METHOD VALIDATION

What is Method Validation?

Method validation is the process of proving that an analytical method is acceptable for its intended purpose.

- verifying that a method is fit-for-purpose, i.e suitable for solving a particular analytical problem
- The process consists of the establishment of the performance characteristics and limitations of the method.

Method validation is necessary in analytical laboratory to **ensure** that **reliable analytical procedures** are used under the defined conditions.

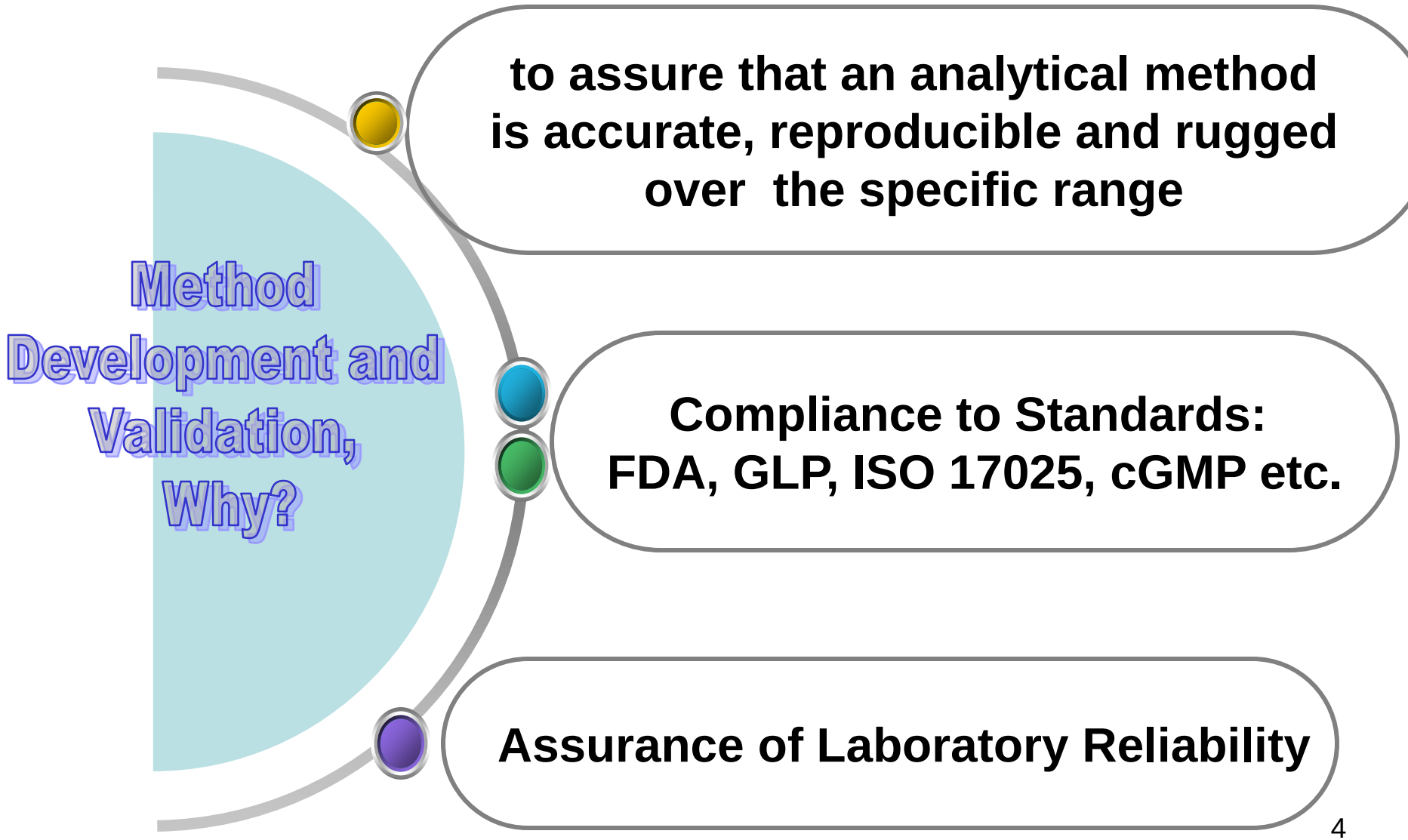
Why Method Validation is Necessary?

