Electron activated dissociation

A new paradigm for mass spectrometry

EAD (electron activated dissociation) provides more informed decisions, accelerating research and development and improving productivity for routine analytical applications.

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Executive summary

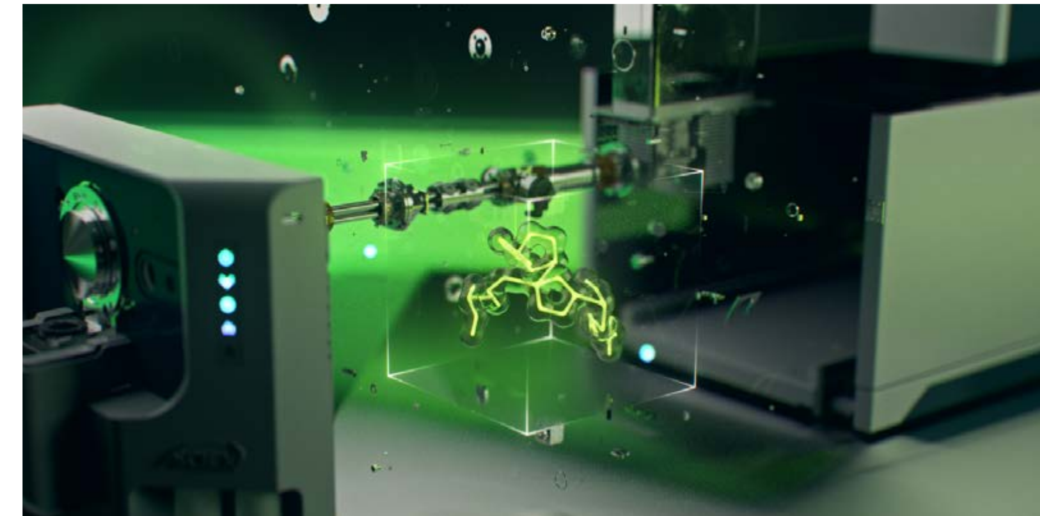
Electron activated dissociation or EAD is a ground-breaking approach for tandem mass spectrometry applications. While the industry standard collision induced dissociation (CID) has proven to be an invaluable tool for MS/MS experiments, the data produced using CID can leave gaps in our understanding of:

- Molecular structure
- Information on positional isomers
- Characterization of larger molecules including post translation modification (PTMs)
- Structural elucidation of entire compound classes that can be difficult or even impossible to fragment

Electron-based fragmentation has been demonstrated to provide vital information that is key for complete molecule identification and characterization. Its utility extends beyond CID, providing new and crucial information even for traditionally difficult compounds.

Now, with the advent of tunable EAD technology, a range of different free-electron based fragmentation mechanisms are provided within one device. The availability of both low and high energy electron fragmentation within the same design extends the utility of the approach for both small (singly-charged) and large (multiply-charged) molecules alike.

Further enhancements serve to significantly increase the sensitivity and speed of fragmentation over traditional electron-based fragmentation mechanisms, making the process compatible with fast separations using UHPLC and with the analysis of complex mixtures containing a range of compound masses and abundances. Continual investments in the development of MS/MS technologies are central to delivering tools and workflows that enable the characterization of an increasingly comprehensive suite of compound classes, molecular structures, and sample types. As such, EAD has proven to be a game-changing technology that can transform MS/MS experiments and enable the acquisition of essential fragmentation data. This white paper provides an overview of the challenges encountered with current MS/MS approaches and the significant advantages that can be realized using new tunable EAD technology. Examples show the power of EAD for structural elucidation of small molecules and metabolites, differentiation and quantification of isomeric compounds, identification and localization of protein modifications and complete characterization of lipids.



Problem statement

Tandem mass spectrometry has evolved as one of the most valuable analytical tools available to modern scientists. Introduced over 50 years ago, tandem mass spectrometry, or MS/MS, enables the identification, structural elucidation and quantification of compounds by breaking them apart and using the pieces to infer details about the original parent molecule.[1]

Most MS/MS applications today use collision induced dissociation, or CID, for inducing fragmentation. CID is the underlying framework supporting most quantitative assays and is responsible for the identification and structural elucidation of countless numbers of compounds. But as with any technology, there are certain limitations to its applicability. With CID, those limitations manifest as insufficient fragmentation of specific molecule classes, sizes, and chemistries that limits their characterization or selective quantification. As a result, there is clearly a need for improved fragmentation mechanisms to address the shortcomings of CID.

Electron activated dissociation, or EAD, addresses those shortcomings by providing a rich assortment of new and different diagnostic fragment ions, and by enabling the dissociation of intractable compounds previously difficult or impossible to fragment using CID. EAD encompasses a range of electron-based fragmentation mechanisms that differ by the kinetic energy of the irradiating electron beam and the charge states of the precursor ions dissociated. Thus, EAD provides the flexibility for fragmenting both singly- and multiply-charged ions and can be applied to the characterization of both small molecules and larger biomolecules. Because EAD fragmentation is both fast and highly sensitive, structural elucidation of low-level compounds and variants is possible, even during fast chromatographic separations. Workflows that traditionally use CID are amenable to EAD such as in-depth analysis of complex biological mixtures, with EAD now delivering new information that can clarify molecular structures.

EAD can be used for the analysis of a wide range of molecules possessing vastly different chemistries and molecular weights. Accordingly, many applications can benefit from EAD, including those within life science research, environmental, food, and forensic analysis, drug development and clinical studies. The new information that EAD provides allows scientists to make faster more informed decisions, accelerating research and development as well as improving productivity for routine analytical applications.

Key benefit

The ZenoTOF 7600 system with EAD combines high sensitivity with high information content to enable the characterization of important, and previously difficult to characterize metabolites.

The Goldilocks principle of MS/MS

There are a variety of MS/MS scan types for qualitative studies in use today, but the most widely used is undoubtedly the product ion scan. The general approach for product ion MS/MS involves selecting precursor ions based upon their mass, fragmenting those ions, and then analyzing the array of resulting product ions. This is accomplished using two stages of mass filtering or scanning with a fragmentation event in between. The masses of the product ions as well as the mass differences between product ions are used in determining the structure or sequence of the original parent molecule. The nature of specific chemical bonds can also be surmised from the energy required for their fragmentation.

For MS/MS to be effective, the fragmentation must be interpretable and reproducible. Additionally, the energy transferred for fragmentation must be "just right" – that is, enough to break the precursor into diagnostic product ions, but not so much that it obliterates the parent molecule into pieces so small that they lose specificity, destroying more informative higher mass fragment ions (Figure 1). Even with the right amount of energy, the fragmentation can still be lacking in many ways. With refractory compounds, there can be a failure to produce enough fragmentation leaving bare areas within the spectrum with no fragment ions for interpretation. Specific diagnostic fragment ions can be missing that are precisely the key for discerning fine details in structure, such as isomers. Important side chains and modifications can be cleaved off from the parent molecule leaving no indication of its location within the molecule. Alternatively, all of the fragmentation energy might be directed to the side chain or modification itself, again leaving little indication of its attachment point.

As a result, many different mechanisms have been used for fragmenting ions over the years including the use of photons, electrons, atoms, molecules, and even solid surfaces, with some methods more compatible than others with specific mass analyzers.[2] But overall, the most common and widespread techniques use atoms, molecules or electrons to impart energy for fragmentation.

