Chapter1 Theory and Instrumentation of Infra-Red Spectroscopy

1.1 Introduction

Infrared (IR) spectrometry deals with the interaction of IR with matter. IR spectrum of a compound can give information about the chemical nature and molecular structure and can be used for organic compounds as well as polyatomic inorganic and organometallic compounds.

- There are 3 regions of Infrared (IR) radiation that ranges from 12800 to 10 cm^{-1} as shown in Table 1.1 below

Table 1.1 IR Spectral Regions

Region	Wavelength (λ, μm)	Wave number ($cm^{-1)}$	Frequencies (Hz)
Near	0.78 – 2.5	12800-4000	$3.8x10^{14}$-$1.2x\ 10^{14}$
Middle	2.5-50	4000-200	$1.2x10^{14}$-$6.0x\ 10^{12}$
Far	50-1000	200-10	$6.0x10^{12}$-$3.0x\ 10^{11}$
Most used	2.5-15	4000-670	$1.2x10^{14}$-$2.0x\ 10^{13}$

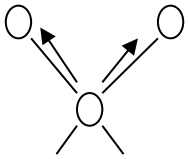
- Nearly all molecules absorb IR radiation with the exceptions of homonuclear diatomic molecules e.g. O_2, N_2, H_2. IR absorptions, emissions, and reflections of molecular species occurrence is due to changes in energy caused by molecular transitions from one vibrational or rotational energy state to another state. IR absorption bands are sharp and characteristic of certain groups within a molecule. In condensed phase, the rotational structure is lost which results into a sharper signal. IR spectrometry can be used to do qualitative and quantitative measurements. But the quantitative measurements and less accurate and precise when compared to UV spectrometry
- An IR spectrum is obtained with gas phase or condensed phase (liquid, solid) molecules. It can be reported as either an absorbance or transmittance ordinate with an abscissa that is reported as wave length, λ, or wave number. The wave number is linear with either the energy or the frequency (ϼ) of radiation ($c/\lambda=$ ϼ)
- IR energy is not high enough to cause transitions between electronic energy levels but between vibrational and rotational energy levels that are very close to each other
- IR absorption can occur with molecules that has a net change in dipole moment as it vibrates and rotates (selection rule)
- Its only molecules with a net dipole moment that can interact with the alternating electrical field of the incoming IR radiation to cause a change in its amplitudes of vibration and rotation. Rotational energy states accompanies each vibrational state

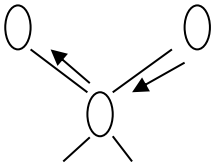
- For example, HCl is a dipole molecule with chlorine having a higher electron density surrounding it ,i.e.it is more electronegative than Hydrogen hence, it becomes a polar molecule. As the molecule vibrates and rotates, it dipole moment changes (net charge and distance between the center of charges) and a field that can interact with the electric field of the incoming IR radiation is created. If the frequency of the radiation coincides with the vibrational/rotational frequencies of HCl molecule, then absorption will occur which causes the amplitude of the vibrational frequency to increase
- Molecules with no net dipole (homonuclear species: N_2, O_2, Cl_2 etc) do not absorb IR and are said to be infrared inactive.
- All molecules with net dipole absorbs IR
- Energy that will cause a rotational transition is small (100 cm^{-1} or $\lambda \geq 100cm$)
- Vibrational and rotational energy states are quantized energy states and hence gives discrete distinct lines in the mid-IR and far IR region respectively
- Rotational levels are closely packed and they therefore, broaden the vibrational spectra and also, especially in liquid and gases intermolecular collisions causes the rotational spectrum to be broadened.

1.2 Types of Molecular Vibrations

- The relative position of atoms in a molecule is not fixed. It fluctuates continuously because of vibrations and rotations about the bonds in the molecule
- A non-linear molecule has 3N-6 fundamental vibrational modes and liner molecules have 3N-5 fundamental vibration modes (N= number of atoms in the molecule) e.g. water is a non-linear molecule and therefore will have 3 fundamental vibrational modes in IR.
- There are two types of vibrations: stretching and bending
- **Stretching:** continuous change in interatomic distances along the bond between two atoms. It could be a symmetric (vibration in the same direction) or asymmetric stretching (vibration in the different direction)
- **Bending:** change in angle between two bonds. There are 4 types of bending vibrations: scissoring, rocking, wagging and twisting

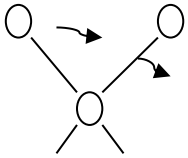
(a) Stretching vibrations

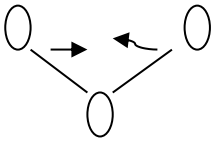




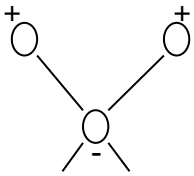
Symmetric stretching **asymmetric stretching**

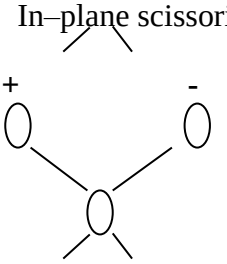
(a) Bending vibrations





In-plane rocking In–plane scissoring





Out-of-plane wagging Out-of-plane twisting

Group frequencies: Certain functional or structural groups give characteristic vibrational frequencies that is independent of the rest of the molecule e.g. the carbonyl group vibrates between 1650-1740 cm^{-1}

1.3 IR Instrumentation

There are three types of IR instruments; (1) dispersive spectrophotometers (using grating monochromators) (2) non dispersive photometers (using a filter or absorbing gas) (3) Fourier Transform spectrometers (FT, uses interferometers)

- In general, FTIR are used mostly for the Far and mid IR measurements because they are fast, reliable, has low signal-to noise ratio (S/N) and very convenient. It is now applied to many spectroscopic techniques because of its higher S/N ratio
- FT instruments are not used for near IR, UV and Visible radiation because the S/N ratio of shot and flicker noise associated with these source of radiation increases with increasing power of radiation. The total noise (detector + shot + flicker noise) are distributed evenly to all the resolution elements therefore, the S/N ratio of the strong peaks in the presence of weak peaks improves after averaging and degrades for weak peaks. Overall, the S/N ratio degrades for all peaks. This is called **multiplex disadvantage**

- FTIR instrument are based on Michelson interferometer design
- Dispersive spectrophotometers are still very common with near IR measurements
- **Advantages of an FT Instrument**: (i)High throughput or Jaquinot advantage- the radiant power reaching the detector is much greater than in dispersive instrument (ii) Fewer optical elements in its compositions (iii) wavelength reproducibility which makes possible the analysis of complex spectra (iv) fast, an entire spectrum can be acquired in ≤ 1s, (v) spectral quality -large number of resolution elements can be achieved in ≤ 1s (vi) higher resolution (<0.1 cm^{-1}) - all elements (n) in the source reaches the detector at the same time (large number of resolution elements reaches the detector at the same time) while they reach the detector one at a time with dispersive instruments. FTIR is useful in the analysis of gaseous mixtures (vii) greater S/N ratio – $(S/N)_n = \sqrt{n}(S/N)_i$ where n= number of resolution elements and i= S/N ratio for one element. $\sqrt{n}$ = Fellget or multiplex advantage (viii) highly accurate and reproducible which makes it good for background subtraction (ix) study of samples with high absorbance's (x) study of samples with weak absorption bands (x) investigations requiring fast scanning such as Kinetic order studies or detection of chromatographic effluents (xi) collecting IR data from very small samples (xii) obtaining reflection spectra (xiii) IR emission studies
- **Operations of an FT Spectrometer**: a conventional spectrometer records changes in radiate power as a function of frequency or wave number and it is

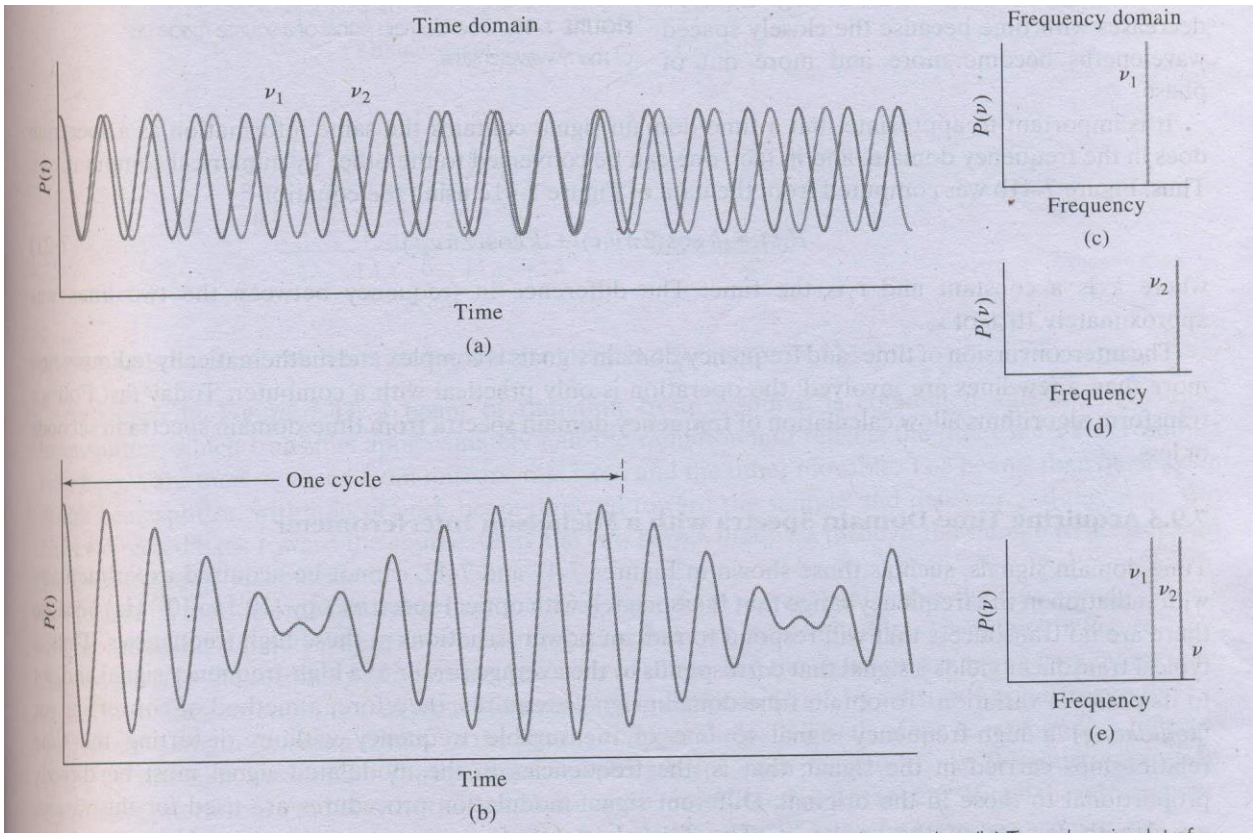


Figure1.1 (a) Time domain plot of two frequencies of the same amplitude v_1 and v_2 (b) Time domain plot of the sum of the two frequencies in (a) (c) frequency domain of plot v_1 (d) frequency domain of the pot of (v_2) (e) Frequency domain plot of the wave for (b)

- described as a frequency domain data. FT is a time domain data because the radiant power is recorded as a function of time (Fig1.2).

