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New Algorithm for $^{15}N/^{14}N$ Quantitation with LC−ESI−MS Using an LTQ-FT Mass Spectrometer

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Abstract: A new algorithm (*QN*) for the $^{15}N/^{14}N$ quantitation of relative protein abundances in complex proteomic samples is described. *QN* takes advantage of the high resolution, mass accuracy and throughput of the hybrid mass spectrometer LTQ-FT MS. Peptide quantitation is based on MS peak intensity (measured in the FT MS), while peptide identification is performed in the MS/MS mode (measured in the LTQ linear ion trap). Accuracy of the protein abundance is enhanced by a novel scoring procedure, allowing filtering of less reliable measurements of peptide abundances. The performance of *QN* is illustrated in the relative quantitative analysis of *M. acetivorans* C2A cultures grown with carbon monoxide vs methanol as substrate. Roughly 1000 proteins were quantitated with an average CV of 9% for the protein abundance ratios. *QN* performs quantitation without manual intervention, does not require high processing power, and generates files compatible with the Guidelines for Proteomic Data Publication.

Keywords: quantitative proteomics • LC−MS • metabolic labeling • software • *M. acetivorans*

Introduction

The emergence of quantitative methodologies in mass spectrometry-based proteomics will have a significant impact on our understanding of biological systems.[1] A number of methods for determining the relative abundances of proteins between various samples have been introduced, using either labeled[2−9] or label-free approaches.[10−14] Although the label-free approaches can be applied to any samples, including human tissue, quantitation after metabolic labeling with stable isotopes, as can occur with cell culture studies, generally provides higher accuracy and reliability. Mixing such labeled cell cultures before lysis ensures identical treatment in all subsequent steps of purification, storage and LC−MS analysis, thus minimizing the influence of variations of experimental conditions and improving the precision of quantitation.[1] A well-established method for metabolic labeling is growing a cell culture in defined media, e.g., with isotope-enriched essential amino acids (SILAC) or salts such as ^{15}N ammonium chloride.[3,6]

Both types of quantitation, label-free and labeled, benefit significantly from the high mass resolution of modern mass spectrometers, e.g., TOF, FTICR, or Orbitrap, because such high resolution can reduce the likelihood of incorrect peak assignment. Specifically, with high mass accuracy: (i) MS peak overlap is decreased, (ii) the charge state of the ion can be easily determined, and (iii) correct identification is improved by the use of accurate precursor masses.

Correct peptide identification is obviously essential for accurate quantitation, and such identification should rely not only on the accurate mass, but also be confirmed by the LC retention time[15] and CID fragmentation. Unfortunately, the scan cycle of high resolution instruments, such as the FT−MS, when alternating between the MS and MS/MS modes, is relatively long, thus compromising the analysis. One solution to this problem is to select differentially abundant precursors quantitated in a series of LC−MS runs and then perform targeted MS/MS identifications of those precursors in a separate run and/or a separate instrument.[13] An improved solution can be obtained with the recently introduced hybrid mass spectrometer that combines the linear ion trap with a high resolution mass analyzer, such as the LTQ-FT MS. This new generation of instrumentation allows concurrent acquisition of high resolution MS spectra and information rich ion trap MS/MS fragmentation spectra at a speed compatible with nanoLC. Thus, both reliable peptide identification and accurate quantitation can potentially be achieved in a single LC run. Obviously, requirements for run-to-run retention time reproducibility are less severe when MS and MS/MS are performed in the same run.

Although quantitation can be performed manually, i.e., MS selection of an appropriate peak followed by software-assisted integration of the chromatographic peak area,[2] such an approach is feasible only if a limited number of proteins are to be quantitated. Several algorithms have been developed for automated high throughput quantitation;[4,5,9,10,14,16] however, using low mass resolution, MS spectra can often compromise quantitation for complex proteomic samples, especially for low S/N and coeluting peaks. High resolution MS spectra can

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greatly improve the reliability and precision of the automated quantitation even for low S/N peaks.

In this paper, we present a new algorithm *QN* ("quantitation of N-15/14") for the determination of relative abundances of proteins between two samples, one of which is metabolically labeled with ^{15}N and the other with ^{14}N. Compared to existing quantitation software,[4,5,16] the *QN* algorithm: (i) utilizes the high mass resolution and mass accuracy of LC−ESI−MS data acquired using the LTQ-FT MS, (ii) maximizes the number of proteins with accurate quantitation by a novel scoring procedure, (iii) operates without manual intervention, and (iv) is compatible with the Guidelines for Proteomic Data Publication.[17]

QN calculates the $^{15}N-^{14}N$ mass differences of all peptides identified by analyzing MS/MS spectra with Sequest, and finds and integrates corresponding peaks in the raw FT MS data. Similar to[16] the algorithm recognizes that the values of the observed $^{15}N/^{14}N$ ratios may vary among several peptides derived from the same protein; however, *QN* uses a novel method of scoring and selection of individual peptide ratios in order to filter out less reliable values that could result from incorrect peak assignments or from low S/N measurements. The implementation and performance of *QN* is demonstrated in the analysis of relative protein abundance in two *M. acetivorans* C2A cultures, one with carbon monoxide (CO) and the other with methanol as the growth substrate.

