Defining Lower Limits of Quantitation

A Discussion of Signal / Noise, Reproducibility and Detector Technology in Quantitative LC/MS/MS Experiments

When analyzing an unknown sample, the analytical chemist's task is to provide an estimate of the concentration of an analyte with acceptable uncertainty (accuracy and precision). Very often, due to the time required to perform a good statistical assessment of instrument performance, the signal-to-noise ratio (S/N) (the ratio of a peak's height to the variability in the background signal) is used as a way of estimating the lowest quantifiable amount (the lower limit of quantitation - LOQ) for the analyte. The LOD (limit of detection) and LOQ (limit of quantitation) are often defined as the concentrations which yield a measure peak with S/N of 3 and 10 respectively. However, as will be described, this method can be misleading and should not be used when evaluating instrument performance, and should particularly be avoided when comparing one instrument model to another.

Differences in the way that an instrument detects ions, processes the ion signal, and treats the resulting data can have a drastic effect on the apparent S/N ratio without necessarily reflecting an instrument's ability to quantify an analyte at that concentration. The only reliable way to estimate a system's performance is through a good statistical approach to experiment design and the calculation of accuracy and reproducibility for several injections of a sample at several levels, above, near and below the LOQ level. The importance of using a statistical evaluation of the data is even more important when comparing instruments which use different methods to collect and store the ion signal. What follows herein is a discussion of the most

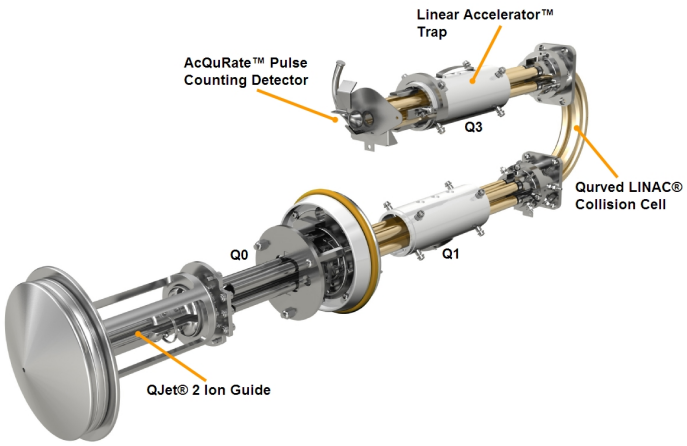

Figure 1. Ion Rail of a QTRAP® 5500 System. The pulse counting detector (the AcQuRate™ Detector) is a key component of this system for providing the highest reproducibility and accuracy of measurements at the lower limit of quantitation in MRM assays.

common practices in the collection, storage and processing of ion signals in modern LC/MS/MS systems and the impact of these practices on the S/N, accuracy and precision of an LOQ determination.

Defining Lower Limit of Quantitation

A more practical and statistically correct way to define the LOQ is as the lowest concentration where the relative uncertainty on a single measurement is reproducible within +/- 20%. In this definition of LOQ, S/N and peak height are still important parameters to consider but are not sufficient to fully define it. Determining the LOQ of a method for an analyte, based on a statistical estimate of uncertainty, requires many injections to adequately characterize the system's response to an analyte and to estimate uncertainty. At least 3-5 injections at each of 5 concentrations, including at least one near and one below the limit of quantitation, must be performed to determine the uncertainty with reasonable confidence. At that concentration, the CV of replicate injections should be <20% and the accuracy of the measurement should be +/- 20%.

Data Collection

Mass spectrometers create ions in the source region, and must detect these ions to form a mass spectrum, or in a quantitative MRM experiment, a signal which is proportional to the flux of ions into the ion path. Almost all triple quadrupole and ion trap mass spectrometers today use some sort of electron multiplier to convert an electron generated by an ion impact to a burst of electrons, which may be measured (Figure 1).

