

# ETD and ECD Mass Spectrometry Fragmentation for the Characterization of Protein Post Translational Modifications

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## 1. Introduction

The introduction of electron capture dissociation (ECD) by McLafferty and co-workers, and further of electron transfer dissociation (ETD), mechanism allows gas-phase fragmentation of multiply charged protein and peptide ions upon capture of a low-energy (<1 eV) electron or electron transfer in a gas phase ion-ion chemistry. The odd-electron species then undergoes rearrangement with subsequent cleavage of N-C<sub>α</sub> backbone. Peptide fragmentation can take place inducing the formation of c- and z- type fragment ions without loss of the information on the PTM localization. The key to the success of this approach is the selection of intact protein molecular ions and its profound potential for PTM characterization as alternative to vibrational excitation techniques. With ECD, that occurs within 10<sup>-12</sup> s, modifications that are rapidly lost upon vibrational excitation, such as phosphorylation, N- and O-glycosylation, sulfation and γ-carboxylation, can be easily established.

In this Chapter, we will illustrate the ECD and ETD protein and peptides fragmentation mechanism, the MS instruments used and the parameters involved and we will provide an overview on the most recent applications of protein PTM characterization.

## 2. Overview

Transcription of DNA to RNA and translation of RNA into protein result in a broad variety of final products so called "proteome" much more complex than the encoding genome system [1]. During or after synthesis protein post-translational modifications (PTMs) can occur resulting in the chemical modification of the amino acid side chains and the amine and carboxy terminus of the protein. PTMs can occur at one or more sites extending the function of a protein at any step of its life cycle [2]. The regulation or the alteration of these processes determine the cellular activity toward protein localization, protein/receptor, protein/protein, protein/DNA, protein/small ligands interactions. More than 200 PTMs, such as phosphorylation, glycosylation, acylation, nitrosylation, etc. are known and they play a pivotal role in the understanding of normal cell functions as well of degenerative diseases, diabetes, hearth diseases or cancer [3]. Proteomic methods strives to gain insights

in the complexity of proteins, protein network, their role and biological functions. Over the last decade the role of mass spectrometry (MS) techniques to study proteins, peptides and other biomolecules was proved of fundamental importance to elucidate physiological pathways [4, 5]. Among the different applications of mass spectrometry to protein analysis, a primary interest is related to the investigation of PTMs able to regulate a variety of biological mechanisms. The development of analytical instrumentations and strategies exhibiting sensitivity, selectivity and accuracy suitable to identify and quantify them is thus demanding [6]. Several publications illustrated the excellent results obtained by MS and tandem MS (MS/MS) experiments enabling the site-specific assignment of PTMs even at the resolution of individual amino acids in proteins [7, 8]. In addition, the miniaturization of liquid chromatography (LC) techniques together with the development of nano-electrospray sources allows in combination with MS/MS methods the analysis of very small volumes of complex protein and peptide mixtures [9]. However, while the modern soft ionization techniques, matrix assisted laser desorption ionization (MALDI) and electrospray (ESI), allow to obtain intact gas-phase protein ions with even a high degree of modification, PTM analysis still remains a challenge.

Usually, proteins are digested into smaller peptides with an enzyme (i.e. trypsin, Lys C, Glu C etc.), that are separated by ionic exchange and/or reversed phase chromatography before mass spectrometry analysis. These peptides ionize via ESI or MALDI to different charge states (generally +1, +2 or +3), which are suitable for sequencing by collision-activated dissociation (CAD) process. Low-energy collision induced dissociation (CID) is the most commonly method used to sequence peptide ions by MS/MS. Upon collision with a neutral gas, fragmentation usually occurs at the amide bond level in the peptide backbone leading to the formation of singly charged b- and y-type fragment ions (Figure 1) [10]. The CID process is highly effective for small (15-20 aa residues), low-charged peptides (+1, +2, +3 charge state), and most of the algorithms for data interpretation prefer singly charged fragment ions. The reliability to identify a peptide involves the generation of an extensive fragmentation of the amino acid backbone. On the other hand fragmentation depends on the protein sequence, the peptide length or the presence of PTMs. In addition, the presence of several basic residues can prevent random protonation along the peptide backbone inducing site specific dissociation and few sequence ions. CAD is a widely used technique able to provide several useful information, however is not suitable for fragmentation of intact proteins and could prevent the detection of PTMs. Several PTMs are labile (i.e. phosphorylation, nitrosylation, sulfonation) and could be difficult to identify the sites of the modification or the relationship between them by CAD. For example, in the case of phosphorylated serine (Ser) or threonine (Thr) residues, the phosphate group competes with the peptide backbone as the preferred site of cleavage. The activation of these peptides upon collision displaces phosphoric acid from the peptide, and frequently results in the peptide's backbone bonds remaining intact [11]. The resultant spectra contain minimal peptide sequence information and may not allow for unambiguous peptide sequence assignment.

While phosphorylation of serine and threonine residues is widely documented a labile chemical modifications, other PTMs such as glycosylation [7], sulfonation [7], and nitrosylation [12] belong to this category. Loss of these labile PTMs during CAD process results in product ion spectra that does not contain sufficient information on the peptide sequence. However, even with this limitation, the methodology of proteolytic digestion followed by CAD MS/MS has successfully provided sequence identifications (including PTM locations) of thousands of peptides with labile PTMs (13-15).

