Basic Techniques in Biochemical Laboratory

Daniela Obitkova MD
CTU in Prague
Faculty of Biomedical Engineering
Department of Health Care Disciplines and population protection

Introduction

Understanding the basic principles of techniques and the theory of instrumental analysis will provide a working knowledge of instruments, applications to patients testing in the clinical chemistry laboratory.

Basic Methods

- ✓ Spectrophotometry
- ✓ Nephelometry
- ✓ Turbidimetry
- ✓ Electrophoresis
- ✓ Chromatography
- ✓ Mass spectrometry

Photometry

Many determinations made in the clinical laborotory are based on radiant energy absorbed, or reflected under controlled conditions.

The principles involved in such measurements are:

- ✓ spectrophotometry
- ✓ nephelometry
- ✓ turbidimetry

Spectrophotometry

Definition

The absorbance A of a solution is the amount of light absorbed by that solution. According to *Lambert's - Beer's Law* the absorbance A varies directly with the concentration of the solution c in question.

$$A = \varepsilon . l . c$$

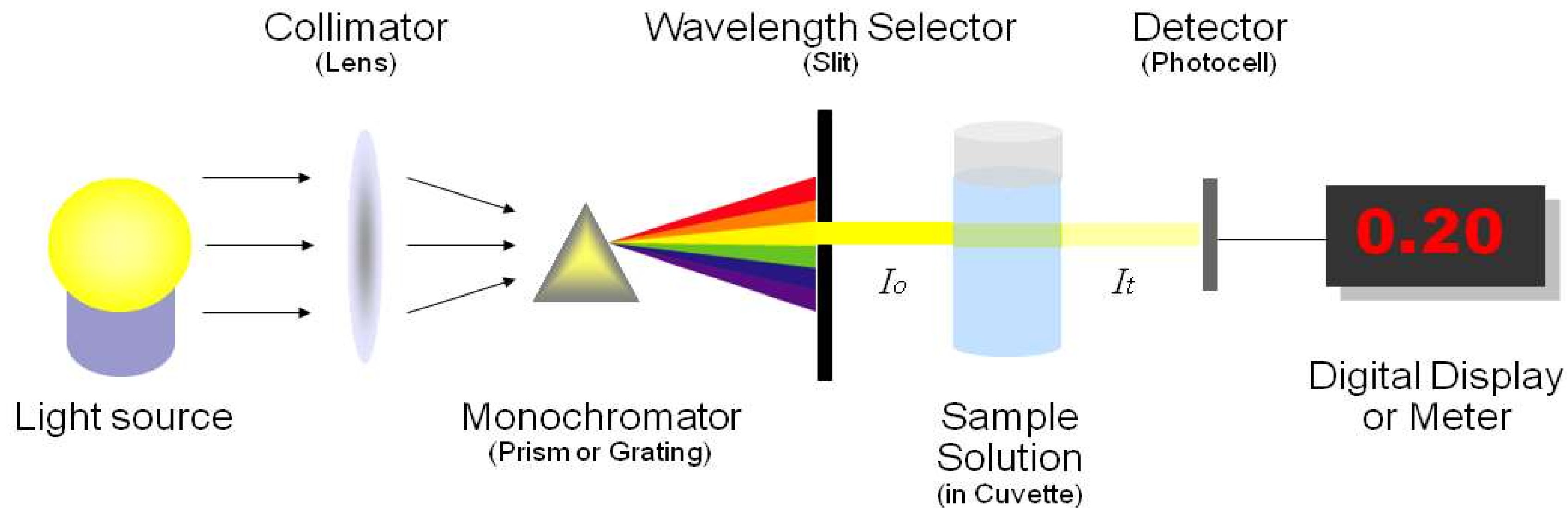
A – absorbance

Ɛ – molar absorptivity [Ɛ] = L .mol.cm^{-1}

c - concentration

Spectrophotometry

Basic design of the spectrophotometer



Spectrophotometry

Basic methods of assesement analytes concentration in biological materials

end-point methods – 1. calibration (standard) curve
2. aone standard technique

kinetic measurements

Spectrophotometry

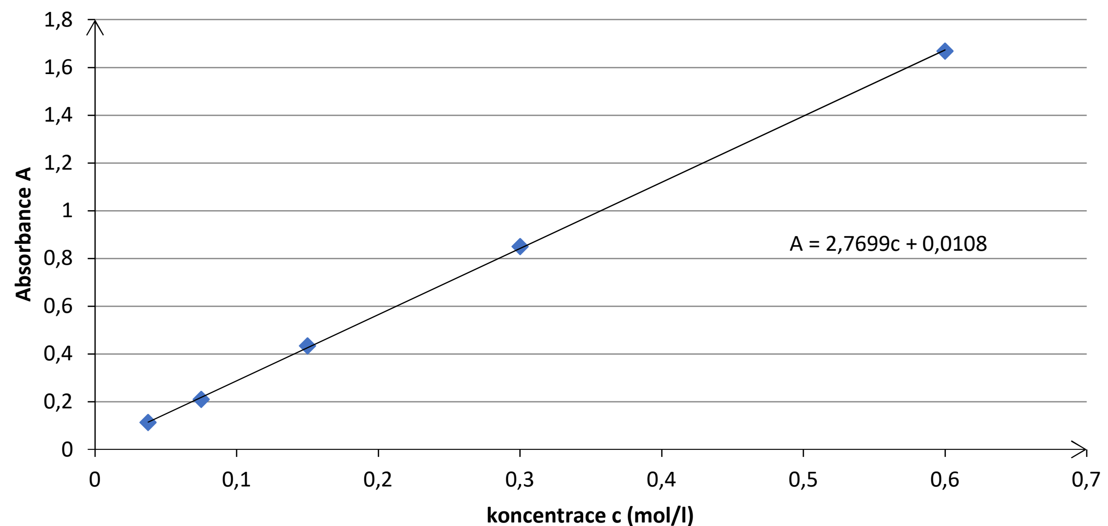
calibration (standard) curve

based on direct proportion of absorbance and concentration

steps:

