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Advances in Mass Spectrometry Fragmentation Techniques for Peptide and Protein Characterization: CID to ETD

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Abstract

Mass spectrometry (MS) has transformed the field of proteomics by enabling the precise analysis of proteins and peptides at the molecular level. The heart of this analysis is the process of fragmentation, which plays a pivotal role in elucidating amino acid sequence, identifying post-translational modifications (PTMs), and characterizing structural features. Peptides and proteins are first ionized and then subjected to fragmentation techniques such as Collision-Induced Dissociation (CID), Electron-Transfer Dissociation (ETD), and Electron-Capture Dissociation (ECD). These techniques induce fragmentation along the peptide backbone, generating characteristic fragment ions (b and y ions, c and z ions) that reflect the sequence of amino acids. The mass-to-charge (m/z) ratios of these fragment ions provide crucial data for de novo sequencing and PTM identification. Furthermore, fragmentation patterns can reveal insights into protein folding, interactions, and dynamics. This article provides the detailed view of mass fragmentation techniques in peptides and proteins, highlighting its importance in advancing proteomic research and its applications in fields ranging from biomarker discovery to drug development.

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Introduction

Mass spectrometry (MS) is a prevailing analytical technique that provides valuable information about the structure and composition of molecules. One of the key processes in mass spectrometry is fragmentation, which occurs when molecules break apart into smaller pieces during analysis. The use of MS in molecular biology has become widely accepted. It allows for the examination of large molecules and their interactions and modifications, as well as the study of organelles, cell lysates, intact cells, cell lines, tissues, and even entire organisms. Its purpose is to analyze the structure of proteins in various physiological or pathological conditions [1], [2]. Mass spectrometry (MS) is a highly effective method used in advanced

therapeutics to detect modifications in proteins. It has become known as an extremely efficient instrument for the analysis of peptides and proteins. Structural elucidation of peptides and proteins has become much simpler with the introduction of tandem mass spectrometry (MS-MS), which offers extremely detailed MS-MS spectra. Recent advancements in MS sensitivity and resolution have enabled the identification of previously unknown categories of tumor-specific proteoforms, such as post-translational modifications (PTMs) and variants resulting from

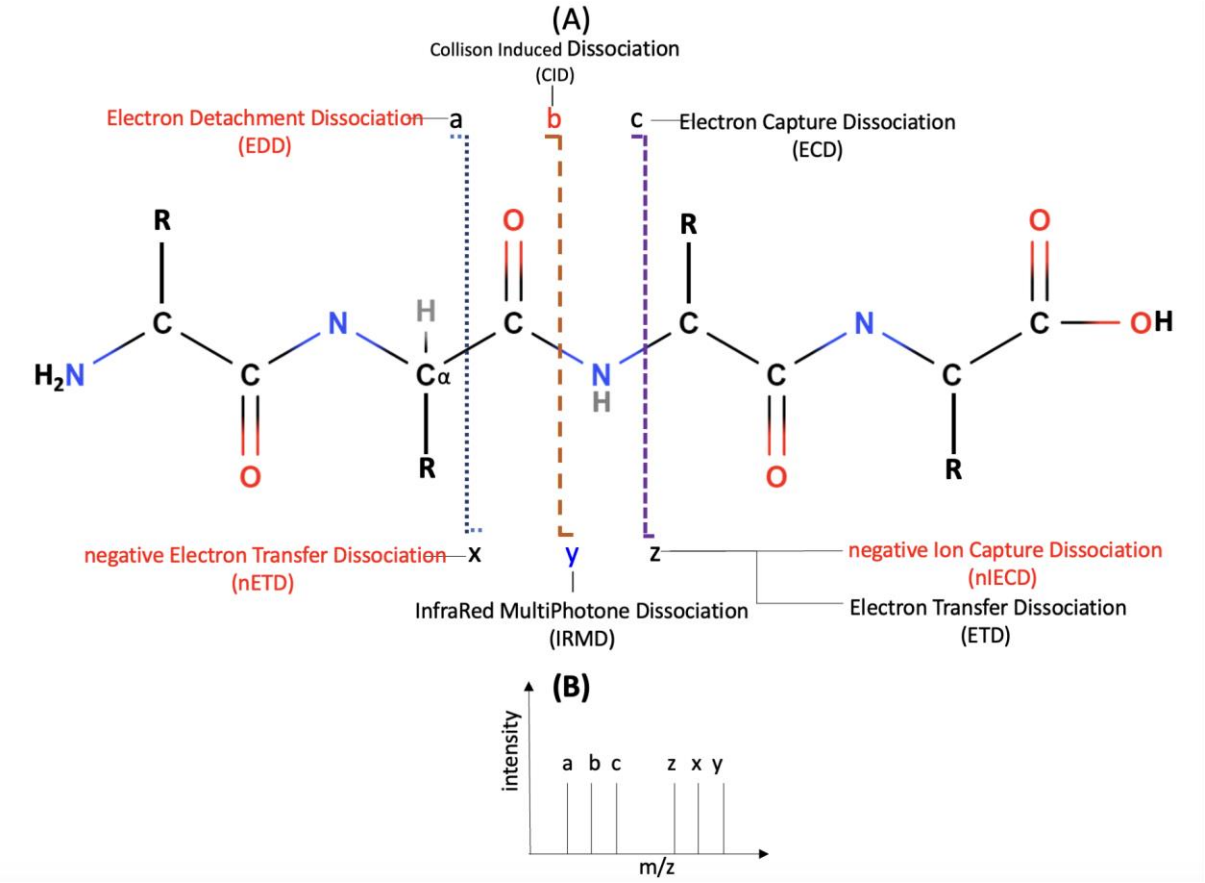

Figure 1 Fragmentation pattern of Peptide backbone: The peptide backbone can break at three different locations for every two adjacent amino acid residues, producing six different kinds of ions. (A). In the theoretical spectrum, each type of ion may produce a peak at the corresponding m/z value (B)

