Computational Principles of Determining and Improving Mass Precision and Accuracy for Proteome Measurements in an Orbitrap

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Precision proteomics requires high-resolution and high mass accuracy peptide measurements. The Orbitrap instrument achieves excellent resolution on a chromatographic time scale and its design is favorable for very high mass accuracy. Here we describe how mass precision for each peptide increases successively by considering all associated measurements, starting from the MS peak and proceeding to its chromatographic elution profile, isotope envelope, and stable isotope pair in SILAC measurements. We extract peptide charge pairs to perform nonlinear recalibration of the Orbitrap mass scale through spline interpolation. The deviation of mass values determined from charge pairs is used to convert mass precision to mass accuracy for subsequent database search. The corrected mass precision is consistent with the mass accuracy independently determined by database identification. Individual mass deviations range from below 100 ppb for peptides with many associated mass measurements and good signal intensities to low ppm for peptides with few mass measurements and signals close to the noise level. This extremely high and individualized mass accuracy is equivalent to a substantial increase in database identification score. (J Am Soc Mass Spectrom 2009, 20, 1477–1485) © 2009 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

Mass spectrometry-based proteomics has become a widely used technology in biologic research. In the most common format, mixtures of proteins ranging from simple complexes to the entire protein complement of the cell are digested with a sequence specific protease and are analyzed by liquid chromatography tandem mass spectrometry [1]. The introduction of high-resolution hybrid mass spectrometers several years ago is part of an ongoing trend towards "precision proteomics" [2]. This has been a great advance for the field, because they allow accurate determination of peptide masses and accurate quantitation by label-free or labeling-based methods, such as ICAT [3], iTRAQ [4], or SILAC [5]. The LTQ-Orbitrap, in particular, has proven to be a very popular instrument in proteomics research, as it combines high-resolution, high mass accuracy, and high sensitivity in a compact and robust instrument. The high-resolution part of the instrument consists of an Orbitrap analyzer, which is a new type of mass spectrometer developed by Alexander Makarov [6, 7]. The Orbitrap is inherently capable of very high mass accuracy because the axial motion of ions along the central spindle only depends on the mass to charge ratio of the ion and not on initial conditions during injection. Furthermore, the central spindle shields ions of the same *m/z* from each other because they orbit the spindle. The mass accuracy achievable with the Orbitrap has been addressed before by Makarov and coworkers, especially with regards to the influence of signal intensity and dynamic range [8]. They found that low parts per million (ppm) mass accuracies could be obtained over at least three orders of magnitude of signal intensity. The LTQ-Orbitrap allows storage of ions in the C-trap used to inject ions into the Orbitrap, which is located between the linear ion trap and the Orbitrap. A few thousand ions of a lock mass (i.e., a readily ionizable background ion from laboratory air) can be stored in this C-trap and injected together with the analyte ions [9]. This procedure removes systematic mass shifts in real time by providing an internal mass calibrant.

The above mentioned studies were mainly concerned with fundamental properties of the ion trap using chemical standards. In proteomics experiments, masses of hundreds of thousands of peptides with different signal intensities need to be determined in very complex mixtures. These precursor masses are then used as constraint for database searches with the tandem mass spectra. Thus, in proteomics experiments, the mass accuracy is an extremely important parameter, whose incorrect determination can lead either to identification statistics that are worse than they need to be (if the mass accuracy window is set too large) or to missed identification (false negatives) if the window is set too narrow. Indeed, as we have argued before, the very concept of mass accuracy in proteomics has not been

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well defined, and various definitions of the term have been used [10]. For example, the precision of particular measurements—often the root mean square of repeated measurements of standards—has been claimed as the accuracy, even though it only represents the precision (repeatability) rather than the accuracy (deviation from the true value) of the measurement. Conversely, proteomics researchers typically set the maximum allowed mass deviation (MMD) very wide to capture identifications at very low intensity, which nevertheless lead to successfully fragmented peptides. This "lowest common denominator" strategy has the unfortunate effect of "in silico" degrading the mass accuracy that the instrument is capable of.

We have recently described a set of computational algorithms, termed MaxQuant, for the analysis of shotgun proteomics data [11]. It deals with the workflow of computational proteomics from peak finding to protein quantitation, and also directly addresses the issue of peptide mass accuracy. In particular, it introduced the concept of individualized mass accuracies for each peptide based on the determination of peptide mass precisions from repeat measurements, and the conversion from these precision values to the mass accuracies to be used in database searches. Here we expand on the principles developed in the MaxQuant algorithms, quantify the increase in the number of mass measurement values obtained by considering whole elution profiles, isotope clusters, and SILAC partners, and further improve our algorithm for nonlinear recalibration of the mass scale. We show that the corrected mass precisions determined from consideration of charge pairs are in fact the mass accuracies obtainable in the Orbitrap instrument, and explain how they should be used in database searches. Importantly, our approach relies entirely on algorithms applied to the data that are acquired in a standard manner—no internal mass standards need to be added to the sample.