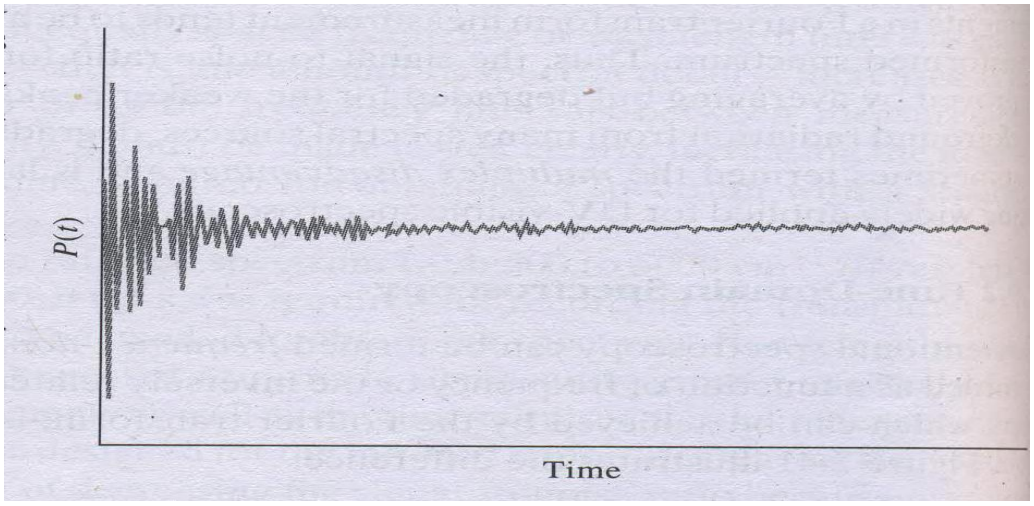


Figure1.2 Time domain signal of a source made up of many wavelengths

Interferometer- A Michelson interferometer is used in FT spectrometer and is shown in Fig. 1.3

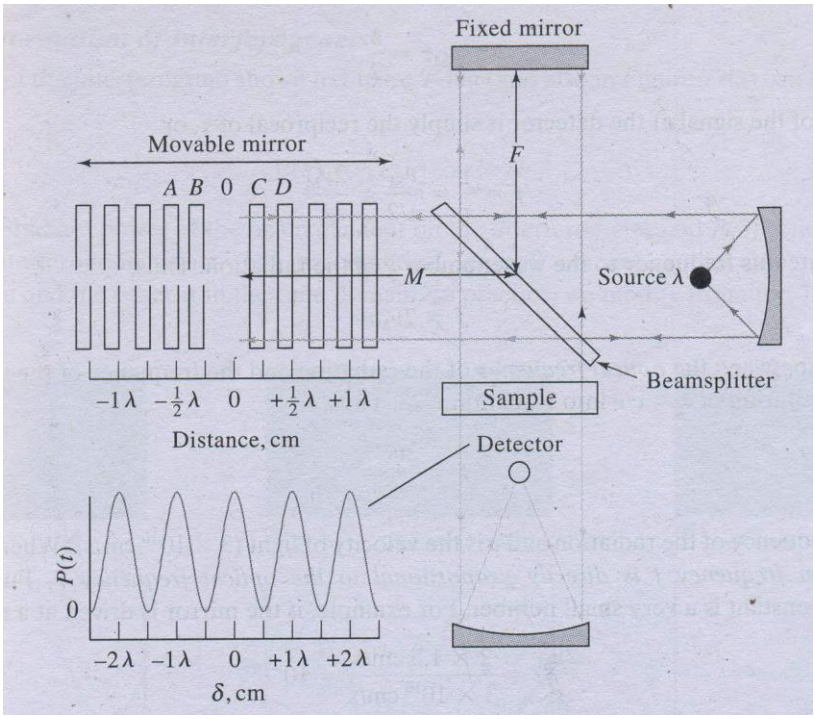


Figure 1.3 Schematic of a Michelson interferometer illuminated by a monochromatic source

- Michelson dispersive design is based on the principle of an interferometer. A beam from a source is splitted into two by a beam splitter. One half is reflected back to the source and the other half is transmitted. The transmitted beam and reflected beams are then reflected by two mirrors (fixed and movable mirror). The two beams again meet at the beam splitter and half of each beam goes to the source and the other two halves to the sample and detector and used for analysis. Horizontal motion of the movable mirror causes the radiant power reaching the detector to fluctuate in a reproducible way. If the two mirrors are equidistant from the splitter, the two recombine beams will precisely be in phase and the signal power is at a maximum. If the source is monochromatic, motion of the movable mirror in either direction by a distance equal to exactly ¼ wavelength (position B or C) will change the path length of the corresponding beam by ½ wavelength (¼wavelength in each direction). At this position, destructive interference (beams are out of phase) will reduce the radiant power of the radiant beams to zero. Positions A or D moves the beam in phase again and a strong signal is obtained. The difference in path length for the two beams, 2(M-F), is called the *retardation, δ.* Plotting output power of the detector, P(t) *vs* δ will result into an *interferogram*. The plot looks like a cosine wave (not a

sine wave because the power is at its maximum when δ = 0). The frequency of the radiation source and that of the interferogram are different. The relationship can be derived from the P(t) *vs* δ plot where it shows that moving the movable mirror at a distance equal to ½ of a wavelength, λ/2. If the mirror moves at a constant speed of v_m and τ is the time to move a distance λ/2, then

$v_m\tau = \lambda/2$ (1). If *f* is the frequency of the beam at the detector, then,

$f = 1/\tau = 2v_m/\lambda$. If $\lambda = c/v$, then $f = \mathbf{2v_m\bar{v} = 2v_mv/c}$

if v_m is constant, then, *f(interferogram frequency)* = optical frequency(*v*)

if $\boldsymbol{v_m}$ **= 1.5 cm/s, then,**

$\mathbf{2v_m/c = 2x\ 1.5\ cm/s/3x\ 10^{10}\ cm/s = 10^{-10}}$ and

$f = 10^{-10}v$. This implies that the visible and IR radiation will be modulated into the audio wave range by a Michelson interferometer. Some visible and IR traducers can follow signal power that falls into the radio frequency range

1.4 Parts of an FT Instruments: (i) Driving mechanism for a moving mirror (produces a good spectra) accompanied with a good sampling and averaging device (ii) moving mirror (iii) source (IR single beam of double beam) (iv)Laser (reference beam) (v) interferometer (vi) mirrors (vii) sample holder (viii) detector (ix) read out. A double beam FT spectrometer is shown in Fig. 3.4. The beam emerging from the interferometer strikes mirror M_1, which in one position directs the beam through the reference cell and in the other position directs it through the sample cell. Mirror M_2, which is synchronized to M_1, alternately directs the reference beam and sample beam to the traducer.

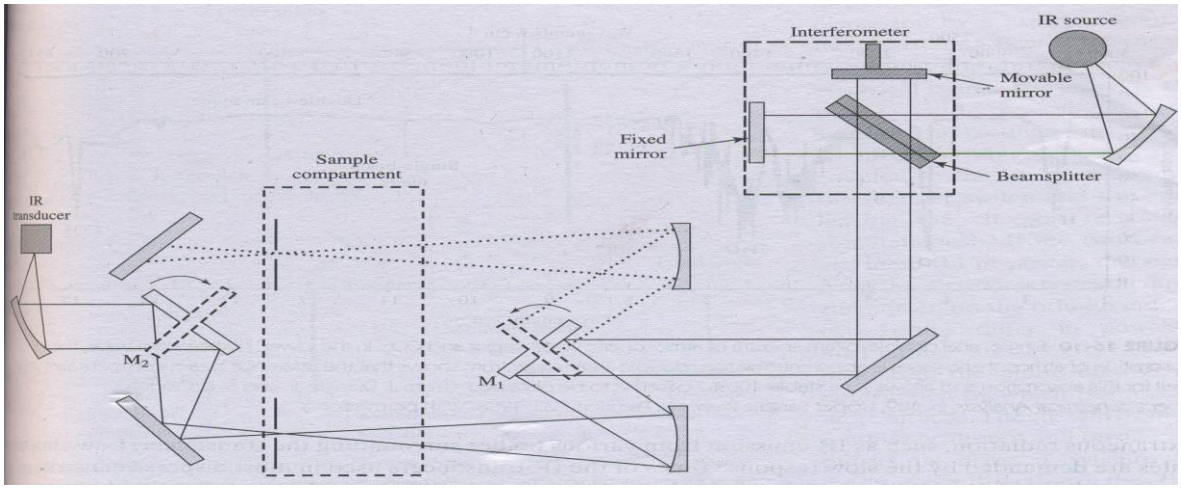


Figure1.4 Double beam FTIR spectrometer

1.5 Dispersive Instruments

Dispersive IR instruments are usually double beam spectrophotometers that use reflection gratings for dispersing the radiation. The double beam design is less demanding with respect to the performance of the sources and detectors (low IR output and sensitivity, need for large amplification) by (i) compensating for all but the most short-term fluctuation in the radiant output of the sources as well as for the drift in the amplifier and the traducer (ii) compensates for the wide variation in source intensity with wavelength (iii) the reference beam compensates for the absorption of atmospheric water and carbon dioxide

