Development of novel chromatographic and mass spectrometric techniques for characterizations of Bispecific antibodies (BsAbs) and ADCs

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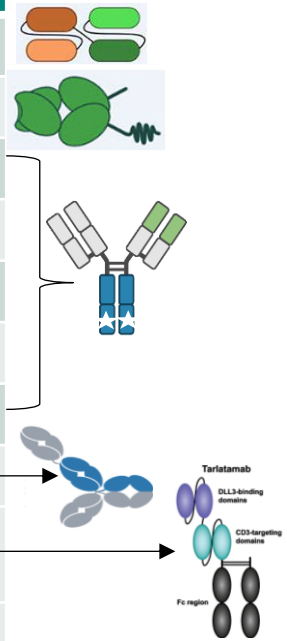
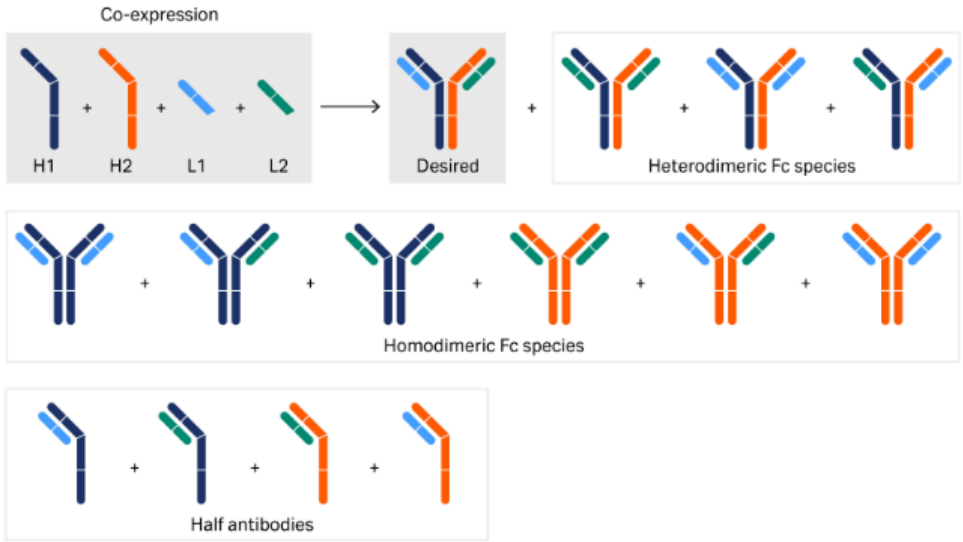
Background – Bispecific antibody (BsAb)

TCE	Target Ag	Indication(s)	FDA approved	Company	Format
BLINCYTO (Blinatumomab)	CD19	R/R B-ALL; B-ALL MRD	2014	Amgen	BiTE
KIMMTRAK (Tebentafusp)	Gp100	Uveal melanoma	2022	Immunocore/AZ	ImmTAC
TECVAYLI (Teclistamab)	BCMA	R/R MM	2022	Janssen/Genmab	DuoBody (Fab/Fab)
LUNSUMYO (Mosunetuzumab)	CD20	R/R folliclular lymphoma	2022	Roche/Genentech	KiH (Fab/Fab)
EPKINLY (epcoritamab)	CD20	R/R DLBCL	2023	Janssen/Genmab	DuoBody (Fab/Fab)
TALVEY (talquetamab)	GPRC5D	MM	2023	Janssen/Genmab	DuoBody (Fab/Fab)
ELREXFIO (elranatamab)	BCMA	R/R MM	2023	Pfizer/Genmab	DuoBody (Fab/Fab)
COLUMVI (glofitamab)	CD20	R/R DLBCL/LBCL	2023	Roche/Genentech	KiH (2:1 Fab/Fab)
IMDELTTRA (tarlatamab)	DLL3	R/R ES-SCLC following Pt-based chemo	2024	Amgen	HLE-BiTE
>60 TCEs at different stages of clinical development					





Mis-paired species are a Critical Quality Attribute (CQA) that need to be characterized

Challenges:

- High similarity between mispairing impurities and the desired BsAb
- Ion suppression by highly abundant BsAb species in RPLC

https://www.cytivalifesciences.com/en/us/Solutions/Bioprocessing/Knowledge-center/Purifying-bispecific-antibodies-in-a-single-step

Workflow for BsAb impurities characterizations

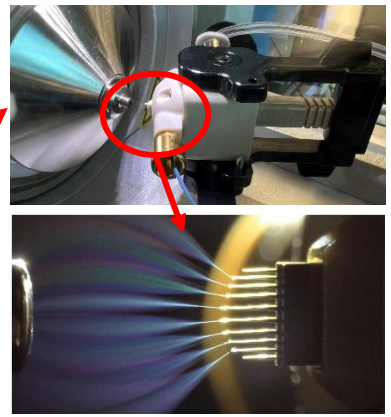
Charge and size, native

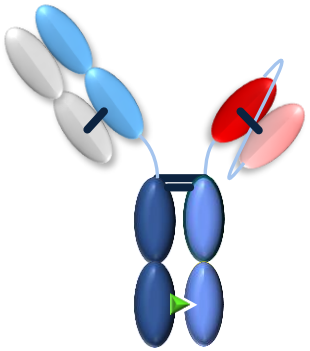
Hydrophobicity, denatured

Native SEC-MS → Native SCX-MS → HIC-RP-MS → RPLC-MS

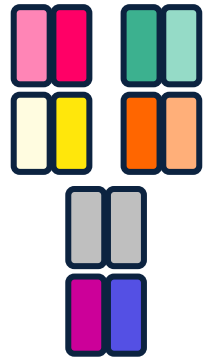
Hydrodynamic radius, native

Hydrophobicity, native





Fab-ScFv-Fc



Fab-Fab-Fc

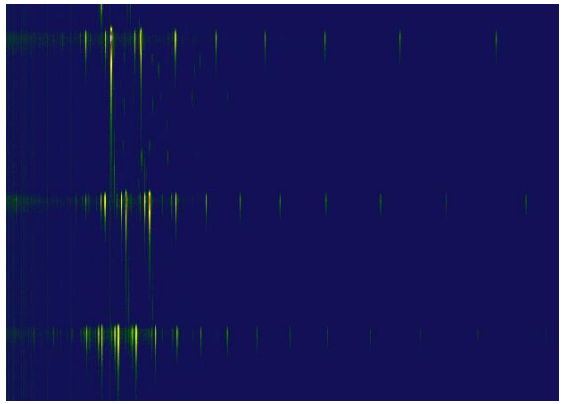
Ma, Fengfei, et al. "Hyphenation of strong cation exchange chromatography to native mass spectrometry for high throughput online characterization of charge heterogeneity of therapeutic monoclonal antibodies." *MAbs*. Vol. 12. No. 1. Taylor & Francis, 2020.

Automated MS-based Characterization of Native Proteins

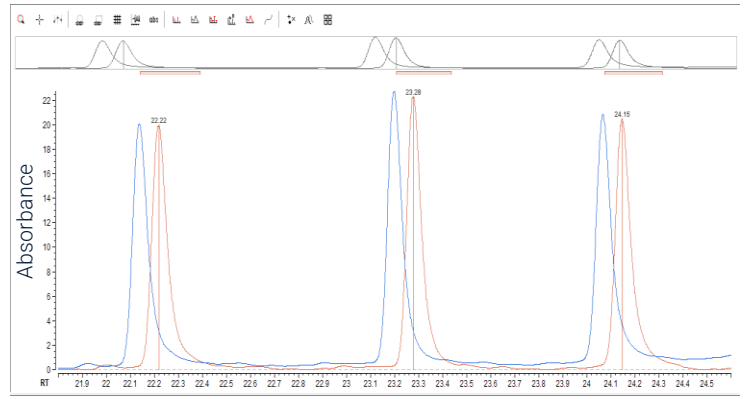
INTACT MS
ON QE HFX OR AGILENT QTOF

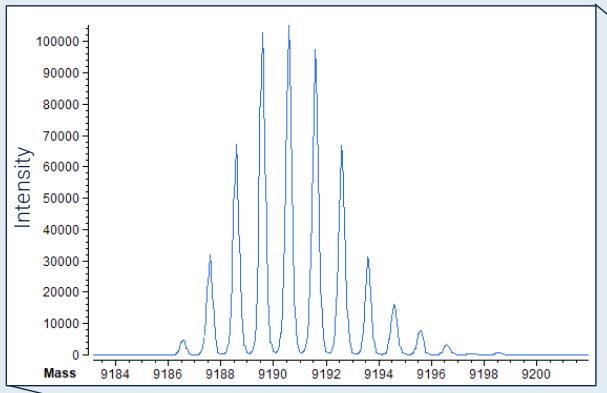
Genedata EXPRESSIONIST

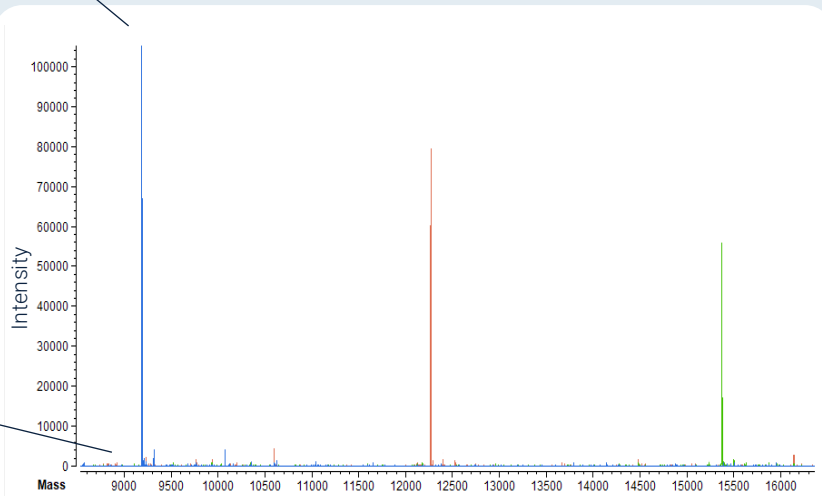
ION MAP – RAW DATA OVERVIEW



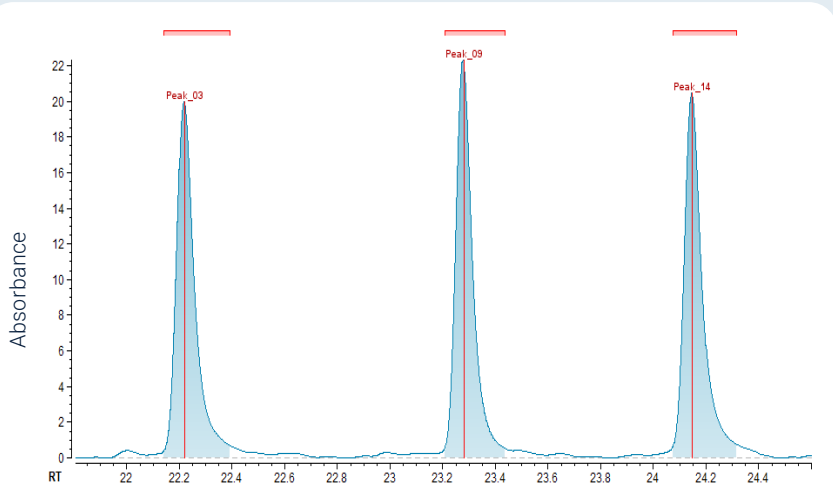
AUTOMATED UV TO MS ALIGNMENT





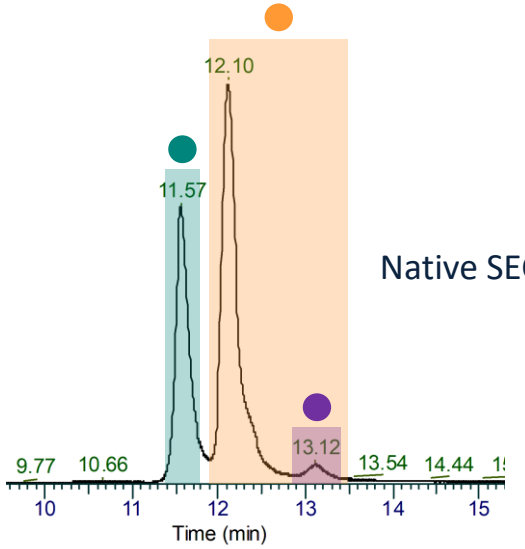


DECONVOLUTED MS DATA

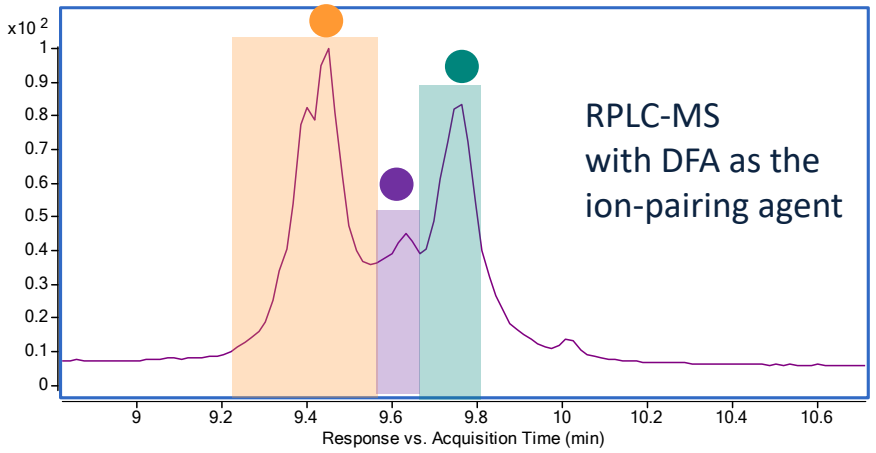


UV PEAKS USED FOR RT RANGE CONDENSING

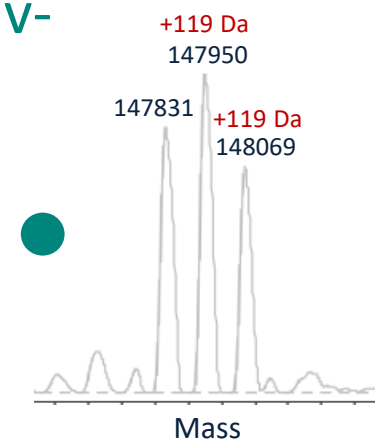
Comparison of Native SEC-MS, HIC-MS and RPLC-MS for Fab-ScFv-Fc impurities separation