Experimental

Methods

Mass spectrometric data. We used an existing dataset from our laboratory [12], which was also analyzed in two recent computational studies [11, 13]. It was obtained from SILAC-labeled [14] HeLa cells, one population of which had been stimulated with growth factor. We performed isoelectric separation of peptides on the OFFGEL apparatus as described [15] in triplicate and analyzed the fractions with nanoLC-MS on an LTQ Orbitrap mass spectrometer. MS scans were acquired with high-resolution (60,000 at *m/z* 400), and MS/MS spectra were obtained at low-resolution as centroid data in linear ion trap mode. The lock mass option was used for all survey scans [9]. MS/MS spectra were filtered by always retaining only the six most intense peaks in each 100 Th interval, and fragment ions were matched with 0.5 Th mass tolerance. Processing of the 72 raw files with our MaxQuant software [11] leads to 461,336 identified MS/MS spectra in the human IPI database at a 1% false discovery rate (FDR).

Computational methods. Data were analyzed with the MaxQuant framework [11], which is written in C# in the Microsoft .NET environment. Algorithmic parts of MaxQuant are available as source code, and the entire program can be freely downloaded as well from www.maxquant.org. Detailed instructions about installation and support programs are also available [16]. The cubic spline function used for parametrizing the nonlinear recalibration of the mass scale was chosen to have one support point per 25 charge pairs that contribute to its determination. The parameters of the spline, including the boundary conditions, are then determined by a fit in the same way as it is done for the polynomial in [11].

Results

Extracting the Maximum Number of Mass Measurements per Peptide

Until recently, the precursor mass in LC MS/MS experiments was estimated solely from the spectrum used by the instrument software to pick the peptide for sequencing. This is illustrated in Figure 1a, the MS/MS spectrum of an example peptide from our dataset, and Figure 1b, the precursor peak for the fragment spectrum. The vertical blue and pink stripes in Figure 1b are one ppm in width. Note that the actual measurement values for the peak (red dots) are about five ppm apart. This immediately suggests that determination of the centroid of the peak is likely to be a major source of experimental mass error. In MaxQuant, the top data points of each peak are fitted with a Gaussian distribution, and the center of this Gaussian distribution is the mass estimate of the peak from this scan.

One problem of estimating the precursor mass only from the ion isotopic peak in a mass spectrum used for subsequent fragmentation is visualized in Figure 1c and d. The peak was picked for sequencing at the start of its chromatographic elution profile (red arrows in the contour plot and 3D plot of the eluting peptide peak), where its intensity was about a tenth of that at the elution peak, leading to suboptimal mass determination and fragmentation. The centroid determined by MaxQuant over the eluting peak is marked by a blue line in Figure 1c. In this case, there are 38 separate values, which are each weighted by intensity and combined for the mass estimate. In the entire dataset, more than 27 million LC-MS isotope peaks were recognized, reducing to 2.7 million isotope patterns and almost 500,000 SILAC pairs. Figure 2a shows that isotope peaks contained in SILAC pairs each contain about 10 mass measurements on average. This value agrees well with a chromatographic half width of about 20 s and a cycle time of