Double beam spectrometers incorporate a low-frequency chopper (5-30 cycles/s) that differentiates between source, stray and IR signals from various bodies around the traducer. IR spectrometers are similar to the UV spectrometers except that the reference and sample cells are placed before the monochromator. A typical double beam IR spectrometer is shown in Fig1.4 with an attenuator in place (optical null type design). Radiation from the source is splitted into two; half passing through the reference cell and the other half passes through the sample cell. The reference beam passes through and attenuator and to a chopper. The attenuator constantly removes a variable part of the reference beam so as to match the beam coming out of the sample. The attenuator is synchronized with the recorder pen in the old spectrometers so that its position gives a measure of the difference between the two beams. In modern instruments a comb-like structure with tapered teeth's is used as the attenuator. The lateral movement of the comb is a measure of the differences between the two beams. The beam from the attenuator is passed through a chopper which reflects the reference beam or transmits the sample beam to the monochromator, the traducer and then converted to an electrical signal. The signal is amplified and passed to a synchronous rectifier (couple to the chopper) which causes the rectifier switch and the beam coming out of the chopper to change simultaneously. If the two beams are identical, the rectifier will produce a constant direct current (dc) if not, a fluctuating alternating current (ac) is produced. The more intense beam determines the phase of the current (ac). The current from the rectifier is filtered and again amplified to drive another synchronous motor in one direction of the other. The direction is determined by the phase of the input current and the motor is connected (mechanically) to the attenuator and the pen drive and causes them to move until a null point is reached. Another synchronous motor simultaneously drives the chart and varies the wavelength (connected to the wave selector)

1.6 Limitations of Double beam IR spectrometers

The limitations of a double beam spectrometer include: (i) attenuator always lag behind the transmittance changes (regions where signal change is fast) (ii) the momentum associated with the mechanical attenuator and the recorder makes the pen drive to overshoot the true transmittance (iii)cannot exactly establish the null position because when transmittance approaches zero, no radiation is are given out. Rounded peaks are shown in this area

1.7 Non Dispersive Spectrophotometers

There are spectrophotometers that use filters as the dispersive element. They are used for quantitative analysis and are more rugged and cheaper to maintain. There are also photometers with no filters designed to run carbon monoxide determinations

1.8 IR Sources and Detectors

1.8.1 Sources: Consist of inert solids heated to 1500-2200 K which produces continuous radiation equivalent to a black body radiation. The maximum radiant intensity at these temperatures occur between 5000- -5900 cm^{-1} (2 and 1.7 μm). At longer wavelengths, the intensity gradually fades up until it is about 1% transmittance. At shorter wavelengths, the intensity decreases very much rapidly. Examples of sources include the Nernst glower, which a cylindrical oxides of rare earth metals (1-3mm diameter and 2-5 cm long), the Globar, which is silicon carbide rod (5cm long and 5mm diameter), mercury arc, incandescent wires (nichrome or rhodium wire), tungsten wire, carbon dioxide laser source etc.

1.8.2 Traducers: There are three types of IR traducers: (i) pyro electric traducers **(ii)** photo conducting traducers (ii) thermal traducers

1.9 Sample Preparation

The preparation of samples for IR spectrometry is the most difficult step in obtaining an IR spectrum because all substances absorb IR radiation at some wavelengths. Because of this, cell windows, cell path lengths and solvents for the wavelength region and sample of interest must be carefully chosen in order to obtain good IR spectrum. Good handling techniques have been developed for gases, liquids and solids.

1.9.1 Gas Sample

A gas sample cell is made up of a cylinder of glass or some metals closed at both ends with an appropriate window material and equipped with valves or stopcocks for sample introduction. For routine analysis, cell with ~10 cm pathlength are used. Long or Multipass (glass are used to make light to pass several times through the sample before exiting the cell) cells are available for very dilute or weakly absorbing species. To resolve rotational structures, the spectrum is taken at reduced pressure

1.9.2 Liquid Samples

Liquid samples are often used to obtain IR spectrum. Often thin films of pure liquids are used when amounts are small or suitable solvents are not available. A drop of the liquid is placed between two NaCl plates which are then clamped together in a demountable cell. In some cases, **solution samples** are used to obtain IR spectrum. Obtaining a suitable solvent is always problems since all common solvent absorb IR but there are wavelength regions where each

solvent is somewhat transparent; also samples must be soluble in the solvent and chemically inert to the sample for it to be useful for IR analysis. Common IR solvents are carbon tetrachloride (CCl_4) carbon disulfide (CS_2) and chloroform ($CHCl_3$). Water is not often used because it absorbs strongly in certain IR regions and it also dissolves most common window materials, hence organic solvents must also be very dry. For aqueous samples e.g. biological samples, insoluble window material or FT IR are often used because it compensates for solvent absorption.

For volatile samples sealed cells are used. The pathlength are often made to be variable or fixed. Cells with pathlength between 0.1 and 1mm are available that can allow quantitative determinations of solution of 0.5-10% concentration range. The thickness of the cell must often be determined because of erosion that occurs with use. The thickness, b, is obtained by obtaining an interference spectrum of a cell in air. The reflection of the cell walls interferes with the IR radiation passing through it to produce an interference pattern. If *n,* interference maxima is obtained within a wavelength region, $\Delta\nu$, then, $b = n/2\Delta\nu$

1.9.3 Solid Samples

Solid samples can be used in the form of mineral oil mulls of KBr disc or pellets. The sample must be finely grounded so that the particle size is smaller than the wavelength of the IR in radiation in order to reduce loss to scattering. Nujol is often used as the mineral oil. It has a stretching vibration at 300 cm^{-1} and a bending vibration at 1400 cm^{-1} and is transparent in other regions. If Nujol absorbs strongly in the area of interest, chlorinated (hexachlorobutadiene) or fluorinated (Florolube) are used as minerals oils. The mull are made by grinding a few milligrams of the sample in a drop of the oil. The paste is then used to obtain the IR spectrum. Similarly, a few milligrams (< 1 mg) are mixed with KBr (100 mg) to make the pellets. KBr is transparent to IR region up to 25 µm. KBr react with some sample, therefore unknown sample is made with the oil. Mulls and pellets are okay for qualitative analysis not for quantitative analysis. Internal standards are often added for quantitative determinations. Qualitative analysis requires a few milligrams of sample while quantitative will require 20 mgs or more of sample.

1.10 Qualitative Analysis and Structure Determination

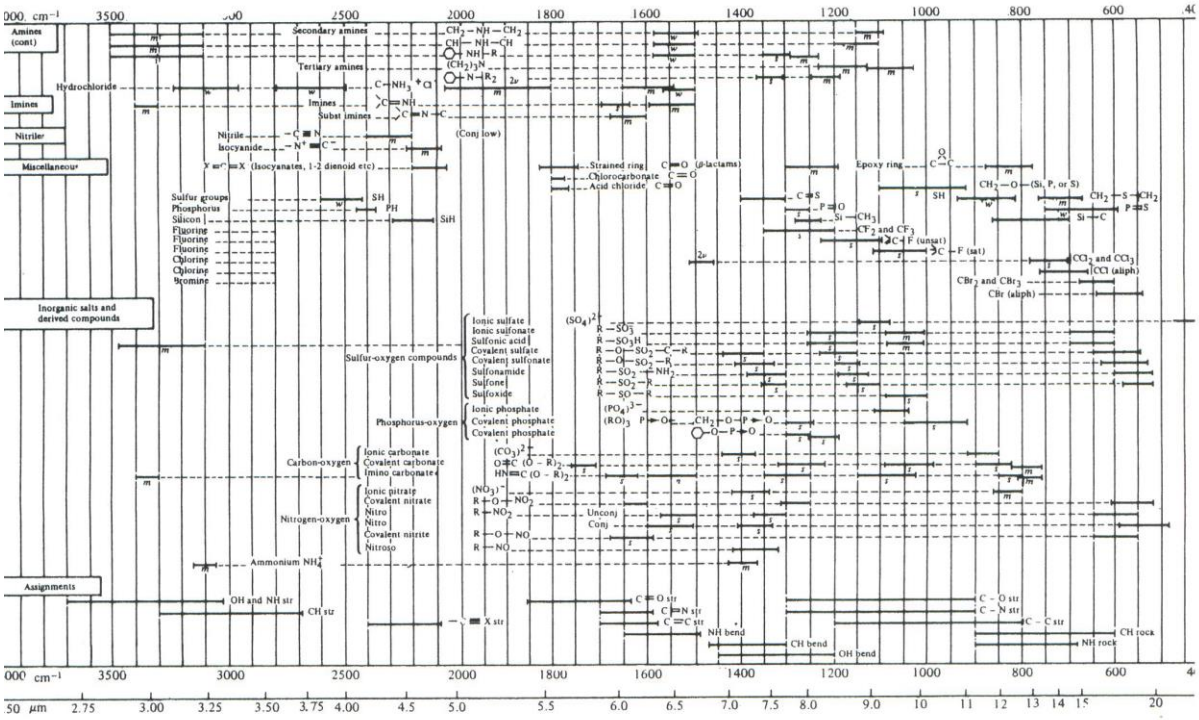
IR is mostly used to do qualitative and molecular structure determinations. I molecular determinations, we have to use pure samples for accurate interpretation of the spectrum. Often the result is combined with other techniques (NMR< MS) for proper structural determinations. Qualitative determinations may use impure samples to acquire functional group information in the sample and then compare with the known spectrum of known compounds.

