Application Note

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Identification of Amino Acid Isomers Using Electron Capture Dissociation in the Agilent 6545XT AdvanceBio LC/Q-TOF System

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Abstract

Accurately determining the amino acid sequence is crucial for understanding a protein's structure and function. However, distinguishing between leucine (Leu) and isoleucine (Ile) poses a challenge, because they are positional isomers and cannot be differentiated using traditional collision-based fragmentation techniques. Electron-based fragmentation offers a promising solution by producing side chain fragments that can be used to distinguish between Leu/Ile and other isobaric residues such as aspartate (Asp) and isoaspartate (isoAsp). This application note demonstrates the identification of isobaric residues in peptides and intact proteins using the Agilent 6545XT AdvanceBio LC/Q-TOF system equipped with an ExD cell for electron capture dissociation. Agilent ExDViewer software enables intuitive analysis of side chain fragmentation in Q-TOF datasets, enhancing the ability to discern between isobaric amino acids.

Introduction

Approximately one-sixth of the human proteome consists of the isobaric amino acids Leu or Ile.[1] Sequence variations involving these amino acids can significantly influence protein structure and function. For example, variations between Ile or Leu within the complimentary determining region of an antibody affect the strength of target binding.[2] Additionally, spontaneous isomerization of Asp to isoAsp is associated with protein aging and various diseases.[3–5] Therefore, reducing ambiguity in protein sequence analysis by accurately determining the identity of isobaric amino acids is crucial for understanding protein function and disease mechanisms.

Agilent's innovative solution for electron dissociation, the ExD cell, enables a more comprehensive and complementary characterization of protein sequences compared to using collision-induced dissociation (CID) alone. Radical z-type ions can undergo secondary fragmentation of amino acid side chains producing w-ions that can be used to distinguish between Leu and Ile.[1,6] The isomerization of Asp to isoAsp results in a carboxyl group shift, which can be detected as a 57 Da shift from the expected c and z-type ions.[4–6]

This application note describes a fast and highly efficient fragmentation of peptides and proteins using the Agilent 6545XT AdvanceBio LC/Q-TOF equipped with an ExD cell for electron capture dissociation. Isobaric amino acids were identified in MS/MS spectra from a synthetic peptide and using intact ubiquitin. While very few freely accessible tools exist for the identification of isobaric residues, ExDViewer makes annotation of side chain fragmentation in peptide and protein spectra simple and easily interpretable.[7] Together, the sensitivity and fragmentation capabilities of the 6545XT AdvanceBio LC/Q-TOF combined with ExDViewer for analysis provide an effective solution for the comprehensive characterization of protein sequence including the identification of isobaric amino acids.

Experimental

Chemical and standards

- Melittin/tune mix tuning standard formic acid, 99.0+%, Optima LC/MS Grade (part number A-117-50), Fisher Chemical
- Acetonitrile, LCMS grade, 99.9%+, OmniSolv (part number AX0156-6), Supelco
- REALLYisoD synthetic peptide (part number 4144889), Bachem
- Bovine ubiquitin (part number U6253), Sigma
- Agilent Tuning Mix (G1969-85000)

Sample preparation

Lyophilized samples were stored at –20 °C before analysis. Before analysis, samples were reconstituted in 15% acetonitrile and 0.01% formic acid. The final concentrations of the samples were 1 µM for the REALLYisoD peptide and 10 µM for ubiquitin.

Instrumentation

- Agilent 6545XT AdvanceBio LC/Q-TOF
- Agilent ExD cell (G1997AA)

Software

- Agilent ExDControl software version, v 3.6
- Agilent MassHunter Acquisition software for LC/TOF and LC/Q-TOF, v. 11.0
- Agilent ExDViewer software, v 4.5.14

Mass spectrometry methods

All samples were directly infused using a 500 µL syringe at a rate of 20 µL/min. A New Era syringe pump (model number 300) was used for infusion. The samples were introduced using PEEK tubing connected with a finger-tight ferrule to the nebulizer inlet of the Agilent Dual Jet Stream (AJS) source. Mass spectrometry was performed using the 6545XT AdvanceBio LC/Q-TOF equipped with the ExD cell. A targeted acquisition method was set up in MassHunter Acquisition v11.0. Fragmentation results were analyzed using ExDViewer v4.5.14. Detailed instrument parameters for the 6545XT LC/Q-TOF are listed in Table 1.

