Application Note: 483

Improving Quantitation of TMT-Labeled Peptides Using Stepped Higher-Energy Collisional Dissociation

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Key Words

- LTQ Orbitrap
- HCD
- TMT
- SNCE
- Quantitation

Introduction

The relative quantification of proteins in cells, tissues, and body fluids is of great interest in proteomics. Over the past decade, numerous mass spectrometric quantification strategies have been developed[1,2]. One of these strategies is tagging with isobaric stable isotopes. It allows multiplexing in relative or absolute proteomic quantification experiments via tandem mass spectrometry[3,4]. A number of isobaric stable isotope tagging technologies have become commercially available, including Tandem Mass Tags (TMT®)[3] and Isobaric Tags for Relative and Absolute Quantification (iTRAQ®)[4]. The general applicability of the TMT approach for quantitative proteomics and biomarker discovery has been demonstrated recently.[5]

The 6-plex TMT Kit is a newly available isobaric tagging reagent set. Each of the six tags is composed of a reporter group, a balance group and a primary amine reactive group, which modifies the N-terminus and lysine ε amino groups of a peptide or protein[3]. The consistent use of only C and N isotopes guarantees that each tag has exactly the same total mass, as well as identical physicochemical properties, which are critical for uniform liquid chromatographic separation and electrospray ionization (ESI). Upon dissociation (fragmentation), each tag generates a unique reporter ion at *m/z* 126, 127, 128, 129, 130 or 131. The relative ratios of these reporter ions can be used for relative quantitation.

Higher-energy collisional dissociation (HCD) is an MS/MS fragmentation technique that operates over a wide *m/z* range. This makes it well suited to identification and quantitation of isobarically labeled peptides[6,7]. For TMT or iTRAQ quantitation, the statistics of reporter ions directly influence precision and accuracy of the analysis. Optimization of HCD acquisition parameters, including collision energy (CE), AGC target for MS/MS scans, and the number of microscans, is a key factor for obtaining high-quality MS/MS spectra.

As one of the most important HCD parameters, collision energy must be optimized to both generate enough fragments for peptide identification and obtain reasonable reporter ion abundance for quantitation. For most peptides, a single CE value is sufficient to produce good MS/MS spectra for both protein ID and quantitation. However, for some peptides, the CE needed to produce abundant reporter ions is higher than that for structural fragments. The implementation of HCD in Thermo Scientific Orbitrap hybrid mass spectrometers allows multiple fills of the C-trap where ions are accumulated prior to injection into the Orbitrap™ mass analyzer. HCD fragments generated at several different collision energies (stepped CE) can all be stored together in the C-trap prior to analysis.

Goal

The goal of the research reported in this application note was to investigate the effects of stepped HCD collision energy on relative quantitation of TMT-labeled peptides.

Experimental

Sample Preparation

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

LC Separation

HPLC System:	Thermo Scientific Surveyor MS Pump equipped 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 (eluent A); 0.1% formic acid in acetonitrile (eluent B)
Gradient:	10% B 10 minutes, 10% – 30% B in 120 minutes
Flow:	300 nL/min

Mass Spectrometry

Mass spectrometer:	Thermo Scientific LTQ Orbitrap XL hybrid MS
MS resolution:	60000
MS2 resolution:	7500
MS AGC target:	$5x10^5$
MS/MS AGC target:	$2x10^5$. For stepped CE, AGC for each step is $1x10^5$. Thus 2e5 AGC target allows for a two-step fill.
Exclusion mass tolerance:	10 ppm
Injection time FT MS/MS:	500 ms
Full MS mass range:	380 – 1300 *m/z*
MS/MS mass range:	100 – 2000 *m/z*
MS/MS events:	Full MS with Orbitrap detection followed by top three Data Dependent HCD and top three Data Dependent CID events in the ion trap
CE for HCD:	45%, stepped CE method used 2 steps with range of 10, resulting in CE of 40% and 50%, respectively

Data Analysis

Results were analyzed and compared using Thermo Scientific Proteome Discoverer 1.1 software with SEQUEST® search. Figure 1 is the TMT quantitation data analysis workflow used in the Proteome Discoverer™ analysis. TMT modification of *m/z* 229.163 on lysine and the N-terminal peptide amino acid were used for database searching. The identified proteins were filtered using medium and high confidence on the peptide level, peptide mass deviation of 10 ppm, and peptide rank one. The "Apply Quan Value Corrections" and "Reject All Quan Values if not All Quan Channels are Present" features in Reporter Ions Quantitizer tool were used.