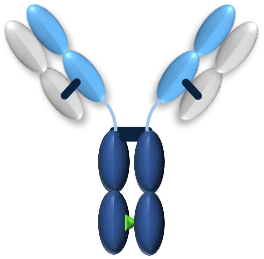


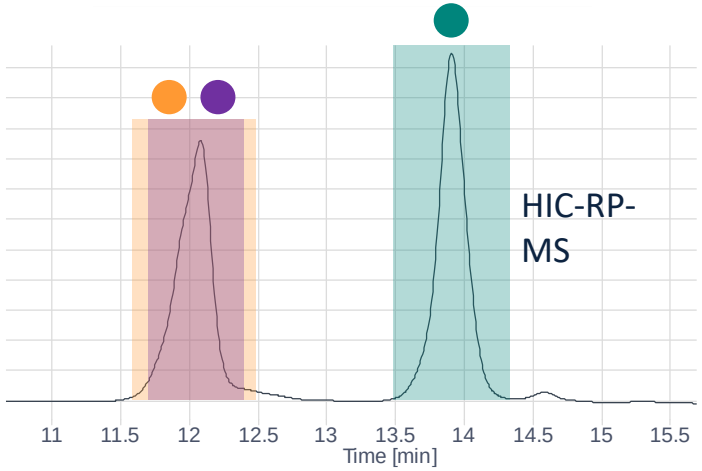
Native SEC-MS



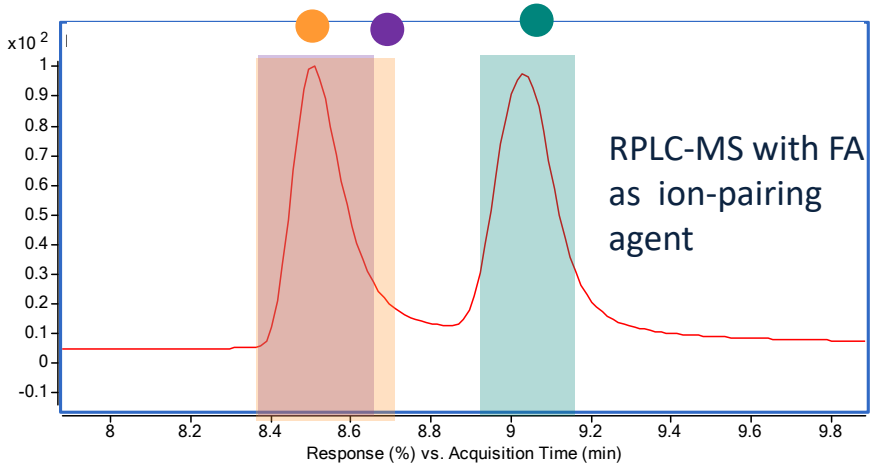
RPLC-MS with DFA as the ion-pairing agent



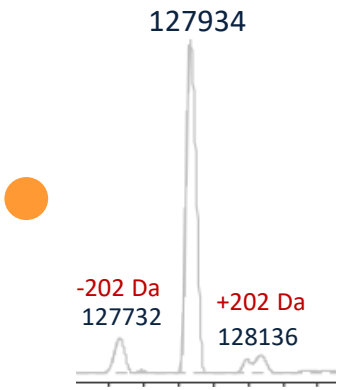


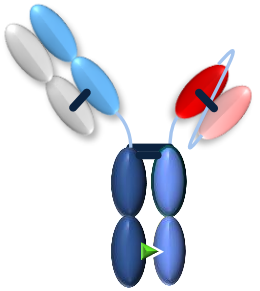


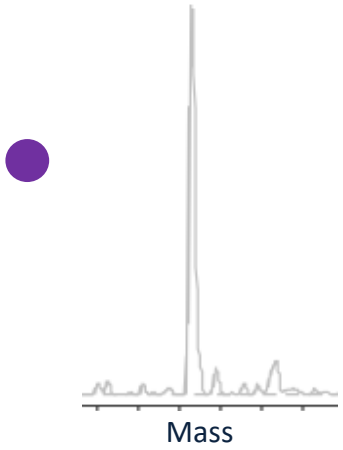
HIC-RP-MS

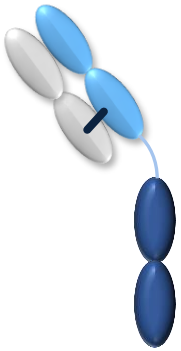


RPLC-MS with FA as ion-pairing agent



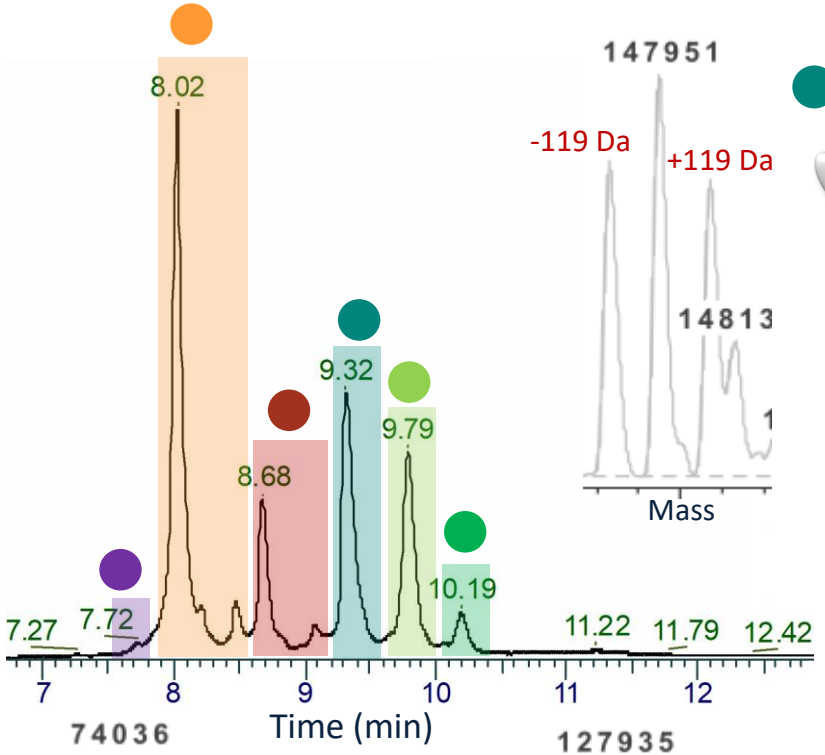


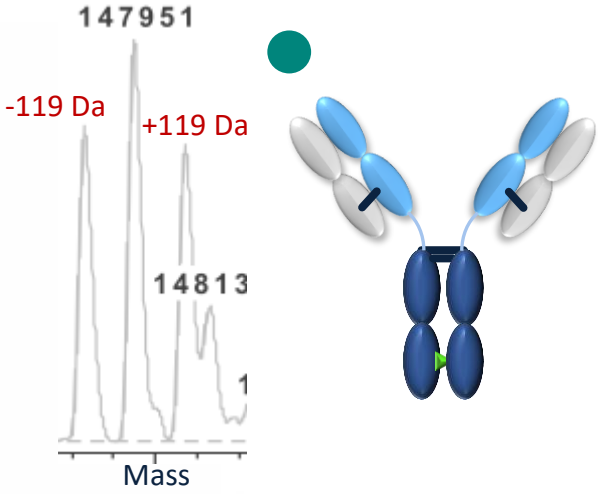


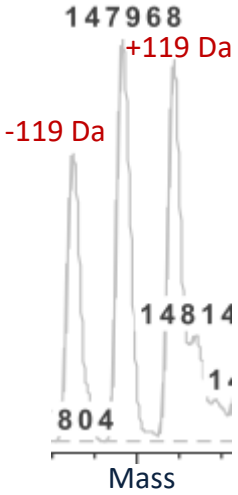


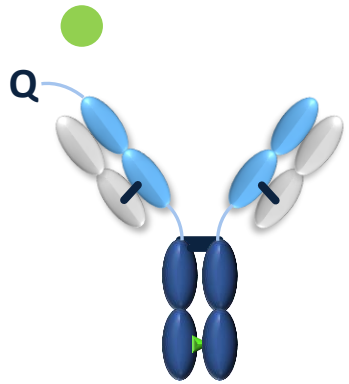
- Three species are observed: **bsAb**, **homodimer**, and **half-mAb**.
- DFA has sightly better resolution than FA.
- Baseline resolution can be achieved, but coelution may affect quantitation.

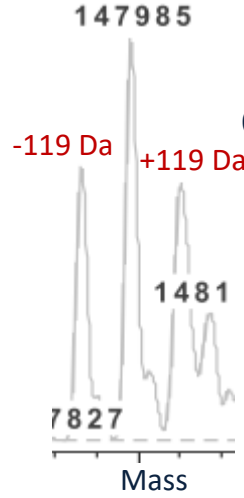
Native SCX-MS provides superior chromatographic separation for Fab-ScFv-Fc

