Chromatography

The separation process involving the interaction of one or more solutes and two phases.

Mobile phase
A gas or liquid that passes through a 'column'

Stationary phase
A solid or liquid which does not move.

Chromatography

Chromatography - "color" and "to write"

Originally described by Tswett - 1906
He devised a method to separate plant pigments using a tube filled with $CaCO_3$.

After adding a plant extract, he was able to produce several colored bands by washing the extract through the column with an organic solvent.

The separation process

Sample components are carried by a mobile phase through a bed of stationary phase.

Individual species are retarded by the stationary phase based on various interactions such as:

- Surface adsorption
- Relative solubility
- Charge

Types of separation

Frontal analysis
Continuously add your sample to the start of the column.

Monitor components as they evolve.

Gives a general measure of how things are retained.

Example - charcoal filtration

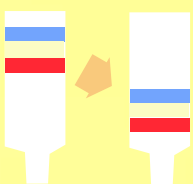
Frontal analysis

Approach can be used to evaluate relative retention. Not useful as a method of separation.

Displacement Analysis

Materials move down a column by being displaced by a more highly retained solute.

Example - ion exchange / water softeners



You can't achieve complete resolution. Making the column longer has no effect

Elution

A solute partitions between two phases (equilibrium).

Separation is based on relative retention.

Making the column longer will increase the degree of separation.

Elution

Many types of competitive attractions can be used.

Example

In GLC it is vapor pressure vs. solute solubility in the stationary phase.

Theories

Two approaches can be taken to explain the separation process.

Plate theory - proposed in 1941 by Martin and Synge. Based on an analogy with distillation and counter current extraction.

Rate theory - accounts for the dynamics of a separation - 1956, J.J. van Deemter.

Each has advantages and limitations.

Plate theory

In plate theory, we treat our chromatographic column as though it was a 'static' system in equilibrium.

Each species exhibits an equilibrium between the mobile and stationary phase.

$A_{\text{mobile phase}} \rightleftharpoons A_{\text{stationary phase}}$

Plate theory

We can define a partition coefficient based on this equilibrium.

$$K = \frac{\text{concentration solute in stationary phase}}{\text{concentration solute in mobile phase}}$$

K is assumed to be independent of concentration. It can be altered by such factors as temperature.

As K increases, our solutes take longer to pass through the column.

Plate theory

For a separation, assume that:

The column is of fixed length.

Flow is held constant.

We can then describe several terms based on how our average sample species performs.

The first thing to do is measure how long it takes for our solute to travel through our column.

Retention time and volume

Retention volume, V_R - volume of mobile phase required to elute a solute to a maximum from a column.

Retention time, t_R, time required to reach the same maximum at constant flow.

V_R **or** t_R

where $V_R = t_R \times$ flowrate

Plate theory

The average linear rate for the solute is:

$v = L / t_R$

where L is the column length.

And for the mobile phase it is:

$u = L / t_R$

This is simply the time required for a non-interacting substance to pass through the column.

Plate theory

$$v = u \times f$$

f is the fraction of time that the solute spends in the mobile phase.

$$f = n\ solute_M / n\ solute_S$$

Since both the mobile and stationary phases have known volumes (V_M, V_S), we can now determine K - our partition coefficient.

Plate theory

$$v = u \frac{C_M V_M}{C_M V_M + C_M V_S}$$

$$= u \left(\frac{1}{1 + \frac{C_S V_S}{C_M V_M}} \right)$$

$$= u \left(\frac{1}{1 + \frac{K V_S}{V_M}} \right)$$

Plate theory

$$v = u \left(\frac{1}{1 + K V_S / V_M} \right)$$

This shows the factors required to have a component elute and how two materials can be separated.

Each material has its own K.

As K increases, elution takes longer.

Other terms affect the overall separation.

Plate theory

Raising

V_S	General increase in retention
V_M	General decrease in retention
u	Increases speed of separation.

