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Effect of Collision Energy Optimization on the Measurement of Peptides by Selected Reaction Monitoring (SRM) Mass Spectrometry

Brendan MacLean^{1,†}, Daniela M. Tomazela^{1,†}, Susan E. Abbatiello², Shucha Zhang³, Jeffrey R. Whiteaker³, Amanda G. Paulovich³, Steven A. Carr², and Michael J. MacCoss^{1,*}

¹Department of Genome Sciences, University of Washington, Seattle, WA, USA.

²Broad Institute of MIT and Harvard, Cambridge, MA, USA

³Fred Hutchinson Cancer Research Center, Seattle, WA, USA.

Abstract

Proteomics experiments based on Selected Reaction Monitoring (SRM, also referred to as Multiple Reaction Monitoring or MRM) are being used to target large numbers of protein candidates in complex mixtures. At present, instrument parameters are often optimized for each peptide, a time and resource intensive process. Large SRM experiments are greatly facilitated by having the ability to predict MS instrument parameters that work well with the broad diversity of peptides they target. For this reason, we investigated the impact of using simple linear equations to predict the collision energy (CE) on peptide signal intensity and compared it with the empirical optimization of the CE for each peptide and transition individually. Using optimized linear equations, the difference between predicted and empirically derived CE values was found to be an average gain of only 7.8% of total peak area. We also found that existing commonly used linear equations fall short of their potential, and should be recalculated for each charge state and when introducing new instrument platforms. We provide a fully automated pipeline for calculating these equations and individually optimizing CE of each transition on SRM instruments from Agilent, Applied Biosystems, Thermo-Scientific and Waters in the open source Skyline software tool (<http://proteome.gs.washington.edu/software/skyline>).

Introduction

Due to its high sensitivity and selectivity, selected reaction monitoring-mass spectrometry (SRM-MS) has become a very attractive technique for measuring target peptides in a complex mixture^{1–6}. This methodology is deeply rooted in small molecule quantitative mass spectrometry where it has been applied successfully for several decades^{7–8}. In proteomics, however, SRM-MS is being used in experimental paradigms ranging from classical quantitative experiments to experiments seeking to measure larger numbers of targeted peptides with less emphasis on precise quantification⁹.

At one extreme, the target assay is thoroughly evaluated for accuracy, precision, linearity, limit of detection, and limit of quantification. To maximize precision, stable isotope labeled internal standards are frequently used to account for errors and losses that can occur during sample handling and ionization in the analysis of small molecules^{7,10–13} as well as for peptides^{1–6}. Because of the rigors of establishing these assays and successfully performing

*To whom correspondence should be addressed. maccoss@u.washington.edu.

†These authors contributed equally to this manuscript.

them in complex matrices¹⁴, they tend to be implemented on only a selected number of analytes in parallel. With only a small number of analytes measured, it is common to expend significant time optimizing tune parameters and collision energies of each analyte individually to attain the highest sensitivity possible.

More recently, SRM-based experiments are being used earlier in the discovery process targeting large numbers of hypothesized candidates¹⁵. In contrast to a quantitative experiment that generally targets tens of compounds for the purpose of quantifying them (usually relying on a stable isotope labeled standard)^{1,2}, discovery-oriented SRM can target hundreds of peptides in a single proteomics experiment^{16,17}. In these experiments, SRM may first be used to confirm that the peptides of interest can be detected within the sample matrix. In addition, exogenous synthetic peptides may be used as internal standards in assays which are differential or semiquantitative without establishing the linearity, response, precision, and accuracy of each analyte. This multiplexed differential assay approach can significantly reduce the list of peptides subsequent to developing a precise and accurate quantitative assay for targets of future studies.

One of the challenges of discovery-oriented SRM proteomics is the need to determine *a priori* a set of general MS instrument parameters that work well with the broad diversity of peptides to be targeted. Synthetic peptide standards are commonly measured by direct infusion to optimize these parameters empirically. This approach, however, scales poorly to experiments where the best peptides for monitoring target proteins may not be known, greatly increasing the number of peptides to be assayed.

Collision energy (CE) is an instrument parameter that is frequently optimized to increase fragment ion intensity. Multiple instrument manufacturers offer automated routines for CE optimization by peptide infusion as part of their instrument tuning software. This optimization technique, however, requires significant human attention during infusion and a relatively pure standard for each peptide targeted.

