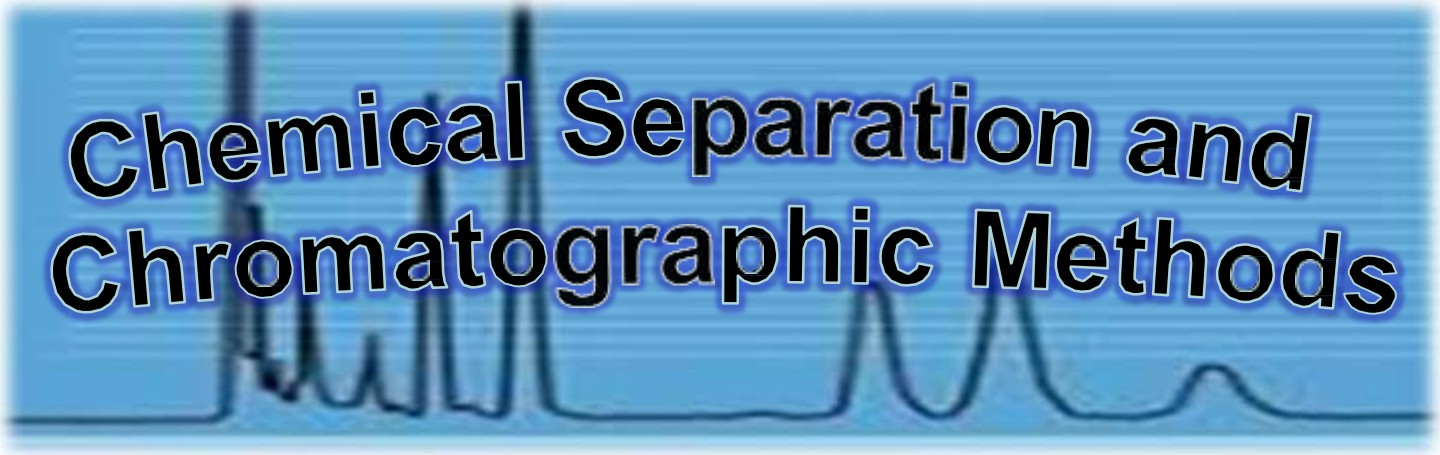


Analytical Performance & Method Validation

Validation is the process that helps establish, by laboratory studies, that the performance characteristics of a method meet the requirements for the intended application. It provides documented evidence that the method performs for the intended purpose.

The true test of any analytical technique is how well it works for real analysis where the samples may be much more complex and difficult to handle than the clean, idealized samples used in the initial evaluation of the method.

In order to develop a method effectively, most of the effort should be spent in method development and optimization as this will improve the final method performance. The method validation, however, should be treated as an exercise to summarize or document the overall method performance for its intended purpose.

Analytical chemistry deals with methods for determining the chemical composition of samples. A compound can often be measured by several methods.

The choice of analytical methodology is based on many considerations, such as chemical properties of the analyte and its concentration, sample matrix, the speed and cost of the analysis, type of measurements i.e., quantitative or qualitative and the number of samples.

Qualitative method yields information of the chemical identity of the species in the sample, while a quantitative method provides numerical information regarding the relative amounts of one or more of the species (the analytes) in the sample. Qualitative information is required before a quantitative analysis can be performed.

A separation step is sometimes a necessary part of both a qualitative and a quantitative analysis.

To help guarantee that a method is readily utilizable by any trained analyst, method validation has been defined by a number of scientific and regulatory bodied. Some documents were generated in close affiliation with governmental agencies [e.g., the United States Pharmacopoeia (**USP**) and United States Food and Drug Administration (**FDA**)], and some are the result of international cooperation between organizations [e.g., International Conference on Harmonization (**ICH**) and International Standards Organization (**ISO**)].

The intention of all the documents generated by these organizations is to give guidance to those analysts involved in the validation of a method. This guidance is meant to produce statistically verifiable and testable results, while at the same time allowing for as much scientific and flexible as possible.

Each parameter in method validation is generated from the statistical analysis (or the comparison of the parameter to an existing statistical limit) of the results generated during the validation run.

Accuracy (Bias)

The accuracy of an analytical method expresses the closeness of agreement between the obtained (by practical experiments) value and the value that is accepted either as a conventional true value or as an accepted reference value. Accuracy is expressed as the %recovery. Typically, it is requested to test three replicates per level at a minimum of three concentration levels covering the entire experimental range. The acceptance criteria are set for the assay range of the main compound and for the range of the impurities.

Accuracy provides a measure of the systematic error of an analytical method. The difference between the obtained result and the expected result is usually divided by the expected result and reported as a percent relative error. To establish the method accuracy, the validation is often run against a standard reference material.

Precision

The precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from multiple samplings of the same sample under the prescribed conditions in the test method. The closer the agreement between individual analyses, the more precise the results, note that a precise method need not be an accurate method. Precision provides a measure of the random error of an analysis. The precision of an analysis is often expressed in terms of the relative standard deviation (in percent, ***%RSD***) or the coefficient of variation (***COV***). These values are calculated from the standard deviation, ***s***, and mean, ***x***, of the data set:

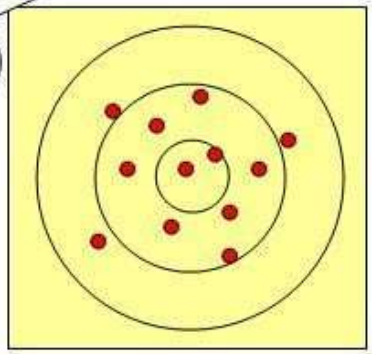
$$\%RSD = COV = 100\,(s / x)$$

Note that the smaller the ***%RSD*** the less variability there is in the data set.