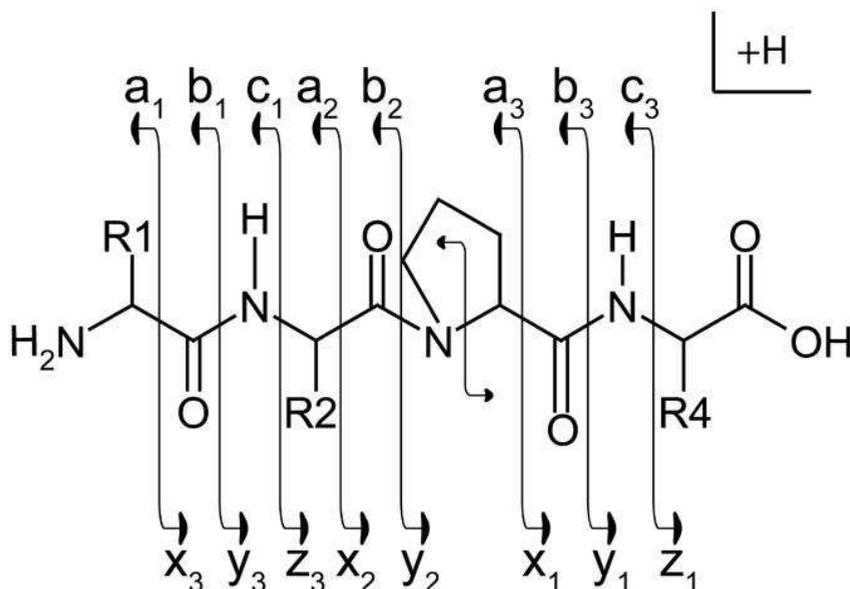


Fig. 1. Roepstorff Nomenclature Scheme. Illustration of fragment ions formed from the backbone cleavage of protonated peptides. Fragment ions retaining the positive charge on the amino terminus are termed a-, b-, or c-type ions. Fragment ions retaining the positive charge on the carboxy terminus are termed x-, y-, or z-type ions. [Ref 10, 54].

An alternative fragmentation technique, the electron capture dissociation (ECD), was introduced by McLafferty and co-workers, based on the gas-phase fragmentation of multiply charged protein and peptide ions upon capture of a low-energy (<1 eV) electron within a penning trap of an FT-ICR (Fourier Transform-Ion Cyclotron Resonance) MS [16]. The odd-electron species undergo rearrangement with subsequent cleavage of N-C<sub>α</sub> backbone (Figure 2) [17]. Peptide fragmentation can take place following a lower energy pathway than CAD, inducing the formation of c- and z- type fragment ions without loss of the information on the PTM localization. The key to the success of this approach is the selection of intact protein molecular ions and its profound potential for PTM characterization as alternative to vibrational excitation techniques. With ECD, that occurs within 10<sup>-12</sup> s, modifications that are rapidly lost upon vibrational excitation, such as phosphorylation, N- and O-glycosylation, sulfation and γ-carboxylation, can be easily established.

To go into the details, in the ECD process multiply charged peptides or proteins are isolated in the trap of a Fourier transform ion cyclotron resonance (FTICR) mass spectrometer and exposed to electrons with near-thermal energies [16]. The capture of a thermal electron by a protonated peptide is an exothermic process (6 eV) that does not involve intramolecular vibrational energy redistribution and is able to induce fragmentation of the peptide backbone by a nonergodic process. The initial step involves the formation of an odd-electron hypervalent species (RNH<sub>3</sub><sup>•</sup>) that dissociates to produce RNH<sub>2</sub> and a hydrogen radical. As described by Zubarev et al. addition of H<sup>•</sup> to the carbonyl groups of the peptide backbone leads to a homologous series of complementary fragment ions of types c and z.

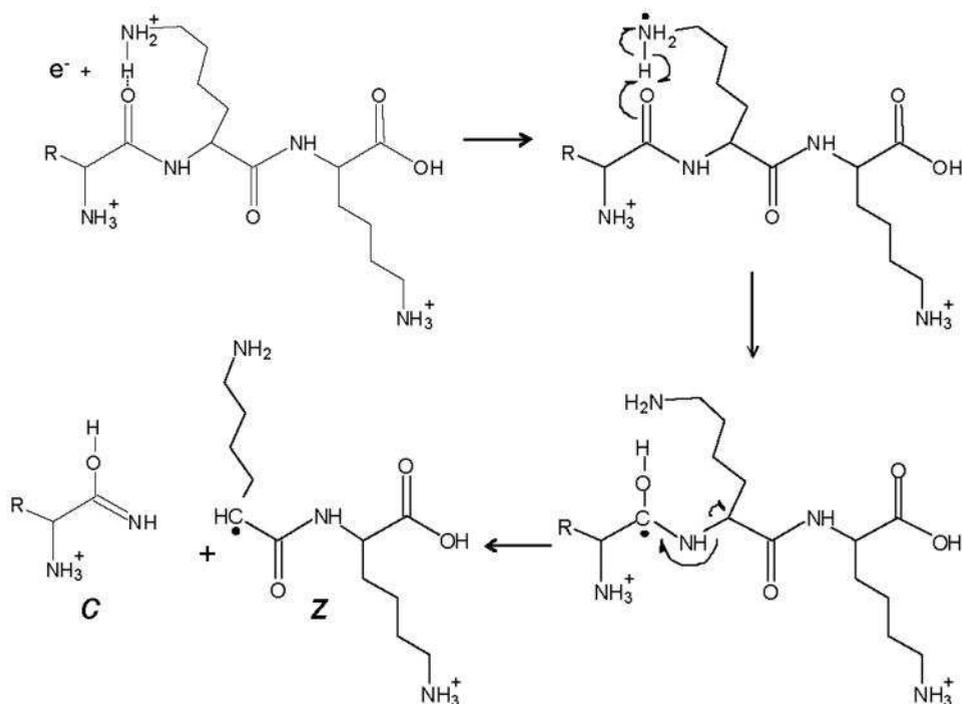


Fig. 2. ETD fragmentation scheme. Fragmentation scheme of a multiply protonated peptide after reaction with a low energy electron to produce c- and z-type ions [17].

ECD process occurs along the peptide backbone and is able to retain PTMs, for this reason was the technique of choice for the analysis of peptide and proteins with FTICR mass spectrometers [18-21]. Unfortunately, the efficiency of the ECD process requires that the precursor sample ions be immersed in a dense population of near-thermal electrons and can be performed only in the FTICR mass spectrometers. Thermal electrons introduced into the RF fields of RF 3D quadrupole ion trap (QIT), quadrupole time-of-flight, or RF linear 2D quadrupole ion trap (QLT) instruments maintain their thermal energy only for a fraction of a microsecond and are not trapped.