Table 1. Q-TOF LC/MS data acquisition parameters.

Parameter	Value
Agilent 6545XT AdvanceBio LC/Q-TOF System	
Ion Source	Agilent Dual Jet Stream Electrospray ionization source
Polarity	Positive
Gas Temperature	325 °C
Drying Gas Flow	5 L/min
Nebulizer	20 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	4,000 V (ubiquitin), 3,200 V (peptide)
Nozzle Voltage	2,000 V
Fragmentor	175 V
Skimmer	45 V
Acquisition Rate	1 spectrum/sec
Isolation Window	Wide (9 *m/z*)
MS1 Spectra Range	100 to 3,200 *m/z*
MS2 Spectra Range	120 to 3,200 *m/z* (ubiquitin) 120 to 2,400 *m/z* (peptide)

ExD cell operation

The ExD cell is an add-on for the 6545XT Q-TOF LC/MS systems that enables electron capture dissociation capabilities. The ExD cell is controlled using the ExDControl software, which is stand-alone software operated alongside MassHunter Acquisition to control ExD cell voltages and filament heating current. ExDControl features an autotune algorithm that automatically adjusts ExD cell voltages to optimize function for transmission or electron capture dissociation (ECD).

The following steps were used to set up the ExD cell isobar analysis. First, an appropriate filament heating current was established and allowed to warm up for 20 minutes. Next, the melittin/tune mix standard was infused from bottle B. An ExDControl autotune was performed on tune mix ions to adjust the ExD cell lens voltages to maximize MS1 transmission.

After optimizing transmission, the melittin 3+ precursor (949 *m/z*) was isolated. ExD cell voltages were optimized for fragmentation by performing an autotune on melittin fragment masses. A cell voltage profile optimized for ECD of melittin works effectively for most peptides, however, additional tuning standards may be needed for optimal ECD of proteins. For ubiquitin, an autotune was performed on a built-in mass list of ubiquitin ECD fragments to maximize the intensities of ubiquitin fragments (Table 2). After establishing MS1 transmission and MS2 ECD profiles in ExDControl, MassHunter was switched to the acquisition context, and targeted acquisition methods were created.

Table 2. A summary of the tune standards used to optimize the ExD cell lens voltages for transmission or ECD. The ExDControl autotune algorithm is installed with several built-in mass lists, including masses for tune mix, melittin, and ubiquitin. However, custom mass lists can also be created to optimize the intensities of a user-defined mass list.

ExD Cell Tuning Standards		
	MS1 Transmission	**MS2 ECD Fragmentation**
REALLYisoD (1.9 kDa)	Tune mix	Melittin
Ubiquitin (8 kDa)	Tune mix	Ubiquitin

Targeted analysis with ExDViewer

ExDViewer is a freely available software tool that enables confident analysis of all polypeptide fragment types in Q-TOF MS/MS data. ExDViewer provides a quick and intuitive method for analyzing complex fragmentation patterns from peptides or large, intact proteins. A key feature of the software is the support for annotating side chain fragmentation patterns and isoAsp ions.

The targeted deconvolution workflow is used to match fragmentation patterns against a known sequence. Target sequences are defined using the target editor where the sequence and minimum and maximum charge states are specified. After defining a target, .d files can be loaded directly into ExDViewer for targeted deconvolution. Figure 1 shows a screenshot of the input page of the target deconvolution workflow. Profile data featured in this document were processed using the restrictive matching presets for ExD fragmentation analysis with iterative multi-pass matching for overlapping ions.