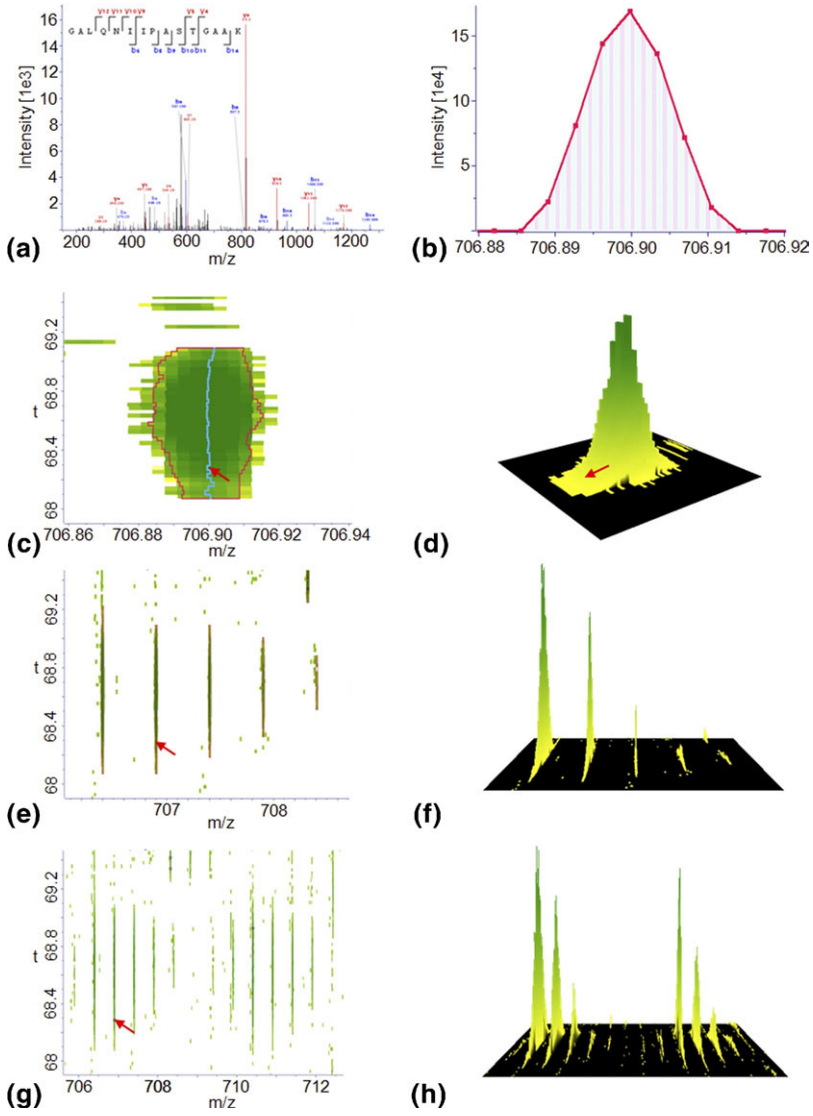

Figure 1. Successive increase of available mass information. (**a**) Annotated MS/MS spectrum leading to the identification of the unmodified peptide. (**b**) Peak in the MS scan at the position where the MS/MS spectrum has been acquired. Vertical stripes indicate 1 ppm intervals. (**c**) Contour plot of the whole elution profile of the LC-MS peak. It consists of 38 mass measurements. The blue line indicates the positions of the individual peak centroids. (**d**) Same as (**c**) but represented as 3D graph. (**e**) Isotope envelope of the light labeled form of the peptide. It contains 154 individual mass measurements. (**f**) Same as (**e**) but represented as 3D graph. (**g**) Whole SILAC pair containing 320 mass measurements. (**h**) Same as (**g**) but represented as 3D graph.

about 2 s. Next, the natural isotopes of the peptides are grouped together (Figure 1e and f), providing an increase in the average number of mass measurements to 27 (Figure 2b). For this step, we assumed that the isotopic composition of the peptide corresponds to that of an average amino acid or "averagine" [11, 17]. Finally, in case of quantitative datasets using SILAC, the mass difference between "light" and "heavy" pair is known, and a single SILAC pair now provides on average more than 50 separate mass measurements (Figure 1g and h and Figure 2c). In principle, it would also be possible to include the mass measurements for possible modified forms of the peptide, such as oxidized forms, if they exist. However, this is not done in MaxQuant because these modified forms are only determined with certainty later in the work flow. The mass estimates for different charge states are also not combined at this point because they will be used for recalibrating the mass scale. Likewise, the mass estimates from separate LC MS/MS raw files are not combined because it would be difficult to determine the false discovery rate for the required matching at this point.

Weighted Mean and Bootstrap Error

This large number of measurements each with high-resolution results in very high precision of average