For this reason, QLT mass spectrometer were modified to enable ion-ion experiments and the capability to perform electron transfer dissociation (ETD) process.

ETD can be either utilized to fragment intact proteins by a top-down approach by direct introduction into the mass spectrometer analyzer. As reported in the literature, proteins or large peptides are isolated and reacted with the fluoranthene radical anion, generated by chemical ionization. McAlister et al. proposed in the 2008 the modification of the quadrupole linear ion trap-orbitrap mass spectrometer to accommodate a negative chemical ionization source for the production of fluoranthene radical anions and the ETD process [22]. The resulting highly charged product ions are reacted with even electron anions of benzoic acid in order to reduce the charge states of the c and z-type fragment ions into predominantly singly charged species. This proton transfer (PTR) charge reduction along with ETD is needed to reduce the complexity of the spectra generated from these highly

charged ( $z > 10$ ) species. ETD followed by proton transfer of larger peptides and proteins typically only generate the first 15–40 amino acids at both the N- and C-termini. Nonetheless, information from current ETD mass spectrometers is more than sufficient to identify the protein. For this purpose, the accurate determination of the precursor charge state is fundamental for a suitable identification of the proteins by using the bioinformatics tools and the sequence data bases.

A complete protocol to isolate and characterize intact proteins by using liquid chromatography, MALDI-TOF-MS and ETD/PTR in a quadrupole ion trap together with different applications were recently provided by Hart SR et al. [23].

To better explain, the ETD process involves two fundamental reactions: proton and electron transfer reaction. The ET reaction allows the formation of products that do not dissociate upon ion/ion-reactions as well as side chain losses from aa residues. Theoretically PTR from multiply protonated species to singly charged anions is a facile process for any negative ion [24]. As reported by Wiesner et al., the ET process can compete with PTR only if the species generating the negative ion has appropriate activation energy and the respective anion has favorable Frank-Condon factors associated with the transition from anion to neutral. Taking into account that the exothermicity for ET process for an anion can be determined by the recombination energy of the cationic species, low (60 kcal/mol) and high (62 kcal/mol) electron affinities hardly provide any probability for ET [25–27]. In case of ion/ion reactions, however, both kinds of reactions (ET and PT reactions) are exothermic, in particular if the peptide is multiprotonated, whereas for the majority of singly charged anions, PT from a multiply protonated peptide is thermodynamically favored over ET (Figure 3).

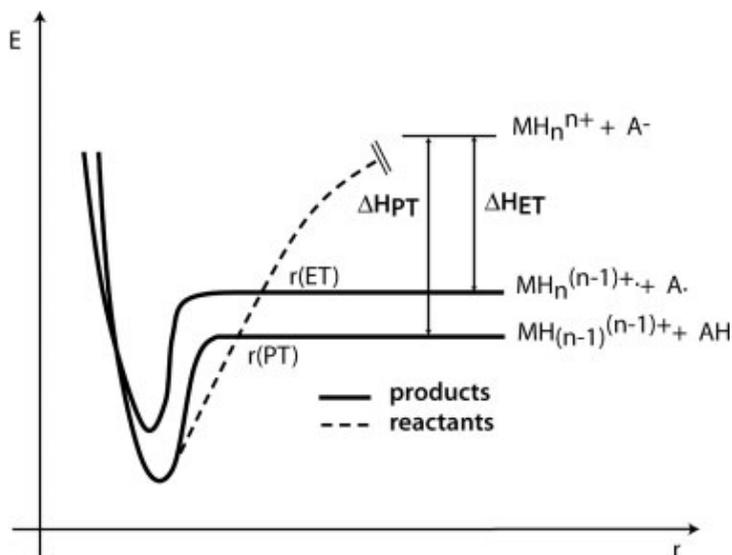


Fig. 3. Schematic potential energy curve of an ion/ion reaction between a peptide precursor  $[MH_n]n1$  and a reagent anion  $A2$ . As the entrance channel crosses the exit channels of either electron or PT, the reaction can occur. With regard to ET happening at greater distances, this reaction is more likely than PT for the typical anion/cation pairs. Slightly modified after [26].

As evident, the ion/ion reaction for ET might be reached before the point at which PT is likely and opens up the possibility for ET. Since fragmentation does not occur after PT, the charge-reduced species are produced. For these reasons, intact ET products should have higher masses than PT products that differ in mass by 1 Da. [28, 29].

The success of ETD experiments depends upon several parameters and Xia et al. provided an excellent overview of the features required by the precursor peptide ions in terms of the effects of cation charge-site identity and position on ETD of polypeptide cations.

Turecek et al. [30], showed the pivotal role played by hydrogen transfer from a neutralized charge site in providing high fragmentation efficiencies, thus peptide cations containing protonated lysine, arginine, and histidine provide a similar degree of ET which is significantly higher than for those peptides having fixed charge sites. However, the backbone fragmentation can still be observed suggesting the occurrence of direct ET to an amide linkage and the formation of some c- and z-type ions. In conclusion, even it is not a strict prerequisite, the presence of multiply charges facilitates the cleavages of bonds. As previously reported [30], among the different charge sites, protonated histidine reveals the highest degree of ET without dissociation while apparently no intact ET products are observed for peptides with protonated lysine and arginine. A possible explanation might be given by the stability of the imidazolium ion that is formed upon electron capture and can delocalize the radical along the aromatic ring of histidine.

Xia et al. demonstrated that all cation types show aa side chain losses with arginine containing peptides yielding the largest fraction and lysine peptides the smallest [28]. This feature fits with the ability to transfer a hydrogen atom from a neutralized charge site to the peptide backbone. Generally, electron attachment is favored at N-terminal lysine sites which reveal the lowest reaction energy value, thus decreased side-chain fragmentation is observed for these species. Apart from its identity, the amount of precursor charges also plays a role for the ETD outcome. ETD contributes a 20- fold higher confidence for the identification of triply charged peptides than doubly charged ones [31]. In addition, the product ratios depends also on the selected anion. Some anions function as strong bases and react exclusively as proton acceptors, whereas others participate in both proton and ET (*e.g.*, 1,3-dinitrobenzene results in a higher degree of PT than azobenzene) [32, 33].