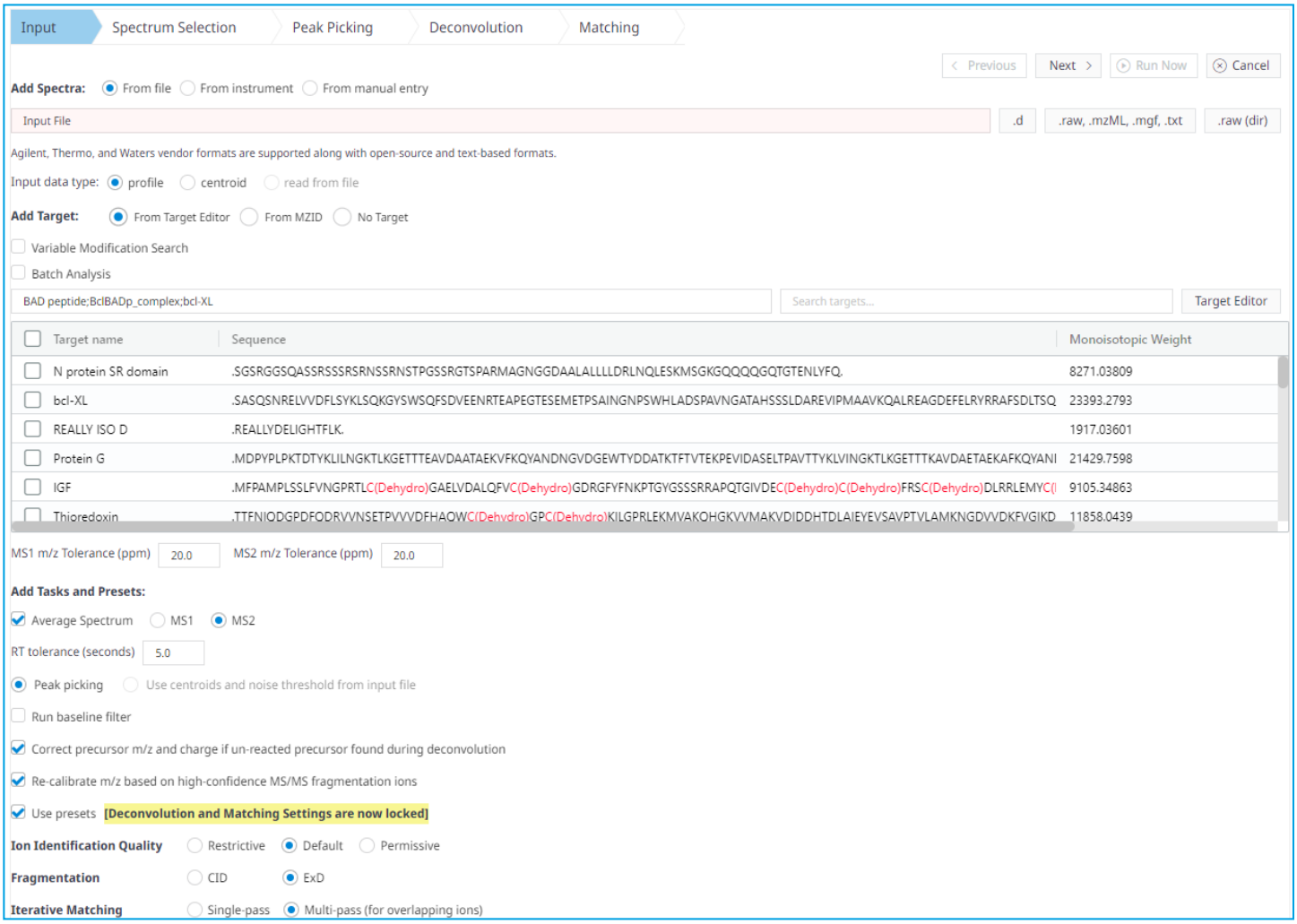

Figure 1. The ExDViewer targeted deconvolution input page. Preset match settings work well for a wide range of peptide and protein analytes.

