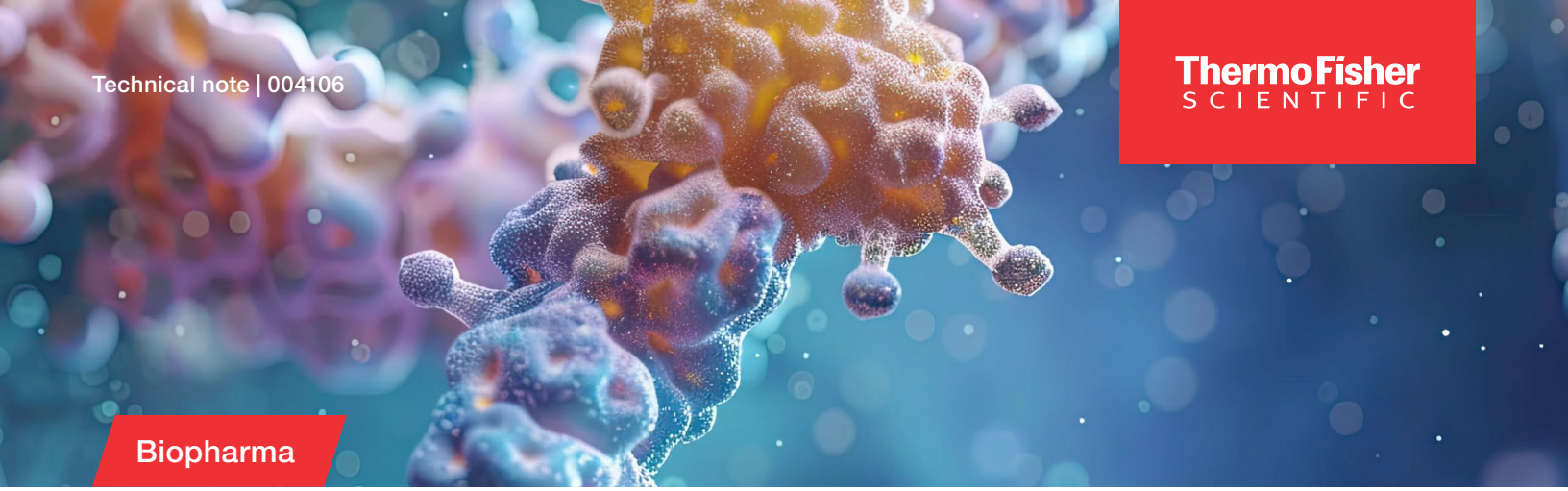

Technical note | 004106

Biopharma

Comprehensive peptide mapping analysis of protein therapeutics

Electron-transfer/higher-energy collision dissociation (EThcD) on the new Orbitrap Excedion Pro BioPharma hybrid mass spectrometer

Authors

Reiko Kiyonami[1], Cong Wang[2], Peter Krueger[2], Roberto Gamez[1], Heiner Koch[2], Min Du[1]; [1]Thermo Fisher Scientific, Lexington, MA; [2]Thermo Fisher Scientific, Bremen, Germany

Keywords

EThcD, peptide mapping, Orbitrap Excedion Pro BioPharma hybrid mass spectrometer, data-dependent MS/MS, PTM characterization, isomeric amino acid differentiation

Goal

Develop a platform data-dependent EThcD MS/MS peptide mapping method on the next-generation Thermo Scientific™ Orbitrap™ Excedion™ Pro BioPharma Hybrid Mass Spectrometer to achieve high protein sequence coverage, accurate identification and localization of low-abundant post-translational modifications (PTMs), and unambiguous differentiation of isomeric amino acids in a single LC-MS run.

Introduction

Peptide mapping is a widely used approach for therapeutic protein characterization. While higher-energy collisional dissociation (HCD) is primarily employed for peptide mapping, it can sometimes cause the loss of labile post-translational modifications (PTMs), such as phosphorylation and glycosylation, due to the high energy involved in the fragmentation process. Additionally, HCD lacks the ability to determine peptide side chain differences and disulfide linkage locations.

Electron-transfer/higher-energy collision dissociation (EThcD) combines the softer electron-transfer dissociation (ETD) with HCD and has emerged as an important tool for peptide mapping in biotherapeutic protein characterization. This includes labile PTM analysis, sequence variant analysis, and de novo sequencing. EThcD is particularly beneficial for identifying and locating PTMs such as asparagine deamidation, aspartic acid isomerization, glycosylation, and disulfide linkages, as it can simultaneously fragment both the peptide backbone and the modified site, providing valuable structural details. EThcD is also very useful for differentiating isomeric amino acids.

The new Orbitrap Excedion Pro MS implements ETD (Figure 1) on a hybrid Orbitrap mass spectrometer platform for the first time, enabling high performance EThcD fragmentation. In this study, we demonstrate that EThcD enabled on the new Orbitrap Excedion Pro MS allows for complete sequence coverage, precise low-level PTM characterization, and differentiation of isomeric amino acid residues in biotherapeutic proteins through peptide mapping analysis (Figure 2).

Experimental

Sample preparation

A commercially available NISTmAb (RM8671) was used as a model biotherapeutic protein. To prepare the sample, 10 µL of NISTmAb standard (10 mg/mL) was denatured using 7 M guanidine HCl and 100 mM Tris and adjusted to a final concentration of 1 mg/mL. The denatured NISTmAb was reduced using TCEP and alkylated using iodoacetamide, then digested with trypsin at 37°C for 2 hours. The trypsin to NISTmAb ratio was 1:10 (w:w). The digestion process was stopped by adding water containing 10% formic acid. The final volume of the digest was adjusted to 120 µL.

