



MULTIDIMENSIONAL LC-MS PLATFORMS FOR STRUCTURE-FUNCTION CHARACTERIZATION OF THERAPEUTIC ANTIBODIES

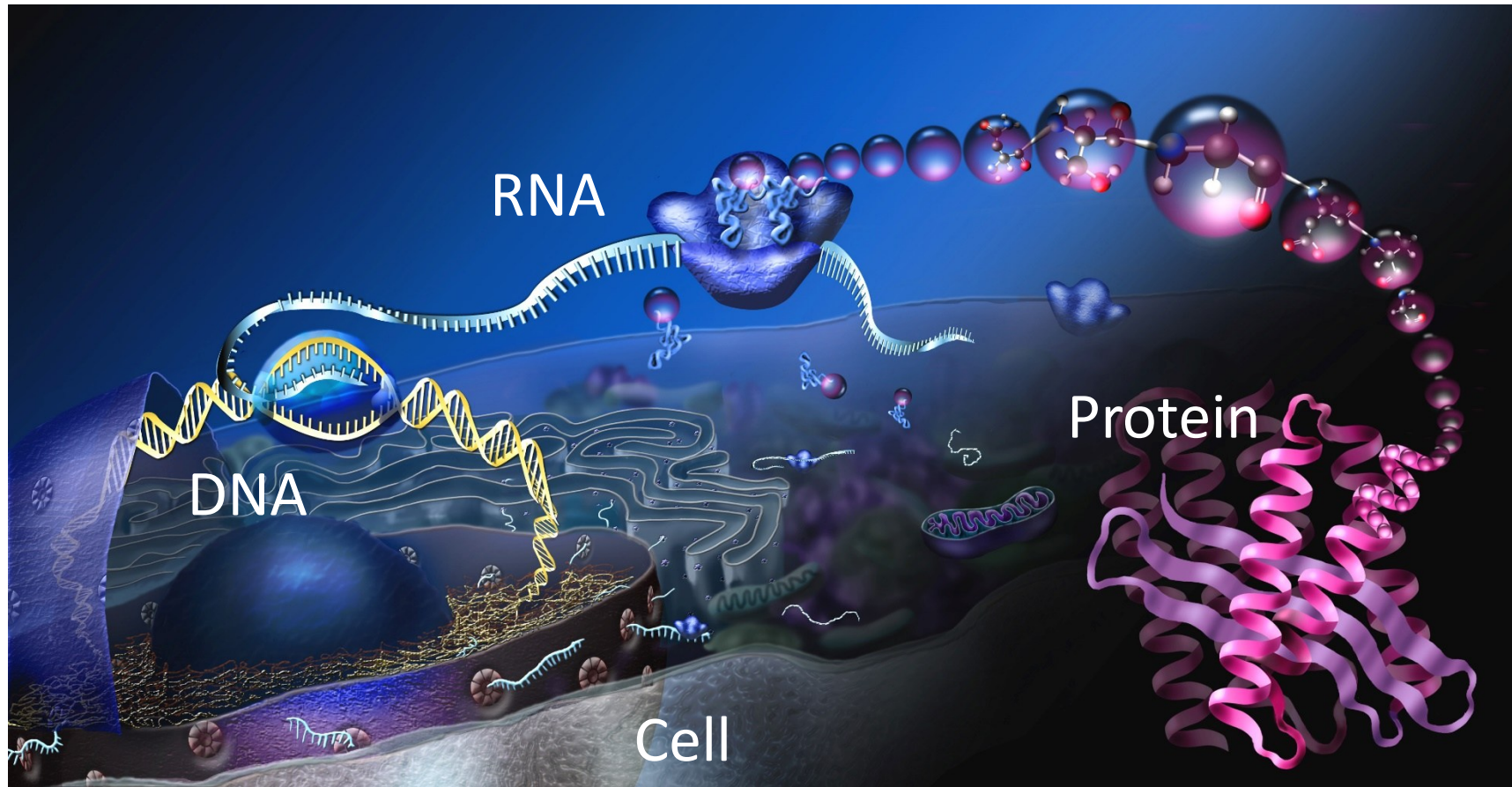
Jelle De Vos

The Multidimensional Chromatography Workshop 2025, Liège

4 February 2025



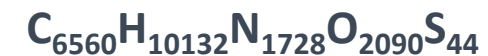
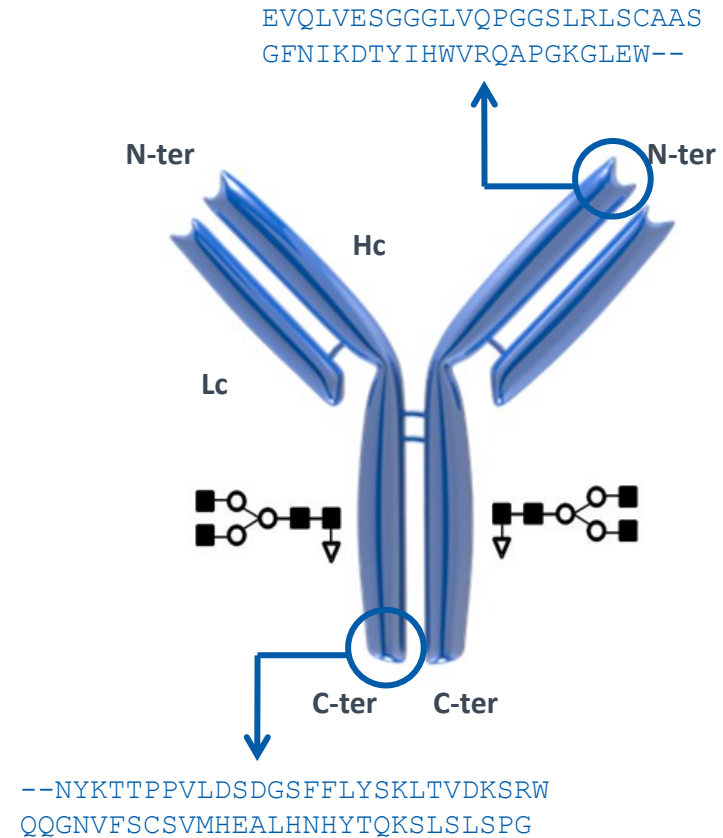
The wonders of nature!



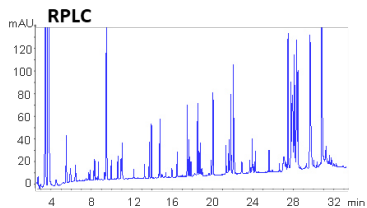


Monoclonal antibodies (mAbs)

- | mAbs have become a core aspect of the pharmaceutical industry
- | Together with a huge therapeutic potential comes an equally great structural complexity
- | mAbs are large ($\pm 150,000$ Da) and heterogeneous (result of biosynthetic process and subsequent manufacturing and storage)



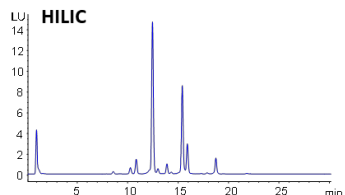
MW: 148,057 Da



DIQMTQSPSSLSASVGDR
VTITCR
ASQDVNTAVAWYQQKPGK
APK
LLIYSASFLYSGVPSR
...

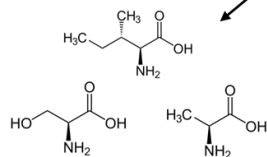
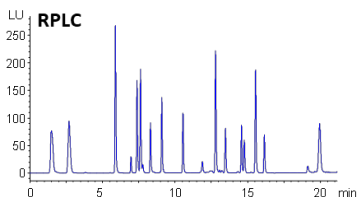
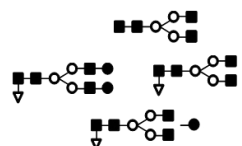
Peptides

Trypsin



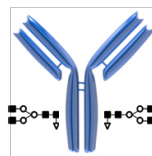
Glycans

PNGaseF
2-AB labeling

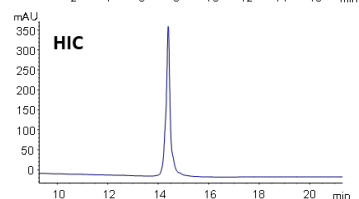
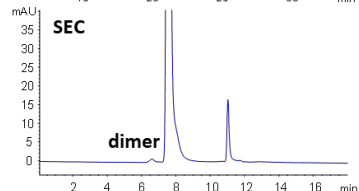
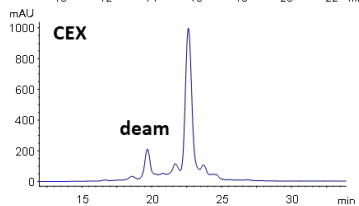
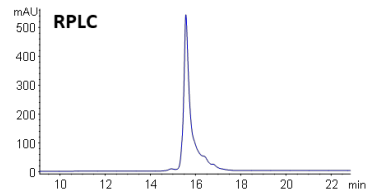


Amino acids

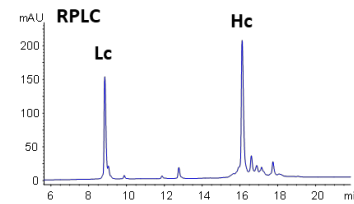
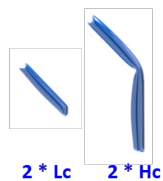
Acid hydrolysis
OPA/FMOC labeling



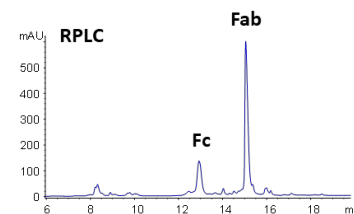
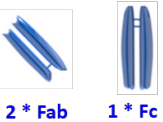
Intact mAb



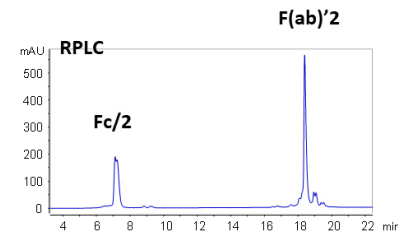
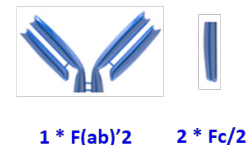
Reduction



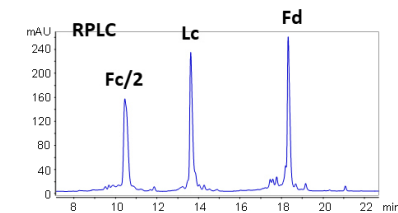
Papain



IdeS



Reduction



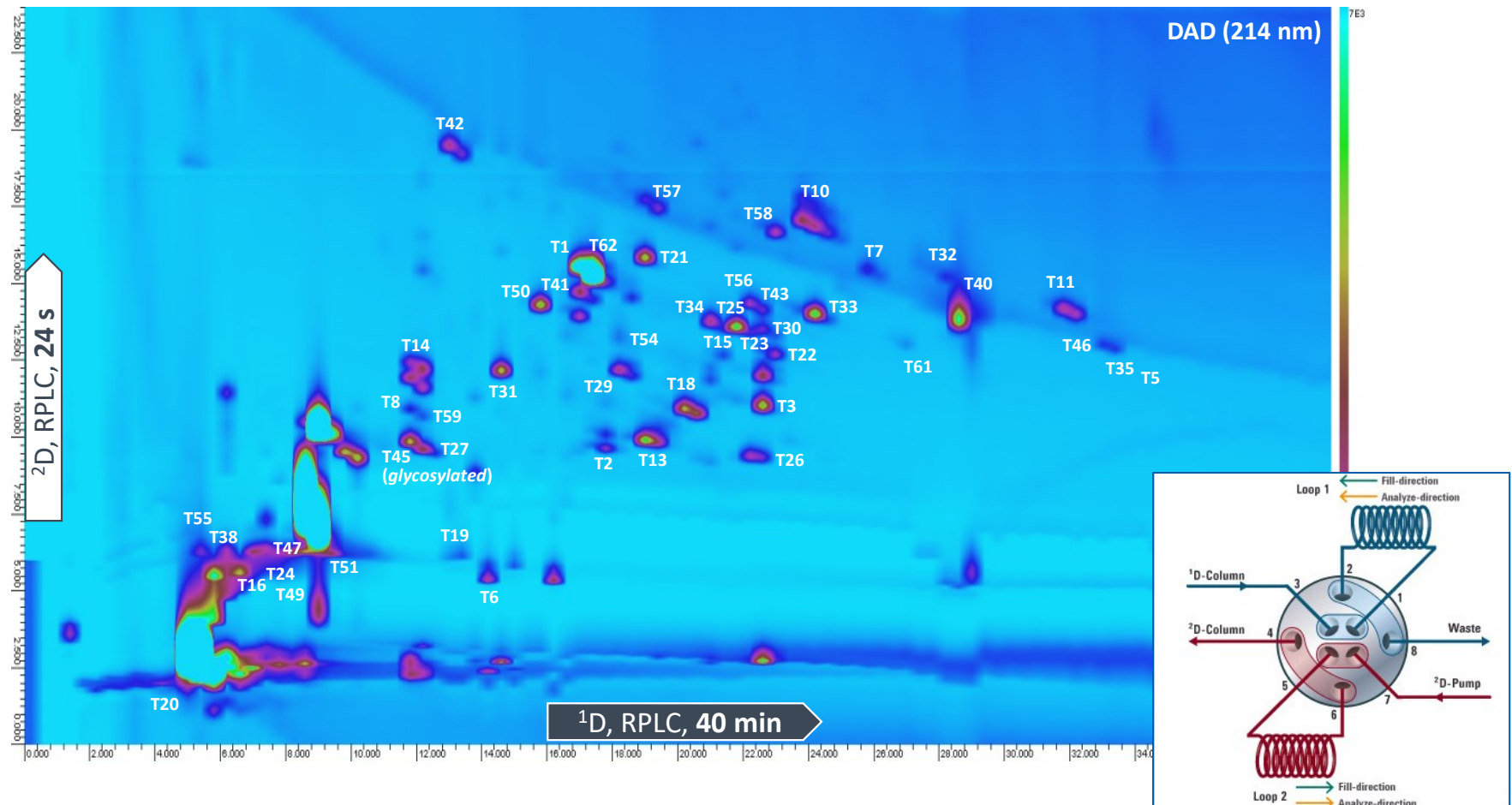
One-dimensional liquid chromatography of trastuzumab (Herceptin)

