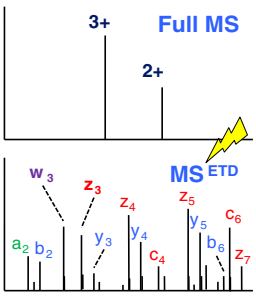
J. Am. Soc. Mass Spectrom. (2017) 28:384–388
DOI: 10.1007/s13361-016-1538-2

APPLICATION NOTE

Electron Transfer Dissociation of All Ions at All Times, MS^{ETD}, in a Quadrupole Time-of-Flight (Q-ToF) Mass Spectrometer

Christian N. Cramer,[1] Jeffery M. Brown,[2] Nick Tomczyk,[2] Peter Kresten Nielsen,[1] Kim F. Haselmann[1]

[1]Novo Nordisk A/S, Protein Engineering, Global Research, Novo Nordisk Park, DK-2760, Måløv, Denmark
[2]Waters Corporation, Stamford Ave., Altrincham Road, Wilmslow, SK9 4AX, UK



Abstract. Data-independent mass spectral acquisition is particularly powerful when combined with ultra-performance liquid chromatography (LC) that provides excellent separation of most components present in a given sample. Data-independent analysis (DIA) consists of alternating full MS scans and scans with fragmentation of all ions within a selected *m/z* range, providing precursor masses and structure information, respectively. Fragmentation spectra are acquired either by sequential isolation and fragmentation of sliding *m/z* ranges or fragmenting all ions entering the MS instrument with no ion isolation, termed broadband DIA. Previously, broadband DIA has only been possible using collision induced dissociation (CID). Here, we report the use of electron transfer dissociation (ETD) as the fragmentation technique in broadband DIA instead of traditional collision induced dissociation (CID) during MS^{E}. In this approach, which we refer to as MS^{ETD}, we implement the inherent benefits provided by ETD, such as discrimination of leucine and isoleucine, in a DIA setup. The combination of DIA analysis and ETD fragmentation with supplemental CID energy provides a powerful platform to obtain information on all precursors and their sequence from a single experiment.

Keywords: Electron transfer dissociation (ETD), Liquid chromatography mass spectrometry (LC-MS), Data-independent acquisition (DIA), MSETD, MSE, Peptide mapping, Leucine/isoleucine discrimination, w-Ions

Received: 19 July 2016/Revised: 2 September 2016/Accepted: 25 September 2016/Published Online: 2 December 2016

Introduction

LC-MS is one of the most widely used techniques in protein characterization, since ultra-high performance LC systems offer very high separation power and MS characterization provides information of the eluting protein or peptide identities, their sequences, and modifications. MS methods that provide precursor mass as well as molecular structure information on the LC scale of all eluting species are attractive since all information is obtained in a single experiment. The choice to obtain such information is to perform either data-dependent analysis (DDA) or data-independent analysis (DIA). In DDA, a fixed number of precursors are selectively subjected to mass selection and fragmentation, based on a preceding full MS survey scan and predetermined rules and filtering criteria. DIA consists of cycles of a full MS scan and a (or multiple) scan(s) with fragmentation of all ions within a preselected *m/z* range. DIA exists in two forms based on how the fragmentation scans are performed, using either (1) sequential wide precursor isolation windows such as Sequential Window Acquisition of all THeoretical fragment ion spectra (SWATH) [1] or (2) no precursor isolation (broadband DIA) such as in-source fragmentation or MS^{E} [2]. Distinct pros and cons can be ascribed to each, such as increased specificity using sequential isolation windows useful when analyzing more complex samples, and increased sensitivity using broadband DIA, which is beneficial with less complex samples. The higher specificity using isolation windows comes at the expense of decreased duty cycle, depending on the size of the isolation windows and the mass range scanned. This also narrows the ultimate mass range that can be interrogated. Broadband DIA, with no precursor mass isolation, offers fragmentation of all ions of the full *m/z* range only decreased by a factor of two compared with a MS only experiment, as a result of the high energy fragmentation scan. Since all (co)-eluting precursors are fragmented without isolation, the sample complexity is the limiting factor using broadband DIA and perhaps less targeted to the proteomics field, but

Electronic supplementary material The online version of this article (doi:10.1007/s13361-016-1538-2) contains supplementary material, which is available to authorized users.

