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iTRAQ reagent-based quantitative proteomic analysis on a linear ion trap mass spectrometer

Timothy J. Griffin^{1,*}, Hongwei Xie¹, Sricharan Bandhakavi¹, Jonathan Popko¹, Archana Mohan², John V. Carlis², and LeeAnn Higgins^{1,3}

¹ Departments of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, MN 55455

² Computer Science and Engineering, University of Minnesota, Minneapolis, MN 55455

³ Center for Mass Spectrometry and Proteomics, University of Minnesota, Minneapolis, MN 55455

Abstract

For proteomic analysis using tandem mass spectrometry, linear ion trap instruments provide unsurpassed sensitivity, but unreliably detect low mass peptide fragments, precluding their use with iTRAQ reagent labeled samples. While the popular LTQ linear ion trap supports analyzing iTRAQ reagent labeled peptides via pulsed Q dissociation, PQD, its effectiveness remains questionable. Using a standard mixture, we found careful tuning of relative collision energy necessary for fragmenting iTRAQ reagent labeled peptides, and increasing microscan acquisition and repeat count improves quantification, but identifies somewhat fewer peptides. We developed software to calculate abundance ratios via summing reporter ion intensities across spectra matching to each protein, thereby providing maximized accuracy. Testing found results closely corresponded between analysis using optimized LTQ-PQD settings plus our software and using a Qstar instrument. Thus, we demonstrate the effectiveness of LTQ-PQD analyzing iTRAQ reagent labeled peptides, and provide guidelines for successful quantitative proteomic studies.

Keywords

quantitative proteomics; iTRAQ; linear ion trap; pulsed-Q-dissociation

INTRODUCTION

Many proteomic studies use linear ion trap mass spectrometers¹ to analyze complex peptide mixtures derived from proteolytic digestion of protein samples via online microcapillary liquid chromatography (μLC) and tandem mass spectrometry (MS/MS). Compared to earlier ion trap instruments, the trapping of ions in an axial fashion in the linear ion trap provides significant improvements, including 15 times higher ion capacity, three times faster scan rate, and improved detection and trapping efficiency². Consequently, these instruments identify proteins by MS/MS from complex biological mixtures with unsurpassed sensitivity^{3, 4}, which, combined with their low maintenance, ease of use, and relatively low cost, have made linear ion traps the instrument of choice for a wide variety of proteomic studies.

*Corresponding author University of Minnesota, 321 Church St SE, 6-155 Jackson Hall, Minneapolis, MN 55455, Tel: 612-624-5249, Fax: 612-624-0432, Email: tgriffin@umn.edu.

One limitation of linear ion traps, commonly known as the “one-third rule”⁵, arises from decreased stability of fragment ions with m/z values less than 30% of the m/z for the precursor peptide selected for fragmentation by collision-induced dissociation (CID) during automated MS/MS analysis. This limitation precludes using these instruments for quantitative proteomic studies with the popular iTRAQ (™ Applied Biosystems) reagent^{6–8}, due to unreliable MS/MS detection of the iTRAQ reporter ions (at m/z values of 114, 115, 116 and 117) used for calculating relative abundance of peptides in the starting mixtures. Analysis of iTRAQ reagent labeled samples has been relegated to relatively slow scanning quadrupole time-of-flight instruments⁶, which have less sensitivity for complex mixture analysis compared to linear ion traps, or in some studies MALDI-TOF/TOF instruments⁹.

Given their respective advantages, combining the iTRAQ reagent with linear ion trap MS/MS analysis could provide a powerful platform for quantitative proteomic studies. In the case of the highly popular LTQ (™ Thermo Fisher) linear ion trap mass spectrometer, the introduction of the new pulsed-Q-dissociation (PQD) MS/MS operating mode¹⁰, hereafter called LTQ-PQD, has made analysis of iTRAQ reagent labeled samples possible. LTQ-PQD fragments peptides during MS/MS through a different mechanism than traditional CID, enabling the routine and reliable detection of ions down to 50 m/z . Peptide fragmentation using PQD occurs on a similar time-scale compared to CID, producing high quality MS/MS spectra peptide identification by sequence database searching¹⁰. However, the effectiveness of iTRAQ reagent labeled samples by LTQ-PQD for quantitative proteomic studies remains in question.

Here, we report results from a novel investigation of LTQ-PQD for the analysis of iTRAQ reagent labeled mixtures. Using a standard yeast lysate mixture, with known relative abundance for every protein of 10:5:2:1 for the 114:115:116:117 iTRAQ reporter ions, we have investigated the effects of 1) relative collision energy (CE) settings on LTQ-PQD fragmentation of iTRAQ reagent labeled peptides; 2) MS/MS repeat count setting; and 3) microscan (μ scan) acquisition. We also developed customized software for calculating accurate relative abundance ratios for proteins identified by LTQ-PQD. Finally, we compared the LTQ-PQD results analyzed by our software against those for the same mixture analyzed using a Qstar quadrupole time-of-flight mass spectrometer and accompanying commercial software, and found the analysis platforms to have similar quantitative accuracy. Collectively, our results demonstrate for the first time the effectiveness of analyzing iTRAQ reagent labeled samples by LTQ-PQD, and provide guidelines for its successful use in quantitative proteomic studies.