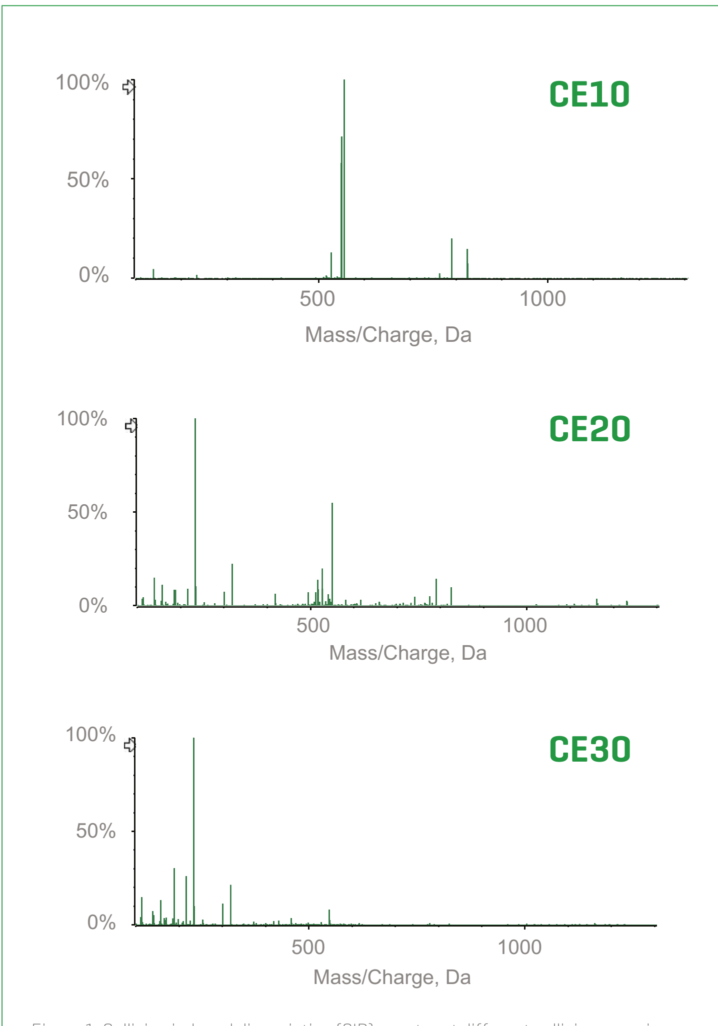


Figure 1. Collision induced dissociation (CID) spectra at different collision energies. As the collision energy is increased during MS/MS, the fragments created will change with large fragments typically observed with lower CE values (top) and smaller fragments observed with higher CE values (bottom).

EAD workflow flexibility

Isomeric amino acids

Glycosylation

Phosphorylation

Disulfides

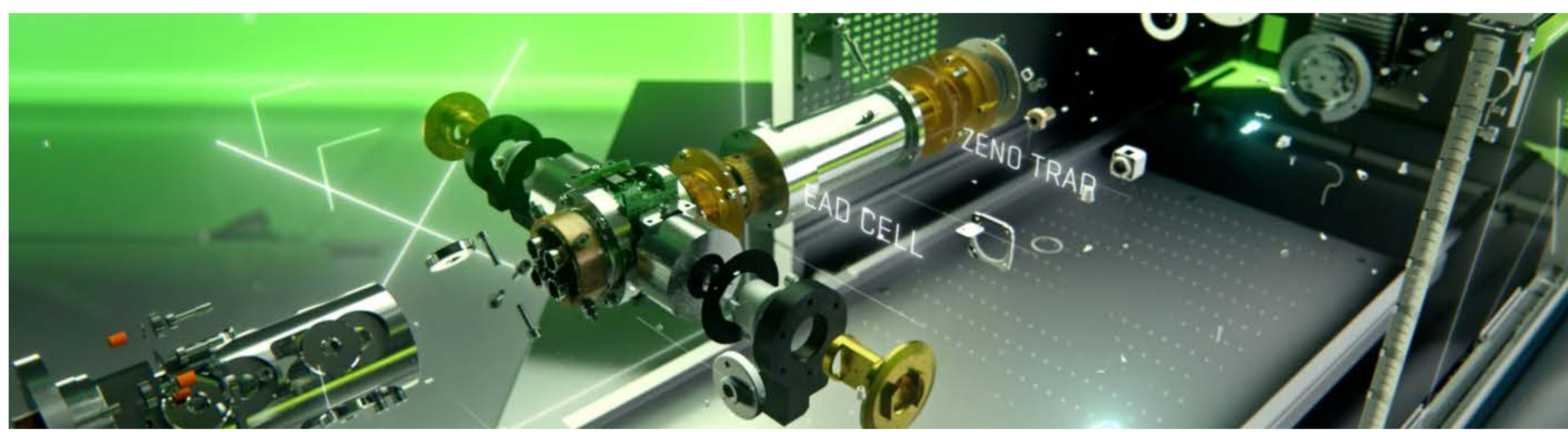
Lipidomics

Met ID

Metabolomics

Pesticides

New approaches to challenging problems



Collision induced dissociation, or CID, is the underlying mechanism used for most research and commercial assays in drug development, food, water and environmental testing, forensic studies, omics research (proteomics, lipidomics, metabolomics), biomarker experiments and clinical assays. But CID does have its limitations with certain molecules and classes of compounds.

Electron-based fragmentation mechanisms can address many of the shortcomings of CID with enhanced qualitative data that can greatly extend the capabilities of tandem mass spectrometry for structural elucidation. One drawback of most commercially available electron-based fragmentation devices is that they support either small molecule analysis or large molecule analysis, but not both. Electron transfer dissociation (ETD) and electron capture dissociation (ECD) both require multiply-charged precursor ions that capture low-energy electrons to induce fragmentation. Conversely, electron impact excitation of ions from organics (EIEIO), and other higher energy electron fragmentation techniques, fragment singly-charged ions. Thus, in some cases where both CID and ETD are available, a third fragmentation technique is used to fill the void, such as ultraviolet photodissociation (UVPD). For simplicity and general applicability, an ideal electron-based fragmentation device would enable a range of EAD electron energies and remove any requirement for a reagent (as in ETD).

Recently, a tunable EAD device was demonstrated on a quadrupole time-of-flight mass spectrometer.[3] The nature of the device allows simultaneous capture of ions and electrons. The ability to tune the device allows the kinetic energy of the electron beam to be adjusted to provide the best fragmentation for the molecule of interest. Large molecule applications using low-energy electron dissociation are possible, as well as small molecule applications better suited to fragmentation using higher electron energies. Thus, both low-energy and high-energy electron voltage fragmentation experiments are possible in addition to CID.

Small molecules

Research and development labs are continuously challenged to characterize small molecules. CID is typically the first choice for tandem mass spectrometry experiments but can sometimes leave a puzzling absence of information when it is most needed. CID can produce limited or non-selective fragmentation, leading to inconclusive spectra and sub-par quantitative assays. With small molecules, the compounds that make up different molecule classes, such as pesticides, metabolites or lipids can be extremely diverse in size, polarity and solubility. And the low levels of these compounds in matrix further compound the problem.

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Challenges with metabolite identification

Identification of drug metabolites is a required step during drug development. Difficulties can arise when trying to fully characterize positional isomers and conjugations. These difficulties can be further compounded because:

- Relevant circulating metabolites are of very low abundance
- Ionization suppression can occur when analyzing these molecules from complex matrices
- Fragmentation using CID is often lacking and unable to provide the informative structural information necessary for complete characterization.

An example is shown in Figure 2. that illustrates the difficulties that can sometimes be encountered with CID. Both the EAD and CID spectra for the drug darunavir are shown. The EAD spectrum produces an important fragment ion with the glucuronide preserved, which allows localization of the glucuronide modification. Additionally, the EAD spectrum is more information-rich with over 250 fragment ions overall, further aiding in the full characterization of the metabolite. As an added bonus, because the EAD was performed on a QTOF-based platform, low-mass fragment ions are preserved, which helps with the structural elucidation. These ions can be lost when performing MS/MS on trapping instruments.

