Application Note

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Differentiation of Isomeric Amino Acids by Electron Capture Dissociation

Analysis of the antigen-binding region of a monoclonal antibody using an Agilent 6545XT AdvanceBio LC/Q-TOF system equipped with ExD cell

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Abstract

Peptide mapping using liquid chromatography-mass spectrometry (LC/MS) is a key analytical tool in the biopharmaceutical industry for studying the primary structure of monoclonal antibodies (mAbs), including sequence and posttranslational modifications (PTMs). Molecular fragmentation by tandem mass spectrometry (MS/MS) using collision induced dissociation (CID) is widely applied in these analyses, yet this approach is limited by its inability to distinguish between isomeric residues. In this application note, electron capture dissociation (ECD) was used to successfully differentiate isomeric amino acids such as leucine/isoleucine and aspartate/isoaspartate in the complementarity-determining region (CDR) of a mAb.

Introduction

Antibodies are an integral part of the immune system, and over the past several decades, their potential to bind antigens has been harnessed to develop therapeutics for treating conditions such as cancer, autoimmune diseases, and infectious diseases.[1] Therapeutic monoclonal antibodies (mAbs) are highly complex glycoproteins with a characteristic molecular architecture. They consist of four polypeptide chains that assemble into an intricate Y-shaped arrangement with a molecular weight of approximately 150 kDa. The complementarity-determining regions (CDRs) that form the antigen binding sites are situated at the tips of the Y-shape, more specifically within the variable domains of both the heavy and light chains. Posttranslational modifications (PTMs) in these CDRs, such as isomerization of amino acids, may have a significant impact on the specificity and therefore the efficacy of mAbs.[2]

Peptide mapping is an advanced analytical methodology used to unravel this challenging microheterogeneity. It involves the digestion of the mAb into peptides that are separated by liquid chromatography (LC) and then detected and sequenced by mass spectrometry (MS) or tandem mass spectrometry (MS/MS) using collision induced dissociation (CID). In CID, accelerated peptide ions collide with a neutral gas (nitrogen). The collisions convert kinetic energy into internal energy, causing peptide (amide) bond cleavage and thereby generating a series of b- and y-ions (Figure 1) that allow the molecular location of different PTMs to be deduced. Labile PTMs such as glycosylation or phosphorylation, however, are often lost during CID and therefore cannot be localized. Likewise, isomeric amino acids such as leucine/isoleucine or aspartate/isoaspartate cannot be discriminated using CID.

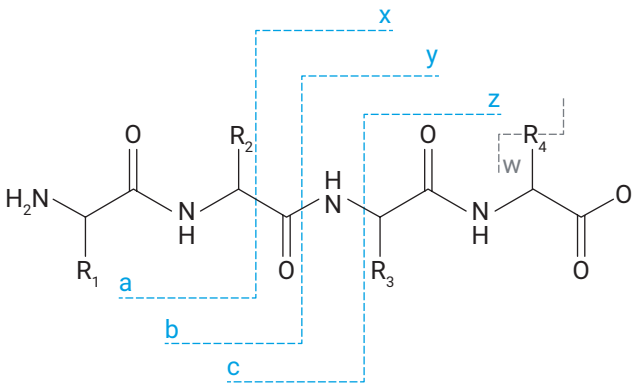

Figure 1. MS/MS fragmentation of a peptide.

Electron capture dissociation (ECD) is a softer fragmentation technique where gas-phase peptide ions capture low-energy electrons, leading to radical-driven peptide backbone cleavage.[3–4] This type of fragmentation mainly produces c- and z-type ions (Figure 1) and can preserve labile PTMs. In addition, radical-driven side chain fragmentation leads to the formation of w-type ions (Figure 1) that enable differentiation of isomeric amino acids.[5–7]

This application note describes the differentiation of isomeric amino acids present in a tryptic peptide associated with one of the CDRs of a mAb using ECD. Isoaspartate formation in CDRs has been shown to alter target affinity, making this modification a critical quality attribute (CQA).[8] Here, a tryptic digest was subjected to reversed-phase separation using superficially porous silica particles loaded with octadecyl stationary phase (C18). The eluting peptides were analyzed on an Agilent 6545XT AdvanceBio LC/Q-TOF system equipped with an Agilent ExD cell. The presence of signature mass shifts in the acquired MS/MS spectra was evaluated using the Agilent ExDViewer software.

