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Answers for Science.
Knowledge for Life.™

CESI-MS a technique for the characterization and analysis of both intact and digested biopharmaceuticals

Dr. Stephen Lock

RUO- MKT-10-3523

For Research Use Only. Not for use in diagnostic procedures

Agenda

- The use of CE in Biopharma
- What is CESI-MS?
- Applications using CESI-MS in BioPharma
  - 100% sequence coverage and PTM detection for monoclonal antibody studies.
  - The use of CESI-MS in intact protein analysis in relation to size and charge heterogeneity profiling.
  - How CESI-MS can be used in glycoprotein analysis in the characterisation of EPO & IFN
- Summary

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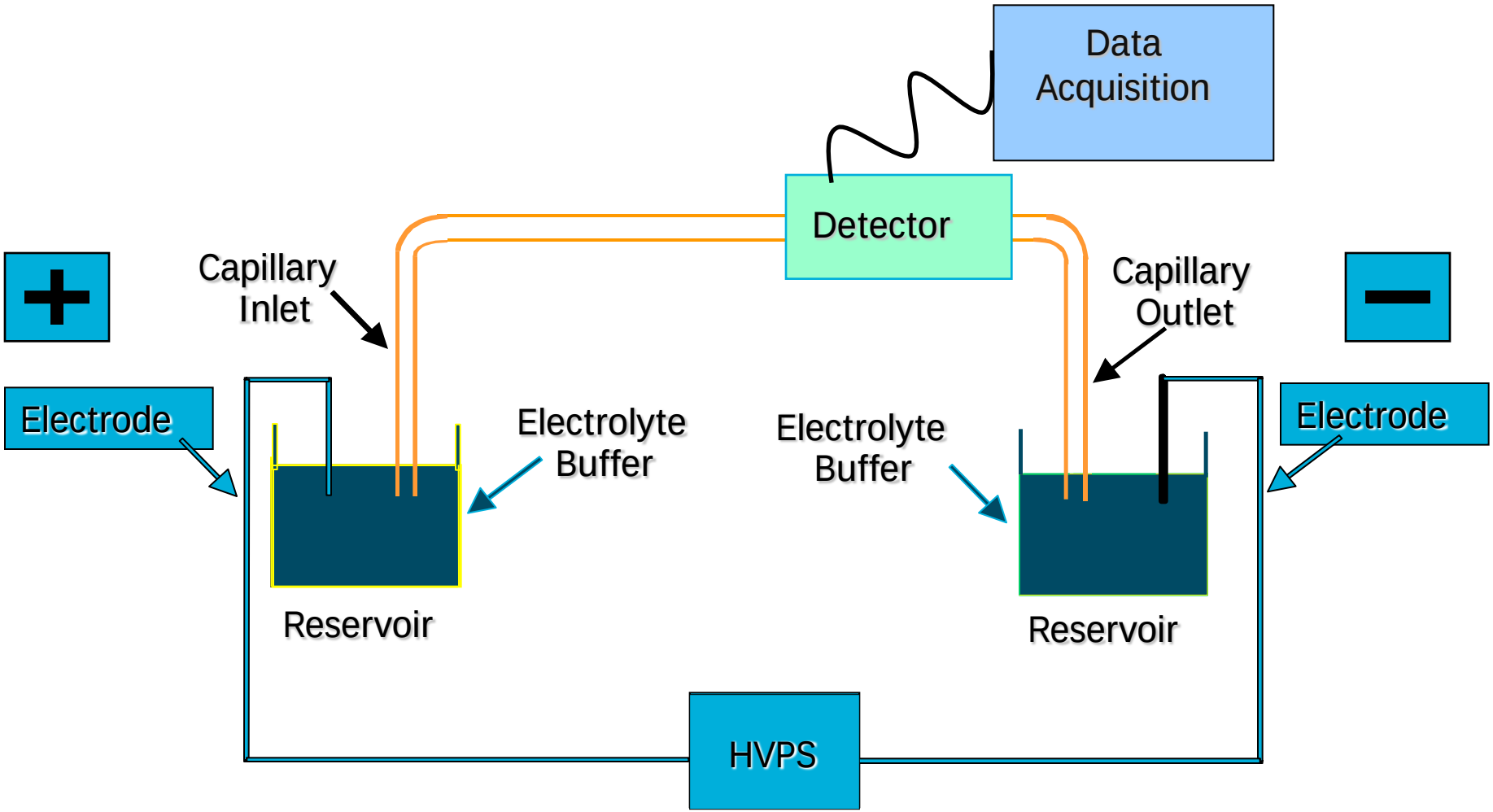
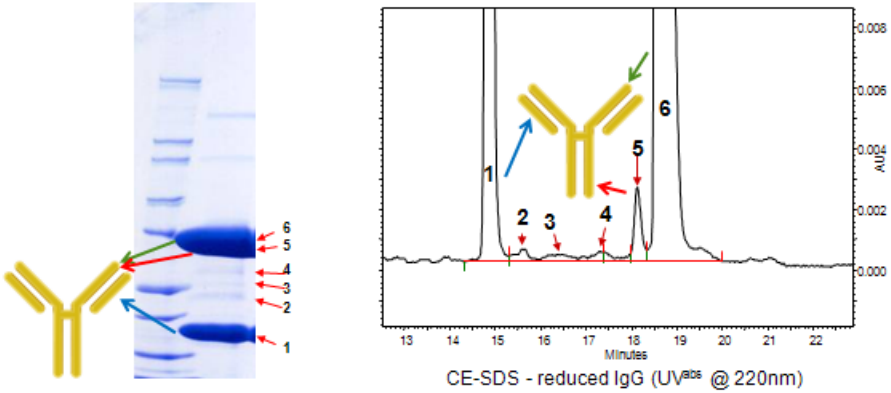
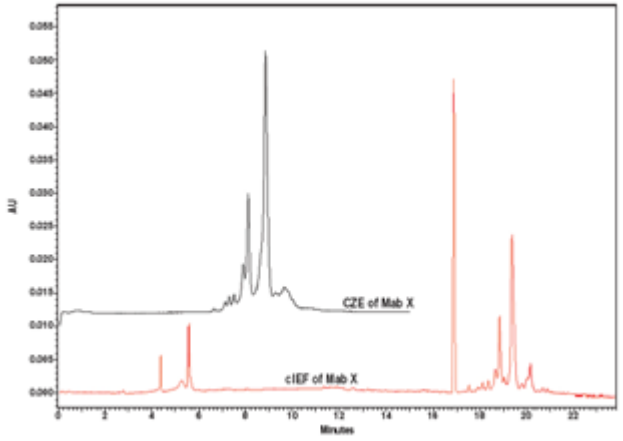
Traditional CE System Schematic



Assessment of IgG Purity and Heterogeneity



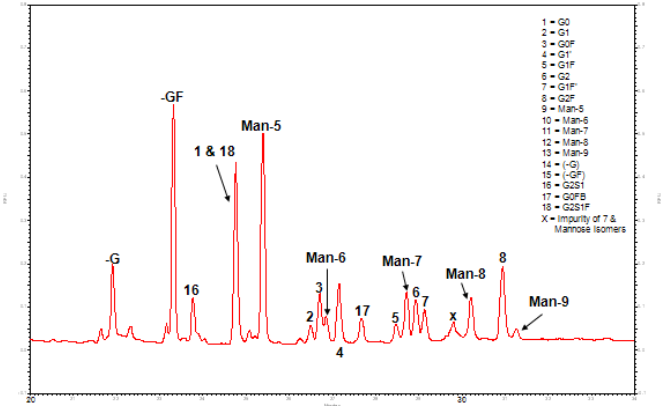
1. CE-SDS = Purity analysis



2. CIEF & CZE = charge heterogeneity analysis

"Capillary electrophoresis uses one or more capillaries as migration channels for electrophoresis and increasingly has become the procedure of choice when an electrophoretic separation method is needed. This is because CE is easier to perform, requires less time, and allows better precision and robustness than PAGE."

USP Guideline for Submitting Requests for Revision to *USP-NF, v3.1*



3. Glycan analysis = microheterogeneity determination

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Multi-site Studies in Industry Illustrate Portability of CE

- 2006: CE-SDS Gel: Chromatographia, 64, 359-368; A series of collaborations between various pharmaceutical companies and regulatory authorities concerning the analysis of biomolecules using CE
- 2011: CIEF: Chromatographia, 73, 1137-1144; Intercompany study to evaluate the robustness of C-IEF technology for the analysis of monoclonal antibodies:
- 2012: Imaged CIEF: J. Separation Science, 335, 3124-3129; Robustness of Imaged Capillary IEF methodology for the analysis of monoclonal antibodies: An Inter-laboratory Study
- 2013: N-Glycan Mapping Study: CE Pharm 2013: Pending Publication
- 2015: Evaluation of capillary zone electrophoresis for charge heterogeneity testing of monoclonal antibodies. Journal of Chromatography B, 983–984 (2015) 101–110

Synopsis:

“CE Methods Can Be Reliable, Robust ,and Transportable Across Sites”

