FDE 301
INSTRUMENTAL ANALYSIS

Calibration of Instrumental Methods
Selection of an Appropriate Analytical Method
Validation of Analytical Methods

Calibration of Instrumental Methods

Calibration of Instrumental Methods

- A very important part of all analytical procedures is the calibration and standardization process.
- **Calibration** determines the relationship between the analytical response and the analyte concentration.
- Usually this is determined by the use of *chemical standards*.
- Almost all analytical methods require some type of calibration with chemical standards. Gravimetric methods and some coulometric methods are among the few *absolute* methods that do not rely on calibration with chemical standards.

Calibration Methods

- External Standard
- Internal Standard
- Standard Addition

External Standard Method

- The **external standard method** is the best known and most widely used calibration method.
- Known solutions of analyte that do not involve the unknown solution are called **external standards**.
- An **external standard** is prepared separately from the sample. By contrast, an **internal standard** is added to the sample itself.
- External standards are used to calibrate instruments and procedures **when there are no interference effects from matrix components in the analyte solution**.

External Standard Method

- The **external standard method** employs a series of standards consisting of at least one blank that contains no known concentration of the analyte, and several non-zero standards containing known concentrations of the analyte and prepared in order of increasing analyte concentration.

- The calibration standards are prepared separate from (external to) the sample matrix, usually in a solvent, e.g., water, and containing the reagents used in sample preparation.

- The measured signals of the blank and non-zero standards are adjusted for the blank signal to yield a signal that reflects the signal due only to the analyte.

External Standard Method

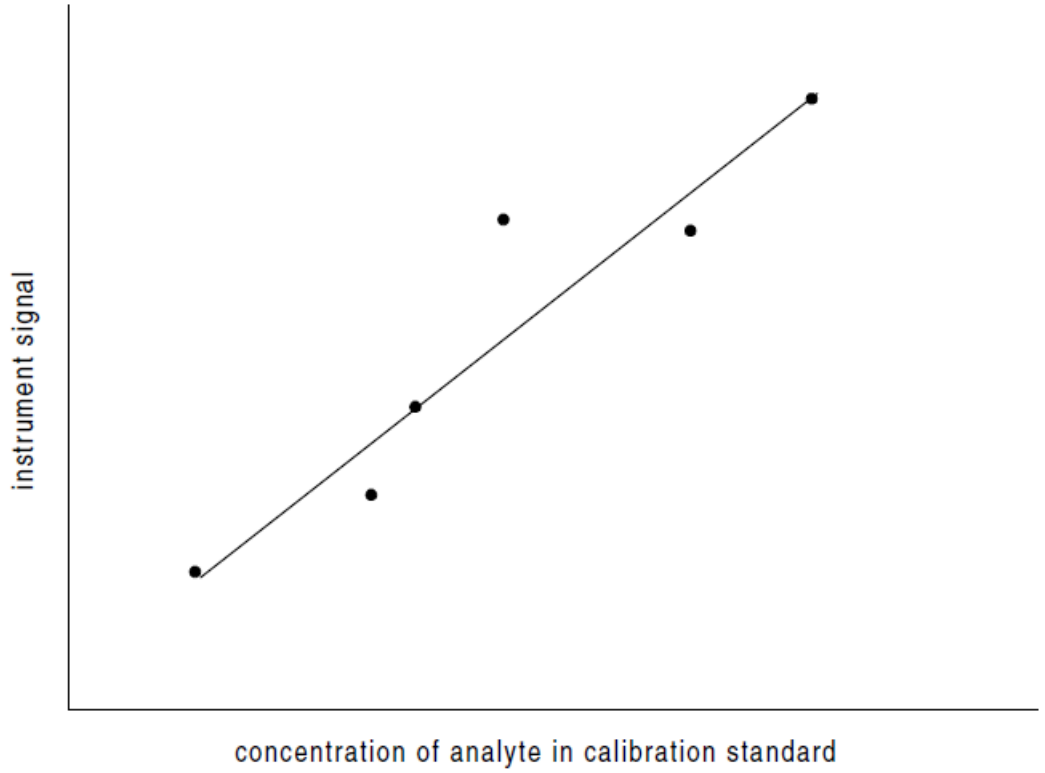
- **Calibration** is accomplished by obtaining the response signal (absorbance, emission intensity, electrode potential, peak area) as a function of the known analyte concentration.

- **A calibration curve** is prepared by plotting the data or by fitting them to a suitable mathematical equation, such as the slope-intercept form used in the **method of linear least squares**.

