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Combined Pulsed-Q dissociation and electron transfer dissociation for identification and quantification of iTRAQ-labeled phosphopeptides

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Abstract

Here, we report a new approach that integrates pulsed Q dissociation (PQD) and electron transfer dissociation (ETD) techniques for confident and quantitative identification of iTRAQ-labeled phosphopeptides. The use of isobaric tags for relative and absolute quantification enables a high-throughput quantification of peptides via reporter ion signals in the low m/z range of tandem mass spectra. PQD, a form of ion trap collision activated dissociation allows for detection of low mass-to-charge fragment ions and electron transfer dissociation is especially useful for sequencing peptides that contain post-translational modifications. Analysis of the phosphoproteome of human fibroblast cells using a sensitive linear ion trap mass spectrometer demonstrated that this hybrid approach improves both identification and quantification of phosphopeptides. ETD improved phosphopeptide identification, while PQD provides improved quantification of iTRAQ-labeled phosphopeptides.

INTRODUCTION

The importance of quantitative measurements in proteomics studies has resulted in numerous creative strategies for quantifying changes in peptide and protein abundances.^{1, 2} Most of the quantitative approaches for mass spectrometry (MS)-based analyses rely on stable isotope labeling to obtain relative or absolute abundance information of peptides with measurable differences in mass. However, detection of these labeled peptides often requires the use of a high resolution mass spectrometer and only a limited number of cellular treatment conditions (two or three in most cases) can generally be combined in a single analysis.^{3–5}

Isobaric labeling techniques^{6, 7} such as isobaric tags (e.g., iTRAQ from Applied Biosystems) for relative and absolute quantitation allow 4–8 samples to be combined within the same analysis. Pooling samples for quantitative measurements (multiplexing) is useful for studying complex biological systems, e.g., following different treatment conditions or longitudinally over time, or for increasing confidence in the results by combining biological replicates and performing inverse labeling to detect and correct system bias.⁸ Although iTRAQ labels have the same mass, the different distribution of isotopes between the reporter and balance groups ensures each labeled peptide is spectrometrically distinct. Upon fragmentation in the mass

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SUPPORTING INFORMATION

Additional information as noted in the text.

spectrometer, the labels dissociate, releasing the reporter groups as singly charged ions with distinct masses, e.g., 114, 115, 116, or 117 for 4-plexed iTRAQ labeling. Quantitative values can then be determined based on the abundance of these reporter ions.

In spite of increasing application of the iTRAQ approach,^{9–12} most MS analyses to date have been performed using quadrupole time-of-flight (Q-TOF) mass spectrometers rather than the more sensitive linear ion trap¹³ (e.g., LTQ, LTQ-FT, or LTQ-Orbitrap) instruments. The preferred use of Q-TOF MS is due to the fact that traditional collision-activated dissociation (CAD) MS/MS in ion traps precludes detection of any fragments ions below $\sim 1/3$ of the precursor m/z (“one-third rule”) and therefore, prevents identification of the iTRAQ reporter ions in many cases.¹⁴ A recently introduced collision activated fragmentation technique referred to as pulsed-Q dissociation (PQD) eliminates the one-third rule for ion trap mass spectrometers and makes analysis of iTRAQ -labeled samples possible with these instruments, as PQD enables routine and reliable measurement of ions down to 50 m/z .¹⁵ Recent studies have demonstrated the superiority of LTQ-PQD for analyzing iTRAQ reagent-labeled peptides in complex samples, showing that LTQ-PQD can detect twice as many proteins compared to Q-TOF instruments with no loss in quantitative precision.^{13, 16} PQD using an LTQ-Orbitrap reportedly outperformed “high energy collision induced dissociation” (HCD) on the same instrument in terms of the lower limit of quantitation.¹⁶