Fekete S, Guillaume D, Sandra P, Sandra K
Anal. Chem. 88 (2016) 480-507



Unsurpassed resolution offered by comprehensive 2D-LC

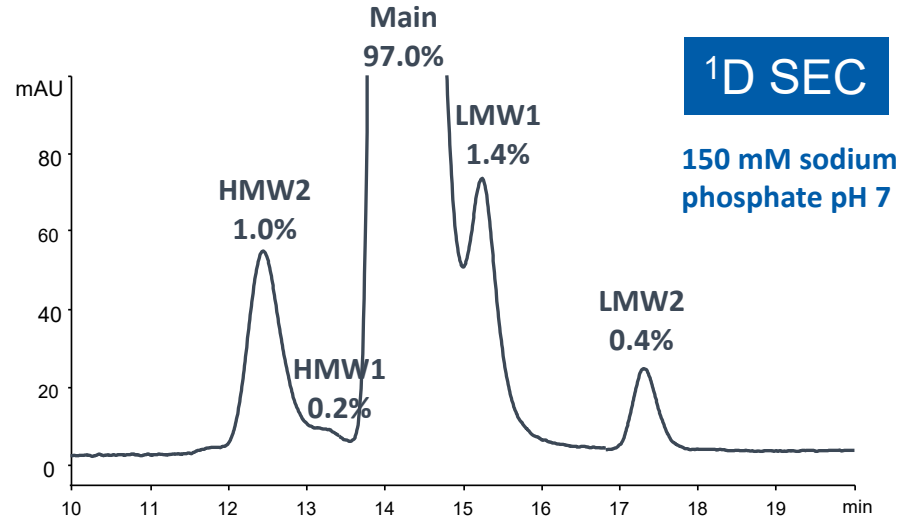
RPLC × RPLC peptide map of trastuzumab (Herceptin)



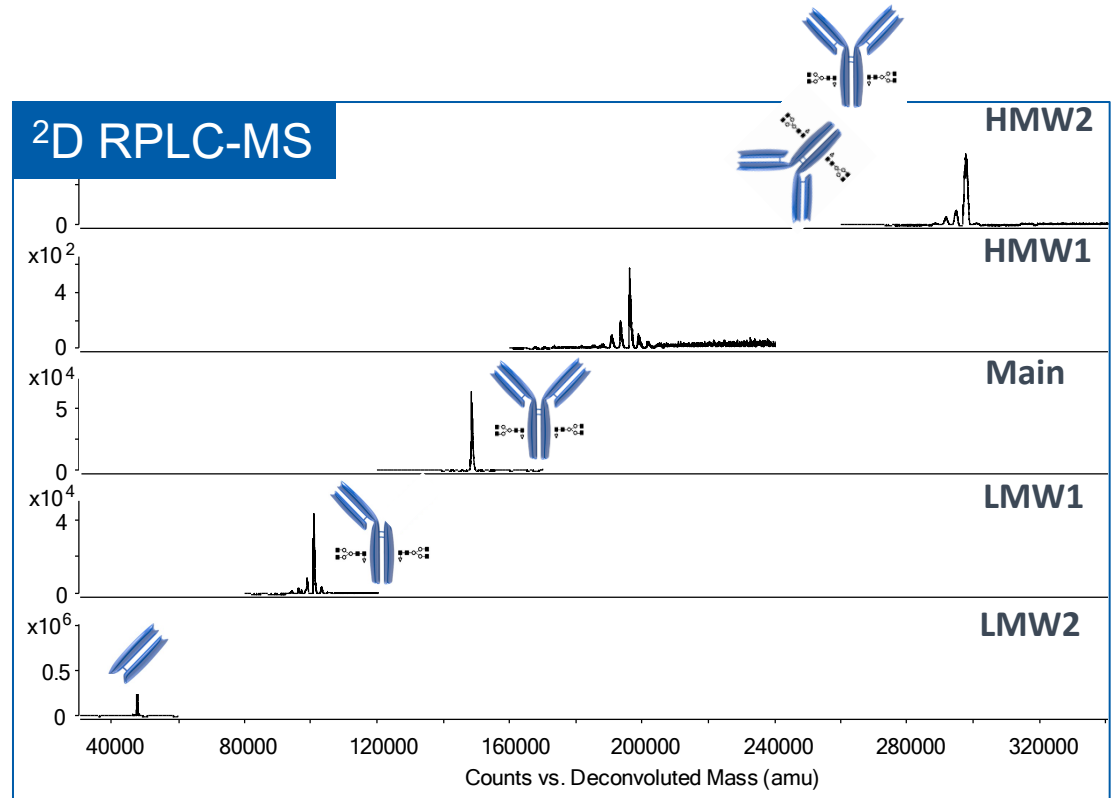
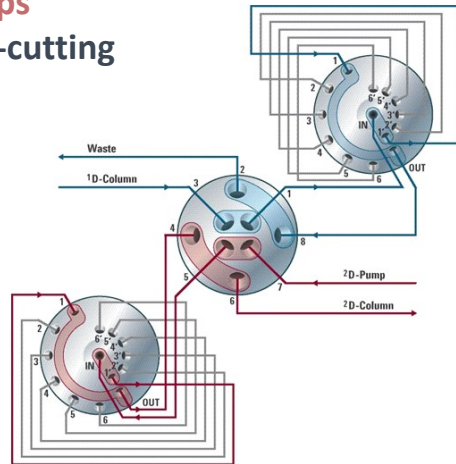
Vanhoenacker G. et al.,
Anal. Bioanal. Chem.
407 (2015) 355-366



Making first dimension MS compatible through multiple heart-cutting 2D-LC

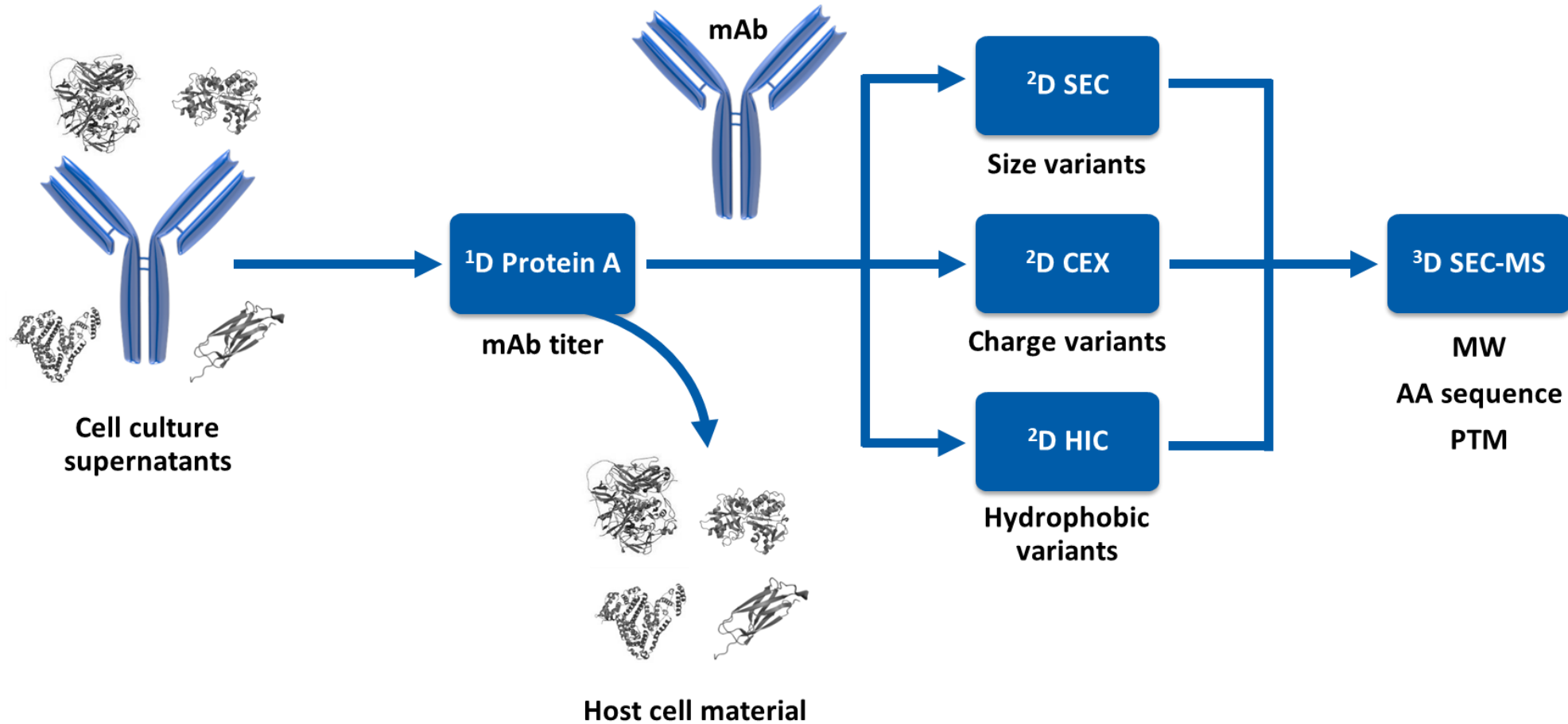


40 μ L loops
Multiple heart-cutting (MHC)





