Fundamentals: How do we calculate mass, error, and uncertainty in native mass spectrometry?

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ABSTRACT: Mass spectrometry (MS) is uniquely powerful for measuring the mass of intact proteins and other biomolecules. New applications have expanded intact protein analysis into biopharmaceuticals, native MS, and top-down proteomics, all of which have driven the need for more automated data processing pipelines. However, key metrics in the field are often not precisely defined. For example, there are different views on how to calculate uncertainty from spectra. This Critical Insight will explore the different definitions of mass, error, and uncertainty. It will discuss situations where different definitions may be more suitable and provide recommendations for best practices. Targeting both beginners and experts, the goal of the discussion is to provide a common foundation of terminology, enhance statistical rigor, and improve automation of data analysis.

Introduction

Native electrospray (ESI) mass spectrometry (MS) has matured from an emerging technology for specialists into an established research tool. Unlike conventional soft ionization MS, which preserves covalent interactions but often disrupts non-covalent interactions with denaturing solvent conditions, native MS uses non-denaturing solvent conditions prior to ionization and often seeks to preserve non-covalent interactions during MS to study biomolecular interactions.[1] As instrumentation and methods have improved, more academic researchers are using native MS to characterize biomolecular interactions and stoichiometry.[1-3] Furthermore, native MS techniques are becoming essential in industry for development and quality control of biotherapeutics.[4,5] The expansion of the field, especially into industrial applications, is transforming the analysis of native MS data from a manual process performed by trained personnel to an automated process performed by sophisticated software from a variety of vendors and research labs.[6-10]

As the field expands, it is important that we discuss best practices for native MS data analysis and interpretation. Community-driven efforts have driven standardization for proteomics,[11] glycomics,[12] and ion mobility spectrometry,[13] in particular with regards to development of data analysis software.[14] A full discussion for native MS is beyond the limited scope of this Critical Insight, but these fields provide helpful recommendations for native MS.

Here, the goal is to start a conversation over how to define and calculate mass, error, and uncertainty. We will focus only on the mass information. Readers interested in intensities are referred to prior work from John Klassen[15,16] and Valerie Gabelica.[17,18] We will also limit our discussion to spectra without isotopic resolution, as spectra with isotopic resolution warrant different consideration. Finally, we will primarily consider homogeneous spectra with easily assignable charge states to simplify our discussion. Heterogeneous spectra can present additional challenges of intrinsic ambiguity and are nicely reviewed by Roland and Prell[19] and discussed previously.[20]

Precise definitions of mass, error, and uncertainty specific to native MS will help ensure reproducibility of results, standardize methods, and guide software development for data analysis. The goal is that this Critical Insight will offer a helpful introduction to beginners and some thought-provoking questions for experienced practitioners. Although we will focus on native MS, the same discussions are also relevant for denatured or conventional ESI analysis of larger molecules and for MS1 spectra in top-down proteomics.

How Do We Calculate Mass?

The primary goal of many MS experiments is to measure the mass of a molecule or complex. Although this appears to be trivial, carefully defining the mass measured by MS without isotopic resolution requires more discussion to carefully define the measurand.[21] Mass spectrometry measures the distribution of ion mass/charge (m/z) values. For our discussions, we will disregard the differences between mass analyzers and consider the distribution of generic intensity with respect to *m/z*. For convenience, we will refer to a single distribution of ions in *m/z* as a peak, which may include adducts.

Several descriptive statistics can be employed to describe the position of the peak in *m/z*: mean, median, and apex. In practice, median is rarely used to describe peaks in MS. The mean is often referred to as the centroid and is defined for MS as the average of *m/z* values (m_i) weighted by their intensities (I_i): $Centroid = \frac{\sum m_i I_i}{\sum I_i}$. The apex is the position in *m/z* of the highest intensity value: $Apex = m_{Max(I)}$.