1.11 Correlation Charts

A correlation chart is a collection of group frequencies of functional groups in a molecule. It is organized according to the type of compound in which the group is found. After obtaining the

spectrum, the strongest absorption bands are selected and the wavelengths of the absorption maxima are identified. This is followed by identifying the moderate and weak bands in the spectrum. It is often impossible to identify all the bands in the spectrum even for a pure compound. Qualitative analysis is often done by making comparison with reference spectra in the instrument library. Other information like boiling point, melting point is used correlation information with IR data.

Correlation charts only indicates the presence of a certain group in a molecule and should be combined with other techniques to ascertain the structures of molecule. Ambiguities may arise due to overlapping frequencies, combination and overtones bands or sample preparation techniques or instrumental factors.



1.12 Spectral Collections and Search System.

Extensive collections of IR data are available for use in spectral matching and searching process. They are available in computer software's which stores absorption and relative intensity information of the spectrums for comparison.

1.13 Applications

IR spectrometry has many applications e.g. it can be interfaced with GC and LC to monitor their effluents at the µg ml^{-1} level. For such interfaces a small volume, long narrow flow through cell

is needed to create a reasonable pathlength for several reflections to occur for adequate IR signal. In general, FTIR has increased IR capability to monitor GC and LC effluents.

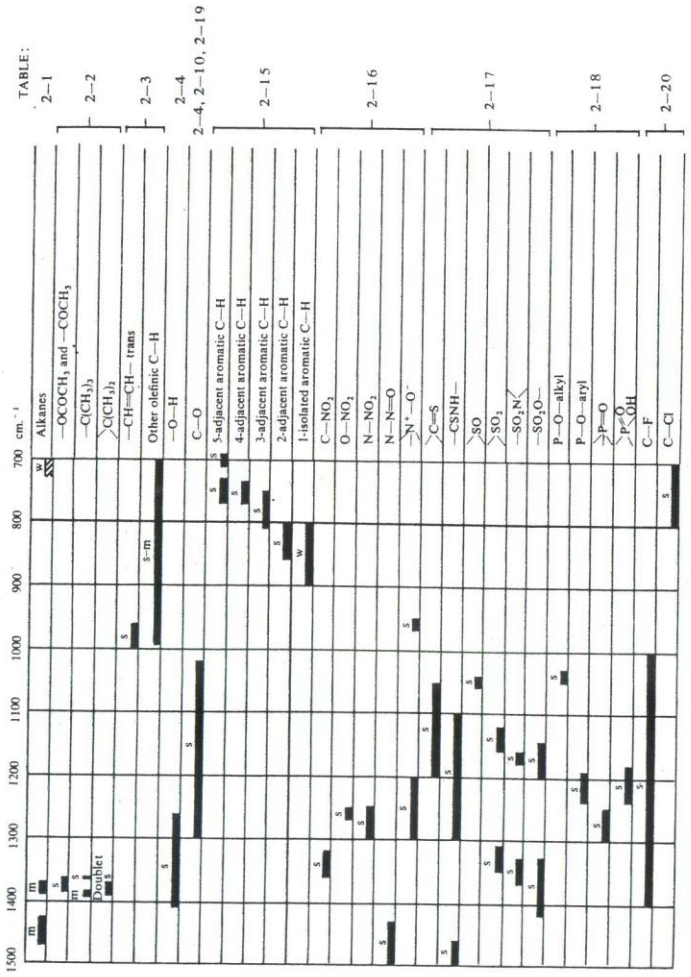


Fig. 2–7. Some characteristic bands in the fingerprint region.

2–8. Absorption Frequencies of Single Bonds to Hydrogen

Table 2–1
Saturated C—H and C—C

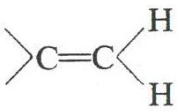
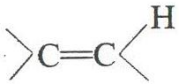
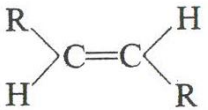
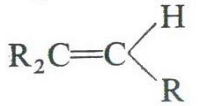
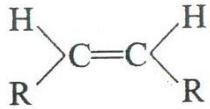
Group	Band	Remarks
>CH_2, —CH_3	2960–2850(s)	Two or three bands usually; >C—H stretching
>CH	2890–2880(w)	
>CH_2, —CH_3	1470–1430(m)	>C—H deformations
—CH_3	1390–1370(m)	—CH_3 symmetrical deformation
>CH_2	~720(w)	>CH_2 rocking

Table 2–2
Miscellaneous C—H

Group	Band	Remarks
Cyclopropane C—H, Epoxide C—H, —CH_2-halogen	~ 3050(w)	C—H stretching; cf. alkenes
—CO—CH_3	3100–2900(w)	Often very weak
—CHO	2900–2700(w)	Usually two bands, one near 2720 $cm.^{-1}$ (see Fig. 2–13a)
—O—CH_3	2850–2810(m)	
—O—CH_2—O—	2790–2770(m)	
N—CH_3 and N—CH_2—	2820–2780(m)	(see Fig. 2–12)
—$C(CH_3)_3$	1395–1385(m) 1365(s)	
>$C(CH_3)_2$	~ 1380(m)	A roughly symmetrical doublet (see Fig. 2–8)

Alkene and Aromatic C—H

See also Table 2–13 and Table 2–14 for the corresponding double bond absorptions, and Table 2–15 for the aromatic C—H out-of-plane bending vibrations.

Group	Band	Remarks
—C≡C—H	~3300(s)	
	3095–3075(m)	C—H stretching; sometimes obscured by the much stronger bands of saturated C—H groups which occur below 3000 cm.$^{-1}$
	3040–3010(m)	(See Fig. 2–12)
Aryl—H	3040–3010(w)	Often obscured (but see Fig. 2–9)
	970–960(s)	C—H out-of-plane deformation. When the double bond is conjugated with, for example, a C=O group this band is shifted towards 990 cm.$^{-1}$
$RCH{=}CH_2$	995–985(s) and 940–900(s)	
$R_2C{=}CH_2$	895–885(s)	
	840–790(m)	
	730–675(m)	

Alcohol and Phenol —O—H

Group	Band	Remarks
Water in solution	3710	
Free —OH	3650–3590(v)	Sharp; O—H stretching (see Fig. 2–8)
H-bonded —OH (solid, liquid and dilute solution)	3600–3200(s)	Often broad but may be sharp for some intramolecular single bridge H-bonds; the lower the frequency the stronger the H-bond (see Fig. 2–8)
Intramolecular H-bonded —OH in chelate form (see also Table 2–10, carboxylic acids)	3200–2500(v)	Broad; the lower the frequency the stronger the H-bond; sometimes so broad as to be overlooked
Water of crystallization (solid state spectra)	3600–3100(w)	Usually a weak band at 1640–1615 $cm.^{-1}$ also; water in trace amounts in KBr discs shows a broad band at 3450 $cm.^{-1}$ (see Fig. 2–9)
—O—H	1410–1260(s)	O—H bending
>C—OH	1150–1040(s)	C—O stretching (see Fig. 2–8)

2–11. Absorption Frequencies of the Double Bond Region

Table 2–10

Carbonyl Absorption $\rangle C{=}O$ *All bands quoted are strong.*

Groups	Band	Remarks
Acid anhydrides —CO—O—CO—		
Saturated	1850–1800 1790–1740	Two bands usually separated by about 60 $cm.^{-1}$. The higher frequency band is more intense in acyclic anhydrides and the lower frequency band is more intense in cyclic anhydrides
Aryl and $\alpha\beta$-unsaturated	1830–1780 1770–1710	
Saturated five-ring	1870–1820 1800–1750	
All classes	1300–1050	One or two strong bands due to C—O stretching
Acid chlorides —COCl		
Saturated	1815–1790	Acid fluorides higher, bromides and iodides lower
Aryl and $\alpha\beta$-unsaturated	1790–1750	
Acid peroxides —CO—O—O—CO—		
Saturated	1820–1810 1800–1780	
Aryl and $\alpha\beta$-unsaturated	1805–1780 1785–1755	

Table 2–9
Cumulated Double Bonds

Group	Band	Remarks
Carbon dioxide O=C=O	2349(s)	Appears in many spectra due to inequalities in path length
Isocyanates —N=C=O	2275–2250(s)	Very high intensity; position unaffected by conjugation
Azides $-N_3$	2160–2120(s)	
Carbodiimides —N=C=N—	2155–2130(s)	Very high intensity; split into an unsymmetrical doublet by conjugation with aryl groups
Ketenes >C=C=O	~2150(s)	
Isothiocyanates —N=C=S	2140–1990(s)	Broad and very intense
Diazoalkanes $R_2C{=}\overset{+}{N}{=}\overset{-}{N}$	~2100(s)	
Ketenimines C=C=N—	~2000(s)	
Allenes C=C=C	~1950(m)	Two bands when terminal allene or when bonded to electron attracting groups, e.g., $-CO_2H$