An alternative to optimizing the CE for each peptide empirically is to predict the optimal CE value from the precursor mass-to-charge ratio (m/z). In this approach, the optimal CE is determined empirically for selected peptides. The resulting precursor m/z , CE pairs are used to derive a linear equation, expressed as $CE = k * (m/z) + b$, for each precursor charge-state^{18,19}. Once the slope (k) and intercept (b) have been calibrated, this equation can be used to estimate the CE for new peptides that may not have standards available. This method of predicting the peptide CE has been used for qualitative analysis of peptides on triple quadrupole⁹ and quadrupole time-of-flight mass spectrometers for more than a decade^{18,19}. Though it is sufficient for qualitative analysis of peptides, the effect of using a predicted CE for measuring the quantitative response of a peptide has not been thoroughly evaluated. Previous papers have reported large improvements in signal intensity^{20,21} when transitions are optimized individually instead of using linear equations to predict the CE based on precursor m/z . These reports, however, have been for small datasets²⁰ or listed as based on “unpublished data”²¹.

A recent paper by Sherwood and co-workers describes a manual “instrument setup workflow,” involving a Perl script and proprietary software, to aid in method optimization of CE in tandem quadrupole instruments (Waters Quattro Premier and ABI 4000 Q Trap)²⁰. This workflow relies on performing “effective collision energy and cone voltage ramps for many precursor–product ion pairs within a single SRM run and without the need for a pure peptide.” Here we report a fully automated workflow for optimized method development, compatible with MS platforms from Agilent (pending a software patch[†]), Applied Biosystems, Waters and Thermo Fisher Scientific. This workflow has been released, with a

step-by-step tutorial in its use, in version 0.6 of the open source Skyline software (<http://proteome.gs.washington.edu/software/skyline>)²². The new Skyline CE optimization pipeline facilitated the efficient collection and analysis of much more data on CE optimization than have been published previously. Using these data, we compare the results obtained with CE values predicted by default linear equations against the results obtained with linear equations optimized on our laboratory instruments. Furthermore, we compare the use of predicted CE values against the use of CE values obtained by optimizing for each transition individually, and show that these equations perform far better than previously reported.

Methods

The data were acquired at three sites (University of Washington, The Broad Institute and Fred Hutchinson Cancer Research Center) by three instrument operators. All data from Thermo Fisher Scientific instruments were acquired at the University of Washington by a single operator, all data from Applied Biosystems and Agilent instruments were acquired by a single operator at The Broad Institute, and all data from the Waters instrument were acquired by a single operator at Fred Hutchinson Cancer Research Center.

Sample Preparation

A tryptic digest of six bovine proteins purchased from Michrom (PN: PTD/00006/63) was used for the collision energy optimization data acquisition study. For the collision energy optimization replicates, the sample was reconstituted and diluted to 50 fmol/μl (or 100 fmol/μl for the 4000 Q Trap) using 97% Water, 3% Acetonitrile, and 0.1% Formic Acid. 2 μl of the digest was injected into the LC system (100 fmol on column) for data acquisition. For the dilution analysis, the sample was initially reconstituted to 200 fmols/μl using the same buffer and diluted to produce the following concentrations: 6.25 fmols/μl, 12.5 fmols/μl, 25 fmols/μl, 50 fmols/μl, 75 fmols/μl and 100 fmols/μl. A 2 μl aliquot of each sample was injected on column for data acquisition. The data for the dilution analysis was acquired on a TSQ Ultra (Thermo Fisher Scientific, San Jose).

LC-MS/MS systems and data acquisition

The following six LC-MS/MS platforms were used for data acquisition:

i) nanoLC-1D+ (Eksigent)/TSQ Ultra (Thermo Fisher Scientific, San Jose, CA)

—The system was equipped with a 20 cm column of 75 μm I.D. fused silica capillary pulled to a 5 μm I.D. tip using a Sutter Instruments P-2000 CO₂ laser puller. The analytical column was packed with C-12 reversed phase chromatography material (Phenomenex, Jupiter 4 μm, Proteo 90 Å) using an in-house constructed pressure bomb and compressed helium gas. Peptides were eluted using a 60 min gradient according to the Supplementary Table 1.

ii) NanoAcquity (Waters, Milford)/TSQ Vantage (Thermo Fisher Scientific, San Jose, CA)

—The system was equipped with a 20 cm column of 75 μm I.D. fused silica capillary pulled to a 5 μm I.D. tip using a Sutter Instruments P-2000 CO₂ laser puller. The analytical column was packed with C-12 reversed phase chromatography material

(Phenomenex, Jupiter 4 μm, Proteo 90 Å) using an in-house constructed pressure bomb and compressed helium gas. The LC system was also configured with a trap column of 100 μm × 3 cm (C-12, Phenomenex, Jupiter 4 μm, Proteo 90 Å). Peptides were loaded into the trap

[†]From personal communication with Dr. Christine Miller, Agilent Technologies.

column for 5 min using a flow rate of 2 $\mu\text{L}/\text{min}$ and were eluted using a 60 min gradient presented in Supplementary Table 1.