1. the solution of analyt with known concentration
2. dilution of this solution to 5 tubes with decreasin concentration
3. measurement of these standards
4. preparing the linear regresion curve
5. the unknown sample measurement
6. the unknown concentration count

Result:



Spectrophotometry

one standard method

Steps:

1. one solution of known concentration standard)
2. measurement of absorbance
3. the absorbance of an anylyte in question is compared with the absorbation of standard
4. concentration of analyte calculation

Result:

$$\frac{A\ standard}{A\ sample} = \frac{c\ standard}{c\ sample}$$

matematical adjustment

$$c = \frac{A_{sample}}{A_{standard}} \cdot c_{standard}$$

general equation used for majority of end-point assessment

[c] = $\frac{mol}{l}$

Spectrophotometry

kinetic measurements

measures the diference of absorbance
in particular time period
used mainly for determination
of enzymes concentration

steps:

1. the assesment of the standard solution
2. save programe to the instrument
3. concentration calculation

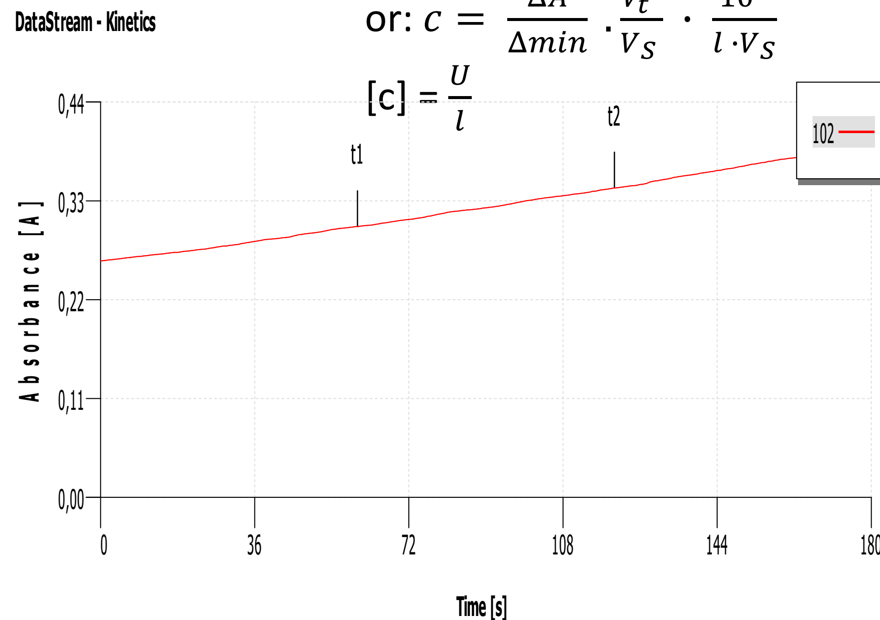
Result:

$$c = \frac{\Delta A}{\Delta t} . \frac{1}{\varepsilon} . 10^6 . \frac{1}{60} . \frac{V_t}{V_S}$$

$[c] = \frac{\mu \, kat}{l}$

or: $c = \frac{\Delta A}{\Delta min} . \frac{V_t}{V_S} \cdot \frac{10^6}{l \cdot V_S}$

