Leverage orthogonal ciEF, icIEF-UV/MS and LC-MS workflows for comprehensive biotherapeutic characterization

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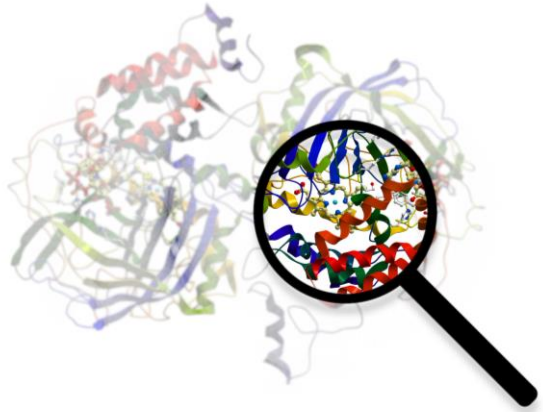
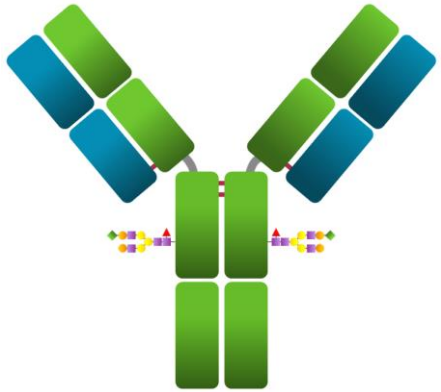
SCIEX
The Power of Precision

Challenges with biotherapeutic characterization

- Sequence confirmation, charge heterogeneity assessment and post-translation modifications (PTMs) analysis are key elements of comprehensive biotherapeutic characterization to ensure the safety, efficacy and consistent quality of drug molecules
- Characterization of modern biotherapeutics with increasing diversity and complexity typically involves multiple analytical assays using different instrument platforms and software packages. This increases the investment in resources, the length of a development cycle and the difficulty of method implementation across labs
- Rapid charge heterogeneity analysis, accurate PTM localization and clear differentiation of amino acids isomers are all challenging using traditional analytical assays





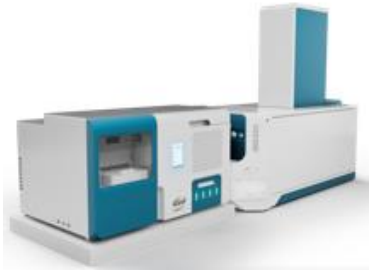
SCIEX portfolio: characterization, screening and QC

ZenoTOF 7600 system

Routine to advanced characterization

- Comprehensive characterization using electron-activated dissociation (EAD)
  - Localization of PTMs such as glycation and glycosylation
  - Differentiation of isomers such as Leu *vs.* Ile and Asp *vs.* isoAsp
  - Disulfide bond mapping
  - Middle-down analysis
  - Single-injection platform method

Intabio ZT system



Routine screening and characterization

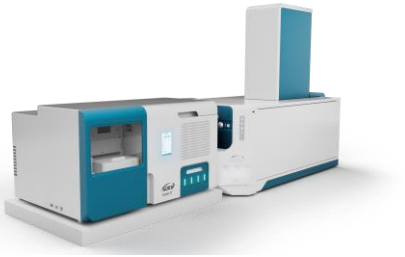
- Rapid charge heterogeneity analysis using icIEF-UV/MS
  - Intact N- and O-glycan distribution
  - Stability profiling
    - For example, deamidation, oxidation, glycation
  - Free thiol/cysteinylation
  - Bioconjugation and distribution

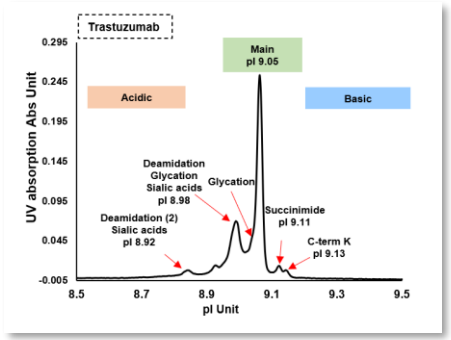
BioPhase 8800 system

High throughput & GMP-ready

- High throughput screening and monitoring using ciEF
  - Charge variant separation and pI determination
  - Low molecular weight impurity sizing
  - Released N-glycan GU determination

A single MS platform to achieve complete characterization





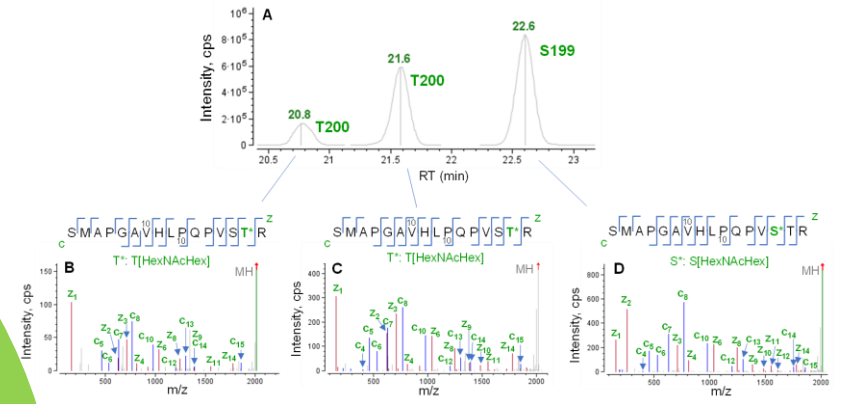
ZenoTOF 7600 system

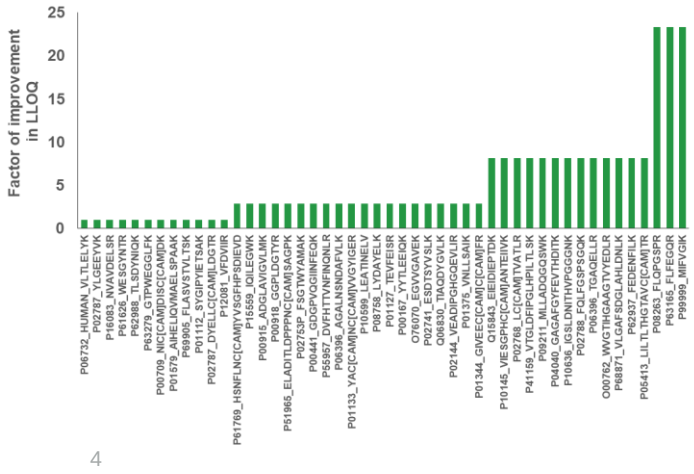
icIEF-UV/MS
Charge variants
Intact proteoform analysis

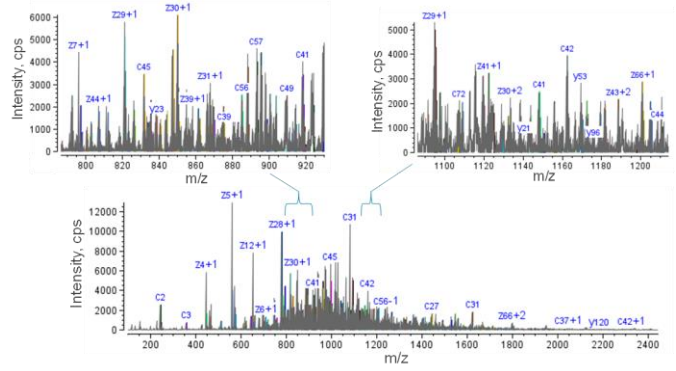
Peptide mapping
Glycoforms, PTMs
Disulfide mapping
Isomer ID

Middle-down
Subunit sequence
Clipping and PTM analyses
Disulfide mapping

Peptide quantitation







The promise of EAD for biomolecules

ELECTRON ACTIVATED DISSOCIATION (EAD)

- Data-dependent acquisition platform method for peptide mapping – no need for optimization
- Improved bottom-up characterization performance to meet the challenges of complex next gen therapeutics
  - Confirmation of PTMs (glycosylation, disulfide-bonds, phosphorylation, sulfation, …)
  - Detailed determination of aa isomers
  - Fragmentation of singularly, doubly and multiply charged ions
  - Comprehensive sequence coverage
- Routine and reliable similar to CID MS/MS: set and forget
- Only alternative fragmentation technique with sensitivity improvements using the **Zeno trap**
- Allows for sequence information directly from the intact molecule (top/middle down)
- Wide range of electron energy adjustments (up to 25 eV) allows for high degree of selectivity for backbone fragmentation and maintenance of side chain
- Potential to support quantification

7600: Solving the sensitivity gap with the zenoTrap

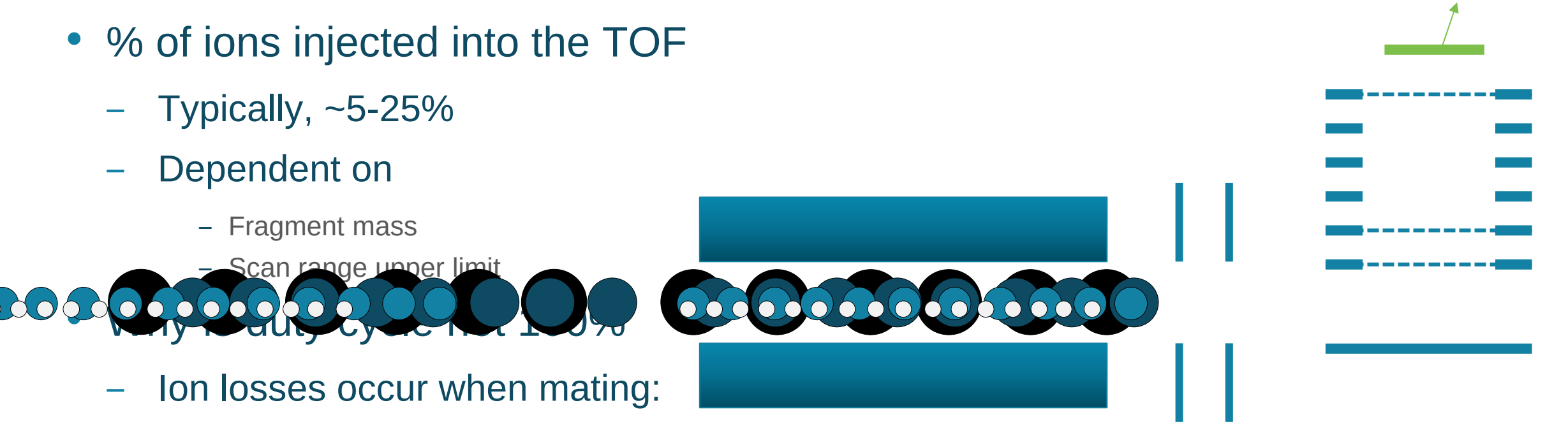
ZENO TRAP

- Given the disperse nature of ion current in EAD, sensitivity enhancement is needed to operate at routine levels
- Normally ions are lost in between TOF pulses primarily due to velocity differences
  - The Zeno trap gains ion transmission back by timing the ion injection from the Zeno gate with subsequent extraction pulses
  - The Zeno trap contains critical technology to enhance MS/MS sensitivity, for both CID and EAD
  - EAD and Zeno trap are designed to work together to significantly improve data quality for alternative fragmentation

What is duty cycle?

… AND WHY IS DUTY CYCLE IMPORTANT?

- % of ions injected into the TOF
  - Typically, ~5-25%
  - Dependent on
    - Fragment mass
    - Scan range upper limit
- Why is duty cycle not 100%
  - Ion losses occur when mating:
    - Pulsed measurement technique
      - TOF
    - Continuous ion beam
      - Quadrupole



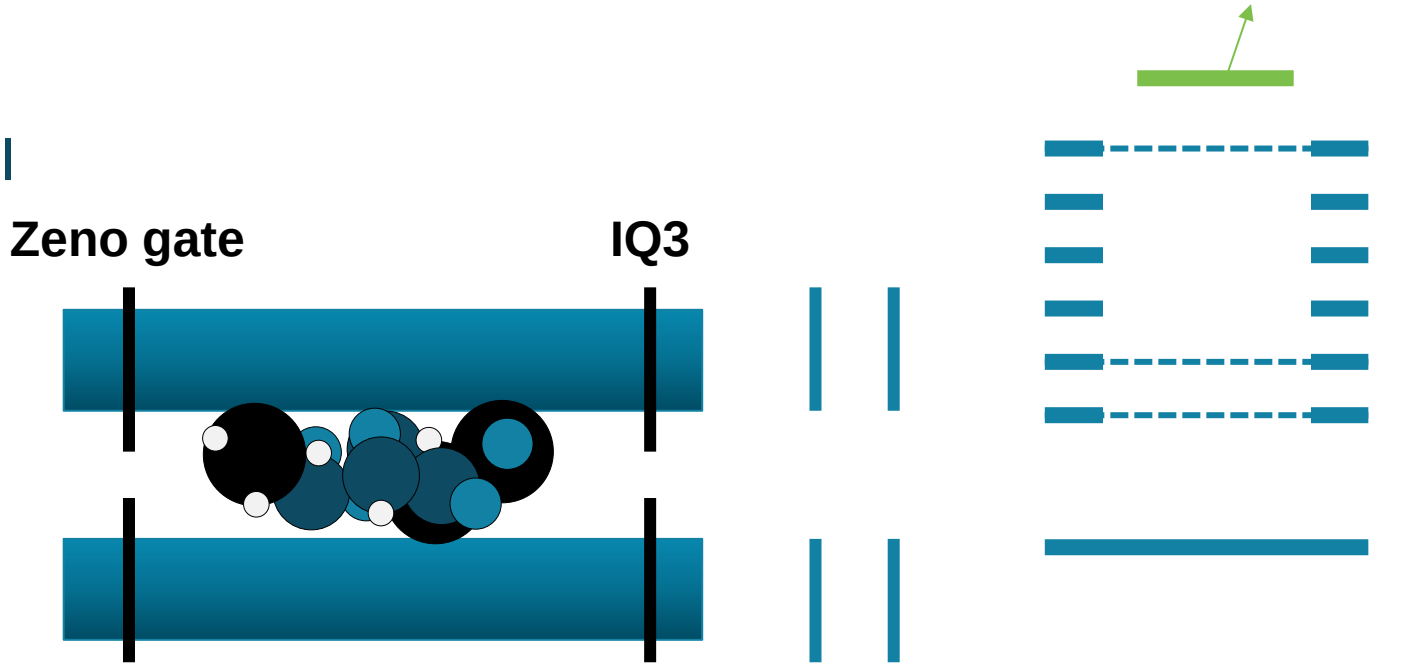
Quadrupole region
Continuous ion beam

TOF accelerator
Ion pulsing

Zeno trap

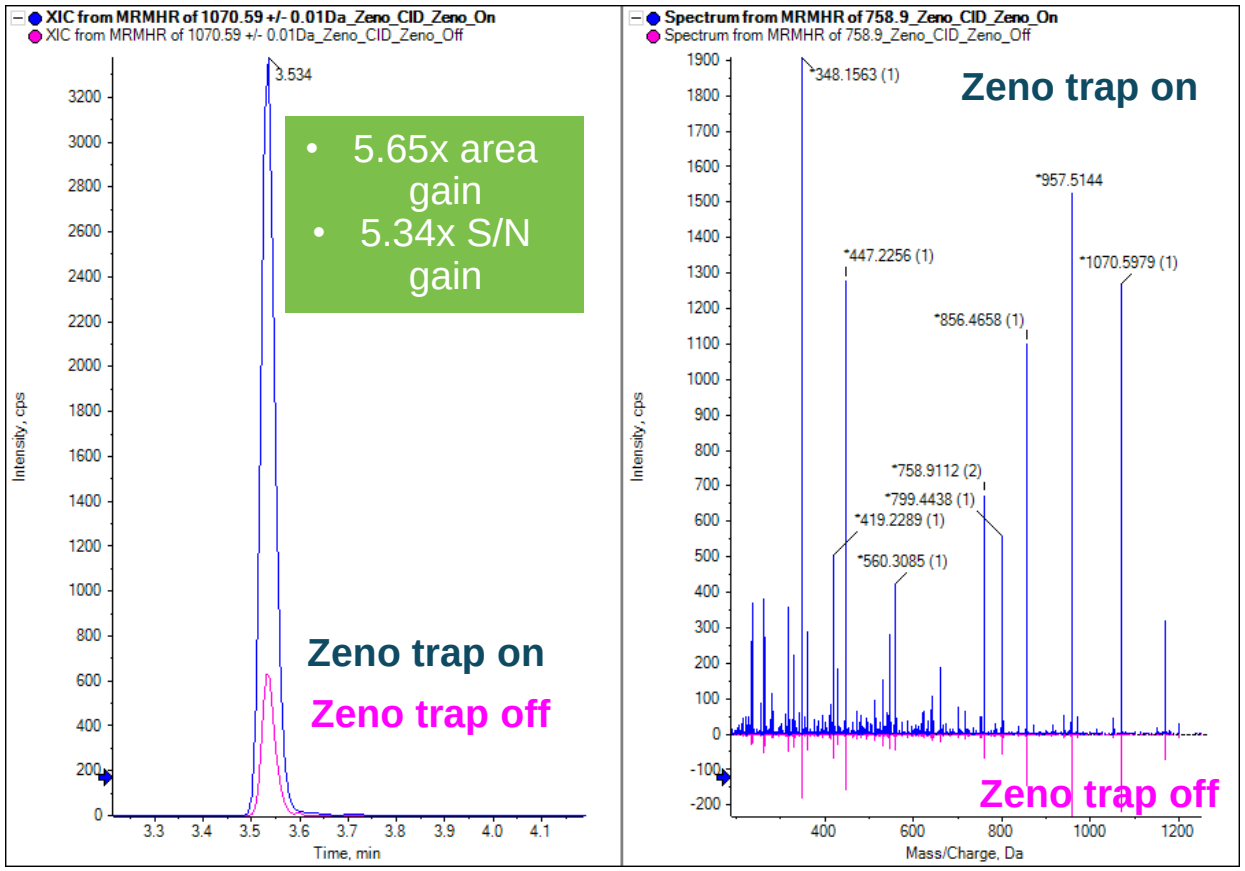
FOR SENSITIVITY GAINS IN MS/MS

- The Zeno trap provides control of the ion beam from the collision cell into the accelerator
- Ions exit the Zeno trap in an ordered release based on potential energy
  - Ions are generally released from a high *m/z* to low *m/z*
  - All ions now arrive in the accelerator at the same time and location

