Model Answer

AS-2545

M. Pharm. (I Semester) Examination, 2013
MODERN REASEARCH METHOD

SECTION-A **[12×2 = 24]**

1

(i) TMS is tetramethyl silane. It is used as a reference because it gives sharp peak and absorb at higher field (shielded) than almost all organic protons. It is chemically inert, symmetrical, volatile and soluble in most organic solvents.

(ii) The essential components of UV spectrometer is as follows –

a) Radiation source (tungsten filament lamp, deuterium discharge lamp)
b) Monochromator
c) Sample compartment
d) Detector (Photomultiplier tube / Photodiode)
e) Amplification and recording devices

(iii) (d)

(iv) (c) or (d)

(v) These cover the wavelength range 340-1000. They absorbs strongly from 300 nm down and is not practically useful for organic work.

(vi) **(**a) – (ii)
(b) – (iv)
(c) – (i)
(d) – (iii)

(vii) (a) - TRUE
(b) - TRUE

(viii) Isoelectric focusing is the focusing of the separated components (proteins) at the point in the pH gradient across the supporting medium in electrophoresis where they carry no net charge.

(ix) Retention time is defined as the time after the point of injection where the maximum point of the peak occurs.

(x) HETP is the height equivalent to a theoretical plate. The height of a column or paper section required for one equilibration called HETP.

(xi) (d)

(xii) Size and shape (volume) of solute molecules.

SECTION-B [Any Four] **[4×14 = 56]**

2

Ans. Technique involves creating gas phase ions from the analyte atoms or molecules, separating the ions according to their mass-to-charge ratio (m/z) and measuring the abundance of the ions.

Mass spectrum is a plot of relative abundance vs m/z. The most abundant peak is known as the base peak. The base peak is scaled to 100. Spectrum shows fragmentation patterns. The m/z values and the fragmentation pattern are used to determine the molecular weight and structure of organic compounds.

Technique can be used for

- Qualitative and quantitative analysis
- Providing information about the mass of atoms and molecules
- Molecular structure determination (organic & inorganic)
- Identification and characterization of materials

Applications of Mass Spectrometry

Mass spectrum is a graph of the mass of each cation vs. its relative abundance. The peaks are assigned abundance as a percentage of the base peak, which is the most intense peak in the spectrum. The base peak is not necessarily the same as the parent ion peak.

Most elements occur naturally as a mixture of isotopes. The presence of significant amounts of heavier isotopes leads to small peaks that have masses that are higher than the parent ion peak.

- M+1 = a peak that is one mass unit higher than M^+
- M+2 = a peak that is two mass units higher than M^+

Easily Recognized Elements in MS

- Bromine:
  - M^+ ~ M+2 (50.5% ^{79}Br/49.5% ^{81}Br)
- Chlorine:
  - M+2 is ~ 1/3 as large as M^+
- Sulfur:
  - M+2 larger than usual (4% of M^+)
- Iodine
  - I^+ at 127
  - Large gap

Fragmentation Patterns

- The impact of the stream of high energy electrons often breaks the molecule into fragments, commonly a cation and a radical.

- Bonds break to give the most stable cation.
- Stability of the radical is less important.

- Alkanes
  - Fragmentation often splits off simple alkyl groups:
    - Loss of methyl $M^+ - 15$
    - Loss of ethyl $M^+ - 29$
    - Loss of propyl $M^+ - 43$
    - Loss of butyl $M^+ - 57$
  - Branched alkanes tend to fragment forming the most stable carbocations.
- Alkenes:
  - Fragmentation typically forms resonance stabilized allylic carbocations
- Aromatics:
  - Fragment at the benzylic carbon, forming a resonance stabilized benzylic carbocation (which rearranges to the tropylium ion)
- Aromatics may also have a peak at m/z = 77 for the benzene ring.
- Alcohols
  - Fragment easily resulting in very small or missing parent ion peak
  - May lose hydroxyl radical or water
    - $M^+ - 17$ or $M^+ - 18$
  - Commonly lose an alkyl group attached to the carbinol carbon forming an oxonium ion.
    - 1^o alcohol usually has prominent peak at m/z = 31 corresponding to $H_2C{=}OH^+$
- Amines
  - Odd M^+ (assuming an odd number of nitrogens are present)
  - a-cleavage dominates forming an iminium ion
- Ethers
  - a-cleavage forming oxonium ion
  - Loss of alkyl group forming oxonium ion
  - Loss of alkyl group forming a carbocation
- Aldehydes (RCHO)
  - Fragmentation may form acylium ion
  - Common fragments:
    - $M^+ - 1$ for
    - $M^+ - 29$ for
- Ketones
  - Fragmentation leads to formation of acylium ion:
    - Loss of R forming
      Loss of R' forming

- Esters (RCO_2R')
    - Common fragmentation patterns include:
        - Loss of OR'
            - peak at M^+ - OR'
        - Loss of R'
            - peak at M^+ - $R^{'}$

Structural Information by MS

MW determination

- nominal
- accurate (elemental composition)
    - Isotope pattern
    - High resolution
    - Fragmentation

3

Ans. 3(a) Thermal conductivity detector [Katherometer or Hot-wire detector]

Thermal conductivity detectors (TCD) were one the earliest detectors developed for use with gas chromatography. The TCD works by measuring the change in carrier gas thermal conductivity caused by the presence of the sample, which has a different thermal conductivity from that of the carrier gas.

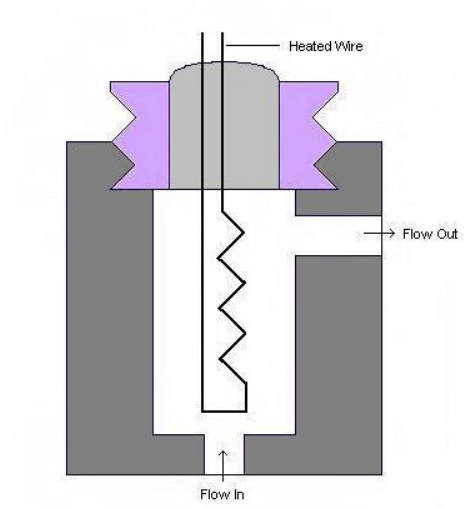
This common detector relies on the thermal conductivity of matter passing around a tungsten-rhenium filament with a current traveling through it. In this set up helium or nitrogen serve as the carrier gas because of their relatively high thermal conductivity which keep the filament cool and maintain uniform resistivity and electrical efficiency of the filament. However, when analyte molecules elute from the column, mixed with carrier gas, the thermal conductivity decreases and this causes a detector response. The response is due to the decreased thermal conductivity causing an increase in filament temperature and resistivity resulting in flucuations in voltage. Detector sensitivity is proportional to filament current while it's inversely proportional to the immediate environmental temperature of that detector as well as flow rate of the carrier gas.

Their design is relatively simple, and consists of an electrically heated source that is maintained at constant power. The temperature of the source depends upon the thermal conductivities of the surrounding gases. The source is usually a thin wire made of platinum, gold etc. The resistance within the wire depends upon temperature, which is dependent upon the thermal conductivity of the gas.

TCDs usually employ two detectors, one of which is used as the reference for the carrier gas and the other which monitors the thermal conductivity of the carrier gas and sample

mixture. Carrier gases such as helium and hydrogen has very high thermal conductivities so the addition of even a small amount of sample is readily detected.

The advantages of TCDs are the ease and simplicity of use, the devices' broad application to inorganic and organic compounds, and the ability of the analyte to be collected after separation and detection. It is also called a universal detector. The greatest drawback of the TCD is the low sensitivity of the instrument in relation to other detection methods, in addition to flow rate and concentration dependency.



Schematic of thermal conductivity detection cell.

Design

- based on electronic circuit known as a *Wheatstone bridge*.
- circuit consists of an arrangement of four resistors with a fixed current applied to them.
- thermal conductivity changes with presence of other components in the mobile phase.
- the voltage between points (+) and (-) will be zero as long as the resistances in the different arms of the circuit are properly balanced
- one resistor in contact with mobile phase leaving column
- another in contact with reference stream of pure mobile phase

As solute emerge from column:

change in thermal conductivity → change in amount of heat removed from resistor → change in resistor's temperature and resistance → change in voltage difference between points (+) and (-).

Considerations

- mobile phase must have very different thermal conductivity then solutes being separated.
- most compounds separated in GC have thermal conductivity of about $1\text{-}4X10^{-5}$.
- H_2 and He are carrier gases with significantly different thermal conductivity values.

