Detection Limits in Microbiology

by

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May 2001

A writing project submitted in partial fulfillment
of the requirements for the degree

Master of Science in Statistics

Introduction

History has dictated the need for detecting earthquakes, fires, pollutants in the environment, and many other natural and unnatural occurrences in the world (Currie, 1988). The need for detection is no less necessary in the chemical and biological laboratories than it is in the real-world. It is important to determine the extent to which laboratory techniques can detect small quantities.

Detection limits indicate how small a quantity is necessary to be considered immeasurable. For example, some analytical techniques can measure chemical concentrations as small as one part per billion, but other techniques cannot measure concentrations less than one part per million. Detection limits already have been established for many of the existing sciences and natural phenomenon.

The terminology associated with detection limits has, in the past, been confusing. Articles published and research done employing a specific detection limit may have been confusing to readers unfamiliar with that particular definition. This is analogous to a situation that often arises in statistics. Many journals site the mean as a measure of center for their data, but a definition of the mean may not be given. Readers are lead to believe that the mean is actually an average, when, occasionally, authors will utilize the median as the measure of center and call it the median. Just as the mean, or average, needs a precise mathematical definition, so too does the detection limit. Detection limits in chemistry have been established and utilized for the analytic chemistry field for years, but a precise, stable definition of the detection limit was not adopted until 1995.

Researching the literature on detection limits, terminology and concepts have sometimes been confusing. The detection limit also is named the limit of detection. In

addition, there are other limits that have been confused with the detection limit (or limit of detection), such as the detection decision and the quantification limit. These quantities are associated with true values of the quantity of interest and are not related with any particular estimated result, as is the detection limit (Currie, 1996). In 1988, Currie reported that a review of literature in the 1960's demonstrated that limit of detection definitions spanned almost three orders of magnitude when used to measure the same quantity.

Microbiologists often utilize detection in their field as well. They use the value of the detection limit to establish how sensitive their laboratory approach may be rather than using it to establish an estimate of the number of bacteria in a suspension containing bacteria. However, a universal definition of the detection limit for microbiological purposes has not yet been established. Because the detection limit is an important characteristic of a microbiological method, it would facilitate communication among microbiologists if there were one accepted definition of the detection limit.

The purpose of this paper is to review the detection limit defined in chemistry and apply the methods from the chemical definition to suggest a definition for use in microbiology.

Detection Limits in Chemistry

Detection limits have been widely discussed for decades, especially in the field of analytical chemistry. The theory of the detection limit began to take shape in the 1920's, but not until the 1960's did it begin to take precedence in research. Detection limits have been introduced into many different aspects of chemistry, including water

analysis and spectrochemical analysis. The most popular definitions for the limit of detection have been based on either hypothesis testing or probability estimation definitions.

In the 1960's, Kaiser used a hypothesis test to compute a detection limit in spectrochemical analysis (Kaiser, 1965). Kaiser discussed testing the null hypothesis that the sample taken is a blank versus the alternative that it is not a blank. If there are multiple samples, the null hypothesis is that the mean of the sample taken is the same as the mean of the blanks. He advocated the testing rule that the null hypothesis is rejected if the sample is more than three standard errors away from the blank mean (Kaiser,1965). Initially, he ignored the false negative error (Type II error), but, after realizing the importance of the error in the discussion of the detection limit, Kaiser began researching the topic more in depth (Currie, 1988). His research and discussion have led many others to think about the detection limit in a more critical manner. Many individuals recognize Kaiser's work in the detection field as ground-breaking and many still utilize his terminology for the limit of detection.

More recently, work in the field of detection limits has focused on probability definitions. In 1995, Currie published a definition of the detection limit that was accepted as the standard by the International Union of Pure and Applied Chemistry and the International Organization for Standardization (Currie, 1996). Currie's definition stems from his work in analytical and radiochemistry. This definition of the detection limit, or the minimum detectable value L_D, is the solution of Equation 1 for L_D, where the random variable $\hat{L}$ is the estimator of the quantity of interest, L is the true quantity

of interest, Lc is the "minimum significant *estimated value* of the quantity of interest", and β is the probability of producing false negatives.

$$\Pr[\hat{L} \leq Lc \mid L = L_D] = \beta . \qquad (1)$$

Note that the numerical value of Lc is established by expert opinion. In chemistry, the default value for β is 0.05 (Currie, 1996).

