Differentiation of leucine and isoleucine using electron activated dissociation (EAD)

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

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Here, an unambiguous differentiation of leucine (Leu) and isoleucine (Ile) in peptides derived from a monoclonal antibody (mAb) therapeutic was achieved utilizing a new fragmentation type based on electron activated dissociation (EAD). This workflow demonstrates the combination of routine characterization and elucidation of challenging residues in one single analysis, without the need for sample specific optimization.

Ensuring drug safety and efficacy is essential for biotherapeutic development, which drives the need for in-depth characterization. Confirmation of the protein sequence is a standard requirement for all protein therapeutics by regulatory agencies. Although mass spectrometry (MS) has been mostly adapted for sequence verification, differentiating Leu/Ile remains a challenge. These two isomeric amino acids have the same molecular weight and an MS/MS spectrum obtained from collision-induced dissociation (CID) cannot tell them apart. Thus, Edman degradation is still widely employed today, which is reagent expensive and time consuming.

Alternative fragmentation techniques have been reported to introduce secondary fragmentation, resulting in different losses of the side chain from Leu and Ile.[1,2,3,4] The derived signature ions can be used to discriminate between these two amino acids. However, previous approaches required extensive pre-characterization in order to perform MS^3 experiments. The use of nano-liquid chromatography (LC), offline fractionation or infusion was also common to enhance sensitivity, but they lacked reproducibility and throughput. With more and more protein therapeutics in the market and in development, the need to distinguish Leu and Ile in an easy manner has dramatically increased. In addition, it is likely that more analytical questions will need to be answered which require technologies better able to elucidate complex structural moieties.

Figure 1. The SCIEX ZenoTOF 7600 system.

The data presented in this work show the streamlined identification of Leu and Ile as part of a general peptide mapping study. Data were acquired in a fast, automatic and sensitive manner using data-dependent acquisition (DDA) with Zeno EAD[5-7], with streamlined data interpretation utilizing Protein Metrics Inc. software.

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, particularly for isomer differentiation such as Leu and Ile differentiation
- **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 EAD fragmentation for both singly- 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 adalimumab sample 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 16h.

Chromatography: 3 µl (4 µg) of the trypsin/Lys-C digest was separated with a CSH C18 column (2.1×100 mm, 1.7 µm, 130 Å, Waters) using an ExionLC 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 350 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*
1.00	*98*	*2.0*
86.00	*55*	*45*
86.50	*20*	*80*
87.00	*2.0*	*98*
87.50	*2.0*	*98*
87.51	*98*	*2.0*
90.00	*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 the alternative fragmentation in the EAD cell was set to a value of 7 eV.

Table 2. MS parameters.

Parameter	MS	MS/MS
Scan mode	*TOF-MS*	*IDA dependent*
Polarity	*positive*	
Gas 1	*50 psi*	
Gas 2	*50 psi*	
Curtain gas	*35 psi*	
Source temperature	*550 °C*	
Ion spray voltage	*5500 V*	
Declustering potential	*80 V*	
Collision energy	*12 V*	*NA*
CAD gas	*7*	
Maximum candidate ion	*10*	
Intensity threshold	*125 cps*	
Charge states	*2 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*
Electron KE	*NA*	*7 eV*
Electron beam current	*NA*	*4750 nA*
ETC	*NA*	*100*
Zeno trap	*NA*	*ON*
Accumulation time	*0.25 s*	*0.10 s*
Time bins to sum	*8*	*12*

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

The what, why and how

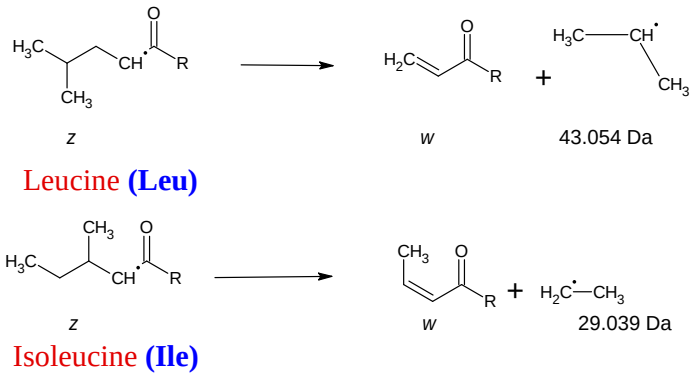

Figure 2. The schemes of secondary fragmentation for Leu and Ile. The *z*-ion of Leu experiences a 43 Da side chain loss, while that of Ile experiences a 29 Da side chain loss, resulting in a *w*-ion.