All instruments will provide the user with a "raw" data file, which is intended to be, or at least most often assumed to be, a verbatim recording of the signal observed at the detector during an analytical experiment. The signal at the detector is a representation of the ion current, as a series of pulses, corresponding to the cascade of electrons created in the electron multiplier when an ion (or ions) strikes the detector. The raw data then is created by recording a series of time specific digital values that in some way reflects the number of ions impinging on the electron multiplier detector per unit time for the duration of the run.

But what is the relationship between the ion current at the detector and the digital values stored in the raw data file? This relationship is determined by the electronics used to collect the ion current, and the software used to store these values. There are two common methods of converting the ion signal into digital values which are discussed below: Analog and Pulse Counting. Both techniques have advantages and disadvantages.

Analog Mode Detection

In Analog mode detection the ion current is measured directly as a voltage, or integrated electronically over small time intervals, and converted into digital values using an analog-to-digital converter (ADC). Because the signal is converted to a voltage prior to the ADC, this mode is more susceptible to noise than frequency based modes. Typical ADC's include circuitry to filter the raw signal, which at low levels is a series of pulses, and provide a stable signal for conversion to a digital value. An advantage of the analog mode is that greater dynamic range is possible since ions arriving simultaneously at the detector can be properly integrated into the measurement. The filtering done during conversion also helps provide a "cleaner" signal, which is then stored as raw data. The main disadvantage of analog detector circuitry is that the raw data does not necessarily represent the actual ion count over small intervals at small signal levels. This is due to the fact that filtering and application of data thresholds to the signal is most often used to reject noise, thus making it impossible to record true single-ion pulses (the lowest signal level possible).

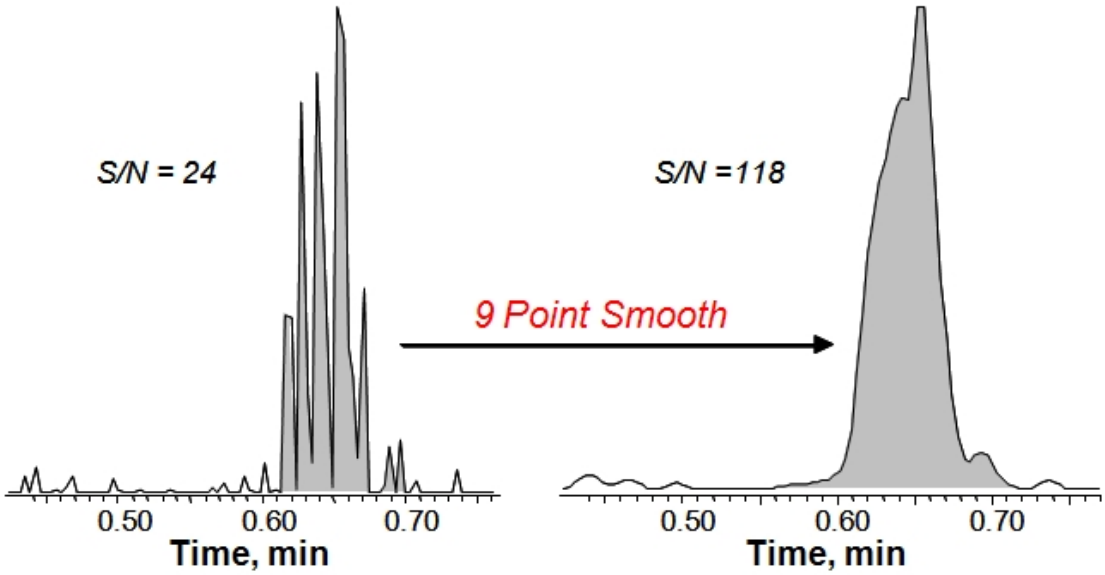

Figure 2. An Example of a Low Level Signal using an Analog Detector. Increase in S/N for a signal that has had a threshold applied is observed when smoothing is applied. Despite a factor of 4x improvement in S/N, no significant improvement in real LOQ is seen.

