

An evaluation of a single injection platform method for advanced characterization of protein therapeutics using electron activation dissociation (EAD)

Featuring the ZenoTOF 7600 system from SCIEX

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This single injection platform method offers superior capability over other fragmentation techniques for detailed biopharmaceutical characterization. A comprehensive evaluation of the electron kinetic energy (KE) of EAD was performed to determine the single optimal condition for peptide mapping. Furthermore, the results highlight the ability to adjust electron KE, a key advantage of EAD, to facilitate the fragmentation of a wide range of peptides.

New biopharmaceutical modalities, such as fusion and multi-specific protein therapeutics, are becoming more complex in their composition and diverse in their modifications. As a result of this complexity, the characterization of biopharmaceutical modalities is challenging for traditional analytical approaches. It has been demonstrated that EAD¹ provides fast and confident peptide mapping^{2,3} and is powerful for the accurate localization of labile modifications and differentiation of isomers.⁴⁻⁶ These experiments can be performed in a data-dependent manner at up to 20 Hz to maintain compatibility with analytical flow liquid chromatography (LC).

In this technical note, the effect of the KE of the electrons on the fragmentation pattern of various peptides was examined in detail. The results demonstrate the ability of EAD as a generic

fragmentation method to meet the needs of efficient bottom-up analysis of protein therapeutics and their candidates.

Key features of the generic platform method for biopharmaceutical characterization

- **Optimized single injection method:** One EAD method provides best-in-class single injection analysis results for biopharmaceutical proteins
- **Ease-of-use:** No optimization necessary for EAD, enabling adoption by walk-up users for efficient, routine analysis
- **Reproducibility and high sensitivity:** EAD provides consistent fragmentation patterns day-in and day-out and high MS/MS sensitivity, boosted by the Zeno trap
- **Tunable electron KE:** For advanced users, the ability to quickly fine-tune electron KE in a method enables characterization of analytes and modifications that pose challenges for traditional electron-induced dissociation (ExD) approaches

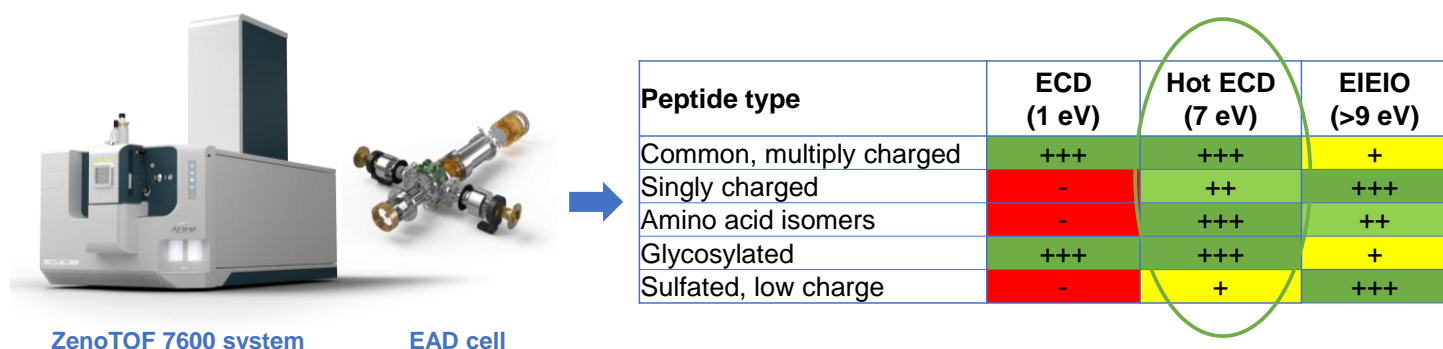


Figure 1. In-depth characterization of peptides using EAD. A platform EAD method²⁻⁶ using an electron KE = 7 eV via hot electron capture dissociation (ECD) within the ZenoTOF 7600 system (left) is the best option for characterization of a wide range of peptides in a single injection. The low-energy EAD (KE = 1 eV) via ECD mechanism is suitable for characterization of common peptides and glycopeptides, while EAD with KE > 9 eV via electron-impact excitation of ions from organics (EIEIO) is particularly powerful for the analysis of singly charged species⁷.