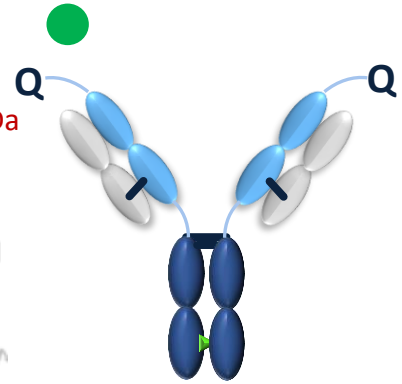


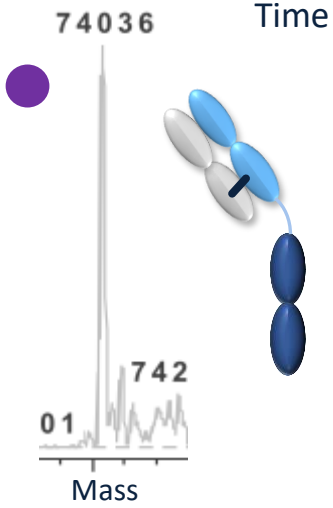


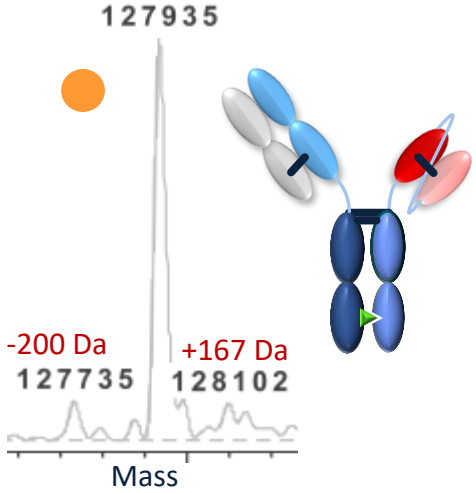


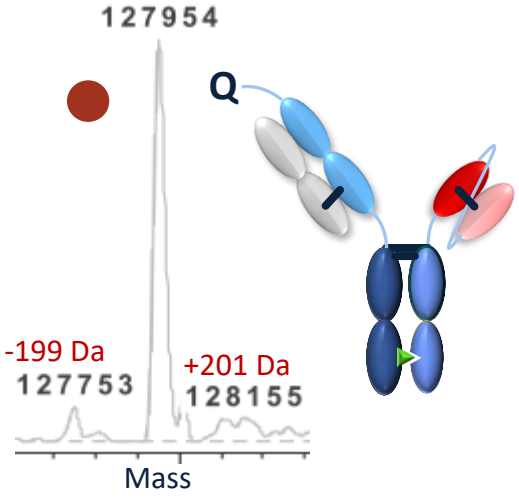






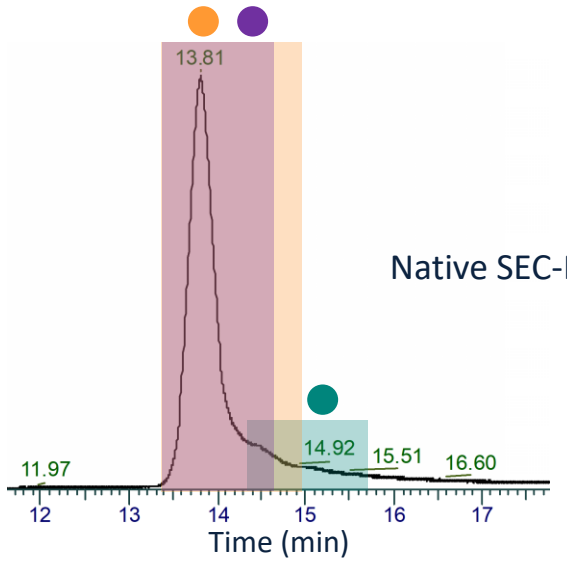




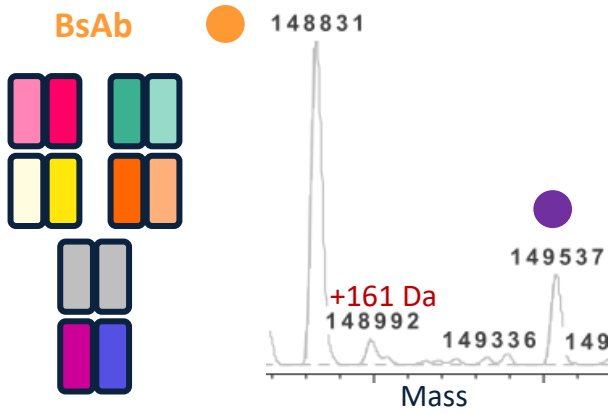


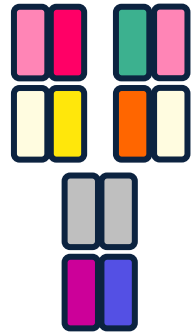
- **Three** more species are observed in addition to the **three** major species.
- Better separation is achieved to resolve all unprocessed N-glutamine forms which cannot be detected by other methods.
- Optimal chromatographic separation (baseline resolved) combined with high MS S/N makes quantitation of all species possible.

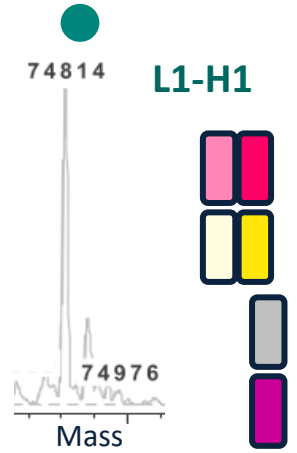
Comparison of Native SEC-MS and RPLC-MS Fab-Fab-Fc impurities separation

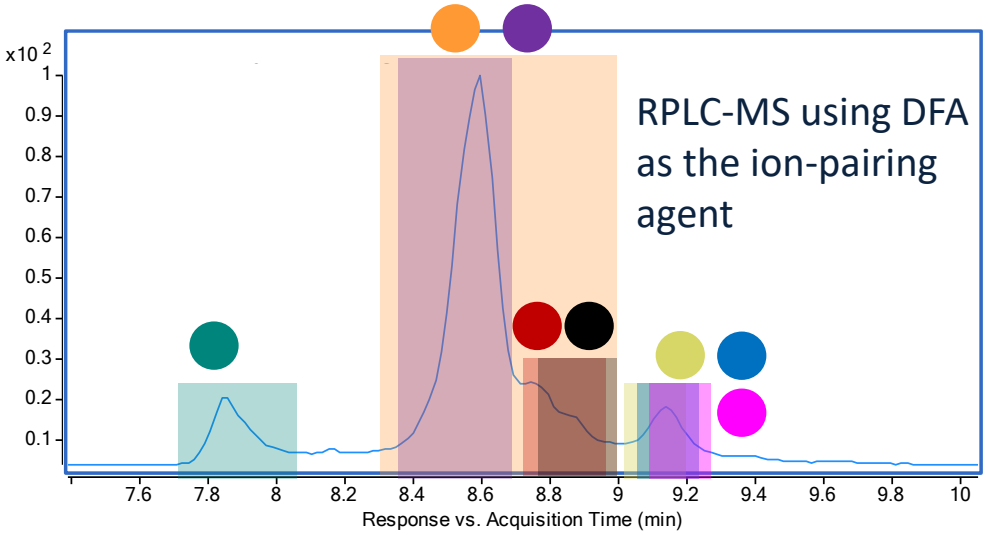


Native SEC-MS

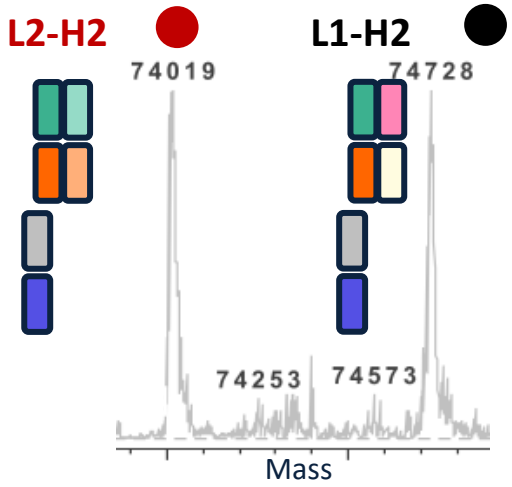


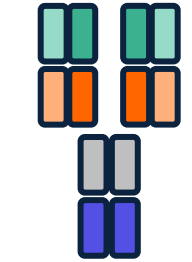


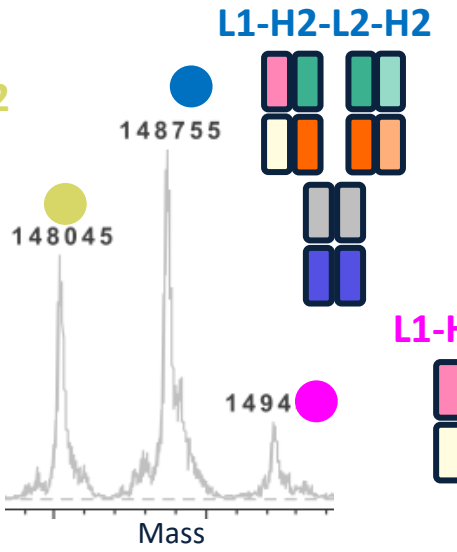


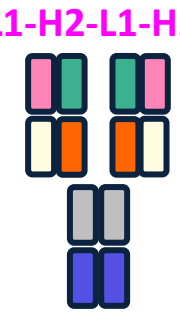


RPLC-MS using DFA as the ion-pairing agent



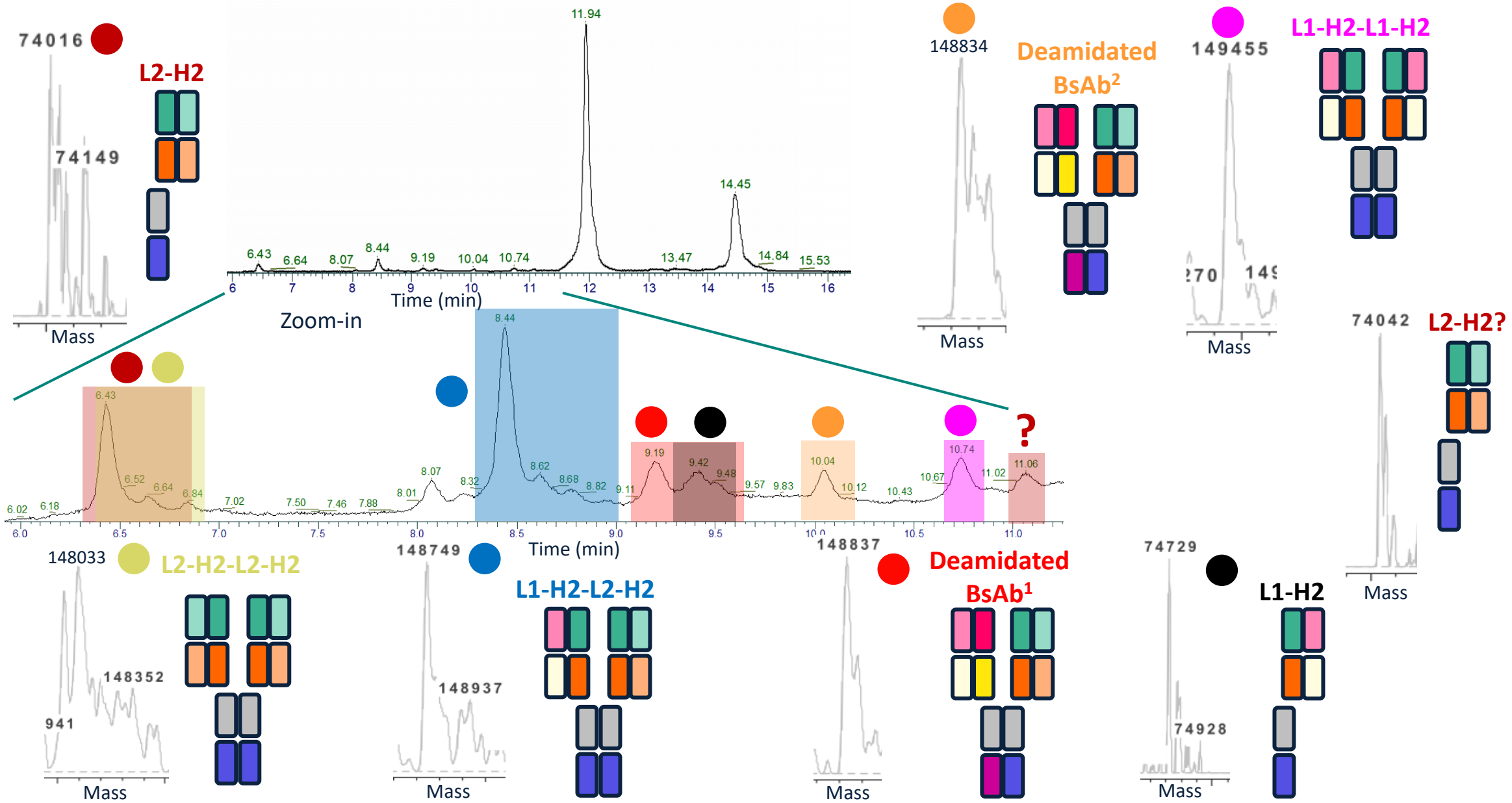




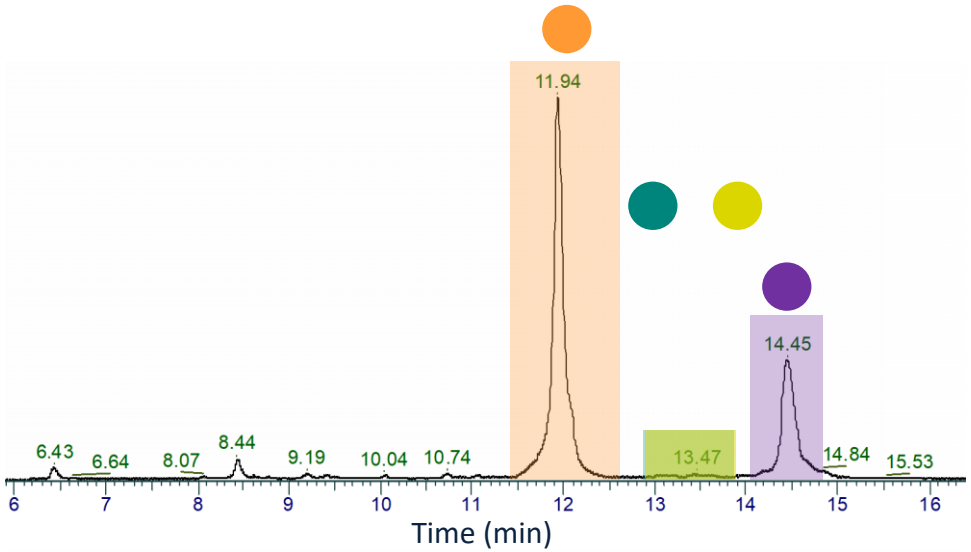


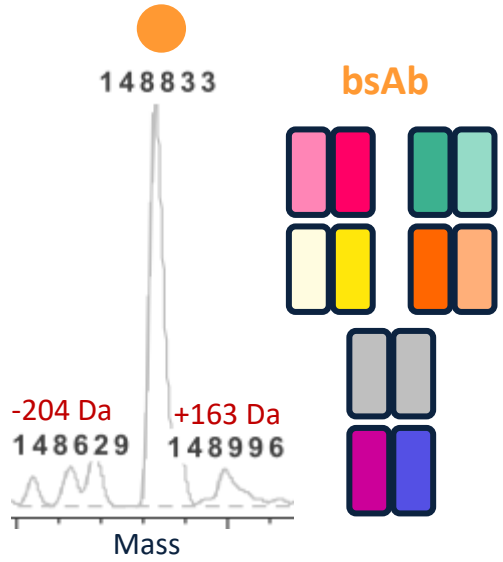
- **Five** more species observed by RPLC in addition to the **three** observed by SEC, demonstrates the importance of chromatographic separation for MS detection.
- Quantitation by RPLC is still challenging due to coelution of multiple species.