Although the sequence of a biotherapeutic is mainly obtained from DNA sequencing of the source cell lines[8], the confirmation is on the protein level and sometimes even *de novo* sequencing is required. During a biotherapeutic's development, the confirmation of the sequence integrity is requested by agencies such as the FDA and EMA. High-resolution mass spectrometry was demonstrated to be a well-accepted approach to provide sequence information with high accuracy and reasonable throughput. However, the discrimination of Leu and Ile remains a challenge because these two amino acids have the same molecular weight. Their *b*- and *y*-ion, but also their *c*- and *z*-ions, are isoelemental and therefore cannot be used for further differentiation. Nevertheless, secondary fragmentation is known to occur for Leu and Ile residues when applying alternative fragmentation techniques, resulting in distinct *w* signature ions, *z* – 43 Da in case of Leu and *z* – 29 Da in case of Ile (Figure 2). With these diagnostic ions, the discrimination of the two amino acids is possible. However, previously described methods show drawbacks in terms of ease-of-use, throughput, and sensitivity, limiting their broad adoption in the biopharma industry.

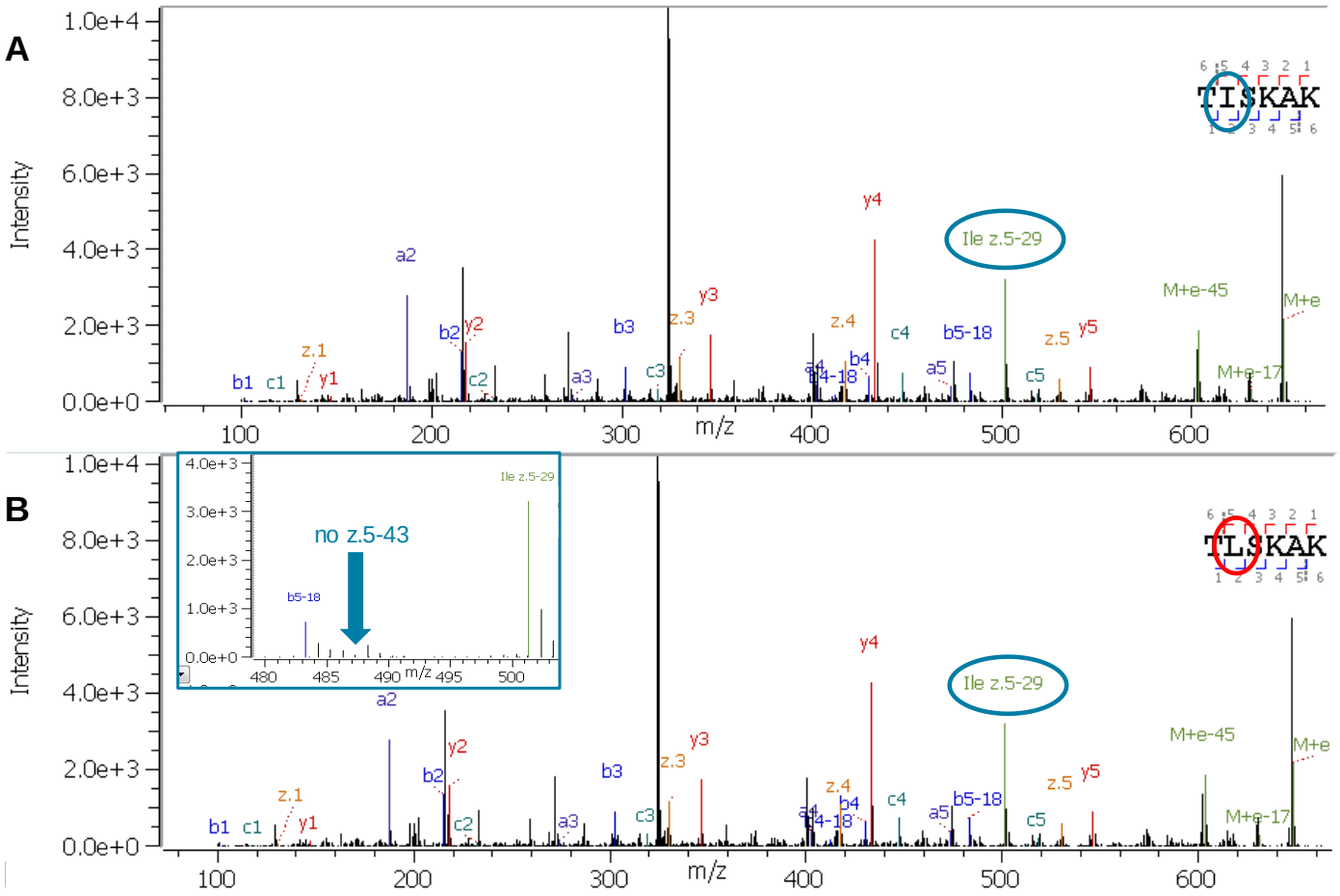

Figure 3 EAD spectra for peptide TISKAK. A: The correct sequence containing one Ile residue was used for data interpretation. The distinct *z*5-29 fragment (encircled) was found proving Ile to be present. B: incorrect sequence containing one Leu was used for data interpretation, still the Ile signature fragment was correctly identified (encircled *z*-29 ion), but no Leu signature fragment (*z*5-43) was detected above the noise level (insert).