Experimental

Materials

Acetonitrile (ACN) (HPLC-S), water (ULC/MS), and formic acid (ULC/MS) were obtained from Biosolve. Guanidine-HCl, DL dithiothreitol (DTT), and 2-iodoacetamide (IAA) were purchased from Merck. Tris-HCl (1 M, pH 7.5) was acquired from Thermo Fisher Scientific, and sequencing-grade trypsin was acquired from Promega. Agilent AdvanceBio Spin columns, Agilent LC/MS ESI-X tuning mix, Agilent ESI-L low-concentration tuning solution, and melittin were also used. The test sample was kindly provided by a biotechnology company.

Sample preparation

In brief, 50 µg of mAb was denatured using 3 M guanidine-HCl in 100 mM Tris HCl (pH 7.5), reduced with 5 mM dithiothreitol (DTT) for 30 minutes at 60 °C, and alkylated for 1 hour at 37 °C using 10 mM iodoacetamide (IAA). The resulting alkylated light and heavy chains were then desalted by gel filtration (spin column) prior to a 4-hour digestion at 37 °C using a trypsin:mAb ratio of 1:25.

Instrumentation

Samples were run on an Agilent 1290 Infinity II bio LC system equipped with an Agilent 1290 Infinity II bio high-speed pump (G7132A), Agilent 1290 Infinity II bio multisampler (G7137A) with integrated sample thermostat, and an Agilent 1290 Infinity II multicolumn thermostat (G7116B) with Agilent InfinityLab Quick Connect bio heat exchanger. Mass spectra were obtained using an Agilent 6545XT AdvanceBio LC/Q-TOF (G6549A) equipped with an Agilent Dual Jet Stream ESI source (G1958-65171) and an Agilent ExD cell (G1997A).

Software

Data were acquired using Agilent MassHunter acquisition software for LC/Q-TOF (version 11.0) and the Agilent ExDControl software (version 3.6). Data processing was performed with Agilent MassHunter Qualitative Analysis software (version 12.0), Agilent MassHunter BioConfirm (version 12.1), and Agilent ExDViewer (version 4.6.31).

ExD cell operation

The ExD cell, placed after the collision cell, is an add-on for the 6545XT AdvanceBio LC/Q-TOF and allows molecular fragmentation of peptides based on ECD. The ExD cell is controlled using the standalone ExDControl software, which operates alongside MassHunter acquisition software to regulate ExD cell voltages and filament heating current. For tuning of the system, the default voltage parameters were used as defined manually during installation.

Before initiating ExD cell tuning, the cell was activated for at least 20 minutes to allow proper thermal stabilization of the electron-emitting filament. First, an autotune algorithm was applied that automatically optimizes all voltages for the different electronic lenses and magnets present in the ExD cell (Figure 2).

After optimizing the electron-emitting filament current, the ExD cell was tuned for transmission using the standard positive ESI-L tuning solution infused from bottle B (1:10 dilution in 95% ACN). The signal intensity for the tuning mix ions was optimized by applying a fine tune for parent ions. Proper CID fragmentation was checked by selecting *m/z* 622.0 while applying a collision energy of 55 V.

Afterwards, the ExD cell was tuned to maximize ECD fragmentation using the ESI-X tuning mix with melittin (25 µg/mL) infused from bottle B (1:2 dilution in 95% ACN). During tuning, ion intensities were optimized for the built-in melittin mass list (not amidated) by first applying a fine tune for parent ions and, next, a fine tune for fragment ions. Finally, proper ECD fragmentation settings were checked by isolating the melittin $[M+3H]^{3+}$ precursor at *m/z* 949.9 and evaluating its c_7^{1+} fragment ion at *m/z* 656.4. The dissociation level was approximately 9%.

