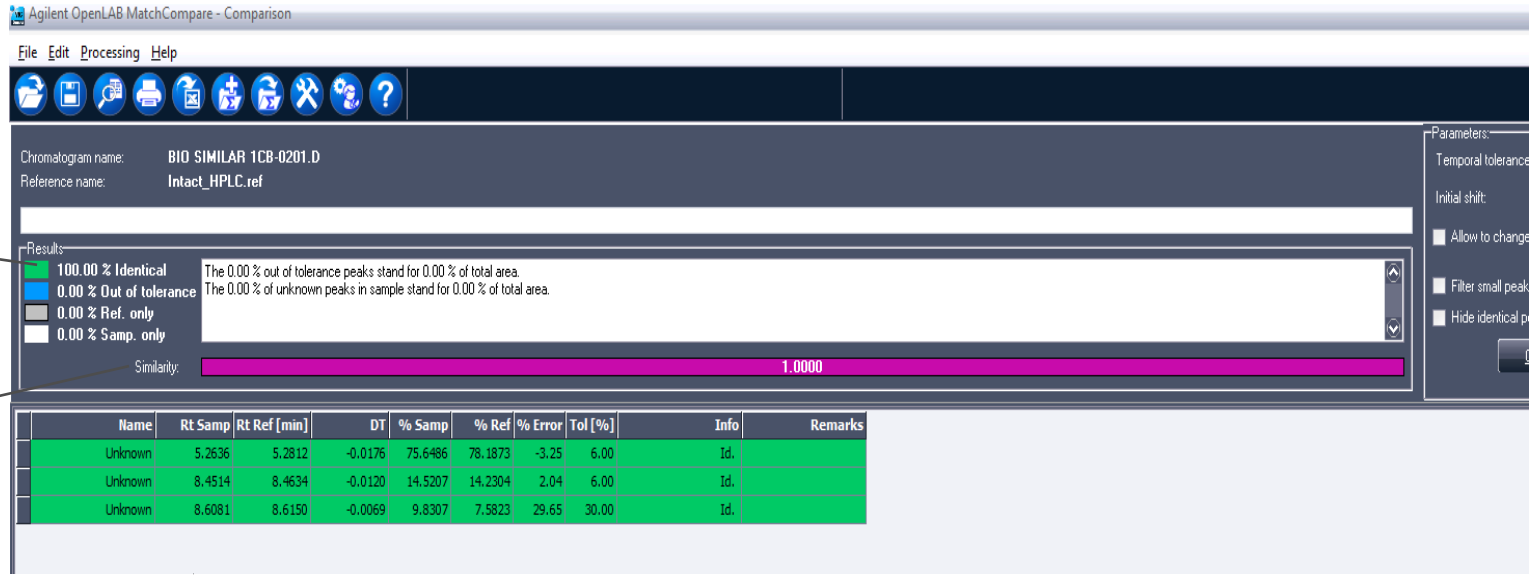
To start the comparison, select “Compare current chromatogram” under the Match Compare menu item.

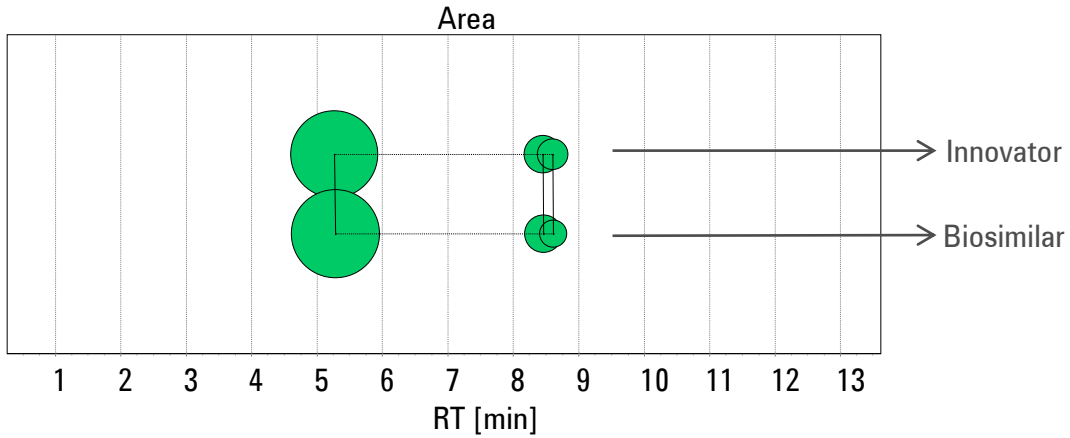


Agilent Match compare analysis of intact mAbs – RP HPLC

100% identical

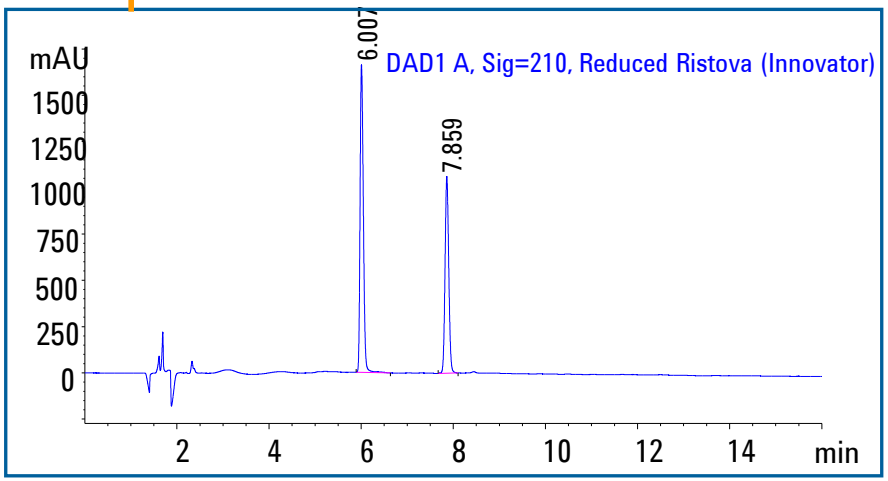
Similarity 1.000

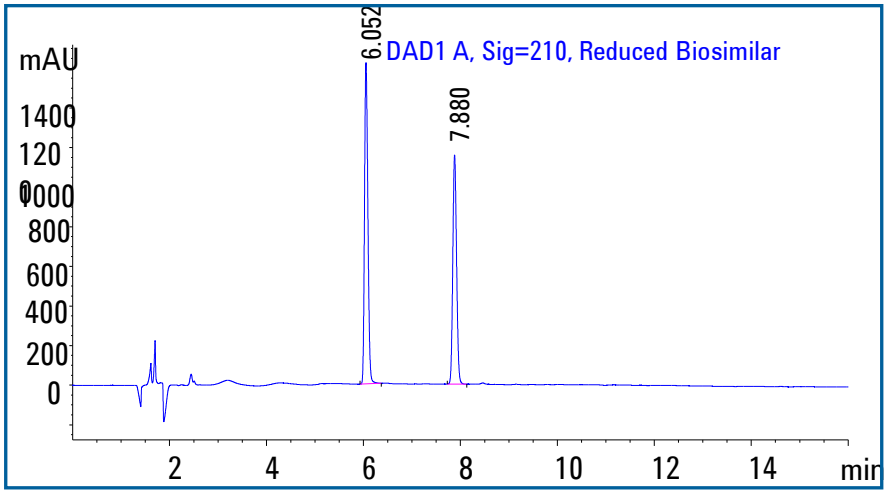


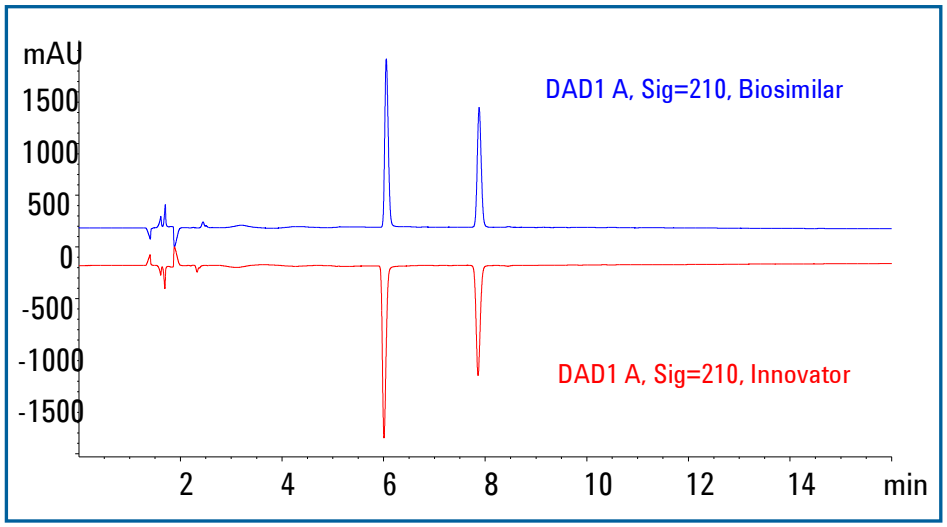


RP HPLC of reduced biosimilar and innovator mAb

Agilent 1260 Infinity Bio-inert LC using Poroshell 120 SB C18 4.6x150 mm, 2.7 µm column