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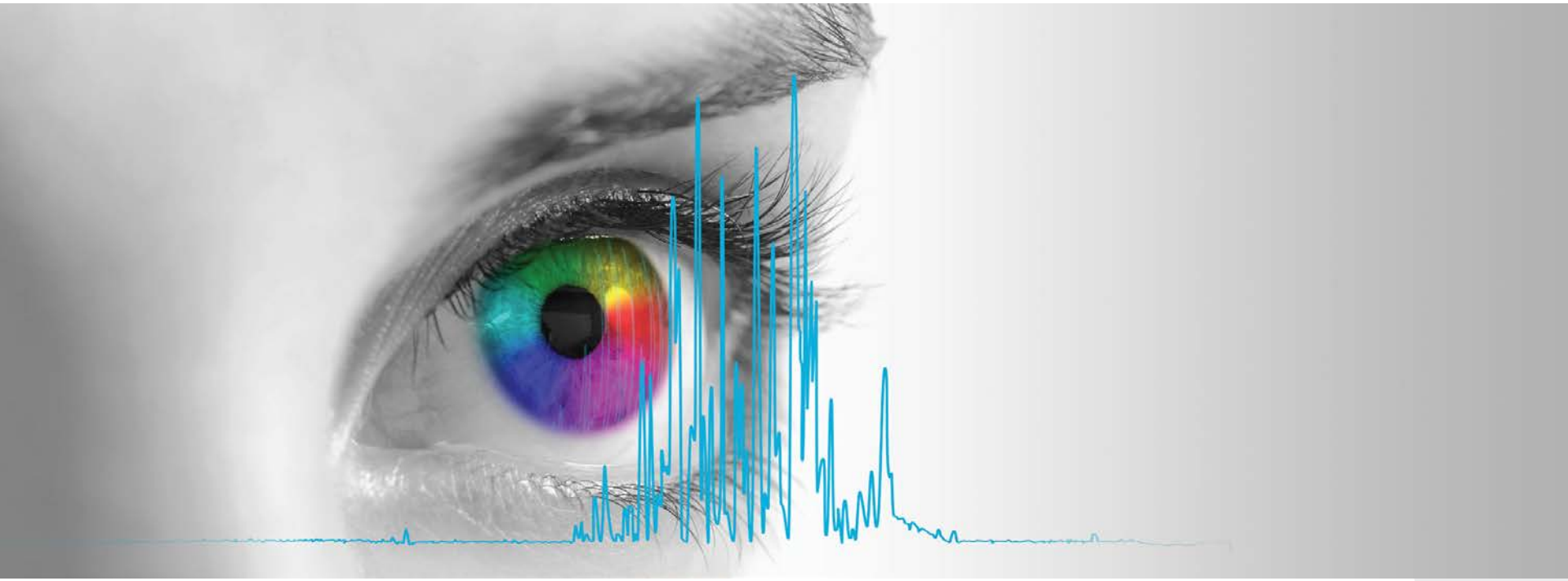


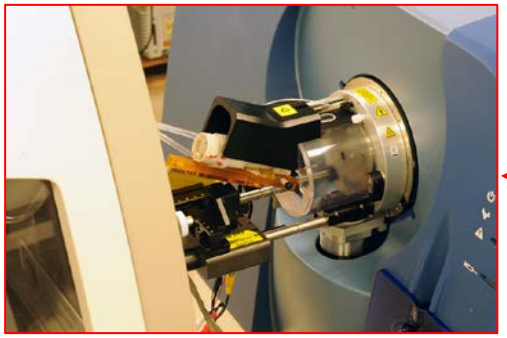
What is CESI – MS?

Combining CE & ESI-Mass Spectrometry

CESI 8000 High Performance Separation-ESI Module

High resolution, ultra-low flow rate CE separations coupled with high resolution, high sensitivity MS

Ultra-low flow rates of < 30nL/min



TripleTOF® 5600+ and 6600 Systems

CESI - "The Integration of Capillary Electrophoresis (CE) with Electrospray Ionization (ESI) Into a Single Dynamic Process Within the Same Device"

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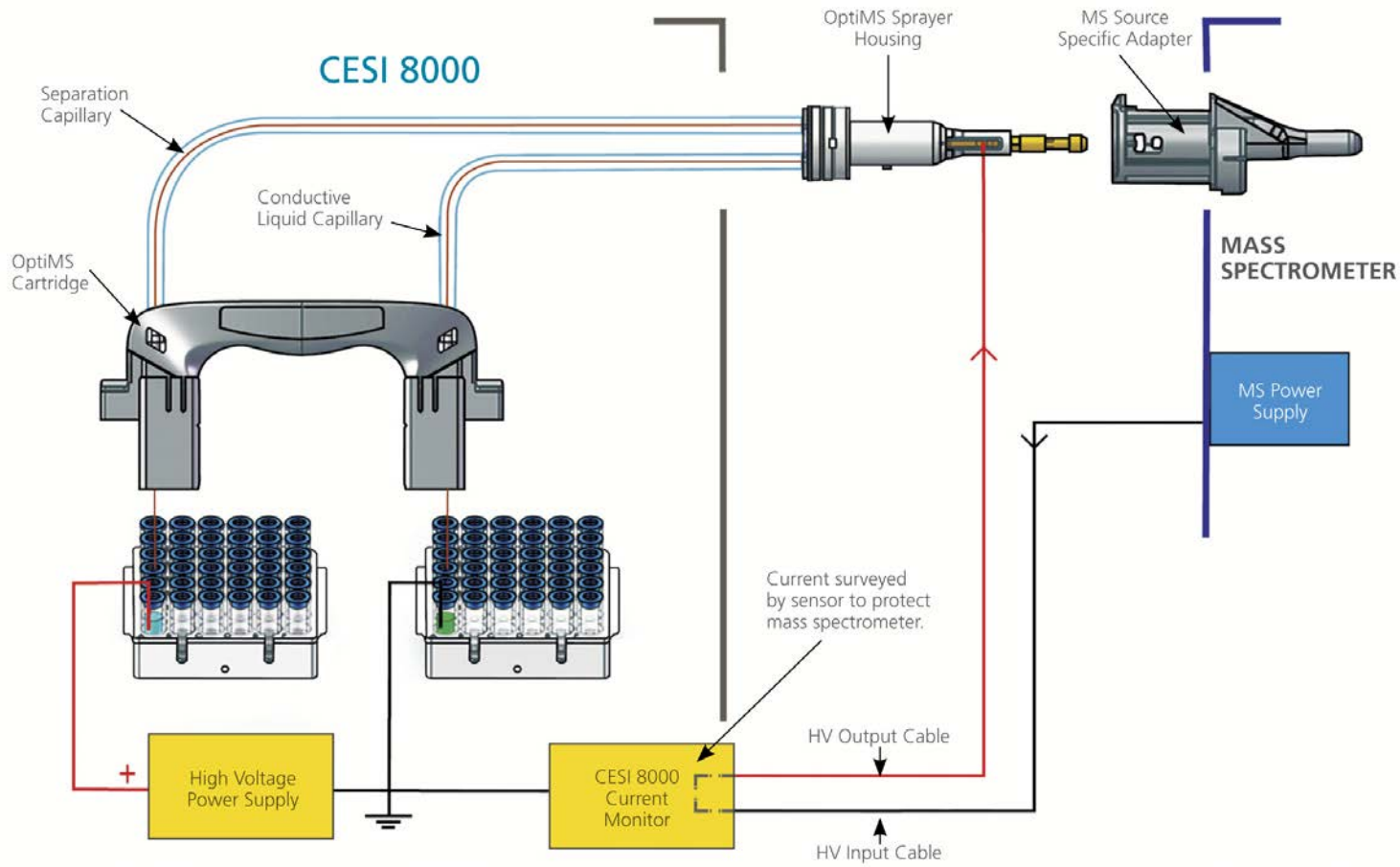
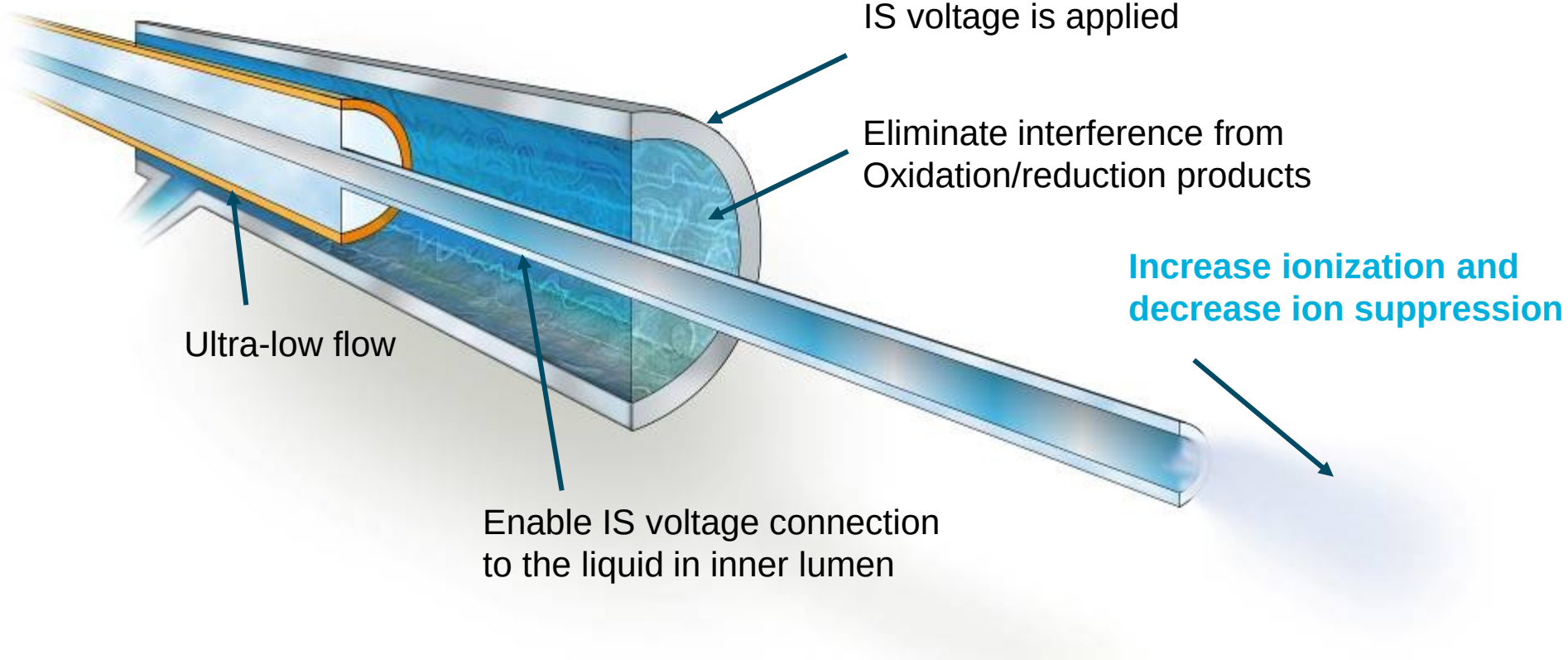
Implementation of CESI-MS Through a Commercial Interface