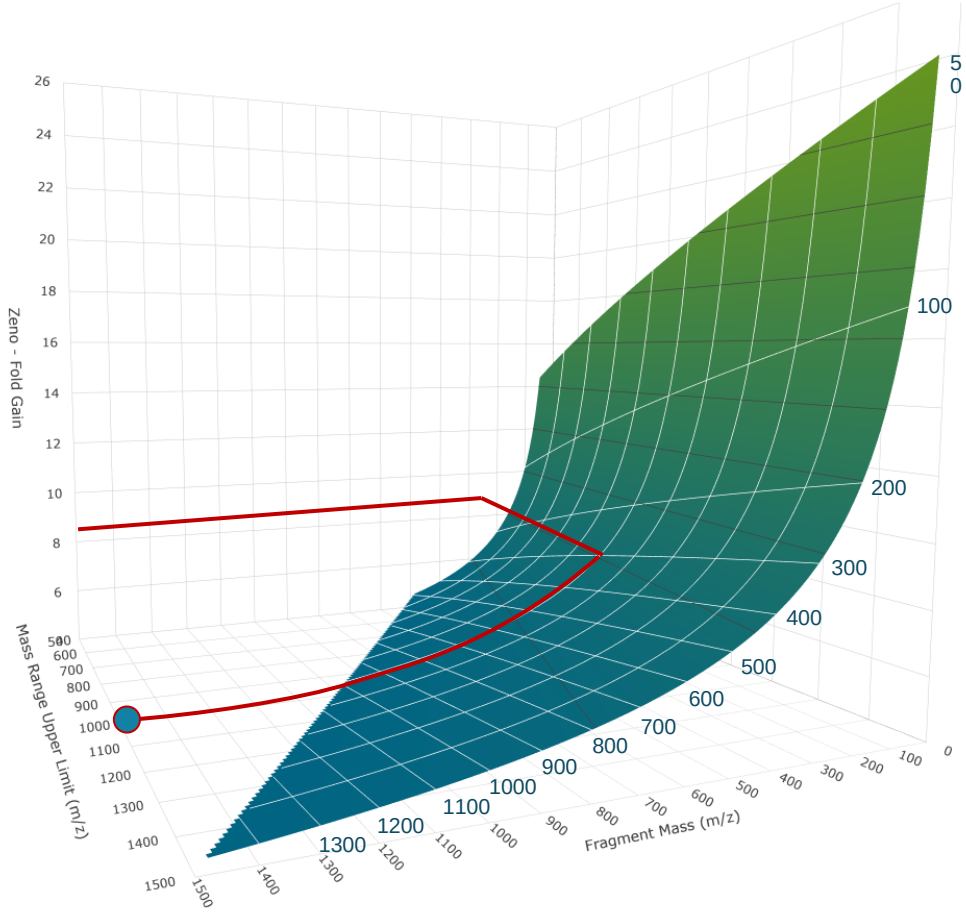


Enhancing sensitivity for time-of-flight MS/MS

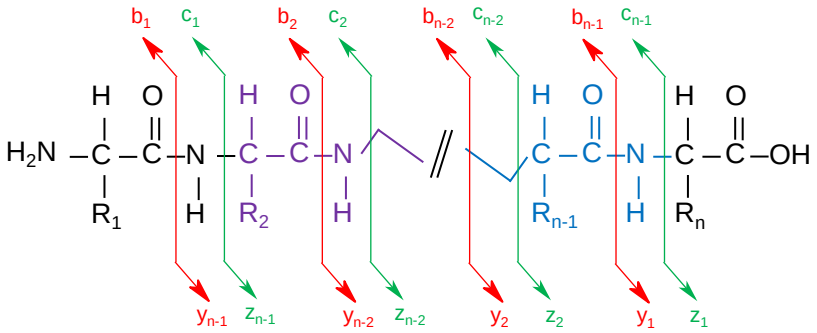
GAIN USING THE ZENO TRAP



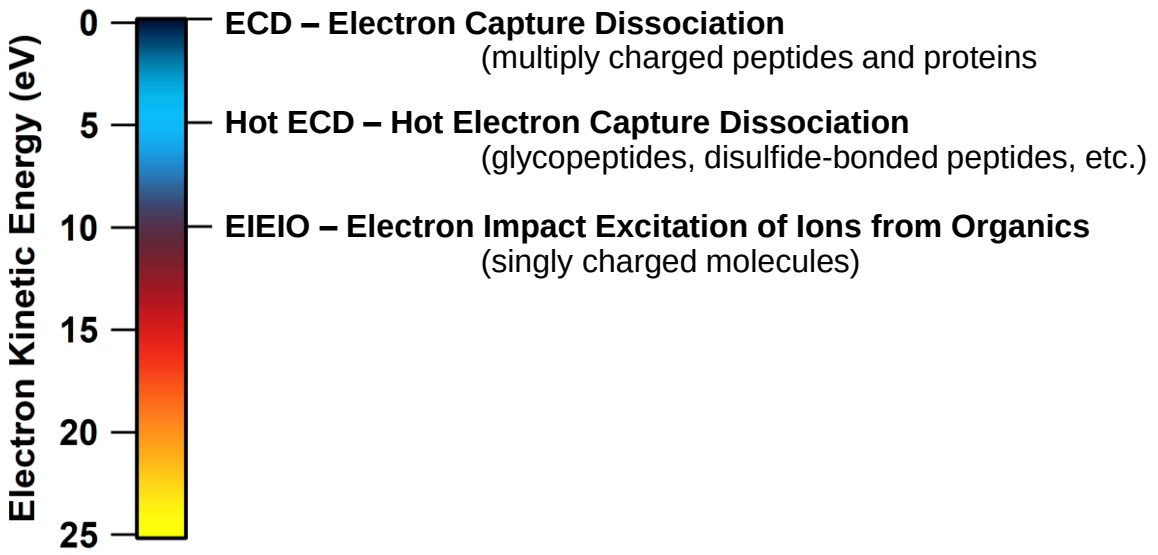
GAIN VS. FRAGMENT *M/Z*



Electron activated dissociation (EAD)



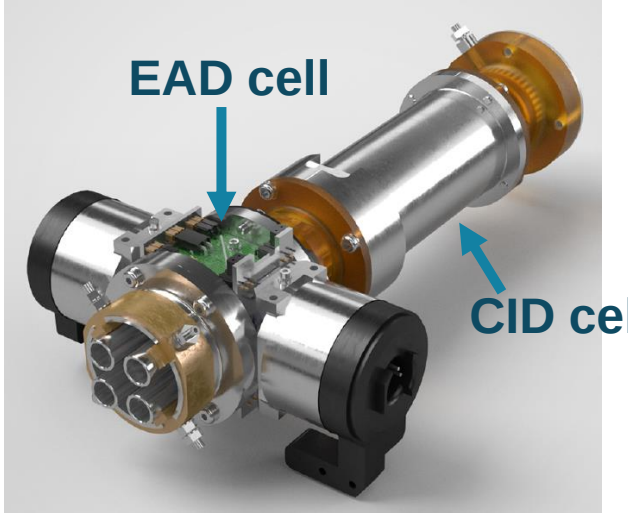
- Free electrons are captured by ions and form a radical state which then fragments
- Electrons introduced with different energies will induce fragmentation in different molecule types
- EAD cell enables you to perform ECD, Hot ECD and EIEIO in single instrument

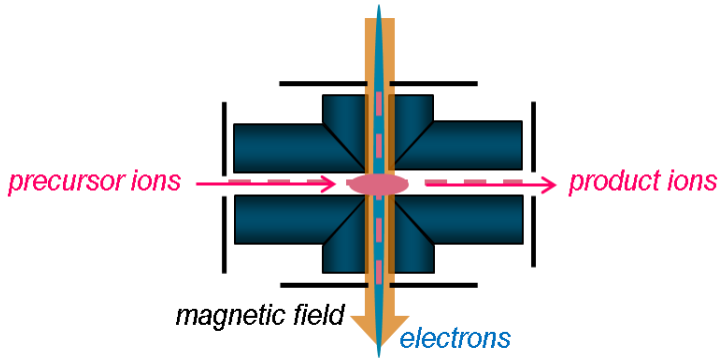


T. Baba *et al. Anal.Chem*. 2014

EAD technology from SCIEX

- **Fast** fragmentation and acquisition[1-3]
  - Electron capture reaction times of 10–30 ms
  - DDA on LC timescale up to 20 Hz
- **Reproducible** at a quantitative level
- **Reagent-free** electron capture dissociation[2]
- **Tunability** of electron kinetic energy (KE) from 0 eV to 25 eV[1-3]
- **Single-injection platform method** for routine to advanced biopharma characterization





[1]Takashi Baba *et al.* (2015) Electron capture dissociation in a branched radio-frequency ion trap. *Anal. Chem.* 87(1):785-792.
[2]Takashi Baba *et al.* (2021) Dissociation of biomolecules by an intense low-energy electron beam in a high sensitivity time-of-flight mass spectrometer. *J. Am. Soc. Mass Spectrom.* 32(8):1964-1975.
[3]Takashi Baba *et al.* (2022) Electron impact excitation of ions from organics on singly protonated peptides with and without post-translational modifications. *J. Am. Soc. Mass Spectrom.* 33(9):1723-1732.

Advantages of EAD for biopharma characterization

Application	CID	EAD	ExD
Single-injection platform method	√	√	×
Full sequence coverage	√	√	×
Labile modifications	×	√	√*
Isomer differentiation	×	√	√*
Di-/tri-sulfide bond mapping	√*	√	√
Long peptides (>5 kDa)	√*	√	√
Singly charged species	√	√	×
Middle-down analysis	√*	√	√*

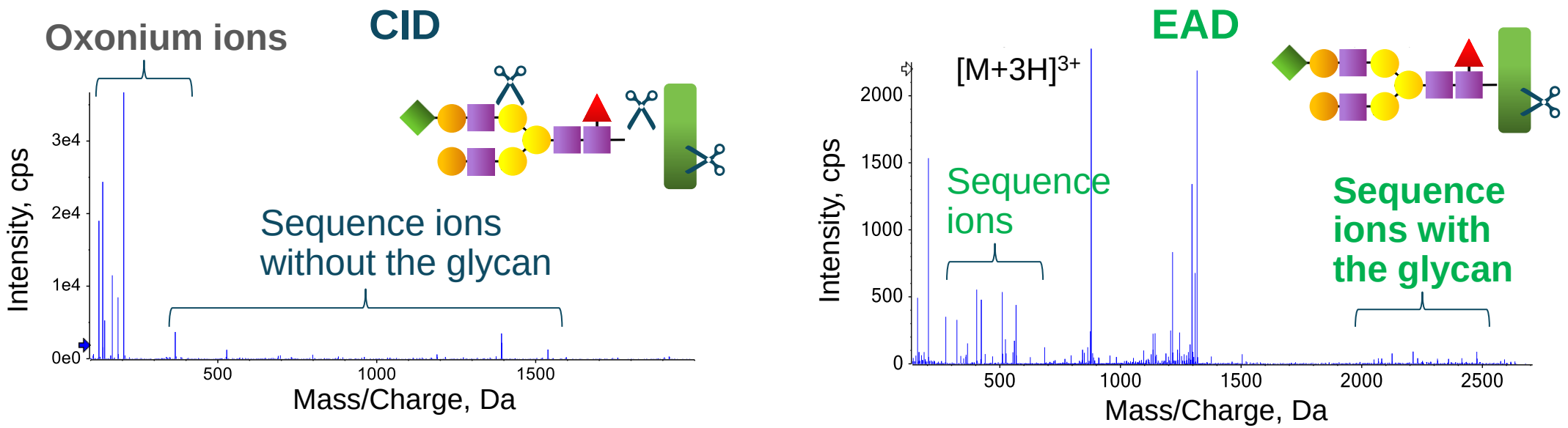
- Accurate localization of labile modifications
- Confident differentiation of amino acid or positional isomers
- Di-/tri-sulfide bond mapping
- High sequence coverage of antibody subunits
- Fast and sensitive platform method
- Quantitatively reproducible
- Tunability of electron KE for challenging molecules

EAD provides superior comprehensive characterization of protein therapeutics as a platform method in a single injection

** Not optimal in certain cases*

EAD vs CID for glycopeptide characterization

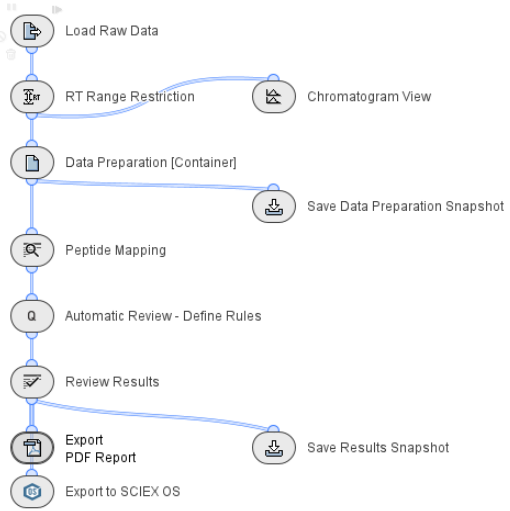
- Complex structures of N-linked glycosylation
- Absence of a consensus sequence and the presence of positional isomers for O-linked glycosylation
- While localization of labile glycan moieties using collision-based MS/MS is challenging, EAD can provide site-specific information about glycosylation[1,2]

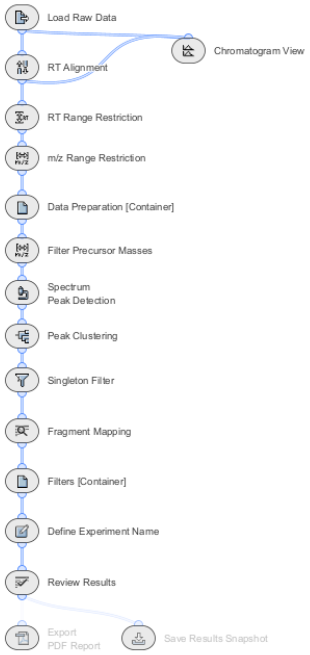


[1]A new electron activated dissociation (EAD) approach for comprehensive glycopeptide analysis of therapeutic proteins. SCIEX technical note, RUO-MKT-02-12980-A.
[2]Comprehensive characterization of O-linked glycosylation in etanercept by electron activated dissociation (EAD). SCIEX technical note, RUO-MKT-02-14921-A.

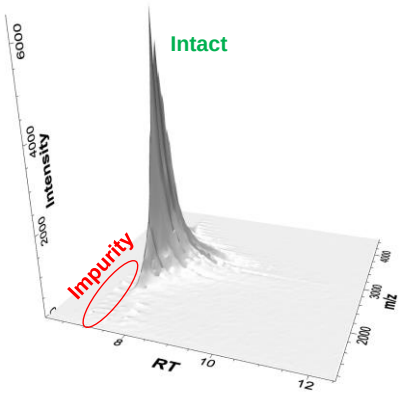
Biologics Explorer software

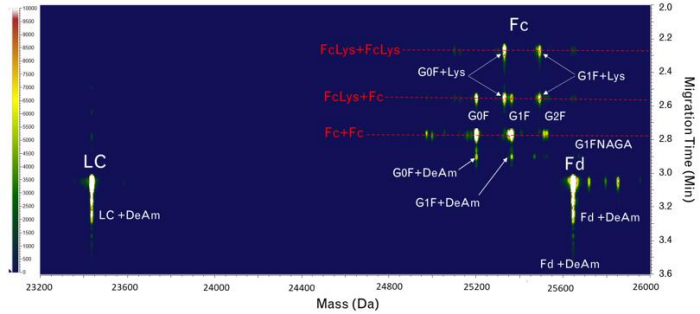
Intuitive workflow templates for intact, peptide mapping, and middle-down data analysis

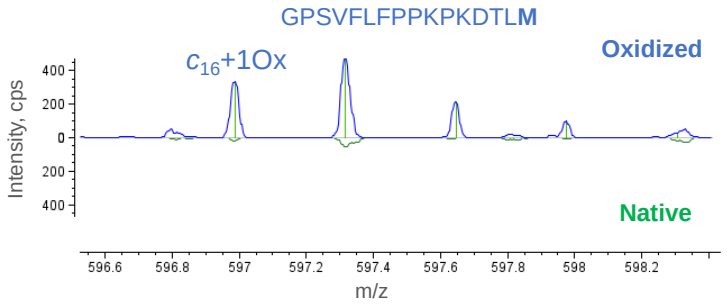


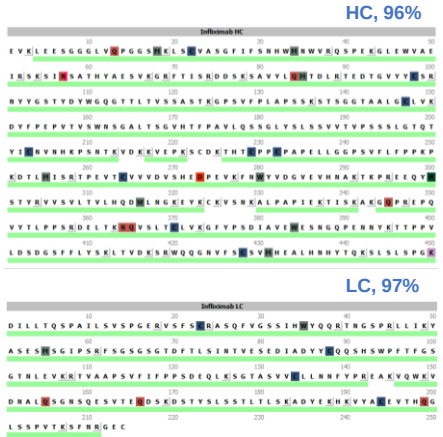


Full toolset for automated data interpretation and results review, comparison, and visualization





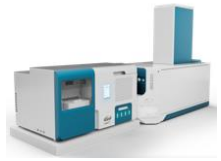


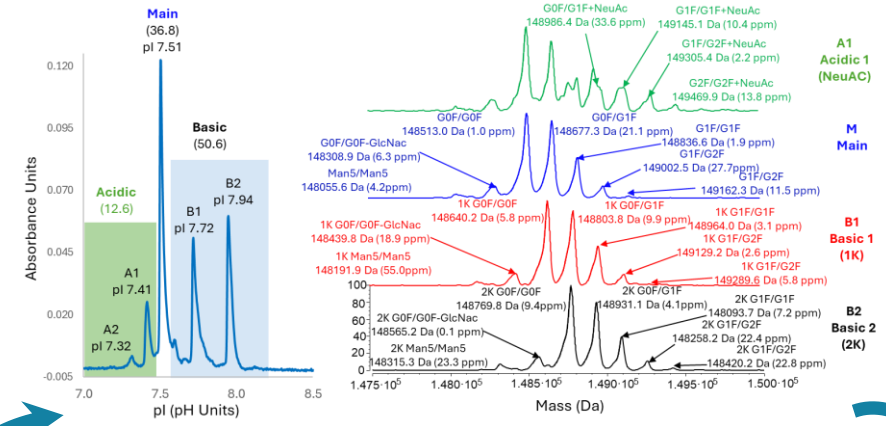


Comprehensive characterization of infliximab using ciEF, icIEF-UV/MS and LC-MS workflows

Variant detection, characterization and monitoring

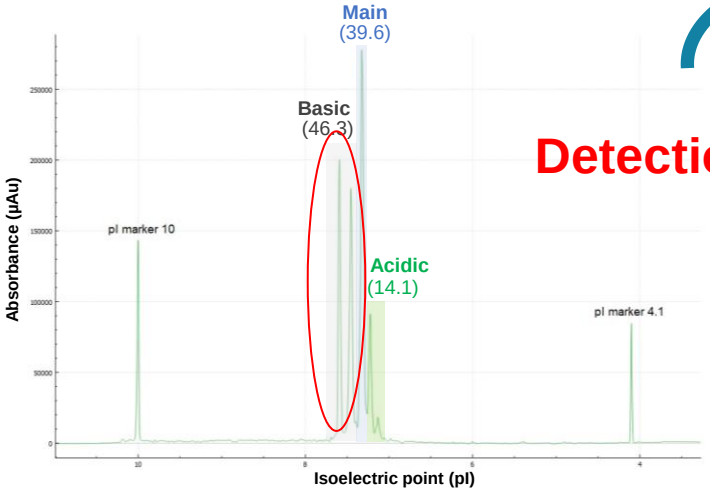
icIEF/UV-MS workflow achieves the same separation as cIEF and identifies the proteoforms contributing to the basic variants



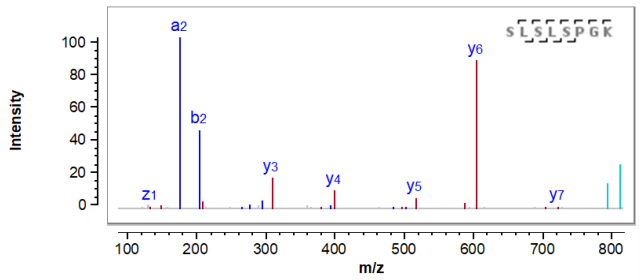


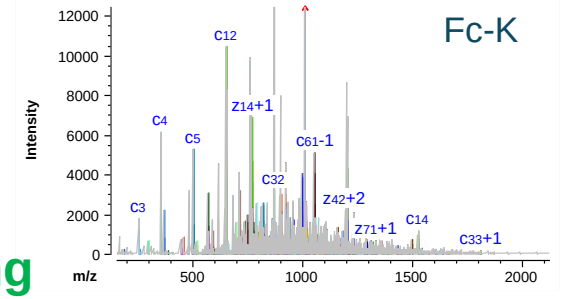
Subunit mass analysis confirms the presence of abundant lysine species on Fc