Chromatography

For all experiments, chromatographic separations were carried out using a Thermo Scientific™ Hypersil GOLD™ Peptide UHPLC Column (Cat. No. 26002-152130) on the Thermo Scientific™ Vanquish™ Horizon UHPLC System, consisting of the following:

- Thermo Scientific™ System Base Vanquish™ Horizon/Flex (P/N VF-S01-A-02)
- Thermo Scientific™ Vanquish™ Binary Pump H (Cat. No. VH-P10-A-02)
- Thermo Scientific™ Vanquish™ Split Sampler HT (Cat. No. VH-A10-A-02)
- Thermo Scientific™ Vanquish™ Column Compartment (Cat. No. VH-C10-A-02)
- Thermo Scientific™ Viper™ MS Connection Kit for Vanquish LC systems (Cat. No. 6720.0405)
- Thermo Scientific™ SureSTART™ 2 mL Polypropylene Screw Top Microvials (Cat. No. 03-452-259)
- Thermo Scientific™ SureSTART™ 9 mm Screw Caps (Cat. No. 03-452-276)

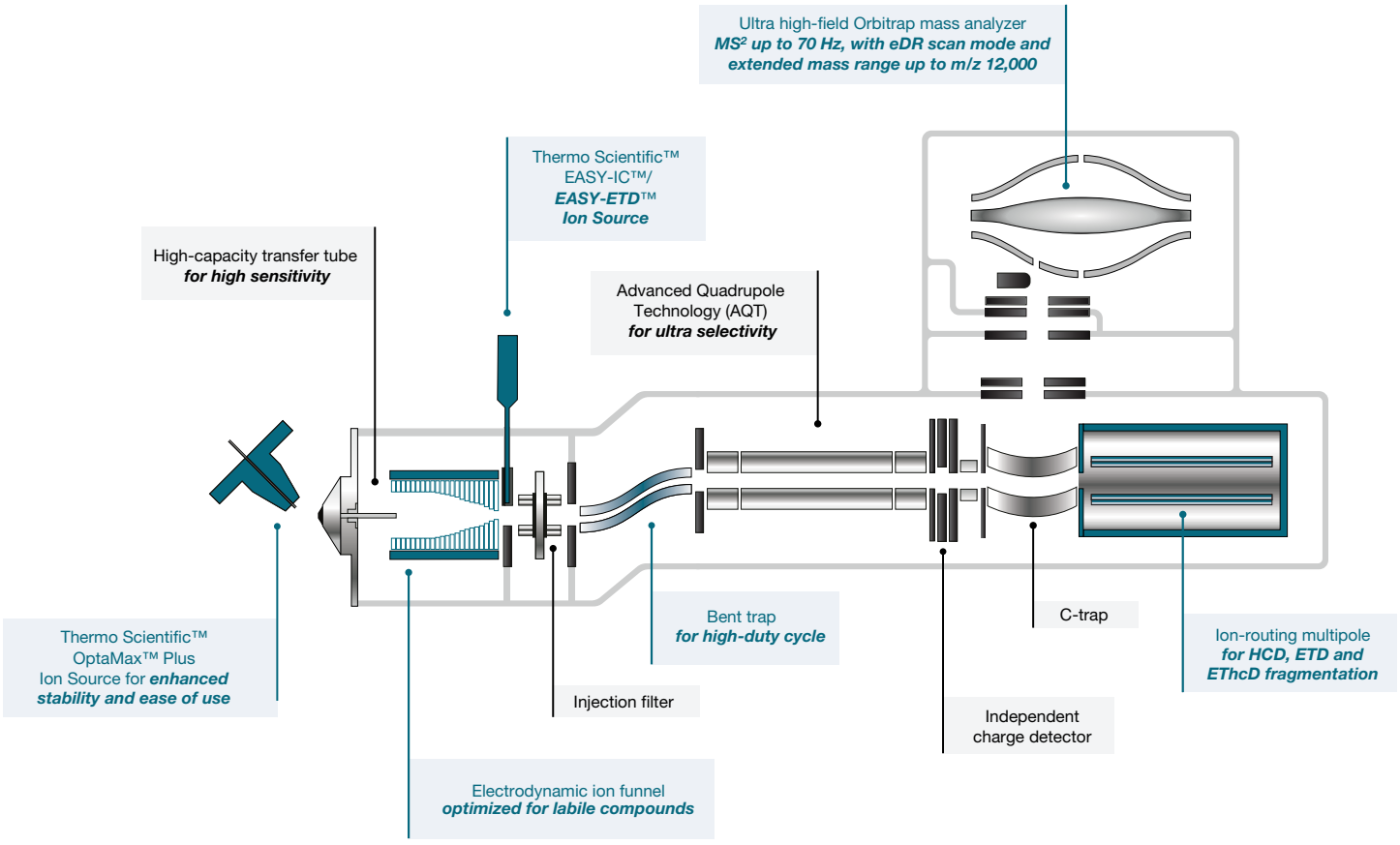

Figure 1. Schematic of the Orbitrap Excedion Pro BioPharma hybrid mass spectrometer.

Table 1 shows the details of chromatographic separation conditions; 5 µL of trypsin digested sample was injected on column.

Table 1. HPLC set-up conditions.