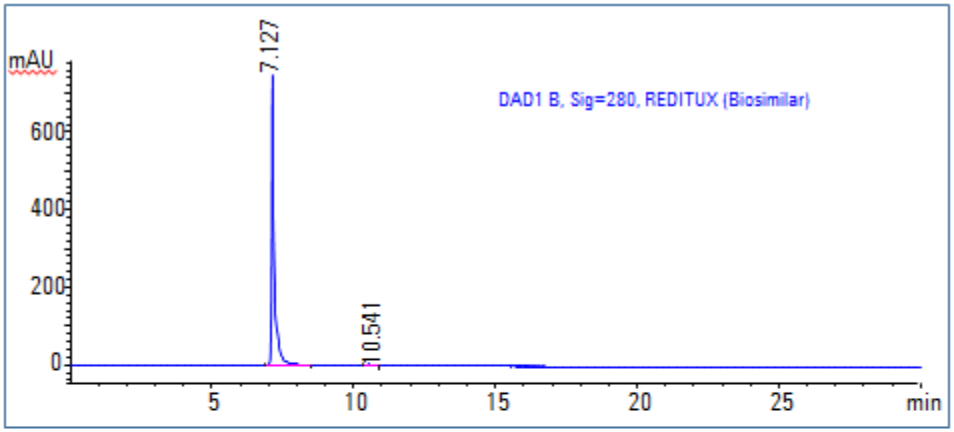


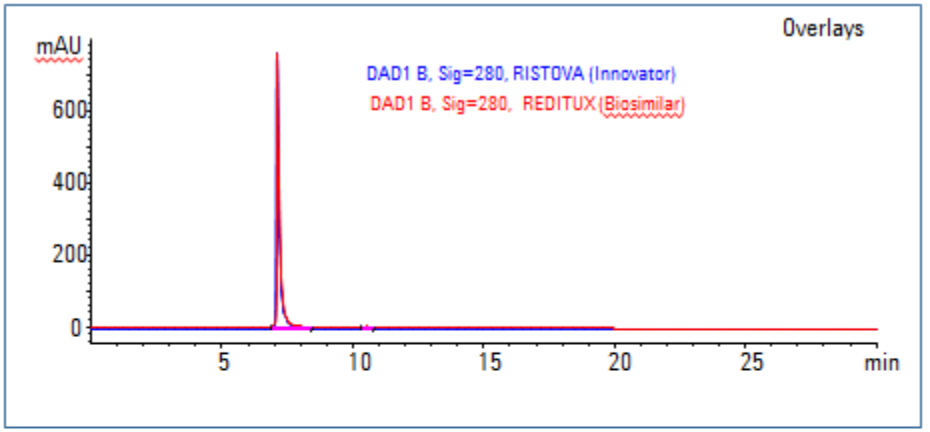


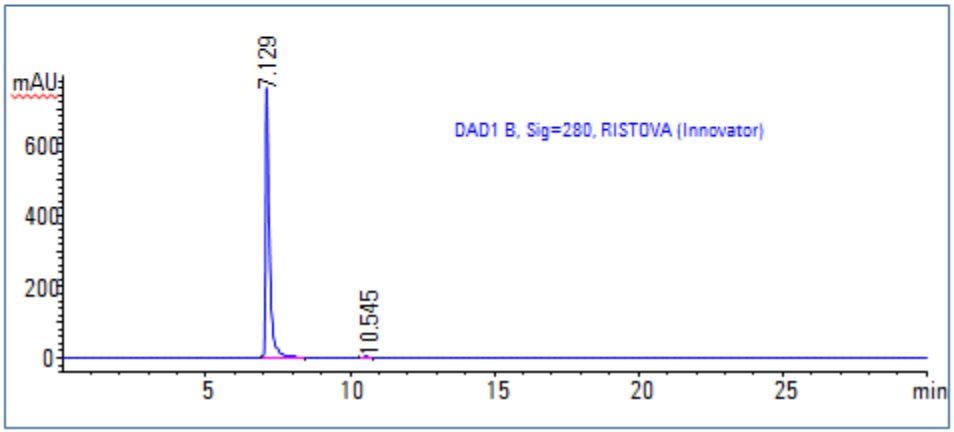


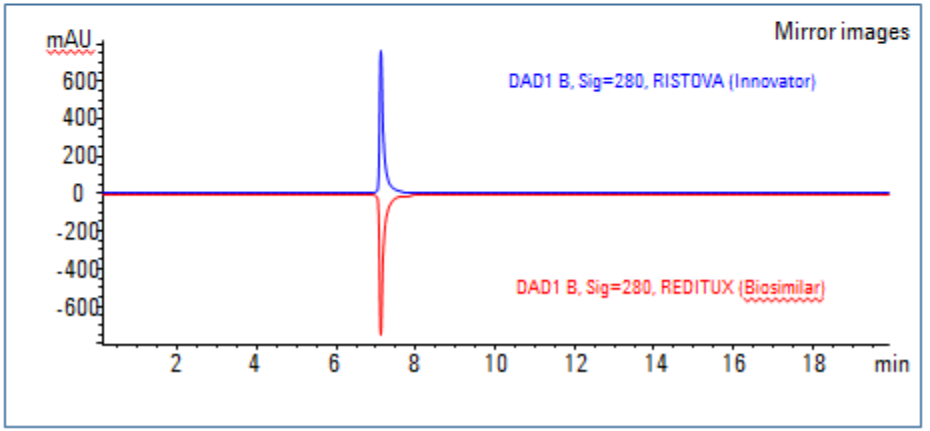
Intact SEC of Biosimilar and Innovator mAb

Agilent 1260 Infinity Bio-LC using a Bio SEC-3, 300Å, 7.8x300 mm, 3 µm



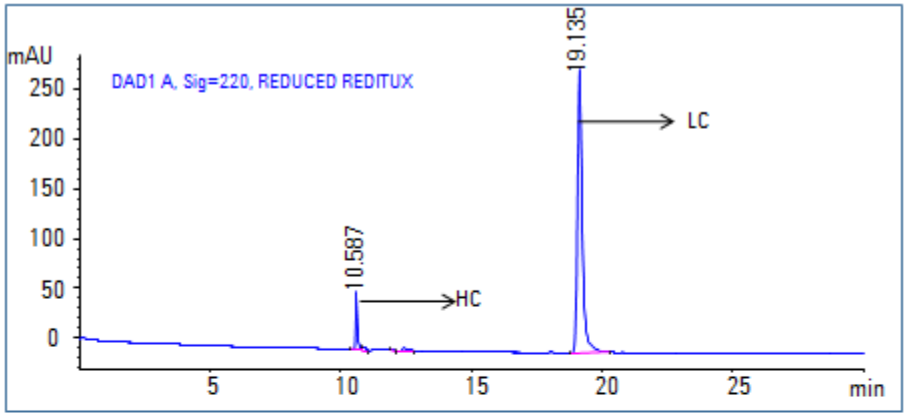


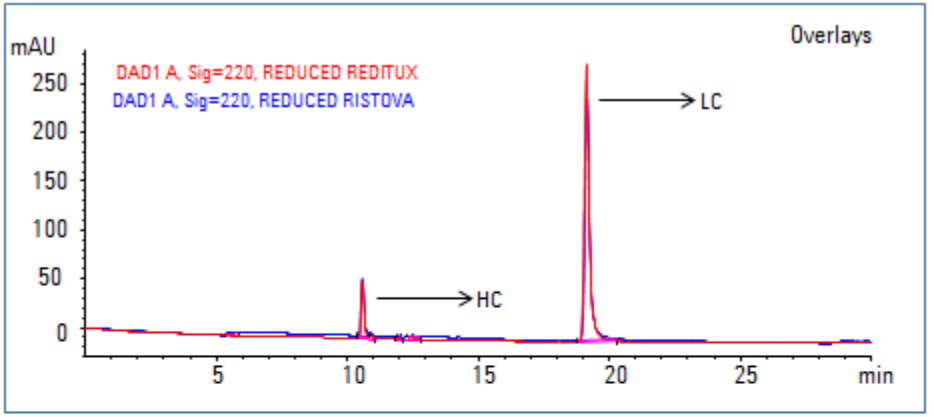


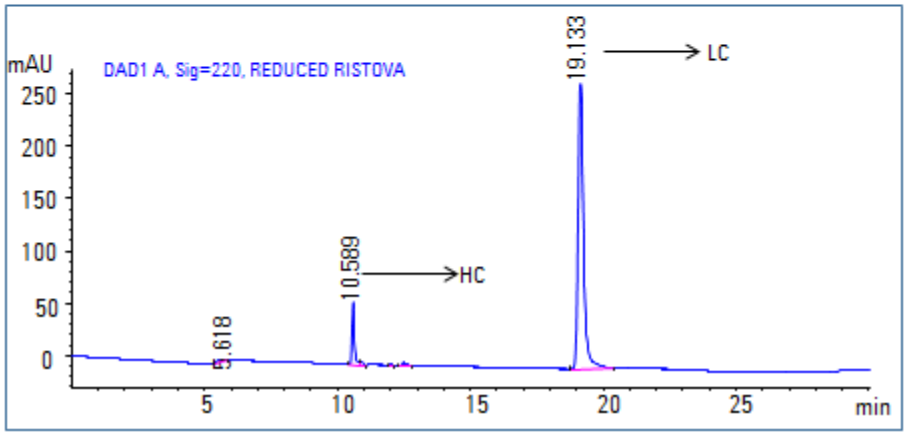


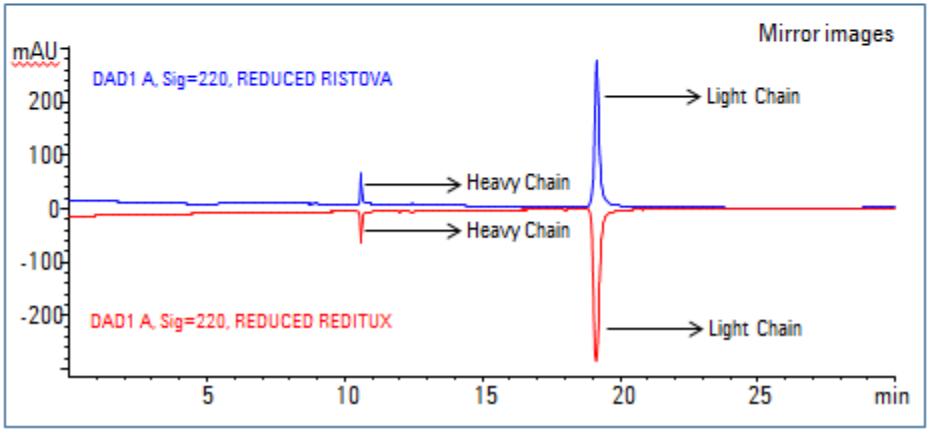
SEC of Reduced Biosimilar and Innovator mAb