As observed by Gunawardena et al., in general, a high degree of spectra similarities can be observed for different kinds of anions reacting with the same peptide [26].

Moreover, the MS instrument parameters, such as the filling of the IT space charge limit with anionic reagent, can affect ETD efficiency [34]. The space charge limit for anions in such instruments is higher than for cations, resulting in a critical cation current: too many will produce space-charge-associated mass shifts, whereas too few will result in low product ion intensities and the necessity of spectral averaging. The integrated automatic gain control (AGC) or ion charge control (ICC) regulate the anion and cation populations [35, 36].

As for instrumentation, a combination (compared with the FTICR MS) of ETD with a high-resolution mass spectrometer was recently introduced (Figure 4) [22]. As proposed for the LIT (LTQ XL) instruments with ETD, an negative CI was implemented at the back side of the instrument. In this way, positive peptide precursors are initially trapped within the first section of the LIT and subsequently, reagent anions are orientated in few ms through the ion optics at the rear side of the trap. After the ion/ion reactions, anions are removed and product ions are either detected within the LIT or with high-resolution within the Orbitrap mass analyzer.

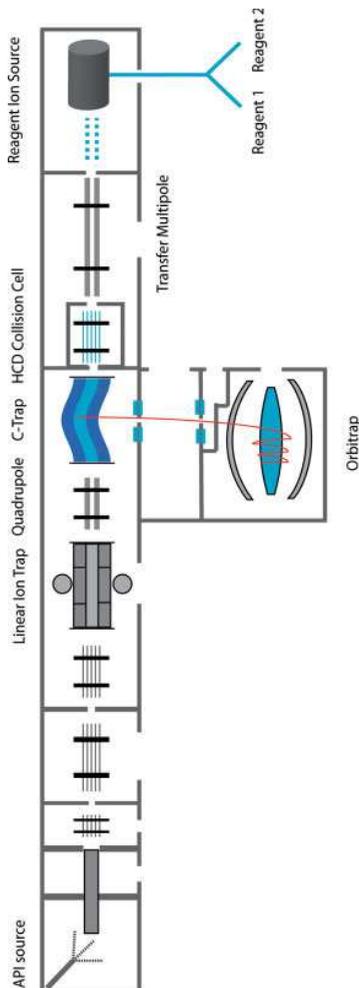


Fig. 4. Scheme of the newly developed LTQ-Orbitrap XL with ETD. The reagent ion source is implemented at the back side of the instrument. Slightly modified after [22].

Frese et al. very recently reported the results obtained by performing ETD experiments on a new generation Orbitrap [37]. A comprehensive experimental comparison of using ETD, ion-trap CID, and beam type CID (HCD) in combination with either linear ion trap or Orbitrap readout for the large-scale analysis of tryptic peptides was carried out. They demonstrated that the combination of fragmentation technique and mass analyzer is able to provide the best performance for the analysis of distinct peptide populations such as N-acetylated, phosphorylated, and tryptic peptides with up to two missed cleavages (Figure 5). They found that HCD provides more peptide identifications than CID and ETD for doubly charged peptides. In terms of Mascot score, ETD FT outperforms the other techniques for peptides with charge states higher than 2. Their data shows that there is a trade-off between spectral quality and speed when using the Orbitrap for fragment ion