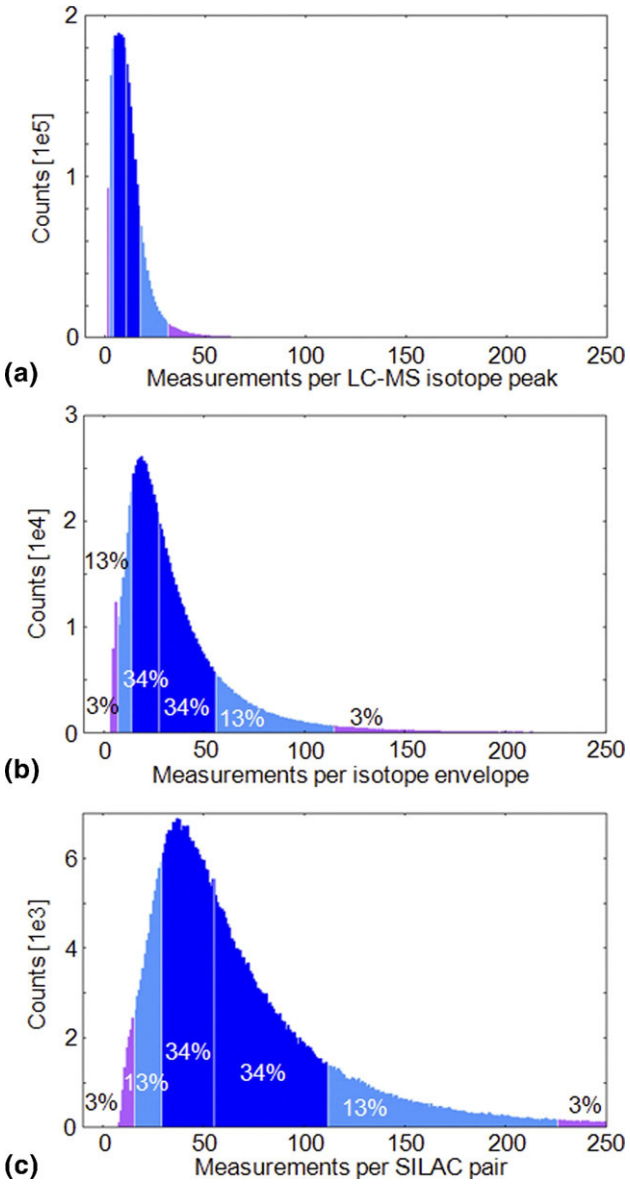

Figure 2. Distribution of the number of mass measurements. (**a**) in LC-MS isotope peaks. (**b**) In isotope envelopes. (**c**) In SILAC pairs. Only peaks and isotope envelopes contained in SILAC pairs were considered for (**a**) and (**b**).

mass measurements for each peptide—typically in the 100 ppb range. To obtain the best estimate for the mass precision, the signal intensity has to be taken into account because more intense signals should contribute more to the mass measurement. Therefore, the mass is determined as the weighted average of the individual mass measurements obtained by fitting the Gaussian curve. In this situation, the error of the mean, which is a nonlinear function of potentially correlated variables, cannot be determined by a simple root mean square or standard deviation. Instead, a statistical procedure called bootstrap is employed [18]. The principle of this method is that the mean is calculated many times on subsamples of the original mass values (allowing repetition of the same procedure). The variation between the subsample averages is then used to calculate the error of the mean.

Low ppb Mass Precision

Figure 3 provides a graphical overview of the process of mass determination. The top panel of the figure depicts the part of the LCMS contour plot in which a particular SILAC pair elutes as well as a three dimensional view. The SILAC pair contains a total of 11 detected isotope peaks, and there are up to 30 MS scans in each isotope state that contribute to the mass measurement. The middle panel shows the peaks together with the determined centroid for each scan. Clearly, the centroid varies less when the intensity of the peak is higher. This is even more apparent in the lower panel that compares the relative mass deviation from the weighted mass estimate for all the isotopes. The mass deviations are relatively high at the beginning and end of each eluting peak but are nearly flat when intensity is high. For low intensity peaks, however, mass deviations are always relatively high.

Altogether, 191 individual mass measurements contribute to the mass estimate of the SILAC pair in Figure 3. The bootstrap error estimate for the mass precision of this peptide is 21 ppb. To our knowledge, this mass precision is unprecedented, at least in large-scale datasets. However, as explained below, this value refers to the repeatability of the measurement and is not yet the true error of our mass determination. It needs to be multiplied by factors, taking account of errors in the mass scale, and for the fact that subsequent mass scans are not statistically independent. This can be seen in the lower panel of Figure 3 (^{13}C isotope of the light SILAC partner, marked by an oval in the figure), where the fluctuation around the zero line is clearly not independent from scan to scan. For similar reasons, the often quoted root mean square errors or standard deviations given for standard substances by manufacturers of mass spectrometers are not necessarily the true mass accuracy. At this point of the workflow, we have improved our mass estimate by extracting the maximum possible number of separate measurements. We find that on average 56 mass measurements are available per peptide.