Column	Hypersil GOLD Peptide UHPLC column, 2.1 mm x 150 mm, 1.9 µm	
Mobile phase A	Water with 0.1% formic acid	
Mobile phase B	Acetonitrile with 0.1% formic acid	
Flow rate	250 µL/min	
Column temperature	45°C	
Gradient	Linear	
	Time (min)	%B
	0	1
	5	1
	6	10
	70	35
	72	90
	77	90
	77.1	1
	83	1

Mass spectrometry

The Orbitrap Excedion Pro MS was used for data collection. The mass spectrometer was operated in a data-dependent mode at a 1.5 second cycle time. EThcD was performed with calibrated charge-dependent reaction time supplemented by 25% HCD activation. The details of ESI and mass spectrometer setups are shown in Tables 2 and 3, respectively.

Table 2. ESI source parameters.

ESI source settings	
Sheath gas (a.u.)	40
Aux. gas (a.u.)	10
Sweep gas (a.u.)	0
Spray voltage (+V)	3,400
Capillary temp. (°C)	320
Vaporizer temp. (°C)	250

Table 3. MS parameters.

General	
Application mode	Peptide
Pressure mode	Standard
RF lens (%)	40
Full MS	
Scan range (*m/z*)	200–2,000
Resolution	120,000 at *m/z* 200
AGC target value (%)	300
Max injection time (ms)	50
dd MS/MS (cycle time: 1.5 s)	
Resolution	30,000 at *m/z* 200
Isolation window (*m/z*)	2
Activation type	ETD
Use calibrated charge-dependent ETD parameters	True
ETD supplemental activation	True
SA collision energy (%)	25
AGC target value (%)	100
Max injection time (ms)	250

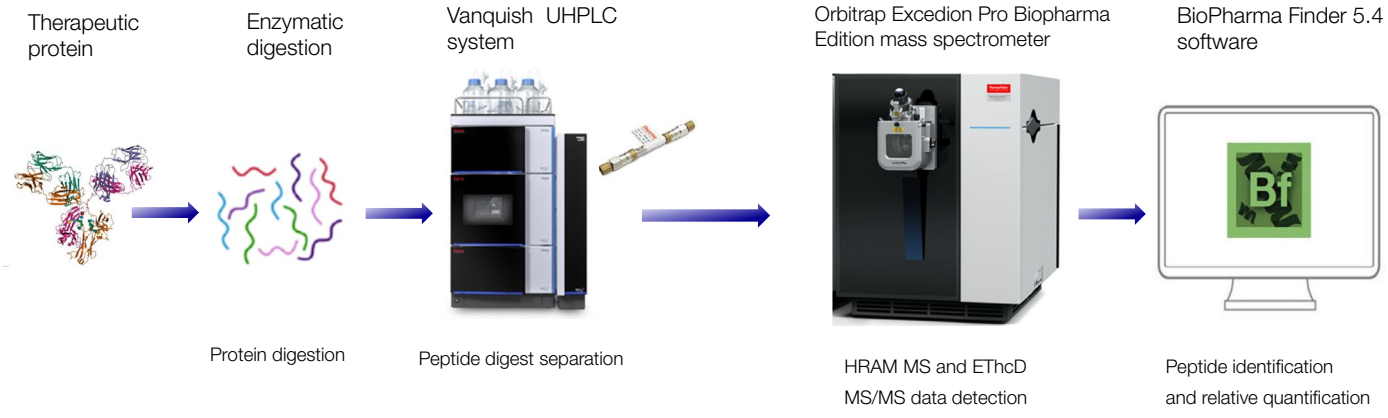

Figure 2. The workflow of peptide mapping using EThcD on the Orbitrap Excedion Pro Biopharma Hybrid mass spectrometer.

Data processing

Data were analyzed using Thermo Scientific™ BioPharma Finder™ 5.4 Software for sequence confirmation, PTM characterization, and differentiation of isomeric amino acid residues. Peptide mapping processing settings are listed in Table 4.

Results

As shown in Figure 1, the Orbitrap Excedion Pro MS performs ETD and EThcD in the ion routing multipole where precursor ions and fluoranthene radical anions are mixed for electron transfer and c/z• fragment ion generation. For performing EThcD, the generated c/z• ions are transferred to the C-trap and accelerated back into the ion routing multipole for supplemental higher-energy collisional activation, further improving c/z• ion fragmentation efficiency and allowing generation of additional HCD type ions (b/y) based on the chosen activation energy. This new design enables EThcD fragmentation with short reaction times while keeping good fragmentation efficiency and instrument sensitivity. The automated setting of charge-dependent reaction times ensures reliable outcomes across various precursor charge states on the peptide level without the need for manual fine-tuning. Figure 3 shows the base peak chromatogram of the NISTmAb digest and the sequence coverage from a single LC-MS/MS run using EThcD, easily achieving 100% sequence coverage.

Combined with the high capacity of the transfer tube and the electrodynamic ion funnel, which allows optimal transfer efficiency for labile compounds, the EThcD on the Orbitrap Excedion Pro MS enables excellent quality EThcD MS/MS data, even for low-abundant peptides with PTMs. This facilitates confident identification and precise localization of low-abundant post-translational modifications. In the LC-MS/MS experiment using EThcD, less than 0.1% of low-abundant PTMs were routinely detected with high confidence from the NISTmAb trypsin digest. Figure 4 highlights the confident identification of a very low-abundant deamidation on the peptide VYACEVTHQGLSSPVTK using EThcD fragmentation. Although the relative quantification ratio of the deamidation was determined to be only 0.026% using the integrated peak areas of native and modified peptide pairs, excellent quality EThcD data were observed for both native and modified peptide pairs, enabling unambiguous identification of the deamidation site. To clearly demonstrate the 0.984 amu difference of the fragment ions between the native and modified pairs of this peptide, the zoomed-in fragment ion range of *m/z* 670–1,400 from the peptide pairs is shown in Figure 5. Taking the c-type ions as an example, a 0.984 amu increase was clearly observed from c_9, c_{10}, c_{11}, and c_{12}, but not from c_7 and c_8, confirming the deamidation occurring on the Q residue of the peptide.