- To increase the value of test results
- To justify customer’s trust
- To trace criminal
- To prove what we claim is true

Examples:

- To value goods for trade purposes
- To support health care
- To check the quality of drinking water

Method Development and Validation



FDA-Food and Drug Administration is an agency of the United States Department of Health and Human. The FDA is responsible for protecting and promoting public health through the regulation and supervision of food safety, tobacco products, dietary supplements, prescription and over-the-counter pharmaceutical drugs (medications), vaccines, biopharmaceuticals, blood transfusions, medical devices, electromagnetic radiation emitting devices (ERED), veterinary products, and cosmetics.

cGMP – current good manufacturing practice

cGMPs provide for systems that assure proper design, monitoring, and control of manufacturing processes and facilities. Example **human pharmaceuticals**. Consumers expect that each batch of medicines they take will **meet quality standards** so that they will be safe and effective.

GLP- good laboratory practice

Outline

1

Quality in Analytical Laboratory

2

Method Development and Validation

3

Method validation selected samples

METHOD VALIDATION

Validation of an analytical method is primarily concerned with:

- the **identification** of the sources of potential errors.

- **quantification** of the potential errors in the method.

METHOD VALIDATION

A method that is valid in one situation could be invalid in another situation.

Validation of analytical procedures is the process of determining the suitability of a given methodology for providing useful analytical data.

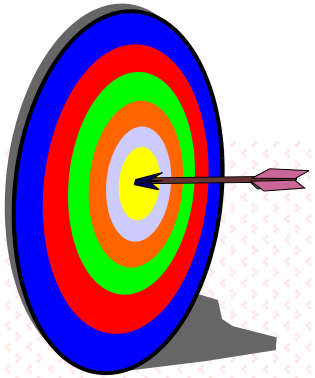
The process of the method validation begins with the planned and systematic collection by the application of the validation data to support the analytical procedures

METHOD VALIDATION

Methods need to be validated or revalidated:

- **before their introduction into routine use.**
- **whenever the conditions change for which the method has been validated, e.g., instrument with different characteristics.**
- **whenever the method is changed, and the change is outside the original scope of the method.**

Method Validation



Method validation data submitted should INCLUDE the following issues:

Method Validation

- Accuracy
- Precision
- Limit of Detection (LOD)
- Limit of Quantitation (LOQ)
- Specificity
- Linearity and Range
- Ruggedness/Robustness
- System Suitability

ACCURACY

Accuracy is the measure of exactness of an analytical method, or the closeness of agreement between the measured value and the value that is accepted as a conventional true value or an accepted reference value.

The true value for accuracy assessment can be obtained in several ways:

(i) compare results of the method with results from an established reference method.

(ii) assessed by analyzing a sample with known concentrations, for example, a certified reference material, and comparing the measured value with the true value as supplied with the material.

(iii) If such certified reference material is not available, a blank sample matrix of interest can be spiked with a known concentration by weight or volume and determined its recovery.

ACCURACY (continued)

The determination of Accuracy usually requires a "**gold standard**" or an **accepted method** to which a new method can be compared.

Accuracy assessment is normally **calculated as Percent Recovery (%R)** and is done by analyzing Matrix Spikes.

$$\% \text{ Recovery} = \frac{\text{amount detected}}{\text{amount spike}} \times 100$$

12

METHOD VALIDATION : ACCURACY

The concentration should cover the range of concern and should particularly include one concentration close to the quantitation limit.

Analyte recovery at different concentrations

Active Ingred. [%]	Analyte ratio	Unit	Mean recovery [%]
100	1	100%	98-102
>=10	10^{-1}	10%	98-102
>=1	10^{-2}	1%	97-103
>=0.1	10^{-3}	0.1 %	95-105
0.01	10^{-4}	100 ppm	90-107
0.001	10^{-5}	10 ppm	80-110
0.0001	10^{-6}	1 ppm	80-110
0.00001	10^{-7}	100 ppb	80-110
0.000001	10^{-8}	10 ppb	60-115
0.0000001	10^{-9}	1 ppb	40-120

PRECISION

The Precision of a method is the **degree of agreement among individual test results**, when the procedure is applied repeatedly to multiple samplings of a homogeneous sample.

Precision is the **closeness of agreement between independent test results** obtained under stipulated conditions. It is a measure of random errors, and may be expressed as **repeatability** and **reproducibility**.

Precision is usually expressed in terms of standard deviation or **Relative Standard Deviation** percent (RSD). It is often expressed as a percentage.

The two most common precision measures are:

“repeatability”
“reproducibility”

14

PRECISION (continued)

How to measure the precision?

At least, **5 or 6 determinations of three different matrices**, at **2 or 3 different concentrations** should be done, and the relative standard deviation was calculated.

The acceptance criteria for precision **depend** very much on the **type of analysis:**

- in pharmaceuticals quality control, precision of better than 1 % RSD
- for biological samples (blood, urine) the precision is more like 10%-15%
- for environmental and food samples, the precision is very much dependent on the sample matrix, the concentration of the analyte and on the analysis technique. It can vary between 2% and more than 20%.

