An Introduction Method validation

Tim McDonald

University of Exeter Medical School

Royal Devon and Exeter NHS Foundation Trust

Exeter Clinical Laboratory International

Method validation

- DULL?
- You will be the people undertaking method validation
- What are the right experiments to do?
- UKAS expectations….
- FRCPath exams!
- Easy to do wrong

Method validation

Validation, is the proof needed to ensure that an analytical method can produce results which are reliable and reproducible and which are fit for the purpose intended. The parameters that need to be **demonstrated** are those associated with the '**Performance characteristics**' together with **robustness, repeatability and reproducibility.**

Many analytical methods appearing in the literature have not been through a thorough validation exercise and thus should be treated with caution until full validation has been carried out. Validation of a new method (new to your laboratory), is a costly and time-consuming exercise, however the result of not carrying out method validation could result in litigation, failure to get product approval, costly repeat analysis and loss of business and prestige.

Validation

Extent of validation

- New methods require complete validation
- Commercial (CE) methods require partial validation (or verification)
- Significant changes mean partial revalidation
  - *equipment changes*
  - *formula changed*
  - *changed suppliers of critical reagentS*

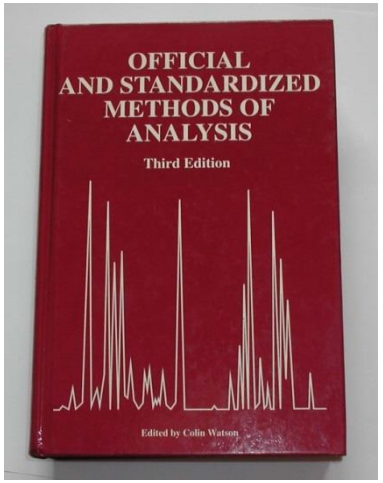
Fit for Purpose?

The cost of producing data can often be reduced by selecting analytical methods and technologies that produce data in accordance with the stated objectives for carrying out the analysis or test. Consider two examples:

The EU current statutory limit for lead in potable water is 25 µg/l. Thus the use of a method to perform the analysis, which only provides a 'less than' value of 100 µg/l, is not suitable for this purpose

A commercial limescale remover states that it contains between 5 to 15% w/v of sulphamic acid. Therefore any analysis for quality control purposes needs only to show that the quantity present is indeed between these two limits and does not need to be accurate to the nearest $\pm$ 0.1%

Choice of Method



In selecting a method you will need to consider the following parameters:

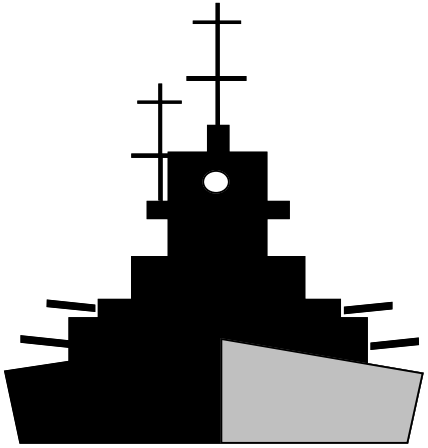
- sample type (matrix) and size (lot or a little)
- data required (qualitative/quantitative)
- expected level(s) of analyte(s)
- precision & accuracy expected
- likely interferences
- number and frequency of samples for analysis.

Method development and verification/validation

'Never attempt to re-invent the wheel!'

Before embarking on the development of a new method, always research the literature to see if a suitable one already exists. If a suitable one is found, it will still be necessary however to perform some method validation (or verification!) to prove that the method can be successfully adapted to **your laboratory, equipment and personnel**. More extensive validation is required for a brand new method(full validation).

Method validation - robustness



Robustness of an analytical method refers to it's ability to remain unaffected when subjected to small changes in method parameters.

For example

In an hplc analysis the mobile phase is defined in terms of % organic modifier, pH of the mobile phase, buffer composition, temperature etc. A perfect mobile phase is one which allows small changes in the composition without affecting the selectivity or the quantitation of the method.

