A comprehensive peptide-level characterization of a multispecific monoclonal antibody (mAb)

Identification and localization for a variety of PTMs with the SCIEX ZenoTOF 7600 system using the Zeno trap in combination with electron activated dissociation (Zeno EAD)

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This work presents a comprehensive bottom-up characterization of a multispecific antibody, using the novel fragmentation technology of electron activated dissociation (EAD)[1,2] as part of a fully automated data-dependent acquisition (DDA) workflow. MS/MS analysis with Zeno EAD provides a significant improvement in protein therapeutic characterization, based on more descriptive fragmentation, high sensitivity, fast speed of acquisition, and reproducibility comparable to CID. In addition, data analysis was automated with Protein Metrics Inc. software.

Multispecific antibodies contain multiple binding sites to different epitopes in one molecule. They are becoming increasingly attractive because of their potential for achieving improved drug efficacy and for enabling new functionalities that do not exist in traditional mAbs.[3] As technology has matured over the years, peptide mapping has become a routine analytical tool in the development, characterization and quality control of next generation biologics. However, traditionally used collision induced dissociation (CID) presents a number of limitations for the characterization, including: the localization of post-translational modifications (PTMs), the ability to determine peptide side chain differences (amino acid isomers), and comprehensive characterization of glycosylations. Previous options for alternative fragmentation, aimed at addressing CID gaps, often struggle with sensitivity, reproducibility and acquisition speed. These fundamental limitations have restrained the widespread adoption of alternative fragmentation as the primary technique for peptide mapping.

Figure 1. The SCIEX ZenoTOF 7600 system.

The rigorous analysis of biopharmaceuticals with EAD is capable of facilitating speeds up to 20 Hz and of offering sensitivity enhancements using the patented Zeno trap instead of traditional TOF pulsing. Data acquisition is done in an automated fashion (DDA), achieving great MS/MS coverages, isomer elucidation, and PTM characterization in a single injection with the SCIEX ZenoTOF 7600 system.[4] In-depth peptide mapping of a multispecific antibody applying the new fragmentation type based on EAD was used here to showcase the capabilities for next gen biologics.

Key features of the SCIEX ZenoTOF system for characterization of next generation biotherapeutics

- **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 monoclonal antibodies (mAbs)
- **Higher levels of structural information:** Changing the mechanism of fragmentation by tuning the electron energy may provide a higher level of structural information, facilitating a comprehensive characterization of next gen biologics.
- **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 multispecific antibody was denaturated with 7.2 M guanidine hydrochloride / 100 mM Tris buffer (pH 7.2), followed by reduction with 10 mM DL-dithiothreitol and alkylation with 30 mM iodoacetamide. Digestion was performed with trypsin/Lys-C enzyme at 37°C for 16 h.

Chromatography: 10 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	*98.0*	*2.0*
5.00	*98.0*	*2.0*
6.00	*90.0*	*10.0*
40.00	*55.0*	*45.0*
44.00	*10.0*	*90.0*
46.00	*10.0*	*90.0*
47.00	*98.0*	*2.0*
50.00	*98.0*	*2.0*
51.00	*10.0*	*90.0*
54.00	*10.0*	*90.0*
55.00	*98.0*	*2.0*
60.00	*98.0*	*2.0*

Mass spectrometry: Data were acquired with an information-dependent acquisition (IDA) method using the SCIEX ZenoTOF 7600 system. The electron energy for the alternative fragmentation in the 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*
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*	*12 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*	*0.2 s*
Time bins to sum	*4*	*4*

Data processing: Data were processed using Byos software (Protein Metrics Inc.). Peptide precursor and fragments mass tolerances were set to 6 ppm and 20 ppm, respectively.

Sequence coverage

The multispecific antibody consists of two identical light chains (LC), and two heavy chains (HC) differing in their sequence: one HC is extended by multiple glycine-containing linker peptides. The multispecific mAb contains two N-linked glycosylation sites located in the Fc region of the heavy chains, resulting in an overall molecular weight of approximately 170 kDa. A dual enzyme (trypsin/Lys-C combination) digestion was performed and the digested peptides were analyzed using an automated DDA method with EAD on the SCIEX ZenoTOF 7600 system. The results show above 96% sequence coverage for every individual protein chain (Figure 2) with rich MS/MS information described in the following sections.