Agilent 1260 Infinity Bio-LC using a Bio SEC-3, 300Å, 7.8x300 mm, 3 µm



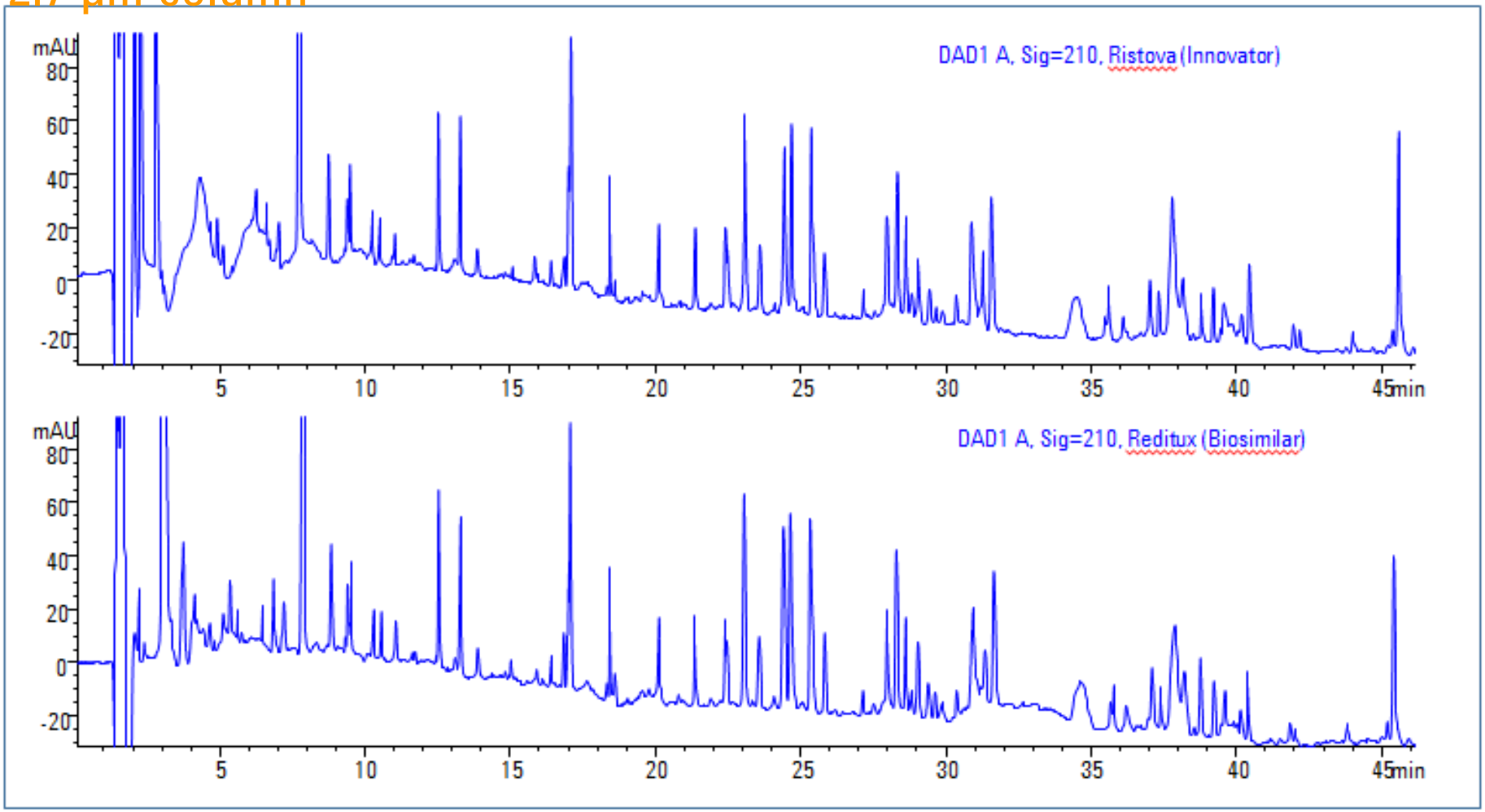






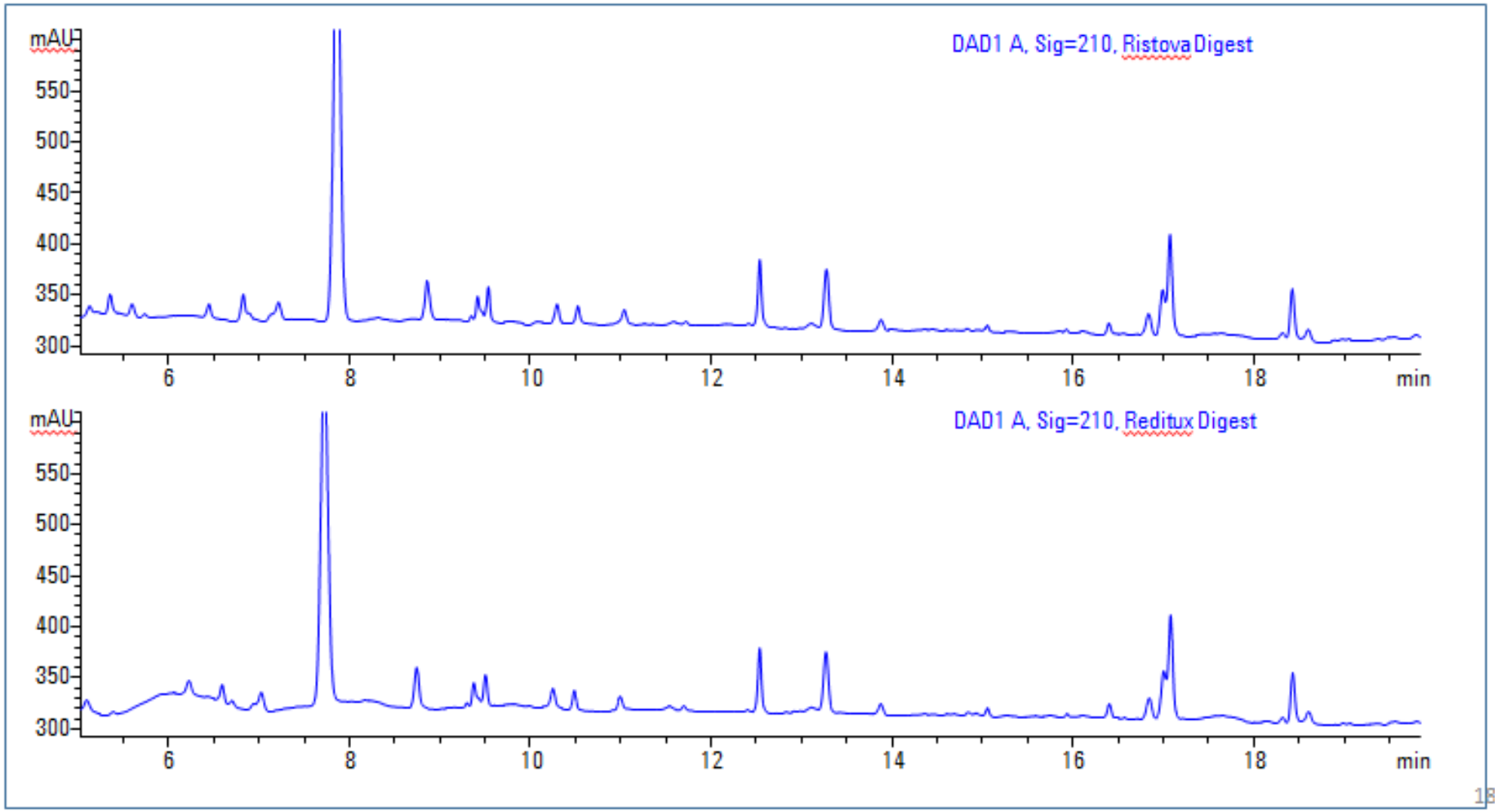
Peptide mapping of biosimilar and innovator mAb

Agilent 1260 Infinity Bio-LC using a Poroshell 120 SB C18 4.6x150 mm, 2.7 µm column



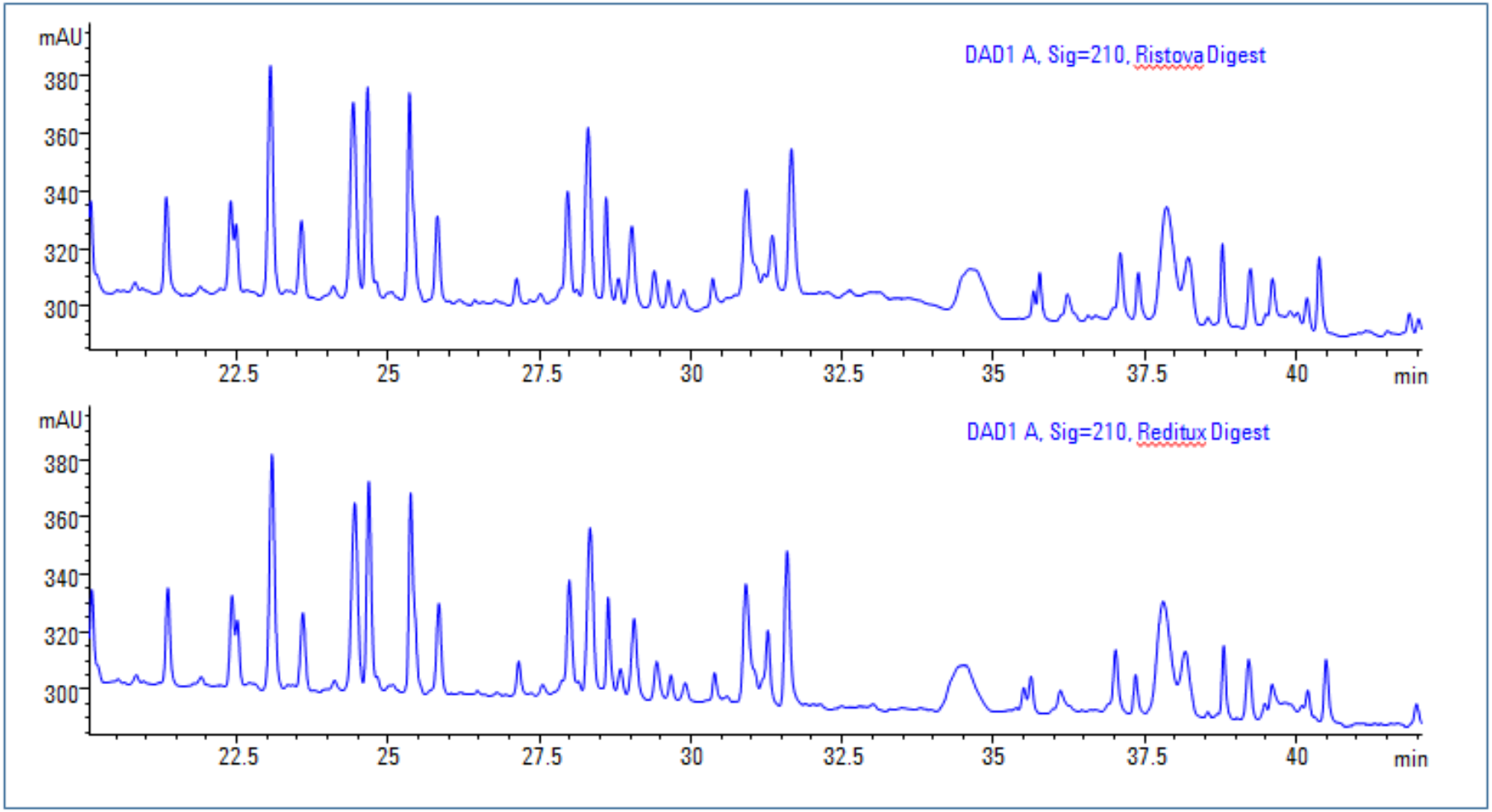
Peptide mapping of Biosimilar and innovator mAb

Zoom in of chromatogram; 5 – 20 min

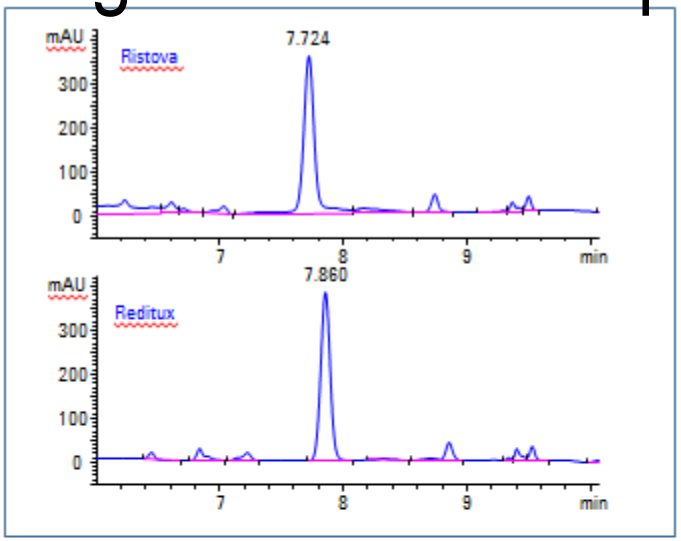


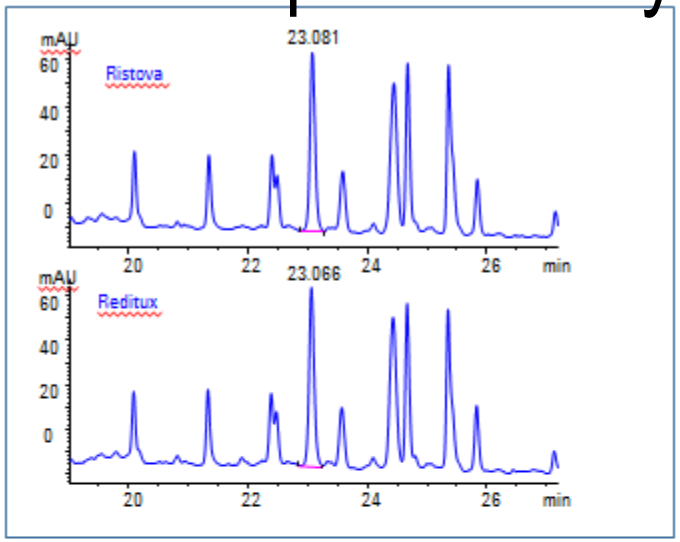
Peptide mapping of Biosimilar and innovator mAb

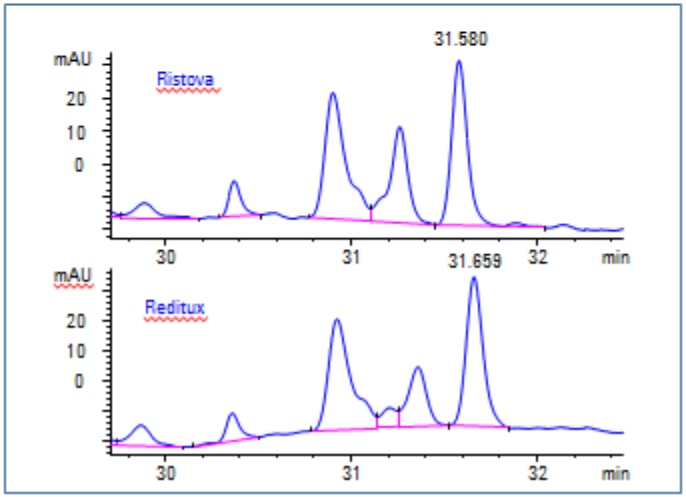
Zoom in of chromatogram; 20 – 40 min

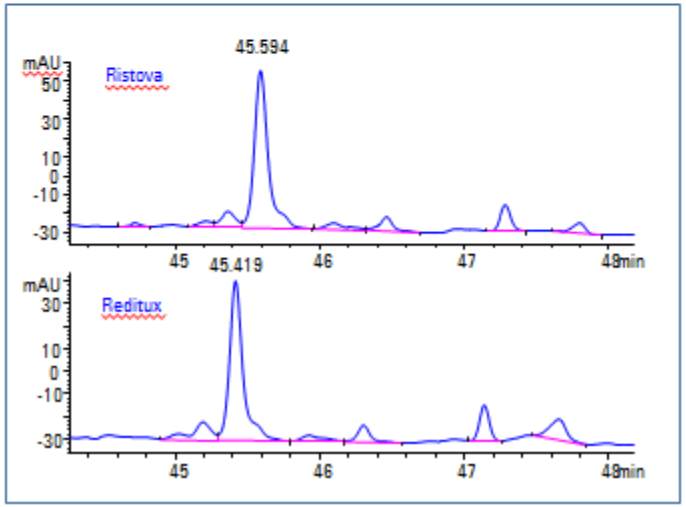


Zoom in of four representative peaks across the chromatogram to show separation reproducibility



