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

Featuring the SCIEX ZenoTOF 7600 system with EAD and Protein Metrics Inc. software

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Here, the characterization of lysine-linked drugs in an ADC sample is demonstrated. A bottom-up approach was chosen to determine the position of the conjugation. Alternative fragmentation using electron activated dissociation (EAD)[1,2] was leveraged for detailed structural information on the drug and linker, along with obtaining peptide backbone information.

With the development in protein engineering, antibodies and their related derivatives become the fastest growing class of therapeutics.[3] ADCs are one of those new modalities. ADCs a are often composed of a 150 kDa monoclonal antibody (mAb) covalently coupled with cytotoxic payloads, or other types of drugs, through synthetic linkers.[4] ADCs show a more complex structure and heterogeneity compared to unconjugated proteins, since the addition of a variable number of payload and linkers can significantly enhance the number of proteoforms.[5] To ensure drug safety and efficacy, an in-depth characterization of ADCs is essential during their development. This includes not only the identification and the localization of post-translational modifications (PTMs) on the mAb, but also a verification of the drug conjugation. Mass spectrometry (MS) has become the most widely employed method for ADC characterization, owing to the rapid advancement of MS technologies. Intact mass analysis is the platform method utilized to determine drug-to-antibody ratio (DAR), while deep characterization of the sites of conjugation usually relies on bottom-up approaches. Most widely-adopted, collision-induced dissociation (CID) is able to provide amino acid sequence confirmation, but the harsh fragmentation technique also breaks the payload into small pieces. The highly complex spectra derived from such an approach can be very difficult to interpret. Alternative fragmentation can provide further insights into such complex samples, but previous techniques suffered from long reaction times, low sensitivity and lack of reproducibility.

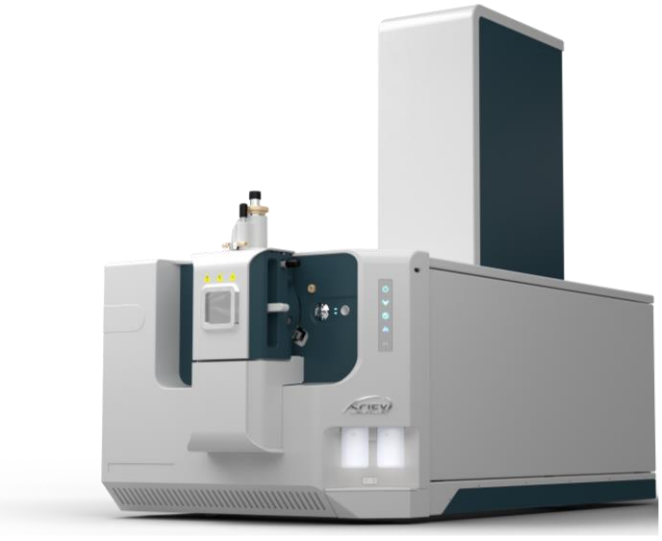

Figure 1. The SCIEX ZenoTOF 7600 system.

A new, highly reproducible fragmentation type based on EAD[1,2] was used to analyze the conjugated peptides from a commercial ADC. The data were acquired with an untargeted 10 Hz rapid data-dependent acquisition (DDA) method and interpreted with Protein Metrics Inc. software. With this workflow, regular and advanced characterization leveraging EAD-based fragmentation is achievable in one injection, enabling a streamlined characterization accessible to every user-level.

Key features of the SCIEX ZenoTOF 7600 system

- **New depths of peptide mapping analysis:** EAD with fast DDA enables alternative fragmentation for routine, in-depth analysis of next generation protein therapeutics and standard mAbs
- **Higher levels of structural information:** Changing the mechanism of fragmentation by tuning the electron energy may provide a higher level of structural information
- **Higher MS/MS sensitivity:** Increased detection of fragments (5 to 10 fold) using the Zeno trap enables higher confidence in data assignment
- **High reproducibility:** Reproducible fragmentation with EAD for singly, doubly, and multiply charged ions enables analysis of more precursors than other alternative and low reproducibility fragmentation techniques
- **Streamlined and easy-to-use:** Fully automated data acquisition in DDA mode using EAD with SCIEX OS software, and automated data interpretation with Byos software (Protein Metrics Inc.) simplifies the entire user experience

Methods

Sample preparation: The lysine-linked ADC sample (ado-trastuzumab emtansine, T-DM1) was denatured with 7.2M guanidine hydrochloride, 100mM Tris buffer pH 7.2, followed by reduction with 10mM DL-dithiothreitol and alkylation with 30 mM iodoacetamide. Digestion was performed with trypsin/Lys-C enzyme at 37°C for 16 h.

