Types of Assays

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1- Chemical Assays: Spectrophotometry, Spectrofluorimetry, Chromatography.

2- Bioassays.

3- Immunoassays (ELISA).

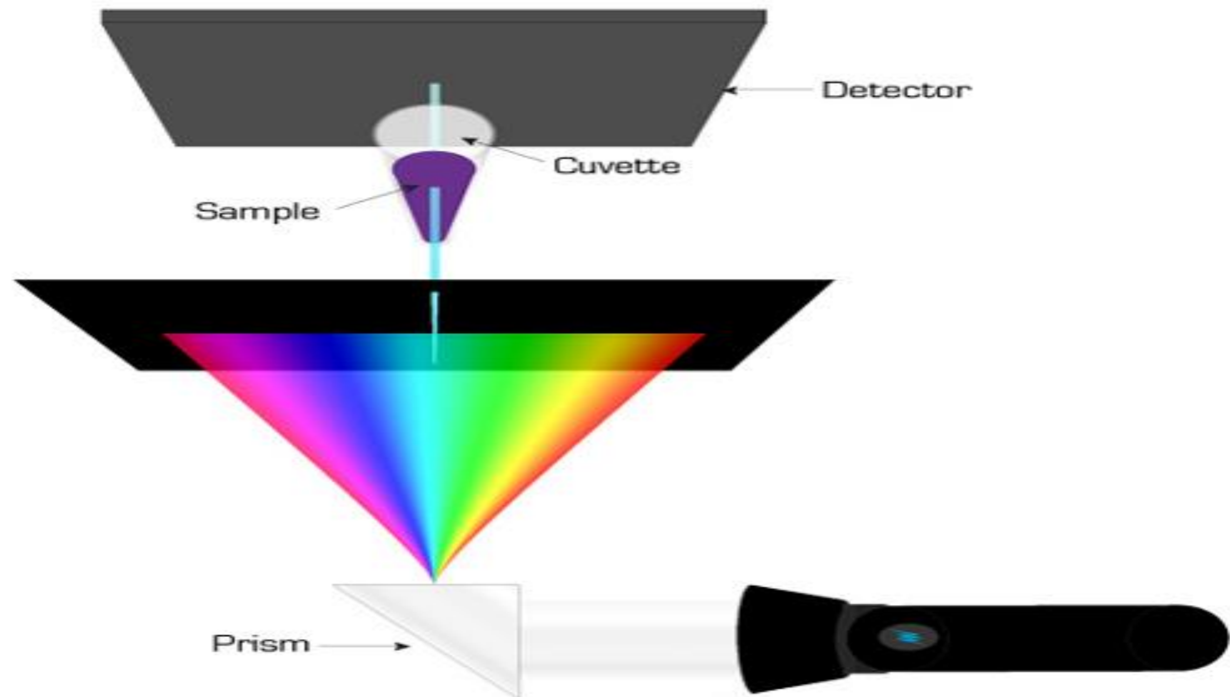
4- Radioimmuno Assay (RIA).

5- Microbiological assays.

1- Chemical Assays

- Spectrophotometry.
- Spectrofluorimetry.
- Colourimetry.
- Chromatography.
- Titrimetry (Aqueous/ Nonaqueous).
- Gravimetry.
- Potentiometry.

1- Spectrophotometry is a method used in qualitative and quantitative analysis in which the light absorption of the substance being examined is measured at a definite wavelength.



➢ The spectral ranges involved in pharmaceutical analysis mainly consist of **<u>3 regions</u>**:

* 200-400 nm ,the ultraviolet region.
* 400-800 nm ,the visible region.
* Above 800 nm, the infrared region.

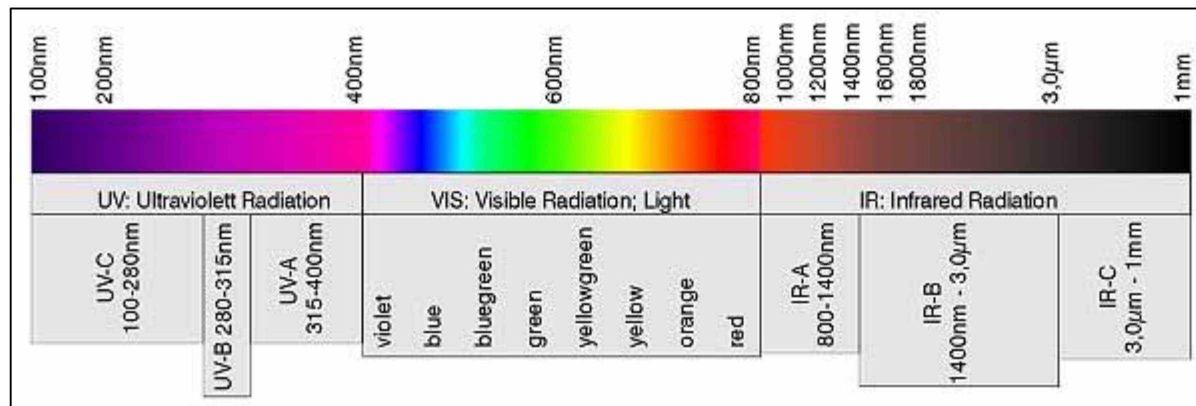
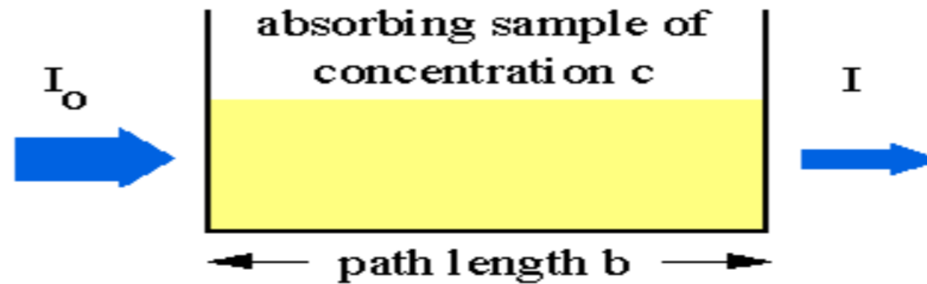


Fig.. Wavelength ranges of electromagnetic radiation.

Lambert-beer Law

The **absorption strength** of the substance based on their own structure, as well as their concentration, which is the principle for absorption spectroscopy.



$$A = \log I_0/I = \varepsilon\, Cl$$

A= Absorbability
I_0= Intensity of incident light
I= transmitted intensity
K= absorbance coefficient transmittance
C= concentration of solution
l= length of the light path (width of the cuvette)

Absorbance coefficient

- 1 g of solute is dissoved in a 100ml solution, the liquid layer thickness is 1cm, the value of absorbability of the solution obtained in a designated wavelength (λmax) and conditions (solvent, pH and temperature) .

$$\varepsilon = A / cl$$

- It can be described as $A^{1\%}_{1cm}\lambda$ or $E^{1\%}_{1cm}\lambda$, the unit is $g.cm^2$。

The value of absorbance coefficient for a certain substance is related with the **solvent, pH value, temperature and the wavelength used**.

The testing conditions should be described in detail. Generally, the maximum wavelength (λ_{max})will be chosen and during a certain range of low concentration for the test.