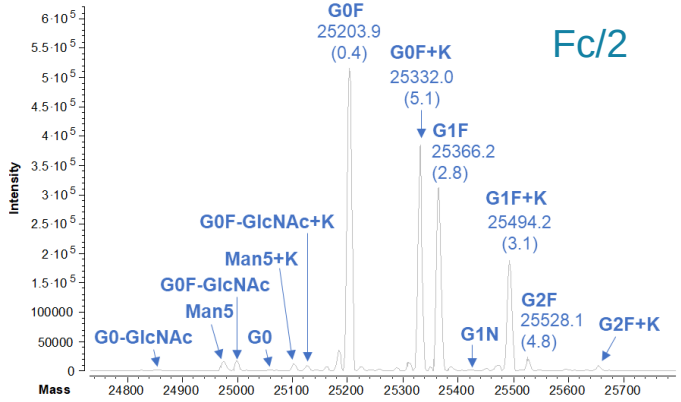
Characterization



Detection





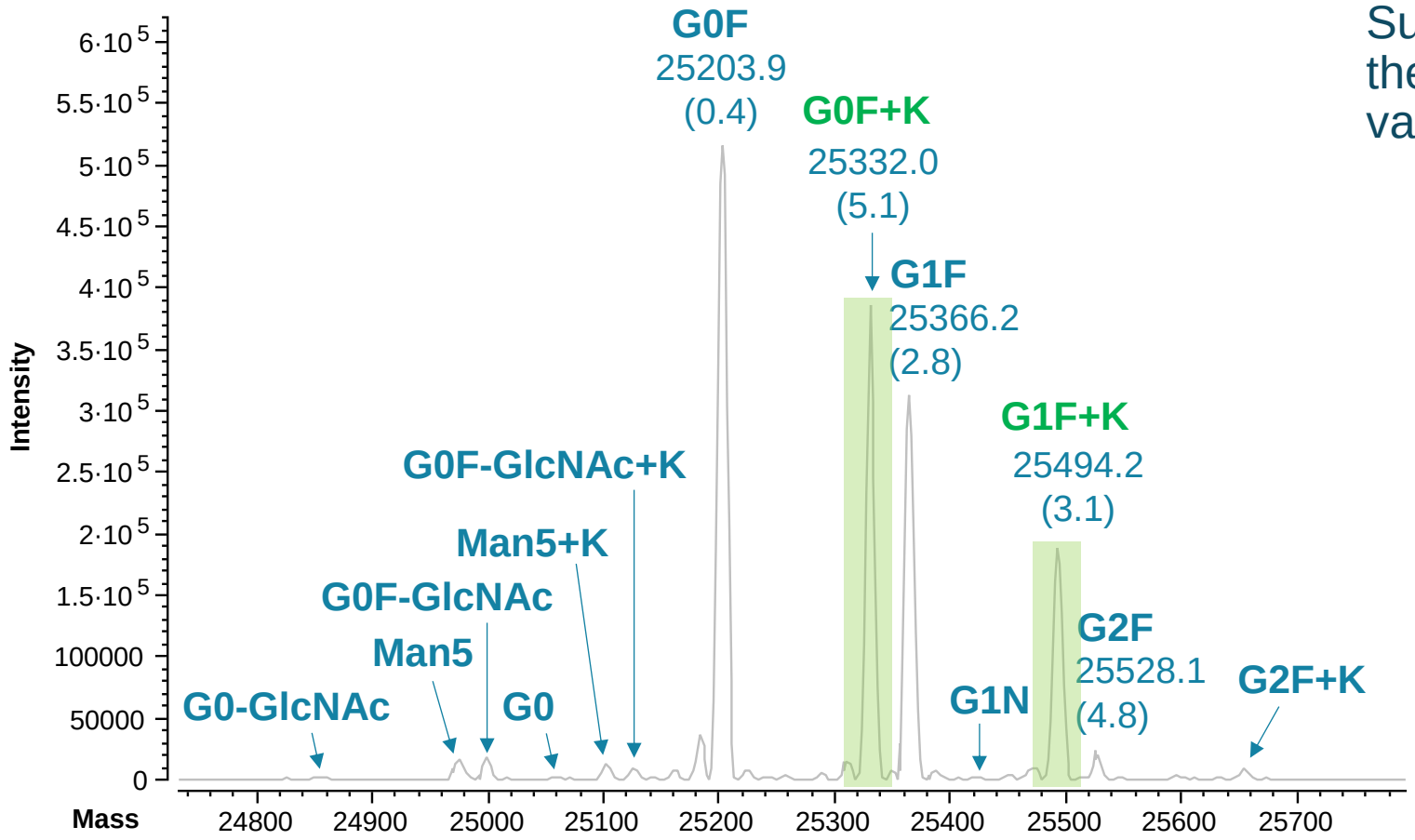


cIEF workflow detects an abnormally high level of basic variants

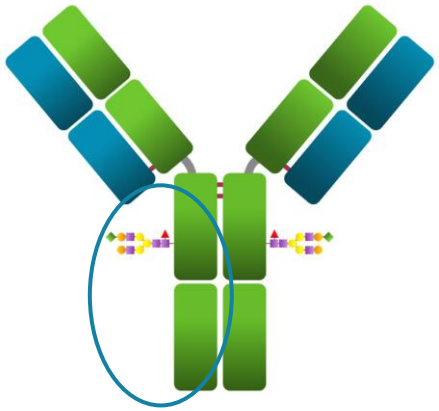
Monitoring

Middle-down and peptide mapping workflows identify the C-terminal lysine variant

Subunit mass analysis



Subunit mass analysis revealed the presence of abundant lysine variants on the Fc/2 subunit



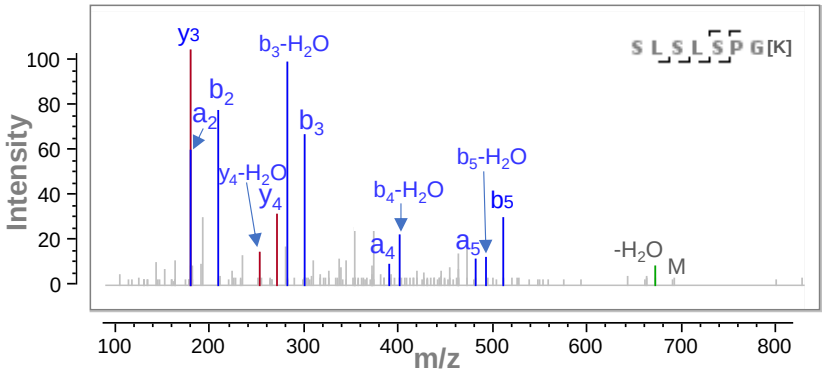
Fc/2

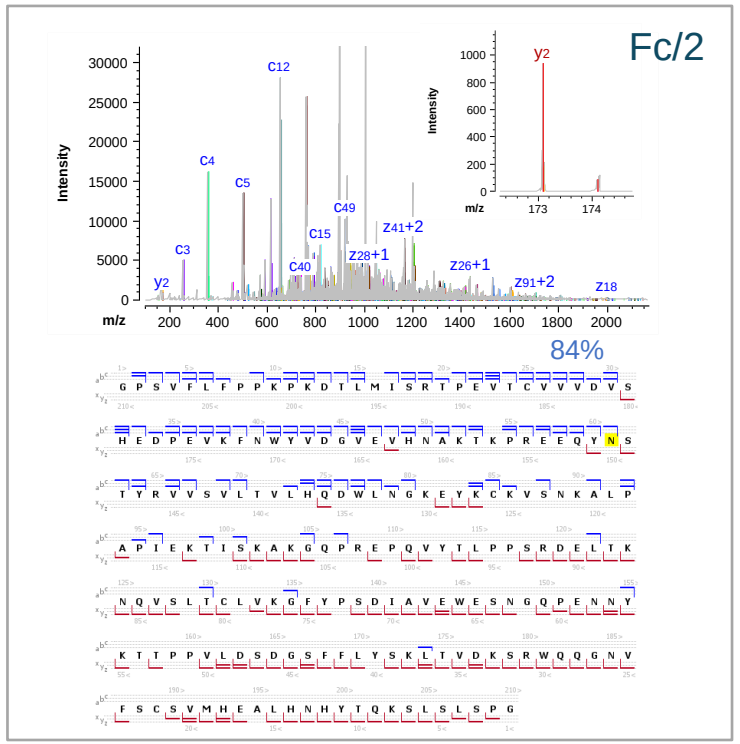
Peptide mapping and middle-down analyses

Peptide mapping

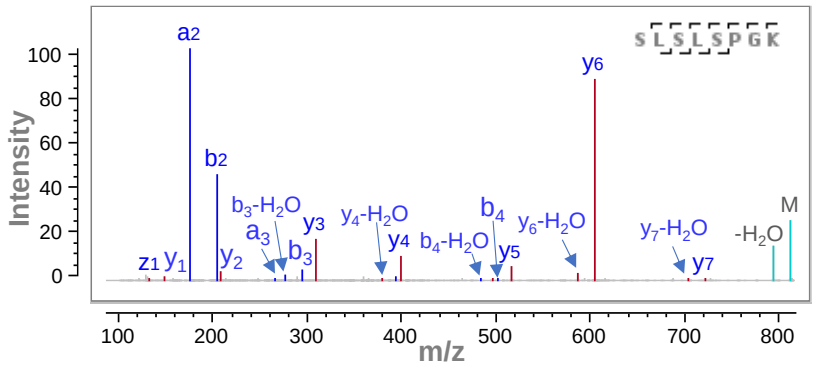
Middle down

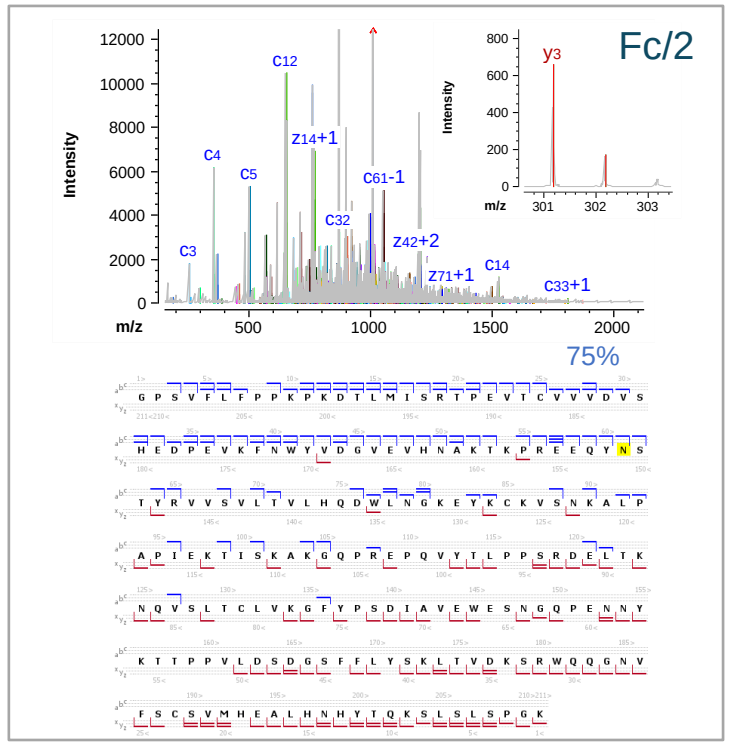
No Lys





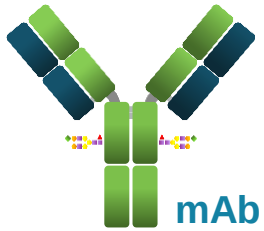
+Lys



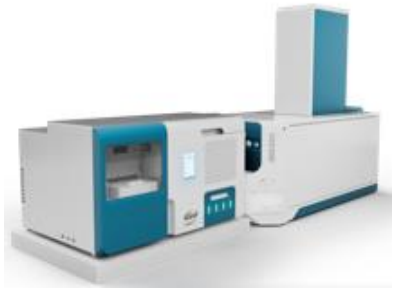


- Middle-down and peptide mapping and workflows provide a multi-level confirmation of the species with or without the C-terminal Lys

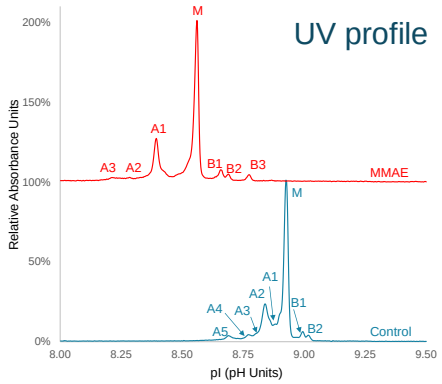
ADC characterization



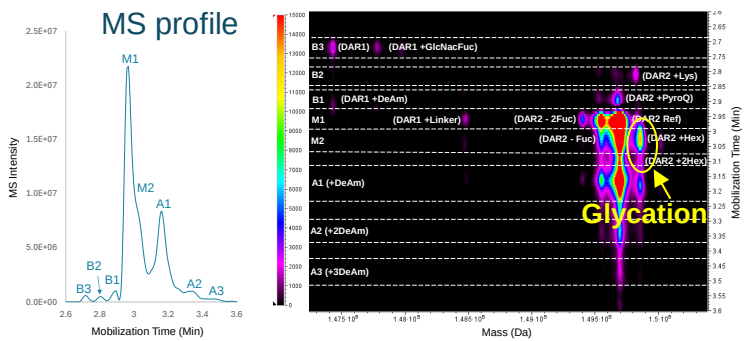
icIEF-UV/MS workflow provides high-resolution separation and sensitive detection of charge variants for rapid screening and monitoring of intact ADCs



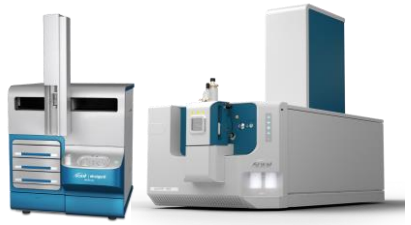
Intabio ZT system



Biologics Explorer software

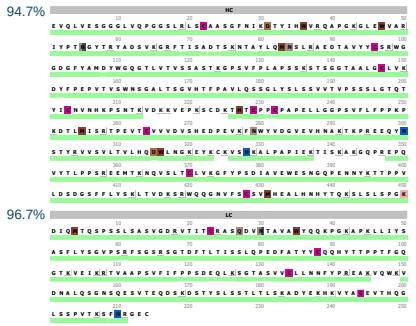


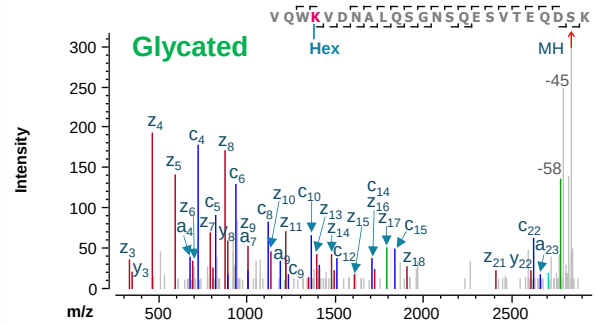
Single-injection EAD based peptide mapping workflow offers high sequence coverage of ADCs for confident variant identification and accurate PTM or payload localization



ZenoTOF 7600 system
MicroLC M5 system

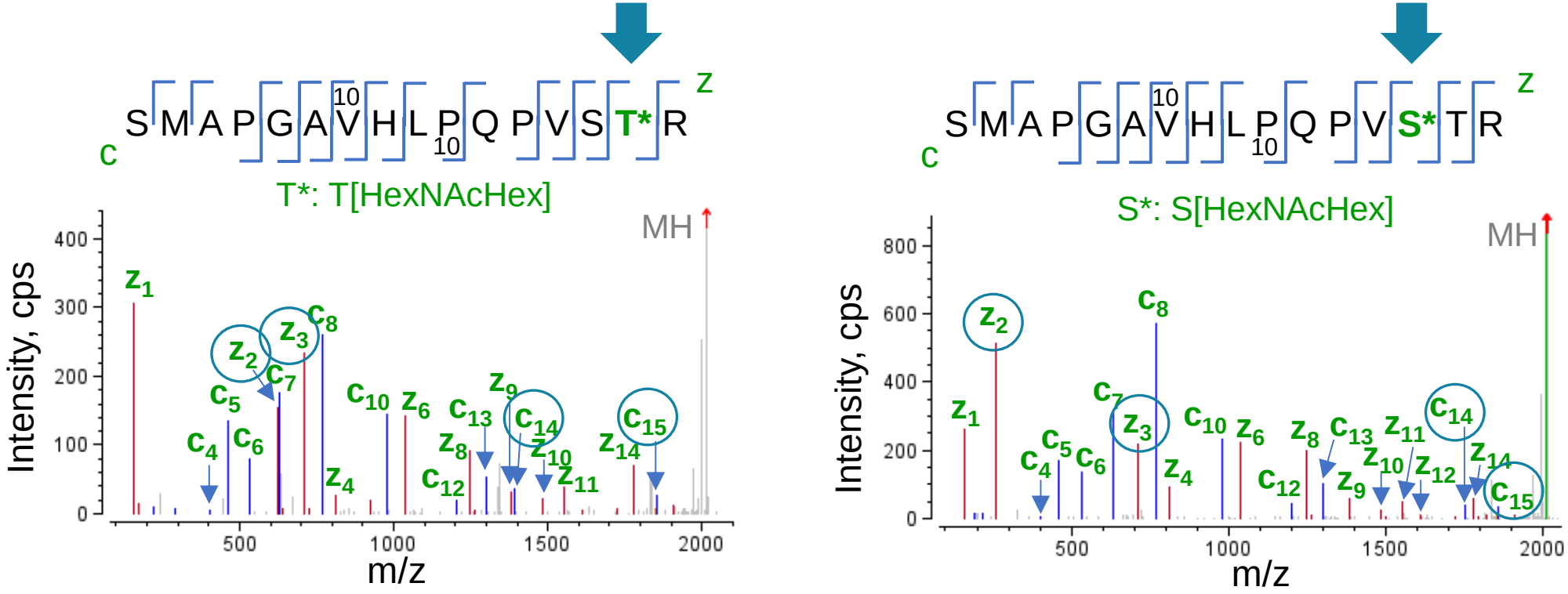
EAD DDA





EAD-based workflows for comprehensive characterization of biotherapeutics

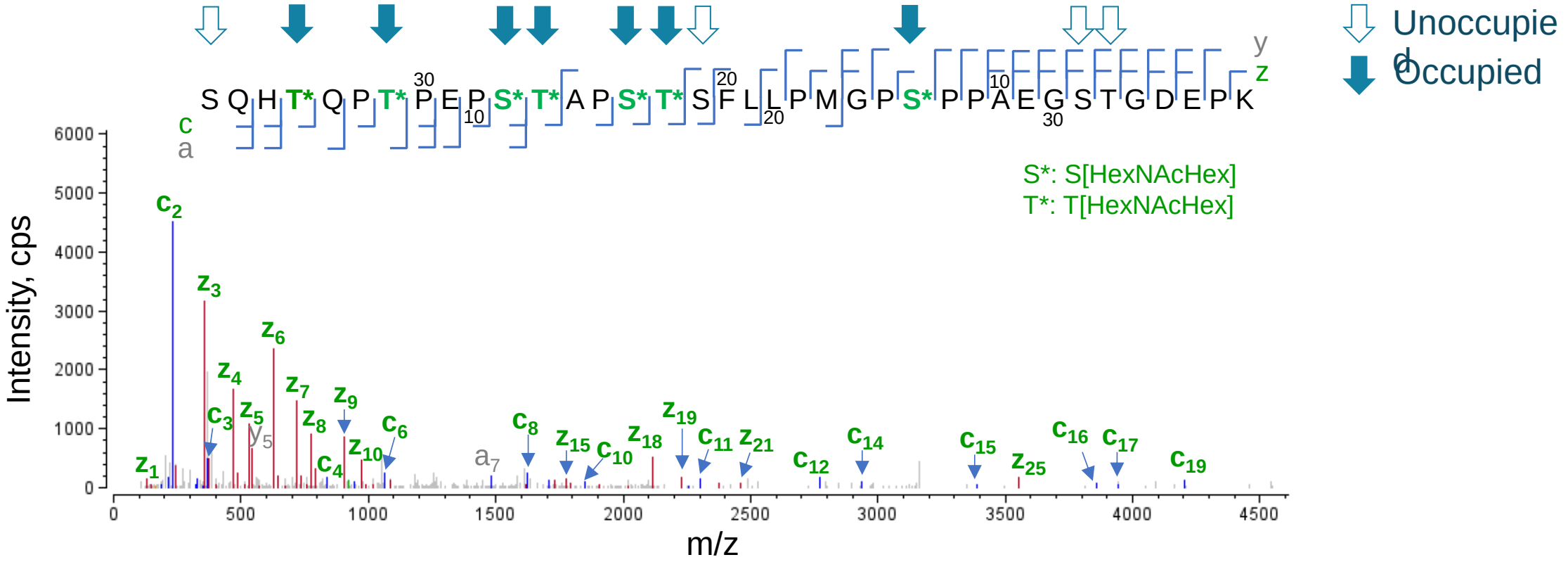
Glycan localization and isomer differentiation



- EAD resulted in excellent fragmentation of the O-glycopeptide backbone and the formation of glycan-containing fragments, allowing accurate localization of the O-glycan and confident differentiation of 2 positional isomers (S *vs.* T glycosylated)[1]

[1]Comprehensive characterization of O-linked glycosylation in etanercept by electron activated dissociation (EAD). SCIEX technical note, RUO-MKT-02-14921-A.

