Comparison of Peptide Parallel Reaction Monitoring via MS^2 and MS^3 Methods

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ABSTRACT

Purpose: Demonstrate the feasibility of MS^3 for quantitation of large numbers of peptides using the ion trap analyzer.

Methods: Targeted MS^2 and MS^3 of 100 peptides, with quantitation of heavy/light PRTC ratios in 200 ng HeLa protein digest background.

Results: MS^3 results were similar or better for 11 of 15 PRTC peptides.

INTRODUCTION

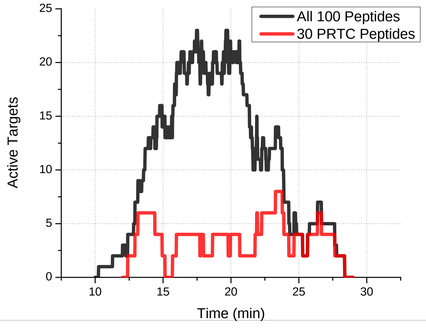
Quantitation of peptide abundance via LC-MS/MS with stable isotope labeled peptide pairs is a well established experiment in proteomics. One of the challenges, especially with nominal mass accuracy/resolution analyzers such as the quadrupole ion trap and mass filter, is that interferences can worsen the quality of the measurements, or at least pose a method development obstacle. MS^3 techniques are a potential solution to the problem, which dramatically increase measurement specificity. While many of the benefits of targeted peptide MS^3 quantitation have previously been demonstrated, these studies were limited to monitoring a small number of compounds, due to a lack of speed and sensitivity[1]. Here we compare targeted MS^2 to a MS^3 method that is an order of magnitude faster than the method reported in the previous studies, allowing for at least 100 peptides to be measured in a 40 minute assay.

MATERIALS AND METHODS

The ion trap analyzer of a modified Thermo Scientific™ Orbitrap Fusion™ Tribid™ mass spectrometer coupled with a Thermo Scientific™ EASY-nLC™ 1000 LC system was used for this study. The LC column was a 500x0.075 mm, Thermo Scientific™ EASY-Spray™ C18, heated to 55 degrees Celsius. The quantified peptides were isotopically labeled heavy and light versions of the Thermo Scientific™ Pierce™ Peptide Retention Time Calibration (PRTC) standards. The light peptides were spiked at 500 amol/µl into 200 ng/µl of Thermo Scientific™ Pierce™ HeLa Protein Digest Standard, and this solution was used to dilute the heavy peptides from 1 amol to 100 fmol. Replicates of 4 injections of 1 µl were made. The LC method was a H2O/ACN gradient of 40 minutes, with a flow rate of 300 nl/min. The retention time windows were 2 minutes wide, maximum cycle time was 1 second, with dynamically determined dwell times and 1e4 AGC target. Precursor isolations were 0.7 Da for MS^2 and 1.3 Da for MS^3. 18,000 unique HeLa peptides were identified from 3x pooled data dependent acquisition (DDA) experiments, and 70 of these from the most intense 50% were randomly chosen to be included. (Figure 1). Typical LC peak widths were 8 seconds wide at the baseline. Skyline software was used to choose transitions and integrate LC peaks for MS^2, while for MS^3 custom software was used, which picked the most intense peaks at the top of the LC peak of a concentrated injection for quantitation. LOD and LOQ were determined by a standard method[2] (see below). Thermo Scientific™ Proteome Discoverer™ 2.1 software was used to identify unique peptides in DDA experiments.

$$LOD = \mu_{blank} + \frac{t_{0.95}\left(\sigma_{blank} + \sigma_{0.001fmol}\right)}{\sqrt{4}} \qquad LOQ = 3xLOD$$

Figure 1. Distribution of elution times for all 100 peptides (HeLa + PRTC) and the quantified PRTC heavy/light peptides pairs.

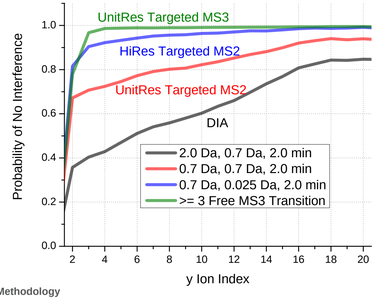


SELECTIVITY CALCULATIONS AND MS^3 METHODS

Selectivity of MS^3 compared to MS^2

To quantitatively assess the selectivity benefits of MS^3 compared to existing MS^2 methods, calculations were performed using a set of 25,000 unique peptide sequences identified from 4x 1 µg HeLa pooled DDA runs, in a manner inspired by Gillet and coworkers[3]. The quadrupole mass filter, mass analysis tolerance, and runtime window form a set of filters which are applied to the sequences. The number of interferences from y-ions, b-ions, and their H_2O and NH_3 losses were counted for each peptides' y-ions, which are the most commonly used for quantification. For each condition, the likelihood of an interference-free transition grows with the length of the peptide fragment (Figure 2). For MS^3, the probability of observing >=3 interference free transitions arising from the indicated y-ion precursor is higher even than the probability of a single free transition for MS^2 analysis with a 0.025 Da extraction window.