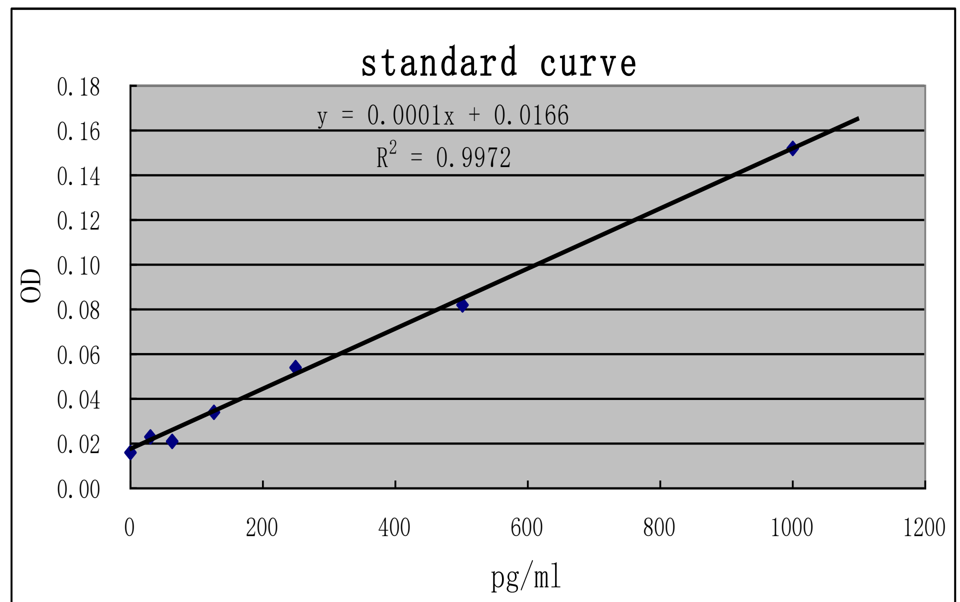
Methods for chemical assays

1. Absorption Coefficient Method:

The absorption coefficient value of the reference substance is obtained in the certain conditions described in some handbook or pharmacopoeia, **the percentage content (%) of the sample is known from the value of the sample tested in the same conditions divided by the value of reference substance.**

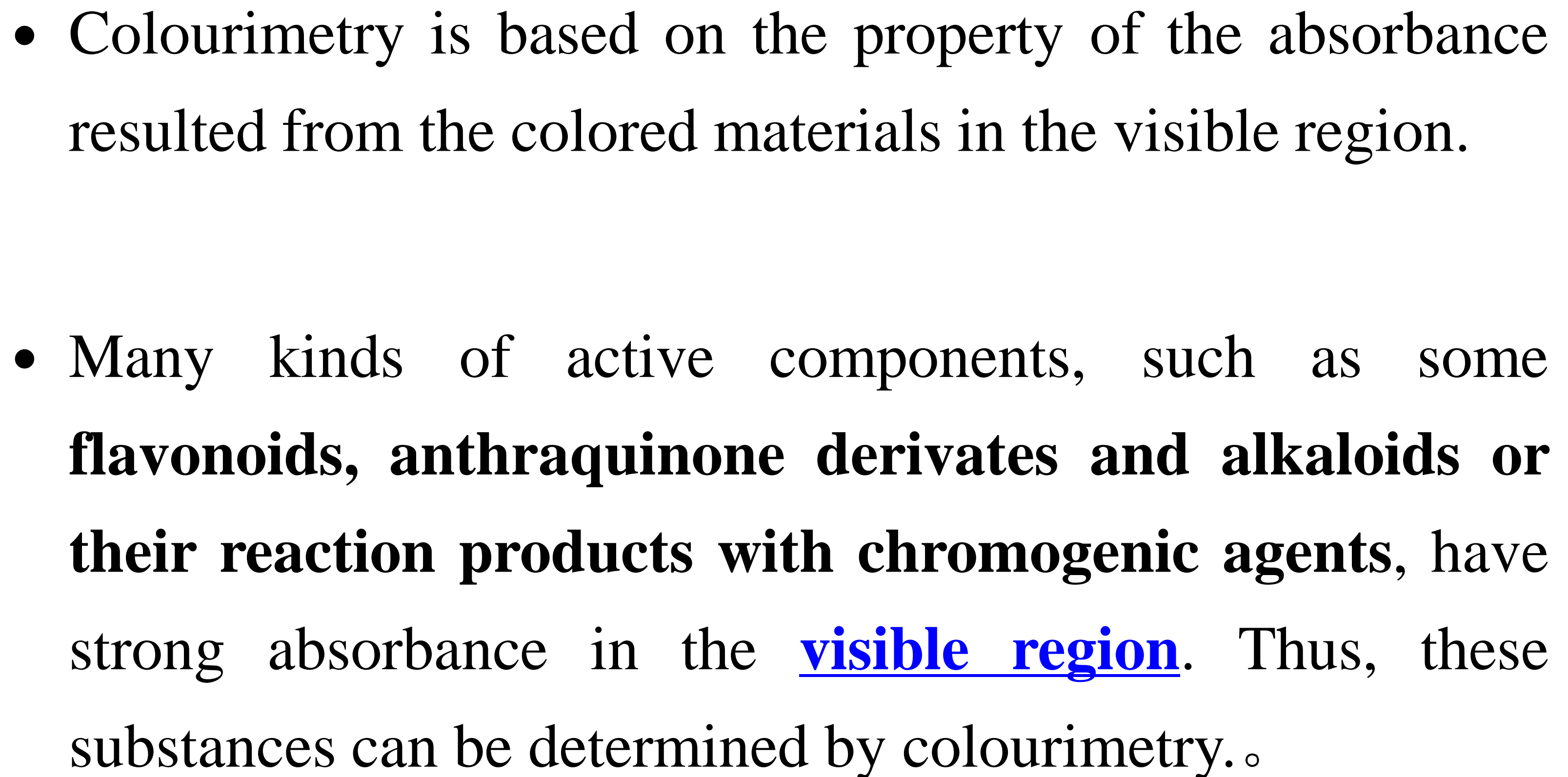
2. Standard Curve Method:

The absorbance of series standard solutions in the maximum wavelength are measured. The standard curve is obtained by plotting concentration versus absorbance. The concentration of the sample solution is obtained through the absorbance value of the sample solution in the same conditions and calculated from the standard curve. 。



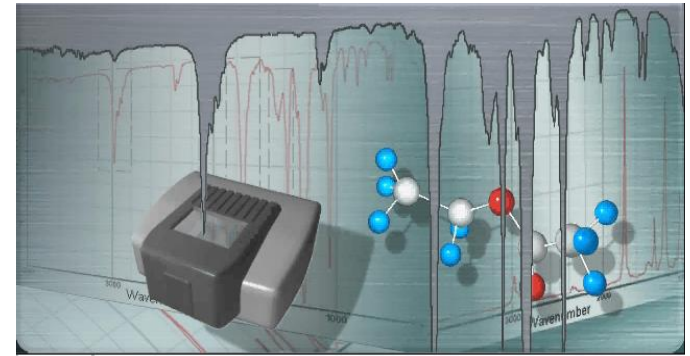
2- Colourimetry (400 to 760 nm)

- Light during this region can be observed by human beings, such as red, orange, yellow and others.
- The variation in color depends on the different absorption properties to the light.
- Such as, the light with the wavelength between 500-560nm (green light) is absorbed by potassium permanganate solution, so it shows the color of prunes.。



- Colourimetry is based on the property of the absorbance resulted from the colored materials in the visible region.
- Many kinds of active components, such as some **flavonoids, anthraquinone derivates and alkaloids or their reaction products with chromogenic agents**, have strong absorbance in the **visible region**. Thus, these substances can be determined by colourimetry.。

3. Infrared Spectrophotometry:



- Near Infrared region， NIR, 0.75μm - 2.5μm
- Middle Infrared region， MIR, 2.5μm - 25μm
- Far Infrared region, FIR, 25μm - 500μm
- MIR are often used.

Infrared spectrophotometry is described as follows:

- A physical optical analysis method used in pharmaceutical analysis, the **object** are exposed with a continuous spectrum of electromagnetic waves in infrared region, the signal of absorbance are recorded as an absorption curve, and it can also be named as infrared absorption spectrum.

- Each drug shows its special character of IR spectrogram because of its difference in conformation and proportion of their components.