Results and discussion

Leu/Ile and Asp/isoAsp identification

Radical-driven side chain fragmentation leads to the formation of w-type ions, which enable Leu/Ile differentiation. Leu w-ions are formed by a radical loss of an isopropyl (z – 43 Da) group while the corresponding w-ion for Ile involves the loss of an ethyl radical (z – 29 Da).

In contrast, isoAsp formation is detected based on a structural change involving the peptide backbone, which results in the shift of 57 Da from the corresponding c or z ion for Asp (c + 57 Da, z – 57 Da).

Here, a synthetic peptide with the sequence REALLYisoDELIGHTFLK was used to demonstrate Leu/Ile and Asp/isoAsp identification using electron-based fragmentation (Figure 2).

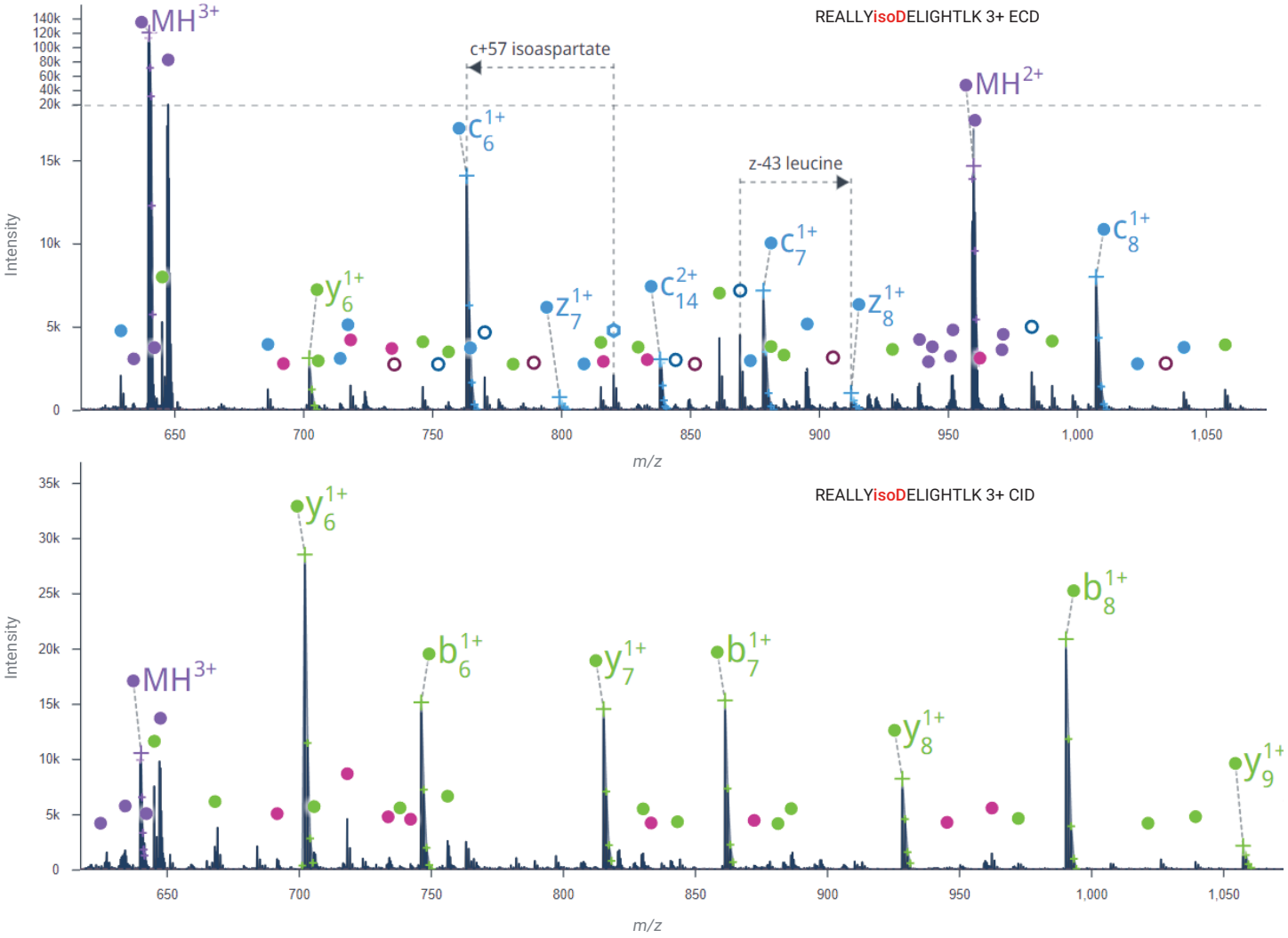

Figure 2. The top mass spectrum is an ECD fragmentation spectrum of the synthetic peptide REALLYisoDELIGHTFLK (1 µM). The Asp has been engineered to an isoAsp and is highlighted in red. Isobar fragment evidence is annotated with text in the spectrum. The bottom figure shows the CID mass spectrum for the same peptide. ECD type fragments are labeled in blue, CID type fragments are labeled in green, precursor ions are labeled in purple, and a-ions are in pink.

REALLYisoDELIGHTFLK has five isobaric Leu/Ile residues and one engineered isoAsp, which yields 64+ possible isobaric sequences with identical theoretical CID spectra. Side chain fragmentation detected with electron fragmentation uniquely enables the identification of the exact peptide sequence with isobar specificity. Figure 2 compares the MS/MS spectrum of the 3+ REALLYisoDELIGHTFLK precursor using ECD or CID fragmentation. Using ExDViewer, diagnostic ions for Leu, Ile, and isoAsp are intuitively annotated in the spectrum. The charge-reduced precursors are also annotated.