Table 4. BioPharma Finder 5.4 software peptide mapping parameters.

Sequence parameters	
Protein sequence	NISTmAb
Category	Peptide mapping
Protease	Trypsin (C-term KR)
Static modifications	Gln→Pyro-Glu (N Term, Q, x1), Carbamidomethlyation (SideChain, C, x16)
Glycosylation	CHO
Variable modifications	Lys (CTerm), Deamidation (N)(side chain), Double oxidation (side chain), Glycation (side chain), NH_3 loss (side chain), Oxidation (MW)(side chain), Isomerization (side chain)
Component detection parameters	
Task to perform	Find all ions in the run
MS noise level	8,000
S/N threshold	50
Typical chromatographic peak width (min)	Automatically determined by software
Maximum chromatographic peak width (min)	
Maximum retention time shift (min)	
Identification parameters	
Search by Full MS only	No
Use MS/MS	Use all MS/MS
Maximum peptide mass	11,000
Mass accuracy (ppm)	5

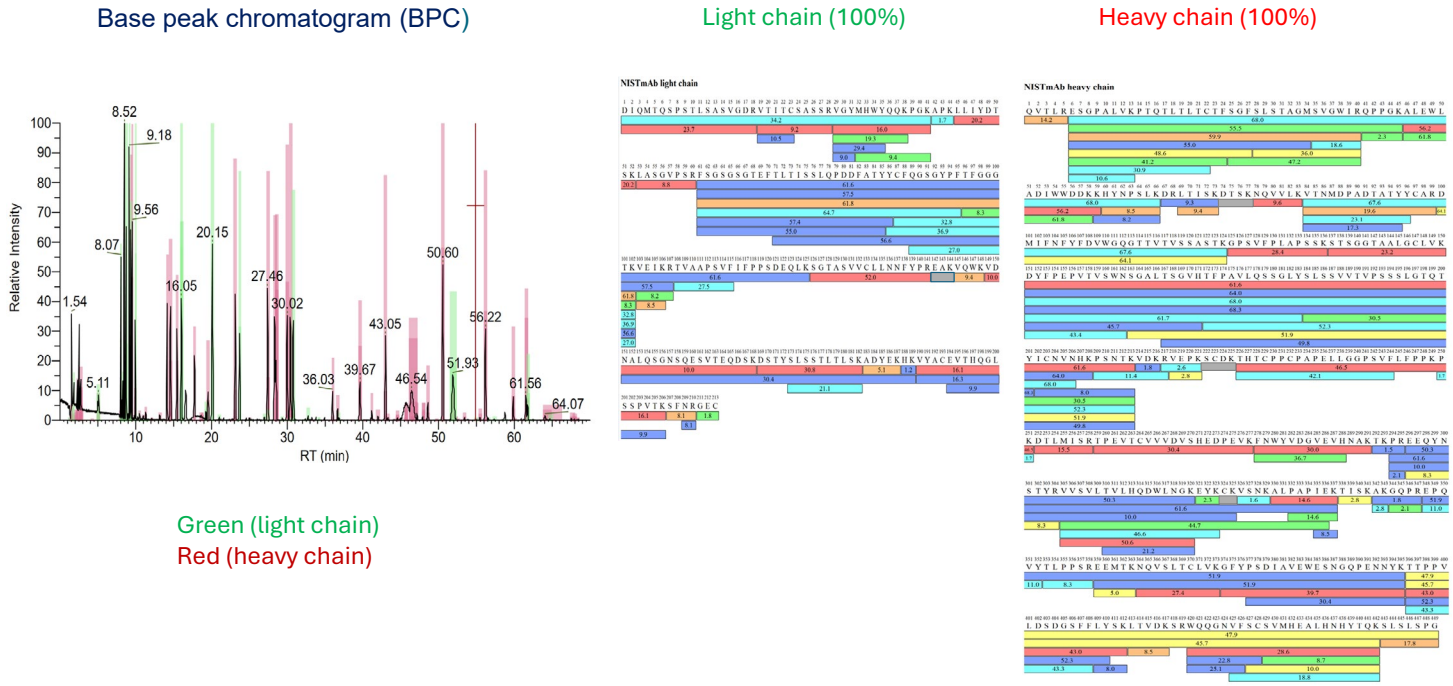

Figure 3. Base peak chromatogram and 100% sequence coverage map of the NISTmAb digest from a single LC-MS/MS run using EThcD.