$[c] = \frac{U}{l}$



Turbidimetry

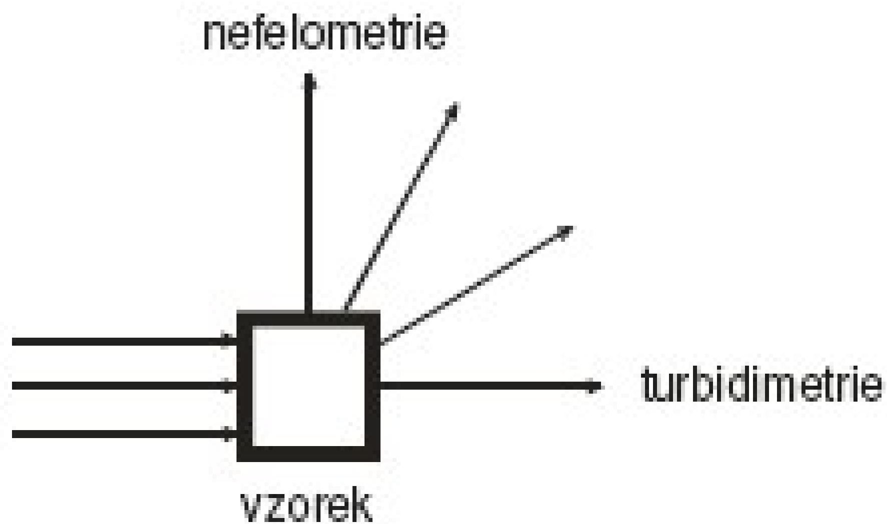
Principle

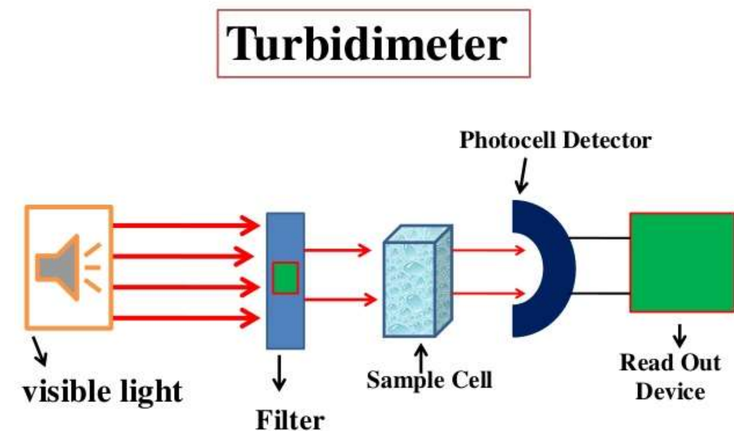
Turbidity is the cloudiness of a fluid caused by large numbers of individual particles that are generally invisible to the naked eye
Turbidity causes the decrease of the
intensity of the light beam as it passes through a solution of particles.

The decrease of the intensity of the light beam is caused by scattering, reflectance, and absorption of the light.

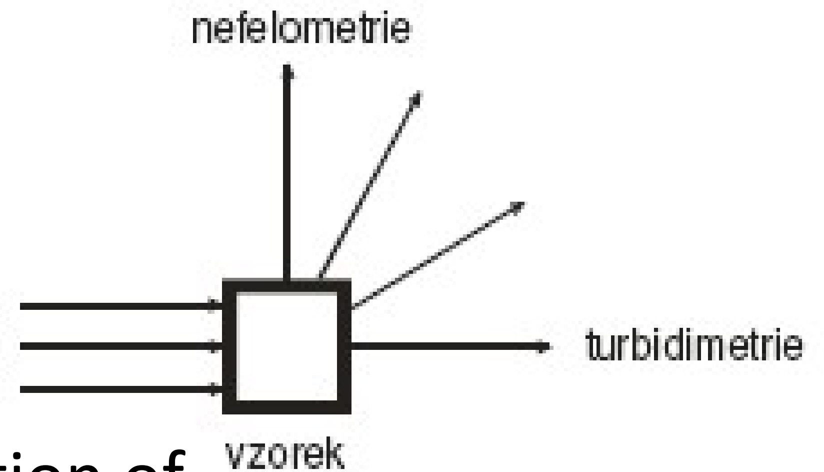
Turbidimetry

The principles of *Lambert's - Beer's Law are used to determine the concentration of analytes*





Nephelometry



Principle

Nephelometry is defined as the detection of light energy scattered or reflected toward a detector that is not in the direct path of the transmitted light.

Common nephelometers measure scattered light at right angles to the incident light.

Nephelometry

the samples are usually koloid solutions or microsuspension

nephelometry is more sensitive than turbidimetry, can measure very low concentrations of analytes

esed in imunology – reactoin antigen-antibody – precipitate – measurement of antigen or antibody concentration

Electrophoresis

Theoretical prerequisites

anode: the positively charged electrode in electrophoresis system.

cathode: negative electrode.

isoelectric point pI - of a molecule:

is the pH at which it has no net charge and will not move in an electrical field

ampholyte or zwitterion: is a molecule that can be

either positively or negatively charged; example: proteins, amino acids.

the net charge depends mainly on the value of pH

Electrophoresis

Chemical species carrying an electrical charge move either to the cathode or the anode in an electrophoresis system, depending on the kind of charge they carry.

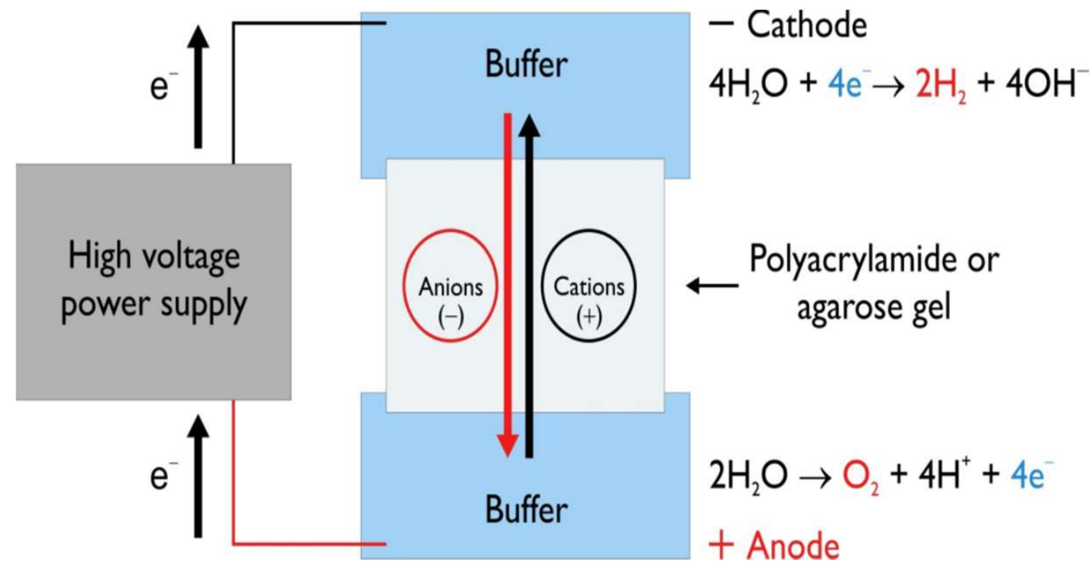
In a solution more acid than the isoelectric point of the solute, an ampholyte takes on a positive charge and migrates toward the cathode. In the reverse situation, it migrates toward the anode.