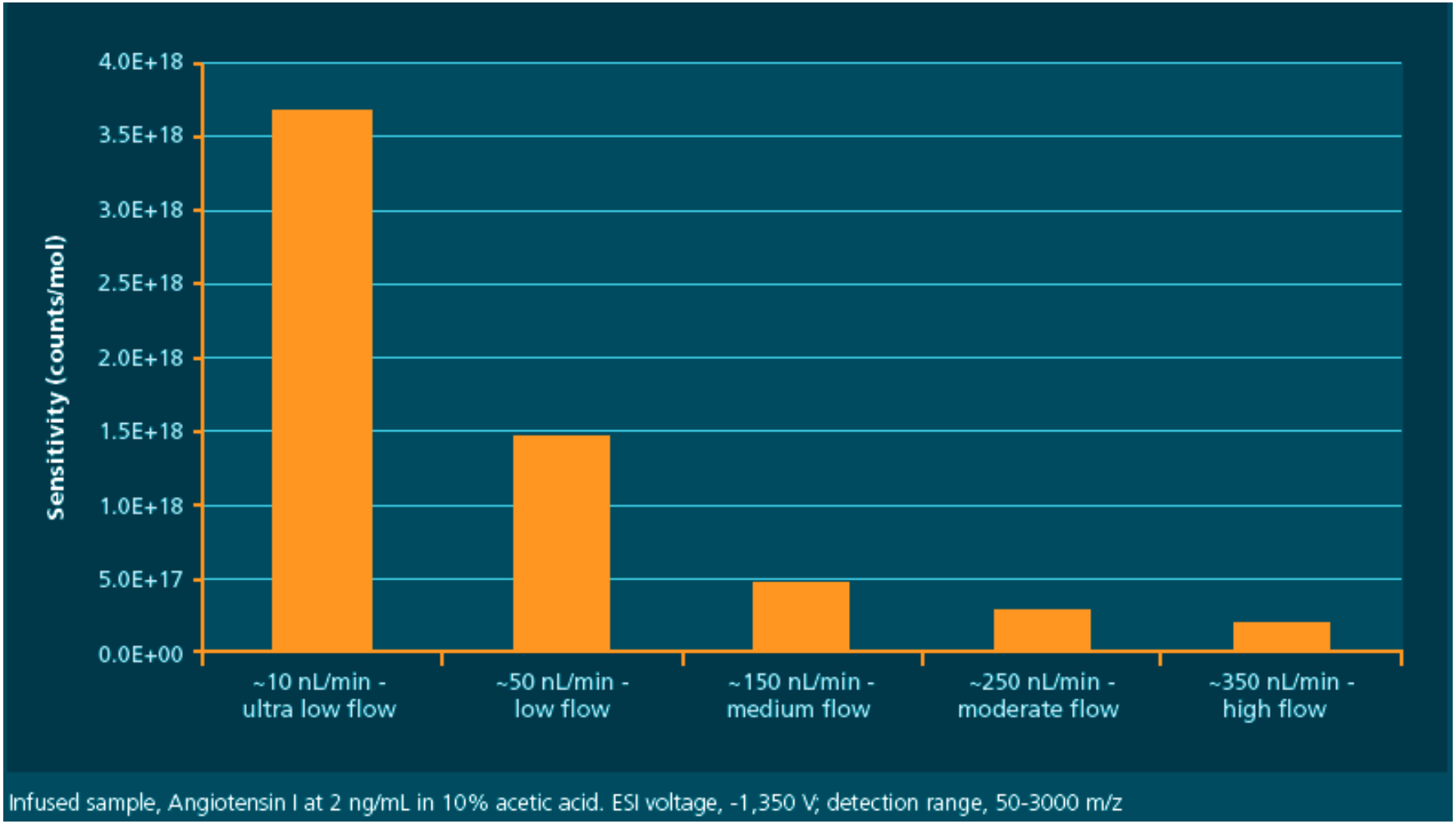
CESI - "The Integration of Capillary Electrophoresis (CE) with Electrospray Ionization (ESI) Into a Single Dynamic Process Within the Same Device"

CE with an Integrated Electrospray Ionization

" The Integration of Capillary Electrophoresis (CE) With Electrospray Ionization (ESI) Into a Single Dynamic Process Within The Same Device"



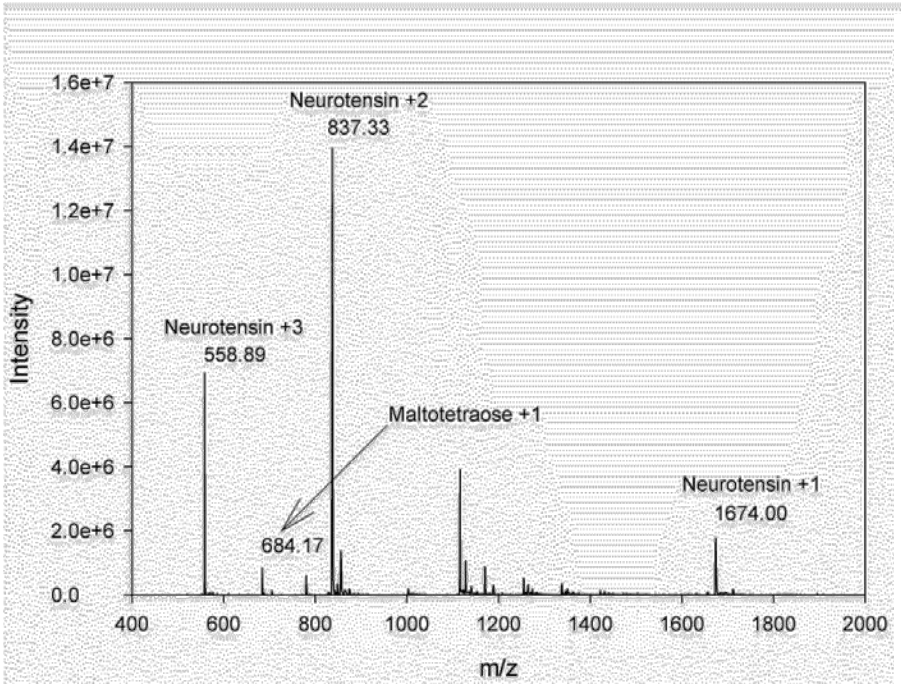
Influence of Flow Rate on Sensitivity

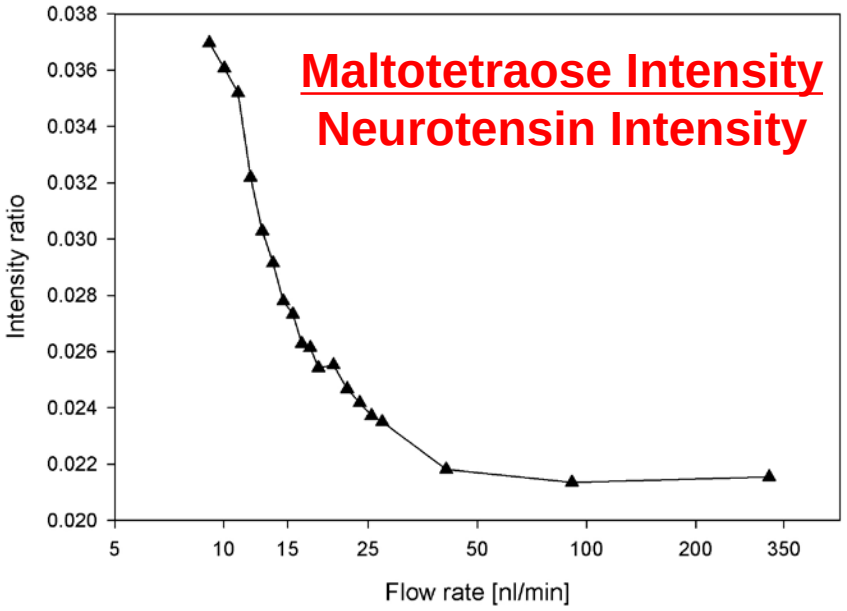


Evolution of the peak intensity of Angiotensin I (a) and detection sensitivity as a function of the flow rate (b) Experimental conditions: capillary electrophoresis; bare fused silica capillary with a porous tip, total length 88.5 cm × 30 *μ*mi.d. × 150 *μ*mo.d.; Infused sample, Angiotensin I at 2 ng/mL in 10% acetic acid. Mass spectrometry; capillary voltage,-1350 V; detection range, 50-3000*m/z*,