Alter all major parameters in order to ascertain when the method ceases to function in accordance with specifications

Method validation - robustness

Characteristics of analytical procedures

- Ruggedness & Robustness
- Variability caused by:
  - Day-to-day variations
  - Analyst-to-analyst
  - Laboratory-to-laboratory
  - Instrument-to-instrument
  - Chromatographic column-to-column
  - Reagent kit-to-kit
  - Instability of analytical reagents

Method performance characteristics

A method's performance is defined by a number of important individual characteristics. There are:

Sensitivity **Precision**

Accuracy **Limit of Detection (LoD)**

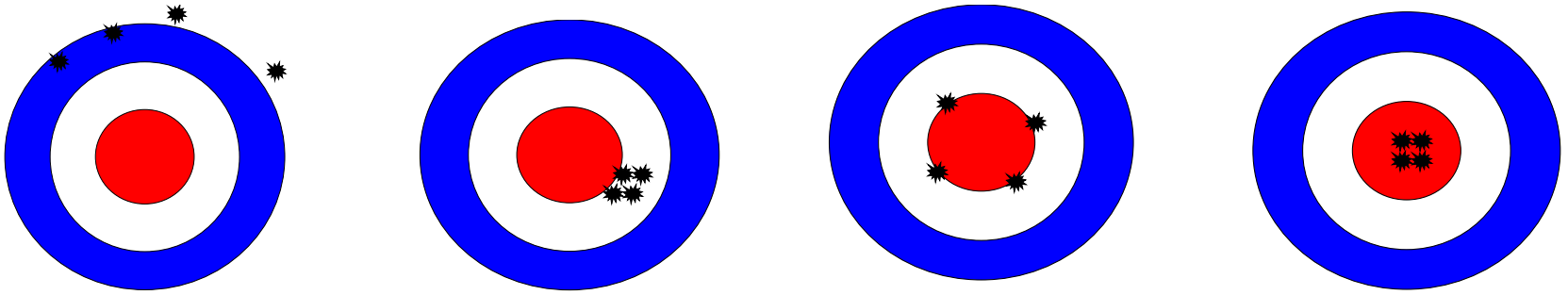
Limit of Determination **Bias**

Selectivity **Linear Range**

Dynamic range

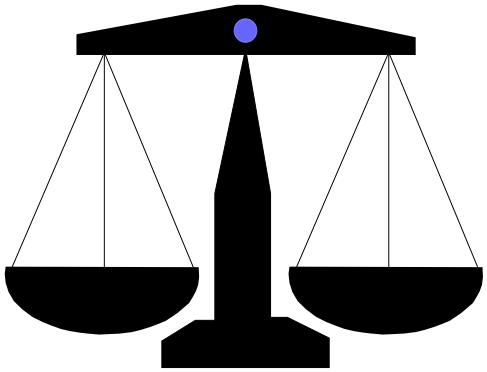
Accuracy and precision

The dictionary definition of both '**accuracy**' and '**precision**' are roughly the same, indicating that these words may be used synonymously. However in 'Analytical Science' they have two separate meanings, the difference between them is best illustrated by using target diagrams



Poor precision poor accuracy	Good precision poor accuracy	Good mean accuracy poor precision	Good accuracy good precision

Accuracy and precision (2)



A set of results can be either **accurate and/or precise** or can be neither **accurate nor precise**. Thus **accuracy** may be defined as:

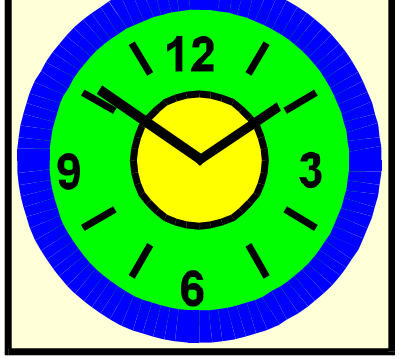
The closeness of the mean value from a replicate set of results to the true or accepted value

Precision may be defined as:

The spread of results from a replicate set of measurements

The difference between the true value and the mean measured value is termed **bias**. The spread of replicate data is measured in terms of **standard deviation (s) or variance (s^2)**

Intra and Inter assay reproducibility



. A replicate set of data produced at a particular **time point** by an **operator** working with a particular set of **equipment** in a given **laboratory** will verify **intra-assay reproducibility**. To show i**nter-assay reproducibility**, the method must produce similar results when **any** of these parameters are changed. The most likely changes are to time and operator.