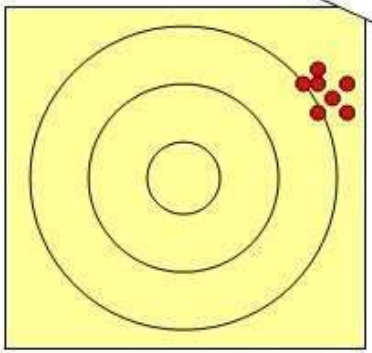
Accuracy vs Precision

Measure of bias

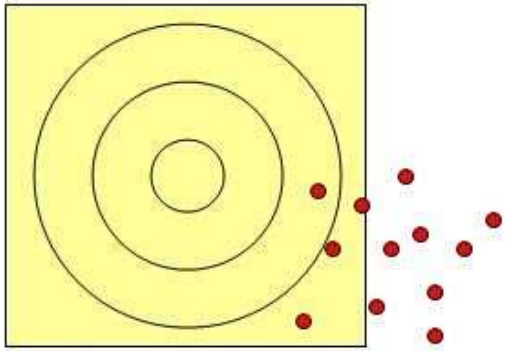
Measure of spread



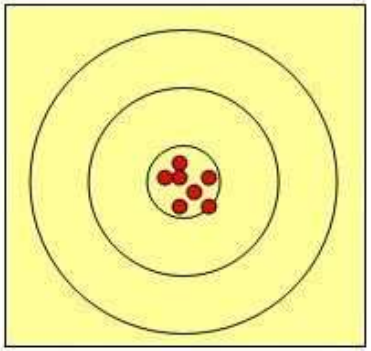
Accurate but not Precise



Precise but not Accurate



Not Accurate or Precise

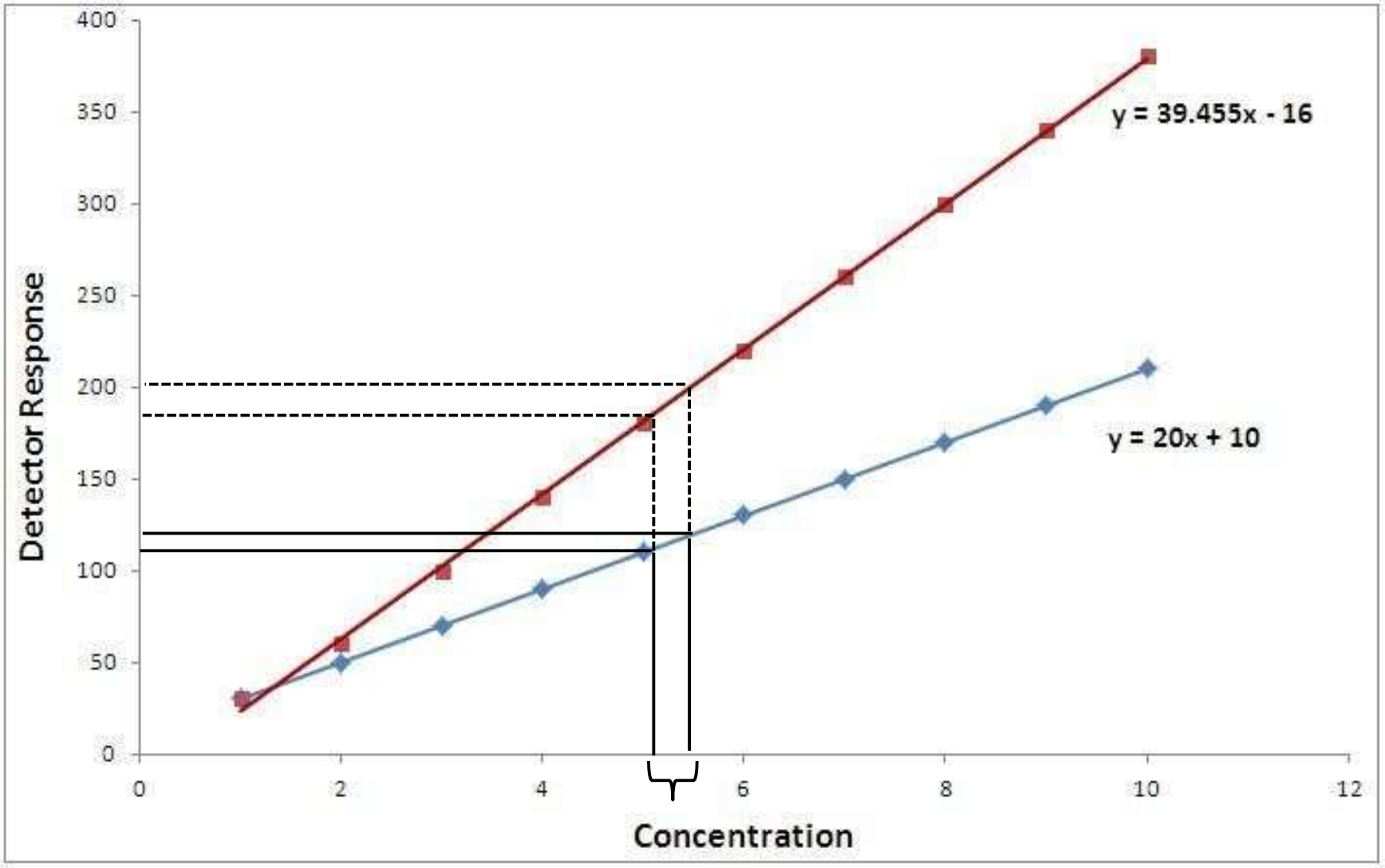


Both Accurate & Precise

Sensitivity (*m*)

A measure of a method ability to distinguish between small differences in analyte concentration. Sensitivity is the change in signal per unit change in the amount of analyte or in other words it's the slope of the calibration curve. Of two methods, the one that has the steeper calibration curve will be the more sensitive. The sensitivity is directly derived from a response versus concentration plot.

The sensitivity is the slope of the line: (Δdetector response/Δconcentration). A very sensitive method has a large slope, so that a small change in concentration results in a large shift in the response. The term sensitivity is often used in place of the limit of detection. A highly sensitive method readily distinguishes between very small differences in sample concentration.


400
350
300
250
200
150
100
50
0
Detector Response
y = 39.455x - 16
y = 20x + 10
0
2
4
6
8
10
12
Concentration

Limit of detection (*LOD*)

A statistical statement about the smallest amount (concentration or mass) of analyte in a sample that can be determined at a known confidence level (not necessarily quantitated as an exact value). More mathematically, it may be defined as that amount of analyte which produces a signal greater than the standard deviation of the background noise by a defined factor. This limit depends upon the ratio of the magnitude of the analytical signal to the size of the noise in the blank signal.

The detection limits estimated as three times the standard deviation of the blank signal. Alternatively, detection limit may be calculated following the equation:

$$C_m = 3\,(s_{bl} / m) = 3\,(S/N)$$

Where: s_{bl} is the standard deviation of the blank, m is the slop.

Limit of quantitation (*LOQ*)

The lowest concentration of analyte in a sample at which quantitative measurements with a suitable level of accuracy and precision can be made. The lower limit of quantitative measurements is generally taken to be equal to ten times the standard deviation of repetitive measurements on a blank:

$$LOQ = 10\,(s_{bl} / m) = 10\,(S/N)$$

Limit of linearity (*LOL*)