Methods

Sample preparation: The leucine (Leu) and isoleucine (Ile) isomers of human leptin (93-105, NVIQISND-X-ENLR, where X = Leu or Ile) were obtained from AnaSpec Inc. The 2 isomeric peptides were analyzed separately or in a mixture.

NISTmAb (reference material #8671, NIST) was denatured by guanidine-hydrochloride, reduced with dithiothreitol and alkylated using iodoacetamide, followed by tryptic digestion. Several peptides in the tryptic digest of NISTmAb were selected for further analyses, including N-glycosylated and singly charged peptides. Different KEs were used as part of an EAD MRM^{HR} experiment to investigate the impact on fragmentation patterns and information gain.

Etanercept, an N- and O-linked fusion protein, was digested with trypsin using the same procedure described above. This digest was further treated with N-glycanase (PNGase F, Agilent Technologies Inc.) for removal of all N-glycans, and reacted with SialEXO (Genovis Inc.) to cleave sialic acids. Selected O-linked glycopeptides were characterized by EAD in an MRM^{HR} experiment.

Chromatography: Peptides were separated using an ACQUITY CSH C18 column (2.1 × 150 mm, 1.7 μm, 130 Å, Waters Corp), which was kept at 60 °C in the column oven of an ExionLC system (SCIEX). Table 1 shows the LC gradient used for separation of peptide isomers at a flow rate of 0.25 mL/min with mobile phases A and B consisting of 0.1% formic acid (FA) in water and 0.1% FA in acetonitrile, respectively. The separation of peptides derived from NISTmAb and etanercept were carried out using a 60-minute gradient described previously.²

Table 1. LC gradients for separation of peptide isomers.

Time [min]	A [%]	B [%]
Initial	95	5.0
2.0	95	5.0
21	50	50
22	10	90
25	10	90
26	95	5.0
30	95	5.0

Mass spectrometry: Peptide mapping on NISTmAb and etanercept digests was performed by the platform EAD method²⁻⁶ using a data-dependent acquisition (DDA) approach on a ZenoTOF 7600 system (SCIEX). Selected peptides from the

DDA experiments were analyzed further with EAD MRM^{HR} using different electron KEs ranging from 1 eV to 11 eV. The key MS and MS/MS parameters are listed in Tables 2 and 3.

Table 2. TOF MS parameters.

Parameter	Value
Ion source gas 1	40 psi
Ion source gas 2	40 psi
Curtain gas	35 psi
CAD gas	7
Source temperature	400 °C
Spray voltage	5500 V
TOF start mass	200 m/z
TOF stop mass	2000 m/z
Accumulation time	0.1 s
Declustering potential	20 V
Collision energy	10 V

Table 3. EAD MRM^{HR} parameters.

Parameter	Value
TOF start mass	100 m/z
TOF stop mass	*
Accumulation time	0.1 s
Q1 resolution	Unit
Zeno trap	ON
Electron beam current	5000 nA
ETC	100%
Electron KE	1 to 11 eV

*Stop masses were m/z 3000 for glycosylated peptides from etanercept/NISTmAb, m/z 2000 for leptin isomers (3+) and m/z 600-800 for singly charged peptides from the NISTmAb digest.

Data processing: The interpretation of the peptide mapping data of NISTmAb and etanercept, from which N- and O-linked glycopeptides were identified and chosen for EAD MRM^{HR}, was performed using the Biologics Explorer software, as described previously.⁸ The EAD MRM^{HR} data of targeted peptides were analyzed using the Explorer module and the BioToolKit add-on in SCIEX OS software.

Confidence in peptide identification

The power of a generic platform method using alternative fragmentation with EAD at 7 eV for confident peptide mapping, differentiation of isomers and accurate localization of glycosylation was highlighted in previous technical notes.²⁻⁶ Here, the effect of electron KE, which is a key parameter for EAD, on fragmentation of isomeric, singly to multiply charged, and glycosylated peptides was studied in detail. The results highlight the benefits of using an electron KE = 7 eV as a generic setting for effective, yet comprehensive bottom-up analysis of protein therapeutics.