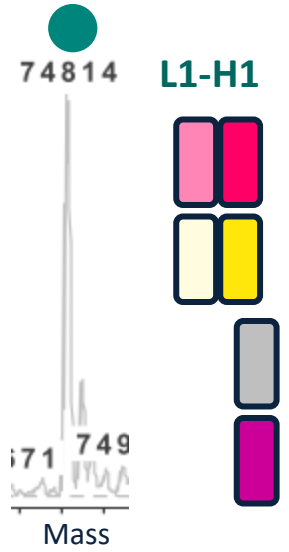
Native SCX-MS provides superior chromatographic separation for Fab-Fab-Fc

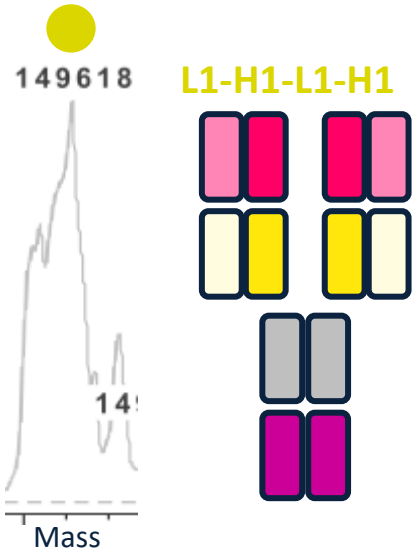


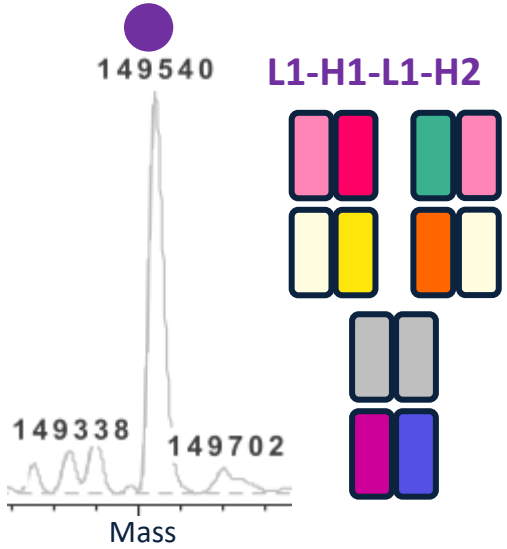
Native SCX-MS provides superior chromatographic separation for Fab-Fab-Fc









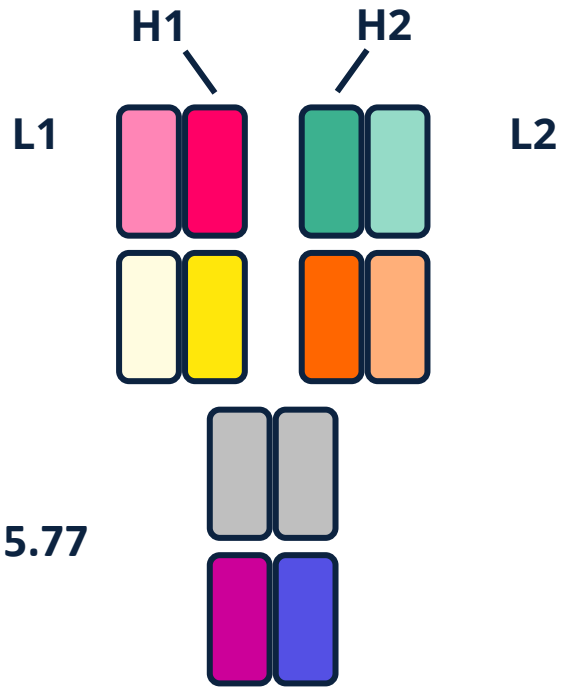


- **One** more species are observed by SCX in addition to the **eight** observed by RPLC.
- Deamidation can be detected by SCX due to the change of PI, highlighting the advantage of SCX to detect impurities as well as their PTMs.
- Although there is still coelution, the high separation efficiency and baseline resolution of SCX makes quantitation possible.

Reliable quantitation can be achieved by SCX

Species	Theoretical Mass (Da)	Observed mass (Da)	Peak area (UV)	Percentage (%)
bsAb (L1-H1-L2-H2)	148832	148833	1371779	**65.09**
L1-H1-L1-H2	149540	149540	538371	**25.54**
L1-H2-L2-H2	148744	148749	60434	**2.87**
L1-H2-L1-H2	149452	149455	15459	**0.73**
L2-H2/ L2-H2-L2-H2	74018/ 148036	74016/ 148033	35690	**1.69**
L1-H1/ L1-H1-L1-H1	74814/ 149628	74814/ 149618	69478	**3.30**
L1-H2	74726	74729	16404	**0.78**

CESDS (NR)		
LMW	Main Peak	HMW
5.8	94.2	0



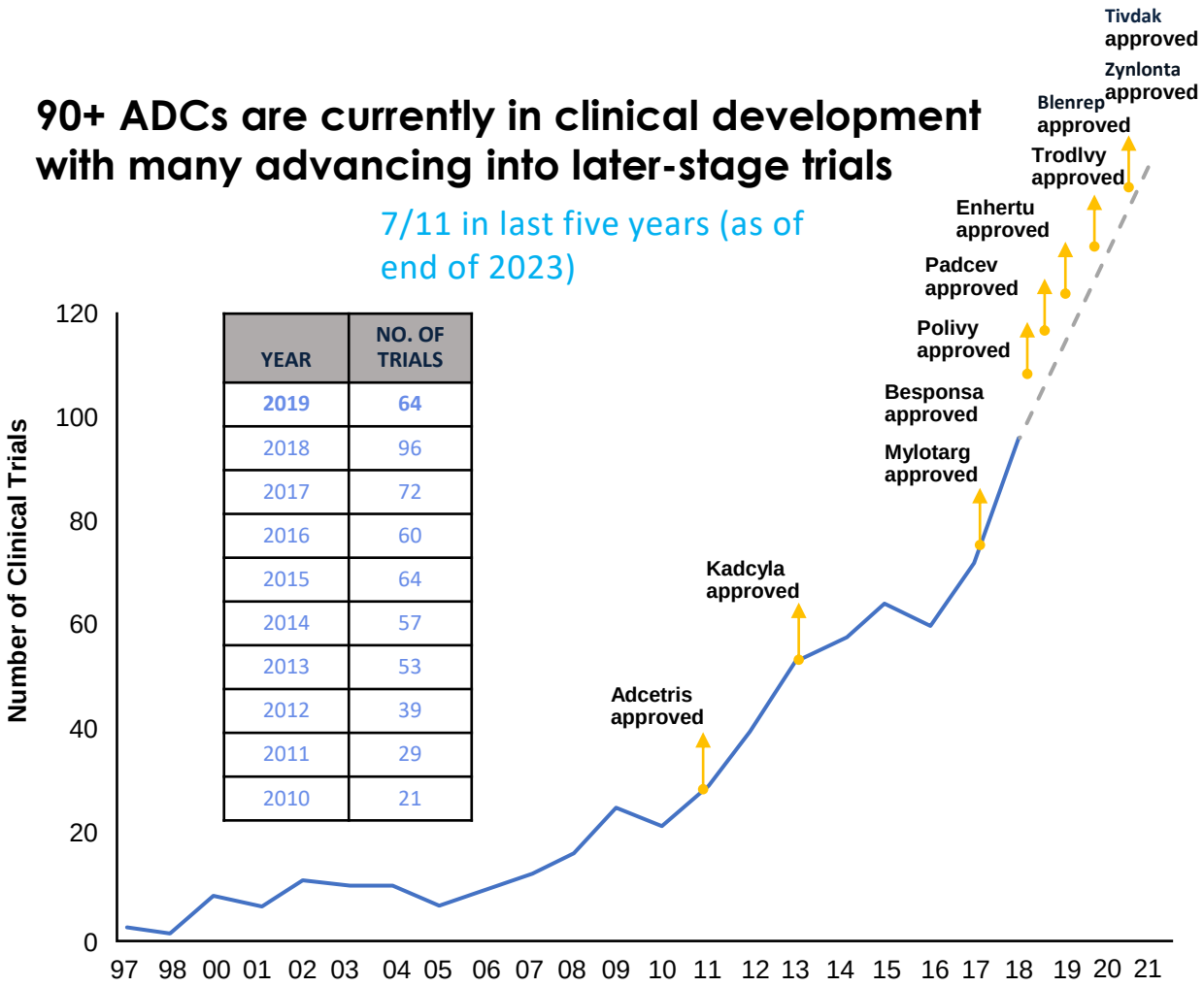
Summary

- Four different methods including SEC, RPLC, HIC and SCX have been assessed and compared to separate bispecific antibodies and their mis-pairing impurities.
- With the increase of the sample complexity, SCX is established to be the method with the highest separation efficiency.
- Replacing formic acid (FA) with difluoroacetic acid (DFA) as the ion-pairing agent for RPLC significantly improves its separation efficiency.
- SCX achieves successful quantitation of all samples, and the results are comparable to traditional CESDS method.
- Selection of unique techniques can be applied more for other modalities such as mAbs, ADCs, and fusion proteins.

Background – ADC

90+ ADCs are currently in clinical development with many advancing into later-stage trials

7/11 in last five years (as of end of 2023)

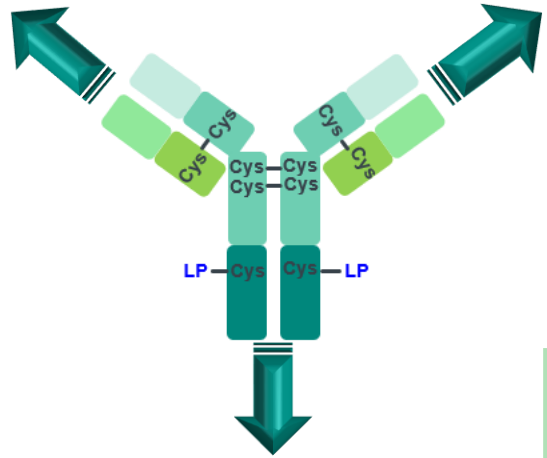


YEAR	NO. OF TRIALS
2019	64
2018	96
2017	72
2016	60
2015	64
2014	57
2013	53
2012	39
2011	29
2010	21

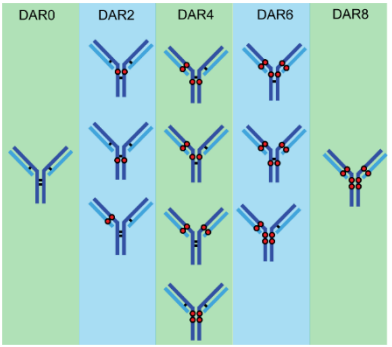
Complexity of ADCs characterizations

Variants in antibody
- During antibody production
- During conjugation process

Impurities in linker-payload
- From drug linker synthesis
- During conjugation process