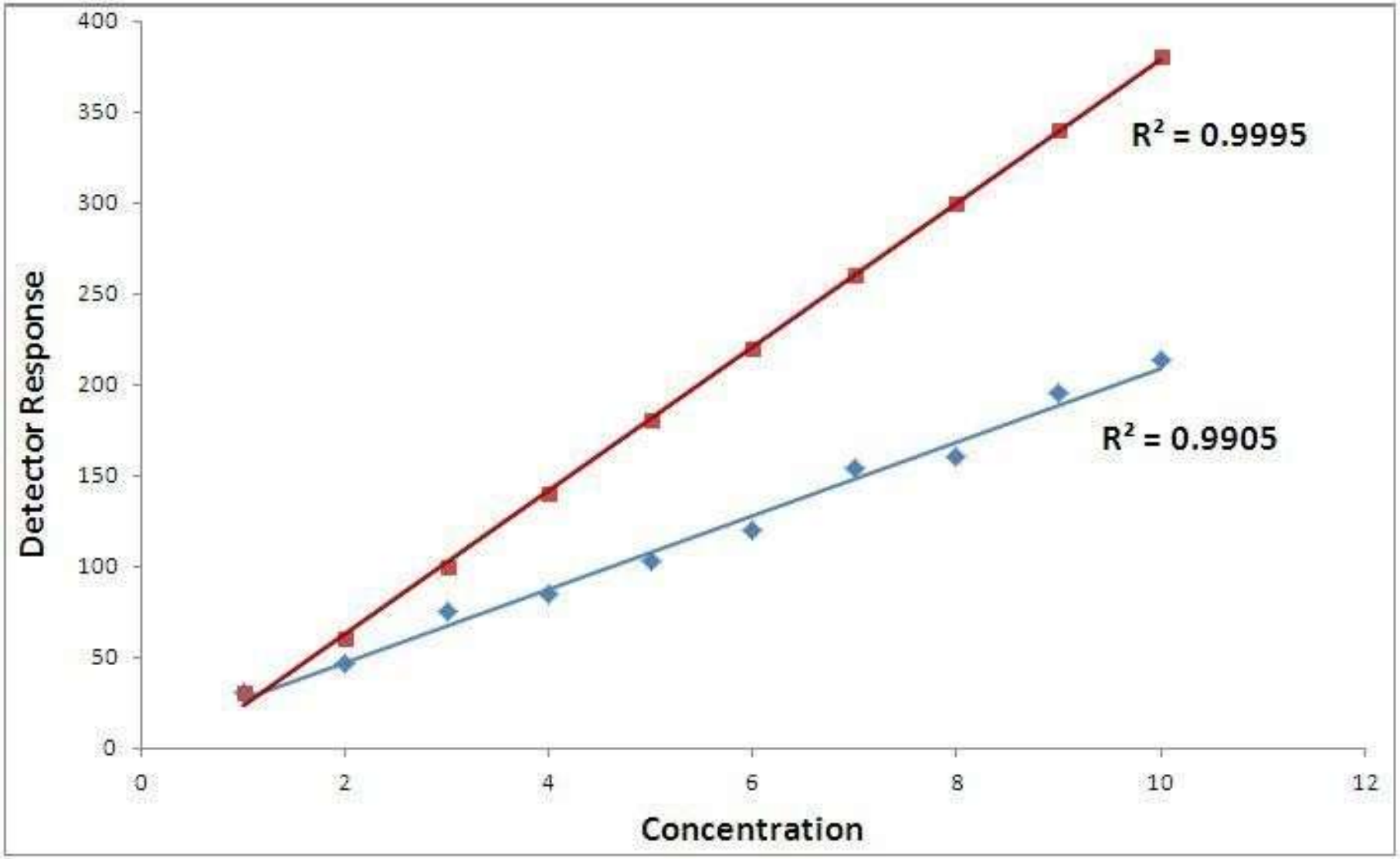
The concentration at which the calibration curve departs from linearity. The extends from the ***LOQ*** to ***LOL*** called the dynamic range. A well defined relationship between detector response and analyte concentration is crucial for quantitative analysis.

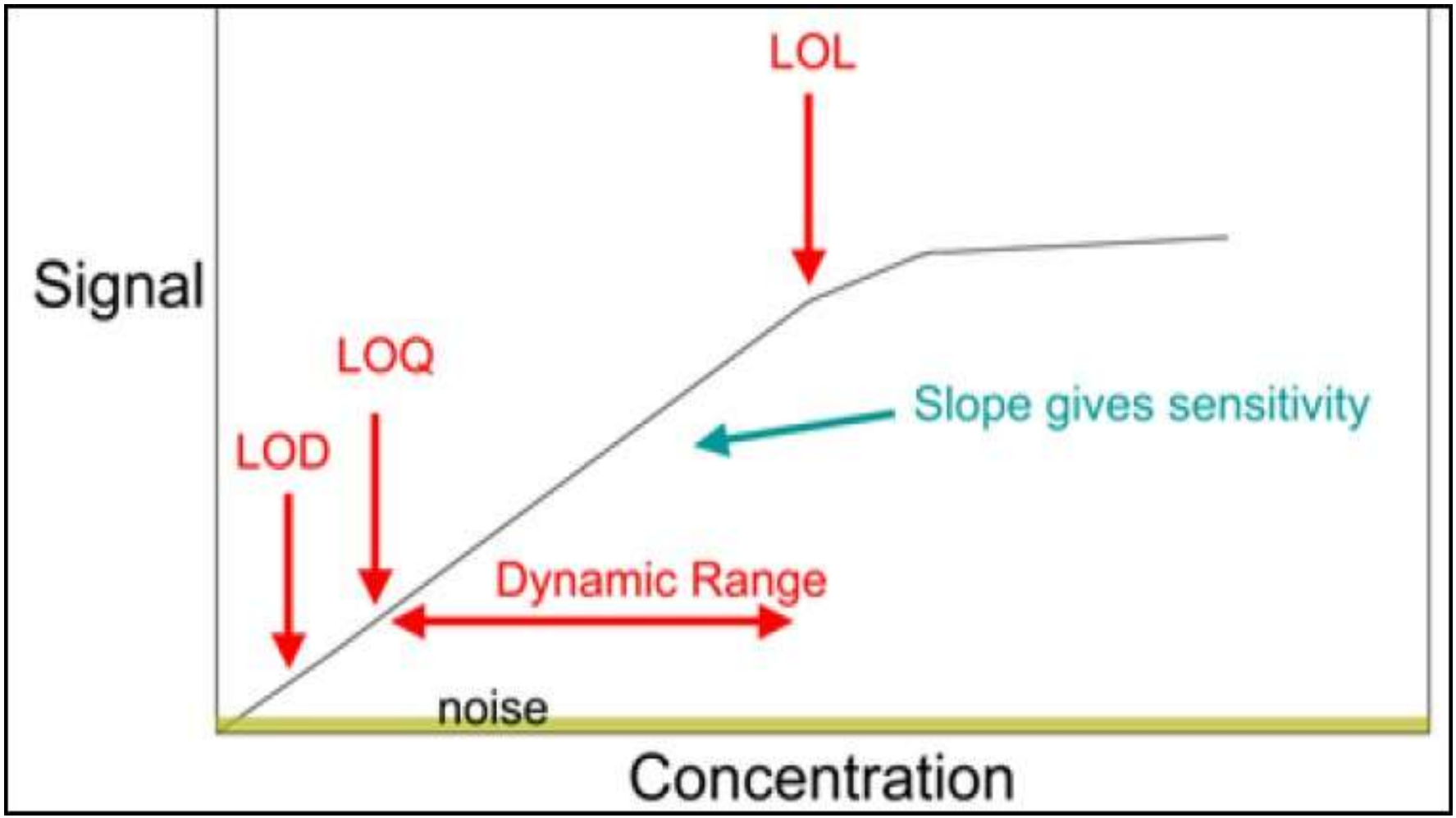
The working range of an analysis usually defines by the linear portion of the response versus concentration curve. The important criterion is that the response concentration relationship is constant and reproducible. Once the linear range is defined, the resulting response versus concentration plots are often referred to as standard, calibration, working or linear curves.

Linearity

Linearity is the ability (within a given range) to obtain an output that is directly proportional to the input. Linearity should be evaluated over the range of the analytical procedure and equipment, **ICH** recommends a minimum of five points to demonstrate linearity by means of statistical evaluation (correlation coefficient, R^2) of the data.

