General
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Method Validation

INTRODUCTION

This document outlines the set of experimental procedures needed to validate a method that measures specific constituent(s) or characteristic(s) in a specific matrix or specific matrices. Method development generally requires three steps: 1) Determination of the Method Detection Limit (MDL), 2) Determination of the Recovery by analyzing independently prepared unknown samples, and 2) A Holding Time Study. Depending on the constituent or characteristic of interest, the matrix, or the necessary instrumentation, other tests and analyses may be needed. This document does not give guidelines about how to develop the actual steps of the method, but how to validate a method once it has been developed. Methods developed at the Water Sciences Laboratory need to be both robust and accurate. Following this general method validation procedure will assist in ensuring that new methods meet the rigorous standards required at the WSL. The actual steps in the method should be determined by the analyst, and are usually based on previous experience, other similar methods, and peer-reviewed scientific papers.

INSTRUMENT DETECTION LIMIT (IDL)

The Instrument Detection Limit (IDL) is defined as the minimum mass amount (e.g. ng or pg quantities) of a substance that can be measured on the instrument and reported with 99% confidence that the constituent amount is greater than zero. It is determined by analyzing a low-level standard containing the constituents(s) of interest at least 8 times, calculating the standard deviation of the measured concentration, and then multiplying by the appropriate student t value. The injected volume is used to calculate the mass of constituent(s) injected from the known concentration of the standard. Typically, the lowest standard of the instrument calibration curve is used to determine the IDL.

- Analyze eight or more injections of the low calibration standard solution.
- From the determined values, calculate the standard deviation of the eight or more samples. See the Data Reduction section for statistical formulas.
- Multiply the standard deviation of the eight or more samples by the appropriate student t value. See Data Reduction section for the relevant formula. See Student t Values Table for t values. Select the value of t for n – 1 degrees of freedom at the 99% confidence level. If eight replicates have been run, the student t value is 2.998.

METHOD DETECTION LIMIT (MDL)

The Method Detection Limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the constituent concentration is greater than zero. It is determined by taking samples containing the constituent(s) of interest and processing them through the

complete proposed analytical method, calculating the standard deviation of the values, and then multiplying by the appropriate student t value.

- Fortify a suitable quantity of matrix (reagent water, sand, etc.) with target compound(s) as to be able to run eight or more samples of known concentration at a level near the estimated MDL. (This can be estimated by consulting the MDL values listed in other similar methods, or by multiplying the blank/noise for the method by three to five.)
- Analyze eight or more portions of this solution by processing it through all the steps of the proposed method over a period of at least three days. (Processing over three or more days ensures the MDL determination is more representative than measurements performed sequentially.)
- From the determined values, calculate the standard deviation of the eight or more samples. See the Data Reduction section for statistical formulas.
- Multiply the standard deviation of the eight or more samples by the appropriate student t value. See Data Reduction section for relevant formulas. See Student t Values Table for t values. Select the value of t for n – 1 degrees of freedom at the 99% confidence level. If eight replicates have been run, the student t value is 2.998.

RECOVERY

The recovery of the target compound(s) in a matrix is the measured value obtained in a fortified sample divided by the calculated concentration. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for the extracted samples at a concentration representative of the concentration of the unknown samples. Known samples can be prepared in the analysts laboratory using either purchased analytical grade reagents, or standards available from the National Institute of Standards and Technology (NIST).

- Prepare or obtain (i.e. from NIST) a sample(s) containing a known amount of the constituent(s) of interest in the appropriate matrix.
- Process the sample(s) through the proposed method.
- Compare the results with the initial known concentration of the sample by dividing the average of the determined results by the initial known concentration, and multiplying by one-hundred. See Data Reduction section for the formula.

HOLDING TIME STUDY

Short Term Stability

Three samples of known concentration should be warmed to room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed. The recovery should then be determined as stated in the Recovery section.

Long Term Stability

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection, and the date of last sample analysis. Long-term stability should be determined by storing at least three samples of known concentration under the same conditions as the study samples. The volume of samples should be sufficient for analysis on three separate occasions. Test the stability of samples on three separate occasions spaced equally apart, for the expected duration of the study. The recovery of the stability samples should then be determined as stated in the Recovery section.

CALIBRATION CURVE/STANDARD CURVE/CONCENTRATION-RESPONSE (IF APPLICABLE)

A calibration curve of a blank sample (matrix sample processed without internal standard, if applicable), a zero sample (matrix sample processed with internal standard, if applicable), and three to eight non-zero samples covering the expected range. The simplest model that adequately describes the concentration-response relationship should be used. Selection of weighting and use of a complex regression equation should be justified. The following conditions should be met in developing a calibration curve:

- 20% deviation of the low standard from nominal concentration
- 15% deviation of standards other than LLOQ from nominal concentration

At least two-thirds of the non-zero standards should meet the above criteria, including the low standard and the calibration standard at the highest concentration. Excluding the standards should not change the model used.

SPECIFICITY (FOR COLUMN CHROMATOGRAPHY)

Specificity is the ability to measure accurately and specifically the constituent(s) of interest in the presence of other compounds that may be expected to be present in the same matrix. It is a measure of the degree of interference from such things as other active ingredients, excipients, impurities, and degradation products, ensuring that a peak response is due only to a single component; that is, that no co-elutions exist. Specificity is divided into two separate categories:

- **Identification Tests:** For identification purposes, specificity is demonstrated by the ability to discriminate between compounds of closely related structures, or by comparison to known reference materials.
- **Assay/Impurity Tests:** For assay and impurity tests, specificity is demonstrated by the resolution of the two closest eluting compounds. These compounds are usually the major component or active ingredient, and an impurity. If impurities are available, it must be demonstrated that the assay is unaffected by the presence of spiked materials (impurities and/or excipients). If impurities are not available, the tests results are compared to a second well-characterized procedure. For assay tests, the two results are compared; for impurity tests, the impurity profiles are compared head to head. See Data Reduction section for resolution equation. Baseline resolution is achieved when $R_S = 1.5$.

DATA REDUCTION

Method Detection Limit Calculations

$$StandardDeviation(S) = \sqrt{\frac{1}{n-1} * \left[\frac{\sum_{i=1}^{n} X_i^2 - \left(\sum_{i=1}^{n} X_i\right)^2}{n}\right]}$$

$$MethodDetectionLimit(MDL) = t_{99\%} * S$$

Recovery Calculations

$$Recovery(\%) = \left(\frac{\left(\frac{\sum_{i=1}^{n} X_i}{n}\right)}{X_{Actual}}\right) * 100$$

Where:
X_i is the method determined concentration for the known sample.
n is the number of replicates of the known sample.
X_{Actual} is the calculated concentration of the fortified sample.

Resolution

$$R_S = \frac{2\left(t_{R,B} - t_{R,A}\right)}{W_A + W_B}$$

Where:
$t_{R,A}$ and $t_{R,B}$ are the retention times of species A and B.
W_A and W_B are the peak widths of species A and B.
R_S is the resolution, and baseline resolution is achieved when R_S =1.5.