Table 2–3

Amine, Imine, Ammonium, and Amide N—H N—H Stretching

Group	Band	Remarks
Amine and imine $>$N—H =N—H	3500–3300(m)	Primary amines show two bands in this range; the unsymmetrical and symmetrical stretching. Secondary amines absorb weakly. The pyrrole and indole N—H band is sharp (see Fig. 2–10)
$—NH_3^+$ Amino acids	3130–3030(m)	Values for solid state; broad; bands also (but not always) near 2500 and 2000 cm.$^{-1}$ (see text below Fig. 2–10)
Amino salts	~3000(m)	
$>NH_2^+$ $>NH^+$ $=\overset{+}{N}H$	2700–2250(m)	Values for solid state; broad, due to the presence of overtone bands, etc.
Primary amide —$CONH_2$	~3500(m) ~3400(m)	Lowered ~150 cm.$^{-1}$ in the solid state and on H-bonding; often several bands 3200–3050 cm.$^{-1}$ (see Fig. 2–11)
Secondary amide —CONH—	3460–3400(m)	Two bands; lowered on H-bonding and in the solid state (see Fig. 2–13a). Only one band with lactams
	3100–3070(w)	A weak extra band with bonded and solid state samples (see Fig. 2–13a)

Table 2–10 *continued*

Groups	Band	Remarks
Saturated	1725–1700	The monomer is near 1760 cm.$^{-1}$ but is rarely observed. Occasionally both bands, the free monomer and the H-bonded dimer, can be seen in solution spectra. Ether solvents give one band near 1730 cm.$^{-1}$
$\alpha\beta$-unsaturated	1715–1690	(See Fig. 2–9)
Aryl	1700–1680	
α-halo-	1740–1720	
Carboxylate ions $-CO_2^-$ For amino acids, see text below Fig. 2–10		
Most salts	1610–1550 1420–1300	Antisymmetrical and symmetrical stretching respectively
Amides —CO—N< (See also Table 2–5 and 2–6 for N—H stretching and bending)		
Primary $-CONH_2$		
In solution	~1690	Amide I; C=O stretching
Solid state	~1650	
In solution	~1600	Amide II; mostly N—H bending
Solid state	~1640	
		Amide I is generally more intense than amide II. (In the solid state amide I and II may overlap.) (See Fig. 2–11)
Secondary —CONH—		
In solution	1700–1670	Amide I (see Fig. 2–13)
Solid state	1680–1630	
In solution	1550–1510	Amide II; found in open chain amides only (see Fig. 2–13)
Solid state	1570–1515	
		Amide I is generally more intense than amide II
Tertiary	1670–1630	Since H-bonding is absent solid and solution spectra are much the same (see Fig. 2–12)
Lactams		
Six- and larger rings	~1670	
Five-ring	~1700	Shifted to higher frequency when the N atom is in a bridged system
Four-ring	~1745	
R—CO—N—C=C		Shifted +15 cm.$^{-1}$ by the additional double bond

Groups	Band	Remarks
C=C—CO—N		Shifted by up to +15 cm.$^{-1}$ by the additional double bond. This is an unusual effect for $\alpha\beta$-unsaturation. It is said to be due to the inductive effect of the C=C on the well conjugated CO—N system, the usual conjugation effect being less important in such a system
Imides —CO—N—CO—		
Cyclic six-ring	~1710 and ~1700	Shift of +15 cm.$^{-1}$ with $\alpha\beta$-unsaturation
Cyclic five-ring	~1770 and ~1700	
Ureas N—CO—N		
RNHCONHR	~1660	
Six-ring	~1640	
Five-ring	~1720	
Urethanes		
R—O—CO—N	1740–1690	Also shows amide II band when non- or mono-substituted on N
Thioesters and Acids RCO—S—R′		
RCOSH	~1720	$\alpha\beta$-unsaturated or aryl acid or ester shifted ~ −25 cm.$^{-1}$
RCOS—alkyl	~1690	
RCOS—aryl	~1710	

Table 2–10 *continued*

Groups	Band	Remarks
Esters and lactones —CO—O—		
Saturated	1750–1735	
Aryl and $\alpha\beta$-unsaturated	1730–1715	
Aryl and vinyl esters		
C=C—O—CO—Alkyl	1800–1750	The C=C stretching band also shifts to higher frequency
Esters with electronegative α-substituents; e.g., >CCl—CO—O—	1770–1745	
α-keto esters	1755–1740	
Six-ring and larger lactones	Similar values to the corresponding open chain esters	
Five-ring lactone	1780–1760	
$\alpha\beta$-unsaturated five-ring lactone	1770–1740	When α-C—H present there are two bands, the relative intensity depending on the solvent (see Fig. 2–14)
$\beta\gamma$-unsaturated five-ring lactone; i.e., vinyl ester type	~1800	
Four-ring lactone	~1820	
β-keto ester in H-bonding enol form	~1650	Keto form normal; chelate type H-bond causes shift to lower frequency than the normal ester. The C=C is usually near 1630(s) cm.$^{-1}$
All classes	1300–1050	Usually two strong bands due to C—O stretching.
Aldehydes —CHO (see also Table 2–2 for C—H). All values given below are lowered in liquid film or solid state spectra by about 10–20 cm.$^{-1}$. Vapour phase spectra have values raised about 20 cm.$^{-1}$		
Saturated	1740–1720	
Aryl	1715–1695	(See Fig. 2–13a) *Ortho* hydroxy or amino groups shift this value to 1655–1625 cm.$^{-1}$ due to intramolecular H-bonding
$\alpha\beta$-unsaturated	1705–1680	
$\alpha\beta,\gamma\delta$-unsaturated	1680–1660	
β-ketoaldehyde in enol form	1670–1645	Lowering caused by chelate type H-bonding

Table 2–10 *continued*

Groups	Band	Remarks
Ketones C=O All values given below are lowered in liquid film or solid state spectra by about 10–20 cm.$^{-1}$. Vapour phase spectra have values raised about 20 cm.$^{-1}$		
Saturated	1725–1705	
Aryl	1700–1680	
$\alpha\beta$-unsaturated	1685–1665	
$\alpha\beta,\alpha'\beta'$-unsaturated and diaryl	1670–1660	
Cyclopropyl	1705–1685	
Six-ring ketones and larger	Similar values to the corresponding open chain ketones	(See Fig. 2–8)
Five-ring ketones	1750–1740	$\alpha\beta$-unsaturation, etc., has a similar effect on these values as on those of open chain ketones
Four-ring ketones	~1780	
α-halo ketones	1745–1725	Affected by conformation; highest values are obtained when both halogens are in the same plane as the C=O
α,α'-dihalo ketones	1765–1745	
1,2-Diketones s-*trans*: (i.e., open chains)	1730–1710	Antisymmetrical stretching frequency of both C=O's. The symmetrical stretching is inactive in the infrared but active in the Raman
1,2-Diketones s-*cis*, six-ring	1760 and 1730	
1,2-Diketones s-*cis*, five-ring	1775 and 1760	
o-Amino- or *o*-hydroxy-aryl ketones	1655–1635	Low due to intramolecular H-bonding. Other substituents and steric hindrance etc. affect the position of the band
Quinones	1690–1660	C=C usually near 1600(s) cm.$^{-1}$
Extended quinones	1655–1635	
Tropone	1650	Near 1600 cm.$^{-1}$ when lowered by H-bonding as in tropolones
Carboxylic acids —CO_2H		
All types	3000–2500	O—H stretching; a characteristic group of small bands due to combination bands etc. (For the appearance of this group see Fig. 2–9)

Table 2–14

Aromatic Compounds

See also Table 2–3 and Table 2–15 for aryl—H vibration frequencies.

Group	Band	Remarks
Aromatic rings	~1600(m)	(See Fig. 2–13)
	~1580(m)	Stronger when the ring is further conjugated
	~1500(m)	This is usually the strongest of the two or three bands

Table 2–15

Substitution Patterns of the Benzene Ring

Group	Band	Remarks
Five adjacent H	770–730(s) and 720–680(s)	Mono-substituted
Four adjacent H	770–735(s)	*Ortho*-disubstituted (see Fig. 2–10)
Three adjacent H	810–750(s)	*Meta*-disubstituted etc. and 1, 2, 3-trisubstituted
Two adjacent H	860–800(s)	*Para*-disubstituted etc. (see Fig. 2–13a)
Isolated H	900–800(w)	*Meta*-disubstituted etc.; usually not strong enough to be useful

Table 2–15

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Three adjacent H	810–750(s)	*Meta*-disubstituted etc. and 1, 2, 3-trisubstituted
Two adjacent H	860–800(s)	*Para*-disubstituted etc. (see Fig. 2–13a)
Isolated H	900–800(w)	*Meta*-disubstituted etc.; usually not strong enough to be useful

Table 2–15

Substitution Patterns of the Benzene Ring

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Four adjacent H	770–735(s)	*Ortho*-disubstituted (see Fig. 2–10)
Three adjacent H	810–750(s)	*Meta*-disubstituted etc. and 1, 2, 3-trisubstituted
Two adjacent H	860–800(s)	*Para*-disubstituted etc. (see Fig. 2–13a)
Isolated H	900–800(w)	*Meta*-disubstituted etc.; usually not strong enough to be useful

Table 2–16

Nitro, Nitroso, etc. N=O

Group	Band	Remarks
$C—NO_2$	~1560(s) ~1350(s)	Lowered ~30 cm.$^{-1}$ when conjugated. The two bands are due to asymmetrical and symmetrical stretching of the NO bonds (see Fig. 2–9)
Nitrates $O—NO_2$	1650–1600(s) 1270–1250(s)	
Nitramines $N—NO_2$	1630–1550(s) 1300–1250(s)	
C—N=O	1600–1500(s)	
O—N=O	1680–1610(s)	Two bands
N—N=O	1500–1430(s)	
$\rangle N^{+}—O^{-}$		
aromatic aliphatic	1300–1200(s) 970–950(s)	Very strong bands
NO_3^-	1410–1340 860–800	

Table 2–11

Imines, Oximes etc. $\rangle C{=}N-$

Group	Band	Remarks
$\rangle C{=}N{-}H$	3400–3300(m)	N—H stretching; lowered on H-bonding
$\backslash C{=}N-$	1690–1640(v)	Difficult to identify due to large variations in intensity and the closeness to C=C stretching region. Oximes usually give very weak bands
$\alpha\beta$-unsaturated	1660–1630(v)	
Conjugated cyclic systems	1660–1480(v)	

Table 2–12

Azo Compounds —N=N—

Group	Band	Remarks
—N=N—	~1575(v)	Very weak or inactive in infrared. Sometimes seen in Raman
$-\overset{+}{N}{=}N-$ $\quad\quad\vert$ $\quad O^-$	~1570	

Table 2–13

Alkenes $\rangle C{=}C\langle$

(See also Table 2–3 for the =C—H absorptions of alkenes.)