Two different operators analysing milk using different pieces of equipment at different times. The laboratory is the same.

Random and systematic errors

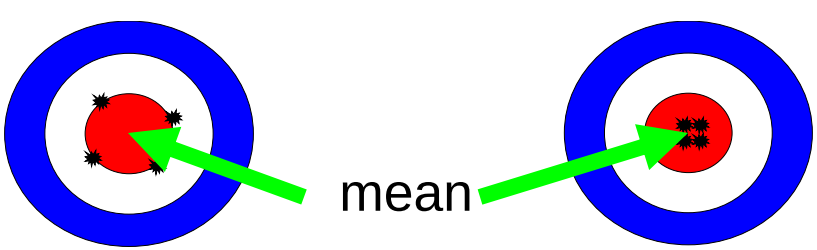
There are 2 types of error for which allowance may be made:

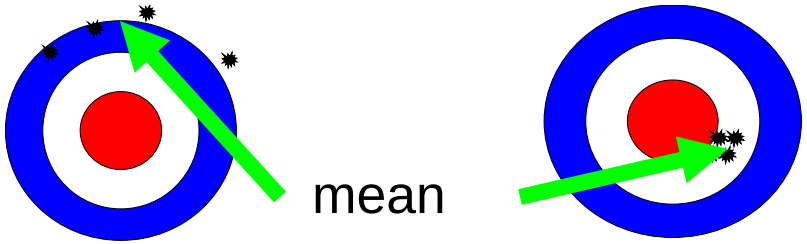
Random error

Random error arises from variations in parameters which are outside the control of the analyst, but which influence the value of the measurement being made. Because these errors are statistically random, the mean error should be **zero** if sufficient measurements are made.

Systematic error

Systematic error remains constant or may vary in a predictable way over a series of measurements and cannot be reduced by making replicate measurements. In theory, if known, this error can be allowed for.
Eg: subtraction of blank values

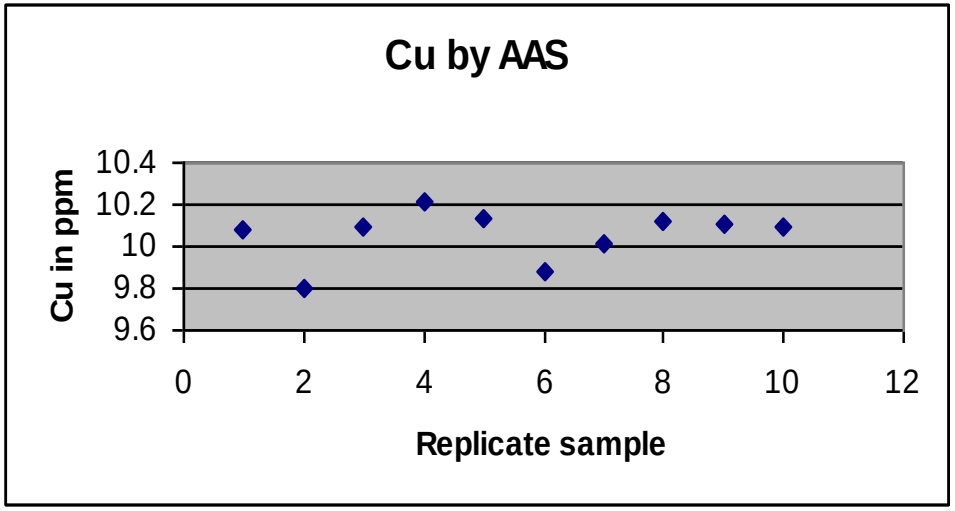




Bias and variance

A solution containing copper was analysed 10 times using atomic absorption spectroscopy.
The results obtained in ppm were:
10.08, 9.80, 10.10, 10.21, 10.14, 9.88, 10.02, 10.12, 10.11, 10.09

We can now calculate the precision of the data as **standard deviation**
If the true value is known to be 10.00 ppm, we can also calculate the **bias**