Experimental Section

Sample Preparation. *M. acetivorans* C2A (DSM 804) was grown in high-salt, minimal media, either under a headspace containing 100% CO or with 250 mM methanol in the media, as described in refs 18−20. In the methanol-grown cells, $^{14}NH_4Cl$ was substituted with $^{15}NH_4Cl$ (98%) (Sigma, St. Louis, MO). The amount of protein extracted from the cell pellets of CO− or methanol-grown cells was determined by the Bradford assay (Bio-Rad, Hercules, CA). The samples were then combined in a 1:1 mass ratio, separated by SDS-PAGE, and silver stained.[21] Finally, each SDS gel lane was cut into 10 bands of approximately similar density, as determined by visual inspection, and then each band was in-gel digested with trypsin, as described in ref 22.

NanoLC−MS Systems. Samples were analyzed using a nanoLC system (Ultimate, Dionex, Mountain View, CA) coupled to a hybrid linear ion trap FTICR MS (LTQ-FT MS, Thermo Electron, San Jose, CA). Approximately 4 μL (∼1.6 μg) of the concentrated peptide extract was loaded onto a 75 μm × 15 cm column packed with 3 μm Magic C18 particles (Michrom BioResources, Auburn, CA), followed by a 75 min linear gradient from 2% to 35% ACN (v/v) in 0.1% formic acid using a 250 nL/min flow rate. Two LC−MS runs were performed for each gel band. In the data acquisition cycle, each high-resolution FT−MS scan (accumulation of 2×10^6 ions) was followed by up to 10 MS/MS spectra in the linear ion trap (accumulation of 3×10^4 ions), with dynamic exclusion set at 1 min. Each data acquisition cycle was completed in approximately 3.8 s. It should be noted that all m/z values were corrected for a +4 ppm systematic mass shift with respect to the default calibration of the instrument.

Protein Identification. The data acquired from nanoLC−MS was searched against the NCBI database of *M. acetivorans* C2A (downloaded, June 2005) using Sequest.[23] The database search was performed twice, once using natural ^{14}N abundance and subsequently using modified amino acid masses corresponding to ^{15}N incorporation. The precursor ion mass tolerance was set to ±1.4 Da, and trypsin was assigned as the proteolytic enzyme with up to 2 missed cleavages. To minimize the rate of false positive identifications, multiple filtering criteria were used. Initially, PeptideProphet[5] was used to assign probabilities to all Sequest search results, and peptides with probabilities higher than 0.8 in either the ^{14}N or ^{15}N searches were selected. Using this subset, peptides identified with X_{corr} values (Sequest) greater than 1.5 (1+), 2.0 (2+), and 2.5 (3+) were considered. ProteinProphet[24] was used to assign probabilities to proteins, and peptides associated with proteins based on probabilities higher than 0.9 were selected. Finally, peptides with a precursor mass within ±15 ppm of the theoretical mass were accepted as correct identifications. Identification was followed by quantitation. Description of the quantitation algorithm is given in the Results and Discussion section.

Results and Discussion

Algorithm Description. The *QN* software was written to determine the ratios of protein abundances in a pair of samples, one of which was metabolically labeled with ^{15}N, and analyzed by LC−ESI−MS using the LTQ−FT−MS. The input data for the quantitation algorithm *QN* were as follows: (i) the raw LC−MS data file (MS peaks collected in the centroid mode) and (ii) a list of identified peptides, specifically, the INTERACT[5] file resulting from an MS/MS database search with Sequest and verified with PeptideProphet (Institute for Systems Biology, Seattle, WA).

QN consisted of 3 modules schematically shown in Figure 1. The first module converted the LTQ−FT−MS raw data set into a set of files of individual mass spectra. The second module assigned peaks from the mass spectrum to identified peptides. The module then searched for the second member of each possible $^{15}N/^{14}N$ peptide pair and, if found, determined the ratio of peak intensities, thus quantitating at the peptide level. The third module quantitated at the protein level by averaging the $^{15}N/^{14}N$ ratios of individual peptides that identified the same protein. Scoring the reliability of peptide abundance ratio measurements and filtering out of less reliable measurements were conducted prior to the averaging of individual peptide abundances for each protein. The first module was written in Visual Basic 6.0; the second and third modules in MATLAB. *QN* is freely available for download from www.barnett.neu.edu.