The range is the interval between the upper and lower limits for a parameter (including these upper and lower limits), for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. **ICH** recommends that a minimum of five concentrations is used to determine the linearity. Typically, acceptance values for the correlation coefficient R^2 are >0.999.


400
350
300
250
200
150
100
50
0
Detector Response
0
2
4
6
8
10
12
Concentration
R² = 0.9995
R² = 0.9905



Typical calibration curve showing ***LOD***, ***LOQ***, ***LOL*** and dynamic range.

Recovery

High recovery of the analytes from the matrix is a desirable outcome of sample preparation, and is therefore an important characteristic of the extraction procedure.

The recovery is the ratio of the found concentration to the added concentration. The recovery can be used to reveal whether sample losses in the extraction are due to matrix effects or to bad extraction.

Specificity and selectivity

Specificity refers to the ability of a method to accurately determine the analyte level in the presence of all other components or materials present in the analytical system (i.e., the sample matrix, solvents used, impurities, degradation products, etc).

Selectivity refers to the ability of a method to separate or resolve a pair of compounds from one another (not to generate the unique identification of the analyte from all compounds as implied by specificity).

Ion selective electrodes are designed to respond to one ion (e.g., fluoride selective electrode responds to F^-) but show response to others (e.g., hydroxide and chloride ions in this example) and therefore are selective but not specific.

Repeatability and reproducibility

Short term precision (e.g., replicate analysis during the same day) is often referred to as the repeatability of the method. While long term precision (e.g., over a week by the same laboratory or between laboratories) is called reproducibility. Usually, repeatability and reproducibility expresses as the relative standard deviation on the measured data.

Robustness and ruggedness

The robustness of a method is typically determined during the method development stage, and is a measure of how consistently a method generates the same analytical result when small deliberate changes in operating parameters are made. Many times it is part of the intralaboratory development and validation process. For example, changeable parameters could include organic level in mobile phase, pH of mobile phase, concentration of mobile phase modifiers, temperature and column.

The concept of ruggedness includes that of robustness but includes the reproducibility of a method when different analysts, laboratories and instruments are used. Ruggedness determination is almost invariably an interlaboratory result. In many cases, a well defined collaborative effort is used in which eight or more laboratories analyze the same sample or a set of samples. It is evident that the potential variability in the method conditions will be significantly greater than in the controlled design robustness testing. In ruggedness test, not only the system parameters tested but also the manner of sample handling, sample and mobile phase preparation, and even analyst interpretation.

Calibration techniques

Calibration is the process to determine the two factors that describe the relation between the method response and the component concentration.

Common calibration techniques:

- **The external standard technique (calibration curve method)**

- **The standard addition technique**

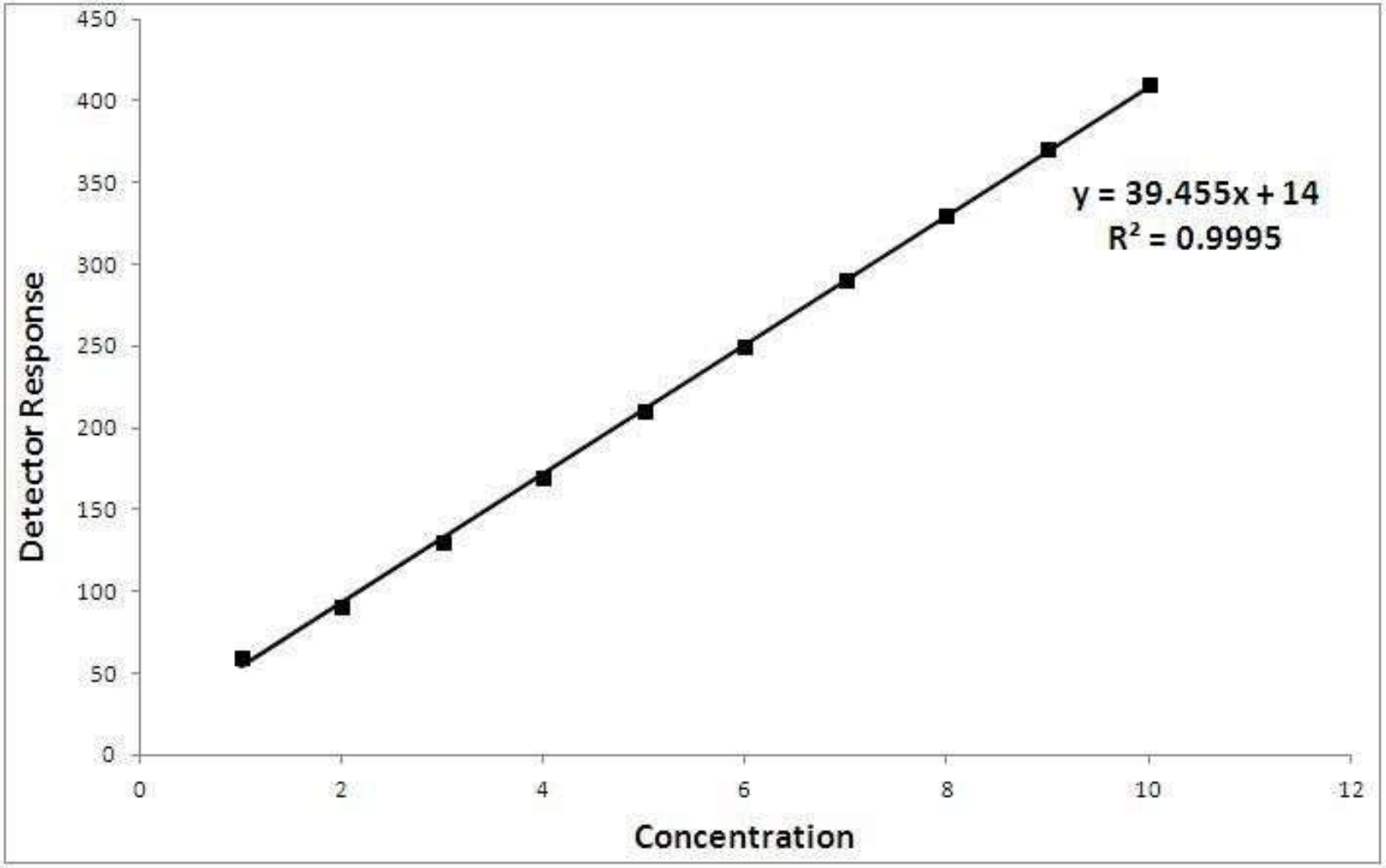
- **The internal standard technique**

External standard method

The use of an external standard procedure is probably the simplest methodology that may be employed. In this situation, a number of samples containing known amounts of the analyte of interest are made up and analyzed. The intensity of the analytical signal from these standards is then plotted against the known concentration of analyte present and a calibration graph is obtained.

It is important that the range of concentrations covered by the standards includes the concentrations encountered in the unknowns, interpolation of the result is required, rather than extrapolation. Care should also be taken to fit the correct form of curve to the calibration data, i.e. to ensure that the data genuinely obey a linear relationship, not a gentle curve, before using linear regression to define the relationship between signal intensity and concentration.