Peptide Charge Pairs Allow Nonlinear Recalibration of the Mass Scale

We next make use of the multiple charge states seen for peptides in electrospray ionization. A typical LC MS file contains several hundred peptides, which occur in at least two charge states. These peptides cover the relevant *m/z* range as well as the relevant elution range as shown in Figure 4a. As an example, panels b and c show the 2^+ and 3^+ of a SILAC peptide pair (marked with red arrows in Figure 4a). SILAC pairs are used for this

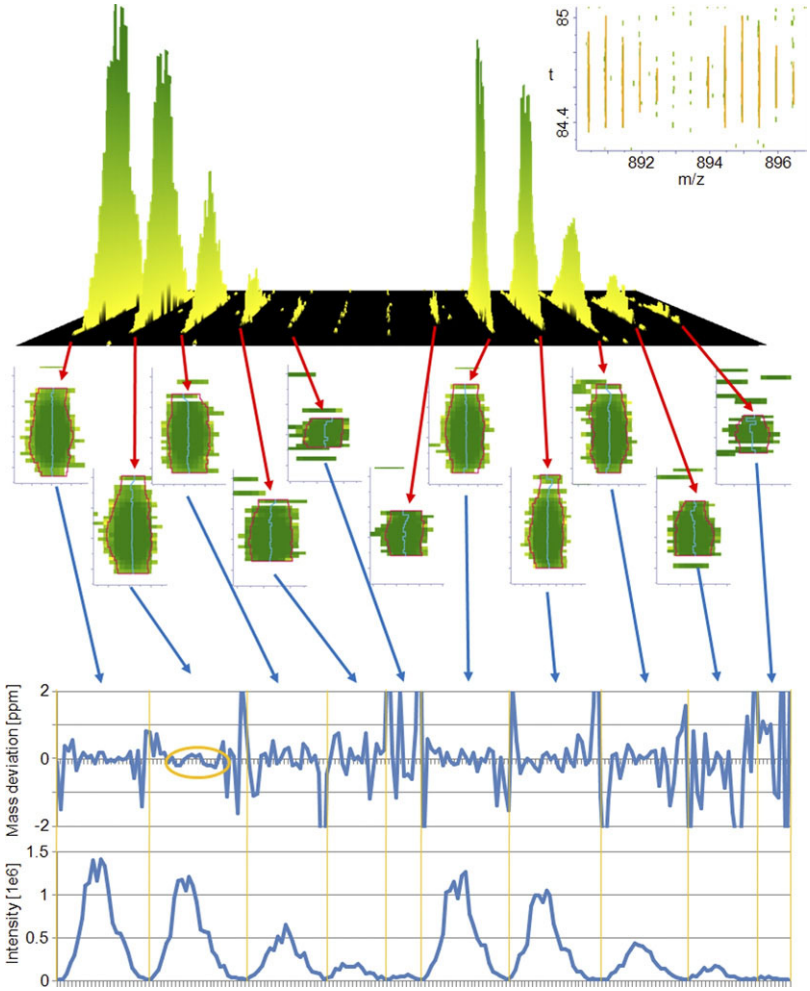

Figure 3. SILAC pair with uncorrected bootstrap mass precision of 21 ppb. The mass deviations in each individual measurement in ppm as well as the peak intensities are plotted over the ten isotope peaks used for estimating the peptide mass. For details see text.

procedure if they are available, but it works equally well for non-SILAC experiments.

The manufacturer's software (Xcalibur), automatically corrects the mass scale by terms accounting for the ion intensity and for nonlinear effects of the Orbitrap [19]. However, even after these corrections, there are clearly mass scale dependent systematic errors as can be seen in Figure 5a, where measured and calculated *m/z* of each peptide are compared.

Starting from the fact that the several hundred charge pairs have to yield the same peptide mass, we calculate a correction curve to eliminate this systematic error. In MaxQuant, a 5th degree polynomial was used [11], however, a comparison of Figure 5c and d demonstrates that a spline function instead of the polynomial leads to superior results. This is presumably because the coefficients in the polynomial affect the function in a non-local way, whereas the spline function better corrects the non-linearities at a local scale. These corrections of the mass scale are necessary to reach sub-ppm mass accuracy (as opposed to mass precision).

Our bootstrap error estimation implicitly assumes that the mass determinations from the different peaks within the isotope pattern and elution profile are independent. If they are not independent, then the true mass accuracy will be worse than appears from the bootstrap error. To determine the needed correction factor, we again employ the charge pairs. If there was no autocorrelation, the mass distribution given by the mass estimate from the doubly charged and the triply charged peptide quantity then the quantity

$$M = \frac{m_1 - m_2}{\sqrt{\Delta m_1^2 + \Delta m_2^2}}$$

where $m_{1,2}$ are the two mass estimates in the charge pair and $\Delta m_{1,2}$ the corresponding bootstrap errors, should be distributed with unit width. In fact, the distribution of M, when plotted for all the charge pairs from all 72 LC MS files, is much wider. The factor needed to convert it to a distribution with unit width must then be the factor that converts mass precision into mass accu-