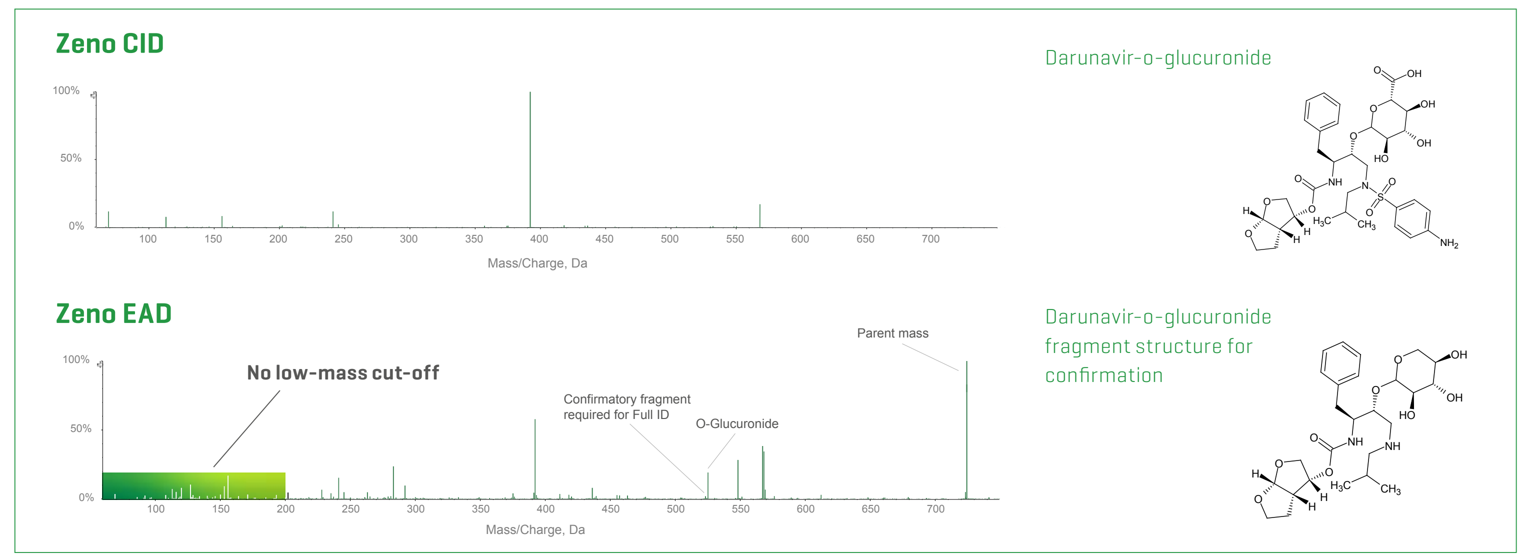
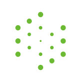


Figure 2. The EAD spectrum. EAD fragmentation of darunavir contains an important fragment ion with the glucuronide preserved, which allows localization of the glucuronide modification. Also, low-mass fragment ions are preserved by using a QTOF-based platform.



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Complete characterization of lipids in a single experiment

Lipids are a complex group of compounds with subtypes that share a similar high-level structure. For example, triglycerides consist of a glycerol group bonded to three long hydrocarbon chains with additional functional head groups attached in some cases. Small but meaningful differences between lipid species, such as the location of a single double bond along the hydrocarbon chain can have important implications for health and disease. In one experiment, EAD provides all of the information for complete lipid characterization that normally requires multiple technologies and experiments.

Complete characterization of lipids involves the identification of:

- Head group
- Backbone
- Regio-isomerism
- Double bonds
- Cis/trans isomerism

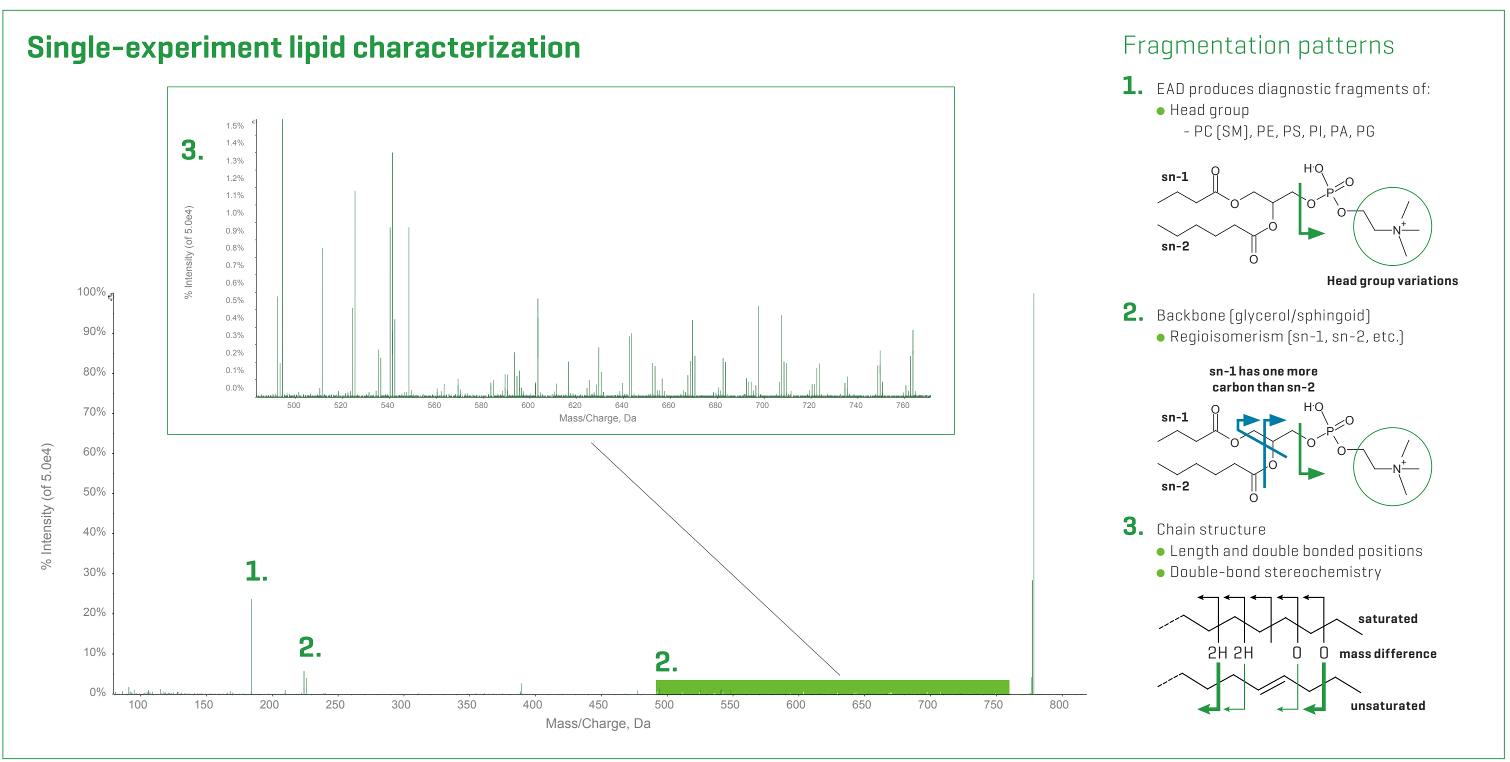
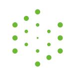


Figure 3. Single-experiment lipid characterization. The complete de novo identification and characterization of the lipid PC 16:0 / 18:1(n-9:cis) in a single experiment.