- Because of the high specialty of IR spectrophotometry, **it is very useful for the elucidation for the chemical structure of substance.**

Sample Processing

A. Gas sample:

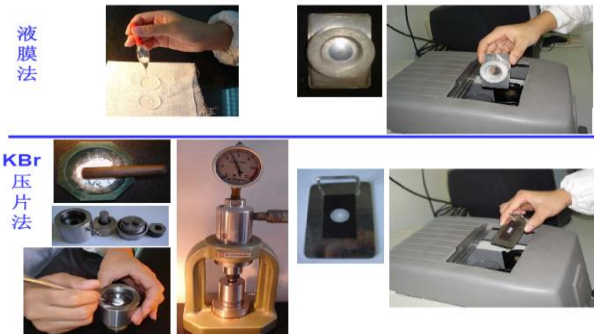
Gas sample can be contained in a glass pneumatic trough to determine.

B. Liquid sample:

Liquid sample can be injected directly to a solvent containing cell, the thickness of the liquid layer should keep in a range between 0.01-1mm. The commonly used solvents are carbon tetrachloride (CCl_4), chloroform ($CHCl_3$), and so on.

C. Solid Sample:

1-2g of fined powder of the sample is directly mixed with potassium bromide and prepared the tablet under the pressure of 50-100MPa, then it was determined by the instrument and obtained an infrared spectrogram.



IR spectrophotometry is used in:

- Structure analysis for chemical drugs.
- Identification for raw drugs and preparations.

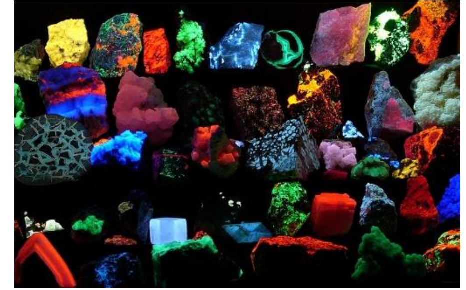
Advantages for using IR in assay:

- The principle for it is Lambert-Beer law.
- More wavelength could be used in the assay.
- The state of the sample are not limited.

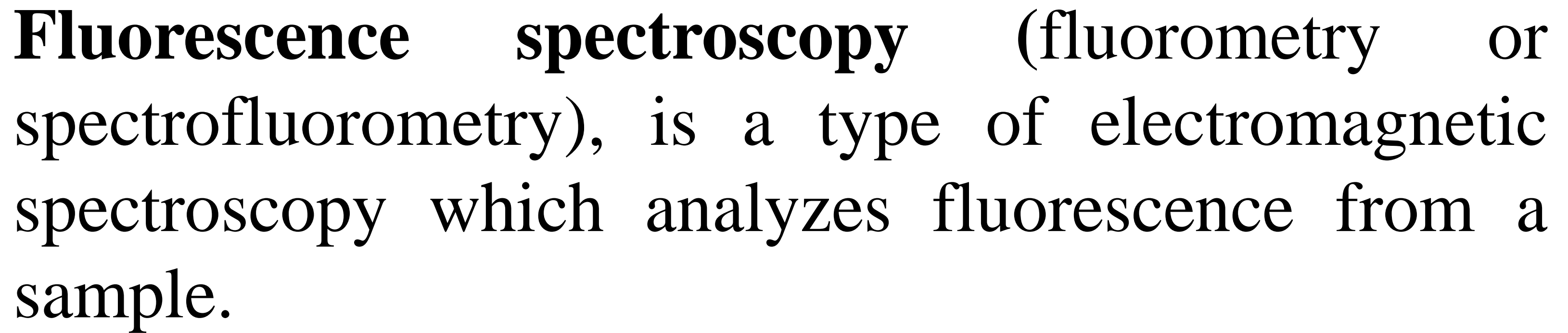
Disadvantage for using IR in assay:

Lower sensitivity than UV.

4. Fluorimetry:



- **Fluorescence** is a luminescence that is mostly found as an optical phenomenon in cold bodies, in which the molecular absorption of a photon triggers the emission of a photon with a longer (less energetic) wavelength.
- The energy difference between the absorbed and emitted photons ends up **as molecular rotations, vibrations or heat.** **Sometimes** the absorbed photon is in the ultraviolet range, and the emitted light is in the visible range, but this depends on the absorbance curve and stokes shift of the particular **fluorophore.**



Fluorescence spectroscopy (fluorometry or spectrofluorometry), is a type of electromagnetic spectroscopy which analyzes fluorescence from a sample.

- It involves using a beam of light, usually ultraviolet light, that excites the electrons in molecules of certain compounds and causes them to emit light of a lower energy, typically, but not necessarily, visible light.
- A complementary technique is absorption spectroscopy.
- **Devices** that measure fluorescence are called **fluorometers or fluorimeters**.

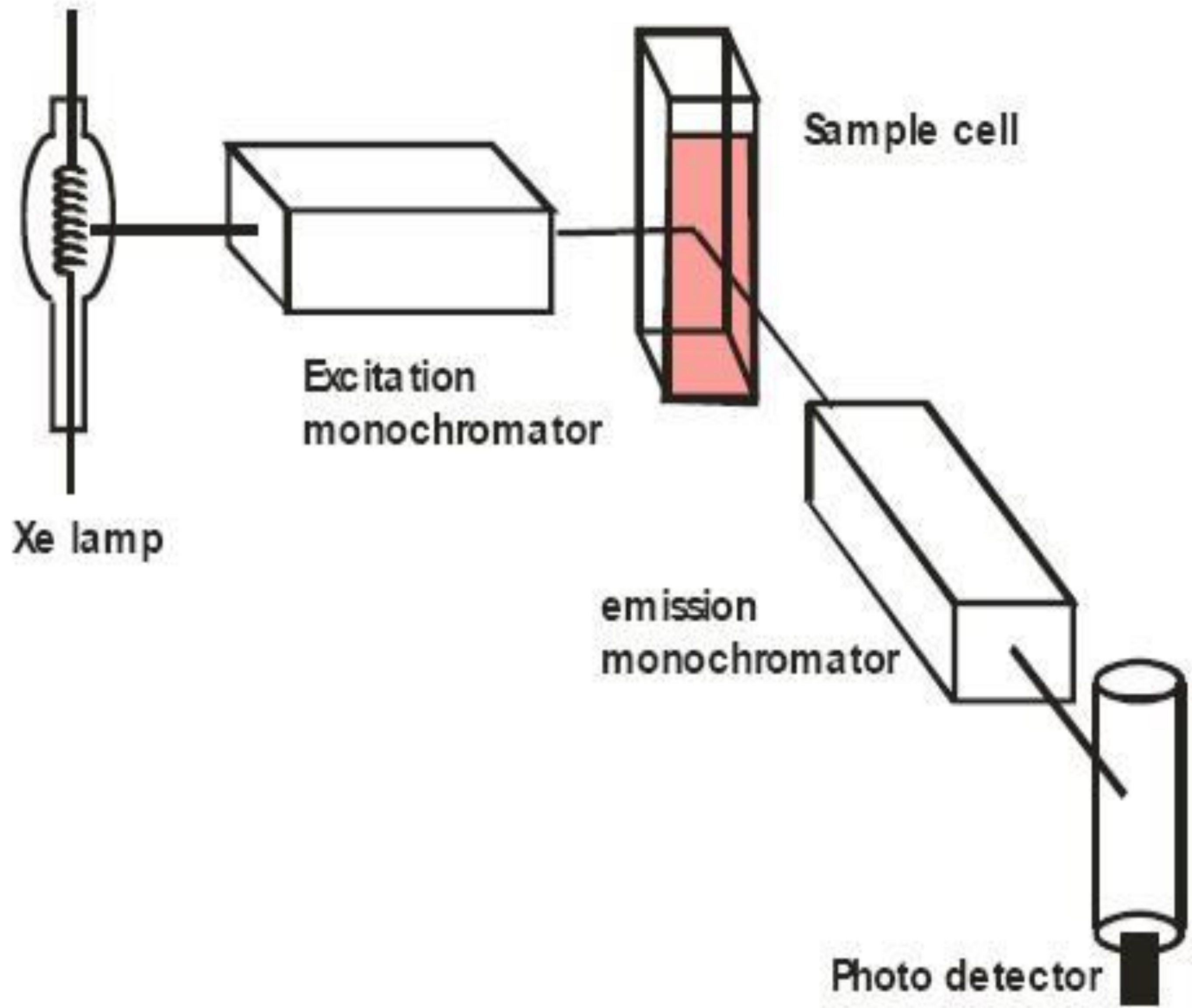
All fluorescence instruments contain three basic items:

-Source of light.

