Analysis of post-translational modifications using fast electron-activated dissociation (EAD)

The Power of Precision

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INTRODUCTION

Characterization of human cellular proteomes enables identification of disease biomarkers and new therapeutic targets. Mass spectrometry-based techniques, such as bottom-up proteomics, are widely used to analyze cellular proteomes. For analysis of protein digests for protein identification, data-dependent acquisition (DDA or IDA) is often used. The ZenoTOF 7600 system, a QTOF platform, uses the Zeno trap to increase duty cycle to ≥90% across the entire fragment ion mass range, resulting in signal gains of 4- to 25-fold in MS/MS mode.[1] These gains enable up to a 40% improvements in proteins identified using DDA workflows.[2]

For proteomics experiments, there are two fragmentation options available on the ZenoTOF 7600 system: collision induced dissociation (CID) and electron activated dissociation (EAD). Unlike CID, which relies on the collision of ions with nitrogen gas to fragment molecules at their weakest bonds, EAD involves the capture of electrons by molecular ions to form a radical state that then fragments. CID and EAD result in different fragments for the same peptide, with CID yielding y and b ions and EAD yielding additional c' and z• ions. EAD fragmentation provides complimentary sequence information to CID and often preserves post-translational modifications (PTMs) that undergo neutral loss in a CID experiment.[3,4] This work focuses on DDA method development and analysis of HeLa digest using EAD, with an emphasis on site-specific localization of phosphorylation sites. In addition to phosphorylated peptides, glycated human peptides were also investigated using EAD.

MATERIALS AND METHODS

Sample preparation: A 100 µg sample of digested HeLa cell lysate was fractionated into 44 samples using high pH RP-HPLC, as previously described.[5] Then, 10% of each fraction was injected for LC-MS/MS analysis. Human plasma was used as the source of human serum albumin (HSA) for glycation modification analysis.

Chromatography: HeLa peptide fractions were separated using a Waters ACQUITY UPLC M-class system with a flow rate of 6 µL/min in direct inject mode. A Phenomenex Kinetex 2.6 µm XB-C18 LC column (100 Å, 150 x 0.3 mm) was used with a rapid linear gradient from 5 – 30% B over 21 minutes to interrogate the fractions. Mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid.

Mass spectrometry: A SCIEX ZenoTOF 7600 system equipped with the OptiFlow Turbo V ion source[6] using a low microflow probe and electrode was used for all data acquisition. DDA parameters that were implemented for all experiments included a TOF MS accumulation time of 250 ms and an exclusion time of 6 seconds. Only precursors with charge states in the range 2-5 with intensities greater than 100 cps were selected for fragmentation. For CID acquisition, the maximum number of candidate ions per cycle was 45 and the accumulation time was 20 ms. For EAD analysis of HeLa digest, the maximum number of candidate ions was 10 per cycle and an accumulation time of 50 ms was used. The reaction time for EAD was 20 ms was used, with an electron beam current of 3000 nA with a KE of 0 eV. For analysis of glycated peptides, 20 candidate ions were used per cycle with a 20 ms reaction time and 25 ms total acquisition time.

Data processing: MASCOT software was used for all data processing. HeLa digest files were searched against the Swissprot_Human database using the SCIEX EAD algorithm for EAD and the ESI-QUAD-TOF algorithm for CID. For both phosphorylated and glycated peptides, high quality figures were generated using Bio Tool Kit in SCIEX OS software.[7]

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Proteomic coverage of HeLa digest obtained using EAD