In the case of an ideal symmetric peak, the centroid and the apex will be the same (Figure 1A). However, unlike denatured MS, peaks in native MS are often not symmetric, typically because adduction of salts, solvent, or other small molecules from solution shifts a subpopulation of ions to slightly higher mass.[7,22-24] These sorts of adducts are important because native MS traditionally produced lower resolution data where adducts are convolved into the same peak and cannot be distinguished. A peak that is Gaussian on the low mass side and Lorentzian on the high mass side, producing a longer tail at higher mass, can be used to model an asymmetric peak distribution caused by unresolvable adduction.[25] Here, the centroid and the apex diverge (Figure 1B). Importantly, the apex is likely a better measure of the true mass with asymmetric peak shapes because the centroid is easily skewed by higher mass adducts.

Peaks in native MS may also be superimposed with baseline and noise. The apex is likely less sensitive to baseline effects (Figure 1D) but more sensitive to noise (Figure 1C). In contrast, the centroid is likely more sensitive to baseline but less sensitive to noise.

It is important to note that peaks are defined relative to a local window. The apex is defined as the local max, and the centroid must ignore intensity from neighboring peaks and from baseline or noise. Determining the window to define peaks is often subconscious for humans, but it must be specified for automated peak detection and analysis. The apex is less sensitive to the definition of the local window than centroid, especially when baseline is present. For example, Figure 1D shows how the centroid can shift an otherwise ideal peak when baseline is included in the centroid calculation and the window is not centered.