-Sample holder.

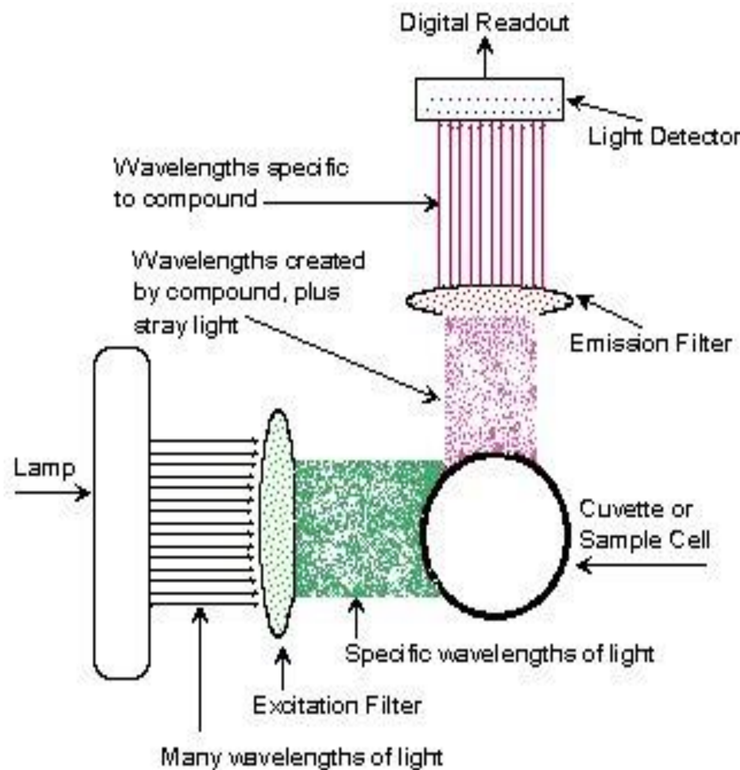
-Detector.

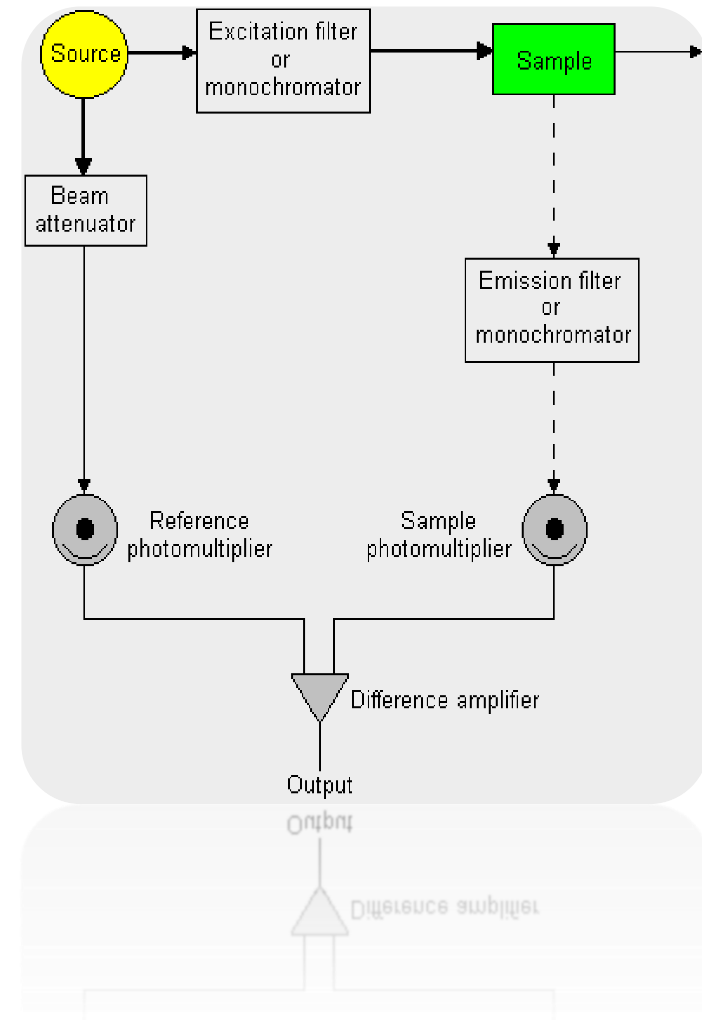
- In addition, to be of analytical use, the wavelength of incident radiation needs to be selectable and the detector signal capable of precise manipulation and presentation. In simple filter fluorimeters, the wavelengths of excited and emitted light are selected by filters which allow measurements to be made at any pair of fixed wavelengths.


Sample cell
Excitation
monochromator
Xe lamp
emission
monochromator
Photo detector

Spectrofluorimetry

Fluorescence Spectrometer Simulation:





Measurement of Fluorescence:

- Fluorescence indicates effective absorbance & re-emission of electromagnetic radiation **often** in the visible range, ~300 - 800 nm, but may involve other ranges, e.g., x - ray.
- If the endpoint is presence of fluorescence, e.g., qualitative staining of a microscopic specimen, it can be directly observed or recorded on film or by digital means.
- If a quantitative test is done, color is usually measured with a spectrofluorimeter.

Factors affecting quantitative accuracy:

- **Non-linearity**

  The proportional relationship between light absorption and fluorescence emission is only valid for cases where the absorption is small.

- **Temperature effects**

  Changes in temperature affect the viscosity of the medium and hence the number of collisions of the molecules of the fluorophore with solvent molecules.

- **pH effects**

  Relatively small changes in pH will sometimes radically affect the spectral characteristics of fluorescence. Accurate pH control is essential.

- **Inner-filter effects**

  Fluorescence intensity will be reduced by the presence of any compound which is capable of absorbing a portion of either the excitation or emission energy.

- **Quenching**

Decrease of fluorescence intensity by interaction of the excited state of the fluorophore with its surroundings is known as quenching and is fortunately relatively rare.

Complications for Spectrofluorimetry:

- Light scattering.
- Photochemical reactions, the system is dynamic.
- Quenching, multiple compounds absorb light energy & alter spectral intensities acting on other compounds present.
- Nonlinearity or spectral impurity of light sources, illumination intensity varies by wavelength.