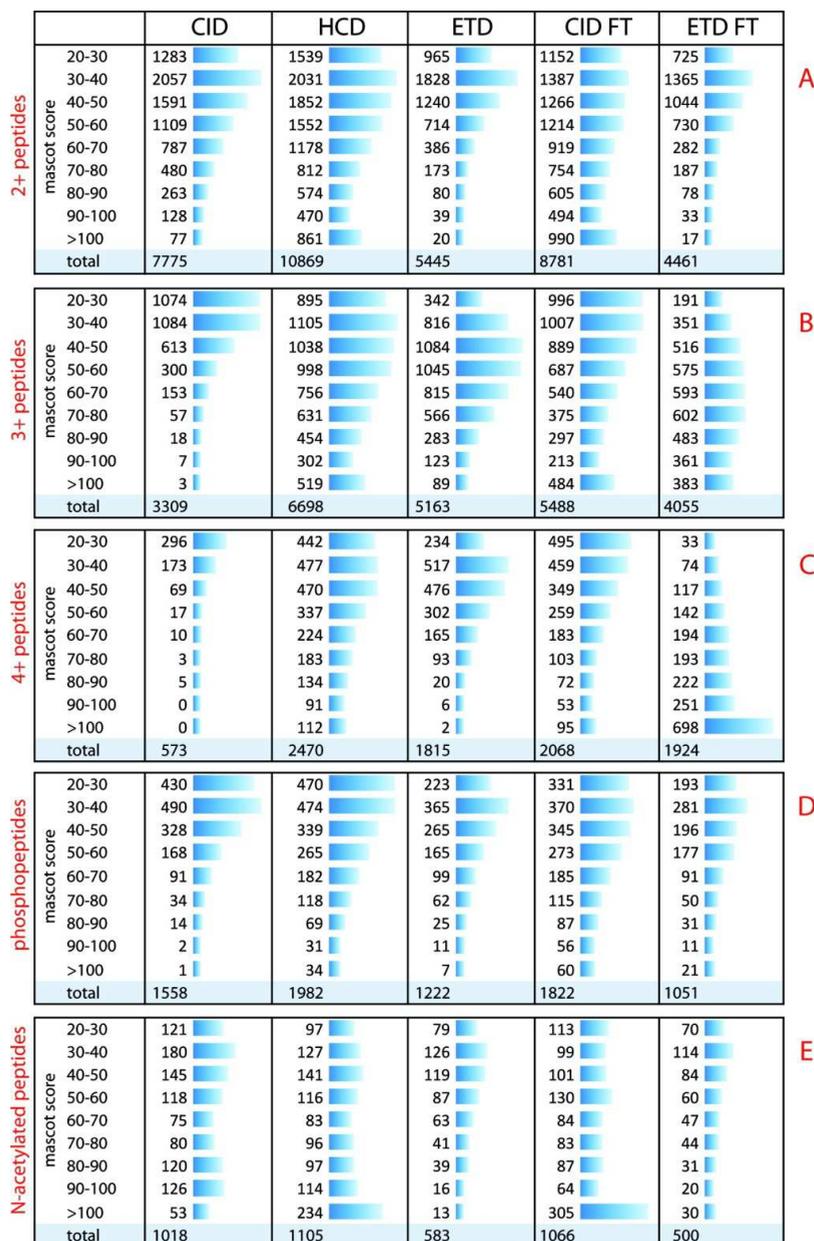


Fig. 5. Histograms illustrating the Mascot ion score distribution of the doubly (A), triply (B), quadruply (C), phosphorylated (D), and N-acetylated (E) peptides that were identified from the analyses of SCX-fractionated tryptic peptides. Samples were analyzed consecutively for five times employing either CID, HCD, ETD, CID FT, or ETD FT. Data bars are normalized to the highest value within each population. [37]

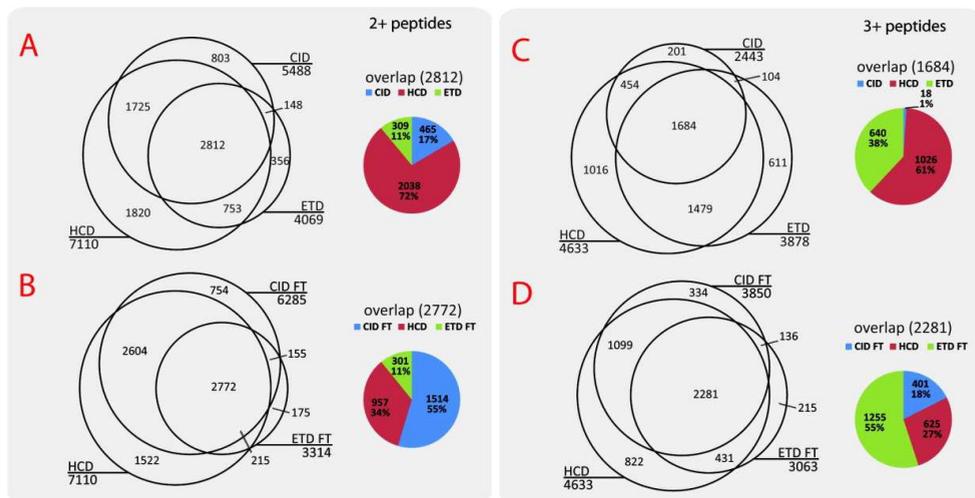


Fig. 6. Overlap of the doubly (A, B) and triply (C, D) charged peptides that were identified from the SCX-fractionated tryptic HeLa digest. Venn diagrams illustrate the overlap of the unique peptides identified between CID, HCD, and ETD (A, C) and between CID FT, HCD, and ETD FT (B, D). Pie charts show the number of unique peptides with the highest Mascot score per fragmentation mode within the peptides that were identified by all three fragmentation modes [37].

detection, concluding that a decision-tree regulated combination of higher-energy collisional dissociation (HCD) and ETD can improve the average Mascot score (Figure 6).

### 3. Applications of ECD/ETD process to PTM characterization

#### 3.1 Phosphorylation

Phosphorylation consists in the addition of a phosphate ( $\text{PO}_4^{3-}$ ) group to one or more amino acid side chain (Ser, Thr and Tyr) of eukaryotic proteins. Protein phosphorylation is a reversible reaction that regulates a wide range of cellular processes and plays a predominant role in biochemistry research. Liquid chromatography-tandem mass spectrometry techniques are commonly used in combination with selective sample enrichment strategies (i.e.  $\text{TiO}_2$  cartridges or immobilized metal affinity chromatography, etc.) to identify phospho-peptides in complex mixtures. Many papers report about the use of CID/CAD strategies based on product-ion scan, neutral-loss scan or precursor ion scan acquisition modes for the identification of both phospho-peptides and phosphorylation sites. Usually, the CID fragmentation of a phospho-serine and -threonine results in the extremely favorite neutral loss of phosphoric acid ( $\text{H}_3\text{PO}_4$ ;  $\Delta 98$  Da) and the mass spectrum is characterized by a base peak corresponding to the loss of phosphoric acid from the parent mass and few low-intense fragments.

By using ETD the fragmentation process follows different rules, as reported above, and it is possible to observe in the mass spectrum the cleavage of the peptide backbone in to the c and z-type ions without loss of the phosphate group improving global protein profiling (Figure 7) [24].

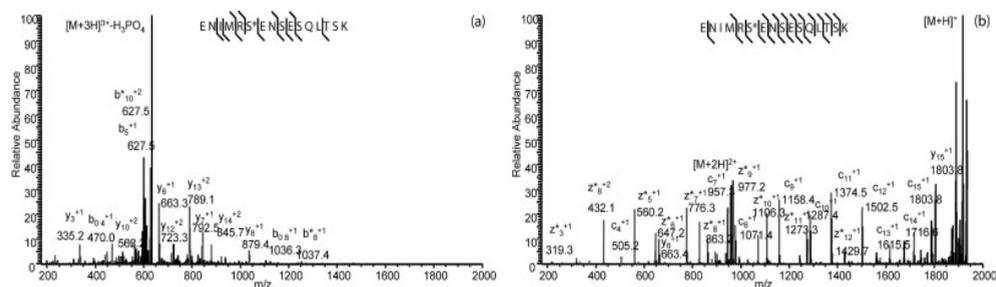


Fig. 7. CID (a) und ETD (b) spectra of a triply protonated synthetic peptide ENIMRS\*ENSESQLTSK with phosphate modification (\*). The spectrum was recorded on an LTQ XL (Thermo Scientific) with ETD by direct infusion of the synthetic peptide [Ref. 24].