Peptide Quantitation Module. The peptide quantitation module read, in addition to the mass spectra, a list of peptides identified by Sequest and verified by PeptideProphet (INTERACT file). This file included (i) the molecular weight and amino acid sequence of each identified peptide, (ii) the name of the protein, (iii) the charge state of the peptide ion, (iv) the MS/MS scan number in which the peptide was identified, and (v) the probability of correct peptide identification, estimated by PeptideProphet.

For each identified peptide (based on the criteria specified in the Experimental Section), the program first calculated the mass difference between ^{15}N and ^{14}N labeled peptides by counting the number of nitrogens in the amino acid sequence. The listed m/z values, MS scan numbers and charge states were then used to assign the ^{15}N and ^{14}N labeled peptides to MS peaks in the spectra. Assignment started with selection of MS spectra acquired within a given retention time window (±30 s) from the MS/MS scan in which the peptide was identified. In the selected spectra, peaks were chosen with centroids

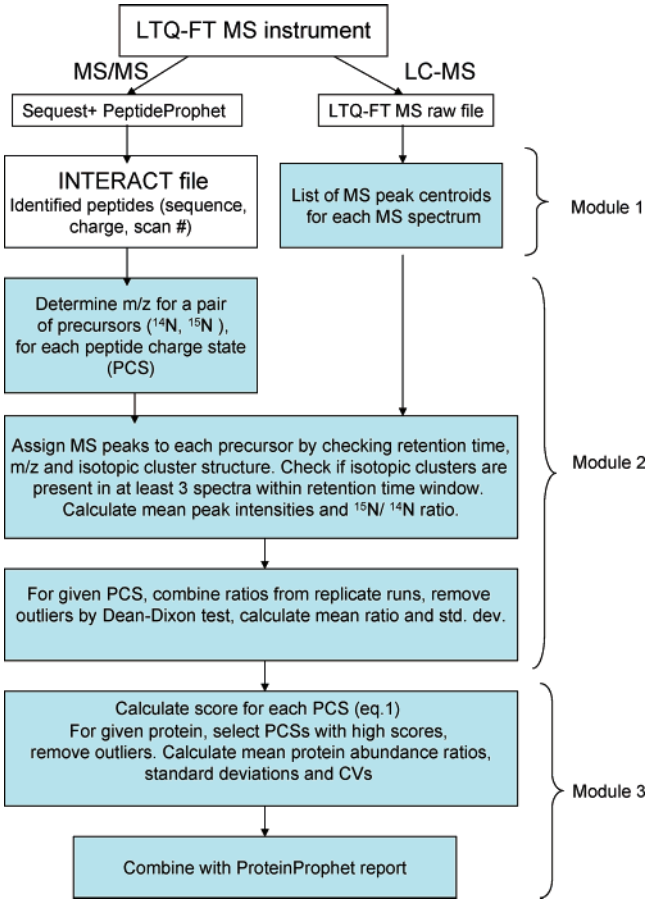

Figure 1. Workflow of the *QN* quantitation algorithm.

within a specified mass accuracy window (±10 ppm) of the m/z of the identified peptide. The correct assignment was confirmed by the presence of two or three isotopic peaks (depending on the molecular weight of peptide) in at least three MS spectra, all possessing a ratio of isotope peak intensities consistent (±30%) with the theoretical values. To calculate the ratio of abundances of ^{15}N and ^{14}N labeled peptides, the intensities of the monoisotopic peaks in these 3 or more spectra were averaged. Monoisotopic peaks were selected as a measure of abundance based on the high level (98% in our experiments) of ^{15}N enrichment and 100% level of incorporation. In the case of lower incorporation levels, an entire isotopic envelope should be used as a measure of abundance as described in ref 25.

The results of peptide quantitation for replicate runs (generated as described above) were then averaged. Different charge states of the same peptide were treated as separate entities, and hereinafter termed peptide charge states (PCSs). The abundance ratios (R_i) for each PCS in replicate analyses were examined for outliers by a Dean−Dixon test (confidence level 90%),[26] then the mean of the abundance ratios for each PCS was calculated.

Protein Quantitation Module. It was recognized that not all measurements of peptide abundance ratios were equally reliable. Errors in individual measurements can result from a number of factors including ion suppression, overlapping of LC−MS peaks and noise. It was assumed that high intensity peaks were measured with higher accuracy, and so such peaks were preferentially selected for quantitation of the parent protein. Similarly, PCSs with intensity ratios that were consistent across several replicates were assumed to be more reliable than PCSs with less reproducible ratios. These assumptions were incorporated in a score Sc_i that was calculated for each PCS as