Challenges with small polar metabolites

The numbers and types of small molecules that make up the metabolome for any given biological system are vast and complex. Although these compounds span a range of different structures, sizes, chemistries and abundance, the metabolome is also composed of a multitude of compounds that differ only slightly in their make-up. As a result, metabolomics studies are particularly challenged in trying to identify and quantify the vast range of very different compounds and in differentiating between extremely similar compounds within one sample.

The CID and EAD spectra for 3,5-cAMP are shown in Figure 4. The power of EAD lies in the production of deeper, more rich fragmentation spectra for structural elucidation/identification. For cAMP, utilizing EAD resulted in an increase from 6 fragment ions to 50 fragment ions.

In addition to the increase in fragment coverage, the alternative selectivity with EAD can be utilized for quantification. While in this case the two isomers of cyclic AMP are fully resolved chromatographically, for 3,5-cAMP, a fragment ion with nominal mass of m/z 165 is generated and, when extracted as an XIC, shows no interference from the 2,3 isomer (Figure 5). The unique diagnostic fragment ions produced by EAD leave no doubt as to the precursor identity.

Comparison of CID and EAD

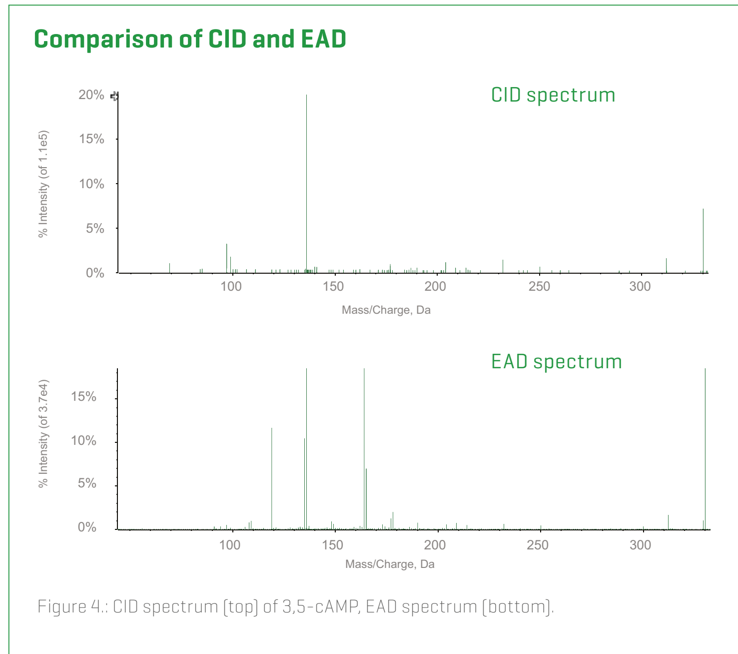


Figure 4.: CID spectrum (top) of 3,5-cAMP, EAD spectrum (bottom).

Quantitative extraction of EAD fragment ions

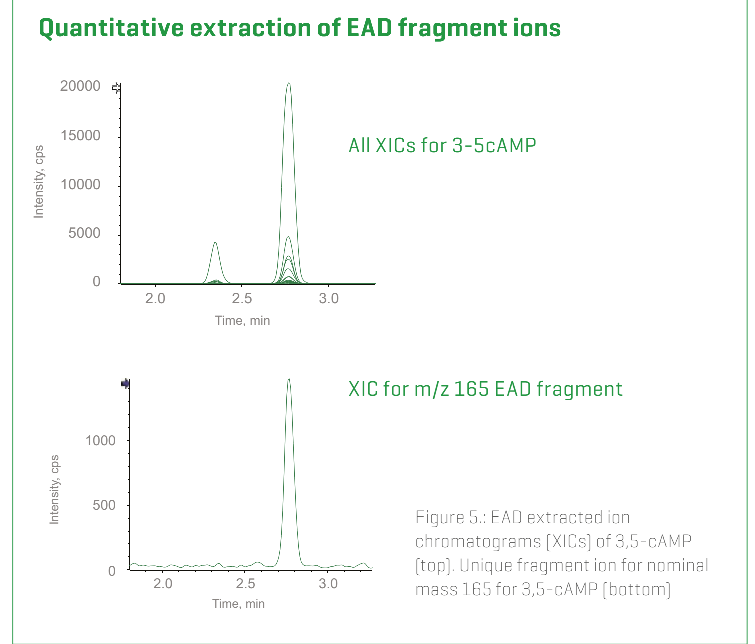


Figure 5.: EAD extracted ion chromatograms (XICs) of 3,5-cAMP (top). Unique fragment ion for nominal mass 165 for 3,5-cAMP (bottom)

Challenges of pesticide identification

There are over 1000 pesticides in use around the world today with many different chemistries and toxicities. Human toxicity will depend upon several factors including the chemical nature of the pesticide, its metabolism within the body, exposure levels and use. Because of the inherent poisonous nature of pesticides, they can also be harmful to the environment if they come in contact with, susceptible plants and animals.. Additionally, contact with sunlight, water and other chemicals can cause certain pesticides to degrade, leading to even more toxic breakdown products within the environment.

While approved pesticides have acceptable residual levels that are set by regulatory agencies, there are no acceptable levels for banned pesticides. Testing for residual pesticides is an important task to ensure the safety of human and animal food products, field crops, such as cotton and grass, and the environment.

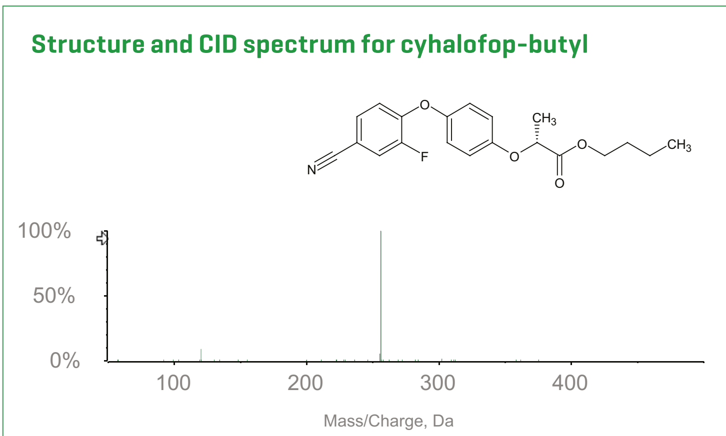


Figure 6. The CID spectrum is shown for cyhalofop-butyl with 1 major and 2 minor fragment ions generated.

Because there are so many different types of pesticides, multi-residue testing is a common workflow for pesticide analysis. Library matching is typically used in order to identify and confirm specific pesticides. Figure 7. compares the EAD and CID LC-MRM^{HR} spectra for the fungicide cyhalofop-butyl. While CID only produces 2 fragment ions, EAD produces a much richer spectrum with over over 60 fragment ions that can be used for vastly improved specificity for library matching and ion ratios.