Group	Band	Remarks
Non-conjugated $\rangle C{=}C\langle$	1680–1620(v)	May be very weak if more or less symmetrically substituted (see Fig. 2–12)
Conjugated with aromatic ring	~1625(m)	More intense than with unconjugated double bonds
Dienes, trienes, etc.	1650(s) and 1600(s)	Lower frequency band usually more intense and may hide or overlap the higher frequency band
$\alpha\beta$-unsaturated carbonyl compounds	1640–1590(s)	Usually much weaker than the C=O band (see, however, Fig. 2–11)
Enol esters, enol ethers and enamines	1690–1650(s)	(See Fig. 2–14)

2–13. Examples of Infrared Spectra

The following spectra show the appearance and relative intensities of the absorption peaks due to a number of functional groups. The wide variety of fingerprints shows the usefulness of this region for identification.

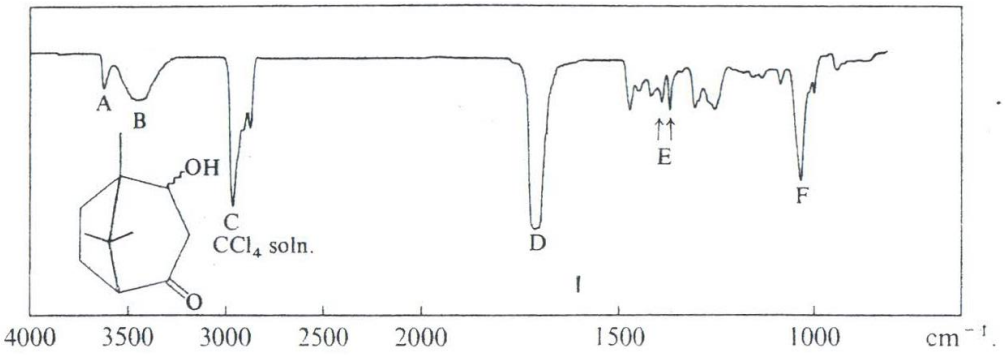

Fig. 2–8

A	3620 $cm.^{-1}$	Free O—H
B	3460 $cm.^{-1}$	Intermolecular and weakly bonded O—H
C	2960 $cm.^{-1}$	Saturated C—H
D	1710 $cm.^{-1}$	Ketone C=O
E	1370 and 1390 $cm.^{-1}$	$>C(CH_3)_2$
F	1035 $cm.^{-1}$	C—O stretching

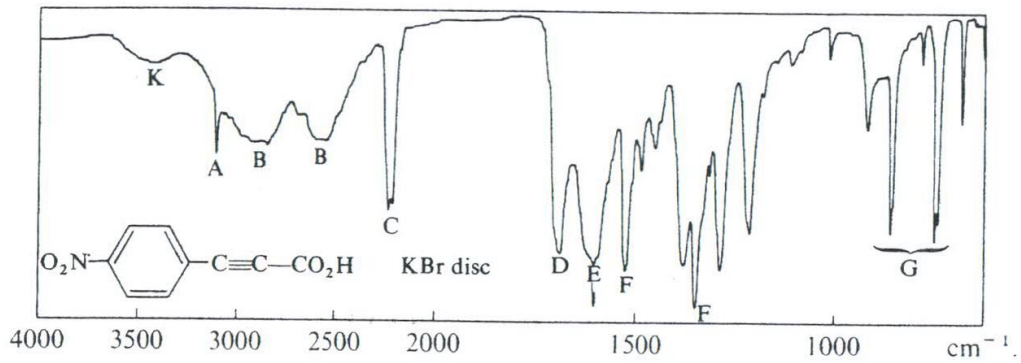

Fig. 2–9

A	3100 $cm.^{-1}$	Aryl C—H stretching
B	3200–2400 $cm.^{-1}$	Characteristic strongly H-bonded O—H of carboxylic acid
C	2225 $cm.^{-1}$	Conjugated C≡C, hence strong
D	1690 $cm.^{-1}$	Conjugated $—CO_2H$
E	1605 $cm.^{-1}$	Benzene ring, unusually broad and unresolved. A band near 1500 $cm.^{-1}$ is masked
F	1520 and 1350 $cm.^{-1}$	Conjugated nitro group—NO_2
G	950–650 $cm.^{-1}$	An example of a substituted ring in which it is not possible to decide with any certainty, due to the large number of bands in the region, in favour of 1, 4-disubstitution. For an example where the assignment can be made with confidence see Fig. 2–10
K		OH of water almost always present even in good KBr discs like this one

2–13. Examples of Infrared Spectra

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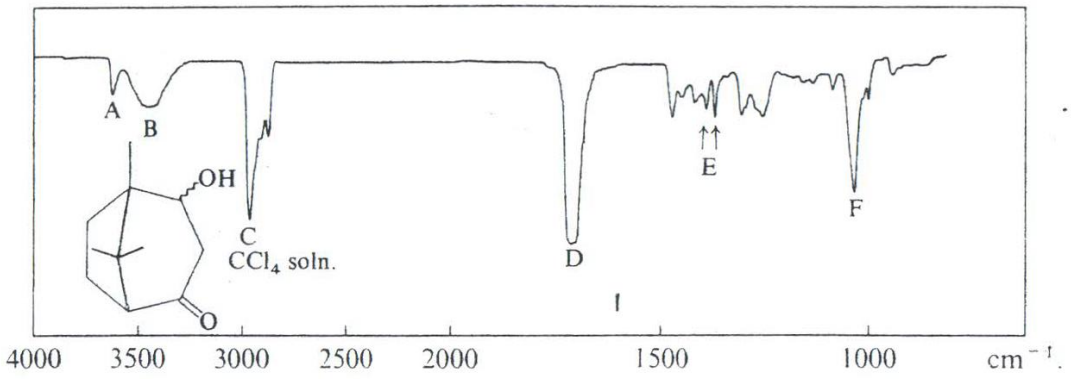

Fig. 2–8

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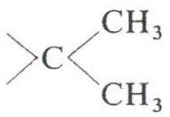
B	3460 $cm.^{-1}$	Intermolecular and weakly bonded O—H
C	2960 $cm.^{-1}$	Saturated C—H
D	1710 $cm.^{-1}$	Ketone C=O
E	1370 and 1390 $cm.^{-1}$	
F	1035 $cm.^{-1}$	C—O stretching

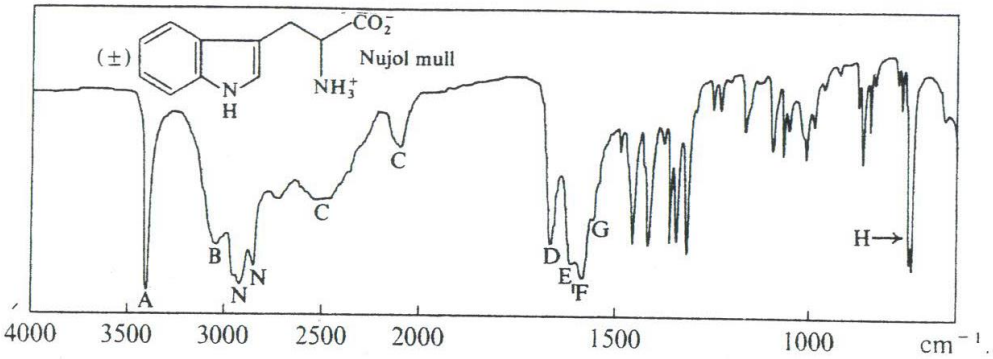


Fig. 2–10

A	3400 cm.$^{-1}$	Indole N—H
B	3040 cm.$^{-1}$	Broad 'ammonium' band due to $—NH_3^+$
C	~2500 and ~2100 cm.$^{-1}$	Two bands, very common with amino acids; also shown by primary amine salts
D	1665 cm.$^{-1}$	Amino acid I; unusually strong
E	1610 cm.$^{-1}$	Possibly aryl group
F	1590 cm.$^{-1}$	Amino acid II; the ionized carboxylate group $—CO_2^-$
G	1550 cm.$^{-1}$	$—NH_3^+$ deformations
H	750 or 740 cm.$^{-1}$	C—H out-of-plane deformations showing a 1, 2-disubstituted benzene ring
N		Nujol peaks

Amino acids show the spectrum of the zwitterionic groups. The primary ammonium $—NH_3^+$ group N—H stretching appears under the peaks of the saturated C—H absorption. The two bands near 2500 and 2000 cm.$^{-1}$ are frequently found when the $—NH_3^+$ group is present, and are due to overtones and combinations. In the double bond region, there are several peaks, including at least one due to N—H bending and one, the strongest, due to the ionized carboxyl group. The highest frequency N—H bending peak is often very weak.