iii) NanoAcquity (Waters, Milford)/TSQ Access (Thermo Fisher Scientific, San Jose, CA)—The system was operated and the peptides chromatographically separated as described above for the NanoAcquity/TSQ Vantage.

iv) nanoLC-2D (Eksigent)/ABI 4000 Q Trap (Applied Biosystems, Concord, ON)—Two systems (A and B) were each equipped with a 12 cm column of 75 μm I.D. fused silica capillary with a 10 μm I.D. tip (Pico frit selfpack column, PF360-75-10). The analytical column was packed with C-18 reversed phase chromatography material (Reprosil-Pur C18-AQ, 3 μm particle size and 120 $\text{\AA}$ pore size) using a pressure bomb and compressed helium gas. Peptides were eluted in an 80 minute gradient according to the Supplementary Table 1. A second iteration of the experiment was run on system B (labeled B') using an initial linear equation derived in the first iteration.

v) NanoAcquity (Waters, Milford)/Xevo TQ (Waters, Milford, MA)—The system was equipped with a nanoACQUITY UPLC BEH analytical column (Waters, C18, particle size 1.7 μm , I. D. 100 μm , length 100 mm, pore size 130 $\text{\AA}$). The LC system was also configured with a trap column of nanoACQUITY UPLC Symmetry trap column (Waters, C18, particle size 5 μm , I.D. 180 μm , length 20 mm, pore size 100 $\text{\AA}$). Peptides were loaded into the trap column for 6 min with 99% mobile phase A using a flow rate of 10 $\mu\text{L}/\text{min}$ and were eluted using a 40 min gradient presented in Supplementary Table 1B. The TQ instrument settings included a capillary voltage of 2.8 kV, a cone voltage of 35 V, an ion source temperature of 150 $^{\circ}\text{C}$, a cone gas flow of 15 L/Hr, and a nanoflow gas flow of 0.20 bar.

vi) 1200 capillary and nano pumps/6460 QqQ with ChipCube Interface (Agilent, Santa Clara, CA)—The 6460 ChipCube system was equipped with a capillary pump for loading the enrichment (trap column, 160 nL volume, Zorbax C18, particle size 5 μm) at 4 $\mu\text{L}/\text{min}$ for 2.5 minutes at initial conditions (97% mobile phase A). The integrated LC column/ESI emitter then switched to analysis mode and the gradient was supplied through the trap column onto the resolving column (Zorbax C18, particle size 5 μm , 75 μm ID, 150 mm length) by the nanopump at 300 nL/min, according to the gradient in Supplementary Table 1B.

Skyline method set up for collision energy optimization

The data were acquired in two steps. In Step 1, Skyline was used for building unscheduled MS methods for the targeted peptides (precursors>fragments) using a minimum of 4 transitions per precursor. Where more than 4 transitions were acquired, only the 4 most intense transitions were retained for statistical analysis. Each site chose 20 doubly charged peptides and 10 triply charged peptides for optimal performance on the instrument being tested. No effort was made to ensure that peptides and product ions corresponded between sites. The information on precursors and fragments selected for the MS platforms investigated are shown in the Supplementary Tables 2A-D. Initial CE linear equations used are shown in Table 1 and are the default equations in Skyline version 0.5. The “Thermo” and “Xevo” default equations for doubly charged peptides are reported in the literature^{19,20} and the linear equation for the triply charged peptides was acquired by personal communication from Drs. S. Peterman and A. Prakash at Thermo Fisher Scientific. The “ABI” default equation was derived in house, prior to the addition of the Skyline CE optimization capabilities, using a limited set of standard peptides measured and optimized

by direct infusion on a 4000 Q Trap. The “Agilent” default equation was acquired via personal communication with Dr. C. Miller from Agilent Technologies.

For step 2, the RAW data from step 1 was loaded into Skyline and scheduled SRM methods were exported for the collision energy optimization study. Scheduling parameters were set to use a time window of 4 or 5 minutes and a maximum number of 132 or 110 allowable concurrent measurements. With these settings, Skyline generated 4 or 6 scheduled methods, depending on LC gradient conditions and system capabilities, for a single set of measurements on all 30 peptides. Collision energy optimization parameters were set to use 5 steps on either side of the value predicted by the default equation (Table 1), with the step size set to 1 V. In total, 11 collision energy voltage values were considered for each fragment ion, yielding 1320 transitions per replicate. Consecutive mass variations of one hundredth of a mass unit were used for each fragment ion as a vendor-neutral method of allowing software tools like Skyline to specify and recognize variation in secondary parameters like CE. The true product m/z was assigned to the CE for the default equation, with no other product m/z value varying more than 5 hundredths from that. This approach is reported in the literature by Sherwood and co-workers²⁰. The data were acquired in ten replicates and then imported into Skyline for peak area integration and for determination of the updated linear collision energy equations. All peak area integration was performed by Skyline, manually reviewed and finalized by a single investigator.