- H_2 reacts with metal oxides present on the resistors, so not used

Advantages:

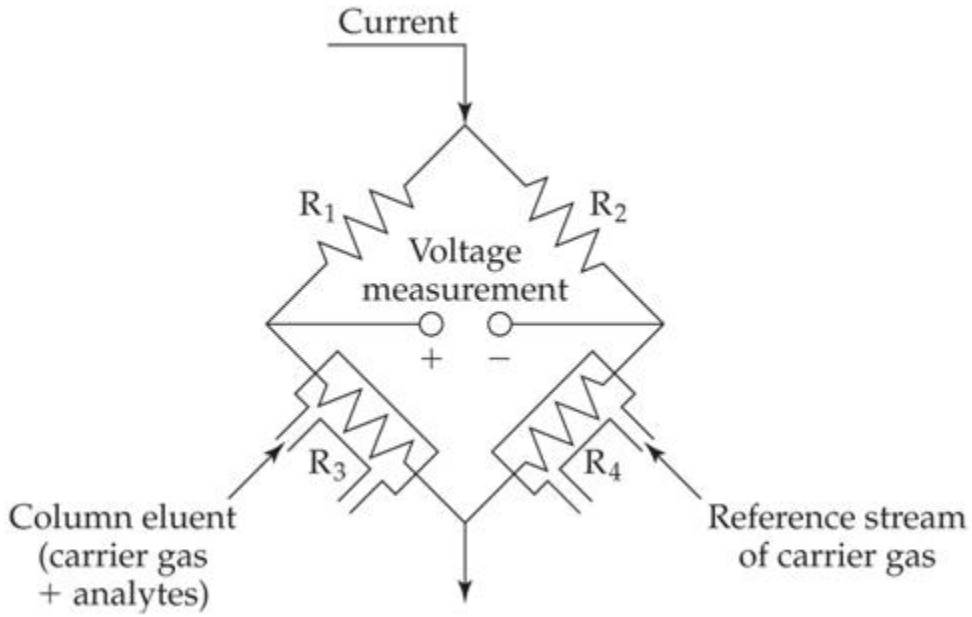
- truly universal detector
  , applicable to the detection of any compound in GC
- non-destructive , useful for detecting compounds from preparative-scale columns, useful in combination with other types of GC detectors

Disadvantage:

- detect mobile phase impurities
- sensitive to changes in flow-rates
- limit of detection, ~ 10^{-7} M , much higher then other GC detectors

- TCD can be used for organic and inorganic analytes.
- The key aspect is the ability of the carrier gas and the analytes to change the conductivity of a wire filament, which will vary with different analytes.
- The carrier gas should have different thermal conductivity of analytes.
- TCD is a non-destructive type of detection that uses a Wheatstone bridge style. Downsides are the response to impurities, leakage in air, and poor response to LOD.

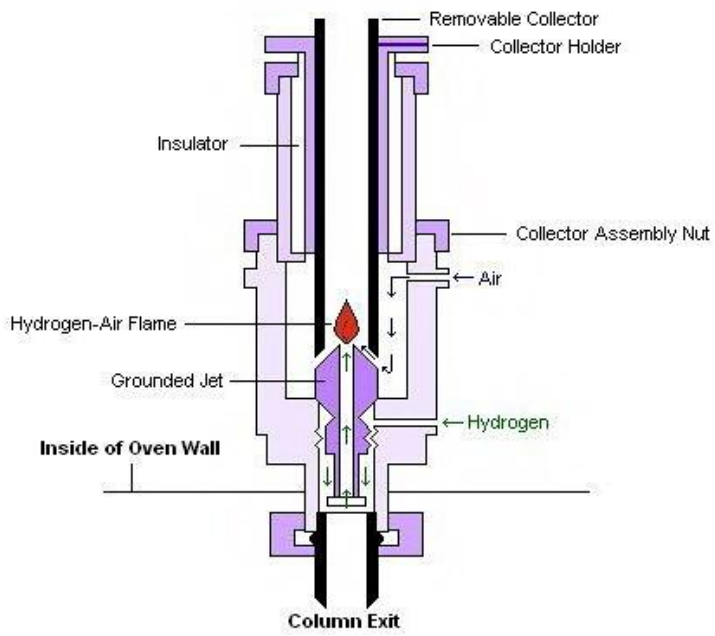
Wheatstone Bridge TCD



Ans. 3(b) Flame Ionization Detector

Flame ionization detectors (FID) are the most generally applicable and most widely used detectors. In a FID, the sample is directed at an air-hydrogen flame after exiting the column. At the high temperature of the air-hydrogen flame, the sample undergoes pyrolysis, or chemical decomposition through intense heating. Pyrolized hydrocarbons release ions and electrons that carry current. A high-impedance picoammeter measures this current to monitor the sample's elution.

It is advantageous to used FID because the detector is unaffected by flow rate, noncombustible gases and water. These properties allow FID high sensitivity and low noise. The unit is both reliable and relatively easy to use. However, this technique does require flammable gas and also destroys the sample.



Schematic of a typical flame ionization detector.

The most commonly used detector is the flame ionization detector (FID) it is a general carbon detector. It does not detect compounds that do not contain carbon such as nitrogen (N_2), oxygen(O_2), or water. The presence of N, O, or S in a carbon compound will tend to decrease the response of the FID.

The Carbon atoms (C-C bonds) are burned in a hydrogen flame. The hydrogen can be supplied either from a cylinder or from an electrolytic hydrogen generator. The hydrogen must be pure to

avoid background noise. A charcoal filter is often placed in the hydrogen supply line to remove any organic contaminants.

The response of the detector depends on the flow of the hydrogen, air and the makeup gas (if it is used). A certain amount of inert gas is needed for optimum response of the detector. Generally the flow from a capillary is too low so a makeup gas is used to provided the inert gas flow. The makeup gas has other beneficial effects such as stabilizing the detector, prolonging the lifetime of the jet, and purging any unswept areas of the detector. It is also very important to adjust the air and hydrogen gas flows for optimum response.

The FID must be heated. There are two main reasons for this. First, the burning of hydrogen in air produces water, which can reduce the detector response and even put out the flame. The second reason for heating the detector is to avoid condensation and deposition of compounds in the detector.

The detector response depends on the ionization of carbon atoms. Only a small portion are actually ionized (about 1 in 10,000), but since there is such a low background signal with the FID, this is enough. The ions carry a charge from the flame to the walls of the detector which surrounds the flame. The charge is electronically amplified and sent to a recording device. The FID is very sensitive down to 10^{-12} g. It has a high linear dynamic range 10^{7} and is very robust and reliable.

Advantages:

- universal detector for organics, doesn't respond to common inorganic compounds
- mobile phase impurities not detected
- carrier gases not detected
- limit of detection: FID is 1000x better than TCD
- linear and dynamic range better than TCD

Disadvantage:

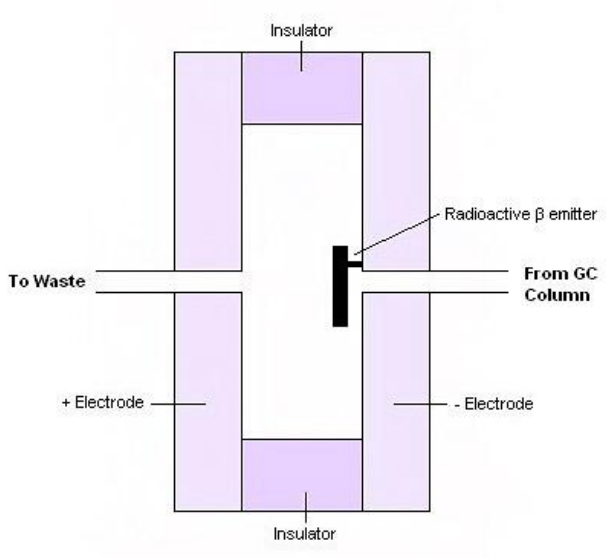
- destructive detector

Ans. 3(c) Electron Capture Detector

Electron-capture detectors (ECD) are highly selective detectors commonly used for detecting environmental samples as the device selectively detects organic compounds with moieties such as halogens, peroxides, quinones and nitro groups and gives little to no response for all other compounds. Therefore, this method is best suited in applications where traces quantities of chemicals such as pesticides are to be detected and other chromatographic methods are unfeasible.

The simplest form of ECD involves gaseous electrons from a radioactive emitter in an electric field, which typically consists of nickle-63 or tritium. The electrons from the emitter ionize the nitrogen carrier gas and cause it to release a burst of electrons. In the absence of organic compounds, a constant standing current is maintained between two electrodes. With the addition of organic compounds with electronegative functional groups, the current decreases significantly as the functional groups capture the electrons.

The advantages of ECDs are the high selectivity and sensitivity towards certain organic species with electronegative functional groups. However, the detector has a limited signal range and is potentially dangerous owing to its radioactivity. In addition, the signal-to-noise ratio is limited by radioactive decay and the presence of O2 within the detector.



Schematic of an electron-capture detector.