PRECISION (continued)

Precision under **"repeatability, r"** conditions:
same method on identical test items, in the same laboratory, by the same operator, using the same equipment, within short time intervals.
e.g: Within-day variability

It gives a **measure of variability** to be expected when a method is performed by a single analyst on the same equipment over a short timescale. Variability is expected when a sample is analyzed in duplicate

Precision under **"reproducibility, R"** conditions:
same method on identical test items, in different laboratories, with different operators, using different equipment.

Usually, the sample is analyzed by a number of laboratories for comparative purposes (Inter-laboratory excercise).

16

PRECISION (continued)

Reproducibility is the closeness of agreement between test results obtained with the **same method** on **identical test material** in **different laboratories** with **different operators using different equipment**.

The objective of conducting reproducibility is to verify that the method will provide the same results within different laboratories.

The **reproducibility** of an analytical method is determined by analyzing aliquots from homogeneous lots in different laboratories with different analysts and by using operational and environmental conditions that may differ from but are still within the specified parameters of the method (inter-laboratory tests).

PRECISION (continued)

Typical variations (factors) affecting a method's reproducibility:

- Differences in room temperature and humidity
- Operators with different experience and thoroughness
- Equipment with different characteristics, e.g. delay volume of an HPLC system
- Variations in material and instrument conditions, e.g. in HPLC, mobile phases composition, pH, flow rate of mobile phase
- Equipment and consumables of different ages
- Columns from different suppliers or different batches
- Solvents, reagents and other material with different quality

Intermediate Precision

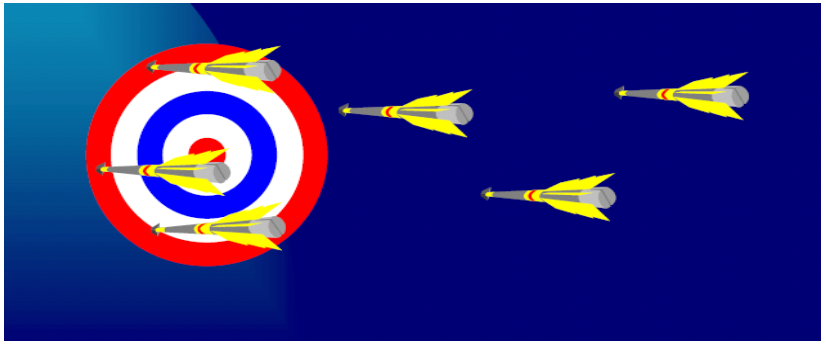
Normally for everyday laboratory practice,

Intermediate precision is performed by repeating testing on the different combinations of analyst, equipment, reagents and time within one laboratory. It is recommended to do 6-10 measurements on each of repeatability study.

For example, calculate the precision under repeatability conditions (same method, same analyst but different equipment; or same equipment same method but different analyst).

Requirements:
According to International Conference on Harmonisation (ICH), both repeatability and intermediate precision should be tested .

Evaluation of Precision



Measurement of **10 samples (n=10)** fo each concentration level.

Calculate mean, $\overline{X} = \frac{\sum X_i}{n}$

The **standard deviation** is a measure of how precise the average is,

Standard deviation, S = $\sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{(n-1)}}$

The **relative standard deviation (RSD)** is often times more convenient. It is always expressed as percentage (%)

RSD (%) = $\frac{100s}{\overline{X}}$

METHOD VALIDATION: PRECISION

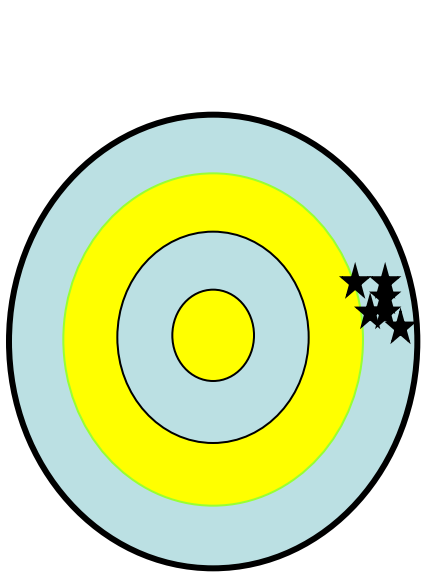
Estimated precision data as a function the of analyte concentration