V_S and V_M can be altered by changing column diameter and length for a specific column packing.

u can be altered by changing the flowrate.

All terms can be found by knowing how the column was prepared.

Capacity factor

We'd like to be able to simply inject a sample and obtain the information we need from the results.

Capacity factor

$k' = K V_S / V_M$ (constant for our column conditions)

so $v = u (1 / (1 + k'))$

We can now expand the equation.

Capacity factor

$$\frac{L}{t_R} = \frac{L}{t_M} \times \frac{1}{1 + k'}$$

$$k' = \frac{t_R - t_M}{t_M}$$

We now have a simple method to determine k values based on elution times.

Selectivity factor - α

Remember, the goal of chromatography is to 'separate' two or more species.

Since each species has its own k value, we need a way to tell if two species can be separated.

Selectivity factor

$K_b > K_a$, $\alpha \geq 1$ $\qquad \alpha = \frac{K_B}{K_A}$

Selectivity factor

We can also use:

$$\alpha = \frac{k'_a}{k'_b} = \frac{t_{RB} - t_M}{t_{RA} - t_M}$$

You can either:

Determine α based on the retention times for you solutes or

Estimate if your species are separable based on α.

Mechanism of component separation

Mobile phase volume is proportional to column length so retention is also increased for longer columns.

However, as peaks travel through the column, they broaden. Width increases with the square root of column length.

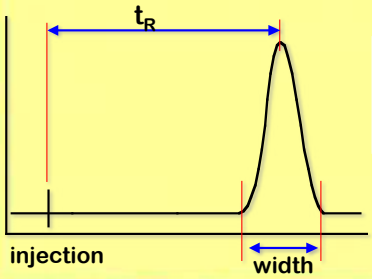
You can't just make a column longer to obtain a 'better' separation.

Theoretical plates - N

- In solvent extraction, a plate is represented by each equilibrium (extraction) we conduct.
- In a chromatographic column, we can't see these plates so they are theoretical.
- We can estimate the number of theoretical plates in our column based on peak retention times and widths.
- Both factors are important in determining if a separation will work.

Determining N

The number of plates can be determined from the retention time and peak width.



It doesn't matter what units are used as long as they are the same.

Determination of N

The number of plates is then calculated as:

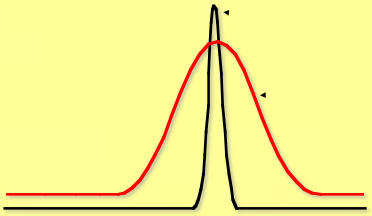
$$N = 16\left(\frac{t_R}{W}\right)^2$$

This approach is taken because peaks evolve as Gaussian-like shapes and can be treated statistically.

In essence, we are taking $\pm$ 2 σ.

Determination of N

In this example, we have materials with the same elution time but different numbers of plates.



N = 1000

N = 100

Determination of N

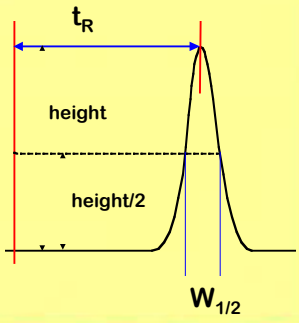
It is often difficult to accurately measure peak width.

- ➔ The peak may co-elute with another.
- ➔ Low detector sensitivity can result in you finding the start or end at the wrong place.
- ➔ We can take an alternate approach.

Determination of N

We can measure the width at half height.

This insures that we are well above background and away from any detector sensitivity limit problems.



Determination of N

Since the peak is Gaussian in nature, we end up with the following modified formula.

$$N = 5.54\left(\frac{t_R}{W_{1/2}}\right)^2$$

For a fixed length column, we can calculate an additional term - h (or HETP)

h = height equivalent of a theoretical plate
= column length / N

Resolution

Knowing how well a column can retain a component is nice but we need to deal with multiple eluents or why bother.