Chromatography: 4 µL (4 µg) of the trypsin/Lys-C digest were separated with a CSH C18 column (2.1×100 mm, 1.7 µm, 130 Å, Waters) using an ExionLC AD system. The mobile phase A consisted of water with 0.1% formic acid, while the organic phase B was acetonitrile 0.1% formic acid. A gradient profile was used at a flow rate of 300 µL/min (Table 1). The column temperature was maintained at 50°C.

Table 1. Chromatography for peptide mapping analysis.

Time [min]	Mobile phase A [%]	Mobile phase B [%]
Initial	*98*	*2.0*
5	*98*	*2.0*
6	*90*	*10*
40	*55*	*45*
44	*10*	*90*
46	*10*	*90*
47	*98*	*2.0*
50	*98*	*2.0*
51	*10*	*90*
54	*10*	*90*
55	*98*	*2.0*
60.0	*98*	*2.0*

Mass spectrometry: Data were acquired with an information-dependent acquisition (IDA) method using the SCIEX ZenoTOF 7600 system. The electron energy for EAD cell was set to a value of 7 eV. Detailed method parameters are summarized in Table 2.

Table 2. MS parameters.

Parameter	MS	MS/MS
Scan mode	*TOF-MS*	*IDA dependent*
Polarity	*positive*	
Gas 1	*40 psi*	
Gas 2	*40 psi*	
Curtain gas	*30 psi*	
Source temperature	*350 °C*	
Ion spray voltage	*5200 V*	
Declustering potential	*20 V*	
Collision energy	*8 V*	
CAD gas	*7*	
Maximum candidate ion	*5*	
Intensity threshold	*100 cps*	
Charge states	*2 to 10*	
Exclusion time	*6 s after 2 occurrences*	
Start mass	*100 m/z*	*150 m/z*
Stop mass	*1,800 m/z*	*2,500 m/z*
Electron KE	*NA*	*7 eV*
Electron beam current	*NA*	*4750 nA*
ETC	*NA*	*100*
Zeno trap	*NA*	*ON*
Accumulation time	*0.25 s*	*0.20 s*
Time bins to sum	*4*	*4*

Data processing: Data were processed using Byos software (Protein Metrics Inc.) using customized modifications.

The what, why and how

Ado-trastuzumab emtansine (T-DM1) was amongst the first ADC therapeutics, receiving approval by the FDA in 2013 for the treatment of human epidermal growth factor receptor 2 (HER2)-positive, metastatic breast cancer. T-DM1 consists of a monoclonal antibody, trastuzumab, which is covalently conjugated to the cytotoxic agent emtansine (DM1) via a non-cleavable linker (Figure 2). Combining target-specificity of a mAb with the high potency of a cytotoxic drug takes advantage of the best of two worlds, minimizing side effects.[3] T-DM1 is linked to amines, such as in the side chain of lysine residues of trastuzumab. Previous intact mass studies show that the average DAR for T-DM1 is around 3.5.[5,6] However there are 88 lysine residues and 4 N-terminal groups in trastuzumab, which could result in more than 4.5 million unique molecules.[5] The site and structure of payload will directly affect drug efficacy and safety, which are therefore classified as critical quality attributes (CQA) and require comprehensive characterization and tight monitoring during development. Currently, bottom-up approaches are the method of choice for the characterization of product quality attributes, enabling the simultaneous identification and localization of modifications. LC-MS/MS utilizing CID is commonly used to verify conjugation sites, as each DM1 will lead to a ~957 amu mass shift. However, besides the dissociation of the peptide backbone, CID results in a series of small fragments from the payload drug, such as *m/z* of 547.221, 485.224, and 453.199, which increase the spectra complexity.[5] Although alternative fragmentation technology such as electron capture dissociation (ECD) is expected to provide orthogonal information on drug conjugated peptides, the application has not been explored extensively.

With the SCIEX ZenoTOF 7600 system, a robust alternative fragmentation technique is introduced, enabling scientists to get an in-depth picture of their samples by using analytical-flow LC separation in combination with a fast scanning DDA method and processing using Protein Metrics Inc. software. This breakthrough technology realizes the dream of answering complex questions in a routine and reproducible manner.