Analyte concentration versus precision within or between days

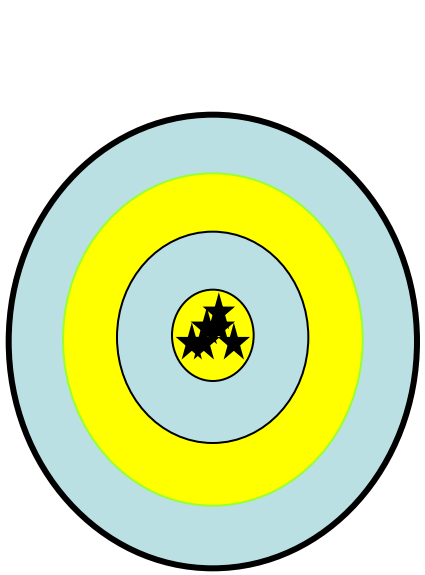
Analyte %	Analyte ratio	Unit	RSD (%)
100	1	100%	1.3
10	10^{-1}	10%	2.8
1	10^{-2}	1%	2.7
0.1	10^{-3}	0.1 %	3.7
0.01	10^{-4}	100 ppm	5.3
0.001	10^{-5}	10 ppm	7.3
0.0001	10^{-6}	1 ppm	11
0.00001	10^{-7}	100 ppb	15
0.000001	10^{-8}	10 ppb	21
0.0000001	10^{-9}	1 ppb	30

The lower the concentration of analyte, the higher the precision obtained

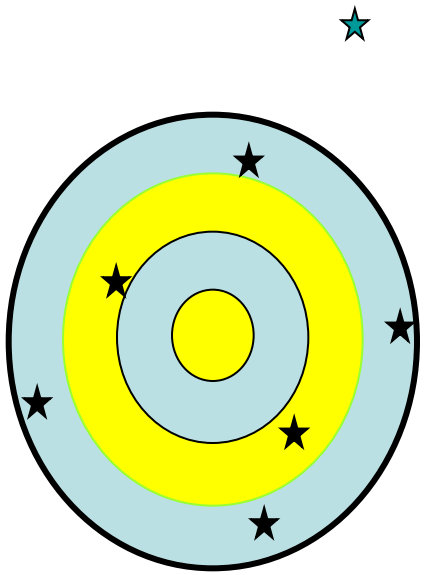
Accuracy and Precision



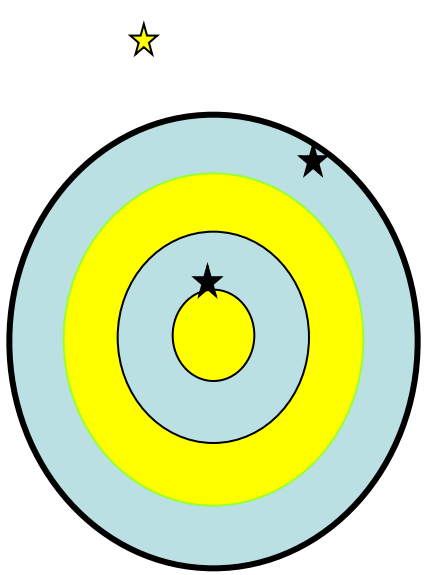
Low accuracy
High Precision



High Accuracy
High Precision



High Accuracy
Low Precision



Low Accuracy
Low Precision

LOD and LOQ

Limit of detection (LOD) and **limit of quantification (LOQ)** are two fundamental elements of method validation that define the limitations of an analytical method.

- **LOD** is the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified, under the stated conditions of the test.

- **LOQ** is the lowest concentration of an analyte in a sample that can be determined with **acceptable precision and accuracy** under the stated conditions of test

LOD (continued)

The Limit of Detection (LOD) of a method may be defined as the concentration of analyte which gives rise to a signal that is significantly different from the negative control or blank.

The LOD is the lowest concentration of analyte that can be distinguished from background.

The results obtained at the Limit of Detection are not necessarily Precise or Accurate or Quantitated.

LOD

Usually is expressed as concentration of analyte generating an instrument response equivalent to **three times the noise (S/N ratio ~ 3)**

In chromatography the detection limit is the injected amount that results in a peak with a height at least three times as high as the baseline noise level.

Methods in estimating LOD and LOQ:

- Signal-to-noise ratio (S/N)
- Blank determination
- Linear regression

Signal-to-noise ratio

The noise value was calculated based on the peak height of the blank (solvent) around the retention time of each analyte.