genomic and epigenomic abnormalities. Mass fragmentation techniques play a crucial role in analyzing proteins and peptides using mass spectrometry by breaking them into smaller, more manageable fragments. The key fragmentation techniques include Collision-induced dissociation (CID), High-energy collision dissociation (HCD), Electron Capture Dissociation (ECD), Electron Transfer Dissociation (ETD), and Ultraviolet Photodissociation (UVPD). These fragmentation techniques are often used in combination with various mass analyzers and separation methods, such as high-performance liquid chromatography (HPLC) and Capillary Electrophoresis (CE), to achieve comprehensive protein and peptide analysis. Recent advancements in this area include the development of machine learning approaches, such as the "ad hoc learning of fragmentation" (AHLF) model, which has shown promise in detecting post-translational modifications and cross-linked peptides based solely on fragmentation spectra [3]. In this article, we discuss the mechanisms of each fragmentation technique and their compatibility with the MS analyzer, as well as the applications of each fragmentation technique in peptide and protein analysis. The article will explain the choice of fragmentation technique based on variables such

as the protein's size/peptide, the presence of PTMs, and the specific information required.

Fragmentation Nomenclature in Peptides and Proteins

The established nomenclature for peptide fragment ions in mass spectrometry was first suggested by Roepstorff and Fohlman in 1984 and later revised by Johnson et al. in 1987. The fragmentation nomenclature for peptides and proteins in MS follows a systematic approach to describe the different types of fragment ions produced during tandem mass spectrometry (MS/MS) experiments. A structured fragmentation nomenclature has been established to classify the various types of fragment ions produced by peptides and proteins. Fragment ions are classified using alphabet letters based on the type of bond cleaved in the peptide or protein backbone. Fragments cleaved from the N-terminus are labeled as a, b, and c ions, while fragments cleaved at the C-terminus are labeled as x, y, and z ions [4].
• **a-ions:** Include the N-terminus and extend to a backbone cleavage before the carbonyl carbon.
• **b-ions:** Include the N-terminus and extend to a backbone cleavage at the carbonyl carbon.
• **c-ions:** Include the N-terminus and extend to a backbone cleavage after the amide nitrogen.
• **x-ions:** Include the C-terminus and extend to a backbone cleavage before the carbonyl carbon.
• **y-ions:** Include the C-terminus and extend to a backbone cleavage at the amide nitrogen.
• **z-ions:** Include the C-terminus and extend to a backbone cleavage after the amide nitrogen.
The charge state is denoted by a superscript. For example, y2+ represents a doubly charged y ion. Loss of water (-18 Da) is observed for fragments containing Ser, Thr, Asp, and is denoted by (O), or Glu. Loss of ammonia (-17 Da) occurs for fragments with Asn, Gln, Lys, or Arg and is denoted by (*) [5]. Satellite ions (d, v, w ions) are formed from radical (a+1) and (z) ions created in charge-remote processes of high-energy CID. They provide additional structural information, especially useful for differentiating between isomeric amino acids like leucine and isoleucine, and aspartic acid and isoaspartic acid.
It is important to note that the most common peptide fragmentation techniques in proteomics focus on producing b/y ions (CID/HCD) or c/z ions (ETD/ECD) due to their more predictable nature and usefulness in sequence determination. The a and x ions, while informative when present, are not typically the primary focus of fragmentation method development [6].

Mass Fragmentation Techniques

Mass fragmentation techniques are methods used in mass spectrometry to fragment ions and analyze their component parts. These techniques are crucial for determining the structure of molecules and identifying unknown compounds. Some common mass fragmentation techniques are discussed below. **Table 1** presents a comparison of different fragmentation techniques, and **Table 2** presents the compatibility of scaffolds with different fragmentation techniques.

Collision-Induced Dissociation (CID)

CID, also known as collisionally activated dissociation (CAD), is a widely used fragmentation technique in mass spectrometry. CID induces fragmentation of selected ions in the gas phase by allowing them to collide with neutral molecules, resulting in bond breakage and the production of fragment ions in non-volatile molecules such as peptides, proteins, and nucleic acids [7].

Mechanism

- **Ion acceleration:** The precursor ions are accelerated in the mass spectrometer, typically by an electric field, to increase their kinetic energy [7].
- **Collision with neutral gas:** In the collision cell, the accelerated ions collide with neutral gas molecules, most frequently argon, nitrogen, or helium.
- **Energy transfer:** Upon collision, some of the ion's kinetic energy is converted into internal energy within the ion. This process is governed by the conservation of energy and momentum.
- **Vibrational excitation:** The increased internal energy causes vibrational excitation of the molecular bonds within the ion [8].
- **Bond breakage:** As the internal energy increases, it eventually exceeds the dissociation threshold of the weakest bonds in the molecule, leading to fragmentation.

- **Fragment ion formation:** The molecule breaks apart, forming fragment ions (product ions) and neutral fragments [7].
- **Mass analysis:** The resulting fragment ions are then analyzed in the mass analyzer to determine their m/z ratios.