Results and Discussion

Stepped normalized collision energy (SNCE) HCD experiments were performed by passing ions from the linear trap to the C-trap and into the collision cell at different collision energy offsets. The fragment ions from several such fills of the HCD collision cell were transferred back to the C-trap, from which they were then ejected into the Orbitrap analyzer[8].

When using SNCE, the MS^n AGC target, central normalized collision energy (CNCE), ion activation time, range of normalized collision energies (NCE), and number of steps must be considered to result in improved quantitation[9]. Each multiple fill consists of 100,000 ions, so the functionality may be used with an MS/MS target of 2e5 or higher. Table 1 shows the general concept for stepped normalized collision energy settings. Figure 2 covers the general steps for the method set-up. The CNCE was set at 45% and the step collision energy was set with two steps and 10% collision energy width, resulting in the effective collision energies of 40% and 50% respectively.

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% Each Step: 15 msec
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

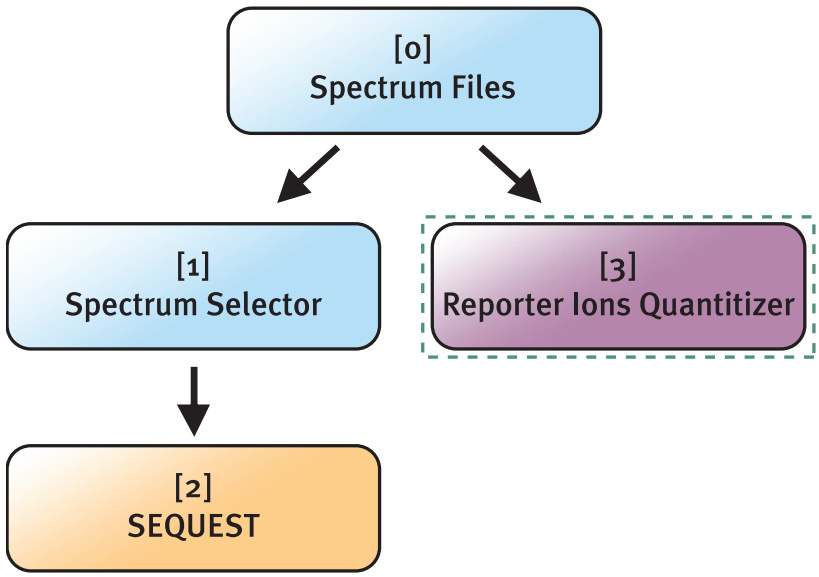

Figure 1: Isobaric labeled peptide/protein relative quantitation workflow in Proteome Discoverer

