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Comparison of Database Search Strategies for High Precursor Mass Accuracy MS/MS Data

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Abstract

In shotgun proteomics, the analysis of tandem mass spectrometry data from peptides can benefit greatly from high mass accuracy measurements. In this study we have evaluated two database search strategies which use high mass accuracy measurements of the peptide precursor ion. Our results indicate that peptide identifications are improved when spectra are searched with a wide mass tolerance window and precursor mass is used as a filter to discard incorrect matches. Database searches with a peptide dataset constrained to peptides within a narrow mass window resulted in fewer peptide identifications but a significantly faster database search time.

Keywords

Proteomics; Database search; Precursor estimation; Mass accuracy

Introduction

Shotgun proteomics is a method of identifying proteins in a complex mixture by digesting a protein mixture to peptides prior to analysis. In a typical shotgun proteomics experiment, the protein sample of interest is enzymatically digested to peptides, separated by microcapillary liquid chromatography (μ LC), and introduced into the mass spectrometer using electrospray ionization. In the mass spectrometer, these ions are observed by the acquisition of a full scan over a wide m/z range. Selected signals detected in the full scan are automatically selected for isolation and fragmentation to generate tandem mass spectra (MS/MS spectra). The resulting MS/MS spectra are then searched against a protein sequence database using a database searching algorithm to produce a peptide spectrum match (PSM)^{1–4}.

High resolution mass spectrometers such as hybrid LTQ-FT or LTQ-Orbitrap combine the strengths of a Fourier transform mass analyzer with a linear ion trap mass spectrometer. These hybrid instruments are capable of recording mass spectra in high resolution while simultaneously acquiring MS/MS spectra at a lower resolution in the linear ion trap. Data acquisition in this manner can be used to acquire the precursor mass of the peptide at high resolution and high mass accuracy while maintaining the speed and sensitivity of the linear ion trap for acquisition of MS/MS spectra⁵.

By acquiring the survey mass spectrum in the Fourier transform mass analyzer of the hybrid mass spectrometer, the high mass accuracy measurements of the peptide precursor can be used to improve peptide database search results. However before using the Fourier transform mass spectrum to improve the analysis of the low resolution MS/MS spectrum several technical

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hurdles need to be overcome. First, the high resolution Fourier transform mass spectrum must be deisotoped to obtain the monoisotopic mass and the charge state for all the isotope distributions in the spectrum. Several algorithms have been developed that are capable of determining the monoisotopic mass and charge state from complicated high resolution mass spectra^{6–9}. Secondly, the correct assignment of the resulting monoisotopic mass and charge state must be made to MS/MS spectra. Because of the wide fragmentation window used for MS/MS on these instruments, it is not uncommon to have multiple isotope distributions and, thus, multiple peptide precursors within the fragmentation window. Additionally, matching correct monoisotopic masses to MS/MS spectra is complicated because peptides are often selected for fragmentation prior to the apex of their elution. The isotope distribution measured in a spectrum prior to the actual chromatographic peak may not be intense enough to confidently determine the monoisotopic mass.

The onboard computer of the LTQ-FT and LTQ-Orbitrap attempts to deisotope and assign monoisotopic masses to each precursor selected for MS/MS. Monoisotopic masses are determined by the instrument firmware performing a fast Fourier transform on a partially acquired transient. The short transient allows the instrument to determine which ions to isolate for data-dependent MS/MS and acquire the MS/MS spectra in the linear ion trap while acquiring the remaining transient in parallel. Using the monoisotopic mass values determined from the short transient requires no post-acquisition computation time beyond reading the appropriate value from the RAW data files. However, because only a short transient is used, the resolution is lower and mass measurements may not be as accurate, especially for those ions of low intensity. A software tool from Thermo Scientific, *extract_msn*, uses complete MS full scans to determine monoisotopic masses which results in more accurate measurements. A shortcoming of *extract_msn* is that only the MS scan preceding the fragmentation event is used to determine the precursor mass.

