

Improving Fragmentation Efficiency of Tandem Mass Tag Labeled Peptides Using Stepped Higher Energy Collisional Dissociation

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Overview

Purpose: Investigate the effects of stepped collision energy during higher energy collisional dissociation (HCD) for relative quantitation of Tandem Mass Tag (TMT) labeled peptides on the LTQ Orbitrap XL mass spectrometer.

Methods: Enzymatic digests of 10 standard protein mixtures labeled with TMT were analyzed under variable experimental conditions using an LTQ Orbitrap XL mass spectrometer.

Results: Collision energy (CE) is one of the most important parameters for quantitation using reporter ions from TMT labeled peptides. It needs to be optimized to generate enough fragment ions for protein identification and reporter ions for quantitation. Using a fixed CE at 45%, we observed good MS/MS spectra for most peptides but some of them did not have detectable or enough reporter ions. Increasing CE to 50% improved the reporter ion abundance but it also caused excessive fragmentation of some peptides at the same time. Here, we used stepped collision energy functionality with two steps at 40% and 50% CE during HCD to improve the reporter ion abundance while preserving peptide structural fragments. The first step (CE at 40%) produced abundant structural fragments while the second step (CE at 50%) added more fragment ions in the lower *m/z* region and also increased the abundance of reporter ions. As a result, richer fragmentation information was obtained along with more abundant reporter ions. Comparison of results from a digested 10 protein mixture showed that the two-stepped CE identified about the same number of peptides but more quantifiable peptides. Since stepped CE increased the reporter ion abundance, it improved overall ion statistics at both the peptide and protein level. We also compared the effects of increasing the number of microscans for quantitation. The results showed that using several microscans did not improve quantitation results.

Introduction

The importance of quantitative proteomics has driven the development of various mass spectrometry based quantitation methods in the past few years. Among them, isobaric stable isotope tagging reagents such as TMT^{1,2} and iTRAQ^{3,4} enable multiplexed quantitation of peptides via reporter ions in the low mass range of MS/MS spectra. Efficient fragmentation with detection over a wide *m/z* range makes the higher energy collisional dissociation (HCD) of the LTQ Orbitrap XL a powerful tool for identification and quantitation of isobaric labeled peptides⁵.

For quantitation using reporter ions from TMT, the ion statistics of reporter ions is an important factor with respect to the precision of quantitation. Instrument parameters including CE of HCD, Automatic Gain Control (AGC) target of MS/MS scans and the number of microscans are the keys for generating good quality MS/MS spectra. As one of the most important parameters, CE not only needs to be optimized to generate enough fragments for peptide identification but also need to be adjusted to get reasonable reporter ion abundance for quantitation. For most peptides, one optimized CE is sufficient to produce good MS/MS spectra for both protein ID and quantitation but for some peptides more energy is needed to generate enough reporter ions for quantitation. In order to improve the quantitation results without losing protein IDs, different CE is needed. With the capability of multiple C-trap fills in the LTQ Orbitrap XL, different CE can be used in one HCD event. Here, we investigated the improvement of results using stepped CE.

Methods

Sample Preparation

Digest of reduced and alkylated 10 standard protein mixture from Sigma was divided into 6 aliquots and each of these was labeled according to manufacturer provided protocol with Thermo Scientific Tandem Mass Tags (126, 127, 128, 129, 130 and 131). Samples were subsequently mixed in one to one ratio, and diluted five times with 5% formic acid before LC-MS/MS analysis.

LC

HPLC System: Thermo Scientific Surveyor MS Pump with a flow splitter
Column: PicoFrit™ column (10 cm x 75 µm id), New Objective, Inc., Cambridge, MA
Mobile Phases: 0.1% formic acid in water; 0.1% formic acid in acetonitrile
Gradient: 10% B 10 minutes, 10% - 30% B in 120 minutes
Flow: 300 nL/min on column

MS

Mass Spectrometer: Thermo Scientific LTQ Orbitrap XL
MS Resolution: 60000
MS2 Resolution: 7500
MS AGC target: 5×10⁵
MS/MS AGC target: 1×10⁵. For stepped CE, AGC is set at 2×10⁵ so that for each step the AGC is 1×10⁵
Exclusion mass tolerance: 10 ppm
Injection Time FTMS/MS: 500 ms
Full MS mass range: 380–1300 *m/z*
MS/MS Mass range: 100–2000 *m/z*
MS/MS Events: Full MS in Orbitrap followed by top three Data Dependent HCD and three CID events in ion trap
CE for HCD: 45%. Stepped CE method used 2 steps with range of 10, CE at 40% and 50%, respectively

Data Processing

The results were compared after data analysis using Thermo Scientific Proteome Discoverer 1.1 software. Figure 1 is the TMT quantitation data analysis workflow set up using Proteome Discoverer™ 1.1 software.

Results

To set up stepped normalized collision energy (SNCE), the central normalized collision energy, the ion activation time, the range of normalized collision energies and the number of steps needed to be considered⁶. Table 1 shows the general concept for stepped normalized collision energy settings.