Bias = Mean value - true value
= 10.06 - 10.00
= **0.06 ppm**

Standard deviation (SD) = 0.12(4)

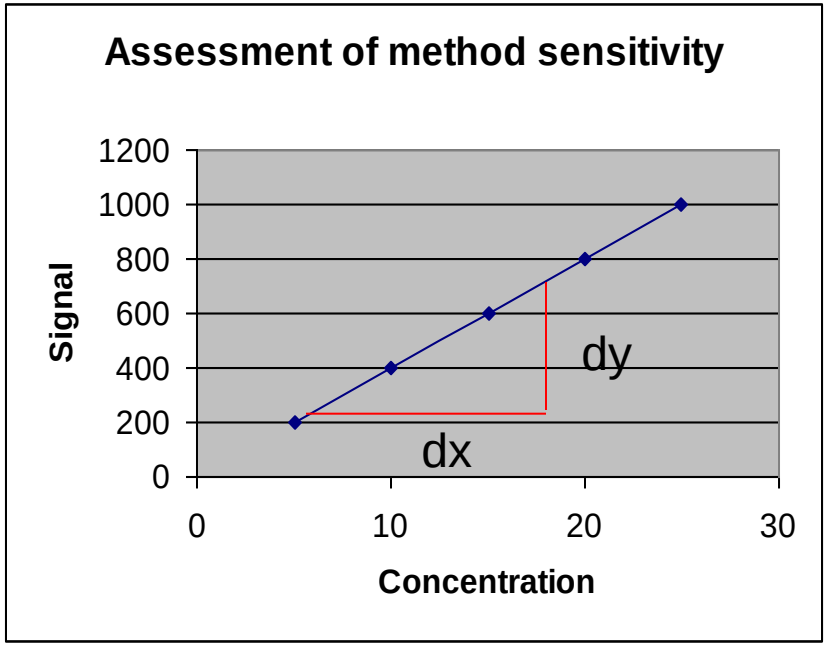
CV= (SD/10.00) x 100 = 1.2%

Conclusion - the method gives both good accuracy (low bias) and acceptable precision CV of 1.2%)

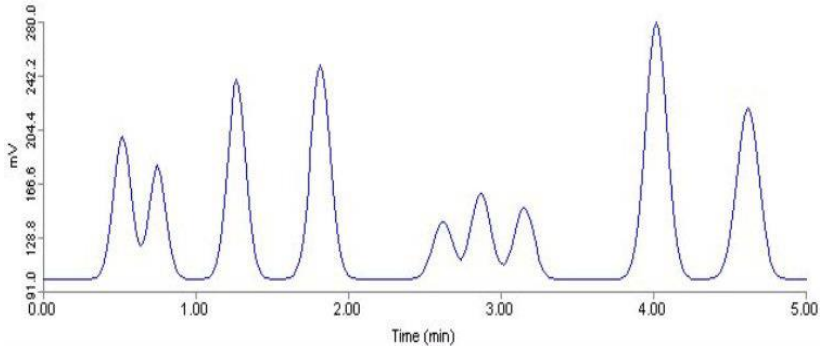
Sensitivity and selectivity

Sensitivity is the change in measured signal for unit change in concentration and can be obtained from the calibration graph

Sensitivity = dy/dx



Selectivity is the ability of a method to discriminate between the target analyte and other constituents of the sample. In many instances selectivity is achieved by high performance separation using chromatographic or electrophoretic techniques.



Hplc chromatogram

Limits of detection (LoD) and determination

These values refer to the statistical limits below which results of detection or accurate quantitative measurements (determination) should not be reported. The levels of both are dependent upon the variability of the signal when a blank containing none of the analyte is being measured. The signal generated under these conditions is mostly signal noise and is assumed to exhibit a normal distribution pattern. Both the **blank signal** and the **standard deviation of the blank signal** need to be measured. From this data we can calculate both limits.