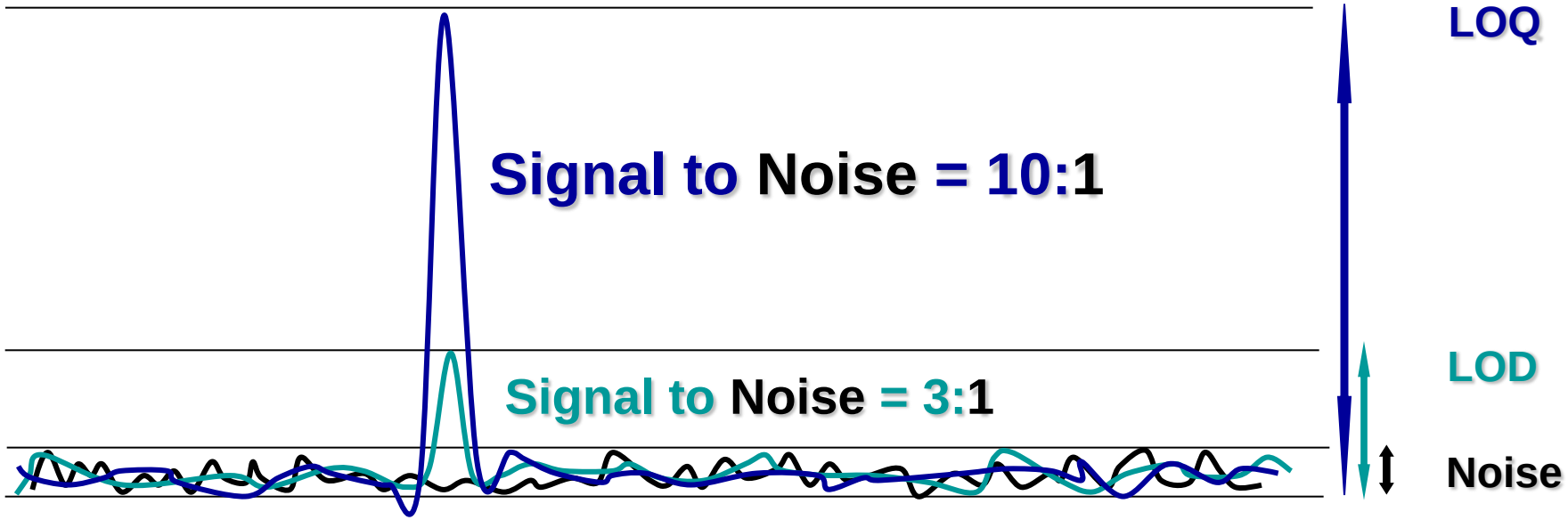
Procedure:

- Run blank and the lowest concentration of analyte
- Run 10 replicates independently
- Measure the peak height values (noise magnitude was measured by auto-integrator)
- Calculate LOD: S/N = 3, and

  LOQ: S/N = 10

Analytical Method Development

LOD, LOQ and Signal to Noise Ratio (SNR)



Blank Determination

- Analysis of independent sample blanks (10 replicates), and calculate the mean concentration and the standard deviations of the blank results
- $LOD \cong \bar{x}_{bi} + 3s_{bi}$

  $LOQ \cong \bar{x}_{bi} + 10s_{bi}$

  $\bar{x}_{b_i} = \text{mean concentration of the blank}$
- $s_{b_i} = \text{standard deviation of the blank}$

Linear Regression

Based on linear calibration curve

$$y = a + bx$$

y: instrument response

x: analyte concentration

a: y-intercept

b: slope

Linear Regression

$$LOD \cong \frac{3s_a}{b}$$

$$LOQ \cong \frac{10s_a}{b}$$

s_a = standard deviation of the blank

b = slope of the calibration curve

Limit of Quantification (LOQ)

LOQ are the lowest concentrations of analyte in a sample or specimen that can be measured with an acceptable level of accuracy and precision.

A typical acceptable signal-to-noise ratio 1:10 (S/N=10)