Detection Limits in Microbiology

The density (the number of bacteria per volume) of viable bacteria in a liquid is typically found by plate counting techniques. Plate counting can be done with the pour plate, the spread plate and the drop plate methods. The spread plate method is the most common. In the spread plate method, a beaker contains a suspension with an initial density of bacteria. A sample is taken from the beaker with a calibrated pipette and placed into sterile diluent because, generally, the initial density is too large to be counted. From this diluted sample, a portion is taken again with a calibrated pipette, placed onto an agar plate, and spread evenly with a miniature hockey stick. When the plate is incubated, the viable bacteria divide and form colonies that are non-overlapping and can be counted. The number of colonies, or colony forming units (cfu) of bacteria, are then counted on the agar plate. After the number of cfu's are counted, this count is scaled up to estimate the number of bacteria in the first dilution and then scaled up by the dilution factor to estimate the number of bacteria in the suspension.

The limit of detection issue arises if, after dilution, there are no colony forming units on the agar plate. This does not necessarily mean that the original suspension has zero bacteria. It is possible that there are bacteria in the suspension, but we have diluted our original suspension to the point in which there are no microbes in the sample

plated. For example, suppose it is known that there are one hundred colony forming units in a beaker of a 10 mL suspension. A sample of one mL taken from the original suspension is placed into a beaker containing 9 mL of sterile diluent. From this 10 mL, a one mL sample is spread onto an agar plate so that the colony forming units can be counted. However, suppose that the technician gets a count of zero cfu's on the agar plate. When the count of zero is scaled up, the estimated number of bacteria in the original sample would be zero (although we know there are 100 bacteria in the original sample). In this example, a detection limit is the number of bacteria necessary to be in the original sample in order to assure non-zero cfu's on the agar plate.

A "detection limit" for microbiology should be established to alleviate these problematic counts of zero and to give microbiologists a method to compare their laboratory sensitivities more precisely. Generally, a count of zero will occur after the first dilution if a count of zero was found for the first dilution. Therefore, we will focus on counts only at the first dilution when determining a detection limit.

Because analytical chemistry already has a universal detection limit definition, our strategy is to adapt the chemistry definition to fit microbiological plate count assays. That definition (Equation (1)), when adapted to counting cfu's, leads to Equation (2), where X_1 is a random variable denoting the number of bacteria counted at the first dilution, ID is the number of bacteria in the initial density of the beaker, β is specified by the microbiologist, and L_D is the limit of detection, which is the minimum of all L_D values that satisfy Equation (2).

$$P[X_1 = 0 \mid ID = L_D] \leq \beta \quad . \qquad (2)$$

Note that L_D depends on β. For a probability of 0.05 ($\beta = 0.05$), the L_D is the largest density of bacteria in the initial concentration for which we will have no more than a 5% chance of seeing zero cfu's at the first dilution.

We will now use Equation (2) to find actual detection limits. Suppose X_1 is a Poisson random variable with parameter Λ. Experience has shown that occasionally X_1 exhibits extra Poisson variability (Bliss and Fisher,1953). To model this variability, we let Λ be a random variable. Suppose that Λ is distributed as a gamma random variable with parameters μ and coefficient of variation CV. The coefficient of variation is the standard deviation of Λ divided by the mean of Λ. It is convenient to let $d = \frac{1}{CV^2}$. Then, the distribution of X_1 can be derived as in equations (3), (4), (5), and (6) using, for convenience, the parameters $\nu = d$ and $\tau = \frac{\mu}{d}$ (Bain and Engelhardt, 1987). Equation (6) shows that X_1 is distributed as a negative binomial random variable with mean $\mu = \nu\tau$ and variance $\nu\tau + \nu\tau^2 = \mu + \frac{\mu^2}{d}$. The term $\frac{\mu^2}{d}$ is the extra Poisson variability.