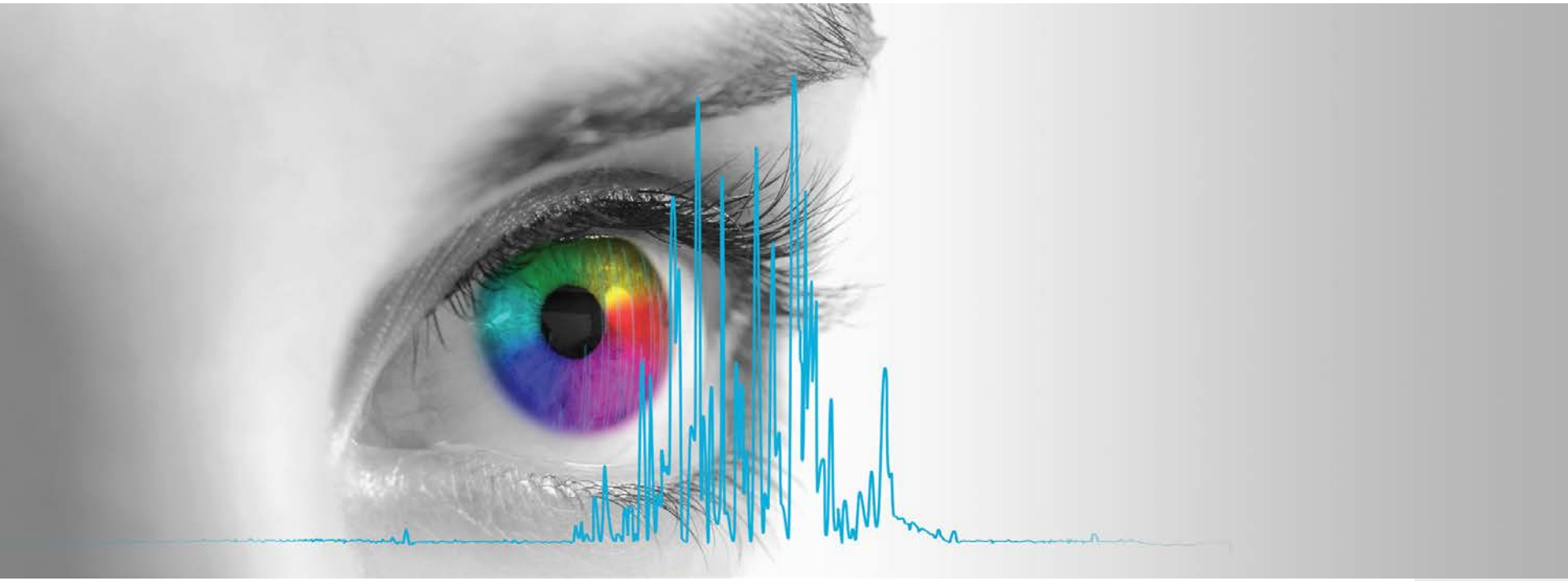
Reducing Ion Suppression Bias at Low Flow Rates

Monitoring maltotetraose suppression in the presence of neutrotensin





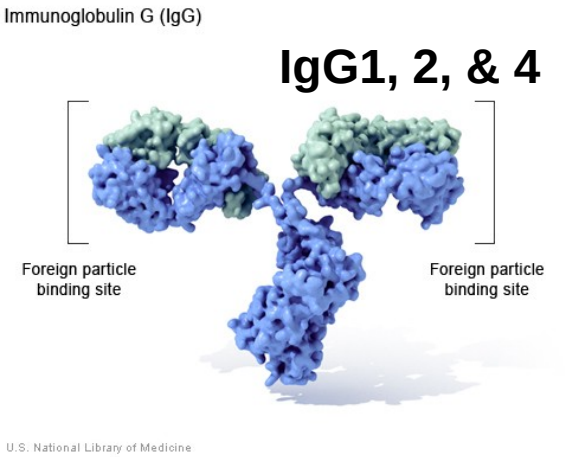
Analyte suppression decreases logarithmically below 50 nL/min



Multilevel characterization of mAbs by CESI-MS

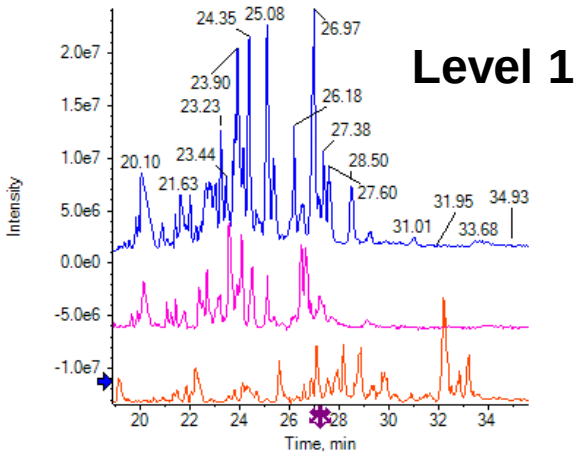
Multilevel characterization of mAbs by CESI-MS – Bottom up analysis

Evaluation of CESI-MS for the comprehensive characterization of mAb forms



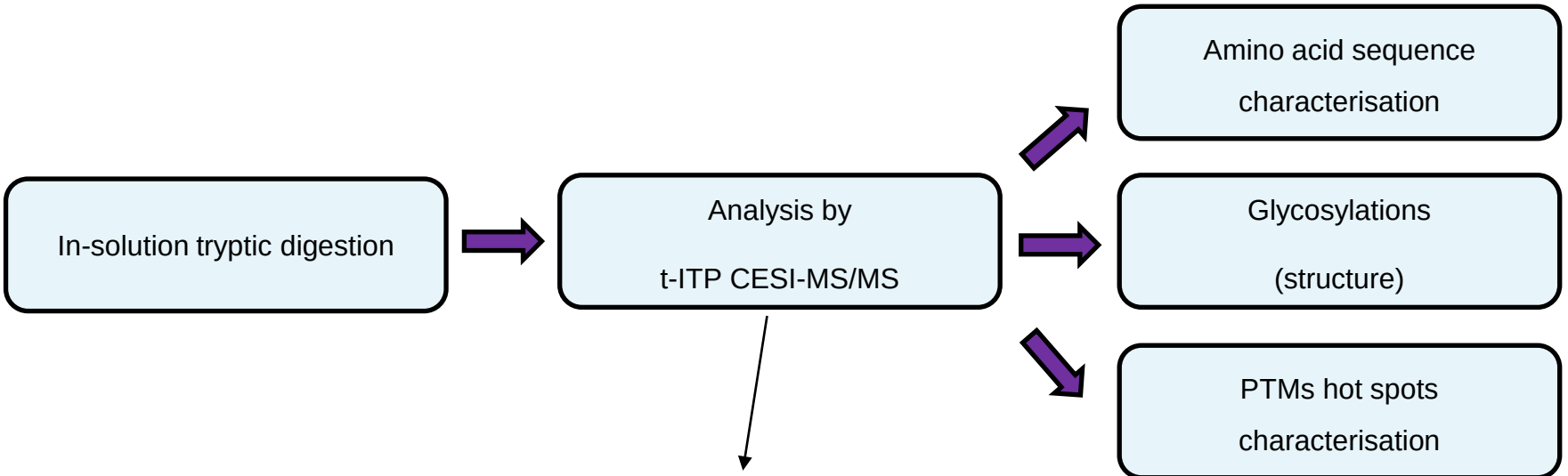
Peptide mapping

Reduce, alkylate & digest



mAb Characterisation Workflow

- Primary structure characterisation workflow based on bottom-up proteomics strategy



CESI8000 coupled to 5600 TripleTOF MS

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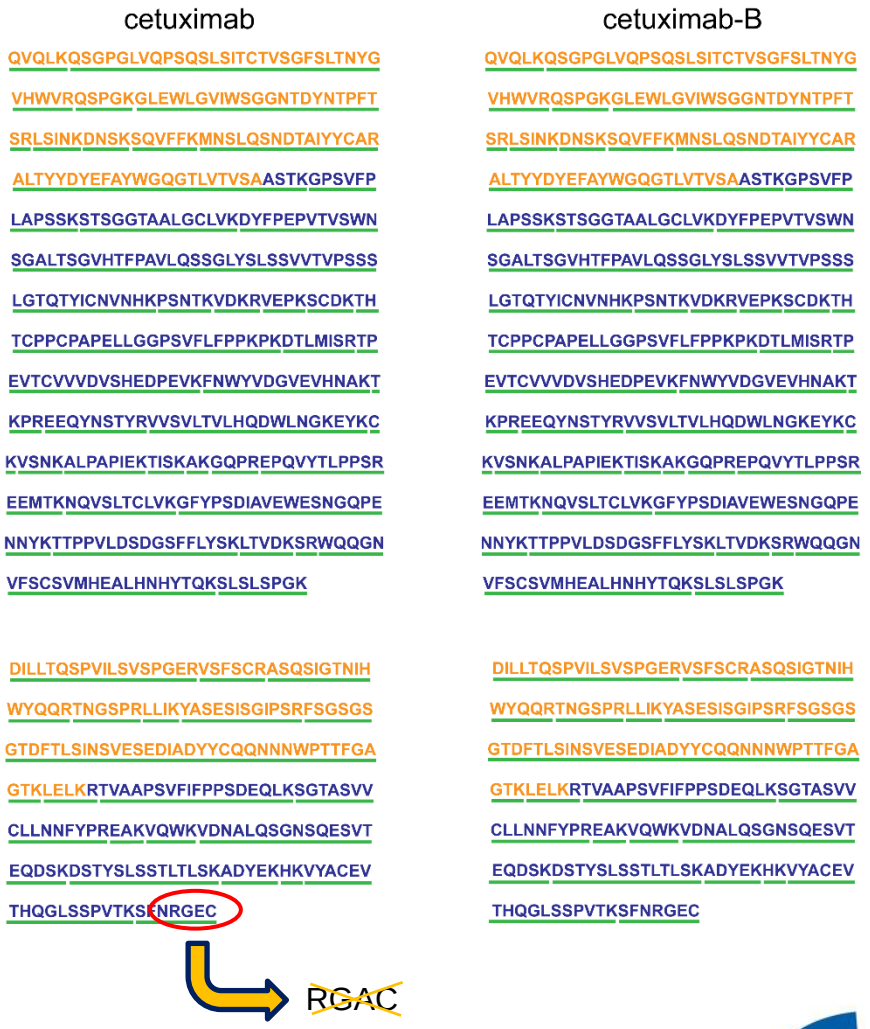
Slide courtesy of Dr Yannis Francois, University of Strasbourg

Biosimilarity Case Study: Cetuximab

- A single analysis of each sample sufficient to conclude on the complete similarity regarding AA sequence
- Complete sequence coverage is obtained through peptides without miscleavages or PTMs
- CESI-MS/MS enabled to confirm an error, recently reported in the litterature