EXPERIMENTAL SECTION

Yeast Protein Preparation and iTRAQ reagent labeling

Protein from the budding yeast *S. cerevisiae* was isolated as described previously¹¹. After digestion with trypsin, the purified peptides (~500 μ g) were divided into four different microcentrifuge tubes, so that each separate tube contained 100, 50, 20 and 10 μ g of total peptides, respectively. Peptides in each tube were brought to equal volume with the iTRAQ reagent labeling buffer, and one unit of iTRAQ reagent (Applied Biosystems, Foster City) was added to the each tube as follows: 114 label to the 100 μ g sample, 115 to the 50 μ g sample, 116 to the 20 μ g sample, and 117 to the 10 μ g sample. The combined mixture thus had known 114:115:116:117 iTRAQ reporter ion intensity ratios of 10:5:2:1.

μ LC-MS/MS analysis by LTQ-PQD

All on-line μ LC separations were done on an automated Paradigm MS4 system (Michrom Bioresources, Inc., Auburn, CA) inline with an LTQ mass spectrometer (Thermo Fisher, San Jose), which has been described previously¹¹. Xcalibur 2.0 software upgraded with the beta

version of PQD operating software was used for instrument operation. For all experiments, 0.5 μg of total peptides from the standard iTRAQ reagent mixture was loaded. Peptides were eluted using a linear gradient of 10–35% HPLC buffer B (0.1% formic acid in solution of 95% acetonitrile and 5% water) over 60 minutes, followed by isocratic elution at 80% buffer B for 5 min with a flow rate of 0.25 $\mu\text{L}/\text{min}$ across the capillary column. Buffer A was 0.1% formic acid in a solution of 95% acetonitrile and 5% water.

For the CE testing experiments, a method was designed such that for each peptide precursor selected, four successive MS/MS spectra were collected at CE settings of 20, 23, 26 and 29%, respectively. For all other subsequent experiments, the CE setting was 22.5%. Also for these experiments, the instrument followed an automated, data-dependent routine, alternating between a single MS scan across the mass range of 400 to 1800, followed by four MS/MS scans, in the order of intensity of precursor peaks. Precursor m/z values were dynamically excluded for 30 seconds. Fragments at m/z values of 50 and above were detected for all MS/MS collected. Settings specific to PQD were a Q activation setting at 0.7, and the activation time of 0.1. The isolation width was set at 2.0, and the minimum precursor intensity for MS/MS acquisition was 10,000 counts. Other relevant instrumental settings were: capillary temperature, 160 $^{\circ}\text{C}$; source voltage, 2.0 kV; source current, 100 μA ; capillary voltage, 9.0 V; Tube lens, 100 V; skimmer offset, 0.0 V; multipole RF amplifier, 400; multipole 00 offset, -4.0 V; Lens 0 voltage, -4.2 V; multipole 0 offset, -4.5 V; lens 1 voltage, -15.0 V; gate lens offset, -35.0 V; multipole 1 offset, -8.0 V, front lens, -5.25 V. The maximum fill time for each MS scan was 50 microseconds, and the maximum fill time for each MS/MS microscan was 100 microseconds. No zoom scans were used.

$\mu\text{LC-MS/MS}$ analysis via quadrupole time-of-flight mass spectrometry

The LC system (LC Packings/Dionex, Sunnyvale, CA) was on-line with a QSTAR Pulsar *i* quadrupole-TOF MS instrument (Applied Biosystems), which was equipped with Protana's (Denmark) nanoelectrospray source, and has been described in detail elsewhere¹². The same amount of peptides from the standard mixture (0.5 μg) was analyzed as in the LTQ-PQD analyses. Peptides were eluted with a linear gradient from 0 – 35% B over 45 min, followed by 35 – 80% B over 2 minutes, and held isocratic at 80% B for 10 minutes. Solvents A and B were the same as those used in the LTQ-PQD analyses. Product ion spectra were collected in an information dependent acquisition (IDA) mode, using continuous cycles of one full scan TOF MS from 400 – 1500 m/z (1.5 seconds) plus three product ion scans from 50 – 2000 m/z (3 seconds each). Precursor m/z values were selected starting with the most intense ion, using a selection quadrupole resolution of 3 Da. The rolling collision energy feature was used, which determines collision energy based on precursor value and charge state. Dynamic exclusion time for precursor ion m/z values was 60 seconds.

Analysis of LTQ-PQD data

The obtained MS/MS spectra were searched using SEQUEST¹³ (Bioworks version 3.2, Thermo Fisher, San Jose, CA) against a database containing protein sequences translated from 6139 open reading frames in the *S. cerevisiae* genome, with a reversed-sequence version of the same database appended to the end of the forward version for the purpose of false positive rate estimations¹⁴. Search parameters included differential amino acid mass shifts for oxidized methionine (+16 Da), static mass shift (+144 Da) at lysine and the n-terminus of all peptides, and differential mass shift of +144 Da for tyrosine, to account for the iTRAQ reagent. Precursor peptide mass tolerance was ± 2.0 Da with trypsin specified, allowing for partially tryptic peptides and up to two internal missed cleavages. Intensity m/z values detected ± 0.5 units of each iTRAQ reporter ion m/z value were extracted and appended to the appropriate column in the summary file (e.g. a centroided peak in a DTA file within the range $113.5 \leq m/z < 114.5$ are assigned to the 114 column, etc.) using our developed software (see Results section). The

HTML summary files with appended iTRAQ intensities were further processed using the tool Interact¹⁵, which also assigned each peptide match a Probability score (P score) using the Peptide Prophet algorithm¹⁶. Peptide matches were filtered using a P score threshold of 0.85 for all datasets, ensuring an estimated false positive rate below 1%¹⁷ calculated by reverse-database searching.