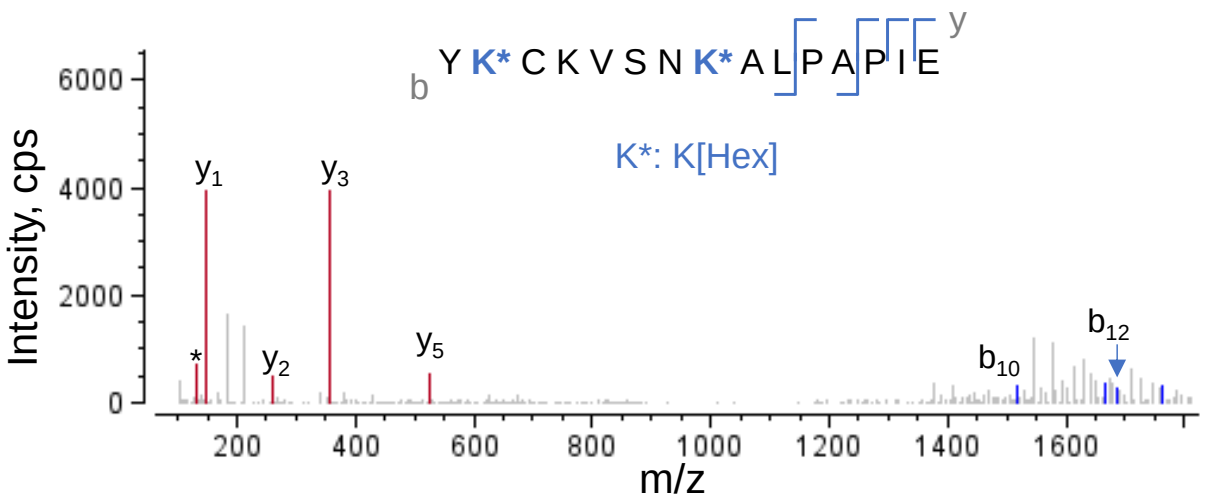
Accurate localization of multiple O-linked glycans



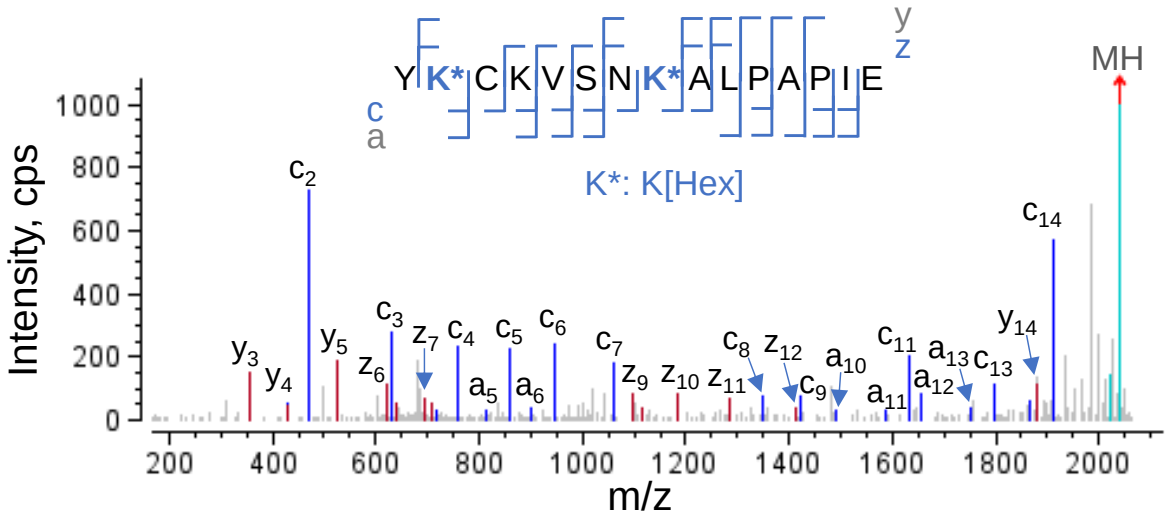
- EAD provides excellent fragmentation of long glycopeptides
- High-quality EAD data led to accurate localization of glycosylation for etanercept glycopeptides carrying as many as 7 O-linked glycan moieties

Challenges with glycation characterization

Poor fragmentation by CID



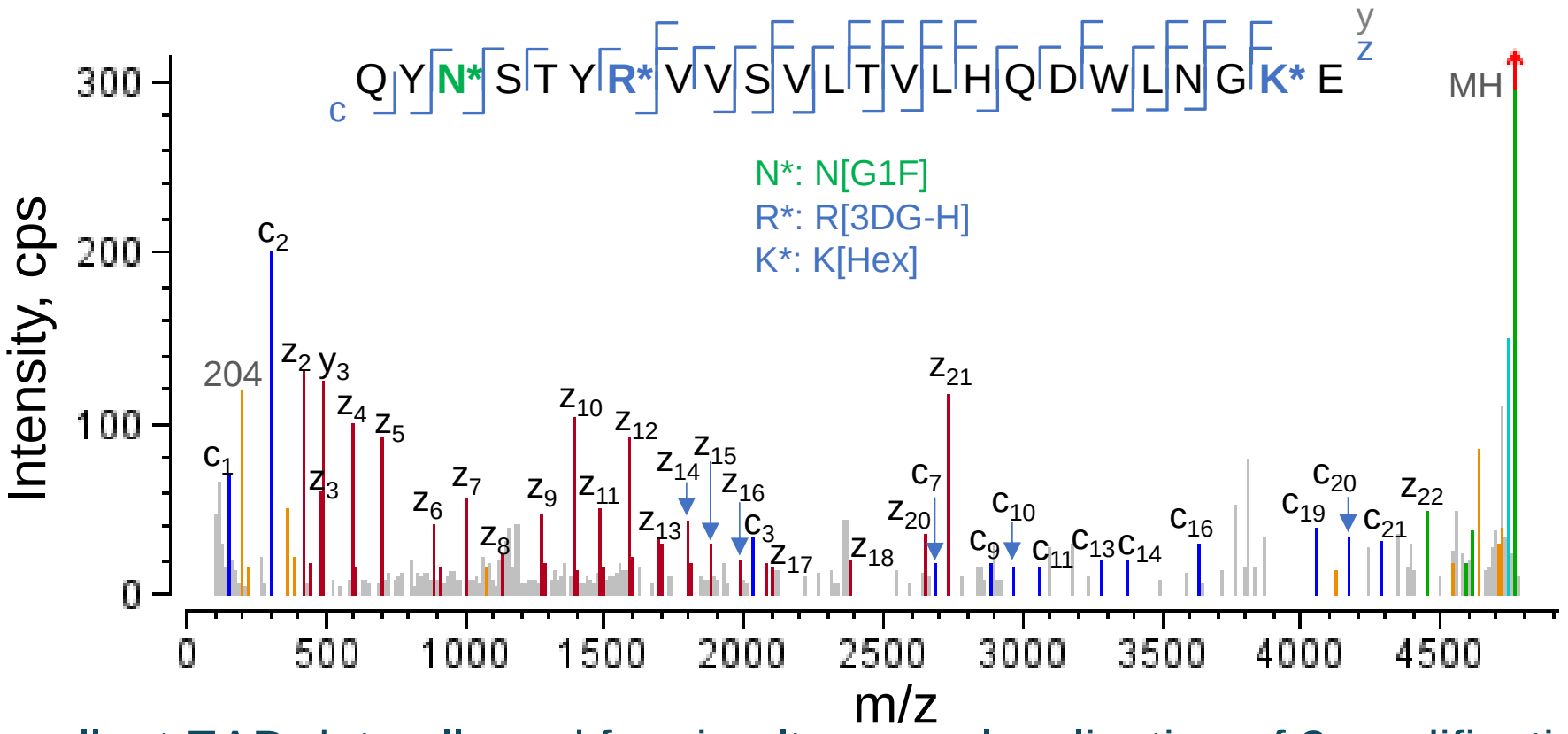
Excellent fragmentation by EAD



- Glycated peptides and advanced glycation end products (AGEs) are difficult to fragment by CID
- CID leads to preferential cleavage of H_2O from the hexose moiety and low yield of sequence ions[1]
- Enzymatic digestion of glycated or AGE species, in which Lys and/or Arg residues are modified, leads to the formation of many long peptides containing the glycation and/or AGE moieties, and the length of these species poses an additional challenge to CID

[1]Comprehensive characterization of glycation in protein therapeutics using electron activated dissociation (EAD). SCIEX technical note, RUO-MKT-02-15020-A.

Simultaneous localization of multiple modifications

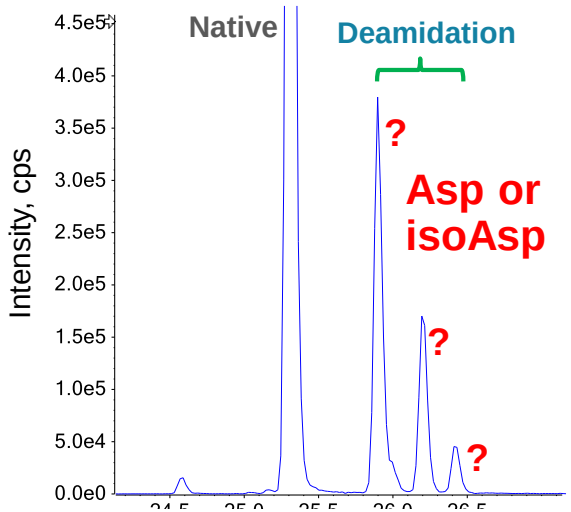


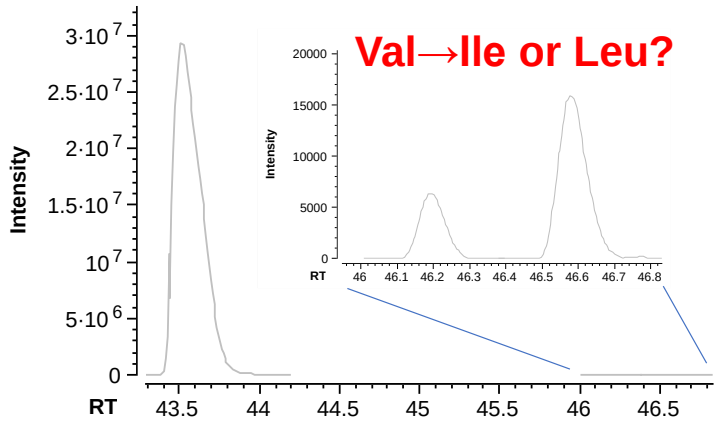
- Excellent EAD data allowed for simultaneous localization of 3 modifications, including 1 N-linked glycan (G1F) on an Asn residue, 1 AGE modification (3DG-H) on an Arg residue and 1 glycation moiety (Hex) on a Lys residue. Such depth of information cannot be achieved using CID[1,2]

[1]Comprehensive characterization of glycation in protein therapeutics using electron activated dissociation (EAD). SCIEX technical note, RUO-MKT-02-15020-A.
[2]Comprehensive characterization of advanced glycation end products (AGEs) in protein therapeutics using electron activated dissociation (EAD). SCIEX technical note, RUO-MKT-02-15088-A.

Differentiation of amino acid isomers using EAD

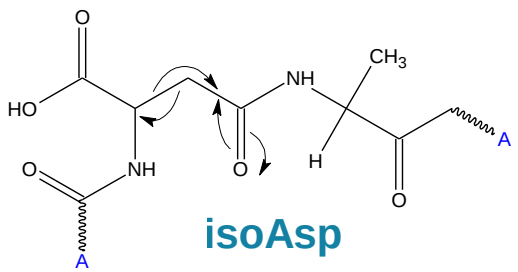
Challenges



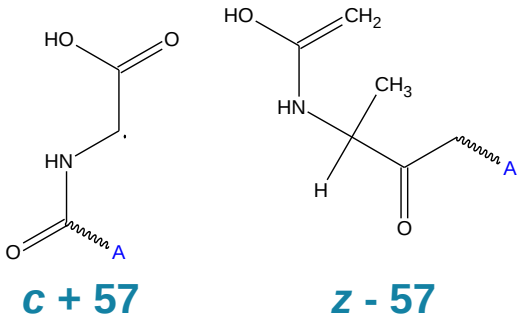


Solution

EAD

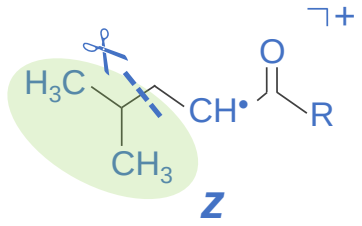


EAD

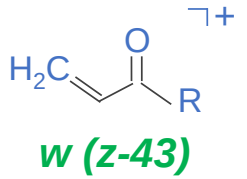


EAD

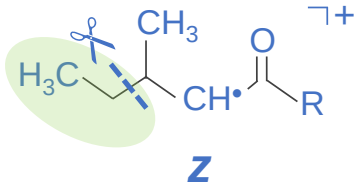
Leu



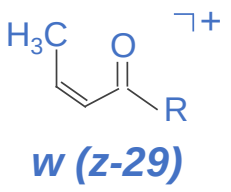
EAD
$-C_3H_7$



Ile

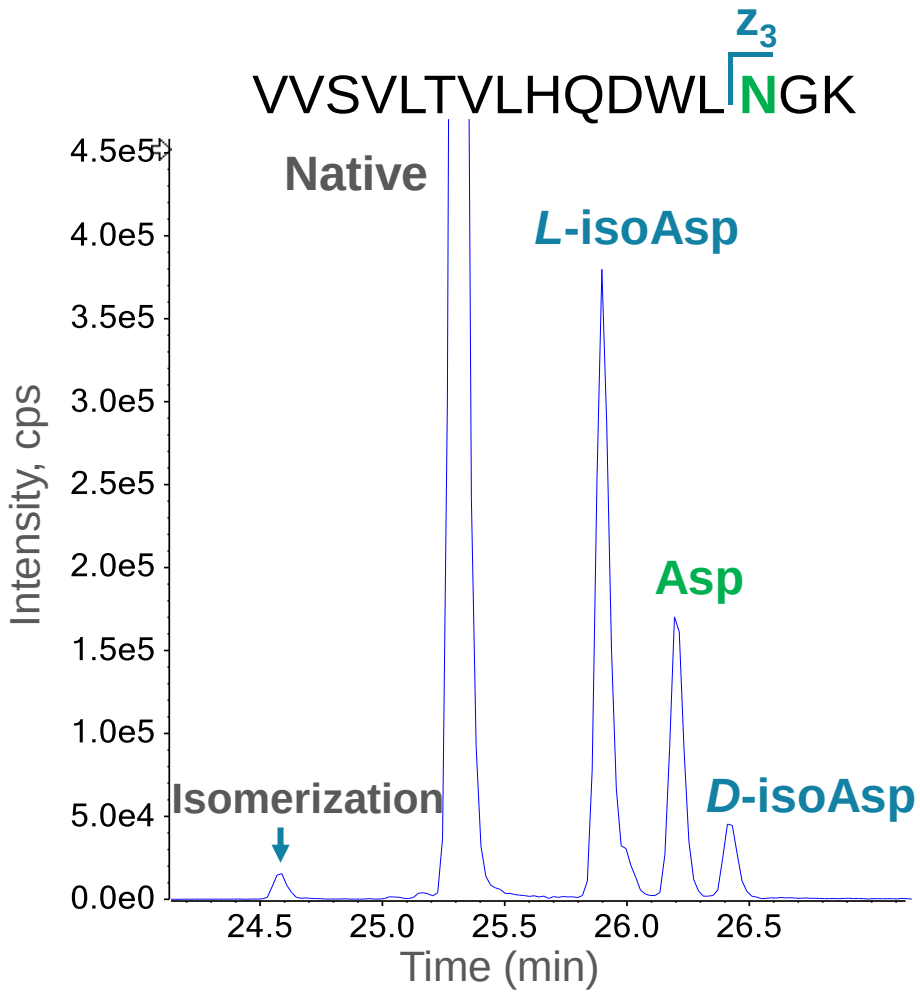


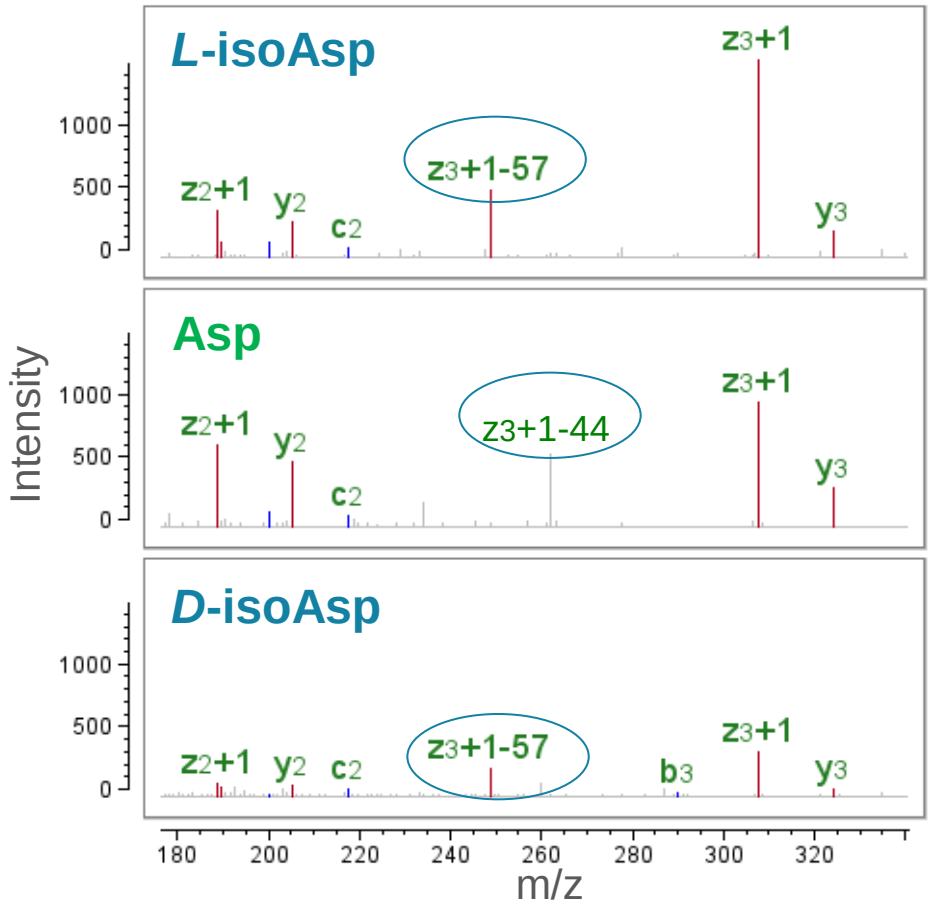
EAD
$-C_2H_5$



- EAD generates diagnostic fragments for clear differentiation of amino acid isomers