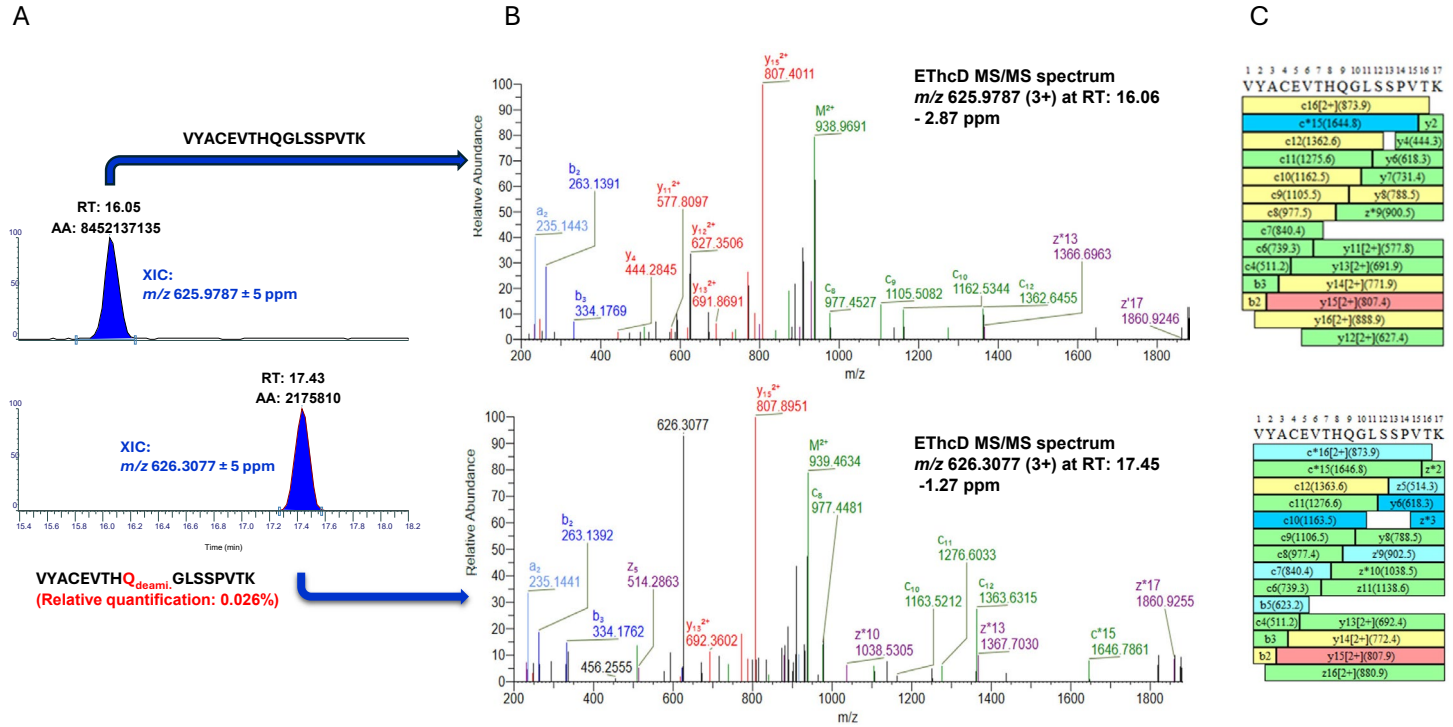

Figure 4. Confident identification and relative quantification of low abundance deamidated peptide. (A) Extracted ion chromatograms; (B) high quality of EThcD MS/MS spectra annotated by Biopharma Finder software; (C) peptide sequence coverage map of the unmodified and deamidated peptide VYACEVTHQGLSSPVTK. The relative quantification of the deamidated peptide was determined as 0.026%.

One benefit that EThcD offers is its capability to differentiate isomeric amino acid residues. For example, the formation of odd-electron z-ions from multi-protonated peptide ions by ETD and their subsequent HCD fragmentation leads to the formation of secondary w-ions. Since the z-ions lose 43.055 mass units (isopropyl radical) in the case of leucine and 29.039 mass units (ethyl radical) in the case of isoleucine, the masses of the corresponding w-ions are different for leucine and isoleucine, allowing reliable identification of these residues[1] (Figure 6). The EThcD fragmentation on the Orbitrap Excedion Pro MS was highly sensitive and efficient, even for precursor ions with low charge states. Figure 7 shows that the diagnostic w-ions (z_7-43Da and z_3-29Da) were clearly detected from a doubly charged precursor ion of the peptide ALPAPIEK, enabling the distinction between leucine and isoleucine.

EThcD also provides diagnostic fragment ions to distinguish between aspartic acid (D) and isoaspartic acid (isoD) isomers. For isoD, EThcD fragments the peptide backbone at the Cα-Cβ bond within the isoD residue, resulting in the formation of c+57 and z-57 ions[2] (Figure 8). In contrast, EThcD produces normal c- and z-type ions for D because D lacks a methylene group in the backbone.

One example for isoD identification using EThcD is shown in Figure 9. Figure 9A shows the extracted ion chromatograms of peptide WQQGNVFSCSVMHEALHNHYTQK (RT: 28.55 min) and its two low abundant deamidated isomer peaks (0.28% at RT: 29.07 min and 0.26% at RT: 29.39 min). The peptide is triply charged and the full scan mass spectrum of three forms of the peptide are shown in Figure 8B. Figure 8C shows the EThcD spectrum acquired from the one of the deamidated peaks, eluted at RT: 29.07 min. Although the two deaminated isomers cannot be distinguished with the full scan MS data, the diagnostic c+57 and z-57 ions were detected clearly from the EThcD spectrum of the deamidated peak eluted at 29.07 min, enabling confident identification and localization of this low abundant isoD (0.28%) modification.