Analysis of Qstar data

Tandem mass spectra were searched using Protein Pilot v1 (Applied Biosystems) which uses the upgraded version of the Interrogator algorithm¹⁸. The same yeast database described above for searching the LTQ-PQD data was used for database searching. The peptide and fragment ion mass tolerances were data dependent, and were determined in real time during the database search; the generic set of variable amino acid modifications were used, including methionine oxidation (+16). Differential iTRAQ reagent labeling of lysine, tyrosine and peptide N-termini were chosen. The minimum protein score threshold was 0.47 (66% confidence, where protein score = $-\log(1 - (\text{confidence}/100))$). Reporter ion intensities for each protein are calculated by averaging of reporter ion ratios for each identified peptide, weighting this average based on individual peptide reporter ion signal intensities. Only proteins identified from at least two distinct peptides were included for quantitative analysis and peptides shared by two distinct proteins were not used in the protein average. The statistical parameters of P-value and Error Factor assigned to each protein and used to determine the 95% confidence interval for the average, were determined by Protein Pilot. All statistical calculations are documented in the Protein Pilot 2.0 Software Help.

RESULTS

A narrow collision energy range optimally fragments iTRAQ reagent labeled peptides using LTQ-PQD

PQD operation involves a different fragmentation mechanism of peptides than normal CID operation on the LTQ¹⁰. Therefore, we first investigated the effect of the relative collision energy (CE) setting in PQD mode, with the objective of defining the CE setting maximizing identification and accurate quantification of iTRAQ reagent labeled peptides. We fragmented each peptide selected for MS/MS in a data-dependent manner using increasing CE (20, 23, 26, and 29%). Figure 1A shows representative spectra from a peptide selected for MS/MS analysis, where both the full MS/MS mass range is shown, as well a zoomed view of the iTRAQ reagent reporter ions in the m/z range of 114–117 (left side of Figure 1A) for each CE setting.

Two important points are apparent from examining these spectra. First, both the intensity of iTRAQ reporter ions and accuracy of their ratios decrease as the collision energy increases. The counts of the most intense iTRAQ reporter ion, shown in each zoomed m/z spectrum, decrease by two orders of magnitude as the collision energy is increased from 20 to 29%. Also, the ratios between the iTRAQ reporter ion intensities vary widely from the known at CE settings above 23%.

Second, unlike normal CID operation on the LTQ, the amount of the peptide backbone fragmentation in PQD operating mode is highly dependent on the collision energy setting. The precursor peptide (indicated by the vertical arrow at m/z 563.3) dominates the MS/MS spectrum at the 20% CE setting, with relatively limited detection of peptide backbone fragmentation ions, necessary for sequence identification. Conversely, the precursor is undetectable at the setting of 29%, and peptide fragment ions are numerous. Meanwhile, the labile iTRAQ reagent is preferentially fragmented at the 20% CE setting, where the reporter ions are detected with highest intensity, and detected at dramatically lower signal intensity at 29%.

The optimal CE setting balances sufficient backbone fragmentation of the precursor peptide, and retains sufficient iTRAQ reporter ion intensity for accurate quantitative measurements. A straightforward measure of this balance is the ratio between the precursor intensity and the intensity of the 114 iTRAQ reporter ion (Precursor/114 ratio) in any given MS/MS spectrum. The Precursor/114 ratio from MS/MS spectra recorded at each CE setting is shown for the representative peptide in Figure 1A, which decreases dramatically from over 30 at 20% CE to zero at 29% CE (i.e. no precursor detected).

To determine the optimal LTQ-PQD CE setting from the data for our standard peptide mixture, we randomly selected and manually examined over 30 peptides from the several hundred identified in our CE testing analysis, and calculated the Precursor/114 ratio at each of the four CE settings tested. We also calculated the ratio between the 114 and 115 iTRAQ reporter ions (114/115 ratio) for each of these peptides at each different CE setting used, to measure the accuracy as a function of CE. Figure 1B shows the results of this analysis, where the average Precursor/114 ratio and the average 114/115 error (as compared to the known ratio of 2.0) are plotted as a function of CE setting. The accuracy, as measured by the 114/115 ratio error, decreases dramatically as the CE is increased above 23%. Meanwhile, at a CE setting of 20%, peptide backbone fragmentation is limited, but iTRAQ reporter ions are strongly detected. The average Precursor/114 ratio dramatically decreases between 20 and 23% CE, indicating a large increase in precursor fragmentation due to this relatively small increase in CE. Based upon this, we defined a relatively narrow region for the optimal CE setting ($20 < \text{CE} < 23$) for LTQ-PQD operation on our system, in which peptide fragmentation and iTRAQ reporter ion detection were balanced ($5 \leq \text{Precursor}/114 \leq 30$), and accuracy was maximized. We used a CE setting of 22.5%, within this optimal range, for all subsequent LTQ-PQD experiments reported here.