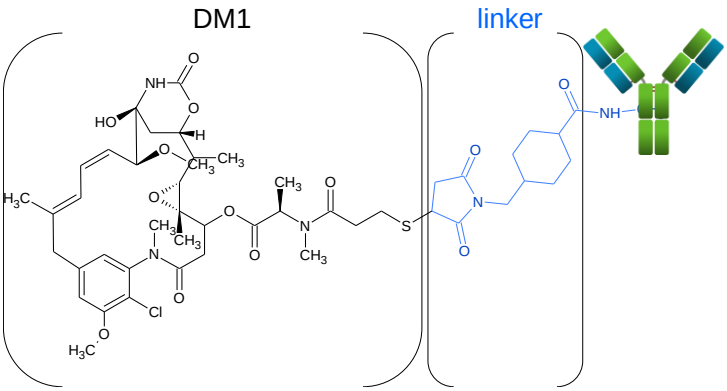

Figure 2. Schematics of cytotoxic payload and linker attached to mAb. T-DM1 consists of DM1 (black) being attached to the mAb through an MCC linker (blue) targeting amine residues.

Analysis of conjugated peptides

The study focused on the characterization of a commercialized ADC: T-DM1. The conjugation reaction between DM1 and trastuzumab is of stochastic nature, targeting amines. Out of the 88 lysine residues on trastuzumab, 40 are solvent exposed[6] and therefore susceptible to conjugation. Multiple approaches on intact and subunit mass have been explored to study the DAR. These approaches, however, cannot reveal the exact sites of the conjugation.

Here, a DDA approach in combination with Zeno EAD was chosen. With this approach, routine peptide mapping analyses can be performed, while EAD enables advanced characterization in the same, single analysis. Furthermore, the detection of fragment ions, and thus the correct identification of low abundant species, is enhanced by Zeno EAD. This approach allowed for the straight-forward data interpretation using Protein Metrics Inc. software. It is the first exploration of SCIEX EAD technology for an ADC sample. Figure 3 demonstrates an example of the fragmentation pattern observed on a conjugated peptide, SCDK[DM1]THTCPPCPAPELLGGPSVFLFPPKPK. The peptide without linker and drug or part of it was not observed in the analysis indicating a full conjugation. High quality MS/MS spectra was achieved for the peptide, allowing for 96.6% MS/MS sequence coverage for this particular peptide. One of the more dominant fragments was derived from the payload with an *m/z* greater than 500 (see labeling in Figure 3). The major cleavage site observed for the payload structure was the COO-C bond of the DM1. This fragmentation pattern is different from previous published data leveraging CID, which produced a series of small fragments.[5] Larger fragments of the drug can be used as signature fragments to confirm the existence of the payload more specifically, and can be leveraged to confirm the payload structure. Furthermore, fragments from the peptide backbone were also well detected by applying Zeno EAD for enhanced fragment ion detection, providing information on the molecular integrity of the peptide. The existence of conjugated drugs on the protein can lead to more missed cleavages during enzymatic sample preparation, due to steric hinderance of the enzyme. In addition, the conjugation process between lysine residues and the payload is a stochastic reaction. The occupancy of the conjugation is not always 100%, which results in diverse and low abundant forms. When multiple potential positions exist in one peptide, it can be a challenge to identify the correct site of linkage. The peptide ASQDVNTAVAWYQQKPGKAPK is another