Although widely employed, the use of external standardization takes no account of matrix effects, i.e. the effect on the analytical signal caused by the interaction of analyte with the matrix in which it is found, or losses of analyte from the unknowns during sampling, storage and work up.



Typical calibration curve

Calibration curve procedure

- Prepare a series of standard solutions (analyte solutions with known concentrations).
- Plot Analytical Signal vs. [analyte].
- Use signal for unknown to find [analyte].

Example: Determination of Pb in Blood by GFAAS

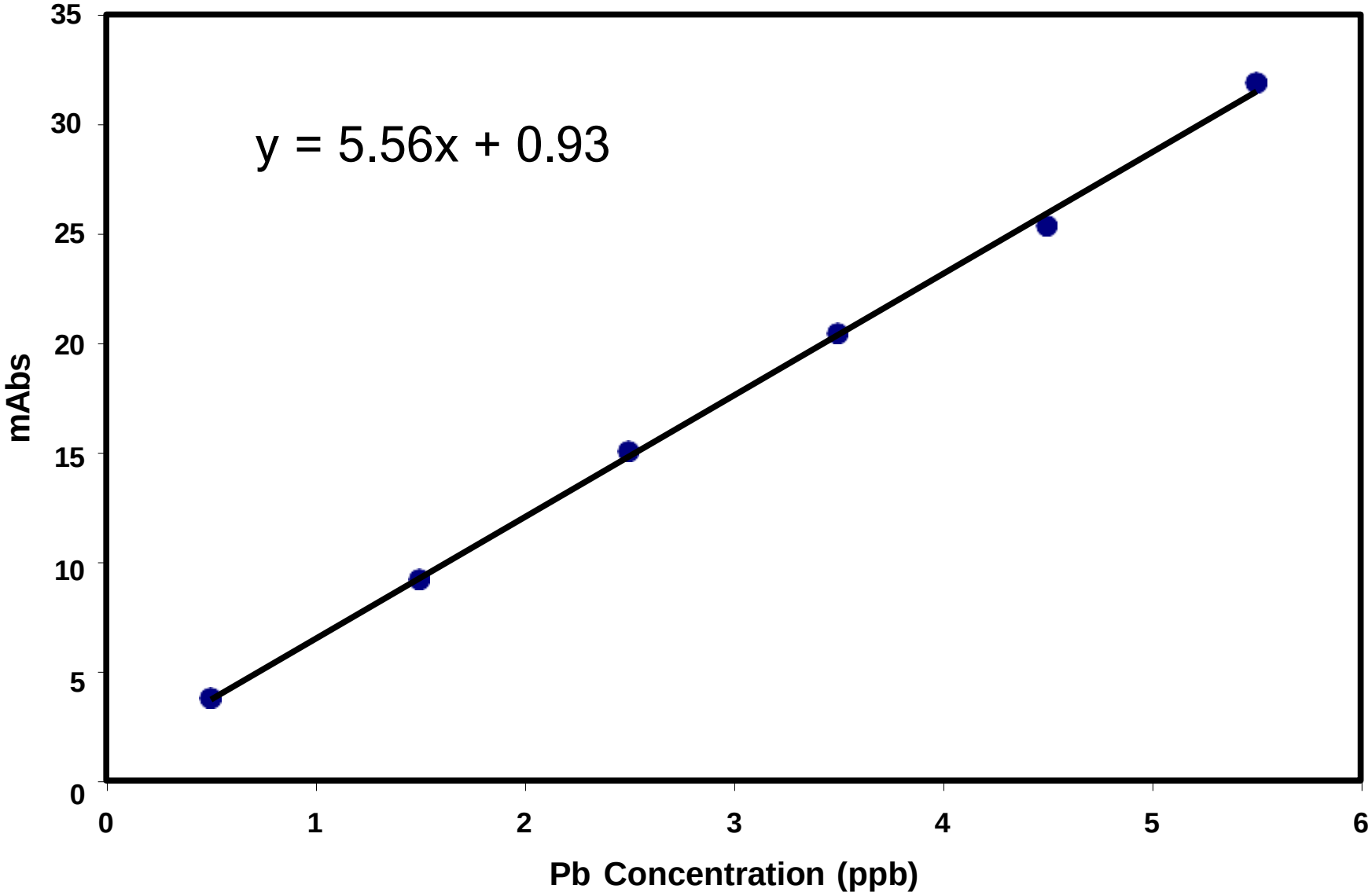
[Pb] (ppb)	Signal (mAbs)
0.50	3.76
1.50	9.16
2.50	15.03
3.50	20.42
4.50	25.33
5.50	31.87

Results of linear regression:

$$S = m\,C + b$$

$$m = 5.56 \text{ mAbs/ppb}$$

$$b = 0.93 \text{ mAbs}$$


y = 5.56x + 0.93
mAbs
Pb Concentration (ppb)
0
5
10
15
20
25
30
35
0
1
2
3
4
5
6

A sample containing an unknown amount of Pb gives a signal of 27.5 mAbs. Calculate the Pb concentration.