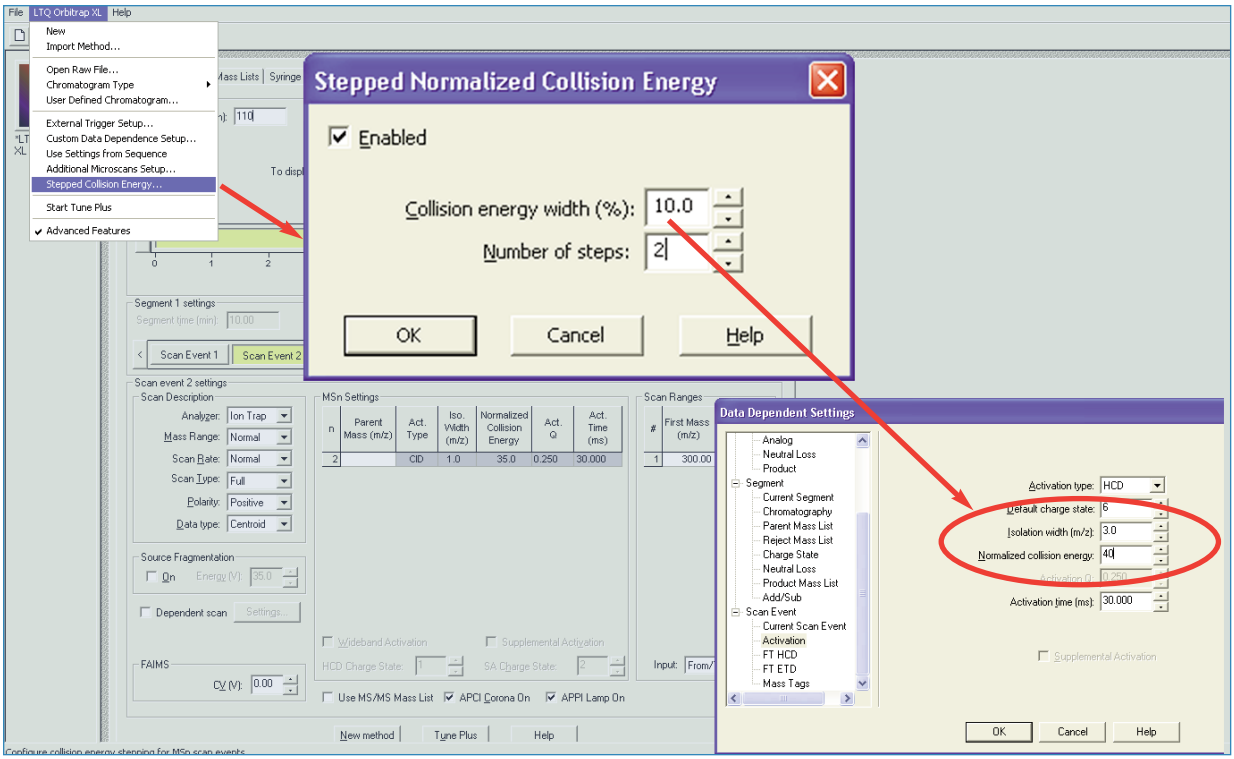

Figure 2: SNCE method set up from Thermo Scientific Xcalibur 2.07 (Tune 2.5.5)

The benefits of the SNCE approach for TMT-labeled quantitation of a ten-protein digest mixture were compared with two other methods: fixed 45% CE with 1 µscan and 2 µscans (see Figure 3). The top panel shows that using 45% collision energy with 1 µs resulted in identification of slightly more peptides than using the other two methods, primarily due to the increased duty cycle of this method. However, more peptides were quantified using the SNCE method, as this method provides improved fragmentation efficiency.