The process typically involves multiple collisions, gradually building up internal energy in the ion until fragmentation occurs. CID is generally considered a low-energy process, favoring the cleavage of the weakest bonds in the molecule. The fragmentation typically occurs at the most energetically favorable points in the molecule, often at the weakest bonds or following specific fragmentation rules. In positive ion mode, the charge is usually retained on the fragment with the higher proton affinity.

The choice of collision gas can influence the fragmentation efficiency and patterns. Heavier gases like argon can deposit more energy compared to lighter gases like helium.

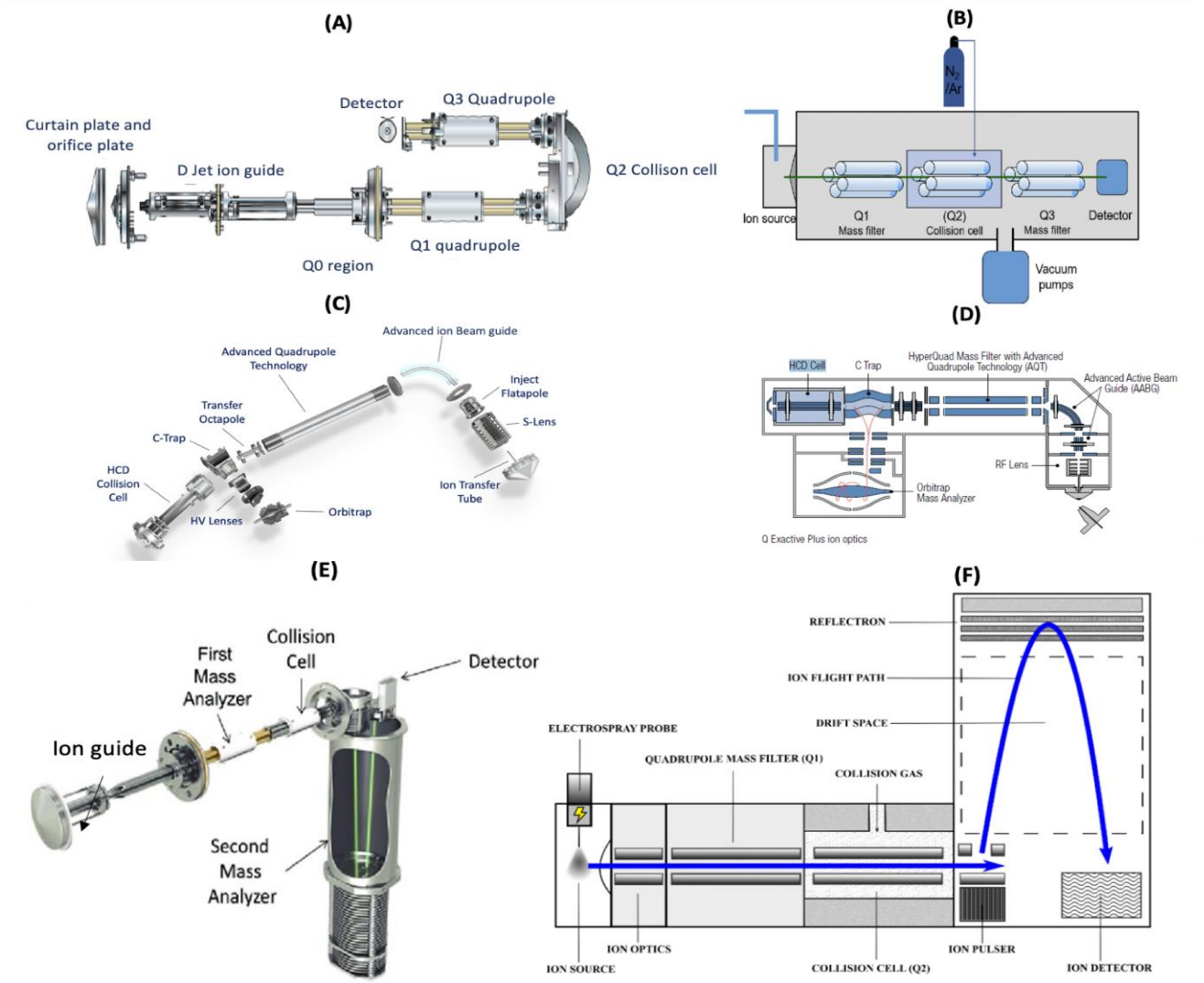

Figure 2 Representative, common mass analyzers used in mass spectrometry analysis of protein and peptides (A) Machined diagram of a triple quadrupole tandem mass spectrometer, image credit; AB sciex user manual (B) Schematic diagram of triple quadruple (Q3) mass spectrometer (C) Machined diagram of Orbitrap mass spectrometer, image credit; Thermofisher training manual
D) Schematic diagram of a Thermo QExactive Orbitrap mass spectrometer with a quadrupole mass filter (E) Machined diagram of a quadrapole/time-of-flight tandem mass spectrometer
(F) Schematic diagram of Quadrupole/Time of Flight mass spectrometer (Q-TOF)

It's important to note that the exact fragmentation patterns depend on various factors, including the molecular structure, collision energy, and instrument parameters. Understanding these mechanisms is crucial for interpreting CID spectra and elucidating molecular structures in mass spectrometry-based analyses.

Instrumentation

In mass spectrometry, CID is a commonly used fragmentation technique, and its instrumentation can vary depending on the specific mass spectrometer design. **Figure 2** represents the machined and schematic diagrams of the most commonly used mass spectrometers with the collision cell position.