The rate of migration **S** dependent on factors such as:

- the net electrical charge of the molecule
- the size and shape of the molecule
- the electric field strength
- the characteristics of the supporting medium
- and the operation temperature

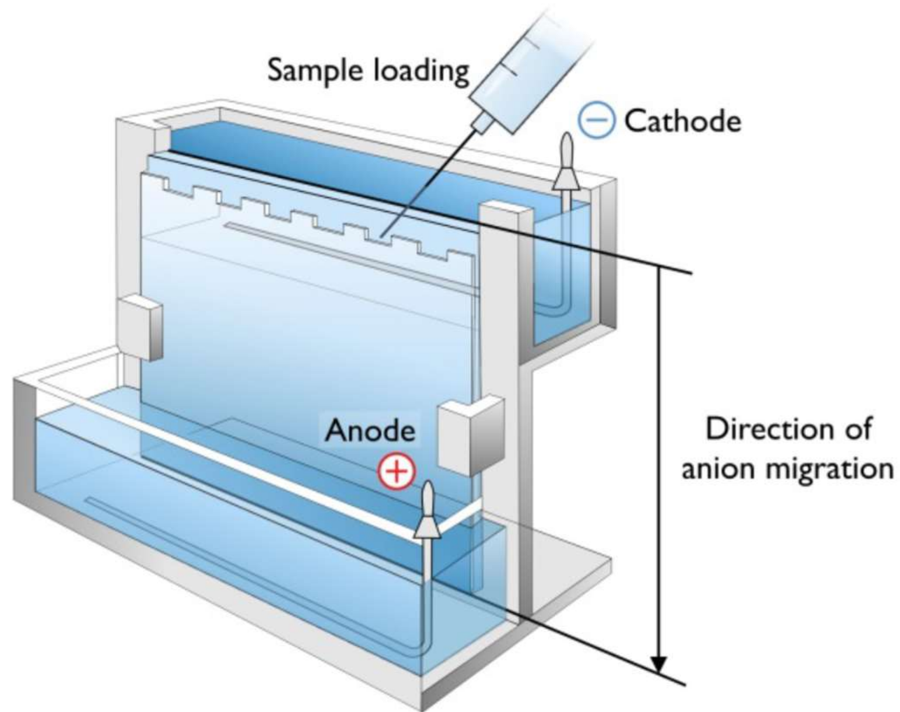
Electrophoresis

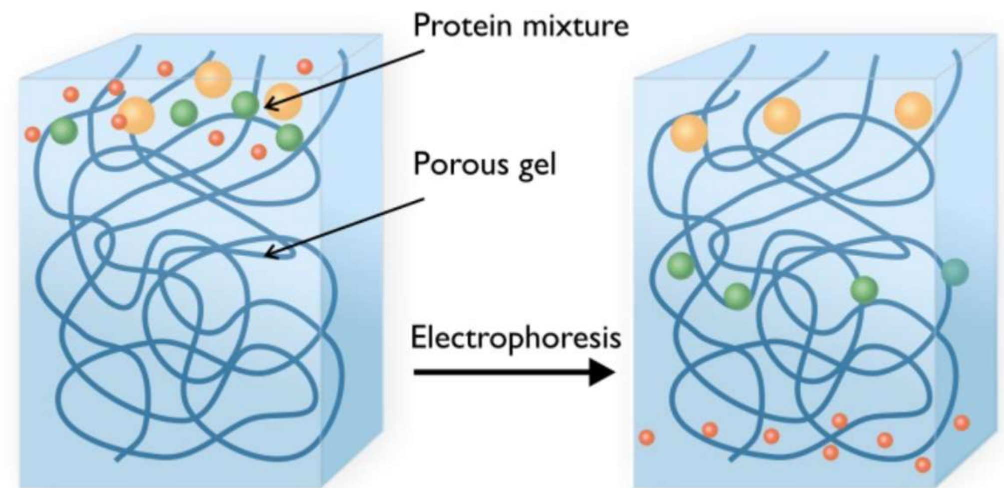
Schematic picture of electrophoresis



The sheme electrophoresis system shows:
two buffer boxes containing
the buffer used in the process.
In each buffer box is an electrode of either platinum or carbon, the polarity of which is fixed by the mode of connection to the power supply.