In order to select the acquisition parameters for Zeno EAD DDA analysis of HeLa digest, a variety of parameters were tested, including electron beam current, electron kinetic energy, reaction time and accumulation time. For peptides, it was determined that using an electron KE of 0 eV and a beam current of 3000 nA promoted fragmentation and yielded high fragment ion intensities. To evaluate the performance of EAD for protein identification, the same fractionated HeLa cell lysate was analyzed with data-dependent analysis using both CID and EAD fragmentation for MS/MS. The analysis identified 52,905 peptides at 1% FDR (Figure 1) using EAD. Fewer total numbers of peptides were identified using EAD due to the slightly slower acquisition speed (2.5x slower). However, the orthogonal fragmentation mode provided identifications of peptides not found using CID, yielding 11% more peptides. This highlights that while slower, the use of EAD is easily fast enough to be compatible with data-dependent acquisition. An example peptide is shown in which nearly complete c and z• (z+1) ion series were detected, with CID fragmentation yielding only partial y and b ions (Figure 2).

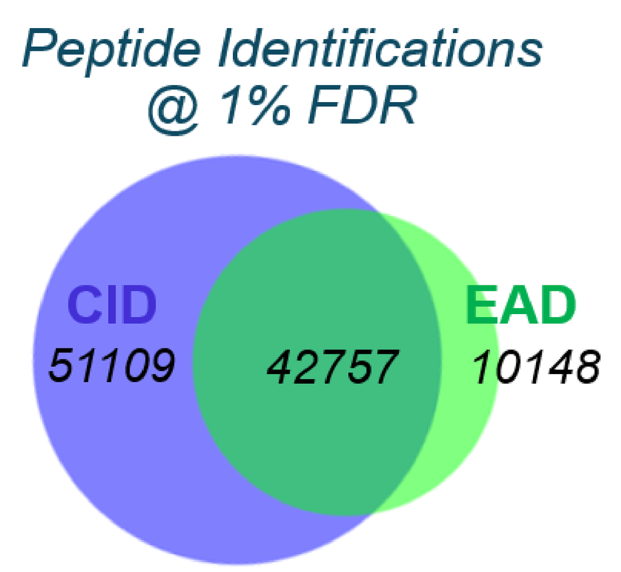


Figure 1. Comparison of peptide identifications at 1% FDR as a function of fragmentation mode on the ZenoTOF 7600 system. Due to the higher frequency of sampling with CID, peptide identifications were approximately 2-fold higher than with the EAD fragmentation mode, but EAD provided over 10,000 new peptide identifications.