*D. Ayoub et al., mAbs **2013**, 5, 699-710*

*Gahoual R. et al., mAbs, **2014** (6), 1464-1473*

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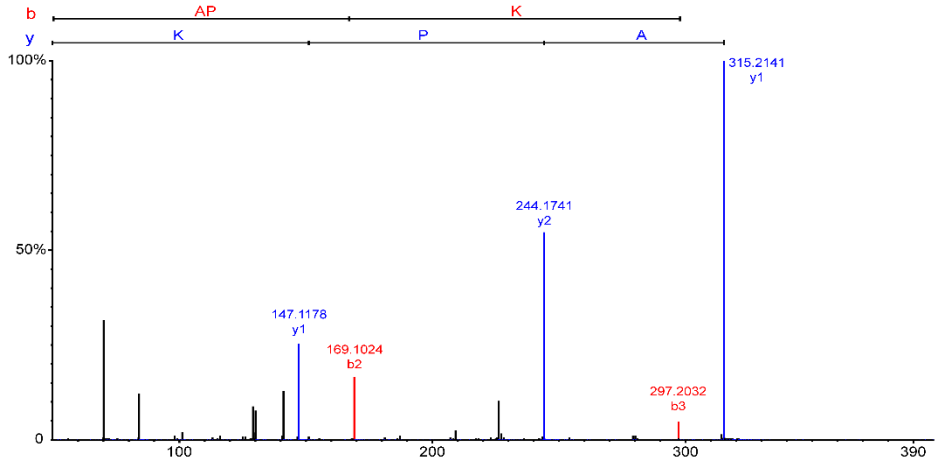
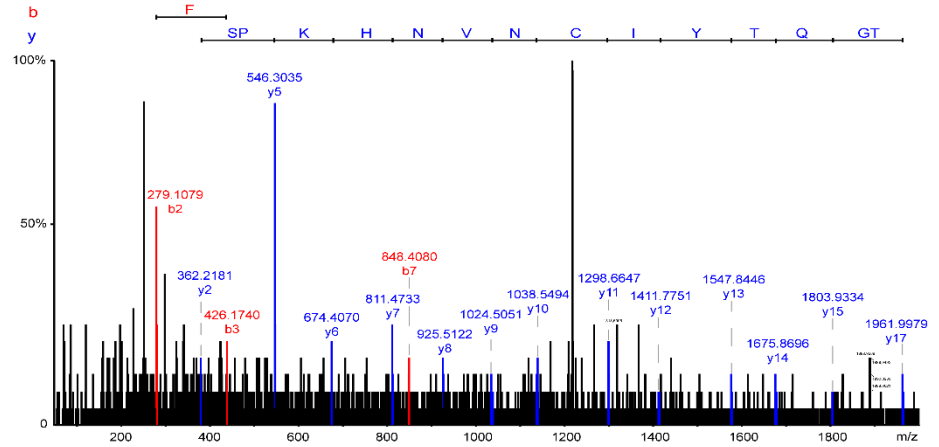
Amino Acid Sequence Characterisation



*MS/MS spectrum of digested peptides **LT04***



*MS/MS spectrum of digested peptides **HT15***

APK

(m/z 315.2039 ; 2+)

CESI-MS detects both very short and long peptides in the same separation

DYFPEPVTVSWNSGALTSGVHTFPAVLQS SGLYSLSSVVTVPSSSLGTQTYICNVNHKP SNTK

(63 amino acids ; m/z 1119.898 ; 6+)

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Slide courtesy of Dr Yannis Francois, University of Strasbourg

Broad Range of Analysis for MAb Glycan ID

Glycopeptides identified as R.EEQYN(Glycan)STYR.V	mAb glycan abbreviation	Glycan mass (Da)	Monoisotopic mass [M+H]* (Da)	Mass accuracy (ppm)	Average migration time (min)[1]	Average relative abundance (%)[1,2]
	FA2G1 (G1F)	1606.5867	2796.0987	-0.41	34.92 ± 0.43	54.49 ± 1.45
	FA2 (G0F)	1444.5339	2634.0459	0.25	34.72 ± 0.42	17.28 ± 0.97
	FA2G2 (G2F)	1768.6395	2958.1515	-0.01	35.19 ± 0.43	8.59 ± 0.40
	A2 (G0)	1298.476	2487.9880	0.67	35.00 ± 041	5.58 ± 0.31
	A2G1 (G1)	1460.5288	2650.0408	0.93	34.88 ± 0.43	4.07 ± 0.09
	FA1G1	1403.5073	2591.0037*	3.04	34.45 ± 0.41	1.52 ± 0.06
	FA2G2S1	2059.7349	3249.2469	0.78	34.45 ± 0.42	1.41 ± 0.11
	FA1	1241.4545	2430.9665	0.40	34.76 ± 0.44	1.25 ± 0.06
	M5 (Man5)	1216.4228	2405.9349	0.09	38.18 ± 0.53	1.13 ± 0.66
	FA2G1S1	1897.6821	3085.1785*	3.10	37.93 ± 0.52	1.02 ± 0.48
	A1G1	1257.4494	2446.9614	0.44	38.03 ± 0.48	0.78 ± 0.40
	FA1G1S1	1694.6027	2884.1147	-3.15	34.39 ± 0.42	0.57 ± 0.03
	A2G2 (G2)	1622.5816	2812.0936	-2.77	34.71 ± 0.39	0.55 ± 0.03
	A1	1095.3966	2284.9086	0.57	41.57 ± 0.64	0.51 ± 0.32
	FA2G2S2	2350.8303	3540.3423	1.77	35.11 ± 0.44	0.44 ± 0.05
	A1G1S1	1548.5448	2738.0568	-2.81	37.90 ± 0.51	0.32 ± 0.15
	FA2BG1	1809.6661	2999.1781	-3.00	35.09 ± 0.44	0.17 ± 0.01
	M4A1G1S1	1710.5976	2900.1096	-1.62	34.83 ± 0.43	0.14 ± 0.01
	M5A1	1419.5022	2609.0142	-1.92	37.95 ± 0.52	0.14 ± 0.07
	A2G2S1	1913.677	3103.1890	-1.74	38.05 ± 0.57	0.06 ± 0.03

[1]Error was calculated as standard deviations from triplicate technical CESI-MS replicate runs.
[2]Relative abundances were calculated using peak areas of glycosylated peptides of the same sequence and charge state (+3).
[M+H] mass has a -18.0106 Da loss due to water neutral loss.

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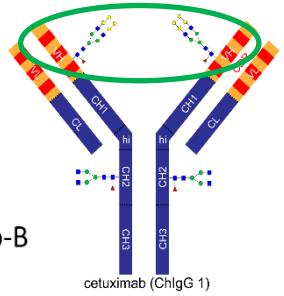
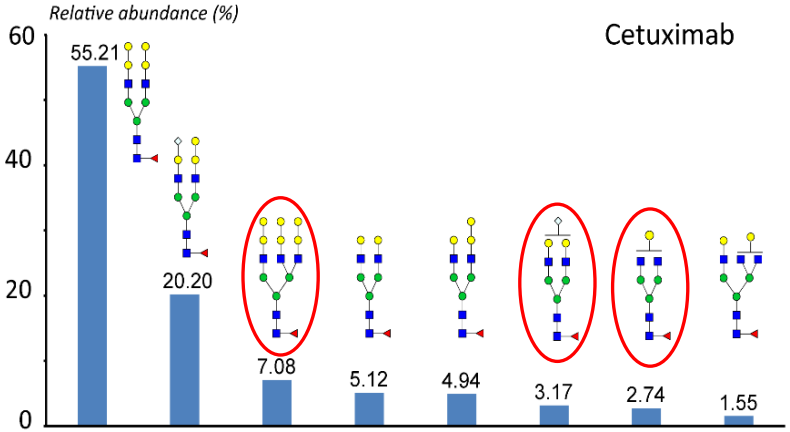
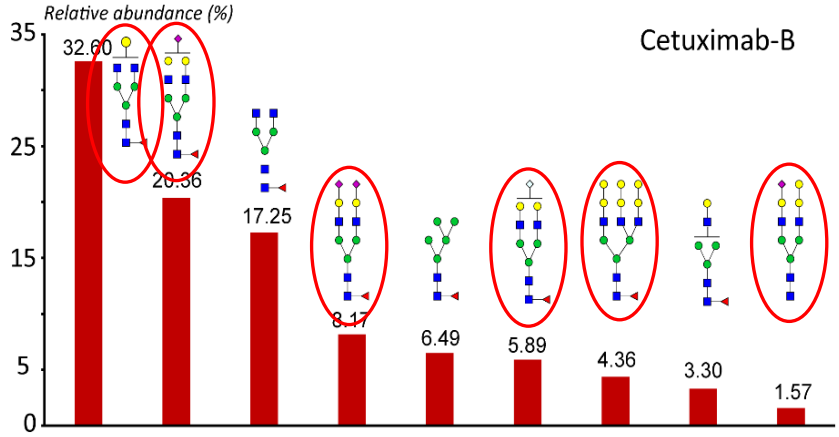
Glycoform Characterisation: Fd Region

- Fd glycosylation site characterisation







Glycoforms exhibited by the candidate biosimilar are significantly different from cetuximab

➢ *30 % of glycans contains N-acetylneuraminic acid* → Rejected as biosimilar

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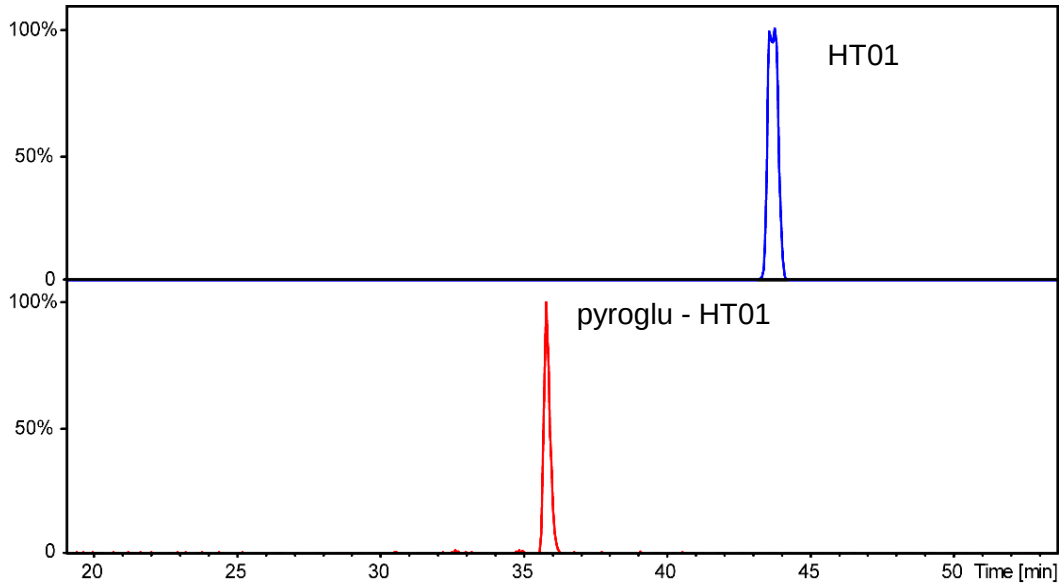
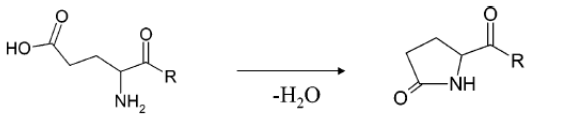
Gahoual R. et al., mAbs, ***2014*** *(6), 1464-1473*