Conjugation heterogeneity
- Conjugation sites
- DAR distributions

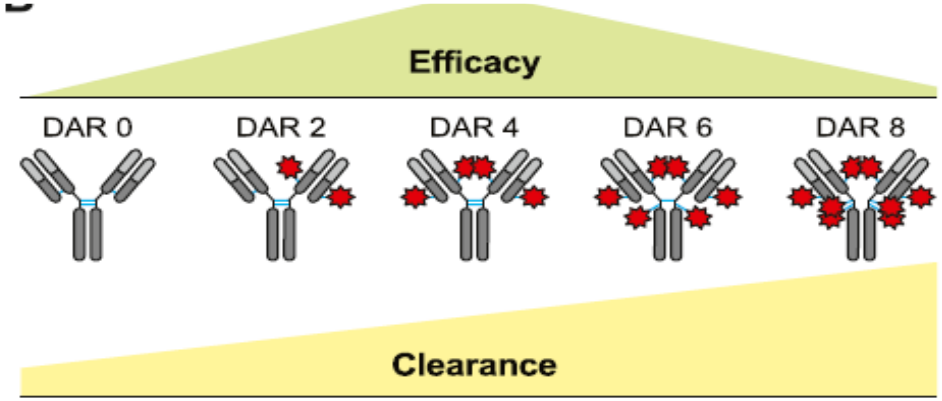


Hydrophobic interaction chromatography (HIC)

- Gold standard for drug distribution of ADCs.
- High salt buffer and low volatility (incompatible with MS).
- The salt reduces the solvation of sample so that hydrophobic region become exposed and adsorbed by media.
- Nondenaturing separation with decreasing salt concentration

Novel native Reversed-Phase Liquid Chromatography (nRPLC)

- LCMS characterization of ADCs.
- Low salt + IPA buffer and MS friendly.
- Lower hydrophobicity of bonded phase.
- Nondenaturing separation

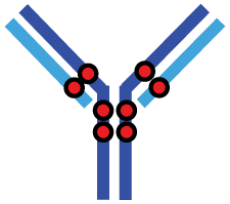


Why is it important to measure Drug to Antibody Ratio (DAR)?

- Low DAR value may indicate decreased efficacy
- Relatively high DAR value may negatively impact safety

Strop, Pavel, et al. "Site-specific conjugation improves therapeutic index of antibody drug conjugates with high drug loading." *Nature biotechnology* 33.7 (2015): 694-696.

Workflow for native Reversed-Phase Liquid Chromatography (nRPLC)-MS



ADC samples

Dionex 3000 UPLC

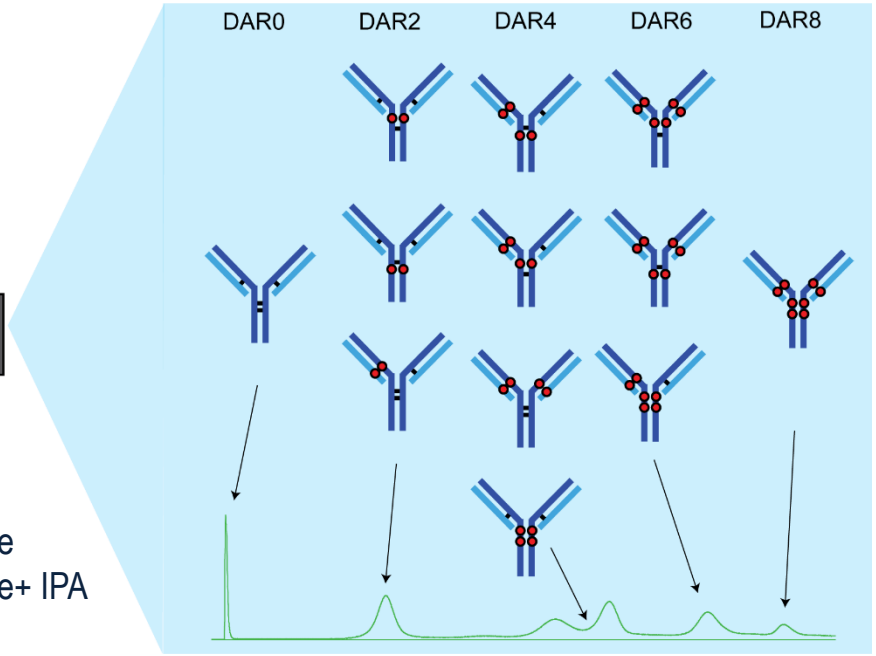
QE HF-X

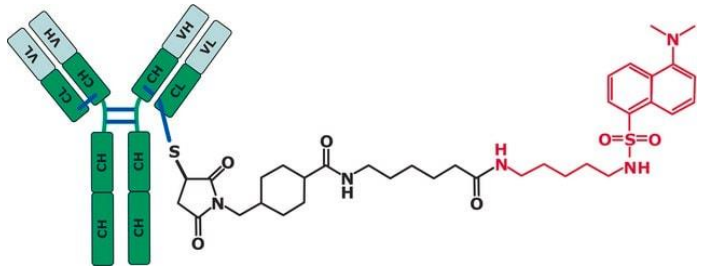
Genedata
solutions in silico

Data analysis

nRPLC-MS
V1 or V2 columns

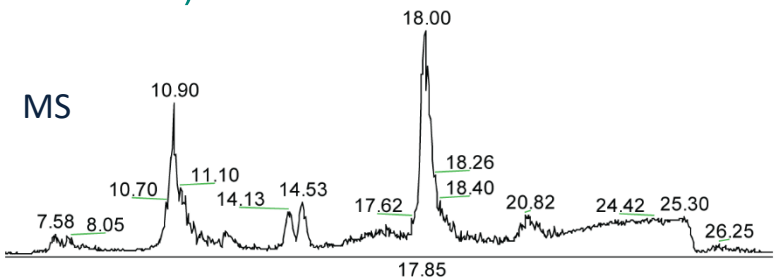
A: Ammonium Acetate
B: Ammonium Acetate+ IPA

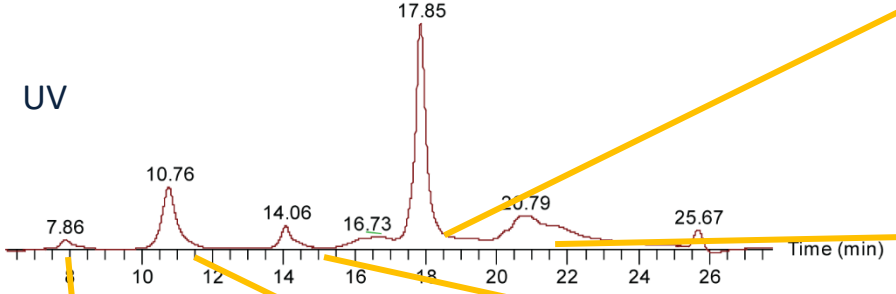


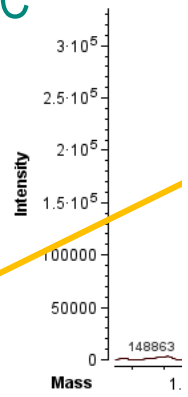


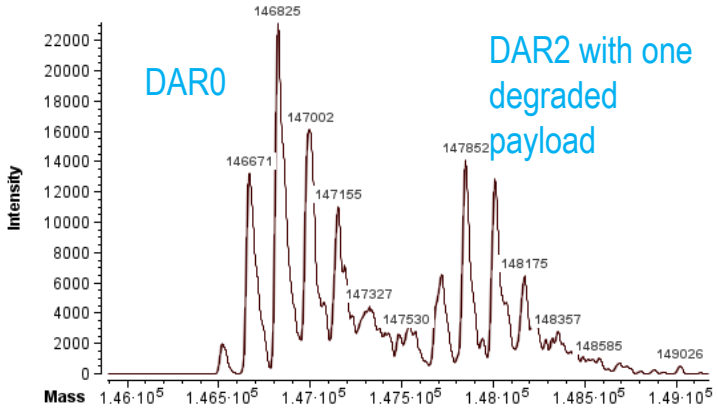
ADC mimic

Online separation and characterization of interchain linked ADC mimic (V1 column)

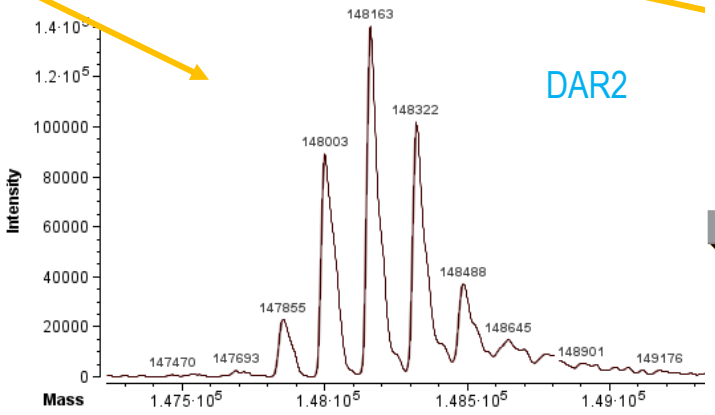








DAR2 with one degraded payload



ARTICLE

https://doi.org/10.1038/s41467-020-19498-y OPEN

Co-administered antibody improves penetration of antibody–dye conjugate into human cancers with implications for antibody–drug conjugates

Guolan Lu[1,7], Naoki Nishio[1,2,7], Nynke S. van den Berg[1], Brock A. Martin[3], Shayan Fakurnejad[1], Stan van Keulen[1], Alexander D. Colevas[4], Greg M. Thurber[5,6] & Eben L. Rosenthal[1✉]

Poor tissue penetration remains a major challenge for antibody-based therapeutics of solid tumors, but proper dosing can improve the tissue penetration and thus therapeutic efficacy of these biologics. Due to dose-limiting toxicity of the small molecule payload, antibody-drug conjugates (ADCs) are administered at a much lower dose than their parent antibodies, which further reduces tissue penetration. We conducted an early-phase clinical trial (NCT02415881) and previously reported the safety of an antibody-dye conjugate (panitumumab-IRDye800CW) as primary outcome. Here, we report a retrospective exploratory analysis of the trial to evaluate whether co-administration of an unconjugated antibody could improve the intratumoral distribution of the antibody-dye conjugate in patients. By measuring the multiscale distribution of the antibody-dye conjugate, this study demonstrates improved microscopic antibody distribution without increasing uptake (toxicity) in healthy tissue when co-administered with the parent antibody, supporting further clinical investigation of the co-administration dosing strategy to improve the tumor penetration of ADCs.

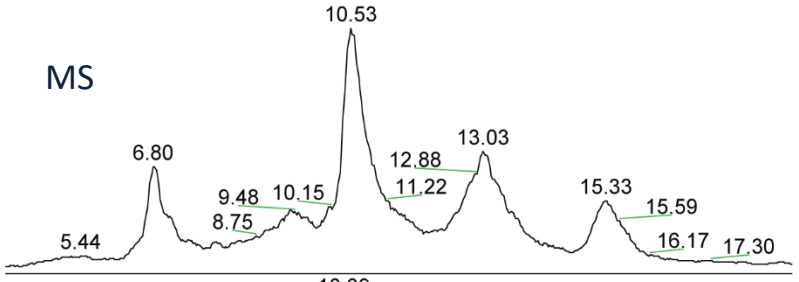
Antibody Co-Administration Can Improve Systemic and Local Distribution of Antibody–Drug Conjugates to Increase *In Vivo* Efficacy

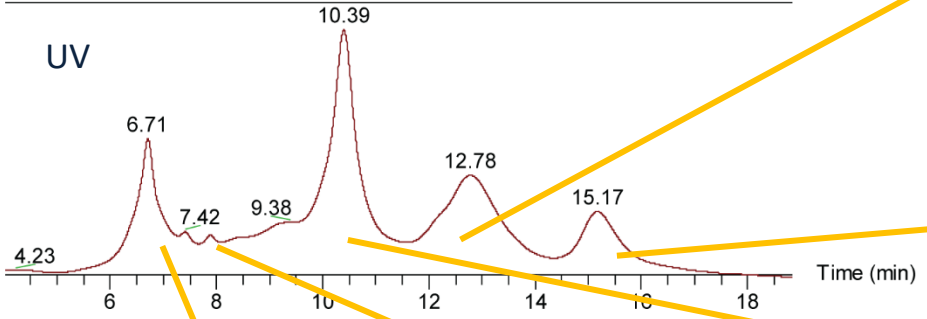
Jose F. Ponte[1], Leanne Lanieri[1], Eshita Khera[2], Rassol Laleau[1], Olga Ab[1], Christopher Espelin[1], Neeraj Kohli[1], Bahar Matin[1], Yulius Setiady[1], Michael L. Miller[1], Thomas A. Keating[1], Ravi Chari[1], Jan Pinkas[1], Richard Gregory[1], and Greg M. Thurber[2,3]