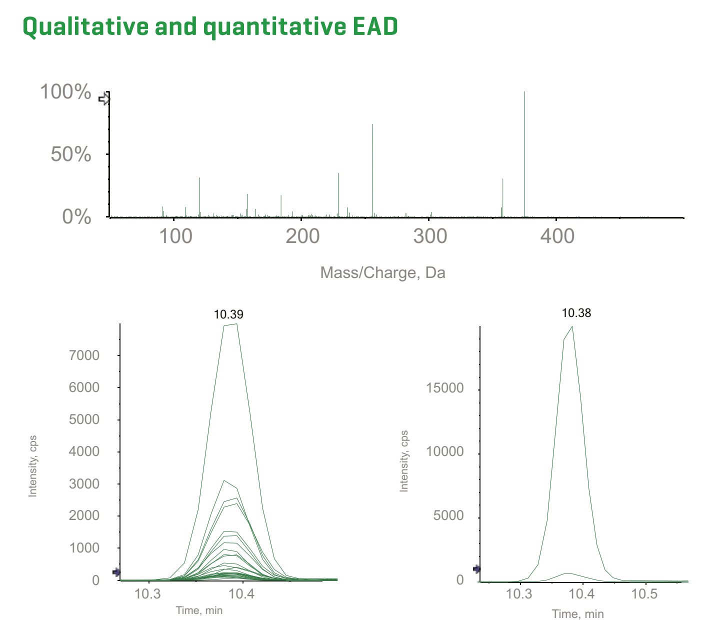


Figure 7. The EAD spectrum (top) is shown with multiple (>60) fragment ions observed. Using EAD with many more choices for targeted MRM^{HR} as shown in the XIC plots (left), compared with only 1 minor and 1 major fragments for targeted MRM^{HR} using CID (right).

Large biomolecules

Proteins as a class come with a rich assortment of varying structures and modifications, often existing as extremely complex and heterogeneous mixtures within biological fluids. As a result, the acquisition of MS/MS data is imperative for their identification, characterization and quantification.

CID has been used extensively for determining the structure and sequence of large biomolecules such as peptides, poly saccharides and oligonucleotides, but full characterization of biomolecules and their modifications can be difficult or impossible using CID.

EAD can provide a more complete picture. With EAD, fragmentation of large multiply-charged precursor ions can be induced by the capture of lower-energy electrons. Different fragment ions are produced than those typically observed with CID.[7] For example, with peptide fragmentation, CID typically produces “b” and “y” ions, while EAD produces “c” and “z” ions. These ions enable sequencing of the peptide amino acid backbone through examination of the mass differences between sequential ions within a series.

Challenges of isomeric amino acid residues

Some amino acids are identical in mass, such as aspartic acid/isoaspartic acid and leucine/isoleucine, making their differentiation impossible using low-energy CID. Using EAD, however, these isomers can be identified from additional fragment ions that are produced. For example, with leucine/isoleucine, a secondary fragment ion called the w-ion, caused by further fragmentation of the backbone z-ion, can be used for identification. With leucine, the z-ion loses 43 Da from the side chain, while for isoleucine the z-ion loses 29 Da from the side chain (Figure 8).

An example of EAD for leucine/isoleucine differentiation is shown in Figure 8. At the top, EAD clearly indicates the identity of two leucine residues within this peptide sequence through the loss of 43 Da from the z6 and z13 ions. At the bottom, loss of 29 Da from the z5 ion identifies an isoleucine within this peptide sequence.

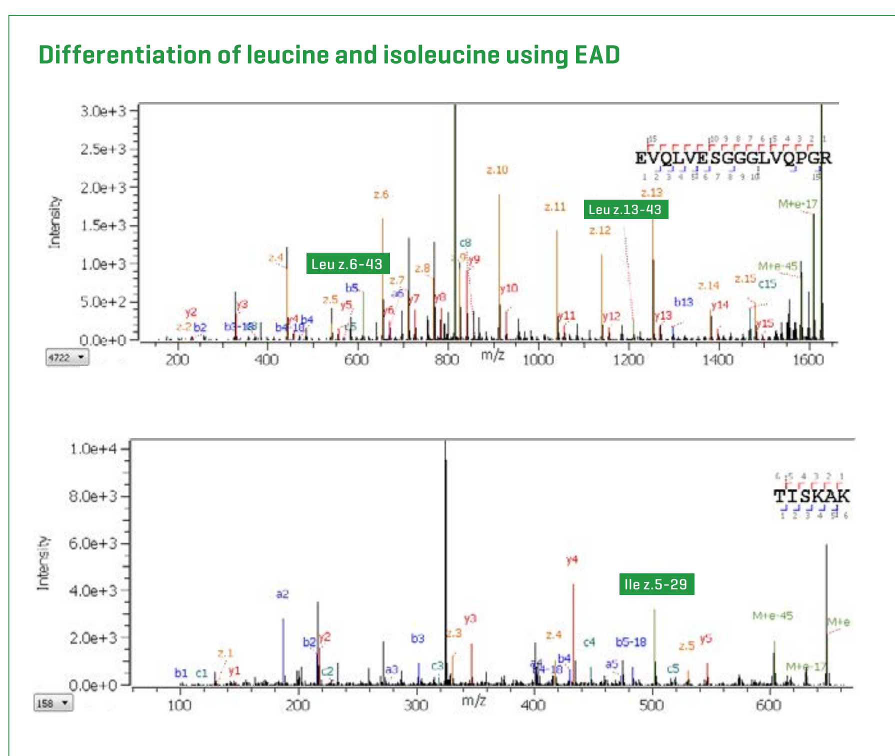


Figure 8. EAD clearly indicates the identity of two leucine residues within this peptide sequence through the loss of 43 Da from the z6 and z13 ions (top). At the bottom, loss of 29 Da from the z5 ion identifies an isoleucine within this peptide sequence.

Leucine

Isoleucine

Key takeaway

EAD enables the differentiation of isomeric amino acids that otherwise cannot be differentiated using conventional low-energy CID.

Challenges of disulfide linked peptide characterization

Disulfide bonding through cysteine residues plays an important role in protein structure and function. However, identifying and locating disulfide bonds, including the peptide sequences that surround both cysteine residues, can be a difficult task using traditional CID LC-MS/MS approaches. With CID, often the peptides will fragment along their backbones until reaching the cysteine residues, providing only partial information for the peptide sequences. This also makes determining the exact location of the cysteines and disulfide bond along the peptide backbone more difficult to identify.

Figure 9 compares the LC-MS/MS spectrum obtained using EAD versus CID for a disulfide-bound peptide from the hinge region of a monoclonal antibody. Fragmentation of this precursor using CID produces both b and y type fragment ions along both peptide backbones, up to the cysteine residue.

At this point, fragmentation stops, as CID typically does not cleave the disulfide bond. This leaves the residual peptide backbones still connected and an absence of data for complete sequencing. In contrast, EAD fragmentation shows a preference to cleave the disulfide bond, leading to superior sequence coverage that enables the identification of the linked peptides and locations of cysteine residues.

EAD and CID comparison of a disulfide peptide

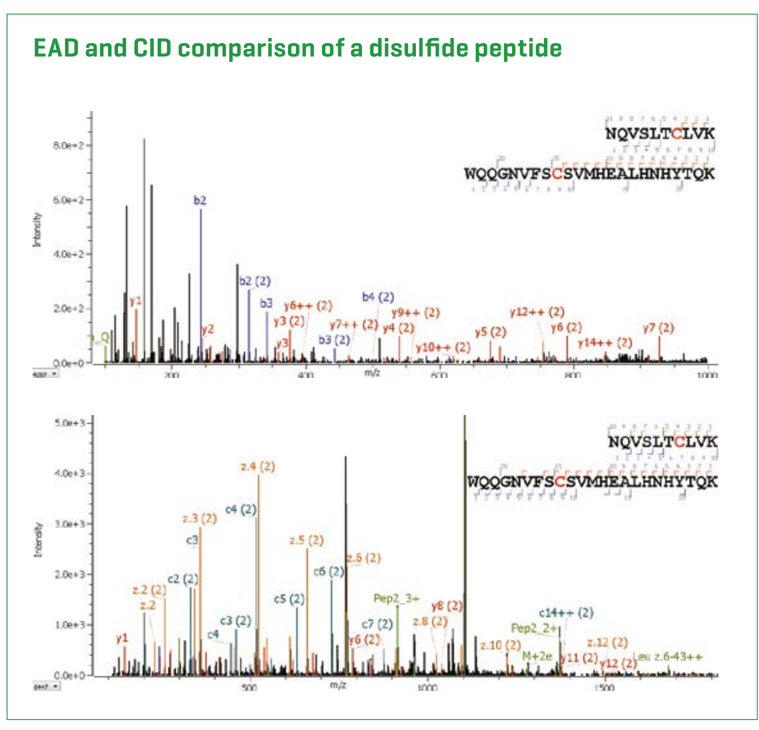


Figure 9. EAD and CID LC-MS/MS of a disulfide bound peptide. The CID spectrum (top) contains fragment ions up to the cysteine residue leaving a lack of information for the complete peptide sequences. In contrast, EAD (bottom) dissociates the disulfide bond linkage providing greater sequence coverage along both peptide backbones.