Multi-attribute analysis of mAbs using 3D-LC-MS with ²D multi-method option

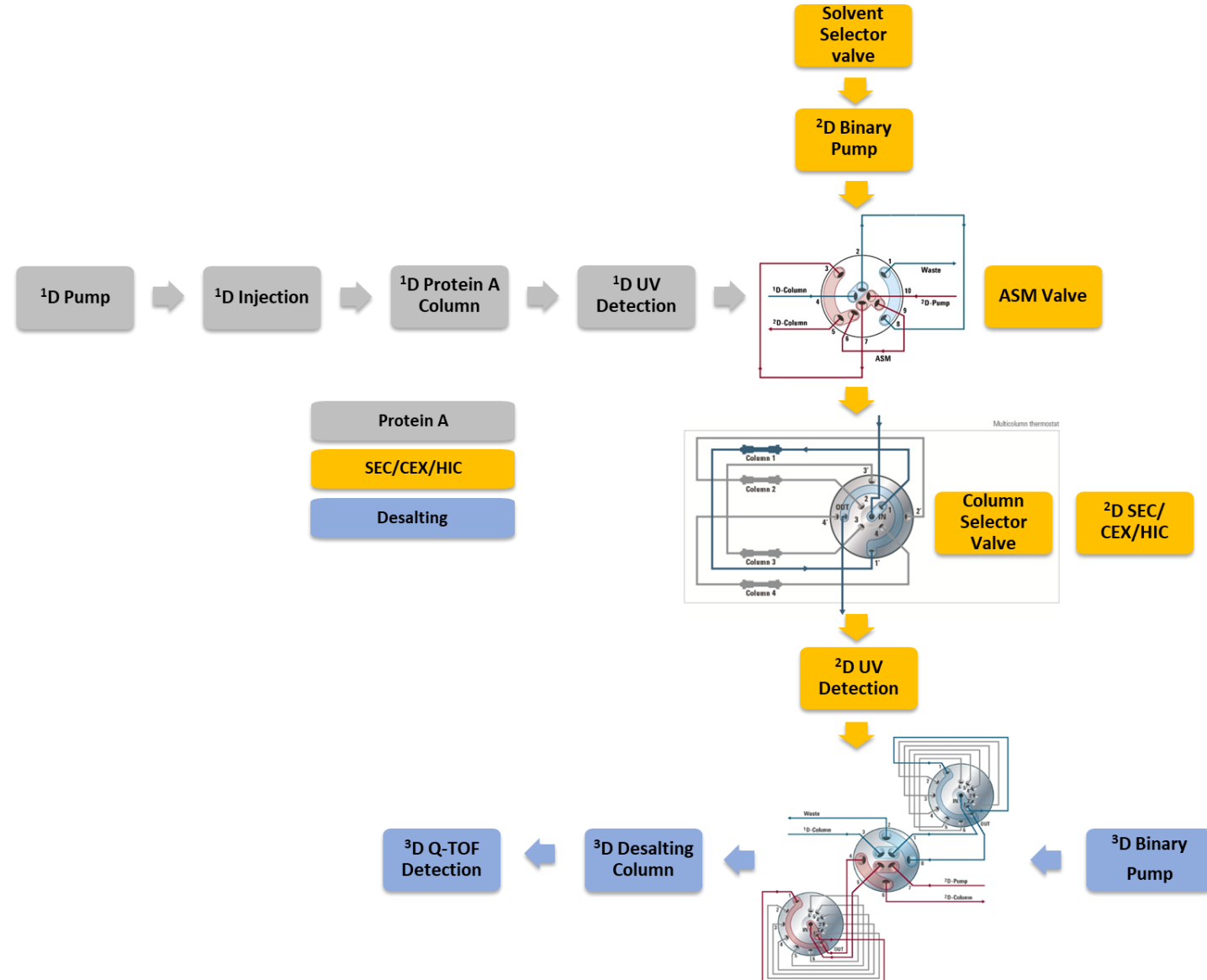




3D-LC-MS with ²D multi-method option

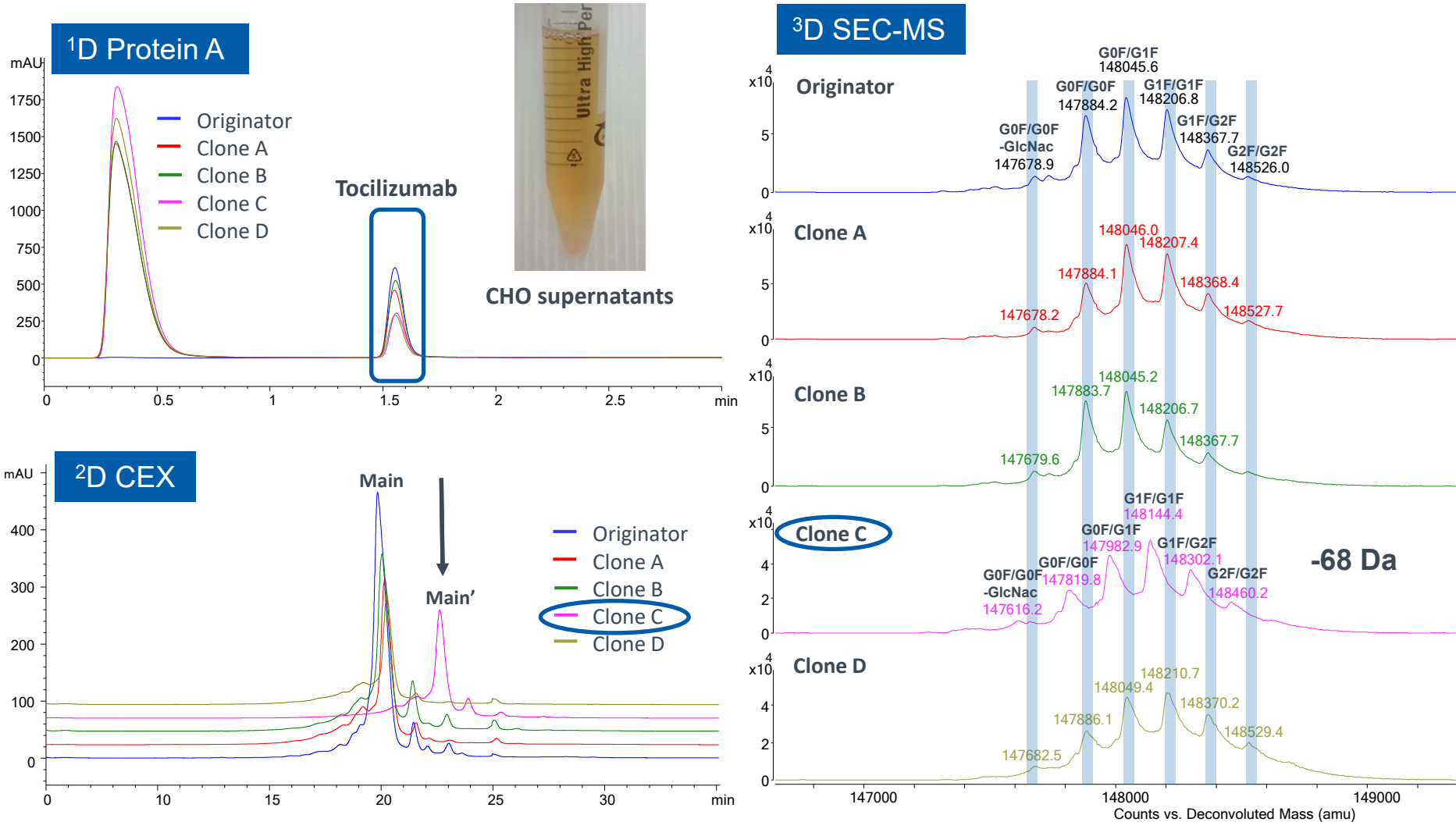


Verscheure L. et al. *Anal. Chem.*
94 (2022) 6502-6511



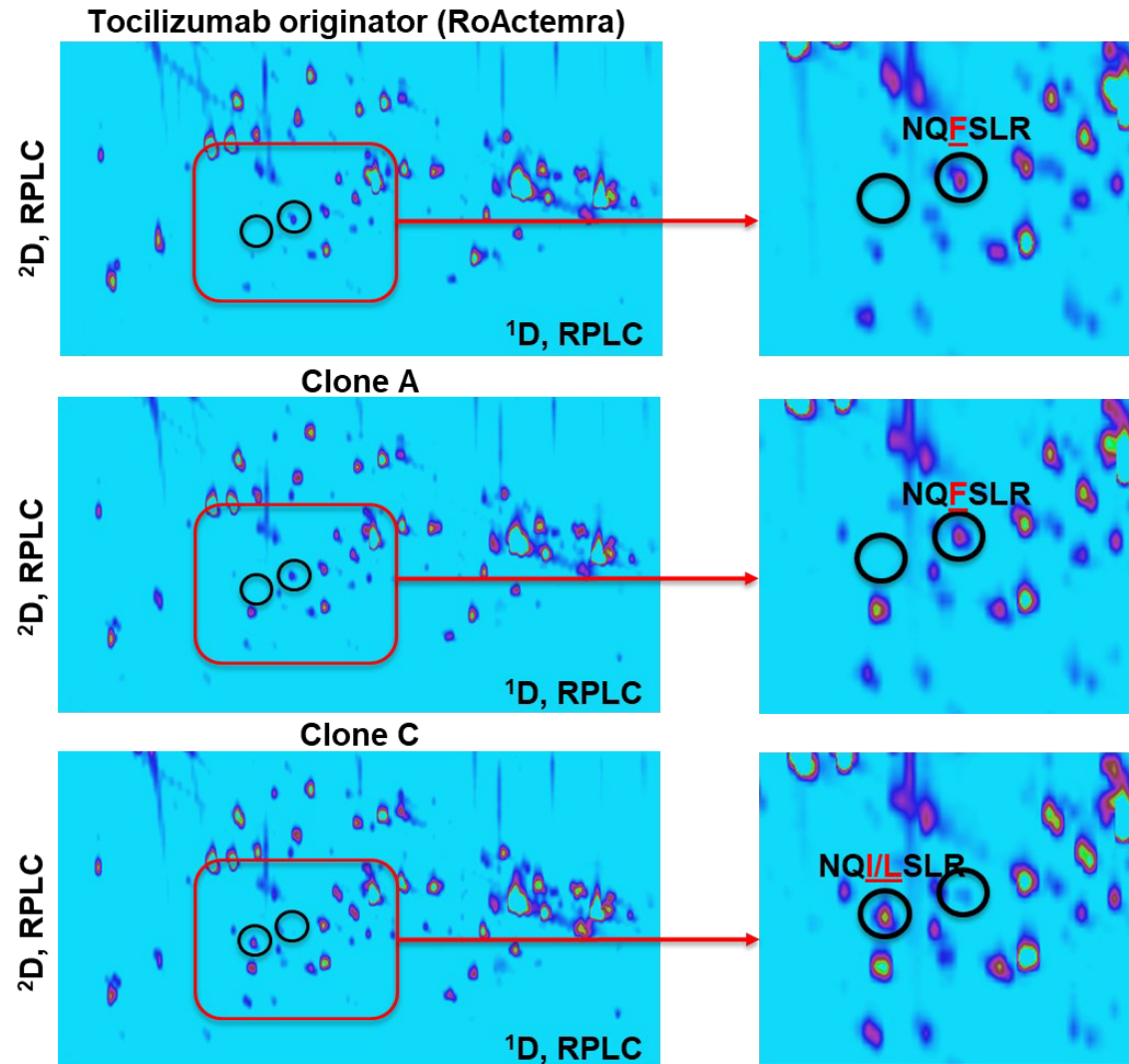


3D-LC-MS with ²D multi-method option – Tocilizumab clone selection

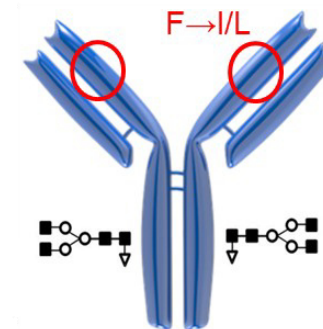




Locating mutation by RPLC × RPLC-MS