Figure 2 displays EAD spectra of the triply charged leptin (Ile) at electron KE = 1 eV and 7 eV. An electron KE = 1 eV (Figure 2A) produced a high-quality EAD spectrum, dominated by *c/z*-type fragments. This result is consistent with typical low-energy ExD experiments and yielded complete sequence coverage of this peptide. A higher electron KE of 7 eV (Figure 2B) leveraged hot ECD¹, a mechanism that led to the formation of additional fragment ions (*a/b*-type) for increased confidence in identification and secondary fragmentation of *z* ions, needed for differentiation between isomers, as will be described more in detail in the following section.

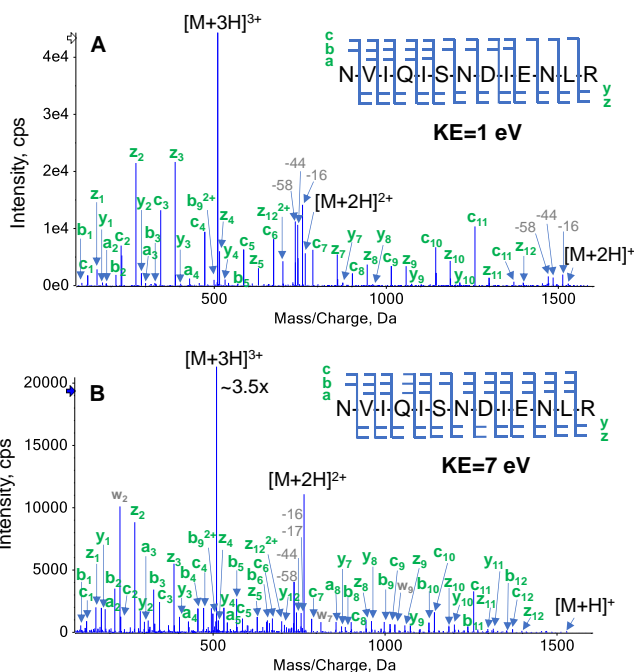


Figure 2. EAD spectra of the triply charged leptin (Ile) at electron KE = 1 eV (A) and 7 eV (B). While an electron KE = 1 eV produced an excellent EAD spectrum dominated by *c/z* ions (A), additional fragments (*a*, *b*, and *y*) were generated at electron KE = 7 eV, providing more fragment information for in-depth characterization of the peptide.

