Evaluation of biotherapeutic sequence coverage using electron activated dissociation (EAD)

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

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The data presented here demonstrate that novel electron activated dissociation (EAD) [1,2] is highly comparable to traditionally used collision induced dissociation (CID) with regards to sequence coverage in a peptide mapping workflow. In addition, streamlined, advanced characterization in one injection is possible through high-speed, highly reproducible, alternative fragmentation. This solution takes peptide mapping experiments to a new level, one previously thought unachievable.

Ensuring drug safety and efficacy is essential for biotherapeutics, which drives the need for in-depth characterization during their development. This includes the verification of the sequence on the MS and MS/MS level, and the identification and localization of posttranslational modifications (PTMs). Bottom-up approaches employing liquid chromatography coupled to mass spectrometry (LC-MS) are still the method of choice for assessing these quality attributes in a streamlined manner. To obtain more detailed information on the order of a sequence in a given peptide or to localize PTMs, MS/MS is performed. CID is considered the gold standard for MS/MS experiments, offering a fast dissociation mechanism, and highly reproducible results. However, CID has known limitations, especially when descriptive fragmentation of larger moieties is required, or when deeper information on the peptides or their potentially labile modifications is needed. Alternative fragmentation techniques filled that gap, but long cycle times and low reducibility have limited the wide adoption as a standard technique for biopharmaceutical characterization.

Figure 1. The SCIEX ZenoTOF 7600 system.

Here, a new fragmentation type based on EAD was evaluated.[1, 2] Compared to traditional fragmentation techniques, such as CID, it provides better sensitivity, reproducibility, and acquisition speeds while maintaining comparable sequence coverage and fragment ion coverage. Powerful data reduction with Protein Metrics Inc. software allows for automatic interpretation in a routine fashion.

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 mAb sample (adalimumab) was denaturated with 7.2 M guanidine hydrochloride, 100mM Tris buffer at pH 7.2, followed by reduction with 10mM DL-dithiothreitol and alkylation with 30mM iodoacetamide. Digestion was performed with trypsin/Lys-C enzyme at 37 °C for 16 h.

Chromatography: 4 µL (2 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	*99*	*1.0*
5.00	*99*	*1.0*
6.00	*90*	*10*
25.00	*75*	*25*
65.00	*60*	*40*
70.00	*40*	*60*
70.50	*10*	*90*
74.00	*10*	*90*
74.10	*99*	*1.0*
75.00	*99*	*1.0*
75.10	*10*	*90*
79.00	*10*	*90*
79.10	*99*	*1.0*
83.00	*99*	*1.0*

Mass spectrometry: Data were acquired with an information-dependent acquisition (IDA) method using the SCIEX ZenoTOF 7600 system. General method parameters were kept the same and are summarized in Table 2. Parameters specific for EAD or CID can be found in Table 3.

Table 2. General MS parameters.

Parameter	MS	MS/MS
Scan mode	*TOF-MS*	*IDA dependent*
Gas 1	*50 psi*	
Gas 2	*50 psi*	
Curtain gas	*35 psi*	
Source temperature	*450 °C*	
Ion spray voltage	*5500 V*	
Declustering potential	*80 V*	
Collision energy	*12 V*	*
CAD gas	*7*	
Maximum candidate ion	*15*	
Intensity threshold	*125 cps*	
Charge states	*1 to 10*	
Exclusion time	*6 s after 2 occurrences*	
Start mass	*200 m/z*	*100 m/z*
Stop mass	*2,000 m/z*	*3,000 m/z*
Accumulation time	*0.25 s*	*
Time bins to sum	*8*	*10*

*specific for EAD/CID; see Table 3.

Table 3. MS parameters for CID and EAD.

Parameter	CID	EAD
Collision energy	*rolling*	*NA*
Electron KE	*NA*	*7 eV*
Electron beam current	*NA*	*4750 nA*
ETC	*NA*	*100*
Zeno trap	*ON*	*ON*
Accumulation time	*0.05 s*	*0.09 s*

Data processing: Data were processed with a modified PTM workflow in Byos software (Protein Metrics Inc.). To achieve side by side comparison, the standard PTM workflow was modified to include two MS/MS Id Byonic processing nodes: one for CID data processing, one for EAD data processing. All other processing parameters were kept the same. Peptide identification and fragments mass tolerance were set to 6 ppm and 20 ppm, respectively. The processed results were filtered to eliminate results with MS/MS scores lower than 100.