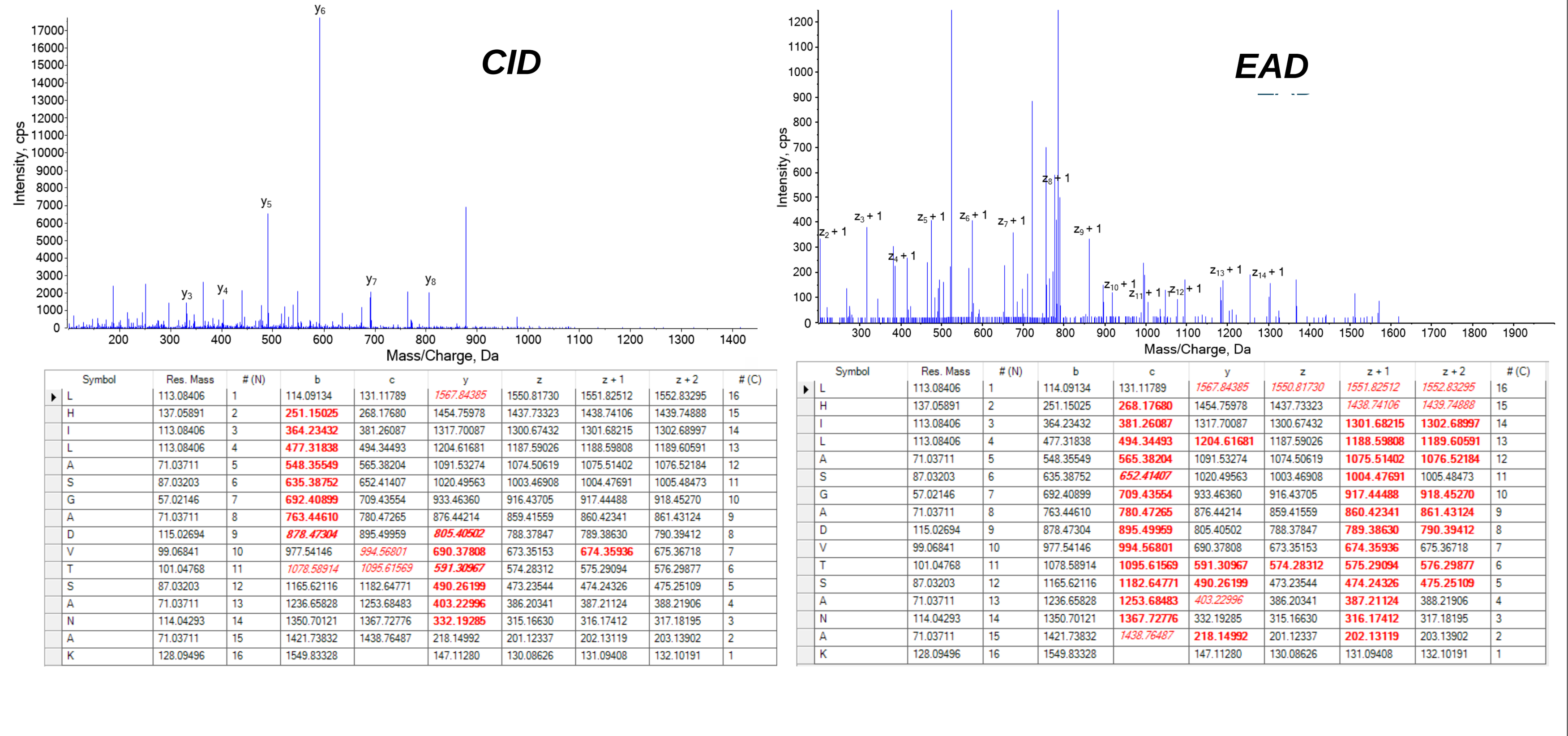


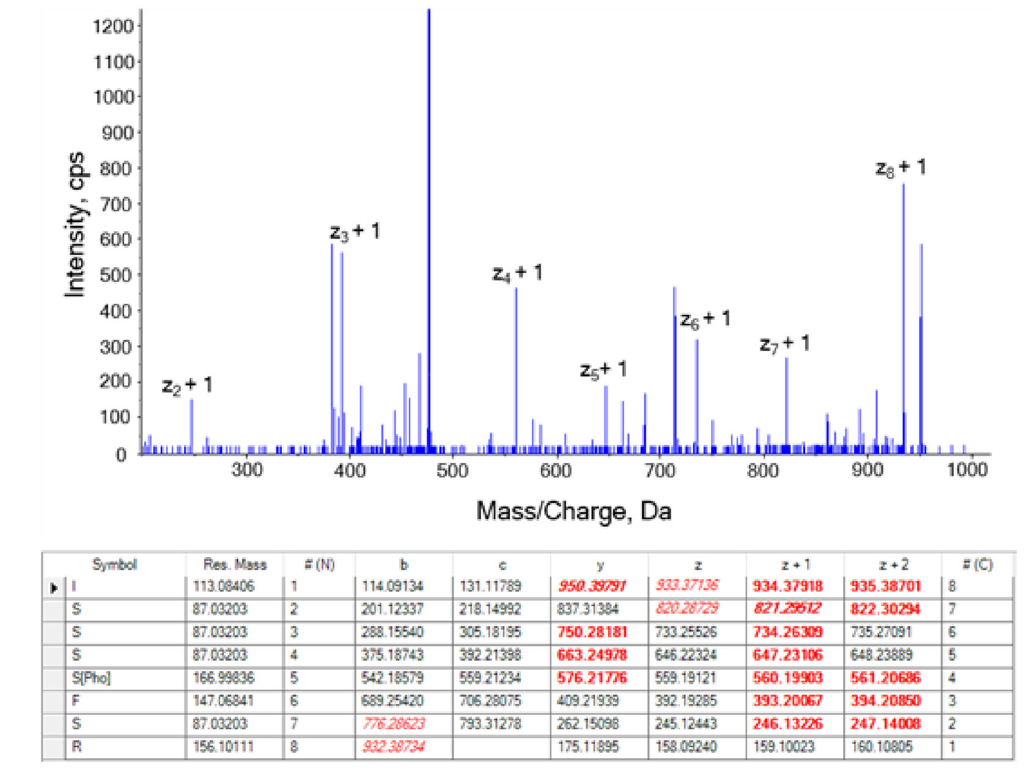
Figure 2. Comparing CID and EAD fragmentation of a non-phosphorylated peptide. (Top) CID fragmentation yielded incomplete y and b ion series for the LHILASGADVTSANAKAK peptide. (Bottom) Nearly complete z• (z+1 in table) and c ion series were detected for the same peptide when fragmented by EAD. Detected target fragment ion masses (+1 charge state) are shown in bold red in the tables below each associated spectrum.