Figure 2. Selectivity of various MS^2 and MS^3 schemes



MS^3 Methodology

The chief disadvantage of MS^3 has traditionally been slow data acquisition speed, because long dwell times were needed to achieve enough ions for reasonable quantitation. The advent of simultaneous isolation of multiple MS^2 fragments and their subsequent fragmentation[4] allows MS^3 to be nearly as fast as MS^2.

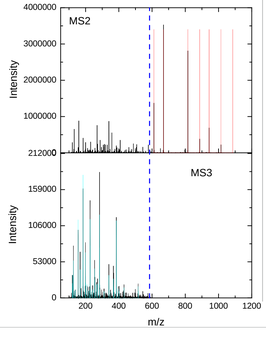
Although sometimes multiply charged MS^2 fragments generate spectacular MS^3 spectra[5], the majority of MS^2 fragments are 1^+ and generate MS^3 products with *m/z* less than their precursor. This was demonstrated by DDA MS^2-MS^3 experiments (not shown), where the MS^3 spectra from identified MS^2 spectra were parsed.

Of the 100 peptides analyzed in this study, several had 2^+ or 3^+ fragments, but none of these generated significant MS^3 fragments.

Therefore the general scheme used was to simultaneously isolate the ~5-7 y-ions with *m/z* > precursor, and quantify using all unique MS^3 ions with *m/z* < precursor and intensity > 5% of the base MS^2 fragment (Figure 3).

Both beam type and resonance type collision induced dissociation modes were investigated (bCID and rCID).

Figure 3. MS^2 and MS^3 spectra of the peptide SAAGAFGPELSR. Red lines are the precursor ions from the MS^2 that were simultaneously isolated before the MS^3 event. Cyan lines in the MS^3 spectrum indicate the fragment ions used for quantitation. The blue dashed line is the precursor m/z.



SIGNAL TO NOISE RATIO

MS^3 Selectivity Enhancement

One fact that makes MS/MS of peptides particularly amenable to unit resolution analysis is that most peptides have many more possible transitions than the ~5 or so that are typically used for quantitation. Thus for example while unit resolution analysis has a higher probability of interference than high resolution analysis (Figure 2), there remains a good probability that at least *some* of the transitions will not be interfered with. The use of stable isotope labeled peptides also lowers the uncertainty of which signals belong to a peptide of interest.

Still, the results demonstrate the expected selectivity enhancement predicted by the calculations in Figure 2. For example, 100 amol of the peptide SFANQPLEVVYSK has an unambiguous MS3 signal with no interference at all, while more care has to be exercised in finding and integrating the MS^2 signal, due to the many nearby peptides that contribute some chemical noise.

This phenomenon is summarized in Figured 5, where signal to noise ratio (S/N) is computed as the LC peak height of the peptide divided by the average of the other points in the 2 minute scheduled window. S/N is clearly higher for the majority of the PRTC peptides. There are only 2 peptides that have much poorer S/N in the MS^3 with both activation techniques, while the other 13 had comparable or better S/N than bCID MS^2.

Figure 4. 100 amol of SFANQPLEVVYSK analyzed by MS^2 and MS^3.

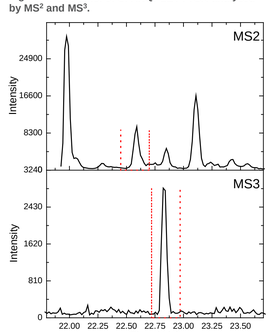
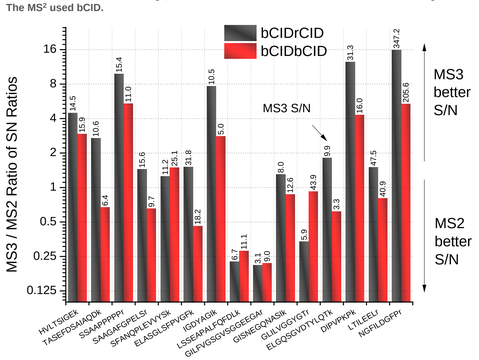


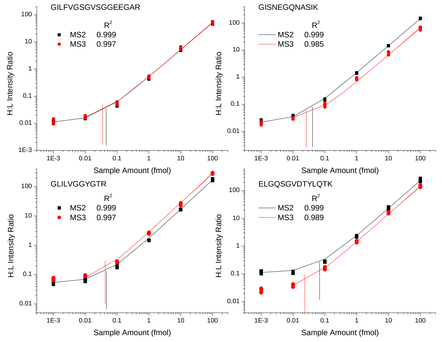
Figure 5. Ratio of signal to noise ratios, MS^3 / MS^2 for 100 amol of the PRTC peptides, where MS^3 uses bCID for the first stage, and either bCID or rCID for the second activation stage. The MS^2 used bCID.