QUADRUPOLE MASS SPECTROMETERS

Triple Quadrupole (QQQ) Mass Spectrometers: These instruments use three quadrupoles in series. The precursor ion is chosen by the first quadrupole (Q1), and the collision cell where CID occurs is occupied by the second quadrupole (Q2) (**Figure 1**A and 1B), while Q3, or the third quadrupole, examines the fragmented ions. This setup allows for precise control over ion selection and fragmentation [8].

Time-of-Flight (TOF) Mass Spectrometers

Quadrupole Time-of-Flight (QTOF) Mass Spectrometers: These hybrid instruments combine a quadrupole mass filter with a time-of-flight analyzer. The quadrupole selects the precursor ion, which then undergoes CID in a collision cell before the fragments are analyzed by the TOF analyzer. This combination provides high resolution and accurate mass measurements [9][10].

Ion Trap Mass Spectrometers

Linear Ion Trap (LIT) Mass Spectrometers: These instruments trap ions in a linear configuration and use CID to fragment the ions within the trap. The resulting fragments are then analyzed, often providing high sensitivity and fast scan speeds. An example is the Thermo Scientific LTQ Velos Pro.

Fourier Transform Ion Cyclotron Resonance (FT-ICR) Mass Spectrometers: These high-resolution instruments use a magnetic field to trap ions and perform CID within the ion cyclotron resonance cell. The FT-ICR MS provides ultrahigh mass resolution for CID fragments, making it suitable for detailed structural analysis.

Orbitrap Mass Spectrometers

This high-resolution instrument can perform CID, as well as other fragmentation techniques like electron transfer dissociation (ETD) and higher-energy collisional dissociation (HCD) (**Figure 1**C and 1D). It is particularly useful for proteomics and the analysis of post-translational modifications.

Ion Mobility Mass Spectrometers

Waters Synapt G2 HDMS: This instrument combines ion mobility separation with CID and other fragmentation techniques. Ion mobility provides an additional dimension of separation based on ion shape, size, and charge, enhancing the analysis of complex mixtures.

Specialized Techniques

Sustained Off-Resonance Irradiation CID (SORI-CID): Used in FT-ICR MS, this method involves cyclotron motion acceleration of ions and pressure-induced fragmentation through collisions. The high vacuum is then restored for analyzing the fragment ions [10].

High-Energy Collision Dissociation (HCD)

HCD is a variant of CID that uses higher collision energies, typically in the range of 1-20 keV. Here are the key aspects of the HCD mechanism. Higher-energy C-trap dissociation, beam-type CID, or high-energy collision dissociation (HCD) is the term for this Orbitrap mass spectrometer-specific method. HCD is typically performed in specialized or dedicated instruments like magnetic sector mass spectrometers or tandem time-of-flight (TOF/TOF) instruments [11]. It provides complementary information to low-energy CID, often yielding more extensive fragmentation and unique structural insights, particularly for larger molecules or those with specific structural features.

In HCD, the precursor ions are accelerated to much higher kinetic energies (1-20 keV) compared to low-energy CID. The high-energy ions collide with neutral gas molecules, often helium or argon. Upon collision, a significant amount of kinetic energy is rapidly converted to internal energy within the ion. This process is more energetic and occurs faster than in low-energy CID. The high internal energy causes rapid and extensive bond breakage, often

leading to more complex fragmentation patterns. HCD yields unique fragmentation pathways and can access fragmentation pathways not available in low-energy CID, including charge-remote fragmentation (cleavage of bonds far from the charge site), side-chain fragmentation in peptides, and formation of radical ions [12].

Applications

Collision-induced dissociation (CID) has several important applications in mass spectrometry and analytical chemistry. These applications demonstrate the versatility and importance of CID in modern analytical chemistry, enabling researchers to gain detailed insights into molecular structures and compositions across a wide range of scientific disciplines [13], [14].

i. **Structural elucidation:** CID is widely used to determine the structure of molecules, especially large biomolecules like proteins and peptides. By analyzing the fragmentation patterns produced by CID, researchers can deduce structural information about the original molecule.

ii. **Proteomics and peptide sequencing:** CID is essential for tandem mass spectrometry (MS/MS) proteomics experiments. Peptic digests from the enzymatic digestion of proteins undergo CID, which produces fragment ions that are specific to a particular sequence. This allows for the identification and amino acid sequencing of proteins/peptides.

iii. **Quantitative analysis:** CID is used in quantitative mass spectrometry to determine the concentration of particular molecules within a sample. This is particularly important in

pharmaceutical research, environmental monitoring, and other fields requiring precise quantification of analytes [15].

iv. **Metabolite identification:** In metabolomics studies, CID helps identify and characterize metabolites by generating fragment ions that provide structural information about these small molecules.

v. **Drug discovery and development:** CID is used to study drug molecules, their metabolites, and potential impurities. This information is crucial for understanding drug behavior in the body and ensuring drug quality and safety.

vi. **Environmental analysis:** CID helps in the identification and quantification of environmental contaminants, pesticides, and other pollutants in complex matrices.

vii. **Forensic analysis:** In forensic science, CID is used to identify and characterize drugs, toxins, and other compounds of interest in criminal investigations.

viii. **Food safety and quality control:** CID can be used to detect and quantify contaminants, additives, and other compounds in food products.

ix. **Isobaric tag-based quantification:** HCD, a variant of CID used in Orbitrap mass spectrometers, is particularly useful for isobaric tag-based quantification as it allows observation of reporter ions.