In Figure 3, the effect of collisional activation before ECD on the detection of diagnostic ions for Leu/Ile and isoAsp was investigated. A range of collision energies was applied to the 3+ REALLIYisoDELIGHTFLK precursor before electron capture. Overall, ion intensities went down with increasing collision energy. The 3+ precursor had a more gradual decline in intensity compared to the 4+ precursor which was more sensitive to collisional activation. These results suggested that using ECD alone was most effective for detecting diagnostic ions for this example peptide.

Top-down MS/MS isobar determination

In contrast to traditional peptide analysis, top-down mass spectrometry involves the sequencing of an entire protein without pre-enzymatic digestion. Avoiding enzymatic digestion reduces sample preparation steps, saving time while minimizing the risk of artifact introduction. Importantly, top-down analysis enables the characterization of unique proteoforms that are unable to be defined using a mixture of digested peptides. Here, top-down analysis was employed to characterize isobars in intact ubiquitin. 10 µM ubiquitin was directly infused and analyzed using a targeted acquisition method. The 11+ precursor was selected for isolation and subjected to ECD in the ExD cell. Data were collected at 1 spectrum/second for a total of 1 minute. Highly efficient fragmentation of the 11+ precursor resulted in 99% sequence coverage and the differentiation of 10/16 of isobaric Leu and Ile residues. Additionally, isoAsp c and z ions were identified that suggest the presence of isoAsp in amino acid positions 39 and 52. Figure 4 shows the sequence coverage map generated in ExDViewer, which enables the user to explore the fragment evidence supporting a target sequence interactively. Figure 5 highlights a 350 *m/z*-wide slice of the ubiquitin fragmentation spectrum featuring abundant ECD ions and w-ions for Leu and Asp identification.