Although reliable quantitation can be achieved with PQD, it is still not clear whether this fragmentation method is suitable for identifying phosphopeptides, an extremely important class of peptides. While PQD allows the detection of low m/z peptide fragment ions, the fragmentation efficiency of PQD is generally less than CAD, even under optimized conditions.^{13, 17} While the hybrid mode of PQD and CAD appears to be advantageous for identifying and quantifying unmodified peptides,¹⁷ fragmentation of phosphopeptides is often ineffective due to the labile nature of the phosphate group.¹⁸ Electron transfer dissociation (ETD) has proven to be an attractive complementary fragmentation technique for phosphopeptide identification.^{19, 20} Recently, the Coon group showed that ETD can be used for quantifying iTRAQ labeled peptides²¹ even though the four different iTRAQ labels (114, 115, 116 and 117 tags) only generate three reporter ions (101, 102 and 104 tags). In addition, this study also showed that fewer ETD scans could be used for reliable peptide quantification (average error <10%) compared to PQD.²¹ In recent work Coon’s group has reported a method using ETD and CAD for 8-channel iTRAQ quantification;²² where additional CAD mode had to be used to take full advantage of the 8 channel iTRAQ reporters. However, to our knowledge, ETD has never been used in combination with a multiplexed quantification strategy for analysis of phosphopeptides.

Considering the importance of and challenges associated with quantitative phosphoproteomics, it is important to notify researchers in the field of significant technology developments and how to best apply these advanced technologies for the accurate identification and quantification of phosphopeptides. In this study, we combined PQD and ETD techniques in liquid chromatography (LC)-MS/MS and evaluated the approach for quantitative phosphoproteome analysis. As part of this evaluation, LTQ settings were optimized for PQD using iTRAQ-labeled peptides from standard protein mixtures. Subsequent analysis of iTRAQ-labeled phosphopeptides from human fibroblast cells using sequential PQD and ETD demonstrated that the hybrid approach provides confident identification and robust quantification of phosphopeptides compared with either technique alone.

EXPERIMENTAL

Standard protein digestion and iTRAQ reagent labeling

Standard proteins – ubiquitin (U6253), β -lactoglobulin A (L7880), β -lactoglobulin B (L8005), β -casein (C6905), α -casein (C6780), carbonic anhydrase II (C3934), BSA, lysozyme (L6876),

and ribnuclease A (M1882) – were purchased from Sigma, and trypsin was obtained from Promega (Madison, WI). A 1 mg/mL stock solution of each standard protein was prepared in water, after which equal volumes of each protein solution were mixed to provide a standard protein mixture for optimizing the PQD collision energy setting for both peptide identification and quantification. Samples were reduced, alkylated, and digested with trypsin, and then desalted using solid-phase extraction. Peptides (~100 µg peptides/tube) were dried down and brought to equal volume with the iTRAQ reagent labeling buffer (~70% ethanol and 0.15 M triethylammonium bicarbonate), and one unit of each 4-plexed iTRAQ reagent (Applied Biosystems, Foster City) was added to each corresponding sample tube at room temperature for 1h according to the manufacturer's directions. Samples were subsequently mixed and concentrated using a SpeedVac. Following lyophilization, each sample was washed three times with ethanol (1 mL/cycle) and once with methanol (1 mL) to further remove any volatile salt,

iTRAQ labeling and enrichment of phosphopeptides from human fibroblast cells

Proteins (~1 mg) extracted from human fibroblast cells were reduced, alkylated, and digested with trypsin and then desalted using solid-phase extraction. The purified peptides (~400 µg) were equally divided into four different microcentrifuge tubes (~100 µg peptides/tube) and dried. The peptides in each tube were subjected to methyl esterification,²³ dried again, and then labeled with iTRAQ (as described above for the standard protein mixture). Next, the mixed samples were subjected to immobilized metal-ion (Fe^{3+}) affinity chromatography (IMAC) for phosphopeptide enrichment using previously described procedures.²³ Two biological replicates of human fibroblast cell samples were independently processed and analyzed in this work.

Liquid chromatography and mass spectrometry

All samples were separated using an automated dual-column phosphoproteome nano-HPLC platform assembled in-house²³ and analyzed using an LTQ-ETD instrument (Thermo Fisher Scientific). All ETD reactions were performed for 125 ms, and a precursor cation AGC target value of 30,000 and an anion AGC target value of 100,000 were used. PQD collision energies were optimized on iTRAQ-labeled peptides from the standard protein mixture, and the optimized normalized collision energy of 35 was used for PQD phosphopeptide identification. Data-dependent data sets were collected for the four most abundant species after each MS scan using sequential PQD and ETD collision modes. Either two or three LC-MS technical replicate analyses were performed for each biological replicate sample of human fibroblast cells.