Results and discussion

The enzymatically digested adalimumab sample was run in six replicate injections on the same instrument using either EAD or CID for fragment generation. The summary of sequence and fragment coverages for all replicates obtained is listed in Table 4. Only matches within the mass tolerance of 6 ppm for MS and 20 ppm for MS/MS and peptides with an MS/MS score above 100 were taken into account for both sets of data. The two fragmentation technologies show highly comparable sequence coverages that are close to 100% for both CID and EAD. Furthermore, EAD showed an improved fragment coverage, especially for the heavy chain (HC) (Table 4). In addition, the repeatability of the sequence and fragment coverage results obtained with EAD was very well aligned with the results achieved with CID. The sequence/fragment coverage map obtained by EAD and CID is shown in Figure 2 (light chain, LC, of adalimumab) and Figure 3 (heavy chain, HC, of adalimumab) as a comparison for one example injection. It highlights the similarity of identification of the same peptides in a comparable number (non-modified forms, modified forms, missed cleaved forms), while showing differences in the number of fragments being detected (hash marks in Figure 2 and 3).

Peptide mapping usually requires identification through MS and MS/MS for a wide range of different peptides. This can include very short, singly-charged peptides to very long, heavily charged peptides. Obtaining comprehensive fragmentation when using CID or even an alternative fragmentation mechanism can be a challenge for such a variety of peptides. However, independent of the charge state, informative MS/MS results can be achieved with EAD (Figure 4 and 5). Even singly-charged peptides can be fragmented well with EAD (Figure 5). A closer look at the sequence coverage maps (Figure 2 and 3) revealed improved fragmentation over CID for large peptides (Figure 4). EAD provided a highly descriptive fragment coverage with overlapping N-terminal and C-terminal fragments—mainly *c*- and *z*- ions—for a peptide with 63 amino acids. CID mainly resulted in fragment ions closer to the N- and C-terminus of the peptide, missing a huge portion of the peptide. Regardless, identification based on the precursor mass and some fragments can be performed. More descriptive information increases the confidence in the correct confirmation of the sequence, supporting the general goal of full fragment coverage for biotherapeutic samples.

Table 4: Overview of the peptide sequence coverage and fragment coverage achieved with CID and EAD (*n* = 6).[#]

	Adalimumab LC		Adalimumab HC	
	Sequence coverage	Fragment coverage*	Sequence coverage	Fragment coverage*
CID	96.88+/-1.24	96.97+/-1.09	96.67+/-0.00	92.26+/-0.63
EAD	97.66+/-0.00	98.14+/-0.00	96.34+/-0.43	96.64+/-0.32

[#] peptide identification was achieved using 6 ppm mass tolerance for precursor and 20 ppm for fragment matching.
* terminal amino acids were considered as fragments, resulting in slightly higher number for fragment coverage calculation compared to sequence coverage in some cases.