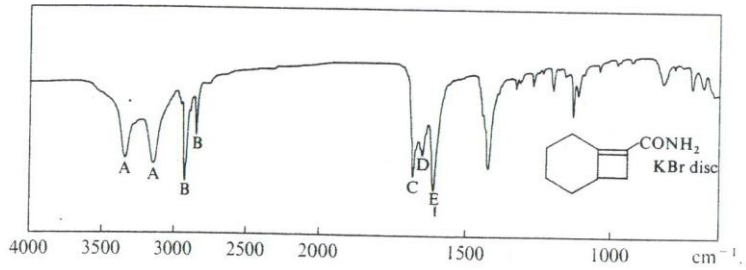


Fig. 2–11

A	3340 and 3140 cm.$^{-1}$	Typical amide —NH_2 pair of bands
B	2840 and 2930 cm.$^{-1}$	Saturated C—H
C	1680 cm.$^{-1}$	Amide I
D	1650 cm.$^{-1}$	Amide II
E	1610 cm.$^{-1}$	Conjugated and strained C=C

This spectrum shows the pair of N—H stretching bands and the pair of bands in the C=O region typical of primary amides in the solid state. The C=C peak appears here as an unusually strong peak. The amide I and II bands are not always so well resolved in the solid state.

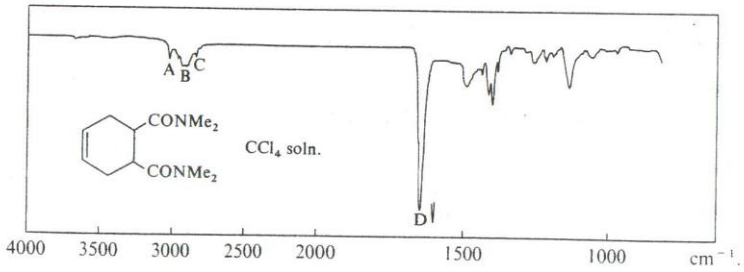


Fig. 2–12

A	3020 cm.$^{-1}$	Olefinic C—H stretch
B	2920 cm.$^{-1}$	Saturated C—H stretch
C	2830 cm.$^{-1}$	N—CH_3 C—H stretch
D	1650 cm.$^{-1}$	Tertiary amide C=O

This spectrum shows the absence of N—H, the strong sharp C=O of a tertiary amide, and, because of the symmetry of the molecule, no C=C stretching absorption.

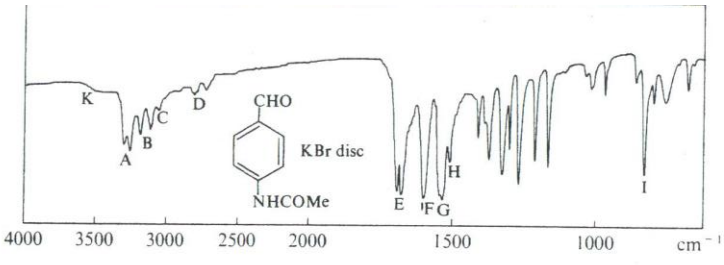


Fig. 2–13a

A	3300 and 3260 cm.$^{-1}$	Secondary amide N—H
B	3190 and 3110 cm.$^{-1}$	Secondary amide bands of unknown origin
C	3060 cm.$^{-1}$	Aryl C—H
D	2810 and 2730 cm.$^{-1}$	Aldehyde C—H
E	1695 and 1680 cm.$^{-1}$	Aldehyde C=O and amide I
F	1600 cm.$^{-1}$	Benzene ring
G	1535 cm.$^{-1}$	Amide II
H	1510 cm.$^{-1}$	Benzene ring
I	835 cm.$^{-1}$	*p*-Disubstituted benzene ring
K		Shoulder of OH band from traces of water in the KBr

This spectrum shows the multiplicity of bands found with secondary amides. The presence of so many bands in the spectra of such compounds as secondary amides is probably caused by the many ways in which such groupings can associate with each other, of which those shown on p. 51 are only two of many possibilities.

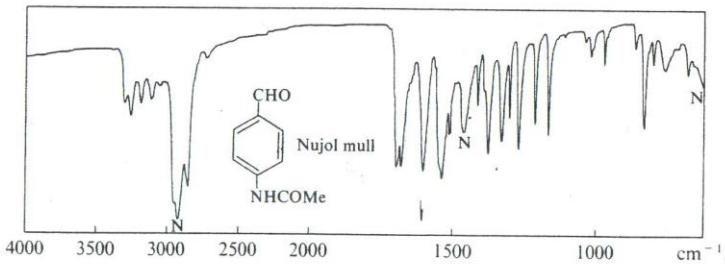


Fig. 2–13b

This spectrum was taken on the same compound as that in Fig. 2–13a, but was done as a Nujol mull. The main features are closely similar, but it can be seen how one of the Nujol peaks (labelled N) can obscure an important peak such as one of those labelled D on Fig. 2–13a. On the other hand, the band marked K on Fig. 2–13a is no longer present.

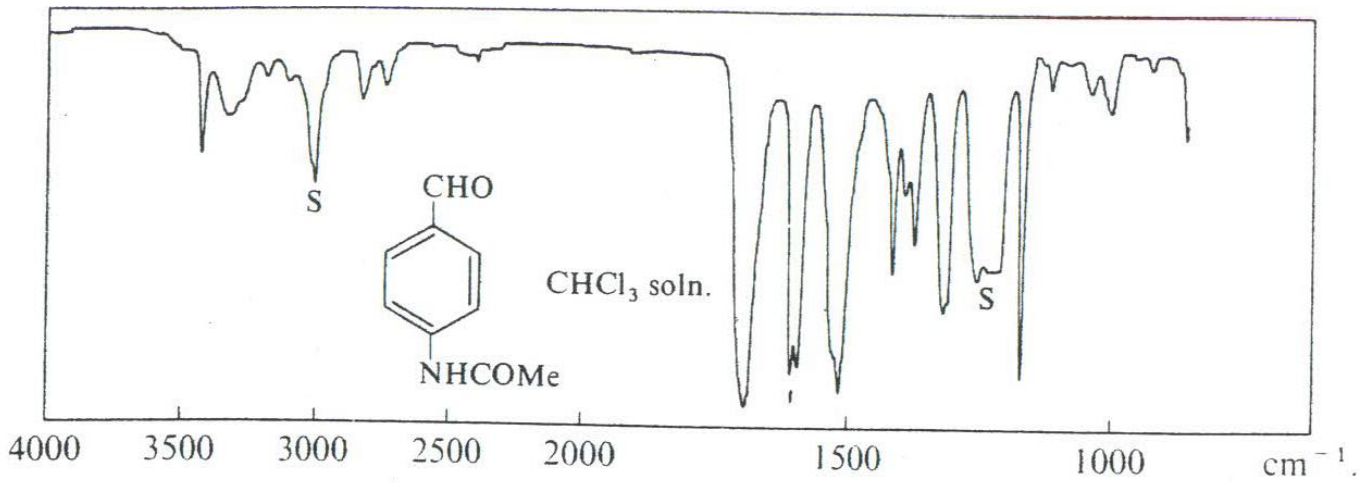


Fig. 2–13c

This is again a spectrum of the same compound, but taken in solution. This time some changes in appearance are apparent. The N—H stretch region is markedly different, and the amide I band has moved to a slightly higher frequency, making it coincident with the aldehyde C=O band. These changes are expected of a secondary amide, where a change in the nature of the intermolecular associations occurs in going from the solid state into the solution state. Such changes most affect the vibration frequencies of the functional groups involved in those associations.

The benzene ring band near 1600 $cm.^{-1}$ is now resolved into the two bands often found here: solution-state spectra are often better resolved than solid-state spectra. On the other hand there are many more strong lines in the fingerprint region in the solid state spectra.

The bands marked S are partly due to the solvent, because the absorption of the solvent has been incompletely cancelled (see p. 37).

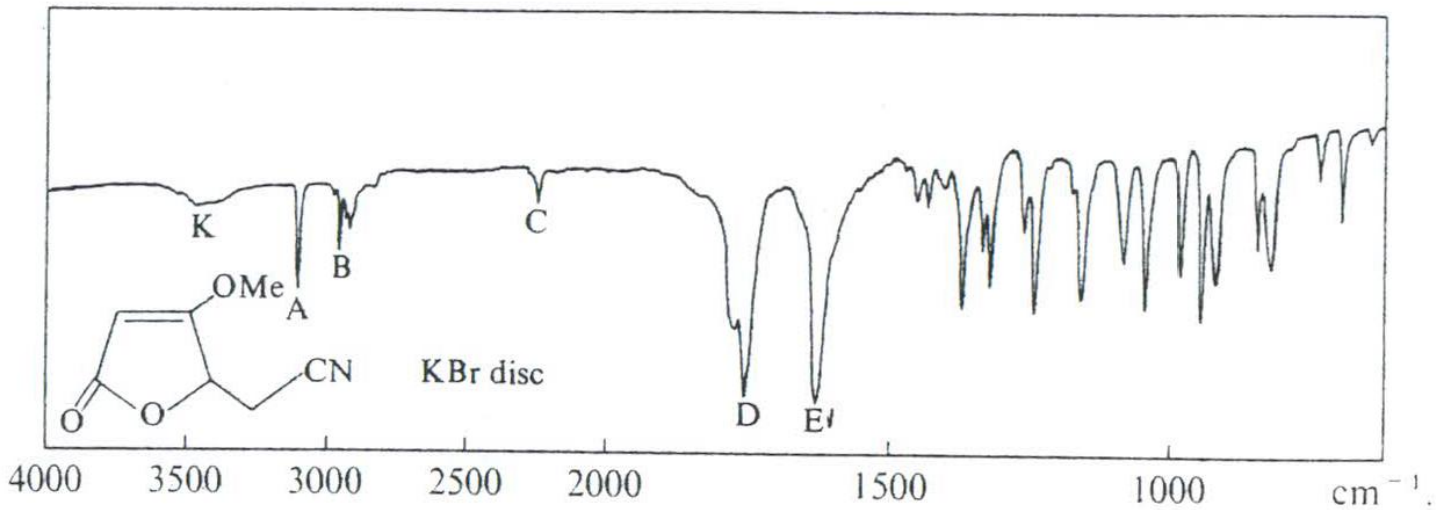


Fig. 2–14

A	3100 cm.$^{-1}$	Vinyl C—H stretch
B	2960–2900 cm.$^{-1}$	Saturated C—H stretch
C	2250 cm.$^{-1}$	Unconjugated C≡N stretch
D	1770 and 1755 cm.$^{-1}$	α,β-Unsaturated-γ-lactone C=O stretch
E	1630 cm.$^{-1}$	Vinyl ether C=C stretch

This spectrum shows how weak the unconjugated C≡N absorption can be, and how $\alpha\beta$-unsaturation, which lowers the frequency of the vibrations of the carbonyl group, combines with the presence of a five-membered ring, which raises the frequency, to give a band at 1755 cm.$^{-1}$, near the normal position of saturated esters and six-membered ring lactones. The extra band at 1770 cm.$^{-1}$ is common with $\alpha\beta$-unsaturated five-membered ring lactones having an α—H.