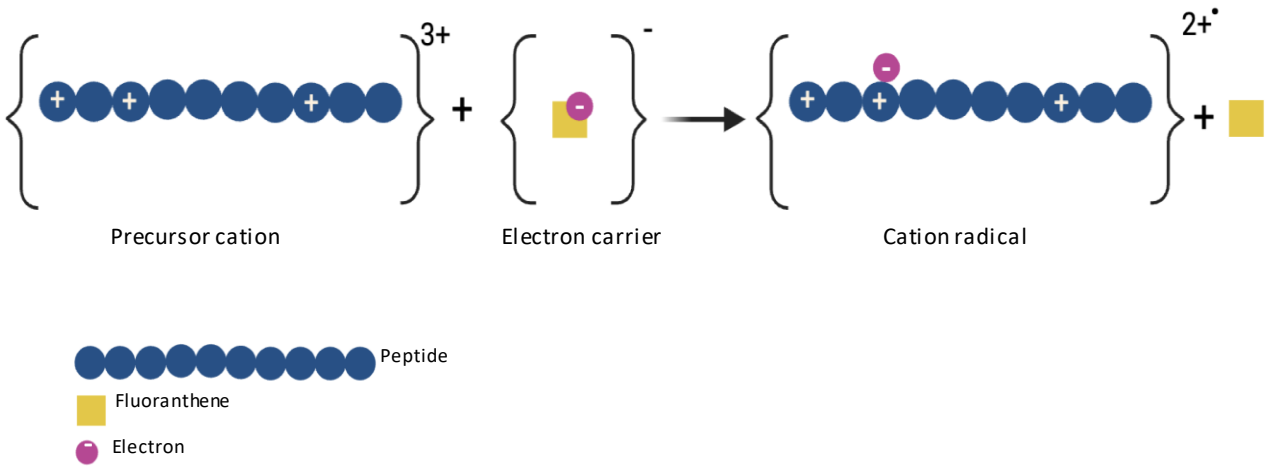

Figure 3 Multiple-charged cation from a protein or peptide precursor reacts with an anion to form a radical cation

x. **Molecular fingerprinting:** The fragmentation pattern produced by CID can serve as a unique "fingerprint" for identifying specific molecules, even in complex mixtures.

Electron Transfer Dissociation (ETD)

ETD is a commonly used method for fragmenting peptides in mass spectrometry. The technique is usually restricted to an ESI source because it needs multiply charged gas-phase cations (z > 2). The process begins with the formation of multiply charged cations (z > 2) of peptides or proteins, typically using electrospray ionization [16].

Mechanism

Ion formation: The process begins with the formation of multiply charged cations (z > 2) of peptides or proteins, typically using electrospray ionization [7].

Electron transfer: The multiply charged precursor cations react with radical anions (often fluoranthene) in an ion trap. An electron is transferred from the anion to the cation, creating an unstable cation radical [19].

Fragmentation: The unstable cation radical then undergoes rapid dissociation, primarily cleaving at the N-Cα bond along the peptide backbone [16], [17].

Product ions: This fragmentation produces c-type and z-type ions:

c-type ions: Even-electron ions containing the N-terminus [16]

z-type ions: Odd-electron ions containing the C-terminus [16]

Random cleavage: ETD randomly breaks apart side chains and post-translational modifications along the peptide backbone [16].

Charge state preference: ETD works best for precursor ions with higher charge states (>+2), making it particularly useful for larger peptides and proteins.

Complementarity to CID: ETD is often used in combination with collision-induced dissociation (CID), as they provide complementary information and work better for different types of precursor ions[18].

The ETD mechanism allows for more widespread sequence coverage and better preservation of labile PTMs compared to traditional collision-based fragmentation methods. This makes it particularly valuable for top-down proteomics and the analysis of post-translational modifications [16].

3.3.2 Instrument Compatibility with ETD

ETD fragmentation instrumentation is designed to provide enhanced peptide sequencing capabilities, improved post-translational modification analysis, and increased protein sequence coverage compared to traditional CID-only instruments. The integration of ETD capability into modern mass spectrometers has significantly advanced the field of proteomics and protein characterization [19].

ETD requires multiply charged precursor ions, so electrospray ionization (ESI) is commonly used to generate these ions. The multiply charged precursor ions are captured and stored in an ion trap. Because of their quick cycle times and large ion storage capacity, linear ion traps are especially well suited for ETD applications.

Radical anion source:

A separate source is needed to generate radical anions, typically using fluoranthene. This source is integrated into the mass spectrometer.

Reaction chamber:

The ion trap serves as the reaction chamber where the multiply charged precursor ions interact with the radical anions.

After fragmentation, the resulting c- and z-type ions are analyzed. Various mass analyzers can be used, including[9]:

1. **Linear ion trap mass spectrometers:**

   The optimum conditions for ETD reactions are created by linear ion traps' quick cycle times and large ion storage capacity. Specifically, the Thermo Scientific LTQ series linear ion traps are mentioned as being compatible with ETD.

2. **Hybrid quadrupole/linear ion trap-Orbitrap instruments:**

   These combine the ETD capabilities of linear ion traps with the high resolution and mass accuracy of Orbitrap analyzers.

The LTQ-Orbitrap ETD is mentioned as an example.