Here, a peptide mapping analysis of a mAb was performed, capable of answering in-depth characterization questions like Leu/Ile differentiation, while maintaining sensitivity and ease-of-use. An analytical flow LC setup and a DDA approach with Zeno EAD on the SCIEX ZenoTOF 7600 system (Figure 1) were used. Robustness and throughput are achieved by using analytical flow rates, while the DDA method set-up further enhance ease-of-use by avoiding compound-specific method optimization. In addition, the Zeno trap enhances the detection of fragments enabling a confident assignment.[6] This breakthrough technology realizes the desire of an easy, and at the same time comprehensive, method for advanced characterization in the biopharmaceutical industry.

Example study on a mAb

The study focused on the characterization of adalimumab, a commercially available antibody. Its sequence consists of 104 Leu and 30 Ile residues. With the enhanced detection of fragments using the Zeno trap, their straight-forward interpretation using Byos (Protein Metrics Inc.) could be achieved. Leu and Ile residues in a peptide digest were identified accurately for the biotherapeutic mAb.

As an example, peptides T**I**SKAK and EVQ**L**VESGGG**L**VQPGR were chosen. They contain one Ile and two Leu, respectively. For both peptides, *b*-, *y*-, *c*- and *z*-ions were found (Figure 3 and 4). The fragment coverage was 100% in both cases with excellent signal-to-noise ratios for most fragments. The red and blue hashmarks indicate the *b*- and *y*-ion coverage in the sequence annotation in Figure 3 and 4. They cannot be used for differentiating between Leu and Ile because of their isoelemental nature, but they show that either could be present. According to the published sequence information, the peptide TISKAK contains one Ile. In this case a *w* fragment derived from the *z*5-ion -29 Da is expected. This signature ion was indeed detected in the spectrum, confirming the correct sequence (Figure 3A). To check the accuracy of the assignment, the same data were processed assuming the sequence was with Leu instead of Ile: T**L**SKAK (Figure 3B). Any method relying on mass spectrometry information alone would rely on distinctive fragment ion information, if peptides were not separated chromatographically. Therefore this “stress test”, while artificial, provides a simulation of what might happen if the sequence were in doubt. Even with a wrong sequence being entered, the data were annotated with an Ile *z*5-29 fragment. No *z*543 fragment was detected in the spectra above the noise level, confirming the correct assignment of Ile for this peptide in a streamlined manner.

For the peptide EVQ**L**VESGGG**L**VQPGR, the comparison of the MS1 and the MS/MS intensities showcases the advantage of using EAD in combination with the Zeno trap. Despite the richer fragmentation spectra including *b*-, *y*-, *c*-, *z*- and w-ions, the overall intensities achieved are very comparable to CID data with significantly fewer fragment ions (data not shown). This enhancement in sensitivity paves the way for accurate identification in an automatic fashion using analytical flow LC systems. The two Leu residues in the peptide could also be clearly discriminated from Ile by the signature w fragments observed in the MS/MS spectra: Leu-4 and Leu-11 were differentiated from Ile with two abundant w-ions, *z*13-43 and *z*6-43, respectively (encircled in Figure 4B). Nevertheless, the wrong sequence was entered for this peptide in order to verify the assignment (Figure 4C). The data derived from EAD and the data interpretation with Byonic enabled the correct assignment as an Leu also in this case (Figure 4C).

Unambiguous differentiation between Ile and Leu in one single DDA run was achieved by using Zeno EAD on the SCIEX ZenoTOF 7600 system with automatic data interpretation by Protein Metrics Inc. software. This workflow proposes a streamlined solution for distinguishing isomers previously thought a challenge by LC-MS/MS for years.