- The next step is the prediction step, where the response signal is obtained for the sample and used to *predict* the unknown analyte concentration, *cx*, from the calibration curve or best-fit equation.

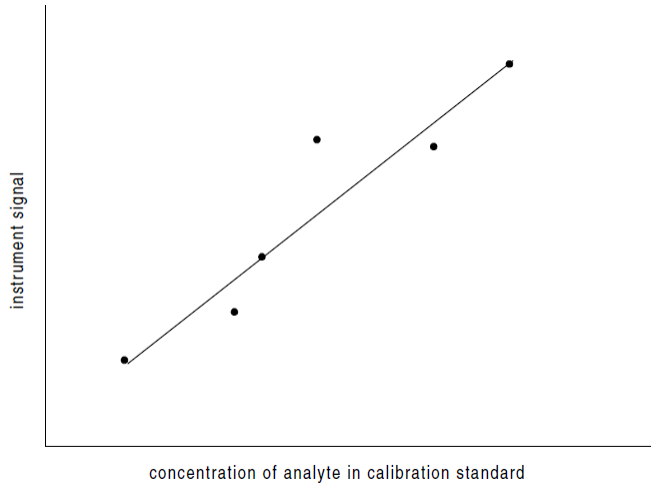
- The concentration of the analyte in the original bulk sample is then calculated from *cx* by applying the appropriate dilution factors from the sample preparation steps.

Typical calibration curve



- **A calibration curve** shows the response of an analytical method to known quantities of analyte.
- The instrument response is measured for a series of calibration standards, which contain a known concentration of analyte.
- The curve is a function that describes the functional relationship between signal and concentration.
- Note that the calibration curve should never be extrapolated (i.e., never extended beyond the range of the calibration measurements).

External Standard Method



- **The ordinate:** dependent variable (i.e. absorbance, the chromatographic peak for glucose)

  The abscissa: independent variable (i.e. concentration of copper, concentration of glucose)
- If the analyte signal-concentration relationship is linear with a slope a, the plot approximates a straight line.
- The method of least squares is often applied to obtain the equation for the line.
- The method of least squares is based on the assumption that there is a linear relationship between the measured response y and the standard analyte concentration x.
- The mathematical relationship that describes this assumption is called the regression model, which may be represented as

$$y = ax + b$$

b = the y intercept (the value of y when x is zero)

a = the slope of the line

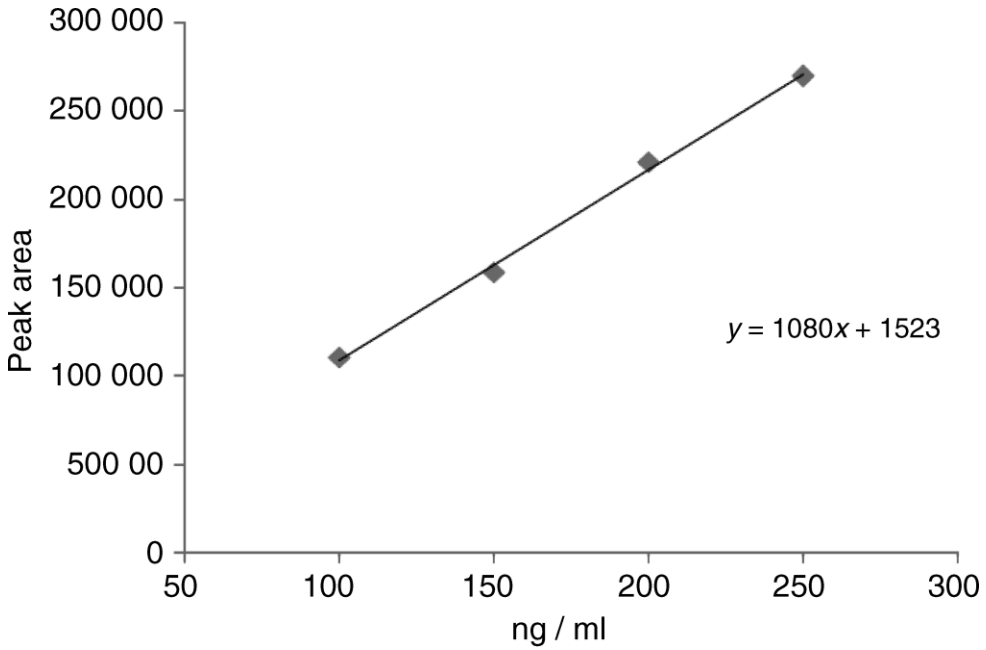
Example of a quantitative determination based on external standard method