PTM Hot Spot Characterization

N-terminal glutamic acid cyclization characterization



Extracted ion electropherograms of peptides HT01 and modified HT01



Glutamic Acid → Pyroglutamic Acid

- CE mechanism separates of peptide with N-terminal glutamic acid cyclization from the unmodified peptide

Results suggest partial modification of sample → Favorable conditions to estimate sample modification level

Gahoual R. et al., Anal. Chem., ***2014*** (86), 9074-9081

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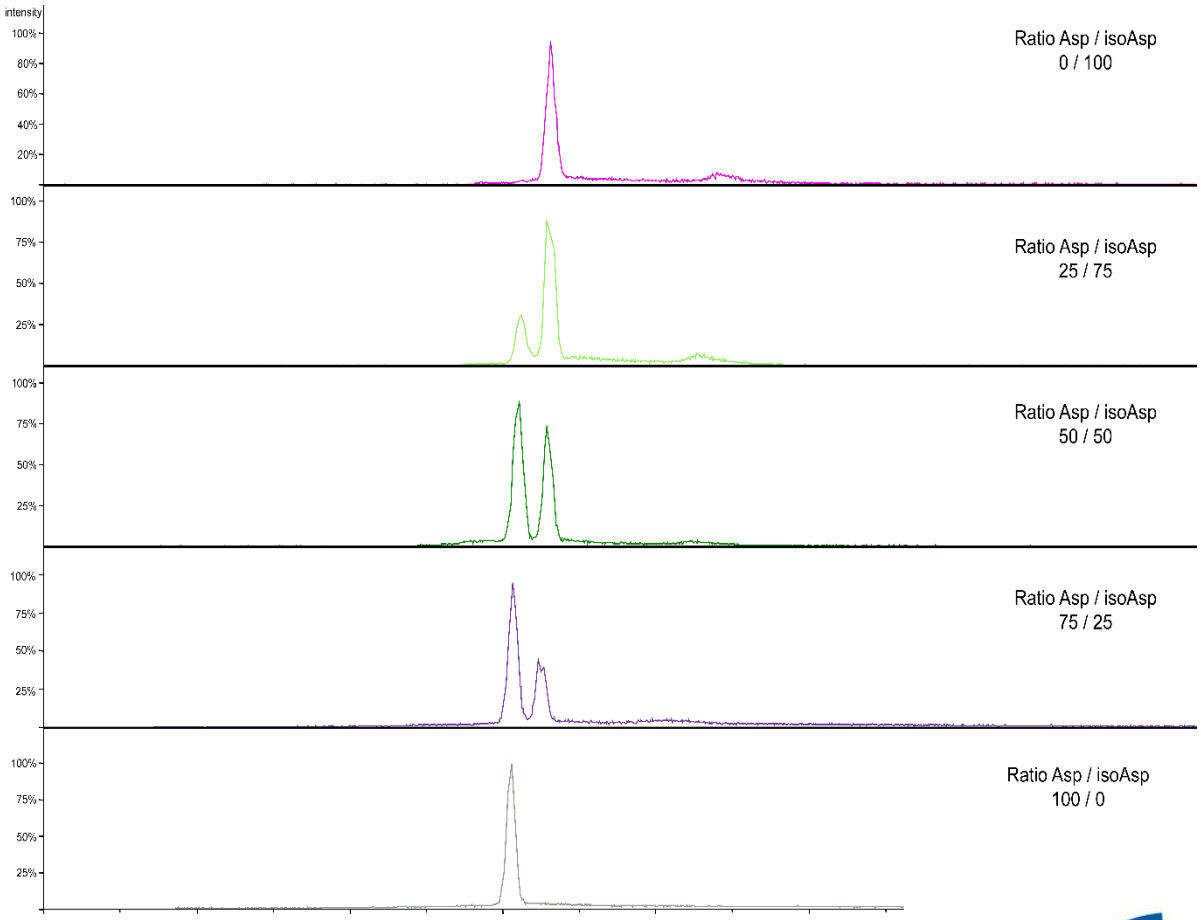
Separation of Aspartic Acid Isomers: Confirmation in Synthetic Peptides

- Aspartic acid isomers separation by CZE confirmed using a synthetic peptide

Synthetic peptides sequences :

NH_2-GLEWIGYISY **D** GTNNYKPSLK-OH

NH_2-GLEWIGYISY **isoD** GTNNYKPSLK-OH



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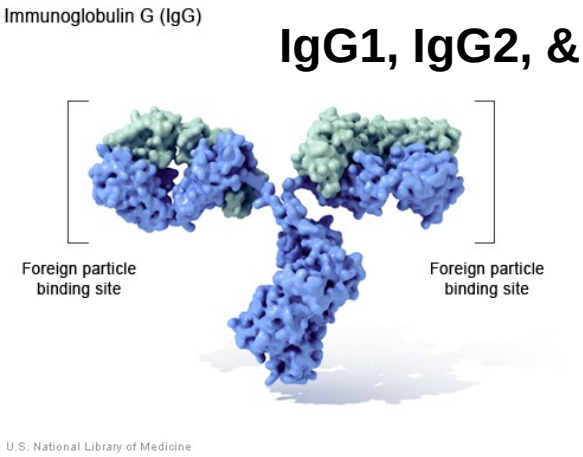
Slide courtesy of Dr Yannis Francois, University of Strasbourg

Multilevel characterization of mAbs by CESI-MS – middle down analysis

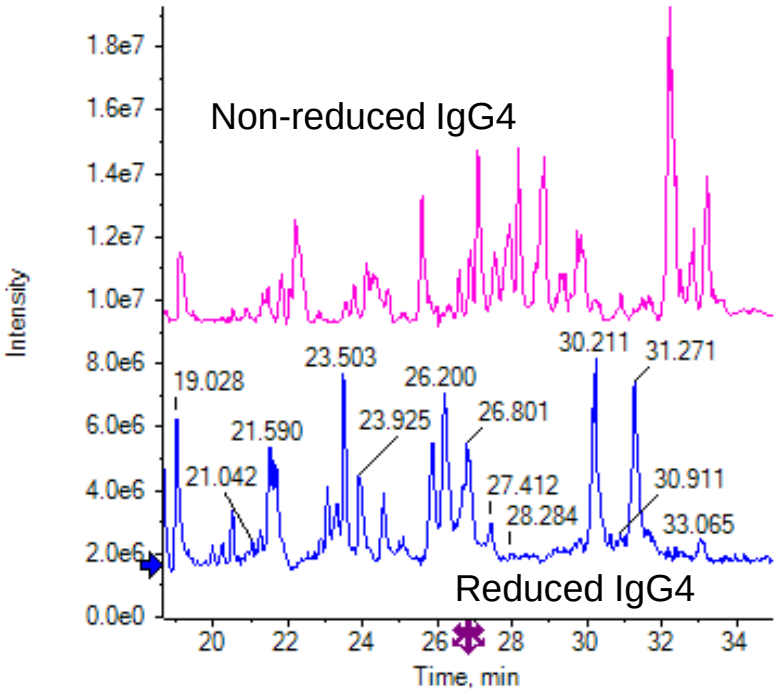


IgG1, IgG2, & IgG4

Disulfide mapping
Alkylate & digest
~25 ng of digest analyzed

*Disulfide scrambling between mAb heavy and light chains can result in functional differences.