Correspondence to: Kim F. Haselmann; *e-mail:* kmfh@novonordisk.com

to single (or few) protein(s) analysis, such as in the biopharmaceutical industry.

To date, CID is the only fragmentation method used for all ion fragmentation DIA as it is easily implemented even on MS systems without mass isolation capabilities (e.g., by in-source CID). However, different fragmentation methods offer different advantages with regards to the information obtained because of different mechanisms and energy regimes involved [3, 4]. Other fragmentation techniques include impacting ions into surfaces [surface induced dissociation (SID)] [5], photon-based fragmentation [e.g., ultraviolet photodissociation (UVPD)], ion/electron and ion/ion reactions [e.g., electron capture dissociation (ECD) [6] and ETD [7], which are frequently employed in non-DIA experiments. Over recent years, ETD has been implemented in MS instruments employing radio frequency (rf) ion traps or rf collision cells. Few examples of combining ETD and DIA have been shown, all using MS/MS by sequential window precursor isolations over narrow *m/z* ranges [8, 9]. In ETD, the dissociation mechanism is fundamentally different from CID as it is based on ion–ion reactions, with transfer of an electron and backbone dissociations through production of unstable charge-reduced radical cations. Particular advantages of ETD include the ability to maintain labile modifications [10], the possibility to cleave disulfide bonds [11], higher sequence coverage of especially larger proteins [12], and generation of reporter fragments for discrimination of isomeric compounds [13], such as leucine and isoleucine [14].

Here, we introduce an expansion of the MS characterization toolbox by combining broadband DIA analysis (i.e., MS^{E}), with ETD, and gain the benefits from both worlds.

Experimental

Reagents and Sample Preparation

Lyophilized albumin from human serum (HSA) (≥99%) was obtained from Sigma-Aldrich (St. Louis, MO, USA), optima LC/MS grade 0.1% formic acid (FA) in water (LC buffer A) and 0.1% FA in acetonitrile (ACN) (LC buffer B) from Fisher Scientific (Geel, Belgium), modified trypsin from Promega (Madison, WI, USA), endoproteinase Lys-C from Roche Diagnostics (Mannheim, Germany), and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4{\cdot}H_2O$) and disodium hydrogen phosphate dihydrate ($Na_2HPO_4{\circ}2H_2O$) used to a make a 10 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.5) from Merck (Darmstadt, Germany).

HSA was dissolved in Milli-Q water (18.2 MΩ.cm) (Millipore, Darmstadt, Germany) to a final concentration of 1 mg/ml. Tryptic digestion of HSA was performed by addition of Lys-C and Trypsin in 1:50 and 1:12.5 enzyme to protein ratios, respectively, to HSA in NaH_2PO_4/Na_2HPO_4 buffer (pH 7.5) with a final HSA concentration of 0.5 mg/mL. The digest reaction was incubated overnight at 37 °C before analysis.

Instrumentation

Tryptic digested HSA (4 µg) were injected onto a Waters Acquity UPLC system. The column used was a Waters Acquity CSH C18 reverse phase column (1*150 mm, 1.7 µm) with a temperature of 55 °C and a flow rate of 100 µL/min. Sample elution was performed with increasing ratio of buffer B with a gradient from 2% to 38% in 77 min for digested HSA employed. The UPLC was connected to a Waters Synapt G2 HDMS mass spectrometer setup to perform either MS^{E} or MS^{ETD} (MS with ETD mode enabled). For both types of acquisition, two functions were acquired for all MS experiments. For the first function, a low energy "CID mode" was selected where both the trap cell and transfer cell collision energies were set at low values to maintain intact precursor ions (referred to as full MS scan). As the second function in MS^{E}, CID fragmentation was achieved by applying collision energy ramps of 20–40 V in the trap collision cell. For the second function in MS^{ETD}, ETD for MS was enabled, and ETD type fragment ions were generated in the trap collision cell pressurized with helium at ~5×10^{-2} mbar. For this second MS^{ETD} function, slightly elevated collision energies (supplemental activation energy ramps of 10–30 V) were applied to the transfer collision cell pressurized at ~1×10^{-2} mbar with argon to improve the overall fragment ion efficiency. The acquisition scan rate for all experiment functions was one spectrum per s.