- The concentration of a compound in a sample solution should be determined. It was expected that the unknown concentration should be in the range of 100–250 ng/ml. Standard solutions containing 100, 150, 200 and 250 ng/ml of the reference standard were prepared and a fixed volume was accurately injected into the chromatographic column. The peak areas of the reference standard read from the chromatograms are:

Concentration of standard (ng/mL)	Peak area
100	110 952
150	159 143
200	221 645
250	270 045

Solution

- The concentrations are plotted against the peak areas yielding a straight line:



- The regression line calculated by the method of least squares is:

$$\underline{y = 1080x + 1523}$$

where **y** is **the peak area** and **x** is **the concentration in ng/ml.**

- The sample solution was analyzed at the same conditions as the standard solutions and the peak area of the analyte was **175 432**.
- The unknown concentration x of the analyte was:

  175 432 = 1080x + 1523

  x = (175 432 - 1523) / 1080 = 161

- The concentration was **161 ng/ml**.

Internal Standard Method

- An **internal standard** is a substance that is added in a constant amount to all samples, blanks and calibration standards in a analysis.

- Calibration then involves plotting the ratio of the analyte signal to the internal-standard as a function of the analyte concentration of the standards.

- This ratio for the samples is then used to obtain their analyte concentrations from a calibration curve.

Internal Standard Method

- An **internal standard** is a known amount of a compound-different from the analyte-that is added to the unknown.
- Signal from analyte is compared with signal from the internal standard to find out how much analyte is present.
- A carefully chosen internal standard will give an analytical signal (such as a chromatographic peak or spectrophotometric absorption) that is well separated from those of the analyte and other species in an unknown.
- The internal standard should be chemically stable and not react with components of the unknown.
- It is helpful for the internal standard to be chemically similar to analyte so that uncontrolled effects of the matrix that increase or decrease the analyte signal might have a similar effect on the signal from the standard.

Internal Standard Method

- **Internal standards are especially useful for analyses in which the quantity of sample analyzed or the instrument response varies slightly from run to run.**

  For example, gas or liquid flow rates that vary by a few percent in a chromatography experiment could change the detector response. A calibration curve is accurate only for the one set of conditions under which it was obtained. However, the *relative* response of the detector to the analyte and standard is usually constant over a range of conditions. If signal from the standard increases by 8.4% because of a change in flow rate, signal from the analyte usually increases by 8.4% also.

- **As long as the concentration of standard is known, the correct concentration of analyte can be derived.**

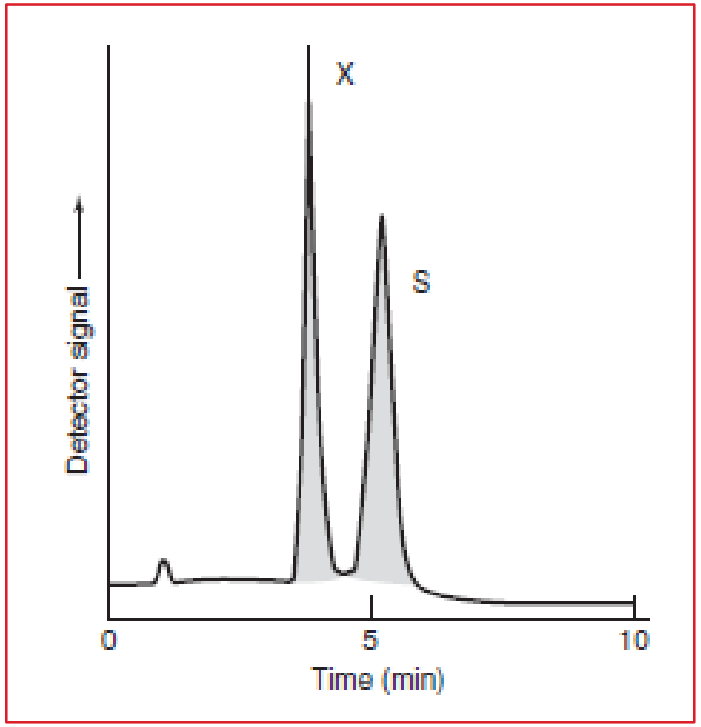
- **Internal standards are used in chromatography because the small quantity of sample injected into the chromatograph is not reproducible.**

- **Internal standards are desirable when sample loss can occur during sample preparation steps prior to analysis. If a known quantity of standard is added to the unknown prior to any manipulations, the ratio of standard to analyte remains constant because the same fraction of each is lost in any operation.**

Internal Standard Method

- To use an internal standard, we prepare a known mixture of standard and analyte to measure the relative response of the detector to the two species.

