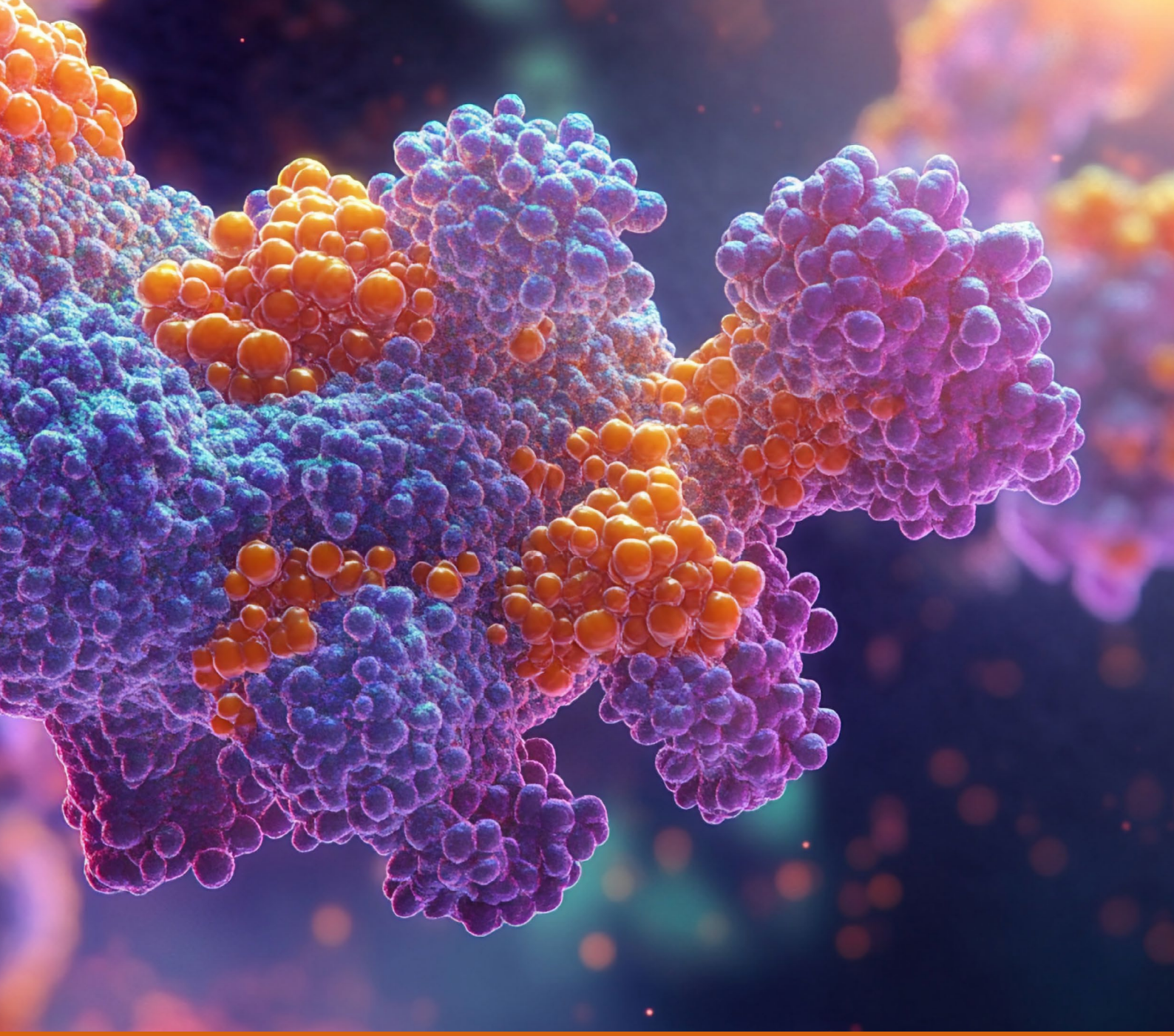

Transformative Workflows for Protein Characterization:

ECD and Multidimensional Separations

Expert Insights

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WILEY Analytical Science

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Contents

Introduction

In recent years, breakthroughs in liquid chromatography–mass spectrometry (LC-MS) have transformed the way scientists characterize peptides and proteins. These innovations have dramatically improved the resolution, specificity, and sensitivity available to researchers, enabling more thorough investigations of protein structures, functions, and the quality of biotherapeutics. Techniques that combine multidimensional separation, advanced fragmentation, and high-performance detection are now essential tools in both proteomics research and pharmaceutical development.

This Expert Insights eBook summarizes two comprehensive publications that highlight critical advances in LCMS-driven peptide and protein analysis.

Beginning with a historical review article by Cooper *et al.* [1], the principles and impact of Electron Capture Dissociation (ECD) in mass spectrometry are explored. ECD provides a unique approach to peptide and protein fragmentation by capturing electrons on multiply charged ions, which results in extensive backbone cleavages while preserving sensitive post-translational modifications. This makes ECD an essential technique for mapping protein modifications and achieving deep sequence coverage for large and complex biomolecules. The summary outlines the core chemistry, main analytical benefits, and the expanding range of instrument platforms now supporting ECD.

The second summary, based on the recent work of Sarin *et al.* [2], presents a state-of-the-art workflow for resolving monoclonal antibody charge variants using a combination of hydrophobic interaction chromatography (HIC), capillary zone electrophoresis (CZE), and high-resolution LC/Q-TOF mass spectrometry. This multidimensional approach allows researchers to unravel the microheterogeneity of therapeutic antibodies, distinguishing between subtle modification patterns that may otherwise go undetected. Their research not only demonstrates the analytical power of these combined methods but also provides practical guidance on applying the workflow using modern LC-MS platforms.

Overall, this Expert Insights eBook captures the dynamic evolution of LCMS methods in protein science. By highlighting foundational techniques alongside recent technological advancements, it serves as both a reference and a guide for anyone looking to apply cutting-edge mass spectrometry in peptide and protein research. To gain a deeper understanding of available options for improving your research, we encourage you to visit Agilent.

Róisín Murtagh
Senior Content Strategist, Wiley

References

[1] Cooper, H.J. *et al.* (2005). The role of electron capture dissociation in biomolecular analysis. Mass Spectrometry Reviews. https://doi.org/10.1002/mas.20014.

[2] Sarin, D. *et al.* (2024). Offline Coupling of Hydrophobic Interaction Chromatography–Capillary Zone Electrophoresis for Monitoring Charge-Based Heterogeneity of Recombinant Monoclonal Antibodies. ELECTROPHORESIS. https://doi.org/10.1002/elps.202400158.

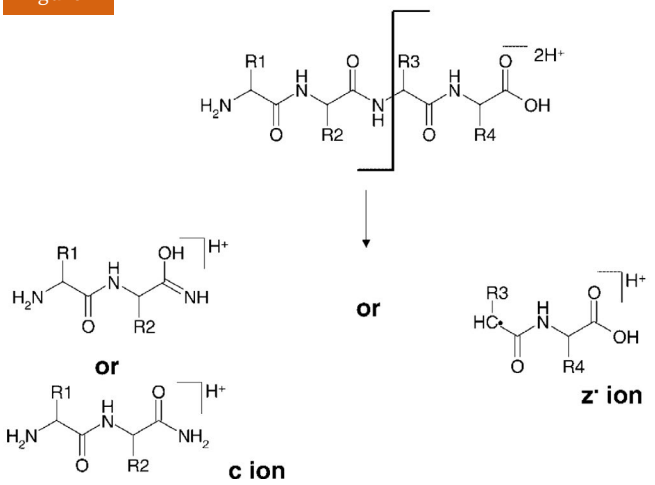
The Role of Electron Capture Dissociation in Biomolecular Analysis

Adapted from Cooper *et al.* [1]

Electron-driven fragmentation has become a powerful tool in mass spectrometry, offering detailed structural insights into peptides and proteins that go beyond the capabilities of traditional MS/MS fragmentation techniques. The field has evolved dramatically since electron capture dissociation (ECD) was first introduced nearly three decades ago, with newer techniques building on its foundational chemistry to meet the growing demands of proteomics and biopharmaceutical research. Nonetheless, this 2004 review article on ECD remains a clear and concise summary of the underlying principles that continue to inform the development of modern electron-based fragmentation techniques.

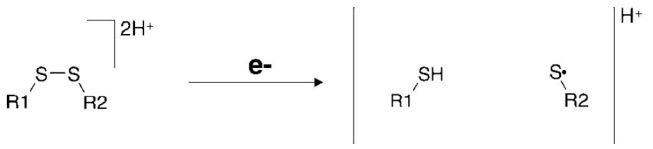
How ECD Works for Peptides and Proteins

Figure 1



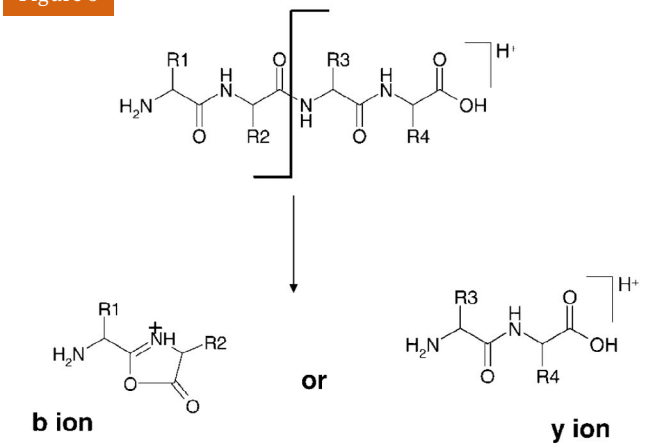
Fragmentation of peptide amine bonds to produce N-terminal c and C-terminal z ions. This type of fragmentation is unique to ECD.

Figure 2



Cleavage of disulfide bonds following electron capture.

