Université de Strasbourg

Licence 3

Analytical Chemistry

Chromatography

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Outline

1. General introduction of Chromatography
2. Fundamentals of Chromatography
3. Gaz Chromatography: Principle and Instrumentation
   - Sample introduction: Injectors
   - Columns
   - Detectors
4. Liquid Chromatography: Principle and Instrumentation
   - Sample introduction: Injectors
   - Columns
   - Detectors
5. LC-GC/MS coupling
6. Quantitative Analysis

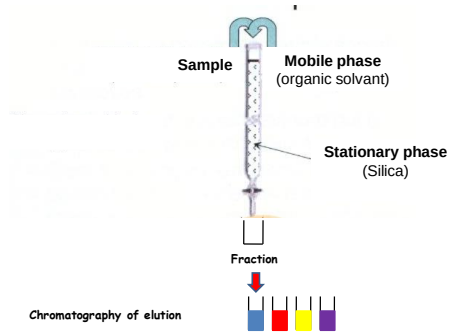
Introduction

History

- 1900: Invention of Chromatography (Michel TSWETT)
- 1938: First thin layer chromatography (Ismailov et Schraiber)
- 1952: Official birth of Gaz Chromatography (Martin et Synge, Nobel 1952)
- 1955 – 1960: Golden Age of Gaz Chromatography
- End of 60: Official birth of Liquid Chromatography
- Nowadays: Instrumental development (Amélioration instrumentale (computarization) and miniaturization (nanotechnology)

Introduction

Principle



Introduction

Principle

- **Separation of complex mixture**
- **Based on equilibrium between the sample and two phases:**
  - ✓ **Stationary phase**
  - ✓ **Mobile phase**
- **Different interactions can come into play:**
  - ✓ **Adsorption**
  - ✓ **Partition**
  - ✓ **Ion Pairing**
  - ✓ **Ion Exchange**
  - ✓ **Steric Exclusion**

Introduction

Principle

- **Strong affinity of the sample for the stationary phase:**
  - ✓ **Sample progresses slowly in the stationary phase**
  - ✓ **Retention time of the sample is long**
- **Low affinity of the sample for the stationary phase:**
  - ✓ **Sample progresses quickly in the stationary phase**
  - ✓ **Retention time of the sample is shorter**
- **Retention time of the sample allows his identification**

Introduction

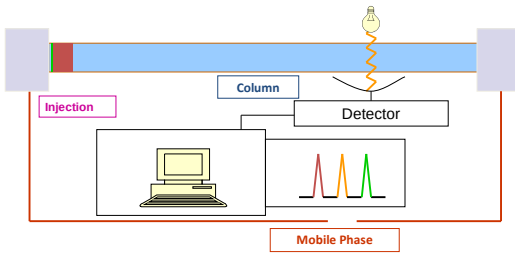
Principle

Constant instrumental development of chromatography:

- **Miniaturization and automation of injectors**
  - ✓ **Injection of small volume**
  - ✓ **Repeatability of injection**
- **Large variety of stationary phases**
  - ✓ **Variation of the types of interaction**
- **Increase of sensitivity of detectors**
  - ✓ **Very low concentration sample (trace)**

Introduction

Principle



Introduction

Fields of Application

- **Nowadays, chromatography is the reference method the most used.**
- **Techniques widely used in the industrial world**
- **Very large field of applicability**

Introduction

Fields of Application

- **Chemical industry:** production, control…
- **Food industry**
- **Cosmetic and perfumes**
- **Pharmaceutical and biopharmaceutical industry**
- **Energy :** oil and gas
- **Environment:** water, atmosphere
- **Space exploration**

Introduction

The questions you will have to ask yourself?

- **What kind of sample?** Solid, liquid, gaseous
- **Partial or complete analysis of the sample?**
- **Precision of the analysis?** Qualitative or quantitative
- **What does one want to quantify?** Major compound, minor or traces
- **Recovery of the sample?**
- **Duration of the analysis?**
- **What will be the cost of the analysis?**
- **Potential environment issues?**

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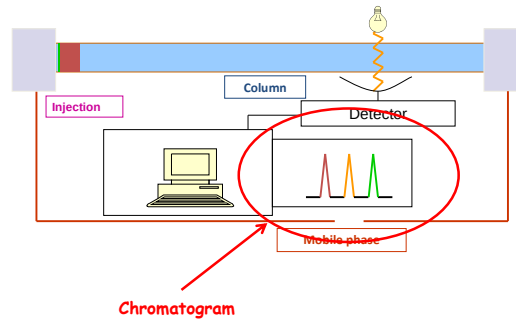
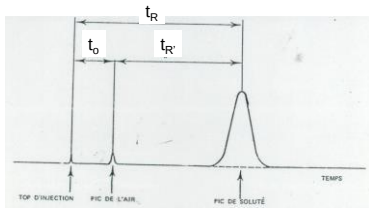
Fundamentals

Chromatography



Fundamentals

The Chromatogram



t_o : Dead time
t_R : Retention time
t'_R : Reduced retention time

Fundamentals

Partition coefficient

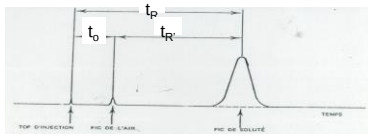
$$K = \frac{C_S}{C_M}$$

✓ C_S : concentration in stationary phase

✓ C_M : concentration in mobile phase

Fundamentals

Physicals: Retention time



t_o (Dead time) : elapsed time for a compound not retained by the column

t_R (Retention time) : elapsed time between injection and the maximum intensity of compound's peak

t'_R (Reduced retention time) : retention time frees from phenomena outside stationary phase

$$t'_R = t_R - t_o$$

Fundamentals

Physicals: Volume

V_M (Dead Volume) : Volume of the moblise phase

$$V_o = t_o \cdot D$$

t_o : Dead time
D: Flow rate

Warning
V_o is different of the column volume **because you need to consider** the porosity ε of the column

V_{Col} = Volume column = $\pi \cdot (d/2)^2$

V_o = Dead volume = $\pi \cdot (d/2)^2 \cdot \varepsilon$

ε = 0,8 for a good column

Fundamentals

Physicals: Volume

V_M (Dead Volume) : Volume of the moblise phase

$$V_o = t_o \cdot D$$

t_o : Dead time
D: Flow rate

V_S (Stationary Volume) : Volume of the stationary phase

$$V_S = V_{tot} - V_o$$

V_{tot} : total internal volume

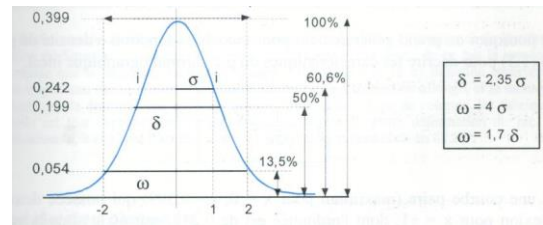
V_R (Retention Volume): Volume of the mobile phase that must be passed to migrate the solute

$$V_R = t_R \cdot D$$

t_R : Rentention time
D : Flow rate

Fundamentals

The ideal chromatogram



Characteristic of the gaussian curve

Fundamentals

Retention factor k'

$$k = \frac{m_S}{m_M} = K\frac{V_S}{V_M}$$

$$k' = K \cdot \frac{V_s}{V_M} = \frac{C_s}{C_M} \cdot \frac{V_s}{V_M} = \frac{n_s}{n_M}$$

- ✓ Independent of the flow rate
- ✓ Independent of the column lenght
- ✓ Defines the behavior of the columns

$t_R = t_0(k' + 1)$ ⟶ $k' = t'_R/t_0$

Fundamentals

Rules: Retention factor k'

- **k' = 0 → $t_R = t_0$ ou $V_R = V_0$**
  - Compound not retained by the stationary phase
- **Low value of k'**
  - Compound bit retained by the stationary phase
  - Fast t_R
- **High value of k'**
  - Compound highly retained by the stationary phase
  - High t_R
- **Too high value of k'**
  - Compound too restrained by the stationary phase
  - Phenomenon of diffusion,

Fundamentals

Rules: Retention factor k'