It is an excellent detector for molecules containing an electronegative group such as Cl or F etc. (or derivitized molecules) It is probably the second most common detector after the FID. It is

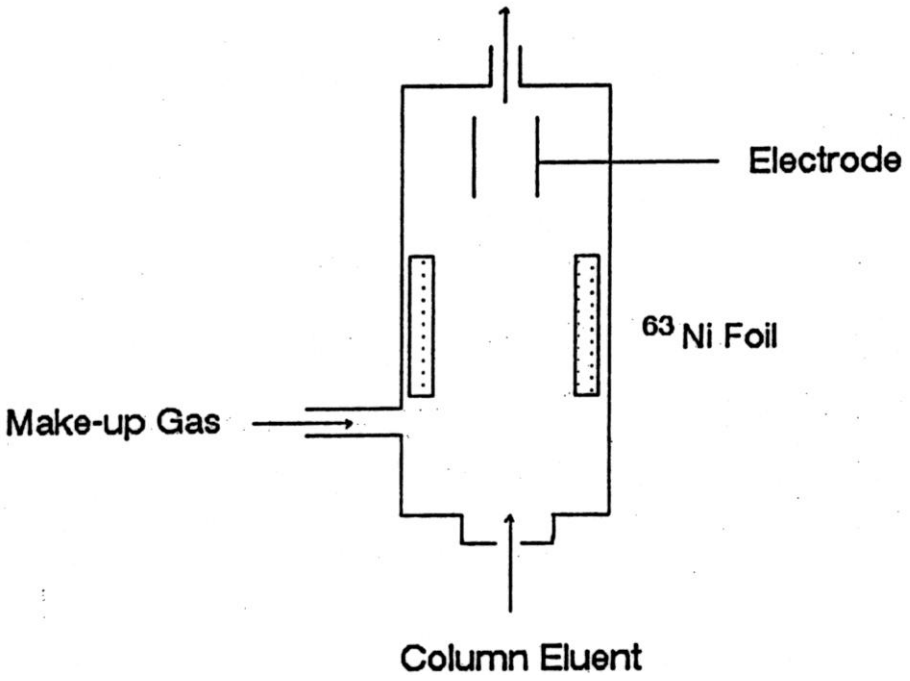
most often used for the trace measurement of halogen compounds in environmental applications for detecting insecticide and herbicide residues.
The ECD uses a radioactive source such as Ni^{63} which produces Beta particles which react with the carrier gas producing free electrons. These electrons flow to the anode producing an electrical signal . When electrophillic molecules are present, they capture the free electrons, lowering the signal. The amount of lowering is proportional to the amount of analyte present. It is sensitive down to 10^{-15} but the dynamic range is only about 10^{4}.

Electron Capture Detector (ECD)

- radiation-based detector
- selective for compounds containing electronegative atoms, such as halogens

Process

- based on the capture of electrons by electronegative atoms in a molecule
- electrons are produced by ionization of the carrier gas with a radioactive s ource, ^{3}H or ^{63}Ni
- in absence of solute, steady stream of these electrons is produced
- electrons go to collector electrode where they produce a current
- compounds with electronegative atoms capture electrons, reducing current



Advantages:

- useful for environmental testing
  - , detection of chlorinated pesticides or herbicides
  - , detection of polynuclear aromatic carcinogens
  - , detection of organometallic compounds
- selective for halogen- (I, Br, Cl, F), nitro-, and sulfur-containing compounds

- detects polynuclear aromatic compounds, anhydrides and conjugated carbonyl compounds

4 (a)

Ion-pair chromatography - form of chromatography in which ions in solution can be "paired" or neutralized and separated as an ion pair on a reversed-phase column. Ion-pairing agents are usually ionic compounds that contain a hydrocarbon chain that imparts a certain hydrophobicity so that the ion pair can be retained on a reversed-phase column. Ion-pairing can also occur in normal-phase chromatography when one part of the pair is loaded onto a sorbent, but this technique is not as popular as the RPC technique.

IPC is a more general and applicable approach that allows the separation of complex mixtures of polar and ionic molecules. The selectivity is determined by the mobile phase: the organic eluent is supplemented with a specific ion-pairing reagent. The IPC reagents are large ionic molecules having a charge opposite to the analyte of interest, as well as a hydrophobic region to interact with the stationary phase. The counter-ion combines with the ions of the eluent, becoming ion pairs in the stationary phase. This results in different retention, thus facilitating separation of analytes. IPC is now an established and reliable technique which provides:

- Reduced separation times
- Highly reproducible results
- Sharper peak shapes
- Simultaneous separation of ionized and non-ionized analytes in one run
- Wide choice of additives to improve separation.

Alkyl sulfonates are a good first choice for basic solutes, whereas quaternary amines are useful for the acidic ones. Halogenated IPC reagents are only suitable for isocratic applications and should not be used in gradient systems.

Ion pair chromatography (IPC) is one technique used to separate charged substances. It is widely used to selectively analyze acids and bases, particularly with reverse phase chromatography.

IPC reagents suitable for cation separation sorted by carbon chain length.

Compound	**Carbon length**
1,2-Ethanedisulfonic acid disodium salt	C2
2-Propanesulfonic acid sodium salt monohydrate	C3
1-Butanesulfonic acid sodium salt	C4
1-Pentanesulfonic acid sodium salt monohydrate	C5
1-Hexanesulfonic acid sodium salt monohydrate	C6
1-Heptanesulfonic acid sodium salt monohydrate	C7
Octyl sulfate sodium salt	C8
Sodium decyl sulfate	C10
Sodium dodecyl sulfate	C12

1-Hexadecanesulfonic acid sodium salt	C16
Sodium 1-octadecanesulfonate	C18

IPC reagents suitable for anionic separation sorted by carbon chain length

Compound	**Carbon length**
Tetramethylammonium sulfate	C1
Dodecyltrimethylammonium hydrogensulfate	C12
Tetradecyltrimethylammonium bromide	C14
Hexadecyltrimethylammonium bromide	C16

Alkyl sulfonate is a typical pair ion used in IPC of substances with a positive charge. Normally, alkyl sulfonate with 5 to 12 carbons is used as a sodium salt. In general, the separation mechanism used in reverse phase ion pair chromatography is explained as consisting of two processes - an ion pair distribution process, which pairs the target components with pair ions and captures them in a solid phase, and an ion exchange process, which retains the target components by ionic interaction with the pair ions hydrophobically adsorbed to the solid phase. However, in the case of alkyl sulfonate, the main mechanism is an ion exchange process. Therefore, the higher the number of carbons in the alkyl sulfonate, the stronger the retention effect on the component. In addition, for the same type of alkyl sulfonate, the lower the concentration of organic solvent in the mobile phase, the stronger the retention. (If an alkyl sulfonate with a large number carbons is used with an extremely low concentration of organic solvent, a pseudo-ion exchange mode occurs, where once equilibrium is reached, target components can be retained without adding pair ions to the mobile phase.) The pair ion concentration affects component retention as well, but surfactants like alkyl sulfonates exhibit a unique relationship between concentration and retention behavior. This relationship is shown in Figure 1. In areas where the concentration is relatively low, component retention increases in a linear manner, but once it becomes saturated at a given concentration (referred to as the "fold over point"), the retention level reverses direction and begins decreasing. This is explained by the alkyl sulfonate forming micelles, resulting in a secondary hydrophobic phase within the mobile phase. Therefore, there is a limited range of pair ion concentration that can be used for IPC.

4 (b) Electrophoresis is a separation technique that is based on the differential migration of charged compounds in a semi-conductive medium under the influence of an electric field.

Capillary electrophoresis

General aspects

Capillary electrophoresis has numerous applications and provides the advantage of high resolution, speed, ease of use, automation and low cost. CE can be applied to a wide range of compounds, ranging from small ions to macromolecules. Numerous CE methods have been developed for pharmaceutical, biological, phytochemical and environmental applications. The quantitative potential of this technique, both in terms of accuracy and robustness, has also been demonstrated.

Instrumentation

The instrumentation needed to perform capillary electrophoresis is very simple. Schematically, CE is composed of a silica capillary with both extremities resting in tanks filled with a buffer solution. Capillaries are usually 30-100 cm long and have an internal diameter of 50 or 75 ìm. An electrical field of up to 30 kV may be applied through electrodes immersed in the electrolyte solution in the two tanks.

The electrolyte solution is generally made up of an aqueous buffer with precise pH and ionic strength. It may also contain specific additives (e.g. cyclodextrins, surfactants, organic modifiers) that enhance the separation of molecules. Prior to use, the electrolyte solution is thoroughly filtered and degassed. In order to avoid Joule heating, the current and the voltage are rigorously controlled and electrophoresis takes place in a thermostatic chamber.

The different compounds separated by electrophoresis are generally detected during the run by a UV-visible absorbance detector or a fluorometer positioned at the cathode end of the capillary. A recent, commercially available system makes it possible to couple CE to a mass spectrometer (MS) in real time. Besides adding sensitivity and selectivity, MS also provides structural information about the separated molecules. This coupled technique can thus be used to analyze pharmaceutically active compounds and their metabolites in any biological fluid (e.g. plasma, urine, saliva). The coupling of the two techniques requires the addition of a specific solution to the effluent from the capillary. The mixture is then vaporized, ionized and finally introduced into the mass spectrometer.

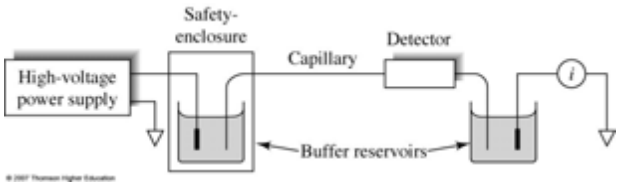
There are two main methods to introduce the sample into the capillary: hydrodynamic injection (by pressure, aspiration or siphoning) and electrokinetic injection. The latter method is particularly useful in the detection of pharmaceutical product present in the ppb range. The injection volumes may be as low as a nanoliter and a few microliters of the sample are sufficient to carry out the entire analysis. Because of such small volumes, very expensive and even exotic additives can be used in developing new electrophoresis methods.

In electrophoresis, strong electrical fields allow very rapid separations. Another advantage of capillary electrophoresis is its capacity to generate 400,000 to 1 million theoretical plates. The efficiency increases with higher voltages. As a rule, macromolecules that normally have lower diffusion coefficients than small molecules yield higher efficiencies.

It can be shown that when the CE injection and detection volumes are very small, the widening of the peaks is essentially caused by axial diffusion or Joule heating generated by the electrical field. In other words, a faster migration of the chemical species within the capillary yields finer peaks.