Data analysis

SEQUEST was used to search MS/MS spectra for peptides from the standard protein mixture. Peptides were then analyzed by applying the PeptideProphet algorithm²⁴ with a PeptideProphet Probability score $P > 0.95$ required for confident identifications. Manual validation was performed for spectra in which phosphopeptides from β -casein and α -casein were identified. MASIC software²⁵ was extended to report PQD and ETD collision modes in each scan, which allowed separate .dta spectra files to be generated for each collision mode. Both PQD and ETD MS/MS spectra were searched against a human protein database to identify phosphopeptides from human fibroblast cells. Searches, including forward and reversed sequences (decoy), were performed using X!Tandem with different parameter files, i.e., either *b*- and *y*-ions (PQD) or *c*- and *z*-ions (ETD). The results were filtered so that a false discovery rate of $< 1\%$ was achieved.

The MASIC software was also updated to extract the maximum peak intensity within ± 0.5 Da of each expected iTRAQ reporter ion (PQD: 114–117 m/z ; ETD: 101, 102 and 104 m/z) from each fragmentation spectrum. As a result, identified peptides were able to be quantified by each collision mode and then compared based on the fact that the PQD scan is one scan

event prior to the ETD scan. Only spectra where all the expected iTRAQ reporter ions (4 for PQD and 3 for ETD in this work) were detected were used for quantification. To quantify peptides across different labeling conditions, such as 114 labeled vs. 115 labeled samples, the individual ratios (e.g., 114/115) from all quantifiable scans for a given peptide were averaged.

RESULTS AND DISCUSSION

PQD optimization

As the normalized collision energy (CE) is reportedly the dominant factor affecting PQD efficiency,^{13, 17} we used the standard protein mixture that contained both unmodified and phosphorylated peptides to determine the optimal CE setting for LTQ-PQD. Based on previous reports of CE settings that ranged from 20 to 45%^{13, 26} for LTQ-PQD analysis of iTRAQ reagent-labeled peptides, we evaluated CE settings at 20, 25, 27, 30, 35, 45 and 50% for their effectiveness for peptide fragmentation (including iTRAQ reporter ions) and identification.

Figure 1 shows the total number of spectra collected that had confident peptide identifications for two MS replicate analyses at each CE setting (27, 30, 35 and 45%). Note that the results for replicate analyses at the same CE setting are consistent. Table 1 summarizes results for iTRAQ reporter ions and includes the average number of identified peptides from two technical replicates at each CE 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 (measure of the precision). These parameters are provided at the peptide level instead of at the protein level because phosphoproteome quantification is routinely performed at the phosphopeptide (i.e., phosphorylation site) level.

From Table 1, optimal quantification using iTRAQ labeling under our experimental conditions is achieved at 35% CE. At this setting, standard deviation/CV is 0.14/0.15, 0.23/0.23 and 0.18/0.17, respectively, for reporter ion ratios of 114/115, 114/116 and 114/117 at the peptide level, which is either similar or better than other reported stable-isotope labeling methods.^{10, 12, 27, 28} The absolute error is < 10% from the expected ratio, indicating good quantitative precision. Moreover, for all collected MS/MS spectra, which includes those with and without peptide identifications, 35% CE yielded the highest percentage of quantifiable spectra with good quantification accuracy and precision for all reporter ions (data not shown) compared to other settings (i.e., 3.6-, 1.8-, and 3.9-fold higher than 27, 30, and 45% CE, respectively). Substantially fewer peptides were identified at CE settings below 35%. At CE settings below the optimal, the precursor ion dominates the MS/MS spectrum and the detection of peptide backbone fragmentation ions is very limited, which results in fewer peptide identifications.^{13, 16} Increasing the CE reduces the number of precursor ions; however, it also decreases the intensity of iTRAQ reporter ions and the accuracy of their ratios.¹³ We also noticed that the number of identifiable peptide spectra decreased at 45% and 50% CE by more than 50% (Figure 1), which indicates significant ion losses at high collisional energies.

