Qualitative and Quantitative determination

Introduction

Chromatography is a separation technique extensively used now to get results for

• Which compounds are present in the mixture?

(It requires identification of compounds normally done by Peaks in a chromatogram and this is **qualitative analysis**.)

•How much of the compounds are Present?

(It requires measuring the amount of component , done by Peak Area and this is **quantitiave analysis.**)

Analytical Goal

- Qualititative :
  - Identification of Sample Component
    - By Retention Time (based on a set of predefined system parameters)
- Quantitative :
  - Concentration of Sample Components
    - By Peak Area/ Height (based on a set of predefined system parameters)

Qualitative determination

- Retention time
  - Total Retention Time
  - Relative Retention Time
  - Retention Indices
- Spectroscopy Technique
  - UV, IR, Fluorescence, MS
- Another instrument analysis

Qualitative determination

- Retention time :
  - The retention Time of unknown component is compared with the retention time of a so-called standard.
  - When the retention times of both compounds are similar, the unknown is considered as Identified.
  - If the analyte itself is not available as a pure Std., identification based on chromatographic results only is not possible.

Quantitative determination

- Principles
- Properties of Quantitative analysis
- Calibration Curve and Response Factor
- Quantitative Calculation methods
  - Normalized 100%
  - External Standard
  - Internal Standard.
  - Standard addition

Quantitative determination

- Peak area / Peak height
- Calibration curve by standard sample
  - External standard
  - Internal standard

Peak Integration

There are 2 ways to measure the relationship b/w the detector response and Concentration.

1) Peak Height
   - It is used when the peaks are symmetrical and having a Gaussian shape with tailing 1.0.
2) Peak Area
   - It is used when the Peaks are not symmetrical and having tailing more then or less then 1.0.

Principles of Calibration

- Comparison of Peak Areas of known Standards with an unknown sample.
- Calculation of sample concentrations using measured “Calibration” factors.
- As we know that the Peak Area is a measure for the amount of a particular component. If one measures in the linear response range of the detector, the size of the area is directly proportional to the amount.
- Outside the linear range the proportion is no longer valid.
- It is important to know about the linear measurement range of detector.

Levels of Calibration

- Single Level Calibration
  - When the calibration curve is a straight line, a single calibration point can be enough, thus 1 std. with a known concentration.
  - With the result of this std. (at least 3 injections) a mean response factor is calculated as : Response Factor = $\frac{\text{Area (mean)}}{\text{Amount}}$
  - The concentration in unknown sample will be calculated as :
    - Amount = $\frac{\text{Area (mean)}}{\text{Rf}}$

Levels of Calibration

- Multi level Calibration
  - In this case a response factor is calculated by determining statistically the best fit of the calibration curve for the data points at different concentrations of Standards.
  - Multi level calibration will improve the accuracy of the method over a wider concentration range of samples.

Quantitative Calculation methods

- **Normalised Area % method**
  - **Simple and Fast**
  - **All components should be identified**
  - **All Response factors known**
  - **Relative method:**
    - **No exact injection volume needed**

In this method the amount of **all** compounds in a mixture are determined.

The amount of a particular component in a mixture is expressed by a relative fraction of the total amount. Like a certain percent of compound A or B in a mixture.

This type of calibration is not often used in HPLC, but can be used in GC analysis of gas mixtures.