$$f_{\Lambda}(\lambda) = \frac{\lambda^{\nu-1} e^{-\lambda/\tau}}{\Gamma(\nu)\tau^{\nu}} \quad ; \tag{3}$$

$$f_{X_1|\Lambda}(x_1 \mid \lambda) = \frac{e^{-\lambda}\lambda^{x_1}}{x_1!} \quad ; \tag{4}$$

$$f_{X_1}(x_1) = \int_0^{\infty} f_{X_1|\Lambda}(x_1 \mid \lambda) f_{\Lambda}(\lambda) d\lambda \quad ; \tag{5}$$

$$f_{X_1}(x_1) = \frac{\Gamma(x_1 + \nu)}{\Gamma(\nu)x_1!}\left(\frac{\tau}{\tau+1}\right)^{x_1}\left(\frac{1}{\tau+1}\right)^{\nu} \quad . \tag{6}$$

Reparameterizing ($d = \nu$ and $\mu = \nu\tau$):

$$f_{X_1}(x_1) = \frac{\Gamma(x_1 + d)}{\Gamma(d)x_1!}\left(\frac{\mu}{\mu + d}\right)^{x_1}\left(\frac{d}{\mu + d}\right)^{d} . \qquad (7)$$

From the definition of the detection limit (Equation (2)), a specific detection limit per dilution factor can be found. Let $k \cdot L_D$ take the place of μ in (7). Then L_D is the smallest value satisfying equations (8) – (10).

$$P[X_1 = 0 \mid k \cdot L_D, d] = \left[\frac{d}{k \cdot L_D + d}\right]^{d} \le \beta \ , \qquad (8)$$

$$\Rightarrow \left(\frac{d}{k \cdot L_D + d}\right) \le \sqrt[d]{\beta} \ ; \qquad (9)$$

$$k \cdot L_D \ge \left(\frac{d}{\sqrt[d]{\beta}}\right) - d \ . \qquad (10)$$

If Λ does not vary, then $CV = 0$, $\lim_{d\to\infty} \frac{\mu^2}{d} = 0$, and X_1 is a Poisson $(mean = \mu)$ random variable. Thus, the Poisson case is derived in display (11).

$$\lim_{d\to\infty}\left(\frac{d}{k \cdot L_D + d}\right)^{d} = \lim_{d\to\infty}\left(1 + \frac{k \cdot L_D}{d}\right)^{-d} = e^{-k \cdot L_D} \Rightarrow k \cdot L_D \ge -\ln(\beta) . \qquad (11)$$

Detection limits for various coefficients of variation can be found in the Table (page 13). The Table shows limits of detection per dilution factor. In order to find the detection limit for a particular dilution factor, one must choose the desired probability of detection (β), specify the coefficient of variation (CV) for the bacterial suspension being analyzed, and the dilution factor. For example, suppose that one would like to have a probability of $\beta = 0.10$ seeing no cfu's at the first dilution. It is believed that, in the running of the experiment, the coefficient of variation is $CV = 0.2$ and it is known that

$k=100$. From the Table, we see that at $CV=0.2$ and $\beta=0.10$, $k \cdot L_D=2.41195$. To calculate L_D, multiply 2.41195 by $k=100$; then $L_D=241$ is the limit of detection. For this type of experiment, there must be at least 241 bacteria in the initial sample in order to have a 90% chance of seeing bacteria at the first dilution.

One popular operational definition of the limit of detection presently used by microbiologists can be found in the Table where $\beta=0.36788$. Under the Poisson model, this choice of β is equivalent to defining the limit of detection as the reciprocal of the dilution factor.

Discussion

For the Poisson model, the bacteria are assumed to be independently and randomly distributed. This is the ideal case, although in reality, bacteria are not always distributed in this way. Sometimes there will be loose clusters of bacteria that will develop in a dilution. When clusters are present, it is possible that a technician could get a sample from pipetting only in a cluster or perhaps not in a cluster at all. The clumping causes the average count of bacteria to vary from sample to sample. This will cause the coefficient of variation of Λ to be non-zero. When there is clumping in the initial density, the extra-variability incurred from sample to sample is extra-Poisson variability.

Another source of this extra-Poisson variability could occur from the variation in pipette volumes (Chase and Hoel, 1975). When we dilute the original sample and dispense a volume of the dilution via a pipette onto an agar plate, we are assuming that all of the volumes taken are the same. Technology has evolved so that we can be accurate when pipetting, but, of course, there will always be some error.