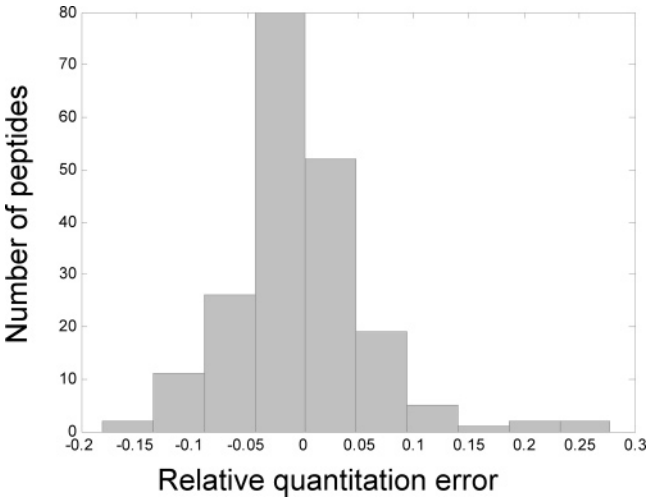

Figure 2. Distribution of relative quantitation error. Comparison of peptide intensity ratios as determined by *QN* and by manual inspection.

$$\mathrm{Sc}_i = \frac{\sqrt{I_{i,14} \cdot I_{i,15}} \cdot \sqrt[M]{\prod_{m=1}^{M} p_m}}{\mathrm{CV}_i^2} \quad (1)$$

where $\mathrm{CV}_i = \mathrm{std}(R_{i,m})/\mathrm{mean}(R_{i,m})$—the coefficient of variation of the abundance ratio R for the i'th PCS in the list; std − standard deviation; $I_{i,14}$and $I_{i,15}$—the mean peak intensities of the ith PCS labeled with ^{14}N and ^{15}N, respectively; p_m—the probability of identification estimated by PeptideProphet; and M—the number of times PCS(i) was identified in all replicate runs, i.e., the number of MS/MS sequencing events. When the given PCS was identified only once (M = 1), eq 1 was modified by substituting the value of CV = 1. Typical CV values were roughly 0.1−0.3, and thus assignment of CV = 1 for a single identification PCS resulted in a significant reduction of the score (~10 to 100 fold). The Sc_i score emphasized reproducibility of R_i measurements (CV_i^2), as well as peak intensity and the probability of correct identification. PCSs with scores lower than 1/100 of the top score for a given protein were excluded, with the exception that PCSs with scores above the threshold, i.e., 3 times the median score, were maintained on the list. After selecting PCSs based on scores, remaining outliers were removed by a Dean−Dixon test (confidence level 90%). It is important to emphasize that the Dean−Dixon test alone was not sufficient to remove outliers. If a protein was represented by two PCSs or two groups of PCSs with significantly different relative abundances, then the Dean−Dixon test would fail to determine which of the two PCSs or two groups of PCSs were correct. On the other hand, eq 1 allowed elimination of the less reliable PCSs with significantly lower scores. The approach differed from the weighted average, used in ref 16, by taking into account not only the CV, but also peak intensity and probability of identification. Scoring, based on eq 1, was important, especially in the case of a low number of measurements for a given PCS where CV may not be representative.