As reported by Palumbo AM et al. [25] the utility of MS/MS strategies for phosphopeptide identification and characterization, including phosphorylation site localization depends on the properties of the precursor ion (e.g. polarity and charge state) and gas-phase mechanisms.

Very recently, Kim MS et al., reported the comparison of CID and ETD phosphopeptide fragmentation of a complex mixture [31]. They reported the identification of 2504, 491, 2584 and 3249 phospho-peptide spectrum from CID alone, ETD alone, decision tree-based CID/ETD, and alternating CID and ETD, respectively. They concluded that although alternating CID and ETD experiments for phosphopeptide identification are desirable for increasing the confidence of identifications, merging spectra prior to database search has to be carefully evaluated further in the context of the various algorithms before adopting it as a routing strategy.

### 3.2 O- and N-linked glycosylation

N- or O-glycosylation are a cell PTM dependent process of paramount importance in cell life, diseases and therapeutics. OGlcNAc was a labile modification not extensively studied by mass spectrometry until the last years mainly because of the analytical methodologies limitations. The introduction of ETD significantly improved the number of information available and thus facilitate the understanding of the role of this PTM.

Zhao P et al reported the use of a combination of HCD and EDT fragmentation techniques for the characterization of O-GlcNAc modified proteins. By enriching O-GlcNAc proteins from HEK293T cells, 83 modified sites were identified with high degree of confidence allowing to provide useful information about O-GlcNAc transferase activity (Figure 8) [38].

As for fragmentation process, a comparison between CAD/HCD and CAD/ETD performances for the characterization of glycopeptides were recently reported by Scott NE et al [39]. They developed a strategy of sample enrichment and LC-MS/MS analysis for the characterization of glycopeptides from gastrointestinal pathogen *C.jejuni* HB93-13. The results showed that CAD/HCD enabled the identification of glycan structure and peptide backbone allowing glycopeptide identification, whereas CAD/ETD enabled the elucidation of the glycosylation sites by retaining the glycan-peptide linkage. This paper reported the first use of HCD fragmentation for the identification of glycopeptide presenting intact glycan. At the end 130 glycopeptides, representing 75 glycosilation sites, were identified by LC-MS/MS using a zwitterionic HILIC (hydrophilic interaction chromatography). In details,

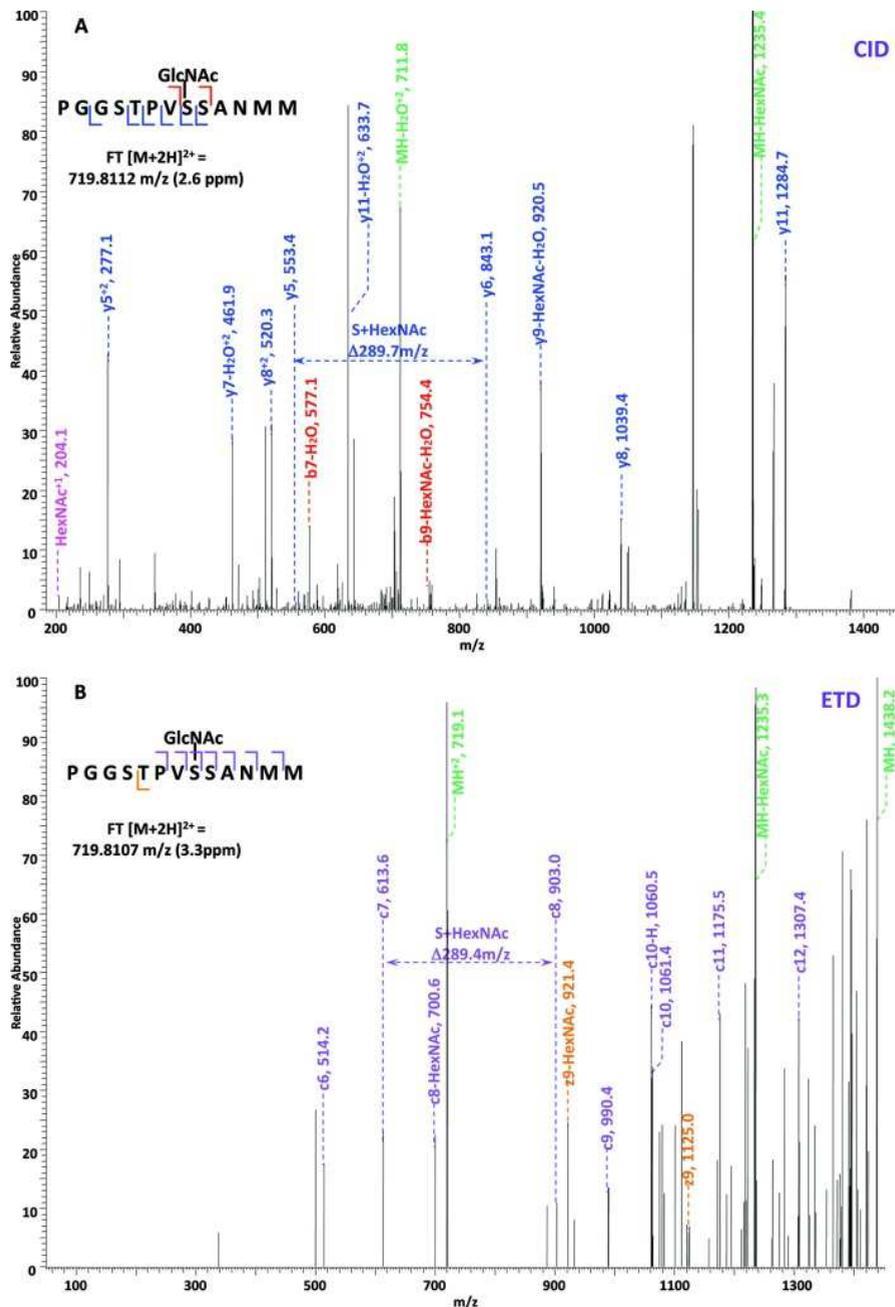


Fig. 8. Respective CID, ETD, and HCD spectra of standard O-GlcNAc modified peptides CKII and BPP. (A-B) CID and ETD spectra of O-GlcNAc modified CKII peptide, respectively [Ref.38].