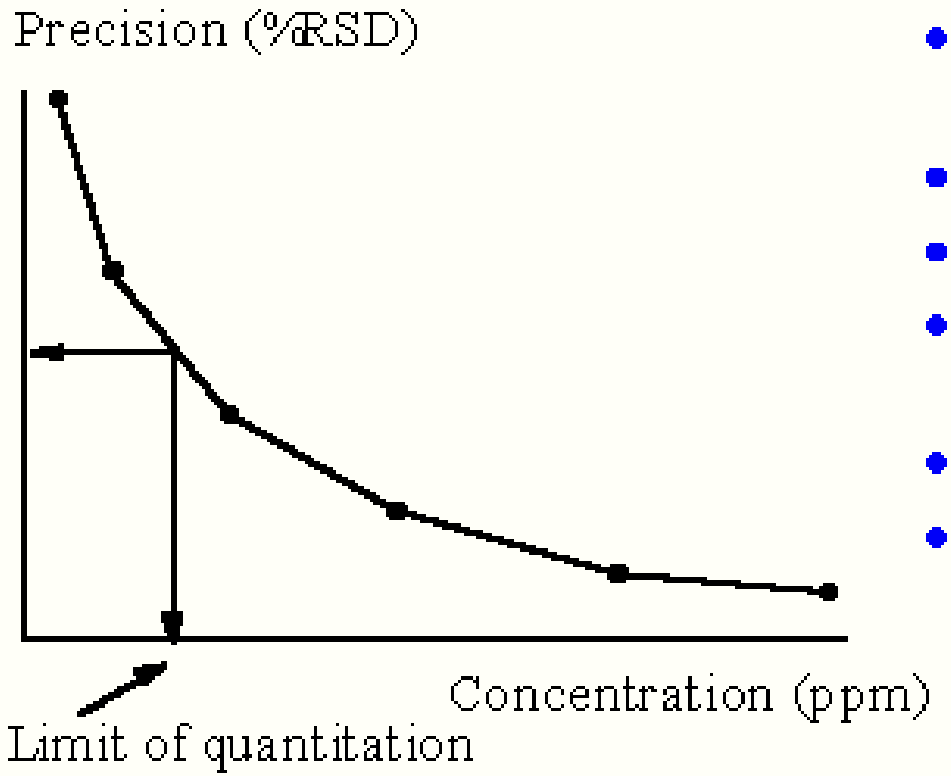
LOQ (continued)

To determine LOQ, if the required precision of the method at the limit of quantitation has been specified:

A number of samples with decreasing amounts of the analyte are injected six times.

The calculated RSD% of the precision is plotted against the analyte amount. The amount that corresponds to the **previously defined required precision** is equal to the limit of quantitation.

METHOD VALIDATION : LIMIT OF QUANTITATION



- Define expected precision for limit of quantitation
- Prepare spiked matrix sample
- Dilute and inject 6 times
- Calculate %RSD for each concentration
- Plot precision vs. concentration
- Concentration at expected precision = limit of quantitation

Limit of quantitation with the EURACHEM method.

SPECIFICITY/SELECTIVITY (continued)

The **Specificity** of a method defines the ability of the method to measure the analyte of interest **to the exclusion of other relevant components.**

Selectivity describes the ability of an analytical method to **differentiate various substances in a sample**.

Specificity

Degree to which the measured response is due to the analyte of interest and not to other substances expected to be present in the sample matrix.

SPECIFICITY/SELECTIVITY

Sometimes, it is difficult to identify a compound by using one test alone. Therefore, another **method** needs to be used to give more information.

Example:

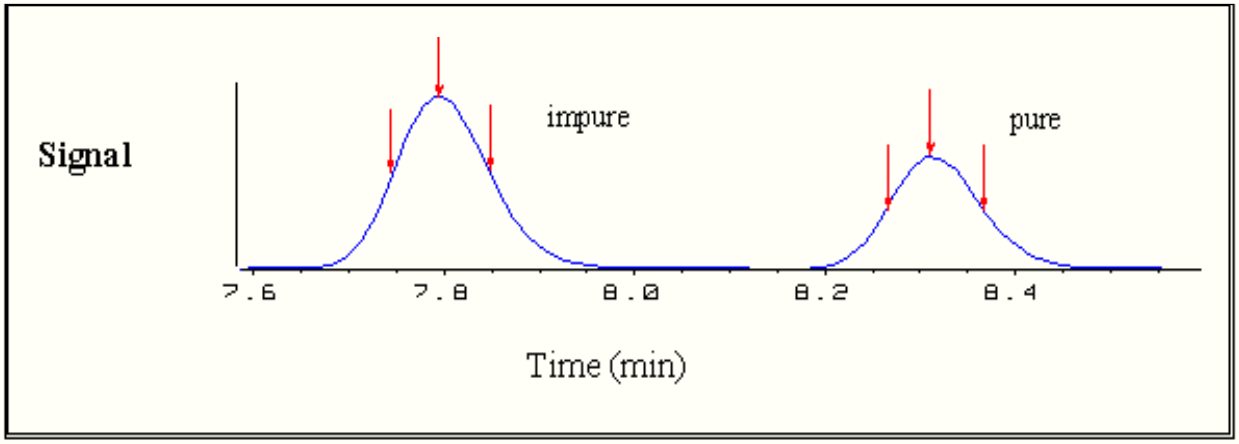
UV/Vis spectra of a drug molecule is used for identification purposes, however, the UV spectra of the drug is the same as the UV spectra of some drug impurities. Thus, the **UV/Vis method on its own is not specific.**