As a word of caution, the optimal CE range determined in these studies should not be taken as the universal setting for all LTQ instruments running in PQD operating mode. We have found that this optimal CE setting can change significantly on the same instrument as a consequence of routine cleaning and re-tuning, and have used an optimal CE setting as high as about 40% in other studies on our instrument under different tune settings. Others have reported on LTQ-PQD analysis of iTRAQ reagent labeled peptides using a range of CE settings, from 27%¹⁹ to 45%²⁰. Thus, we recommend optimization of the CE setting using a standard iTRAQ reagent labeled mixture and our method described above, which provides a reliable means to determine the optimal CE setting on any given system.

Customized software development for quantitative analysis of LTQ-PQD data

To efficiently evaluate, and eventually use routinely, LTQ-PQD for the analysis of iTRAQ reagent labeled samples, we developed software for automated quantitative analysis of large-scale datasets. Figure 2 outlines this software, which avoids labor-intensive manual examination of data. Initially, MS/MS peak lists, in the form of centroided DTA files, are searched using SEQUEST¹³, and the matched peptide sequences are organized into an HTML summary using a publicly available software script (out2summary, http://sashimi.sourceforge.net/software_curation.html). We developed software (A Perl script called 'iTRAQ reporter ion counter') which extracts the reporter ion intensities at m/z 114–117 from corresponding DTA files, and appends these to the peptide sequences in the HTML summary file. The appended HTML file is then processed using the Interact tool¹⁵. We also developed software (An executable program called 'Protein quantifier, MS/MS peptide counter') which further processes the Interact output, calculating iTRAQ reporter ion ratios from the appended intensities for each identified protein. This software also appends information on the total number of MS/MS and total unique peptide sequences matching to

each protein identified in the dataset, important for assessing the confidence of the quantitative measurements for each protein. Our software is available upon request.

Importantly, our software works on centroided peak list data contained in the DTA files, calculating ratios directly from the intensities measured at each iTRAQ reporter ion m/z value. Other software that exists for quantitative analysis of iTRAQ reagent data, specifically the recently described i-TRACKER software²¹, the Multi-Q software⁷, and the commercial Protein Pilot software package marketed by Applied Biosystems, utilizes non-centroided data, and are not currently amenable to the data outputted from the LTQ.

Accurately calculating relative abundance ratios from LTQ-PQD data

Having established an optimized CE setting for iTRAQ analysis by LTQ-PQD, and with working software for data analysis, we next tested two methods for calculating iTRAQ reporter ion ratios. Table 1 shows the results for a representative set of proteins from this initial analysis, containing proteins identified from numerous MS/MS spectra and peptide sequences, and also proteins identified from a single peptide match (i.e. “single hits”). Peptides matched to each protein are shown, along with the iTRAQ reporter ion intensities, and calculated reporter ion ratios.

Using the data in Table 1, we first evaluated averaging of each peptide reporter ion ratio to calculate an overall ratio for the protein, similar to methods used with other stable isotope methods in quantitative proteomics^{22, 23}. From this analysis, we observed a large amount of variability in reporter ion ratios between different peptides, even between MS/MS spectra matching to the same peptide sequence, which resulted in large errors in calculated protein ratios (see % error values columns on far right side of Table 1 for “Averaging” calculation method). The peptide sequence FELSGIPPAPR shown in Table 1, matching to the protein SSA1, and identified from multiple MS/MS spectra, exemplifies this variability. Although the exact reason for the observed variability is difficult to identify, one possible contributing factor is local space charging effects, which are known to especially affect ions of similar m/z values (i.e. iTRAQ reporter ions), introducing slight mass shifts when detecting these ions²⁴. Localized space charging effects can vary from MS/MS to MS/MS, even for the same peptide, and thus this effect could result in variability in measured iTRAQ reporter ion ratios. With some exceptions, we also observed that reporter ion ratios which show the largest inaccuracies are many times from peptides in which the reporter ions were detected at relatively low intensities of less than 100 counts, resulting in poor ion detection statistics. Regardless of the cause, the importance of this observed variability was the relatively large errors in calculated protein ratios compared to the known ratios when averaging the ratios from MS/MS matching to a protein.

Given this inaccuracy, we pursued an alternative method for more accurately calculating protein reporter ion ratios. For this method, we rationalized that a summation of signals across peptides matched to a given protein should accurately reflect the overall relative abundance of the protein from the separate mixtures being compared. Summing also provides the added advantage of stronger reporter ion signal intensities being weighted more heavily in the calculation than weaker signals. Thus, we summed together all of the signal intensities for each iTRAQ reporter ion recorded for each peptide matched to a given protein in Table 1, and then calculated the reporter ion ratios for each protein from these summed intensity values.

Summation of reporter ion intensities increased the quantitative accuracy significantly compared to the ratio averaging method. For example, for the 114/115 protein ratios calculated for the three proteins in Table 1 identified from multiple peptide hits (SSA1, RPS12 and NOP1), the average error was about 315% for the averaging method, compared to about 7% for the summing method. In addition, we have analyzed our standard mixture in triplicate to test

reproducibility of measured protein abundance ratios using our summing method. The average CV was $10 \pm 7\%$ for twenty different, randomly selected proteins identified from multiple MS/MS and quantified in each of the three parallel analyses, indicating good reproducibility when using our method. Therefore, our software (Figure 2) calculates and displays overall protein abundance ratios via the summing method.