For the CE settings evaluated in our study, 35% CE provided the best quantification accuracy, as well as the largest number of total peptide identifications, as shown in Figure 1 and Table 1 (3379 total peptides, the average from two MS replicates). Moreover, confident identifications of phosphopeptides from casein proteins were only observed at 35% CE with good quantification accuracy. These findings showed that phosphopeptides and unmodified peptides have similar optimized PQD settings. This fact was further confirmed by infusion experiments performed using iTRAQ labeled peptides from both the β -casein digest and phosphopeptides from enolase (examples shown in supplementary Figure 2). Importantly, all four reporter ions (114–117 m/z) were detected in ~77% of spectra with peptide identifications, indicating that quantification across four sample conditions can be achieved at this setting. It should be noted that even at this optimal setting, the number of peptide identifications is still

much lower (i.e., <~40–50%, data not shown) than can be obtained using CAD with 35% CE. However, because CAD fragmentation efficiency for phosphopeptides is low due to the labile nature of the phosphate group,¹⁸ we opted to use ETD as the phosphopeptide fragmentation technique to integrate with PQD.

Technically, the PQD optimization should involve optimization of the parameters: collision energy, activation Q, delay time t, AGC target, and the number of microscans, all of which have substantial influence on the PQD efficiency.¹⁶ Among these variables, the CE, Q and t are the major factors.¹⁷ However, these 3 factors are closely related to each other. From infusion experiments of iTRAQ labeled β -casein digest, we have clearly observed that at each Q and t combination, there is an optimized CE (supplementary Figure 1). At their optimal values, each combination of CE, Q and t yields very similar results for iTRAQ quantification in terms of the absolute intensity of iTRAQ reporter ions and the quantification accuracy. This may explain why the published papers have different optimized CE, Q and t settings.^{13, 16, 17, 21} In this work, we have used the default Q and t settings (Q=0.7, t=0.1 ms), and optimized the CE for the iTRAQ quantification; similar to the Coon group approach.²¹ Moreover, the results shown for the peptides from the standard protein digest indicated that good iTRAQ quantification accuracy and precision were both achieved when applying our settings.

The parameters of higher AGC target and number of microscans will improve the precision of iTRAQ quantification; however, the sampling rate will be decreased as a result. From our infusion experiments, we compared our setting (AGC target=1e4 and 1 microscan) to others' settings¹⁶ (AGC target=5e4 and 2 microscans) for several iTRAQ-labeled β -casein peptides. Our setting showed similar results for the quantification (supplementary Figure 2). Moreover, the sampling rate using our setting is ~2-fold compared to the reported setting.¹⁶

A previous report¹³ indicated the optimized PQD setting might change on different instruments and even on the same instrument as a consequence of routine cleaning and re-tuning. We also observed this PQD setting change in the present work when our infusion experiment was performed at a different time. We believe that the main reason for this PQD variability is related to the variation of ion trap vacuum pressure. Pressure variability in the ion trap is mainly due to the MS interface conditions. A dirty, heated inlet capillary would result in a relatively lower ion trap pressure than a clean, heated capillary. The optimum CE value for efficient PQD decreases as the ion trap pressure decreases.

Phosphopeptide identification and quantification using PQD-ETD

While analysis of the standard protein digest indicated that reliable quantification is achievable using PQD for unmodified peptides, the complexity of real biological samples and the introduction of additional sample preparation steps can complicate quantitative MS-based analyses, particularly for peptides containing low abundance, labile, post-translational modifications, such as phosphopeptides in a complex biological system. To test the applicability of multiplexed quantification of phosphopeptides in complex systems and build the basis for future work, we applied LTQ-PQD-ETD to the analysis of iTRAQ labeled phosphopeptides, prepared from a sample that mimicked biological complexity, but provided an expected reporter ion ratio of 1:1:1:1, as detailed in the Experimental section. Briefly, protein digests from human fibroblast cells were desalted and split into four equal aliquots for labeling with the four iTRAQ reagents. The differentially labeled peptides were then recombined prior to IMAC phosphopeptide enrichment. In future experiments this procedure will be performed using differentially treated samples; however, our goal in this study was to test the quantitative accuracy of our workflow. Therefore, only one sample condition labeled with four tags was prepared and analyzed in order to provide an accurate assessment of the quantitative variability inherent to the iTRAQ process as applied to phosphoproteome studies. Samples consisted of

two independent biological replicates of human fibroblast cells, and for each enriched sample, 2–3 technical replicate LC-MS/MS analyses were performed applying sequential PQD-ETD.