- **Order of magnitude of k' : Between 1 to 10**
- **The best compromise:**
  - Fast analysis
  - Good separation

$$2 < k' < 6$$

Fundamentals

The theoretical plate model

- **Theoretical plate model is probably the best theory to explain the phenomena of chromatographic separation.**
- **Equilibrium modeling: Sample / Stationary phase / Mobile phase in the form of plate**
- **Limitations :**
  - ✓ **Absence of diffusion phenomena consideration**
  - ✓ **Absence of kinetic consideration (speed of exchanges between the two phases)**

Fundamentals

Theoretical Efficiency N

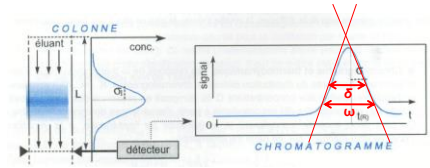
- **Parameter N: Number of Theoretical Plates**
- **Parameter H: Height equivalent to one theoretical plate**

H = L/N

- N : relative parameter, depends of the sample and the operating conditions

Fundamentals

Theoretical Efficiency N



Dispersion of a sample in the column and translation on the chromatogram

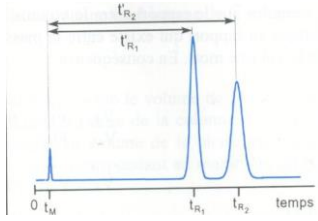
$$N = \frac{t_R^2}{\sigma^2}$$

$$N = 5,54\frac{t_R^2}{\delta^2} \quad \textit{or} \quad N = 16\frac{t_R^2}{\omega^2}$$

Fundamentals

Selectivity α

- **The measurement of the selectivity of a separation between two compounds is carried out using the selectivity factor α**
- **The selectivity factor α describes the position of two adjacent peak, relative to each other.**



Fundamentals

Selectivity α

- **The selectivity factor α can be expressed using the retention parameters:**

• Using Retention Times:

$$\alpha = \frac{t'_{r2}}{t'_{r1}} = \frac{t_{r2} - t_o}{t_{r1} - t_o}$$

• Using the Volumes:

$\alpha = (V_{R2} - V_0)/(V_{R1} - V_0)$

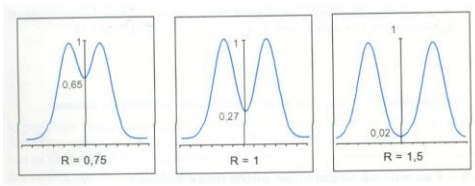
✓ $V_{R2} = V_0 + K_2V_S$
✓ $V_{R1} = V_0 + K_1V_S$

$$\alpha = \frac{K_2}{K_1}$$ **Or** $K_i = k'_i \cdot V_0/V_S$ $$\alpha = \frac{k_2'}{k_1'}$$

Fundamentals

Resolution: R or Rs

- **The resolution factor R makes it possible to numerically translate the quality of the separation between two peaks.**

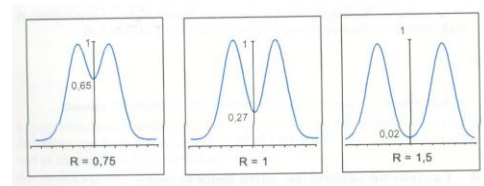


- **Resolution calculation**

$$R_s = 2 \cdot \frac{t_{r2} - t_{r1}}{\omega_2 + \omega_1}$$

Fundamentals

Resolution: R or Rs



Resolution is considered as good when:

$$R_s > 1,5$$

Fundamentals

Relation between R and N

- **For two homologous peak :** $\frac{\omega_2 + \omega_1}{2} = \omega_1$

Or $\omega_1 = \frac{4 \cdot t_{r1}}{\sqrt{N}} = \frac{4 \cdot t_0 \cdot (1 + k'_1)}{\sqrt{N}}$

$$R_s = \frac{\not{4}_0 \cdot (1 + k'_2) - \not{4}_0 \cdot (1 + k'_1)}{4 \not{4}_0 \cdot (1 + k'_1)} \cdot \sqrt{N}$$

$$R_s = \frac{(k'_2 - k'_1)}{1 + k'_1} \cdot \frac{\sqrt{N}}{4}$$

Fundamentals

Application

- **Separation of two compounds A and B on a 2 meters column:**
- Calculate the separation resolution R?
  - Experimental data:
    - ✓ t_{RA} = 400 sec
    - ✓ t_{RB} = 420 sec
    - ✓ t_0 = 50 sec

ω_A = 19,5 sec
ω_B = 20,5 sec

R = 2.(420-400)/(19,5+20,5) = **1**

→ **Bad separation**

Fundamentals

Application

- What column length would be needed to have R = 1.5?

$$\frac{L_2}{L_1} = \frac{N_2}{N_1} = y \qquad \sqrt{N} \rightarrow \frac{R_{S2}}{R_{S1}} = \sqrt{\frac{N_2}{N_1}} = \sqrt{y}$$

- Calculation:

$R_2/R_1 = 1,5/1 = 1,5 = \sqrt{y}$

↳ y = 2,25

↳ **L_2 = 2,25 . 2 m = 4,5 m**

Fundamentals

The theoretical plate model

- **Theoretical plate model is probably the best theory to explain the phenomena of chromatographic separation.**
- **Equilibrium modeling: Sample / Stationary phase / Mobile phase in the form of plate**
- **Limitations :**
  - ✓ **Absence of diffusion phenomena consideration**
  - ✓ **Absence of kinetic consideration (speed of exchanges between the two phases)**

↳ **Kinetic theory**

Fundamentals

The kinetic theory

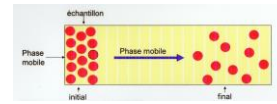
- **The kinetic theory considers the chromatographic peak as representative of the statistical distribution of the retention times of the molecules of a given substance on the column.**
- **The kinetic theory considers diffusion phenomena and mass transfers**

Fundamentals

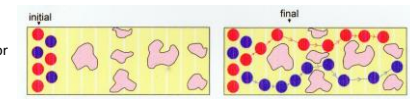
The kinetic theory

Diffusion phenomena:

Molecular longitudinal diffusion



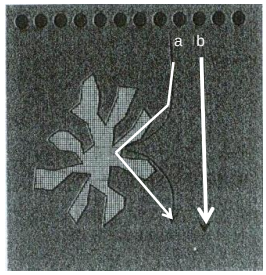
« Eddy diffusion » or unequal pathway



Fundamentals

The kinetic theory

Mass transfert



✓ t_0, the molecules a and b of the same substance are on the same line

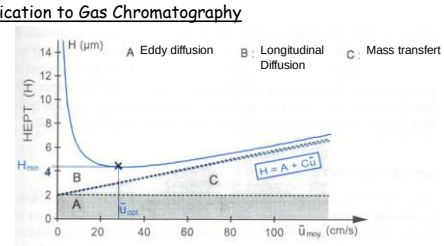
✓ t_i, a will stay in the grain pore of the stationary phase and b in the mobile phase

✓ t_p b will go faster than a

Fundamentals

The kinetic theory

Application to Gas Chromatography



Van Deemter Equation

$$H = A + B/\bar{u} + C.\bar{u}$$

ū = average linear velocity of the mobile phase

Fundamentals

The kinetic theory

Solutions to minimize diffusion phenomena:

- **Improve the homogeneity of the phase:**
  - ✓ **Absence of heterogeneities**
  - ✓ **Absence of bubbles**
- **Reduce of particule diameters d_p**
- **Homogenize the flow of the mobile phase**
- **Decrease pore size of the particules**

Fundamentals

The kinetic theory

Summary:

- **Particules**
  - ✓ **Small size**
  - ✓ **Weak porosity**
- **Separation**
  - ✓ **Fast**
  - ✓ **With miniaturized stationary phases**
- **Experimental condition**
  - ✓ **At low temperature**
  - ✓ **With a reduction of the dead volumes**

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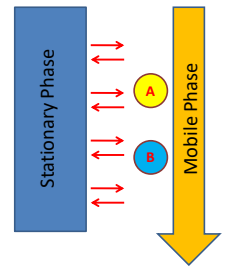
Gas Phase Chromatography (GC)