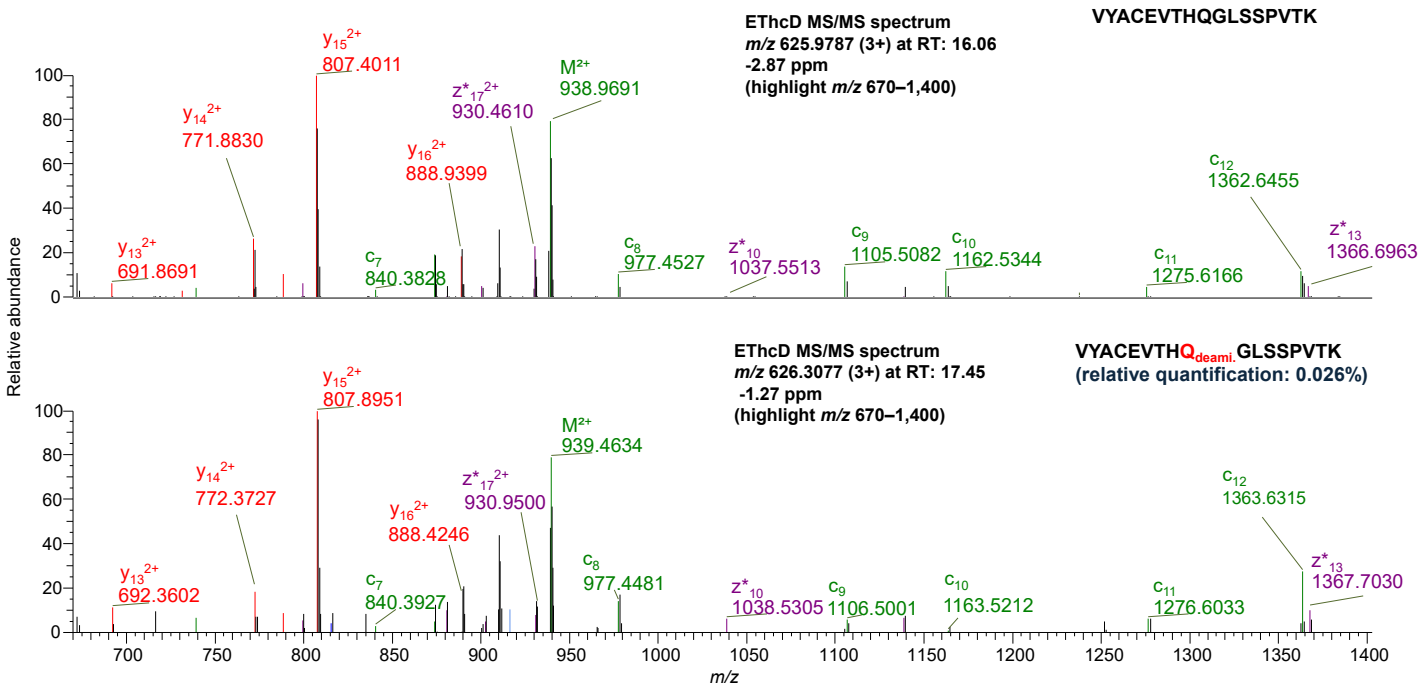

Figure 5. Comparison of zoomed-in EThcD MS/MS spectra across *m/z* 670–1,400 of unmodified and deamidated peptide VYACEVTHQGLSSPVTK. A 0.984 mass shift was clearly detected from the fragment ions observed in the peptide pair.

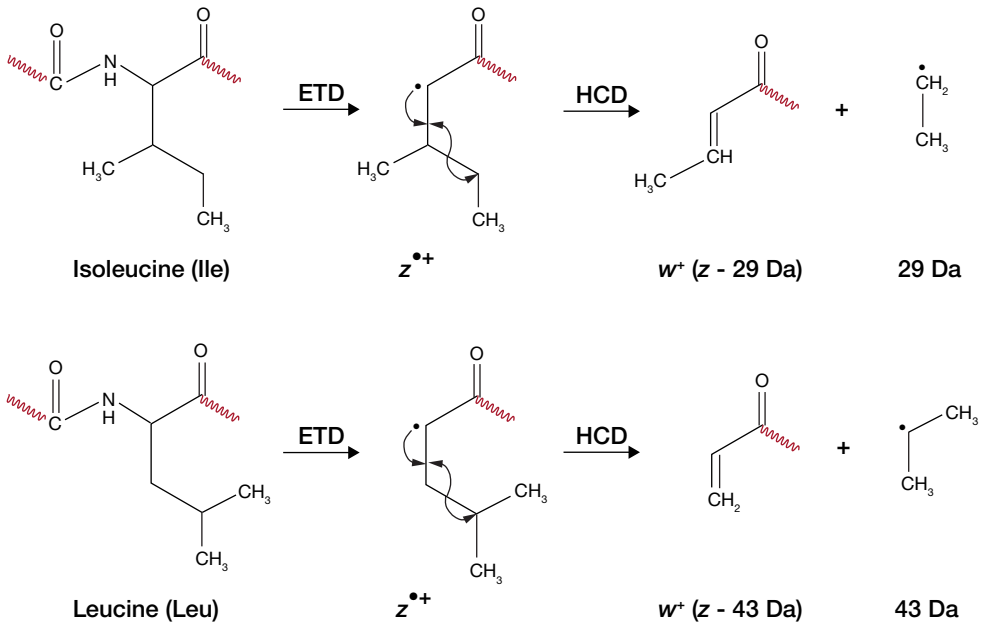

Figure 6. EThcD fragmentation generates diagnostic w-type ions that differentiate leucine and isoleucine.