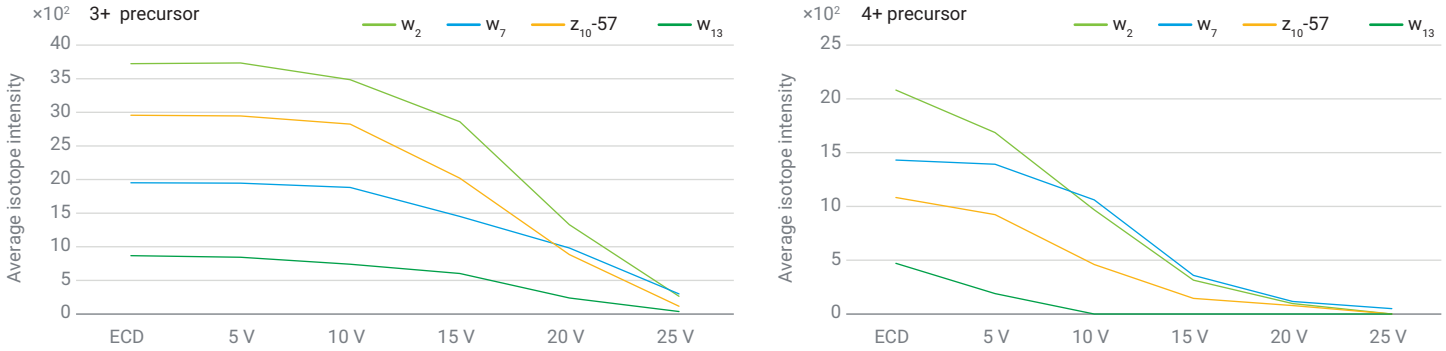

Figure 3. REALLYisoDELIGHTFLK isobar diagnostic ion intensities. Average ion intensities for w_2, w_7, z_{10}-57, and w_{13} were plotted as a function of collision energy.

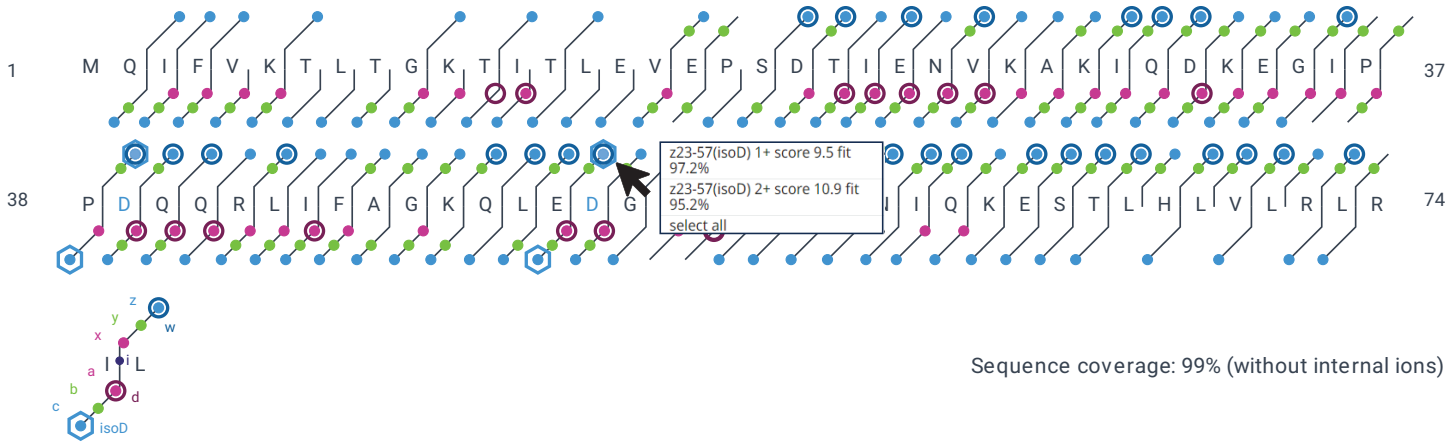

Figure 4. Sequence coverage map generated in ExDViewer. Each color dot represents a different type of ion. CID and ECD type backbone fragments ions are annotated along with side chain fragmentation and c/z isoAsp ions. Informative tool tips indicate the type of ion, charge states, and score for each fragment detected. Here, the tool tip is indicating that there were two charge states detected for the z_{23} -57 isoAsp ion.