The ETD reagent anion was 1,4-dicyanobenzene (Sigma-Aldrich, St. Louis, MO, USA) with a set mass of *m/z* 128 and was ionized using the glow discharge needle. The ETD reagent refill scan time was 0.1 s and found adequate to fill the trap collision cell for all experiments. The trap cell rf amplitude was 350 V and found as an optimum set point for keeping the low mass region of the mass scale abundant. The ETD mode parameters were: discharge current of 80 µA, make-up gas flow 35 mL/min, the trap cell T-wave height set to 0.3 V, and the trap T-wave velocity left at its default value of 300 m/s. In the full MS scan, the trap cell T-wave height was set to 5.0 V to ensure transmission of intact precursor and avoid possible reactions with remaining reagent ETD anions in the trap cell. To summarize, the trap cell T-wave height switches between 5.0 V and 0.3 V for the full MS and ETD scans, respectively, and with 0.1 s of ETD reagent refill only performed prior to the ETD scan (Figure 1).

No separate calibration lock mass source was used in the MS^{ETD} mode, as this additional scan function was found to hamper the ensuing ETD scan. Instead, for accurate mass calibration, post-acquisition lock mass adjustment to known background

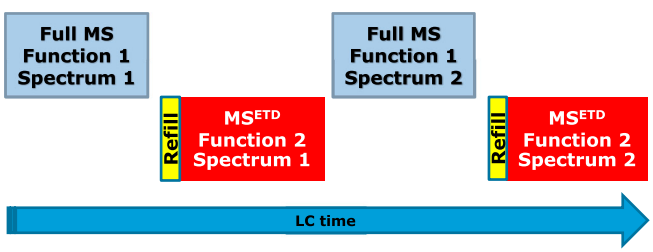


Figure 1. Graphical representation of the timing of the alternating full MS and MS^{ETD} scan functions. Refill of ETD reagent is performed prior to each MS^{ETD} scan

ions was applied when necessary. The instrument was controlled, and data analyses were performed using the MassLynx 4.1 software (Waters, Wilmslow, UK).

Results and Discussion

The method design of MS^{ETD} is analogue to the traditional MS^{E} with two alternating functions; one with no fragmentation and one introducing fragmentation. However, here the high energy trace consists of ETD fragmentation, as previously described. The output is acquisition of two parallel TIC traces in the same LC run with equal scan frequencies, as seen in Figure 2a and b. Linked by the retention time dimension, information of all eluting precursors and their corresponding fragment ions can be obtained due to the previously discussed *fragmentation of all ions at all times*-principle [2]. The strength of the MS^{ETD} methodology is in the simplicity of the method setup and that no information is needed about charge states and/or abundances of the samples to record and obtain data on all sample species without biased filtering criteria. This is especially desirable when analyzing protein digestions for peptide mapping, or for profiling of chemical degradation products in stability studies of protein therapeutics. Here, the LC separation provides specific elution of all species at ideally distinct retention times and the MS^{ETD} setup provides simultaneous information of all precursor and fragment masses. An example is the tryptic digestion of HSA shown in Figure 2. The TIC trace of the full MS function contains all the precursor masses of all the LC separated HSA digest fragments (Figure 2a) and locked in the LC time dimension to the corresponding ETD fragments as recorded in the MS^{ETD} function (Figure 2b). All information is obtained in the same sample injection, eliminating the chance of drifting retention times between different injections. This is analogous to when doing regular MS^{E} experiments. To compare the results obtainable from MS^{ETD} experiments with MS^{E}, MS^{E} was also recorded. The TIC trace of the second function from a MS^{E} experiment of the same sample is shown in Figure 2c. A difference in relative intensities of eluting peaks is evident between the full MS trace in Figure 2a and the two TIC traces of MS^{ETD} and MS^{E} in Figure 2b and c, which relates to differences in the ETD and CID reaction efficiencies, respectively. For ETD reactions, particularly highly charged fragments will have greater electron affinities and undergo higher ETD reaction rates [15].