A review of the spectra indicated that PQD did not result in as many phosphopeptide identifications as in the ETD. Overall, 251 unique phosphopeptides were identified from ETD spectra, of which > 84% (214 unique) phosphopeptides yielded quantitative information over all four conditions; conversely, only 21 unique phosphopeptides were confidently identified from the PQD spectra, 6 of which were also identified from ETD spectra, and 18 yielded quantitative information over the four conditions. Two factors might account for the limited number of phosphopeptide identifications obtained from the PQD spectra. One possibility is that the precursor ion still dominates the PQD MS/MS spectrum at 35% CE, which can lead to limited detection of the peptide backbone fragmentation ions. A second possibility is that when no precursor ion is detected, the ions with the neutral loss of phosphate from the precursor ion dominate the PQD MS/MS spectra, similar to results obtained from CAD. Of the 21 unique phosphopeptides identified from the PQD spectra, 12 were each identified with a 2+ charge state and 9 were each identified with a 3+ charge state. On the other hand, none of the 251 unique phosphopeptides identified from ETD spectra were identified with a 2+ charge state, but 168 peptides were identified as 3+, 82 were identified as 4+, and 16 were identified with a charge state of 5+. These results demonstrate the ability of the integrated PQD-ETD approach to identify phosphopeptides having different charge states;²⁹ however, this integrated approach may suffer slightly from its ability to identify peptides with a 2+ charge state due to the ineffectiveness of PQD to identify tryptically digested phosphopeptides of this charge.

Figure 2 shows PQD and ETD spectra for the phosphopeptide: QGSGREpSPSLASR obtained during a sequential PQD-ETD scan. In terms of peptide identification, the ETD spectrum shows almost complete, complementary *c*- and *z*-ions for the given phosphopeptide, allowing confident identification. However, the corresponding PQD spectrum displays only a few ions that can be matched to theoretical *b*- and *y*-ions for this peptide. In fact, a database search did not result in any confident peptide identification for this PQD spectrum. However, this PQD spectrum provides more reliable quantitative information than the ETD spectrum for this phosphopeptide, as evidenced by the near 1:1:1 ratio of the iTRAQ reagent reporter ions in the zoomed view of Figure 2a. Although ETD cleavage of the N-C α bond of the iTRAQ tag reportedly results in fragment ions that can be used for quantitation,²¹ quantitative information cannot be obtained across all four conditions since the reporter ions are the same for the 116 and 117 tags (see zoomed view of the iTRAQ reagent reporter ions in Figure 2b).

Table 2 summarizes the quantification results for phosphopeptides identified using ETD and quantified using either PQD reporter ions or ETD reporter ions. Note that since the ETD reporter ion (104) is the same for iTRAQ tags 116 and 117, the expected ratio of 101/104 is 0.50 instead of 1. Additionally, the overall absolute errors for quantification using ETD reporter ions are high, i.e., 0.47 for 101/102, as are the CVs, 0.45 for both 101/102 and 101/104. The CVs improve to < 30% for phosphopeptides quantified using PQD reporter ions, indicating more reliable quantification using the iTRAQ-PQD approach with complex samples.

When comparing ETD alone with PQD-ETD hybrid analysis for the same sample, approximately 20% fewer phosphopeptides were identified in the hybrid mode and not surprisingly, since it is true that any hybrid platform will sacrifice part of the instrument cycle. However, for all the obtained spectra, PQD has more (2.3-fold more) quantifiable spectra (with all the expected iTRAQ reporter ions detected) than ETD. For ETD identified phosphopeptide spectra, PQD has >1.4 fold more quantifiable spectra than ETD. This indicates that PQD-ETD is superior to ETD alone in obtaining more quantifiable spectra for phosphopeptides.

For the work presented here we have optimized the ETD setting for peptide identification. From our infusion experiments, by optimizing the ETD conditions for several peptides with charge states 3+ and above, we did not find an extensive difference between our setting and the setting other groups have used for peptide quantification, as shown in our supplementary data. None of the ETD settings either showed good quantification results or reporter ions were simply not detected (supplementary Figures 3). This might be due to the relatively low levels of these peptides (full MS signals $\sim 7\text{--}8\text{e}5$). Another possible explanation according to Coon et al.²¹ is that ETD does not result in preferential cleavage of the N-C α bond of the iTRAQ tag. However, for all these peptides, PQD spectra show very good quantification accuracy. This is due to the fact that iTRAQ tagging reagents were designed specifically so that CAD/PQD would preferentially cleave this bond, producing intense reporter ions. This is again consistent with what we found in this work, confirming that PQD can provide more quantifiable spectra than ETD and it is extremely important for the application of quantification methods, in addition to good quantification accuracy.