Pulse Counting Detection

Pulse counting detectors, as the name implies, count the pulses produced by ions impinging on the electron multiplier for an accurately timed period, thus directly creating a digital representation of the ion flux. Because the duration of the pulses is extremely short, high speed electronics are required to count the pulses fast enough that the counter doesn't miss them. The main advantage of pulse counting detectors is that they are virtually free of electronic noise, and careful attention to ion path design can effectively eliminate internal noise. This has the effect of rendering accurate measurements of single ion pulses. The main disadvantage of pulse counting detectors (aside from increased cost) is limited dynamic range, as the detection circuitry, which counts pulses, cannot typically resolve ions arriving coincidently at the detector at the highest concentrations of analyte.

Smoothing, S/N and Thresholded Signals

Great care must be taken when looking at S/N calculations for a chromatogram, especially when looking at data collected with an analog detector. Some pulse counting and most analog detector systems use 'thresholding' to eliminate low level noise from a chromatogram. Thresholding is simply the elimination of all signals below a certain level. This will generally create a signal that will appear very flat, with occasional 'spikes'. Small peaks will also tend to look 'spiky'. An example of what an unsmoothed, thresholded chromatogram would look like is shown in Figure 2a. Small peaks close to the noise will often show accuracies which are significantly below 100%. This is a result of eliminating signal below the threshold level, which results in losing a fraction of the actual peak area (see Figure 6c). Despite a calculated S/N value of close to 30, this peak cannot be considered to be representative of an LOQ since the accuracy is a factor of 2 (CV 40% accuracy, 10 replicate injections, data not shown) lower than the acceptable limits as outlined above.

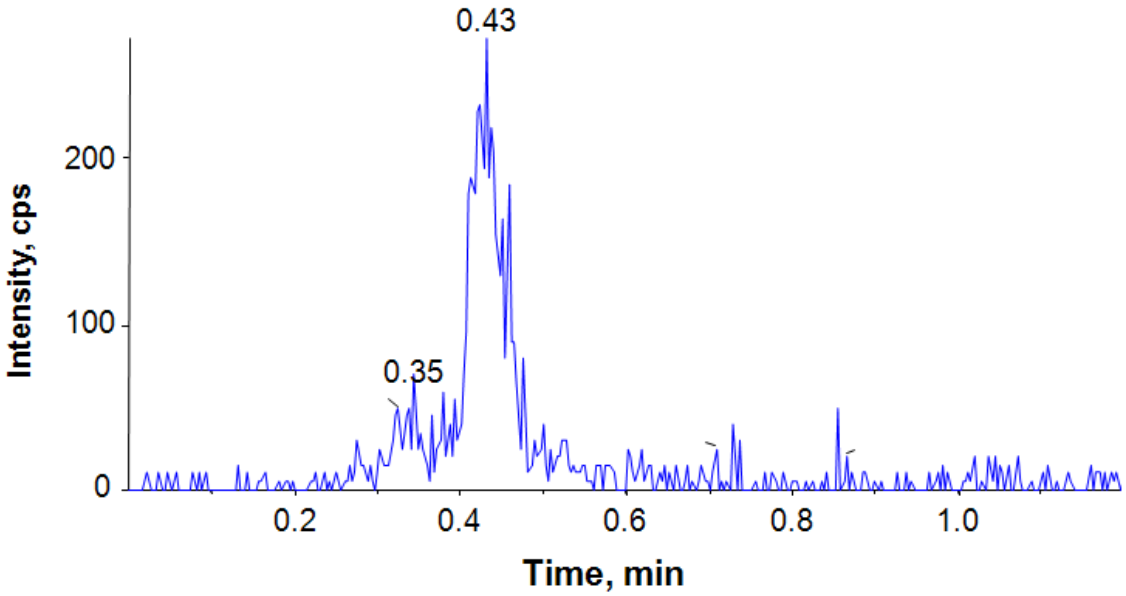

Figure 3. An Example of a Pulse Counting Detector Signal with a S/N of about 30, with No Threshold and Unsmoothed. Even though these data look noisier than the thresholded example of Figure 1, the actual data produces better results. Accuracy was >90% for 10 replicate injections (data not shown).