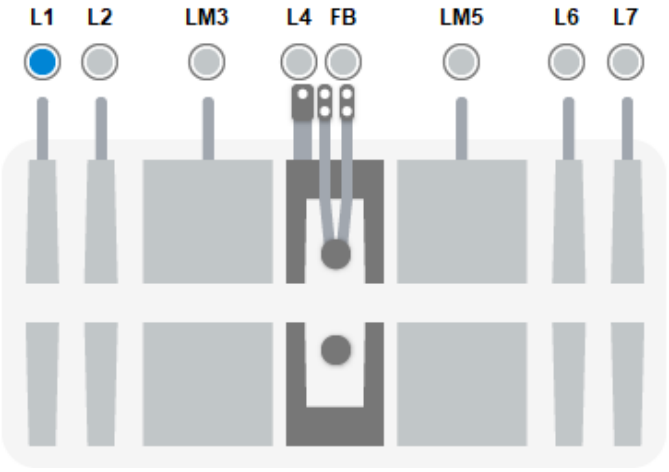

Figure 2. Agilent ExDControl software schematic illustrating the different electronic lenses (L), magnets (LM), and filament beam (FB) in the ExD cell.

Method parameters

The method parameters are summarized in Table 1.

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

LC	
Column	Agilent AdvanceBio Peptide Mapping C18 column (2.1 × 150 mm, 2.7 µm)
Mobile Phase	A) 0.1% formic acid in water B) 0.1% formic acid in acetonitrile
Autosampler Temperature	5 °C
Flow Rate	0.3 mL/min
Injection	8 µL, needle wash 3 s with 100% acetonitrile using flush port
Jet Weaver Volume	100 µL
Gradient	Time (min) %B 0 to 2 1 2 to 35 1 to 45 35 to 36 45 to 90 36 to 41 90 41 to 42 90 to 1 42 to 56 1
Column Temperature	60 °C
Q-TOF	
ESI Source	
Ionization	Positive
Drying Gas Temperature	300 °C
Drying Gas Flow	8 L/min
Sheath Gas Temperature	350 °C
Sheath Gas Flow	8 L/min
Nebulizer Pressure	35 psi
Capillary Voltage	3,500 V
Nozzle Voltage	1,000 V
Fragmentor Voltage	175 V
Acquisition MS	
Acquisition Mode	Extended dynamic range (2 Hz)
Mass Range	*m/z* 300 to 3,200
Data Acquisition Rate	3 spectra/s
Acquisition MS/MS	
Acquisition Mode	Auto MS/MS (CID or ECD separate)
Mass Range	*m/z* 100 to 3,000
Data Acquisition Rate	3 spectra/s
Isolation Width	Medium (4 amu)
Collision Energy (CID)	Formula for all charge states: (4 × (*m/z*))/(100 – 4.8) V
Collision Energy (ECD)	0
Max Precursors per Cycle	5
Precursor Selection	– Precursor threshold: 2,000 counts/0.01% – Mass error tolerance: 20 ppm – Isotope model: peptides – Charge state: 2, 3, > 3 – Sort precursor by abundance only – Active exclusion after 2 spectra (released after 0.1 min)

Data analysis with ExDViewer

ExDViewer software can be used to easily process complex fragmentation patterns observed in MS/MS spectra (CID, ExD such as ECD, ETD, EAD). This freely available software uses an algorithm that recognizes and matches the isotopic distribution for all fragment ion types related to a specific peptide: a, b, c, x, y, and z, as well as w-ion side chain fragmentation, which may distinguish isomeric amino acids (Figure 3). In this study, a targeted deconvolution workflow was used to match MS/MS data to the peptide of interest added to the Target Editor. An *m/z* tolerance of 0.5 was applied to select precursor ions, and the doubly charged precursor ion was selected at the corresponding elution times (21.1 and 21.3 minutes). Two spectra were combined for fragment annotation.

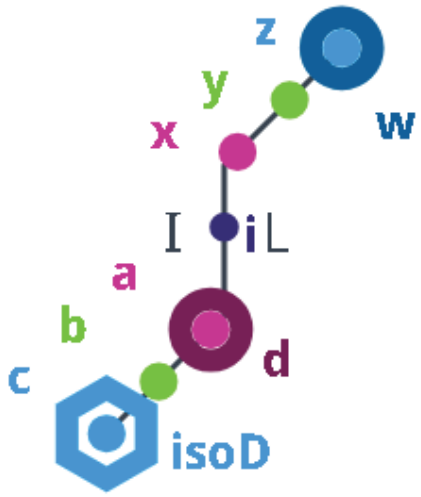

Figure 3. MS/MS fragmentation annotation in Agilent ExDViewer software.