The high level of information obtained from MS^{ETD} is illustrated and compared with MS^{E}, by the full MS, MS^{ETD}, and MS^{E} spectra of the tryptic HSA peptide, T50, in Figure 3. As seen in the figure, full sequence coverage was obtained both with MS^{ETD} and MS^{E}. In addition to the ETD *c*- and *z*-fragments spanning the whole peptide backbone in MS^{ETD}, some *b*- and *y*-ions are also observed. These CID fragments stem from the supplemental energy applied in the transfer cell of the instrument. The function of this relatively low supplemental energy is triple: (1) to dissociate non-covalent interactions after the ETD reaction in the trap cell, (2) to generate b- and y-ions at most preferential CID sites, and (3) to generate diagnostic w-ions for leucine and isoleucine discrimination. The fragments most readily observed with slightly elevated collision energies were from the outer termini and cleavage N-terminal to proline residues. Since ETD fragmentation does not cleave N-terminal of proline residues due to the side chain pyrrolidine, the complementary information obtained from ETD and supplementary CID is a powerful combination. This pattern is evident from

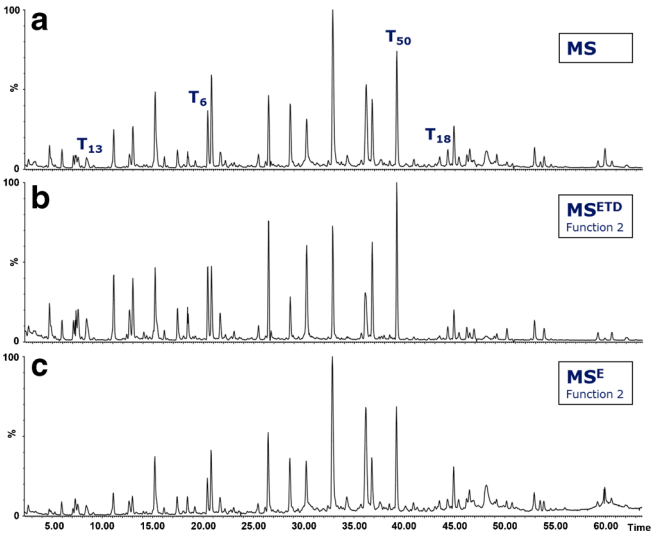


Figure 2. MS^{ETD} and MS^{E} peptide maps of tryptic digested HSA. Both the MS^{ETD} and MS^{E} data were recorded with two alternating functions containing precursor and sequence information, respectively. For the MS^{ETD} experiment **(a)** the full MS TIC trace, and **(b)** the MS^{ETD} TIC trace are shown. For the MS^{E} experiment, only the TIC trace of the second function with elevated CID is shown in **(c)**. Selected peptides present in varying abundances are annotated in the figure

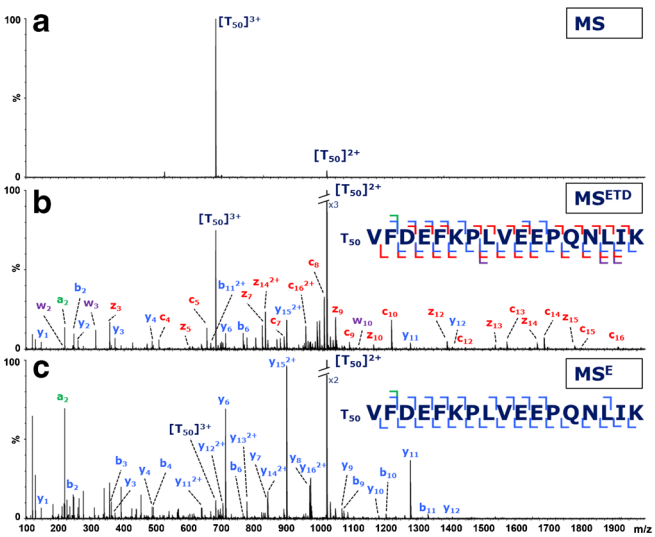


Figure 3. Mass spectra of the T50 peptide from the tryptic digest of HSA. The **(a)** full MS, **(b)** MS^{ETD}, and **(c)** MS^{E} spectra consist of two summed scans at the retention time of the T50 peptide elution indicated in Figure 2. Fragment ions are annotated by the following colors; green: a-ions, blue: b-/y-ions, red: c-/z-ions, and purple: w-ions. All identified fragment ions are illustrated in the insets of the sequence coverages in **(b)** and **(c)**