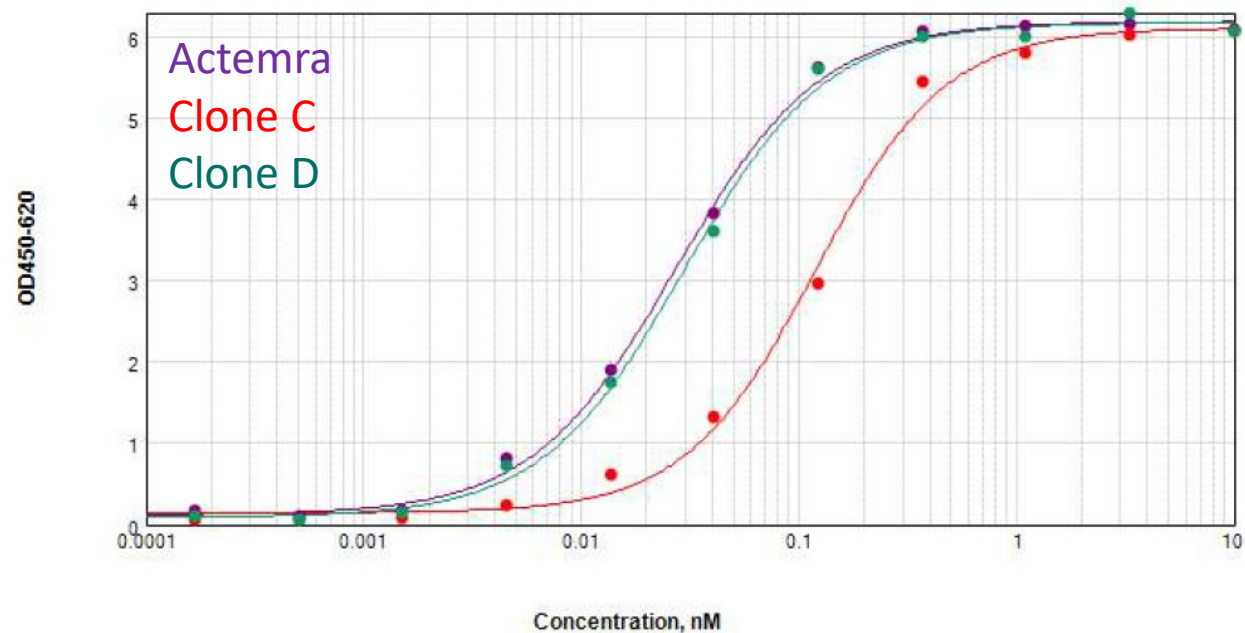
F→I/L:	-34 Da
2 Hc:	-68 Da





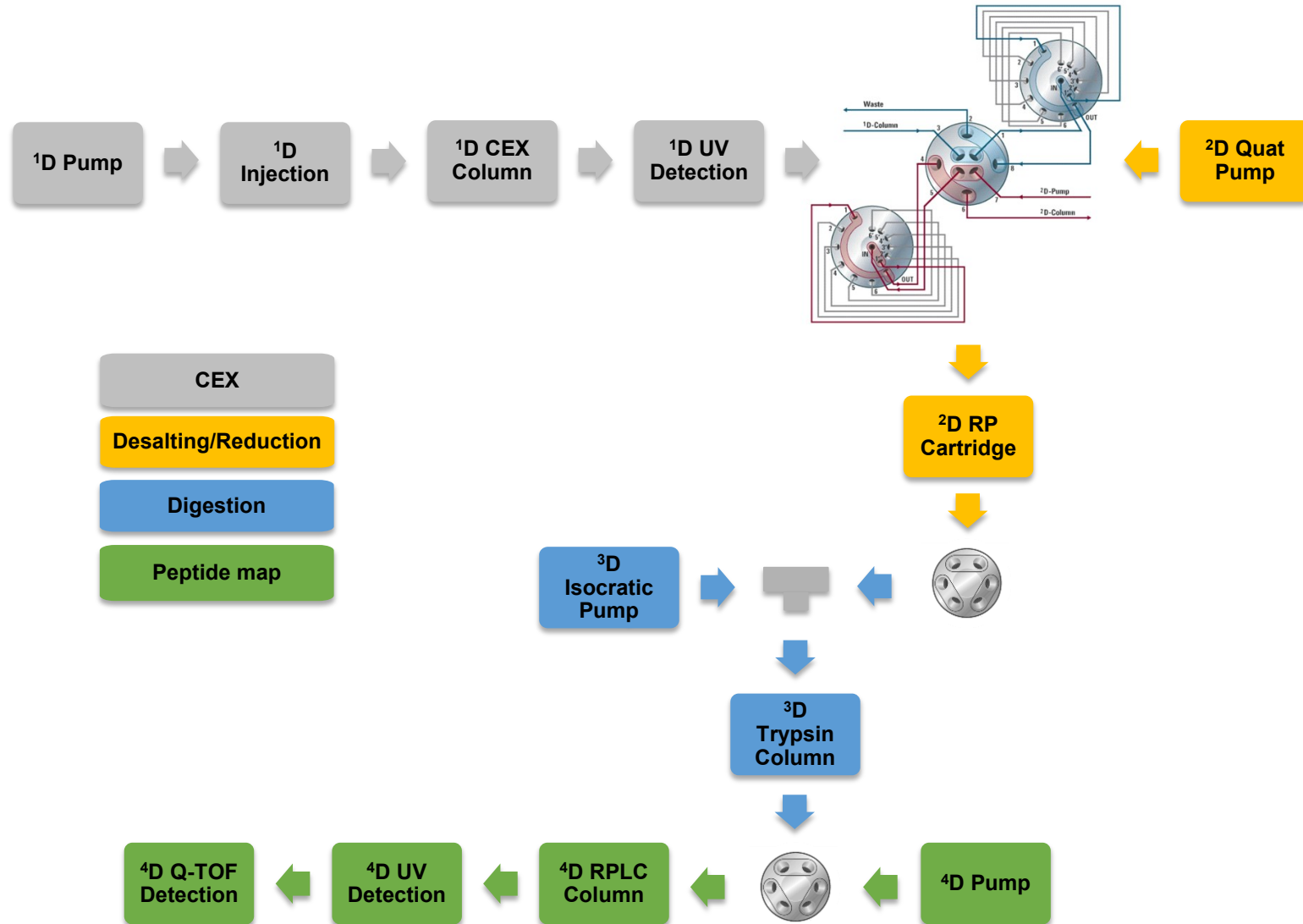
Functional ELISA

Sample	EC50, nM	EC50 relative to Actemra
Actemra	0.032	NA
Clone A	0.033	103%
Clone B	0.032	100%
Clone C	0.119	441%
Clone D	0.030	111%





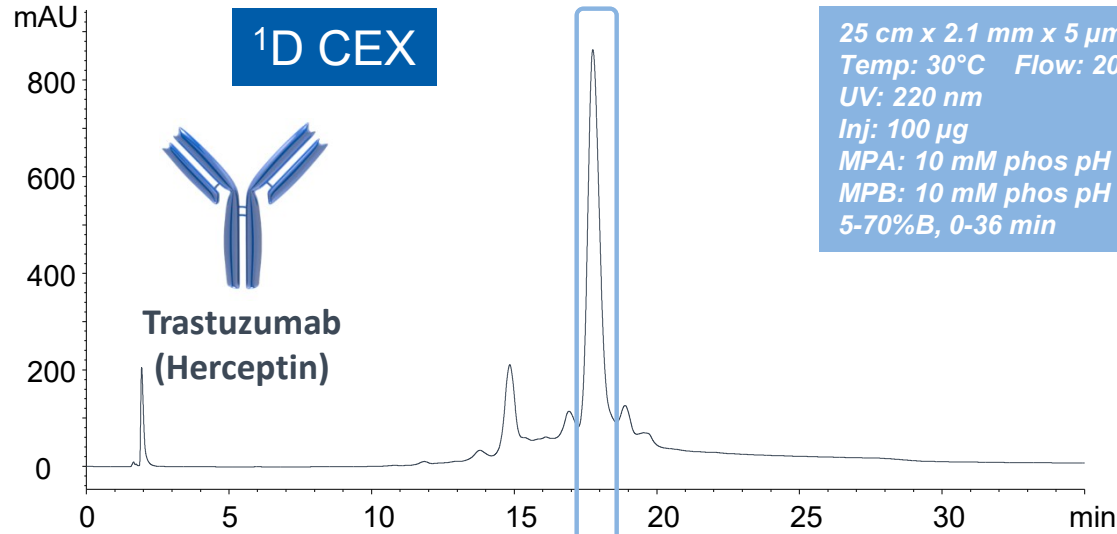
MD-LC-MS – implementing on-line reduction and digestion



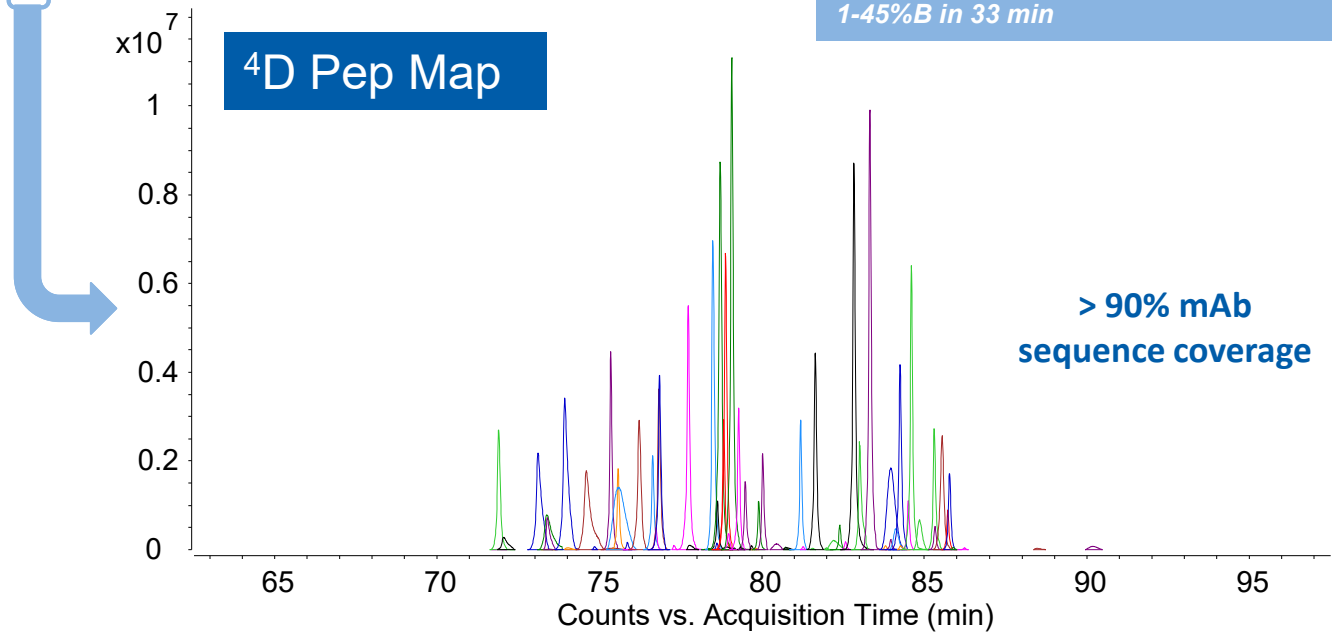
Verscheure L. et al., *J. Chrom. A* 1653 (2021) 462409