As a diagnostic technique, electrophoresis relies on the difference in the mobilities of two distinct substances subjected to an electrical field. It is thus complementary to liquid chromatography and plays an important role among the different analytical techniques currently available.

Capillary Electrophoresis Instrumentation
• Capillary
– Fused silica
– Diameter: 20 to 100μm
• Length 10 -100 cm
• Typical voltages: 10-30 kV
• Current 300μA
• Temperature control to control EOF and Joule heating
• Buffer (10 – 100 mM): pH and conductivity control
• Detection: UV, fluorescence, refractive index, etc.



Instrumentation for Gel Electrophoresis
• Power Supply
– 200-500 V
– 400 μA -100 mA
• Electrophoresis Chamber with Buffer Reservoir
• Electrodes in buffer reservoir
• Mini Gel 8 cm x 8 cm
• Larger gel 40 x 20 cm

Gel Media
• Agarose
– 1g in 50 ml (2%)
– Dissolve, heat, cool, pour in casting stand
– Large pore size (e.g. 150 nm for 1%): no sieving
– Charged surfaces: EOF present

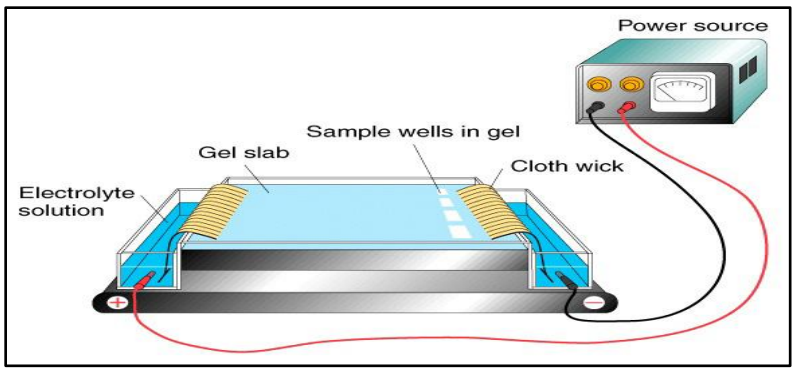
Polyacrylamide Gel
• Polyacrylamide
– Copolymerization of acrylamide and N,N'-methylene-bisacrylamide
– Initiator: TEMED (*N*,*N*,*N*′,*N*′-Tetramethylethylenediamine) and Ammonium

Persulfate($(NH_4)_2S_2O_8$)
– Sieving effect present: pore size depends on total gel concentration (%T: 5 -20%) and degree of cross linking
– SDS for denaturinf and coating proteins
– Mercaptoethanol for reducing dissulfide bonds

Sample Preparation
• Buffer:
– Tris (pH 8), Tris-glycine (pH 9.1)
– 50-100 mM
• Amount of sample: μg
• Sample volume: depends on size of well (μL to mL)
• Denaturing agent: SDS
• β-mercaptomethanol: to reduce disulfide Bonds

Visualization and detection
• Staining
– Coomasie brillant blue (alchoholic solution of dye)
– Silver nitrate
• Fluorescent reagents
– Ethidium Bromide for DNA

SDS PAGE
• Proteins are totally denatured and coated SDS charge (negative): rod-like shaped
• Charge on protein depend on its size: constant net charge per unit mass: same electrophoretic mobility
• Separation based on size/ molecular weight
• Sample preparation: heating (90) in the presence of SDS and β-mercaptoethanol
• Used to determine MW of proteins
• Molecular weight standards used



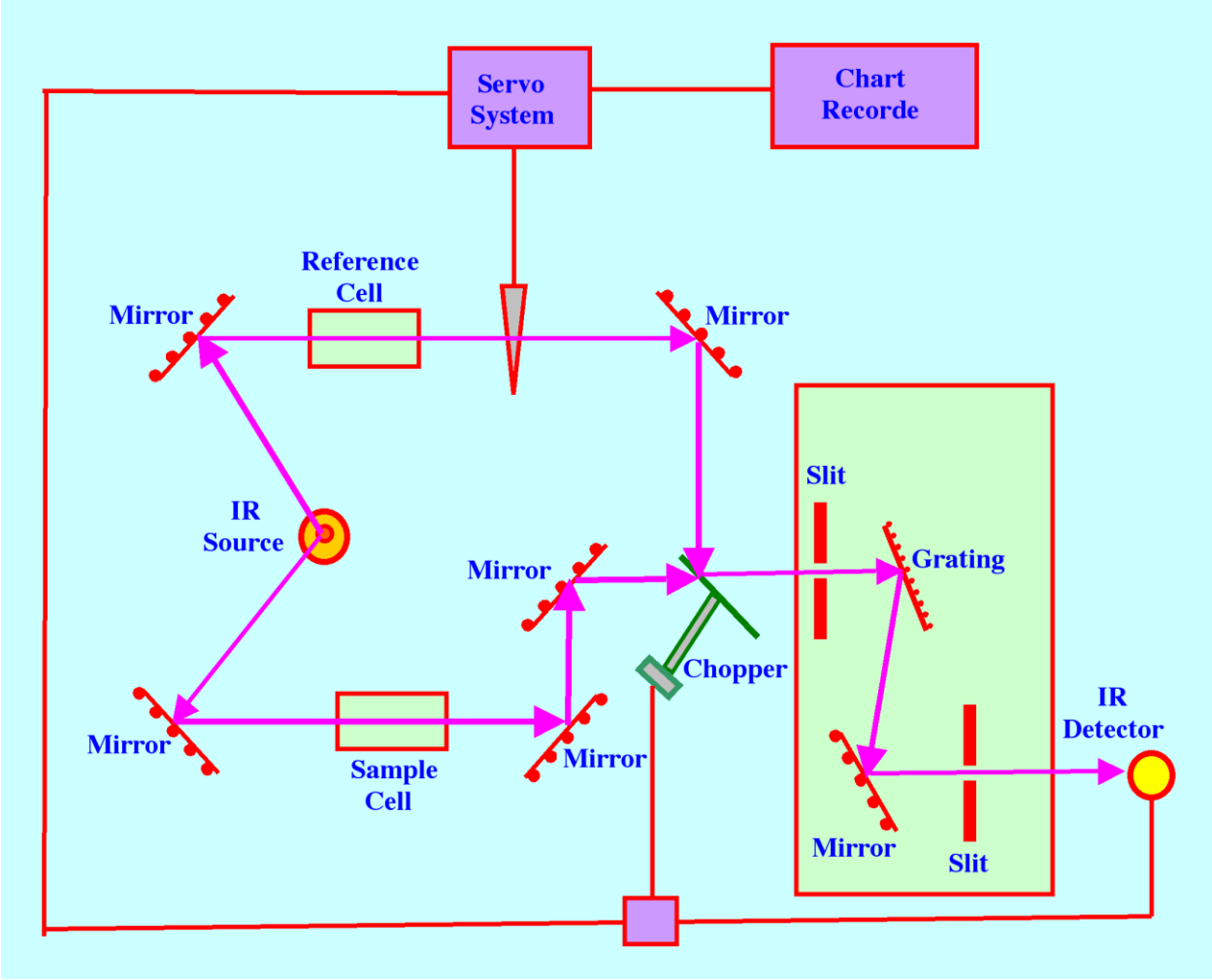
5

(a)

Simple Grating IR spectrometer

Light from the IR source is reflected from a mirror, through the reference cell, through an attenuator system and onto a second mirror, By imposing a simple device in the path of the reference light beam that removes a continuous. but controllable, fraction of the light allows the reference beam to be attenuated. The attenuator can take the form of a comb, the teeth of which are cut so that the amount of light attenuated is linearly related to the lateral movement of the comb through the beam. After passing through the attenuator to the second mirror, the attenuated light passes through a chopper. The chopper has reflecting and transmitting surfaces and, thus, alternately allows light to pass from the reference cell to the grating and then light from the sample cell to the grating. At the grating light of a specific wavelength is selected to pass to the IR detector.

As a consequence, the chopper arranges for the sensor to alternately receive light that has been transmitted through the cell, and light that has passed through the attenuator. A servomechanism controls the attenuator that is actuated by the output from the sensor and adjusts the light transmission until both beams have the same intensity. The amount of light that is absorbed is indicated by the position of the attenuator. In other words, the amount of light, of a particular wavelength, which is absorbed by the sample, is measured by attenuating the reference beam until its intensity is equivalent to that of the beam transmitted through the sample. The resolution is controlled by the width of the slit, which is adjustable. In the older versions of this type of IR spectrometer, an analogue plotter, mechanically associated with the attenuator, recorded the Spectrum. More modern techniques acquire the signals from the attenuator and grating position by a compute and produce a Spectrum relating absorbance to wavelength (frequency, or wave number).