CAD/HCD provided the majority of the identifications (73 sites) compared with ETD (26 sites) showing the capabilities of different approaches.

### 3.3 Nitrosylation

Among more than 200 different types of PTMs, S-nitrosylation, i.e. the replacement of the hydrogen atom in the thiol group of cysteine (Cys) residues by a -NO moiety (SNO), has emerged as a key reaction in several metabolic processes occurring in animals and in plants [40, 41]. The study of NO signalling in animals or human endothelial cells resulted in the identification of more than a hundred modified proteins [42] even though the technical limitations in characterizing this modification due to its low stability caused a delay in appreciating its biological involvement. Different indirect methods were proposed for the identification of such PTM [43]. Among these, the Biotin Switch method, consisting in the selective reduction and substitution of the SNO groups with a biotin moiety, was successfully applied in mammalian cells and opened new insights on the S-nitrosylation of plant proteins [44]. However the sensitivity and specificity of this method are still a matter of debate. A modified version of the Biotin Switch called SNOSID (SNO Site Identification) has been proposed for high-throughput identification of SNO-Cys residues in complex protein mixture from tissues or cells [45-46]. More recently, a His-Tag switch method was introduced as a novel approach to identify proteins modified by nitric oxide allowing the unambiguous localization of the modified cysteine residues by MS [47]. While numerous published studies on protein S-nitrosylation are based on indirect labelling techniques, the direct MS measurement of nitrosylated cysteines is still poorly represented in the literature, due to the difficulty of preserving the integrity of the SNO groups during the analysis. The crucial point rely with the lability of the S-NO bond that readily undergoes fragmentation in the gas phase of a mass spectrometry source [48]. Most of the reported examples focus on single purified proteins, preferably *in vitro* overnitrosylated by chemical agents. ETD dissociation can represent an attractive alternative technique to retain the nitrosilation modification.

As an example, in the literature nitrosylated bovine insulin beta chain was analyzed by CAD and ETD as a model of this type of PTM [49]. The majority of the signal in the CAD spectrum of the  $(M+5H)^{+5}$  of FVNQHLnCGSHLVEALYLIVnCGERGFYTPKA corresponded to the neutral loss of both NO groups on the cysteine residues  $(M+5H-2NO)^{+5}$ . Minimal peptide backbone fragmentation is obtained as only a few product ions are observed above 5% relative abundance ( $y_3$ ,  $y_{13}-NO+2$ ,  $b_{16}-NO+2$ ,  $b_{17}-NO+2$ , and  $b_{24}-NO+2$ ). In the ETD spectrum of the same peptide, the following charge reduced (electron transfer without fragmentation) species with and without losses of NO were observed:  $(M+4H-NO)^{+4}$  (may also be  $z_7$ ),  $(M+3H)^{+3}$ ,  $(M+3H-NO)^{+3}$ ,  $(M+3H-2NO)^{+3}$ ,  $(M+2H)^{+2}$ ,  $(M+2H-NO)^{+2}$ , and  $(M+2H-2NO)^{+2}$ . The loss of NO from the charged reduced species may be acting as its own proton transfer reagent directing mostly charge reduction of the nitrosylated insulin as opposed to fragmentation. However, 6-7 low level c and z-type ion were observed (2% or less of the largest ion in the spectrum) demonstrating the retention of NO on the insulin product ions after ETD.

### 3.4 Disulfide linkage

The disulfide linkage of two cysteine residues plays an important role in the correct protein folding and thus its function. ETD fragmentation can be successfully used for the determination of disulfide linkages instead of CAD process.

Different examples are reported in the literature. In particular, Wu SL et al. reported the results of an LC-MS CAD (MS2), ETD (MS2) and CAD of isolated product ion derived from ETD (MS3) combined strategy for the characterization of disulfide linkages of recombinant human proteins [50]. In the case of human growth hormone (Nutropin), disulfide bond was not broken by using CAD process, whereas ETD fragmentation resulted in two separated peptide ions along with typical fragmentation pattern of the backbone cleavages (c and z ions) with several high intensity ions consisting of charge-reduced species of the precursor ion ( $[M+3H]^{2+}$ ,  $[M-NH_3+3H]^{2+}$ ,  $[M+3H]^+$ ,  $[M-NH_3+3H]^+$ ,  $[M-2NH_3+3H]^+$ ).

As previously reported, charge-reduced species rather than the disulfide-dissociated peptides are dominant product ions in the ETD spectrum for precursor ions with  $m/z > 900$  (Figure 4B and 3B) [51, 52]. The generation of several charge-reduced species with high intensities in the ETD spectrum allowed the determination of the charge state of the precursor ion (4+). Additionally, the Authors observed that the disulfide-dissociated peptides became the major ions in the MS3 step. Final suggestions are the use of combined fragmentation approach and high resolution mass spectrometers (Q-TOF or Orbitrap) for the characterization of multi- and close-disulfide bonds in proteins.

Wang Y et al. proposed the use of ETD process for the characterization and comparison of disulfide linkages and scrambling patterns in therapeutic monoclonal antibodies [53].

### 3.5 Sulfonation

Very few examples are reported in the literature about the use of ETD dissociation and characterization of O-sulfonation, a PTMs involved in protein assembly and signal transduction. CAD fragmentation of sulfonated peptides results in the neutral loss of  $SO_3$  as predominant product ion. ETD process allows to retain the  $SO_3$  group and to observe c and z-type ion series (Figure 9) and thus the amino acid localization [54]. Mikesh et al. reported that the isobaric modifications, sulfonation from phosphorylation, differentiated by their different CAD neutral loss patterns (80 amu vs. 98 amu), can be detected by using a ETD modified LTQ for sequential CAD-ETD analysis.