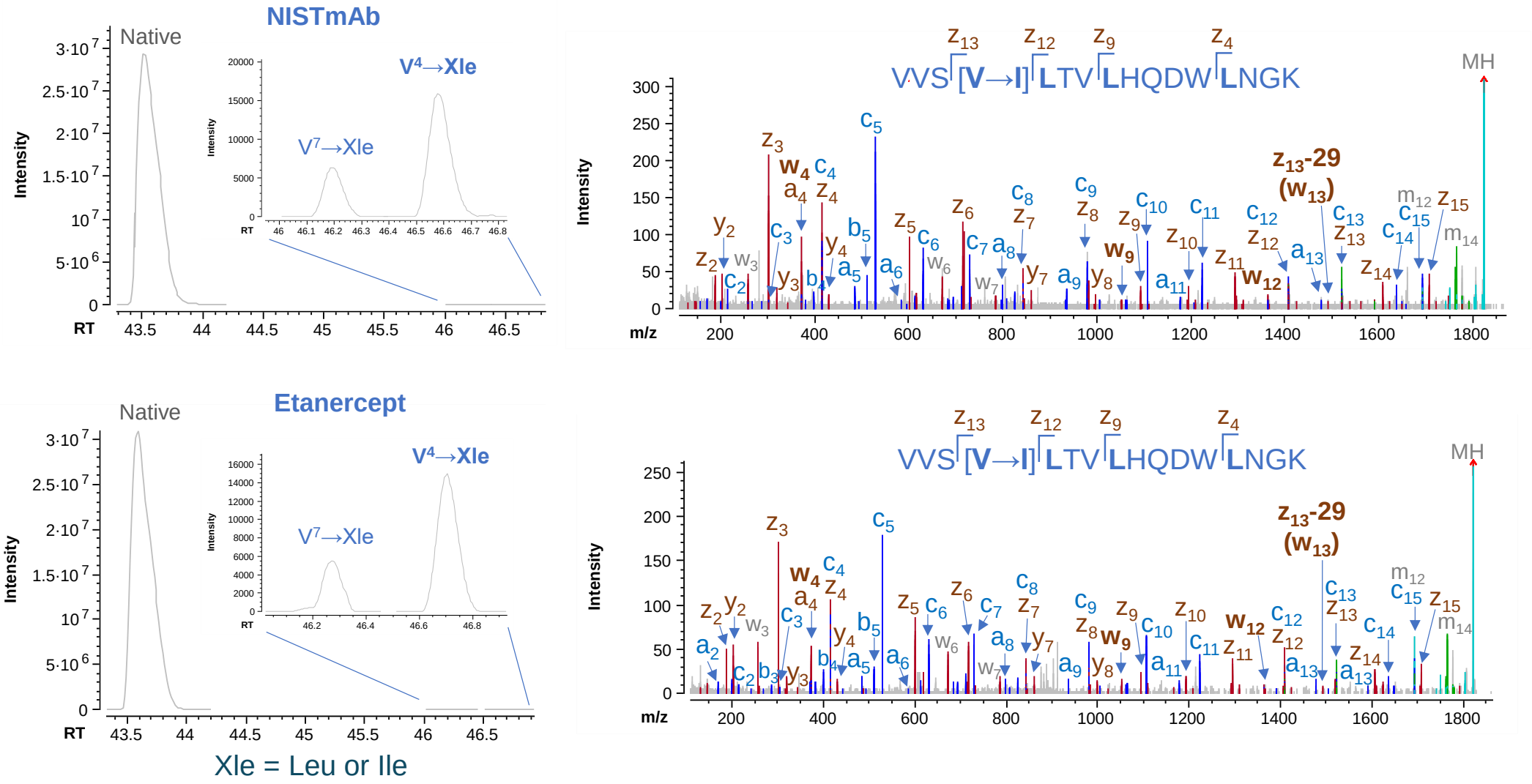
Deamidation isomers in heat-stressed NISTmAb





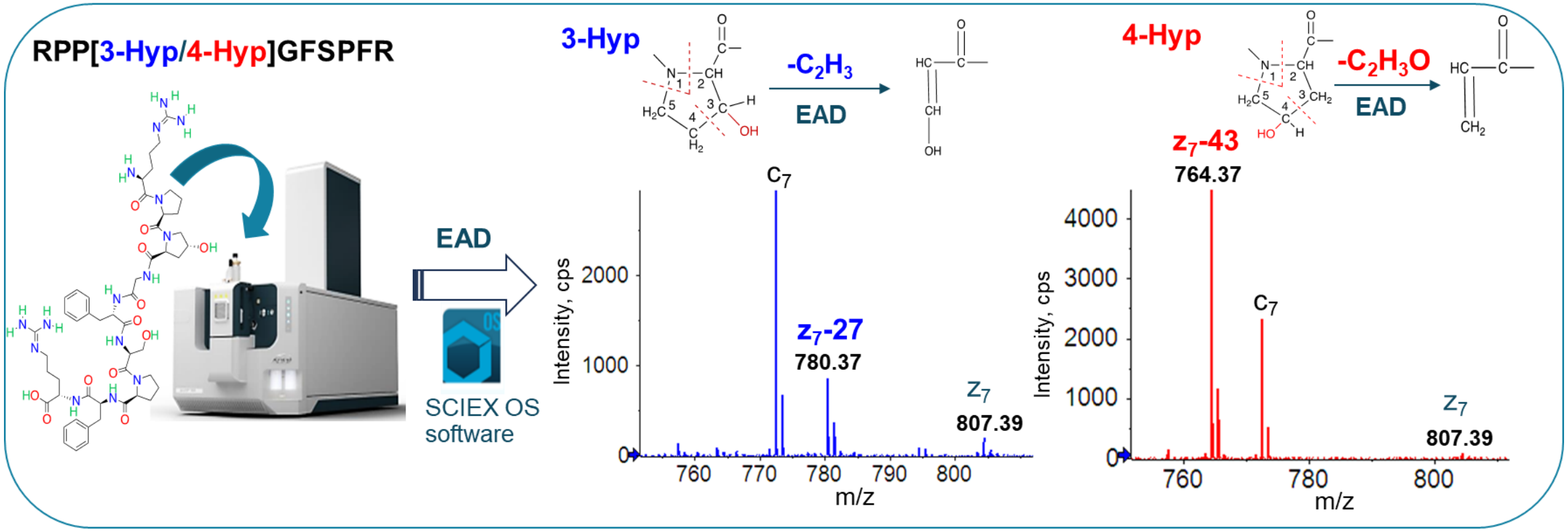
Comprehensive differentiation of deamidation isomers from forced degradation by electron activation dissociation (EAD). SCIEX technical note, RUO-MKT-02-14730-A.

Isomer differentiation for sequence variant analysis



Differentiation of leucine and isoleucine for enhanced sequence variant analysis using electron activated dissociation. SCIEX technical note, MKT-30799-A.

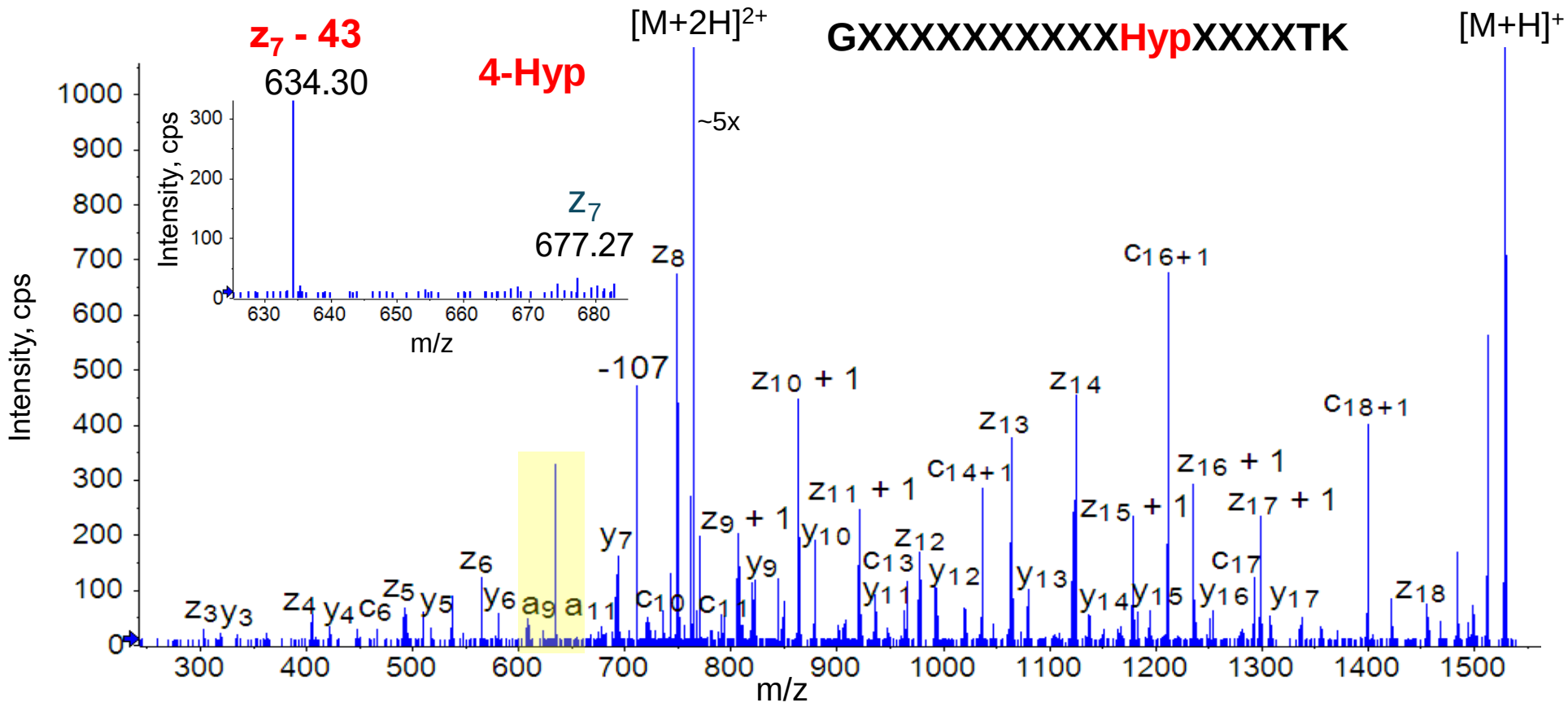
Differentiation of 3- and 4-hydroxyproline



- EAD generates signature *z* - 27 and *z* - 43 fragments for 3- and 4-hydroxyproline (Hyp) isomers, respectively, for the unambiguous differentiation of these 2 Hyp isomers

Site-specific differentiation of hydroxyproline isomers using electron activated dissociation (EAD). SCIEX technical note, MKT-30610-A.

Hyp species in a real-world sample



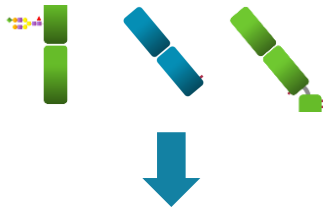
- EAD led to confident identification of a 4-Hyp species in a real-world sample due to the detection of a *z* - 43 fragment

Site-specific differentiation of hydroxyproline isomers using electron activated dissociation (EAD). SCIEX technical note, MKT-30610-A.

Single-injection EAD-based middle-down workflow

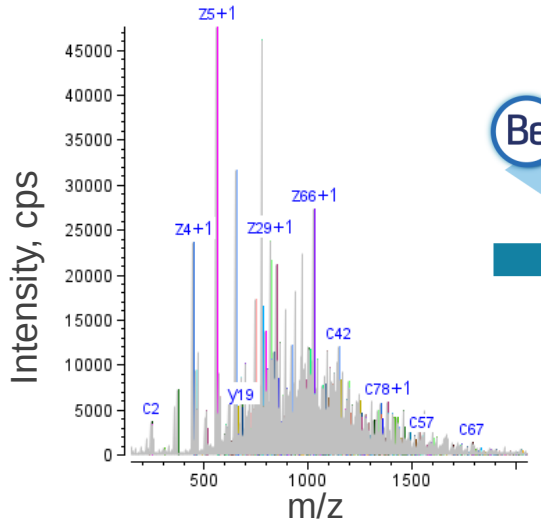
Informative, reproducible and streamlined

mAb subunits



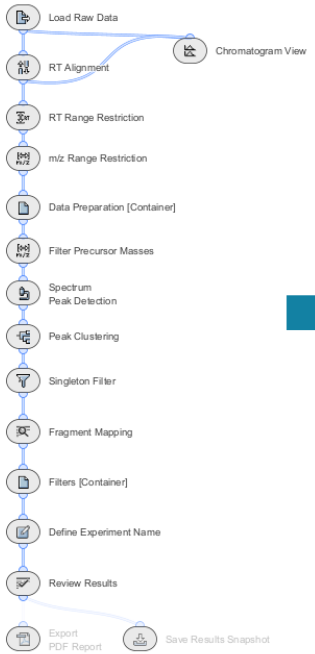
ZenoTOF 7600 system

Zeno EAD

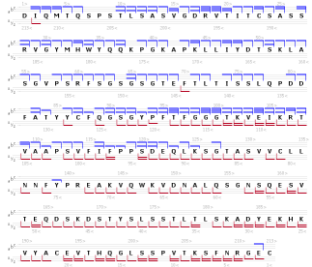


Be

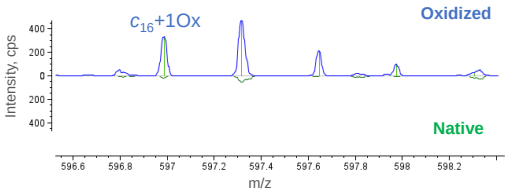
Biologics Explorer software



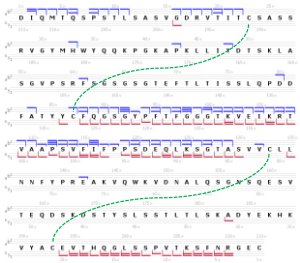
High sequence coverage (70%-90%)[1-5]



Confident sequence and PTM analyses[1-5]

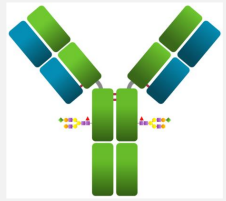


Rapid disulfide bond mapping[4,5]



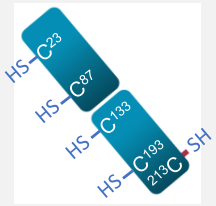
[1]Obtaining high sequence coverage and confident post-translational modification (PTM) analysis of biotherapeutics using an electron activated dissociation (EAD)-based middle-down workflow. SCIEX technical note, MKT-27223-A.
[2]Comparative analysis of biotherapeutics using an electron-activated dissociation (EAD)-based middle-down workflow. SCIEX technical note, MKT-27427-A.
[3]Confident sequence analysis of a trispecific antibody using an electron-activated dissociation (EAD)-based middle-down workflow. SCIEX technical note, MKT-27784-A.
[4]Achieving ultrahigh sequence coverage and high-confidence disulfide bond mapping of biotherapeutics using an electron-activated dissociation (EAD)-based middle-down workflow. SCIEX technical note, MKT-28565-A.
[5]An enhanced single-injection middle-down workflow to achieve high sequence coverage and disulfide mapping of antibody subunits. SCIEX technical note, MKT-30575-A.