Level 2



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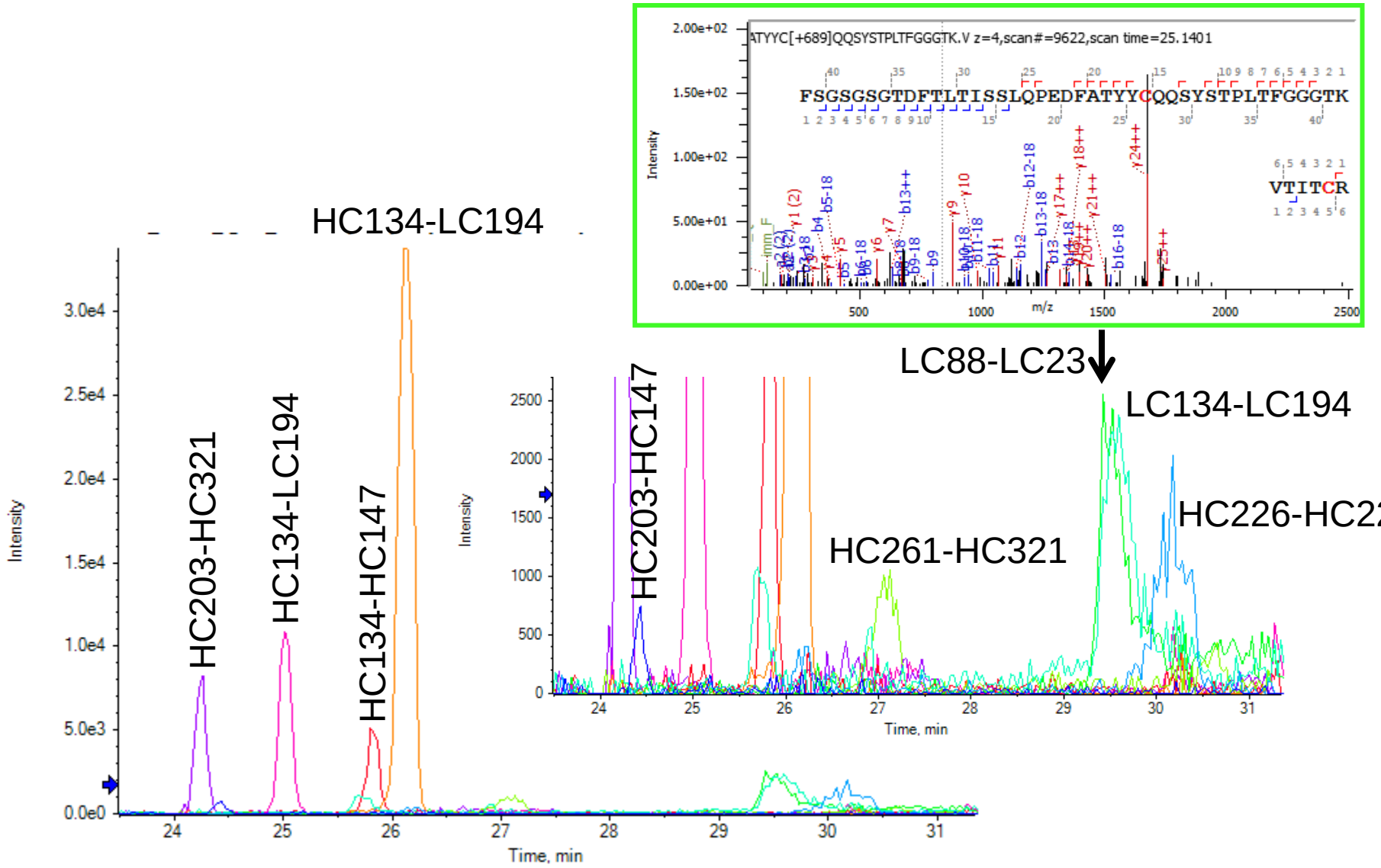
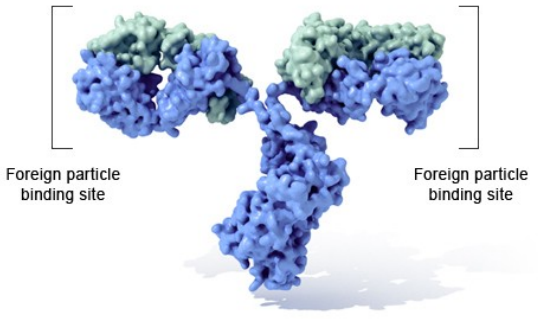
Disulfide linkages identified on IgG4



Multilevel characterization of mAbs by CESI-MS – top down analysis

Immunoglobulin G (IgG)

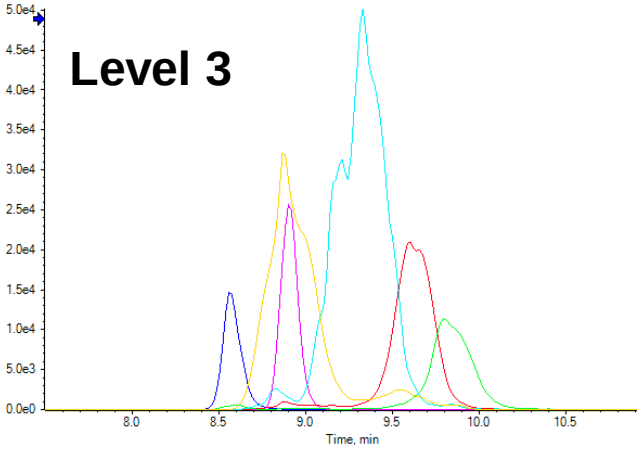
IgG1, 2, & 4



U.S. National Library of Medicine

Intact IgG
Charge Heterogeneity
& Reduced Analysis

Level 3



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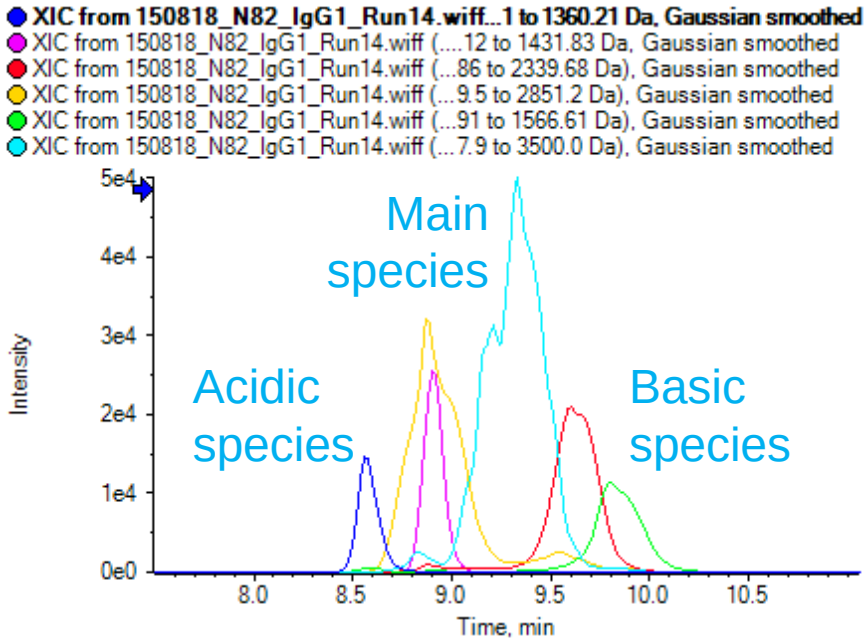
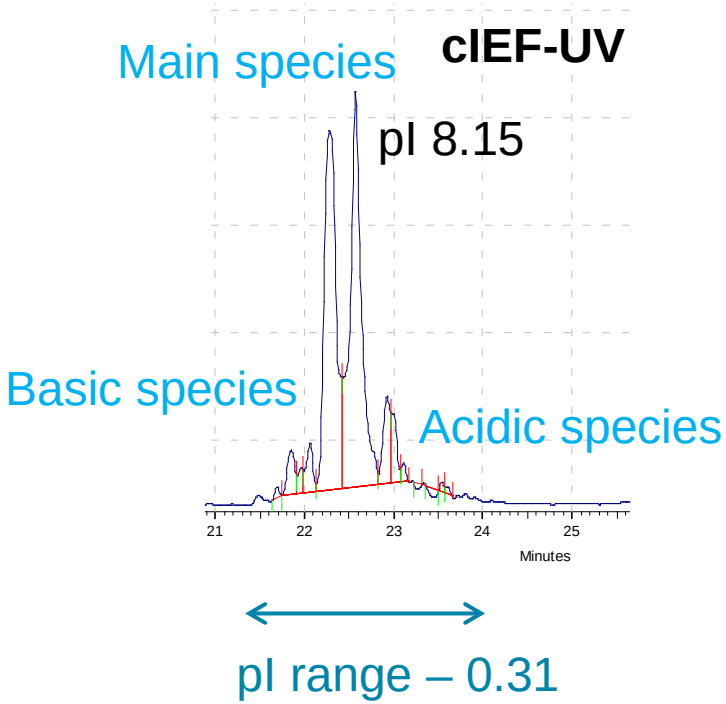
Charge heterogeneity analysis of IgG1 by CESI-MS

CESI-TripleTOF® 6600 MS





~ 10 mg/mL sample desalted into 50 mM ammonium acetate, pH 4

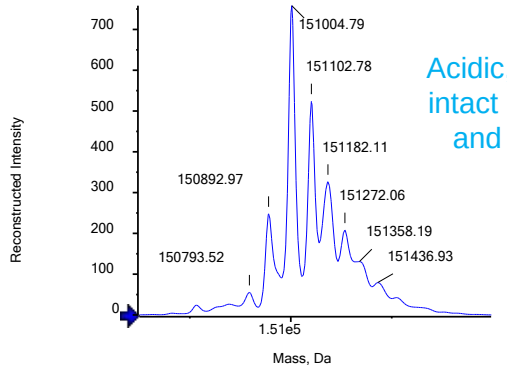
BGE – 3% acetic acid (tITP-CZE mode)

~3.5 nL injection → ~ 35 ng injected

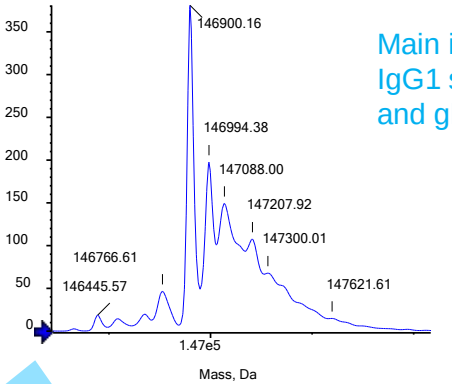
***Reverse migration order**

Charge heterogeneity analysis of IgG1 by CESI-MS

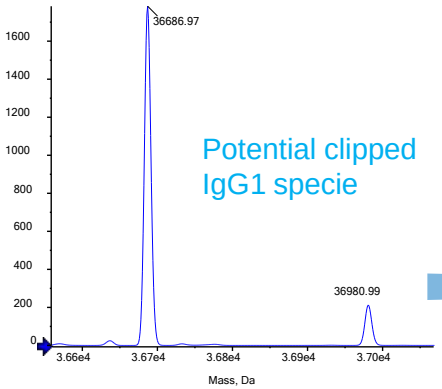
Single analysis at the intact level unifies multiple inferred analyses