Resolution, R_s

A measure of how completely two neighboring peaks are separated from each other.

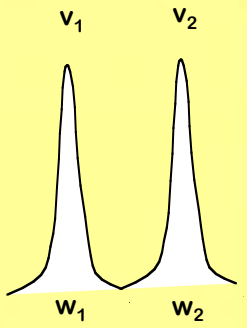
Resolution

$$R_s = \frac{v_2 - v_1}{(w_2 + w_1)/2}$$

since $w = 4\,v / N^{1/2}$

$$R_s = \frac{N}{2}\,\frac{v_2 - v_1}{v_2 + v_1}$$

Note: times or any representative unit can be used in place of v and w terms.



Resolution

Since $v = V_m (1 - k)$

resolution in terms of k is:

$$R_s = \frac{N}{2}\,\frac{k_2 - k_1}{2 + k_2 + k_1}$$

Remember that the relative volatility, α, is

$$\alpha = k_2 / k_1$$

Resolution

$$R_s = \frac{N}{2}\,\frac{\alpha - 1}{\alpha + 1 + 2/k_1}$$

Finally, if we define k as $(k_1+k_2)/2$ - average k

$$R_s = \frac{N}{4}\,\frac{\alpha - 1}{\alpha + 1}\,\frac{k}{1 + k}$$

This version of the resolution equation will be most useful with LC.

Approximate resolution equations

The exact resolution equation is:

$$R_s = \frac{N}{4}\,\ln\left(1 + \frac{k_2 - k_1}{1 + k_1}\right)$$

$k_2 \cong k_1$, when $\frac{k_2 - k_1}{1 + k_1}$ is small compared to 1.

and $\ln (1 + x) \cong x$

For a small x, $R_s = \frac{N}{4}\left(\frac{k_2 - k_1}{1 + k_1}\right)$

Approximate resolution equations

Since $\alpha = k_2/k_1$, we get

$$R_s = \frac{N}{4}\,(\alpha - 1)\,\frac{k_1}{1 + k_1}$$ Knox equation

Similarly, if we start with

$$R_s = -\frac{N}{4}\,\ln\left(1 - \frac{k_2 - k_1}{1 + k_1}\right)$$

This will lead to the Purnell Equation

$$R_s = \frac{N}{4}\,\frac{\alpha - 1}{\alpha}\,\frac{k_2}{1 + k_2}$$

Approximate resolution equations

Each version of the equation will yield R_s values that deviate from the exact solution.

Knox - R_s values will be higher

Purnell - R_s values will be lower
(a more conservative estimate)

If we assume that the peaks are Gaussian, we can us:

$$R_s = 1.18 \frac{t_{R\,2} - t_{R\,1}}{W_{1/2\,2} + W_{1/2\,1}}$$

A 'working' form of the resolution equation.

Example

Determine the k, N and HETP for toluene in the following analysis

Solute	t_R, min	$W_{1/2}$, min
air	1.5	
benzene	7.45	1.05
toluene	10.6	1.45

Column length = 10 meters
Flow rate = 30 ml/min
isothermal conditions

Example

k

$t_R / t_M = 1 + k$

$k = t_R / t_M - 1$

$= 10.6 / 1.50 - 1$

$= 6.07$

Example

N

$N = 5.54 (t_R / W_{1/2})^2$

$= 5.54 (10.6 / 1.45)^2$

$= 296$

HETP

$h = l / N = 1000 \text{ cm} / 296$

$= 3.38 \text{ cm}$

Example

Finally, calculate the resolution between the benzene and toluene peaks.

$$R_s = 1.18 \frac{(t_{toluene} - t_{benzene})}{(W_{1/2\ toluene} + W_{1/2\ benzene})}$$

$= 1.18 (10.6 - 7.45) / (1.05 + 1.45)$

$= 1.48$ (quantitative separation)

Rate theory of chromatography

Plate theory assumes that a column is mathematically equivalent to a plate column.