Figure 3

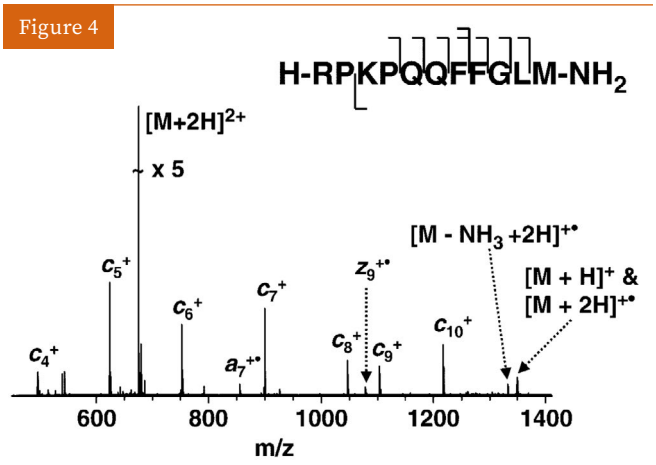


Backbone fragmentation of peptides via collision-induced dissociation (CID), producing N-terminal b ions and C-terminal y ions. Shown here for comparison with ECD fragmentation patterns.

Mechanism

- **Electron Capture:** Multiply charged peptide or protein cations are irradiated with low-energy electrons.
- **Fragmentation:** Upon electron capture, these ions experience cleavage mainly at the N–Cα bond, generating complementary c and z fragment ions, with an extra "radical" electron on one or the other (usually the z ion)
- **Non-ergodic Process:** ECD is distinct from "slow-heating" methods (e.g., CID); fragmentation happens before the energy disperses across the molecule, preserving delicate structural features.

Figure 4



Electron capture dissociation (ECD) mass spectrum of the model peptide substance P ($[M + 2H]^{2+}$), illustrating generation of c and z ions typical for ECD-based peptide backbone fragmentation.

Key Fragment Types

Fragment Ion Type	Structural Detail Provided
c ions	N-terminal sequence information, complementary to b ions
z ions	C-terminal sequence information, complementary to y ions
Charge-reduced ions	Confirmation of precursor, less structural info
Side-chain fragments	Diagnostic for amino acid modifications

Distinct Advantages in Protein and Peptide Analysis

1. Preservation of Labile Modifications

ECD excels at maintaining post-translational modifications (PTMs), such as phosphorylation or glycosylation, during fragmentation. This preservation is essential for pinpointing PTM sites and understanding biological regulation.

2. Sequence Coverage and Mapping

- **Random Backbone Cleavage:** ECD induces random backbone cleavage with minimal bias toward labile modifications, resulting in extensive and informative sequence coverage.
- **Top-Down Analysis:** ECD makes top-down methods significantly more informative by providing greater sequence coverage for large proteins compared to collision-based fragmentation.

3. Disulfide Bond and Cross-link Analysis:

ECD efficiently cleaves disulfide bonds, even within intact proteins, enabling direct mapping of cross-links and folding patterns important for protein structure-function studies.

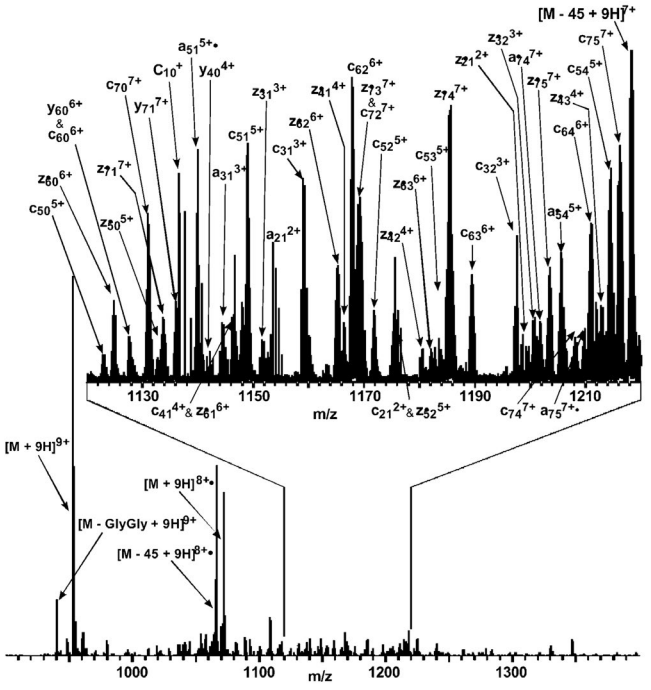
4. Minimal Side-chain Losses

Compared to collision- or photo-based dissociation, ECD produces far fewer small neutral losses (e.g., H_2O, NH_3), which simplifies spectra and improves modification localization.

5. Complementary fragment ions

Additional fragment ion types complement the sequence information obtainable via CID, leading to greater confidence in assigned sequence and extended sequence coverage – especially helpful for sequence characterization *de novo*.

Figure 5



ECD mass spectrum of the intact protein ubiquitin ($[M + 9H]^{9+}$, ~8.6 kDa), demonstrating extensive sequence coverage by c and z fragments in top-down protein analysis.

Applications in Peptide and Protein Research

- **Comprehensive Sequence Mapping:** ECD is now a mainstay in top-down and middle-down proteomics, supporting the analysis of intact proteins, large peptides, and proteoforms.
- **Precise PTM Mapping:** By conserving labile modifications, ECD allows unambiguous assignment of functional groups.
- **Structural Biology:** Disulfide mapping and cross-link analysis inform protein folding and complex assembly.

Recent Advances (Wiley editorial addition)

- **Instrumental Advances:** ECD capabilities are increasingly available on high-resolution hybrid mass spectrometers, widening accessibility in research and industry.
- **Variant Techniques:** Newer processes—such as activated ion ECD and plasma ECD—expand ECD's reach to even broader molecular classes and challenging analytical scenarios.

Addendum/Developments post-2005 (Wiley editorial addition)

Agilent's ExD Cell

Agilent Technologies has significantly advanced the accessibility and practicality of ECD for peptide and protein analysis with the 2024 introduction of the ExD Cell for their 6545XT AdvanceBio LC/Q-TOF platform. This innovation marks an important shift, bringing ECD—once limited mostly to FT-ICR mass spectrometers—directly into widely used, benchtop quadrupole time-of-flight (Q-TOF) instruments.

Key Features of Agilent's ECD Implementation

- **ExD Cell Add-On:** The ExD cell is a field-installable module that integrates with Agilent's 6545XT AdvanceBio LC/Q-TOF. It enables ECD fragmentation alongside traditional collision-induced dissociation (CID), broadening the instrument's utility for protein and peptide research.
- **Enhanced Top-Down and Middle-Down Workflows:** With ECD, Agilent's 6545XT LC/Q-TOF system can cleave proteins and peptides to yield c and z fragments, preserving labile post-translational modifications (PTMs) and providing deeper sequence coverage—vital for the structural study and quality control of biotherapeutics.
- **ExDViewer Software:** Agilent's ExDViewer software is designed to facilitate the interpretation of ECD spectra, supporting researchers in efficiently mapping modifications, performing sequence analysis, and processing complex datasets.
- **Compatibility and Upgrade Path:** The ExD cell is designed for compatibility with existing 6545XT systems and can be retrofitted in the field. This expands ECD access without the need for entirely new high-end platforms.

Impact on Peptide and Protein Analysis

- **Broader Access:** By integrating ECD into Q-TOF platforms that are common in many laboratories, Agilent is helping democratize advanced top-down and middle-down workflows previously limited by instrument availability.
- **Improved Characterization of Difficult Proteins:** ECD on the 6545XT supports the analysis of large proteins, fragile PTMs, isomeric residues, and conformational variants—molecules that often challenge CID methods.
- **Structural Biology and Biotherapeutics:** These recent developments enhance the capabilities of structural biology, biopharmaceutical analytics, and related fields where confident identification of PTMs, glycoforms, and sequence variants is crucial.

Conclusion

ECD has emerged as a transformative technique for peptide and protein analysis, enabling detailed structural and functional characterization that had previously been out of reach. Its capacity to preserve modifications, comprehensively map sequences, and profile structural features has made it indispensable across proteomics, structural biology, and the development of biotherapeutics. The principles of ECD, once rooted in FT-ICR experimentation, now guide advances across the entire field of biomolecular mass spectrometry, with contemporary implementations making these powerful capabilities more accessible to the broader research community.

Reference

[1] Cooper, H.J. *et al.* (2005). The role of electron capture dissociation in biomolecular analysis. Mass Spectrometry Reviews. https://doi.org/10.1002/mas.20014.

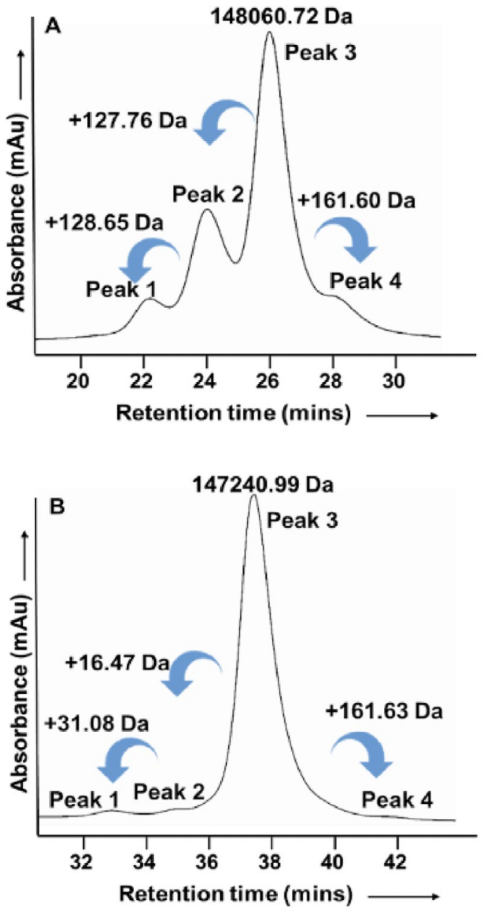
Offline Coupling of Hydrophobic Interaction Chromatography–Capillary Zone Electrophoresis for Monitoring Charge-Based Heterogeneity of Recombinant Monoclonal Antibodies

Adapted from Sarin *et al.* [1]

Monoclonal antibodies (mAbs) are critical biotherapeutics, but their structural heterogeneity—arising from post-translational modifications (PTMs) and subtle microheterogeneities—necessitates rigorous analytical characterization. Charge variants, resulting from modifications such as deamidation, isomerization, oxidation, and C-terminal lysine clipping, are particularly important critical quality attributes (CQAs). Traditional approaches have relied on one-dimensional (1D) techniques, but the complexity of mAb heterogeneity demands more powerful, multidimensional strategies.