But, by combining UV/Vis with another method ie. HPLC, the compound of interest was resolved from any other components of the sample and that the peak of interest wasn't overlapped with another interfering peak

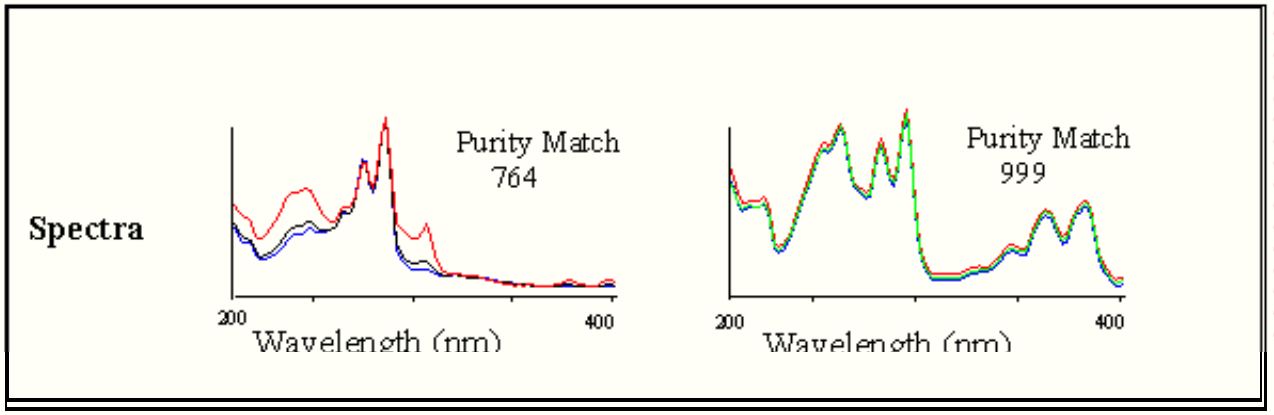
Other examples: Application of GC/MS, LC/MS

SPECIFICITY/SELECTIVITY (continued)

Examples of pure and impure HPLC peaks



The chromatographic signal does not indicate any impurity in either peak. Spectral evaluation, however, identifies the peak on the left as impure.



METHOD VALIDATION: LINEARITY AND RANGE

LINEARITY

The linearity of a method is **its ability to provide measurement results that are directly proportional to the concentration of the analyte**, or are directly proportional after mathematical transformation.

RANGE

The **range** of the method is the area **between the lower and the upper limits** of quantitation that is also linear.

Within the range of the method, results are accurate, precise and “linear”.

LINEARITY AND RANGE (continued)

HOW TO DETERMINE LINEARITY AND RANGE?

(i) **Five concentration levels** are required to allow detection of curvature in the plotted data. Standard should be prepared and analysed a minimum of three times. Measure the responses.

(ii) **Plot the graph** analyte conventration vs responses.

(iii) The line generated should be submitted, together with slope, intercept and correlation co-efficient data.

LINEARITY AND RANGE (continued)

HOW TO DETERMINE LINEARITY AND RANGE?

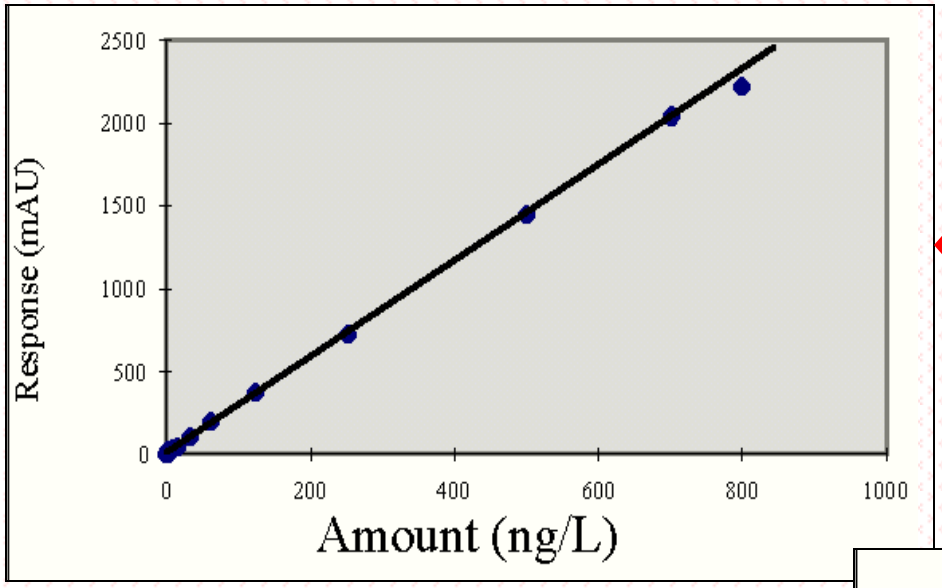
The measured slope should demonstrate a clear correlation between response and analyte concentration.