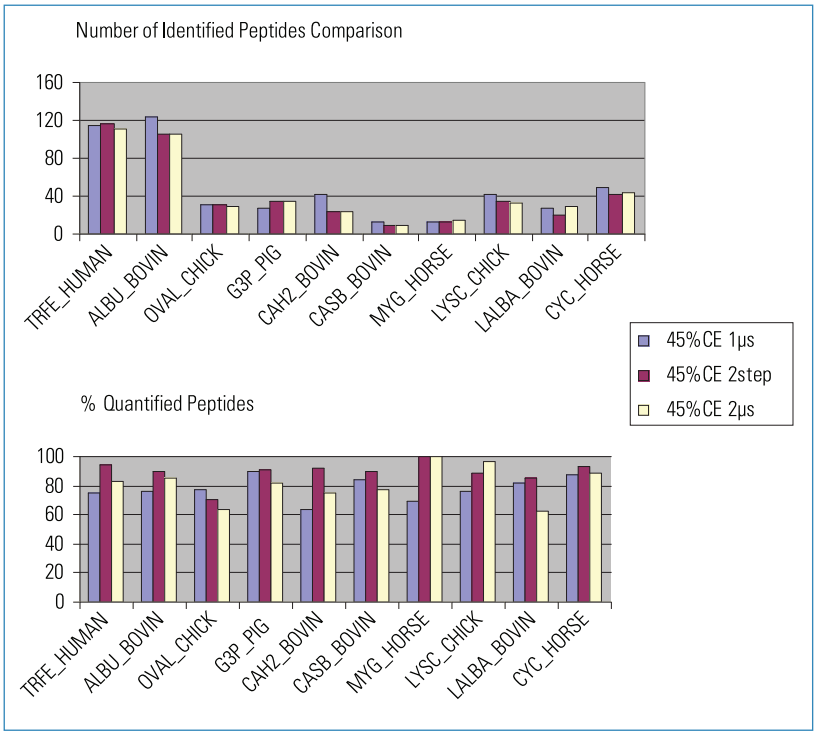

Figure 3: Identification and quantification comparison with different methods. Top panel: identified peptides. Bottom panel: percentage of quantified peptides for all 10 proteins.

Figure 4 shows examples of HCD MS/MS spectra quality from data acquired using the three different methods. For a 4+ peptide precursor ion, using the stepped collision energy approach resulted in a two-fold improvement of the reporter ion intensity relative to the other two methods. The interference peaks in the reporter ion region were well resolved, as shown in Figure 5, resulting in improved quantitation accuracy.

It is likely that larger, multiply charged peptides require a wider range of collision energies to produce a rich fragmentation pattern simultaneously with abundant reporter ions as compared to smaller peptides with fewer charges. The top panel of Figure 6 shows that among the three methods, the stepped CE yielded the lowest % RSD (best reproducibility), benefitting from the better ion statistics of the reporter ions. The bottom panel shows the quantification precision results for cytochrome C per individual reporter ion channel. The SNCE method provided a greater advantage for identification and quantification of peptides with charge states 3+ or higher. Figure 7 shows that by using a two-stepped SNCE approach it was possible to identify slightly more multiply charged peptides and a significantly higher number of identified peptides were amenable to quantitation.