Significant smoothing can be performed on a peak such as this one to improve its appearance and S/N ratio, as shown in Figure 2b, but it will have little effect on the accuracy and the ultimate LOQ. Contrast the chromatogram shown in Figure 2a to the chromatogram shown in Figure 3, which is typical unsmoothed data from a pulse counting detector. A key feature of the data is the natural looking noise signal which forms the baseline of the chromatogram. For small peaks, these data will not suffer from low accuracy as the entire peak area may be integrated easily and reproducibly.

The data from the pulse counting detector shown in Figure 3 looks much noisier than the thresholded data of Figure 2a, but when 10 replicate injections are performed using thresholded anaolog detection vs. pulse counting detection, the pulse counting data provide superior results, accuracies > 90%, compared to 40% for the thresholded data (Figure 4 and 5). Because an artificially large area of the baseline has zero noise signal in the thresholded analog data, the signal to noise ratio is also artificially increased. The pulse counting data more accurately represents the true baseline noise signal, and therefore gives a lower S/N ratio for similar peak heights. The key observation here is that comparing signal to noise ratios from different software packages and different instrument detector signal collection methods is not a good way to assess the true instrument performance on low level signals.

The Effect of Smoothing on S/N

Smoothing of raw data is often performed either during acquisition or in post-acquisition processing. While the appearance of a chromatogram, and in the case of thresholded data the calculated S/N, can be dramatically improved using smoothing, it will do little to improve either reproducibility or accuracy, and will therefore have little effect on the real LOQ. Figure 4 and Figure 5 show the relationship between Signal-to-Noise ratio, accuracy and precision for both thresholded analog data and non-thresholded pulse counting data.

The smoothing of data can have a dramatic effect on the apparent S/N, but it does nothing to improve precision or accuracy of the result. This was demonstrated in Figure 4, where the effect of subsequent smoothing on the S/N reported by the software is drastic, which suggests that the baseline “noise” is not random data. In addition, the precision and accuracy performance is poor, even at an apparently high signal-to-noise ratio. When this simple experiment is repeated on another LC/MS/MS system equipped with a Pulse-counting detector, the results are dramatically different (Figure 5). The S/N ratio displayed in the software is significantly lower than the previous instrument, but the accuracy and precision results are significantly improved. Furthermore, the S/N values are not drastically affected by applying SW smoothing to the data.

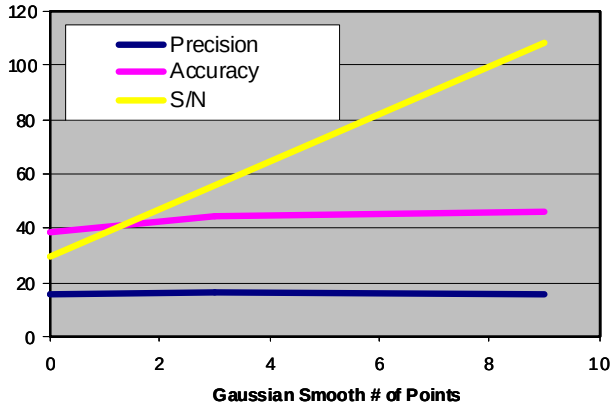

Figure 4. Effect of Smoothing on Accuracy and Precision of a Low Level Thresholded Signal near the LOQ from an Analog Detector. S/N can be dramatically improved through smoothing, but accuracy and precision are not significantly improved. The real LOQ, determined statistically, is therefore not improved with smoothing. For peaks close to the noise, accuracies will be low, due to a significant fraction of the peak being eliminated by thresholding the signal. Despite an unsmoothed S/N ratio of 30, the accuracy was only 40% in this case).