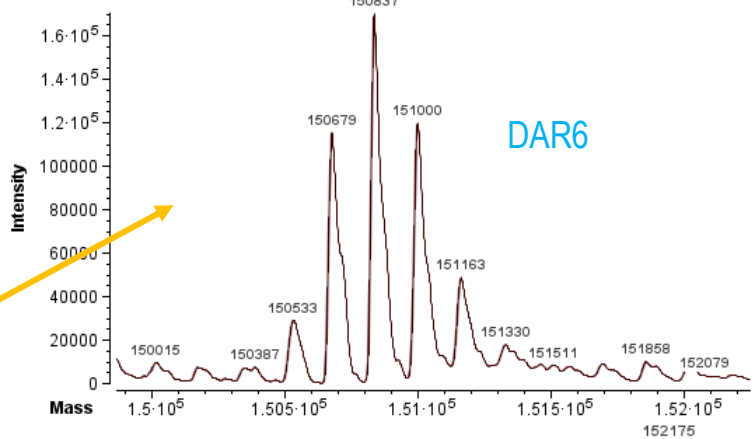
ABSTRACT

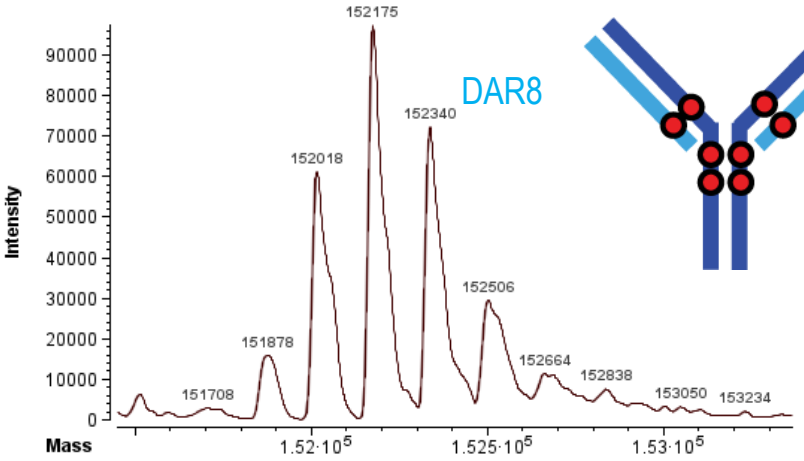
Several antibody–drug conjugates (ADC) showing strong clinical responses in solid tumors target high expression antigens (HER2, TROP2, Nectin-4, and folate receptor alpha/FRα). Highly expressed tumor antigens often have significant low-level expression in normal tissues, resulting in the potential for target-mediated drug disposition (TMDD) and increased clearance. However, ADCs often do not cross-react with normal tissue in animal models used to test efficacy (typically mice), and the impact of ADC binding to normal tissue antigens on tumor response remains unclear. An antibody that cross-reacts with human and murine FRα was generated and tested in an animal model where the antibody/ADC bind both human tumor FRα and mouse FRα in normal tissue. Previous work has demonstrated that a "carrier" dose of unconjugated antibody can improve the tumor penetration of ADCs with high expression target-antigens. A carrier dose was employed to study the impact on cross-reactive ADC clearance, distribution, and efficacy. Co-administration of unconjugated anti-FRα antibody with the ADC-improved efficacy, even in low expression models where co-administration normally lowers efficacy. By reducing target-antigen–mediated clearance in normal tissue, the co-administered antibody increased systemic exposure, improved tumor tissue penetration, reduced target-antigen–mediated uptake in normal tissue, and increased ADC efficacy. However, payload potency and tumor antigen saturation are also critical to efficacy, as shown with reduced efficacy using too high of a carrier dose. The judicious use of higher antibody doses, either through lower DAR or carrier doses, can improve the therapeutic window by increasing efficacy while lowering target-mediated toxicity in normal tissue.

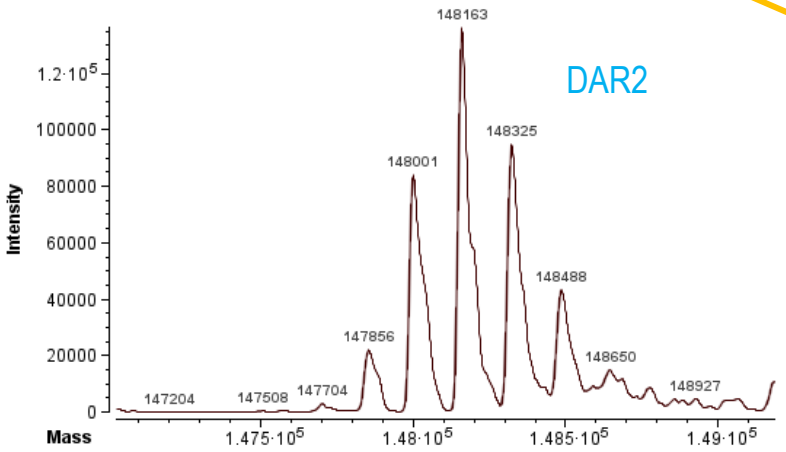
Online separation and characterization of interchain linked ADC mimic (V2 column)

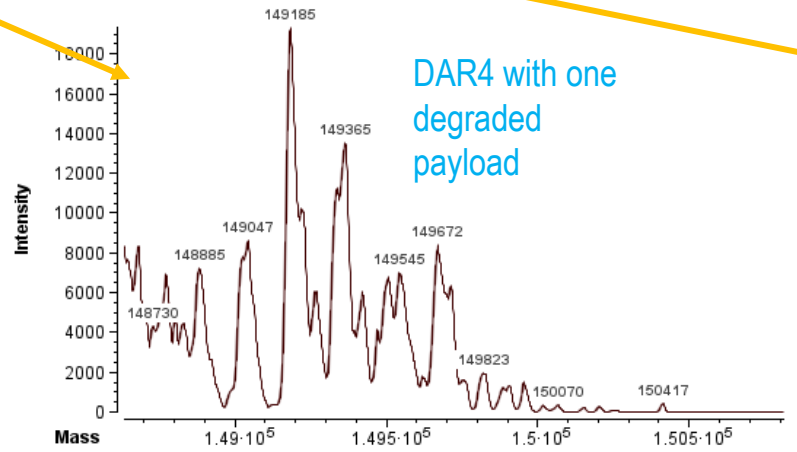


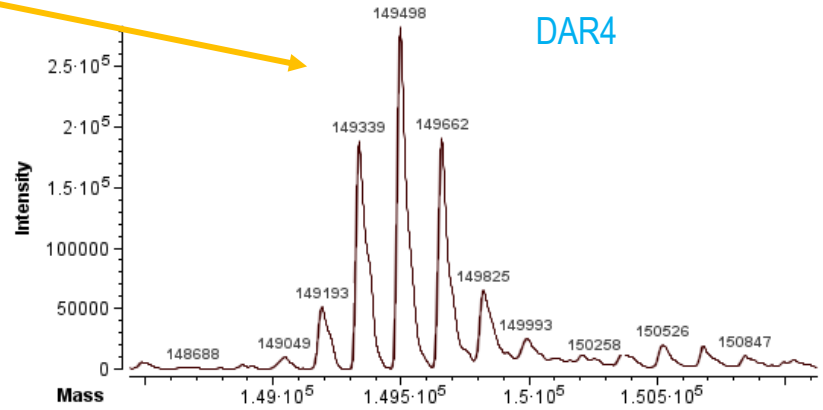




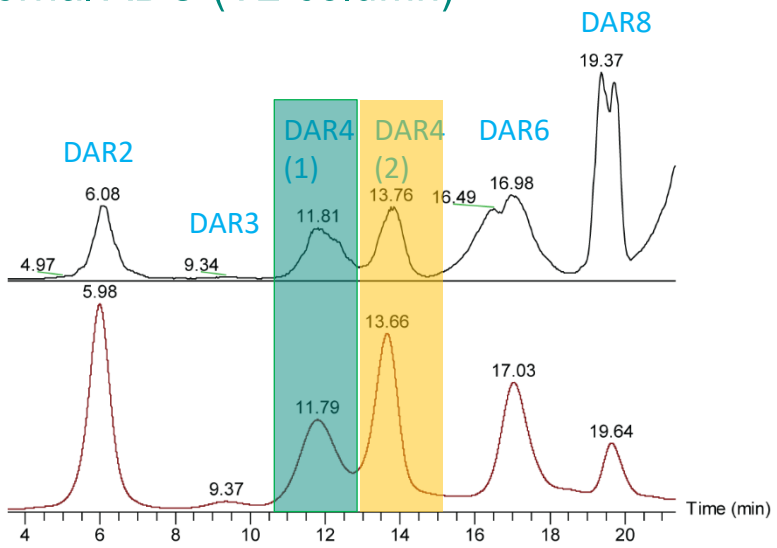


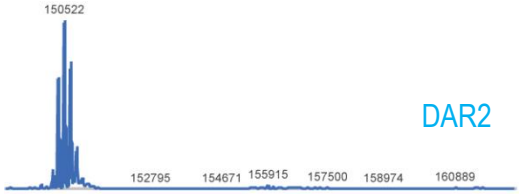


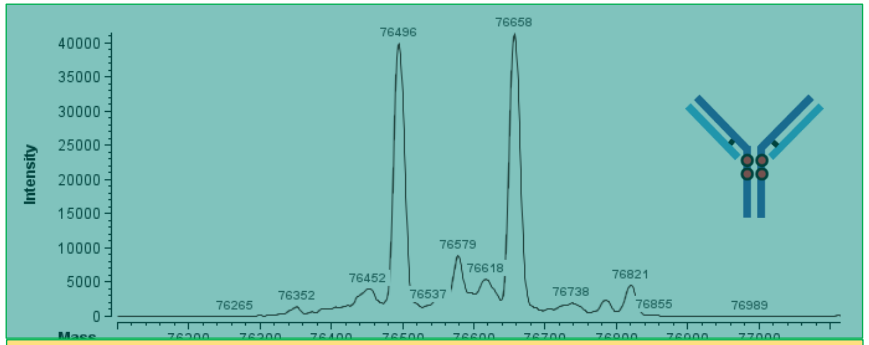


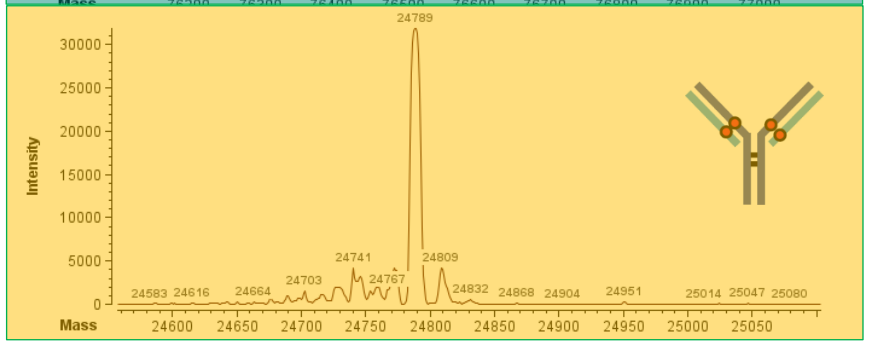


Online separation and characterization of interchain linked internal ADC (V2 column)

