# A novel alternative fragmentation approach for comprehensive glycopeptide analysis of therapeutic proteins

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## INTRODUCTION

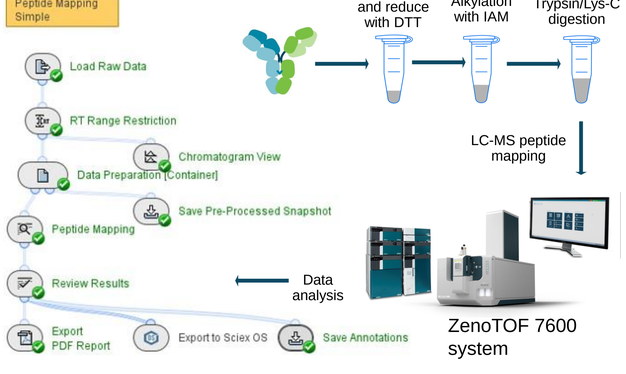
The glycosylation profile of biotherapeutics is quite heterogenous, and is a key area that requires multiple methodologies to ensure full characterization as the complexity poses analytical challenges. The CID-based mass spectrometry approach for peptide mapping has continued to have difficulty with glycopeptides, as it does not allow for consistent identification and localization of glycans on peptides.
Here, a newly developed dissociation technique that creates MS/MS fragment ions of the peptide backbone, while maintaining the intact glycosylation on the fragments, was evaluated. A second technology called Zeno trap was used in conjunction to enhance the duty cycle for MS/MS fragment ions and improve the overall sensitivity and coverage of peptides.

## Key features of the SCIEX solution

- Electron activated dissociation (EAD) with fast data-dependent acquisition (DDA) enables alternative fragmentation for routine, in-depth analysis of next generation protein therapeutics and standard mAbs
- The tunable electron energy provides a higher level of structural information for glycopeptide characterization
- Increased detection of fragments (5 to 10-fold) using the Zeno trap
- Reproducible fragmentation with EAD for singly, doubly, and multiply charged ions
- Fully automated DDA using EAD with SCIEX OS software, and automated data interpretation with the new Biologics Explorer software from SCIEX.

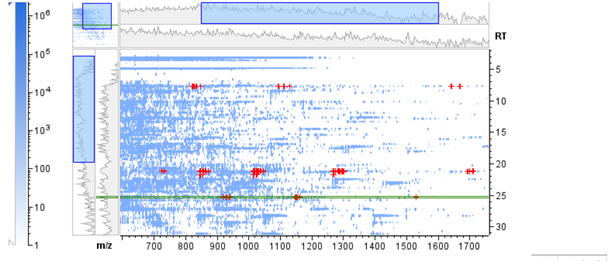
## MATERIALS AND METHODS

Trastuzumab was prepared and digested with trypsin before injection into the LC-MS/MS system. Data was acquired using DDA on a QTOF system (ZenoTOF 7600 system, SCIEX) with either CID or EAD, a novel type of ExD.[1-4]










| Peptide | Modifications | Mod. Locations | Glycans | Calc. Mass | Group Id | RT | Adduct States | Length | Delta [ppm] |
|---|---|---|---|---|---|---|---|---|---|
| TKPREEQYNSTYR | G1F | [N300] | Fuc1Gal1 | 3277.39 | 156 | 7.67... | M+4H : M+4H+(... | 13 | 2.68697 |
| TKPREEQYNSTYRVVSVLTVLHQDWLNGK | G1F | [N300] | Fuc1Gal1 | 5066.38 | 24 | 21.1... | M+7H : M+7H+(... | 29 | 3.18368 |
| TKPREEQYNSTYRVVSVLTVLHQDWLNGK | Deamidated, G1F | [Q314] [N300] | Fuc1Gal1 | 5067.36 | 175 | 21.7... | M+6H : M+6H+(... | 29 | 4.56704 |
| EEQYNSTYRVVSVLTVLHQDWLNGK | G1F | [N300] | Fuc1Gal1 | 4584.08 | 149 | 25.279 | M+5H : M+5H+(... | 25 | 2.84793 |

**Figure 1. Heat map of peptides containing G1F.** All peptides containing G1F were selected and listed in the table (below) showing modification, mass and retention time. Above in blue are all *m/z* identified while red crosses show clusters of charge states and adducts for all 4 peptides containing G1F.