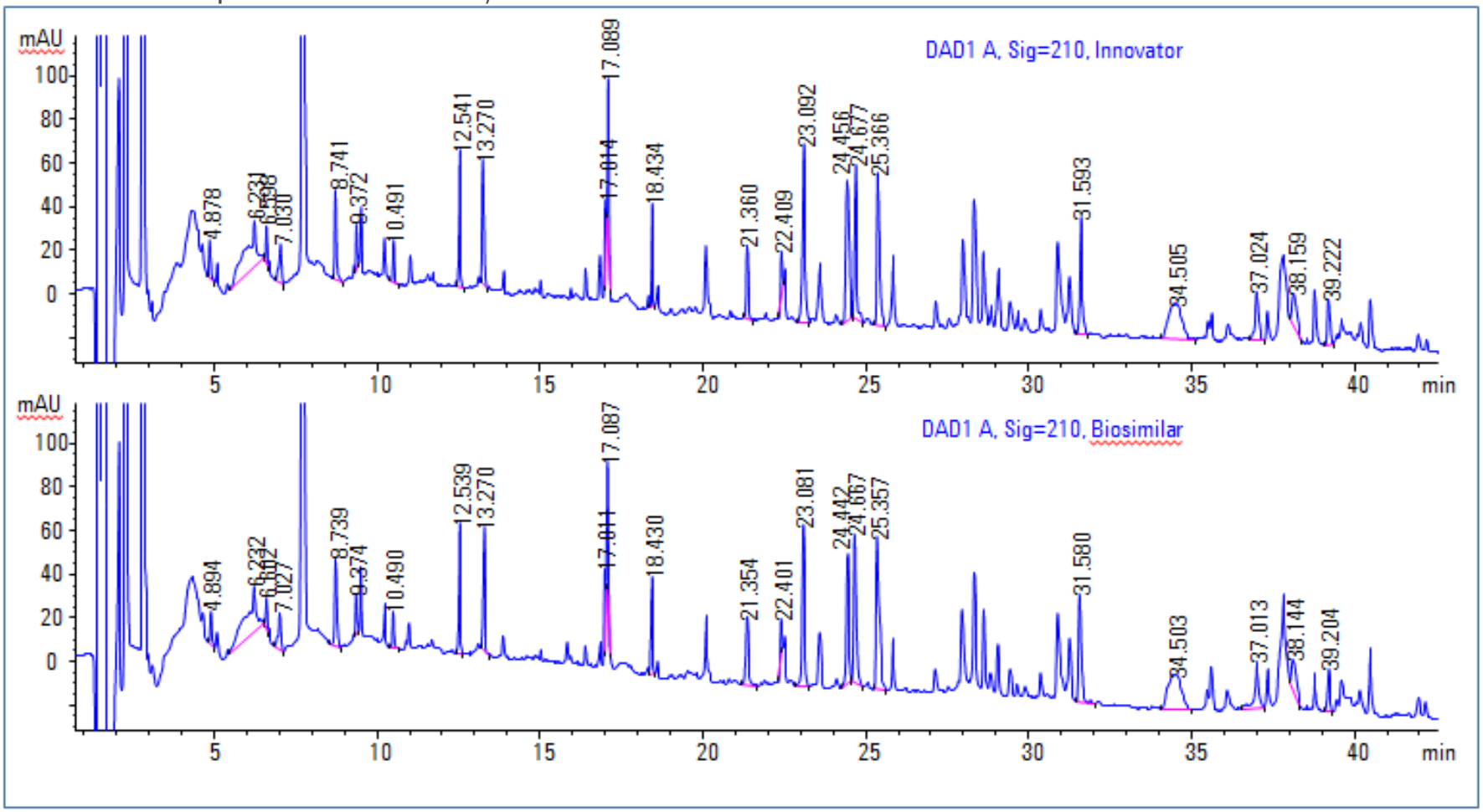






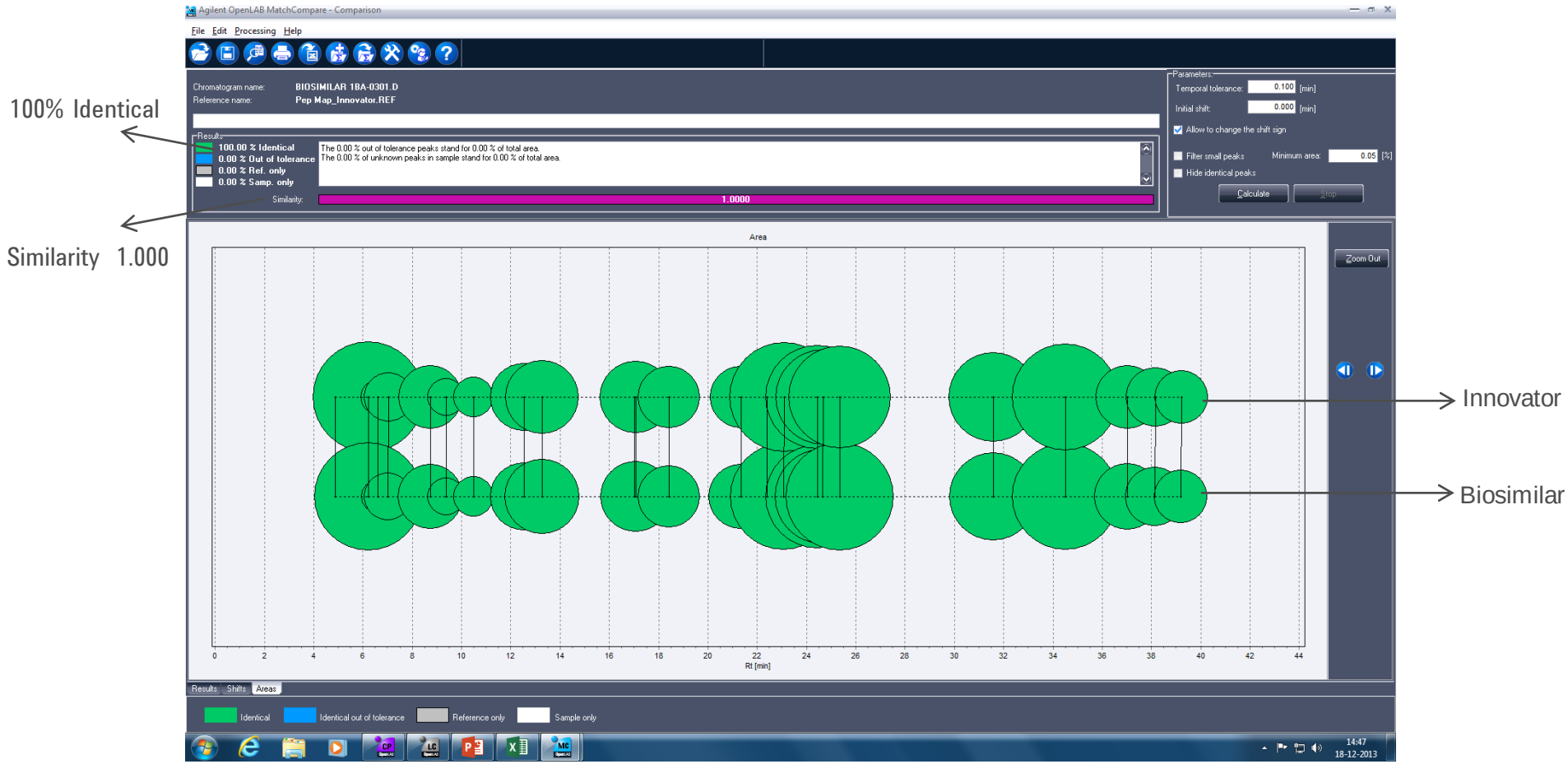
Peptide mapping of Biosimilar and innovator mAb

Comparison of peptide maps of innovator and biosimilar mAb using Agilent OpenLab Match Compare Software (Peaks selected for comparison are annotated)

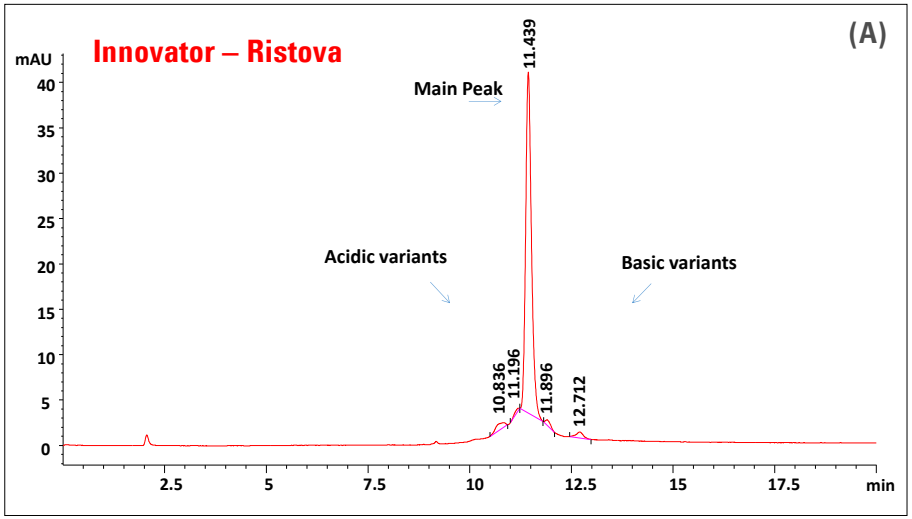


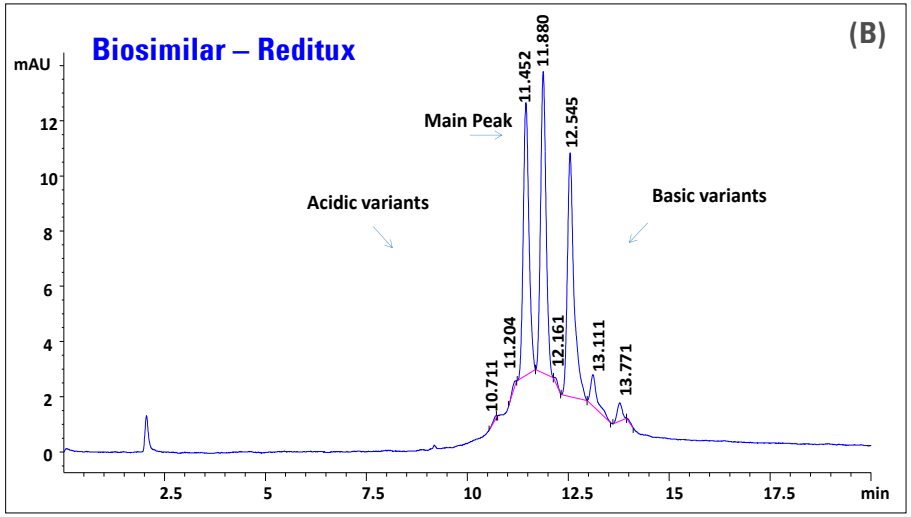
Peptide mapping of Biosimilar and innovator mAb

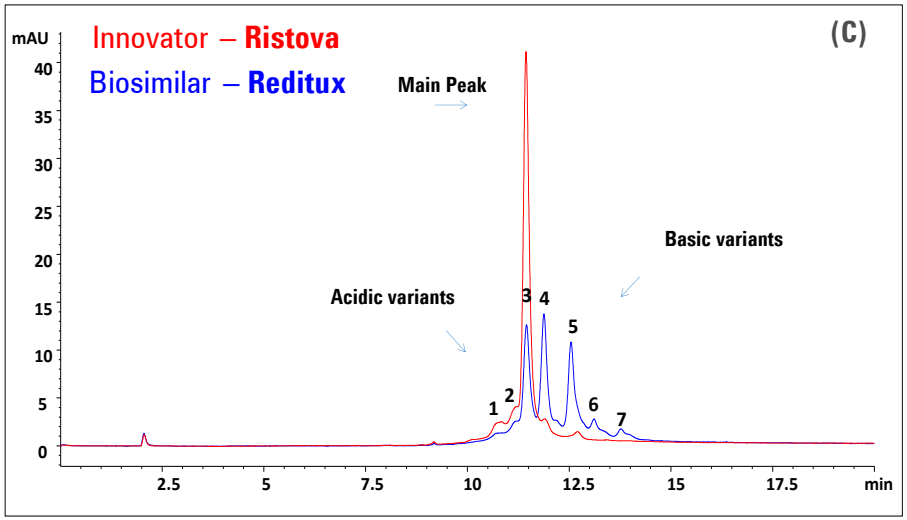
Match Compare result



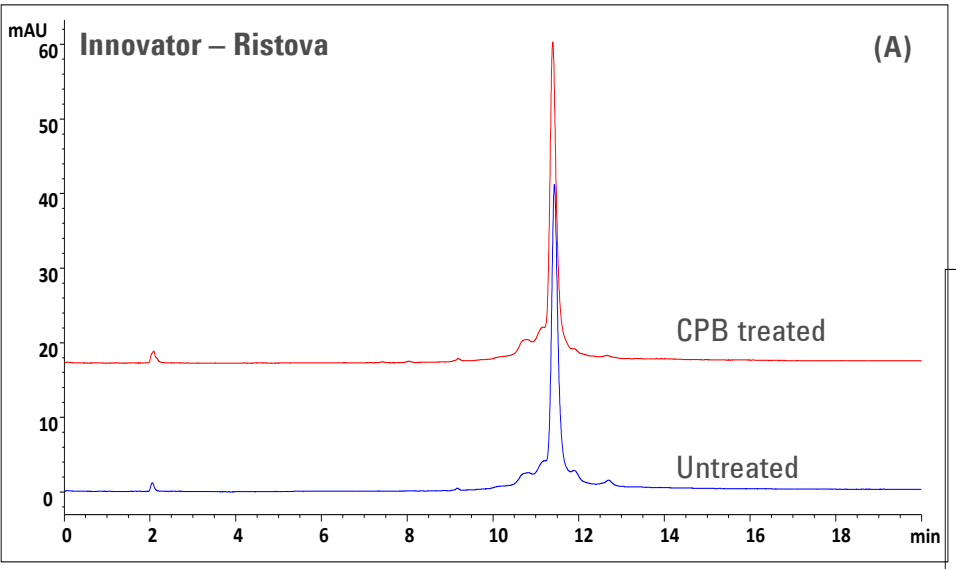
Charge Variant Profile of Innovator and Biosimilar Agilent BioMAb Column, 4.6x250mm, 5um