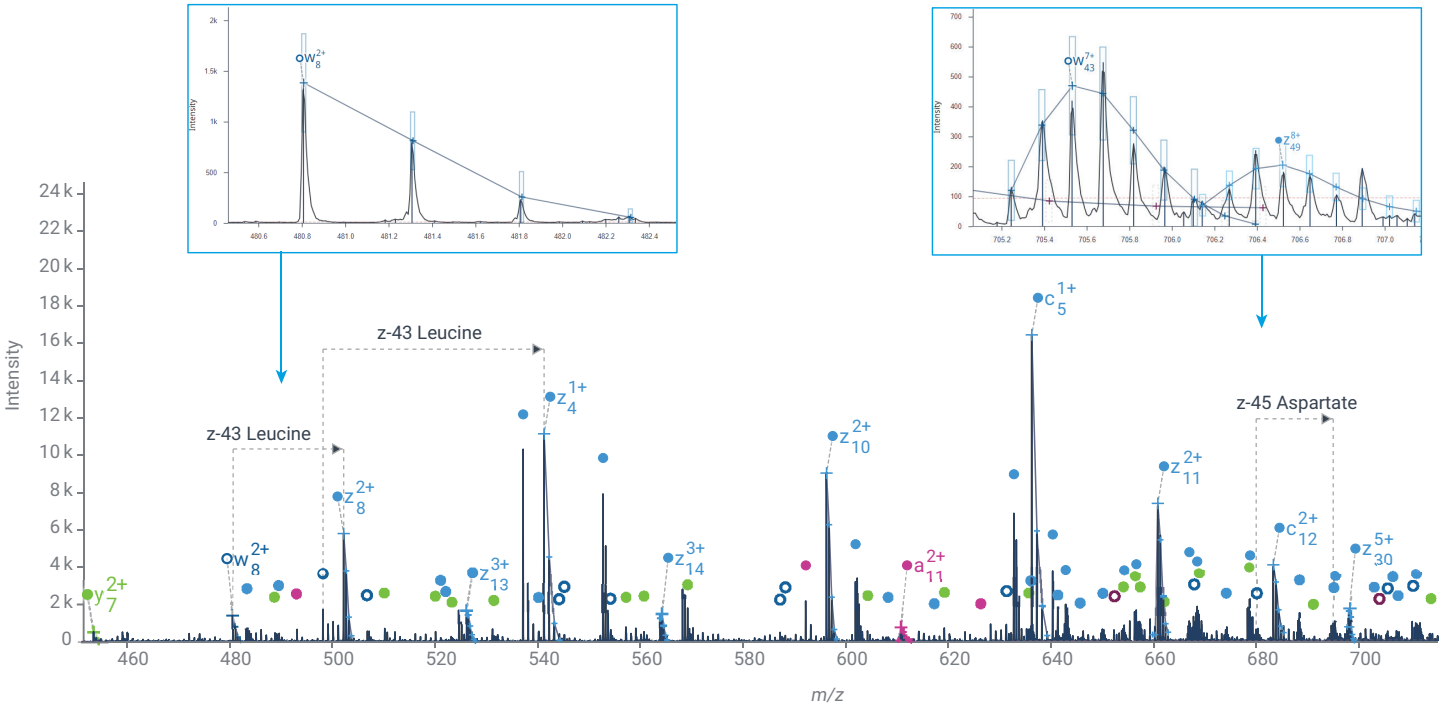

Figure 5. The ExD fragmentation spectrum of 11+ ubiquitin. Side chain fragment evidence identifying Leu and Asp is featured in the insets. Isobar evidence is automatically annotated with dashed lines and labels.

Conclusion

This application note describes the analysis of isobaric amino acids using an Agilent 6545XT AdvanceBio Q-TOF LC/MS system equipped with an Agilent ExD cell and the Agilent ExDViewer software tool for analysis. Electron capture dissociation is a powerful fragmentation technique that provides complementary information to CID fragmentation. These methods can be applied to investigate peptide backbone and side chain fragmentation for a range of molecules, reducing ambiguity in protein sequence analysis. The sensitivity of the Q-TOF to detect ECD fragments combined with ExDViewer for fragment analysis provides an effective solution for targeted amino acid isobar identification.

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