Results and discussion

The total ion current (TIC) and extracted ion chromatogram (EIC) for the target tryptic peptide located in the CDR of the mAb are shown in Figure 4. At retention time 21.3 minutes, a signal corresponding to the nonmodified peptide (native peptide) is present. A prepeak with identical *m/z* and isotopic pattern is observed at 21.1 minutes (peak area of 10.4%).

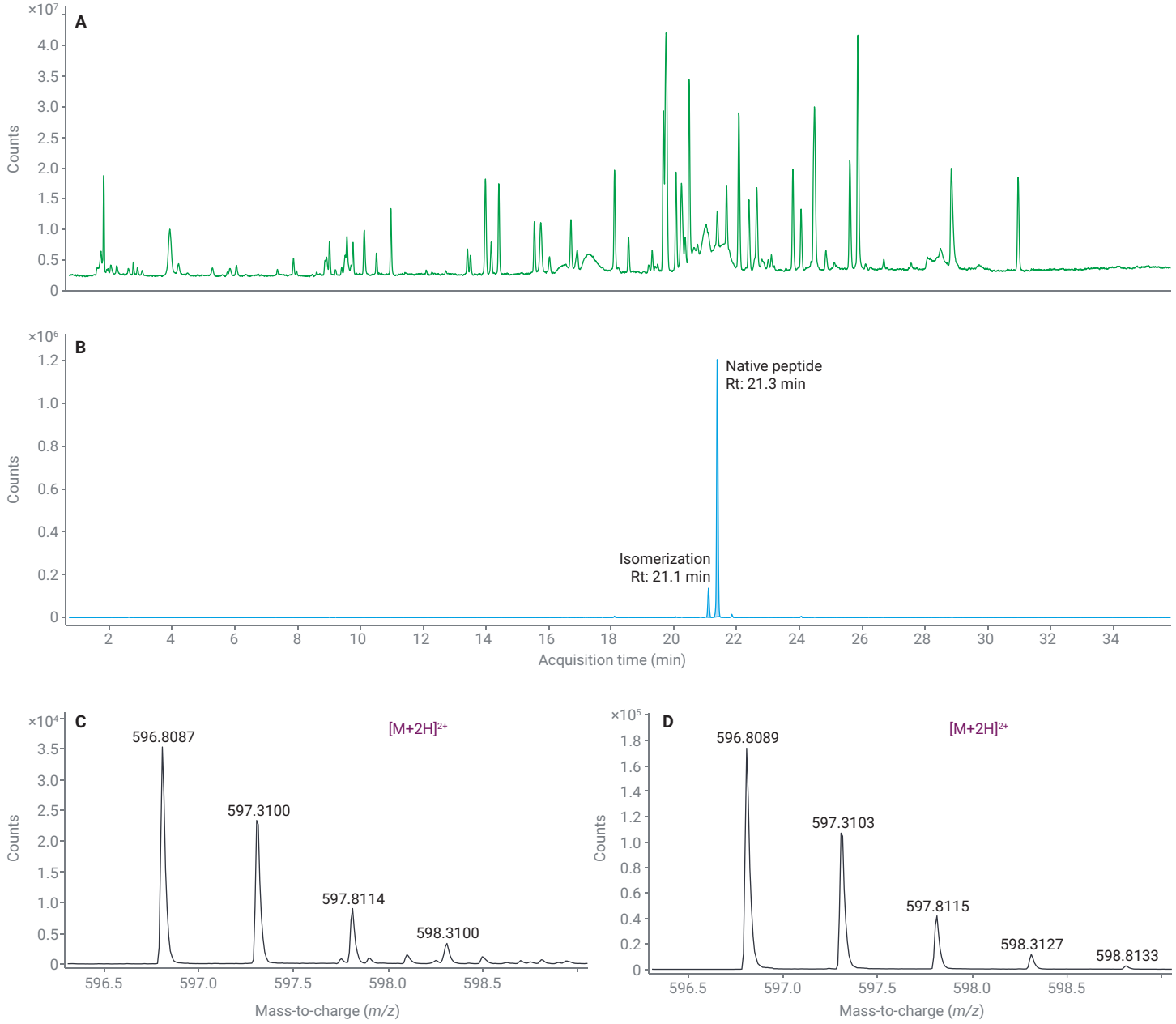

Figure 4. (A) TIC profile of the tryptic digest and (B) EIC of the target tryptic peptide located in the CDR of the mAb, extracted at 20 ppm mass accuracy. The isotopic patterns of the doubly charged tryptic peptides for the (C) isomeric and (D) native peptides are also shown.