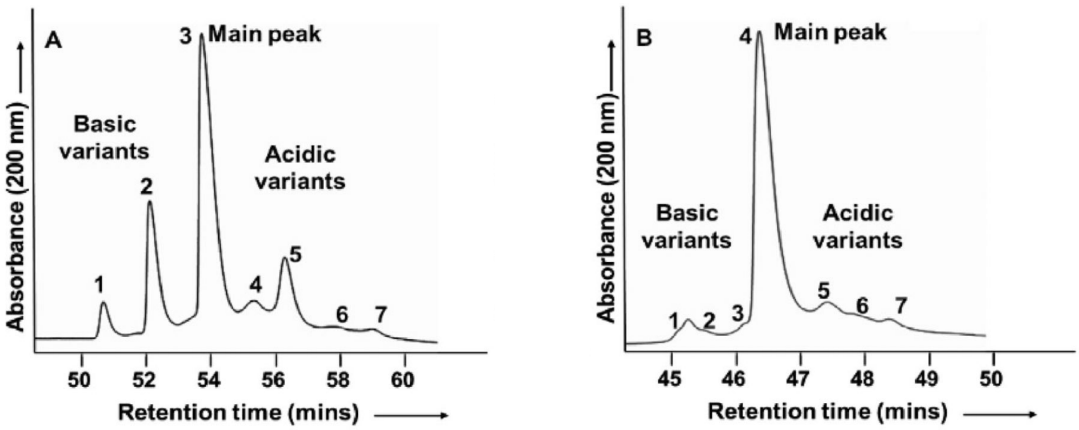
This study by Sarin *et al.* leverages the strengths of hydrophobic interaction chromatography (HIC) and capillary zone electrophoresis (CZE–UV) in a multidimensional workflow to resolve and characterize charge variants in two widely used mAbs—trastuzumab (mAb A) and rituximab (mAb B). The workflow is further complemented by high-resolution intact mass and peptide mapping using the Agilent 6545XT AdvanceBio LC/Q-TOF, celebrated for its sensitivity and performance in large molecule analysis.

Figure 1



HIC chromatograms for (A) mAb A and (B) mAb B. mAb, monoclonal antibody; HIC, hydrophobic interaction chromatography.

Figure 2



CZE electropherograms obtained for (A) mAb A and (B) mAb B. CZE, capillary zone electrophoresis; HIC, hydrophobic interaction chromatography; mAb, monoclonal antibody.

Analytical Challenge and Multidimensional Separation

Limitations of 1D Approaches

Standalone techniques—such as HIC, weak-cation exchange chromatography (WCX), and CZE—each provide partial resolution of mAb charge variants. HIC distinguishes variants by hydrophobicity (Fig. 1), while CZE excels in resolving variants by their net charge-to-mass ratio (Fig. 2). Two-dimensional LC (2DLC) systems, which couple two chromatographic separations, extend peak capacity but remain limited when the variants are indistinguishable in one or both chromatographic dimensions.

Concept of Multidimensional (Orthogonal) Separation

The study introduces a multidimensional platform that first separates main hydrophobic species by HIC, then resolves each hydrophobic fraction into charge-based variants by offline CZE–UV. This orthogonal strategy dramatically increases the number and clarity of resolved peaks. Key elements include:

- **HIC:** Separates mAb variants based on hydrophobicity, such as glycoforms and oxidized forms.
- **Fraction Collection:** Major peaks from HIC are collected, concentrated, and further analyzed.
- **CZE–UV:** Each HIC fraction is injected onto a CZE–UV system, resolving variants by their charge and hydrodynamic properties.

This sequential, multidimensional workflow unlocks populations of variants that elude standalone or 2DLC methods.

Experimental Strategy

Sample Preparation

Trastuzumab and rituximab samples were subjected to HIC using an Agilent AdvanceBio HIC column. Key HIC peaks were collected as fractions, concentrated, and assessed for purity. Each purified fraction was then analyzed by CZE–UV using a bare fused silica capillary and optimized electrolyte composition.

Mass Spectrometry

To confirm the assignment of charge variants and probe their molecular structure, the study used the Agilent 6545XT AdvanceBio LC/Q-TOF. Both intact mass analysis and peptide mapping were performed:

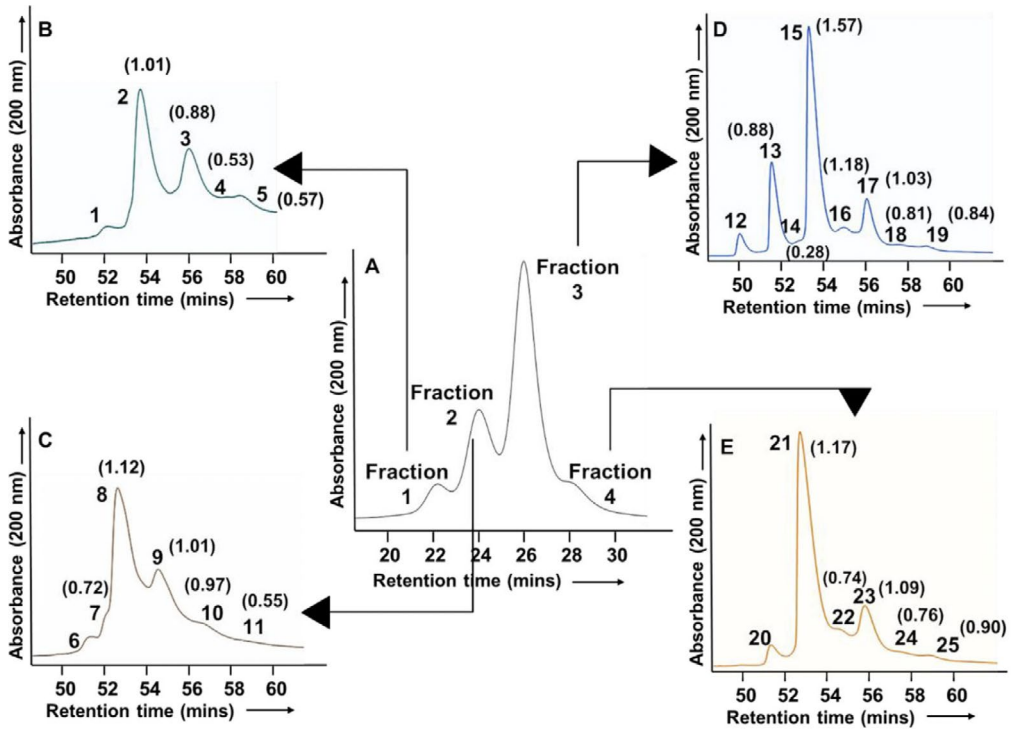
- **Intact mass:** Rapid determination of molecular weights of collected HIC fractions.
- **Peptide mapping:** Following enzymatic digestion (trypsin and carboxypeptidase B), peptides were separated and analyzed to identify PTMs and modifications responsible for charge heterogeneity.

Major Findings

HIC and CZE–UV as Standalone Methods

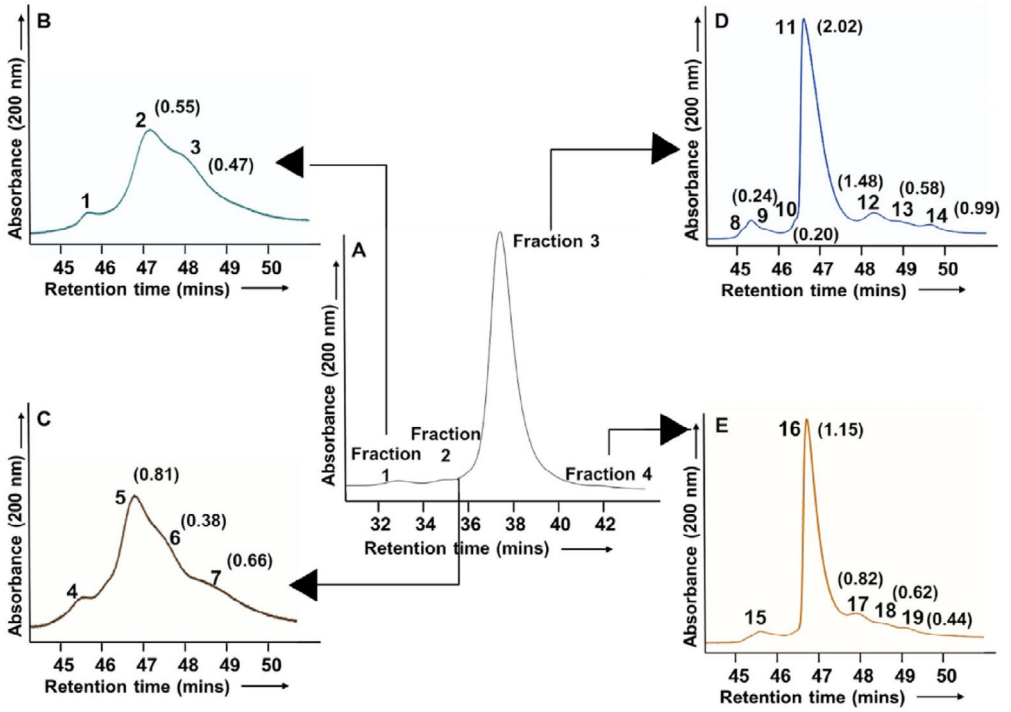
- **HIC** alone resolved four main hydrophobic variants per mAb (e.g., glycoforms, lysine variants, oxidized forms) as shown in Figure 3.
- **CZE–UV** as a secondary separation identified over a dozen major charge variants per mAb (Fig. 4).