- In the Figure, the area, *A*, under each peak is proportional to the concentration of the species injected into a chromatography column. However, the detector generally has a different response to each component. For example, if both analyte (X) and internal standard (S) have concentrations of 10.0 mM, the area under the analyte peak might be 2.30 times greater than the area under the standard peak. We say that the **response factor,** *F*, is 2.30 times greater for X than for S.



Response factor:

$$\frac{\text{Signal from analyte}}{\text{Concentration of analyte}} = F\left(\frac{\text{signal from standard}}{\text{concentration of standard}}\right)$$

$$\frac{A_X}{[X]} = F\left(\frac{A_S}{[S]}\right)$$

Chromatographic separation of unknown (X) and internal standard (S).
A known amount of S was added to the unknown. The relative areas of the signals from X and S allow us to find out how much X is in the mixture. It is necessary first to measure the relative response of the detector to each compound.

Response factor:

$$\frac{\text{Signal from analyte}}{\text{Concentration of analyte}} = F\left(\frac{\text{signal from standard}}{\text{concentration of standard}}\right)$$

$$\frac{A_X}{[X]} = F\left(\frac{A_S}{[S]}\right)$$

This equation is predicated on linear response to analyte and standard.

- If the detector responds equally to standard and analyte, F =1.
- If the detector responds twice as much to analyte as to standard, F = 2.
- If the detector responds half as much to analyte as to standard, F = 0.5.

- Sometimes a signal is an area (as in chromatography) and other times the signal might be a height rather than an area.
- [X] and [S] are the concentrations of analyte and standard after they have been mixed together.

Example of a quantitative determination based on internal standard method-1

- In a preliminary experiment, a solution containing 0.0837 M X and 0.0666 M S gave peak areas of $A_X = 423$ and $A_S = 347$. To analyze the unknown, 10.0 mL of 0.146 M S were added to 10.0 mL of unknown, and the mixture was diluted to 25.0 mL in a volumetric flask. This mixture gave the chromatogram, for which $A_X = 553$ and $A_S = 582$.

  Find the concentration of X in the unknown.

Solution

- First use the standard mixture to find the response factor.
- *Standard mixture*:

$$\frac{A_X}{[X]} = F\left(\frac{A_S}{[S]}\right) \Rightarrow \frac{423}{0.083\,7} = F\left(\frac{347}{0.066\,6}\right) \Rightarrow F = 0.970$$

- In the mixture of unknown plus standard, the concentration of S is:

$$[S] = \underbrace{(0.146\ M)}_{\text{Initial concentration}}\underbrace{\left(\frac{10.0}{25.0}\right)}_{\text{Dilution factor}} = 0.058\,4\ M$$

- Using the known response factor, substitute back into **Equation** to find the concentration of unknown in the mixture:
- *Unknown mixture*:

$$\frac{A_X}{[X]} = F\left(\frac{A_S}{[S]}\right) \Rightarrow \frac{553}{[X]} = 0.970_0\left(\frac{582}{0.058\,4}\right) \Rightarrow [X] = 0.057\,2_1\ M$$

- Beacuse X was diluted from 10.0 to 25.0 Ml when the mixture with S was prepared, the original concentration of X in the unknown was (25.0 mL/10.0 Ml)(0.0572 M) = **0.143 M**

Multipoint Calibration Curve for an Internal Standard

- The previous example uses a single mixture to find the response factor. If there were no experimental error, this "one-point calibration curve" would be sufficient to give an accurate response factor.

- There is always experimental error, so a multipoint calibration curve is preferred to average out some experimental variability. For this purpose, we rearrange the equation so that the signals are on one side and the concentrations are on the other side:

Equation for internal standard calibration curve

$$\frac{\text{Signal from analyte}}{\text{Signal from standard}} = F\left(\frac{\text{concentration of analyte}}{\text{concentration of standard}}\right)$$

$$\frac{A_X}{A_S} = F\left(\frac{[X]}{[S]}\right)$$

- Then construct a graph in which the signal ratio on the left side of Equation above is plotted as a function of the concentration ratio on the right side. The graph should be linear with a zero intercept. The slope of this graph is the response factor.