$S = m\,C + b$

$C = (S - b) / m$

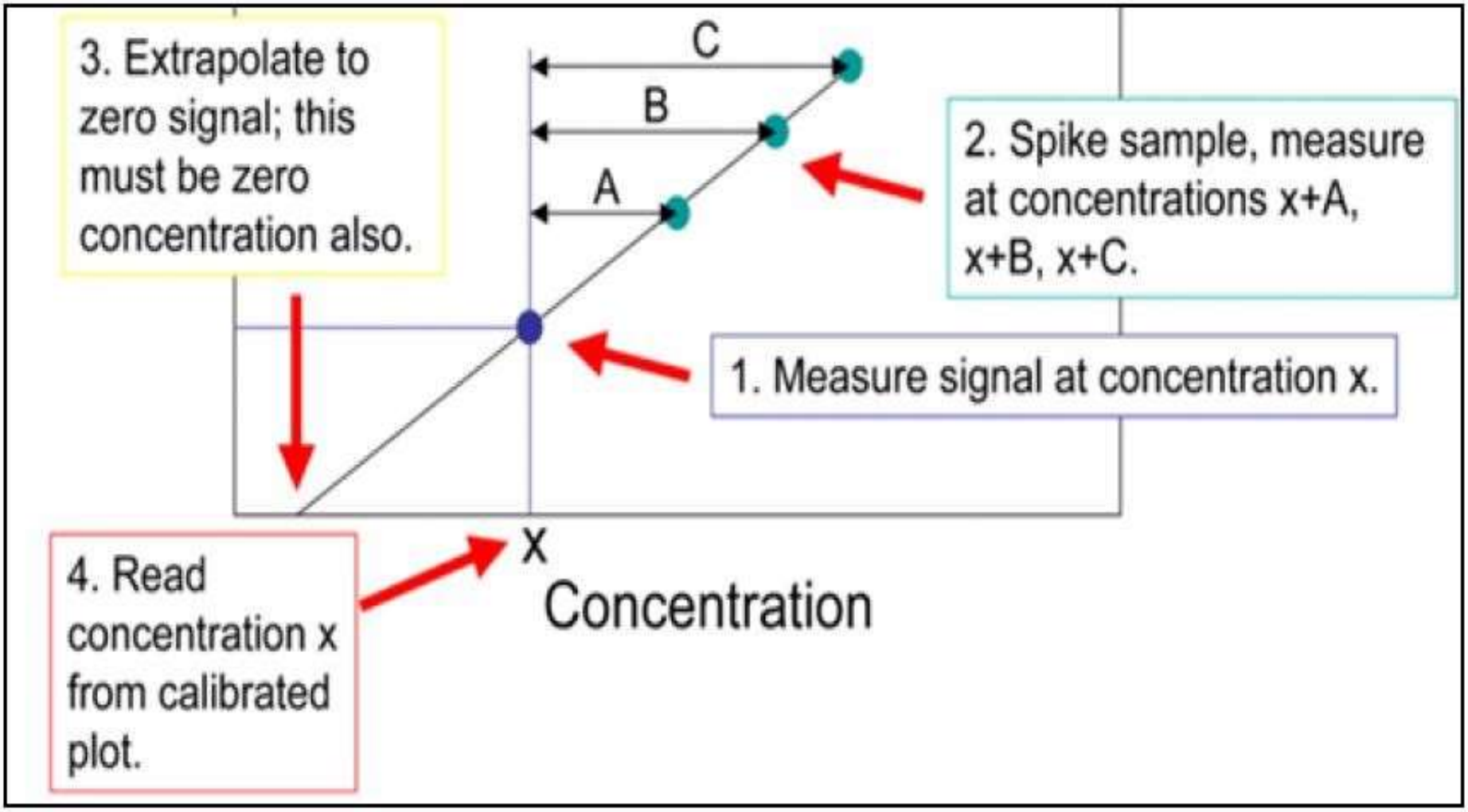
$C = (27.5 - 0.92) / 5.56$

$C = 4.78$ ppb

Standard addition method

This method addresses the influence of matrix effects. The standard is again the analyte itself. An analytical measurement is made on the unknown and the signal intensity noted. A known amount of the analyte is then added to the unknown and a second analytical measurement made. From the increase in analytical signal, a response factor, i.e. the signal per unit concentration, can be calculated. The concentration of analyte in the original sample may then be obtained by dividing the signal from the original sample by the response factor.

A result obtained from a single determination of the response factor is liable to a greater imprecision than had it been obtained from multiple additions, and it is more normal to add further known amounts of analyte and determine the analyte signal after each addition. A graph may then be drawn. The concentration of analyte in the original sample may then be obtained by extrapolation (using the equation of the linear regression straight line) of this graph to intercept the x-axis. It should be noted that this method assumes that the matrix has the same effect on added analyte as it had on the analyte in the unknown, but this is not always the case.



Typical standard addition curve.

Standard addition procedure

- Add one or more increments of a standard solution to sample aliquots of the same size. Each mixture is then diluted to the same volume.

- Prepare a plot of Analytical Signal versus: volume of standard solution added, or concentration of analyte added.

- The x-intercept of the standard addition plot corresponds to the amount of analyte that must have been present in the sample (after accounting for dilution).

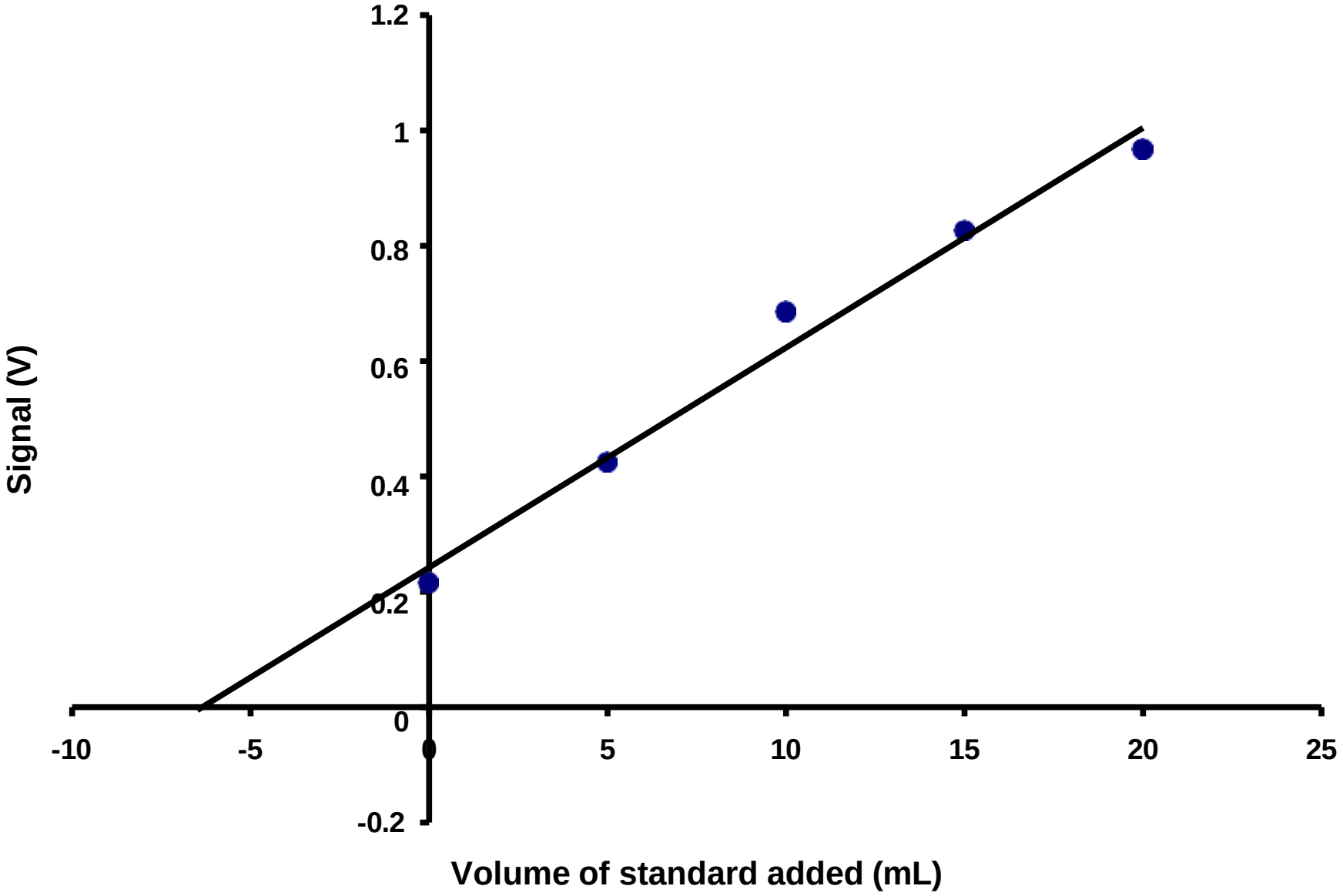
- The standard addition method assumes:
the curve is linear over the concentration range
the y-intercept of a calibration curve would be 0

Example: Determination of Fe in drinking water

Sample volume (mL)	Standard volume (mL)	Signal
10	0	0.215
10	5	0.242
10	10	0.685
10	15	0.826
10	20	0.967

The concentration of the Fe standard solution is 11.1 ppm

All solutions are diluted to a final volume of 50 mL


1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-10
-5
0
5
10
15
20
25
Signal (V)
Volume of standard added (mL)

[Fe] = ?