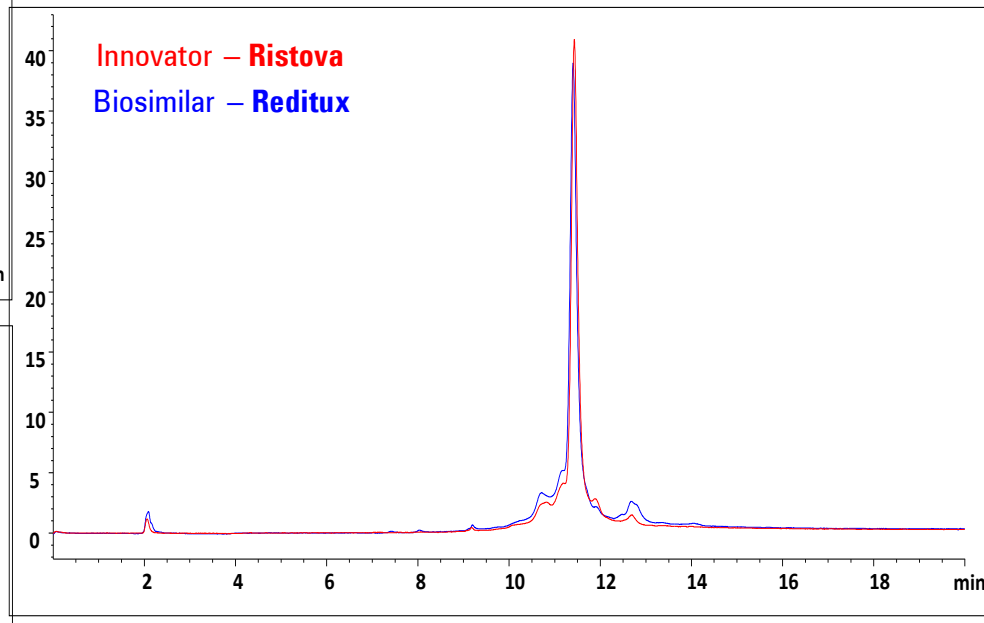


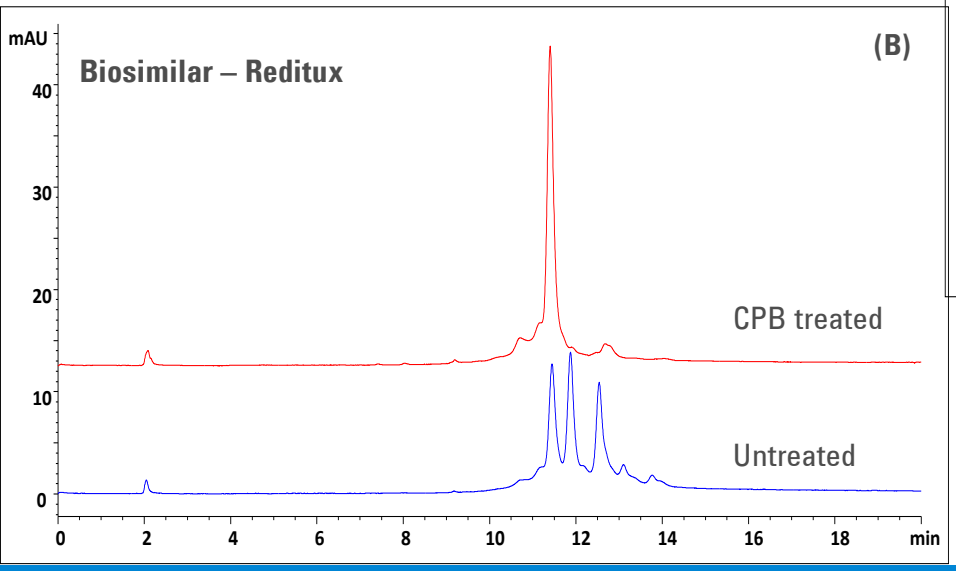




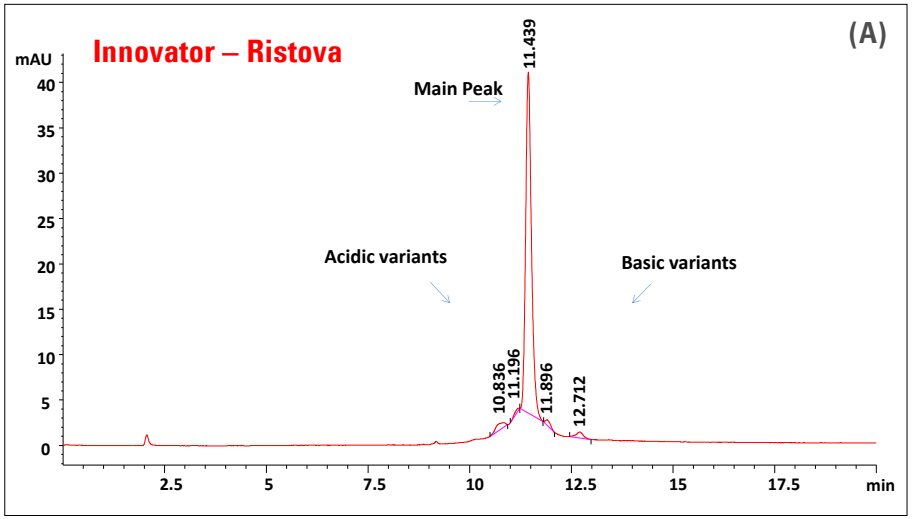
CpB Treatment of Innovator and Biosimilar Rituximab

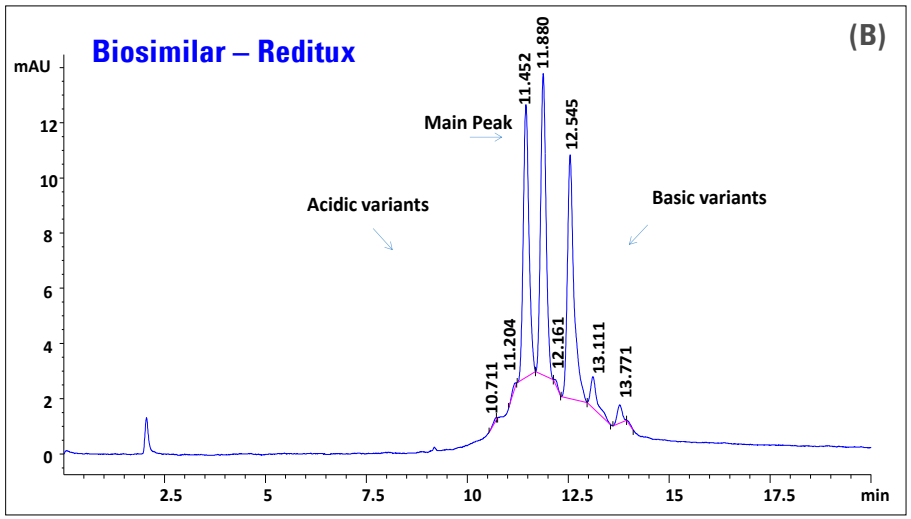






Charge Variant Profile of Innovator and Biosimilar IEC vs. Agilent CZE





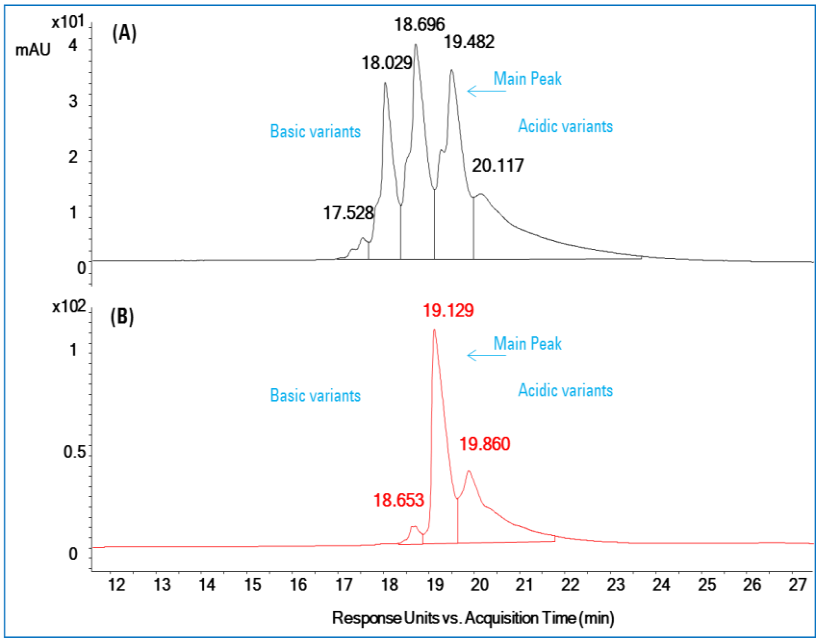


Figure 1: CZE separation of charge heterogeneity of (A) Biosimilar - Reditux and (B) Innovator - Ristova

CE parameters	Conditions
Capillary:	PVA, 56 cm, 50 µm id
Sample:	Rituximab Innovator and Biosimilar mAbs
Injection:	5s @ 50 mbar
Buffer:	400 mM EACA–acetic acid pH 5.7+0.05 % HPMC+2 mM TETA
Voltage:	30 kV
Temperature:	20 °C
DAD:	214 nm

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Biopharma Columns Development Manager
Agilent Technologies, Wilmington Delaware
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January 28, 2015