Increasing repeat count and microscan acquisition improves quantitative results at the expense of protein identification

Given the variability in ratios between MS/MS spectra (even for the same peptide sequence selected multiple times for MS/MS), proteins quantified from a single MS/MS spectrum are not trustworthy. Thus, to increase the number of MS/MS matched to each protein, we increased the repeat count setting to acquire two MS/MS spectra for each selected precursor peptide. This increased the number of MS/MS spectra matching to each protein, and summed together for calculation of abundance ratios, while decreasing slightly (by about 10%) the number of total proteins identified, compared to using the usual setting of one repeat count.

We next investigated the effect of increased microscan (μ scan) acquisition on the quality of quantitative measurements using our standard iTRAQ reagent labeled mixture. Data from each μ scan collected during an MS/MS scan are averaged together, improving signal to noise and detector statistics. Because of the ability of the linear ion trap to isolate larger numbers of ions compared to earlier models of ion trap instruments², generally only one μ scan is used to achieve high quality MS/MS spectra³. This provides the shortest MS/MS duty cycles (each μ scan is 100 milliseconds or less in length), ensuring maximized numbers of proteins identified. We separately analyzed our standard mixture by LTQ-PQD using μ scan settings of one, two, three and five, while maintaining the same optimized values for other settings (22.5% CE, repeat count of two). iTRAQ reporter ion ratios were calculated from proteins identified from two or more MS/MS spectra, using our summing calculation method. Table 2 shows iTRAQ reporter ratios averaged across all identified proteins for each μ scan setting, along with the absolute error relative to the known ratio (measure of the accuracy), and standard deviation and coefficient of variation (CV) for these averages (measures of the precision). Supplementary Table 1 online shows in detail all of the data summarized in Table 2.

Our results demonstrate two important points on the effects of μ scan acquisition. First, increased μ scan acquisition provides improved precision for protein abundance ratios, while having no effect on accuracy. The CV values for each average protein ratio show a general decreasing trend for all iTRAQ reporter ratios with increasing μ scan acquisition (last column, Table 2). Figure 3 graphically displays this trend, plotting the CV for the 114/115 protein ratios against the number of μ scans acquired. For all μ scan settings, the 114/117 ratios show the highest CV, not surprising given the 117 labeled sample was an order of magnitude less in total protein quantity than the 114 labeled sample, leading to many 117 reporter ion peaks with weak signal intensities. Meanwhile, the absolute errors for each average, a measure of accuracy, are about 20% or less, regardless of the μ scan setting, or the reporter ions being compared.

Second, increased μ scan acquisition trades improved precision for fewer proteins identified. This trade-off, clearly evident in Figure 3, is not surprising, given the longer duty cycle of each MS/MS with more μ scans acquired which effectively leads to less total MS/MS being acquired per unit time, hence fewer peptides (and resulting proteins) identified within complex mixtures. As shown in Figure 3, the drop in the number of proteins identified is roughly linear with increased μ scans, with an average of about 10% fewer proteins identified for each additional μ scan acquired.

These findings support a need to balance the improved precision afforded by increased μ scan acquisition with the negative consequence of fewer proteins identified. Based upon our results,

we determined a setting of two μ scan acquisitions reasonable for the analysis of complex mixtures, providing a CV under 30% when measuring quantitative changes of less than an order of magnitude, much like other reported stable-isotope labeling methods^{23, 25}. This setting still offers duty cycles on the time scale of hundreds of microseconds, providing for MS/MS acquisition on multiple precursor ions within a one second time window.

LTQ-PQD is comparable to the Qstar for analyzing iTRAQ reagent labeled samples

As a final test, we compared the results from our standard mixture analyzed by LTQ-PQD to data for the same mixture analyzed using a quadrupole TOF instrument (Qstar, Applied Biosystems). The Qstar instrument is the gold-standard in terms of quantitative accuracy for iTRAQ reagent labeled samples^{6, 26}, given its unique capability of amplifying low mass fragments in MS/MS mode, improving the signal strength of iTRAQ reporter ions and accuracy of relative abundance measurements²⁷. Qstar data was analyzed using the Protein Pilot software (Applied Biosystems), which assigns each quantified protein (considering only those identified from at least two unique peptides) an error factor (EF), providing a measure of the confidence of each ratio. The EF calculation is based upon criteria including signal intensity of reporter ions and variability in ratios from peptides from the same protein. We used a relatively tolerant threshold for the EF value in our data comparison, considering any proteins with an error factor less than 100 (Ratios with EF 2 are considered highly confident). This was done to increase the number of quantified proteins found in common between the LTQ-PQD and Qstar datasets for our comparison. Also, we observed that proteins with larger error factors did not have significantly more inaccurate reporter ion ratios than those assigned smaller values (the average 114/115 error was 3.8% for protein with $EF \leq 2.0$, and 5.5% for those with $EF > 2.0$). For this analysis, we also calculated reporter ion ratios from the Qstar data both with and without correcting for isotope effects due to incomplete incorporation of stable isotope atoms (e.g. C^{13}) in the different iTRAQ reagents^{6, 21}. Because our software does not currently correct for these isotopes effects, we were interested in comparing the LTQ-PQD data to the Qstar data analyzed in both ways.