Figure 3



Schematic representation of the HIC–CZE–UV peaks in mAb A: (A) chromatogram obtained from HIC of mAb A; (B–E) electropherograms obtained from CZE–UV of HIC fractions 1–4 of mAb A, respectively. Resolution of peaks is provided in brackets near each peak.

Figure 4



Schematic representation of the HIC–CZE–UV peaks in mAb B: (A) chromatogram obtained from HIC of mAb B; (B–E) electropherograms obtained from CZE–UV of HIC fractions 1–4 of mAb B, respectively. Resolution of peaks is provided in brackets near each peak.

Dramatic Multiplication of Variant Resolution

By combining HIC fractionation with CZE–UV analysis:

- **29 distinct variants** were resolved for trastuzumab (mAb A) and **23 variants** for rituximab (mAb B).
- This number exceeded what was possible with either technique alone or with previous 2DLC workflows.

Types of Variants Characterized

The multidimensional workflow distinguished and attributed variants to specific modifications based on mass and peptide mapping results:

- **C-terminal lysine variants** (basic, hydrophobic)
- **Deamidation and isomerization** (acidic/basic)
- **Glycoforms** (mass shifts due to varied glycosylation)
- **Oxidized forms** (increase in mass, altered hydrophobicity)
- **N-terminal pyroglutamate** (mAb B, acidic)

Robustness and Reproducibility

The method's reproducibility was confirmed by duplicate runs:

- Variation in migration/retention times was <5% (trastuzumab), <3% (rituximab).
- Peak area variation was <12% for both mAbs.

Intact Mass and Peptide Mapping with Agilent 6545XT

The Agilent 6545XT AdvanceBio LC/Q-TOF played a central role in:

- **Unambiguous identification** of variant masses and PTMs.
- Assigning specific peaks (from HIC and CZE) to structural modifications using accurate mass and sequence evidence.

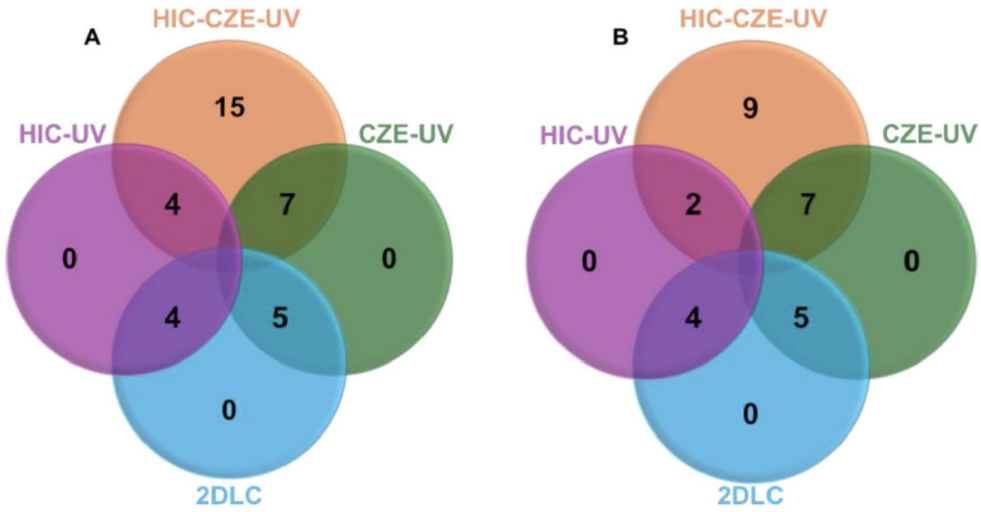
The 6545XT's sensitivity, resolution, and compatibility with complex protein mixtures were crucial for confidently mapping these complex patterns of mAb heterogeneity.

Key Summaries and Comparative Data

Table 1. Comparative Performance Across Analytical Approaches

Method	Trastuzumab (mAb A): Variants Resolved	Rituximab (mAb B): Variants Resolved
HIC–UV	4	4
CZE–UV	7	7
2DLC	10	11
HIC–CZE–UV	29	23

Figure 5



A Venn diagram representing the variant distribution of (A) mAb A and (B) mAb B. Orange, purple, green, and blue represent the variants exclusive to the HIC–CZE–UV, HIC–UV, CZE–UV, and 2DLC methods, respectively. The faded pink, purple, green, and blue patches represent variants common to HIC–UV and HIC–CZE–UV, HIC–UV and 2DLC, CZE–UV and HIC–CZE–UV, and CZE–UV and 2DLC, respectively. 2DLC, two-dimensional liquid chromatography; CZE, capillary zone electrophoresis; HIC, hydrophobic interaction chromatography; mAb, monoclonal antibody.

Practical Insights

- **Higher Peak Capacity:** The multidimensional approach surpasses the variant-resolving power of conventional 2DLC.
- **Orthogonality:** Combining hydrophobic and charge-based separations is especially powerful for dissecting CQAs in complex mAb formulations.
- **LC/Q-TOF Synergy:** MS-based intact mass and peptide mapping approaches are indispensable for confirming assignments, enabled here by the Agilent 6545XT.

Conclusions

This work demonstrates the efficacy of a multidimensional analytic pipeline, combining HIC, CZE–UV, and high-resolution Q-TOF mass spectrometry, for charge variant analysis in monoclonal antibodies. The workflow achieves:

- **Unprecedented separation** and mapping of charge/hydrophobicity-based mAb variants.
- **Comprehensive variant characterization**, essential for regulatory compliance and product development.
- **Robust and reproducible results**, with clear practical implications for biopharmaceutical analytics.

The Agilent 6545XT AdvanceBio LC/Q-TOF is central to this achievement, providing the sensitivity and reliability necessary for advanced protein characterization. This study establishes a new benchmark for multidimensional protein variant analysis in the biotherapeutics field.

Reference

[1] Sarin, D. *et al.* (2024). Offline Coupling of Hydrophobic Interaction Chromatography–Capillary Zone Electrophoresis for Monitoring Charge-Based Heterogeneity of Recombinant Monoclonal Antibodies. ELECTROPHORESIS. https://doi.org/10.1002/elps.202400158.

Application Note

Biopharma

Agilent

Trusted Answers

Comprehensive Characterization of Multiple GLP-1 Analogs

Using an Agilent 6545XT AdvanceBio LC/Q-TOF with electron capture dissociation and ExDViewer software

Authors

Rachel Franklin, Mike Hare, Thomas Walker, and Joseph Meeuwsen
Agilent Technologies, Inc.

Abstract

GLP-1 analogs are synthetic peptides that mimic the naturally occurring peptide hormone known as glucagon-like peptide-1 (GLP-1). The development of GLP-1 analogs is important due to their potential for managing diabetes and obesity. Characterization of synthetic peptides using Agilent high-resolution LC/Q-TOFs provides critical quality information about GLP-1 analogs. Implementing electron-based fragmentation in LC/MS workflows is an effective technique for sequence and modification analysis of proteins and peptides. This application note describes the characterization of three GLP-1 analogs—liraglutide, semaglutide, and tirzepatide using an Agilent 6545XT AdvanceBio LC/Q-TOF system equipped with an Agilent ExD cell for electron capture dissociation. Agilent ExDViewer is an effective and accessible software tool for analyzing all types of fragmentation data resulting from proteins and peptides. This application note uses ExDViewer's targeted deconvolution workflow to demonstrate in-depth sequence analysis, characterize custom modifications, and highlight powerful tools to visualize fragmentation trends and data quality.

Introduction

GLP-1 is an insulin-stimulating hormone that binds to the GLP-1 receptor.[1,2] In 2019, the first GLP-1 analog was approved by the U.S. Federal Drug Administration (FDA) for type 2 diabetes, and in 2020, GLP-1 analogs were approved for obesity management.[3,4] With the increasing use of GLP-1 analogs, it is important to have precise mass spectrometry (MS) methods for structural characterization, purity, and counterfeit analysis.

LC/MS/MS analysis of synthetic peptides using electron capture dissociation (ECD) provides detailed sequence, modification, and purity information. In contrast to collision-induced dissociation (CID), ECD is considered a "gentler" fragmentation approach that retains modifications such as the fatty acid modifications in GLP-1 analogs. This allows their mapping and characterization, while still effectively cleaving the peptide backbone (Figure 1). Also, secondary fragmentation of amino acid side chains via ECD provides additional evidence of sequence assignments and enables the investigation of amino acid isomers such as aspartate and isoaspartate.[5,6] The Agilent ExD cell and Agilent ExDViewer software provide a powerful approach for the detailed characterization of synthetic peptides with complex structures.

This application note describes LC/MS/MS analysis of three GLP-1 analogs: semaglutide, liraglutide, and tirzepatide. The analysis was performed using a 6545XT LC/Q-TOF equipped with an ExD cell to enable ECD. ExDViewer was used to analyze MS/MS fragmentation spectra using the targeted deconvolution workflow, which considers unique aspects of electron fragmentation such as hydrogen transfer and side chain fragmentation.[7] ExDViewer also has useful tools for visualizing fragmentation trends and understanding data quality in both ECD and CID experiments. By combining ExD hardware and software, these methods enabled fast and in-depth characterization of peptide sequences with nonstandard residues and characterization of custom modifications.