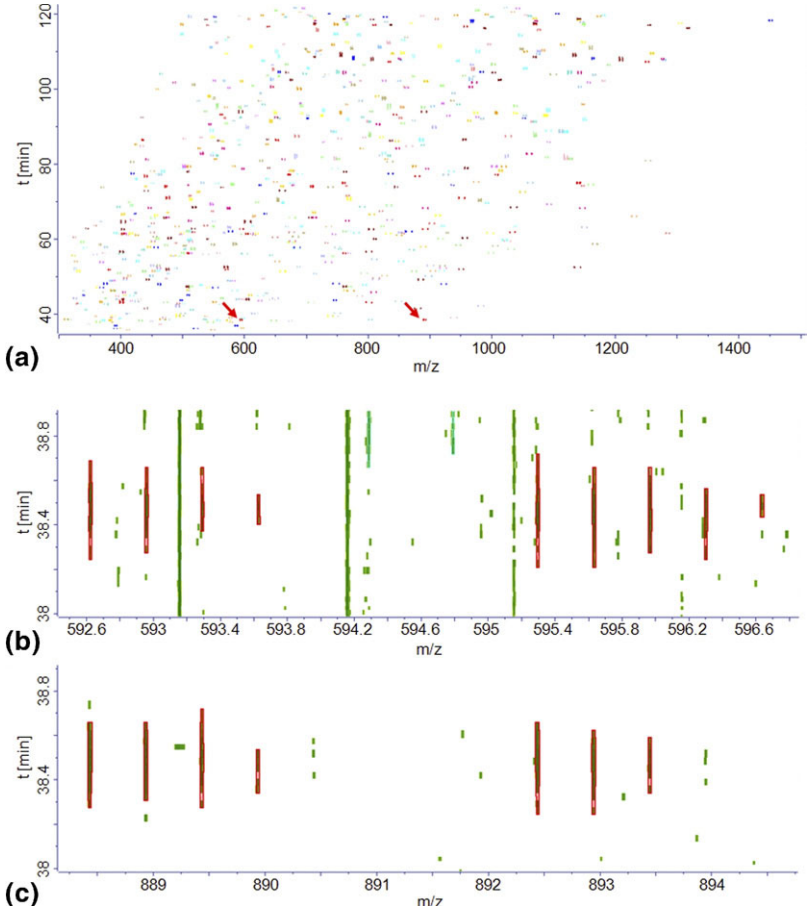

Figure 4. Peptide charge pairs. (**a**) Peptides that were detected as SILAC pairs in at least two charge states are indicated as colored spots in the *m/z*-retention time plane. There were 373 detected charge pairs in this single LC-MS run. (**b**) SILAC pair of the charge 3^+ version of the peptide marked in (**a**). (**c**) Charge 2^+ version of the same peptide.

racy. For most data files, its inverse turns out to be around two to three. Thus, our hypothesis is that the corrected mass precision, obtained by multiplying the mass precision of each peptide or SILAC pair by the correction factor, will yield the correct experimental mass accuracy, which should be used as the basis for database searches.

Experimental Test of the Calculated Corrected Mass Precision

To empirically test the correction factor, we performed a database search with much wider maximum allowed mass deviation (up to 7 ppm), and plotted the mass deviations of the peptide identifications as a function the corrected mass precision (Supplementary Figure 1b, which can be found in the electronic version of this article). Hits from the forward database are plotted in blue and hits from the reverse database are plotted in red. If our calculated, corrected mass precision is indeed the mass accuracy, the true hits should be in an area of the plot where the mass deviation is within several sigma of the corrected mass precision. The inverted triangle (outlined in green in the figure) visualizes this condition allowing four σ-deviations from the corrected mass precision. Hits outside the calculated mass accuracy should be random and therefore should have the same number of hits from the forward and reverse database. Supplementary Figure 1b and c show that this is indeed the case, and we conclude that our calculation of the mass accuracy is correct.

Actual database searches in MaxQuant are performed with the mass accuracy determined as described above, which is a property of each peptide. To capture as many true peptide identifications as possible, we perform the search at 4 σ of the distribution. That is, if a peptide has a mass accuracy of 0.3 ppm, peptide matches that are 1.2 ppm away from the experimentally determined mass estimate would still be allowed.