The CID and ECD spectra of this putative isomeric peptide (21.1 minutes) acquired in data-dependent acquisition mode (auto MS/MS) are presented in Figure 5. The CID spectrum is predominantly populated with b- and y-ions, with the latter covering 100% of the sequence. In addition, trace amounts of a few a-type ions are seen. In contrast, ECD generates mostly c- and z-ions, along with limited low-abundance b- and y-ions. Upon combining all ions, full sequence coverage is again achieved. An interesting observation is the difference in spectral intensity. While the more abundant ions in the CID spectrum can be traced back to daughter ions, the most dominant signal in the ECD spectrum corresponds to the parent ion. The relative abundances of the various ion types present in the CID and ECD spectra are illustrated in Figure 6. For the ECD spectrum, the c- and z-ions accounted for 26.1% of the signal intensity, while b- and y-ions had a cumulative abundance of 3.0%. Ions related to the precursor ions still accounted for 46.8% of the total signal.

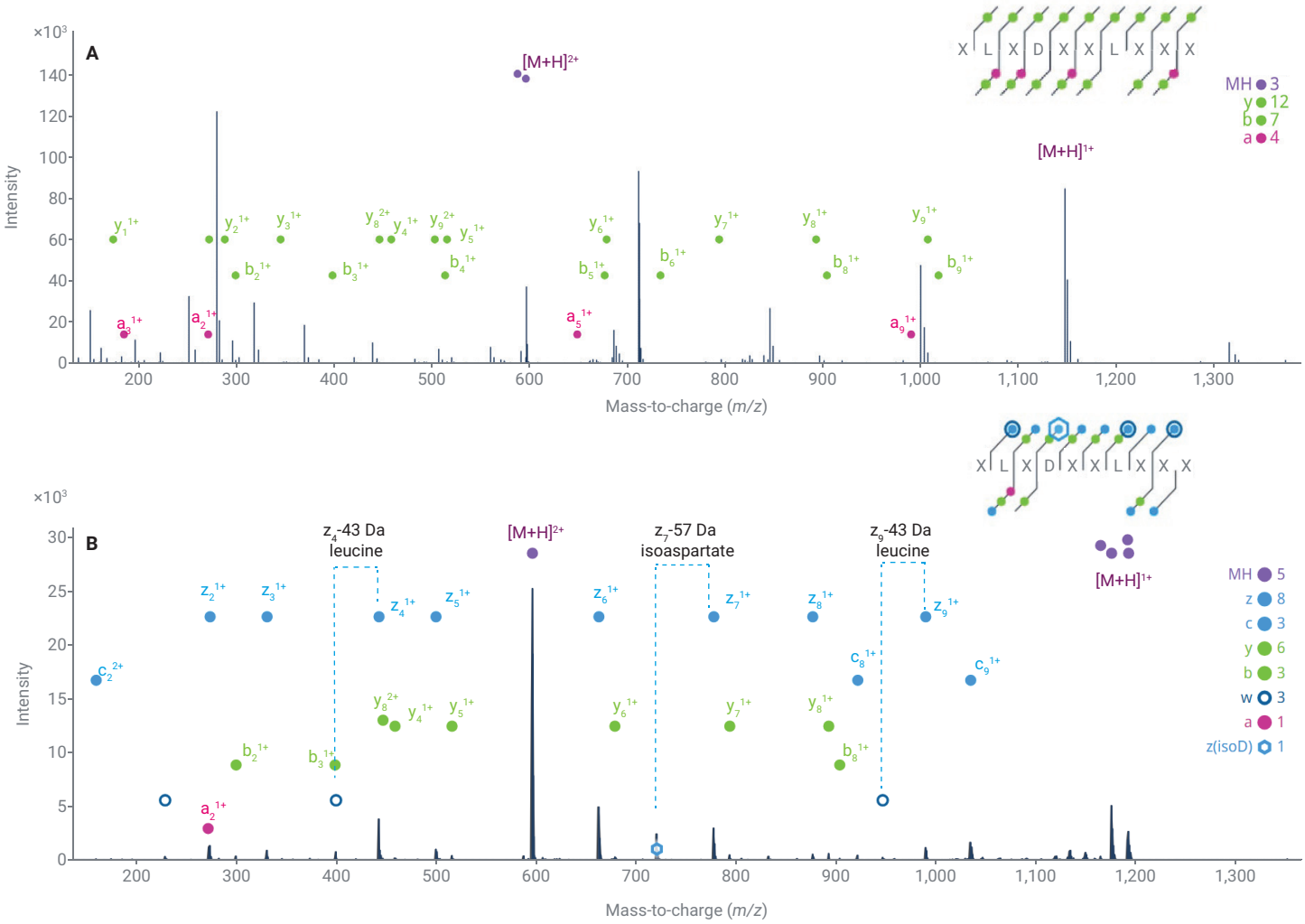

Figure 5. MS/MS spectra acquired after (A) CID and (B) ECD for the doubly charged precursor ion at retention time 21.1 minutes (Figure 4). Ion annotation is enabled by Agilent ExDViewer. CID- and ECD-type ions are labeled as green and blue, respectively, while precursor and a-ions are labeled as purple and magenta, respectively.