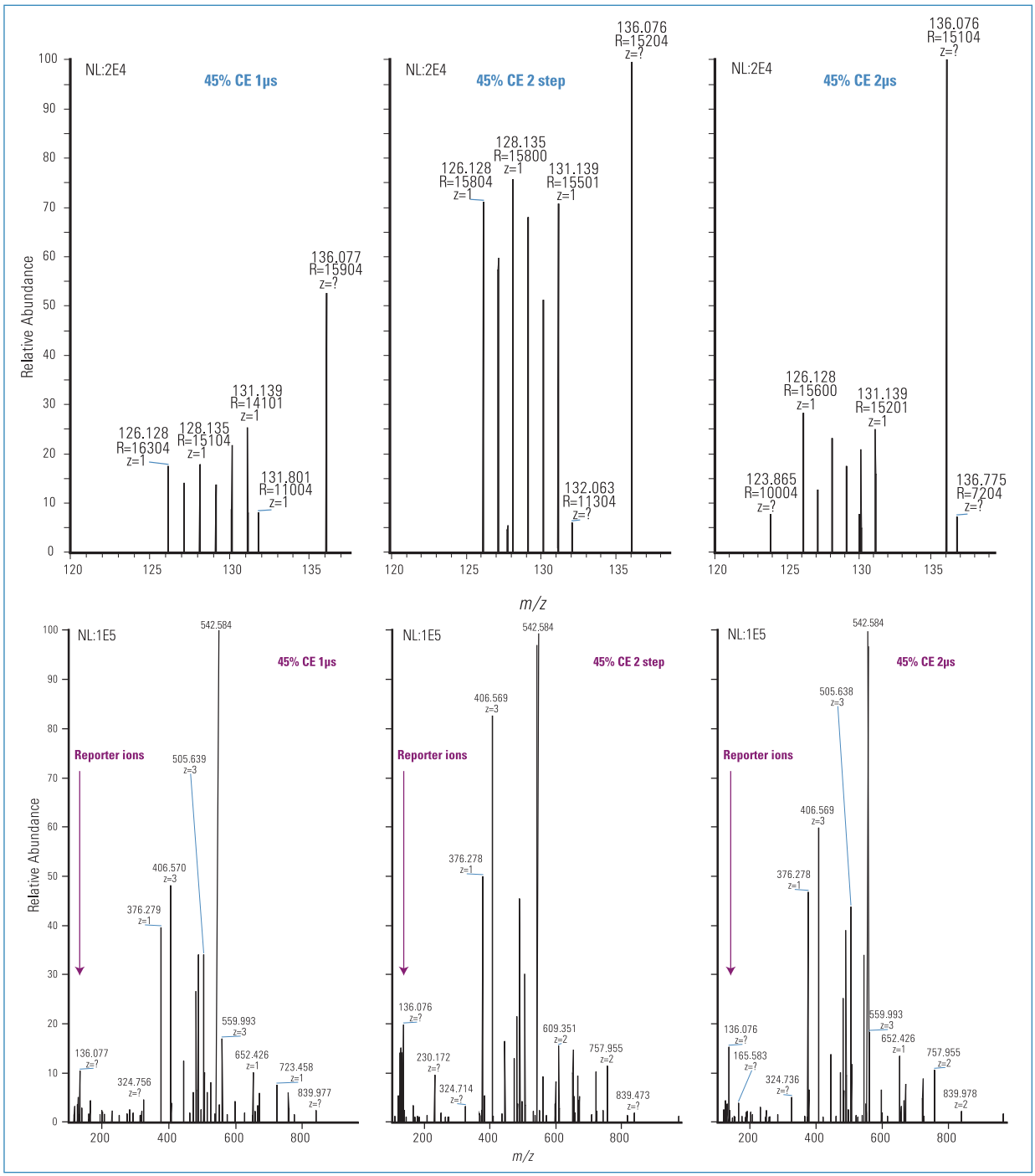

Figure 4: Effect of HCD collision energy on MS/MS spectra of peptide **K*TEREDLIAYLK*** (4+) from cytochrome C. Left column: HCD with single collision energy and 1 microscan. Middle column: HCD with 2-step collision energy. Right column: HCD with single collision energy and two microscans. Top spectra show normalized intensity of TMT reporter ions. Bottom spectra show normalized intensity of HCD MS/MS spectra.