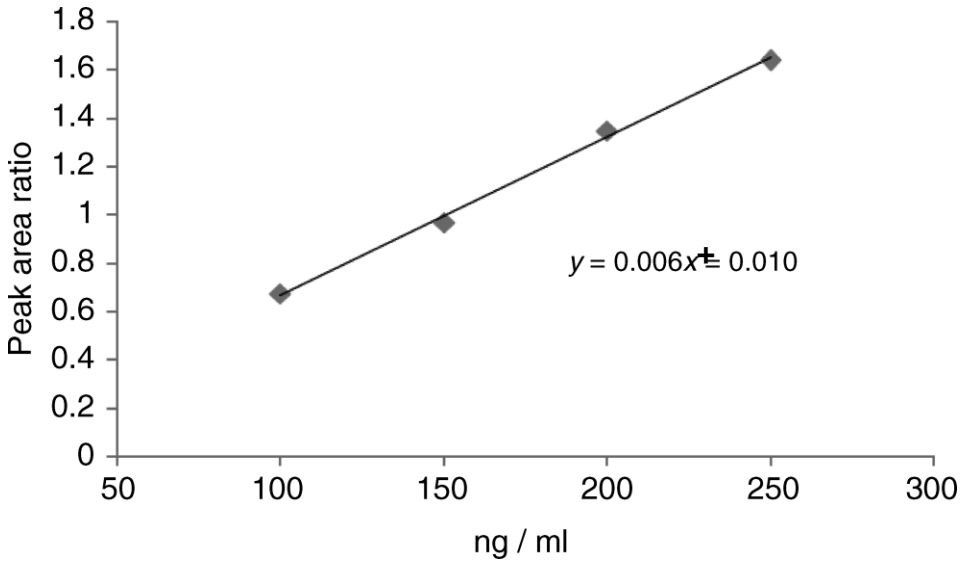
Example of a quantitative determination based on internal standard method-2

- It was expected that the unknown concentrations were in the range of 100-250 ng/ml. Standard solutions containing 100, 150, 200 and 250 ng/ml of the reference standard were prepared and added an internal standard to a final concentration of 150 ng/ml. After sample preparation a fixed volume was accurately injected into the chromatographic column. The peak areas of the reference standard and the internal standard were read from the chromatograms and analyte/internal standard peak area ratios were calculated:

Concentration of standard (ng/mL)	Peak area of the reference standard	Peak area of the internal standard	Peak area ratio
100	110 952	164 302	0.675293
150	159 143	163 997	0.970402
200	221 645	164 204	1.349881
250	270 045	164 289	1.643719

Solution

- The calibration curve is a plot of concentration versus peak area ratios::



- The regression line **y = 0.0066x + 0.0102** where x is the concentration in ng/ml and y is the peak area ratio was used for calculation of analyte concentration in sample solutions.

- **Sample solutions** were added internal standard to a final concentration of 150 ng/ml and were processed equally to the standard solutions. The sample solution was analyzed at the same conditions as the standard solutions.

- In one of the sample solutions **the peak area of the analyte was 175 432** and **the peak area of the internal standard was 164 105**. **The peak area ratio 1.069023 was used to determine the unknown concentration x of the analyte:**

1.069023 = 0.0066x + 0.0102

x = (1.069023 - 0.0102) / 0.0066 = 160.4
The concentration was 160 ng/ml.

Standard Addition Method

- In **standard addition**, known quantities of analyte are added to the unknown. From the increase in signal, we deduce how much analyte was in the original unknown. This method requires a linear response to analyte. The higher precision can be achieved when standards are added by mass instead of volume.

- Standard addition is especially appropriate when the sample composition is unknown or complex and affects the analytical signal. In such case, it is impossible or difficult to create standards and blanks whose composition matches that of the sample. If standards and blanks do not match the composition of the unknown sample, a calibration curve is not reliable.

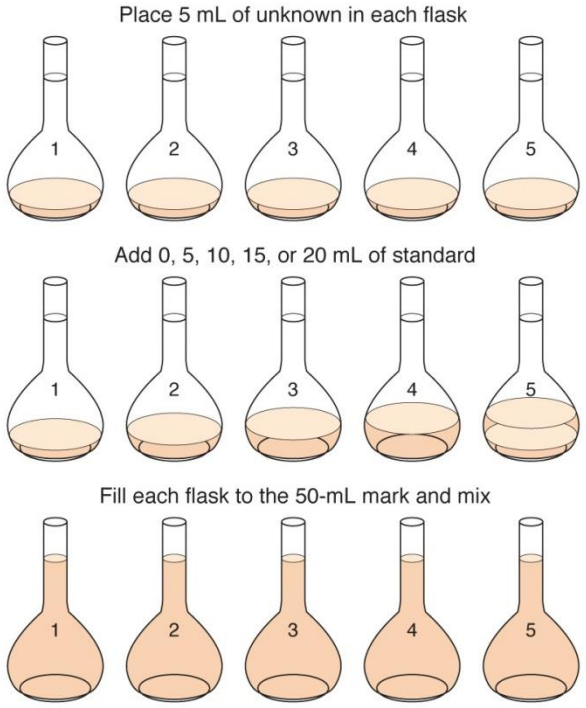
- The **matrix** is everything in the unknown, other than analyte.