An equilibrium is established for the solute between the mobile and stationary phases one each plate.

It is a useful theory and can predict many aspects of chromatographic performance.

Rate theory of chromatography

Plate theory neglects the concepts of solute diffusion and flow paths.

Rate theory accounts for these and can be used to predict the effect on column performance factors such as:

- phase properties
- solute diffusivitivites
- partition coefficients
- phase velocity
- phase thickness
- support size
- support porosity
- flow rates

Rate theory of chromatography

A partial differential equation set up by van Deemter for a linear isotherm resulted in an effluent concentration function.

It is based on a Gaussian distribution similar to that of plate theory.

He was attempting to account for the dynamics of the separation process.

van Deemter equation

$$H = 2\lambda d_p + \frac{2\gamma D_g}{u} + \frac{8}{\pi^2}\frac{k d_f^2}{(1+k)^2 D_l} u$$

- λ - factor characteristic of packing
- d_p - particle diameter
- γ - factor for irregularity of interparticle spaces
- D_g - diffusion coefficient of compound in gas
- D_l - diffusion coefficient of compound in liquid
- k - capacity ratio
- d_f - liquid phase effective film thickness
- u - linear gas velocity
- H - height of a theoretical plate

van Deempter equation

The equation consists of three basic terms.

$2\lambda d_p$	Packing related term
$\frac{2\gamma D_g}{u}$	Gas (mobile phase) term
$\frac{8}{\pi^2}\frac{k d_f^2}{(1+k)^2 D_l} u$	Liquid (stationary phase) term

van Deemter equation

We commonly group the various constants into single terms and reduce the equation to:

$$H = A + B/u + Cu$$

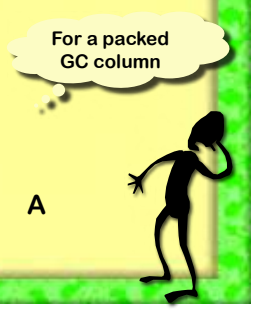
- A - multipath or eddy diffusion
- B - molecular diffusion
- C - resistance to mass transfer

Note that A, B and C are constants but the effect of B and C is dependent of the velocity of the mobile phase.

van Deemter equation

The goal is to find H_{min} for optimum column performance.

$$H = A + B/u + Cu$$



van Deemter terms

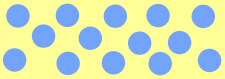
You don't need to calculate H for each column/eluent combination to be able to use this relationship.

An understanding of the effects of each term will help you design/select appropriate columns and optimum flows.

A term

Multipath or eddy diffusion

This term accounts for the effects of packing size and geometry.



fastest

slowest

The range of possible solute paths results in a minimum peak width.

A term

Once the column is packed, nothing can be done to reduce the A term.

Its effect can be reduced by using

- regular sized packing
- small diameter packing
- not allowing any loose packing or dead space in the column

B term

Molecular diffusion

Represents broadening due to diffusion in the mobile phase.

Reverse diffusion is more significant than forward to to mobile phase movement.

trailing tail broadening

B term

The effect of the B term is flow dependent. As you increase the flow, the time for diffusion is reduced.

You should keep the flow as high as possible within the limits imposed by the instrument and the C term.

C term

Resistance to mass transfer.

It takes time for a solute to reach an equilibrium between the mobile and stationary phases.

Thick or viscous stationary phases have larger C terms

C term

You can minimize the effect of the C term by:

Using “thin” coatings of the stationary phase on a solid support.

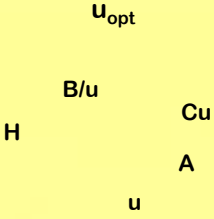
Use less viscous phases.

Keep the flow as low as possible - limited by the effect of the B term.