MD-LC-MS of Trastuzumab

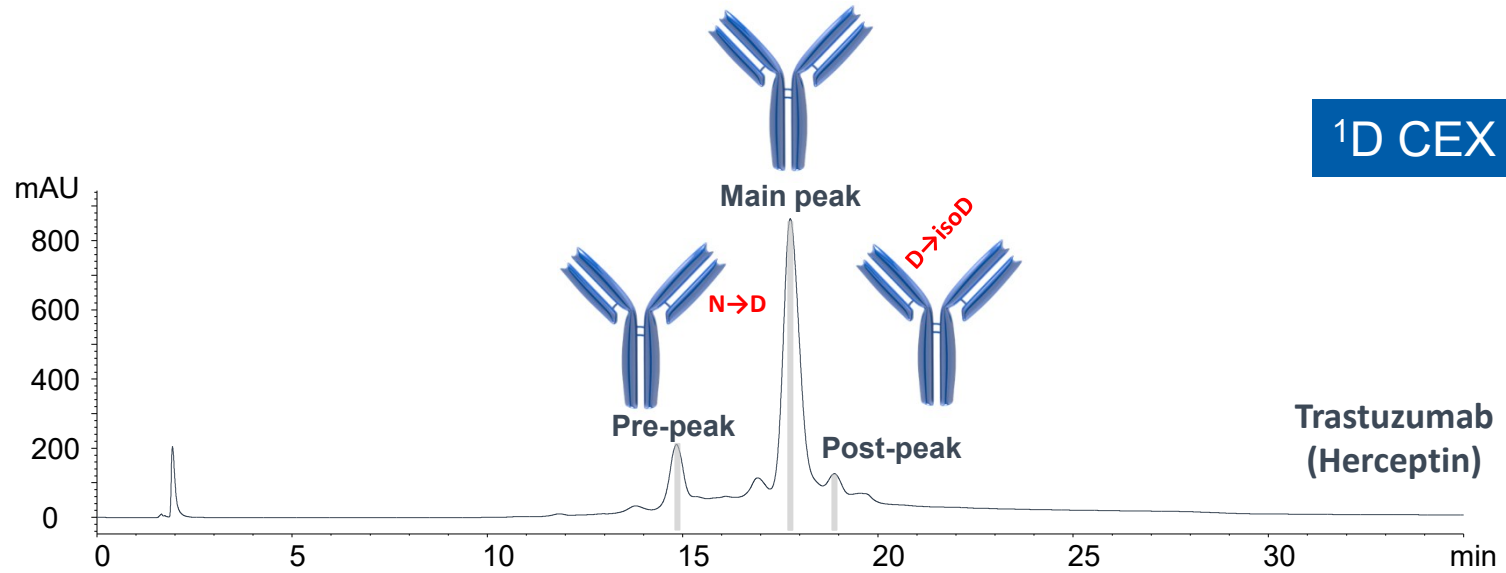


On-line reduction and digestion

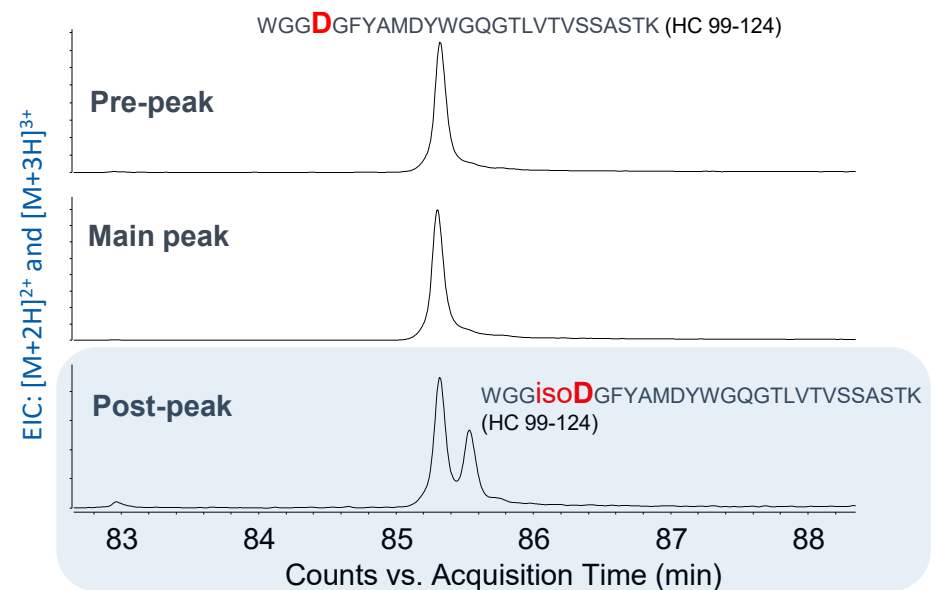
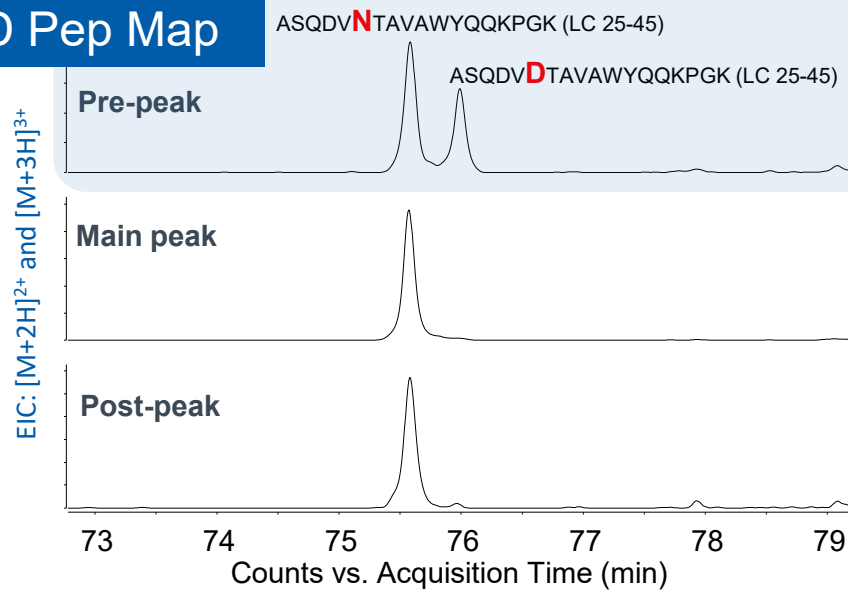




MD-LC-MS of Trastuzumab

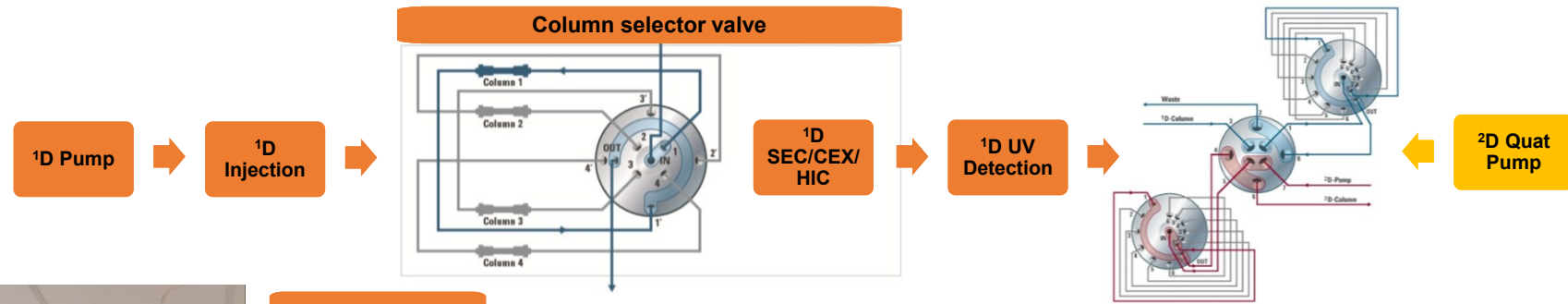


4D Pep Map

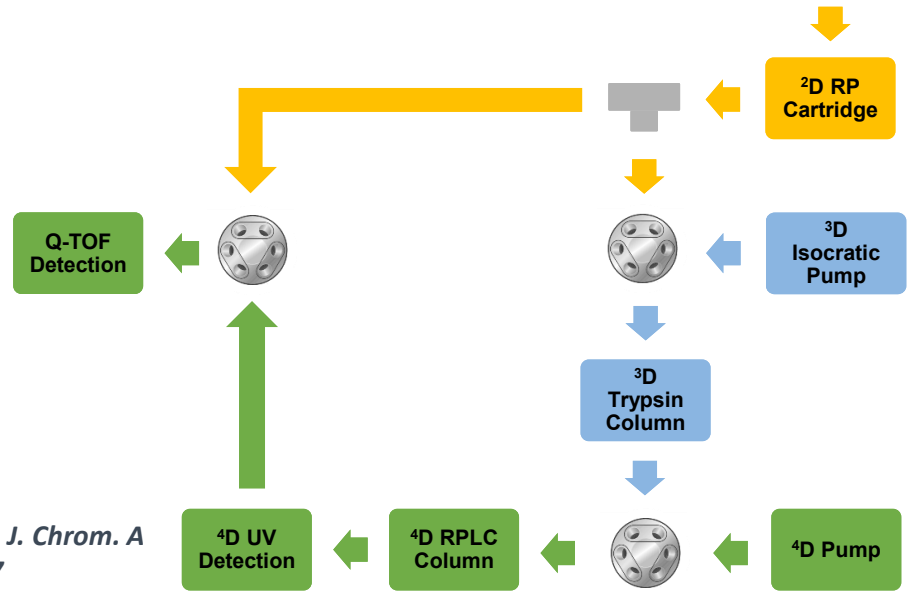




MD-LC-MS with ¹D multi method option and parallel middle-up and bottom-up analysis



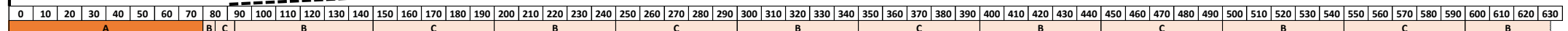
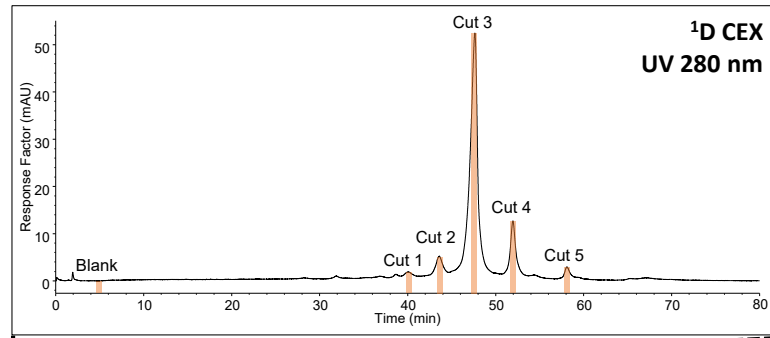
- SEC/CEX/HIC
- Desalting/Reduction
- Digestion
- Peptide map



Verscheure L. et al., J. Chrom. A 1726 (2024) 464947



MD-LC-MS with ¹D multi method option and parallel middle-up and bottom-up analysis



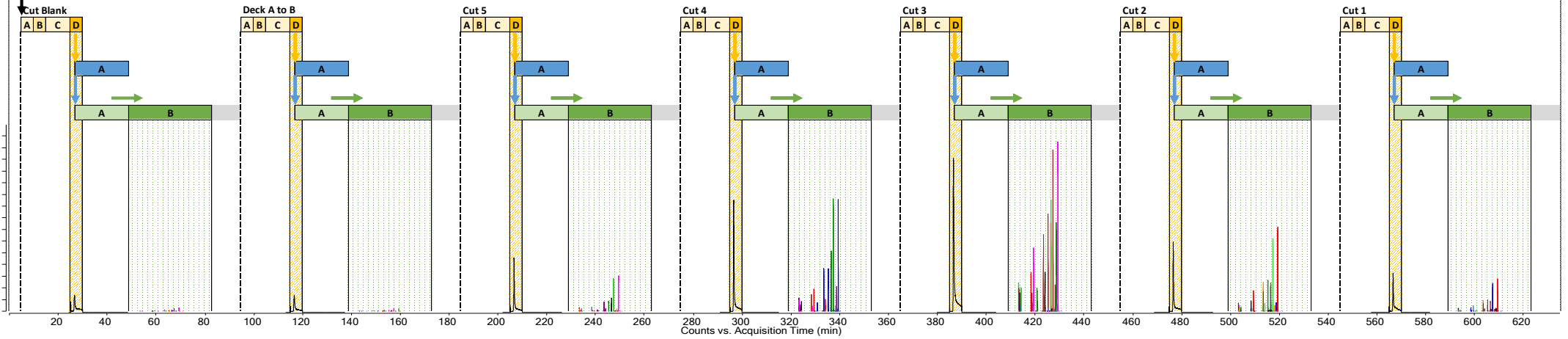
MHC	Time (min)	Event
	4.73	Cut Blank
	40.33	Cut 1
	44.03	Cut 2
	48.48	Cut 3
	52.38	Cut 4
	58.25	Cut 5