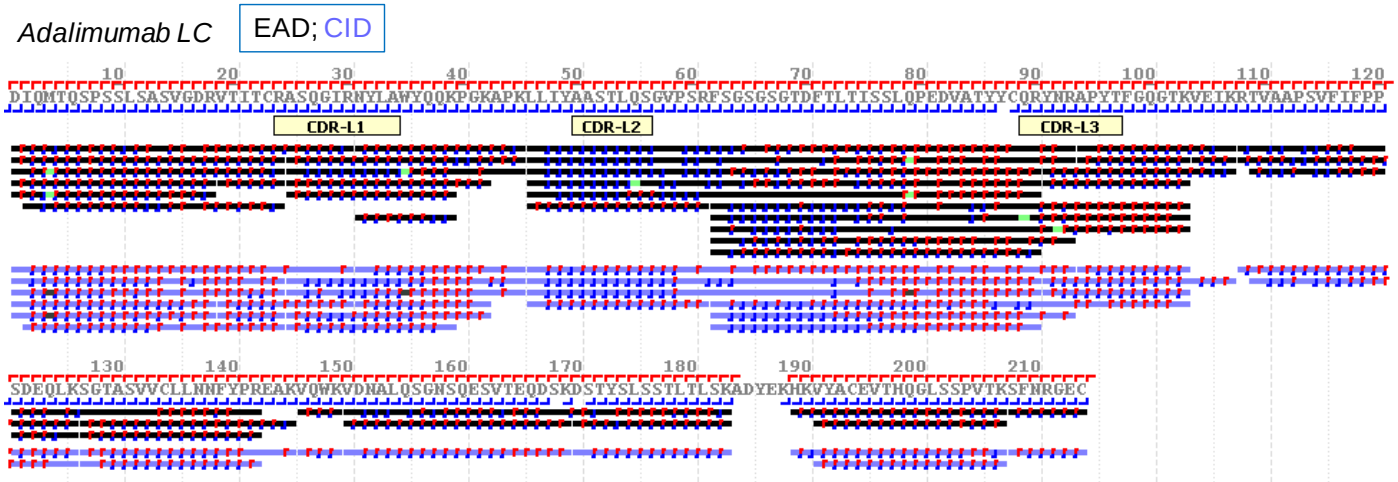

Figure 2. Comparison of adalimumab LC fragment coverage obtained with CID and EAD. Bars below the protein sequence illustrate the identified peptides (blue bars represent data collected using CID and black bars represent data collected using novel EAD). Identified fragments are indicated with blue and red hash marks. AA residues carrying modifications are highlighted in green.