the graphical fragment maps shown as insets in the figure. The potential of this could thus be exploited to analyze a wide range of targets with different characteristics, where trade-off decisions normally would be made between CID versus ETD, data richness versus data analysis, etc. A distinct advantage of MS^{ETD} compared with MS^{E} is the potential of discriminating between the isobaric leucine and isoleucine residues. Leucine/isoleucine discrimination by MS has previously been demonstrated by w-ion formation in MS^3 experiments (ETD-HCD), since the side chain loss from the intermediate radical z-ion is different for leucine ($C_3H_7^{\cdot}$ loss) and isoleucine ($C_2H_5^{\cdot}$ loss) [14]. In MS^{ETD}, diagnostic w-ions can be generated in the same transmission by the ETD and subsequent supplemental CID fragmentation, without the intermediate z-ion isolation step. As seen for the MS^{ETD} fragmentation of the T50 peptide in Figure 3b, all three cases of leucine/isoleucine are discriminated by w-ion generations.

Additional MS^{ETD} and MS^{E} fragmentation spectra of the tryptic HSA peptides T18, T13, and T6, which are present in varying abundances as annotated in Figure 2, are shown as examples in Supporting Figures S1–S3, respectively. These spectra show full sequence coverage of the individual peptides, both using MS^{ETD} and MS^{E}. Except for the leucine residue in the T6 peptide located as N-terminal and thus not able to undergo z-ion formation, all leucine/isoleucine occurrences were discriminated and mapped by w-ion formations using MS^{ETD}. Extensive double coverage by pairs of b-/c- and y-/z-ions is also observed using MS^{ETD}. The observation of b/c and y/z ion-pairs not only increase the confidence of the assignments, but can also aid in de novo sequencing due to the mass difference of b/c ions of 17 and y/z ions of 16, previously reported as the 'golden sets' approach [16].

Depending on the aim of a specific study, other potential benefits of the ETD fragmentation in the MS^{ETD} approach include mapping of labile post-translational modifications (e.g., glycosylations) and isoaspartic acid occurrences (i.e., isomerization of aspartic acid following asparagine deamidation), which cannot otherwise be detected during classical MS^{E} experiments.

The MS^{ETD} method, as other DIA methods, faces limitations with regards to co-eluting peaks from the LC separation, which will complicate the interpretation. However, automated processing of the data by software able to directly read and assign the MS^{ETD} format would be beneficial in the future. Such software already exists for deconvolution of MS^{E} data (BiopharmaLynx/UNIFI, Waters, Wilmslow, UK), which can provide specific fragmentation spectra even when species nominally co-elute. This only fails when species have exactly the same elution profile, which is fairly rare with UPLC separations. Despite the current limitation, MS^{ETD} offers easier data handling and experiment setup than DDA and other fragmentation studies, and provides precursor and sequencing information in a single LC run. Furthermore, the experiment described is highly sensitive due to the transmission type nature of the ETD method, despite the less deterministic control of the reaction time compared with ETD in ion trapping instruments.

Conclusion

In this work we have introduced the broadband DIA method MS^{ETD} as a novel and easy way of running MS^{E} by exchanging the conventional collision-based fragmentation with ETD for the "high energy" step. This setup provides all the benefits of the MS^{E} type experiment but for ETD mode, with unbiased data acquisition and no prior knowledge of the sample needed. The powerful combination of transmission-type ETD and low-energy supplemental activation CID is able to discriminate between the isobaric leucine and isoleucine residues by w-ion formation, and generates both b-/y- and c-/z-ion-pairs, which not only increases the confidence of the sequence assignments but also provides complementary information due to the different fragmentation mechanisms of ETD and CID. The most prominent example hereof is the lack of ETD fragmentation N-terminal to proline residues, which on the other hand is one of the most preferential CID cleavage sites. The richness of information of precursors and their ETD- and CID dissociations all in a single run and the simplicity of the method setup and relatively easy data analysis altogether represents a novel and powerful way of performing DIA.