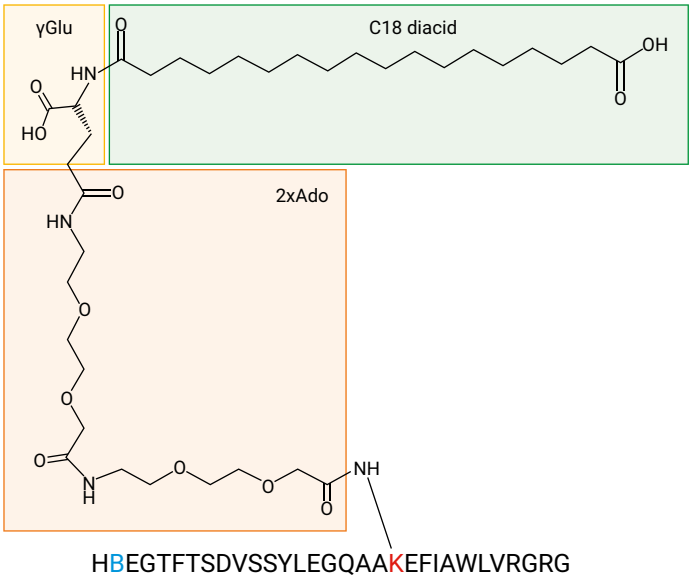


Figure 1. The structure of semaglutide contains the noncanonical amino acid 2-amino isobutyrate in amino acid position 2, indicated by the letter B. Lysine 20 is modified by a C18 fatty acid connected through a bis-aminodiethoxyacetyl (2xAdo) and gamma glutamate linker. Liraglutide and tirzepatide lysine modifications have similar structures with variations in the linker and fatty acid group.

Experimental

Chemicals and standards

- Agilent ESI-L tuning mix (part number G1969-85000) containing 2.5 µg/mL melittin (part number G1997-85001)
- Formic acid, 99.0+%, Optima LC/MS-grade, Cat. no. A-117-50, Fisher Chemical, (part number A-117-50)
- Acetonitrile, 99.9%+ LC/MS-grade, Supelco OmniSolv (part number AX0156-6)
- Liraglutide, Sigma (part number SML3925)
- Semaglutide, AstaTech (part number AT35750)
- Tirzepatide, AstaTech (part number AT40456)

Sample preparation

Each sample was prepared at 10 µM in 15% acetonitrile with 0.1% formic acid. For LC experiments, an equal volume of the three samples was mixed together.

HPLC column

- Agilent AdvanceBio Peptide Mapping 120Å, 2.1 × 150 mm, 2.7 µm (part number 653750-902)

Instrumentation

- Agilent 1290 Infinity II Bio LC System
  - Agilent 1290 Infinity II high-speed pump (G7120A)
  - Agilent 1290 Infinity II multisampler (G7167B)
  - Agilent 1290 Infinity II column compartment (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF MS with Agilent ExD cell

Software

- Agilent MassHunter acquisition software for LC/Q-TOF, version 11.0
- Agilent ExDControl software, version 3.6
- Agilent ExDViewer software, version 4.6.12

Liquid chromatography methods

The LC methods described in this application note were used for most ECD experiments. However, direct infusion was used for tuning the ExD cell and to investigate charge state-dependent fragmentation. Direct infusion was accomplished using a 500 µL syringe and an infusion rate of 10 to 20 µL/min. PEEK tubing was used to transfer the sample from the infusion syringe to the nebulizer inlet of the Agilent Dual AJS source. Figure 2 shows the chromatographic separation of a mixture of all three GLP-1 analogs.

Table 1. Liquid chromatography methods used for GLP-1 analogs.

Agilent 1290 Infinity II Bio LC System	
Column	Agilent AdvanceBio Peptide Mapping 120Å, 2.1 × 150 mm, 2.7 µm
Mobile Phase A	LC/MS-grade water + 0.1% formic acid
Mobile Phase B	Acetonitrile + 0.1% formic acid
Flow Rate	0.400 mL/min
Injection Volume	10 µL
Column Temperature	60 °C
Gradient Program	Time (min) %B 0 20 20 60 20.10 80 21.90 80 22 20

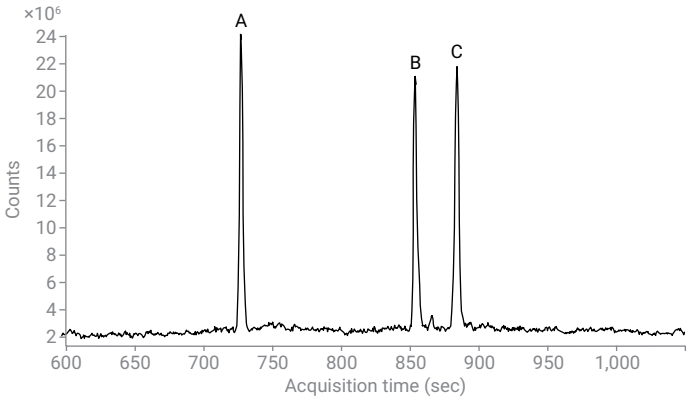


Figure 2. The chromatogram of a mixture of GLP-1 analogs. The peak identities are: (A) semaglutide, (B) liraglutide, and (C) tirzepatide.

Mass spectrometry methods

Table 2. LC/Q-TOF parameters.

Agilent 6545XT Q-TOF MS system	
Ion Source	Agilent Dual Jet Stream ESI source
Polarity	Positive
Gas Temperature	325 °C
Drying Gas Flow	10 L/min
Nebulizer	35 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	2,000 V
Fragmentor	175 V
Skimmer	75 V
Acquisition Rate	2 spectra/sec
Acquisition Mode	Extended Dynamic Range (2 GHz)
Isolation Window	Medium (4 amu)
MS Spectrum Range	*m/z* 120 to 3,200
MS/MS Spectrum Range	*m/z* 120 to 3,200

Table 3. Auto MS/MS data acquisition parameters.

Parameter	Value
Precursor Selection	Preferred list for 4+ precursors: – *m/z* 938.2 (liraglutide) – *m/z* 1,028.7 (semaglutide) – *m/z* 1,203.6 (tirzepatide)
Intensity Threshold (Abs)	2,000
Intensity Threshold (Rel)	0.01%
Mass Error Tolerance	20 ppm
Static Exclusion	*m/z* 100 to 400
Isotope Model	Peptides
Use Preferred Ion List Only	False

Targeted ECD MS workflow

The ExD cell lenses and internal filament were controlled by ExDControl software, version 3.6, which operates as a separate application alongside MassHunter acquisition software. An optimal filament current of 2.55 A was determined by an automated filament tune, followed by manual adjustment to optimize melittin ECD fragments. The lens profile for MS1 transmission of intact ions was determined by autotuning on tune mix ions. Melittin ECD fragment ion peaks were used to autotune the lens profile used for ECD in MS2. For LC experiments, an auto MS/MS acquisition method was set up in MassHunter acquisition software with the parameters described in Tables 2 and 3. For direct infusion experiments, a targeted MS/MS acquisition was used to investigate individual charge states.

Data analysis

GLP-1 analog molecular weights were determined using ExDViewer's MS1 deconvolution workflow. Raw .d files were loaded directly into ExDViewer and default parameters were used for deconvolution. MS1 spectra were averaged over each chromatographic peak. Decharged spectra were generated by toggling the spectrum view in the spectrum window. Files were saved as .svg files and imported into Microsoft PowerPoint for customization of the figure elements.

Sequence and modification analysis was accomplished using ExDViewer's targeted deconvolution. First, the GLP-1 analog sequences were defined in the target editor and searched against the MS/MS results. Default parameters were used for fragment matching, except for characterizing lysine modifications. For modification analysis, the ion score threshold was raised to 10 to observe only the highest quality modification containing fragment matches.

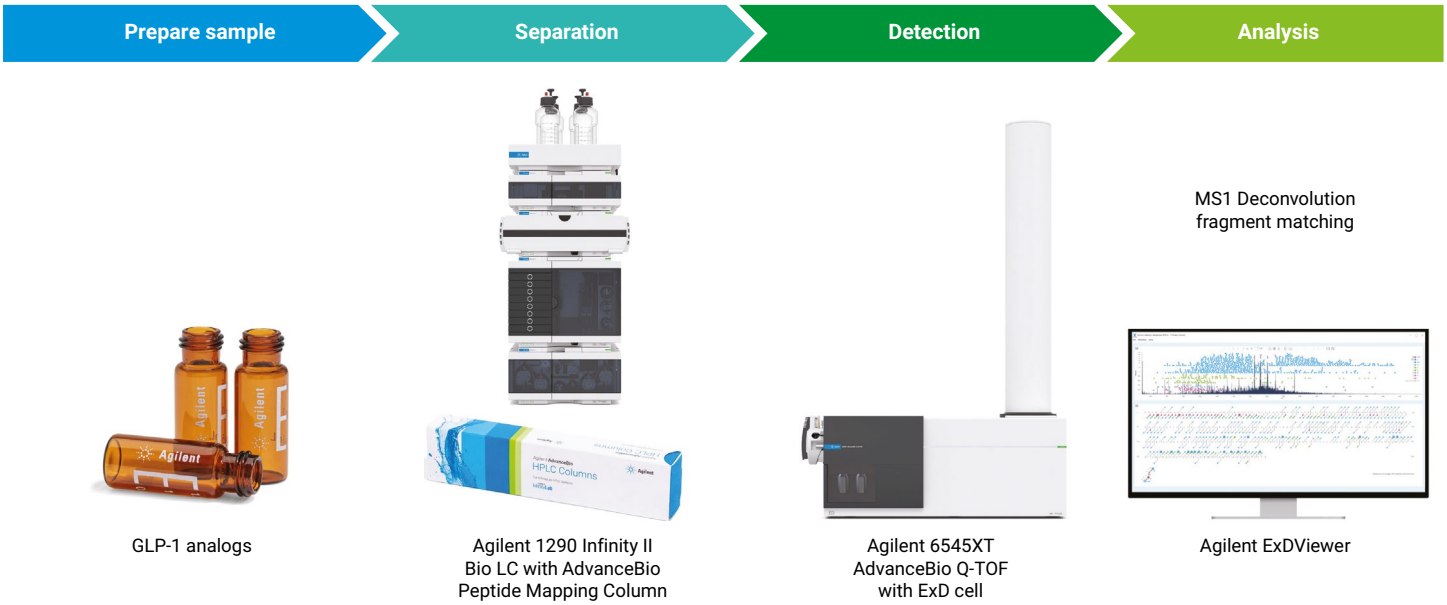


Figure 3. Workflow for the LC/MS/MS analysis of GLP-1 analog synthetic peptides.