Recently, several tools have been developed that process high resolution data and assign monoisotopic mass information to MS/MS spectra^{10–13}. These tools address the challenges of correctly deisotoping Fourier transform mass spectra and assigning precursor masses to the low resolution peptide MS/MS spectra. Previous works investigating peptide database search improvements with high accuracy mass measurements have focused primarily on the improvements obtained when narrowing mass tolerance windows^{10, 12, 13}.

Beausoleil *et al.*¹⁴ noted an advantage of searching with wider mass windows to increase the number of correct PSMs. In this work we have expanded on this observation and we present results from the evaluation of two different database search methods to determine the optimal approach for the use of high mass accuracy. The first method limits the precursor mass in the database search to only those that are within a very narrow mass window surrounding precursor monoisotopic mass, as has been shown previously. The second method uses a wide mass tolerance in the database search and then uses the accurate mass as a post-filter to discard all matches that possess a mass difference that is outside a narrow mass window. We have also examined the potential database search speed increases obtained from reducing the number of peptides searched. Our results indicate that the greatest sensitivity can be obtained by using the accurate mass as a post-search filtering parameter as opposed to a constraint during the database search. A consequence of maximizing peptide identifications with post-search mass filtering is that none of the benefits of reduced database search times are obtained.

Materials and Methods

Sample Preparation

The yeast *Saccharomyces cerevisiae* strain S228C was grown in 5 ml YPD (2% dextrose, 2% peptone, 1% yeast extract) overnight. Yeast cells were harvested by centrifugation (1,500 × g,

5 min) and resuspended in 0.1% Rapigest (Waters) and 50 mM Ammonium bicarbonate pH 8. Cells were lysed with 0.5 mm glass beads by vortexing for 2 minutes. Cell lysate was centrifuged at $20,000 \times g$ for 10 minutes. The supernatant was removed and the protein concentration was determined by a BCA protein assay (Pierce). The protein from the supernatant was treated with 5 mM DTT for 30 minutes at 60 °C to reduce disulfide bonds. Free sulfhydryl bonds were alkylated with 15 mM iodoacetamide (IAA) for 30 minutes at room temperature. A total of 50 µg of protein was digested with 0.5 µg trypsin for 1 hr at 37 °C. The digestion was quenched and the Rapigest hydrolyzed by the addition of 5 M HCl to a final concentration of 50 mM and incubating the sample at 37 °C for 45 min.

All reagents were obtained from Sigma-Aldrich or Fisher Scientific unless noted otherwise.

LC-MS/MS Analysis

The HPLC system used was an Agilent 1100 Quaternary Pump with a vented flow system as described¹⁵, to achieve an approximate flow rate of 200 nl/min. 2 µg of peptides were separated over a homemade 40 cm fused silica capillary (75 µm inner diameter) column packed with Jupiter Proteo (Phenomenex) reverse phase resin. The mobile phase gradient used was a 200 minute linear gradient from 95:5 Buffer A:Buffer B to 68:32 Buffer A:Buffer B. Buffer A consisted of 5% acetonitrile and 0.1% formic acid. Buffer B consisted of 80% acetonitrile and 0.1% formic acid. The mass spectrometer used was a Thermo Scientific LTQ-FT Ultra. Spectra were acquired using a cycle of one MS scan (400 – 1,400 m/z, 50,000 FWHM at m/z 400, profile mode, automatic gain control target of 2×10^6 , maximum ion time of 1 second) in the FTICR followed by five data-dependent MS/MS spectra of the most intense peaks in the LTQ ion trap. The dynamic exclusion feature was activated with an exclusion window of 1 m/z below the peak and 2 m/z above the peak for 30 seconds.

Database Searching and Analysis

MS/MS spectra were searched using SEQUEST³ against a protein fasta database downloaded from the *Saccharomyces* Genome Database. The search was performed with no enzyme specificity and a static modification of 57.02146 Da applied to cysteine to account for carbamidomethyl modifications. For low resolution database searches, spectra were searched at charge states of +1 or +2 and +3, using a ± 3 Da mass tolerance window around the precursor ion mass. The SEQUEST parameters were adjusted to use average mass for the precursor and monoisotopic mass was used for the product ions.