Optimum velocity

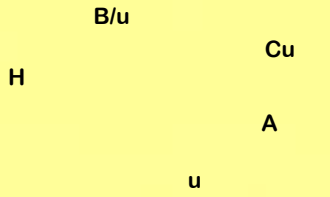
The best velocity is a function of the Van deemter equation and practical conditions. You need to have a useable analysis time.

Also, since the effects of B are greater than C, it is best to set the flow a little on the high side in case it changes slightly during the analysis.



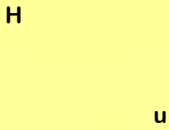
Van Deemter plots

This plot is for a typical packed column GC analysis. Gases have high diffusions and column packings are relatively large.



Capillary columns

Not much effect from A or C. There is no packing and the phase is very thin

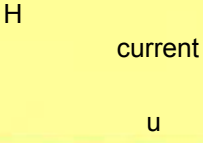


Liquid chromatography

At first, LC relied on irregular packing. Now the packings are pretty good so the A term is very low.

originally

The B and C terms are low because liquids diffuse much more slowly than gases



Qualitative analysis

We typically think of GC and LC as quantitative tools.

In general, chromatography is a ‘blind’ method. It indicates the presence of a substance but not what it is.

Qualitative data can also be obtained even with non-discriminating detectors.

Qualitative analysis

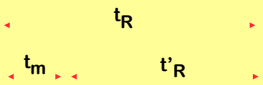
Retention data - can be used for some qualitative work. The t_R is characteristic of a substance, compared to a standard. To be useful, some problems must be addressed.

- Reproducibility of absolute retention data depends on several experimental conditions.
- Is t_R, v_R, v'_R or t'_R best to use?

Retention time

Retention time - t_R - time elapsed from point of injection to maximum of peak.

Adjusted t_R - t'_R - time from maximum of unretained peak to maximum of eluent.



Hold up time - t_M - time required for mobile phase to traverse the column.

Retention volumes

If the flowrate (F_c) is constant and known then:

Retention volume	$= V_R$	$= t_R \ F_c$
Adjusted V_R	$= V'_R$	$= t'_R \ F_c$
Hold up volume	$= V_m$	$= t_M \ F_c$

Retention relationships

Retention volume or time may be used for identification.

For a homologous series, V_R can be accurately determined by:

$$\ln V'_n = a + bn$$

where V'_n = adjusted retention volume
n = carbon number
a, b = fit parameters

and $V'_n = V_n - V_m$

Retention relationships

To determine an unknown carbon number:

$$x = n_1 + (n_2 - n_1) \frac{\ln V_x - \ln V_{n1}}{\ln V_{n2} - \ln V_{n1}}$$

$$n_2 > x > n_1$$

This can only be used for straight chain compounds and the unknown must fall between n_1 and n_2.

Absolute retention index

For non-paraffins, we can simply calculate an index value like it was a paraffin. The value does not need to be a whole number.

$$I_p = n_1 + \frac{\ln V_x - \ln V_{n1}}{\ln V_{n2} - \ln V_{n1}}$$

$$n_2 > x > n_1$$

n_2 and n_1 are reference paraffins.

Kovat's retention index

A modification of the absolute index where:

$$I_K = 100\, I_P = 100n_1 + 100\, \frac{\ln V_x - \ln V_{n1}}{\ln V_{n2} - \ln V_{n1}}$$

This index has been determined at different temperatures for a large number of compounds. Tables are also available.

The value can be used to compare related separations.

Relative retention data

One practical approach for your own data is the use of relative retention.

$$r_{i,std} = \frac{t'_{R(u)}}{t'_{R(std)}} = \frac{V'_{R(u)}}{V'_{R(std)}} = \frac{k_{(u)}}{k_{(std)}}$$

This is the most common approach. It only requires a single standard.

Relative retention data

To be useful

Standard should be a part of the sample or added to it - internal standard

It should be something that elutes near center of an analysis - although you can use more than one.

Sample size should be small.