The peak areas, CE values and other identifying information were exported in tabular comma separated value (CSV) format, using a customized Skyline report. Further statistical analysis was performed on these CSV files, using a custom C# program, to calculate summary linear equations, and to simulate various approaches for choosing CE values as described in the results section and the pseudo-code in Supplement Text 1.

Differential Experiment

A separate differential validation experiment was conducted to compare the impact of CE optimization on an experiment with normal run-to-run peak area variance. The experiment used a single set of measurements for each transition and CE value, gathered in step 2 with 4000 Q Trap instrument A. This instrument output was imported into Skyline, where it was used to export two new unscheduled SRM methods for the same instrument. The methods were identical except for the CE values used, which were derived from the imported replicate as:

1. Values predicted by linear equations calculated from all precursors;
2. Values that produced the maximum area for each transition.

Two of the peptides were given the linear equation predicted CE value for both methods.

These methods were then run in succession with randomized order over 8 replicates, using the same instrument conditions as the original data set, though not the same column. The results were then imported into Skyline for integration processing. Integrated peak areas were exported to a Skyline report for further statistical analysis in Excel.

Results

CE optimization workflow and data acquisition

The results for CE optimizations were acquired in a two step process. Support for these steps is implemented in the Skyline software application as illustrated in Figure 1. In the first step, 30 tryptic peptides (20 doubly charged and 10 triply charged), containing four transitions each (120 transitions total), were measured using an unscheduled SRM method. The

measurements acquired in Step 1 were imported into Skyline and used to determine the retention time range for each peptide. Scheduled SRM methods for Step 2 were exported from Skyline. These methods directed the instrument to monitor each precursor and product ion transition at 11 different CE voltages during specific retention time periods. The collection of data at 11 different CE voltages required multiple scheduled methods to limit the number of transitions measured concurrently.

Data analysis

The raw data from Step 2 were imported into Skyline for data processing and analysis. A single set of measurements for the CE optimization chromatograms acquired for the doubly charged peptide VLVLDTDYK at m/z 533.3 is shown in Figure 2A. Each colored line represents the total ion current (TIC) for the sum of the four transitions (533.3>853.4, 754.4, 641.3 and 526.2) selected for eleven different collision energy voltages. In this specific example, the red line corresponds to the CE value of 21 V predicted by the default CE linear equation for doubly charged peptides: $CE = 0.034 * (m/z) + 3.314$ (TSQ Ultra, Thermo Fisher Scientific). A total of 10 other CE values in 1 volt intervals were measured above and below the center CE value predicted by the linear equation. These voltage values are represented in the Figure 2 legends as steps +5, +4, +3, +2, +1, -1, -2, -3, -4 and -5.

Reproducibility

Figure 2B shows the reproducibility of the relative peak areas, calculated by integrating the TIC chromatograms from ten replicate analyses, for the doubly charged peptide VLVLDTDYK. The areas obtained at each voltage step are normalized within each replicate relative to the peak area value obtained at the reference voltage setting (red bar, 100%) predicted by the linear equation used in creating the method. In this example, the data show that the relative intensities of the peak areas at different CEs are fairly consistent between technical replicates, even if absolute peak areas vary. The CE value that yields the largest measured peak area is reported as either 2 or 3 voltage units below the default CE value predicted by the linear equation. This maximum area is on average 13.7% higher than the area measured using the default CE value.

Although we collected our data from 10 technical mass spectrometry replicates, most laboratories derive “optimal” CE values from a single measurement. In these cases, peptides are injected once, and intensities are measured over a range of CE values. The CE yielding the maximum intensity, by peak area, is chosen for all future experiments. For this approach to work, the effect of CE on peak area must be reproducible enough that a single measurement produces an accurate representation of the optimal CE value.

The reproducibility of these intensity measurements depends on the amount of peptide loaded on column. Peak area measurements, acquired on a TSQ Ultra, are shown in Figure 3 for varying amounts injected on-column (12 - 200 fmol) of the doubly charged peptide YLGYLEQLLR at m/z 634.3, under the same mass spectrometry conditions used to acquire the data in Figure 2. For the injection with less than 50 fmol on column, the area measurements for this peptide show no discernible trend. The maximum peak area is measured 4 to 6 volts below the CE values that yield maximum area at higher concentrations. Such poor precision makes it impossible to determine the CE that maximizes signal intensity from a single measurement. This observation highlights the importance of having a minimum amount of the peptide injected on-column to perform CE optimization.