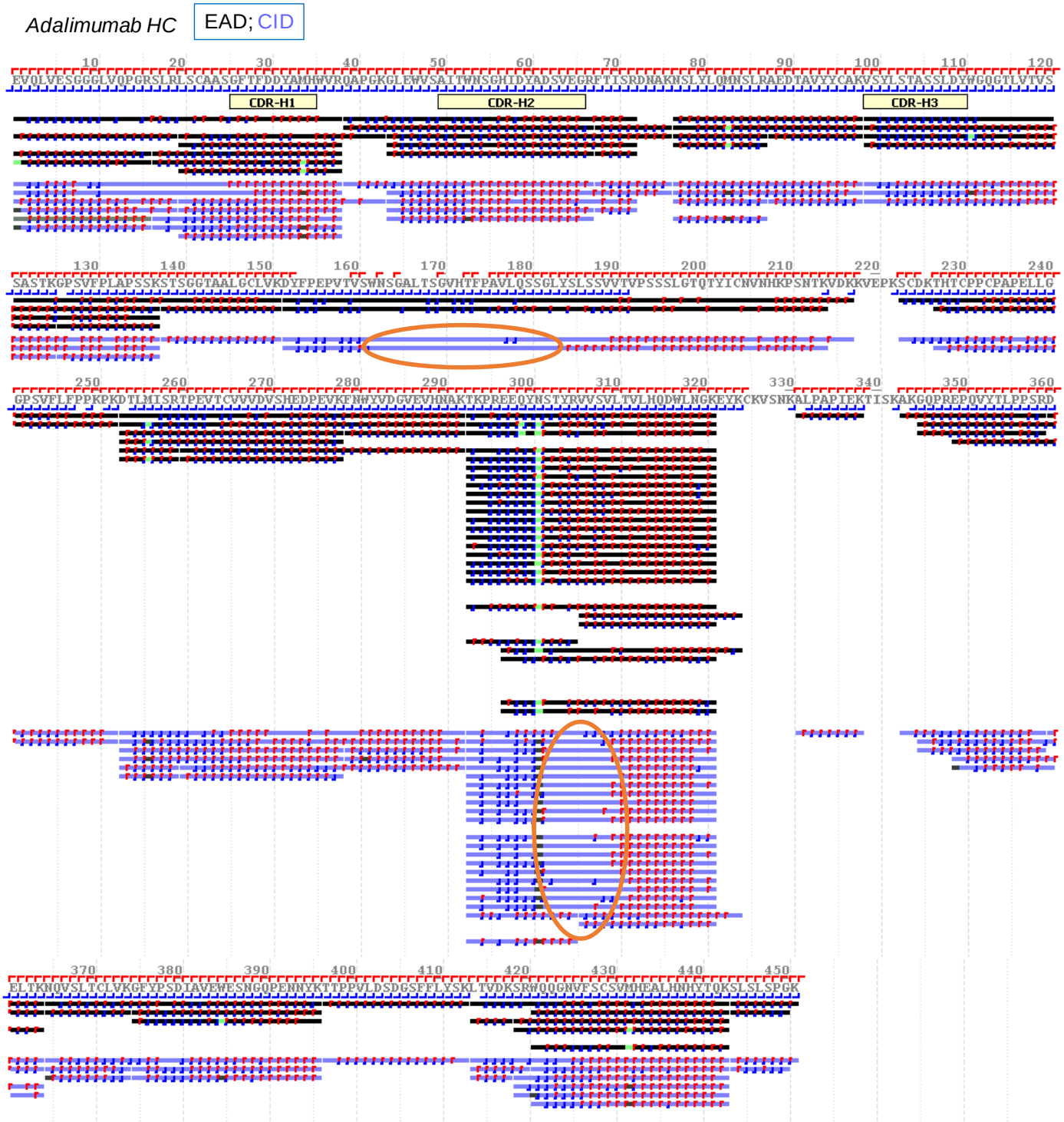

Figure 3. Comparison of adalimumab HC sequence coverage with CID and EAD. Bars below the protein sequence illustrate the identified peptides (blue bars represent data collected using CID and black bars represent data collected using novel EAD). Identified fragments are indicated with blue and red hash marks. AA residues carrying modifications are highlighted in green. CID does not provide fragment coverage towards the middle of very long peptides or peptides with fragile modifications (encircled parts), whereas EAD shows higher fragment coverages for such challenging cases.