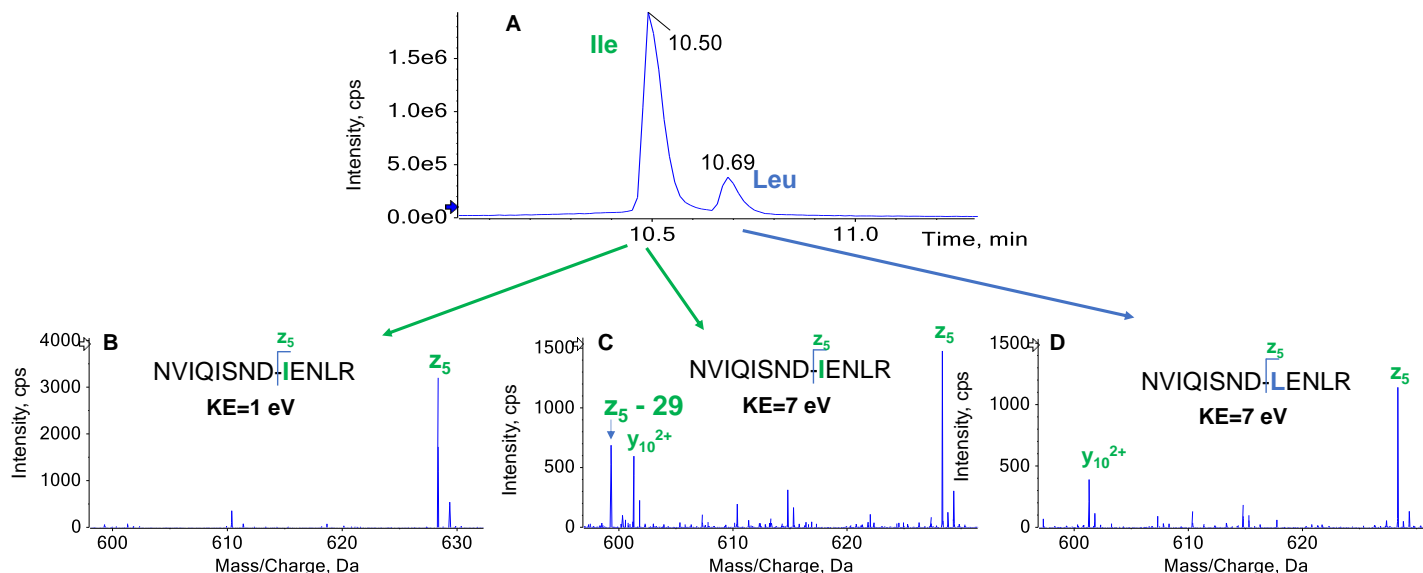


Figure 3. EAD spectra of triply charged leptin Leu/Ile isomers. Although the Leu/Ile isomers can be chromatographically separated (A), the differentiation of these isomers is not possible using MS1 or CID-derived MS/MS data. EAD generated a signature ion (*z*₅-29) for the Ile isomer at electron KE = 7 eV (C), but not at KE = 1 eV (B). By contrast, this fragment was not detected for the Leu isomer (D) at KE = 7 eV as expected.

Differentiation between isomers

As described previously, EAD allows differentiation of Leu/Ile isomers by generating a signature z-29 fragment for Ile and a z-43 ion for Leu via the losses of $\text{CH}_3\text{CH}_2\bullet$ and $(\text{CH}_3)_2\text{CH}\bullet$ from the side chains of Ile and Leu, respectively. Therefore, the Leu/Ile isomer peptides of leptin, which were separated chromatographically (Figure 3A), can be confidently assigned. A z₅-29 fragment was detected for the Ile isomer (Figure 3C), but was absent in the EAD spectrum of the Leu isomer (Figure 3D), as expected. The generation of this signature fragment was dependent on the electron KE applied. While an electron KE = 7 eV produced an abundant z₅-29 for the Ile isomer (Figure 3C), this peak was barely visible at KE = 1 eV (Figure 3B) despite the presence of an intense z₅. This indicates that electrons with KE = 1 eV were not “hot” enough to induce secondary fragmentation of the z-ion needed for the differentiation between isomers. The detailed effect of electron KE on the formation of z₅ and z₅-29 from the Ile isomer is illustrated in Figure 4. It is worth noting that the significant drop in intensity of z₅ between KE = 1 eV and 3 eV can be attributed to a change of the EAD mechanism, from the low-energy condition, typically used in ExD, to hot ECD, capable of providing rich sequence information.¹ The transition from low-energy to hot ECD likewise results in the ratio of z₅-29 to z₅ (gray trace) to increase with KE. Although this ratio continues to increase with electron KE (gray trace in Figure 4), the absolute intensities of the z₅ and z₅-29 fragments dropped at high KE values (blue and orange traces in Figure 4, respectively). An electron KE = 7 eV provided a balanced outcome for generating the sequence-specific z and Ile-

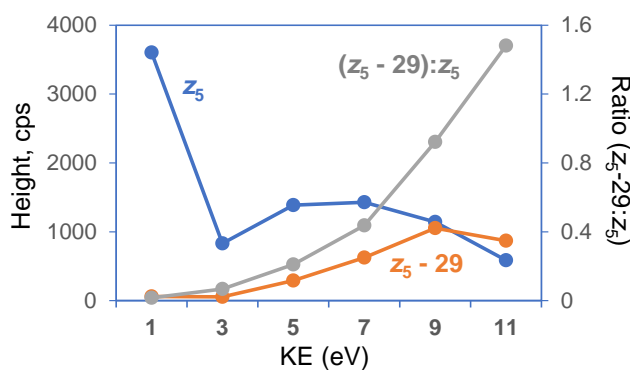


Figure 4. Effect of KE on the formation of z₅ and z₅ – 29 ions from the Ile isomer of leptin. The relative ratio of z₅-29 to z₅ increased with the increasing KE (from 1 eV to 11 eV) (gray trace). The absolute intensity of z₅-29 also showed an upward trend until KE = 9 eV (orange trace). By contrast, the intensity of z₅ dropped significantly from 1 eV to 3 eV and showed a decrease for values > 7 eV (blue trace). An electron KE = 7 eV provided a good balance for generating the sequence-specific z₅ and Ile-diagnostic z₅-29. EAD with higher KEs (> 7 eV) resulted in an increasing degree of internal and secondary fragmentation (see Figure 5).