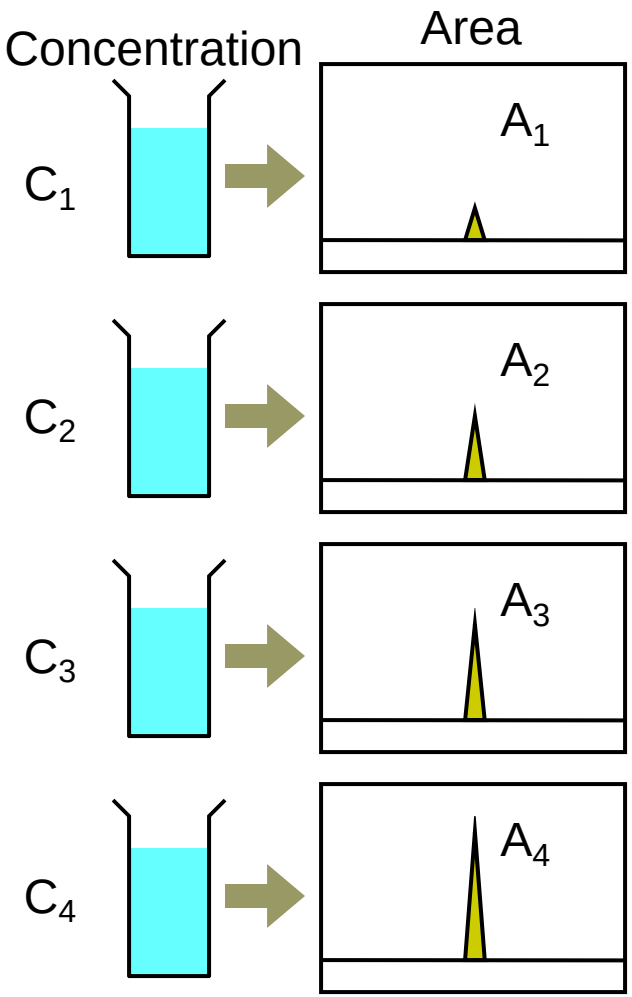
Quantitative Calculation methods

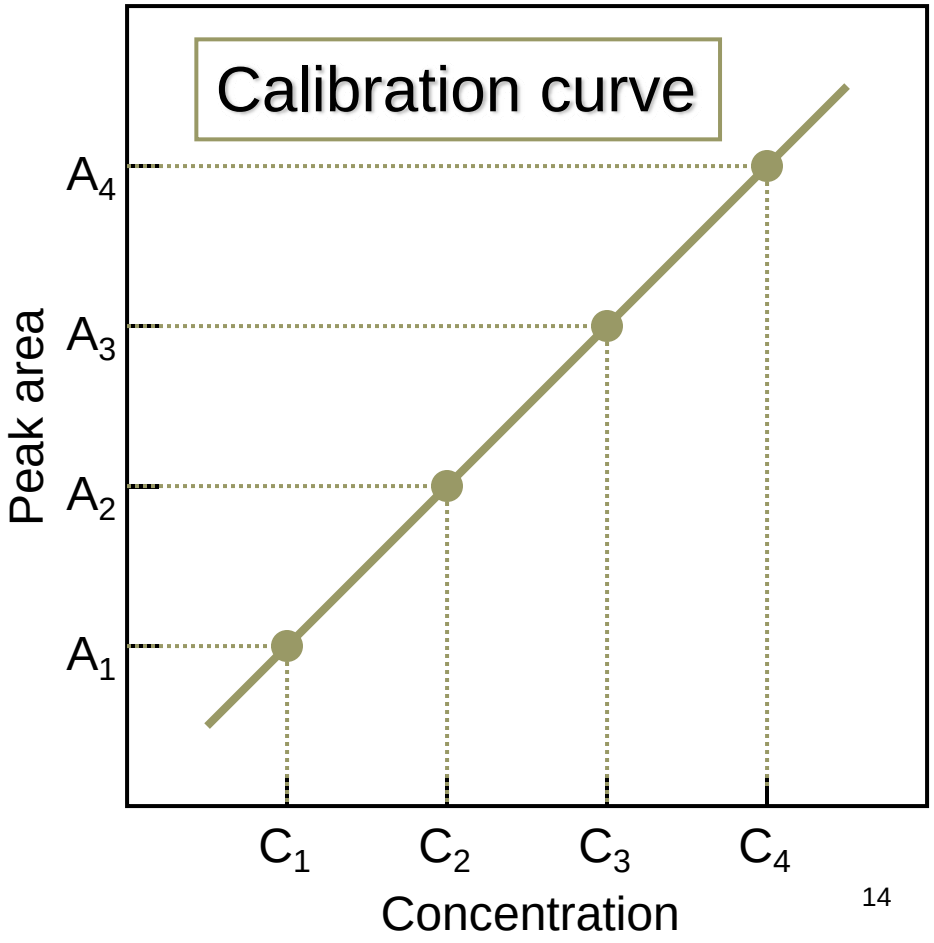
- **External standard method:**
  - **Comparison of Peak Areas of Standard with the same components as in the samples.**
  - **Identical conditions for both the Standards and samples.**

In this method the absolute concentration is determined by comparing the Peak area of an unknown sample with peak areas of known amounts of separately injected standards of the same compound.