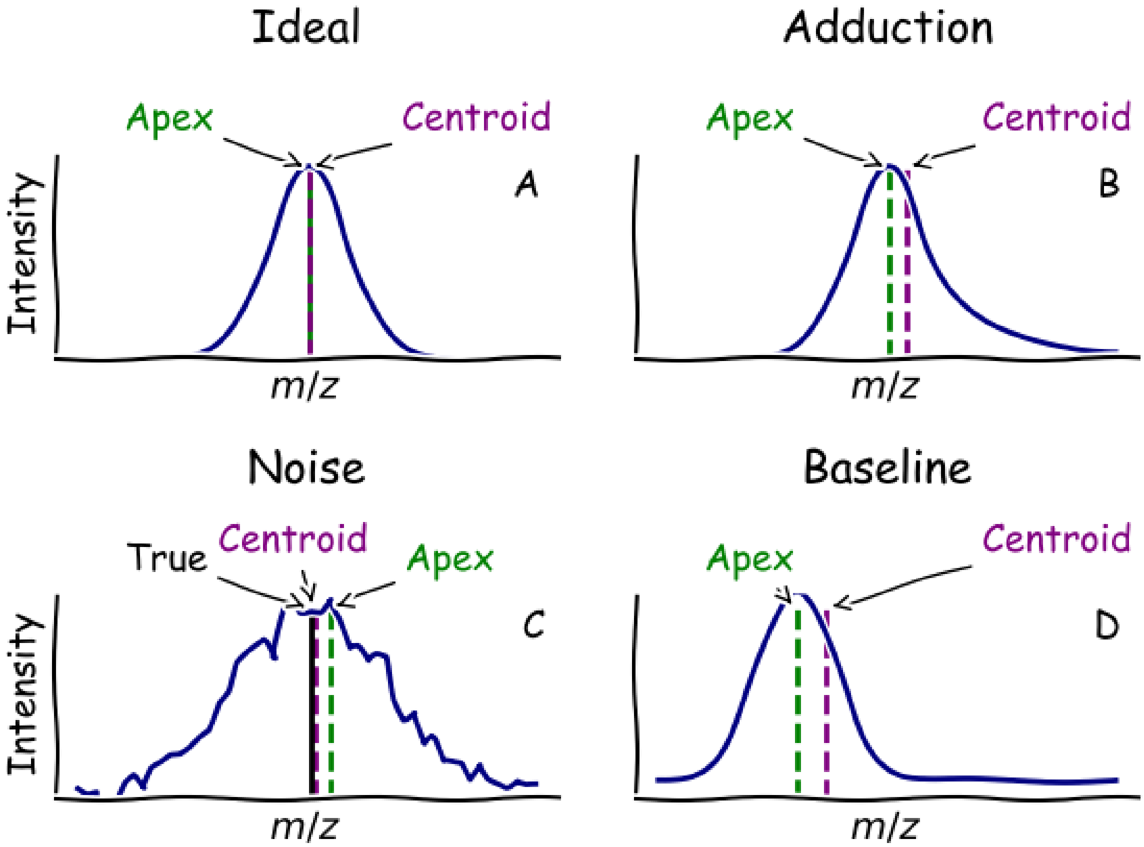

Figure 1. Sketch of an ideal peak (A) along with peaks suffering from adduction (B), noise (C), and baseline (D). The apex (*green*) and centroid (*purple*) are annotated along with the true peak (*black*, C only). For illustration, the width of the Gaussian peak in C has been doubled, and the window in D has been shifted.

Here, we propose that the apex is generally a more reliable measure of mass than centroid in native MS because it is less sensitive to adduction and baseline. It is also easier to calculate by hand or computer, in part because it is less sensitive to definitions of local window.

In spectra where noise is a major issue, centroid may be more suitable, and the centroid of the intensities above a specific threshold is a useful compromise to avoid adducts causing a systematic shift towards higher masses. In UniDec,[9,10] the peaks are generally defined as the apex, but tools allow calculation of the centroid for intensities above a 0, 10, or 50% threshold. In any case, as instrumentation continues to improve, enhancements in resolution, desolvation, and data quality will lead to the convergence of centroid and apex.[26-28] For example, improvements in resolution sometimes allow adducts to be resolved. In this case, adducts can be treated as distinct peaks and assigned independently as an analyte plus a specific number of bound adducts.

Computational techniques such as smoothing and baseline subtraction can be used for spectra with noise and baseline, respectively. A detailed discussion of different algorithms is outside the scope of this article. However, when used carefully, both approaches can be used safely to remove potential artifacts in the mass measurement without distorting the underlying data. To apply these methods appropriately, start by understanding the sources of noise/baseline, which differ between instrument platforms. Next, learn how the algorithms work and what potential biases they may have. Finally, use the least amount of smoothing/subtraction needed.

Our discussion has centered on a single peak, but ESI MS generally produces multiple charge states for a single species. Thus, the mass is calculated from the separate *m/z* values from each charge state and must be combined to determine the average mass. The simplest calculation is the average of the masses measured from each individual charge state.[29] However, the unweighted average may be skewed from minor charge states at the fringes of the distribution with lower signal-to-noise ratios. Thus, we propose that the average weighted by intensity is a more reliable approach to combine peaks from multiple charge states. In fact, many deconvolution algorithms, including UniDec, use this approach implicitly to assemble a zero-charge mass spectrum, which is the weighted average of all data after transformation from *m/z* into mass. The mass definitions described above are equally applicable to the zero-charge spectrum and should converge to the same result as calculating the weighted average of individual charge state peaks.

How Do We Calculate Error?

In contrast to uncertainty/precision (discussed below), error or accuracy in native MS is easy to define and compute. The IUPAC definition of error is as "the result of a measurement minus the true value of the measurand."[30] Thus, if we measure a molecular weight ($M_{measured}$) and compare it with a predicted molecular weight ($M_{calculated}$), we define:

$$Error = M_{measured} - M_{calculated}$$

Hidden in this definition is a key insight into MS experiments: the reason to measure mass is to determine the identity of an ion. The power of MS is that mass is easy to predict from a candidate molecule. With good standards ranging from 8-800 kDa,[31] known molecules can be used to evaluate error, but known masses can also be calculated for potential species to help assign an experimental peak. However, adduction can cause systematic biases towards positive error that should be considered when interpreting the data, especially for larger protein complexes where adducts are more difficult to remove.