FIGURE 1. TMT quantitation workflow in Proteome Discoverer software

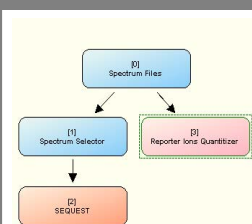


Table 1: Stepped normalized collision energy (SNCE) setting concept

Range	Steps	Collision Energy	Activation Time	Resulting SNCE
20	3	40	30 msec	SNCE: 30%, 40%, 50% Each Step: 10 msec
20	2	40	30 msec	SNCE: 30%, 50%
20	3	40	60 msec	SNCE: 30%, 40%, 50% Each Step: 20 msec
20	3	30	60 msec	SNCE: 20%, 30%, 40% Each Step: 20 msec

In Figure 2, The top panel shows that 45% collision energy with 1 microscan (µs) identified slightly more peptides than the other two methods. It is mainly because each scan takes longer time in the other two methods. For quantitation, it is clear that more peptides were quantitated using two step CE method. It is mainly due to the improved fragmentation efficiency. Figure 4 is one of the examples showing HCD MS/MS spectra quality using the three different methods.

FIGURE 2. Comparison of peptide identification and quantitation results using different CE settings. Top panel: number of peptides identified from three different conditions. Bottom panel: percentage of quantified peptides for all 10 proteins.

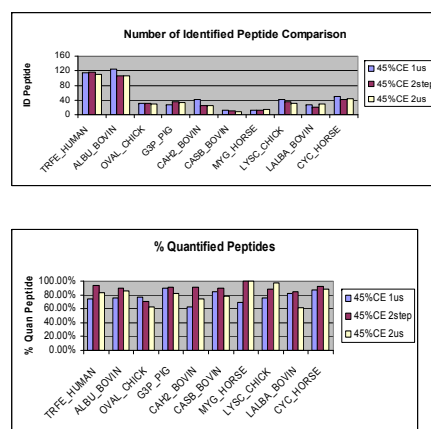
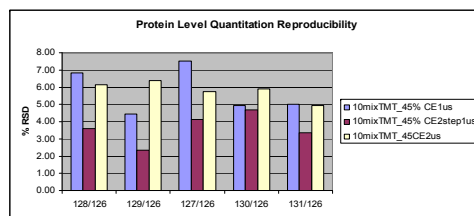


FIGURE 3. Comparison of reporter ion ratio reproducibility at protein level



Differently labeled protein digests were mixed at a 1:1 ratio. We compared the reporter ion ratios across all ten proteins (Figure 3). The results showed that among the three methods, stepped CE showed the lowest %RSD (best reproducibility). This method produced more reporter ions, which improved the ion statistics.

For the particular 4+ peptide shown in Figure 4, stepped collision energy generated more than twice amount of reporter ions than the other two methods. Also, the interference peaks in the reporter ion region were well resolved in all three methods. One of the biggest advantages of HCD is that the high resolution spectra obtained from Orbitrap increases the accuracy of quantitation.

FIGURE 4. HCD MS/MS spectra of peptide K TEREDLIAYLK* (4+) from cytochrome C using three different experiment conditions. Top panel: Normalized TMT reporter ion intensity. Bottom panel: Normalized intensity of HCD MS/MS spectra

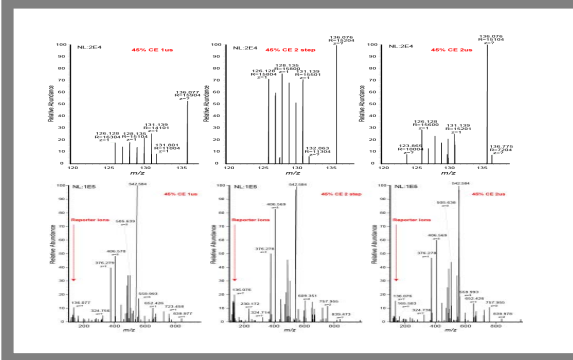
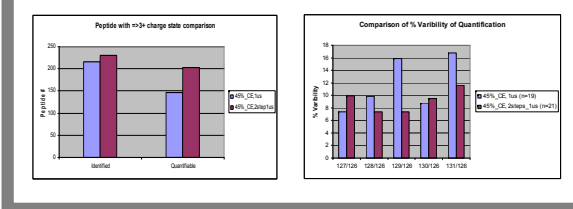


FIGURE 5. The number of identified and quantitated peptides with charge state at 3 or higher using one-step or two-step CE.



The effects of CE on high charge state peptides (>=3) were also investigated. The results showed that stepped CE provided a big improvement in the number of peptides that are identified and quantified.

Conclusion

- A fixed, optimized collision energy (CE) can produce good identification and quantitation results for most peptides.
- For peptides that do not yield good quantitation results at a fixed CE, a stepped CE method can be used.
- Stepped CE showed overall improvement of quantitation results, including number of peptides quantitated and reproducibility.
- Increasing the number of microscans will improve the quality of spectra but it does not significantly improve quantitation results.

References

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