Principle



- Separation method of gas compound or likely to be vaporized without decomposition
- EXCHANGE of GAZ molecules with stationary phase
- Stationary Phase: Liquid of Solid
- Mobile Phase: GAS

Gas Phase Chromatography (GC)

Mobile Phase or Carrier Gas

- Nature : Inert Gas
  - ✓ Helium
  - ✓ Nitrogen
  - ✓ Argon
  - ✓ Hydrogen…
- Property: inert to analytes and stationary phases
- Choice of carrier gas
  - ✓ Nature of the detector
  - ✓ Running cost…

No interaction between the carrier gas and the stationary phase

No interaction between the carrier gas and analytes

Gas Phase Chromatography (GC)

Purity of the carrier gas

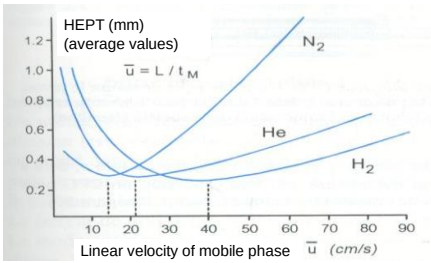
- Imperative: gas must be of very high purity
- Reference A.0 (or NA0), A indicates the number of 9 present in the purity number:
  - ✓ 6.0 (or N60) is a purity of 99.9999%
  - ✓ 3.5 (or N35) is a purity of 99.95%

In GC, we usually use a purity of 5.0 (or N50)

Gas Phase Chromatography (GC)

Mobile Phase or Carrier Gas

- **The nature and the linear velocity of the mobile phase contribute to the quality of the separation**



Gas Phase Chromatography (GC)

Temperature T

Temperature T is a major parameter in GC

- **Retention time t_R varies according the equation:**

$Log(t_R) = (a/T) + b$

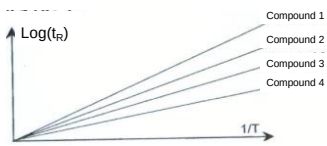
If T increase → retention time decrease

Gas Phase Chromatography (GC)

Temperature T

- **Representation of the equation for a serie of homologous compounds**

$Log(t_R) = (a/T) + b$



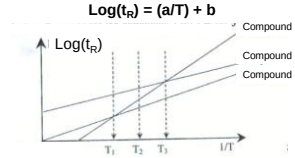
- **The higher the temperature, the faster the separation**
- **At very high temperatures, there is no more separation**

Gas Phase Chromatography (GC)

Temperature T

- **Representation of the equation for a serie of heterogeneous compounds**

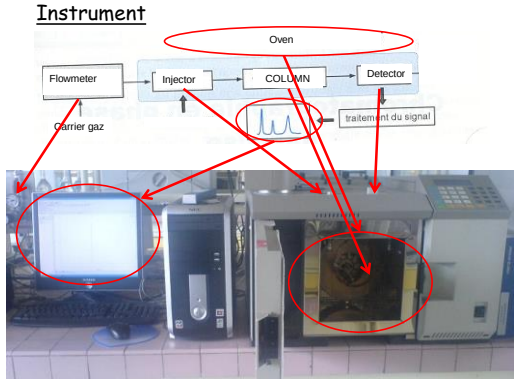
$Log(t_R) = (a/T) + b$



- **At T_1, the compounds 1 and 3 are not separated**
- **At T_3, the compounds 1 and 2 are not separated**
- **At T_2, all compounds are separated**

Gas Phase Chromatography (GC)

Instrument



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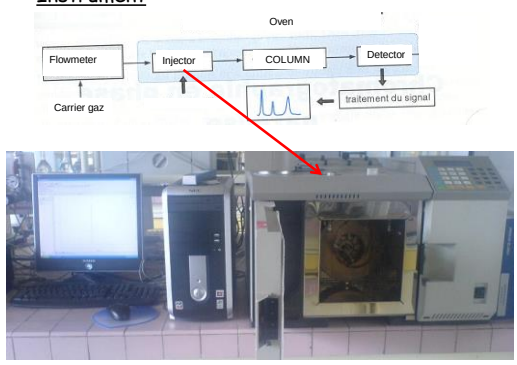
Gas Phase Chromatography (GC)

Instrument



Injection system

ROLE :

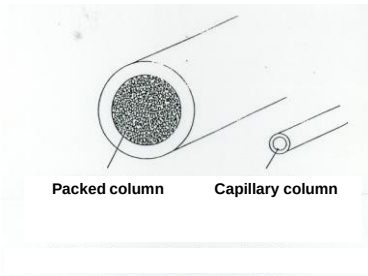
- **Sample-chromatograph interface**
- **System of vaporization**
- **Part of the instrument allowing the tranfer in the column**

IDEAL :

- **Representative recovery of the sample without discrimination**
- **Allow quantitative analysis**
- **Allow analysis of trace**
- **Repeatatility of manual injection**
- **Automation…**

Injection system

Two types of column in GC



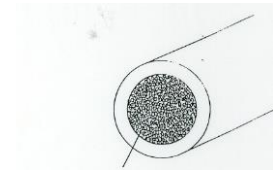
Packed column Capillary column

Injection system

Choice of injector following the column nature

Packed column

- **Classic injector with septum**
- **Automated injector**
- **Injector for solids**



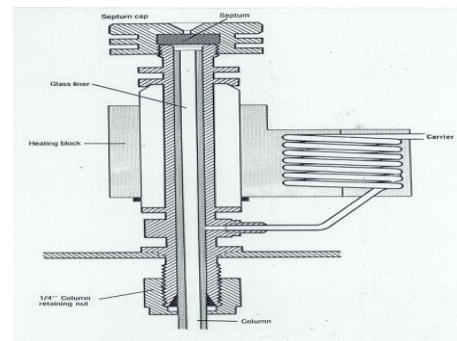
Injection system

Choice of injector following the column nature

Capillary columns

- **Classic injector with septum**
- **Split injection**
- **Splitless injection**
- **On column injector**

Injection system



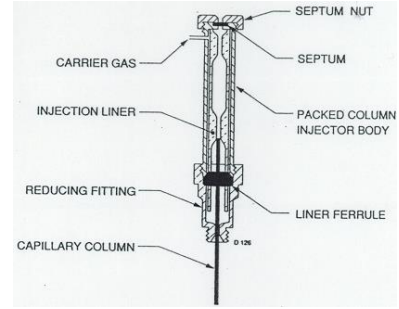
Schema of an injector

Injection system

Direct injector

- **System of first generation**
- **Used for packed and capillary columns**
- **Small injected volume**
- **Variation of the liner diameter according to the type of column**
  - ✓ **Large diameter for packed column**
  - ✓ **Small diameter for capillary column**

Injection system



Direct Injector

Injection system

Direct injectro : Example

High concentration sample:

- **For an 0.1 µL injection (minimum possible) of a product with a concentration of 10%, then there will be saturation of a capillary column**

↳ Poor efficiency, bad separation

Low concentration sample

- **Sample volume limited by the volume of the liner. If you are working on traces and the detector is not sensitive, then the chromatogram will be flat.**

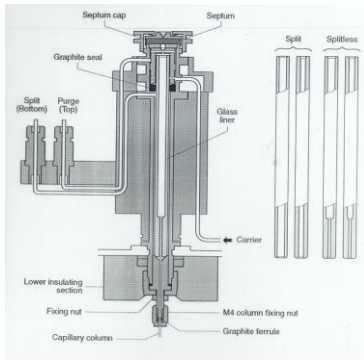
↳ No separation

Injection system

Split/splitless injector

- **Most common system for capillary column separation**
- **Sample volume injection consistent with the amount of stationary phase in the column**
- **Split: System for high concentrated samples**
  - ✓ **Use of a split valve to split the total injected volume**
  - ✓ **Reduction of the injected volume into the column**
- **Splitless: System for low concentrated samples**
  - ✓ **Use of the purge to concentrate the sample**
  - ✓ **Concentrate at the top of the column**

Injection system



Split/Splitless Injector

Injection system

Injection split/splitless

Split mode:

- **Injecting a total volume of sample into the liner using a syringe**
- **The injector being heated, the sample is instantly vaporized**
- **The sample is then divided into two parts by a split valve**
- **The smallest part is injected into the column**
- **The largest is evacuated by what is called the purge**
- **olumn carrier gas flow rate into the split vent flow rate**

Split ratio R = Split vent flow/column carrier gas flow

Injection system

Injection split/splitless

Example Split mode :

- **Carrier gas flow: 52 ml/min**
- **Split vent flow: 50 ml/min**
- **We deduce the flow of the column carrier gas to 2 ml/min**

R = Split vent flow/column carrier gas flow

R = 50/2 = 25

If we inject 1 µL, then we introduce 1/25 de µL in the column

Injection system

Injection split/splitless

Splitless mode:

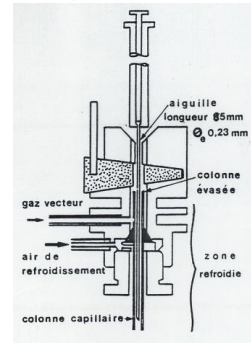
- **The split valve is closed**
- **Introduction of maximum sample volume l'échantillon (max 3 µL)**
- **We wait a few tens of seconds**
  - ✓ **Focalisation in the few cm of the column**
- **The purge valve is open to purge the injector**

Injection system

On column injector

- **Especially used for the analysis of compounds with a high boiling point**
- **For easily degradable compounds**
- **Introduction directly into the column without prior vaporization**
- **Progressive vaporization in the column**

Injection system



On column injector

Injection system

On column injector

advantage :

- **No discrimination and thermal degradation**
- **No breakdown of fragile substances**
- **No septum**

Drawback :

- **Requires to use diluted solutions because we do not divide the sample**
- **Pollution of the column by non-volatile compounds**
- **No possibility to automate**

Injection system

Liner

Liner

Injection system

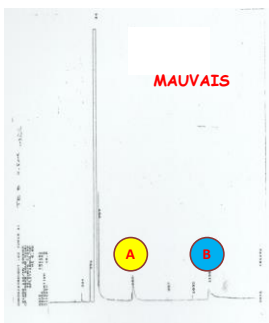
Liner

- First point of contact of the sample with the chromatograph
- Allows to change the quality of the injection
  - ✓ Different diameters
  - ✓ Possibility to modify them chemically
  - ✓ Online derivation of samples
- Large number of liner proposed on the market
  - ✓ The choice of the ideal liner is very complicated (compromise)
- Ability to chemically activate or disable the liner
  - ✓ Increase the field of applications

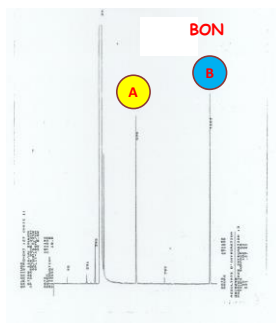
Injection system

Injection system

Injector and column inlet fouled

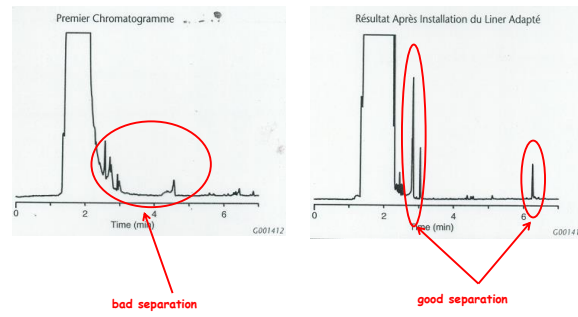


Injector and column inlet cleaned

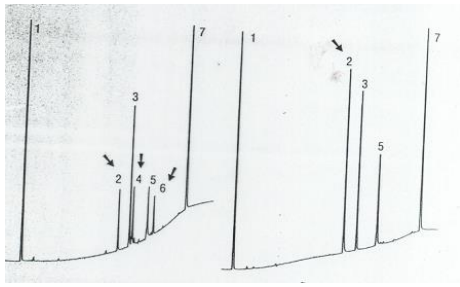


Injection system

Choice of the liner



Injection system



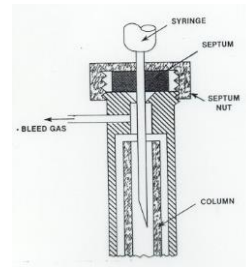
1. 2,4,5,6-tetrachloro-m-xylene
2. Endrin
3. 4,4'-DDT
4. Endrin aldehyde
5. Methoxychlor
6. Endrin ketone
7. decachlorobiphenyl

Activated liner
62 % of endrin decomposition

Disable liner
1 % of endrin decomposition

Injection system

The septum



Injection system

The septum

- **The septum must allow injection and also seal the system**
- **materials of confection:**
  - ✓ **Easy drilling**
  - ✓ **Good sealing**
  - ✓ **High thermal resistance**
- **Increase the separation:**
  - ✓ **Use Septum in teflon (Inert, low temperature)**
  - ✓ **Condition before use (Oven at 250°C)**

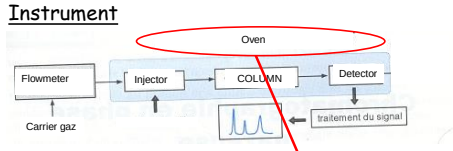
Injection system

The septum

- **very variable life time. Depends of:**
  - ✓ **Injector temperature**
  - ✓ **Syringe needle diameter**
  - ✓ **Clamping**
- **Drawback:**
  - ✓ **Leakage from the injection chamber**
  - ✓ **Septum fragments in the inlet liner can also lead to ghost peaks**

Gas Phase Chromatography (GC)

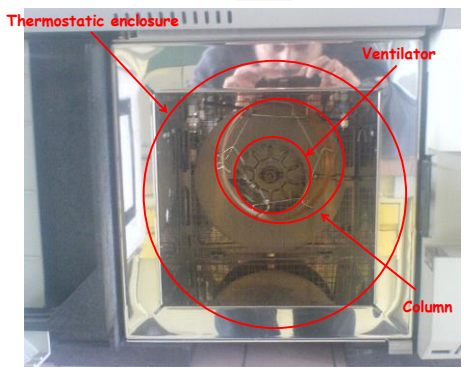
Instrument



Oven

- **Essential element for modern chromatographs because must have excellent thermal stability (until 450°C)**
- **Uniformity of the temperature ensured by a ventilator**
- **Temperature programmer**
- **Must heat and cool very quickly (gradient mode)**

Oven



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   - **Columns**
   - **Detectors**
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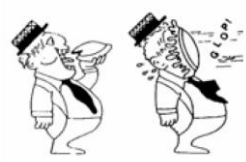
6. **Quantitative Analysis**

Choice of the column

- Two types of chromatography
  - Adorption chromatography
  - Partition chromatography (absorption phenomena)

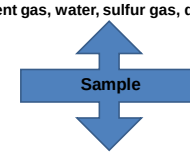


Absorption Adsorption

Column

Adsorption Chromatography

Permanent gas, water, sulfur gas, diols, glycols



Complex mixture of organic compounds of more than 5 carbons

Partition Chromatography

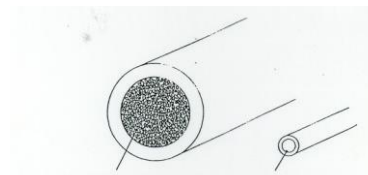
Column

Two types of column in GC

- **Packed column**
  - ✓ **Glass, metal**
  - ✓ **Short (1 à 15 m) and thick (1 à 4 mm)**
- **Capillary column**
  - ✓ **Bare fused silica**
  - ✓ **Lengthy (15 à 100 m) and with low diameter (100 à 250 µm)**

Column

Two types of column in GC



Packed column **Capillary column**

Column

Capillary Columns

- **Not packed with stationnary phase**
  - ✓ Good repeatability
  - ✓ Possibility of long lengths
  - ✓ Thin film of stationary phase = high speeds of exchange
  - ✓ Fast analysis
  - ✓ Drawback: low sample volume injected
- **High permeability of the capillary**
  - ✓ Decrease of the mass transfer
  - ✓ At equal flow rate, it is necessary to apply a pressure of :
    - ❑ 1 bar for 100 m of capillary column
    - ❑ 1 bar for 1 m of packed column