Protein name	Coverage summary	Coverage percent%
mAb LC	207 of 215	96.28
mAb HC1	430 of 446	96.41
mAb HC2	679 of 701	96.86

Figure 2. Sequence coverage of different protein chains of the multispecific mAb. The coverage was calculated based on MS/MS confirmed peptide matches.

N-linked glycosylation

The multispecific mAb has one *N*-linked glycosylation site located on each of the heavy chain. Accurate and detailed identification of glycopeptides was performed using the Byos software (Protein Metrics Inc.). MS data were matched with a tolerance of 6 ppm. Subsequent data interpretation of MS/MS spectra included the identification of glycan-derived fragments (oxonium ions), peptide backbone fragments, as well as glycan-containing peptide fragments (Figure 4). Since glycosylations are

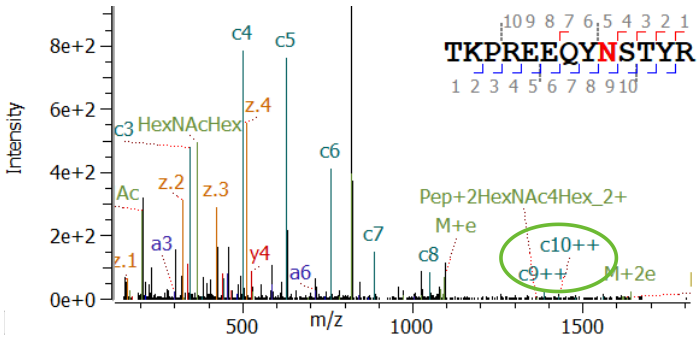

Figure 4. Identification of *N*-linked glycosylation site. The peptide from the HC was identified to contain one G1F (HexNAc(4)Hex(4)Fuc(1)) glycosylation. Peptide fragments containing the intact glycan provide evidence of the localization (encircled fragments).

fragile modifications, it can be a challenge to obtain comprehensive data on the peptide backbone at the same time as gathering information to localize the fragile modification and obtaining data on the signature ions derived from the glycosylation itself. EAD was able to provide this level of information in a generic peptide mapping method that is suitable for general sequence coverage assessment as well as in-depth analysis of modifications for comprehensive characterization. A summary of the identified *N*-glycans can be easily viewed in the report (Figure 3), with automated color-coding indicating their relative abundances. All of the glycopeptides shown were confirmed with MS/MS data within a single injection, highlighting the applicability of EAD as a general tool for peptide mapping. Quite often different glycosylation on the same peptide do not separate well under reversed-phase conditions using C18 material, which drives the need for a fast acquisition speed to confidently identify such peptides reproducibly. The fast reaction time of EAD of 10 ms enables in-depth characterization of

			MS Id ←	1
			MS Alias name ←	
Sequence (unformatted) ↑	Mod. Names ↑	Glycan Short Name ↑		
EEQYNSTYR	NGlycan/1241.4545	G0F-GlcNAc		11.3
	NGlycan/1403.5073	G1F-GlcNAc		9.02
	NGlycan/1444.5339	G0F		12.6
	NGlycan/1606.5867	G1F		37.6
	NGlycan/1768.6395	G2F		24.6
	NGlycan/2059.7349	G2FS1		4.9

Figure 3. Identification of *N*-linked glycosylations in the HC of the multispecific mAb. The heatmap shows the %area for each identified glycan type.

challenging PTMs. This can be done alongside a general peptide mapping approach for sequence confirmation and assessment of less challenging PTMs, such as oxidations, in one run. In addition, enough data points across peaks can be obtained for reproducible assessments of modification percentages based on the same data, as shown previously.[5]

Isomer differentiation

Confirmation of the protein's sequence is a standard requirement for all protein therapeutics by regulatory agencies. Although mass spectrometry has been mostly adapted for sequence verification, differentiating leucine (Leu) and isoleucine (Ile) remains a challenge. These two amino acids have the same molecular weight, but also their *b*- and *y*-ions are isoelemental and therefore cannot be used for further differentiation. Applying alternative fragmentation techniques, such as EAD demonstrated here, can result in distinct *w* signature ions *z*-43 Da in the case of Leu and *z*-29 Da in the case of Ile.[6,7] The short peptide shown contains both, Leu and Ile, and the location of each amino acid can be confidently confirmed with the respective diagnostic ions (encircled ions in Figure 5A and 5B).