To obtain high accuracy precursor mass assignments, MS/MS data was processed with an in-house developed software program called *Bullseye*, described in Supplemental Materials. For database searching of this dataset, search settings were modified to use monoisotopic mass and mass tolerance windows of 5, 10, 50, 200, or 1,000 ppm.

For the filtering of search results by mass, database searching was done as described above. In the results file, for each spectrum, the mass deviation from the measured monoisotopic mass and the theoretical monoisotopic mass of the top ranked peptide match was calculated. If the mass deviation was greater than 5 ppm, the XCorr score for the peptide match was assigned a value of 0. Filtering and rescoring was done by an in-house software script.

The false discovery rate (FDR) was calculated as described previously¹⁶. The FDR is defined as the ratio of the number of decoy peptide-spectrum matches (PSMs) greater than a given XCorr divided by the target peptide-spectrum matches greater than the same XCorr. To generate the decoy database, amino acid sequences for each protein in the target database were shuffled. Target and decoy database searches were done separately. The FDR was calculated

independently for each charge state and the number of positive PSMs in each FDR bin was combined afterwards.

Results

Comparison of two search methods using accurate precursor mass

Research efforts in shotgun proteomics have focused on maximizing the number of positive peptide identifications while minimizing the false discovery rate. With the availability of mass spectrometers with high mass measurement accuracy, we were interested in determining the most effective way to use this information to discriminate between true and false positives. The first search strategy tested was the restriction of the mass search tolerance to a narrow ppm window (Figure 1A). By restricting the mass tolerance window, the number of candidate peptides that were scored against an MS/MS spectrum was reduced. As a result, fewer incorrect peptides were considered by SEQUEST's scoring algorithms because their precursor mass was outside the tolerance window. This decreased the chances of an incorrect peptide scoring higher than a correct peptide and reduced the FDR for a given number of positive peptide identifications.

The second search strategy we examined was the use of mass accuracy as a feature to discriminate between true and false positives post-search (Figure 1B). For this method, MS/MS spectra were searched using a wide mass tolerance window. The peptide results were analyzed to determine the mass difference between the observed monoisotopic mass and the theoretical mass of the highest scoring peptide. Peptide hits with mass differences outside a narrow mass tolerance window were deemed incorrect. We used XCorr values as our scoring metric, so to indicate a PSM was incorrect, its XCorr value was rescored to 0.

The dataset used was acquired as described in *Materials and Methods*. This dataset was processed with an in house developed software program called *Bullseye*. *Bullseye* uses full scan MS spectra to identify an accurate precursor monoisotopic mass for MS/MS spectra. Precursor mass assignments done by *Bullseye* are more accurate than the mass assignments by the instrument data system. A more detailed discussion of the *Bullseye* software program can be found in Supplemental Materials.

For the first database search method, searches were done with mass tolerance windows of ± 5 , 10, 50, 200 and 1,000 ppm. Figure 2A shows a plot of the positive peptide spectrum matches versus false discovery rate for ± 5 , 10, 50, 200 and 1,000 ppm windows. For comparison, the search results of an MS/MS dataset with the mass from the center of the precursor isolation window assigned as the precursor mass value was included ("fragment-precursor"). This dataset was analogous to data that would be acquired on a lower resolution instrument where the monoisotopic mass of the isotopic distribution cannot be resolved and the center of the isolation window was assigned as the precursor mass. Because the monoisotopic mass is ambiguous, this data was searched with a mass tolerance window of ± 3 Da.

As shown in Figure 2A, narrowing the mass tolerance window around an accurately assigned precursor mass increased significantly the number of positive PSMs. A database search using a ± 3 Da mass tolerance on the fragmentation-precursor mass values yielded 864 PSMs at a 0.001 FDR. When narrowing the mass tolerance to $\pm 1,000$ ppm around a *Bullseye* determined precursor mass, 1,278 PSMs were identified. Further narrowing the mass tolerance to ± 5 ppm increased the PSMs to 1,963, an increase of 54% over the $\pm 1,000$ ppm search. At a 5 ppm mass tolerance 94.9% of PSMs were fully tryptic peptide fragments, 5% were semi-tryptic and 0.1% were non-tryptic.