Sequence analysis and disulfide bond mapping



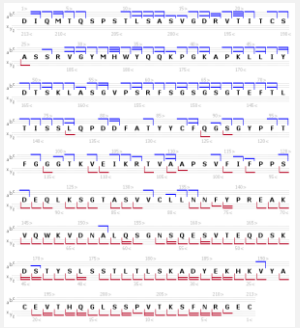
Full reduction

LC

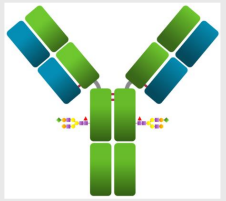


-SH

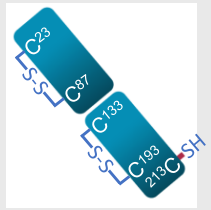
EAD



~80%

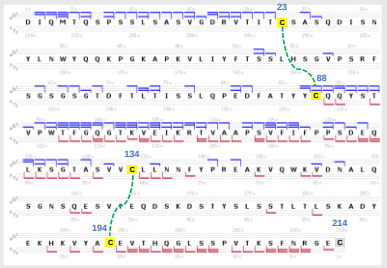


Partial reduction

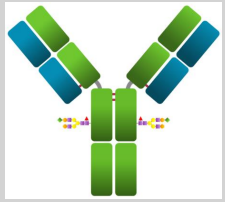


SS

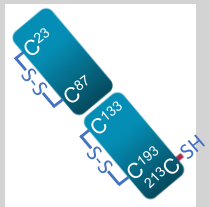
EAD



Disulfide bond mapping

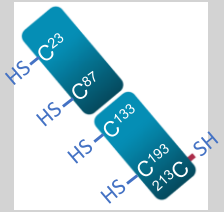


Controlled reduction



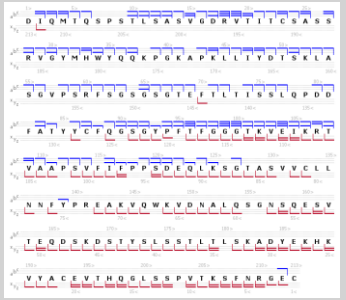
-SH

+



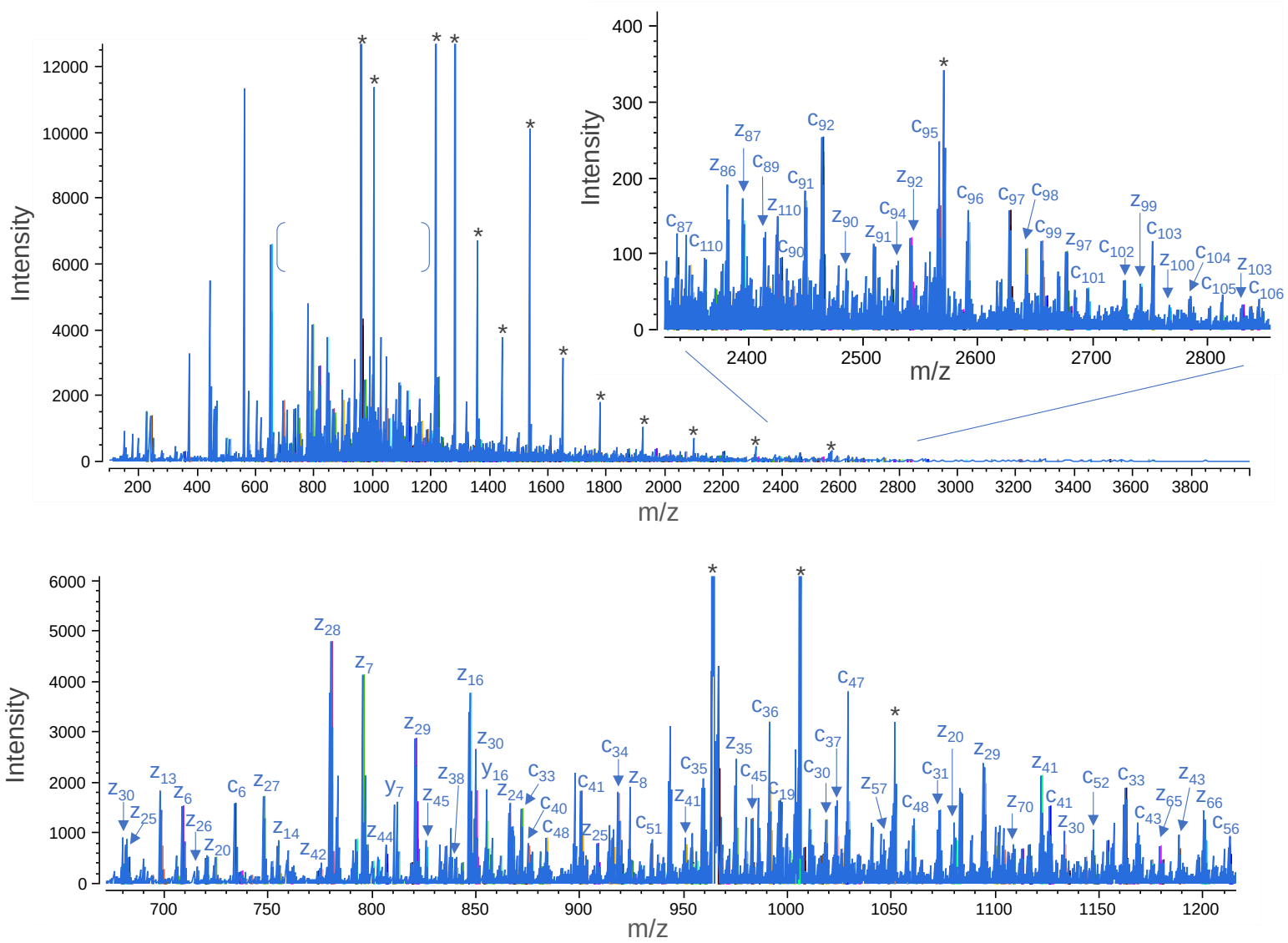
SS

EAD



88% and disulfide bond mapping

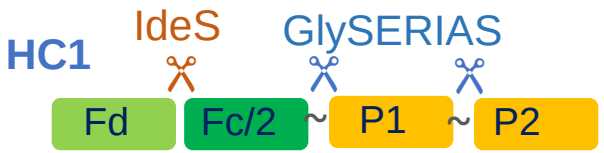
High-quality middle-down data from EAD



- EAD leads to extensive fragmentation of fully reduced or disulfide-linked subunits for enhanced middle-down characterization of biotherapeutic subunits

Achieving ultrahigh sequence coverage and high-confidence disulfide bond mapping of biotherapeutics using an electron-activated dissociation (EAD)-based middle-down workflow. SCIEX technical note, MKT-28565-A.

EAD-based middle-down analysis of a trispecific antibody



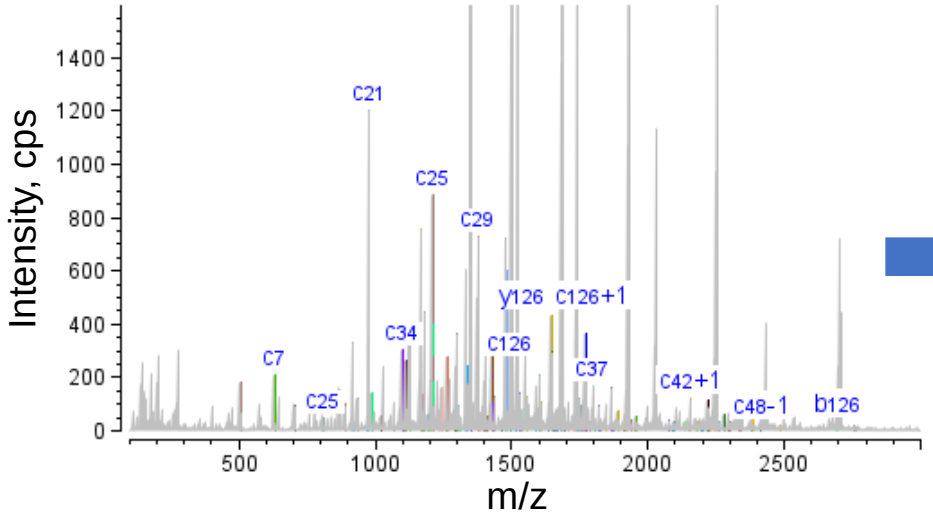
TOF MS

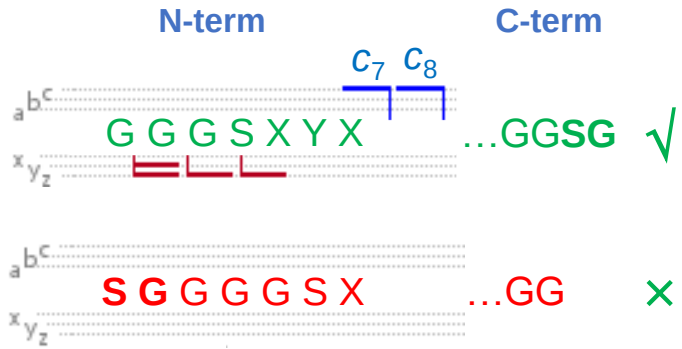
GGGSX…GG**SG**

SGGGGSX…GG

2 candidate sequences for the HC1 P1 subunit

EAD





Confident sequence analysis of a trispecific antibody using an electron-activated dissociation (EAD)-based middle-down workflow. SCIEX technical note, MKT-27784-A.

Disulfide bond mapping

Comprehensive mapping of disulfide linkages in etanercept using an electron activated dissociation (EAD) based LC-MS/MS methodology

Featuring the ZenoTOF 7600 system and Biologics Explorer

Zhengwei Chen and Lei Xiong
SCIEX, USA

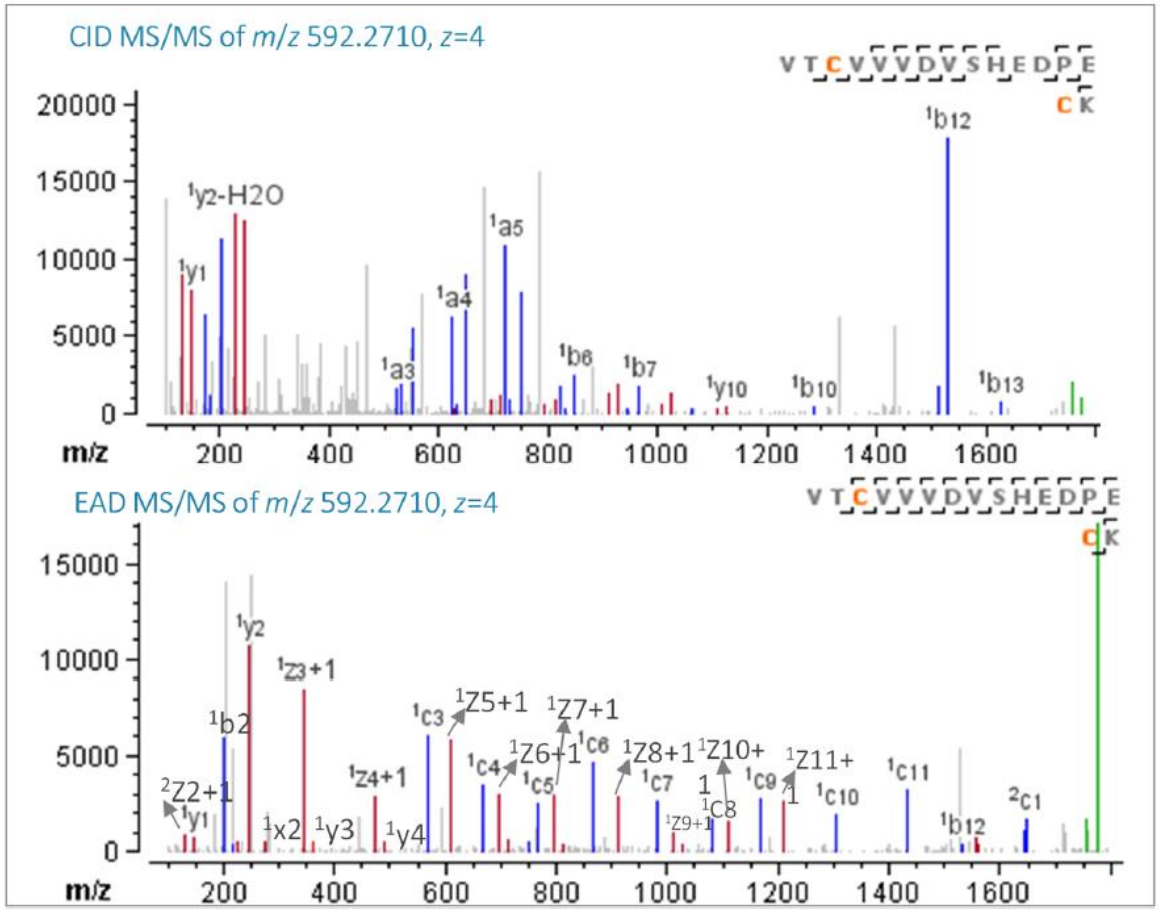


Figure 2. CID and EAD MS/MS spectra of disulfide peptide VTCVVVDVSHEDPE/CK (Cys281-Cys341).

ADC characterization

Comprehensive characterization of an antibody-drug conjugate (ADC) using electron activated dissociation (EAD)

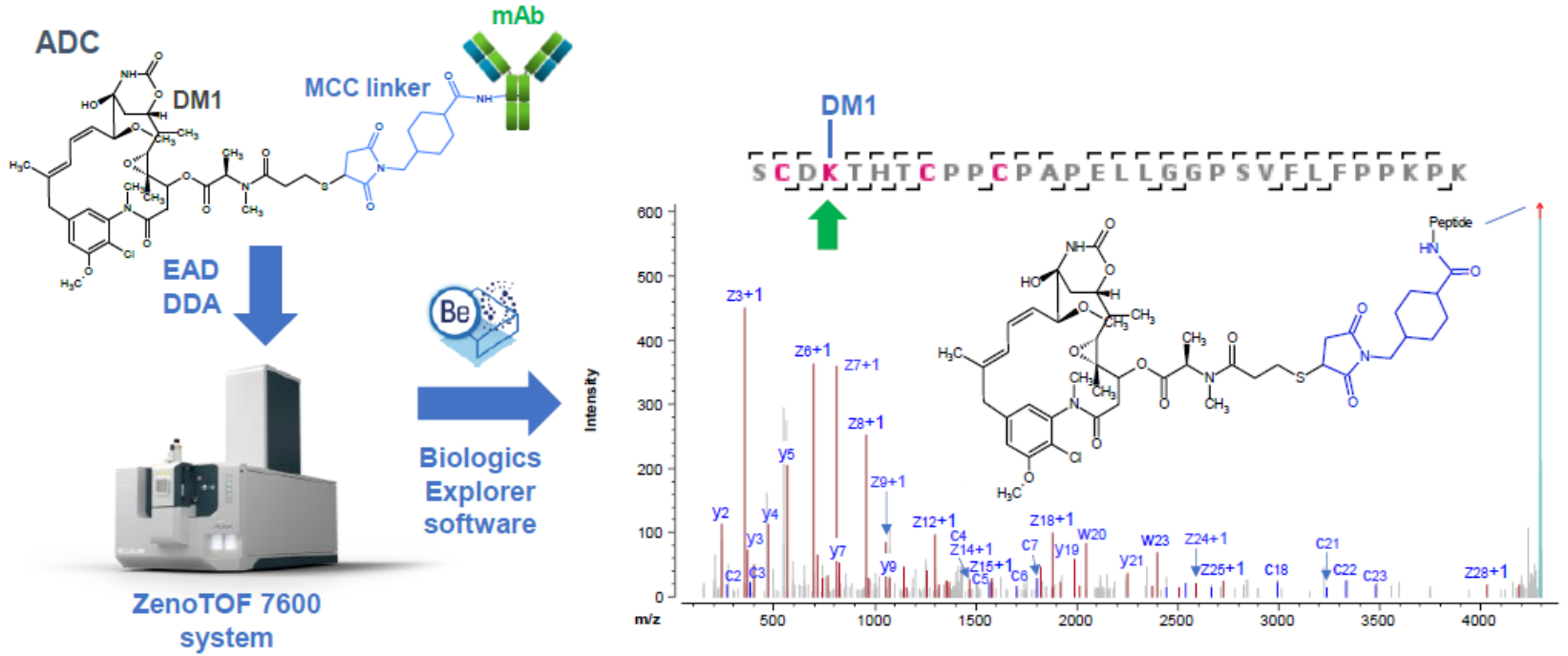


Figure 1. The EAD-based peptide mapping workflow offered by the ZenoTOF 7600 system enables confident sequence confirmation and accurate determination of the payload conjugate sites in T-DM1. The cytotoxic payload, DM1, was retained in the EAD fragments, leading to its accurate localization in the ADC using Biologics Explorer software. MCC: maleimidomethyl cyclohexane-1-carboxylate.

EAD publications

Quantitative structural multiclass lipidomics using differential mobility: electron impact excitation of ions from organics (EIEIO) mass spectrometry[3]

Takashi Baba,[1,*] J. Larry Campbell,* J. C. Yves Le Blanc,* Paul R. S. Baker,* and Kazutaka Ikeda[†]

Sciex,* Concord, Ontario L4K 4V8, Canada; and Center for Integrated Medical Sciences,[†] RIKEN, Tsurumi, Yokohama, 230-0045, Japan

ORCID IDs: 0000-0001-7092-8953 (T.B.); 0000-0002-4496-7171 (J.L.C.); 0000-0001-8342-0278 (K.I.)

Abstract We report a method for comprehensive structural characterization of lipids in animal tissues using a combination of differential ion mobility spectrometry (DMS) with electron-impact excitation of ions from organics (EIEIO) mass spectrometry. Singly charged lipid ions in protonated or sodiated forms were dissociated by an electron beam having a kinetic energy of 10 eV in a branched radio-frequency ion trap. We established a comprehensive set of diagnostics to characterize the structures of glycerophospholipids, sphingolipids, and acylglycerols, including glycosylated, plasmalogen, and ester forms. This EIEIO mass spectrometer was combined with DMS as a separation tool to analyze complex lipid extracts. Deuterated quantitative standards, which were added during extraction, allowed for the quantitative analysis of the lipid molecular species in various lipid classes. We applied this technique to the total lipids extracted from porcine brain, and we structurally characterized over 300 lipids (with the exception of *cis*/*trans* double-bond isomerism in the acyl chains). The structural dataset of the lipidomes, whose regioisomers were distinguished, exhibit a uniquely defined distribution of acyl chains within each lipid class; that is, *sn*-1 and *sn*-2 in the cases of glycerophospholipids or *sn*-2 and (*sn*-1, *sn*-3) in the cases of triacylglycerols.—Baba, T., J. L. Campbell, J. C. Y. Le Blanc, P. R. S. Baker, and K. Ikeda. Quantitative structural multiclass lipidomics using differential mobility: electron impact excitation of ions from organics (EIEIO) mass spectrometry. *J. Lipid Res.* 2018. 59: 910–919.

Supplementary key words electron induced dissociation • complex lipids • brain extract

lipids' actions can be related to their structures, with small structural differences in isomeric lipid molecules having dramatic impact on their physiological functions [e.g., the diastereomers prostaglandin-D2 and prostaglandin-E2 (5, 6)]. Recently, a slight permutation in double-bond position in one of the acyl chains of a phospholipid was shown to be a marker of breast cancer (7), and the presence of a Δ7 vs. a Δ9 double bond in a specific molecular species of triacylglycerol (TG) was correlated with adverse cardiovascular outcomes (1). For these reasons, differentiation of lipids by complete structural characterization is "a real promise in achieving true molecular level lipidomics analysis generation" (8). Not only qualitative structure characterization of each lipid molecule, but also quantitative analysis across many lipid classes in tissues, will be the next target in lipidomics to elucidate cross-class lipid metabolism.

Conventionally, NMR is used as a tool for full characterization of lipid structures (9), but it needs a reasonably large amount (approximately milligrams) of purified samples and very long analysis times (approximately days), which results in low throughput. Mass spectrometry (MS) is used widely as a high-throughput method for versatile biomolecular identification (10). Conventional techniques for lipid analysis in MS are based on a combination of separation techniques (liquid chromatography, gas chromatography, ion mobility spectrometry, etc.) and collision-induced dissociation (CID) in the gas phase (1). This approach,

Journal of Lipid Research. 59, 2018

Journal of proteome research

pubs.acs.org/jpr Article

Localization of Multiple *O*-Linked Glycans Exhibited in Isomeric Glycopeptides by Hot Electron Capture Dissociation

Takashi Baba,* Zoe Zhang, Suya Liu, Lyle Burton, Pavel Ryumin, and J. C. Yves Le Blanc

Cite This: *J. Proteome Res.* 2022, 21, 2462−2471 Read Online

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ABSTRACT: We describe a method to obtain a comprehensive profile of multiple glycosylations in glycopeptide isoforms. We detected a wide range of abundances of various *O*-glycoforms in isomeric glycopeptides using hot electron capture dissociation (hot ECD) in liquid chromatography−tandem mass spectrometry. To capture low abundant glycosylated species, a prototype of a ZenoTOF 7600 system incorporating an efficient electron-activated dissociation device to perform hot ECD was operated in targeted or scheduled high-resolution multiple reaction monitoring workflows. In addition, Zeno trap pulsing was activated to enhance the sensitivity of the time-of-flight mass spectrometer. Sixty-nine *O*-glycopeptides of the long *O*-glycopeptides in tryptic bovine fetuin digest were obtained with a relative abundance range from 100 to 0.2%, which included sialylated glycans with Neu5Ac and Neu5Gc.