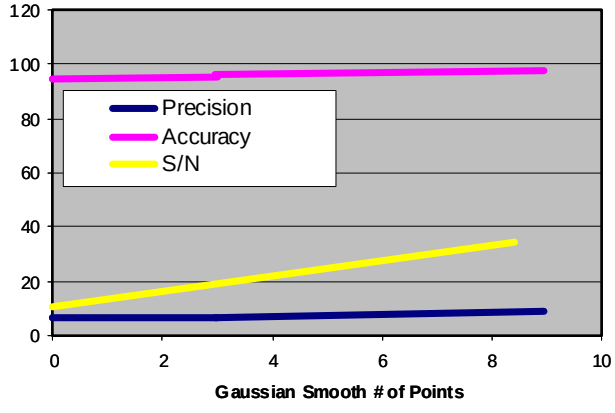

Figure 5. Effect of Smoothing on Accuracy and Precision of a Low Level signal near the LOQ using a Pulse Counting Detector System. S/N is not as dramatically improved with smoothing as with a thresholded signal. Accuracy and precision are also not significantly improved. In contrast to the thresholded signal from the analog detector, the real LOQ is not improved with smoothing. Accuracy is close to 100% for a peak with a S/N of ~10.

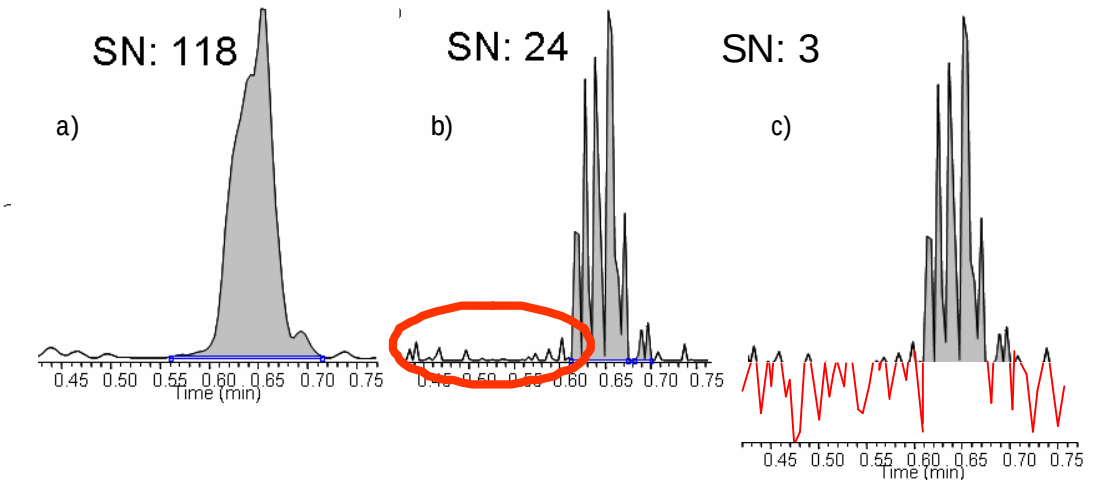

Figure 6. S/N Levels for Various Stages of Smoothing and Thresholding. A) 9 point smooth, B) smoothing filter turned off to see "raw" data, but unusual noise response only has positive noise spikes, never going below zero, which suggests that background thresholding is being used to mask the noise. C) re-creation of true raw noise baseline obscured by thresholding. The real signal to noise ratio of this peak is probably more like 3, but in a system where thresholding is used (b) during collection, the user may never see the real data.

The bottom line is that one needs to always look at the true raw data, understand how the data are collected and what happens to the raw data during processing. Figure 6 shows how the various filters, thresholding and smoothing can affect the appearance of the data. Figure 6b is the 'raw data' collected from an analog detector with a calculated S/N of 24. However, a closer inspection of the noise reveals that there is thresholding occurring on these data before display. The real signal-to-noise ratio of this peak is probably more like 3, (re-creation of 'raw data without thresholding Figure 6c) but depending on the software, the real raw data may not be accessible.

As shown previously, the other potential filter is a smooth applied to the data (Figure 6a), which can greatly improve the appearance and S/N of the analog data but have no effect on the actual LOQ.