A very interesting example comes from Cook et al. that investigated the dissociation behavior of phosphorylated and sulfonated peptide anions using metastable atom-activated dissociation mass spectrometry (MAD-MS) and CID. In this paper phosphorylated and sulfonated peptides in the 3- and 2- charge states were exposed to a beam of high kinetic energy helium metastable atoms. Unlike CID, where phosphate losses are dominant, the major dissociation channels observed using MAD were C( $\alpha$ )-C peptide backbone cleavages and neutral losses of  $CO_2$ ,  $H_2O$ , and  $[CO_2 + H_2O]$  from the charge reduced (oxidized) product ion. Regardless of charge state or modification, MAD provides ample backbone cleavages with little modification loss, which allows for unambiguous PTM site determination resulting a complementary dissociation technique to CID, ETD, ECD for peptide sequencing and modification identification. MAD offers the unique ability to analyze highly acidic peptides that contain few to no basic amino acids in either negative or positive ion mode [55].

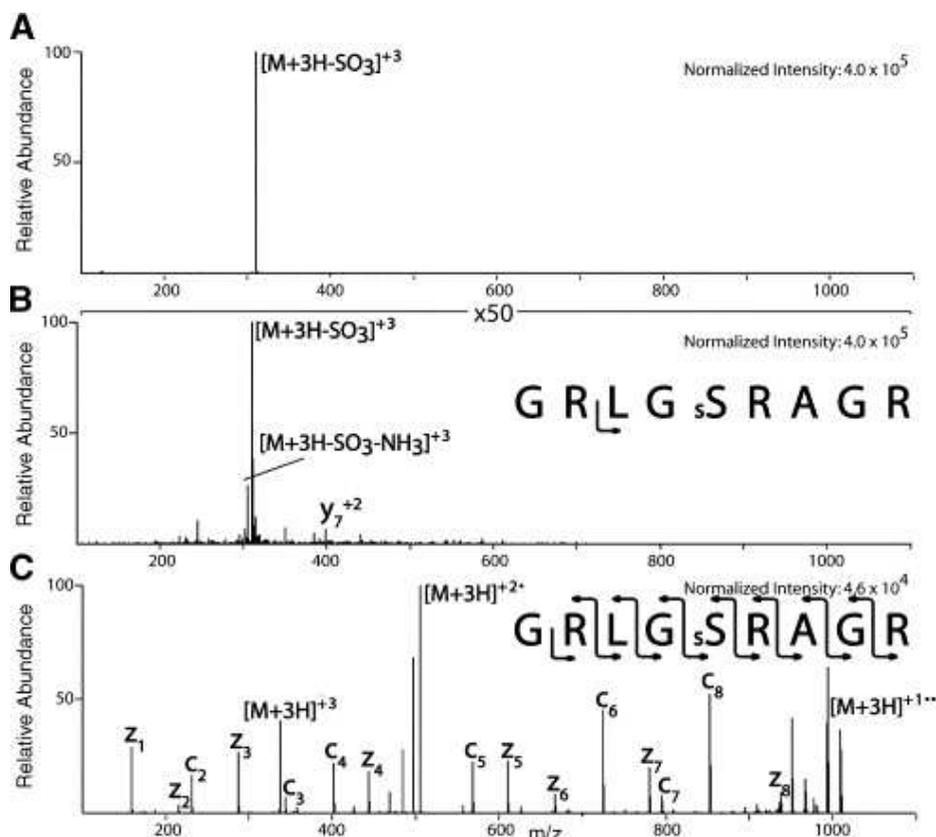


Fig. 9. Comparison of CAD vs. ETD spectrum of a sulfonated peptide. Sulfonation of peptides was achieved by reacting the peptide with 5% chlorosulfonic acid in neat trifluoroacetic acid (TFA) for 20 min at room temperature. The reaction was terminated by the addition of water and was purified by RP-HPLC. Mass spectrometry analysis before and after sulfonation confirmed reaction. Sulfonated peptides (1 pmol/ $\mu$ l) were infused at a flow rate of 60 nl/min into a ThermoElectron LTQ ion trap mass spectrometer modified to perform ETD. (A) The CAD spectrum of the illustrated sulfonated peptide contains one major ion corresponding to the neutral loss of  $SO_3$  from the  $(M + 3H)^{+3}$  precursor ion. (B) Magnification of the spectrum shown in A by 50 $\times$ . (C) Fragmentation of the sulfonated peptide by ETD where a complete c and z-type ion series was observed with no detectable loss of  $SO_3$  from the precursor ion or the peptide backbone. [Ref.54].

#### 4. Conclusions

This chapter describes that ETD dissociation is able to generate product ions from peptides carrying PTMs, multiply charged peptides and intact proteins.

Whereas CID/CAD peptide dissociation usually induces significant loss of labile chemical groups, i.e. phosphoric acid, NO or  $SO_3$ , the ETD process allows to preserve these weak chemical bonds thus providing reliable and accurate AA site assignment.

Additionally, ETD has the capability to fragment intact proteins providing useful information on sequences, presence of disulfide bonds, protein isoforms.

Several application of ETD in proteomic research are reported in the literature involving many life-science areas, i.e. clinical diagnosis, biomarker discovery, cancer and degenerative diseases, cell signaling etc.. In these years we are assisting to a significant improvement in the mass spectrometry instruments that will turn in the increasing the ETD capabilities and applications.

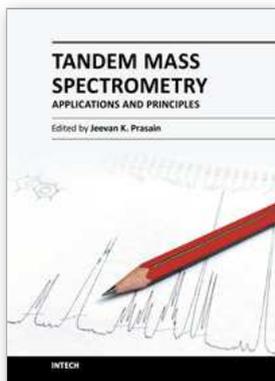
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