3. **Quadrupole time-of-flight (QTOF) mass spectrometers:** These hybrid instruments combine a quadrupole with a time-of-flight analyzer, allowing for accurate mass measurement and ETD fragmentation.
4. **Ion trap instruments alongside ETD capability:** For example, the Agilent 6340 Ion Trap LC/MS is mentioned as being equipped with an ETD source.
5. **Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometers:** While initially developed for electron capture dissociation (ECD), these instruments can also perform ETD analysis.
6. **Modified quadrupole linear ion trap-Orbitrap mass spectrometers:** To enable the generation of the fluoranthene radical anions required for ETD, these have been modified to accept a negative chemical ionization source.

Application of ETD

Post-translational modification: ETD is particularly useful for studying labile PTMs like phosphorylation, glycosylation, and ubiquitination. It preserves these modifications better than collision-induced dissociation (CID) or higher-energy collisional dissociation (HCD).

Phosphorylation: ETD is particularly useful for analyzing phosphorylation sites on peptides and proteins. It preserves labile phosphate groups better than collision-induced dissociation (CID), as neutral losses are preferential over peptide backbone fragmentation in terms of energy efficiency, allowing for more accurate localization of phosphorylation sites. In fact, one of the main driving forces behind the initial development of ETD was this collisional activation deficit. Decision tree methods that combine ETD and collision-based fragmentation are widely used in modern phosphoproteomic experiments to increase the number as well as assurance of phosphopeptide identifications. More sophisticated decision tree processes have been developed to initiate ETD MS/MS scans that utilize phosphoric acid neutral losses as well as evaluation of phosphosite assignments in collisional activation spectra. The supplementary activation methods of EThcD and AI-ETD have proven to be highly advantageous for ETD-based phosphoproteomic experiments. The capacity to recognize and precisely locate phosphosites in phosphopeptides is significantly enhanced by increases in the coverage of the peptide backbone and the number of sequencing ions offered by both techniques, particularly for low charge density precursors. EThcD and AI-ETD have both been applied to analyses of intact phosphoproteins. However, AI-ETD has demonstrated better performance in locating phospho-sites in the approximately 23.5 kDa, eight-site multiply phosphorylated protein α-casein. N-phosphorylation, such as that of arginine and lysine, is also characterized by ETD [18].

Glycosylation: ETD can preserve glycosidic bonds, making it valuable for studying glycosylated peptides and proteins. This is especially useful for analyzing N-linked and O-linked glycosylation. Glycan microheterogeneity, the ability of multiple glycans to modify a single glycosite, means that analyzing intact glycopeptides is essential to characterizing the glycoproteome. Glycan identity at a particular site is therefore critical to understanding the biological context of the modification. The glycan component of the precursor is typically the only information revealed by collision-based fragmentation of intact glycopeptides; little sequence information about the peptide backbone is provided. Peptide sequence clarification and site-specific glycan modification analysis are made possible by ETD, which causes almost limited dissociation of the peptide backbone while maintaining the glycan moiety integral[18].

Ubiquitination: ETD significantly outperforms CID and HCD in identifying ubiquitylation sites. In K-ε-GG peptide-enriched samples, it can result in an approximately two-fold increase in ubiquitylation site identifications.

SUMOylation: Similar to ubiquitination, ETD is effective for studying SUMOylation, another important regulatory PTM.

Acetylation: ETD can preserve acetyl groups on lysine residues, allowing for better characterization of acetylation sites.
Methylation: ETD is useful for studying methylation on lysine and arginine residues, as it preserves these modifications during fragmentation.

Citrullination: ETD can help identify citrullination sites, which involve the conversion of arginine to citrulline.

Disulfide bonds: While not strictly a PTM, ETD can be used to study disulfide bond arrangements in proteins, as it tends to cleave peptide backbone bonds while leaving disulfide bonds intact [18][18]. An EThcD workflow was created expressly to offer a precise, yet general method for mapping disulfide bonds. The ETD reaction preferentially causes the S-S bonds to cleave in EThcD of disulfide-bonded peptides (in addition to a few fragmentations of the peptide backbone). The disulfide-cleaved peptides' unreacted and charge-reduced precursor ions are subsequently dissociated by additional HCD activation, resulting in additional peptide backbone fragmentation [20].

ETD in Sequencing of Larger Peptides and Proteins or Top-down Proteomics

Whole proteins and peptides with over twenty amino acids can be efficiently fragmented using ETD. It is especially useful for top-down proteomics approaches analyzing intact proteins [21][18]. Proteins were eluted in gradient mode into the mass spectrometer for this particular type of experiment, where they were separated and interacted with fluoranthene, a radical anion. The spectrum is then simplified by reacting the resulting highly charged product ions with the even-electron anions of benzoic acid, which reduces the different charge states of the c- and z-type product ions to primarily singly charged species [21].Ion−ion reactions like ETD can be used for structural characterization in cross-linked peptides, ion mobility, native proteomics, and hydrogen-deuterium exchange.

Electron Capture Dissociation (ECD)

Electron capture dissociation (ECD) is a powerful fragmentation technique used in mass spectrometry for the structural characterization of proteins, peptides, and other biomolecules. Developed in 1998 by Roman Zubarev and Neil Kelleher in Fred McLafferty's lab at Cornell University, ECD has become one of the most widely used methods for activating and dissociating mass-selected precursor ions in tandem mass spectrometry (MS/MS) [22]. Since electron dissociation methods are low-energy fragmentation techniques, they allow molecules to be broken up in a way that preserves more labile bonds. This is especially helpful when examining larger peptides and proteins, leading to a significantly improved understanding of protein structure. ECD was limited to FTMS instruments until recently and then to some newer mass spectrometers that were not FT-based, like ion trap mass spectrometers [23][23].