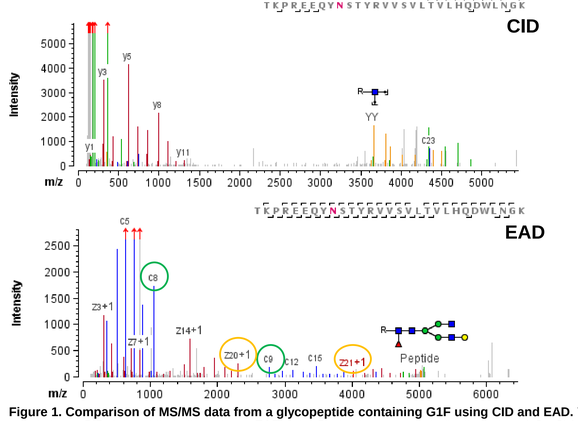
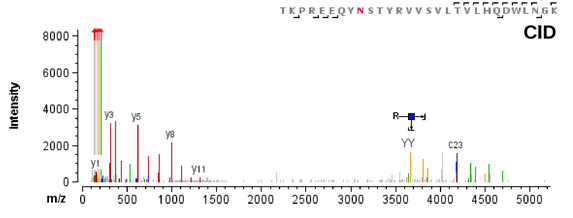



**Figure 1. Comparison of MS/MS data from a glycopeptide containing G1F using CID and EAD.** Top panel shows CID data, while bottom shows EAD data. Circled in green are indicative *c*-ions (*c*8, *c*9) for the glycan while circled in orange are the indicative *z*-ions (*z*20, *z*21). Blue hash (*c*- and *b*-ions) and red hash (*z*- and *y*- ions) indicate the ions identified.



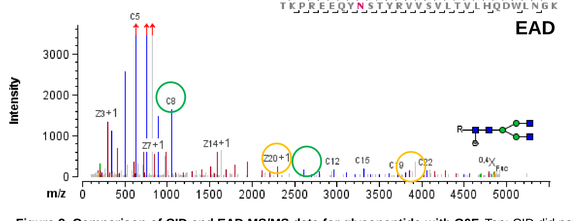




**Figure 3. Comparison of CID and EAD MS/MS data for glycopeptide with G0F.** Top: CID did not provide descriptive fragment information, neither positional information for the glycosylation. Bottom: close to 100% fragment coverage was achieved with EAD and diagnostic fragment ions confirm the localization of the glycosylation (encircled ions *c*8/*c*9 and *z*20/*z*21);

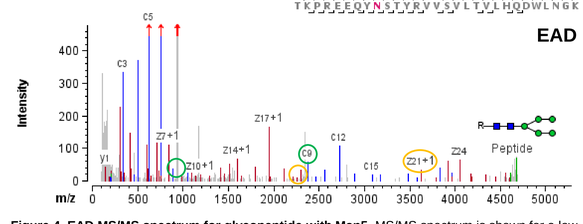



**Figure 4. EAD MS/MS spectrum for glycopeptide with Man5.** MS/MS spectrum is shown for a low-abundance glycopeptide (*z* = +5) of ~3% relative abundance. EAD achieved extensive fragment coverage and localization of the glycosylation. Diagnostic fragments *c*8/*c*9 and *z*20/*z*21 are circled in the spectra.

| Modifi... | 20210130 Herceptin IDA ECD ... | 20210130 Herceptin IDA ECD ... | 20210130 Herceptin IDA ECD Most intense _3 [%] |
|---|---|---|---|
| G0 | 3.23 | 3.20 | 3.13 |
| G0-GlcNAc | 0.57 | 0.58 | 0.60 |
| G0F | 46.08 | 46.09 | 45.48 |
| G0F-GlcNAc | 6.03 | 6.26 | 6.42 |
| G1 | 1.08 | 1.07 | 1.03 |
| G1F | 34.88 | 34.60 | 35.17 |
| G2F | 4.36 | 4.32 | 4.41 |
| Man5 | 3.27 | 3.35 | 3.24 |
| Man6 | 0.42 | 0.43 | 0.40 |

**Figure 5. Identification summary of N-linked glycosylations in trastuzumab at N300.** The table summarizes the identified glycan species based on MS/MS with EAD and the relative abundance based on the XIC of the MS1 for three replicate injections. G0F and G1F are the most abundant glycoforms observed. Trace level of high mannose were also detected. The table confirms high reproducibility from high-abundance modified peptides to very low-abundance forms.

## CONCLUSIONS

- The robust, reproducible and easy-to-use alternative fragmentation mechanism of EAD enables users to identify, fully characterize, and relatively quantify glycopeptides along with a general peptide mapping analysis in a single injection
- Excellent fragment coverage and localization of fragile modifications can be achieved with Zeno EAD with very high reproducibility, allowing for full confidence in peptide ID
- MS/MS fragment detection can be significantly enhanced compared to traditional MS/MS analyses, enabling collection of high-quality data for confident fragment assignment utilizing Zeno EAD
- Automatic data processing enables the routine and advanced characterization of complex biotherapeutics and standard mAbs in a reproducible manner using the new Biologics Explorer software from SCIEX

## REFERENCES

1. Baba T et al. (2015) Electron capture dissociation in a branched radio-frequency ion trap, *Anal Chem*, 87, 785−792.
2. Baba T et al. (2021) Dissociation of biomolecules by an intense low-energy electron beam in a high sensitivity time-of-flight mass spectrometer. Accepted by *JASMS*.
3. Comprehensive peptide mapping of biopharmaceuticals utilizing electron activated dissociation (EAD). SCIEX technical note, RUO-MKT-02-12639-B.
4. A comprehensive peptide level characterization of a multispecific monoclonal antibody (mAb). SCIEX technical note, RUO-MKT-02-12975-B.

## TRADEMARKS/LICENSING