diagnostic z-29 ions, while avoiding excessive internal or secondary fragmentation. As illustrated in Figure 5, EAD with an electron KE = 9 eV resulted in a higher level of internal and secondary fragmentation compared to the platform method with electron KE = 7 eV. Taken together, the results described above demonstrate the capability and suitability of the EAD platform method for confident peptide identification and differentiation of isomers in a single injection.

Localization of N- and O-linked glycosylations

The capability of EAD for accurate localization of glycosylation was demonstrated previously.³ The effects of the electron KE on the fragmentation of an N-linked glycopeptide from NISTmAb (EEQYNSTYR) and an O-linked glycopeptide from etanercept (THTCPPCPAPELLGGPSVFLFPPKPK) were illustrated in Figure 6 and 7, respectively. The electron KEs of 1 eV and 7 eV both generated high-quality EAD data, which enabled unambiguous localization of G0F in the N-linked glycopeptide (Figure 6) and HexNAcHex in the O-linked glycopeptide (Figure 7).

Similar to the observation made for the unmodified peptide (Figure 2), an electron KE = 7 eV produced more fragment ions that aided in the identification of the glycopeptides, while still providing confident localization information of the glycosylation.

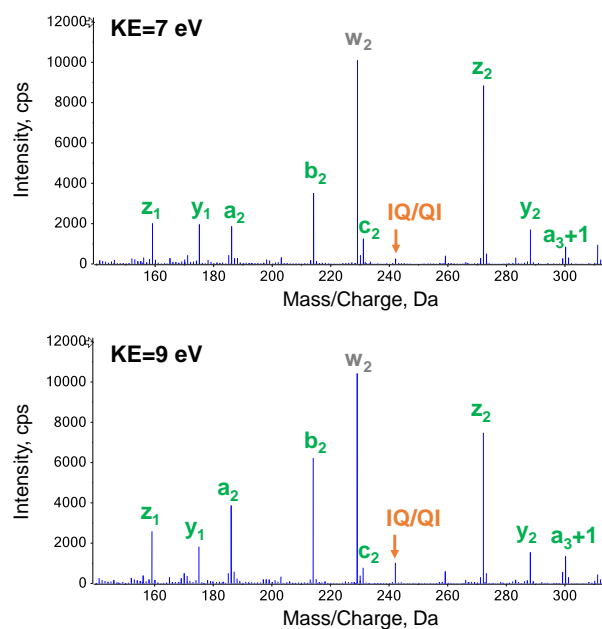


Figure 5. Expanded view of EAD spectra of leptin (Ile) at KE = 7 eV and 9 eV. Increasing electron KE from 7 eV (A) to 9 eV (B) led to increased abundance of smaller a/b ions (a₂ and b₂) and internal fragments, such as IQ/QI.