For post-search mass accuracy filtering, the dataset was searched with SEQUEST using mass tolerances of ± 10 , 50, 200 or 1,000 ppm. The search results were then filtered and rescored as described in *Materials and Methods*. A post-search mass filter of ± 5 ppm was used because it produced the best results with narrow mass window database searches. A plot of the positive PSM versus FDR for a segment of the results from this search method is shown in Figure 2B. For comparison, the search results without mass difference filtering are also included.

As the initial search tolerance increased, the number of positive PSMs after mass filtering increased. At a FDR of 0.001, 1,965 positive PSMs were found with the 10 ppm search window. With a search window of 1,000 ppm, 2,048 positive PSMs were found, a 4% increase. The increase in PSMs was more pronounced at higher FDR thresholds. At a FDR of 0.05, the 10 ppm search found 3,201 positive PSMs while at the 1,000 ppm search identified 3,845 positive PSMs, an increase of 20%. In all cases, mass difference filtering resulted in increased positive PSMs compared to database search results without filtering.

Database search speed improvements

With the large amount of data that can be generated in a proteomics experiment, a bottleneck in most data analysis pipelines is the amount of computational time needed to process the data. Two factors that can affect database search times are the number of spectra that need to be searched and the number of peptides that are scored per spectrum. As the precursor mass tolerance for database searching is lowered in the above search methods, the number of peptides that are within the mass window decreases.

To assess the performance of different precursor mass tolerances, we measured the average time needed for each database search to determine if any significant reduction in search time is obtained (Table 1). The fragment-precursor dataset was searched with a large precursor mass tolerance window of ± 3 Da and, as expected, this search had the longest execution time of 154.62 minutes. The high number of spectra searched was because of the inability to discriminate between charge states at low resolution -- resulting in each MS/MS spectrum having to be searched multiple times at different charge states. In contrast, searches performed using the monoisotopic mass from either the RAW file header or *Bullseye*, were searched only at their determined charge-states -- significantly reducing the number of searches performed on each spectrum.

When the mass tolerance window was narrowed in the *Bullseye* dataset searches, a dramatic improvement in search time was observed. The search with a wide mass tolerance of $\pm 1,000$ ppm was completed in an average of 74.94 minutes. Search times gradually decreased as the mass tolerance is decreased. At ± 5 ppm, the search processing time averaged 7.16 minutes. The time required for post search mass filtering and XCorr rescoring was negligible.

Discussion

Software tools have been developed recently that are capable of assigning precursor monoisotopic masses to fragmentation spectra at greater accuracy than what has been available previously. Because these tools operate on data post-acquisition, they have access to data on the profile of an isotope cluster over the course of a peptide's chromatographic elution. The additional data points increase the number of isotope distributions that can be identified, the confidence that the isotope distributions detected belong to peptides and improve the accuracy of precursor mass assignments.

In this work, we have compared two different approaches for using high mass accuracy precursor ion information. The two search methods were either to constrain the database search

to a narrow window around the monoisotopic precursor mass or to perform the database search with a wide window first and then to filter the data based on mass error, post-search.

Our results demonstrate that post-search filtering is superior to constrained database searches in obtaining more positive peptide spectrum matches. We hypothesize that in post-search filtering, in a wide mass tolerance search, incorrect PSMs are more likely to be matched to peptides with mass differences distributed throughout the search window while correct PSMs will possess mass differences within the narrow mass filter window. During filtering, PSMs outside the filter window are removed and by removing a greater number of incorrect PSMs than correct PSMs, sensitivity is improved. This is in contrast to a constrained database search strategy where the search space is reduced. In this case, sensitivity is improved because with a smaller search space, incorrect PSMs have a lower chance of matching to a peptide with a high score, while the higher scoring peptides from correct PSMs will remain in the search space.