We compared the quantitative results from the two instruments in several ways. First, as shown in Figure 4A, we compared the 114/115 protein ratios, plotted on a logarithmic scale, for 22 proteins identified by both instruments. Table 3 shows a summary of this data, including the average protein 114/115 ratios, absolute errors and CV values for each dataset. Supplementary Table 2 online shows detailed data on all of the proteins compared in Figure 4. The Qstar data was plotted both with and without correction for isotope effects. For the data plotted in Figure 4A, the LTQ-PQD data actually shows better precision (CV of 8.7%) compared to the Qstar data, which had one outlier (protein YGL123W) which heavily contributed to the larger CV (removal of this outlier decreases the CV to 17%). Overall, the Qstar data is slightly more accurate than the LTQ-PQD data, as measured by the absolute errors. Also, the LTQ-PQD 114/115 ratios were consistently slightly below the known value, possibly due to isotope effects not accounted for with our software at present. The Qstar data supports this possibility, as isotope correction increased the 114/115 ratios by an average of 10%, as shown by comparing the dotted blue line (no isotope correction) and the solid blue line (with isotope correction) in Figure 4A. However, even without isotope correction, the absolute error was still below 20% for the LTQ-PQD data.

Second, we compared the 114/117 ratios between the two datasets, to assess the relative ability of the instruments to measure the 10-fold abundance difference between these samples. As shown in Figure 4B, and the right side of Table 3, the results again indicate comparable results between the instruments, with the Qstar data having slightly better accuracy and precision for these ratios. The Qstar data shows an average ratio exactly matching the known value of 10 (0% error); meanwhile the average for the LTQ-PQD data shows an 11% error from the known.

The almost two-fold larger CV for the LTQ-PQD data compared to the Qstar is largely due to the single outlier protein (YOR96W). Inspection of the reporter ion signal intensities from the two LTQ-PQD MS/MS spectra used to calculate this protein ratio indicated that these were relatively weak signals (less than 100 counts), which may contribute to the inaccuracy of the calculated ratio.

Third, we compared the variability in calculated ratios between peptides matching to the protein Enolase 2 (Eno2, YHR114W), which were identified by both instruments. Peptides from Eno2 were matched to 17 MS/MS spectra by Qstar analysis, and 22 MS/MS spectra by LTQ-PQD analysis. The log of the 114/115 ratio for each of the MS/MS spectra, listed in random order from each instrument, is plotted in Figure 4C. Interestingly, only four peptide sequences from Eno2 were identified on both instruments, reflecting differences in peptide ionization and detection between these instruments. The CV values for the LTQ-PQD and Qstar data (40% and 36%, respectively), demonstrates that the precision of both instruments is comparable for peptides identified from this single protein.

DISCUSSION

These results demonstrate that LTQ-PQD analysis of iTRAQ reagent labeled samples with our software is effective, and comparable to the Qstar analysis platform, currently the gold-standard in iTRAQ reagent-based quantitative proteomic analyses. Our results also define guidelines for successful analysis of iTRAQ reagent labeled samples by LTQ-PQD:

1. **Relative collision energy setting.** The CE setting is critical for maximizing detection and accuracy of iTRAQ reporter ion ratios. The optimal CE setting can be determined via analysis of a standard mixture of iTRAQ reagent labeled peptides fragmented at increasing CE, and measurement of the Precursor/114 intensity ratios for representative peptides at each setting. The CE setting producing an average Precursor/114 ratio in the range of ~5–30 is optimal. The optimal CE setting occurs across a relatively narrow range (< 3 CE units).
2. **Collision energy optimization.** The optimal CE setting is instrument-dependent, and may even change on the same instrument after routine cleaning and re-tuning. We suggest frequent testing the optimal CE setting, especially after re-tuning of the instrument, using our described method with an iTRAQ reagent labeled standard mixture.
3. **μ scan setting.** Increasing the number of μ scans acquired for each MS/MS improves the precision of the quantitative measurements from the iTRAQ reporter ions. This improved precision comes at the expense of fewer proteins identified in complex mixtures, due to the increased duty cycle. A suggested setting of two μ scans still provides acceptable precision, and limits the drop in proteins identified for complex samples. For less complex samples, increasing to four or five μ scans acquired is advisable for the best results.
4. **Reporter ion ratio calculation.** We found that calculation of overall protein reporter ion ratios via summing of all iTRAQ reporter intensities obtained from individual LTQ-PQD MS/MS matched to each protein was most accurate. This method is simple, and inherently weights higher intensity reporter ion signals more heavily than low intensity signals, which are generally more apt to provide inaccurate ratios. We have developed customized software for this calculation, which is available upon request.
5. **Repeat count setting.** Increased MS/MS spectra matching to each protein maximizes the accuracy of our data summing calculation method. Therefore, a repeat count setting of at least two is suggested, to maximize the number of MS/MS spectra used

to calculate abundance ratios for each protein. An increase from a setting of one to two repeat counts results in slightly fewer (~10%) proteins identified from a complex mixture.