Naturally Fluorescent Molecules

1- Proteins:

Heme proteins (hemoglobin, myoglobin, cytochrome C, hemocyanin)

Redox & photoproteins (phytochromes, phycoerythrin, phycocyanin, green fluorescent protein phycobiliproteins, rhodopsin, ferredoxins)

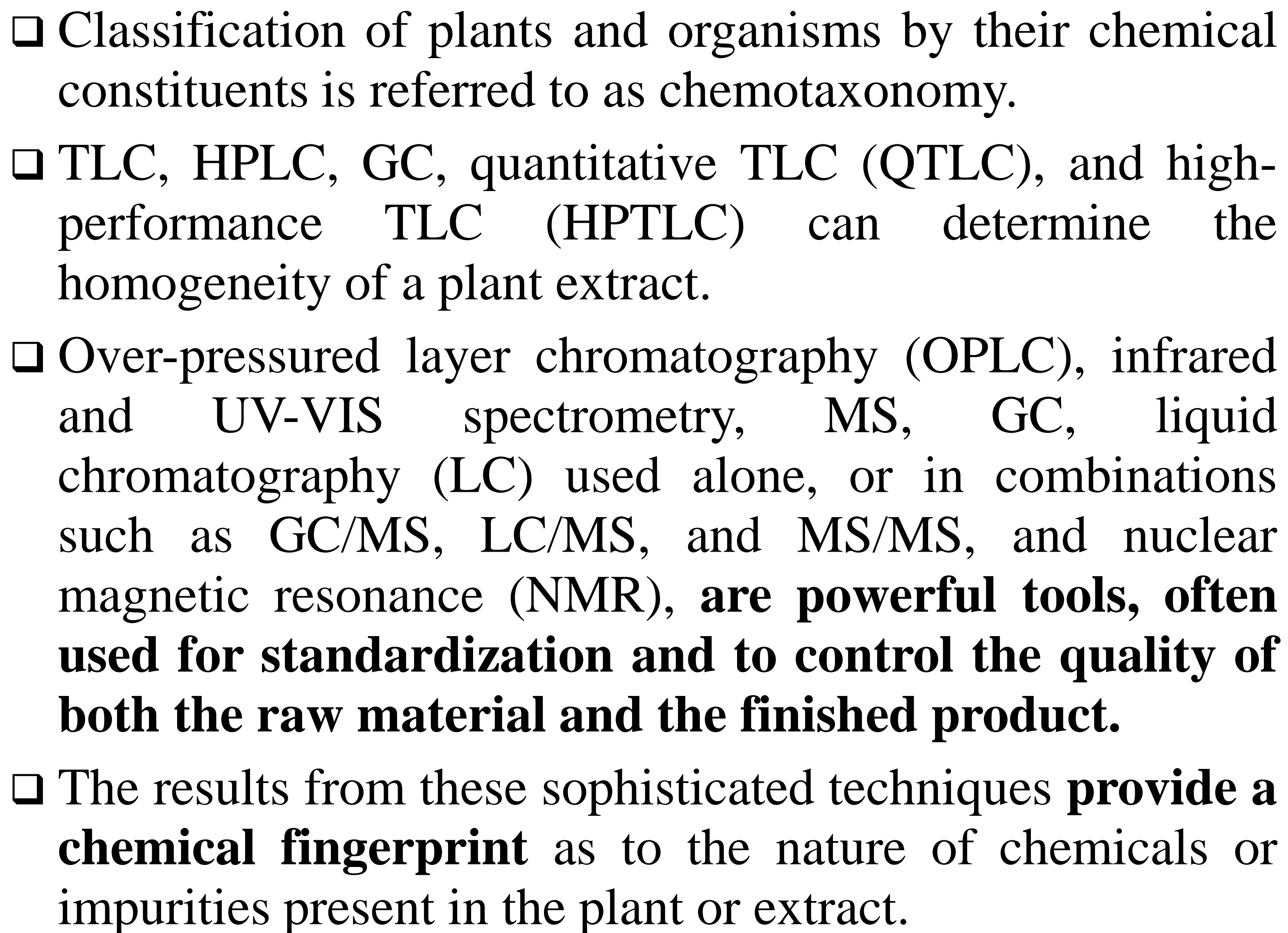
2- Pigments:

flavins, stilbenes, tryptophan, tyrosine, purines.

3- Minerals, metallic chelates

5- Chrmatographic Method of Assays:

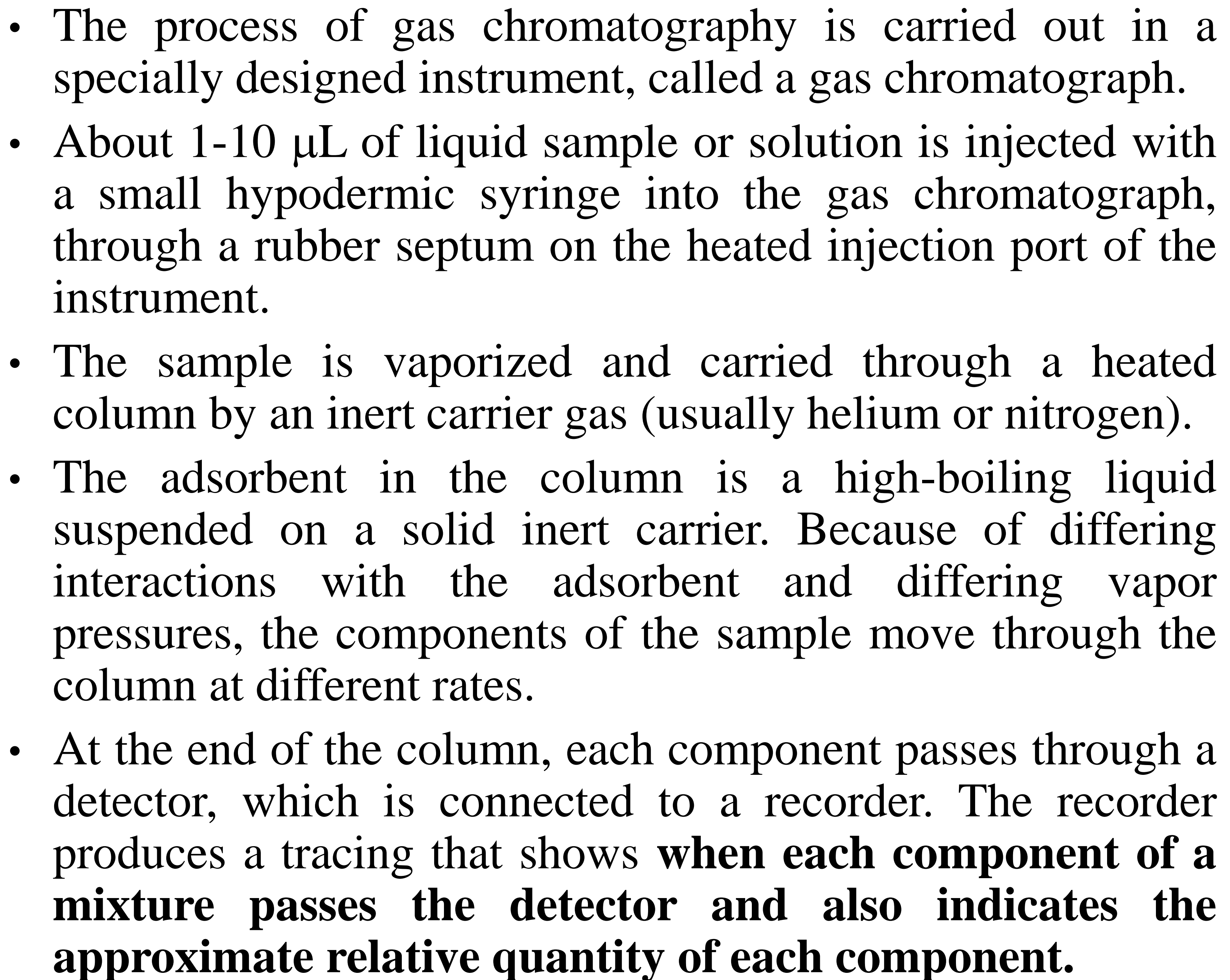
- The **quantitative** determination of constituents has been made easy by recent developments in analytical instrumentation.
- Recent advances in the isolation, purification, and structure elucidation of naturally occurring metabolites have made it possible to establish appropriate strategies for the determination and **analysis of quality and the process of standardization of herbal preparations.**



- Classification of plants and organisms by their chemical constituents is referred to as chemotaxonomy.
- TLC, HPLC, GC, quantitative TLC (QTLC), and high-performance TLC (HPTLC) can determine the homogeneity of a plant extract.
- Over-pressured layer chromatography (OPLC), infrared and UV-VIS spectrometry, MS, GC, liquid chromatography (LC) used alone, or in combinations such as GC/MS, LC/MS, and MS/MS, and nuclear magnetic resonance (NMR), **are powerful tools, often used for standardization and to control the quality of both the raw material and the finished product.**
- The results from these sophisticated techniques **provide a chemical fingerprint** as to the nature of chemicals or impurities present in the plant or extract.