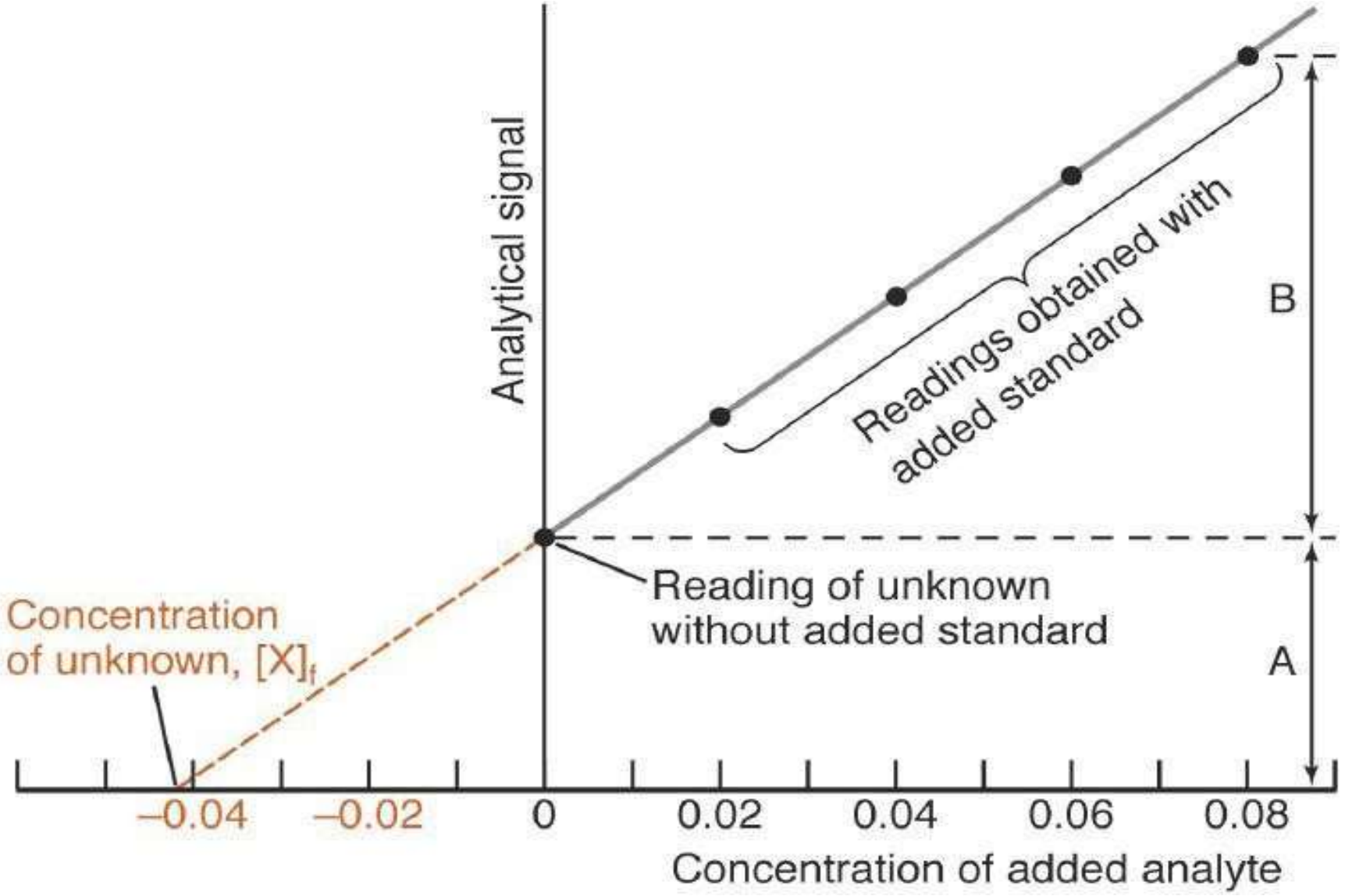
x-intercept = -6.08 mL

Therefore, 10 mL of sample diluted to 50 mL would give a signal equivalent to 6.08 mL of standard diluted to 50 mL.

$V_{sam} \times [Fe]_{sam} = V_{std} \times [Fe]_{std}$

10.0 mL x [Fe] = 6.08 mL x 11.1 ppm

[Fe] = 6.75 ppm


Analytical signal
Readings obtained with added standard
B
A
Reading of unknown without added standard
Concentration of unknown, [X]f
–0.04
–0.02
0
0.02
0.04
0.06
0.08
Concentration of added analyte

Neither of the two methods described above take into account the possibility of loss of analyte between sampling and analysis. They may therefore, provide a precise measurement but the result obtained may not give an accurate indication of the amount of analyte present in the original sample. The use of an internal standard is designed to overcome this major source of inaccuracy and also to improve precision.

Internal standard method

An internal standard is a suitable compound added to the sample as early in the analytical procedure as is possible, ideally at the sampling stage. The internal standard method requires the addition of a known amount of a compound that is easily distinguished from the analyte but which exhibits similar chemical properties. Analytical signals from both the analyte and internal standard are measured during each determination of both standards and unknowns, and it is the ratio of these two signal intensities that are used to generate the calibration graph and to determine the amount of analyte present in each of the unknowns. In order for this methodology to have validity, losses of the analyte must be exactly and/or reproducibly mirrored by losses in the internal standard and for this reason the choice of internal standard is crucial to its success.

Examples for sample and internal standard

Sample	Internal standard
Anthracene	Naphthalene
Dopamine	3,4-Dihydroxy benzylamine
Vitamin D3	Vitamin D2
Na	Li

Internal standard procedure

- Prepare a set of standard solutions for analyte (A) as with the calibration curve method, but add a constant amount of a second species (B) to each solution.

- Prepare a plot of S_A/S_B versus [A].

- The resulting measurement will be independent of sample size and position.