One potential limitation of iTRAQ reagent-based quantitative proteomic studies which is currently not accounted for using LTQ-PQD is the possibility of multiple precursor peptides with similar m/z values (within 1–2 Da of each other), being simultaneously isolated and fragmented in the ion trap. Such “cross-talk” between closely massed precursors could introduce inaccuracies into the iTRAQ reporter ion ratios, as the reporter ion intensities would have contributions from both of the isolated peptides, and not accurately reflect the relative abundance of the single peptide sequence identified by database searching. Although MS/MS containing fragments from multiple precursors many times will not result in a high scoring match to a peptide sequence, manual examination of the high resolution TOF-MS data from the Qstar analysis of our standard mixture indicated that about 3% of peptides matching to proteins with high confidence did show two distinct precursor peptides within the quadrupole selection mass window, which were both isolated and fragmented. In order to minimize potential inaccuracies due to cross-talk effects when using LTQ-PQD, a narrow isolation width may be advisable. Because of the increased ion capacity of the linear ion trap, we have found that decreasing the isolation width from 2.0 (which we used in the studies described here) to 1.0 does not result in a decrease in the number of proteins identified from our standard mixture, or the overall signal strength (data not shown). Inclusion of a zoom scan could also provide high resolution data for precursor peptides necessary to automatically identify MS/MS spectra which contain multiple precursor peptides.

Finally, the guidelines described here for LTQ-PQD operation should be applicable to the newest hybrid LTQ-Orbitrap instrument, which offers the added benefit of high mass accuracy and resolution²⁸ for automated MS/MS analysis. This instrument would provide high quality data amenable to the automated identification and exclusion of MS/MS spectra derived from multiple precursors. The high quality data derived from the hybrid LTQ-Orbitrap, without sacrificing the speed and sensitivity inherent to linear ion trap instrumentation, gives this instrumental platform tremendous potential for quantitative proteomic studies when coupled with PQD operation and the highly versatile iTRAQ reagents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A.) Representative MS/MS of an iTRAQ reagent labeled peptide fragmented at increasing relative collision energy (CE), as indicated. The relative intensity ratios measured against the 114 intensity are shown at each CE value for each iTRAQ reporter ion. The expected 114:115:116:117 ratio is 10:5:2:1. The ratio of the precursor peak intensity to the iTRAQ 114 reporter ion intensity (Precursor/114) is also shown at each CE setting. B.) Plot of the average Precursor/114 ratio and the average 114/115 error versus the CE setting calculated from over thirty randomly selected peptides. The optimal range of CE values is shown in brackets in the plot.

Figure 2. Overview of our data processing method for iTRAQ reagent samples analyzed by LTQ-PQD. Our customized software is shown in bold and described in the text.

Figure 3.
Plot of proteins identified and CV for the average 114/115 protein ratios as a function of number of microscans acquired per MS/MS using LTQ-PQD.

Figure 4.

A.) Plot of the $\log(114/115)$ ratios for proteins identified in common by LTQ-PQD and the Qstar instrument. Proteins are listed by their ORF accession codes. Data from the Qstar is shown both with and without correction for isotope effects. The dotted horizontal line drawn indicates the known $\log(114/115)$ ratio. B.) Plot of the $\log(114/117)$ ratios for protein identified in common by LTQ-PQD and the Qstar instrument. The dotted horizontal line is drawn through the known $\log(114/117)$ ratio. C.) Plot of $\log(114/115)$ ratios for MS/MS matched to peptides from the protein Enolase 2 (Eno2) from the LTQ-PQD and Qstar analyses. The first four MS/MS spectra (indicated by an asterisk in the x-axis labels) matched to the same peptide sequences from Eno2. The other plotted ratios were obtained from MS/MS spectra on each instrument which matched to different Eno2 peptide sequences, and are displayed in random order.

Representative results from LTQ-PQD analysis of a standard iTRAQ reagent labeled yeast protein sample