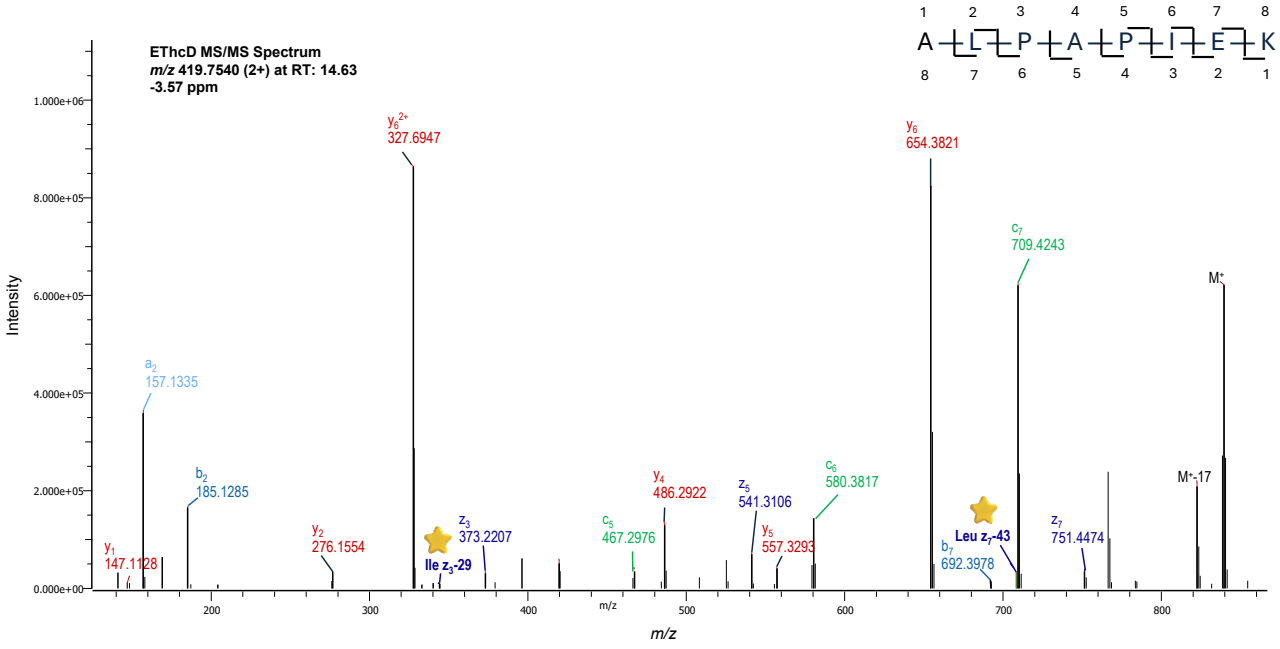

Figure 7. EThcD MS/MS spectrum of peptide ALPAPIEK. The leucine and isoleucine residues can be clearly differentiated by the diagnostic w-ions, z_7-43Da and z_3-29Da, respectively.

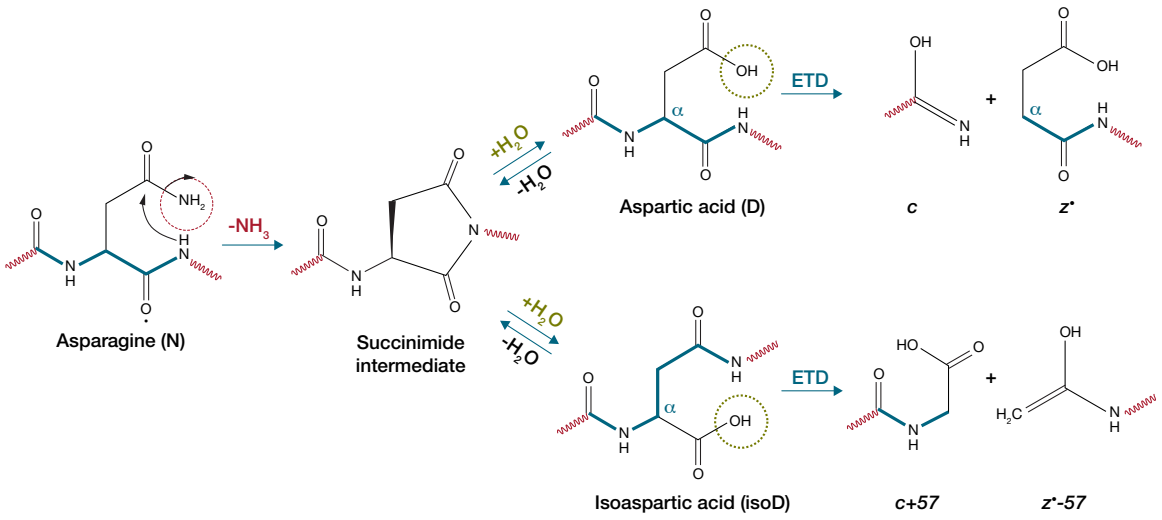

Figure 8. Diagnostic ETD fragment ions differentiate isoD and D isomers, which are formed during the process of asparagine deamidation.