Although the definition of error in MS—how closely the measurement matches a predicted mass—is straightforward, establishing whether a certain amount of error allows us to confirm the identity of a peak requires a metric for uncertainty, which is more challenging to define.

How Do We Calculate Uncertainty?

To determine how well a measurement matches a prediction, we need to know the uncertainty of the measurement, defined as "the dispersion of values that could reasonably be attributed to the measurand."[21] However, in contrast to error, uncertainty or precision of the mass measurement is poorly defined for native MS. Here, we will weigh the advantages and disadvantages of three metrics for Type A uncertainty (uncertainty that can be statistically evaluated)[21] of mass measured by ESI: 1) the weighted or unweighted standard deviation of the mass calculated from different charge states, 2) the peak width, and 3) replicate measurements. In homogeneous spectra where charge states are clearly assignable, we will assume essentially zero uncertainty in the charge state. Heterogeneous spectra[19,20] with uncertainty in the charge state assignments will be partially captured by the metrics described below but may require additional considerations not explored here.

Standard Deviation of Charge States

Perhaps the earliest definition of ESI uncertainty is the standard deviation of the mass calculated from different charge states (Figure 2A).[29] The mass of each peak is typically defined as: $M_i = m_i z_i - M_a z_i$, where M_i is the mass of the peak, m_i is the *m/z* value of the peak, z_i is the assigned charge state of the peak, and M_a is the mass of the ESI charge carrier, typically hydrogen in positive ionization mode. After assigning N peaks as belonging to the same charge state series, M_{avg} can be calculated as the average of the set of all M_i values in the charge state series, and the standard deviation can be calculated:

$$Standard\ Deviation = \sqrt{\frac{1}{N}\sum_i^N \left(M_i - M_{avg}\right)^2},$$

as shown in Figure 2A. Noise, background, and broader peaks (see Figure 1) will increase the standard deviation because the masses from each peak (either the apex or centroid) will vary more between charge states under these conditions.

However, the standard deviation of charge states presents some significant limitations, primarily due to difficulties in defining which peaks to include. For example, overlapping peaks in more complex spectra present challenges for identifying the *m/z* value and thus the mass for these peaks. Even in a relatively simple spectrum with a single charge series, it can be challenging to decide which charge states to include when peaks at the fringes of the charge state distribution have low signal-to-noise ratios, such as points M_1 and M_5 in Figure 2A. Overall, the standard deviation of charge states is relatively easy for humans to calculate but is more challenging to define precisely for algorithms.

Using a weighted standard deviation solves some of these challenges because the most abundant peaks are the most influential. Fringe peaks, like M_1 and M_5 in Figure 2A, will have less influence on the weighted standard deviation. However, very narrow charge state distributions present a significant challenge for weighted standard deviation and to a lesser degree with un-