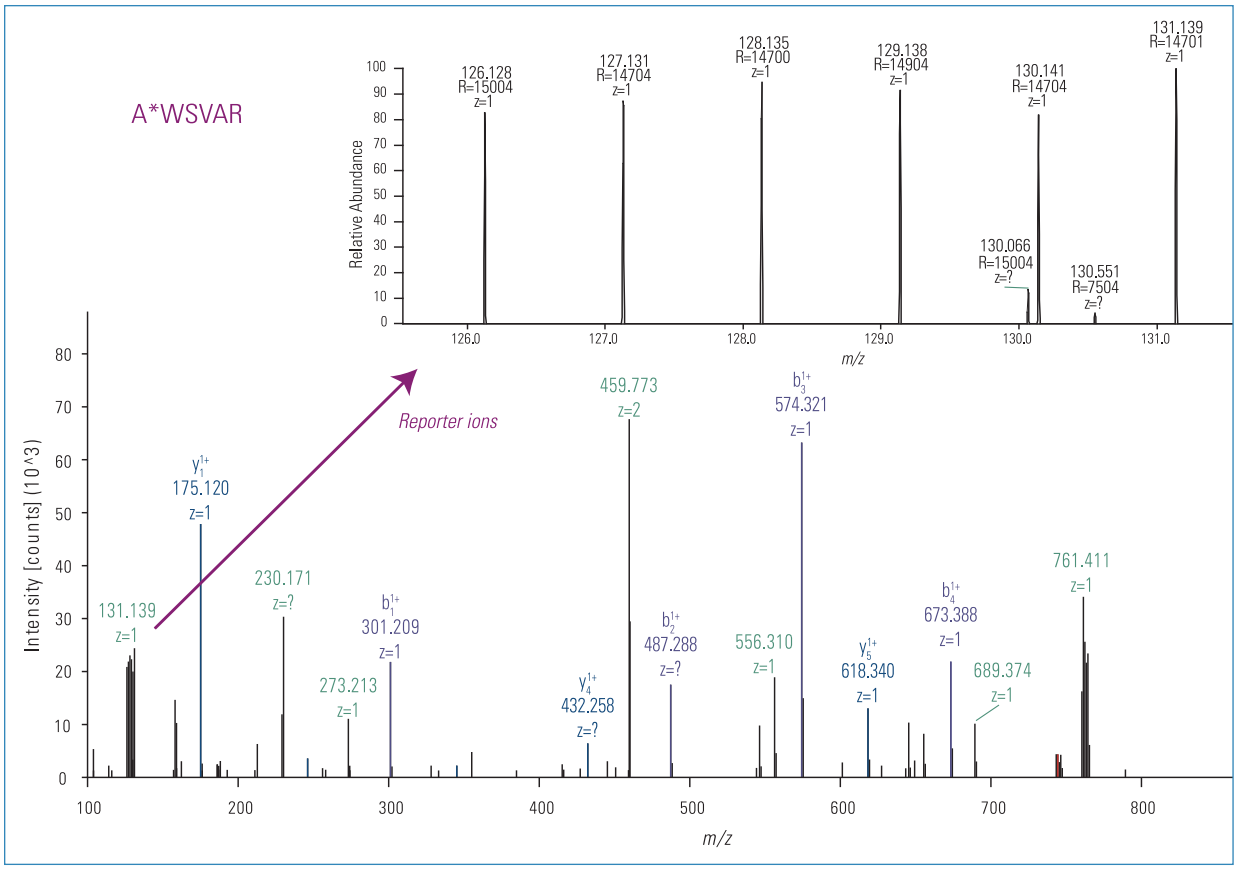

Figure 5: Resolving TMT isobaric interferences with Orbitrap mass spectrometry. The relative abundance ratios are very close to the actual 1:1:1:1:1:1 mixing ratio. Reporter ions and interferences are clearly resolved.