Homogeneous carrier gas velocity throughout the column

How to choose a column

- **Nature of the sample**
- **Nature of the stationary phase**
- **Column diameter**
- **Column length**
- **Thickness of the thin film of stationary phase**

Important parameter: Choice of the column

Stationary phase

- **Polymeric film that covers or is grafted on the inner wall of the capillary column**
- **Equilibrium of interactions between samples and stationary phase depending on the nature of the phase**

Selection parameters of the stationary phase

- ✓ **Nature**
- ✓ **Polarity**
- ✓ **Stability**

Stationary Phase: Polarity

Non polar compound

- **A non-polar stationary phase is suitable for the separation of non-polar compounds: only composed by C and H**
- **The interactions of non-polar compounds and a non-polar stationary phase are "dispersive", ie the molecules enter the stationary phase and come out randomly.**
- **Separation is then only based on the boiling point**

Composé polaire

- **A polar stationary phase is suitable for the separation of polar compounds: mainly composed by C, H + 1 or more heteroatoms of Br, Cl, F, N, O, P, S…**
- **In addition to the "dispersive" interactions, there exists between polar compounds and polar stationary phase interaction of dipole-dipole or acid-base**

Stationary Phase: Polarity

Global rules

Birds of a feather flock together

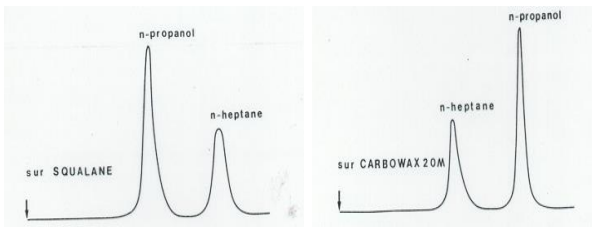
- **On non-polar stationary phases, non-polar compounds will be the most retained and therefore will come out the column with a high retention time**
- **On polar stationary phases, polar compounds will be the most retained and therefore will come out the column with a high retention time**

Stationary phase: Polarity

TYPE OF MOLECULES	EXAMPLES	STATIONARY PHASES
Non-polar molecules Hydrogen-carbon C-C liaison	hydrocarbon	Non polar
Polar molecules Hydrogen-carbon+Br or Cl,F, N,O,P,S	Alcohol, amine, carboxylic acid Diols, ester, cetone, thiols	Polar
Polarizable molecules Hydrogen-carbon C=C liaison	Hydrocarbon aromatic	Medium polarity

Stationary phase: Polarity

Example



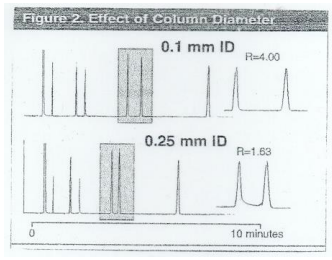
Effect of polarity on the retention time order

- **Squalane is a non-polar phase**
- **Carbowax 20M is a polar phase**

How to choose a column

- **Nature of the sample**
- **Nature of the stationary phase**
- **Column diameter**
- **Column length**
- **Thickness of the thin film of stationary phase**

Choice of column diameter



- **Fast analysis time**
- **Loss of Efficiency (N) and Resolution (R)**
- **Increase of injected volume**

Choice of column diameter

Effect of inner diameter on the GC column characteristics

Internal Diameter	Sample Capacity (ng) (each component)	Efficiency (theoretical plates/meter)
0.20mm	5-30	5000
0.25mm	50-100	4170
0.32mm	400-500	3330
0.53mm	1000-2000	1670
0.75mm	10,000-15,000	1170
2mm (packed column)	20,000	2000

How to choose a column

- **Nature of the sample**
- **Nature of the stationary phase**
- **Column diameter**
- **Column length**
- **Thickness of the thin film of stationary phase**

Influence of column length

The longer the column:
- ✓ More resolution is increase
- ✓ But more the analysis time increase
- ✓ More the column is expensive

Example

- **With a 60 m column, one gains 40% in resolution compared to 30 m column**
- **With a 60 m column, one doubles analysis time compared to 30 column**

The resolution increases proportionally to the square root of the length of the column

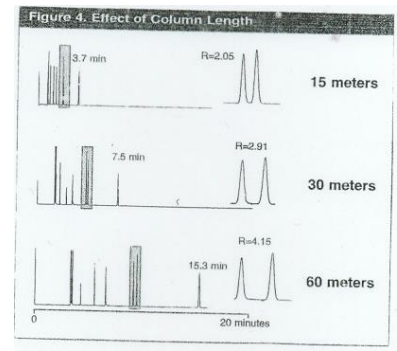
Influence of column length

Effect of the length of the column on efficiency (N) and retention time
(inner diameter fixed at 0.25 mm)

Column Length (m)	Plate Number (N)	nC13 Retention Time (min)
30	155,000	15.2
60	304,000	36.8
120	550,000	82.4
150	719,000	125.0

SPB-5 columns, 0.25mm ID x 1.0µm film (β = 62.5), Col. Temp.: 145°C

Influence of column length



How to choose a column

- **Nature of the sample**
- **Nature of the stationary phase**
- **Column diameter**
- **Column length**
- **Thickness of the thin film of stationary phase**

Influence of thin film of stationary phase

Effects of the thickness of the film of stationary phase and the inner diameter of the column are interdependent

$$\beta = \frac{\text{Volume of Mobile phase}}{\text{Volume of Stationary phase}}$$

- **The lower the value of β, the higher the capacity and the retention of the compound.**
- **Application according to β:**
  - ✓ **For β < 100** - **Very volatile compounds, low masses**
  - ✓ **For β between 100 and 400** - **Usual analysis**
  - ✓ **Pour β > 400** - **High molecular masses**

Influence of thin film of stationary phase

Film thickness and sample capacity

Column ID (mm)	Film Thickness (µm)	Phase Ratio (β)	Sample Capacity (ng/analyte)
0.20	0.10	500	10-20
	0.20	250	30-40
	0.80	63	200-300
0.25	0.10	625	30-40
	0.25	250	100-150
	0.50	125	200-300
	1.0	63	400-500
	2.0	31	700-800
0.32	0.10	800	50-70
	0.25	320	100-200
	0.50	160	200-300
	1.0	80	400-500
	2.0	40	700-900
	4.0	20	1500-2000
0.53	0.10	1325	50-100
	0.25	530	200-300
	0.50	265	500-700
	1.0	133	1000-1500
	1.5	88	1500-2000
	3.0	44	4000-5000
	5.0	27	8000-10,000

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Detectors

ROLE :

- **Physicochemical measuring device that reacts only to the passage of samples or specific species of sample**
- **Signal processed after amplification by an acquisition computer system**

IDEAL :

- **Sensitive**
- **Universal**
- **Robust**
- **Large linearity**

Detectors

3 types of detectors

- **Universal detectors**
- **Semi-universal detectors**
- **Specific detectors**

Detectors

How to choose a detector?

DETECTEUR	TCD Catharomètre	FID Ionisation de flamme	FPD Photomètre de flamme	ECD Capture d'électons	FTD-NPD Thermo-ionique
Type	C	M	M	C	M
Linéarité	10^5	10^6	P 10^6 S 10^3	10^4	10^5
Limite de température	400 °C	400 °C	350 °C	350 °C	400 °C
Gaz vecteur	He, N_2	He, N_2	N_2	N_2	He, N_2
Applications principales	Tous composés	Composés organiques carbonés	Composés contenant S ou P	Composés ayant une forte affinité pour e⁻	Composés contenant N ou P

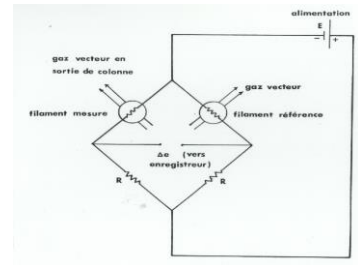
C : détecteur dont la réponse est proportionnelle à la concentration

M : détecteur dont la réponse est proportionnelle au débit massique

Le catharometer

Principle :

The difference in conductivity between the carrier gas alone and the carrier gas charged with sample is measured



The catharometer

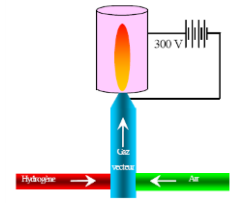
advantages and limitations

- **First generation of detector**
- **The more universal**
- **Easy to use**
- **Considered as low sensitive detector**
- **Last advances: Improved sensitivity thanks to miniaturization**

Flame ionization detector (FID)

Principle :

Measures the ionic current generated in a hydrogen flame at the passage of the sample



Flame ionization detector (FID)

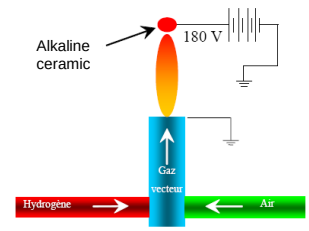
Advantage and Drawback:

- Described for the first time in 1958
- The most commonly used (Reference detector)
- High sensitivy
- Good linearity
- Low dead volume
- Almost universal
- Destructive

Nitrogen-Phosphorus Detector (NDP)

Principle :

Features a small ceramic cylinder doped with an alkaline salt (rubidium or cesium).