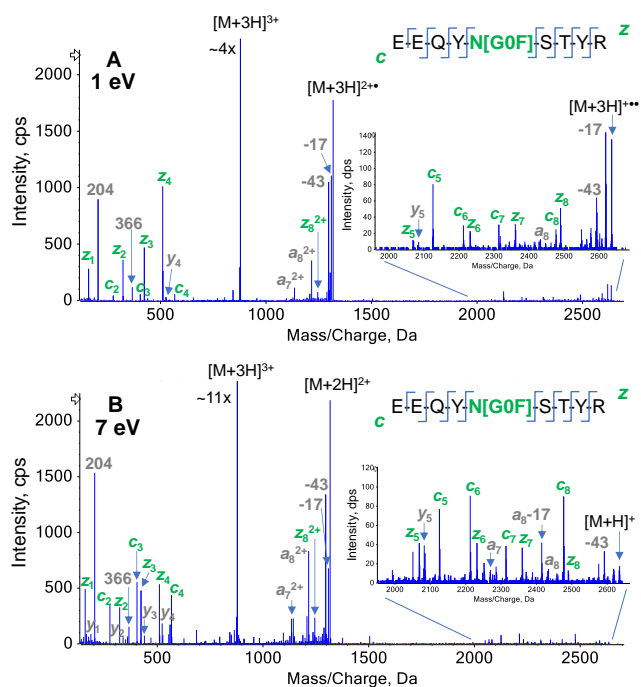


Figure 6. EAD spectra of an N-linked glycopeptide from NISTmAb. Accurate localization of GOF in EEQYNSYR was achieved using an electron KE = 1 eV (A) or 7 eV (B). Compared to an electron KE = 1 eV, the electron KE = 7 eV used in the EAD platform method yielded more y-ions, higher abundant oxonium ions at *m/z* 204, and a more even distribution of *a/z*-ions containing the glycosylation GOF. These results from the KE = 7 eV experiment facilitated glycopeptide identification and characterization.

It should be noted that only 1 of the 3 potential sites (2 T and 1 S) in the analyzed O-linked glycopeptide was found to be occupied. These results demonstrate that the EAD platform method using electron KE = 7 eV is powerful for the identification of N- and O-linked glycopeptides, due to extensive peptide backbone fragmentation, and enables accurate localization of glycosylations within peptides with multiple potential glycosylation sites.

Characterization of singly charged peptides

The analysis of singly charged peptides has been a long-standing challenge for traditional ExD approaches and has limited their adoption as a general characterization tool, compared to CID. In this work, the ability of EAD of singly charged peptides was explored. Low electron KEs of 1-5 eV were not sufficient to produce fragment ions for singly charged peptides (data not shown). By comparison, the EAD platform method generated predominantly y/z-type ions for complete sequence coverage of the singly charged qVTLR (q = pyroglutamic acid) from the N-terminus of NISTmAb heavy chain (Figure 8A). Similar results were obtained for other singly charged peptides (for example, SFNR and SLSLSPG) in the NISTmAb digest (data not shown).