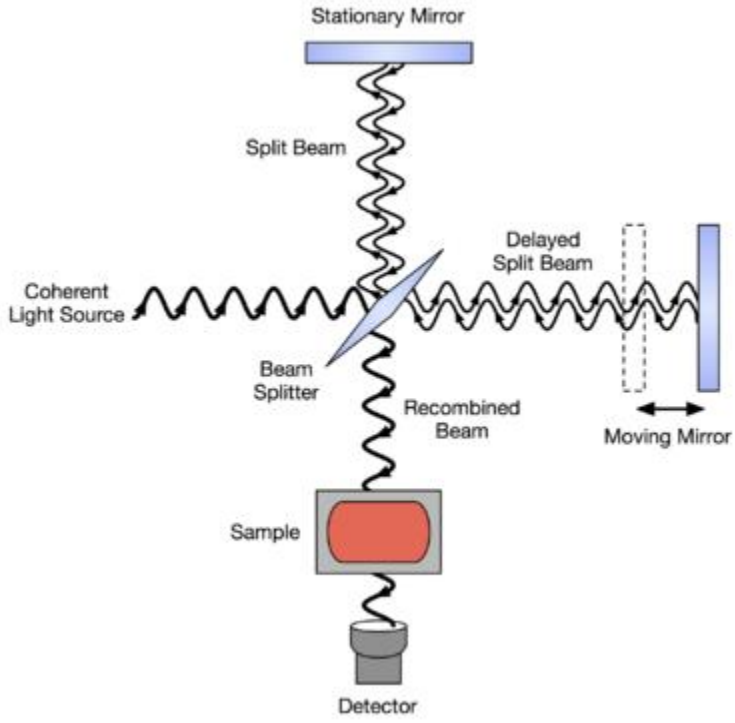
Michelson interferometer

In a Michelson interferometer adapted for FTIR, light from the polychromatic infrared source, approximately a black-body radiator, is collimated and directed to a beam splitter. Ideally 50% of the light is refracted towards the fixed mirror and 50% is transmitted towards the moving mirror. Light is reflected from the two mirrors back to the beam splitter and (ideally) 50% of the original light passes into the sample compartment. There, the light is focused on the sample. On leaving the sample compartment the light is refocused on to the detector. The difference in optical path length between the two arms to the interferometer is known as the retardation. An interferogram is obtained by varying the retardation and recording the signal from the detector for various values of the retardation. The form of the interferogram when no sample is present depends on factors such as the variation of source intensity and splitter efficiency with wavelength. This results in a maximum at zero retardation, when there is constructive interference at all wavelengths, followed by series of "wiggles". The position of zero retardation is determined accurately by finding the point of maximum intensity in the interferogram. When a sample is present the background interferogram is modulated by the presence of absorption bands in the sample.

There are two principal advantages for an FT spectrometer compared to a scanning (dispersive) spectrometer.

1. The multiplex or Fellgett's advantage. This arises from the fact that information from all wavelengths is collected simultaneously. It results in a higher Signal-to-noise for a given scan-time or a shorter scan-time for a given resolution.
2. The throughput or Jacquinot's advantage. This results from the fact that, in a dispersive instrument, the monochromator has entrance and exit slits which restrict the amount of light that passes through it. The interferometer throughput is determined only by the diameter of the collimated beam coming from the source.

Other minor advantages include less sensitivity to stray light, and "Connes' advantage" (better wavelength accuracy), while a disadvantage is that FTIR cannot use the advanced electronic filtering techniques that often makes its signal-to-noise ratio inferior to that of dispersive measurements.



Schematic diagram of a Michelson interferometer, configured for FTIR

(b)

Beer's law relates the absorption to the concentration of the absorbing solute, and Lambert's law relates the total absorption to the optical path length.

Lambert's law states that when monochromatic light passes through a transparent medium, the rate of decrease in intensity with the thickness of the medium is proportional to the intensity of

the light. This is equivalent to the stating that the intensity of the emitted light decreases exponentially as the thickness of the absorbing medium increases arithmetically.
The law may be expressed by the differential equation
$-dI/dl = k\,I$
where I is the intensity of the incident light of wavelength λ,
l is the thickness of the medium and
k is proportionality factor.
I = Io when l = 0, then
$\ln I_o/I_t = kl$
or
$I_t = I_o e^{-kl}$
Io is the intensity of the incident light falling upon an absorbing medium of thickness l, It is the intensity of the transmitted light, and k is constant for the the wavelength and the absorbing medium used.
By changing to the common logarithm,
$I_t = I_o \times 10^{-Kl}$

$I_t / I_o = 10^{-Kl}$
The ratio I_t / I_o is the fraction of the incident light transmitted by a thickness l of the medium and is called the transmittance T. and the absorbance A of the medium
$A = \log I_o / I_t$
Beer's law states intensity of a beam of monochromatic light decreases exponentially as the concentration of the absorbing substance increases arithmetically.
$I_t = I_o e^{-k'c}$
$I_t = I_o \times 10^{-K'c}$
c is concentration and k' and K' are constant.
$I_t = I_o \times 10^{-acl}$
or
$\log (I_o/I) = acl$
this is the fundamental equation and is known as Beer-Lambert law.

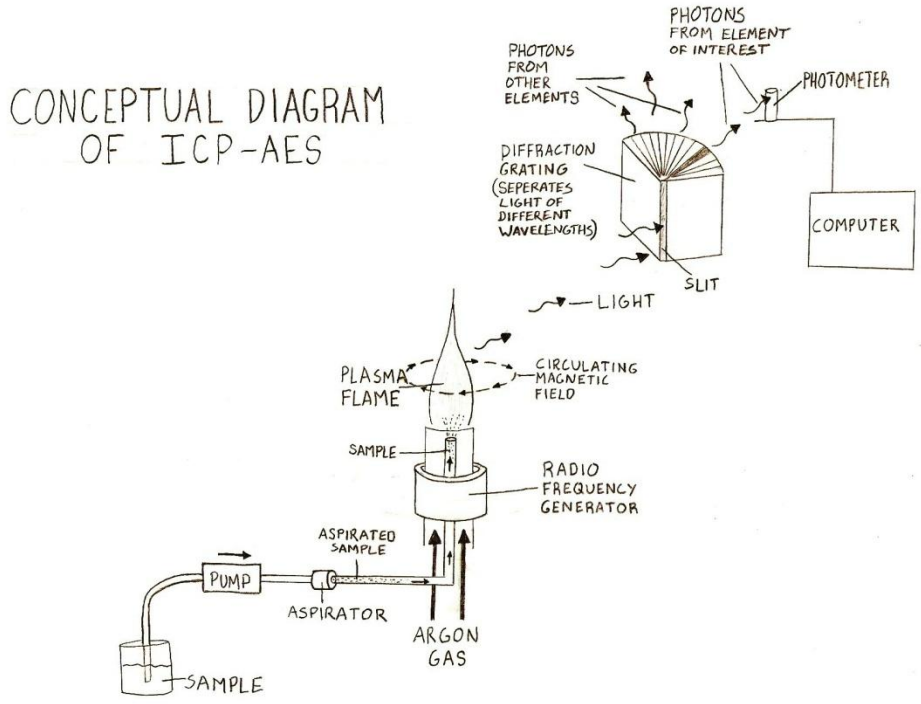
6
(a)

In Plasma Emission Spectroscopy, the exciting medium/ agent is plasma. The term `Plasma' in Physics means a very hot/ high temperature ionised gas, sometimes described as the fourth state of matter (the other three being solid, liquid and gas). In this method of sample analysis, a `Plasma Source' is the basic component. (Plasma Emission Spectroscopy is similar to Optical Emission Spectroscopy in principles but differs in mechanism. In OES, the sample excitation is

done by electric arc or spark mechanism in the temperature range of 3000-5000 degrees Celcius. In PES, the temperature range is 7000-9000^{o}C.

The sample solution or the analyte solution is supplied to the `Plasma Source' by a `Nebulizer' assembly. On reaching the high temperature (7000-9000 degrres Celsius) plasma, the solution attains this high temperature and all types of molecular bonds break down. Free atoms or ions (electrically charged atoms) are produced, which emit their characteristic spectra. A `Monochromator' assembly is in position after `Plasma Source' for isolation of the desired analyte line (or spectrum). The intensity of an analyte line is directly proportional to the concentration of that particular analyte (element being analysed) in the sample solution. The `detector & read-out' assembly gives us the concentration of the particular element in the sample. Modern instruments being computer controlled, the analysis is very fast and needs little operator skill. (Depending on the technique of production, plasma may be of three classes — viz., D.C. argon plasma, Microwave plasma and Inductively coupled plasma (ICP). The ICP is the most widely used due to mechanical simplicity and operational advantages.

Therefore, plasma Emission Spectroscopy is the method of analysis of various elements in a sample, by passing the sample/ analyte solution through a plasma (high temperature ionised gas) source, thereby exciting the outer orbital electrons of the analyte with simultaneous emission of electromagnetic radiations (in the form of lights), which are analysed by means of a spectrograph, which separates various wavelengths of analytes in the sample.



(b)

In emission spectroscopy, a sample is normally excited by thermal energy. Atoms in the sample absorb thermal energy causing outer orbital electrons to become excited from their ground state to higher orbital energy level. After a short lifetime (10{+-}{+6} to 10{+-}{+9}s), the excited electrons return to the ground state. Simultaneously, electromagnetic radiations (normally in the form of light in the UV-visible region) are emitted. The emitted radiations are analysed by means of spectrograph, which separates various wavelengths.

In Plasma Emission Spectroscopy, the exciting medium/ agent is plasma. The term `Plasma' in Physics means a very hot/ high temperature ionised gas, sometimes described as the fourth state of matter (the other three being solid, liquid and gas). In this method of sample analysis, a `Plasma Source' is the basic component. (Plasma Emission Spectroscopy is similar to Optical Emission Spectroscopy in principles but differs in mechanism. In OES, the sample excitation is done by electric arc or spark mechanism in the temperature range of 3000-5000 degrees Celcius. In PES, the temperature range is 7000-9000 {+o}C.