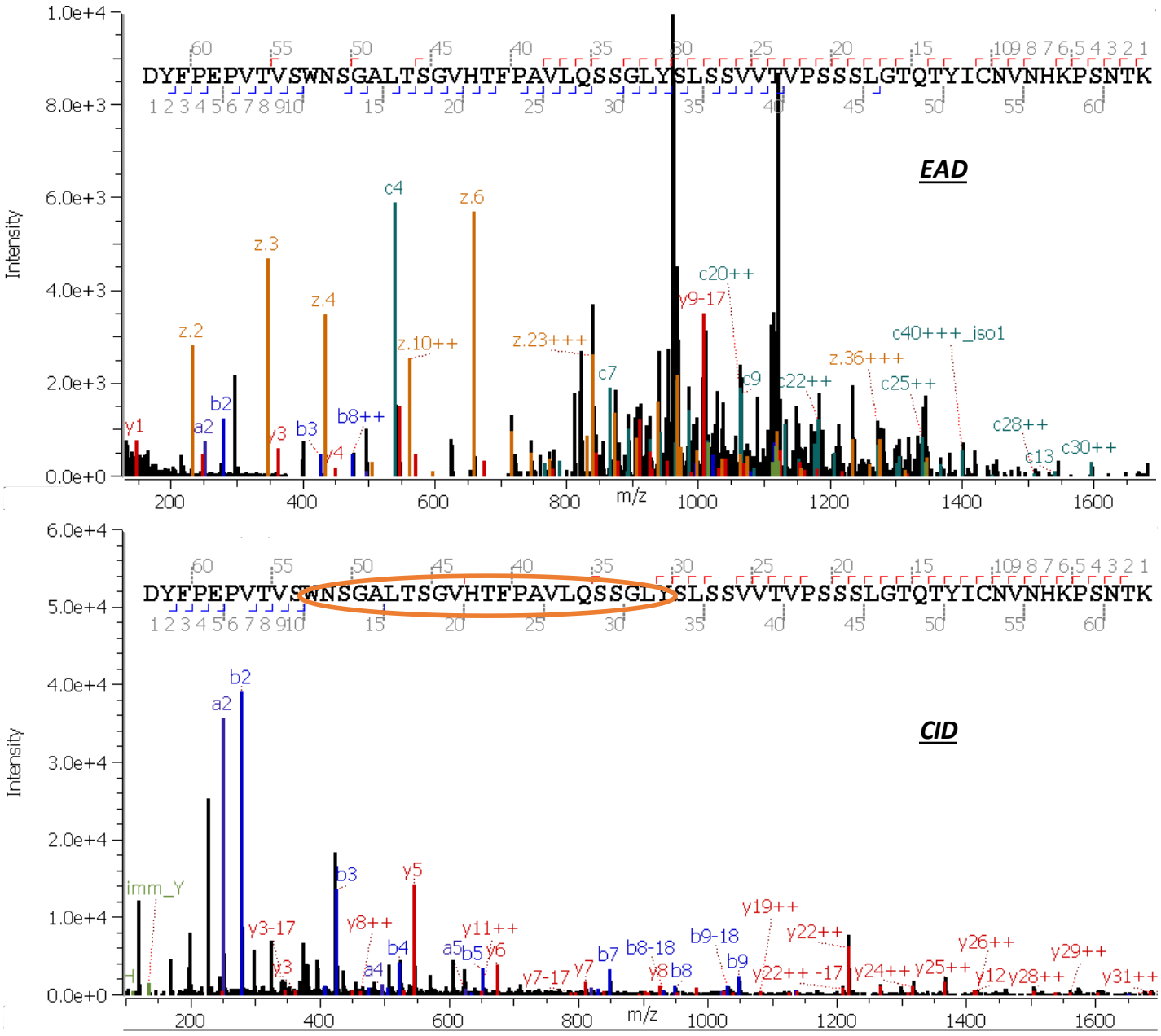

Figure 4. Comparison of MS/MS spectrum obtained with EAD and CID on a peptide from the HC with 63 AA residues.. Blue and red hash marks depict fragment ion coverage. The circle highlights a part of the peptide with missing fragment coverage when using CID technologies. Summing scan feature from Byos software (Protein Metrics Inc.) was employed for optimal results in each case.

Most antibody therapeutics contain at least a few long peptides without any tryptic cleavage site in the HC. With the market shifting to new modalities and more complex next generation biologics, such cases might become even more common, driving the need for more flexible and highly reproducible analytical tools. Since the amount and variety of fragments achieved with EAD can be significantly higher than with CID, the need for enhancing the fragment detection is a necessity to produce meaningful data. The Zeno trap enables significant enhancement of 5-10x in MS/MS mode, building the foundation for EAD as a widely applicable fragmentation technique. For peptides containing a fragile modification such as the glycosylated Fc region, the fragment coverage was increased using EAD compared to CID (see encircled part in Figure 3), which increases the confidence in the correct identification of the peptide and its modification. Previous studies using EAD for such peptides have shown that the glycosylation remained intact on the fragments allowing for simultaneous localization.[3]

In summary, Zeno EAD provides comparable sequence coverage and improved fragmentation coverage compared to Zeno CID. Its excellent reproducibility supports the applicability of EAD as a routine method for peptide mapping within the biopharmaceutical environment. In addition, details of structural information, not achievable with CID, can be determined, which might describe critical quality attributes.[3-8]

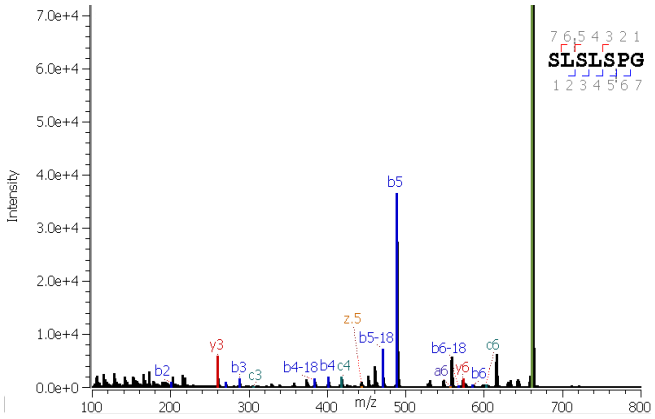

Figure 5. MS/MS spectrum using Zeno EAD on a singly charged peptide SLSLSPG. Blue hashmarks on the AA sequence indicate the identified N-terminal ions (*b*- and *c*- ions) while the red hashmarks indicate the identified (*y*- and *z*-ions).

Conclusions

- Excellent sequence coverage including fragment coverage with very high reproducibility can be achieved with EAD, a novel fragmentation technique
- Sequence and fragment coverage obtained with EAD were highly comparable, or exceeded the results obtained with traditional CID
- MS/MS fragment detection was significantly enhanced compared to traditional high resolution MS/MS analysis, delivering remarkable data quality for confident fragment assignment. When using the Zeno trap, this is true even for precursors with medium or very low intensities, such as modified peptides.
- The robust, reproducible and easy-to-use alternative fragmentation enables users to answer challenging analytical questions along with a general peptide mapping analysis in one single injection
- Automatic data processing using Protein Metrics Inc. software enables the routine and advanced characterization of complex biotherapeutics and standard mAbs in a reproducible manner

References

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