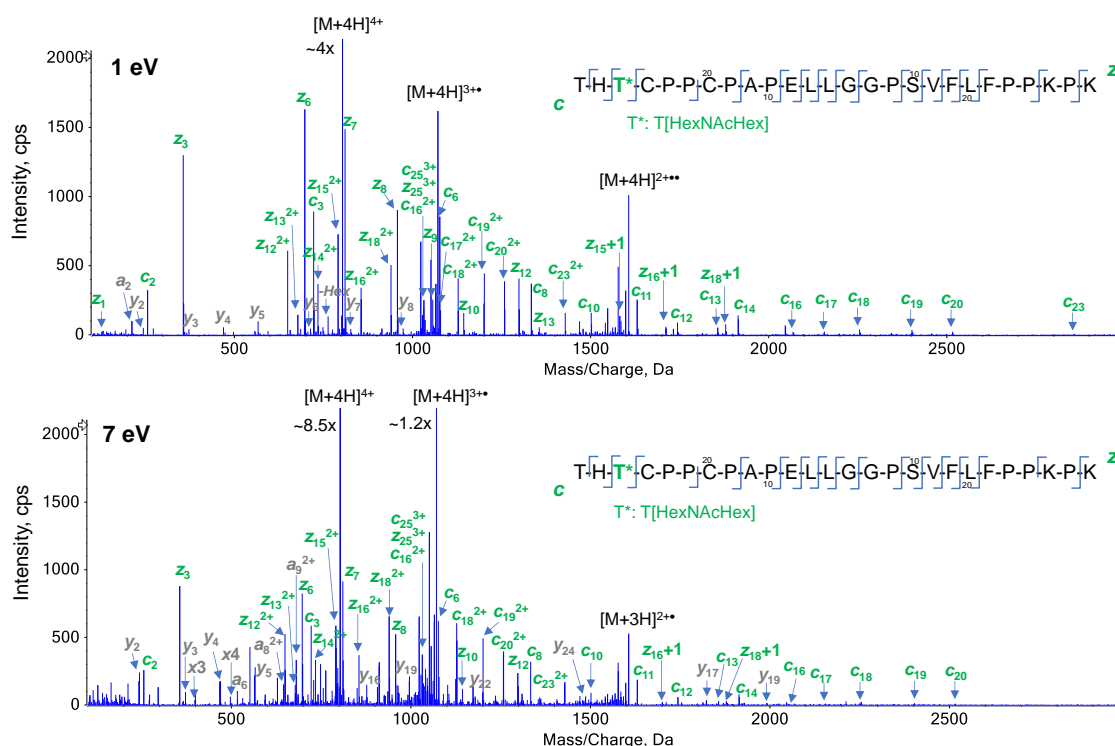


Figure 7. EAD spectra of an O-linked glycopeptide from etanercept. The location of O-glycosylation (HexNAcHex) can be confidently assigned to T3, instead of T1 or S17, based on the presence of abundant *c*₂ and *c*₃ ions. Not all ions were labeled for spectral clarity.

Interestingly, increasing electron KE to 10 eV yielded the optimal outcome for singly charged peptides in terms of the number and intensity of the sequence ions detected (Figure 8B). However, further increase of electron KE to >10 eV resulted in excessive internal and secondary fragmentation, without providing any information gain such as additional sequence information (data not shown).

Conclusions

- Efficient and comprehensive characterization of a wide range of peptides in a single injection can reliably be achieved with the optimized platform method using an electron KE = 7 eV
- Confident peptide identification, differentiation between isomers, accurate localization of glycosylations and the characterization of singly charged peptides with EAD enable an unprecedented level of information, not achievable with CID or traditional ExD techniques in a single injection
- The ability to fine-tune electron KE offers additional mechanisms of fragmentation (low-energy ECD or EIEIO) for full flexibility on the fragmentation achieved for specific analytes

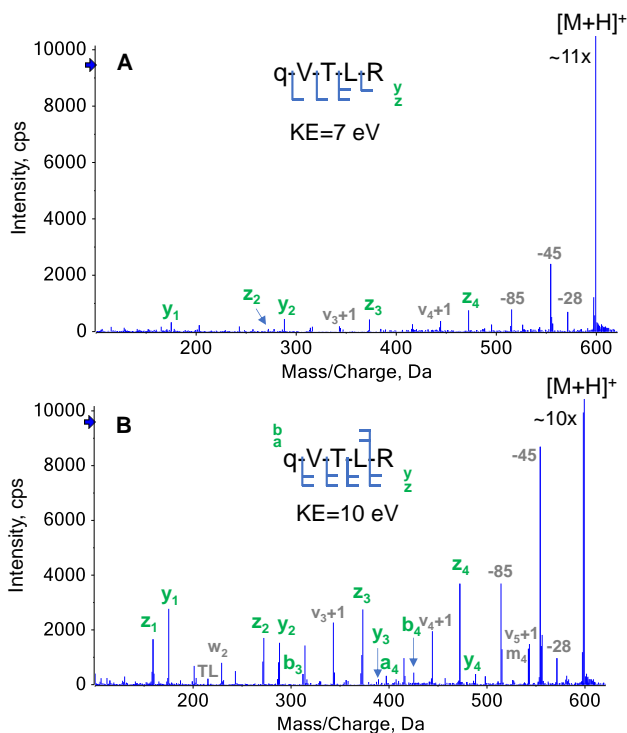


Figure 8. EAD spectra of a singly charged peptide detected in the NISTmAb digest. q = pyroglutamic acid. An electron KE = 7 eV was sufficient to generate enough sequence ions for confident identification of singly charged qVTLR (A). However, further increase of electron KE to 10 eV yielded the optimal outcome for this peptide in terms of the number and intensity of the sequence ions (B).

These results demonstrate that the generic EAD platform method was able to confidently identify singly charged peptides together with other peptides in a single injection. In summary, the EAD platform method provides a significant advantage over existing platform methods, such as CID for identification and localization of labile modifications and differentiation between isomers. In addition, it offers advantages over previously available ExD methods in terms of usability, efficiency and throughput for a wide range of peptides. The standardized KE setting can be incorporated in a DDA workflow for in-depth characterization of protein digests, without the need for further optimization.

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