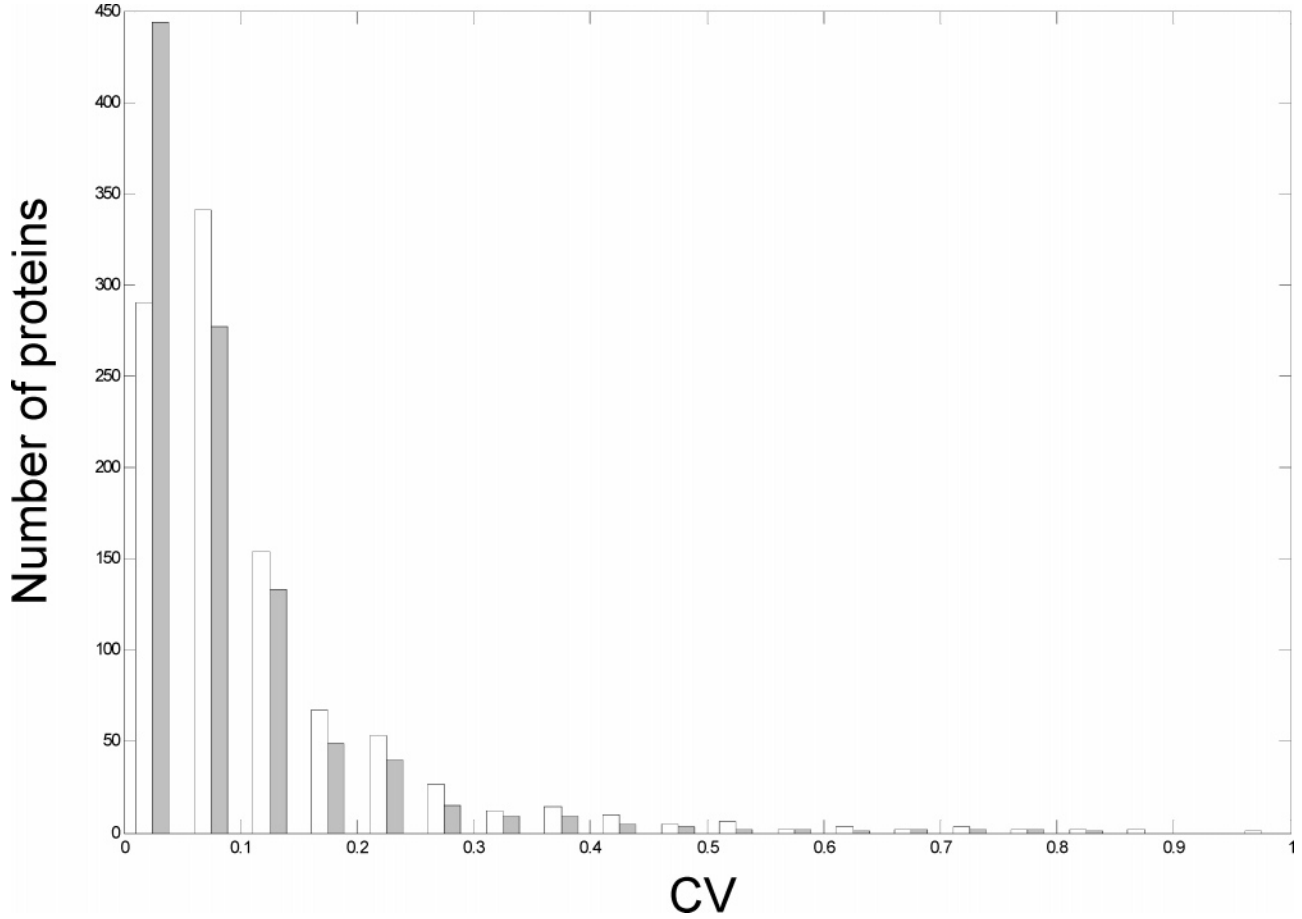

Figure 3. Distribution of the coefficient of variation calculated by *QN* for protein abundance ratios. Open bars: ratio calculated from all PCSs; Solid bars: ratio calculated after excluding PCSs with low score and applying Dean-Dixon test for outliers. The number of proteins with CV < 5% was increased by a factor of 1.5 due to scoring of PCSs and outlier removal, with the number of proteins with higher CV values being reduced. The total number of proteins before and after scoring and outlier removal (open bars vs solid bars) was constant.

The mean value of the PCSs for each identified protein was then calculated and included in the output file, together with the standard deviation and coefficient of variation. Additionally, the ProteinProphet tables were included in the output file, to facilitate comparison of quantitation performed by *QN* with measurements based on the number of MS/MS sequencing events ("spectral counting",[27,28] see later). Finally, to comply with the Guidelines for Proteomic Data Publication,[17] the output file included all information on the MS peaks used in quantitation: raw file name, scan number, molecular weight of the peptide, charge state, peptide sequence, protein name, m/z values and intensities of ^{14}N and ^{15}N labeled peptides, PeptideProphet probability, X_{corr} and ΔC_n parameters from the Sequest search, together with a listing of whether the peak intensity was used in the calculation of the protein abundance ratio or the reason it was not included (e.g., outlier, or low score).

Performance of the *QN* Algorithm and Software. The performance of *QN* was examined in the determination of the relative protein abundances of *M. acetivorans* C2A cultures, grown with different substrates, and analyzed as described in the Experimental Section. Specifically, the combined ^{15}N and ^{14}N lysates (equal amounts of total protein) were separated by SDS-PAGE, followed by cutting the gel lane into 10 bands for analysis. Two replicate (same sample injected twice) LC−MS/MS experiments (a total of 20 runs), each followed by separate sequential Sequest searches for ^{15}N and ^{14}N peptides, resulted in ~11 000 identifications of 4830 unique peptides associated with 1279 proteins. For more than 95% of the precursors, the mass accuracy was within ± 6 ppm, enabling reliable assignment of MS/MS identifications (reported in the INTERACT file) to peaks in the MS spectra. The complete procedure of quantitation of 1000 proteins, represented by 11 000 PCSs observed in 10 gel bands, was performed on a single PC (Pentium IV, 2 GHz) in about 3 h of CPU time.