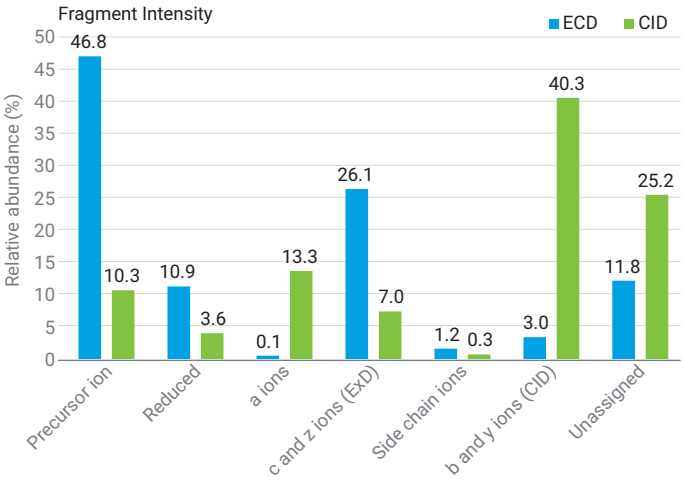

Figure 6. Relative abundances of the various ions observed in the CID and ECD spectra of the doubly charged target ion.

The tryptic decapeptide of interest contains two residues with a mass corresponding to leucine or isoleucine and one putative isoaspartate. The CID spectrum does not allow unambiguous sequence assignment. However, with ECD, these isomers can be distinguished by the presence of radical-driven side chain fragmentation that leads to the formation of w-type ions. For leucine, a secondary w-ion is formed by the radical loss of an isopropyl group (z-ion −43 amu), while the corresponding isoleucine involves the loss of an ethyl radical (z-ion −29 amu).[5] The ECD MS/MS spectrum clearly reveals z-ions indicative of the presence of leucine (Figure 7A and B). The presence of isoaspartate is evident in the ECD spectrum of the target tryptic peptide eluting at 21.1 minutes, based on a structural change involving the peptide backbone that results in a shift of 57 amu relative to the corresponding z-ion for aspartate (z-ion -57 amu; Figure 7C).[6–7] The ECD spectrum of the doubly charged native peptide appearing at 21.3 minutes clearly does not show this deviation. In contrast, a neutral loss of 44 amu is observed, indicating the presence of aspartate (Figure 7D).[9]