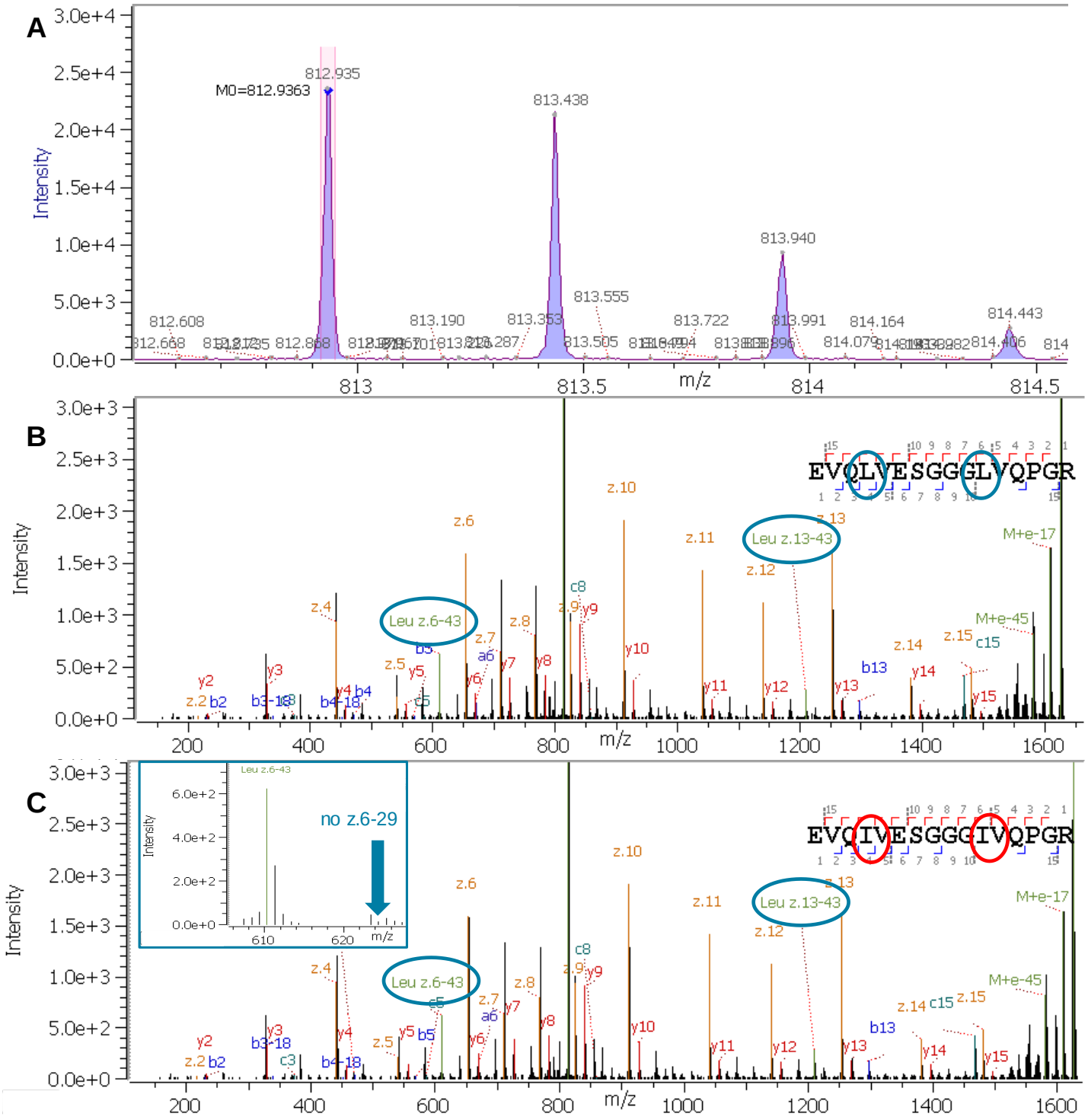

Figure 4. Spectra for peptide EVQIVESGGGIVQPGR. A: MS spectrum of precursor. B: EAD derived MS/MS spectrum using the correct sequence containing two Leu residues for data interpretation. Distinct fragments (*z*-43) were found, proving Leu to be present (encircled ions). C: EAD derived MS/MS spectrum using the incorrect sequence containing two Ile was for data interpretation, still Leu signature fragments were identified (encircled *z*-43 ions), but no Ile signature fragments (*z*-29) was above the noise level (insert for *z*6-29).

Conclusions

- Unambiguous differentiation of Leu and Ile in peptides was achieved with a novel fragmentation technique: EAD
- MS/MS fragment detection was significantly enhanced compared to traditional high-resolution MS/MS analysis, enabling confident fragment assignment even for precursors with medium or very low intensities when utilizing the Zeno trap
- The robust, reproducible and easy-to-use alternative fragmentation enables users to answer challenging analytical questions in a streamlined manner with the SCIEX ZenoTOF 7600 system controlled by SCIEX OS software
- Automatic data processing facilitates answering complex analytical questions in a routine way when using Protein Metrics Inc. software

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