Electrophoresis





Electrophoresis

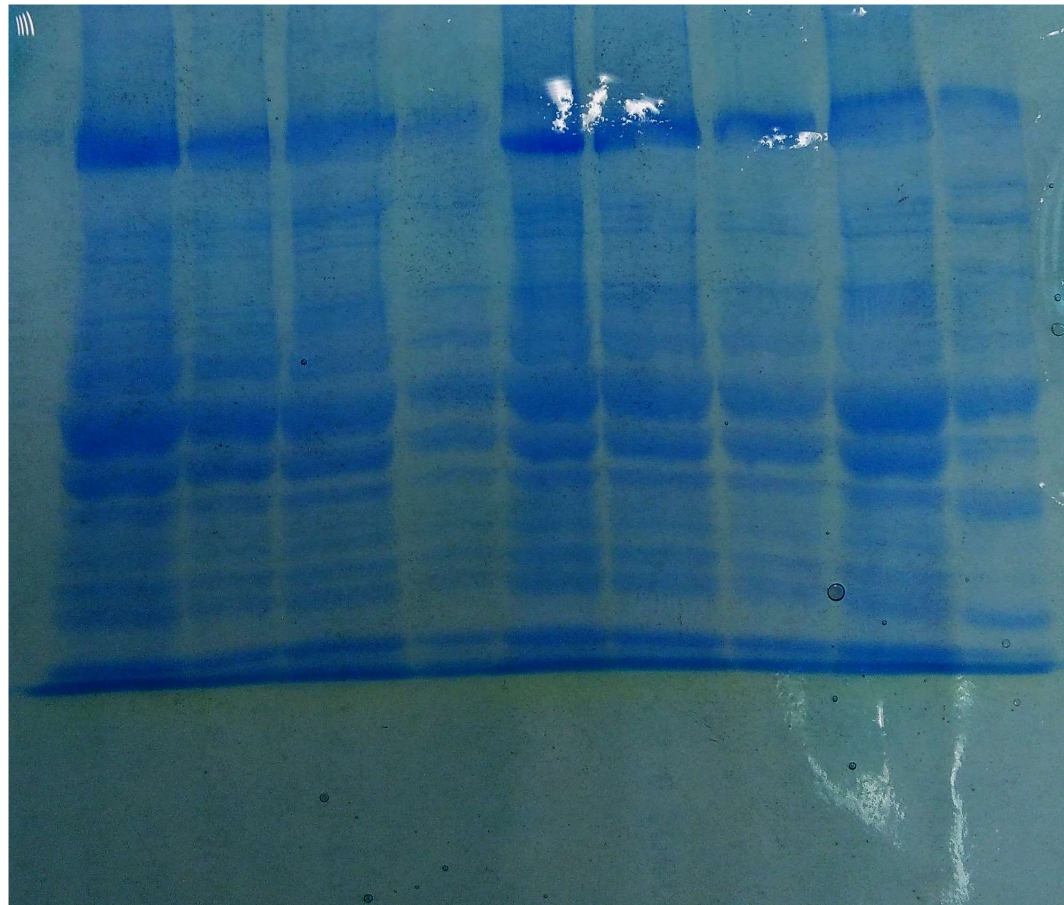
Polyacrylamis gel electrophoresis PAGE/SDS
PAGE

arrangement – *denaturation of proteins*

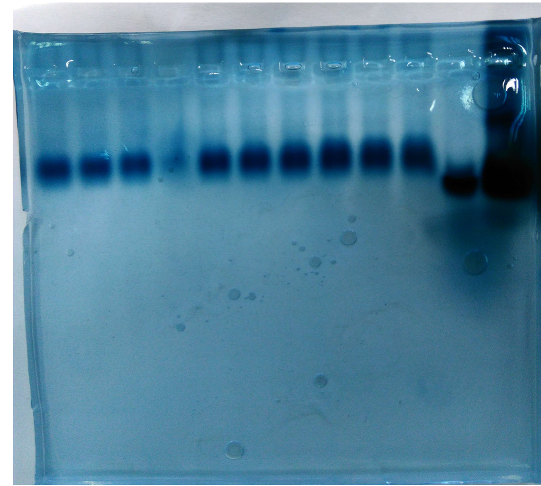
denaturation steps – **high temperature**, **chemical denaturation** (beta mercaptoetanol)
SDS – dives the negative chargé all proteins than have the same chargé – separation according to the molecular mass

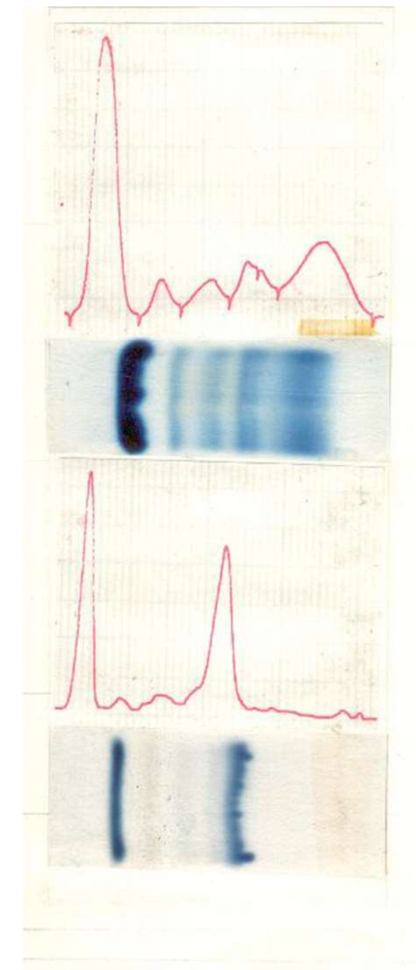
native proteins no denaturation – the net chargé of individual molecules differ – separation according to the net charge

Electrophoresis



Electrophoresis





Agarose gel electrophoresis

It is a convenient method of electrophoresis that uses a purified, essentially neutral fraction of agar called agarose as a medium. It has been successfully applied to the analysis of serum proteins, hemoglobin variants, isoenzymes, lipoproteins fractions and other substances.
The advantages of agarose gel include its lower affinity for proteins and its native clarity after drying, which permits excellent densitometric examination

Chromatography

Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (mobile phase) movesnin the definite direction.

Chromatography

Basic concepts and definition

The primary goal of the chromatographic process is to separate a mixture into its individual components, which are called solutes or analytes.
A chromatographic separation requires a sample to be introduced into a flowing stream of gas or liquid (mobilephase) that passes through a bed, layer , orcolumn containing the stationary phase.

If the mobilephase is a gas, the technique is known as gas chromatography (GC), ifaliquid, it is called liquid chromatography (LC).