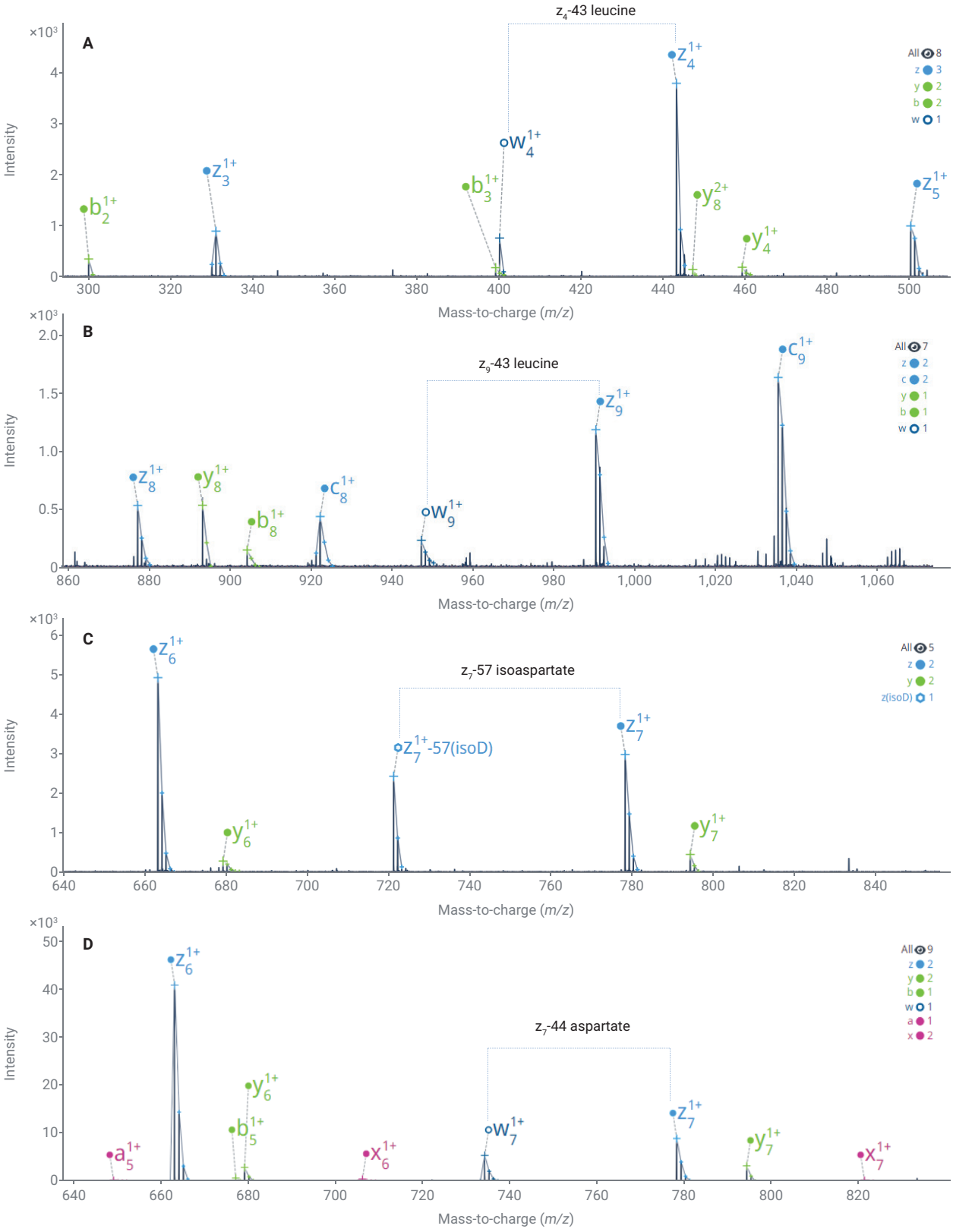

Figure 7. Zoomed-in ECD spectra highlighting the characteristic w-ions related to leucine at (A) position 7 and (B) position 2, as well as the characteristic w-ion (or z-57 amu) for (C) isoaspartate at position 4. The latter ion is absent in the ECD spectrum of the (D) native peptide.

Conclusion

This application note describes the confirmation of isomeric amino acid identities in the complementarity-determining region (CDR) of a mAb using an Agilent 6545XT AdvanceBio LC/Q-TOF system equipped with an Agilent ExD cell. It was shown that ECD provides complementary information to CID, demonstrating the presence of isoaspartate in mAbs or biologics in general. Isoaspartate may alter binding affinity and is therefore an important liability to assess during development. In addition, monitoring isoaspartate formation is of prime importance during stability studies and for the corresponding shelf-life determination of the product. Furthermore, differentiation of leucine/isoleucine is key during de novo sequencing of antibodies.

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DE-014217

Printed in the USA, April 28, 2026
5994-9164EN

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