Results and discussion

Isotopically resolved spectral deconvolution using ExDViewer

The analysis of GLP-1 analogs using Agilent LC/MS/MS workflows enables the measurement of molecular weight, signal heterogeneity, and sequence in a single experiment. ExDViewer's untargeted deconvolution algorithm effectively processes signals from isotopically resolved spectra, commonly obtained during peptide analysis. MS1 deconvolution allows the determination of the molecular weight of synthetic peptides.

Figure 4 illustrates the MS1 and deconvoluted spectra for each GLP-1 analog from the mixture shown in Figure 2. The deconvoluted spectra reveal that the primary signal from each chromatographic peak corresponds to the GLP-1 analogs, along with some less abundant salt-adducted peaks (Na^+/K^+). Deconvolution of isotopically resolved signals with ExDViewer offers a complementary approach to Agilent MassHunter BioConfirm maximum entropy deconvolution for larger protein ions.

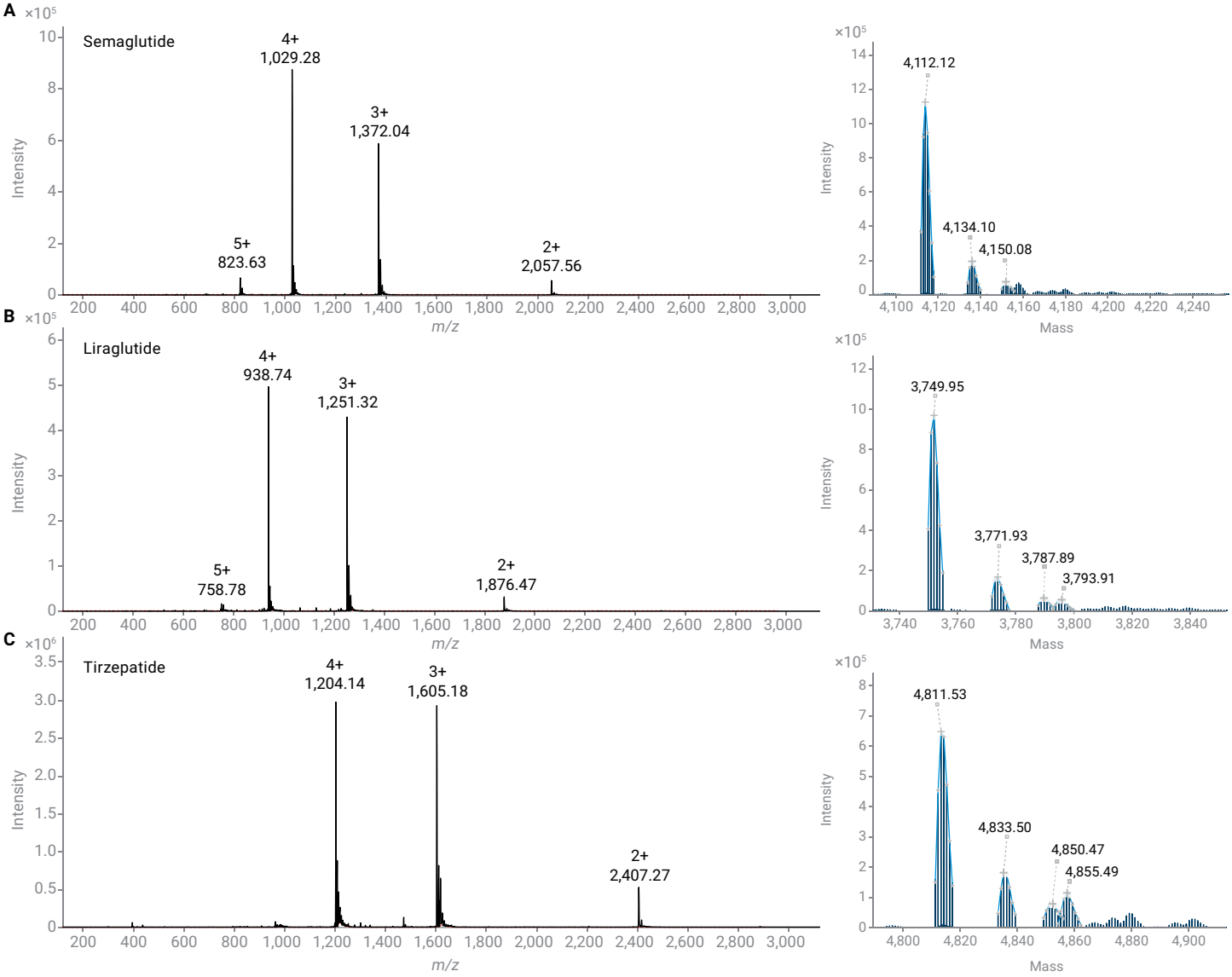


Figure 4. The MS1 spectra (left) and corresponding deconvoluted spectra (right) of the LC-separated GLP-1 analogs (A) semaglutide, (B) liraglutide, and (C) tirzepatide. The monoisotopic masses are annotated in the deconvoluted mass spectra.

Sequence analysis with noncanonical amino acids

Gas phase fragmentation using ECD induces precise breaks in the peptide backbone, which yields clean and reproducible fragmentation spectra that are easily interpreted using ExDViewer. Semaglutide and tirzepatide contain noncanonical amino acids, which can be defined as custom amino acids in the Building Block editor within ExDViewer. In the featured analysis, the noncanonical amino acid is defined as 2-amino isobutyrate, abbreviated as the single-letter code B. Using a single targeted MS/MS spectrum collected at 2 Hz, 100% sequence coverage was obtained for the 4+ precursors of liraglutide, semaglutide, and tirzepatide. Multiple CID and ECD fragment ion types (a,b,c,d and w,y,z) were detected, providing complimentary evidence for sequence confirmation (Figure 5).

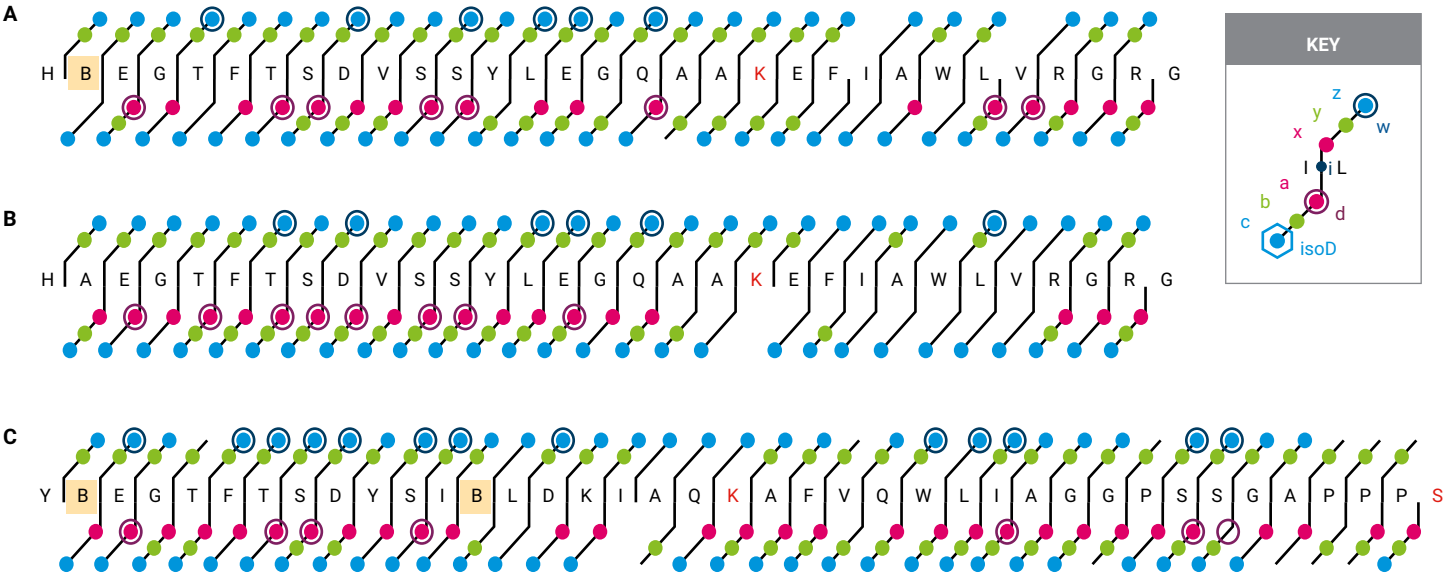


Figure 5. The sequence coverage maps for (A) semaglutide, (B) liraglutide, and (C) tirzepatide. The nonstandard B amino acid is highlighted in yellow, while the modified lysine is indicated in red text. Several complimentary c/z and b/y-type ions were identified. Default parameters were used for Agilent ExDViewer fragment matching.