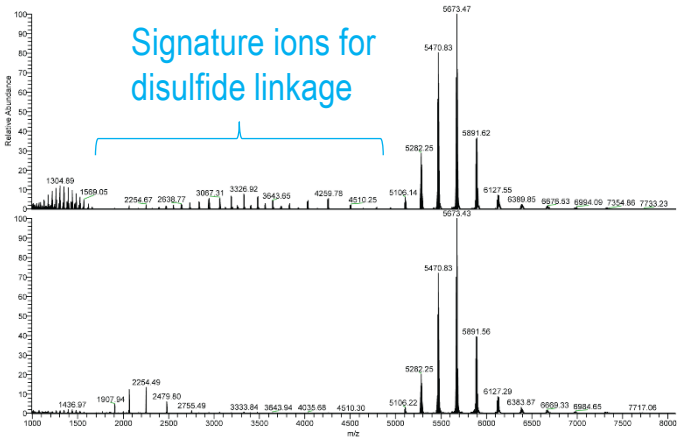




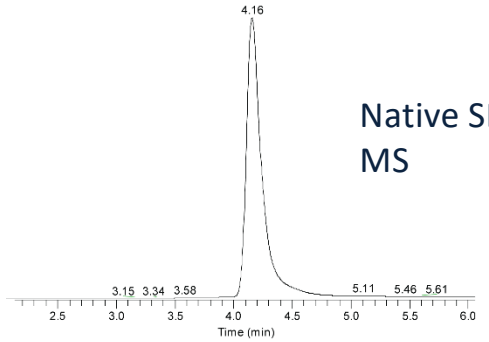




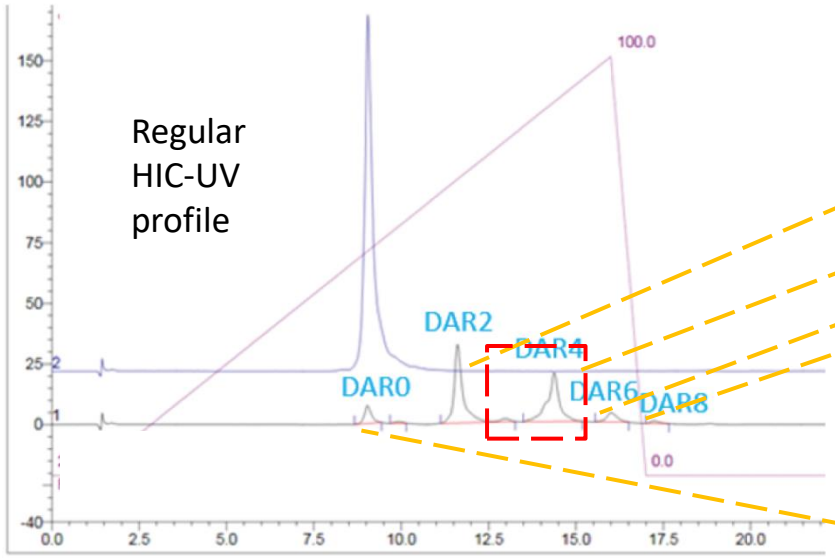
Signature ions for disulfide linkage



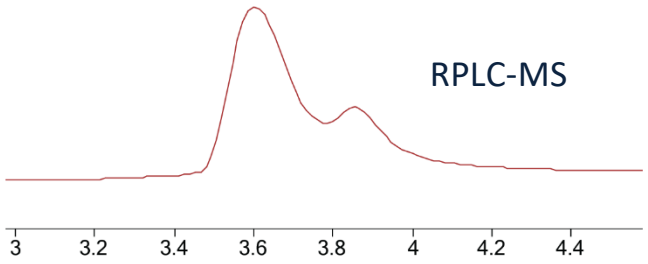
nRPLC-MS analysis provides comparable performance to regular HIC method



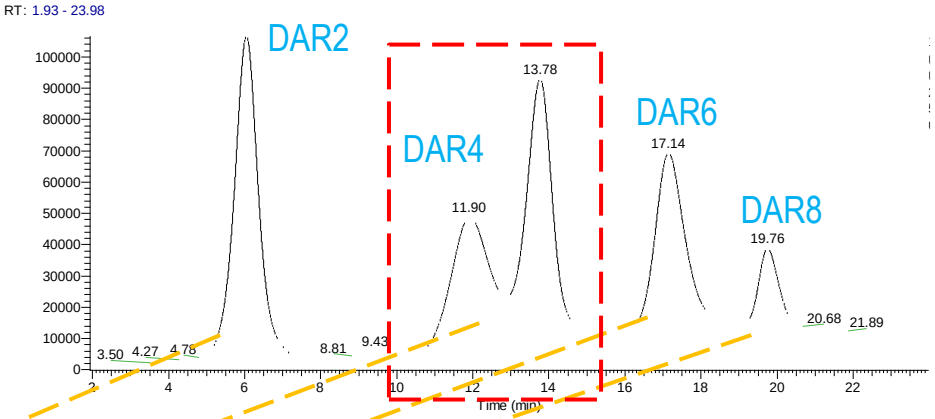
Native SEC-MS



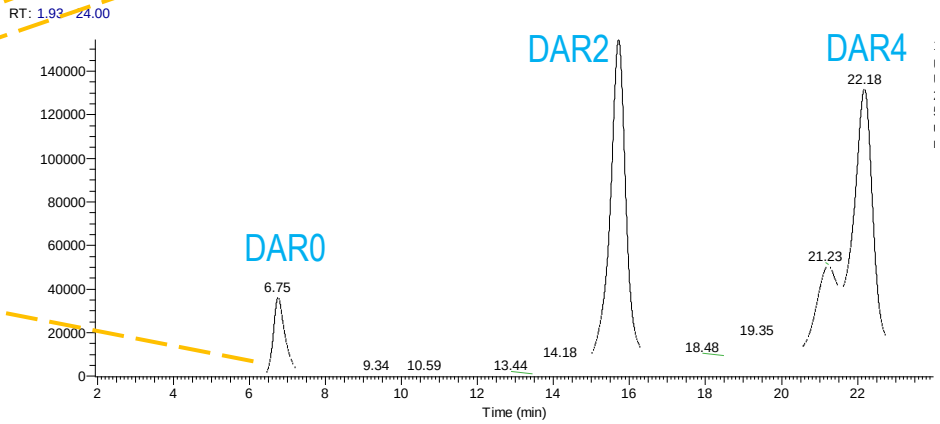
Regular HIC-UV profile



RPLC-MS

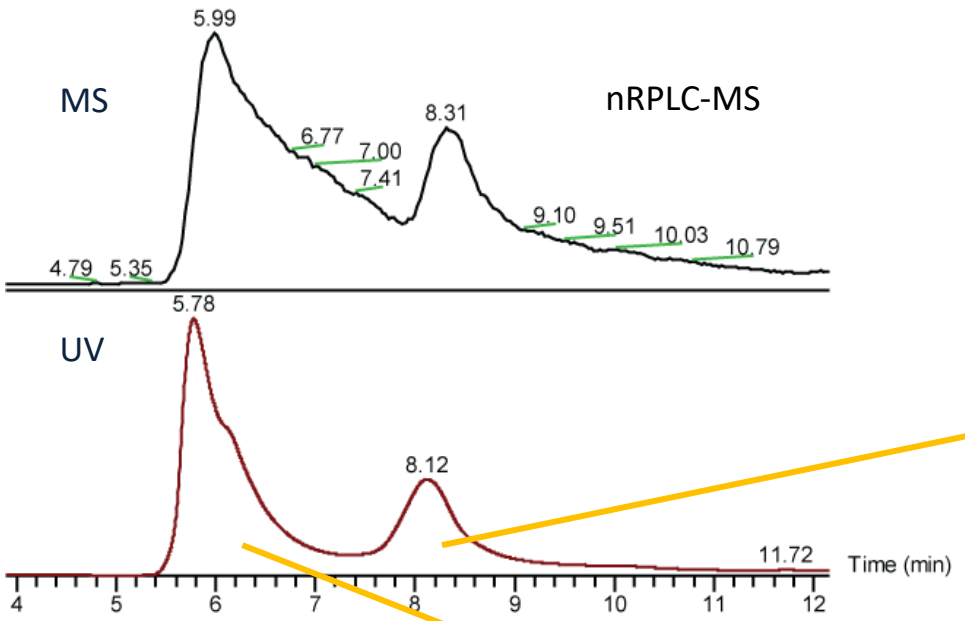


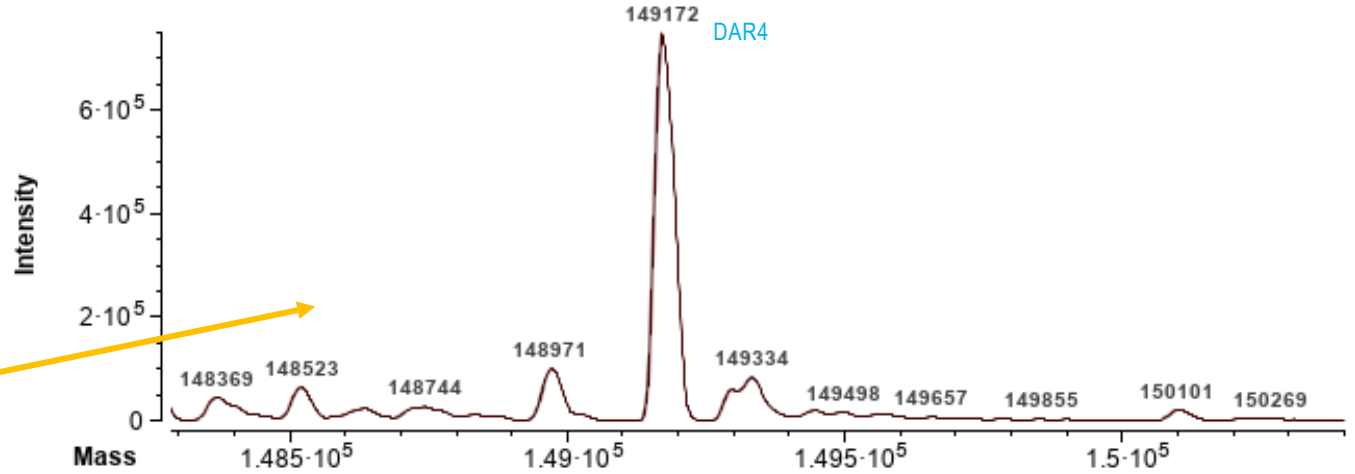
V2 column, more hydrophilic based bonded phases

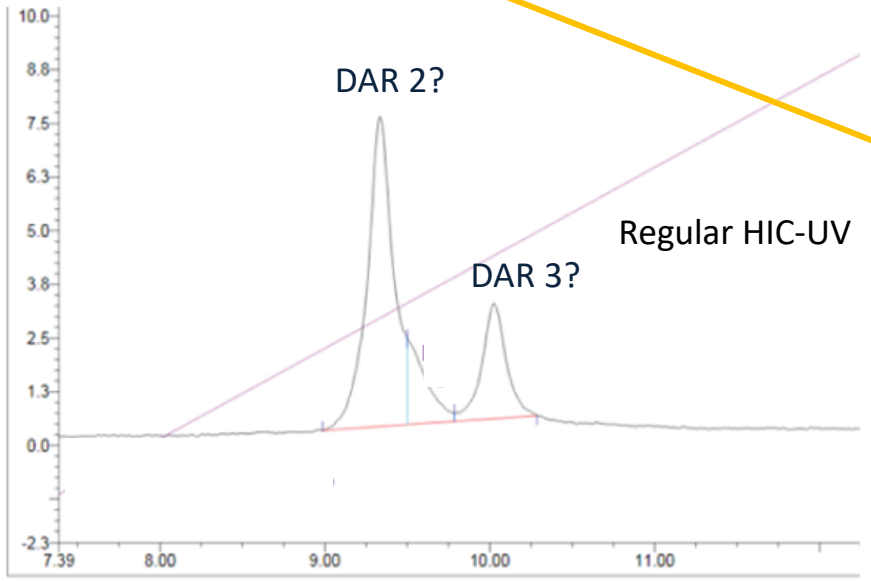


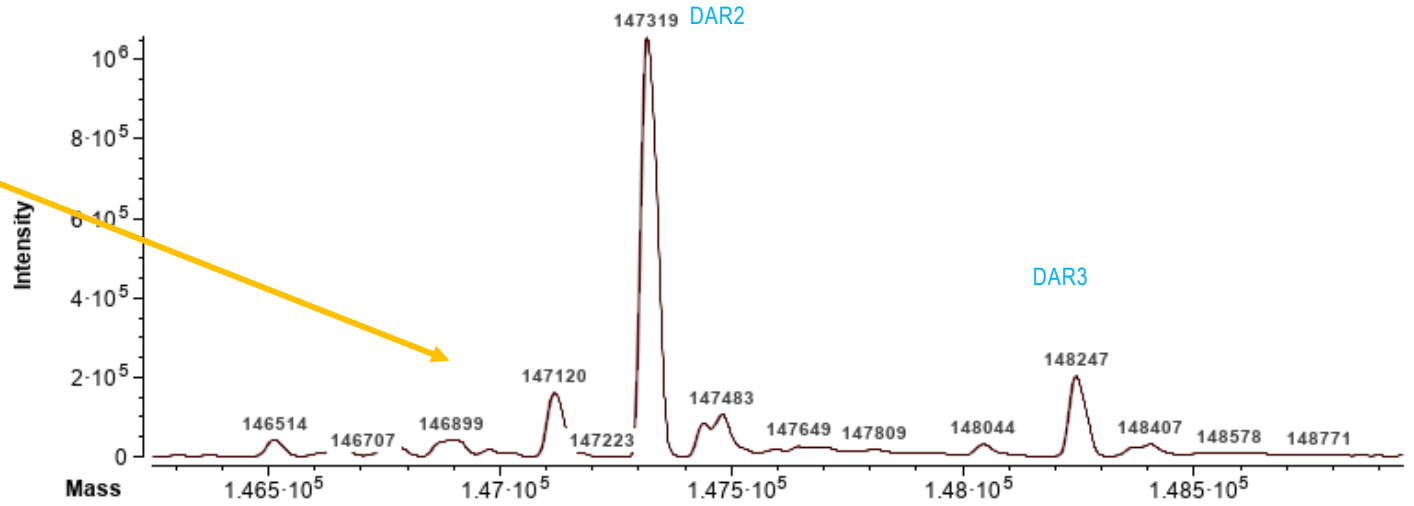
V1 column, more hydrophobic based bonded phases