Example: In an analysis of trace Mn by plasma emission spectrometry the following data were obtained:

- mean blank (Bl) signal 4
- SD of blank signal 12
- 500 ppb Mn 2000

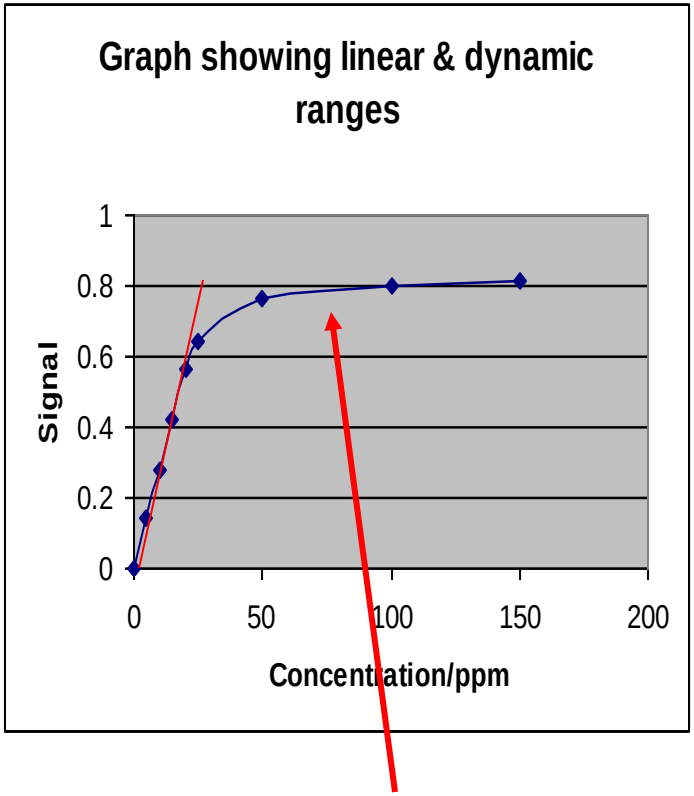
LoD = Bl signal + 3(SD of Bl)
= 4 + 3(12)
= 40

This equates to: [40/2000] X 500 ppb
= **10 ppb Mn**

The **limit of determination** uses a similar formula, replacing the 3 SD's by 10. This gives the limit of determination as **31 ppb Mn**

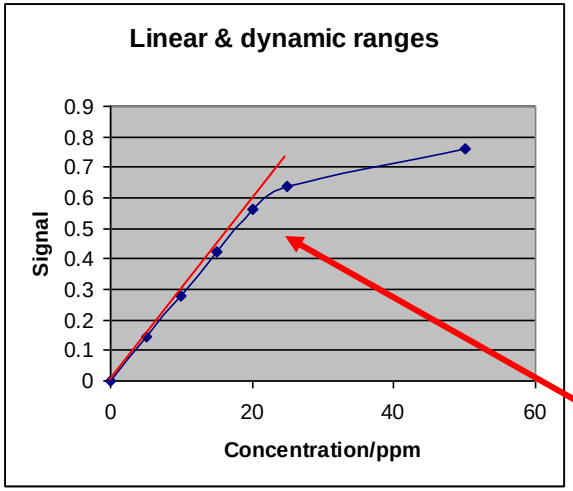
Linear and Dynamic ranges

These terms refer to the extent to which the method may be used to produce accurate quantitative data



Extent of dynamic range

From the graph, it would appear that the data is linear to about 25 ppm and dynamic until about 75 ppm. After 75 ppm there is only minimal increase in signal for increased concentration.



Expanding the lower section of the graph however, shows that non-linearity starts at around 20 ppm.