Peptide Quantitation. The accuracy of peptide quantitation by *QN* was verified by examining 190 randomly chosen ^{15}N /^{14}N pairs. First, all peak assignments, observed by manual inspection of the MS spectra, were in agreement with the automated procedure. These 190 pairs of chromatographic peaks were then manually selected and integrated, using the software Xcalibur (Thermo Electron, San Jose, CA). Figure 2 shows the distribution of the relative differences between *QN* and manual quantitation, which hereinafter is termed the relative quantitation error. For 90% of the pairs, the quantitative results of the two methods agreed within ±10%, and for 65% the agreement was within ±5%. The largest relative errors of 28% and 25% were observed for two peptides with abundance ratios below 0.10 (0.075 and 0.080 from *QN* compared to 0.093 and 0.102 from Xcalibur, respectively). For both methods, quantitation of low-intensity peaks would be expected to be less accurate due to low S/N (near the level of intensity thresholding

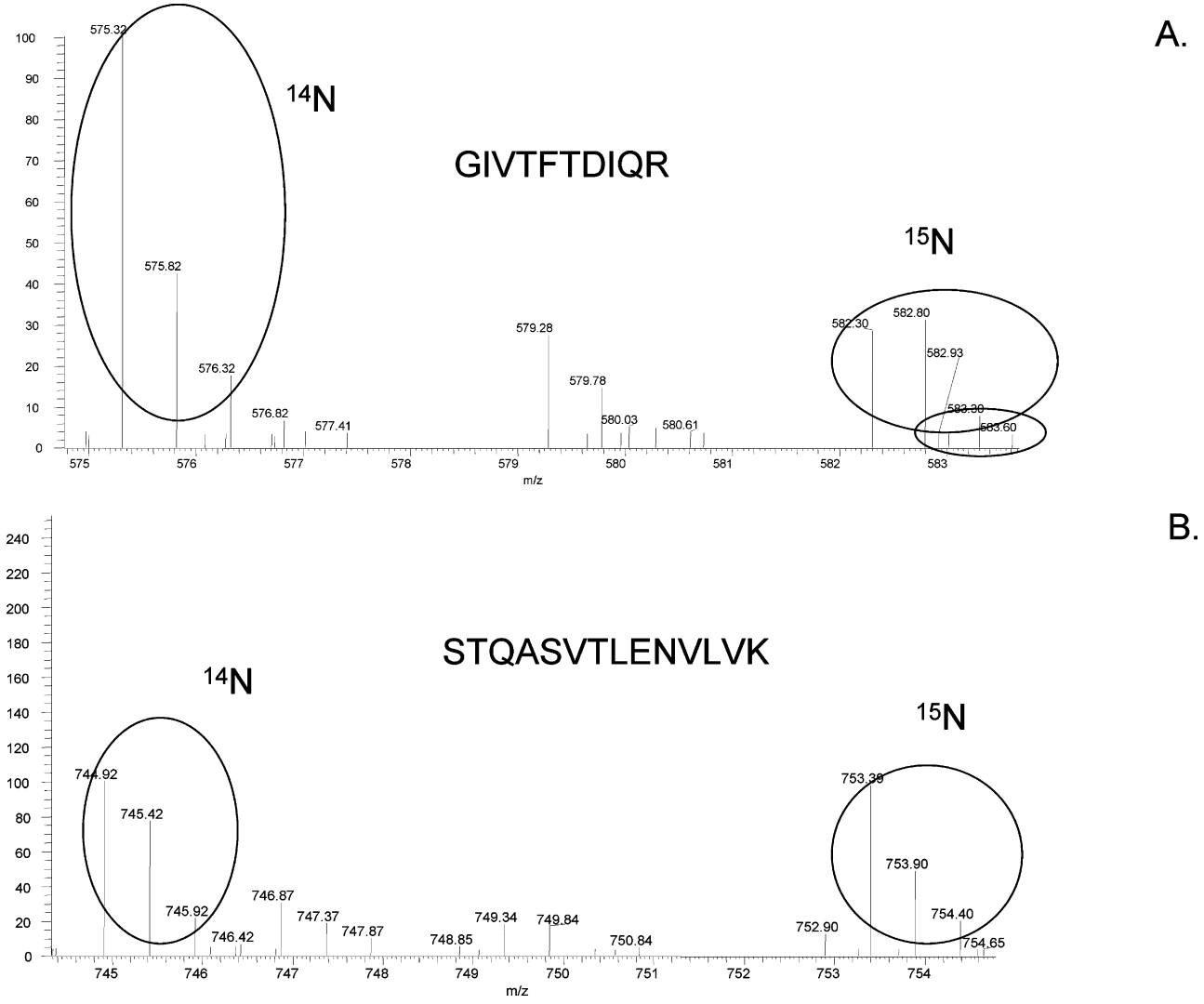

Figure 4. Example of a low score PCS that was excluded by *QN*. (A) MS spectrum (centroid) of the doubly charged peptide GIVTFTDIQR—a low score peptide excluded by *QN*. Note that the structure of the isotopic cluster of ^{15}N labeled peptide is incorrect. (B) MS spectrum of a doubly charged peptide STQASVTLENVLVK—a high score peptide used by *QN* for quantitation. Note that the relative abundances of the isotopic clusters of ^{14}N and ^{15}N labeled peptides are similar.

performed by the LTQ-FT MS instrument software). However, even in these worst cases, the difference between the two methods of quantitation was less than 30%. Importantly, peptide abundance quantitation was performed by *QN* without manual intervention, whereas quantitation with Xcalibur used manual selection, a time-consuming procedure. The complete results of *QN* and manual quantitation of abundance ratios are presented in Supporting Information Table 1.