Mechanism

Electron capture: A source of low-energy electrons is crucial for ECD. This is often an electron gun or a heated filament that produces electrons with energies typically in the range of 1-5 eV. The process begins when a multiply protonated peptide or protein captures a low-energy electron (typically 1-5 eV) at one of its protonated sites, such as lysine, arginine, histidine side chains, or the N-terminus [22].

Formation of a radical species: Upon electron capture, a radical species is formed, which is highly unstable and prone to fragmentation [22].

N-Cα bond cleavage: The primary fragmentation pathway in ECD involves the cleavage of the N-Cα bond along the peptide backbone, resulting in the formation of c- and z-type fragment ions. **Free radical cascade:** After the initial N-Cα bond cleavage, secondary fragmentation can occur through a process known as the free radical cascade, resulting in the production of more fragment ions [24].

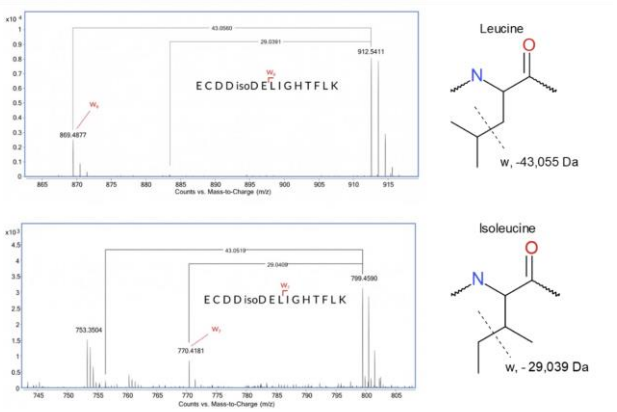

Figure 4 MS ECD spectra of Leucine and Isoleucine

Instrumentation

ECD was initially developed for and primarily used in FT-ICR mass spectrometers. Efforts have been made to implement it in other kinds of mass spectrometers, including quadrupole ion traps and

hybrid instruments [25]. This has led to variations in the specific instrumentation used, but the core principles of trapping ions and introducing low-energy electrons remain consistent across implementations. Some implementations combine ECD with time-of-flight (TOF) mass analyzers for high-resolution mass analysis of fragment ions [26].

Application

Disulfide bond analysis, de novo sequencing, top-down confirmation of DNA-predicted protein sequences, and a top-down/bottom-up combination analysis of post-translational modifications are anticipated to be the primary applications of ECD [25]. At higher electron energies, ECD can distinguish between isoleucine/leucine and Asp/isoAsp due to specific side chain fragments.

Leucine/Isoleucine Differentiation:

In contrast to CID, ECD can distinguish between leucine and isoleucine due to the production of particular side chain fragments. This makes it possible to identify the amino acid as leucine, which loses 43 Da when forming the w-ion, or isoleucine, which loses 29 Da to form a w-ion, without any ambiguity (**Figure 4**) [23].

Aspartic acid/iso Aspartic acid differentiation

Protein aging is a common issue in biopharmaceutical analysis. The deamidation of asparagine residues, which produces a mixture of aspartic acid and iso aspartic acid, is a common observation in this situation.

These two residues cannot be distinguished by CID because they have the same mass and share the same bonds with the residues next to them. With ECD, the presence of iso aspartic acid can be determined directly, thanks to a distinctive z-57 fragment.

This typically eliminates the need to create reference peptides, which take time, in order to compare the results to the reference standards for LC-MS analysis[23].