However, the coefficient of variation of Λ will be zero if the bacteria are disaggregated and perfectly mixed in the initial density. No clustering and no variation in pipette volumes suggest that there would be no additional variability and thus the detection limit will follow a Poisson distribution.

If there is extra-Poisson variability, we have assumed that Λ follows a gamma distribution. A consequence is that the counting variable X_1 follows a negative binomial distribution. If this is not the correct distribution, the calculations for the limit of detection may be incorrect. It is conventional, however, to use the negative binomial distribution to represent extra-Poisson variation in microbiology (Jones, Mollison and Quenouille, 1948).

In 1996, Currie proposed precise mathematical definitions for detection and quantification limits. Also he provided equations for "the very special circumstances where the distribution of $\hat{L}$ can be taken as Normal." By suggesting that the normal calculations are one distribution used in chemistry, he implied that other distributions are used to calculate detection limits. He left readers to decide what distribution is best for their purposes. This indicates how even in the chemistry field, the definition of the detection limit is often dependent on expert opinion concerning the probability model. It will be necessary for microbiologists to estimate the coefficient of variation for their particular microbiological process. The technician may have to run their experiment several times to collect data and then find estimates of the mean, standard deviation, and coefficient of variation. Some processes have been run for many years and so estimates already exist. Expert opinion also will be useful to obtain these estimates.

Further work in the area of detection limit could include determining a detection limit for the upper portion of counts. For example, suppose that a technician observes too many bacteria to count at every dilution. This is generally reported as "too numerous to count." This upper detection limit could be treated with the same approach as we have discussed for counts at the first dilution.

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Acknowledgements
Robin Gerlach kindly translated the German language paper by Kaiser.

Table. Values of $k \cdot L_D$ for several combinations of CV and β. (Notation defined in text.)

CV	$d = \frac{1}{CV^2}$	β = 0.05	β = 0.10	β =0.15	β = 0.20	β = 0.25	β =0.30	β =0.35
0.9	1.23457	12.74035	6.73647	4.50502	3.31197	2.56019	2.03919	1.65491
0.8	1.56250	9.06618	5.25806	3.69915	2.81434	2.23186	1.81397	1.49676
0.7	2.04082	6.81663	4.26591	3.12952	2.44973	1.98462	1.64060	1.37277
0.6	2.77778	5.38933	3.58574	2.72149	2.18045	1.79773	1.50705	1.27575
0.5	4.00000	4.45897	3.11312	2.42743	1.98140	1.65685	1.40480	1.20047
0.4	6.25000	3.84357	2.78400	2.21653	1.83566	1.55207	1.32776	1.14314
0.3	11.11111	3.43846	2.55854	2.06870	1.73184	1.47649	1.27162	1.10102
0.2	25.00000	3.18261	2.41195	1.97096	1.66237	1.42545	1.23343	1.07218
0.1	100.00000	3.04106	2.32930	1.91523	1.62246	1.39595	1.21125	1.05535
0	Poisson	2.99573	2.30259	1.89712	1.60944	1.38629	1.20397	1.04982

CV	$d = \frac{1}{CV^2}$	β = 0.367879	β = 0.40	β =0.45	β =0.50	β =0.55	β = 0.60	β =0.65
0.9	1.23457	1.54063	1.35869	1.12272	0.92989	0.76908	0.63273	0.51550
0.8	1.56250	1.40075	1.24618	1.04224	0.87239	0.72830	0.60422	0.49602
0.7	2.04082	1.29044	1.15657	0.97726	0.82540	0.69462	0.58044	0.47962
0.6	2.77778	1.20369	1.08550	0.92511	0.78729	0.66705	0.56081	0.46598
0.5	4.00000	1.13610	1.02973	0.88379	0.75683	0.64482	0.54488	0.45484
0.4	6.25000	1.08444	0.98686	0.85176	0.73304	0.62736	0.53228	0.44598
0.3	11.11111	1.04638	0.95513	0.82790	0.71522	0.61421	0.52275	0.43924
0.2	25.00000	1.02027	0.93329	0.81140	0.70285	0.60504	0.51608	0.43452
0.1	100.00000	1.00502	0.92050	0.80170	0.69556	0.59963	0.51213	0.43171
0	Poisson	1.00000	0.91629	0.79851	0.69315	0.59784	0.51083	0.43078