Chromatography

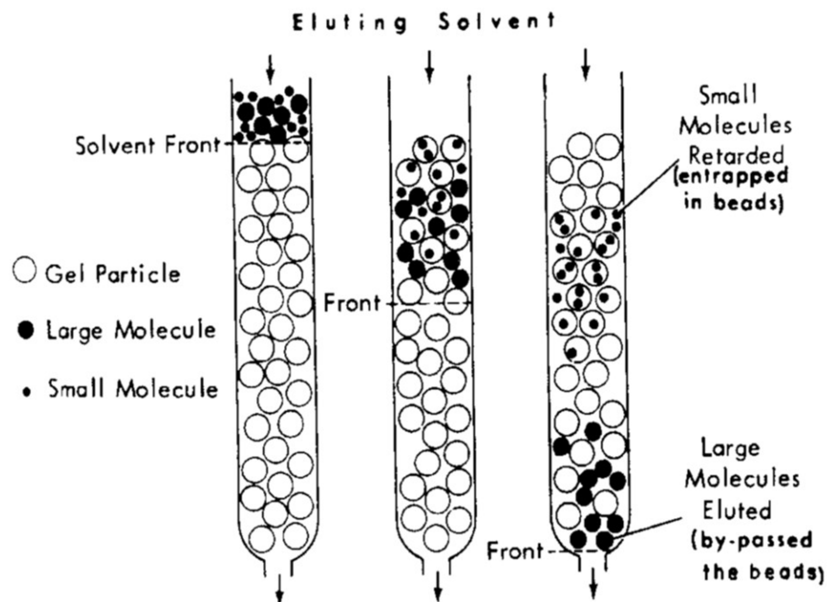
Gel filtration chromatography

It is also known as gel-permeation, size exclusion, molecular exclusion, molecular sieve chromatography and separate solutes on the basis of their molecular size.

A variety of materials have been used as stationary phases:
cross link dextran (Sephadex),
polyacrylamide (Bio- Gel)
agarose (Sepharose)

Gel filtration chromatography

arrangement



Molecules too large to enter the pores remain exclusively in the mobile phase

- rapidly elude from the column.

Molecules tha tare

intermediate sinsize(and

small molecules) have

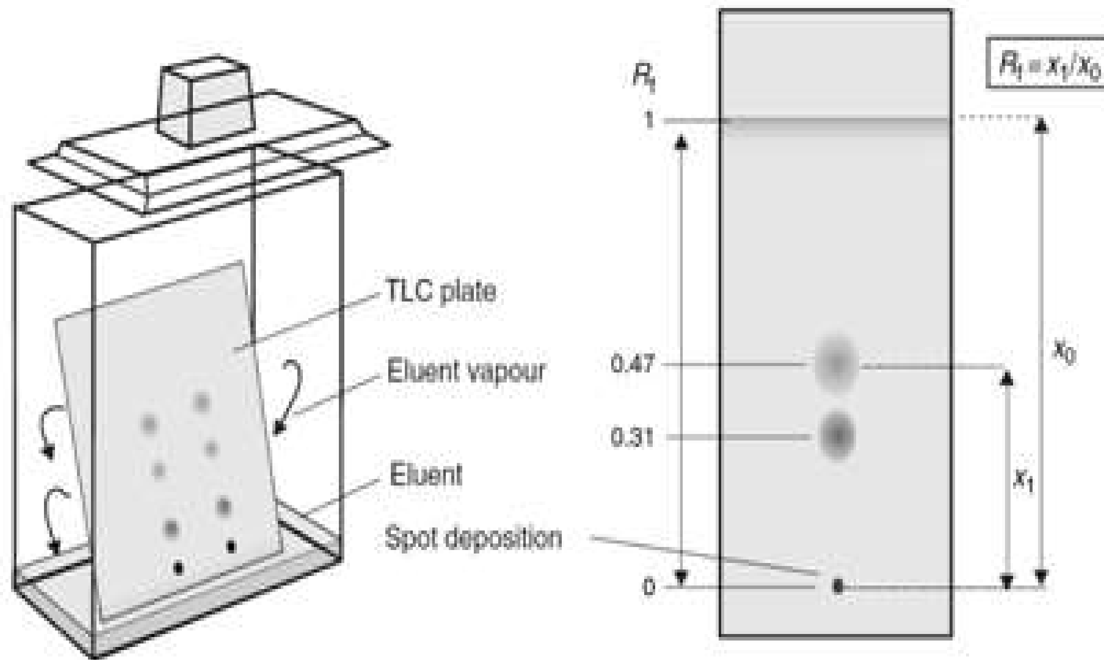
access to various fractions

of the pore volume andelude slowly.

Chromatography

Thin layer chromatography

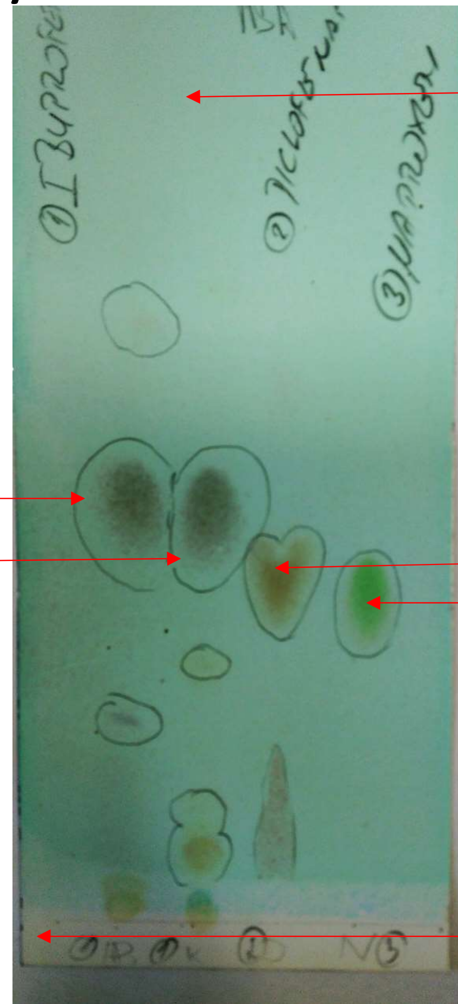
arrangement



- In partition chromatography (also called thin-layer chromatography)
- a thin film of liquid is
- adsorbed onto the surfaces of support particles.
- Separation is based on differences in the relative solubility of solute molecules in this film and the mobile phase.