Top of linear range

Linear and Dynamic ranges Using reference material

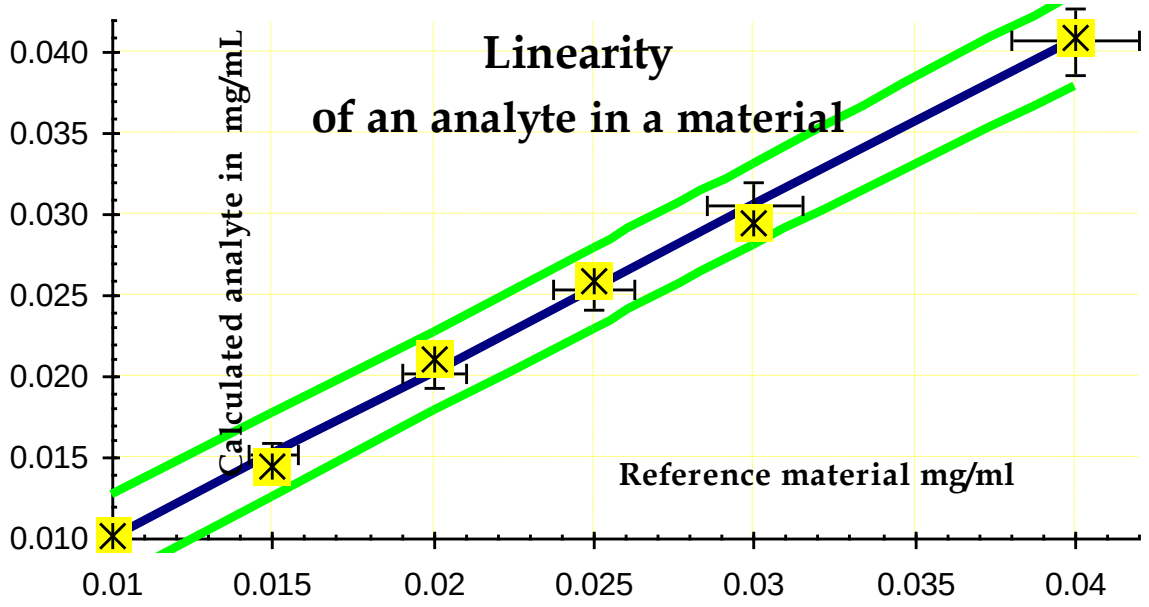


Table of values (x,y)

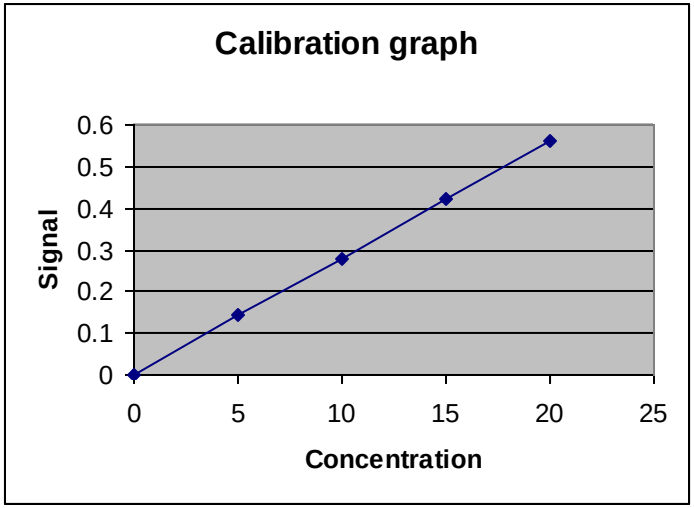
	x	y
#	Reference material mg/ml	Calculated mg/ml
1	0.0100	0.0101
2	0.0150	0.0145
3	0.0200	0.0210
4	0.0250	0.0260
5	0.0300	0.0294
6	0.0400	0.0410

Hook effect?

Method validation -

linearity

Check linearity between 50 - 150% of the expected analyte concentration



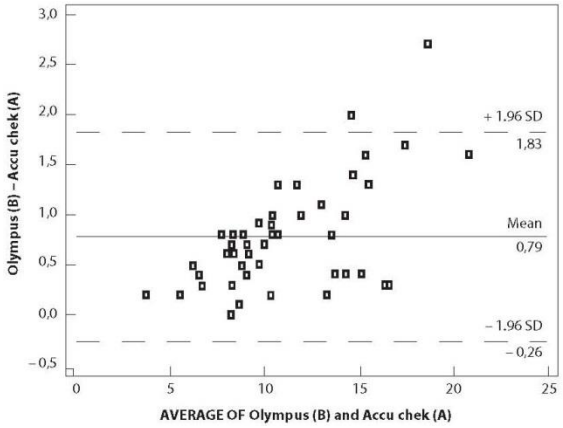
Linearity

Where possible calibration data should show a linear relationship between analyte concentration and measured signal, however it is acceptable under some circumstances, to use a non-linear relationship up to the limit of the dynamic range.