Oxidation

Oxidation within a therapeutic protein often occurs on surface-exposed methionine (M) and tryptophan (W) residues during their production, purification and storage. This modification can potentially impact the binding of its target and therefore might affect its safety and efficacy. Characterization of oxidation, including the localization, is critical for antibody quality control. Here, the confident identification of an oxidized methionine and its location is shown (Figure 5). The identification of the oxidation on the doubly charged peptide was performed within the same injection as part of a general bottom-up workflow using EAD.

Deamidation

Deamidation of asparagine (Asn) can generate either iso aspartic acid (isoAsp) or aspartic acid (Asp) through a succinimide intermediate.[8] Since the isomerization of Asp introduces an additional methylene group in the protein backbone, it can lead to an impact on the protein folding, which can dramatically affect a drug's efficacy and safety. It has been reported that isomerization of Asp in the complementary determining regions (CDRs) of antibodies decreased binding efficiency.[9] Therefore a clear differentiation between Asp and isoAsp can be crucial. The traditional CID derived *b*- and *y*-ion fragments do not provide enough information to distinguish these isomers. On the contrary, using EAD, isoAsp undergoes electron rearrangements, leading to a bond breakage between the α-carbon and the methylene group in the peptide backbone,

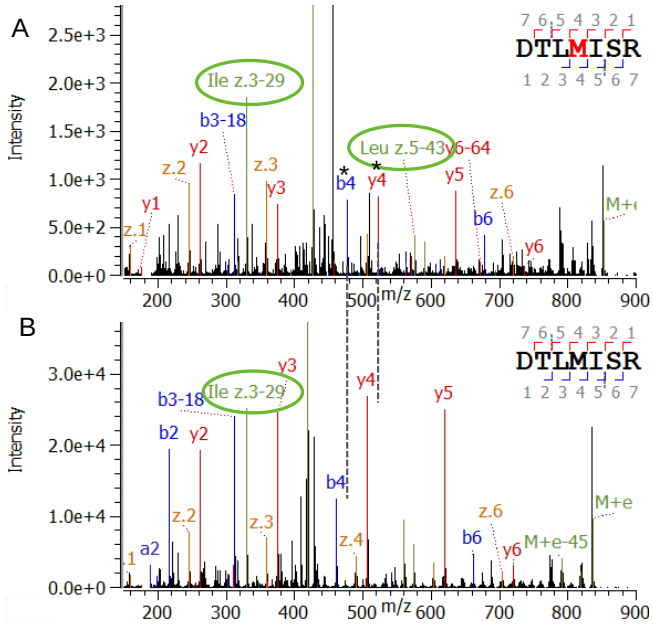

Figure 5. Zeno EAD spectra for of a short peptide (*z* = +2) with and without modification. A) with methionine oxidation. B) Non-modified wild type peptide. The comparison clearly shows the mass shift on the *b*4 and *y*4 ions due to M oxidation (* and dotted lines for comparison). The spectra also shows the distinction between Leu (Leu *z*.5-43) and Ile (Ile *z*.3-29) based on their unique side chain fragment, respectively (encircled fragments).

producing *c*+57 and *z*-57 fragments. These ions are not present when fragmenting Asp due to the lack of a methylene group in the side chain.[10,11] This additional level of information can be used to clearly distinguish Asp from isoAsp (Figure 6).