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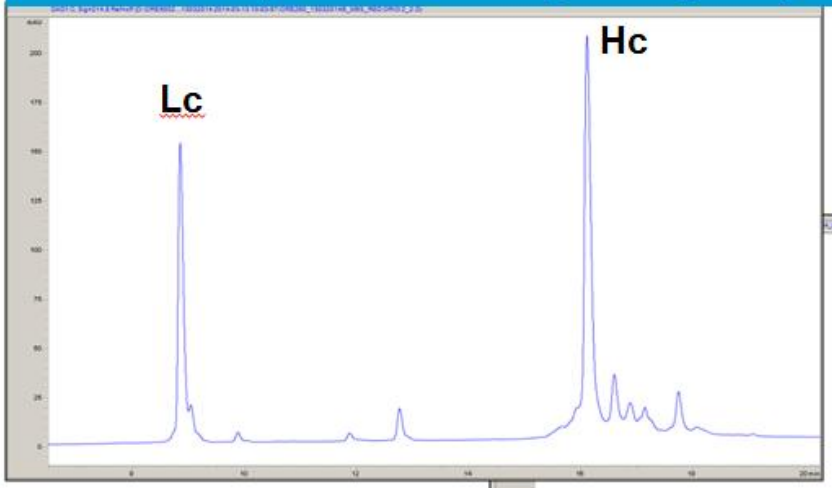
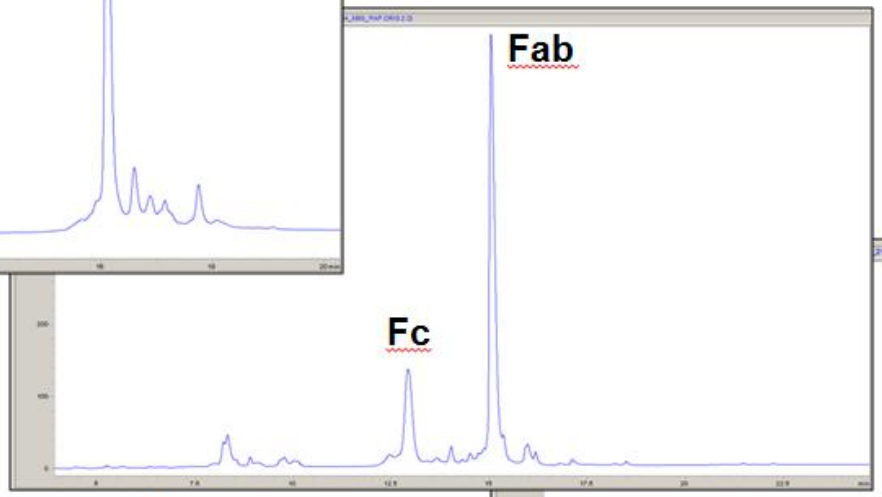
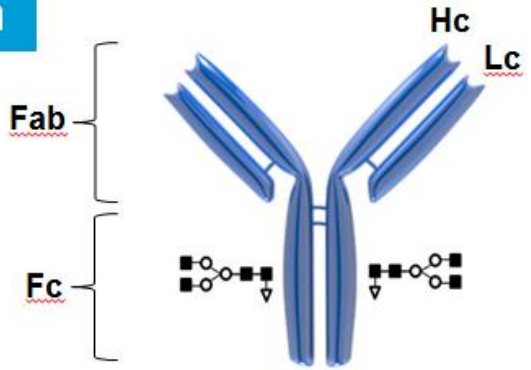
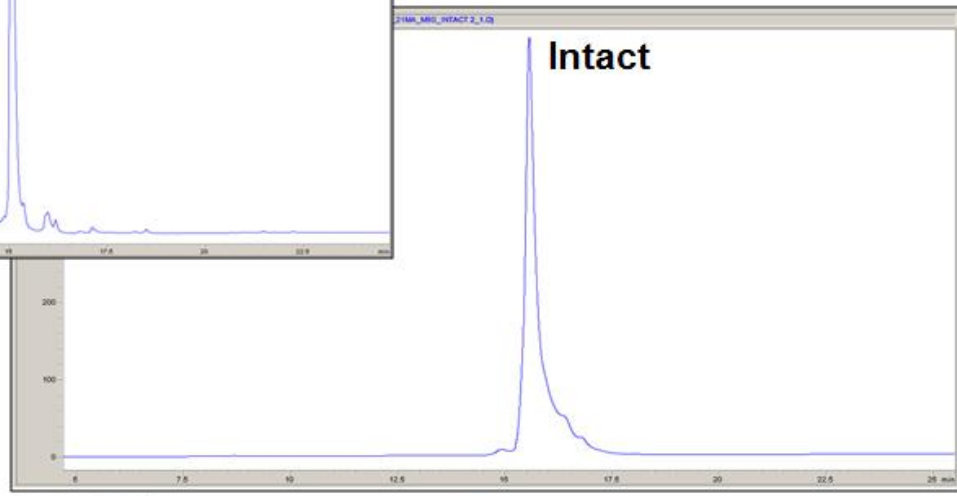
Reversed-phase U/HPLC

RPLC analysis for identity and purity determination of Herceptin









10 cm x 2.1 mm x 1.8 µm Zorbax 300 SB-C8
Temp: 80°C Flow: 200 µL/min
UV: 214 nm
Solv A: 0.1% TFA
Solv B: 0.1% TFA in ACN
30-38.6%B, 2-25 min

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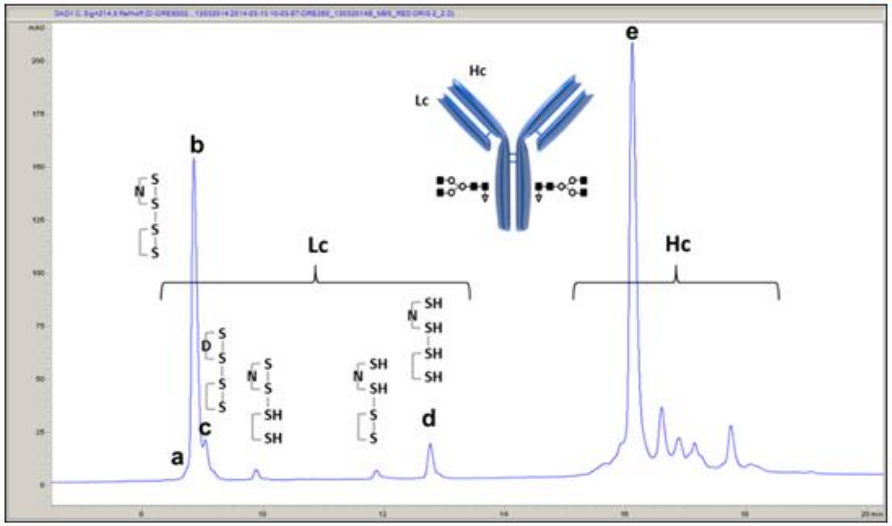
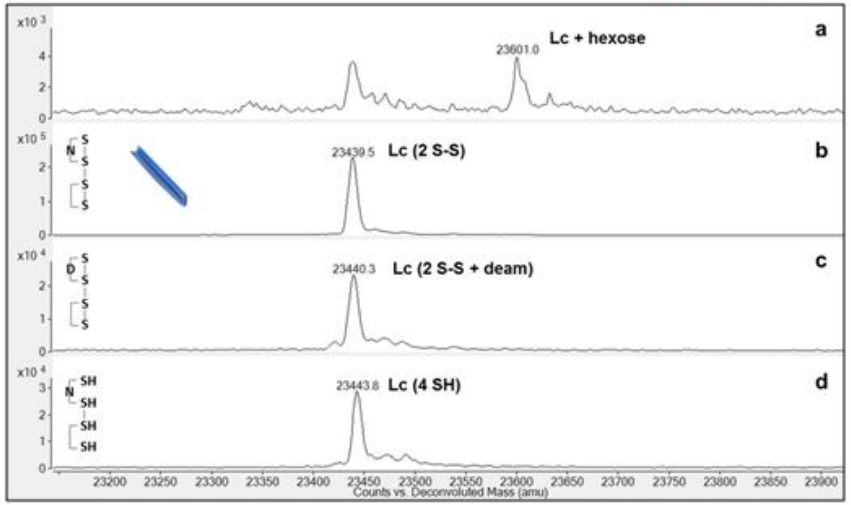
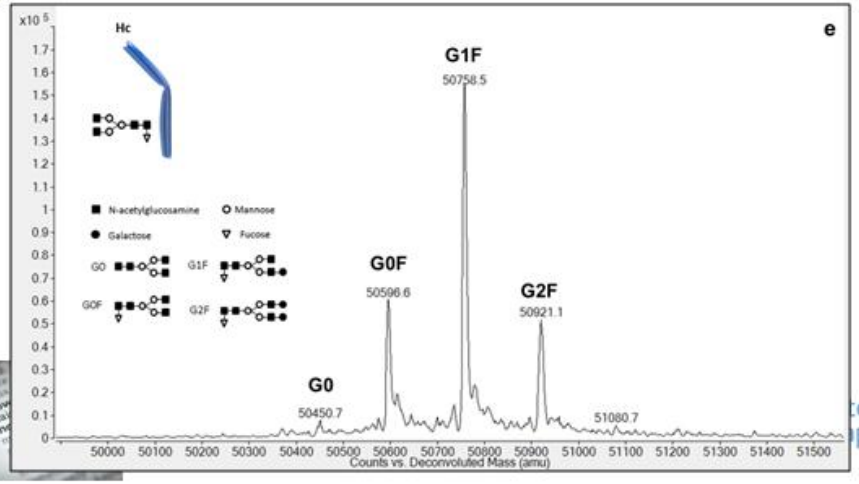
Reversed-phase U/HPLC – Mass Spectrometry