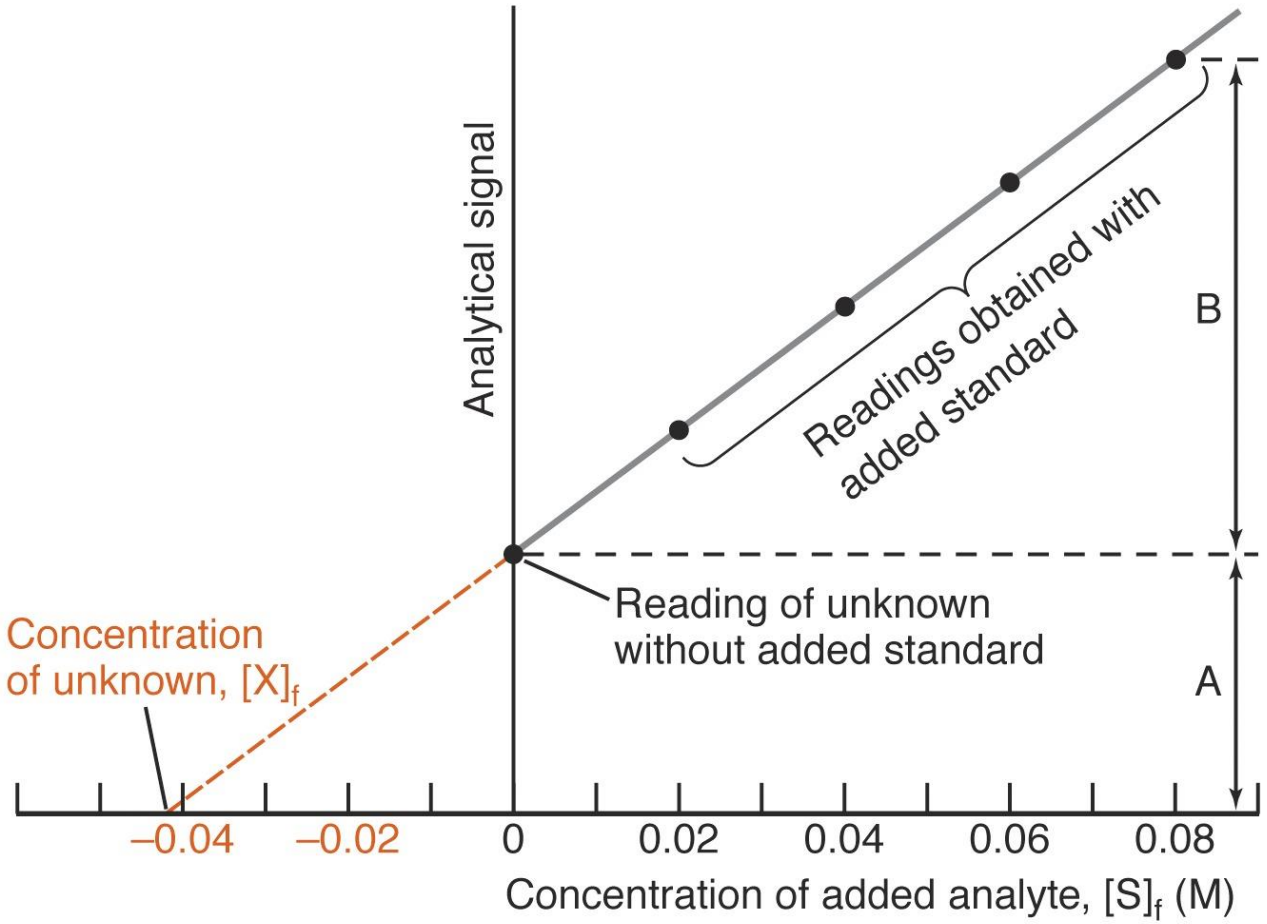
- A **matrix effect** is a change in the analytical signal caused by anything in the sample other than analyte.

Standard Addition Method

- **Standard addition** is a calibration method used to determine the concentration of an analyte in a complex analytical matrix that contains substances that may interfere with the detector response of the analyte. Because of interference, a calibration curve based on pure analyte samples gives an incorrect determination.