Table 1

Protein (total MS/MS)	Peptide	114	115	Intensities ¹	116	117	114/115	114/116	114/117	Calculation Method ²	114/115 (2.0) ³	114/115 % error	Overall Protein Ratios 114/116 (5.0) ³	114/116 % error	114/117 (10.0) ³	114/117 % error
SSA1 (23)	K.ATAGDTLHGGEDFDNR.L	1541.4	551.2	276.0	562.4	2.8	5.6	2.7	Averaging: Summing:		18.1	802.6	6.2	24.2	188.8	1788.3
	K.DAGTIAGLNVLRI	1019.6	332.1	123.7	29.4	3.1	8.2	34.7			1.9	4.2	4.9	1.2	8.4	15.7
	K.ELQDIANPMSK.L	847.6	449.6	222.1	70.8	1.9	3.8	12.0								
	K.ETAESYLGAK.V	1121.1	790.1	79.8	81.9	1.4	14.0	13.7								
	K.FELSGIPPAPR.G	326.9	76.3	79.0	1.0	4.3	4.1	326.9								
	K.FELSGIPPAPR.G	173.4	376.8	9.8	101.0	0.5	17.7	1.7								
	K.FELSGIPPAPR.G	813.0	346.5	125.9	124.8	2.3	6.5	6.5								
	K.FELSGIPPAPR.G	106.6	95.7	12.6	1.0	1.1	8.5	106.6								
	K.FELSGIPPAPR.G	1819.8	875.4	372.7	296.2	2.1	4.9	6.1								
	R.IINEPTAAAIAYGLDKK.G	582.7	179.5	120.6	14.1	3.2	4.8	41.3								
	R.IINEPTAAAIAYGLDKK.G	409.2	283.5	82.6	48.8	1.4	5.0	8.4								
	R.IINEPTAAAIAYGLDKK.G	494.0	194.7	82.9	23.8	2.5	6.0	20.8								
	R.LSKEDIEK.M	3570.6	1816.8	684.6	1.0	2.0	5.2	3570.6								
	K.LVTDYFNGKE	356.9	1.0	244.7	41.4	356.9	1.5	8.6								
	K.LVTDYFNGKE	742.0	348.4	358.5	81.1	2.1	2.1	9.1								
	K.LVTDYFNGKEPNR.S	851.8	788.5	154.4	116.8	1.1	5.5	7.3								
	K.NFTPEQISSMVLGK.M	60.2	25.4	6.9	14.7	2.4	8.7	4.1								
	K.NFTPEQISSMVLGK.M	51.0	67.5	9.6	13.8	0.8	5.3	3.7								
	K.NFTPEQISSMVLGK.M	76.2	10.7	16.5	1.0	7.1	4.6	76.2								
	K.NFTPEQISSMVLGK.M	74.1	28.1	11.3	1.0	2.6	6.6	74.1								
	K.NQLESIAYSLK.N	141.4	91.2	20.9	51.6	1.6	6.8	2.7								
	R.VDIANDQGNR.T	1019.9	731.3	181.2	226.8	1.4	5.6	4.5								
	K.VNDAAVTVPAYPENDSQR.Q	10.6	1.0	5.5	17.4	10.6	1.9	0.6								
RPS12 (4)	Summed intensities:	16210.0	8461.3	3281.8	1921.8				Averaging: Summing:		2.7	37.0	158.5	3069.7	310.2	3002.4
	K.LVEGLANDPENKVLPIK.V	101.5	59.3	37	1	1.7	2.7	101.5			1.9	4.2	4.1	17.3	8.0	20.2
	K.LVEGLANDPENKVLPIK.V	1329.4	271.8	150.3	288.7	4.9	8.8	4.6								
	K.QLGEWAGLGKI	620.4	185.1	1	107.7	3.4	620.4	5.8								
NOP1 (2)	K.QLGEWAGLGKI	1129.1	1124.4	580.7	1	1.0	1.9	1129.1	Averaging: Summing:							
	Summed intensities:	3180.4	1640.6	769	398.4											
	K.DQGGVVISIK.A	579.6	407.4	304.2	99.1	1.4	1.9	5.8			4.1	105.3	9.7	93.2	4.1	58.9
SMT3 (1)	R.ISVPEPSKEDGVPTPK.V	1524.0	518.3	241.6	113.1	2.9	6.3	13.5	--	--	2.3	13.6	3.9	22.9	9.9	0.9
	Summed intensities:	2103.6	925.7	545.8	212.2											
	K.VSDGSSIEFFKI	435.6	173.1	116.8	34.0	2.5	3.7	12.8			2.5	25.8	3.7	25.4	12.8	28.1
DPM1 (1)	R.VAIGEVPFTFGVR.T	22.3	22.6	1.5	8.6	1.0	14.9	2.6	--	--	1.0	50.7	14.9	197.3	2.6	74.1

¹ An intensity value of 1.0 means the signal is not detected above noise, thus ratios to these reporter ions are relative to the noise level.

² The calculation methods are described in the text

³ Numbers shown in parentheses are the known iTRAQ reporter ion ratios for our standard mixture

Table 2
Dependence of LTQ-PQD results on number of microscans acquired per MS/MS

No. microscans	Total proteins identified (2 or more MS/MS)	iTRAQ ratios ^J	Average (all proteins)	Abs error (%)	Standard deviation	CV (%)
1	140	114:115 (2.0)	1.9	5.8	0.8	44.9
		114:116 (5.0)	4.3	13.7	1.3	30.1
		114:117 (10.0)	12.1	20.7	7.7	64.0
2	123	114:115	1.8	10.1	0.4	22.1
		114:116	4.3	13.7	1.3	29.0
		114:117	10.5	4.9	5.0	47.9
3	116	114:115	1.8	11.4	0.4	19.9
		114:116	4.2	16.2	1.0	23.3
		114:117	11.0	10.4	4.6	41.2
5	88	114:115	1.8	12.3	0.2	11.5
		114:116	4.3	13.3	0.7	15.8
		114:117	10.4	3.8	2.5	24.1

^J Known ratios are shown in parentheses

Table 3
Comparison of iTRAQ reporter ion ratios for proteins identified in common by LTQ-PQD and Qstar mass spectrometry

	Qstar (corrected) ^I	114/115 ratios		114/117 ratios	
		Qstar (not corrected) ^I	LTQ-PQD	Qstar (corrected)	LTQ-PQD
Average	2.1	1.9	1.7	10.0	11.1
Absolute error (%)	5.0	5.0	15.0	0.0	11.0
Standard Deviation	0.8	0.5	0.1	2.5	6.0
CV (%)	38.7	27.9	8.7	24.9	53.4

^I „corrected” or “not corrected” refers to correction for isotope effects between iTRAQ reporter ions using Protein Pilot (see text)