Key takeaway

EAD fragmentation of disulfide-linked peptides exhibits a preference to cleave the disulfide bond leading to superior sequence coverage and peptide identification.

Challenges of identifying and localizing phosphorylation

Another area where EAD excels is in the characterization of post-translational modifications on peptides. Some types of post-translational modifications can be quite labile and fragmentation using CID can cause cleavage of these labile groups from the backbone peptide, thereby losing their attachment point and making their full characterization difficult. Alternatively, EAD fragmentation can retain these modifications. Thus, while CID can provide basic sequence information and indicate a modification exists somewhere within the peptide, EAD can identify both the type and site of a modified residue, confirming exactly which residue along the backbone is modified.[8]

Figure 10 shows the LC-MS/MS EAD spectrum of a phosphopeptide acquired using a ZenoTOF 7600 system. The location of the phosphorylated residue is easily identified and confirmed with multiple c- and z- ion series fragments and 100% sequence coverage along the peptide backbone. Because the phosphoserine residue is lost as an intact unit, rather than simply losing the phosphate group from the serine (as often happens with CID), both the identification of serine as the modified amino acid, and the location as the 10th residue from the N-terminus, are provided.

Previous studies using external trapping devices for electron based fragmentation have shown they can increase sensitivity without increasing the probability of adverse side reactions such as neutralization, loss of larger fragment ions, and production of charge reduced species.[9] As shown in Figure 10, the spectrum acquired using the Zeno trap shows increased sensitivity versus the spectrum acquired without the Zeno trap. This added sensitivity is particularly useful when characterizing post-translationally modified peptides such as phosphopeptides as their abundances are typically far lower than unmodified peptides.

Zeno EAD of a phospho peptide

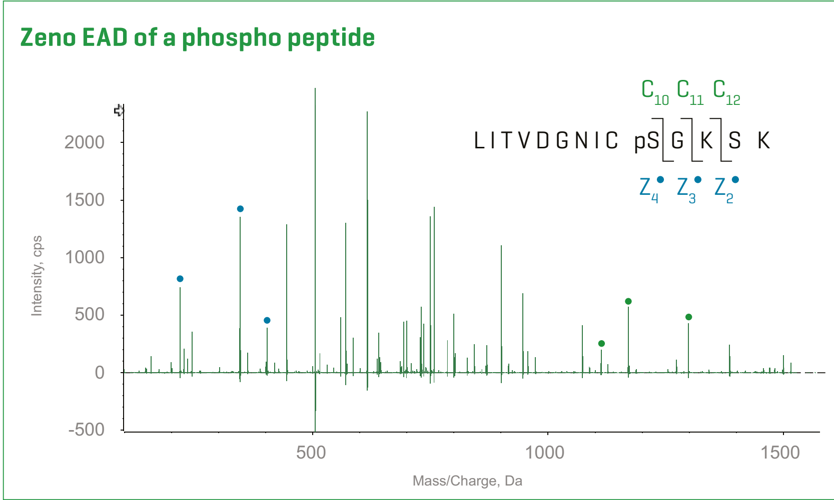


Figure 10. EAD spectrum of a phosphopeptide from bovine bovine NDUFA10 subunit of Complex I (P34942) using a 7eV electron energy. Extensive c- and z- fragment ions are produced with 100% sequence coverage enabling the site and type of modification to be deduced. Site differentiating ions are labeled. The spectrum on the top uses the Zeno trap to increase sensitivity while the mirror spectrum on the bottom shows the comparison without the use of the Zeno trap.

Key takeaway

Phosphorylation, an important regulatory modification on proteins can not only be identified but also localized using EAD.

Challenges of identifying and localizing glycosylation

Protein glycosylation is another important post-translational modification that impacts biological activity. Glycosylation can affect protein folding, protein stability, solubility, cell adhesion and is important in various regulatory processes. While CID of glycopeptides can provide information about the structure of the glycan modification, fragment ions that show loss of the modification still attached to the backbone residue are typically missing.

Yet, this specific fragment ion is necessary for localizing the attachment point of the glyco group on the peptide backbone. In contrast, EAD spectra are often contains this fragment ion along with other peptide backbone fragment ions, making localization straightforward.

Additionally, the mass difference between fragment ions on both sides of the attachment point enable calculation of the glycan molecular weight. Thus EAD and CID are quite complementary for glycopeptide analysis.

Zeno EAD IDA of an N-glycopeptide

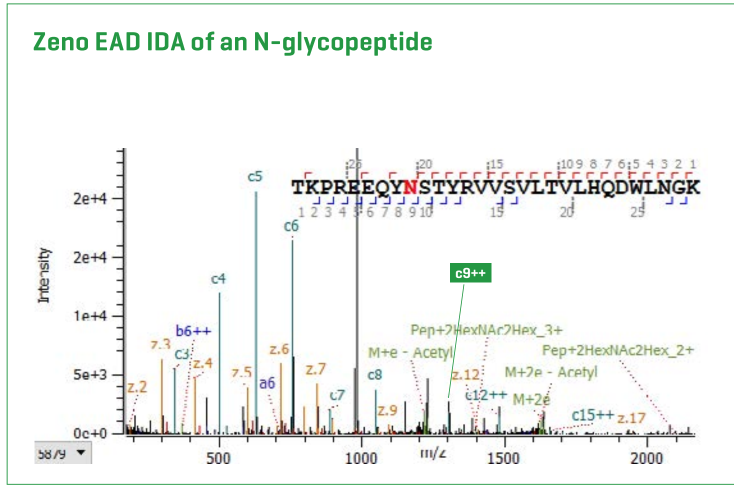


Figure 12. EAD provides both peptide sequence and glycan localization (c9++ ion).

Zeno CID IDA of an N-glycopeptide

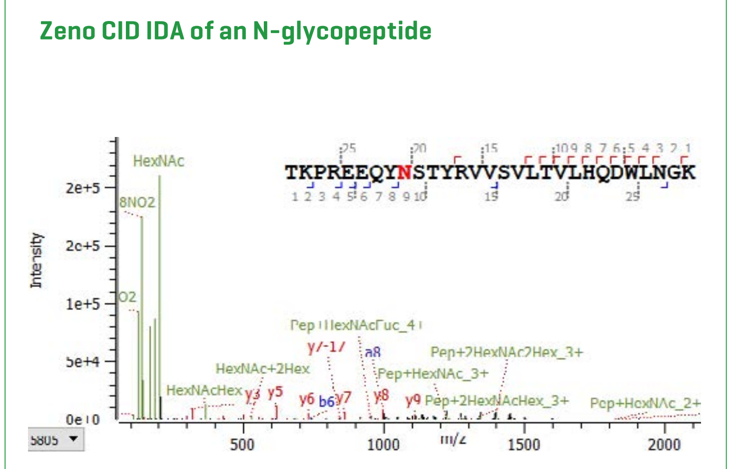


Figure 13. CID only shows glycan loss or peptide backbone information, not both in the same MS/MS spectrum in a descriptive manner; localization is not possible.