Phosphorylation site localization using Zeno EAD MS/MS spectra

Overall, 52,905 peptides were identified at 1% FDR from the EAD data, including 158 phosphopeptides. The sample preparation for the digest was not optimized for preservation of modifications and no enrichment step was performed. EAD spectra from phosphopeptides were used to identify the sequence and site of phosphorylation in peptides, including peptides with multiple phosphorylated serines (Figure 3).

Other challenging phosphorylation sites were located with EAD spectra, including on a threonine residue near the peptide N-terminus (Figure 4) and on a peptide with 17 amino acids and a molecular weight of 1889.88 Da (Figure 5). The associated c and z+1 ion series clearly detailed the location of the phosphorylation sites on the peptides. EAD fragmentation provided a means to identify challenging phosphorylation sites otherwise unattainable with CID.

Figure 3. Localization of phosphorylation site in a peptide with multiple serines. An EAD spectrum for peptide sequence ISSSS[Pho]FSR is shown (right) with a nearly complete z• (z+1) ion series. The associated fragment ion masses are shown in the table below the spectrum for reference and detected +1 fragment ions are shown in bold red.



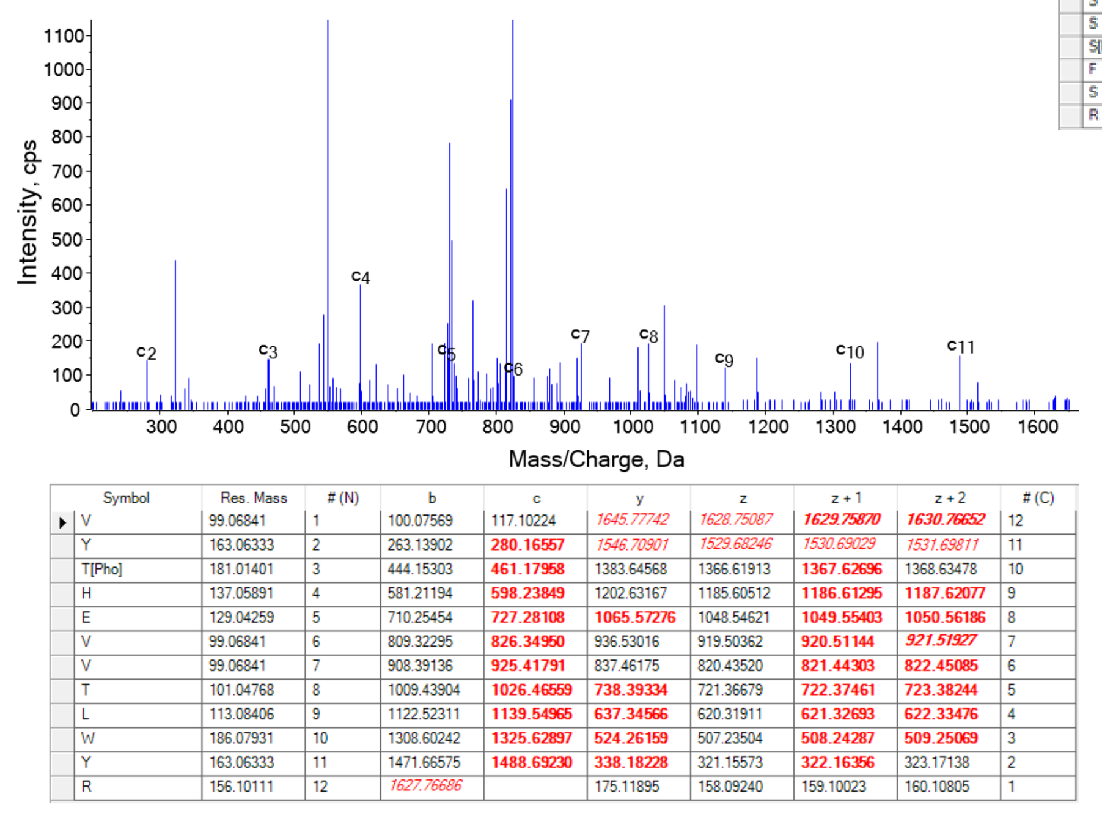


Figure 4. Localization of phosphorylation site near N-terminus of peptide with tyrosine and threonine. The c fragment ion series shown (left) enabled localization of a phosphorylated threonine adjacent to a tyrosine. Both c and z• (z+1) ion series are highlighted in the table below the EAD spectrum for +1 charge state and detected fragments are highlighted in bold red.

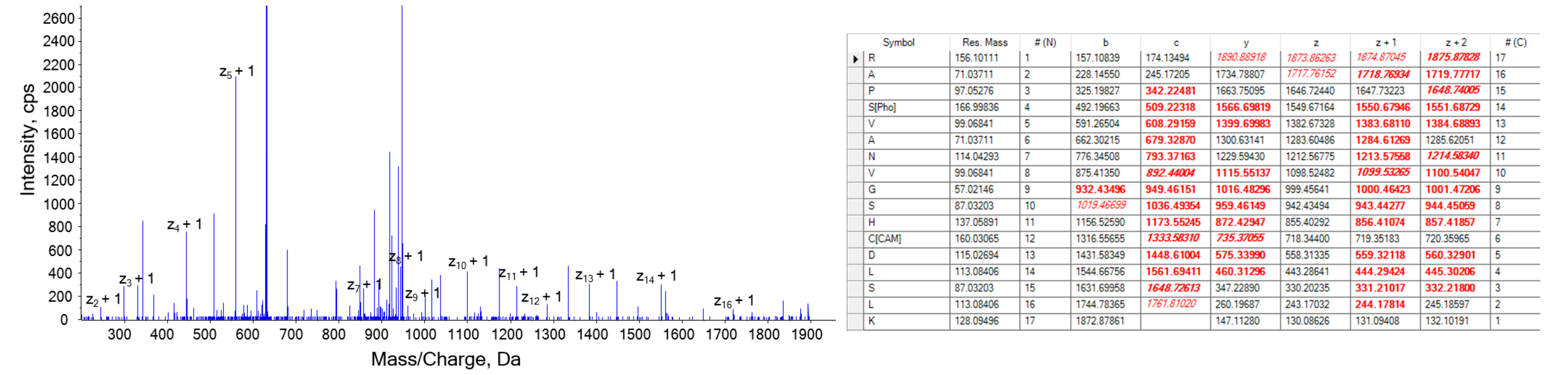


Figure 5. Identification of phosphorylation site in a 17-residue peptide. c and z• (z+1) fragment ions enabled identification of a phosphorylated serine in the sequence of an 1889.88 Da peptide. Detected fragment ions for +1 charge state are tabulated in bold red.