Values will remain pretty constant between runs - may vary wildly with a new column.

Retention time, t_R

Simple retention time data is adequate for simple assays like process quality control.

- You already know what is there.
- There are only a few components in the sample (or only a few of interest).

If a true unknown is observed, you can't do much more than note its presence!

Quantitative analysis

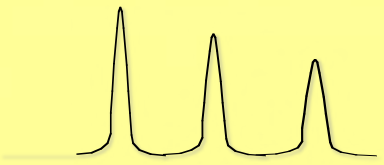
All chromatographic detectors produce a signal that drives a meter, recorder, integrator or A/D converter.

While the detectors used for GC and LC are not the same, quantitation methods are identical.

Each detector will produce a response/unit concentration. This is substance dependent so standards must always be used.

Peaks

Each method of quantitation assumes that you have one or more reasonably resolved peaks.



You must be able to find the beginning and end of each peak as well as it's maximum.

Peak height

In some cases, you can assume that peak height is proportional to concentration.

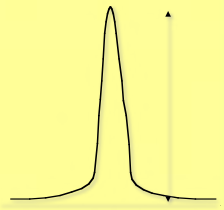
Advantages

Simplicity

Rapid calculations

Disadvantages

Height is more variable than area



Typically used only with capillary columns

Peak area

This is the major approach for establishing a relationship between peaks and concentration.

area α concentration

Area is determined from a large number of measurements and detectors usually have very large dynamic ranges. This results in a very reliable measurement.

Peak area

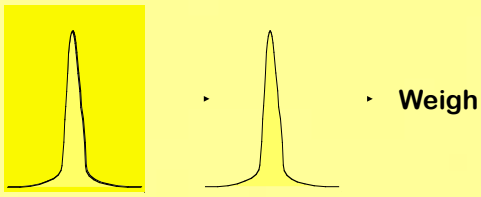
Major problem

If the peak is approximately Gaussian, how do we accurately measure its area?

Manual	Automated
Cut & Weigh	Integrating recorder
Planimeter	Digital integrators
Triangulation	Computer systems

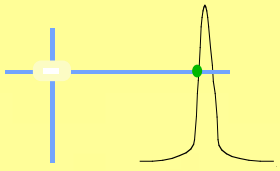
Cut and weigh

With this approach, each peak is cut from the recording paper and weighted. Weight is then considered proportional to area.



Planimeter

A device used to trace the peak. It produces a number that is proportional to peak area.



Triangulation

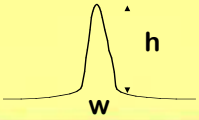
Main manual method.

Assumes that each peak approximates a triangle. Area can be determined by

area = peak height x width

or

area = peak height x 2 $W_{1/2}$

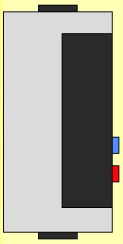


Integrating recorders

A special two pen recorder.

The first pen tracks the chromatographic signal.

The second traces a series of zigzags.



Integrating recorders

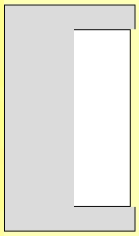
The larger the peak response gets, the more rapidly the second pen sweeps back and forth.

The total number of zigs and zags can then be related to the peak area.

If the peak gets too large, the second pen stops moving. The peak must be kept within the working range.

Digital integrators

Rely on A/D conversion of detector response.



Digital integrators

Caution!

Peaks are typically processed for area on the fly.

This includes not only peak detection but may also handle other methods for dealing with poorly shaped or resolved components.

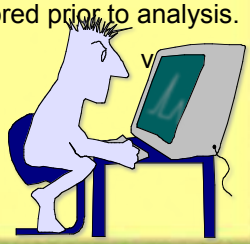
If a peak is missed, the run must be repeated.

Computer systems

Include the same methods of peak detection and integration as integrators.

Major advantage is that the entire chromatographic run is stored prior to analysis.