Fast and comprehensive LC-MS/MS glycan characterization

Alpha-2-HS-glycoprotein, or fetuin, is a protein that contains both N- and O-linked glycosylation. Tryptic digestion of this protein produces a complex mixture of glycopeptides including a large 61-residue peptide with four O-linked glycosylation sites that could be modified in a wide variety of glycan combinations. The difficulty of analyzing O-linked glycopeptides in general, coupled with the difficulty in fragmenting a large 61-residue peptide that is just one of many within a complex mixture, make the characterization of fetuin glycosylation particularly challenging.

Recently, EAD was demonstrated for the site-specific analysis of bovine fetuin O-linked glycosylation.[11] While previous studies have partially characterized fetuin O-linked glycopeptides using infusion and long ETD accumulation times,[12] the current study used UHPLC and fast EAD on a ZenoTOF 7600 system to fully characterize 57 O-glycopeptides with different glycosylation patterns and partially characterize another 22 O-glycopeptides.

Figure 11 shows an example for one O-linked glycopeptide from fetuin that contains two glycans. Here, EAD enables both the identification of the glycans as well as the localization to specific residues along the peptide backbone.

Today, fast fragmentation and MS/MS acquisition on newer instrumentation such as the ZenoTOF 7600 system allows EAD reaction times on the order of 10-30 msec.[13,14] Importantly, high resolution is maintained which is extremely important for structural elucidation, particularly for larger mass compounds. Practically speaking this means that data dependent LC-MS/MS experiments using EAD are now compatible with fast UHPLC chromatography and can be used for the in-depth analysis of complex biological mixtures.

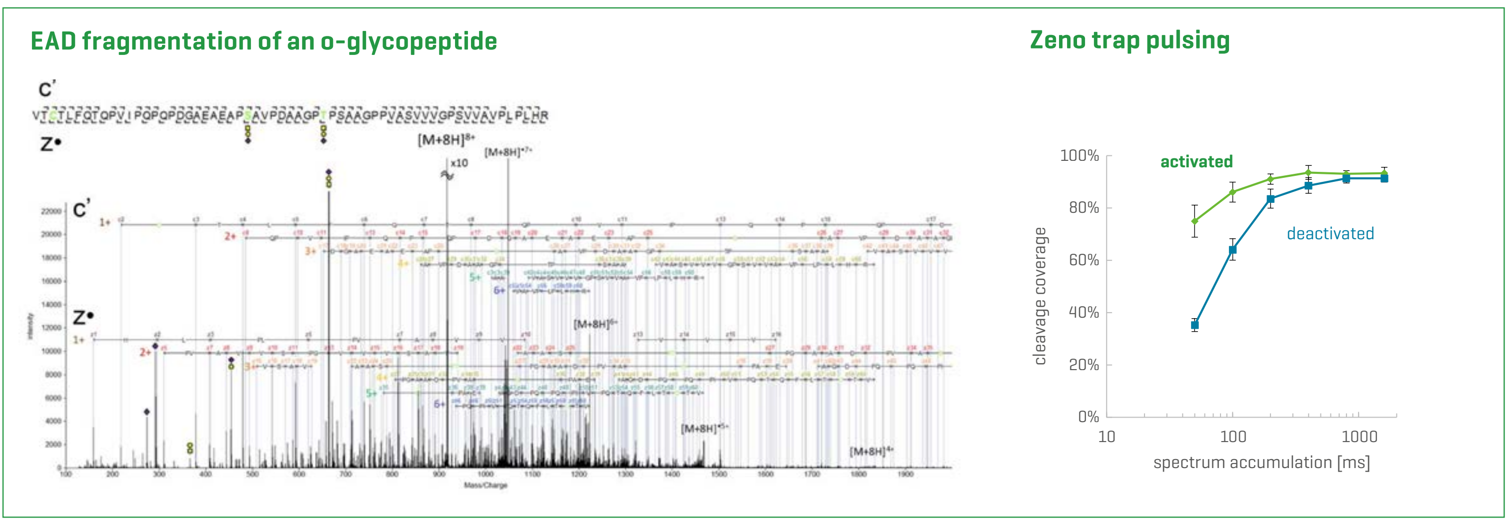


Figure 11. O-glycosylation requires comprehensive fragment coverage to confirm the glycan and also the site localization. Higher energy EAD (hot EAD) for a complex O-glycopeptide shows near complete sequence coverage and both glycans localized in the centre portion of the peptide . (Right) Activation of Zeno trap for significant sensitivity improvements provides high peptide cleavage coverage even at fast spectrum accumulation times.

N-glycosylation

N-linked glycosylation is directed by the specific consensus sequence NXS/T along the peptide backbone. This is defined as an asparagine (N), any residue except proline (X), and either a serine (S) or threonine (T). This makes localization of attachment points easier as it limits the number of N residues that comply with the attachment rules.

Figure 14 shows the assignment of an N-glycosylation site on a peptide from the enzymatic digestion of alpha-2-macroglobulin using an error tolerant database search (Mascot), a workflow typically used in protein identification and characterization experiments. Near complete c- and z-series fragment ions pinpoint asparagine at position 1424 (N1424) as the site of glycosylation with a Hex(5) HexNAc glycan attached.

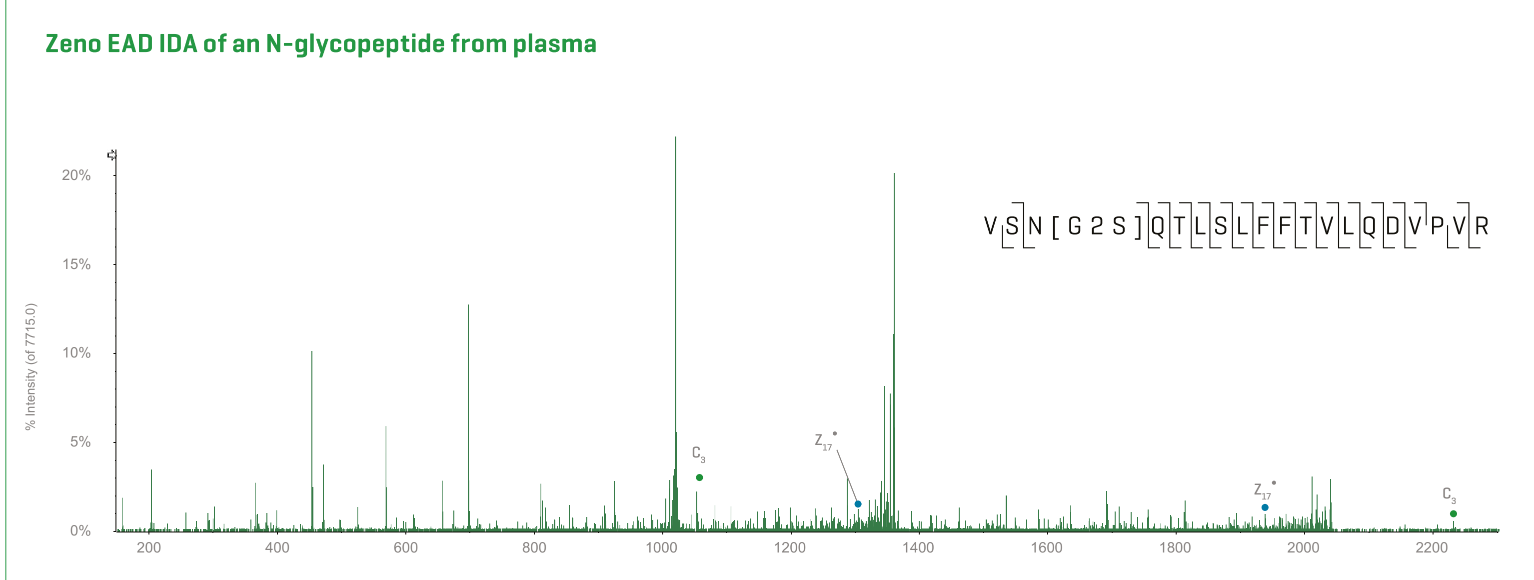


Figure 14. EAD of an N-glycosylated peptide. EAD LC-MS/MS analysis of an N-linked glycopeptide from the tryptic digestion of alpha-2-macroglobulin shows near complete c- and z-series fragment ions that identify and localize the type and site of modification as Hex(5) HexNAc on N1424 through the loss of N+1914 Da.