Inductively coupled plasma atomic emission spectrometry is a method for determining the elemental constituents of a sample.

This instrument embodies a few very cool physical principles on electromagnetism; plasma; and atomic emission. The most overtly impressive feature of the ICP is the plasma flame, which has a temperature of up to 10,000 degrees Kelvin (Kelvin units are the same as Celsius except 0°K is -273°C). This is hotter than the surface of the sun, which is less than 6,000 K.

In short, the process of determining the elements in an aqueous solution , The solution is injected into the machine and aspirated—broken apart into an aerosol of fine particles—into a plasma flame. The elements in the solution, when exposed to the high energy of the plasma, are excited and emit light. Each element emits light of a wavelength (color) specific to that element. A photometer counts the number of photons of whichever wavelength you prescribe and gives you a value, which is used to determine how much of that element is present in the solution.

Plasma is the state of having 1% or more of the electrons in the substance disassociated from any atoms. This is an extremely high energy state as the electrons are free to wreak havoc on any molecules that contact the plasma.

Induction of the plasma: Argon gas flows through a vertical tube—the flame will be formed from the gas at the top of the tube. Radio waves are generated in rings around the tube. A radio wave is, like light, an electromagnetic wave. The electromagnetic field extends beyond the rings. A spark knocks a few electrons off of the argon gas atoms. Electrons are negatively charged, so they get caught in the electromagnetic loop circulating around the gas tube. These electrons smash into atoms of the gas knocking more electrons loose until more than one percent of all the electrons are zipping around smashing into atoms. The result is a blue flame between 5,000° and 10,000° K—blue because that is the color emitted by excited argon atoms.

When atoms are excited, their electrons jump to a higher energy state. This is an unstable state and the electrons always drop back down to their ground state. When they drop down, the excess energy is emitted as light. The wavelength, or frequency, of light emitted is proportional

to the difference in energy between the excited state and the ground state. The different elements all have different structures and the degrees to which the nucleus pulls the electrons inwards from the outer levels varies. Therefore for each element the electrons will be excited to different degrees when exposed to the argon plasma. So each element will release a signature wavelength.

7

(a) Autoradiography

autoradiography - producing a radiograph by means of the radiation emitted from the specimen being photographed

au·to·ra·di·o·graph
An image recorded on a photographic film or plate produced by the radiation emitted from a specimen, such as a section of tissue, that has been treated or injected with a radioactively labeled isotope or that has absorbed or ingested such an isotope. Also called *autoradiogram*.

History

The first autoradiography was obtained accidently around 1867 when a blackening was produced on emulsions of silver chloride and iodide by uranium salts. Such studies and the work of the Curies in 1898 demonstrated autoradiography before, and contributed directly to, the discovery of radioactivity. The development of autoradiography as a biological technique really started to happen after World war II with the development of photographic emulsions and then stripping film made of silver halide. Radioactivity is now no longer the property of a few rare elements of minor biological interest (such as radium, thorium or uranium) as now any biological compound can be labelled with radioactive isotopes opening up many possibilities in the study of living systems.

Radioisotopes,

The mass of the atomic nuclei can vary slightly (=isotopes) for a particular element although the number of electrons remains constant and all the isotopes have the same chemical properties. The nuclei of radioactive isotopes are unstable and they disintegrate to produce new atoms and, at the same time, give off radiations such as electrons (beta rays) or radiations (gamma rays). Naturally occurring radioisotopes are rare because of their instability, but radioactive atom can be produced in nuclear reactors by bombardment of stable atoms with high-energy particles. The disintegrations can be detected in 3 ways. These detection methods are extremely sensitive and every radioactive atom that disintegrates can be detected.

Detection

(i) *Electrical:* This depends on the production of ion pairs by the emitted radiation to give an electrical signal that can be amplified and registered: used in Geiger counter, ionisation counter and gas flow counter

(ii) *Scintillation:* Some materials have the property of absorbing energy from the radiation and re-emitting this in the form of visible light. In a *scintillation counter* these small flashes of light are converted into electrical impulses. Both of these techniques count the pulses of the disintegrating atoms. They are fast and quantitative.

(iii) Autoradiography differs from the pulse-counting techniques in several ways. Each crystal of silver halide in the photographic emulsion is an independent detector, insulated from the rest of the emulsion by a capsule of gelatin. Each crystal responds to the charged particle by the formation of a latent (hidden) image that is made permanent by the process of development. The record provided by the photographic emulsion is cumulative and spatially accurate. It provides information on the localisation and distribution of radioactivity within a sample (i & ii do not do this). Thus there is little point on doing autoradiography on a specimen that is homogeneously labelled. Although it can be quantitative, autoradiography is a much slower and more difficult approach.

Nuclear emulsions have a very high efficiency for b particles (electrons of nuclear origin), particularly those with low energies. Many of the isotopes of interest to biologists have suitable isotopes, e.g. tritium (= hydrogen-3), carbon-14, , sulphur-35 and iodine-125. The effective volume of the detector emulsion in the immediate vicinity of the source may be as little as 100 cubic microns.

Autoradiography Method

- Living cells are briefly exposed to a ‘pulse’ of a specific radioactive compound.
- The tissue is left for a variable time.
- Samples are taken, fixed, and processed for light or electron microscopy.
- Sections are cut and overlaid with a thin film of photographic emulsion.
- Left in the dark for days or weeks (while the radioisotope decays). This exposure time depends on the activity of the isotope, the temperature and the background radiation (this will produce with time a contaminating increase in ‘background’ silver grains in the film).
- The photographic emulsion is developed (as for conventional photography).
- Counterstaining e.g. with toluidine blue, shows the histological details of the tissue. The staining must be able to penetrate, but not have an adverse affect on the emulsion.
- Alternatively, pre-staining of the entire block of tissue can be done (e.g. with Osmium on plastic sections coated with stripping film [or dipping emulsion] as in papers by McGeachie and Grounds) before exposure to the photographic emulsion. This avoids the need for individually (post-) staining each slide.
- It is not necessary to coverslip these slides
- The position of the silver grains in the sample is observed by light or electron microscopy Note: the grains are in a different plane of focus in the emulsion overlying the tissue

section. Often oil with a x100 objective is used for detailed observation with the light microscope.
- These autoradiographs provide a permanent record.
- Full details on the batch of emulsion used, dates, exposure time and conditions should be kept for each experiment.

Types of photograhic detection systems

Stripping film consists of an **even layer** of photographic emulsion on a supporting gelatin membrane (e.g. Kodak AR10), it is floated on water and then wrapped around the slide and forms very close contact as it dries (Rogers, Chap 15). This was once widely used but is now no longer made. It has the major advantage of uniform thickness but the disadvantage that the supporting membrane prevents counterstaining of the section and therefore the tissue block must be pre-stained before sections are coated.

Liquid photographic emulsion. This is the method routinely used today (see details below). It is simpler and much quicker to do, but the layer of liquid emulsion (e.g. Kodak NB2) can be **slightly uneven** in thickness as it flows down to the bottom of the slide as it is withdrawn: for most purposes this slight variation is not important, unless the number of grains are being strictly counted and compared across one slide (Rogers, Chap 16).

Method for coating and developing dipping emulsion

Coating the slides

- Wear gloves and work in the darkroom (using only a red safety light)
- Allow Kodak-NB2 emulsion (which comes as a thick white gel and is stored in the dark at 4^{o}C) to come to room temperature for 2 hours.
- Mix equal volumes of Kodak-NB2 and double distilled water together (say 5ml of each), place in a water bath at 37^{o}C and shake gently for about 15 mins.
- Dip slides vertically into a small amount of emulsion (about 2ml is all that is required) in a holder designed to take one slide at a time (economises on the amount of emulsion)
- Place horizontally for about 15 minutes to air dry. Then stand vertically for at least 2 hours to dry.
- Transfer to a black, light free box and store in the fridge (4^{o}C) with dessicant.
- Allow exposure time as specified e.g. 2 weeks. Or remove test slides at various times to determine optimal exposure time for your particular situation.

Developing the film

- Use a dark room.
- Use Kodak D19 developer mixed 50:50 with water. Immerse slides for 4 mins.
- Wash in gently running tap water.
- Wash in double distilled water

- Use Ilford Hypan Rapid Fixer (leaflet T1812). Mix 40ml+160ml double distilled water +2ml Hypan hardener. Immerse slides for 5mins.
- Wash in gently running tap water.
- Note: once it is fixed can turn the lights on and tidy up. But make sure all sensitive film is put away before you do this.