Protein Quantitation. The protein quantitation results presented below were from 997 proteins identified by 2 or more MS/MS sequencing events. The remaining 282 single-peptide identifications were listed separately by the program (information that could be used with additional verification of MS/MS spectra), but are not further discussed in this paper.

Theoretically, all PCSs belonging to the same protein should have similar abundance ratios. However, variation in these ratios may occur due to ion suppression from the coeluting peptides, digestion efficiency differences, recovery differences of peptides during sample preparation and separation, protein modifications, etc. As described, the protein quantitation module used a novel scoring method to select the most reliably quantitated PCSs for determination of the protein abundance ratio. Removal of roughly 9% of the PCSs (4% due to low score and 5% as outliers) improved the precision of the resulting protein abundance ratios. Figure 3 compares the distribution of CVs with and without PCS selection. Importantly, the number of abundance ratios determined with high precision (CV < 5%) increased from 293 to 446 (from 29% to 45% of the total), while the number low precision ratios (CV > 30%) correspondingly decreased by a factor of 2 (from 7.6% to 3.9%), when PCS selection was implemented. Figure 4 presents an example of a low score PCS (double charged peptide GIVTFTDIQR) that was automatically filtered out due to the scoring procedure. It can be seen that the structure of the lower intensity isotopic cluster of the ^{15}N labeled peptide in Figure 4A does not follow the theoretical distribution. The protein abundance ratio changed by 25% due to removal of the low score PCS, thus improving the accuracy of the quantitation.

Overall protein quantitation by *QN* was verified by comparison with manual quantitation of a randomly selected group of 40 proteins (see Supporting Information Table 2). Manual quantitation (manual precursor selection) was performed using

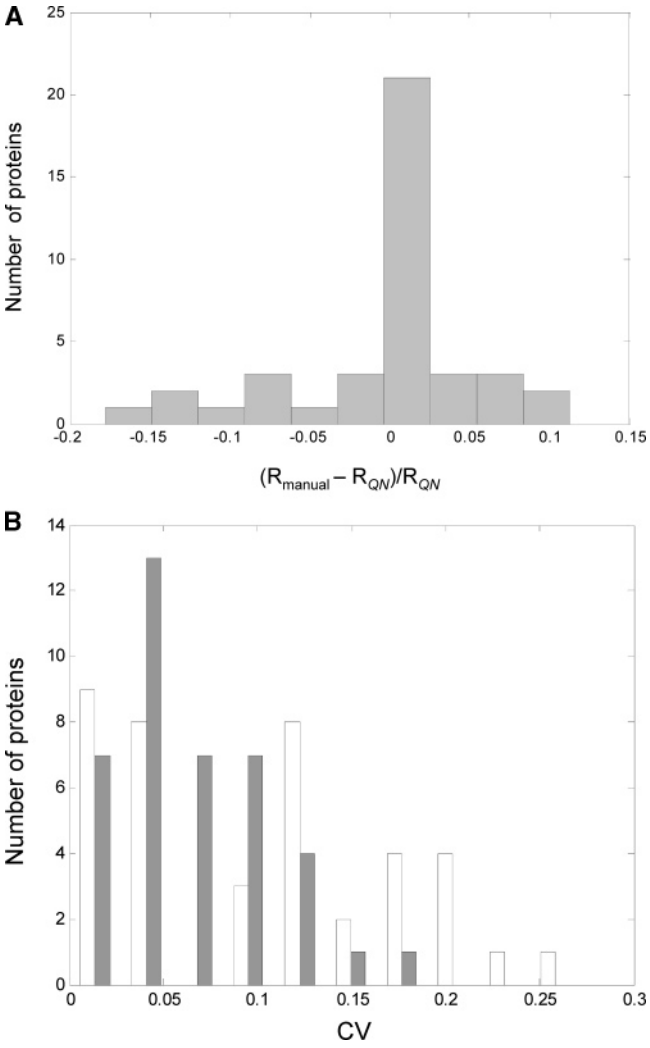

Figure 5. Comparison of the manual and *QN* quantitation of 40 randomly selected proteins. (A) Protein number distribution of the relative deviation between manual (R_{manual}) and *QN* quantitation (R_{QN}). (B) Protein number distribution of CV for protein quantitation. Open bars—manual. Solid bars—*QN*.

the 3 highest intensity PCSs for the given protein, while quantitation by *QN* utilized all available PCSs (average number of PCSs used for quantitation of a protein was 5, while the highest was 50). Figure 5A presents a histogram of the difference in relative protein abundance determined manually and by *QN*. For 68% of the verified proteins, the deviation was within ±5%, and for 88% within ±10%. Thus, good agreement between manual and *QN* quantitation of proteins was found. Figure 5B presents a comparison of CV distributions for manual and *QN* quantitation. The maximum CV in the case of manual quantitation was 27%, while with *QN*, the CV was significantly lower at 17%. Similarly, average CV values for manual and *QN* quantitation were 9.9% and 6.3%, respectively.