Chromatography

TLC result



front line

ibuprofen standard

ibuprofen

diclophenac

naproxene

start line

Chromatography

Gas chromatography

Basic concepts

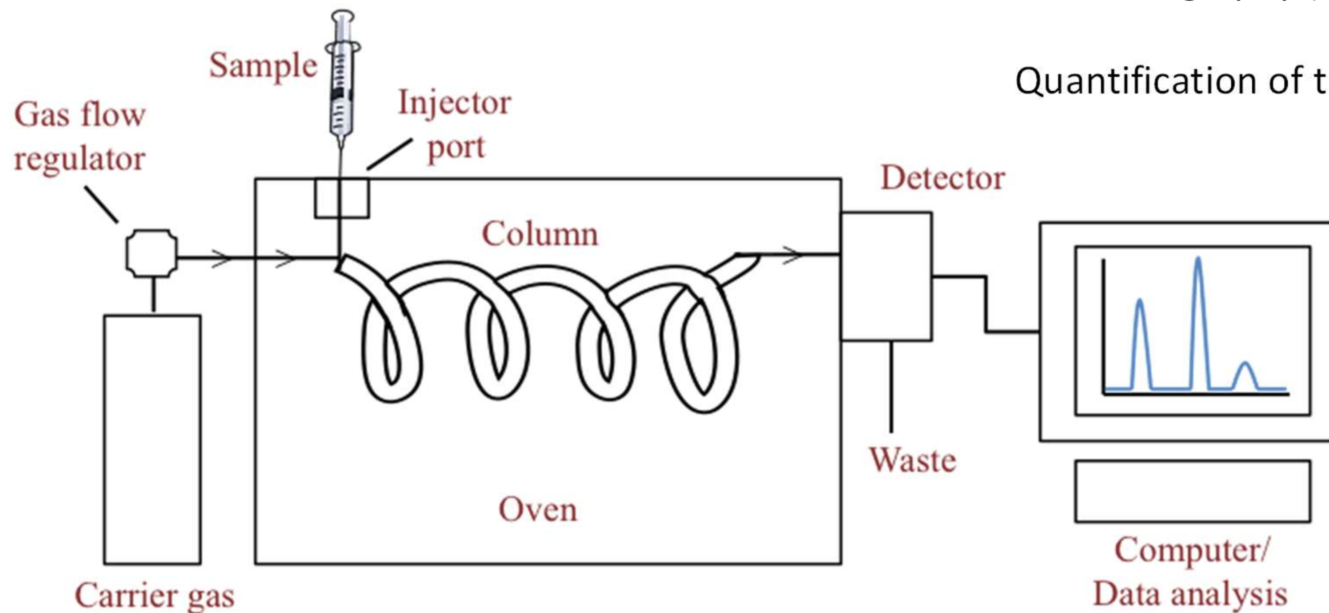
GC is a process by which a mixture is separated into its constituent components by forcing a gaseous
mixture of it and mobile phase (carrier gas) through a column containing the stationary phase. Separation of the solutes in the mixture is based on the relative differences in their vapor pressures and their interaction with the stationary phase.

A compound with a high vapor pressure will be eluted more rapidly than compounds with lower vapor pressures.

Chromatography

Gas chromatography

Arrangement



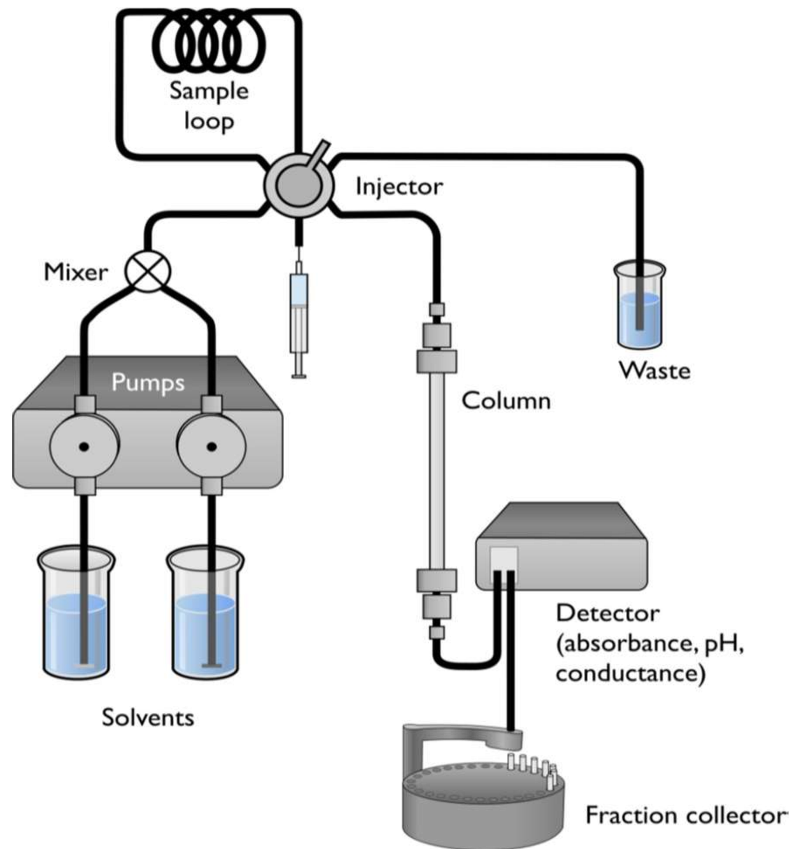
The effluent from the column carries the separated sample constituents to the detector which produces a signal that is displayed as a series of peaks

Peak size (area or height) is proportional to the amount of the compound detected and can be used to quantify the amount of analyte in the sample.
Depending on the nature of the stationary phase, GC techniques are divided into two categories: gas-solid chromatography (GSC) and gas-liquid chromatography (GLC).