Each standard is injected separately. The Peak Area of the standard and the sample can be compared, **if the injection volume are constant**.

External standard



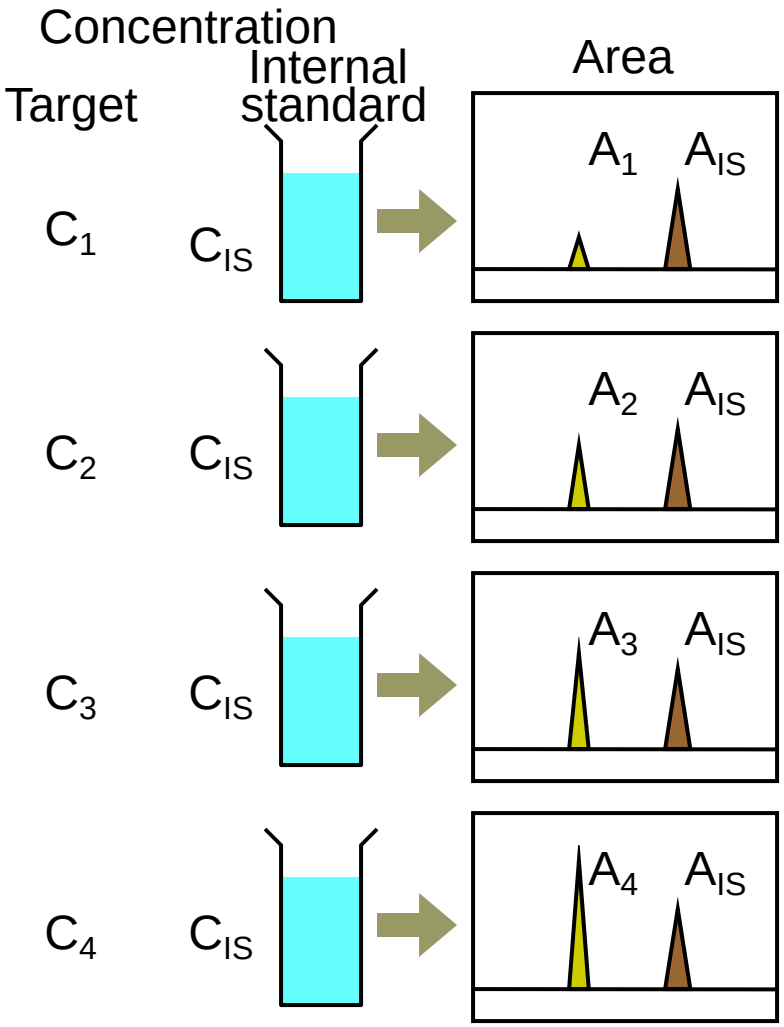


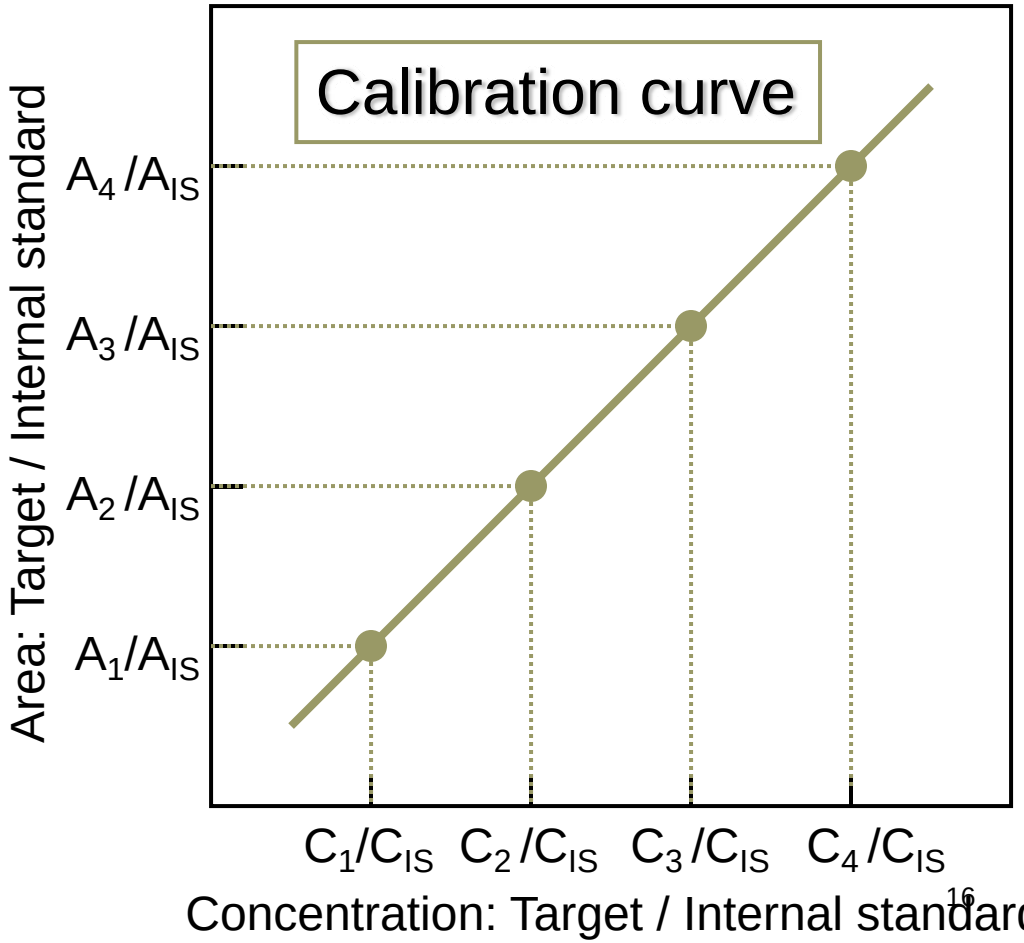
Quantitative Calculation methods

- **Internal standard method:**
  - **Addition of a known internal standard to both the sample and the standard.**
  - **Internal Standard must have comparable physical and chromatographic properties as the compound of interest.**
  - **Important in combination with sample preparation (dilution and extraction).**

In this method a certain amount of internal Std. is added to the sample which should be pure and that is not present in the samples to be anlayzed. The amount of sample peak can be calculated by comparing the sample peak with the standard peak..

Internal standard





Internal standard calculation

Standard Solution :

$$RF_{relative} = \frac{RF_{I.S.}}{RF_{X}} = \frac{\text{Conc. x X Peak Area}_{I.S.}}{\text{Conc. I.S. X Peak Area x}}$$

Sample Solution :

$$\text{Conc. x} = RF_{relative} \times \text{Conc. I.S X } \frac{\text{Peak Area x}}{\text{Peak Area I.S}}$$

With the relative response factor b/w the internal standard and the target compounds, the Conc. of target compounds can be calculated from the measured Peak Areas in the sample, to which exactly the same amount of internal Std. is added.

Internal Standard

- For the selection of internal standard a compound is selected from the same class of compounds as the target analytes.
- The isomers of the compounds are excellent candidates for an internal standard.
- In any case the internal Standard should have the same chemical, physical and chromatographic properties as the compound of interest. Of course the peak of the internal standard must not overlap with any of the others peaks form the sample.
- Examples : Vitamin D2 for Vitamin D3, Triazolam for Alprazolam, Pronethalol for propanolol

Standard addition method

- Sample itself used as standard
- For samples with complex matrices, effecting the detector response (matrix effect)
- Addition of one or more amounts of the compounds of interest to the sample matrix.
- Original concentration to be calculated by subtraction of Peak Areas.

Standard addition method

- In this method a known amount of the target compound itself is added to a sample that already contains the compound.

- This method normally applied where the composition of the sample matrix has an effect on response factor of detector. Also it can be used when there is no blank sample available.