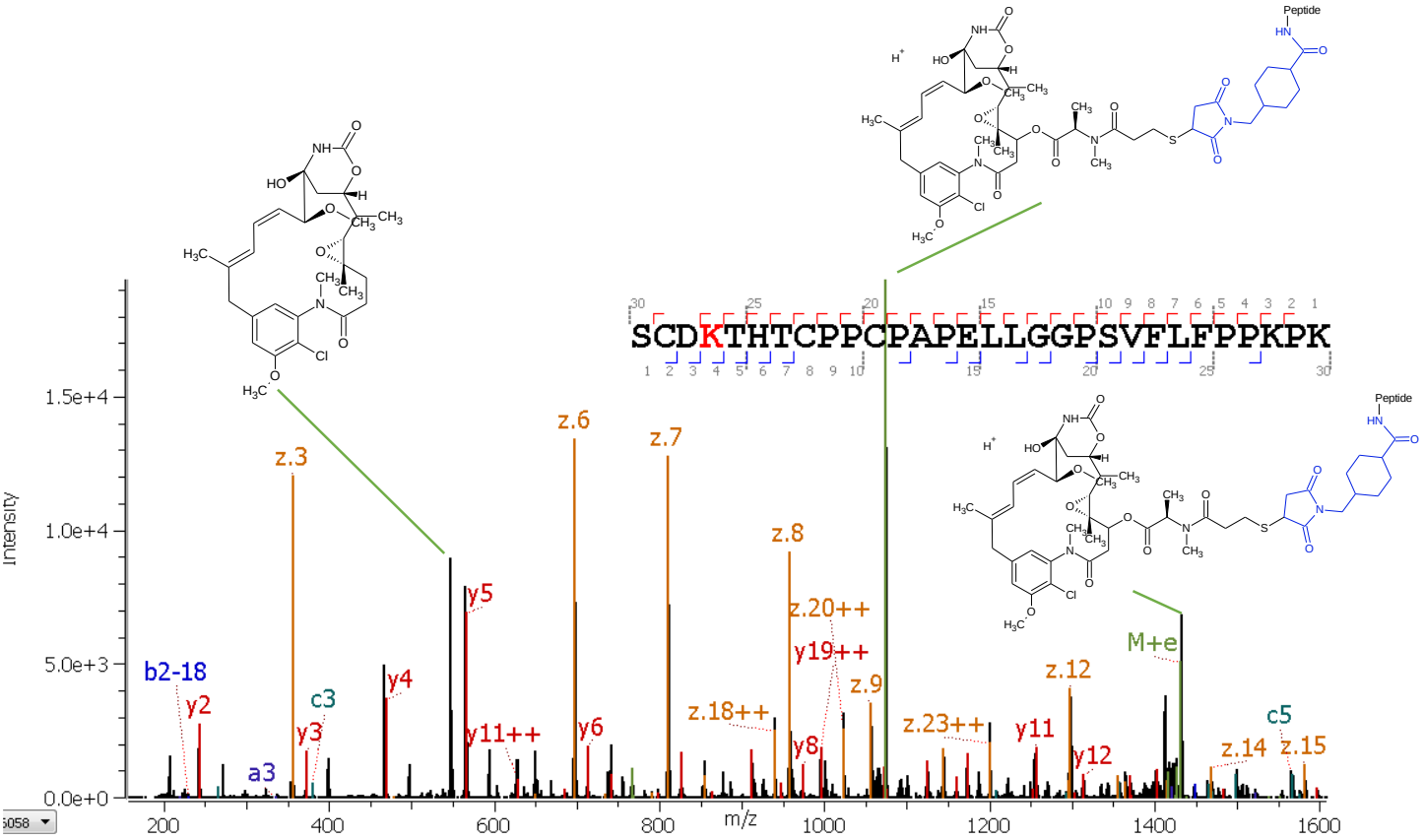

Figure 3. Fragmentation data from the conjugated peptide SCDK[DM1]THTCPPCPAPELLGGPSVFLFPPKPK (*z* = +4) using Zeno EAD. Full scan MS/MS data of conjugated peptide with assigned ions from the peptide backbone, as well as the fragment from the payload.

example of this type of challenging peptide (Figure 4). It contains a miscleavage site and a lysine N-terminal to a proline, leading to multiple options for the site of conjugation. However, with the rich, high-quality MS/MS spectra derived from EAD, an automatic assignment of the drug localization was achieved (Figure 4A). Because the payload is close to the C-terminus of the peptide, more abundant *c* ions are detected than *z* ions (Figure 4A), while the unconjugated peptide shows abundant fragments from both the C-terminus and N-terminus (Figure 4B). A full series of *c* fragments from *c*3 to *c*17 was detected, except for *c*15, since it is well known that electron capture dissociation techniques do not dissociate the N-terminal side of prolines.[7] This provides solid evidence that K15 is not linked to the drug. In addition, *z*4, *z*5 and *z*7 show that K18, not K21, is the correct site of drug conjugation.

This is further supported by the fact that conjugated lysines are not cleavable with trypsin[5], excluding K21 as the site of attachment. The most dominant species in the spectrum in Figure 4A (*m*/*z* 547.221) can be linked to the dissociation of the payload. The isotope pattern supports this finding since it fits a compound containing a halogen element (Cl). The associated counterpart linked to the peptide was also observed (Figure 4A). Unambiguous characterization, including the identification and localization of conjugated peptides in a single DDA run, was achieved with the SCIEX ZenoTOF 7600 system and Zeno EAD. This is an example of how ADC analysis, which was previously thought a challenge by LC-MS/MS, can be simplified by Zeno EAD.