¹ D	Method
A	CEX Analysis
B	CEX Conditioning and Preparing

² D	Method
A	Desalting and Focusing
B	Reduction
C	Desalting
D	Elution and RPC-MS

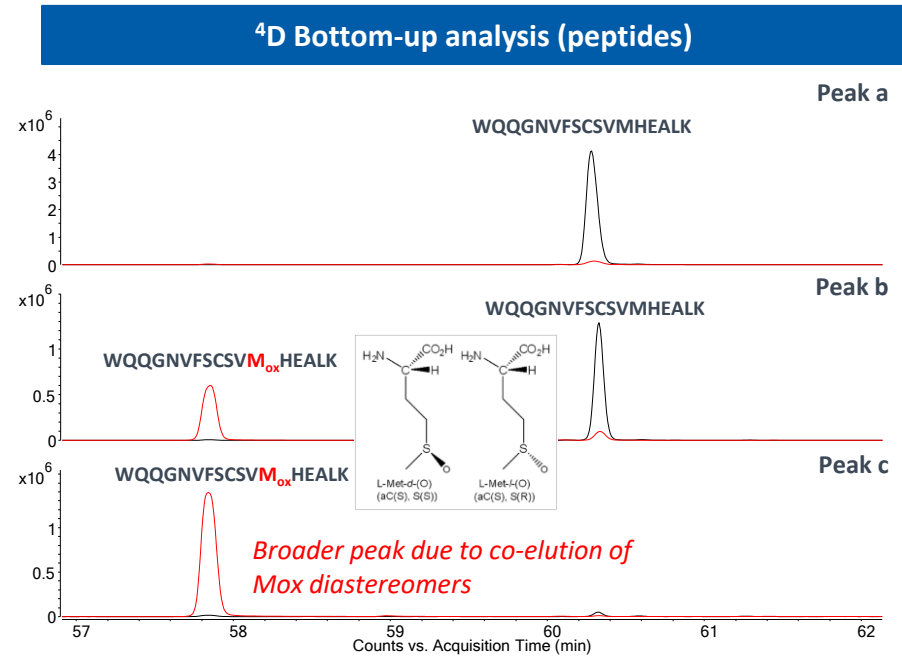
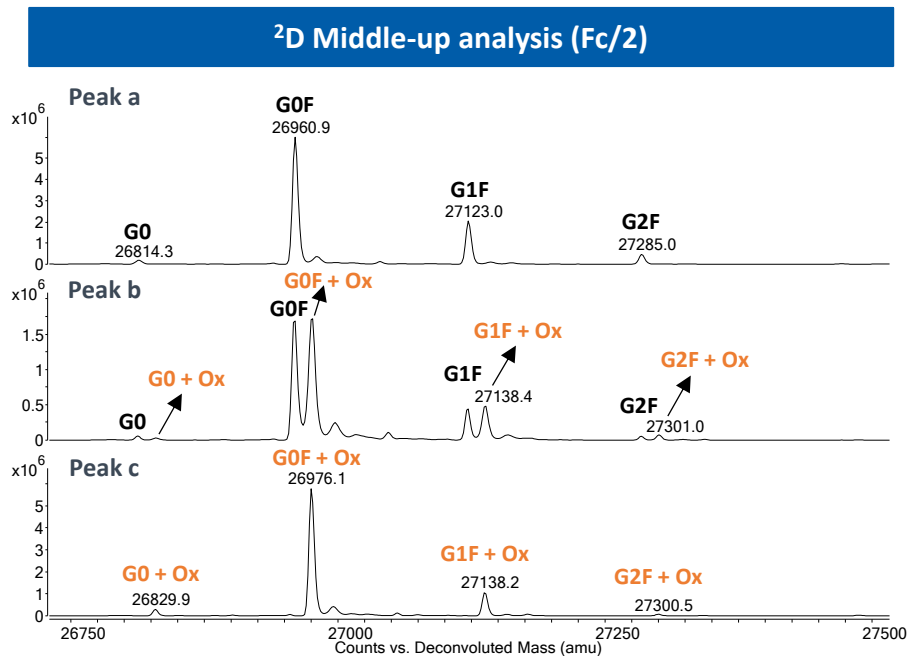
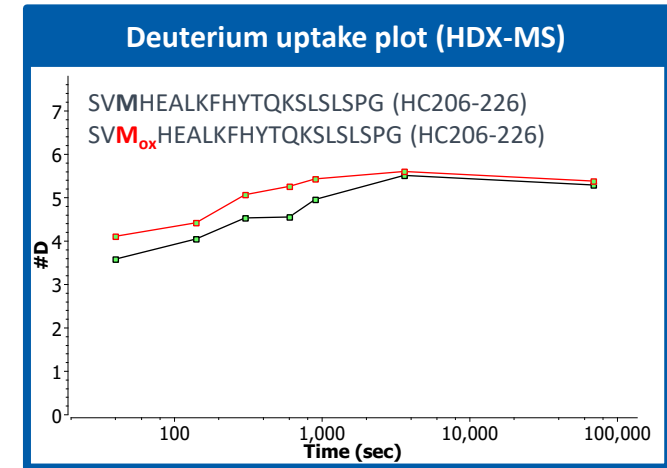
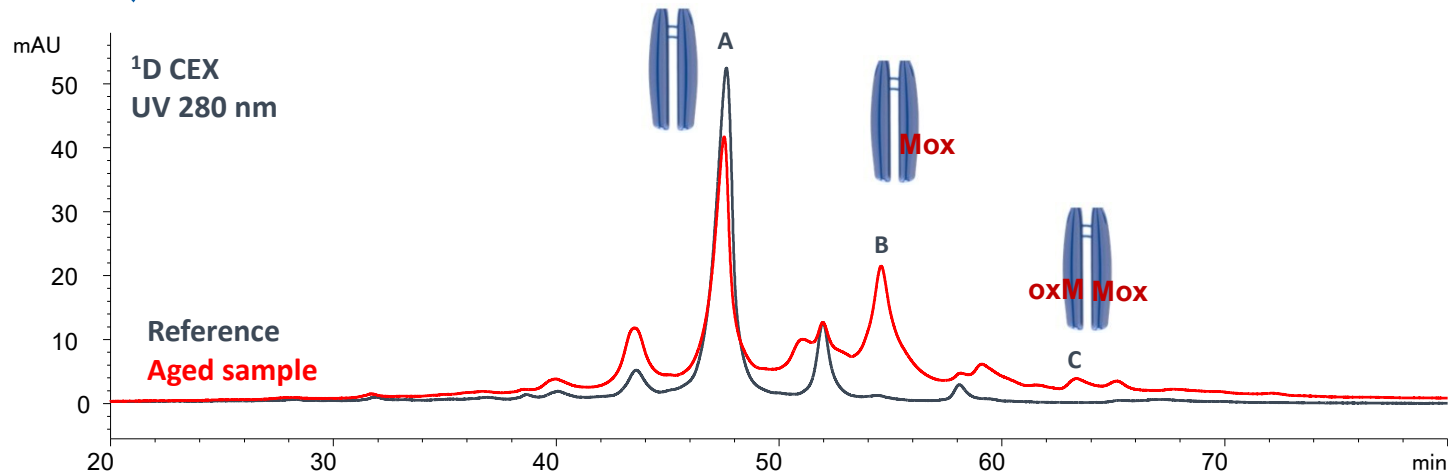
³ D	Method
A	Digestion

⁴ D	Method
A	Load digest on Peptide map column
B	Peptide mapping





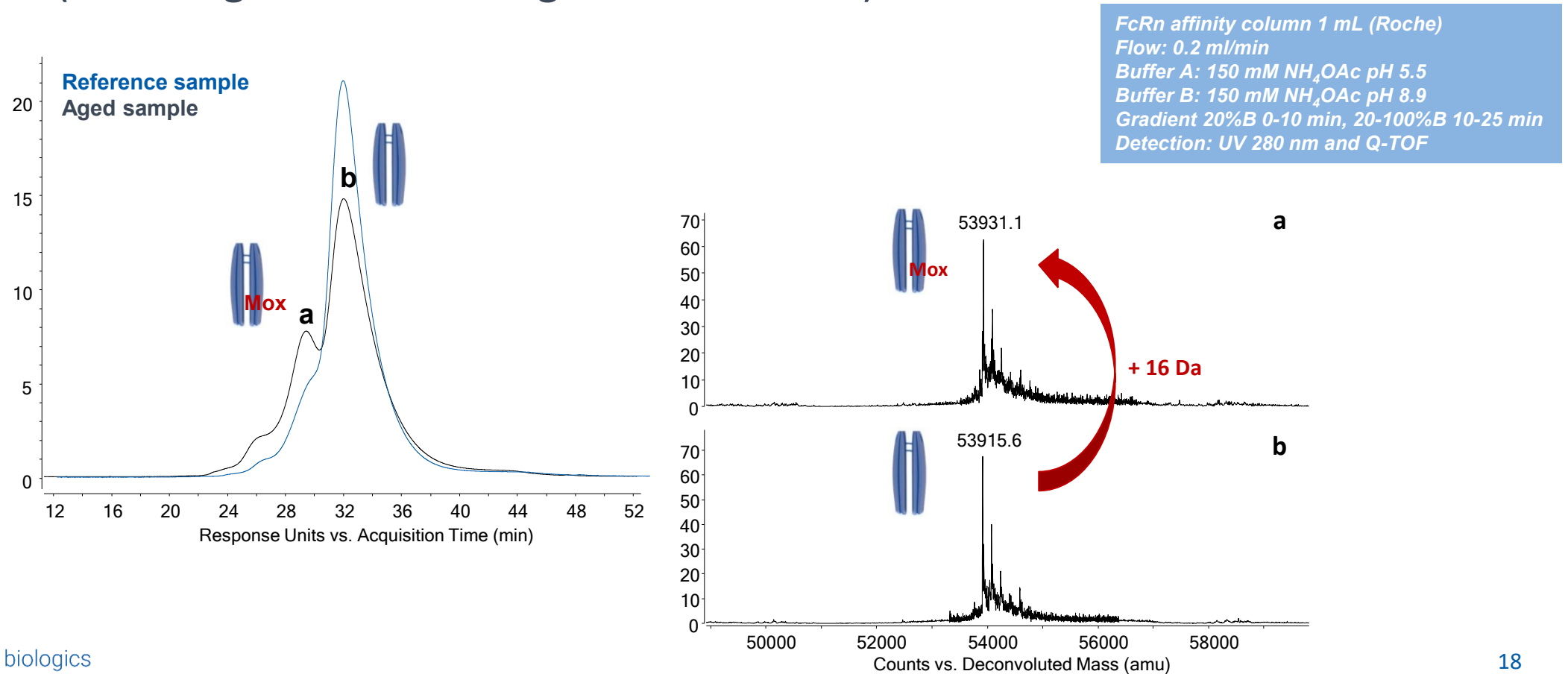
Next generation MD-LC-MS of therapeutic Fc fragment





FcRn affinity chromatography-native MS

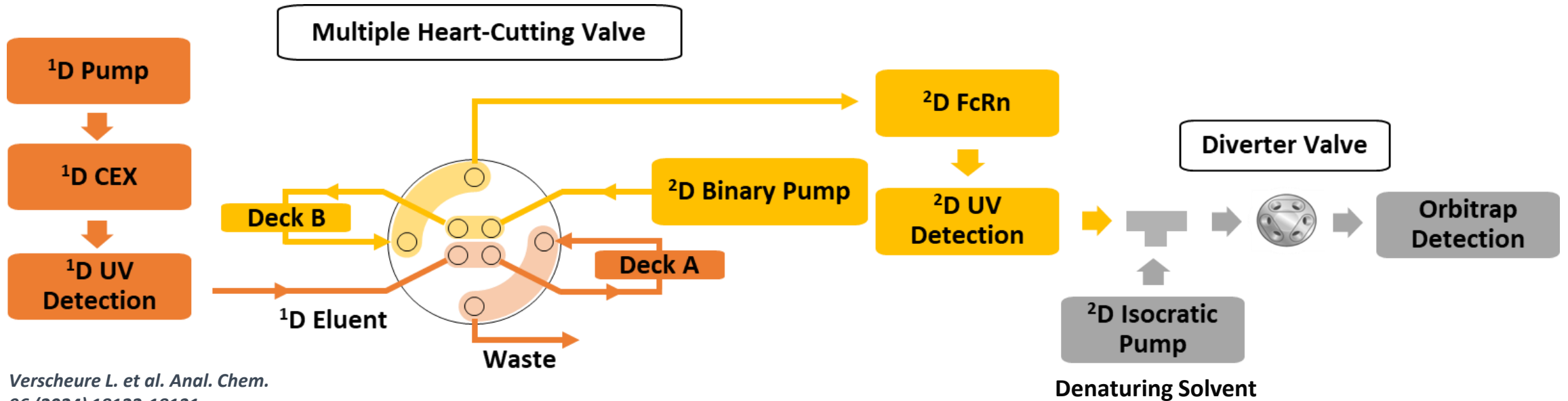
- Therapeutic Fc fragment binds FcRn thereby blocking IgG recycling and increasing IgG clearance (including disease-causing autoantibodies)





2D-CEX-FcRn-MS

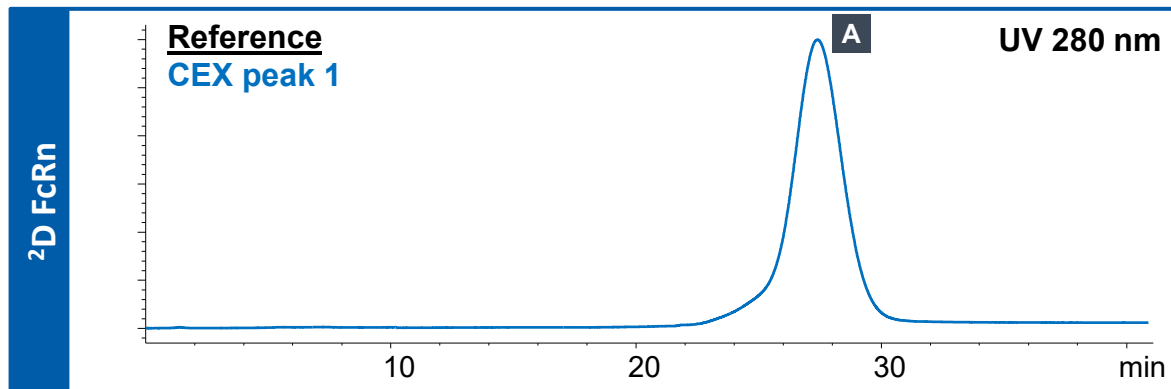
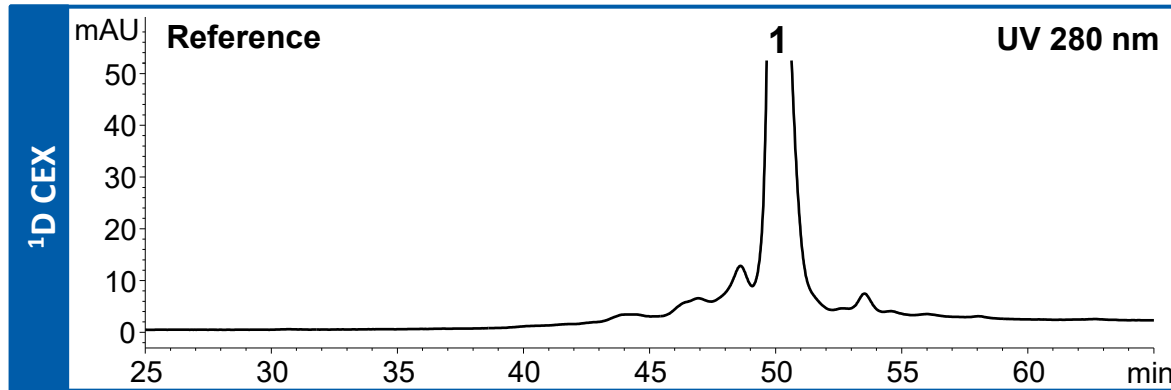
I Fully automated system to study FcRn binding of charge variants