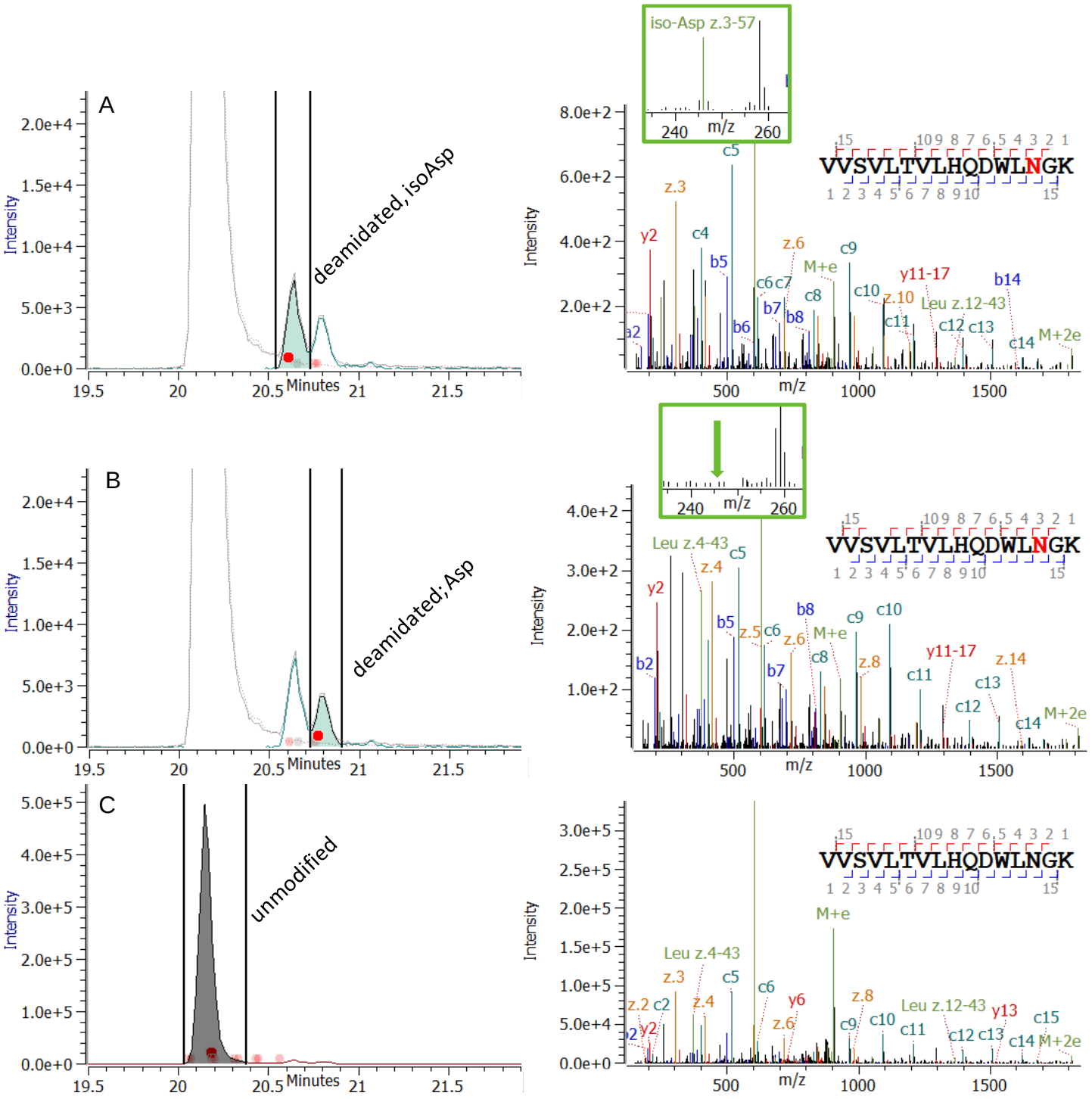

Figure 6. Data of peptide VVSVLTVLHQDWLNGK with deamidation-based modification (*z* = +3). Extracted ion chromatograms with red dots indicating the time point for MS/MS data acquisition on the left and associated EAD spectra on the right for A) the deamidated peptide with iso-aspartic acid with diagnostic ion shown as insert: *z*3-57; B) deamidated peptide with aspartic acid lacking of isoAsp-related diagnostic ions and C) non-deamidated main form.

Confirmation of long linker peptides

In the extended part of the second heavy chain (HC2), the protein carries multiple domains containing a series of glycine (G) residues. As a result, the trypsin/LysC digest generates large peptides with more than twenty amino acids, which are difficult to be ionized and fragmented. Despite this challenge, results obtained with Zeno EAD show great fragmentation coverage on these peptides (Figure 7). Even though a high dispersity of fragments was obtained, the spectral quality in terms of S/N was high due to applying the Zeno trap technology, which accumulates ions in-between each TOF pulse for duty cycles up to 95%. The confident characterization of such challenging peptides with traditionally used CID did not result in as descriptive information (data not shown).