We observed during post-search filtering that the improvement in peptide identifications is dependent on the FDR limit and the width of the search window. At each FDR tested, there was a threshold where increasing the search width no longer resulted in increased positive PSMs. In our dataset, when peptide results were limited to a FDR of 0.001, the improvement in peptide IDs with post-search mass filtering reached a maximum at a search window of ± 200 ppm. When limiting peptide results to a FDR of 0.01, the search window at which the number of peptide IDs reached a maximum is at ± 600 ppm. At a FDR limit of 0.05, the maximum amount of positive IDs was obtained at a search window of ± 900 ppm (Figure 3). With increasing search windows, eventually a greater number of PSMs from the target search are filtered out than from the decoy search and the number of positive PSMs will begin to decrease.

Post-search filtering offered an improvement in the number of positive PSMs that could be made, however there were two notable advantages to database searches that were constrained to narrow mass tolerances. First, mass constrained database searches had the advantage of greatly reducing the computational time to perform the search. Second, constrained database searches allowed for the search of spectra with multiple precursor masses that differed by only a few ppm. Because of the unique advantages of each method, the preferred method to use should be determined by the availability of computational resources and the complexity of the sample being analyzed.

Mass accuracy represents only one discriminating feature that allows for distinguishing correct and incorrect PSMs. Other features such as enzyme cleavage specificity¹⁷ and predicted retention times^{18–20} can be used to discriminate incorrect PSMs and when used in conjunction with mass accuracy should result in the best peptide identification results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Illustrations of two peptide database search methods using accurate precursor mass measurements. A) The peptide list used for database searching is constrained to a narrow mass tolerance window. B) The mass tolerance used to limit the peptide list, is set to a large value. After database searching, peptide results that have a mass difference greater than a narrow mass tolerance window are given an XCorr value of 0.

Figure 2.

Results of different search methods on peptide spectrum matches. The number of peptide spectrum matches from the target database search are plotted versus false discovery rates. False discovery rates are calculated as described in *Materials and Methods*. A) The results from narrowing the mass tolerance for peptides used in database searching. B) The results for the database search of the *Bullseye* dataset with wide mass tolerances followed by filtering the results with a narrow mass tolerance window.

Figure 3.

The number of peptide spectrum matches obtained from database searches using mass tolerances from 5 ppm to 1,300 ppm are shown at an FDR thresholds of 0.001, 0.01 and 0.05. The sensitivity improvement obtained when post-search filtering is used is dependent on the FDR threshold and the mass tolerance used during database searches.

Table 1

Database Search Results.

Spectra Set	Database Search Mass Tolerance (precursor mass type)	Positive PSM (0.001 FDR)	Positive PSM (0.01 FDR)	Positive PSM (0.05 FDR)	Number of Spectra Searched ^a	Average Number of Peptides Searched per Spectrum ^a	Search Time (min) ^{ab}
Fragment-precursor	± 3 Da (Average)	864	1440	2040	32003	157054	154.62
Bullseye Matched	± 5 ppm (Monoisotopic)	1963	2543	3093	14774	1563	7.16
Bullseye Matched	± 10 ppm, (Monoisotopic)	1898	2463	2978	14774	3032	8.61
Bullseye Matched	± 50 ppm, (Monoisotopic)	1665	2111	2587	14774	13788	15.01
Bullseye Matched	± 200 ppm, (Monoisotopic)	1600	2057	2492	14774	25716	22.25
Bullseye Matched	± 1000 ppm, (Monoisotopic)	1278	1671	2202	14774	90821	74.94
Bullseye Matched	± 10 ppm, (Monoisotopic). 5 ppm filtered	1965	2545	3201	14774	3032	8.61
Bullseye Matched	± 50 ppm, (Monoisotopic). 5 ppm filtered	2019	2680	3380	14774	13788	15.01
Bullseye Matched	± 200 ppm, (Monoisotopic). 5 ppm filtered	2054	2712	3471	14774	25716	22.25
Bullseye Matched	± 1000 ppm, (Monoisotopic). 5 ppm filtered	2048	2825	3845	14774	90821	74.94

^a Results from target search.

^b Average of three replicate searches. SEQUEST processes were distributed to 4 cores of an AMD 64-bit 2.5 Ghz processor.