A- Gas Chromatography (GC)

- Gas chromatography (GC) is known by a variety of other names: gas-liquid chromatography (GLC).
- In gas chromatography, the stationary phase is a high-boiling liquid and the mobile phase is an inert gas.
- Gas chromatography is generally used as an **analytical tool rather than as a means of purification**; however, like TLC, this technique can also be used to separate small quantities of compounds.



- The process of gas chromatography is carried out in a specially designed instrument, called a gas chromatograph.
- About 1-10 μL of liquid sample or solution is injected with a small hypodermic syringe into the gas chromatograph, through a rubber septum on the heated injection port of the instrument.
- The sample is vaporized and carried through a heated column by an inert carrier gas (usually helium or nitrogen).
- The adsorbent in the column is a high-boiling liquid suspended on a solid inert carrier. Because of differing interactions with the adsorbent and differing vapor pressures, the components of the sample move through the column at different rates.
- At the end of the column, each component passes through a detector, which is connected to a recorder. The recorder produces a tracing that shows **when each component of a mixture passes the detector and also indicates the approximate relative quantity of each component.**

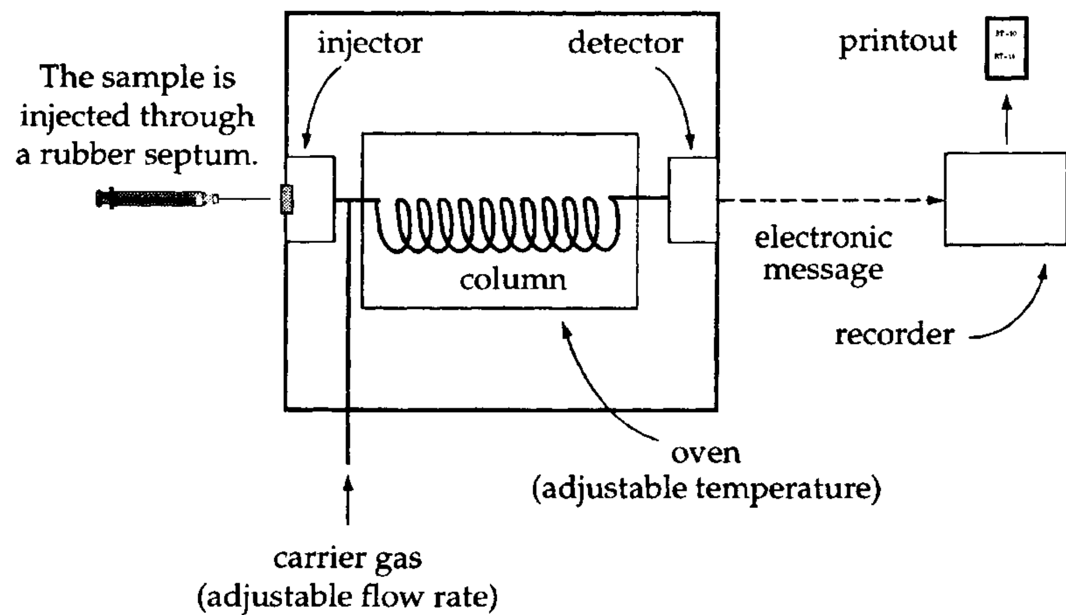
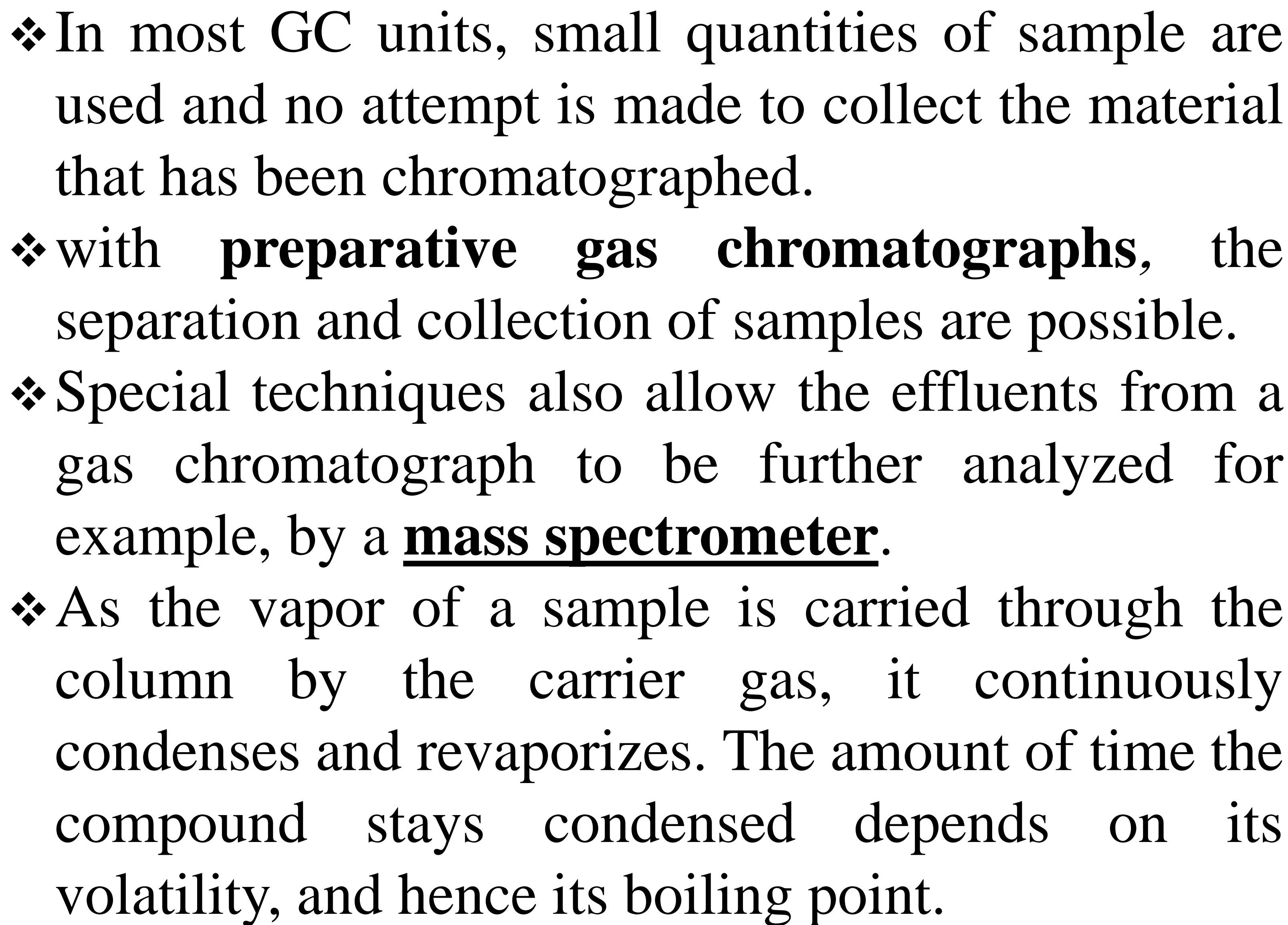


Figure 13.1 The basic components of the gas chromatograph. The vaporized sample is carried through the column by an inert carrier gas. The detector senses the passage of organic compounds, and the recorder graphs this information.