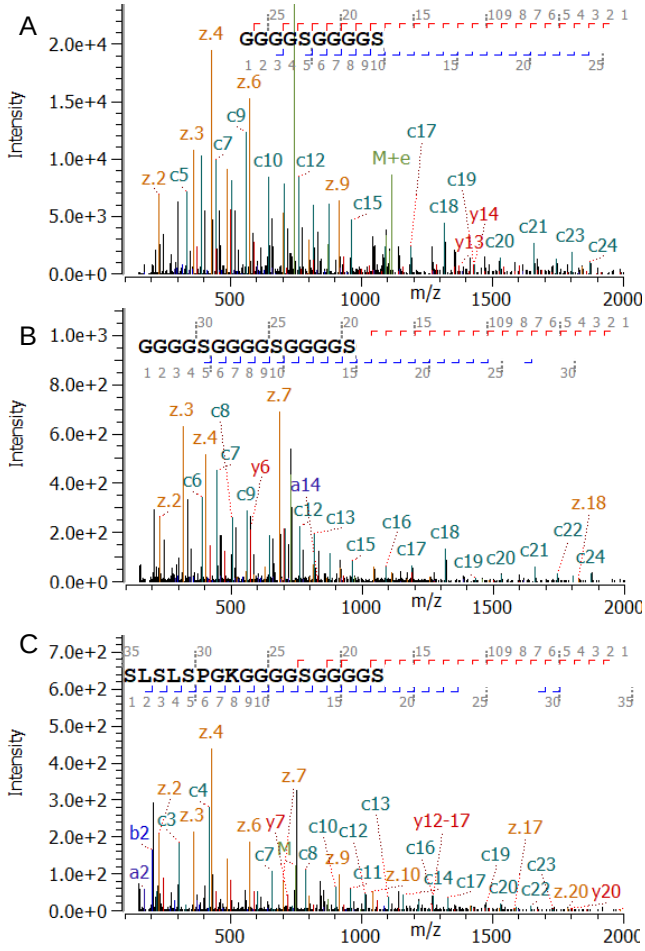

Figure 7. EAD spectra for long linker peptides. A) peptide with 27 amino acids; B) peptide with 34 amino acids; C) peptide with 35 amino acids, with fragment coverage on the sequence indicating the identified fragment ions. High fragment coverages were obtained for all three peptides.

Conclusions

- Highly effective peptide mapping of a multispecific antibody is enabled through descriptive fragmentation using Zeno EAD in an automated DDA workflow
- Great sequence coverages and PTM identification including the exact localizations for *N*-linked glycosylations, deamidations and oxidations, and amino acid isomers (Leu vs. Ile and Asp vs. isoAsp), significantly improved the characterization and quality assessment of the multispecific antibody over traditional CID-based approaches in a single injection
- Significant enhancement of sensitivity combined with high acquisition rates allowed for the correct assignment of challenging, low abundant modification and diagnostic ions by Zeno EAD
- Applying alternative fragmentation in a routine manner enables scientists to find answers for challenging questions more quickly with the SCIEX ZenoTOF 7600 system and intuitive SCIEX OS software for data acquisition
- Byonic software from Protein Metrics Inc. offers a complete biopharma processing suite that delivers accurate and automated data assessment, enabling a user to streamline the way from Zeno EAD raw data to customizable reports

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 timeof-flight mass spectrometer. Accepted by *JASMS*.
3. Amaral M, et al. (2020) Engineered Technologies and Bioanalysis of multispecific antibody formats. *J Appl Bioanal 6(1),26-51 (2020).*
4. Comprehensive peptide mapping of biopharmaceuticals utilizing electron activated dissociation (EAD). SCIEX technical note, RUO-MKT-02-12639-B.
5. Combining in-depth characterization with a routine multi-attribute methodology (MAM) in one shot using electron activated dissociation (EAD). SCIEX technical note, RUO-MKT-02-13110-A.
6. Differentiation of leucine and isoleucine using electron activated dissociation (EAD). SCIEX technical note, RUO-MKT-02-12605-B.
7. Zhokhov Sergey et al. (2017) An EThcD-based method for discrimination of leucine and isoleucine residues in tryptic peptides*. J Am Soc Mass Spectrom 28:1600-1611*.
8. Ni, Wenqin et al. (2011) Analysis of isoaspartic acid by selective proteolysis with Asp-N and electron transfer dissociation mass spectrometry. *Anal Chem, 82(17):7485–7491.*
9. Harris Reed et al. (2001) Identification of multiple sources of charge heterogeneity in a recombinant antibody. *J Chromatography B, 752(2001):233-245.*
10. DeGraan-Weber et al. (2016) Distinguishing aspartic and isoaspartic acids in peptides by several mass spectrometric fragmentation methods. J Am Soc Mass Spectrom, 27(12):2041-2053.
11. Differentiation of aspartic and isoaspartic acid using electron activated dissociation (EAD). SCIEX, RUO-MKT-02-12550-B.

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