- Adding known concentrations of a chemical reference standard of the analyte to the unknown solution can solve the problem. This calibration principle is called **standard addition**.


Place 5 mL of unknown in each flask
1
2
3
4
5
Add 0, 5, 10, 15, or 20 mL of standard
1
2
3
4
5
Fill each flask to the 50-mL mark and mix
1
2
3
4
5


Analytical signal
Readings obtained with added standard
B
Reading of unknown without added standard
A
Concentration of unknown, $[X]_f$
–0.04
–0.02
0
0.02
0.04
0.06
0.08
Concentration of added analyte, $[S]_f$ (M)

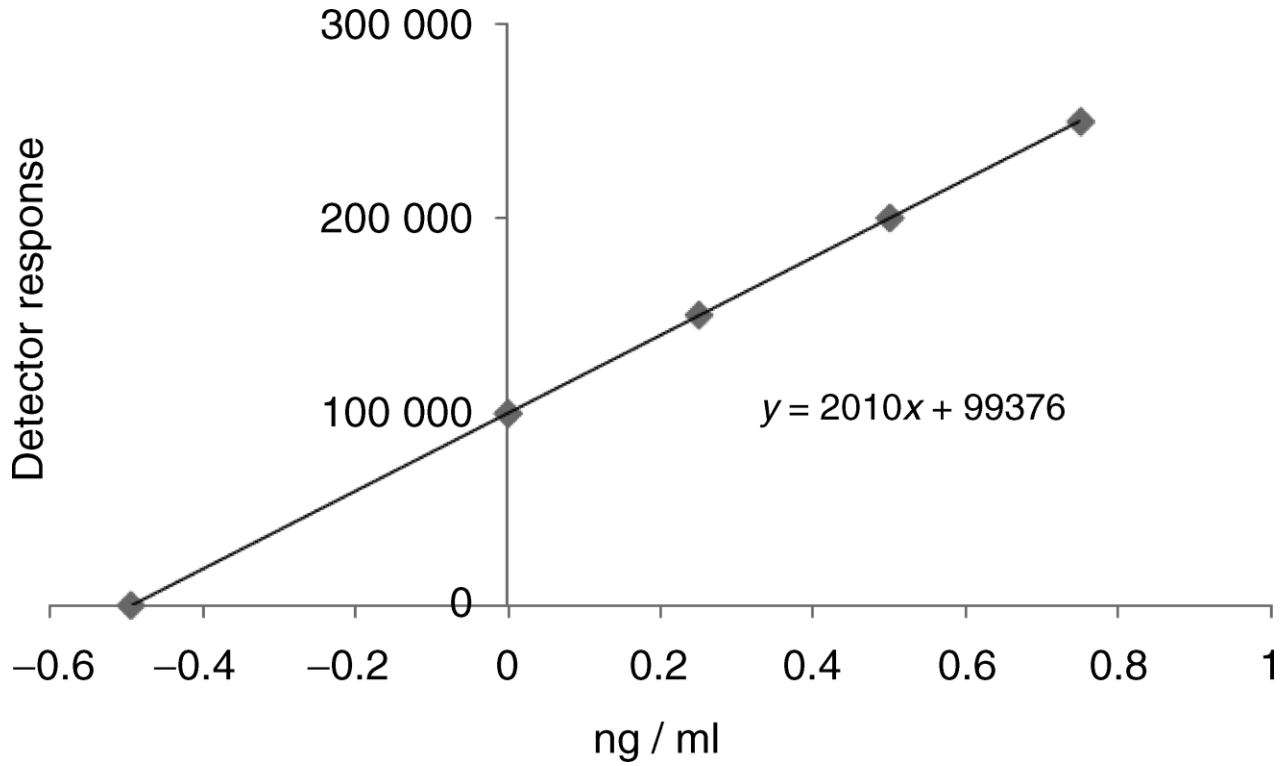
Example of a quantitative determination based on standard addition method

- The concentration of an unknown sample solution is expected to be ca. 1.0 ng/ml. To determine the unknown concentration four standard addition solutions (SAS) were prepared from the sample solution. Volumes of 5.00 ml were taken from the sample solution and added to four 10-ml volumetric flasks. SAS 1 was prepared by diluting 5.00 ml of the sample solution to 10.00 ml. SAS 2, 3 and 4 were prepared by mixing 5.00 ml of the sample solution with respectively 0.25, 0.5 and 0.75 ml of a reference solution followed by dilution to 10 ml. The reference solution contained 10 ng/ml of a chemical reference standard of the analyte. The detector responses, which are the sum of the unknown concentration (x) and the added concentrations, were:

Solution	Concentration of analyte in the standard solution (ng/mL)	Detector response
SAS 1	x	99 151
SAS 2	x + 0.25	149 987
SAS 3	x + 0.50	200 132
SAS 4	x + 0.75	249 889

Solution

- The standard curve is a plot of concentration versus detector response:



- The calibration curve calculated according to the method of least squares was:
  y = 201072x + 99376
  where y is the detector response and x is the concentration (ng/ml) in the standard solution.