Analog vs. Pulse Counting Detectors

So which is the best mode for quantitative performance and why? The key is to perform experiments to statistically analyze the performance of the machine not only for S/N, but for precision and accuracy (much more important parameters for meeting the goals of the actual experiment) and this can only be determined with replicate injections at or close to the LOQ level. Remember that detection limit estimated by the S/N at 3 times the standard deviation of the noise is based on an assumption that the noise is correctly represented in the data. If the non-thresholded raw data is unavailable then this S/N evaluation is no longer valid.

Figure 7 shows the result of multiple injections at 3 different concentrations spanning 3 orders of magnitude. The API 5000™ LC/MS/MS system used in this experiment possessed a pulse counting detector and showed excellent reproducibility and accuracy at 10 fg (1fg/µL – 10 µL injection) of the compound on column. The other instrument used for comparison, possessed an analog detection system, and reported higher S/N ratios because the determined accuracy was considered to have at least 10x less sensitivity than the API 5000™ system.

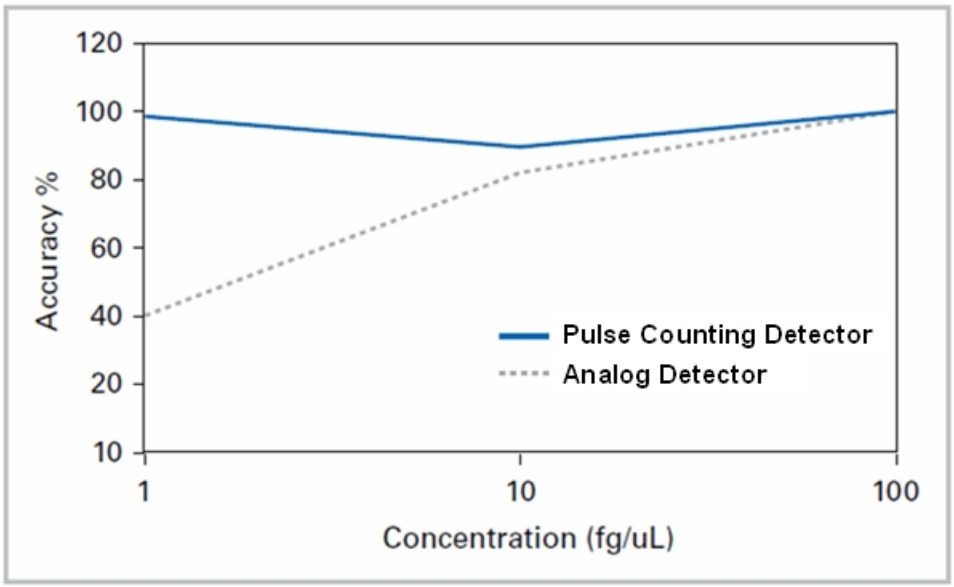

Figure 7. Accuracy Comparison at the LOQ between a Pulse Counting Detector and an Analog Detector. Replicate injections at each concentration level near the LOQ were performed on instruments with either a Pulse Counting detector or an Analog detector. The accuracy of the data points at each level were determined from the standard concentration curve and compared.

Summary

Method LOQs for an analyte are often estimated based on S/N ratios for a single injection. This method can be very misleading, and with analog detectors will suggest a much better LOQ than what can actually be achieved. This effect is compounded by the fact that smoothing can cause the S/N ratio for a thresholded signal to be significantly improved without any improvement in accuracy or reproducibility.

Proper method and instrument evaluation should be performed using multiple injections per concentration over a range of concentrations with at least one level at or near the LOQ. Accuracy for the concentration at the LOQ should be determined using a linear regression with no weighting. Any weighting will tend to artificially improve the accuracy close to the LOQ.

References

1. "Guidance for Industry - BioAnalytical Method Validation" May 2001, http://www.fda.gov/cder/guildance/index.htm
2. Viswanathan et al. (2007) "Workshop / Conference Report – Quantitative Bioanalytical Methods Validation and Implementation: Best Practises for Chromatographic and Ligand Binding Assays", AAPS Journal, 9(1), E30 – E42.

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