With precision as poor as the 50 fmol injection shown in Figure 3, even our 10 replicates would be too small a sample size to derive an optimal CE from the entire data set. To simulate the selection of an optimal CE by an operator from a single data set, we discarded

all precursors where at least 3 out of 10 replicates showed no clear trend around the CE with the maximum signal intensity. Depending on the instrument platform, this left anywhere from 13 to 18 doubly charged precursors, and from 7 to 9 triply charged precursors for subsequent calculations (Table 1).

Comparing Regression Coefficients

A comparison of the default and optimized linear equations for our selected peptides, acquired on a TSQ Ultra, is shown in Figure 4. These best fit linear regressions were computed separately for the doubly and triply charged peptide precursors. The solid lines represent simple linear regressions of the experimental values obtained during the CE optimization study. The dashed lines represent the plots for their respective default linear equations used for data acquisition (Table 1). The same plots were generated for each of the instrument platforms used in our analysis and are included in Supplementary Figures 1A-G.

We performed significance tests of the difference in the regression coefficients between the doubly charged and triply charged precursors for each platform. The resulting p-values for the null hypothesis that the tested sample sets represent the same regression between precursor m/z and CE are summarized in Supplementary Table 3A. Our data show that linear equations for CE should be derived separately for each charge state, as previously suggested by Sherwood, *et al.*²⁰. Of the 9 data sets we collected, 6 had p-values ≤ 0.05 for the comparison between precursors with different charge states.

We performed the same tests between pairs of instrument platforms for peptide precursors of matching charge state, with the resulting p-values summarized in Supplementary Tables 3B and 4C. The variety of instrument platforms and configurations, on which our measurements were performed, provides new information on the types of changes that warrant recalibration of a linear equation. Changes in collision gas pressure from 1.0 mTorr to 1.5 mTorr do not appear to have a significant enough impact on performance to warrant recalibration (p-values charge 2+: Ultra 0.11, Vantage 0.15 and charge 3+: Ultra 0.03, Vantage 0.21 in Supplementary Tables 3B and 3C). It does appear, however, that the TSQ Vantage would benefit from using coefficients different from those of the TSQ Quantum Ultra (p-values charge 2+: 1.0 mTorr 0.02, 1.5 mTorr 0.006, and charge 3+: 1.0 mTorr 0.07, 1.5 mTorr 0.17 in Supplementary Tables 3B and 3C). The optimized equations we calculated here for the two 4000 Q Trap instruments are not statistically distinguishable (p-values charge 2+: 0.12, charge 3+: 0.17 from Supplementary Tables 3B and 3C), indicating reproducibility among instruments of the same model.

In evaluating the initial two Q Trap data sets, we concluded that their failure to differentiate between doubly charged and triply charged precursors might be due to a bias from the CE range we chose to measure. For some peptide precursors, our 11 V range might not have measured the optimal CE, because the maximum peak area was observed at one edge of the CE voltage range. Examples of this for a peptide precursor and an individual y-ion transition are shown in Supplementary Figures 2A and 2C. Supplementary Figures 2B and 2D plot the fraction of the peptide precursors and transitions respectively where the maximum observed peak area was measured at one edge of the CE range.

To evaluate the impact of this range restriction, a second iteration of the optimization process was run on the 4000 Q Trap instrument B (labeled B'), starting with the optimized equation from a single replicate of the first round instead of the default linear equation. For the second iteration on 4000 Q Trap instrument B, the percentage of optimal values measured at the edge of the range fell to only 2.1%. The optimal CE values for doubly charge precursors barely changed (p-value 0.98 from Supplementary Table 3B, Table 1 and Supplementary Figure 1G). While the changes also did not produce a significantly different

equation for triply charged precursors (p-value 0.29 from Supplementary Table 3C), this new data set did show a significant difference between doubly charged and triply charged precursors (p-value 0.01 from Supplementary Table 3A). These data suggest that the charge 3 equation for the Agilent 6460 may also benefit from a second iteration, though not performed for this manuscript.

Comparing Approaches

To compare the relative effect of different approaches for choosing CE values on the sensitivity of measurement, we simulated these approaches using our data. Because CE values are commonly chosen from a single set of measurements, we first chose one of our 10 replicates, designating it the training set. The training set was used to calculate CE values for the remaining 9 replicates, designated as test sets. The four approaches for calculating the CE we tested were:

1. the CE value predicted by the default linear equation;
2. the CE value predicted by a new linear equation calculated from the training set;
3. the CE value that produced the maximum total peak area for the current precursor in the training set;
4. the CE value that produced the maximum peak area for the current transition in the training set.