Online separation and characterization of site-specific ADC (V1 column)









Summary

- Successful establishment of native RPLC-MS method for ADC characterization
- Effective separation of ADC with different DAR species (DAR0-8)
- Comparable results with regular HIC profile.
- Applicable for analysis of both interchain linked and site-specific conjugations
- Positional isomers with different conjugation linkages (conjugation in either the hinge region or between heavy chain and light chain) can be chromatographically resolved and verified

Acknowledgements

Analytical R&D

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Christian Morales

Kanaka Hettiarachchi

Mike Seganish

Discovery Biologics

Mehabaw Derebe

Esther Kofman

Laurence Fayadat-Dilman

Phenomenex

James Song

Carl Sanchez

Genedata

Can Cui

Aude Tartiere

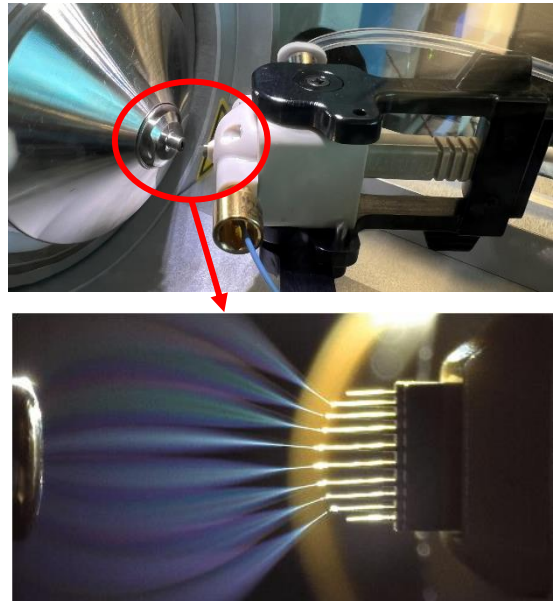
Maurizio Bronzetti

Thank you

- Back up

Method development – SEC and SCX

High-resolution orbitrap



SEC method
Column: Waters BEH200 SEC 4.6 × 300 mm, 200 Å, 1.7 µm

Time (min)	Flow rate (ml/min)	%A (50mM AA)
0	0.2	100
24	0.2	100

Time (min)	Flow rate (ml/min)	%A (20mM AA with acetic acid)	%B (140mM AA, 10mM AB)
0	0.2	100	0
2	0.2	100	0
13.5	0.2	0	100
16	0.2	0	100
16.5	0.2	100	0
22	0.2	100	0

SCX method
Column: Thermo MABPac SCX-10 RS 2.1 mm × 50 mm, 5 µm

Ma, F., Raofi, F., Bailly, M., Fayadat-Dilman, L., Tomazela, D. (2020). mAbs. 12(1)

Method development – RPLC and HIC

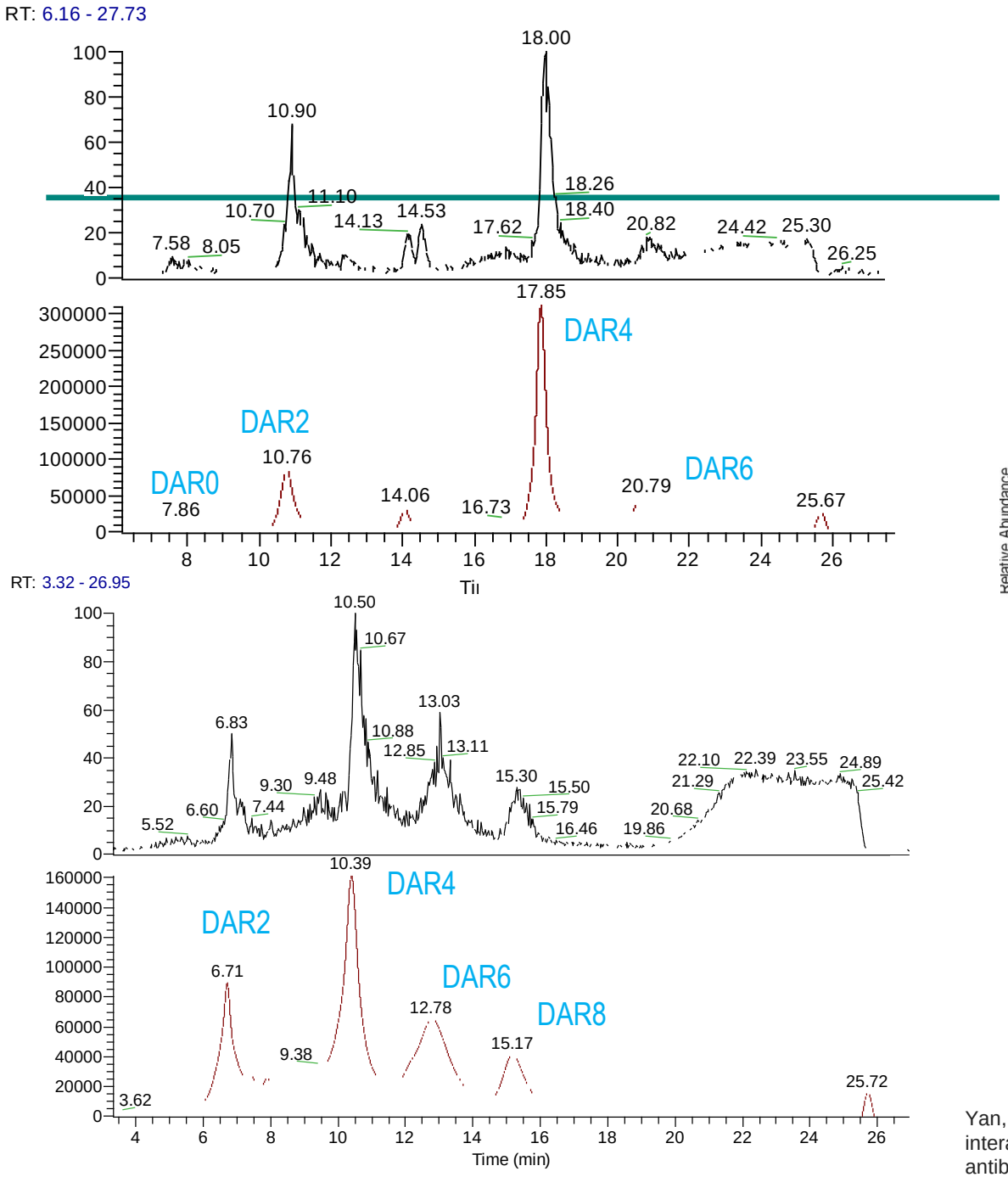
Low-resolution
Q-ToF

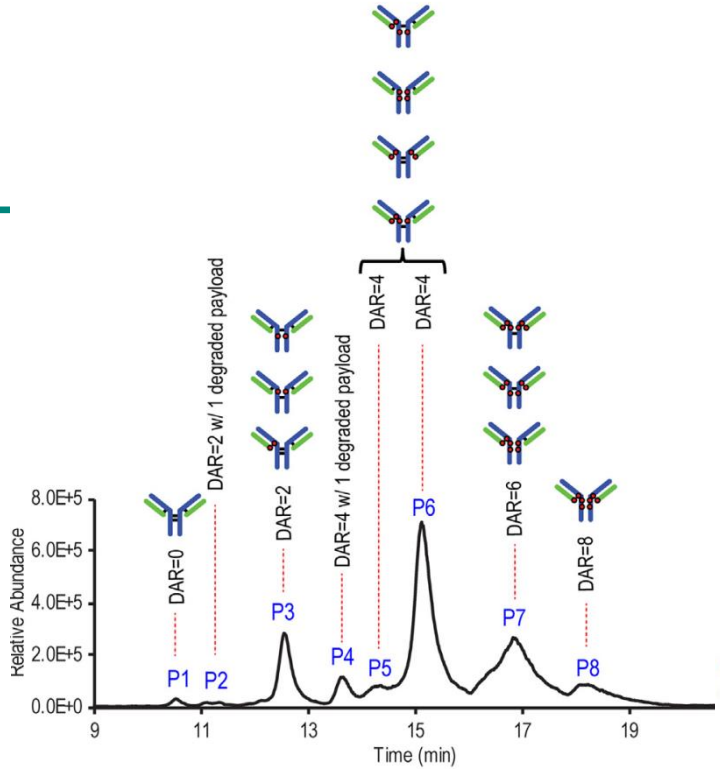
RPLC method
Column: Waters Bioresolve mAb 2.1 x 50mm, 450 Å, 2.7 µm

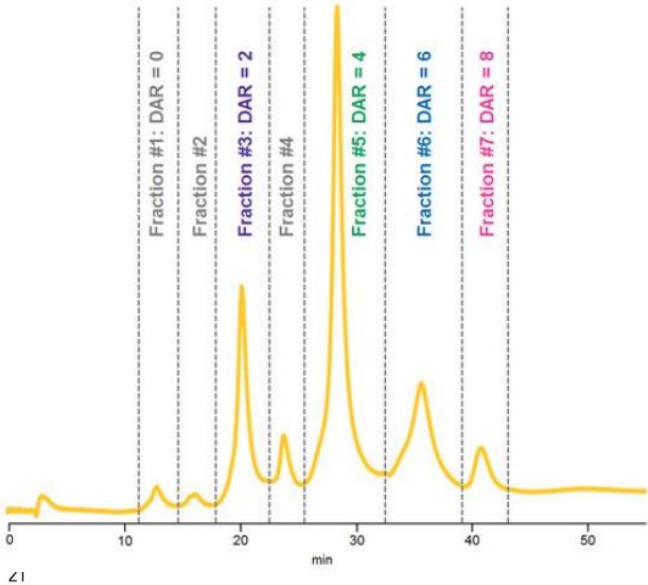
Time (min)	Flow rate (ml/min)	%A (Water with 0.1% DFA)	%B (50% ACN/50% IPA with 0.1% DFA)
0	0.45	80	20
2	0.45	80	20
8	0.45	67	33
10.5	0.45	57	43
17.5	0.45	20	80
19.5	0.45	20	80
20	0.45	80	20

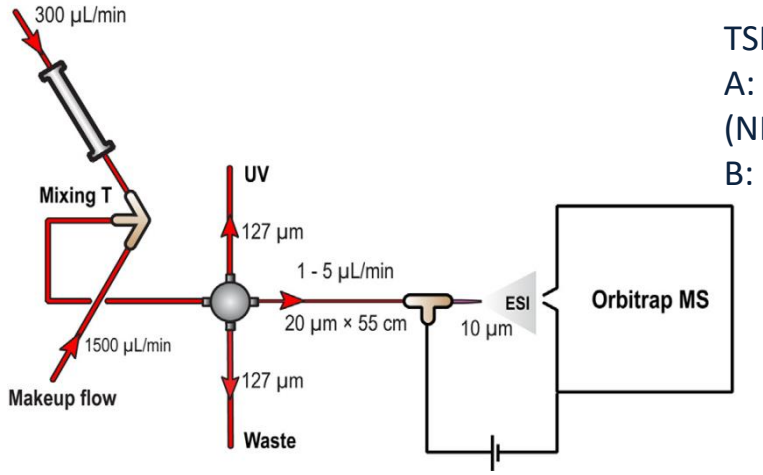
HIC method
Column: Thermo MABPac HIC-10 4.6 x 100mm, 1000 Å, 5 µm

Time (min)	Flow rate (ml/min)	%A (1.5M $(NH_4)_2SO_4$ with 50mM $NaPO_4$)	%B (50 mM $NaPO_4$)
0	0.5	90	10
16	0.5	0	100
20	0.5	0	100
21	0.5	90	10
31	0.5	90	10









TSKgel Butyl-NPR 100 x 4.6 mm (Tosoh)
A: 50 mM PO4 pH 7.0 , 1.5 M (NH4)2SO4 , 5% IPA
B: 50 mM PO4 pH 7.0 , 20% IPA

Yan, Y., Xing, T., Wang, S., Daly, T. J., & Li, N. (2020). Online coupling of analytical hydrophobic interaction chromatography with native mass spectrometry for the characterization of monoclonal antibodies and related products. *Journal of Pharmaceutical and Biomedical Analysis*, *186*, 113313.

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