A semiquantative method for determining protein abundance ratios was introduced in ref 27. This method of "spectral counting" compares the number of precursors submitted for MS/MS analysis ("sequencing events") for each protein in a set of two or more samples. Since this method has been used as an estimate of relative protein abundance, it was also decided to compare results from this method with *QN*. Importantly, the number of proteins suitable for quantitation

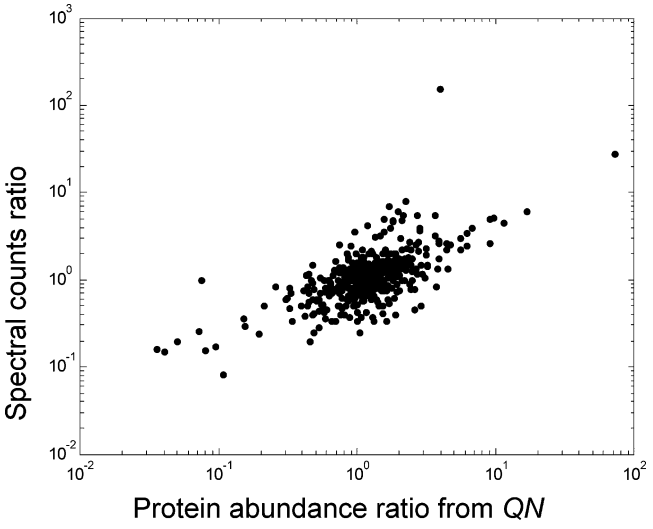

Figure 6. Comparison of protein abundance ratios determined by *QN* and by spectral counting as in ref 27.

by spectral counting (both ^{15}N and ^{14}N proteins being identified) was 498, half the proteins quantitated by *QN* (997). Figure 6 compares the abundance ratios for proteins determined by both methods, and good agreement between the two methods can be seen.

Supporting Information Table 3 presents a representative list of PCS pairs tabulated with their observed m/z, charge state, retention time, and a note whether the PCS was used for protein quantitation or why it was not. Supporting Information Table 4 presents a partial list of the proteins quantitated in this work, tabulated with their observed abundance ratios, standard deviations and CVs. A complete list of all proteins quantitated will be published separately, in a study of biological changes observed using different growth substrates.

Conclusion

In this paper, we have described a new algorithm, *QN*, for ^{15}N /^{14}N quantitation from LC−ESI−MS data acquired using a hybrid high-resolution LTQ-FT MS. *QN*, which does not require manual intervention at the data analysis stage, improves the accuracy and precision of quantitation by taking advantage of the high mass accuracy and resolution of the LTQ-FT MS instrument. A new procedure has been introduced for scoring the reliability of the ^{15}N /^{14}N ratios observed for individual peptides. The performance of *QN* has been illustrated in the comparative analysis of a pair of complex biological samples, *M. acetivorans* C2A cultures grown with CO and separately methanol as a growth substrate. Roughly 1000 proteins were quantitated based on the intensity ratios of two or more ^{15}N /^{14}N pairs of precursors. The average CV of the observed protein abundance ratios was 9%, and about 2/3 of all identified proteins were quantitated with a CV of less than 5%. The output files generated by *QN* contain all of the information required by the Guidelines for Proteomic Data Publication. It should be emphasized that *QN* can readily be modified to perform quantitative analysis using other high-resolution instruments, e.g., LTQ-Orbitrap MS and Q-TOF MS, and other stable isotope labeling methods cICAT, SILAC, or ^{18}O /^{16}O. The source code for *QN* is available for download from the authors' web site (www.barnett.neu.edu).

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Supporting Information Available: Comparison of *QN* and manual quantitation of abundance ratios (Supporting Information Table 1). Overall protein quantitation by *QN* verified by comparison with manual quantitation of a randomly selected group of 40 proteins (Supporting Information Table 2). Representative list of PCS pairs tabulated with their observed m/z, charge state, retention time, and a note whether the PCS was used for protein quantitation or why it was not (Supporting Information Table 3). Partial list of the proteins quantitated in this work, tabulated with their observed abundance ratios, standard deviations, and CVs (Supporting Information Table 4). This material is available free of charge via the Internet at http://pubs.acs.org.

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