In each of the 9 test sets, the peak areas corresponding to the calculated CE values were selected, separately for each approach, summed by precursor and normalized by the sum of the maximum measured areas for the precursor transitions in the test set. This process was repeated, using each of the 10 replicates as the training set, to produce a total of 90 trials for each peptide.

A pseudo-code function documenting how these calculations were performed can be found in Supplement Text 1. The mean and standard deviation of the normalized area percentage values, for each approach, on each platform, are illustrated in Figure 5.

The differences are significant between the relative intensities for triply charged precursors using default linear equations (blue bars, in Figure 5) and those using the equations derived from the test sets (green bars, in Figure 5). Because neither of the default linear equations we used had empirically derived coefficients for triply charged precursors, this observation further corroborates the previous suggestion that linear equations for CE should be derived separately for each charge state. Supplementary Figure 3 displays all of the statistics in Figure 5 separated by charge state.

For doubly charged precursors, the TSQ Ultra (1.0 mTorr) had similar relative intensities using the default equation (red bars, in Figure 5) to those using the equations derived from the test sets (green bars, in Figure 5). This observation is reassuring, because the original equation coefficients were calculated under these same conditions. The fact that these equations perform so similarly suggests that this method generalizes well, since the equations were calculated using different peptides and several years apart from one another.

The “Maximum Transition” values (cyan bars, in Figure 5) show the performance of per-transition CE optimization relative to the maximum measured peak area for each peptide in each test set. The fact that these values are less than 100% shows a single CE does not always produce the maximum area for each transition. These values approximate the maximum relative intensity achievable through CE optimization, against which we compare the other CE calculation methods.

The “Maximum Precursor” values (purple bars, in Figure 5) show a similar metric for empirical CE optimization using a single CE value for all transitions of each peptide precursor. This approach could have the benefit of producing relative intensities most similar to MS/MS spectral libraries, which have been proven useful in peptide identity confirmation in SRM19:23. In the acquisition of a full-scan MS/MS spectrum, it is not possible to assign different CE values for each fragment ion. To evaluate the impact of using different CE values for each transition, we used a dot-product similarity metric^{24,25} to compare the fragment ion intensity ratios between using a single optimal CE for each peptide and using an optimal CE for each individual transition. For the TSQ Vantage at 1.5 mTorr gas pressure we calculated a 0.9997 ± 0.0005 (mean $\pm$ stdev) dot-product correlation, indicating that the adjustment of CE for each transition had minimal effect on fragment ion intensity ratios. The data set for the 4000 Q Trap (A) produced the lowest correlation of 0.998 ± 0.004 (mean $\pm$ stdev) and contained one peptide with a dot-product of 0.98. For this peptide, where the two CE optimization approaches were found least similar, we had monitored two doubly charged fragment ions and two *b*-ions (Supplementary Figure 4). This observation suggests that linear equations, calculated using mostly singly charged *y*-ions, may not be representative of optimal CE values for doubly charged or *b*-ions.

The relative intensities for the newly calculated linear equations (green bars, in Figure 5) provide an approximation of the best possible performance using such equations, because they are trained on the target peptides themselves. This approach on average achieves $93.5 \pm 1.2\%$ (at 95% confidence) of full per-transition optimization. However, the relative intensities of the default equations for doubly charged precursors (red bars, in Figure 5) may better measure the effect of training a linear equation on one set of peptides and using the resulting equation on other peptides. Excluding the TSQ Vantage, for which we believe the default linear equation has never before been properly calibrated, the default equations for doubly charged precursors achieve $89.7 \pm 1.7\%$ (at 95% confidence) of per-transition optimization.

Detecting the Difference

In our final experiment, we investigated the impact of using a predicted CE, versus optimizing each transition, on proteomics experiments where measurements must be compared across multiple sample injections. The data sets reported above have all compared measurements of a peptide separated by milliseconds within a single LC-MS analysis. In this final experiment, peptides were measured in replicate using both the linear equation CE values and per-transition optimized CE values (Supplementary Tables 4A & 4B), as described in the Methods section. The resulting peptide peak areas were normalized by the sum of two peptides for which CE was held constant over all replicates (Supplementary Tables 4C & 4D). Using the normalized values, T-tests were performed on each peptide between the measured groups. After applying a Bonferoni correction to these multiple comparisons, only 4 out of 25 peptides achieve a p-value lower than 0.05 between the two data sets (Supplementary Table 4E). All of the peptides that had a significant signal improvement using the transition optimized CE values are triply charged, and the set includes the peptide with doubly charged and *b*-ion products (Supplementary Figure 4). For the more common doubly charged precursors, however, the impact of per-transition optimization of CE becomes insignificant in multi-replicate proteomics experiments. With the introduction of biological variance, the significance would be diminished even further.