Overall Mass Distribution of Mass Accuracies

Figure 6 is a histogram of the determined corrected mass precisions of the entire experiment. The curve is roughly log normal, that is, it has a long tail accommo-

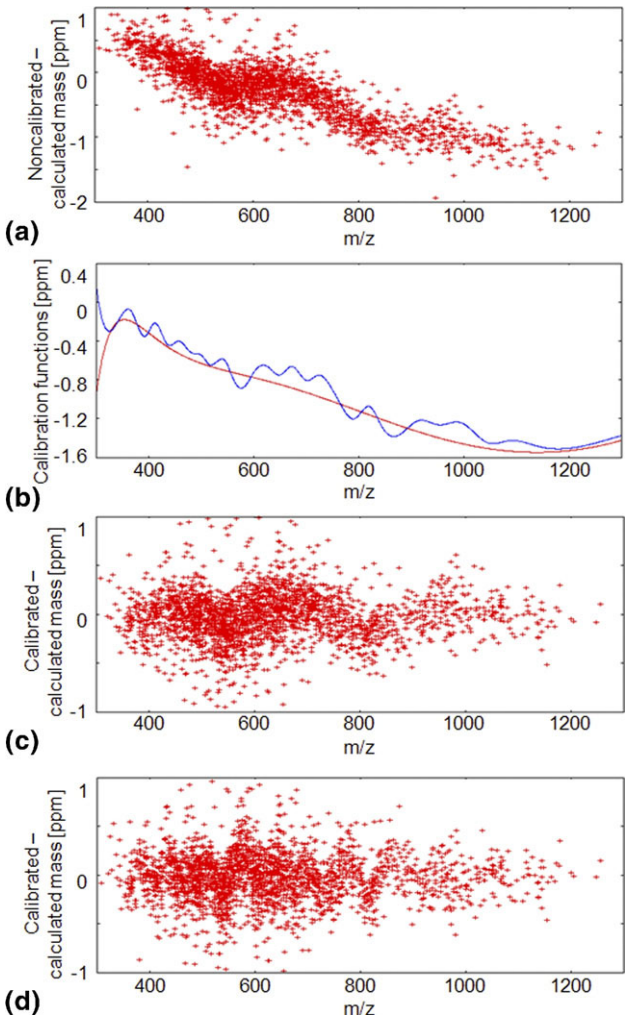

Figure 5. Nonlinear recalibration of the mass scale. (**a**) Relative mass errors in ppm of the identified peptides as a function of *m/z*. (**b**) Nonlinear recalibration functions based on the polynomial (red) and spine (blue) fits. (**c**) Relative mass errors after polynomial recalibration as a function of *m/z*. (**d**) Relative mass errors after spline recalibration as a function of *m/z*.

dating low mass accuracies. A total of 16% of all measurements have a corrected precision worse than 800 ppb and 3% worse than 2.1 ppm. The relatively low mass accuracy of these peptides compared with the others is typically caused by the fact that there are few measurements, which are also of low intensity. As an example, the peak shown in the right hand panel in Figure 6 was only measured eight times in the most abundant isotope peak, and the centroid fluctuates by several ppm between scans. Conversely, the peak in the leftmost panel was measured in many scans and is a member of an extensive isotope cluster. The centroid over most of the peak hardly fluctuates at all, which leads to a corrected mass precision of 60 ppb. As a result, the calculated mass of a potential hit in the database for this peptide has to be within 4 × 60 ppb or 320 ppb of what its measured mass is.

Discussion

Here we have analyzed the major factors necessary to reach sub-ppm mass accuracy in proteomics experiments. The best mass estimate is obtained by weighing the signal by intensity over the LC peak and by combining the mass information from different isotopes as well as from SILAC partners if they are present. On average, this led to about 60 separate mass measurements for a peptide in our dataset. In the absence of SILAC labeling, we expect that half of this number of mass measurements is available per peptide, but that all the presented methods are still applicable. The use of weighted average necessitates bootstrap estimation of the mass precision. These precisions can be extremely high, even in complex mixture analysis, and our data contain examples of peptides measured with a precision better than 20 ppb.

To correct remaining non-linearities in the mass scale and to correct for dependencies in subsequent mass scans (autocorrelation), we used the fact that charge pairs have to lead to the same mass. Consideration of the mass error distributions from the different charge states showed that mass accuracy is substantially lower—by an average factor of two to three—than mass precision. This was also tested experimentally by performing searches with wide windows of maximum allowed mass accuracy and by determining the distribution of forward and reverse database hits. These factors may hold true for other instruments as well and should be explicitly considered.

Overall, we found that mass accuracies for the several hundred thousand peptides in our dataset are lower than one ppm on average. The most important outcome of our treatment is that the mass accuracy of each peptide is known individually and before database search. This means that the results of a database search can be considered individually for each peptide, too. For example, the mass of a peptide with a very good corrected mass precision will have to match the mass of a database peptide within less than a ppm to be considered. Conversely, if a peptide is of extremely low abundance and has only been measured in a few scans, its corrected mass precision can be in the ppm range and a peptide with that mass difference can still be a legitimate match.