QUANTITATION

The linearity and precision of the measurements with both MS^2 and MS^3 were excellent, as demonstrated in Figure 6. Only the TASEFDSAIAQDK response saturated at 100 fmol, for all activation techniques. An instrumental reason hasn't been found for this saturation, so our hypothesis is that this compound is not able to efficiently be ionized at >= 100 fmol.

Figure 6. Example response curves of PRTC peptides with MS^2 (bCID) and MS^3 (bCIDbCID). LOQ's for MS^2 and MS^3 are marked with vertical lines.



Summary of Limits of Quantification

The LOQ's for bCID MS^2 and bCIDbCID MS^3 were in general both quite good, with median LOQ of 55 amol and 41 amol for MS^2 and MS^3 respectively (Figure 7). Similar studies using depleted plasma as a background instead of HeLa reported LOQ's about 3x higher[6]. In general the MS^3 data were similar or better than the MS^2 data, however there were three exceptions that had quite good MS^2 response, and average MS^3 response (Figure 8).

Figure 7. Histograms of limits of quantification of PRTC in 200 ng HeLa background for MS^2 (bCID) and MS^3 (bCIDbCID).

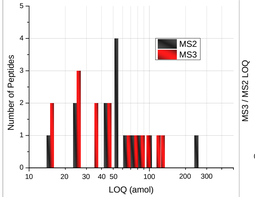
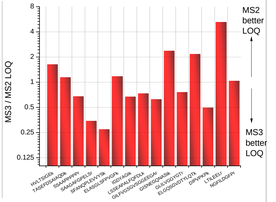


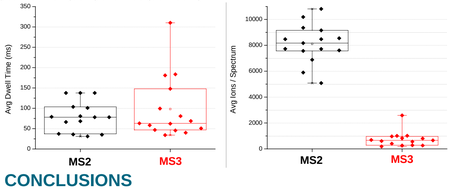
Figure 8. Comparison of LOQs for MS^2 (bCID) and MS^3 (bCIDbCID).



TRADEOFFS BETWEEN MS^2 and MS^3

The average dwell time and number of ions per spectrum in the points across the LC peak for the 500 amol standard in 4x runs were compared between MS^2 and MS^3 (Figure 9). While the dwell times were very similar, the number of ions per spectrum is 10x lower for MS^3. Counting statistics dictate that to achieve an RSD of say 10%, at least 100 ions need be integrated across the LC peak $\frac{\sigma}{\mu} = \frac{\sqrt{100}}{100} = 0.1$. Clearly MS^3 will run out of capacity for good quantitation before the MS^2, but for moderate numbers of peptides in an assay (~100), especially in complex backgrounds, MS^3 offers higher selectivity and better quantitation sensitivity. When the goal is to quantitate as many peptides as possible, MS^2 is today likely the better choice.

Figure 9. Average dwell time and number of ions per spectrum in points across 500 amol LC peak for MS2 (bCID) and MS3 (bCIDbCID).



CONCLUSIONS

- Unit mass analyzers can perform excellent peptide quantitation with both MS^2 and MS^3 in a complex HeLa background.
- MS^3 provided slightly better LOQs for the PRTC peptides than MS^2, with a median of 41 amol versus 55 amol for MS^2.
- The higher selectivity of MS^3 naturally yields higher S/N LC peaks than unit resolution MS^2 analysis, making it more valuable for complex matrices.
- Using multiplexed isolation of MS^3 precursors allows for enough MS^3 fragment ions to be formed for quantitation of at least 100 peptides in a 40 minute run, with capacity for at least 2x this amount.
- MS^2 is faster and produces 10x more ions per spectrum than MS^3, allowing it to be applied to assays with many more compounds.

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ACKNOWLEDGEMENTS

We thank Michael MacCoss for helpful discussions about MS^3, and Sue Abbatiello, who was extraordinarily helpful by providing samples, LC troubleshooting assistance, and practical advice on targeted peptide analysis.

TRADEMARKS/LICENSING

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