Discussion

In these experiments we have used a fully automated workflow, implemented in version 0.6 of the open source software Skyline, to collect and analyze a relatively large dataset for evaluating the effect of individually optimizing CE values using the four main triple

quadrupole vendor instrument platforms. By comparing peak areas at different CE values for different peptide sequences and precursor charge-states we have quantified the improvement in sensitivity obtained by optimizing each transition for each peptide individually compared to using a simple linear equation to predict CE based on the peptide precursor mass. Additionally, we have derived optimized linear equations for predicting CE values that in some cases differ significantly from the default equations recommended by the manufacturer or reported in the literature. These new equations for predicting CE are available in the latest release of Skyline. For operators of the four major triple quadrupole instrument platforms who wish to optimize individual transitions, Skyline makes that task easy and robust.

Understanding the variance in the transition peak area to CE relationship is critical to understanding how any peak area optimization using CE will perform. The Skyline Peak Area View charts provide immediate visual information to assess these data. These charts made it obvious that low concentrations of certain peptides resulted in measurements that were too noisy to support CE optimization. At these levels, the large coefficient of variance makes detecting a difference in peak area intensity between similar CE values impossible. The optimal CE value would be difficult to determine without making a lot of measurements. A similar effect occurs when using an optimal CE value determined with a standard at high abundance to measure a low abundance species in a real sample. Even if the measurement is made with the optimal CE, the improvement in sensitivity over a less optimal CE will be indistinguishable at low intensity because the variance from the measurement shot noise will overwhelm the small improvement in peak area. Skyline automatically sums the peak areas measured over multiple replicates to increase the accuracy of its choice of optimal CE.

One of the goals of these experiments was to assess the impact of optimizing CE for each transition compared with using a single CE value for all the transitions of a single peptide. We hypothesized that optimizing by transition might have an impact on transition area ratios that are critical for peak validation with spectral libraries^{19,23}. The data showed that optimizing each transition produced slightly higher overall peak areas and insignificant impact on ion ratios compared with optimizing by precursor. The majority of our measurements were performed using singly charged y-ion fragments in our CE optimizations. It should be noted, however, that optimizing each transition separately produced the greatest change in the ion ratios for a triply-charged peptide where the transitions monitored included two doubly charged ions and two b-ions. The impact of CE values on doubly charged ions and b-ions has not been fully explored.

This project focused on determining how well linear equations for predicting CE performed within and among triple quadrupole mass spectrometers. Our data indicate that all linear equations should be derived empirically for each respective charge-state, rather than using linear equations derived from doubly charged precursors across charge-states. With well optimized linear equations, we have shown gains of only $7.8 \pm 1.6\%$ (at 95% confidence) in total peptide peak area on average compared with fully optimizing each transition, contrary to previously published work implying that CE optimization frequently results in large gains in signal intensity^{20,21}.

To ensure best results, we have shown linear equations should be reassessed on new instrument platforms. We observed a difference in optimal CE values between the TSQ Ultra and TSQ Vantage. These two instruments have identical collision cells as reported by the manufacturer. Therefore, we had not expected the differences we observed in the optimal CE for the same peptides at the same collision gas pressure. A potential explanation for the difference is that the ion source region differs between the two instruments. It is likely that the ions are entering the collision cell at different energies and are, thus, affected differently

by a CE voltage offset. Reassuringly, we found no evidence of significant variation in optimal CE between instruments of the same type, or even varying gas pressure (within the range tested) on a single instrument.

By simulating a differential proteomics experiment, where the only difference between groups of technical replicates was the method of choosing the CE values, we showed that the loss in peak signal intensity from using an optimized linear equation as opposed to optimizing each peptide was rarely distinguishable in this type of experiment. To determine whether optimization of CE values by transition might still yield significant improvement to studies with larger sample sizes and thoroughly evaluated methods employing labeled internal standards will require further experiments. It is our conclusion that linear equation predictors should be used exclusively at least until such high-accuracy and precision studies become necessary.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Workflow for experimental collision energy optimization using Skyline.

Figure 2: A).

Skyline chart of collision energy (CE) optimization chromatogram for the doubly charged peptide VLVLDTDYK at m/z 533.3. The colored lines correspond to the Total Ion Current (TIC) for the sum of four transitions ($533.3 > 853.4$, 754.4 , 641.3 and 526.2) at eleven different voltage steps. The red line corresponds to the CE value calculated using the instrument default equation (TSQ Ultra, Thermo Fisher Scientific); the positive-value steps represent 1V increments above the red line CE value and the negative-value steps represent 1V decrements.

Figure 2: B).

Skyline Peak Area View shows reproducibility of the measurement of the relative peak areas under the TIC chromatograms for ten replicate analyses for the doubly charged peptide VLVLDTDYK. The areas obtained at each voltage step were normalized within each replicate relative to the peak area values obtained at the reference voltage setting (red bar, 100%) calculated using the default CE equation (TSQ Ultra, Thermo Fisher Scientific).