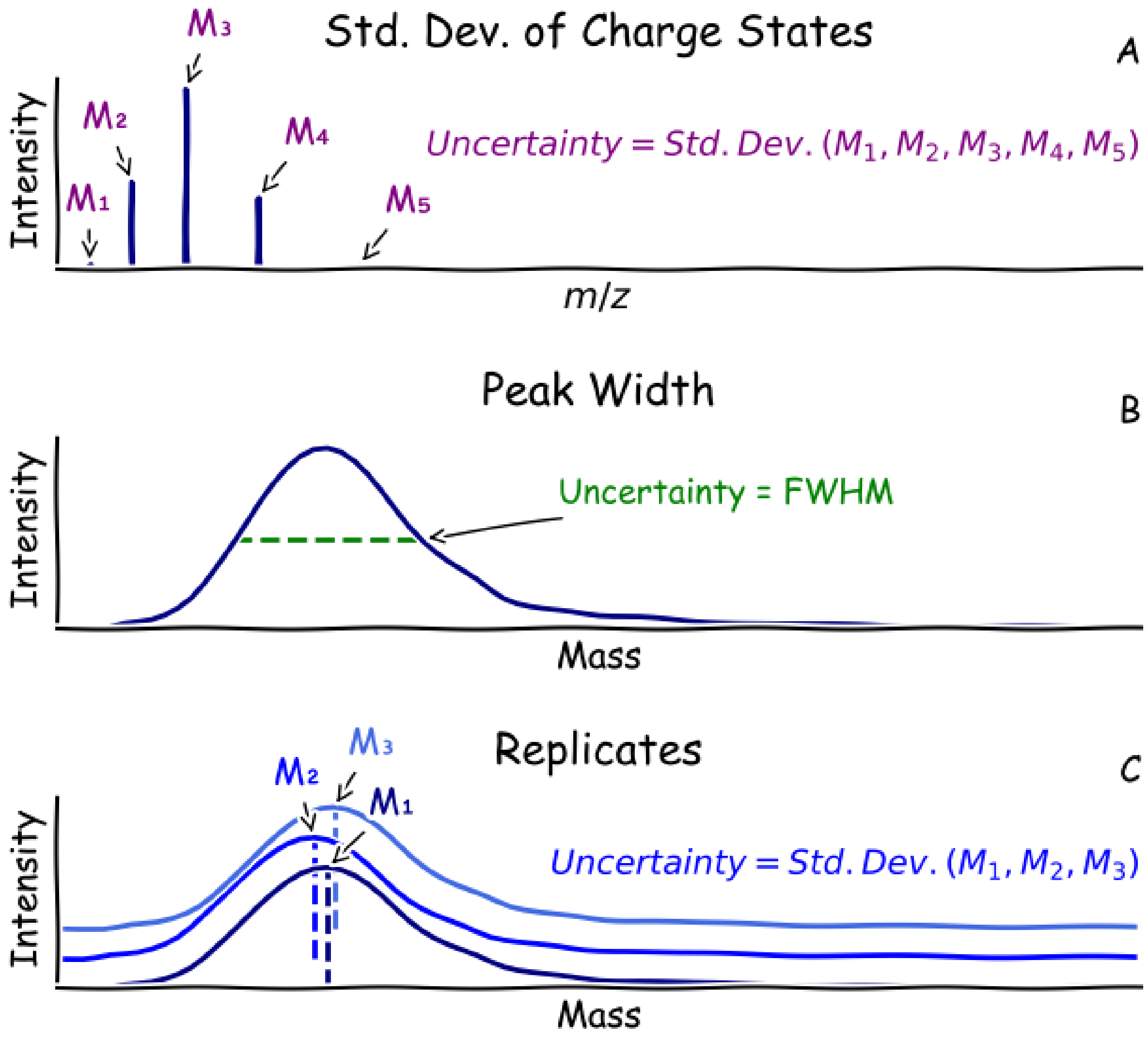

Figure 2. Three different measures of uncertainty: A) the standard deviation of the masses, M_i, measured from each different charge state in *m/z*; B) the peak width such as the full width at half maximum in mass; and C) replicate mass measurements from three samples.

weighted standard deviation. It is not uncommon for small proteins to have only one or two dominant charge states in native MS,[32,33] which will limit the statistical utility of defining uncertainty from weighted standard deviation of the charge state distribution.

Peak Width

Another way to define uncertainty is to consider the peak as a statistical distribution of masses and use a measure of the peak width as the uncertainty (Figure 2B). By fitting the peak to a Gaussian distribution, the standard deviation of the peak distribution can be used as a metric of uncertainty. For asymmetric peaks that do not fit a clear statistical model (Figure 1B), full width at half max (FWHM) can be used instead. Peak width, however defined, is useful as a metric for uncertainty because it is easily defined for complex spectra with overlapping peaks, which can be computationally separated by deconvolution or fitting.[9,34-37] It also avoids issues with trying to decide which fringe charge states to include. Thus, it is easier for computers to calculate but is more challenging for humans to calculate precisely by hand.