O-glycosylation

In contrast to N-linked glycosylation, no unambiguous consensus sequence exists for O-linked glycosylation, which can theoretically occur on any serine (S) or threonine (T) residue. Additionally, O-linked glycosylation can occur as dense clusters with many serines and/or threonines modified in close proximity to one another.

Figure 15 contains the assignment of an O-glycosylation site on a peptide generated from the enzymatic digestion of inter-alpha-trypsin inhibitor using a Mascot error tolerant search. The nearly complete c- and z-series fragment ions identify T653 as the site of glycosylation with a Hex(1) HexNAc(1) NeuAc(1) glycan attached (656 Da).

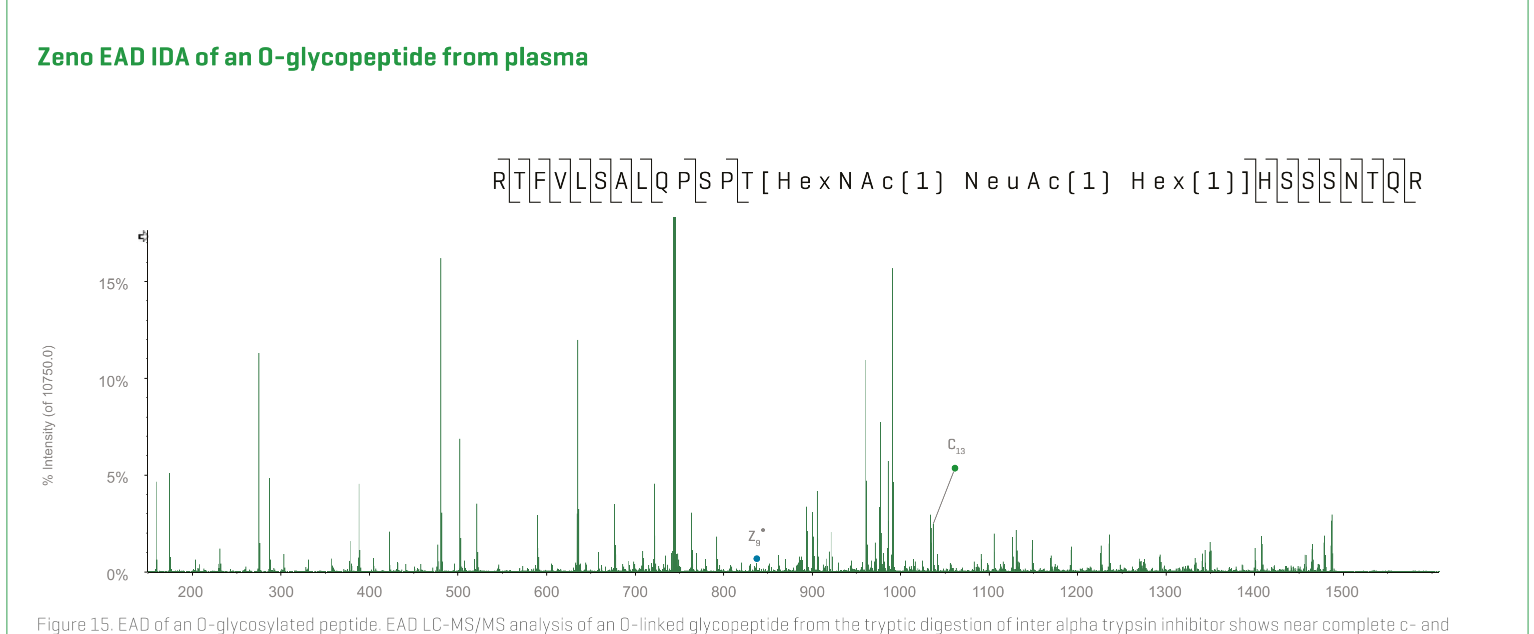
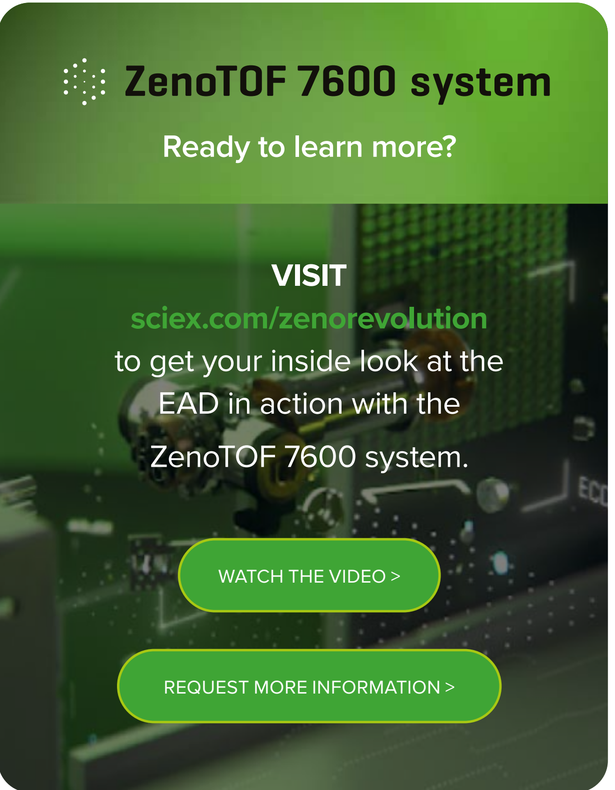


Figure 15. EAD of an O-glycosylated peptide. EAD LC-MS/MS analysis of an O-linked glycopeptide from the tryptic digestion of inter alpha trypsin inhibitor shows near complete c- and z-series fragment ions that identify and localize the type and site of modification as Hex(1) HexNAc(1) NeuAc(1) on T653 through the loss of 656 Da.

Summary

EAD is an exciting and innovative new technology for tandem mass spectrometry applications. The development of a fast, sensitive and tunable EAD device enables the acquisition of a richer data set versus traditional CID approaches. With EAD, critical information is provided that is often missing from CID MS/MS experiments. This information leads to the characterization of previously intractable compounds, greater clarity of molecular structures, differentiation of isomers, localization
of modifications and identification of low-level variants. Entire application areas and compound classes are now accessible due to the benefits that MS/MS can provide through EAD.

The electron energies used in the novel tunable EAD device on the ZenoTOF 7600 system can be adjusted to suit the precursor of interest. Thus, both singly-charged precursors, such as lipids and metabolites, as well as multiply-charged precursors, such as peptides, can be analyzed. The ability to rapidly adjust the electron energy makes EAD compatible with fast UHPLC chromatography. Additionally, the sensitivity provided by the Zeno trap of the ZenoTOF 7600 system enables in-depth analysis of complex mixtures containing compounds with a wide variety of abundances, molecular weights and chemistries. These developments in EAD technology will allow more scientists and researchers to take advantage of the benefits of electron based fragmentation for structural elucidation and quantitative assays.



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