Glycation site localization using Zeno EAD MS/MS

A panel of human peptides was analyzed to evaluate hexose modifications using Zeno EAD DDA. While a hexose modification could not be localized using CID, EAD enabled its unambiguous localization on a lysine residue of a long, 24-amino acid peptide (Figure 6).

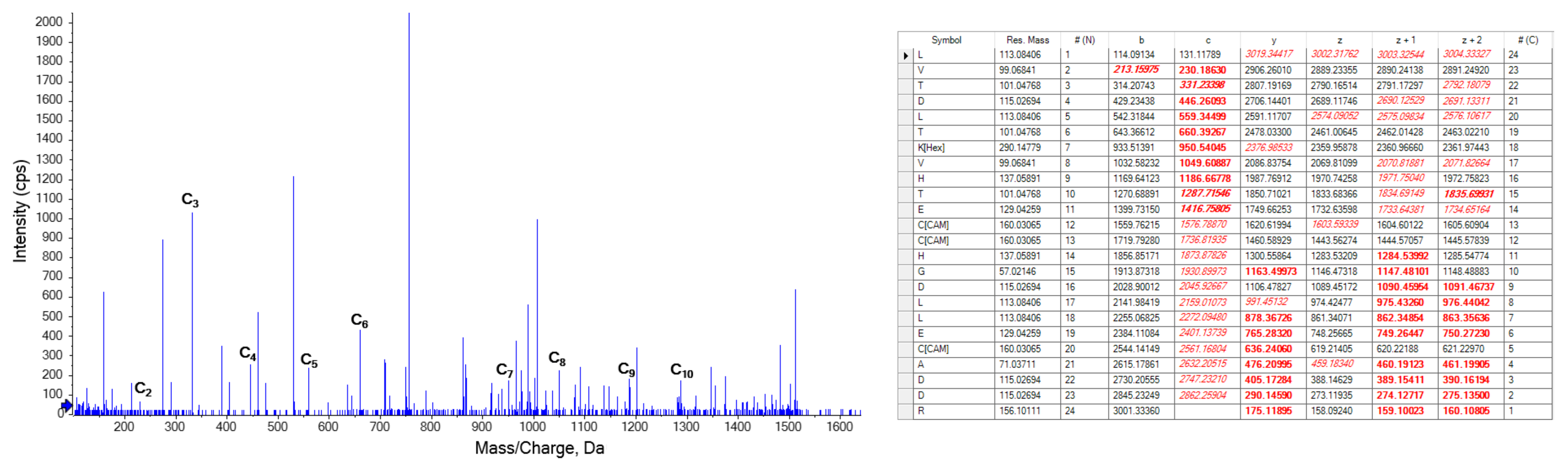


Figure 6. EAD fragmentation of a glycated peptide from human serum albumin (HSA) protein. A hexose modification was localized on a lysine using a nearly complete c ion series. Detected fragment ion masses are shown in bold red in the table to the right of the EAD spectrum for +1 charge state, with fragments matching to a different charge state shown in red italics.

CONCLUSIONS

The Zeno EAD DDA workflow developed in this work enabled robust identification of thousands of unique peptides in HeLa digest. High pH fractionation of HeLa digest followed by LC-MS/MS analysis with the ZenoTOF 7600 system generated a large-scale Zeno EAD DDA data set for interrogation. The Zeno trap was activated to further improve MS/MS sensitivity and yield high fragment ion intensities. EAD provided an alternative fragmentation option for the study of proteomic samples and enabled better characterization of some types of peptides vs. CID, such as long tryptic or mis-cleaved peptides and post-translational modified peptides, especially those modified with labile PTMs. In EAD spectra evaluated here, intact z• and c ion series were readily detectable for sequencing and localization of challenging phosphorylation and glycation sites.

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