It is important to note that the standard deviation of charge states and the peak width are fundamentally different statistical measures. The standard deviation of charge states represents the standard deviation of the mean of the ion population. In other words, how precise is the measurement of the mean mass? The peak width represents the standard deviation of the population of ions, which may include adducts as mentioned above. In other words, how wide is the spread of masses in a packet of ions? These can give very different results and mean fundamentally different things.

Replicate Measurements

The primary limitation of both charge state and peak width definitions of uncertainty is that they use a single spectrum to estimate uncertainty. Even when multiple charge states are present and multiple scans are collected, we propose that a single spectrum should be considered a single measurement and is insufficient to fully estimate the uncertainty. Instead, replicate measurements are a better way to determine the uncertainty. Our proposal is that the best definition of uncertainty is the standard deviation of the measured mass between replicate samples of the analyte (Figure 2C). Standard deviation of multiple replicates is easy for both humans and computers to calculate from either raw or deconvolved data. It sufficiently captures the uncertainty caused by poor spectra. Finally, more scans, charge states, and replicates will decrease the uncertainty of the measurement, as one would expect.

In practice, researchers will have to define the scope of what “replicate samples” means based on the questions they seek to answer, the properties of the analyte, and the sample preparation procedures. For example, in our research with native MS of nanodisc complexes, we found that the primary source of variance is the nanodisc assembly process. Replicate measurements of the same sample of nanodiscs show lower standard deviations than replicate samples prepared under different assembly reactions.[10] Thus, we define replicate samples to mean replicate assemblies. However, soluble protein complexes such as myoglobin are less sensitive to sample preparation. For mass spectrometry studies using myoglobin as a model protein, we found it was sufficient to define replicate samples of myoglobin as simply replicate buffer exchange columns from the same starting stocked prepared by dissolving protein from the bottle in ammonium acetate solution. However, if we were seeking to answer larger biological questions related to myoglobin, more rigorous biological replicates might be necessary.

At a minimum, we propose that uncertainty from MS measurements should be determined from separate injections of replicate samples. The manual clipping of the homemade capillary needles that are common in native MS can introduce variance between injections due to different tip diameters,[25,38] so the use of replicate needles is a reasonable minimum to capture potential uncertainty in the measurement. Again, each researcher needs to define the appropriate replicates for their system, which may go beyond the minimum level proposed here.

Finally, it is important to note that each of these metrics captures primarily random effects. There can be additional systematic effects on uncertainty and error that each of these will miss. For example, poor calibration of the mass spectrometer will introduce error that is not reflected in the uncertainty metrics discussed here. Changes to sample preparations and instrument parameters can also shift the ion population by reducing adduction, which may underestimate the uncertainty in the ion population if we broaden our definition of the ion population to consider alternative solution- and gas-phase treatments. Capturing these forms of error and uncertainty will require Type B evaluations uncertainty, which do not use statistical analysis but instead use other forms of knowledge, such as comparison against true values from standards.[21]

Conclusions

Here, we have discussed the definitions of mass, error, and uncertainty specific to native MS. Error is easy to define and calculate. In contrast, there are two primary definitions of mass: centroid and apex. Both should yield similar results for ideal peaks, but we propose that apex is a more straightforward definition in most cases. However, centroid above a certain threshold is more useful with noisy spectra.

For uncertainty, three primary definitions are used in the field, and they all tell us different things. Charge state uncertainty captures the standard error of the mean within a single spectrum. FWHM captures the standard deviation of the ion population, also from a single spectrum. Replicate measurements captures the standard error of the mean across multiple measurements. We propose that the best measure of uncertainty of the mass is from replicate measurements, although FWHM is also useful to report on the standard deviation of the population.

Although precise definitions of mass, error, and uncertainty are admittedly not the most exciting topic, they are important to advancing native MS and intact mass analysis. As the field advances to more automation and sophisticated data analysis, having common definitions will support ongoing software development and common standards in analysis.

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