This allows you to test out methods of integration on a single run and to reanalyze data if a peak is missed.



Summary

Method	Time, min	Precision, %
Planimeter	15	4.1
Triangulation	10	2.5 - 4
Cut & weigh	20	1.7
Int. Recorder	5	1.3
Integrator	N/A	0.44
Computer	N/A	0.44

Quantitative interpretation

OK, now you have all of your peak areas.

Let's assume you knew what you were doing and all the areas were measured properly.

Big deal!

A relationship between concentration and area must be established or we're just spinning our wheels.

Determining concentration

Several approaches can be used. Use the one that is most appropriate for your method.

Methods we'll cover

External standard method
Internal standard method

External standard method

Requirements for proper use:

Standard solution containing all eluents to be quantified.

Standard eluents should be of similar concentration as unknowns.

The standard and sample matrix should be as similar as possible

Analysis conditions must be identical - stable instrument, same sample size ...

External standard method

You either assume that response is linear over the entire concentration range or measure it. Then:

$$conc_{unknown} = \frac{Area_{unknown}}{Area_{known}} \; conc_{known}$$

This is assuming that the same injection volume was used for both the unknown and standard.

External standard method

Example - determination of X in $MeCl_2$

Prepare a standard of X
(20.0 mg in 100 ml $MeCl_2$) - 0.200 μg/μl

Use an injection volume of 5 μl for both the standard and the unknown.

Measure the areas produced by both the sample and the unknown.

Area X_{std} = 2000 units
Area X_{unk} = 3830 units

External standard method

Now, determine the concentration of X in you unknown.

$$conc_{unknown} = \frac{Area_{unknown}}{Area_{known}} \; conc_{known}$$

$$conc_{unknown} = \frac{3830}{2000} \; 0.200 \; \mu g / \mu l$$

$$= 0.384 \; \mu g / \mu l$$

You can now convert to a more appropriate concentration if required.

Internal standard method

Overall, the most reliable approach.

Basis

A known substance is added at a constant concentration to all standards and samples - **internal standard**.

Since the internal standard is always present at a constant amount, it can be used to account for variations such as injection volume during an analysis.

Internal standard method

Requirements for an internal standard.

- Must be present at a constant concentration in all samples and standards.
- Must be stable and measurable under the analysis conditions.
- Must not interfere with the analysis or co-elute with sample components.

Internal standard method

Three common approaches are used

Classical method - weighed portions of the standard and sample are combined

Stock solution - a known volume of the sample is 'spiked' with a known volume of the standard

Calibration plot - a series of standards are run and a curve plotted based on corrected peak areas.

Internal standard method

Regardless of the method for introducing the standard or calibrating, the calculations are the same.

They are the same as with the detector response factor method.

Our NORM substance is now predetermined and a fix value.

Internal standard method

$$C_{ISTD} = f_{ISTD}\, A_{ISTD}$$

$$C_{unk} = f_{unk}\, A_{unk}$$

Since the internal standard is assigned a value of 1.00 and is held constant, we can correct for run to run variations by:

$$C_{unk} = \frac{A_{ISTD1}}{A_{ISTD2}} \frac{A_{unk}}{A_{known}} C_{known}$$

known & ISTD1 are obtain from the standard, unk & ISTD2 from the unknown

Internal standard method

It is assumed that variations in the internal standard area are representative of the whole analysis.

Accounts for factors such as:

Sample injection errors or changes
Slow detector variations
Slow column changes

Internal standard method

Example

Prepare a standard that contains 11.3 mg of X and 12.00 mg of ISTD.

Make several 2 μl injections and calculate an average response for each component.

Component	Average area
X	635
ISTD	1009

Internal standard method

Now, inject your unknown.

$Area_X$ = 990

$Area_{ISTD}$ = 1031

C_X = (1009/1031) (990/635) x 11.3 mg

= 17.24 mg X in the unknown.