KEYWORDS: *electron-activated dissociation, hot electron capture dissociation, mass spectrometry, O-linked glycopeptides, fetuin, time-of-flight mass spectrometer, scheduled high-resolution multiple reaction monitoring, Zeno trap pulsing*

Electron-Activated Dissociation and Collision-Induced Dissociation Glycopeptide Fragmentation for Improved Glycoproteomics

Kyle L. Macauslane, Cassandra L. Pegg, Amanda S. Nouwens, Edward D. Kerr, Joy Seitanidou, and Benjamin L. Schulz*

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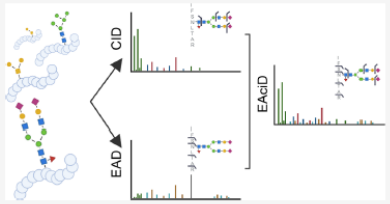
ABSTRACT: Tandem mass spectrometry coupled with liquid chromatography (LC-MS/MS) has proven a versatile tool for the identification and quantification of proteins and their post-translational modifications (PTMs). Protein glycosylation is a critical PTM for the stability and biological function of many proteins, but full characterization of site-specific glycosylation of proteins remains analytically challenging. Collision-induced dissociation (CID) is the most common fragmentation method used in LC-MS/MS workflows, but the loss of labile modifications renders CID inappropriate for detailed characterization of site-specific glycosylation. Electron-based dissociation methods provide alternatives that retain intact glycopeptide fragments for unambiguous site localization, but these methods often underperform CID due to increased reaction times and reduced efficiency. Electron-activated dissociation (EAD) is another strategy for glycopeptide fragmentation. Here, we use a ZenoTOF 7600 SCIEX instrument to compare the performance of various fragmentation techniques for the analysis of a complex mixture of mammalian *O*- and *N*-glycopeptides. We found CID fragmentation identified the most glycopeptides and generally produced higher quality spectra, but EAD provided improved confidence in glycosylation site localization. Supplementing EAD with CID fragmentation (EAciD) further increased the number and quality of glycopeptide identifications, while retaining localization confidence. These methods will be useful for glycoproteomics workflows for either optimal glycopeptide identification or characterization.



ACS Partner Journal

Journal of the American Society for Mass Spectrometry

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Characterization of Phosphorylated Peptides by Electron-Activated and Ultraviolet Dissociation Mass Spectrometry: A Comparative Study with Collision-Induced Dissociation

Marion Girod,* Delphine Arquier, Amanda Helms, Kyle Juetten, Jennifer S. Brodbelt, Jérôme Lemoine, and Luke MacAleese

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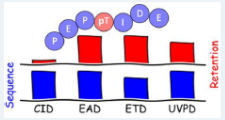
ABSTRACT: Mass-spectrometry-based methods have made significant progress in the characterization of post-translational modifications (PTMs) in peptides and proteins; however, room remains to improve fragmentation methods. Ideal MS/MS methods are expected to simultaneously provide extensive sequence information and localization of PTM sites and retain labile PTM groups. This collection of criteria is difficult to meet, and the various activation methods available today offer different capabilities. In order to examine the specific case of phosphorylation on peptides, we investigate electron transfer dissociation (ETD), electron-activated dissociation (EAD), and 193 nm ultraviolet photodissociation (UVPD) and compare all three methods with classical collision-induced dissociation (CID). EAD and UVPD show extensive backbone fragmentation, comparable in scope to that of CID. These methods provide diverse backbone fragmentation, producing a/x, b/y, and c/z ions with substantial sequence coverages. EAD displays a high retention efficiency of the phosphate modification, attributed to its electron-mediated fragmentation mechanisms, as observed in ETD. UVPD offers reasonable retention efficiency, also allowing localization of the PTM site. EAD experiments were also performed in an LC-MS/MS workflow by analyzing phosphopeptides spiked in human plasma, and spectra allow accurate identification of the modified sites and discrimination of isomers. Based on the overall performance, EAD and 193 nm UVPD offer alternative options to CID and ETD for phosphoproteomics.

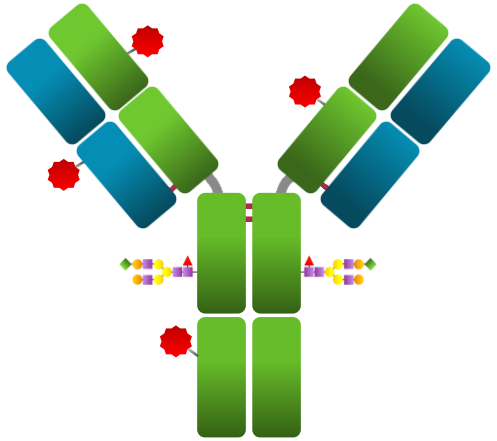


KEYWORDS: *electron-induced dissociation, photofragmentation, phosphorylation modifications, fragmentation method, EAD, ETD, UVPD*

Isoelectric focusing electrophoresis combined to the 7600 for the analysis of ADC’s

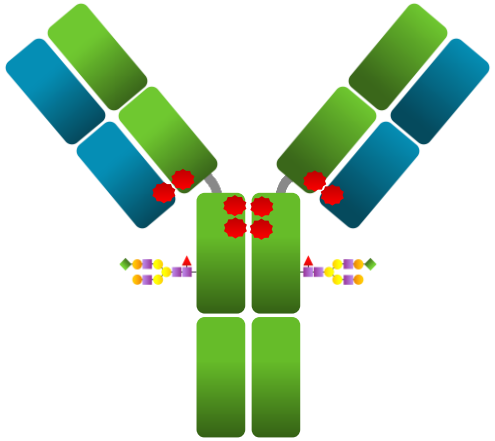
ADCs characterized

Trastuzumab emtansine (Kadcyla, T-DM1)



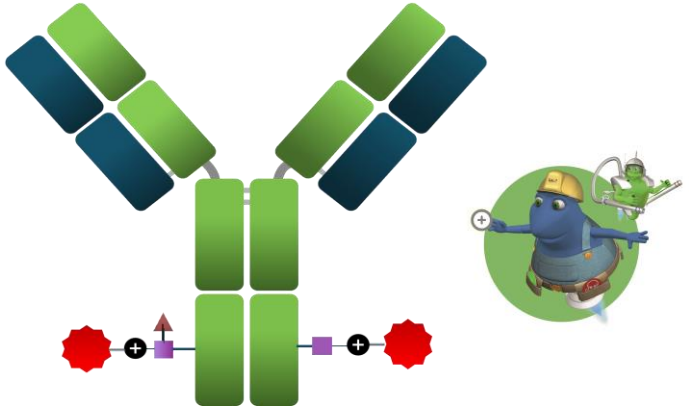
Lys-conjugated
Non-specific
Avg. DAR of ~3.5

Trastuzumab deruxtecan (Enhertu, T-DXd)



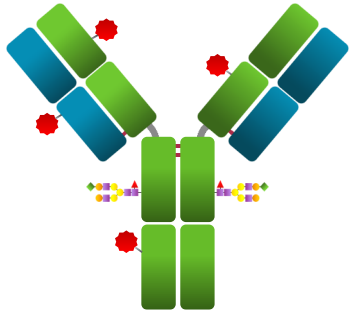
Cys-conjugated
Site-specific
Fixed DAR of 8

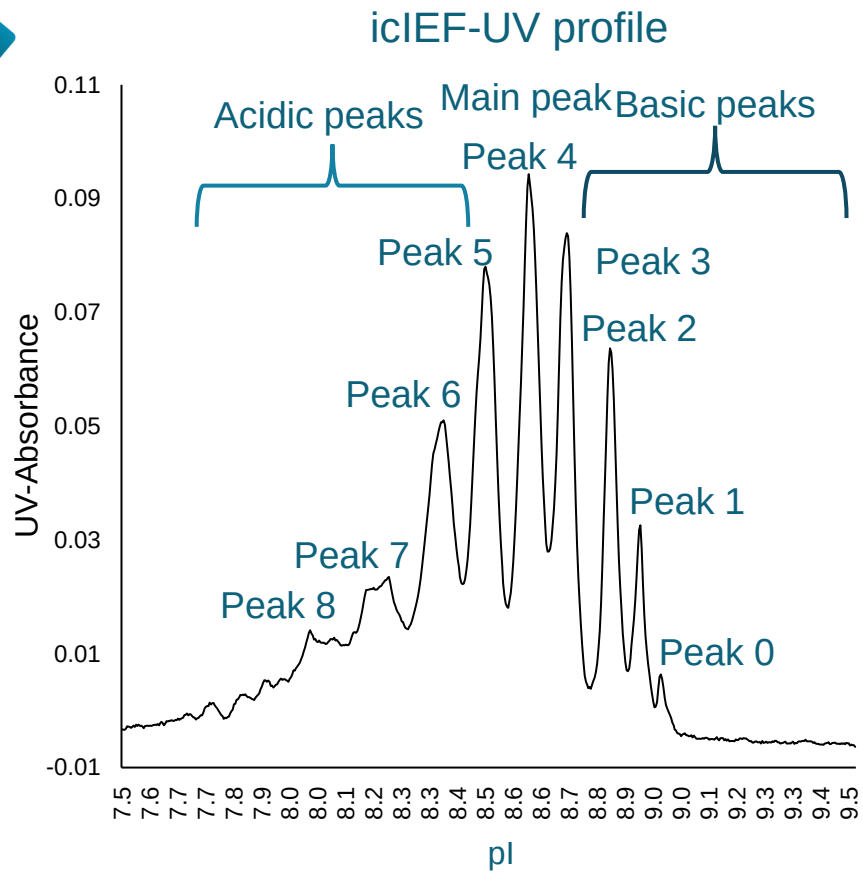
Genovis ADC

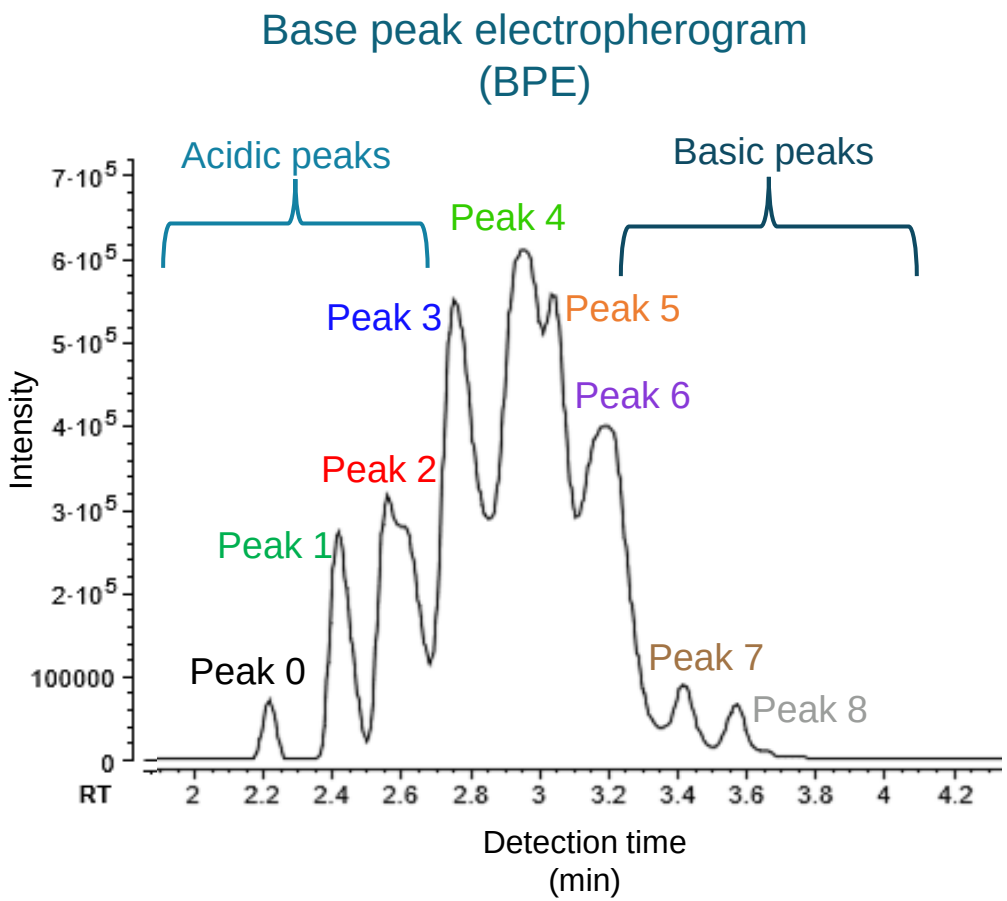


Conjugated via GlyCLICK®
(property of Genovis AB)
Site-specific
Fixed DAR of 2

icIEF-UV/MS analysis of T-DM1







The separation of charge variants is maintained after chemical mobilization.

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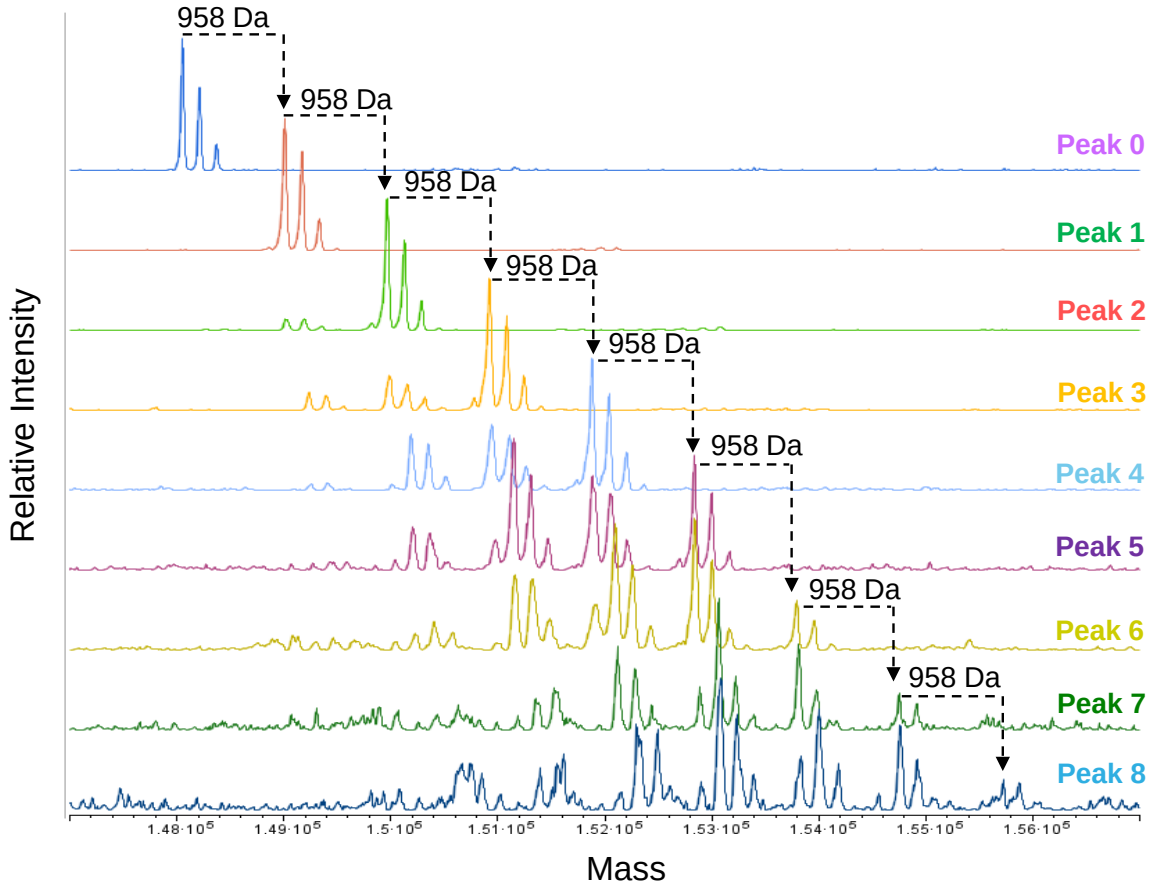
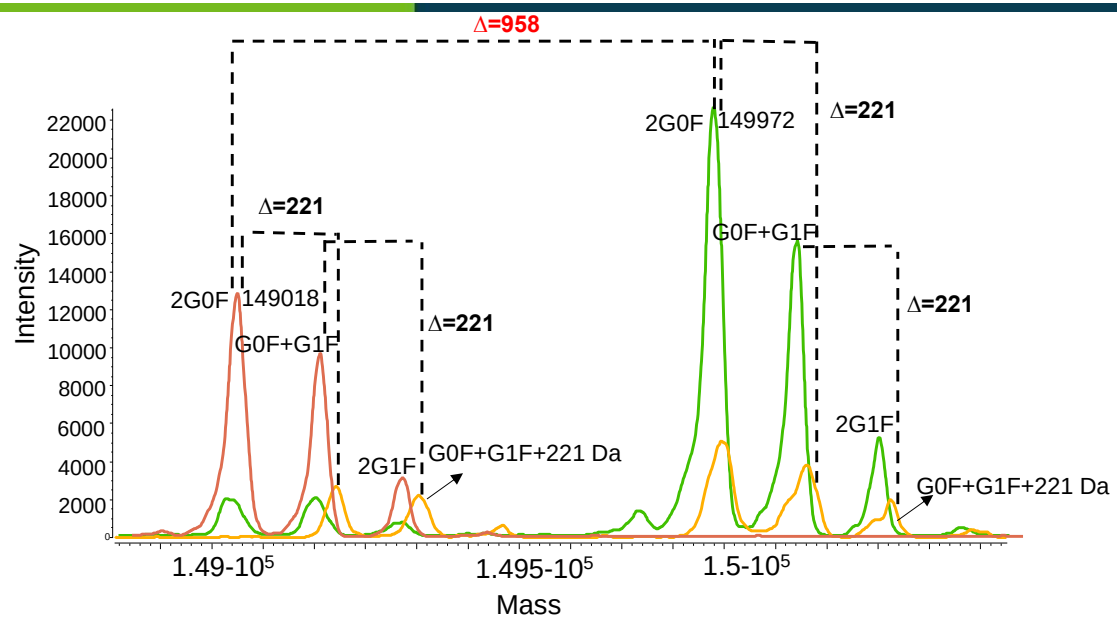
Deconvoluted mass spectra





- The major peaks identified were G0F and G1F glycoforms of T-DM1 carrying 2-6 payloads. A mass difference of +958 Da measured between the adjacent major peaks corresponds to the DM1 payload conjugated with the MCC linker
- Low-abundant peaks with a mass difference of +221 Da can be attributed to the ADC conjugated with 1 free linker but not the DM1 payload

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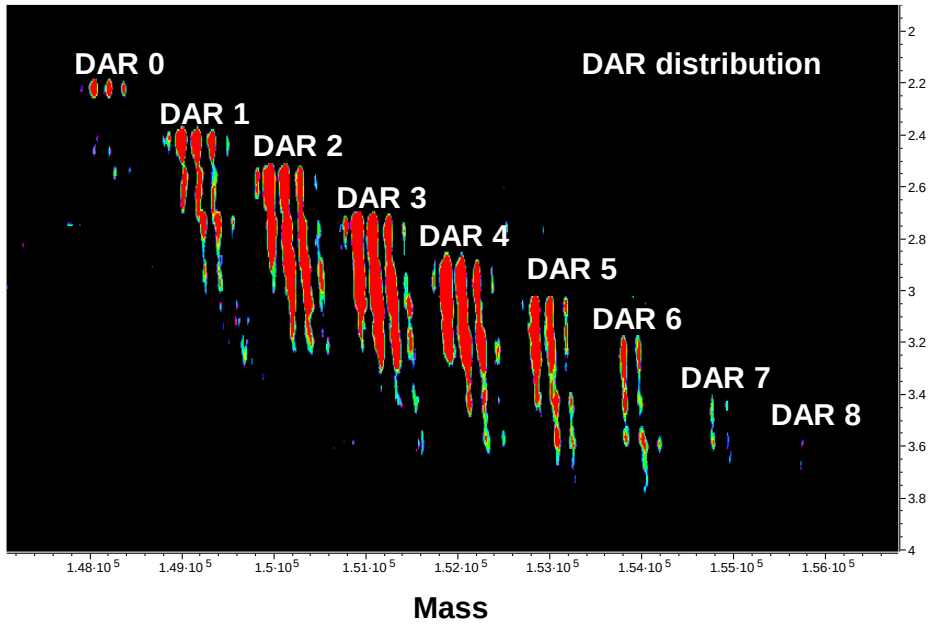
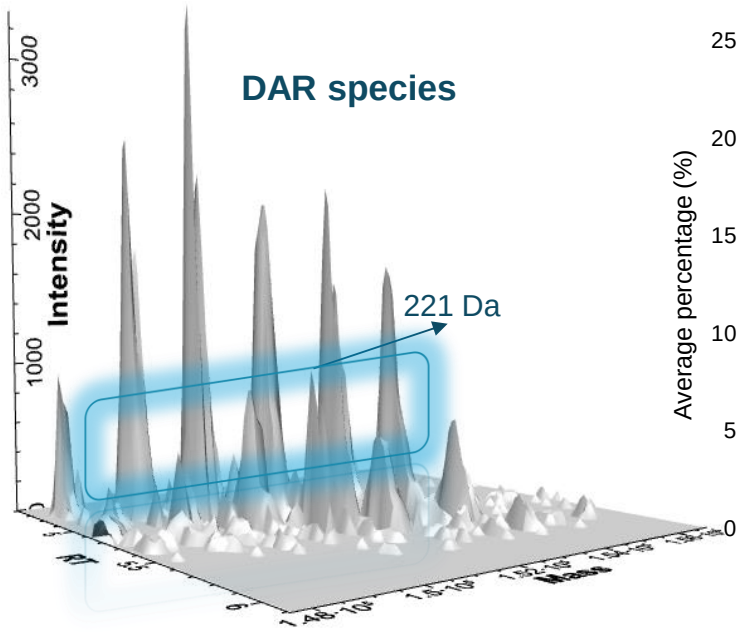
Results visualization within Biologics Explorer software