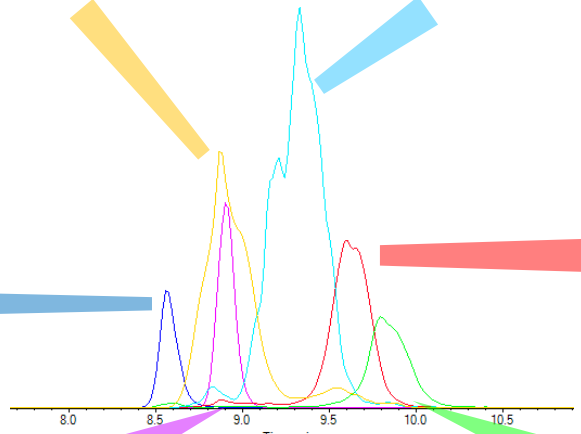
Acidic, higher MW intact IgG1 specie and glycoform

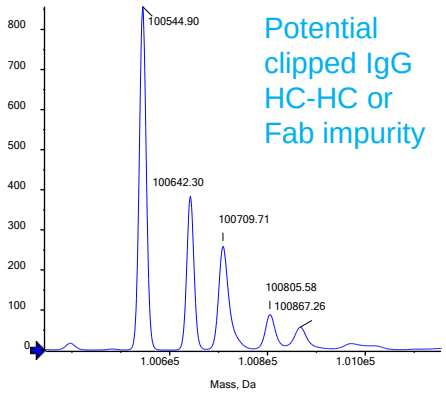


Main intact IgG1 specie and glycoform

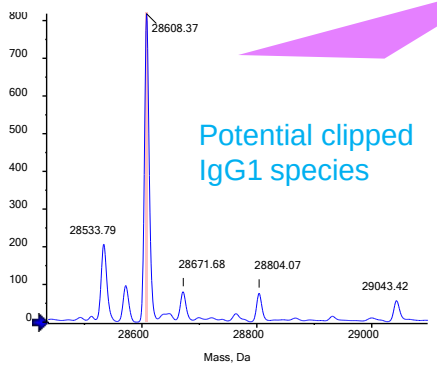


Potential clipped IgG1 specie

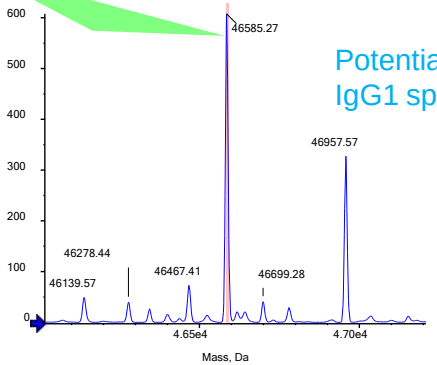




Potential clipped IgG HC-HC or Fab impurity



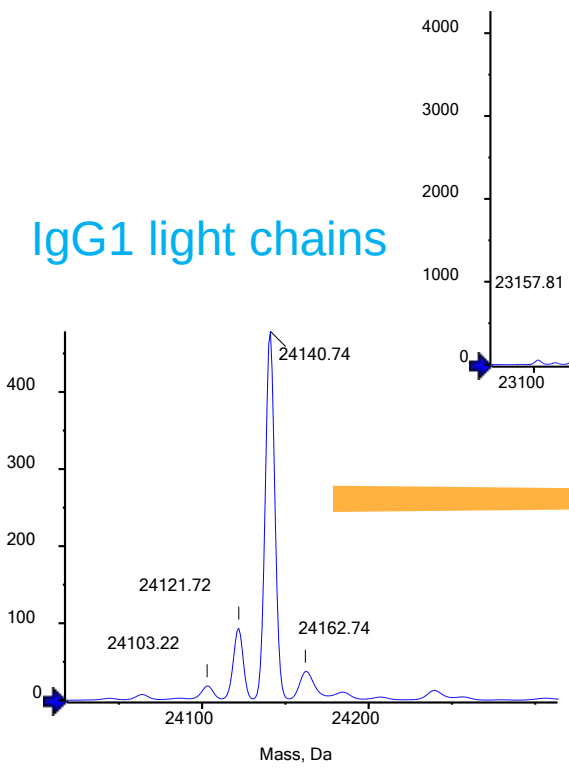
Potential clipped IgG1 species

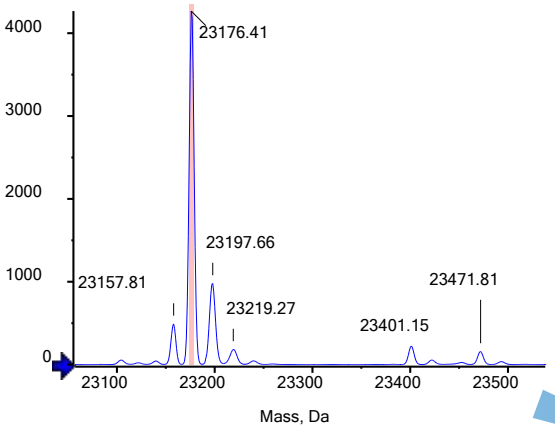


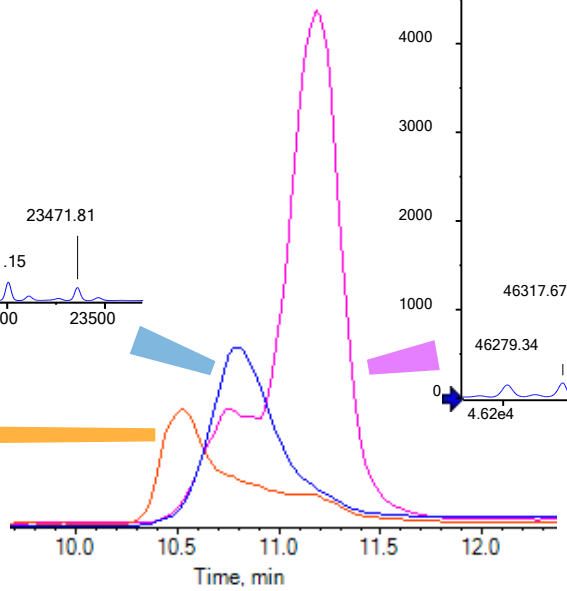
Potential clipped IgG1 species

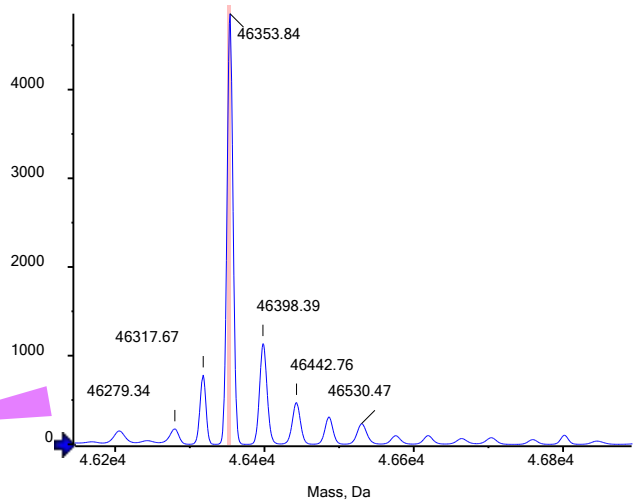
Reduced IgG1 CESI-MS analysis

IgG1 light chains









IgG1 heavy chain

EPO Glycoform Heatmap by CESI-MS of Intact Protein

	SiA_7	SiA_8	SiA_9	SiA_{10}	SiA_{11}	SiA_{12}	SiA_{13}	SiA_{14}	SiA_{15}
$Hex_{14}HexNAc_{11}Fuc_3$	25222								
$Hex_{15}HexNAc_{12}Fuc_3$	25587								
$Hex_{16}HexNAc_{13}Fuc_3$	25952	26243							
$Hex_{17}HexNAc_{14}Fuc_3$	26317	26608							
$Hex_{18}HexNAc_{15}Fuc_3$	26682	26973	27264	27555					
$Hex_{19}HexNAc_{16}Fuc_3$		27338	27629	27920	28211				
$Hex_{20}HexNAc_{17}Fuc_3$		27703	27994	28285	28576	28867			
$Hex_{21}HexNAc_{18}Fuc_3$			28359	28650	28941	29232	29523		
$Hex_{22}HexNAc_{19}Fuc_3$			28724	29015	29306	29597	29888	30179	
$Hex_{23}HexNAc_{20}Fuc_3$			29089	29380	29671	29962	30253	30544	
$Hex_{24}HexNAc_{21}Fuc_3$			29454	29745	30036	30327	30618	30909	31200
$Hex_{25}HexNAc_{22}Fuc_3$				30110	30401	30692	30983	31274	31565
$Hex_{26}HexNAc_{23}Fuc_3$				30475	30766	31057	31348	31639	31930
$Hex_{27}HexNAc_{24}Fuc_3$				30840	31131	31422	31713	32004	32295
$Hex_{28}HexNAc_{25}Fuc_3$					31496	31787	32078	32369	32660
$Hex_{29}HexNAc_{26}Fuc_3$						32152	32443	32734	
$Hex_{30}HexNAc_{27}Fuc_3$							32808	33099	
$Hex_{31}HexNAc_{28}Fuc_3$							33173	33464	
$Hex_{32}HexNAc_{29}Fuc_3$								33829	

Relative intensity (%)

0.016
0.031
0.063
0.13
0.25
0.50
1.0
2.0
4.0
8.0
16
32
64
100

74 distinct glycoforms detected

Sensitive Glycoform Profiling of β-Interferon-1α (Avonex) and recombinant human Erythropoietin by CESI-TOF-MS – Haselberg et al.

The CESI 8000 Plus – a new range of detection capabilities . . .

LIF

UV/Vis

PDA

CE Assays & Method Development

MS Analysis

SCIEX

Summary

- CE is a robust and reliable technique – well integrated into Biopharma development and control processes
- CESI-MS – Effectively integrates capillary electrophoresis with electrospray ionization serving to reduce/eliminate ion suppression and increase ionization efficiency for:
  - Insulin Impurities
  - Peptide quantitation
  - Multi-level mAb characterization
    - 1) Intact mAb/protein analysis
    - 2) Reduced mAb analysis
    - 3) Peptide mapping and PTM characterization
  - Proteoform/glycoform characterization
- We are just starting to see where this technique will take analytical chemistry.
- Take Home Message - “Flow Matters”

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