Abundant amino acid side chain fragments are commonly produced using the ExD cell, which provides additional information about isomeric amino acids such as leucine and isoleucine. Secondary fragmentation of amino acid side chains from ECD radical ions are referred to as w-ions.[4] ExDViewer automatically annotates side chain evidence, simplifying the analysis of amino acid isomers.[7] Ions specific to isoaspartate formation, an important modification for assessing protein quality, are also annotated.[6,7] In Figure 6, the w_{23}^{2+} ion is annotated, providing additional evidence of aspartate in this position. Hovering over the w_{23}^{2+} ion opens an informative tooltip that describes the percentage hydrogen transfer, mass, side chain loss formula, score, and number of matched isotopes.

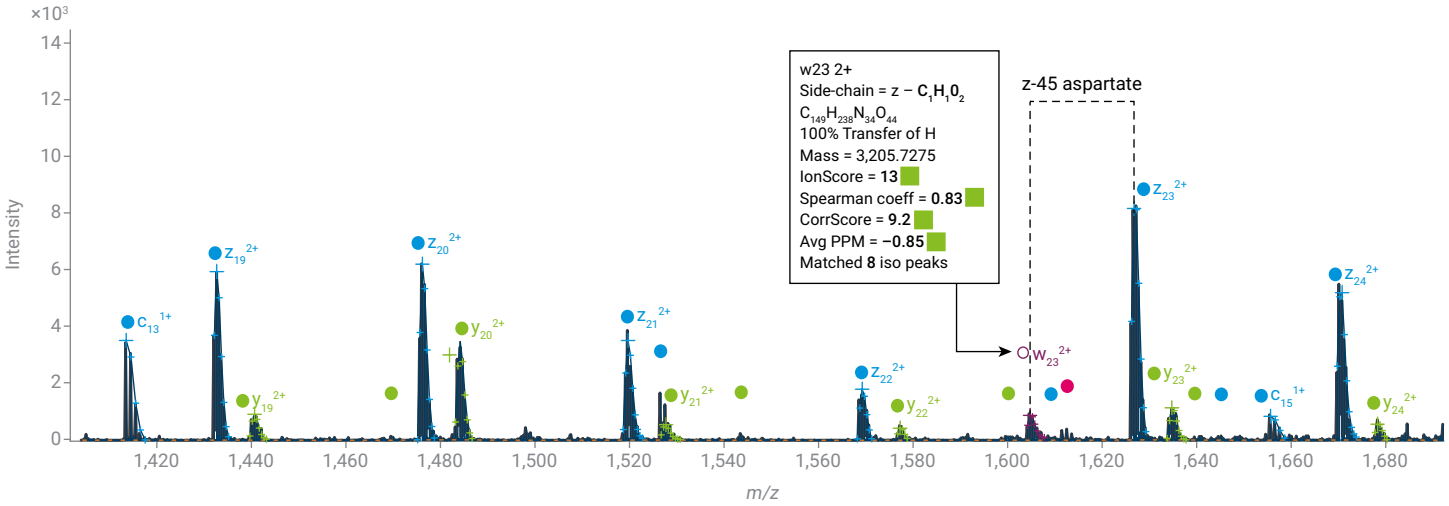


Figure 6. A representative fragmentation spectrum from liraglutide showing the automatically annotated fragment evidence for isomeric amino acids such as aspartate and isoaspartate. Aspartate is identified by the loss of a CHO_2 group from the z_{23}^{2+} ion.

Visualizing fragmentation trends with ECD and CID

Automatically generated plots in ExDViewer illustrate fragmentation trends resulting from various experimental conditions. Visualizing fragmentation trends is a valuable way to assess data quality. For example, the ion intensities graph shows the signal detected for each ion type, including internal and unassigned ions. These graphs are also useful for evaluating the suitability of method parameters such as collision energy. In Figure 7, the ion intensity graphs are used to demonstrate the charge state-dependent fragmentation patterns of semaglutide using ECD or a combination of CID and ECD.

With zero collision energy, ECD effectively achieved 100% sequence confirmation for the 5+ and 4+ semaglutide precursors. However, the 3+ precursor was more challenging to fragment using ECD alone. Adding 35 V of supplemental collision energy helped improve sequence coverage from 57 to 93% for the 3+ precursor (Figures 7C and 7F). Notably, the summed fragment ion abundance for 3+ semaglutide was similar with and without CID, indicating that the increased sequence coverage upon adding CID reflects a different fragmentation pathway rather than a general increase in fragment ion abundance. Therefore, combining ECD with low-level activation can be used as a tool to achieve more

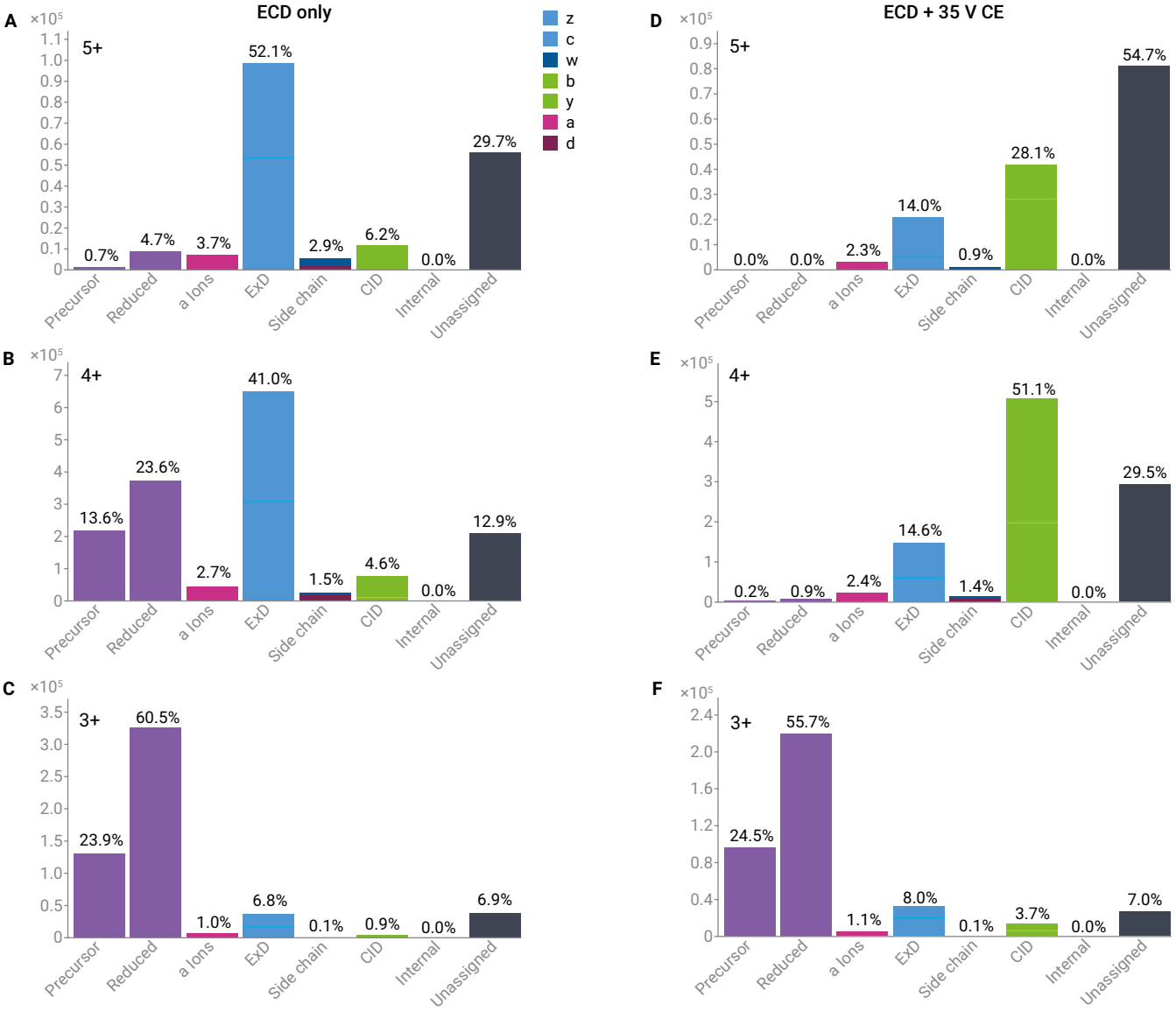


Figure 7. Fragmentation pathways of the semaglutide 3, 4, and 5+ precursor ions. Panels A, B, and C show the ion intensities under ECD-only conditions. Panels D, E, and F show ion intensities resulting from ECD combined with 35 V of collisional activation (ECD+CID). Internal fragment ions were not considered during the analysis of these data sets.

complete sequence coverage even for low-charge peptides. However, adding too much collision energy can result in a high percentage of unassigned ion signals, presumably due to over-fragmentation (Figure 7D).

Characterization of GLP-1 analog modifications

As new GLP-1 analog derivatives are created, it is important to have effective methods to characterize their modifications. Here, ExDViewer was used for fragment analysis to identify the site and structure of synthetic modifications in three GLP-1 analogs. The custom chemistries of the GLP-1 analog modifications were defined in ExDViewer's Modifications Editor.

The ExDViewer fragment matching algorithm assigns each isotope cluster an ion score based on *m/z* and intensity match, along with the surrounding noise levels. The default ion-score setting is effective for many sequence analysis applications. However, for modification characterization, strict spectral quality is essential for pinpointing the modification location. Figure 8 highlights several examples of modification-containing ECD ions detected for tirzepatide, demonstrating the range of ion match quality that can be observed in a fragmentation spectrum.