While a previous paper²¹ showed that ETD provides more accurate quantification than PQD for unmodified peptides, our results for phosphopeptide analysis indicate that ETD alone is not suitable for multiplexed quantitative phosphopeptide analysis. PQD is clearly superior to ETD for quantification of phosphopeptides, in terms of both quantification accuracy and the number of quantifiable spectra. However, due to the relatively low fragmentation efficiency of PQD¹⁷ for phosphopeptides, by combining PQD with other fragmentation methods (e.g., PQD plus ETD with multistage activation) generally proved to be more effective for phosphopeptide analysis and an integrated approach will be needed for the comprehensive identification and quantification of iTRAQ-labeled phosphopeptides.

CONCLUSIONS

We have developed an optimized integrated PQD-ETD pipeline using an LTQ mass spectrometer for iTRAQ-labeled quantitative phosphoproteome profiling. While either PQD or ETD alone can provide identification and quantification for iTRAQ-labeled phosphopeptides, PQD does not fragment phosphopeptides efficiently, and ETD generates less quantifiable spectra for phosphopeptides and does not provide good quantification accuracy and precision for phosphopeptides. The results presented in this work show the advantage of combining PQD and ETD for quantitative phosphoproteome analysis: the use of ETD provides an excellent fragmentation technique for phosphopeptides while the reporter ions, generated from iTRAQ tags, in PQD spectrum provide reliable quantitative results. This integrated approach provides both robust and confident quantitative peptide identifications with broad potential for biological and biomedical phosphoproteome studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Optimization of PQD normalized CE settings using peptides from standard protein mixture. Total identified peptides were plotted with different PQD normalized CE for two MS technical replicates.

PQD Scan #7994 NL: 6.83E4 ms² 529.14**R.Q|G|S|G|R|E|pS|P|S|L|A|S|R.E** ETD Scan #7995 NL: 1.70E4 ms² 529.14**R.Q|G|S|G|R|E|pS|P|S|L|A|S|R.E** **Figure 2.**

MS/MS spectra produced from the $[M+3H]^{3+}$ ion of phosphopeptide QGSGREpSPSLASR (pS indicated that this particular S residue was phosphorylated). (a) MS/MS using PQD. (b) MS/MS using ETD. The zoomed view shows the measure quantitative information using either PQD (2a) or ETD (2b). The expected ratio is 1:1:1 for 114–117 tags (2a) and 1:1:2 for 101, 102 and 104 tags (2b).

Table 1
Optimization of PQD conditions for peptide identification and quantification.

	Total identified peptide per analysis	Total unique peptides: identified/quantified	Peptide ratios	Expected iTRAQ reporter ion ratio	Measured iTRAQ reporter ion ratios (average value from 2 MS analyses)			
					Average	Absolute error	Standard Deviation	CV
PQD27	320	44/13	114/115	1	0.93	0.11	0.14	0.15
			114/116	1	0.93	0.07	0.24	0.26
			114/117	1	1.18	0.19	0.28	0.23
PQD30	1135	148/60	114/115	1	0.93	0.07	0.15	0.16
			114/116	1	0.98	0.04	0.23	0.23
			114/117	1	1.09	0.11	0.23	0.22
PQD35	3379	173/106	114/115	1	0.94	0.06	0.14	0.15
			114/116	1	0.99	0.02	0.23	0.23
			114/117	1	1.07	0.09	0.18	0.17
PQD45	1561	209/87	114/115	1	0.93	0.07	0.23	0.25
			114/116	1	0.93	0.07	0.20	0.21
			114/117	1	1.01	0.03	0.23	0.23

Table 2
Comparison of PQD and ETD-based quantification on iTRAQ-labeled peptides identified from ETD spectra.

Quantification approach		Expected	Average value of 2 biological Replicates analyses		
PQD-based Quantification	Peptide ratios	iTRAQ ratio	Average	Absolute error	Standard deviation
	114/115	1	1.01	0.02	0.31
	114/116	1	0.93	0.07	0.27
	114/117	1	1.13	0.13	0.34
ETD-based Quantification	101/102	1	0.54	0.47	0.24
	101/104	0.50	0.44	0.07	0.20