Nitrogen-Phosphorus Detector (NDP)

Advantage and Drawback:

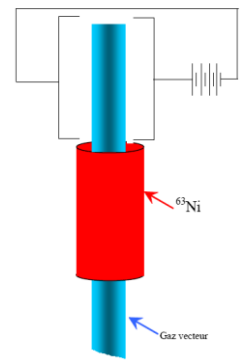
- High sensitivity
- Good linearity
- Low dead volume
- Specific to compounds with nitrogen (N) and phosphorus (P)

Electron Capture Detector (ECD)

Principle :

- Ionization of analytes by β particules emitted by a radioactive source of ^{63}Ni
- When analytes arrive, they will be ionized by electron capture:

  $A + e^- \rightarrow A^-$
- During the passage of the analytes, current decreases
- This current tracking is translated in a chromatogram

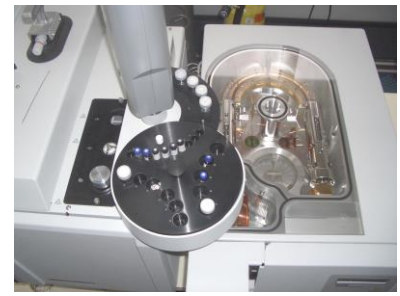


Electron Capture Detector (ECD)

Advantage and Drawback:

- Described by Lovelock in 1960
- Very selectif to electronegative compounds
- High sensitivity for halogens
- Detection of nitril, cyanates, polyaromatic compounds
- No detection of alcohol
- Widely used for the detection of organochlorine pesticides
- Unusual detector in a laboratory because of the presence of radioactive material (important control)

Mass spectrometry



Structural information

Détecteur MS

Advantage and Drawback:

- High sensitivity
- Low dead volume
- Not Universal
- Expensive
- Destructive

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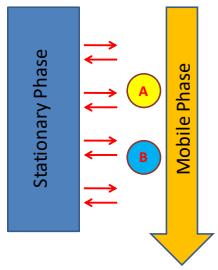
High Performance Liquid Chromatography (HPLC)

Principle



- **Separation method of non-volatile compounds**
- **EXCHANGE of LIQUID molecules with stationary phase**
- **Stationary Phase: Solid**
- **Mobile Phase: LIQUID**

High Performance Liquid Chromatography (HPLC)

Mobile Phase

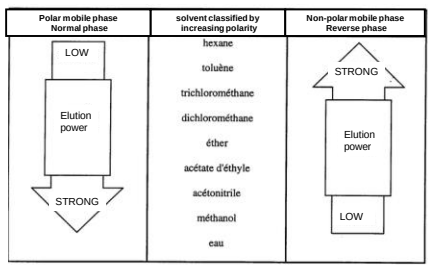
- **Aqueous of organic solvents**
  - ✓ **Water**
  - ✓ **Acetonitrile**
  - ✓ **Methanol**
  - ✓ **Ethanol…**
- **Property: Inverse polarity compared to stationary phase**

There is interaction between the mobile phase and the stationary phase

There is interaction between the mobile phase and the samples

High Performance Liquid Chromatography (HPLC)

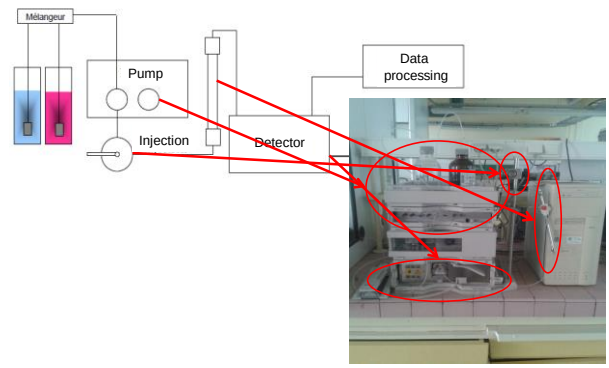
Mobile Phase



Elution power of the mobile phase in HPLC

High Performance Liquid Chromatography (HPLC)

Instrument



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Injection system

ROLE :

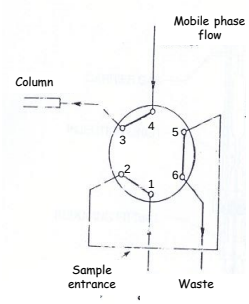
- Sample-chromatograph interface
- Part of the instrument allowing the tranfer in the column

IDEAL :

- Representative recovery of the sample without discrimination
- Allow quantitative analysis
- Allow analysis of trace
- Repeatatility of manual injection
- Automation…

Injection system

Six way Valve

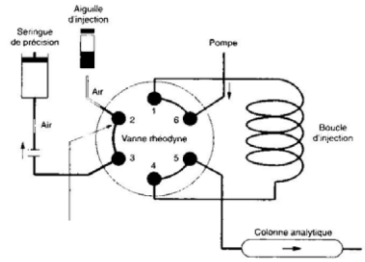


Injection system

Six way Valve

Step 1:

- Washing the loop

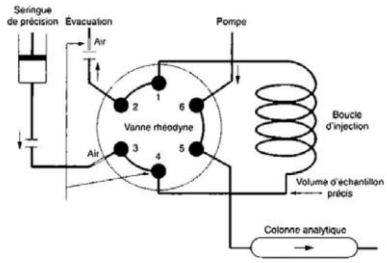


Injection system

Six way Valve

Step 2:

- **Filling the loop with the sample**

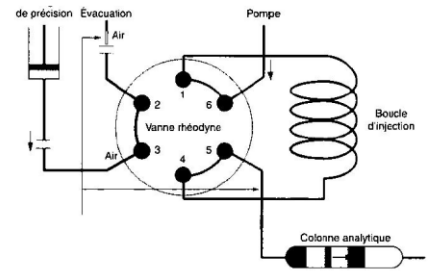


Injection system

Six way Valve

Step 3:

- **Injection of the sample in the column**



Outline

1. **General introduction of Chromatography**
2. **Fundamentals of Chromatography**
3. **Gaz Chromatography: Principle and Instrumentation**
   - **Sample introduction: Injectors**
   - **Columns**
   - **Detectors**
4. **Liquid Chromatography: Principle and Instrumentation**
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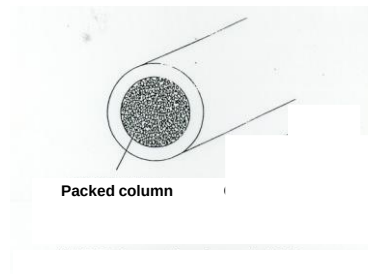
   - **Detectors**
5. **LC-GC/MS coupling**
6. **Quantitative Analysis**

Column

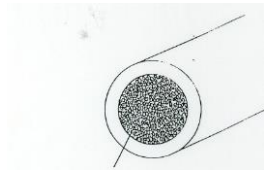
One type of column in HPLC



Column

Packed column

- **Inox of verre**
- **Length: 15 to 30 cm**
- **Diameter: 1 to 6 mm**



How to choose a column

- **Nature of the sample**
- **Nature of the stationary phase**
- **Column diameter**
- **Column length**

Important parameter: Choice of the column

Stationary phase

- **Equilibrium of interactions between compouds and different phases depending on the nature of the stationary phase**

Selection parameters of the stationary phase

- ✓ **Nature**
- ✓ **Polarity**
- ✓ **Stability**

Stationary Phase: Polarity

Polar column also called « normal phase »

- Normal phase columns are columns whose stationary phase is polar and acidic.
- The most used normal phase is based on silica gel: on its surface are silanol groups (-OH) and siloxane groups (-O-). These groups allow the silica to retain the compounds to be analyzed by hydrogen bonds.