RPLC-UV-MS of Herceptin Lc and Hc

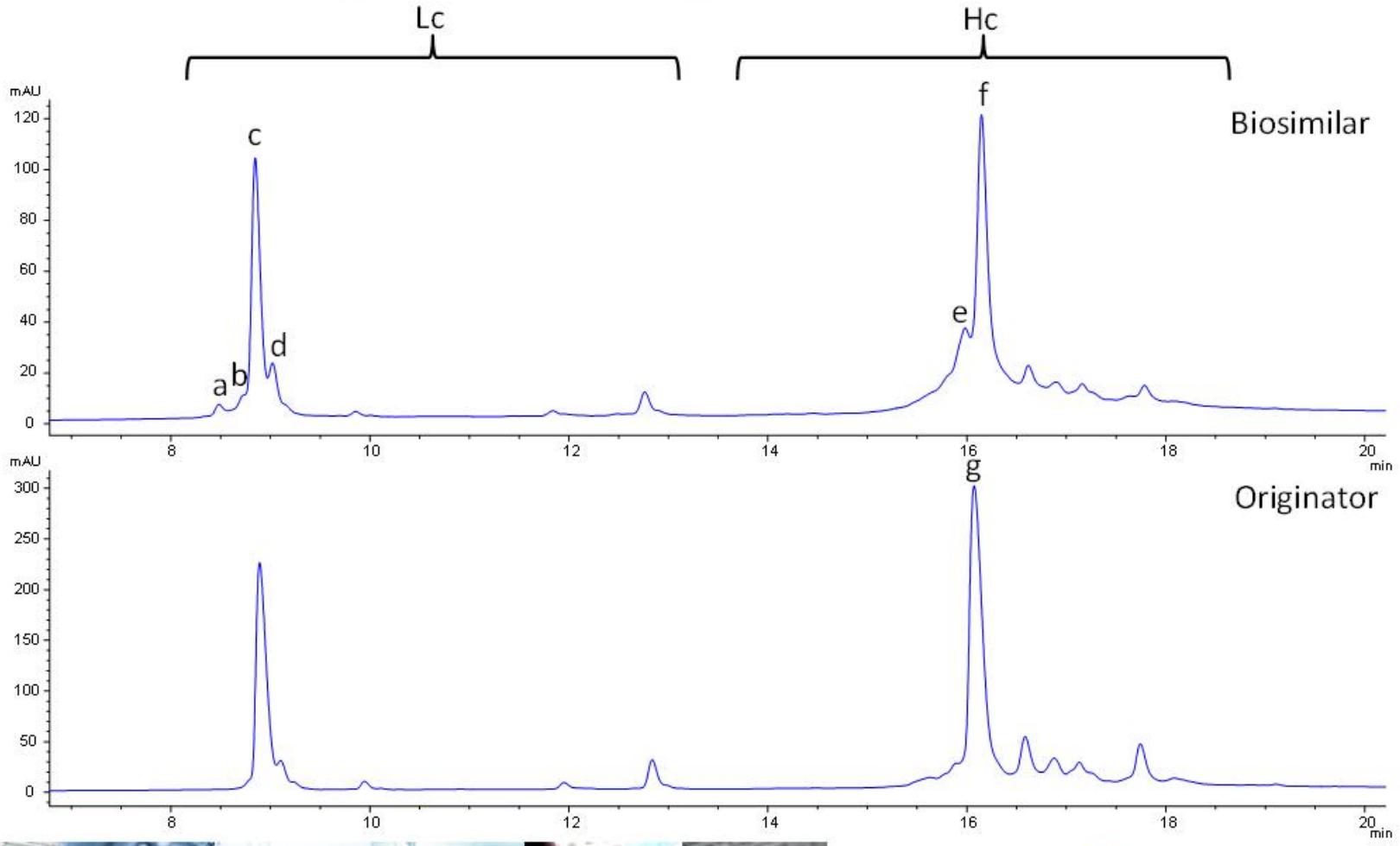


Deconvoluted spectra

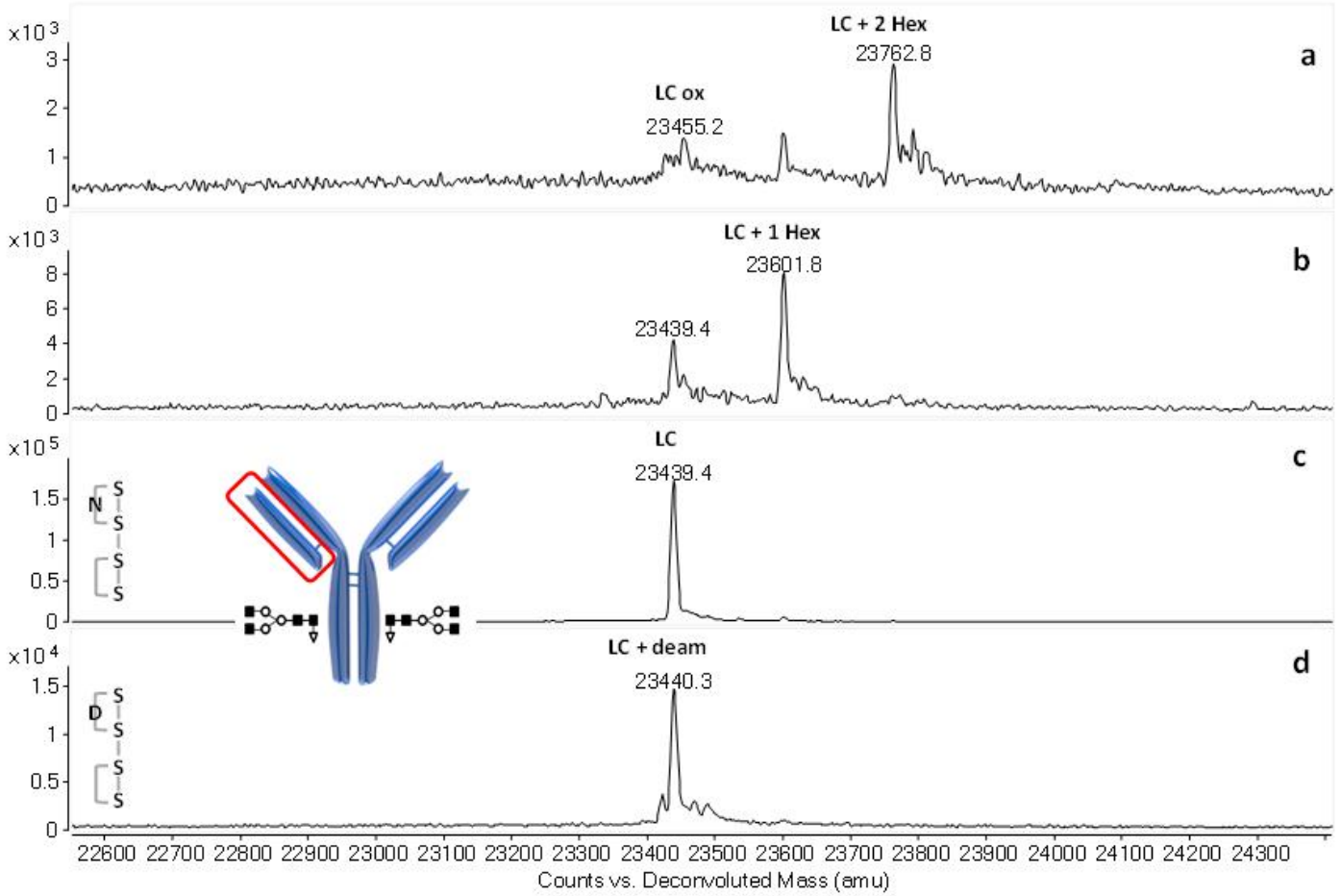




Reversed-phase U/HPLC for comparability assessment



Reversed-phase U/HPLC for comparability assessment



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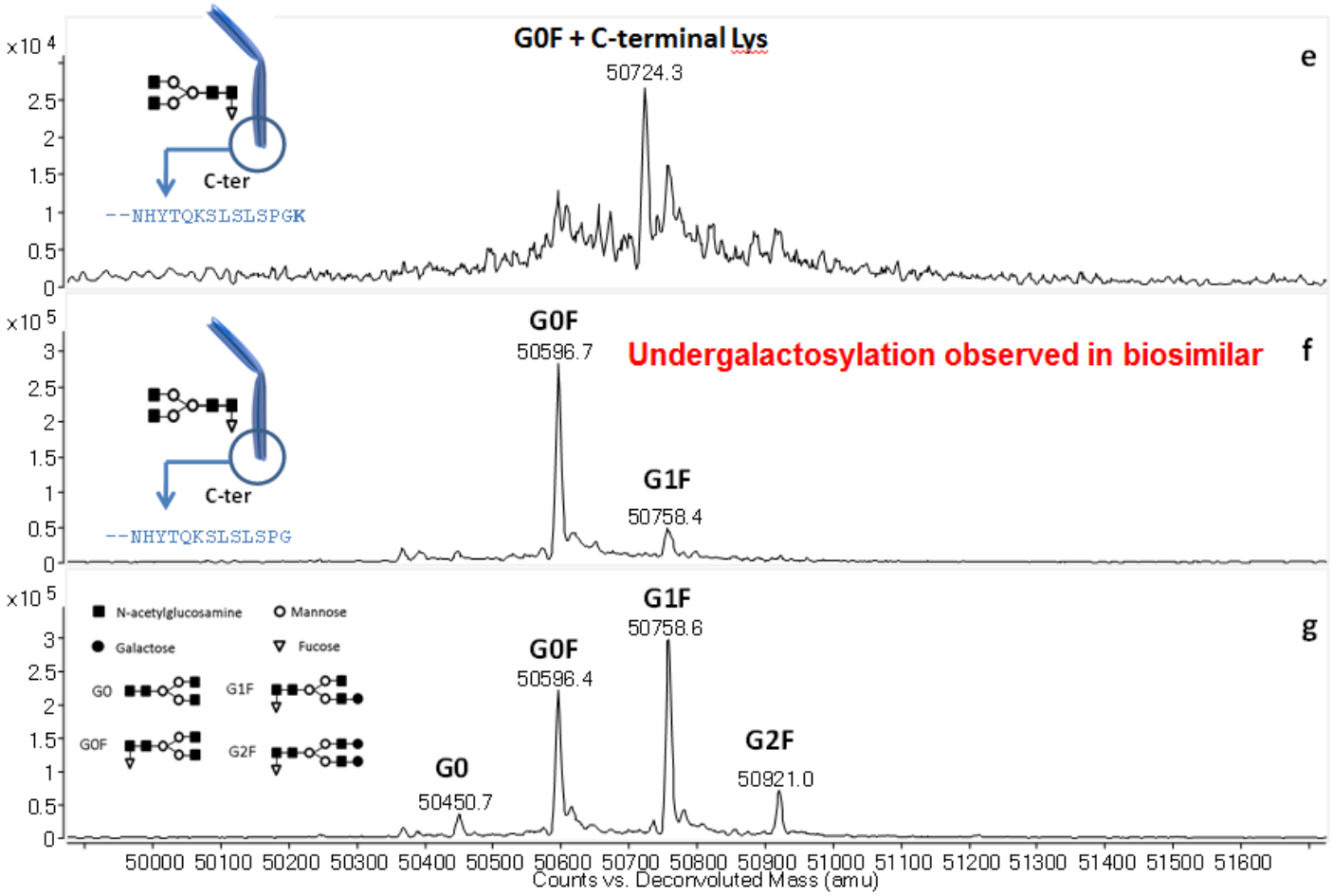
Reversed-phase U/HPLC for comparability assessment



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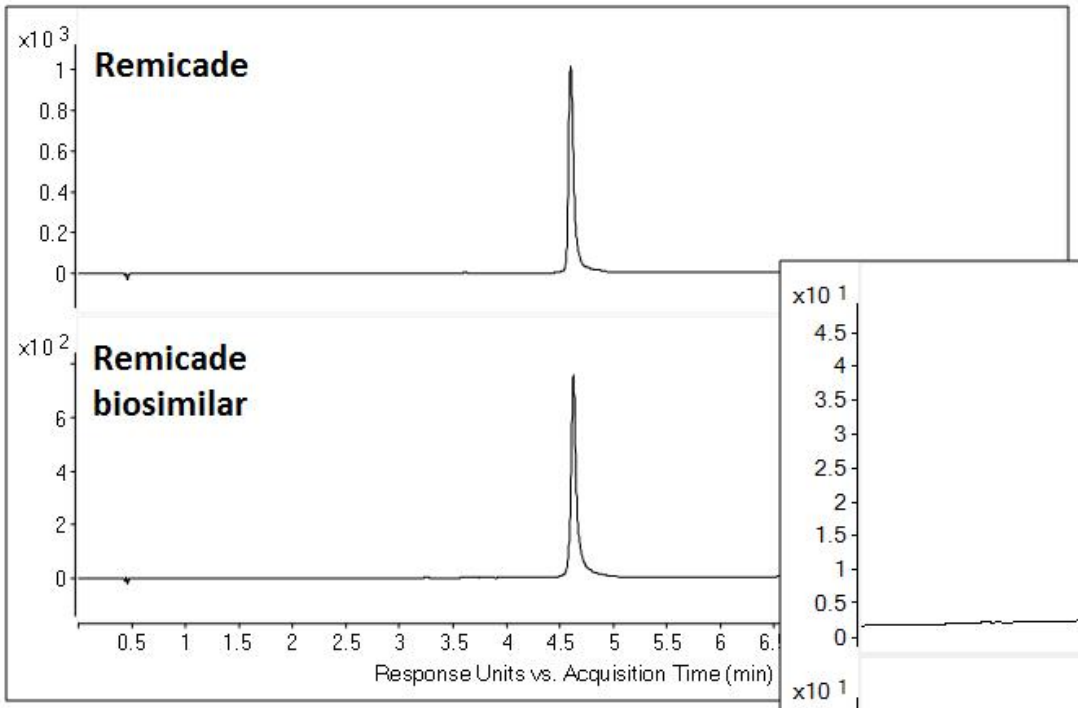
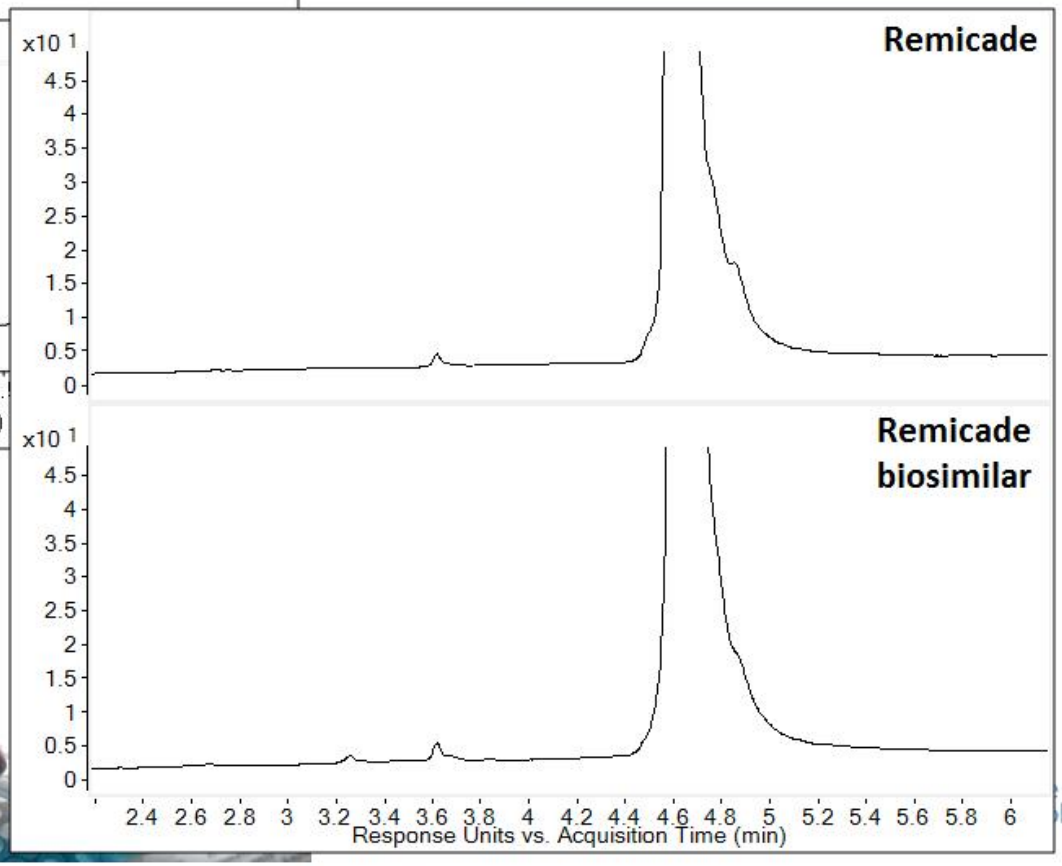
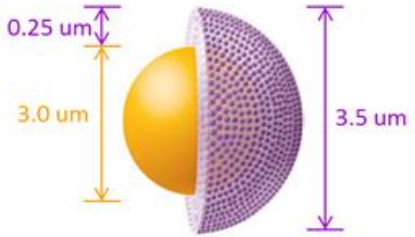
Widepore Poroshell for comparability assessment