Figure 3. Skyline Peak Area View shows concentration dependence of the peak area measurement reproducibility for peptide YLGYLEQLLR under the same conditions described in Figure 2. Varying on-column amounts of peptide YLGYLEQLLR were injected (12 - 200 fmol). Reproducible area measurements were observed at 50 fmol on-column or above.

Figure 4.

Examples of collision energy regression lines generated automatically by Skyline. In this example, the regression lines are for 18 doubly charged and 9 triply charged peptides at 1.5mTorr of pressure in the collision cell. The dashed line represents the default linear equation (TSQ Ultra, Thermo Fisher Scientific) and the dark-blue line represents the regression calculated from the experimental values obtained during the CE optimization study.

Figure 5.

Results of simulated CE optimization as the fraction of the maximum peak area measured for all transitions of each precursor, covered by four possible CE calculation strategies: a) the CE value predicted by the default linear equation (charge 2 and 3 separated); b) the CE value predicted by a new linear equation calculated from regression of a single optimization data set; c) the CE value that produced the maximum total peak area for a precursor in a single optimization data set; d) the CE value that produced the maximum peak area for a transition in a single optimization data set. (Note: 4000 Q Trap B' represents a second experiment on 4000 Q Trap B starting with linear equation coefficients from the first experiment.)

Table 1

Default and updated CE equations with 95% confidence intervals for doubly and triply charged peptides for different MS platforms. Displayed in parenthesis following each equation is the number of precursor measurements used to calculate the regression, after discarding peptides which lacked a discernable trend in peak area over the measured CE range for 3 or more replicates.

	Charge 2 peptides	Charge 3 peptides
	Starting Linear Equations for Predicting Peptide CE (Default equations)	
TSQ Ultra TSQ Vantage	$CE=0.034*m/z + 3.314$	$CE=0.044*m/z + 3.314$
4000 Q Trap	$CE=0.043*m/z + 4.756$	$CE=0.043*m/z + 4.756$
Xevo	$CE=0.034*m/z + 1.314$	$CE=0.034*m/z + 1.314$
6400	$CE=0.036*m/z - 4.8$	$CE=0.036*m/z - 4.8$

	Calculated Linear Equations	
TSQ Ultra 1.0 mTorr	$CE=0.055\pm0.02*m/z + -8.01\pm11.5$ (n = 18)	$CE=0.027\pm0.022*m/z + 4.492\pm10.379$ (n = 9)
TSQ Ultra 1.5 mTorr	$CE=0.036\pm0.008*m/z + 0.954\pm4.818$ (n = 18)	$CE=0.037\pm0.007*m/z + 3.525\pm3.384$ (n = 9)
TSQ Vantage 1.0 mTorr	$CE=0.041\pm0.01*m/z + -3.442\pm5.765$ (n = 18)	$CE=0.04\pm0.005*m/z + 0.773\pm2.214$ (n = 9)
TSQ Vantage 1.5 mTorr	$CE=0.03\pm0.005*m/z + 2.905\pm3.151$ (n = 18)	$CE=0.038\pm0.004*m/z + 2.281\pm1.808$ (n = 9)
TSQ Access 1.0 mTorr	$CE=0.049\pm0.009*m/z + -5.75\pm5.428$ (n = 16)	$CE=0.039\pm0.012*m/z + 3.314\pm5.835$ (n = 7)
4000 Q Trap (Instrument A)	$CE=0.052\pm0.008*m/z + -2.919\pm5.514$ (n = 13)	$CE=0.036\pm0.008*m/z + 4.106\pm4.444$ (n = 9)
4000 Q Trap (Instrument B)	$CE=0.057\pm0.01*m/z + -4.815\pm6.384$ (n = 14)	$CE=0.035\pm0.015*m/z + 6.49\pm8.615$ (n = 9)
4000 Q Trap (Instrument B')*	$CE=0.057\pm0.009*m/z + -4.256\pm5.752$ (n = 18)	$CE=0.031\pm0.018*m/z + 7.082\pm10.3$ (n = 9)
Xevo	$CE=0.037\pm0.009*m/z + -1.066\pm5.024$ (n = 14)	$CE=0.036\pm0.004*m/z + -1.328\pm2.088$ (n = 7)
Agilent 6460	$CE=0.051\pm0.009*m/z + -15.563\pm5.759$ (n = 20)	$CE=0.037\pm0.009*m/z + -9.784\pm5.336$ (n = 7)

* Note: Instrument B' represents a second experiment on Instrument B starting with linear equation coefficients from the first experiment.