GC is commonly used for

1) Checking the purity of volatile-liquid samples, such as distillation fractions.
2) Checking the identity of a substance by comparison of its GC with that of a known.
3) Analyzing a mixture for the presence or absence of a known compound (such as alcohol in blood).



- In most GC units, small quantities of sample are used and no attempt is made to collect the material that has been chromatographed.
- with **preparative gas chromatographs**, the separation and collection of samples are possible.
- Special techniques also allow the effluents from a gas chromatograph to be further analyzed for example, by a **mass spectrometer**.
- As the vapor of a sample is carried through the column by the carrier gas, it continuously condenses and revaporizes. The amount of time the compound stays condensed depends on its volatility, and hence its boiling point.

Chromatogram

- The recorder receives the electrical information from the detector and produces a graph—the chromatogram—of the components passing through the detector.
- The time it takes for a particular compound to pass through the column is called the **compound's retention time (RT).**
- The retention time is **a function of** the physical properties of the compound, the rate of gas flow, the temperature, the liquid phase, and the length and diameter of the column.

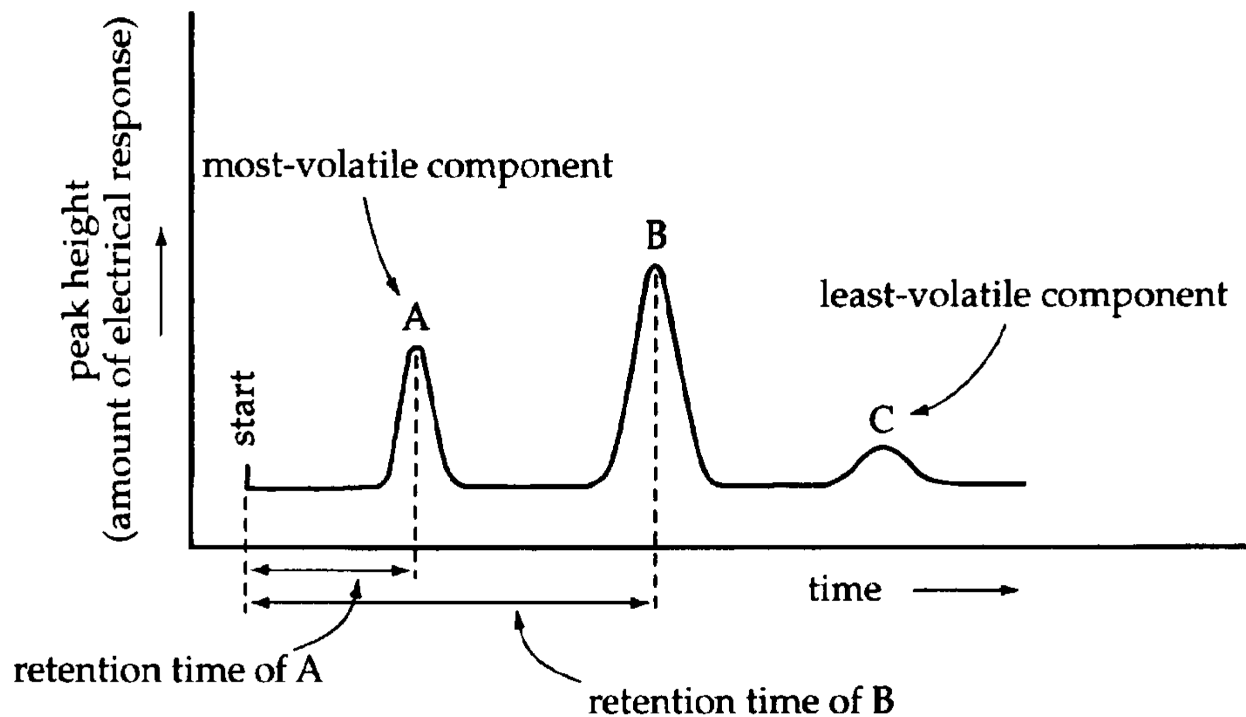
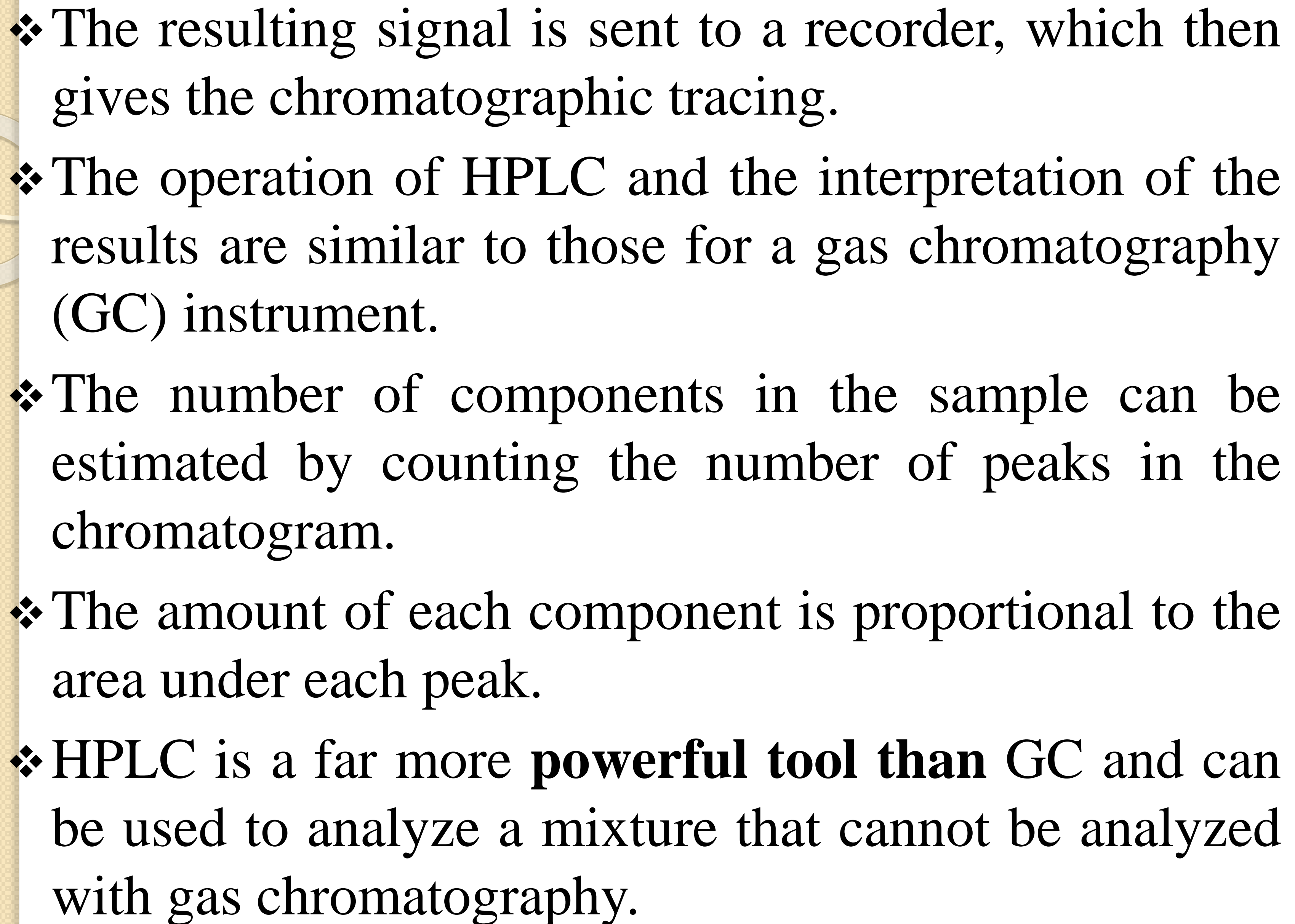


Figure 13.6 A typical gas chromatogram. The retention time of a compound is measured as the distance between the start point and the top of the compound's peak. The areas under the peaks are determined by the integrating recorder, the computer program, or manual triangulation.