While mass accuracies of low ppm are quoted for several instruments, including the LTQ-Orbitrap employed here, the maximum allowed mass deviations in a database search in the proteomics experiments currently reported are always much higher. Indeed in practice they can easily be above 10 ppm or more. These large mass tolerances obviously negate the inherent capabilities of a precision device, such as the Orbitrap. Here we have shown how to make optimal use of the mass accuracy that is obtainable from the instrument.

In this paper, we have not quantified the statistical utility for peptide database searches of the increased and individualized mass accuracy. However, a rough

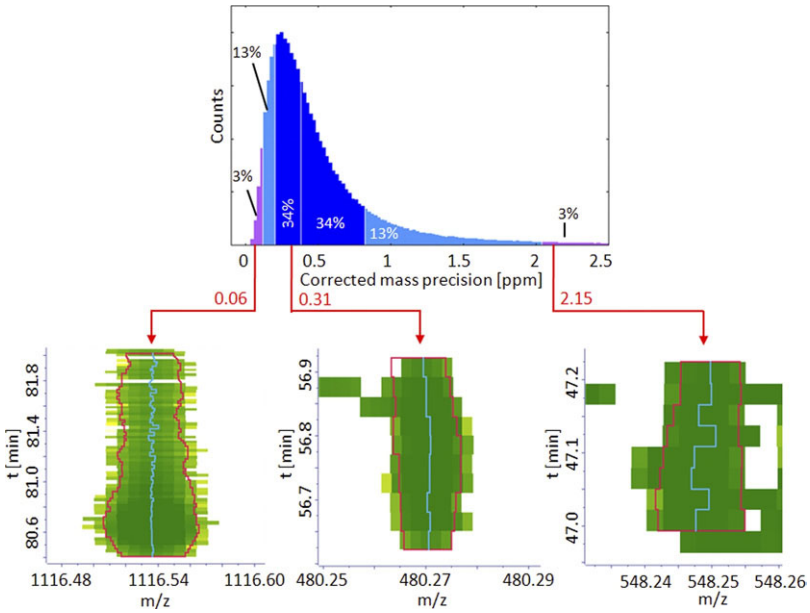

Figure 6. Histogram of the corrected mass precision together with three examples of peptides with high, medium, and low accuracy. The most intense isotope peak is shown for each peptide.

calculation will serve to estimate what is gained by the increase in mass accuracy. Suppose a peptide is identified with a Mascot score of 15 in a database search with a maximum allowed mass deviation of 10 ppm (the lower end of what is reported today). MaxQuant determines masses with sub-ppm accuracy on average and maximum allowed mass deviation, even at 4 σ, is generally around 1 ppm. This means that the chance for a false positive identification is lowered by about a factor 10 for each peptide. To obtain the same improvement through a better MS/MS fragmentation spectrum, the probability score would have to increase 10-fold, which in this case means that the MASCOT score would have to rise from 15 to 25 (probability scores are usually defined as -10*Log(P), where P is the probability for a false positive match). This improvement comes without any cost in experimental design, as it does not add any complexity to the experiment once it is coded into the software.

How close have we come to the theoretically desirable and possible mass accuracy in proteomics experiments? For small peptides with many mass measurements, the results demonstrated here often restrict the peptide to a single composition and, therefore, the highest useful accuracy. However, for peptides with few measurements or for larger peptides up to an order of magnitude improvement would still be desirable. This factor can partly be gained by improving signal to noise for the low abundance peptides. For peptides with good signals, we believe that the mass accuracy reported here is close to the hardware limits of the Orbitrap in its current implementation. Further progress would likely require higher resolution, better control of the injected ion package, and perhaps improvements in the mechanical properties of the trap. More immediate and dramatic gains can be made in the mass accuracy of the fragments. These are currently measured at low-resolution and mass accuracy in the ion trap. However, there is no principle reason that they should not be acquired in the Orbitrap—for example by HCD—once the sensitivity of that technique is increased [20]. This will raise the MS/MS mass accuracy about 100-fold for each fragment peak, with dramatic consequences for the certainty of identification [21].

In conclusion, we hope to have shown how to retrieve the maximum possible mass accuracy from Alexandre Makarov's precision mass spectrometer, the Orbitrap, which is turning out to be a great gift to proteomics researchers.

Acknowledgments

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Appendix A Supplementary Material

Supplementary material associated with this article may be found in the online version at doi:10.1016/j.jasms.2009.05.007.

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