Radioisotopes are used to trace molecules in cells and organisms

Tracer studies: Radioisotope labelling is uniquely valuable as a way to distinguish between molecules that are chemically identical but have different histories - for example those that differ in their time of synthesis. The earliest uses of autoradiography were for tracer studies e.g. radioactivity was used to label various molecules such as amino acids and then the way they were assembled into proteins over time throughout the cell could be followed. This technique was essential to understand:

- oxidative respiration,
- photosynthesis,
- the control of protein synthesis by nucleic acids,
- the timing of events throughout the cell cycle,
- the fate of populations of cells - i.e number of cell divisions, migration, relationship of stem cells to the final differentiated phenotypes (by tritiated thmyidine labelling of replicating cells - see lecture on Cell Replication).
- Comparison of experimental treatments on events such as above

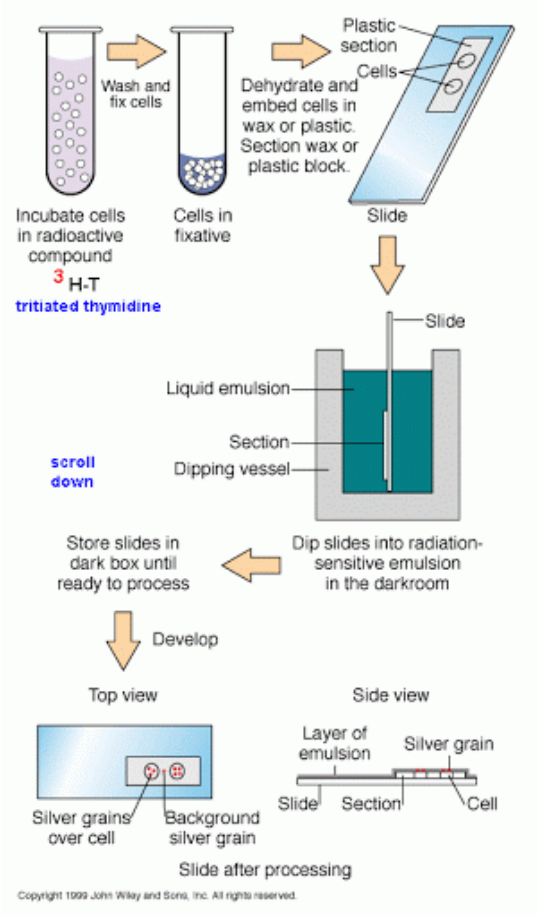
Pulse chase is used to sharpen the resolution of timing in many of these experiments.

Analytical techniques: Radioactive labelling of various molecules enables the binding of these molecules (as markers of other molecules) to be accurately monitored by radioisotope cytochemistry e.g:

- enzyme inhibitors,
- antibodies
- nucleic acid probes

In molecular biology experiments, S^{35}, P^{32} (and I^{125}) are widely used to label nucleic acid probes to detect mRNA by *in situ* hybridisation on tissue sections and also for quantitation by Northern analysis on gels. Radioisotope labelling has great sensitivity but the disadvantage that each time a hybridisation is performed, the probe has to be labelled with fresh radioisotope (since it decays rapidly) and this can be tedious and expensive. Furthermore radioisotopes are dangerous (especially I^{125}). For these reasons digoxygenin is now often favoured for labelling probes for *in situ* hybridisation studies (it is detected by an antibody and a colour reaction), particularly since digoxygenin-labelled probe is stable for many months.

Ingestion: Radioactive isotopes are also used to track the distribution and retention of ingested materials. Exotic radioisotopes with very short half-lives are used clinically.



Two General Types of Experiments:

1)

In-vivo autoradiography - receptors are labeled in intact living tissue by systemic administration of the radioligand (like in PET). Tissue is removed, processed, and visualized.

2)

In-vitro autoradiography - slide-mounted tissue sections are incubated with radioligand so that receptors are labeled under very cont rolled conditions

Autoradiographic film is typically composed of 4 layers.

1- An upper protective coat.

2- A layer of gelatin that contains silver halide (AgBr, AgC l, or AgI) crystals. (The type and proportions of the different silver halides determining the speed of the film.)

3- The film base, usually made from a flexible polymer.

4- An anti-halation backing to prevent light from reflecting back onto the emulsion.

(b) Statistical treatment of data

Statistical treatment of data involves the use of the following parameters.

Mean, arithmetic mean, and average are synonyms.

$$\bar{x} = \frac{\sum_{i=1}^{N} x_i}{N}$$

Median: *is the middle result when replicate data are arranged in order of size.*

Central tendency

When reporting central tendency for a series of measurements, we often use the mean or average value. The symbol for the mean is the variable with a line over the top. The formula for the mean, for example of *x*, is

$$\bar{x} = \frac{1}{N}\sum_{i=1}^{N} x_i$$

where the x_i are the individual measurements and *N* the total number of replicate measurements of a value. The capital sigma indicates that we sum all of the *x* values. The range of values to be summed is from 1 to *N*.

The mean of replicate measurements is a good indication of the central tendency of the measurement value.

Range
The range of a data set is the absolute maximum difference observed in the data. It is calculated as the difference between the maximum, x_{max}, and minimum, x_{min}, values

$$R = \left| x_{max} - x_{min} \right|$$

The vertical bars indicate absolute value, *i.e.*, positive values. For the weight measurement data set, the maximum and minimum values are x_{max}=25.234 g and x_{min}=24.983 g. The range is thus *R* = 25.234-24.983 = 0.251 g. The range is useful for qualitative evaluation of errors.

Measurement variance
The formula for measurement variance, s^2, is

$$s^2 = \frac{\sum_{i=1}^{N} (x_i - \bar{x})^2}{N-1}$$

The symbols are the same as those used in the mean calculation formula. The units of variance are the squared measurement units.

Measurement standard deviation

The measurement standard deviation is more often used to indicate precision or probable error. The greater the standard deviation, the less precise the data. The measurement standard deviation is simply related to the measurement variance through

$$s = \sqrt{s^2}$$

Units of the measurement standard deviation are the same as those of the measurements that it is based on. For the weight measurements used above, the standard deviation is s=0.11038 g.

Relative standard deviation

The RSD is the ratio of the measurement standard deviation to the mean of the quantity being measured

$$RSD = \frac{s}{\bar{x}}$$

Notice that for constant random errors, the RSD decreases with measurement size. RSD are unitless quantities. They are often reported in %, parts-per-thousand (ppth), parts-per-million (ppm), etc., errors. Multiply the RSD by 100 to get the % error; by 10^3 to get the error in parts-per-thousand (ppth); by 10^6 to get the error in parts-per-million (ppm).

Degrees-of-freedom

The sum over the squared-differences in the variance is divided by N-1. The factor "N-1" called the degrees-of-freedom of the calculation. In this case, the degrees-of-freedom is one less than the total number of measurements because the variance calculation is based on all of the x_i replicate measurements *and* the mean. Since the mean is also based on all the replicate measurements, the summation in the measurement variance formula is effectively "double counting" one of the values. In fact, all N replicate measurements could be determined with knowledge of the mean, and only N-1 of the x values. Dividing by the N-1 degrees-of-freedom in the variance formula is a way to account for this double counting.

Keep in mind that the degrees-of-freedom is one less than the number of measurements when doing calculations. You will need this information to get the correct Student's-t value for calculating the in confidence intervals, as discussed below. Degrees-of-freedom are not always one less than the number of measurements. The degrees-of-freedom depend on how many parameters are calculating from the data. The more parameters calculated, the more degrees of freedom we use up. In the case above, only one parameter was calculated from the data.

Confidence intervals

In the real-world where *N* is less than infinity, errors associated with estimates of the "true" mean and standard deviation result in more uncertainty in the confidence interval than is indicated by the Gaussian distribution. In general, the fewer the measurements, the less the confidence level that can be assigned to a particular interval. Similarly, fewer measurements also means larger confidence intervals for a given confidence level.

These concept are quantitatively expressed in the "Student's-*t*" statistic. The Student's-*t* number is an integral over a distribution function similar to the Gaussian. It indicates probabilities that the mean found from a finite number of measurements will differ from the "true" mean by a given amount. A useful form of Student's-*t* formula is

$$\mu = \bar{x} \pm \frac{ts}{\sqrt{N}}$$

where *t* is the Student's-*t* number. One looks up *t* in a table for a given confidence and number of degrees-of-freedom.

Ideally, one would really like to report the "true" mean. But, due to random errors, it is not possible to specify the "true" mean as a single number. Instead, one uses the Student's-*t* formula in the form given above to specify the "true" mean. The "true" mean is reported as the measurement mean, or average, and the confidence interval for that reported value, at a particular confidence level. For example

$$\mu = \bar{x} \pm \frac{ts}{\sqrt{N}} @95\%$$

is the way one reports the "true" mean at the 95% confidence level.

Significance testing

A number of statistical tests are available to check for significant differences between measurement values. Two common tests used in measurement science are the "*Q*-test", for rejecting suspect data points, and the "Student's-*t* test", for determining differences between means.

Q-Test

Very often, when examining the results of a set of measurements, one finds that there is one datum that appears to be different than the others. The question is; should this datum be rejected? If a reason for rejection cannot be found after critical examination of the evidence (hopefully as recorded in a laboratory note book), then one must resort to statistical tests. The *Q*-test is a statistical test used to determine whether or not a suspected datum can be rejected from a data set when the total number of measurements is less than 10.

The *Q*-test is based on the ratio of the interval between the suspect datum and the datum of a value closest to the suspect point, to the range of the data set. The range is the difference between the minimum and maximum data points. The ratio of these differences is the *Q* statistic

$$Q = \frac{Difference}{Range}$$

In performing the test, one "formulates a null hypothesis" and then checks to see if the hypothesis is invalid. (One cannot prove that it is true; it can only be shown to be false) In this case, the null hypothesis is that the *Q* value calculated from a data set including the suspect datum is not statistically different from an extreme *Q* value from a normally behaved data set. If the calculated *Q* value is less than that of the regular data, then the null hypothesis is true. In this case, the datum cannot be rejected based on statistical evidence. If, on the other hand, the calculated *Q* is greater than that for normal data, then the null hypothesis is false. This means that the suspect datum is not from a normal data set and may be rejected. *Q* values for normal data are tabulated according to confidence level and number of measurements. The *Q*-test is outlined below.