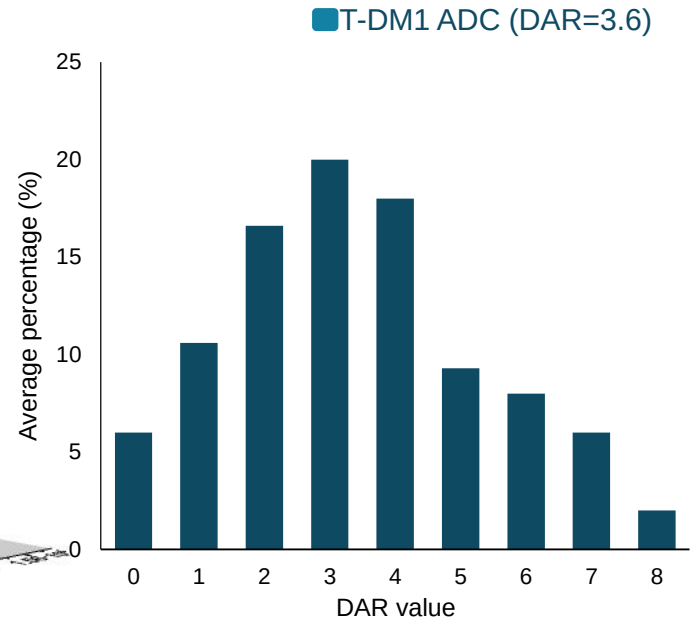
Ion map



3D view

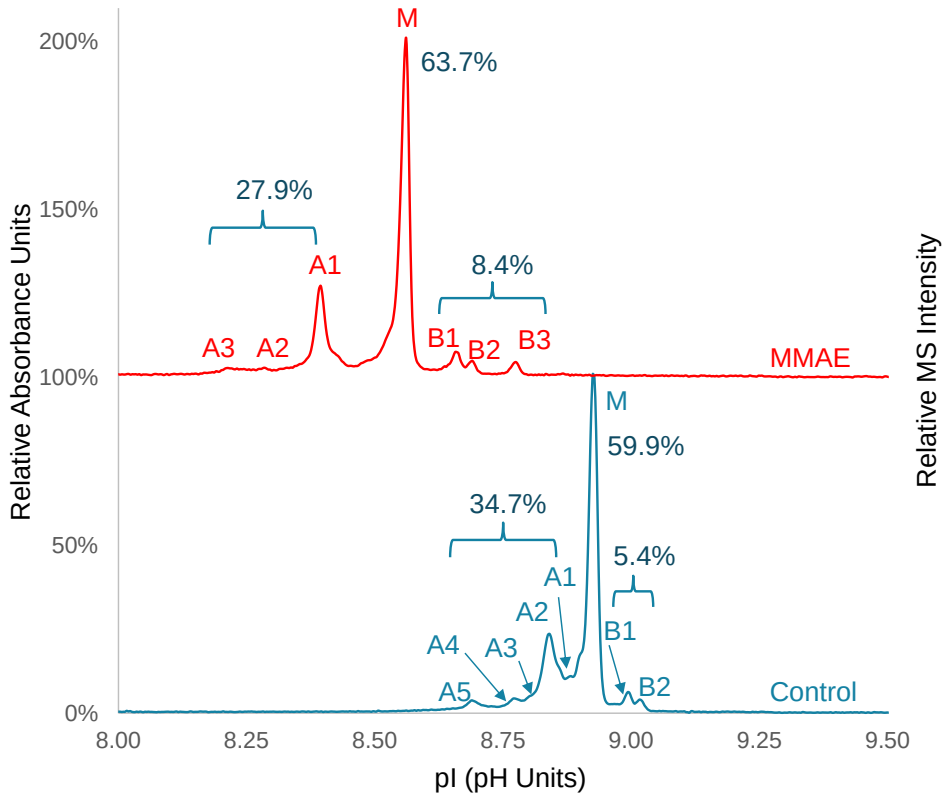


Automated DAR calculation

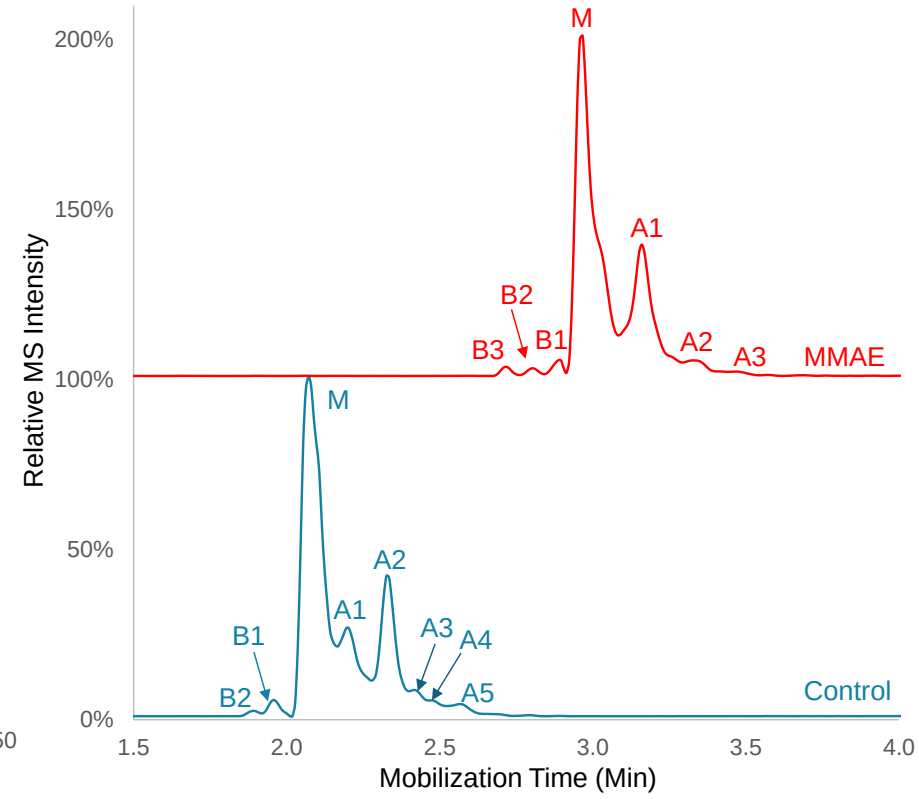


icIEF-UV/MS analysis of Genovis ADC

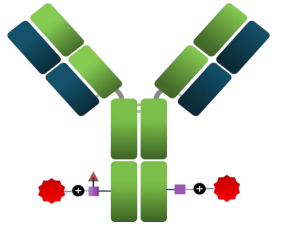
icIEF-UV



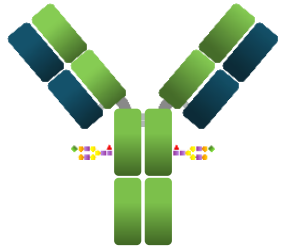
BPE



GENOVIS



ADC



mAb

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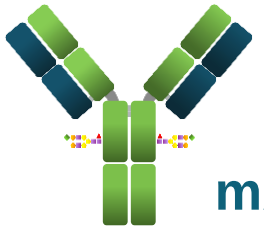
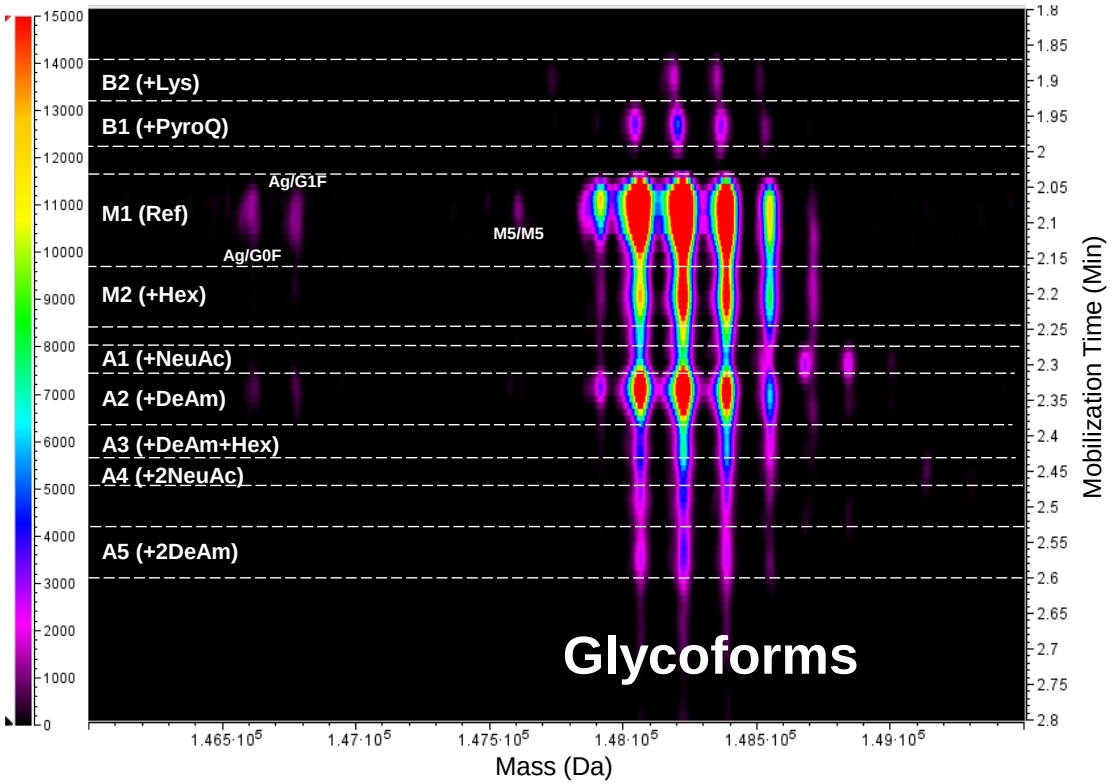
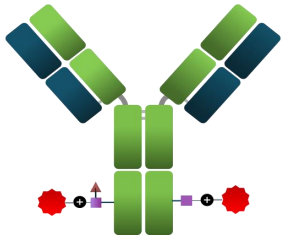
Charge variants identified by icIEF-UV/MS

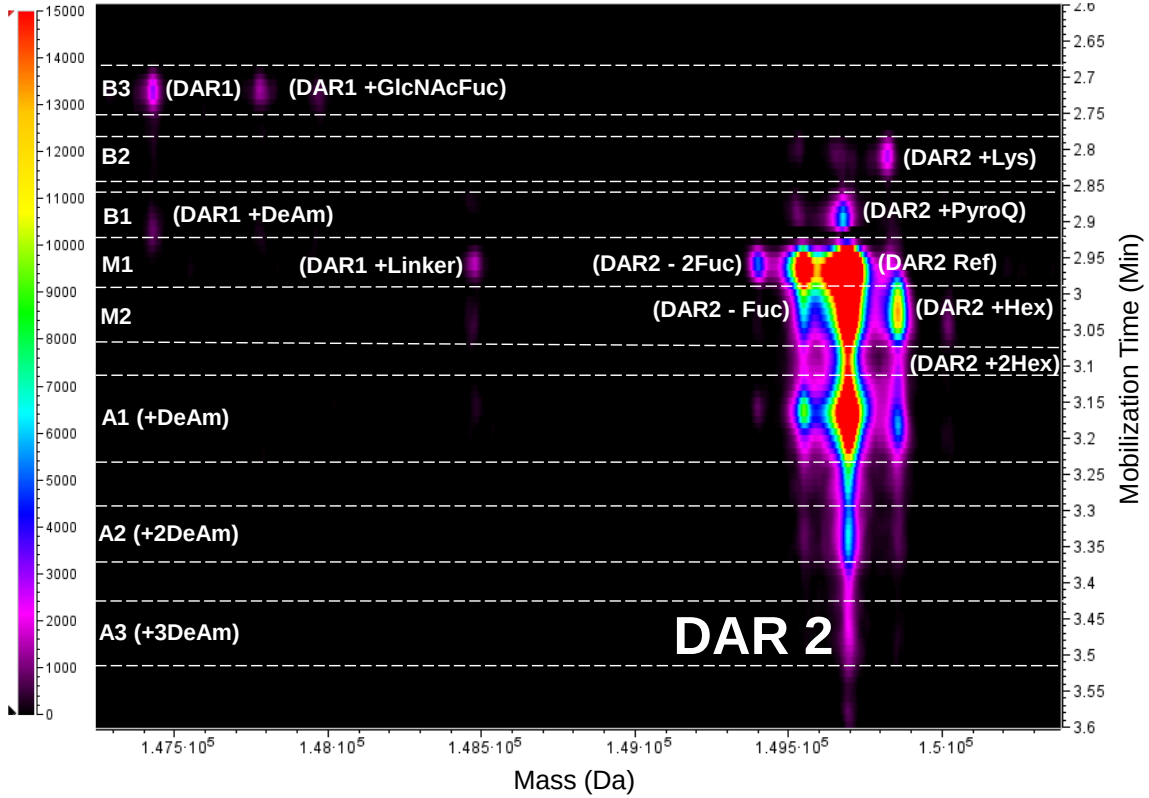


mAb





ADC



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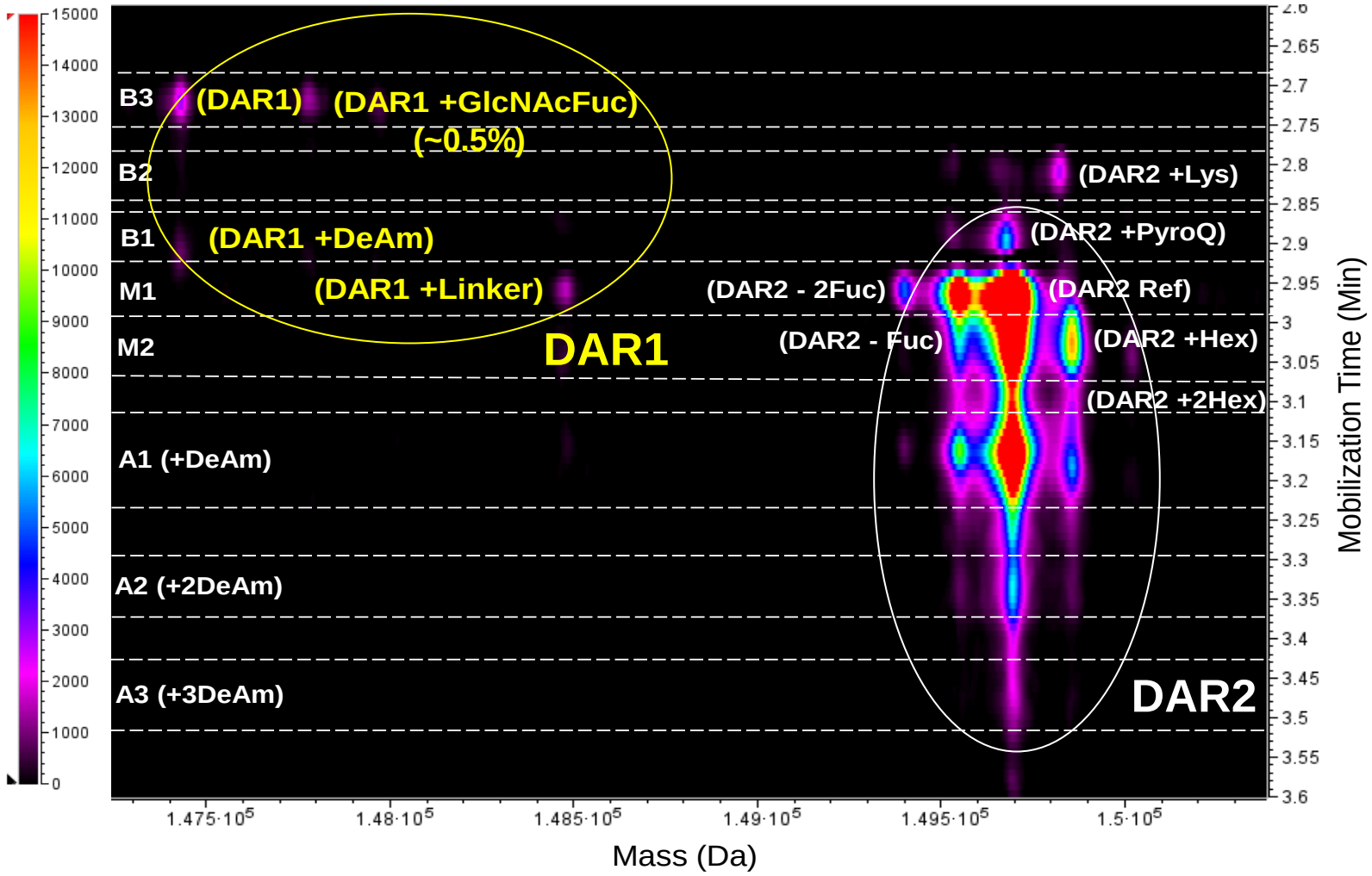
Impurity assessment



- DAR1+GlcNAcFuc was measured to be ~0.5%, demonstrating the high sensitivity of icIEF-UV/MS and the high efficiency of payload conjugation using GlyCLICK

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Conclusions

- SCIEX offers a complete analytical solution for comprehensive characterization of biotherapeutics and their charge variants using a single MS platform
- Orthogonal, streamlined cIEF, icIEF-UV/MS, EAD-based peptide mapping and middle-down workflows provide an in-depth charge heterogeneity analysis of mAbs and ADCs, allowing confidence in assignments beyond traditional workflows such as fraction collection and CID-based peptide mapping
- icIEF-UV/MS workflow provides high-resolution separation, sensitive detection and confident identification of biotherapeutic charge variants
- EAD-based peptide mapping workflow leads to excellent peptide fragmentation, high sequence coverage and accurate PTM localization in a single injection
- EAD-based middle-down workflow allows rapid sequence confirmation, PTM localization and disulfide bond mapping

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  - Philip Widdowson

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Thank you!

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