This phase thus serves mainly to separate polar compounds

Non-Polar column also called « reverse phase »

- Reverse phase is a normal phase on which alkyl chains (or others according to the desired polarity) have been grafted at the silanol groups. In general, the stationary phase is mainly composed of small silica particles on which chemical functions have been grafted, most often 8 or 18 carbon alkyl chains.

This phase thus serves mainly to separate non-polar compounds

Stationary Phase: Polarity

Global rules

Birds of a feather flock together

- On non-polar stationary phases, non-polar compounds will be the most retained and therefore will come out the column with a high retention time
- On polar stationary phases, polar compounds will be the most retained and therefore will come out the column with a high retention time

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   - Detectors
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Detectors

ROLE :

- Physicochemical measuring device that reacts only to the passage of samples or specific species of sample
- Signal processed after amplification by an acquisition computer system

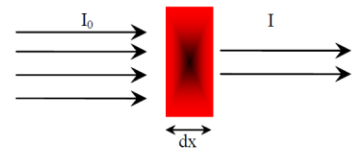
IDEAL :

- Sensitive
- Universal
- Robust
- Large linearity

UV Detection

Beer Lambert Law

Detection by absorbance



dI is proportional to I
dI is proportional to c
dI is proportional to dx
K = proportionality coefficient

$$dI = -K \cdot I \cdot c \cdot dx$$

UV detection

$$dI = -K \cdot I \cdot c \cdot dx$$ molar attenuation coefficient

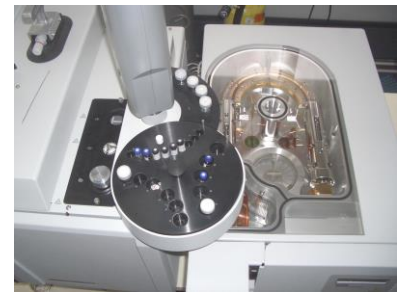
$$\frac{dI}{I} = -K \cdot c \cdot dx$$ ε = Molar attenuation coefficient

$$\ln\left(\frac{I_0}{I}\right) = K \cdot c \cdot L$$

$$\log\left(\frac{I_0}{I}\right) = \varepsilon \cdot c \cdot L$$

$A = \varepsilon.c.L$

Mass spectrometry



Structural information

Détecteur MS

Advantage and Drawback:

- **High sensitivity**
- **Low dead volume**
- **Not Universal**
- **Expensive**
- **Destructive**

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   - **Detectors**
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6. **Quantitative Analysis**

Why an LC-GC / MS coupling?

Despite the analytical power of MS, this technique has strong limitations in the study of very complex mixture (natural products, complex matrices ...)

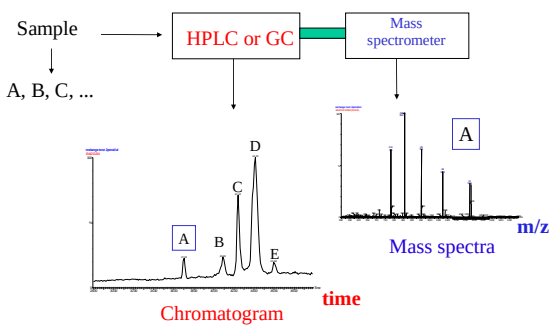
- Loss of signal due to too many compounds to be analyzed
- Loss of sensitivity
- Loss of resolution

"Simplify" complex mixtures
To allow their passage in MS in an optimal way

Interest of LC-GC / MS coupling

- Separation of a mixture to obtain an identification of all constituents
- Have the highest sensitivity
- To be universal, ie to detect all the eluted substances
- Provide as much structural info as possible
- Be selective (identification of a targeted constituent)
- Allow quantitative analysis

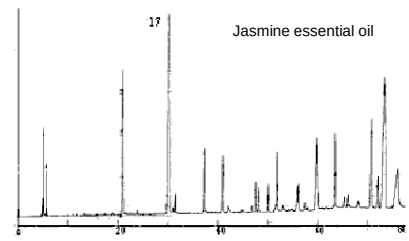
LC-GC/MS coupling



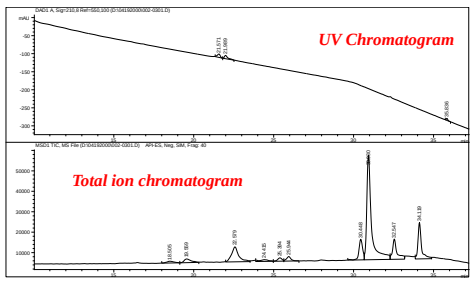
LC-GC/MS coupling

Aquisition mode (chromatogram) :

- Plot of a total ion chromatogram using the mass spectrometer
- Represents the intensity of the ratio m/z ion determined as a function of time



Where the UV detector stops, the mass spectrometer is at ease …



Agilent Technologies

LC-GC/MS coupling

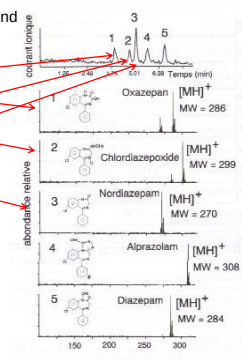
Acquisition mode (mass spectrum):

- Using the ion chromatogram, we determine the mass spectrum of each constituent present in the peaks
- Integration of each peak corresponds to the spectrum of the compounds present in the peak

LC-GC/MS coupling

Acquisition mode (mass spectrum):

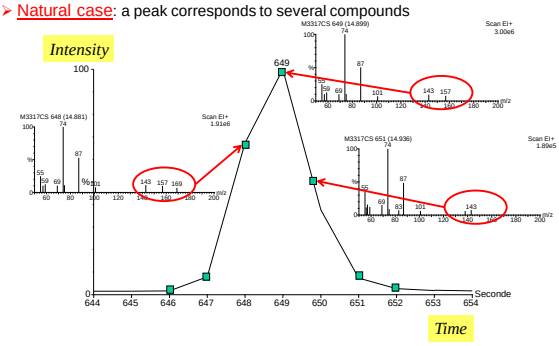
- Ideal case: a peak corresponds to a compound
  - Peak 1 : Oxazepan
  - Peak 2 : Chlordiazepoxide
  - Peak 3 : Nordiazepam
  - etc…



LC-GC/MS coupling

Acquisition mode (mass spectrum):

- Natural case: a peak corresponds to several compounds



GC/MS coupling

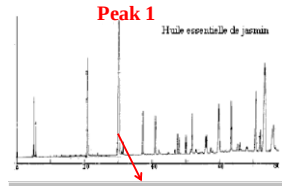
GC-MS coupling

- Study of volatile compounds (small molecules)
- Compatibility with EI and CI sources
- Compatible only with capillary columns
- Carrier gas: helium

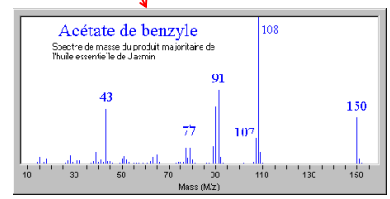
Simple enough to put in place because the ions arrive in the source in the gaseous state

GC/MS coupling

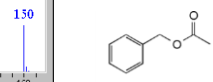
Example



Ion Chromatogram of Jasmine Essential Oil

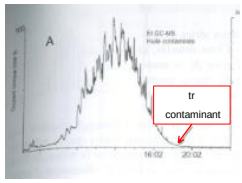


Mass Spectrum of the Main Product (Peak 1) m/z 150 Th

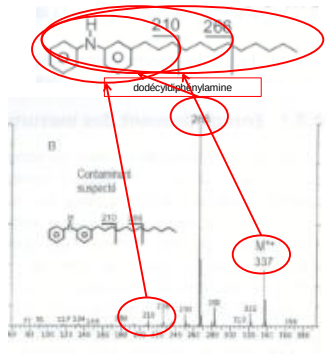


GC/MS coupling

Exemple : Search contaminant in oil using EI/GC-MS/MS



Ion chromatogram of oil



LC/MS coupling

LC/MS coupling

- Study of non-volatile compounds
- Compatibility with ESI source
- Essential choice of mobile phase nature (compatibility with the analyzer)
- Solvent removal, flow compatibility

More complicated to implement, but very powerful

LC/MS coupling

Choice of the mobile phase:

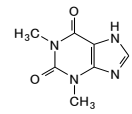
Compatibility Issues for HPLC Eluents with MS

- need to adapt LC methods for LC-MS.
  - The eluting phases must be relatively volatile and free of salts.