Quantification of the amount of the sample – GC- FID
- GC- MS

Chromatoggraphy

High-Performance Liquid Chromatography (HPLC)



In LC, separation is based on the distribution of the solutes between a liquid mobile phase and amstationary phase. When an efficient column is used in a liquid chromatograph, the technique is HPLC Because column efficiency is inversely related to the particle size of the column packing, relatively high pressure is required to pump liquid through an efficient column.

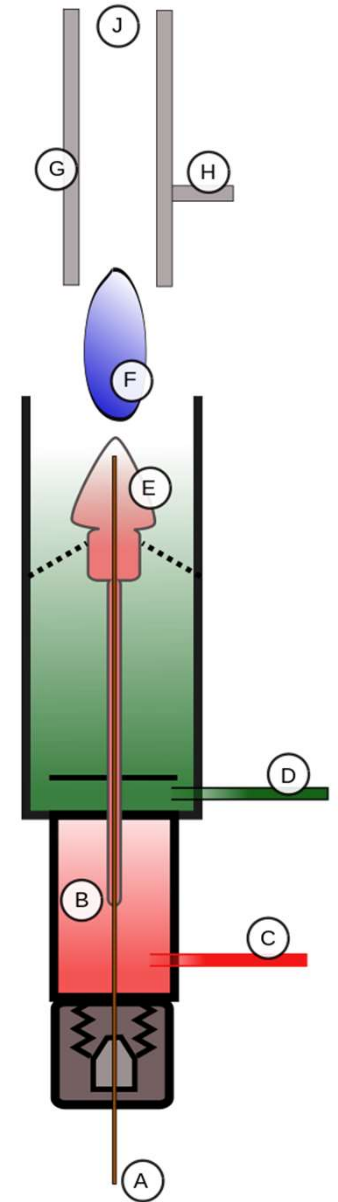
Detectors for chromatography

GC – FID
GC-MS

FID – flame ionization detector

measures analytee in a gas stream.
It is frequently used as a detector in gas chromatography.
The measurement of ion per unit time make this a mass sensitive instrument.

FID Schematic: A) Capillary tube; B) Platinum jet; C) Hydrogen; D) Air; E) Flame; F) Ions; G) Collector; H) Coaxial cable to Analog to Digital converter; J) Gas outlet



Detectors for chromatography

GC – MS
HPLC _MS

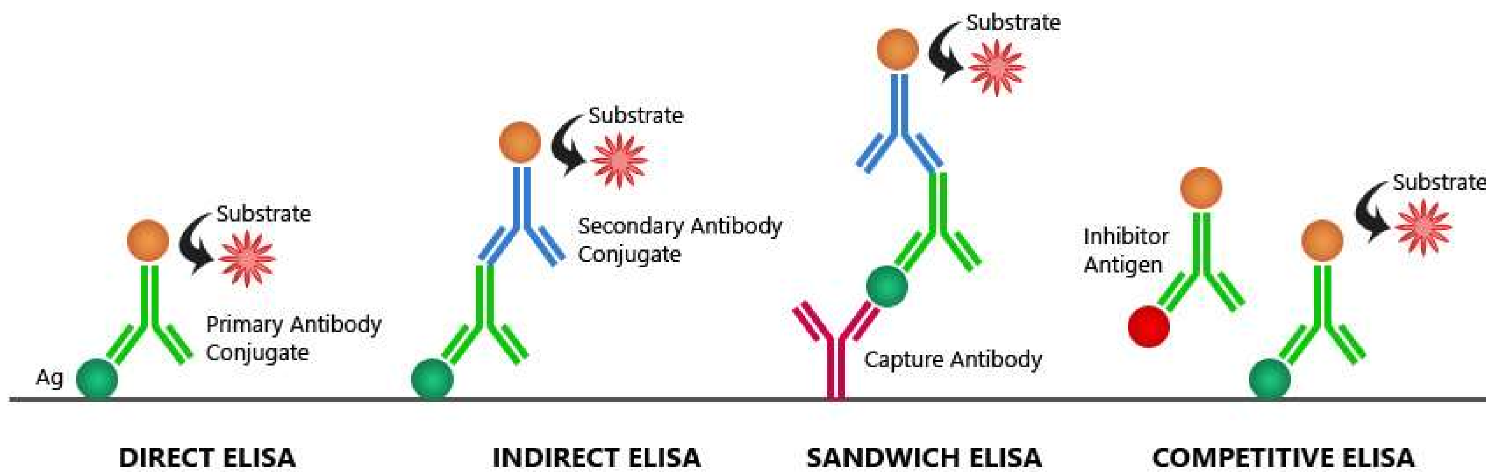
Mass spektrometry

mass spectrometry is an analytical *technique that first ionizes a target molecule and then separates and measures the mass of a molecule or its fragments. Mass analysis is the process by which a mixture of ionic species is identified according to the mass-to-charge (m/z) ratios (ions).*

Immunochemical methods

ELISA Enzyme-Linked Immunosorbent assay

Arrangement



Thank you for your attention