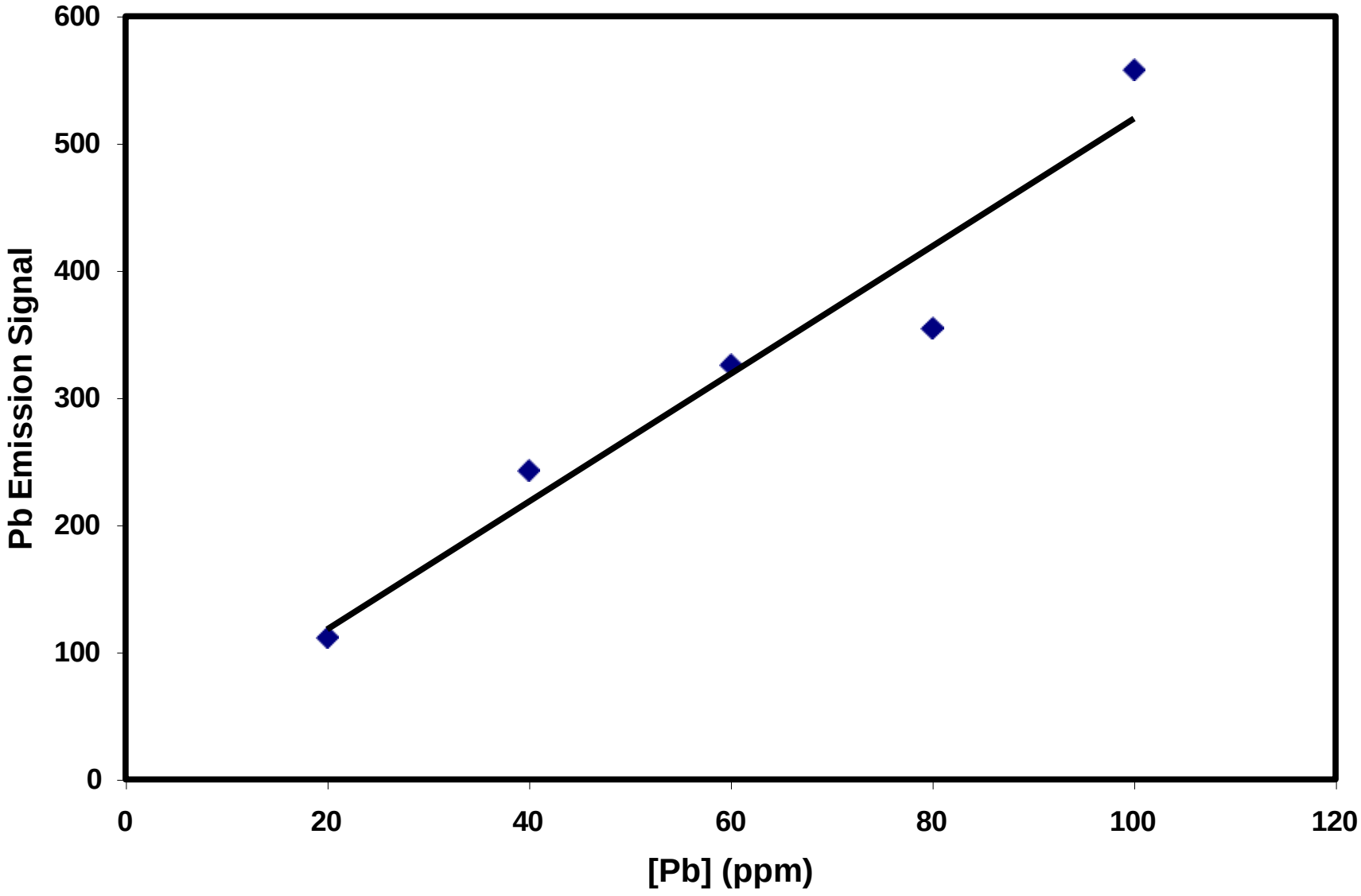
- Species A & B must not produce signals that interfere with each other. Usually they are separated by wavelength or time.

Example: Determination of Pb by ICP emission

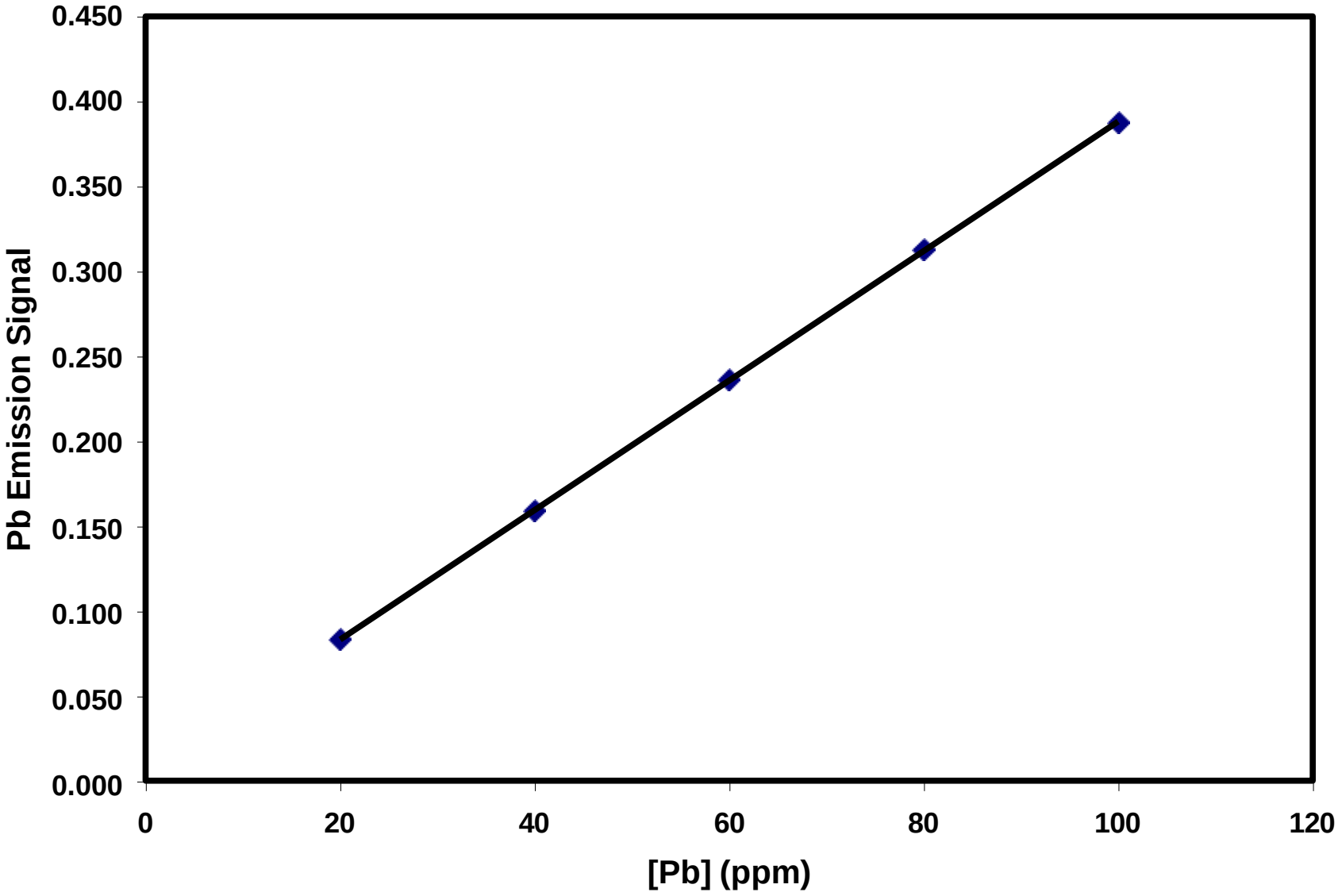
Each Pb solution contains 100 ppm Cu.

[Pb] (ppm)	Signal		
	Pb	Cu	Pb/Cu
20	112	1347	0.083
40	243	1527	0.159
60	326	1383	0.236
80	355	1135	0.313
100	558	1440	0.388

No Internal Standard Correction



Internal Standard Correction




Thank You!