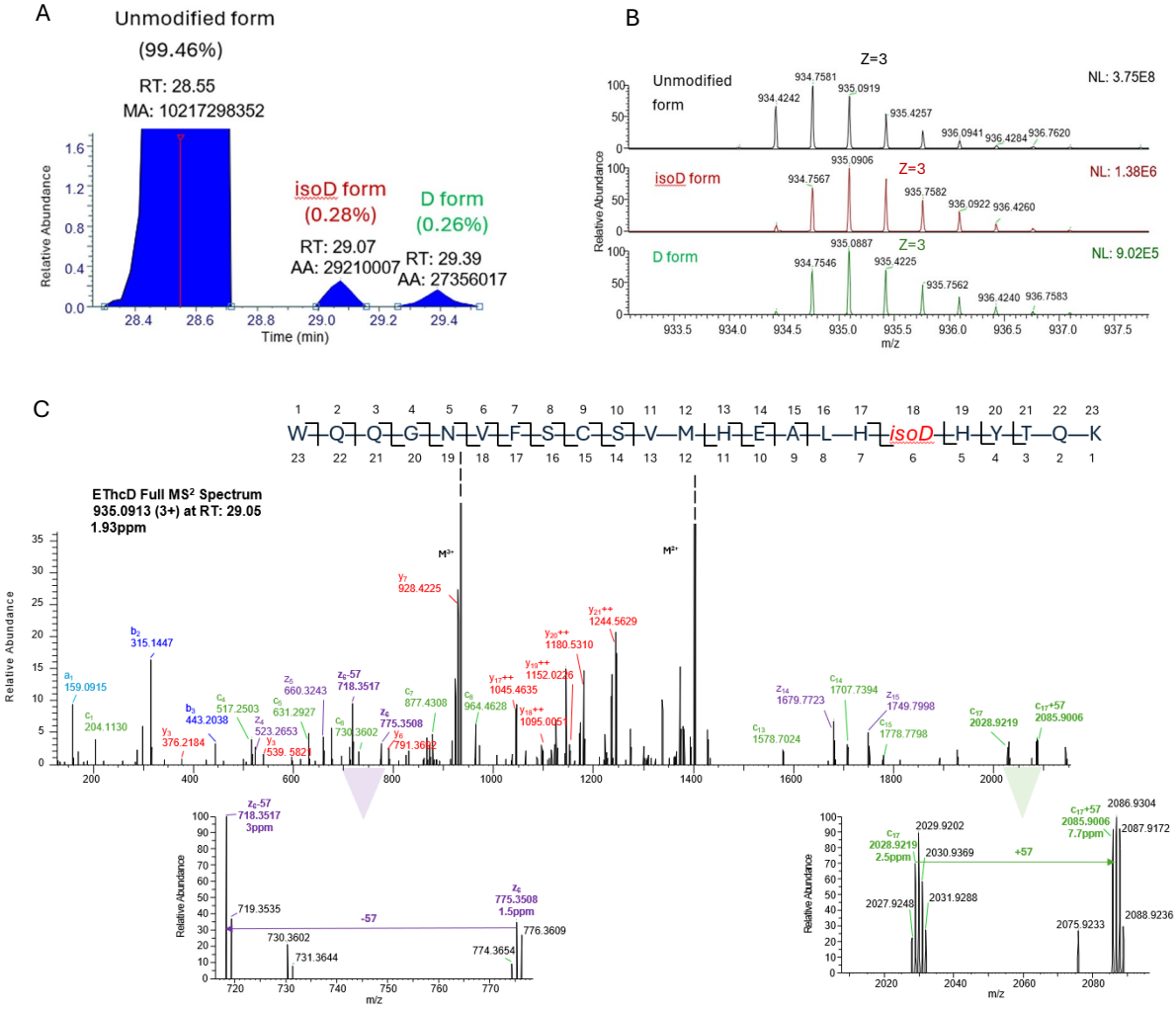

Figure 9. Confident isoaspartic acid identification using EThcD. (A) Extracted ion chromatogram of unmodified and deamidated peptide, WQQGNVFSCSVMHEALHNHYTQK. (B) Full MS spectra of triply charged peptides. (C) EThcD full MS^2 spectrum of 935.0913 (3+) at RT 29.05 minutes. The mass deviation of the parent ion is 1.93 ppm. The information-rich EThcD MS/MS spectrum confirmed the peptide sequence with high sequence coverage. Zoomed-in insets show high mass accuracy (<8 ppm) for diagnostic fragments (z_6-57 and c_{17}+57) for unambitious and confident identification of isoaspartic acid.

Conclusion

- EThcD on the Orbitrap Excedion Pro Biopharma hybrid mass spectrometer offers combined fragment ion types from both ETD and HCD with high sensitivity and fast acquisition rates, enabling extensive sequence coverage. A single HPLC-MS/MS run using EThcD provided 100% sequence coverage for the NISTmAb trypsin digest.
- The high sensitivity of EThcD on the Orbitrap Excedion Pro Biopharma hybrid mass spectrometer enables excellent quality EThcD MS/MS data even for low-abundant peptides with PTMs, allowing confident identification and precise localization of low-abundant post-translational modifications. Deamidation as low as 0.026% was unambiguously identified from the NISTmAb digest using EThcD fragment ions.
- EThcD is particularly useful for differentiating isomeric amino acid residues, such as leucine and isoleucine, as well as aspartic acid and isoaspartic acid, by providing unique diagnostic fragment ions. EThcD on the Orbitrap Excedion Pro Biopharma mass spectrometer was able to generate the diagnostic w-type ions ($z^{\bullet}$-43 and $z^{\bullet}$-29) to differentiate leucine and isoleucine even for a small double-charged peptide. Very low-abundant (0.28%) isoaspartic acid was confidently identified from the NISTmAb digest by detecting diagnostic fragment ions (c+57, $z^{\bullet}$-57).
- Overall, EThcD with automated reaction time scaling is easy to use and enhances the depth and accuracy of peptide mapping, making it a powerful technique for comprehensive and sensitive peptide mapping analysis.

References

1. Kjeldsen, F. et. al., Distinguishing of Ile/Leu amino acid residues in the PP3 protein by (hot) electron capture dissociation in Fourier transform ion cyclotron resonance mass spectrometry. *Anal. Chem.* **2003**, 75, 1267-1274.
2. Hurtado, P.P. et. al., Differentiation of isomeric amin acid residues in proteins and peptides using mass spectrometry. *Mass Spectrometry Reviews*, **2012**, 31, 609–625.

Learn more at **thermofisher.com/proteintherapeutics**