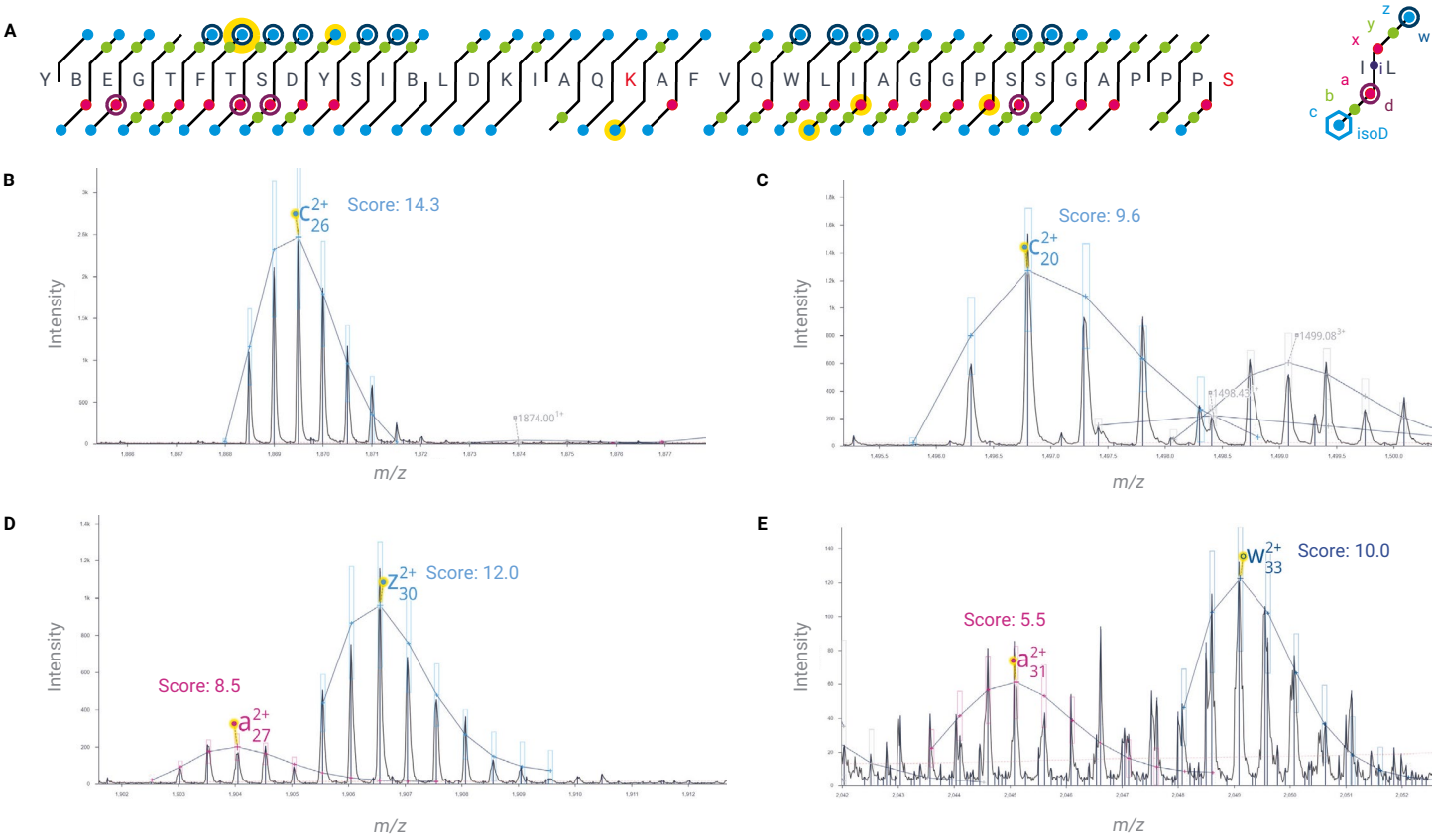


Figure 8. Representative tirzepatide fragments with various ion scores. Each ion is assigned a score based on the *m/z* and predicted intensity match, as well as the surrounding noise levels. (A) Tirzepatide sequence coverage map where only ions with a score of 5 or greater were considered. (B–E) Examples of modification containing fragments and their corresponding ion scores. The modified lysine is indicated in red text. The c_{20}^{2+} ion is a site-specific localizing fragment, while the other highlighted ions are modification-containing peptides fragmented at various positions in the peptide backbone.

Unlike collisional dissociation, ECD is a gentle technique that fragments the peptide backbone while preserving important labile chemical modifications. Comparing ion scores of GLP-1 analogs analyzed with ECD or CID reveals that ECD fragmentation generates more high-quality, modification-containing fragments than CID (Figure 9).

High-quality matches on both sides of the modification enhance localization confidence. In many cases, ECD fragmentation alone provides detailed structural information, circumventing the need for collision energy optimization, which saves time in sequence analysis.

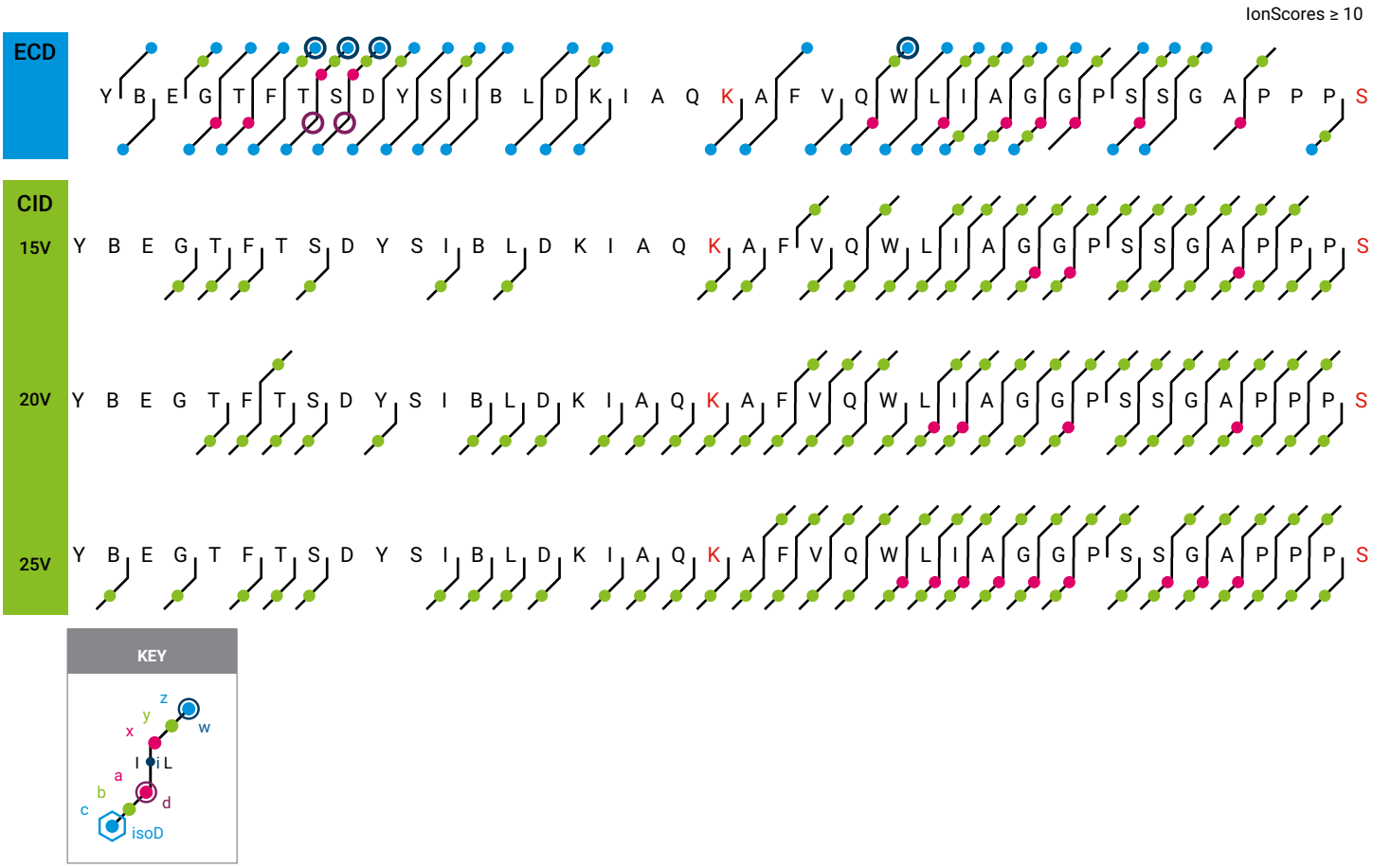


Figure 9. Tirzepatide fragments identified in ECD or CID experiments with ion scores of 10 or greater. ECD provided more high-quality modification-containing fragments, particularly on the N-terminal side of the modification. ECD yielded a more complete ion series with several complimentary ion types. The exception to this is the proline-rich region because ECD does not cleave on the N-terminal side of proline.

Conclusion

This application note demonstrates the comprehensive sequence analysis and localization of modifications in three synthetic GLP-1 analogs by electron dissociation using an Agilent 6545XT AdvanceBio LC/Q-TOF with Agilent ExDViewer for fragment analysis. Fragmentation of amino acid side chains offers additional evidence for identifying amino acid isomers such as aspartate and isoaspartate. High-quality fragment matches from ECD data enable effective modification characterization and localization, saving time as it does not require optimization of collision energy. ExDViewer provides powerful tools for visualizing fragment pathways and data quality for both ECD and CID experiments. The methods presented in this application note provide a framework for the analysis of GLP-1 analogs and should also be used as a starting point for investigating new GLP-1 analog derivatives and impurities using ECD.

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