- The unknown concentration in SAS 1 is found at y = 0:
  x = 99 376 / 201 072 = **0.494**

- The concentration in SAS 1 was 0.494 ng/ml. As SAS 1 was prepared by diluting 5 ml of the sample solution to 10 ml, the unknown concentration, c, of analyte in the sample solution was:

- **c** = 0.494 x 10/5 = **0.99**

- The sample solution contained **0.99 ng/ml** of analyte.

Selection of an Appropriate Analytical Method

Selecting An Appropriate Instrumental Method

The analytical technique selected depends on;

- **the property to be measured**
- **the type of food to be analyzed**
- **The reason for carrying out the analysis**

Criteria For Selecting a Method

- **Precision:** A measure of the ability to reproduce a result by a specific analyst (or group of analysts) using the same equipment and experimental approach keeping other conditions unchanged.

- **Reproducibility:** A measure of the ability to reproduce result using the same experimental approach in same as well as different laboratories using same/different equipment.

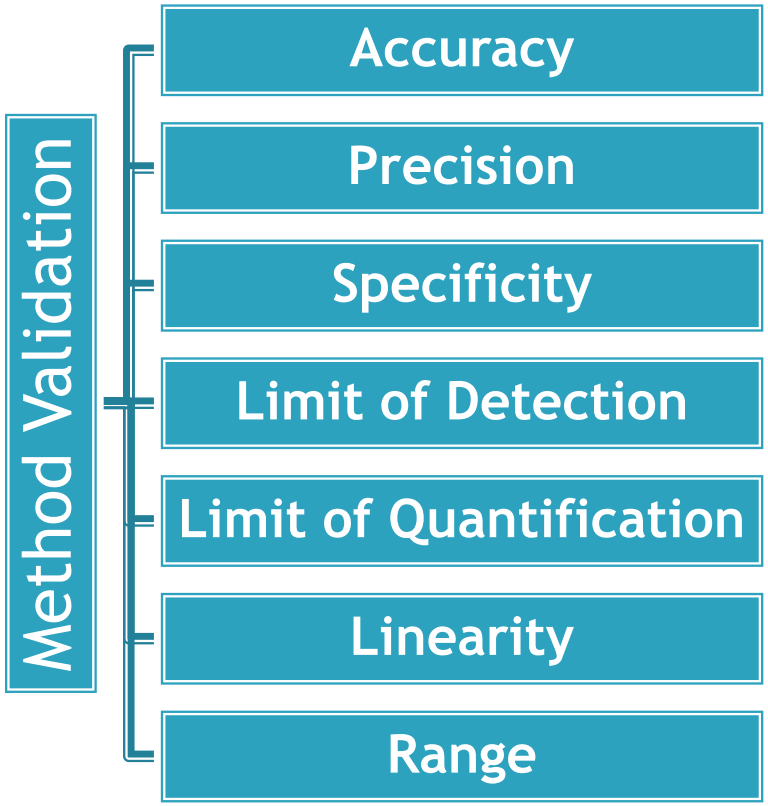
- **Accuracy:** A measure of how close one can actually measure the value to the true value of the parameter being measured.

Selecting An Appropriate Instrumental Method

- **Simplicity of operation:** A measure of the ease with which relatively unskilled workers may carry out the analysis.
- **Speed:** Analysis of single sample or the number of samples in a given time.
- **Sensitivity:** A measure of the lowest concentration of the component that can be detected by a given procedure.
- **Specificity:** A measure of the ability to detect and quantify specific components within a food material, even in the presence of other similar components e.g., Fructose in the presence of sucrose or glucose.
- **Nature of food matrix:** The composition, structure and physical properties of the matrix material surrounding the analyte often influences the type of method that can be used to carry out an analysis e.g. whether the matrix is solid or liquid, transparent or opaque, polar or nonpolar.

Validation of Analytical Methods

Method Validation



- Analytical characteristics used in validation of an analytical procedure