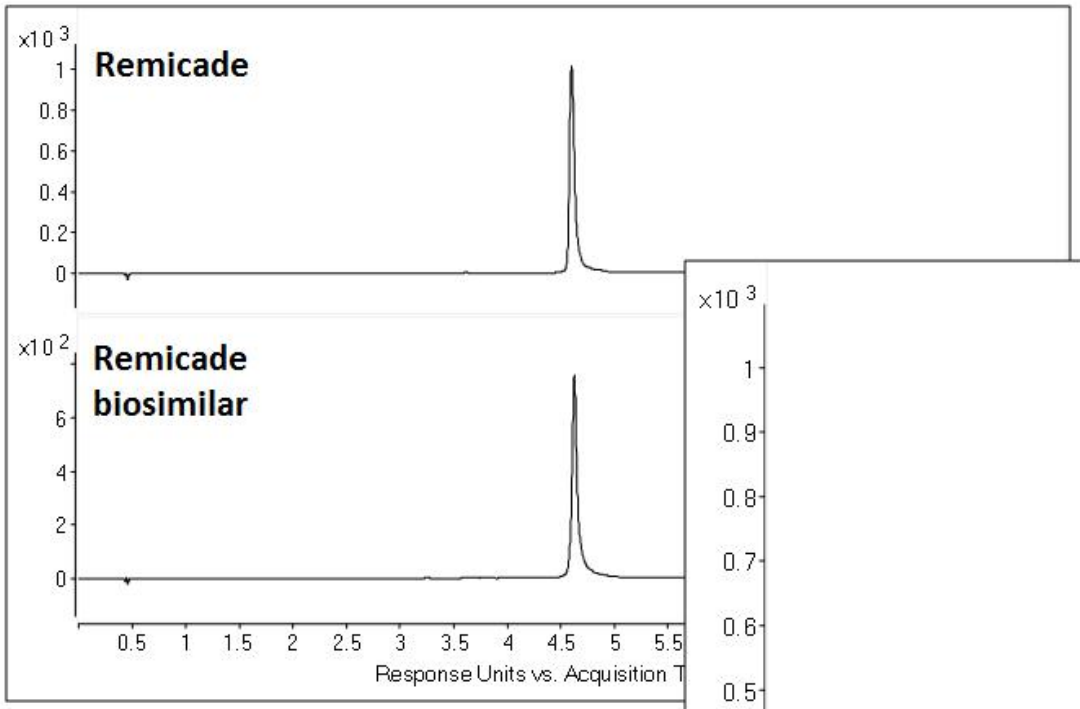


Advance Bio RP-mAb
5 cm x 4.6 mm x 3.5 µm 450Å C4
Temp: 80°C Flow: 1 mL/min UV: 214 nm
Solv A: 0.1% TFA
Solv B: 0.1% TFA in 90% ACN
30-42.5%B, 0-6 min

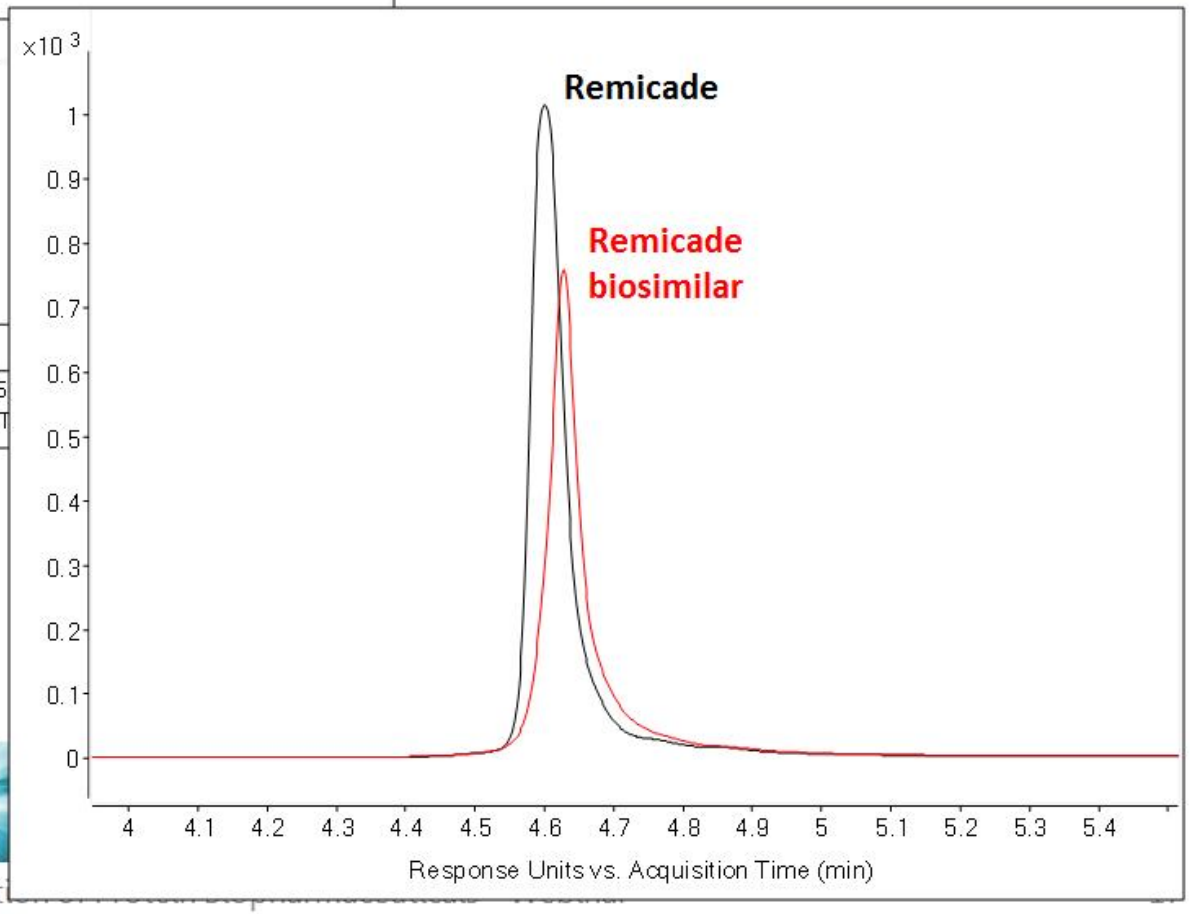




Widepore Poroshell for comparability assessment

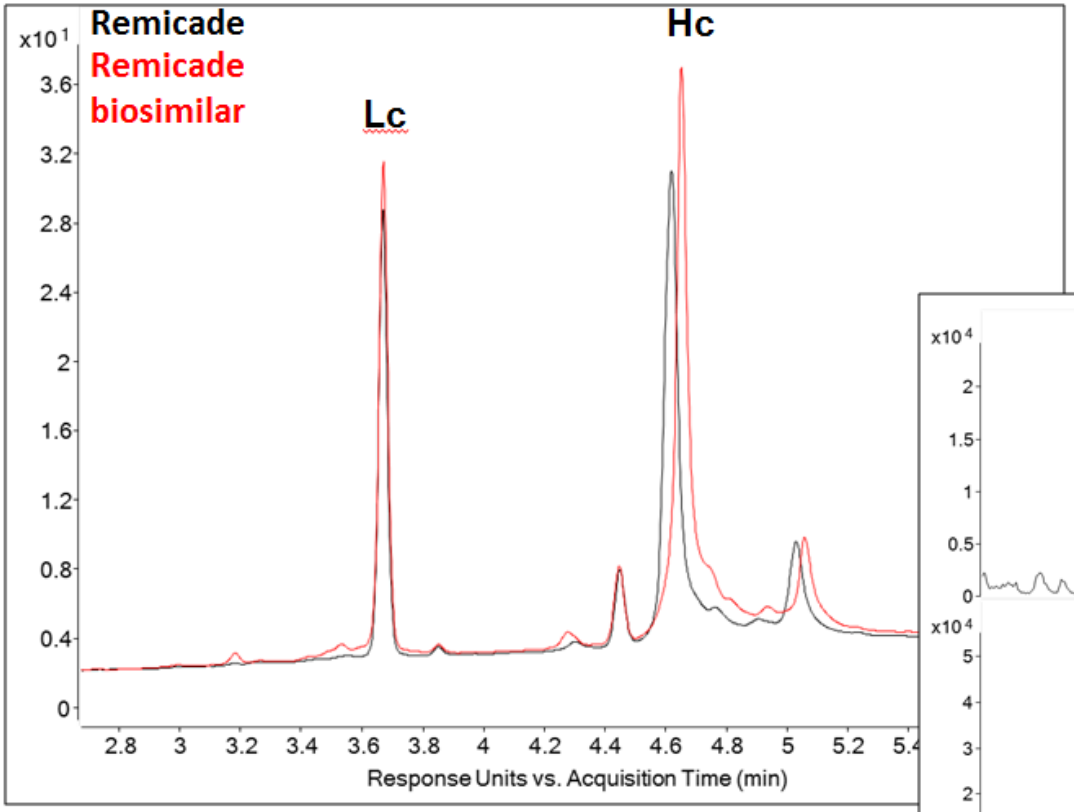


Advance Bio RP-mAb
5 cm x 4.6 mm x 3.5 µm 450Å C4
Temp: 80°C Flow: 1 mL/min UV: 214 nm
Solv A: 0.1% TFA
Solv B: 0.1% TFA in 90% ACN
30-42.5%B, 0-6 min

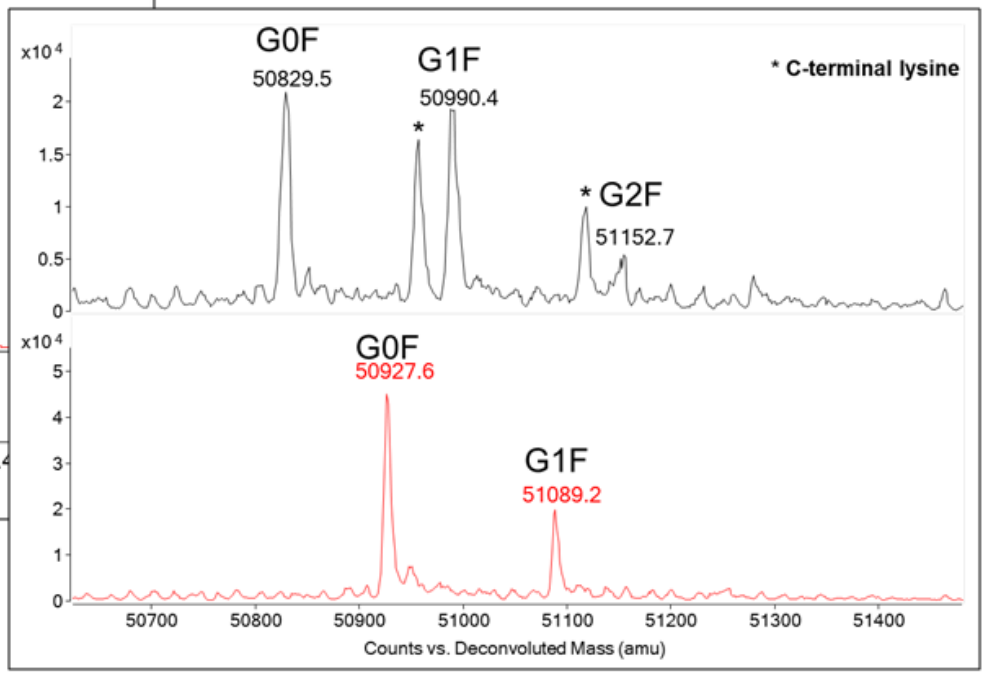


Differences in hydrophobicity due to a 2-point mutation in the AA sequence of the biosimilar

Widepore Poroshell for comparability assessment



Advance Bio RP-mAb
5 cm x 4.6 mm x 3.5 µm 450Å C4
Temp: 80°C Flow: 1 mL/min UV: 214 nm
Solv A: 0.1% TFA
Solv B: 0.1% TFA in 90% ACN
30-42.5%B, 0-6 min



Differences in hydrophobicity due to a 2-point mutation in the AA sequence of the biosimilar compared

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Summary

- The big switch in Pharma
- Biopharma customers have different challenges
- The best kept secrets in Agilent
  - Bioanalyzer – The Lab-on-a-Chip
  - TapeStation – The Next-gen electrophoresis platform
  - OFFGEL Fractionator – A novel sample prep tool
  - CE and CE/MS in biopharmaceutical analysis
  - HPLC-Chip Technology
- Biosimilars
  - Definitions & Regulations
  - How similar is similar enough
  - Case studies: Comparability data between a biosimilar and its innovator reference
- Summary

Thank you for your attention!