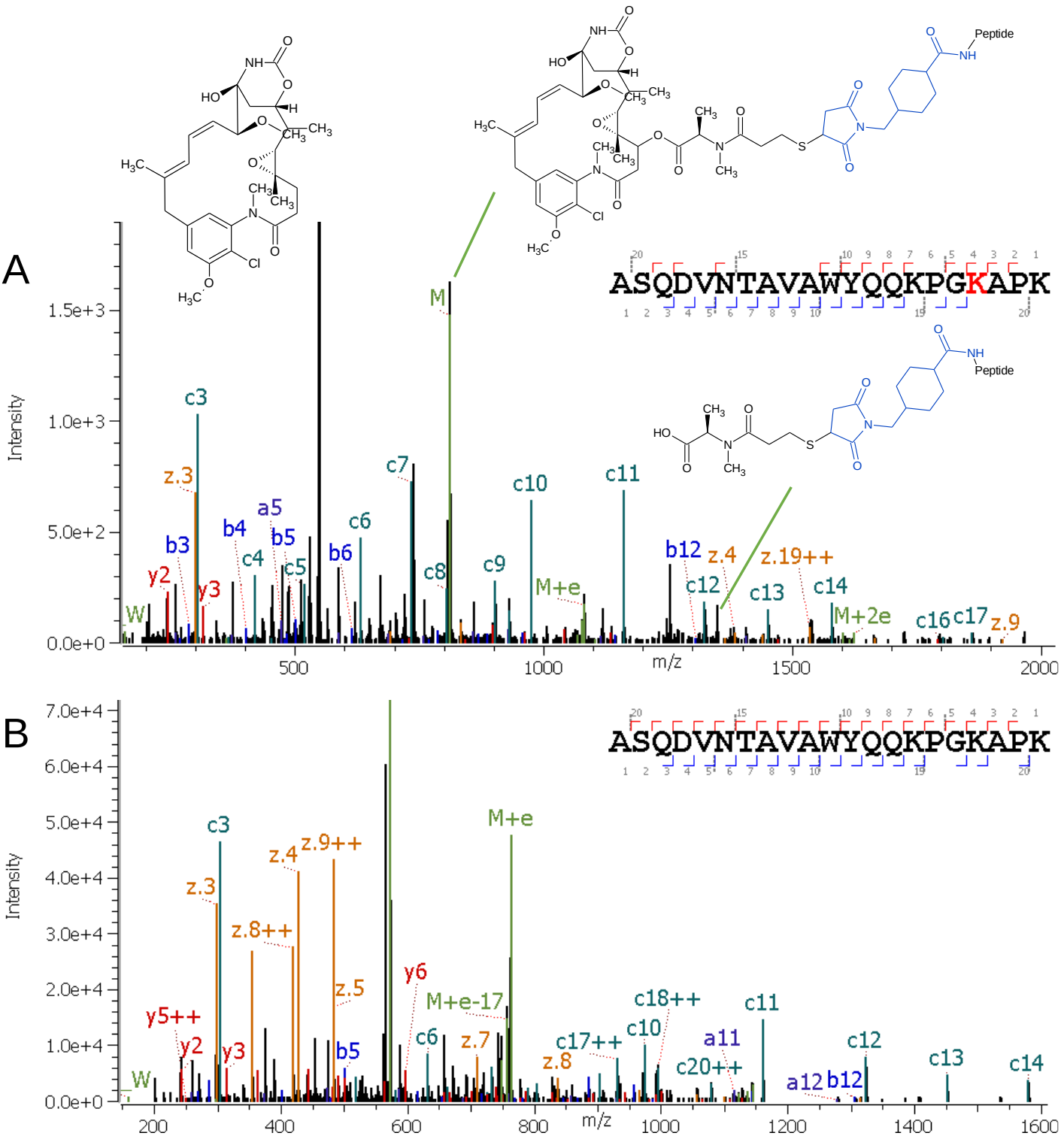

Figure 4. Fragmentation data from the conjugated/naked peptide ASQDVNTAVAWYQQKPGK[DM1]APK (*z* = +3) using Zeno EAD. A: Full scan MS/MS data of conjugated peptide with assigned fragment ions from the peptide backbone, as well as the fragments from the payload. B: Full scan MS/MS data of naked peptide with assigned ions from the peptide backbone. The linker is depicted in blue, the DM1 drug in black

Conclusions

- The exact site of drug conjugation in peptides with multiple potential locations was achieved with the novel fragmentation technique of EAD
- MS/MS fragment detection was significantly enhanced compared to traditional MS/MS analysis. Utilizing Zeno EAD resulted in remarkable data quality for confident fragment assignment, even for precursors with medium or very low intensities, such as low abundant conjugated peptides
- The robust, reproducible and easy-to-use alternative fragmentation of the SCIEX ZenoTOF 7600 system enables users to answer challenging analytical questions in a streamlined manner
- Automatic data processing using Protein Metrics Inc. software enables the reproducible, routine and advanced characterization of complex biotherapeutics

References

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