B- High-Performance Liquid Chromatography (HPLC)

- In HPLC, the solvent is pumped through the column at high pressure (6000 psi; 400 atm).
- The sample is "injected" into the solvent stream and passes through the column.
- The components in the sample are separated from one another as they pass through the column.
- The principles behind their separation are the same as those of column chromatography.
- As the components in the sample are eluted from the column, they pass through a detector, which converts their passage to electrical potential.


❖The resulting signal is sent to a recorder, which then gives the chromatographic tracing.
❖The operation of HPLC and the interpretation of the results are similar to those for a gas chromatography (GC) instrument.
❖The number of components in the sample can be estimated by counting the number of peaks in the chromatogram.
❖The amount of each component is proportional to the area under each peak.
❖HPLC is a far more powerful tool than GC and can be used to analyze a mixture that cannot be analyzed with gas chromatography.

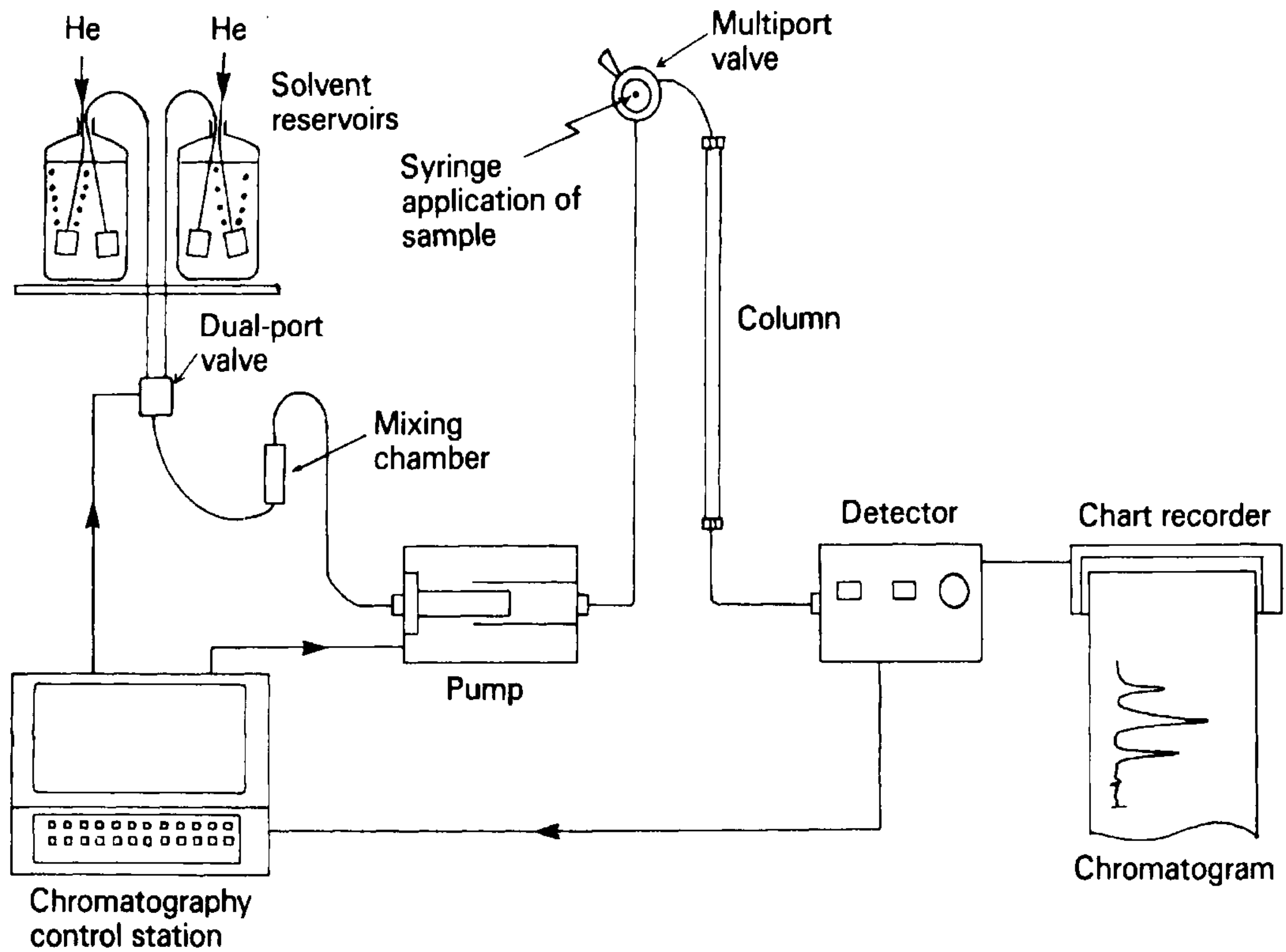


Fig. 6.2 Low-pressure mixing gradient HPLC system

Example of ethnomedicinal herb has safety problems

- ***Echinacea purpurea*** - Australian Adverse Drug Reaction Bulletin, v.18, No.1, 1999:
  - Popular in many countries for prophylaxis and treatment of common cold.
  - In Australia 37 reports of ADRs associated with *Echinacea* in 2 years:
    - **21 allergic reactions, 9 bronchospasm, 8 dyspnoea, 5 urticaria, 4 angina.**
    - **12 patients had previous history of asthma, allergic rhinitis, conjunctivitis, hay fever.**
  - **Conclusion:** *Echinacea* may be dangerous in those patients who have history of allergic diseases.

Drug adulteration

Adulteration involves a number of different conditions:

- ❖Inferiority.
- ❖Spoilage.
- ❖Deterioration.
- ❖Admixture.
- ❖Sophistication.
- ❖Substitution.

From the standpoint of present day commerce, inferior, spoiled or deteriorated drug represent the greatest percentage of case of drug adulteration.

1- Inferiority:

Inferiority refers to any substandard drug or substance. The more restricted definition as applied to foods, drugs and materials produced by nature indicates a naturals substandard condition.

2- Spoilage:

Spoilage refers to a form of substandard drug in which the **quality or value** or usefulness of the article has been impaired or destroyed by the action of **fungi and bacteria** as to render the article unfit for human consumption.

All drugs which are unfit for human or animal consumption are **legally** considered as adulterated.

3- Deterioration:

It is applied to any impairment of the quality or value of an article by the **abstraction or destruction of valuable constituents** by distillation, extraction, aging, moisture, heat, fungi, insects.

4- Admixture:

It is the addition of one article to another through **accident**, **ignorance** or **carelessness.** If the admixture is done **intentionally** to **defraud**, it is sophistication.

5- Sophistication:

Is addition of a spurious or inferior material to any article with **intent to defraud.**

6- Substitution:

It is occur when an **entirely different article** is used or sold in place of the one required or requested. A **complete substitution**, even through **intentional** and **fraudulent**, is none of the true article is present.

Too often we hear the expression:" Since herbal products are natural, they are safe". Unfortunately, this is not always true, due to a myriad of factors including the following:

1- Factual information about many herbs is often lacking.

2- A portion of the information available about many herbs currently on the market may be exaggerated or misleading.

3- The lack of adequate quality control present in some herbal product companies around the world leads to variability in the quantity and quality of the product's content.

- Fortunately, situation is now changing, as some herbal organizations are now establishing good manufacturing practices (GMP), as well as safety checks and controls concerning herbal products.

4- Some herbal products originating in Asia, Africa or South America have not adequately tested for purity, safety or efficacy, prior to their introduction in the market.

5-Serious intoxications have occurred around the globe due to mislabeling of herbal products or erroneous identification of some plant species.

6- Some plants have not been adequately studied with respect to their use by special populations including pregnant or lactating women, small children and the elderly.