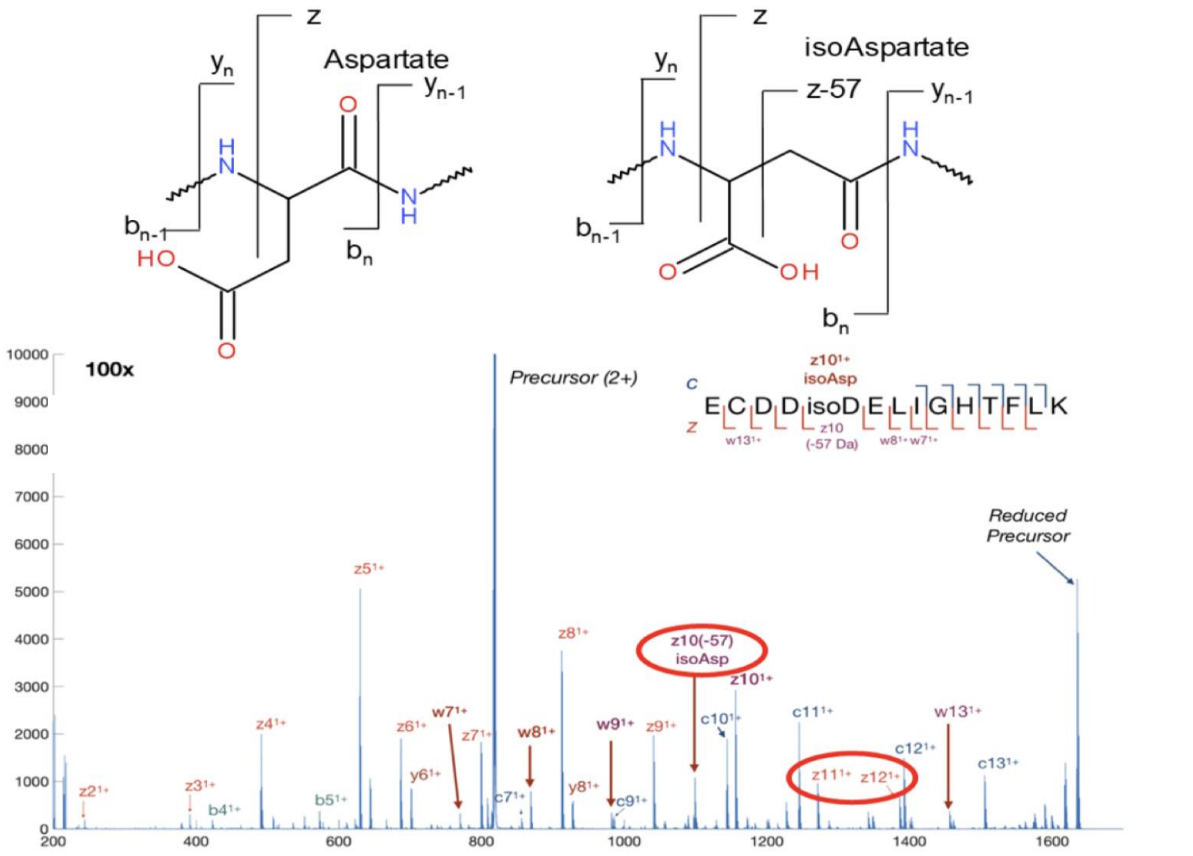

Figure 5 MS ECD spectra of Aspartate and Iso Aspartate

Table 1 Comparison of ECD, CID, ECD and ETD

Feature	CID	HCD	ETD	ECD
Full name	Collision-Induced Dissociation	Higher-energy Collisional Dissociation	Electron transfer Dissociation	Electron Capture Dissociation
Mechanism	Collision with neutral gas molecules	Collision with neutral gas molecules	Electron transfer from reagent anions	Capture of low-energy electrons
Charge State Preference	Lower (2+, 3+)	Lower to medium	Higher (3+ and above)	Higher (3+ and above)
Energy Range	Low to high energy	Typically low energy (<100 eV)	Low energy	Low energy
Fragment process	Slow, stepwise	Rapid, non-equilibrium	Rapid electron transfer	Rapid electron capture
Peptide back bond cleavage/ Fragmentation Type	b- and y-type ions	b- and y-type ions	c- and z-type ions	c- and z-type ions, d- and w-type side chain fragmentation in peptides
Instrument Compatibility	Ion traps, quadrupoles	Orbitrap mass spectrometers	Ion traps, Orbitraps	FTICR mass spectrometers
Labile modification retention	Poor	Poor	Excellent	Excellent
Preservation of PTMs	Often lost	Often lost	Preserved	Preserved
Sequence coverage	Moderate	Good	Excellent for larger peptides	Excellent for larger paptides
Advantages	Widely used, well-understood	Richer fragmentation spectra	High sequence coverage, PTM preservation	High sequence coverage, PTM preservation
Disadvantages	Loss of labile modifications	Loss of labile modifications	Requires multiply charged ions	Requires multiply charged ions
Application	Peptide sequencing, small molecules	Peptide sequencing, small molecules	PTM (Phosphorylation, glycosylation analysis)	Glycan, Isomeric species analysis

Table 2 Search Engine Compatibility with different fragmentation techniques

Name	CID	HCD	ECD	ETD
Proteome Discoverer	Yes	Yes	Yes	Yes
BioPharma Finder	Yes	Yes	Yes	Yes
Mascot	Yes	Yes	-	Yes
Peak	Yes	Yes	-	Yes
Zcore	-	-	-	Yes

Conclusion

Fragmentation techniques play a crucial role in mass spectrometry, enabling detailed structural elucidation and identification of molecules. CID remains a widely used and versatile technique, especially useful for peptide analysis and structural characterization. It offers broad fragmentation coverage and is well-suited for various instrument setups, including triple quadrupole and Q-TOF systems. HCD, a variant of CID, provides higher energy fragmentation, often resulting in more comprehensive sequence coverage for peptides and improved detection of low-mass fragment ions when used with orbitrap mass spectrometers. ETD and ECD are particularly valuable for analyzing larger biomolecules and post-translational modifications. These techniques preserve labile modifications and provide complementary fragmentation patterns to CID and HCD, enhancing the overall structural information obtained. The choice of fragmentation technique significantly influences the observed fragmentation patterns and the resulting structural information. CID and HCD typically produce b- and y-type ions for peptides, while ETD and ECD generate c- and z-type ions. The integration of multiple fragmentation techniques in modern mass spectrometry instruments allows for more comprehensive analyses, combining the strengths of each method to provide a full image of molecular structure and composition. As mass spectrometry evolves, the refinement of existing fragmentation techniques and the development of new methods will improve our ability to analyze complex molecular systems. The combination of advanced fragmentation techniques and sophisticated data analysis algorithms provides the potential to expand mass spectrometry's capabilities in both qualitative and quantitative analyses across a wide range of scientific disciplines.

Ethical Approval

No ethical approval was necessary for this study.

Author Contribution

All authors made substantial contributions to the conception, design, acquisition, analysis, or interpretation of data for the work. They were involved in drafting the manuscript or revising it critically for important intellectual content. All authors gave final approval of the version to be published and agreed to be accountable for all aspects of the work, ensuring its accuracy and integrity.

Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

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