Acceptability of linearity data is often judge by examining the correlation coefficient r^2 and y-intercept of the linear regression line for the response versus concentration plot.

($r > 0.999$ is generally considered as evidence of acceptable fit of the data to the regression line) over the range (nominal ± 20%).

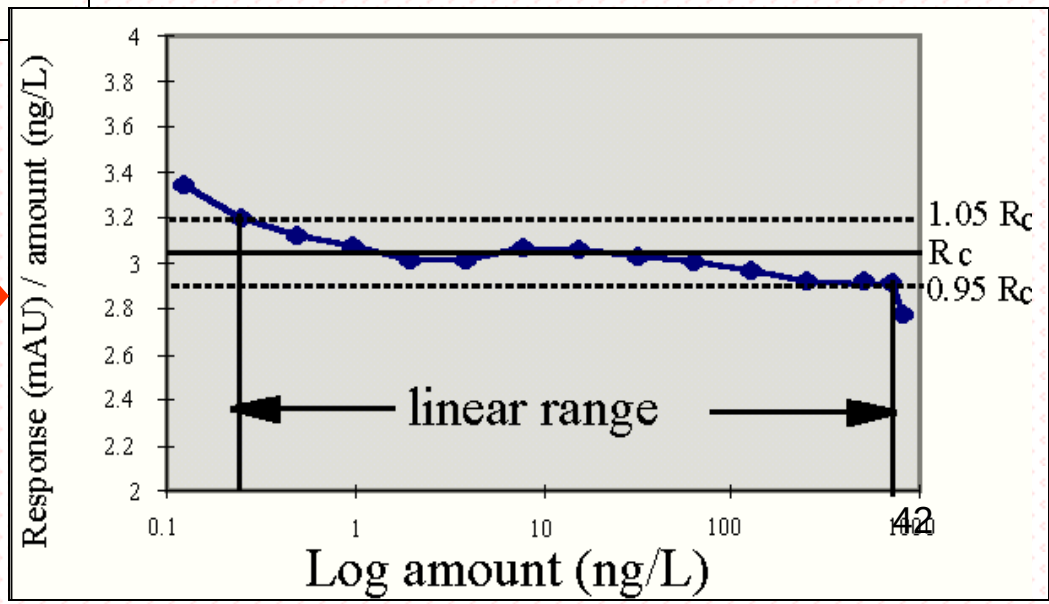
LINEARITY AND RANGE

Graphical presentations of linearity plot of a caffeine sample using HPLC.



Plotting the sensitivity (response/amount) gives clear indication of the linear range.

Plotting the amount on a logarithmic scale has a significant advantage for wide linear ranges. Rc = Line of constant response.



ROBUSTNESS/RUGGEDNESS

Robustness can be defined as a measure of its capability to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

- (involves internal factors to the method)

Ruggedness of a method is the degree of reproducibility of test results obtained by the analysis of same samples under a variety of conditions, such as different laboratories, analysts, instruments, different lot of reagents, days etc.

- (involves external factors to the method)

ROBUSTNESS/RUGGEDNESS (continued)

Ruggedness is the of reproducibility of the test results obtained for identical samples under normal (but variable) test conditions eg: different lab, analyst, assay temperature …..

- involves external factors to the method

The Robustness of a procedure is a measure of its capacity to remain unaffected by small but deliberate variations in the method parameters and provides an indication of its reliability in normal usage eg: mobile phase composition, temperature, flow rate ….

- involves internal factors to the method

RUGGEDNESS /ROBUSTNESS (continued)

How to test the robustness of method:

Example:

Effects of the following changes in chromatographic conditions will be determined:

- Methanol content in mobile phase adjusted by + 2%
- Mobile-phase pH adjusted by + 0.1 pH units
- Column temperature adjusted by + 5^{o}C.

If these changes are within the limits that produce acceptable results, they will be incorporated in the method procedure or if the changes is insignificant, then the method is robust.

System Suitability

System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns.

To check system (equipment, electronics, samples, technique) is working properly before any samples are analysed

Parameters such as plate count, tailing factors, resolution and reproducibility (%RSD retention time and area for six repetitions) are determined and compared against the specifications set for the method.