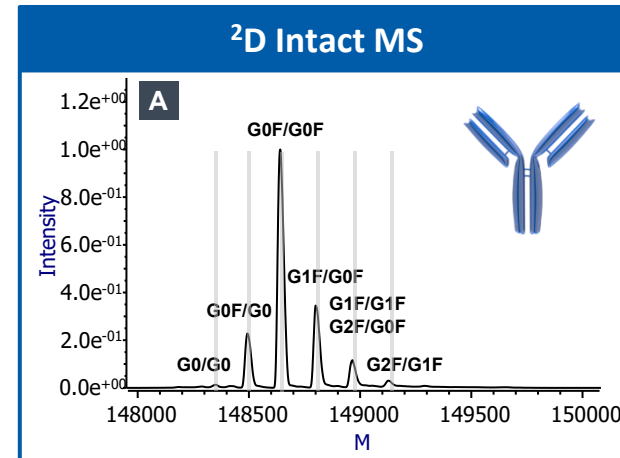
Verscheure L. et al. Anal. Chem.
96 (2024) 18122-18131



2D-CEX-FcRn-MS

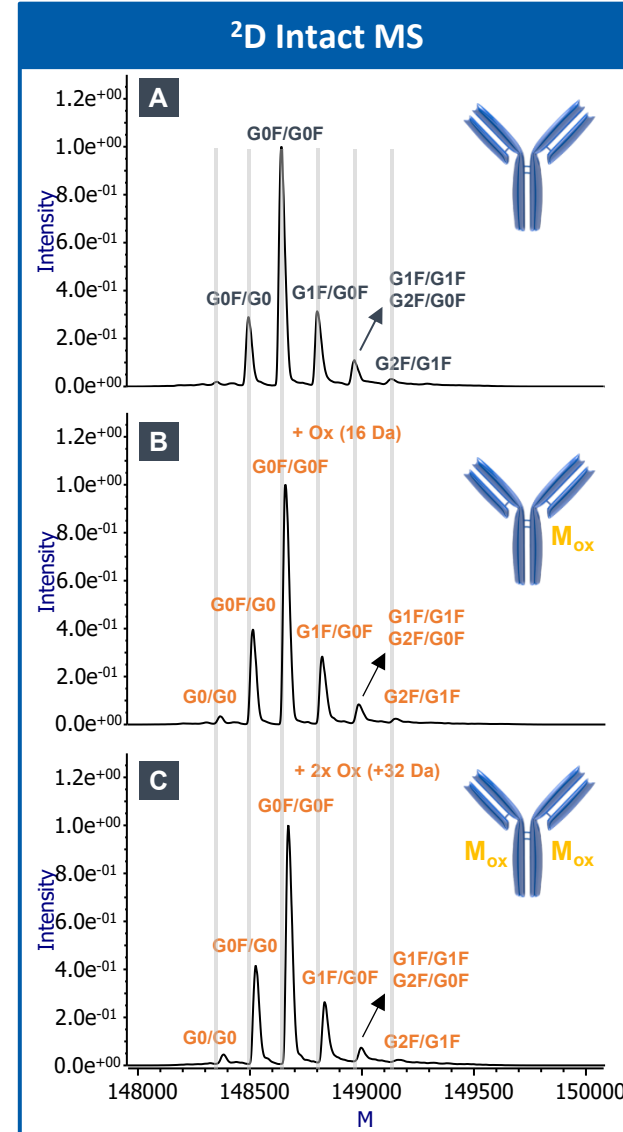
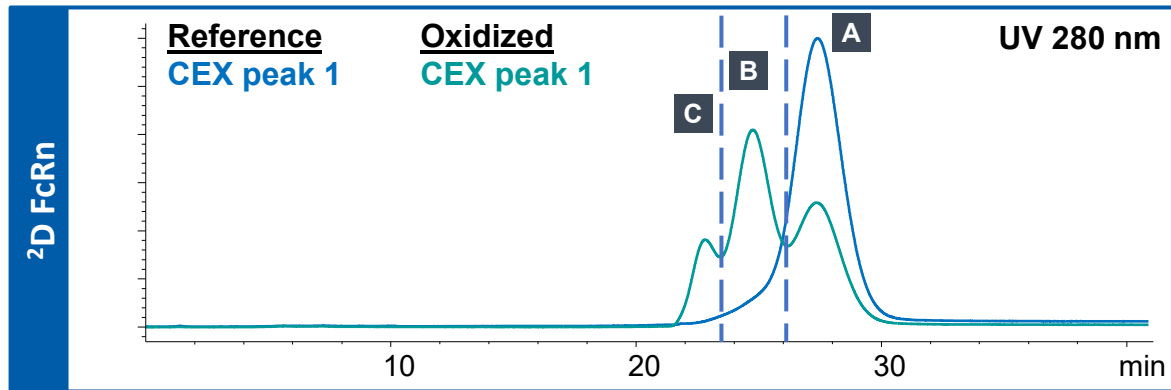
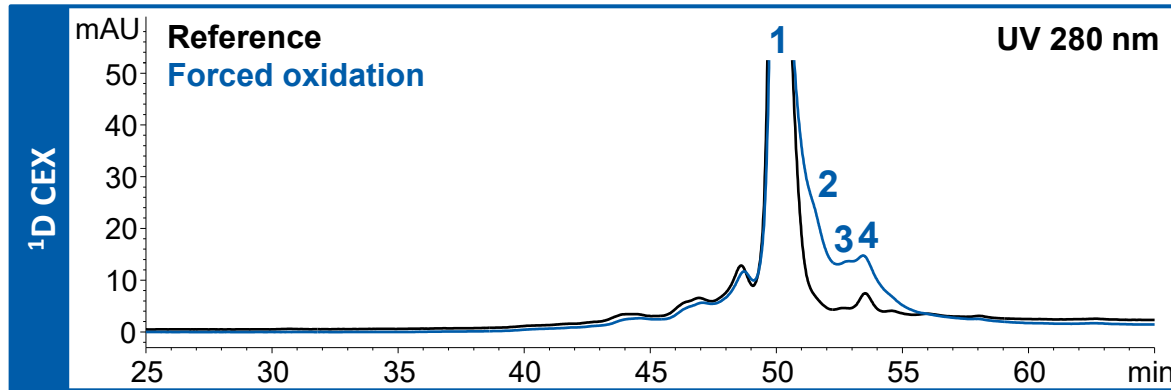


MABPac SCX-10, 2 x 250 mm (ThermoFisher Scientific)
Flow: 0.15 mL/min
Buffer A: 20 mM MES pH 5.8
Buffer B: 20 mM MES pH 5.8 + 300 mM NaCl
Gradient 15%B 0-1 min, 15-43%B 1-67 min
Load: 50 µg
Detection: UV 280 nm



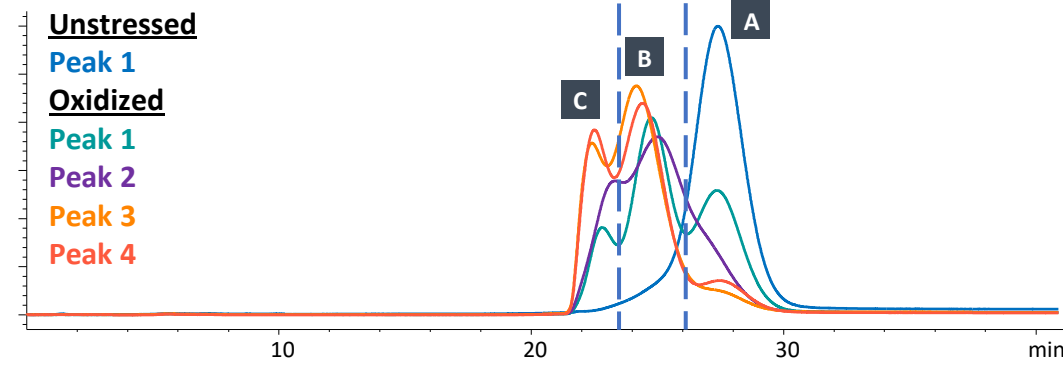
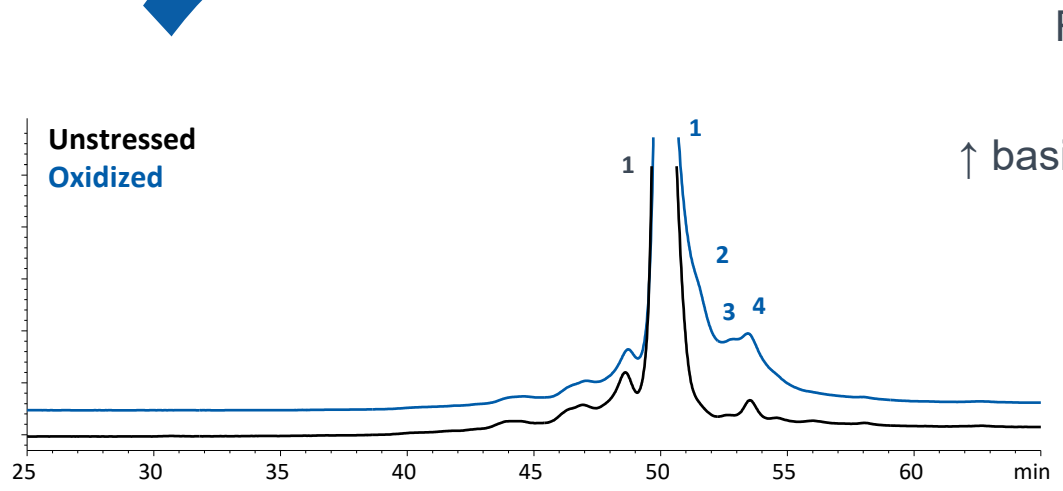