Acknowledgments

This work, a part of the industrial PhD project for C.N.C., was supported by The Danish Agency for Science, Technology, and Innovation and the Novo Nordisk STAR program.

References

1. Gillet, L.C., Navarro, P., Tate, S., Rost, H., Selevsek, N., Reiter, L., Bonner, R., Aebersold, R.: Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. Mol. Cell. Proteomics **11**, O111.016717 (2012)
2. Bateman, R.H., Carruthers, R., Hoyes, J.B., Jones, C., Langridge, J.I., Millar, A., Vissers, J.P.: A novel precursor ion discovery method on a hybrid quadrupole orthogonal acceleration time-of-flight (Q-TOF) mass spectrometer for studying protein phosphorylation. J. Am. Soc. Mass Spectrom. **13**, 792–803 (2002)
3. Oh, H.B., Moon, B.: Radical-driven peptide backbone dissociation tandem mass spectrometry. Mass Spectrom. Rev. **34**, 116–132 (2015)
4. Mayer, P.M., Poon, C.: The mechanisms of collisional activation of ions in mass spectrometry. Mass Spectrom. Rev. **28**, 608–639 (2009)
5. Dongre, A.R., Somogyi, A., Wysocki, V.H.: Surface-induced dissociation: an effective tool to probe structure, energetics, and fragmentation mechanisms of protonated peptides. J. Mass Spectrom. **31**, 339–350 (1996)
6. Zubarev, R.A., Kelleher, N.L., McLafferty, F.W.: Electron capture dissociation of multiply charged protein cations. A nonergodic process. J. Am. Chem Soc **120**, 3265–3266 (1998)
7. Syka, J.E., Coon, J.J., Schroeder, M.J., Shabanowitz, J., Hunt, D.F.: Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. Proc. Natl. Acad. Sci. U. S. A. **101**, 9528–9533 (2004)
8. Sweredoski, M.J., Pekar Second, T., Broeker, J., Moradian, A., Hess, S.: High resolution data-independent acquisition with electron transfer dissociation mass spectrometry: multiplexed analysis of post-translationally modified proteins. Int. J. Mass Spectrom. **390**, 155–162 (2015)

9. Carvalho, P.C., Han, X., Xu, T., Cociorva, D., Carvalho Mda, G., Barbosa, V.C., Yates III, J.R.: XDIA: improving on the label-free data-independent analysis. Bioinformatics **26**, 847–848 (2010)
10. Wiesner, J., Premsler, T., Sickmann, A.: Application of electron transfer dissociation (ETD) for the analysis of post-translational modifications. Proteomics **8**, 4466–4483 (2008)
11. Chrisman, P.A., Pitteri, S.J., Hogan, J.M., McLuckey, S.A.: SO2-* electron transfer ion/ion reactions with disulfide linked polypeptide ions. J. Am. Soc. Mass Spectrom. **16**, 1020–1030 (2005)
12. Coon, J.J., Ueberheide, B., Syka, J.E., Dryhurst, D.D., Ausio, J., Shabanowitz, J., Hunt, D.F.: Protein identification using sequential ion/ion reactions and tandem mass spectrometry. Proc. Natl. Acad. Sci. U. S. A. **102**, 9463–9468 (2005)
13. Sargaeva, N.P., Lin, C., O'Connor, P.B.: Differentiating N-terminal aspartic and isoaspartic acid residues in peptides. Anal. Chem. **83**, 6675–6682 (2011)
14. Lebedev, A.T., Damoc, E., Makarov, A.A., Samgina, T.Y.: Discrimination of leucine and isoleucine in peptides sequencing with Orbitrap Fusion mass spectrometer. Anal. Chem. **86**, 7017–7022 (2014)
15. Rose, C.M., Rush, M.J., Riley, N.M., Merrill, A.E., Kwiecien, N.W., Holden, D.D., Mullen, C., Westphall, M.S., Coon, J.J.: A calibration routine for efficient ETD in large-scale proteomics. J. Am. Soc. Mass Spectrom. **26**, 1848–1857 (2015)
16. Horn, D.M., Zubarev, R.A., McLafferty, F.W.: Automated de novo sequencing of proteins by tandem high-resolution mass spectrometry. Proc. Natl. Acad. Sci. U. S. A. **97**, 10313–10317 (2000)