Step 1, calculate a *Q* value using

$$Q_{calc} = \frac{\left|x(closest) - x(suspect)\right|}{\left|x_{max} - x_{min}\right|}$$

The suspect datum will be one of the terms in the range calculation since it is suspect because of its extreme value.

Step 2, look up the value of *Q* in the table corresponding to the number of measurements at a confidence level. This value is the extreme *Q* value expected from data with random errors

Step 3: If $Q_{calc} > Q_{table}$, then the suspect value can be rejected. All other statistical quantities such as the mean and the standard deviation are then calculated from the remaining values. If the opposite is true, ($Q_{calc} \leq Q_{table}$) then the suspect datum remains in the data set.

Keep in mind that the rejected datum may be valid (anything is possible in statistics). But, although valid, including this datum would unduly influence calculations of mean and standard deviation. There is thus good reason to apply the *Q*-test when a datum is suspected of being different. Also, use this test only once. There are better tests for rejecting more than one datum. Multiple application of the *Q*-test may lead to serious errors.

Q (rejection quotient) 90% confidence	
N	*Q*
3	0.94

4	0.76
5	0.64
6	0.56
7	0.51
8	0.47
9	0.44
10	0.41

Student's-*t* test

The Student's-*t* statistic is useful for comparing data sets of finite number that have random errors characterized by a Gaussian distribution. It is the correct metric for comparing most real data and can be used for a variety of tests. For example, it may be used to compare measured means obtained in different experiments, or to determine to what confidence level two estimated "true" means are the same. It may also be used to test whether or not a suspect point may be rejected from a data set by using sub-sets. The use of Student's-*t* to test for differences between measured and "true" means will be illustrated here.

As with the *Q* test, Student's-*t* tests require formulation of a null hypothesis. Under the null hypothesis, all data are the same. The data can then be manipulated as a combined set under this assumption. A value for Student's-*t* is calculated from the combined data and compared to table values which are based on normal, Gaussian-distributed data. If the calculated Student's-*t* is statistically different than the table value, then the null hypothesis is false, at a particular confidence level. A false null hypothesis indicates that the two sets of data are different.

A different form of the Student's-*t* formula is needed for the test. Rearranging the formula for reporting the "true" mean with confidence interval

$$t = \left|\mu - \overline{x}\right| \frac{\sqrt{N}}{s}$$

This formula is used to calculate a value for *t* based on a "true" or comparison mean, and the measurement mean, standard deviation, and number of data. The actual "test" is performed using the following steps.

Step 1: Determine a t_{calc} using the above formula with the "true" mean and the measurement statistics using the formula given above.

Step 2: Compare the calculated *t* (t_{calc}) to one from the table of Student's-*t* values (t_{table}) for a particular confidence level, and the degrees-of-freedom of the measurement.

Step 3: Test the null hypothesis by comparing the two *t* values. If the calculated value is greater than the table value ($t_{calc} > t_{table}$), then the null hypothesis is false to within the confidence level of

the table value. In this case, the means are different. That is, the variation from the reported value is greater than you would expect from random error alone, and something is likely wrong with your experiment. Else, ($t_{calc} \leq t_{table}$), the null hypothesis is not shown to be false, and the two means are not different at the chosen confidence level.

(c) 13C-NMR

Introduction to Carbon NMR

The chemical shift of carbons is caused by the same phenomenon as the chemical shift of hydrogens, i.e., the electrons in the molecule generate small magnetic fields that affect the net field experienced by each carbon nucleus. In general, electrons surrounding an atom move in such a way so as to create a field at the atom that tends to counteract the applied magnetic field. The electrons thus "shield" the carbon nucleus from the applied magnetic field and this means that less energy is necessary to excite the carbon nucleus from one spin state to another and therefore its chemical shift comes at a lower frequency than it would otherwise. For example, the carbon atom in a carbonyl group has a relatively low electron density around it, and thus is relatively "deshielded" and consequently has a higher chemical shift than most other types of carbons.

Carbon-12 atoms do not have a nuclear spin, and hence don't show up in the NMR. When we take a carbon NMR we are looking only at carbon-13 atoms. Only 1% of naturally occuring carbon atoms are carbon-13, so the sensitivity of natural abundance carbon NMR is lower than that for proton NMR. Another consequence of this low abundance, is that we don't normally observe coupling between adjacent carbon atoms (like we do between adjacent protons in H-NMR) since 99% of the neighboring carbons are carbon-12 and don't have a nuclear spin. However, protons attached to a carbon atom will cause splitting of the carbon signal. This splitting will lower the signal to noise ratio, so carbon NMR spectra are usually obtained under conditions of **proton decoupling**. Under these conditions each nonequivalent carbon atom in a molecule will appear as a single peak in the carbon NMR.

Another difference between proton and carbon NMR is that carbon NMR spectra are not normally integrated. This is due to the fact that unless a long delay is introduced between acquisitions the carbon intensities don't accurately reflect the relative numbers of carbon atoms. For example, what you will usually observe is that carbon atoms with no hydrogens attached to them (e.g., carbonyl carbons) will be less intense than those that do have hydrogens attached. Nevertheless, for carbon atoms that do have hydrogens attached the relative height of the NMR peak usually can be used to estimate the relative number of carbon atoms. For example, in the carbon NMR of isopropanol, the 2 methyl carbons are equivalent and will show up as a peak that is approximately twice as high as the methine (one H-attached) carbon peak.

The 13C NMR is directly about the carbon skeleton not just the proton attached to it.

a. The number of signals tell us how many different carbons or set of equivalent carbons

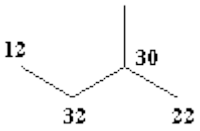
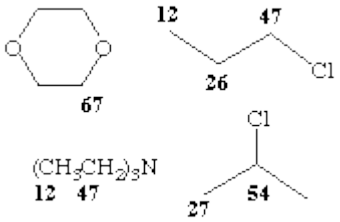
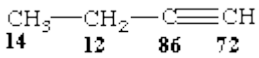
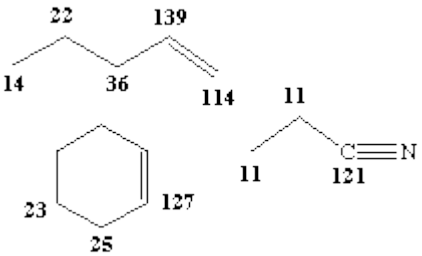
b. The splitting of a signal tells us how many hydrogens are attached to each carbon. (N+1 rule)

c. The chemical shift tells us the hybridization (sp3, sp2, sp) of each carbon.

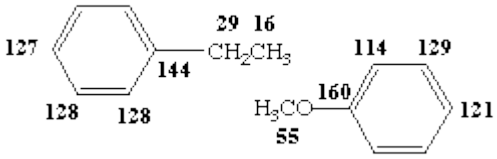
d. Integration: Not useful for 13C NMR

Chemical Shift in 13C NMR spectrum arises in the same way as in the proton NMR spectrum. Each carbon nucleus has its own electronic environment, different from the environment of other, non-equivalent nuclei; it feels a different magnetic field, and absorbs at different applied fields strength. Electronegative atoms and pi bonds cause downfield shifts. 13C chemical shift range 0-250 ppm. In 13C NMR spectrum, the more electronegative group bonded to carbon atom deshielding increases.

Typical C-NMR Shift Ranges

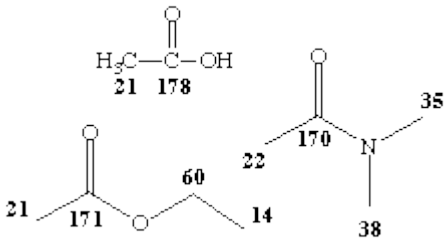
Chemical Shift (□) Type of Carbon	***Examples (Chemical shift in ppm.)***	***Comments***
10-40 ppm *Alkane C's*		*In general the greater the substitution on the carbon the further downfield (higher frequency) the resonance occurs.*
40-70 ppm *Adjacent to an electronegative atom*		*The more electronegative the atom the greater the chemical shift.*
65-90 ppm *sp carbon of an alkyne*		
110-140 ppm *sp2 carbon of an* ***alkene*** *or sp of a* ***nitrile***		

115-150 ppm
Aromatic



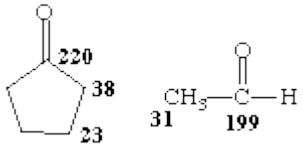
The aromatic carbon atom attached to a substituent does not have a hydrogen attached to it, so usually appears less intense than other carbons in the molecule. It also normally appears at a higher chemical shift.

160-185
Carbonyl carbon of acid derivatives



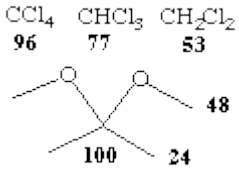
Since carbonyl carbons do not have a hydrogen attached to them they often appear less intense than other carbons in the molecule.

190-220 ppm
carbonyl carbon of ketones and aldehydes



Since carbonyl carbons do not have a hydrogen attached to them they often appear less intense than other carbons in the molecule.

Multiple functional groups



Chemical shift effects are approximately additive. In molecules where important resonance forms are possible, these will often influence the electron density around a carbon and hence cause changes in the chemical shift.