2D-CEX-FcRn-MS

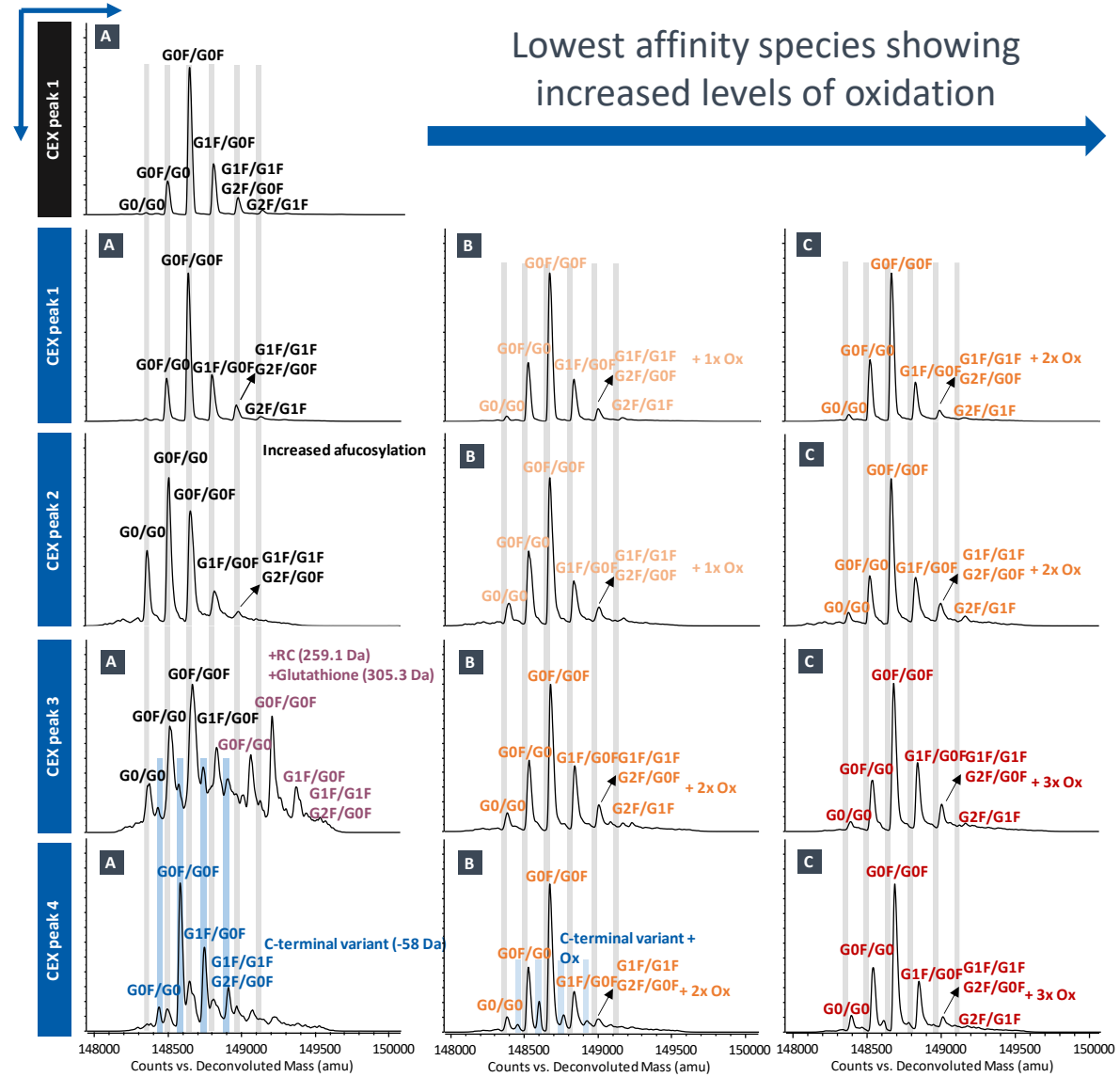




2D-CEX-FcRn-MS



FcRn affinity ↓

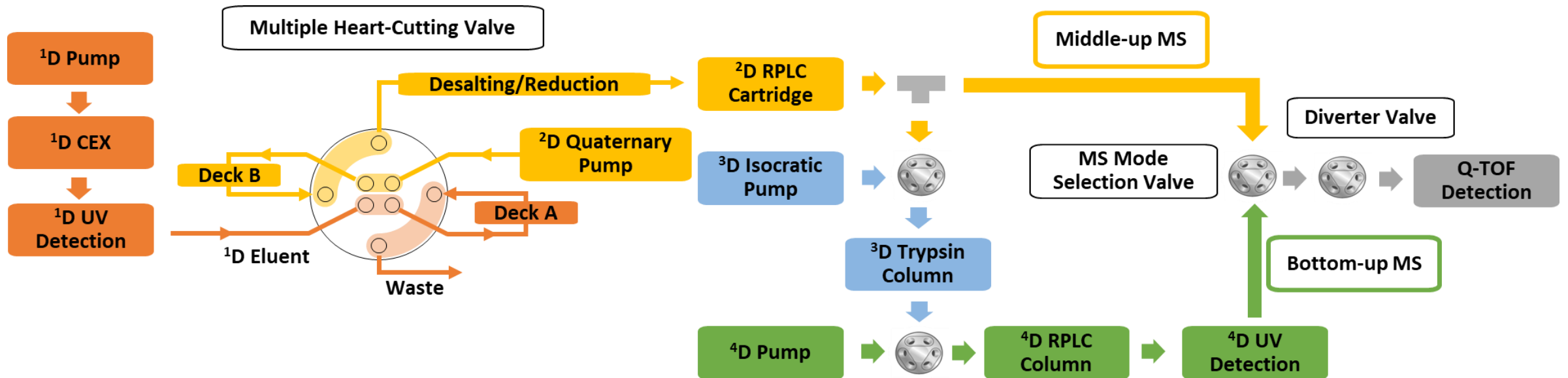




MD-LC-MS with ¹D CEX and parallel middle-up and bottom-up analysis

MD-LC-MS provides next level of structural detail

- Bottom-up LC-MS: identification of oxidation sites
- Middle-up LC-MS: reveals if one or both HCs are oxidized in case of multiple oxidation events

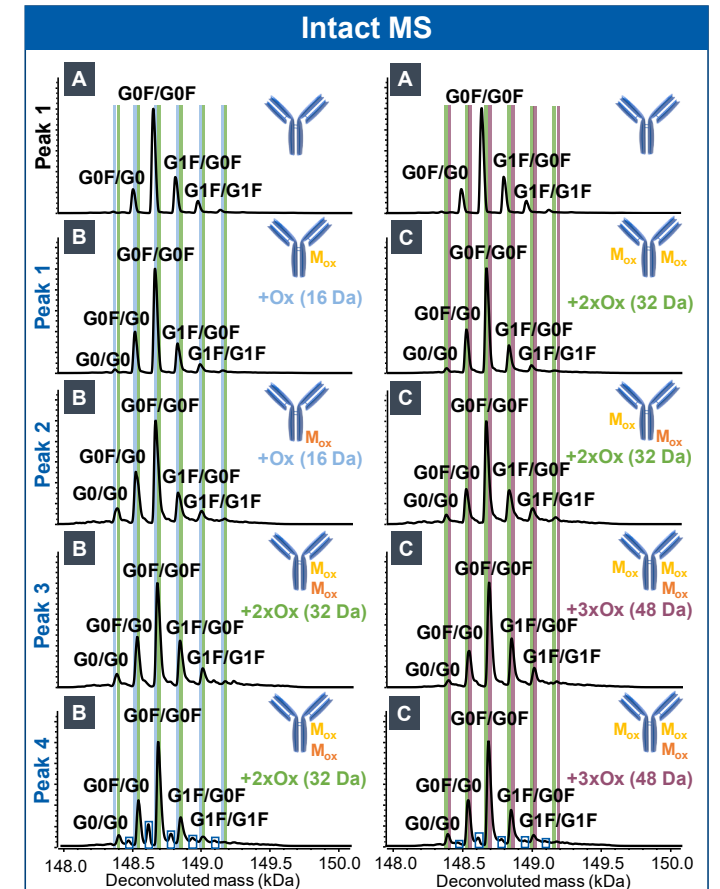
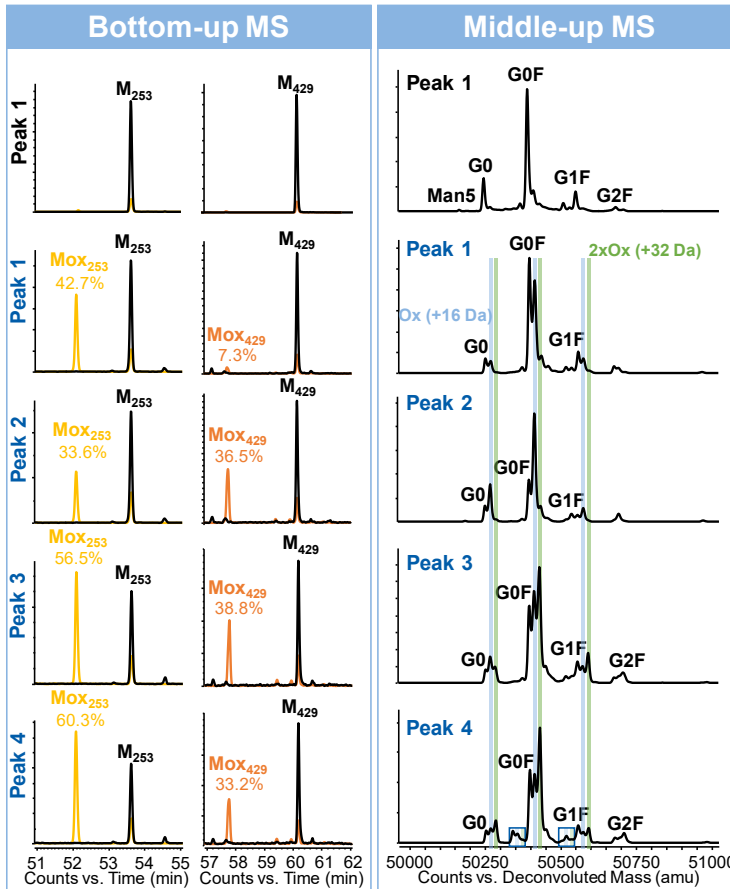
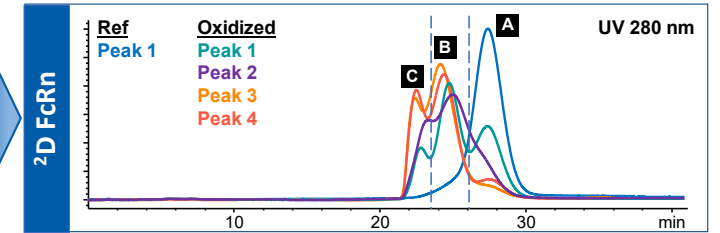
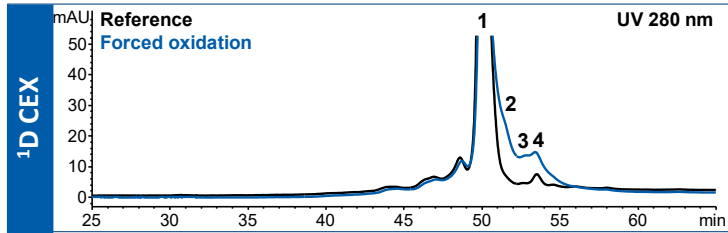




2D-CEX-FcRn-MS and mD-LC-MS

Peak identification and structure/function assessment is based on:

1. Intact MS data acquired with the 2D-CEX-FcRn-MS set-up
2. Bottom-up and middle-up MS data acquired with the MD-LC-MS set-up
3. Retention time differences in ²D FcRn

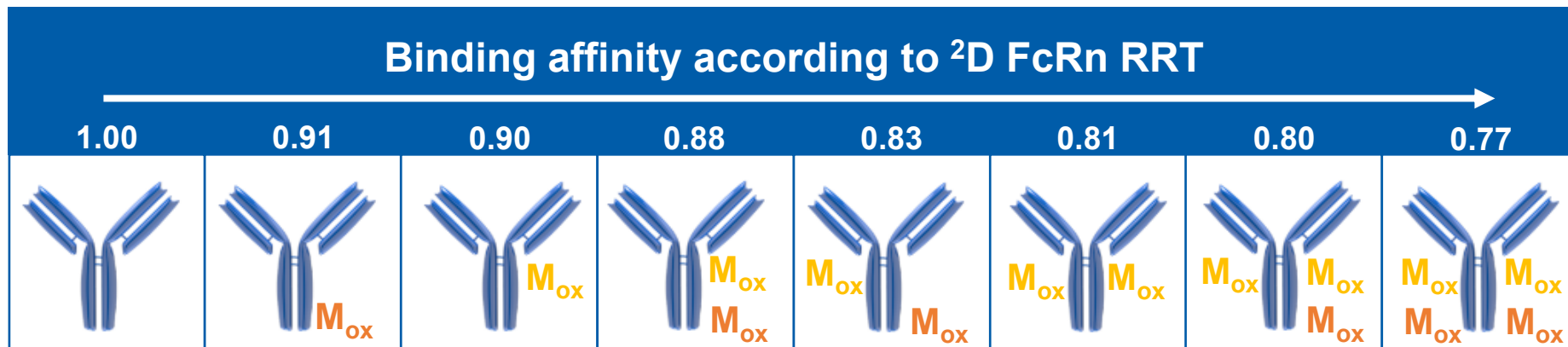




Ranking based on 2D-CEX-FcRn-MS and MD-LC-MS

I Ranking of oxidized (charge) variants

- Oxidation of HC M253 and M429 has an impact on FcRn binding with more impact of M253
- Oxidation of both HC has a bigger impact on FcRn binding compared to single chain oxidation





Acknowledgement

- | Liesa Verscheure, Shauni Detremmerie, Isabel Vandenheede, Eline De Rore, Gerd Vanhoenacker, Mabelle Meersseman, Pat Sandra, Koen Sandra
(RIC group, Belgium)
- | Hilde Stals, Valerie Hanssens, Kris Meerschaert, Sylvia Van Dorpe, Filip Borgions
(argenx, Belgium)
- | Sonja Schneider, Udo Huber
(Agilent Technologies)
- | Frederic Lynen
(Ghent University, Belgium)



RIC

biologics

YOUR MOLECULE. OUR ANALYTICS. NO SECRETS.

www.RIC-biologics.com