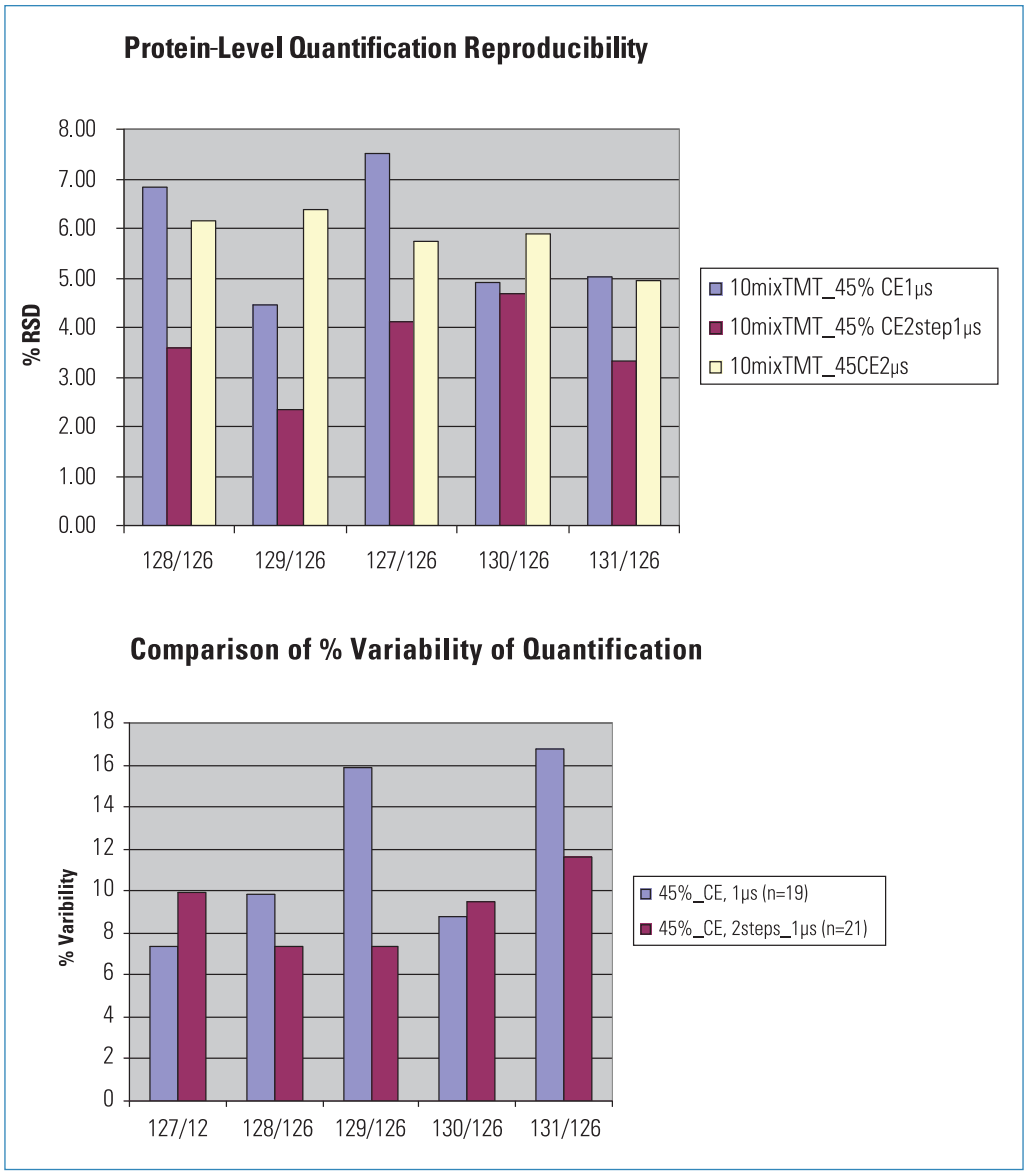

Figure 6: Quantification precision for proteins and peptides. Top panel: reporter ion ratio reproducibility at the protein level. Lower panel: reproducibility of peptide quantification from cytochrome C, calculated using at least 19 peptides.

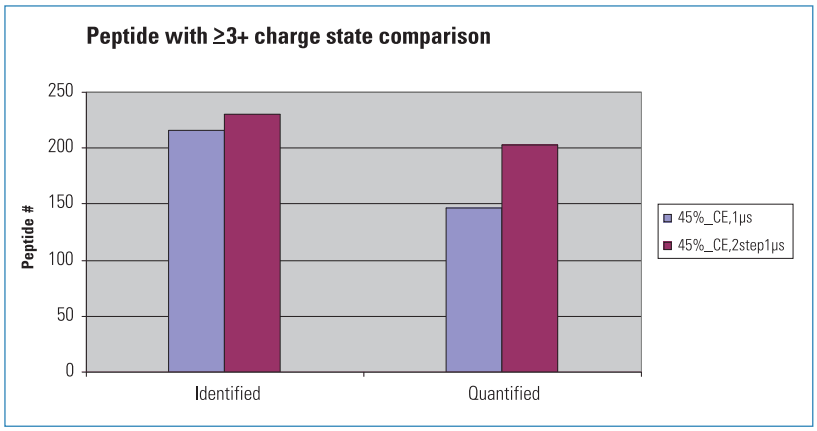

Figure 7: Effect of HCD collision energy on peptides identification and quantification for 3+ and higher-charge-state precursors.

Conclusion

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

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