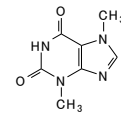
LC/MS coupling

Choice of the mobile phase:

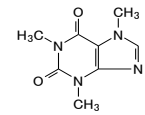
Example of an analysis with non-volatile eluent and volatile eluent



Theophylline (TP)
M.W=180.17
pKa <1, 8.6



Theobromine (TB)
M.W=180.17
pKa <1, 10.0

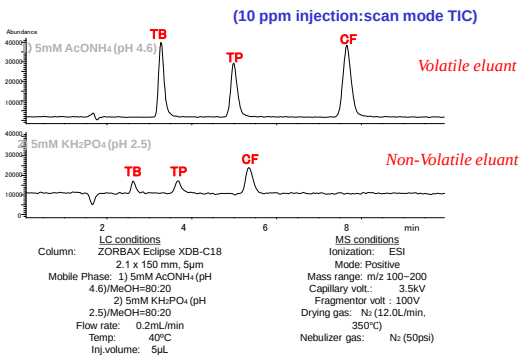


Cafeine (CF)
M.W=194.19
pKa = 14

LC/MS coupling

Choice of the mobile phase:

Example of an analysis with non-volatile eluent and volatile eluent



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Qualitative aspect

The qualitative aspect of a chromatography consists just to identify a compound

- **Comparison of retention times with standards**
- **Determination of k' and α**

Qualitative aspect

If the comparison with standards is not enough:

- **Some detectors may additionally provide information on the nature of the product**
  - ✓ **Mass spectrometry**
  - ✓ **NMR**

No ambiguity regarding the nature of the detected compound

Quantitative aspect

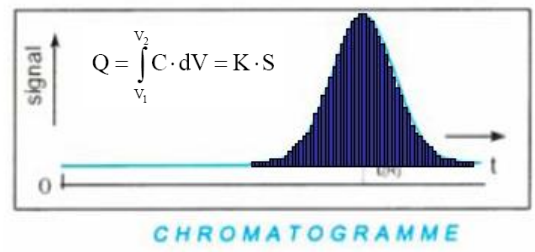
The quantitative aspect of a chromatography consists of identifying a compound and **quantifing it**

- **For a UV detector, it is assumed that there is a linear relationship between the area of a chromatographic peak and the amount of compound responsible for this peak**
- **The amount of product injected into the column is distributed over the entire surface of the chromatographic peak**
- **The calculation of the sample quantity is done by integrating the peak area.**

$$Q = \int_{V_1}^{V_2} C \cdot dV = K \cdot S$$

Quantitative aspect

Integration of the peak



CHROMATOGRAMME

Drawback : the value of the response coefficient K, which links the surface to the quantity injected, is not known

Quantitative aspect

How to quantify a compound?

Using the calibration technique

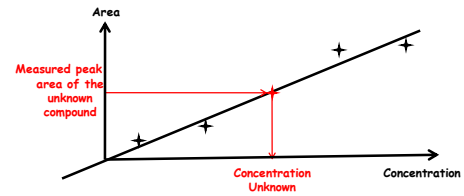
- **External calibration**
- **Internal calibration**
- **…**

Quantitative aspect

External calibration

Principle :

Standard solutions are injected at different concentrations



Quantitative aspect

External calibration

Limitation

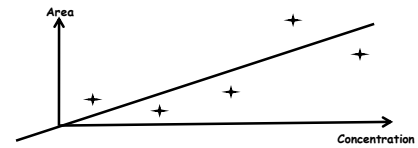
- There may be problems of repeatability of the injected volume
- Standards are essential and may be unavailable commercially

Quantitative aspect

Internal calibration

Rappel :

The major issue of external calibration is based on the non-repeatability of the injection



Result :

Calibration curve not exploitable because unrepresentative

Solution: internal calibration

Quantitative aspect

Internal calibration

Nomenclature :

E_i : internal standard
E_{ech} : unknown sample
E_{St} : standard sample

Principle :

- **Use ab E_i at a fixed concentration**
- **Optimize the separation of the two compounds E_i et E_{ech}**
- **Achieve a calibration curve varying the concentration of E_{St} and keeping a fixed concentration of E_i.**
- **Represent the calibration curve:**

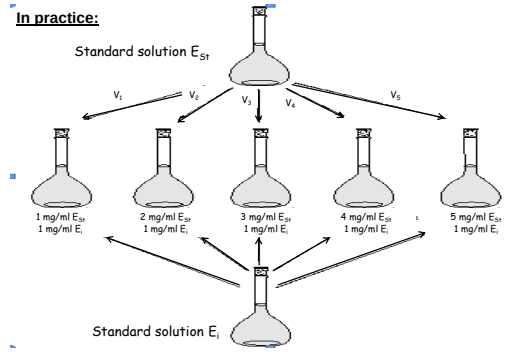
$$\text{Area } E_{ech}/\text{Area } E_i = f([E_{ech}]/[E_i])$$

- **Injected the mixture containing E_i and E_{ech}**
- **Refer the measured value on the curve and deduce $[E_{ech}]$**

Quantitative aspect

Internal calibration

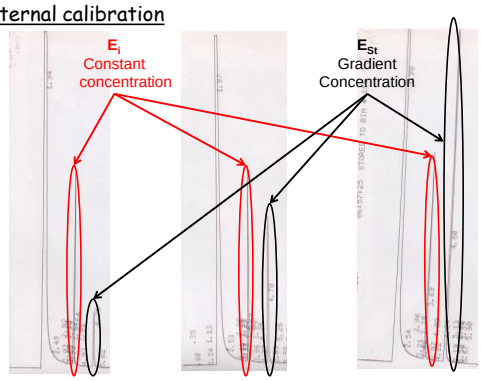
In practice:



Quantitative aspect

Internal calibration

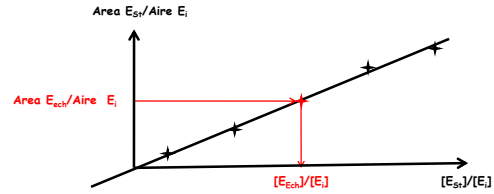
Result :



Quantitative aspect

Internal calibration

Result:



$$[E_{ech}] = (A_{Eech}/A_{Ei}).[E_i]$$

Quantitative aspect

Internal calibration

Properties of E_i:

- **Very close chemical structure**
- **Very close retention time**
- **Very close concentration**

Purpose of using aE_i:

- **Use a concentration ration**
- **Correct injected volume repeatability errors**

Quantitative aspect

Quality control

In chromatographic analysis as for other measurement techniques, the statistical calculation is unavoidable

An analysis result has value only if it is accompanied by an estimate of the possible error

To be acceptable, a measure must be repeated:

Statistic is then possible

Quantitative aspect

Quality control

- **Average $\overline{x}$ on n measurement:**

$$\overline{x} = \frac{x_1 + x_2 + ... + x_n}{n} = \frac{\sum x_i}{n}$$

- **Standard deviation:**

$$s = \sqrt{s^2} = \sqrt{\frac{\sum (x_i - \overline{x})^2}{n-1}}$$

- **Relative standard deviation (RSD):**

$$CV = 100 \times \frac{s}{\overline{x}}$$

Good indication of the dispersion of results