Method validation - specificity

Loss of specificity can be due to **interferences/ cross reactivity** and **matrix**

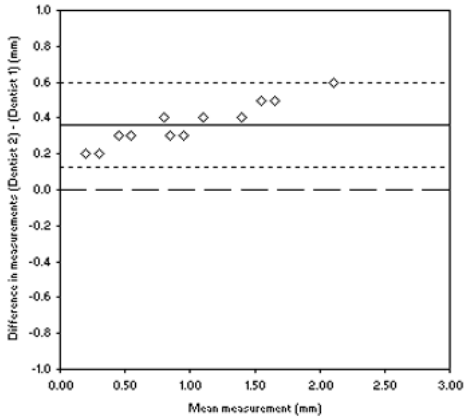
All likely interferences should be investigated and their effects on analyte response determined over a range of concentrations. Measures may then be put into place to **mask**, **eliminate** or **separate** them from the analyte.



Standard addition procedures can be used to identify matrix effects

Also:

- Spike and recovery
- Method comparison



Method validation -
What materials to use?

Selection of reference materials from LGC

The performance of an assay can be tested in a number of ways

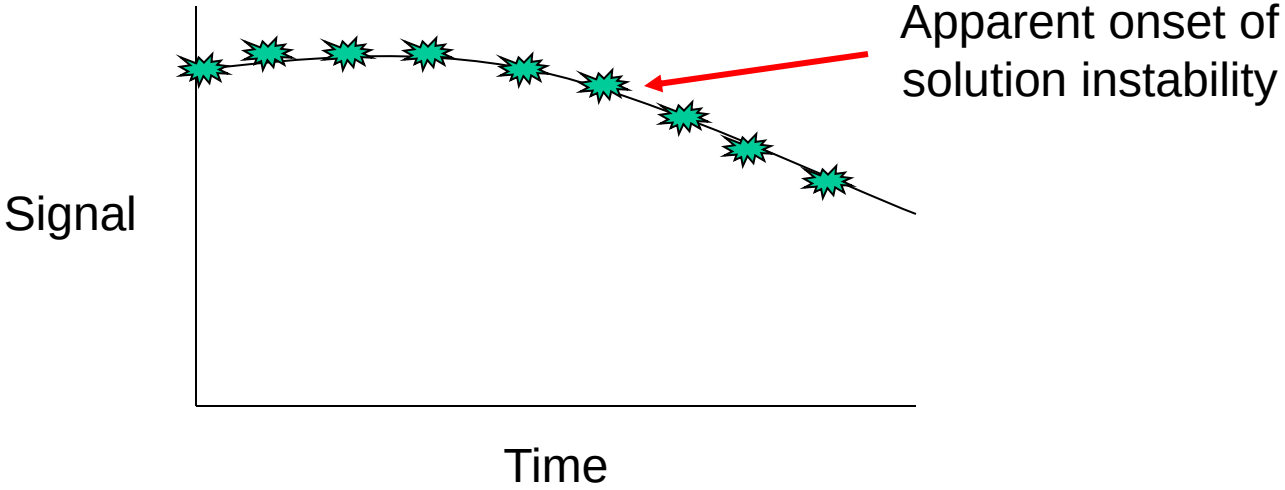
Test results from the new method against an existing method which is known to be accurate (patient samples)

The best way of demonstrating accuracy is to analyse a reference material or certified reference material (CRM) if one is available

Add a known quantity of pure analyte (spike) to a real sample or real sample matrix and check that all of the added substance can be measured (recovered)

Method validation - establish stability

In routine analysis where numerous samples and standards are measured each day, it is essential to assess the stability of the prepared solutions. Stability of these solutions should be tested by repeat analysis over at least a 48 hour period. What about patient samples?



Method validation - additional reading

- An article entitled “A Practical Guide to Analytical Method Validation” was published in Analytical Chemistry in 1996 [Anal. Chem. (68) 305A-309A]
- Westgard method validation
- ACB guidelines on verification (guide to number of samples etc)