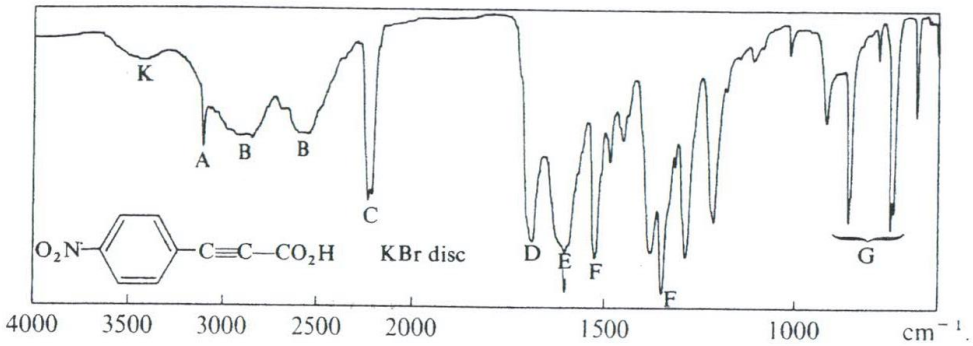


Fig. 2–9

A	3100 cm.$^{-1}$	Aryl C—H stretching
B	3200–2400 cm.$^{-1}$	Characteristic strongly H-bonded O—H of carboxylic acid
C	2225 cm.$^{-1}$	Conjugated C≡C, hence strong
D	1690 cm.$^{-1}$	Conjugated —CO_2H
E	1605 cm.$^{-1}$	Benzene ring, unusually broad and unresolved. A band near 1500 cm.$^{-1}$ is masked
F	1520 and 1350 cm.$^{-1}$	Conjugated nitro group—NO_2
G	950–650 cm.$^{-1}$	An example of a substituted ring in which it is not possible to decide with any certainty, due to the large number of bands in the region, in favour of 1, 4-disubstitution. For an example where the assignment can be made with confidence see Fig. 2–10
K		OH of water almost always present even in good KBr discs like this one

2.12. Groups Absorbing in the Fingerprint Region

Table 2–17

Sulphur Compounds

Group	Band	Remarks
—S—H	2600–2550(w)	S—H stretching; weaker than O—H and less affected by H-bonding. This absorption is strong in the Raman
>C=S	1200–1050(s)	
>C(=S)—N<	~3400	N—H stretching; lowered to ~3150 $cm.^{-1}$ in the solid state
	1550–1460(s)	Amide II
	1300–1100(s)	Amide I
>S=O	1060–1040(s)	
$>SO_2$	1350–1310(s) 1160–1120(s)	
$—SO_2—N<$	1370–1330(s) 1180–1160(s)	
$—SO_2—O—$	1420–1330(s) 1200–1145(s)	

Table 2–18

Phosphorus Compounds

Group	Band	Remarks
P—H	2440–2350(s)	Sharp
P—Ph	1440(s)	Sharp
P—O-alkyl	1050–1030(s)	
P—O-aryl	1240–1190(s)	
P=O	1300–1250(s)	
P—O—P	970–910	Broad
P(=O)—OH	2700–2560	H-bonded O—H
	1240–1180(s)	P=O stretching

Table 2–19

Ethers

Group	Band	Remarks
>C—O—C<	1150–1070(s)	C—O stretching
=C(—)—O—C<	1275–1200(s) 1075–1020(s)	
C—O—CH_3	2850–2810(m)	C—H stretching; aryl ethers at higher end of the range
>C(—O—)C<	~1250 ~900 ~800	

Table 2–20

Halogen Compounds

Group	Band	Remarks
C—F	1400–1000(s)	
C—Cl	800–600(s)	
C—Br	750–500(s)	
C—I	~500(s)	

Table 2–21

Inorganic Ions

Group	Band	Remarks
Ammonium	3300–3030	All bands strong
Cyanide, Thiocyanate, Cyanate	2200–2000	
Carbonate	1450–1410	
Sulphate	1130–1080	
Nitrate	1380–1350	
Nitrite	1250–1230	
Phosphates	1100–1000	

Table 2–3

Alkene and Aromatic C—H

See also Table 2–13 and Table 2–14 for the corresponding double bond absorptions, and Table 2–15 for the aromatic C—H out-of-plane bending vibrations.

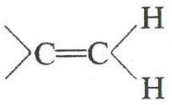
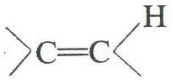
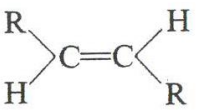
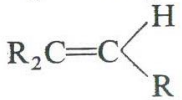
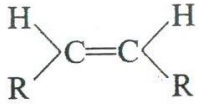
Group	Band	Remarks
—C≡C—H	~3300(s)	
	3095–3075(m)	C—H stretching; sometimes obscured by the much stronger bands of saturated C—H groups which occur below 3000 $cm.^{-1}$
	3040–3010(m)	(See Fig. 2–12)
Aryl—H	3040–3010(w)	Often obscured (but see Fig. 2–9)
	970–960(s)	C—H out-of-plane deformation. When the double bond is conjugated with, for example, a C=O group this band is shifted towards 990 $cm.^{-1}$
$RCH{=}CH_2$	995–985(s) and 940–900(s)	
$R_2C{=}CH_2$	895–885(s)	
	840–790(m)	
	730–675(m)	

Table 2–4

Alcohol and Phenol —O—H

Group	Band	Remarks
Water in solution	3710	
Free —OH	3650–3590(v)	Sharp; O—H stretching (see Fig. 2–8)
H-bonded —OH (solid, liquid and dilute solution)	3600–3200(s)	Often broad but may be sharp for some intramolecular single bridge H-bonds; the lower the frequency the stronger the H-bond (see Fig. 2–8)
Intramolecular H-bonded —OH in chelate form (see also Table 2–10, carboxylic acids)	3200–2500(v)	Broad; the lower the frequency the stronger the H-bond; sometimes so broad as to be overlooked
Water of crystallization (solid state spectra)	3600–3100(w)	Usually a weak band at 1640–1615 $cm.^{-1}$ also; water in trace amounts in KBr discs shows a broad band at 3450 $cm.^{-1}$ (see Fig. 2–9)
—O—H	1410–1260(s)	O—H bending
>C—OH	1150–1040(s)	C—O stretching (see Fig. 2–8)

Chapter 2 Principles of Capillary Electrophoresis

2. 1 Introduction

Electrophoresis is an analytical separation method that uses differential rate of migration of charge species in the presence of a buffer solution and a dc electric field. The method is applicable to inorganic ions, amino acids, catecholamine's, drugs, vitamins, carbohydrates, peptides, proteins (enzymes, hormones, and antibodies), nucleic acids (DNA, RNA), nucleotides etc.

It has the unique ability to separate charged macromolecules with good resolutions. For example it has been used to separate polynucleotides with 200-500 bases that differ by only one nucleotide and had been the power house for the Human Genome project. A typical electrophoresis set up is shown in Figure 5.1

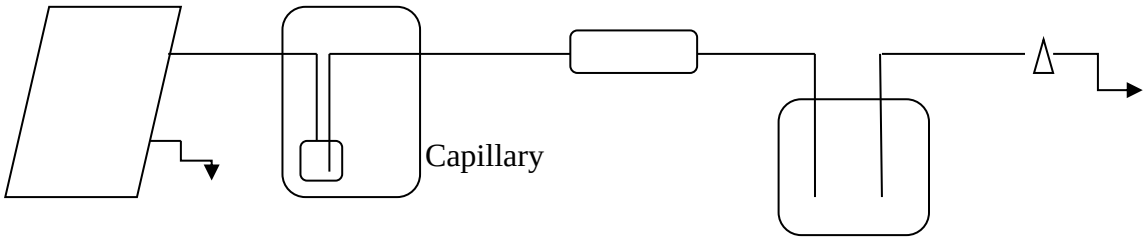


Figure 2.1 Schematic of an Electrophoresis set up

2.2 Types of Electrophoresis

There are two major types of electrophoresis: (i) slab electrophoresis (SE): a thin flat layer of gel containing an aqueous buffer within its pores. Separated species is stained and identified much like that of thin layer chromatography. Quantitation is hard to do with this method (ii) capillary electrophoresis (CE) is the instrumental version of electrophoresis using capillary tubes filled with buffer. The sample is introduced at one end and the separated ions are detected at the other end. Quantitation is possible with this method

2.3 Method of Separation

Samples are spotted on a flat porous support (cellulose acetate paper or a gel) or at one end of a narrow tube containing a buffer. A high voltage electric field is applied across the length of the buffer by electrodes located at each end of the buffer. Electrical field will cause the sample ions to migrate towards one of the electrodes and the *rate of migration depends on the charge/size ratio* of the sample ions. If the charge/size ratio is large the sample ions migrates very fast in the electric field. *For ions of the same size but different charges, sample ions with the larger charge moves faster than the one with the smaller charge. If the ions are of the same charge but*

different sizes, the smaller ion will experience less frictional retardation and hence will move faster. Only *one phase is used in electrophoresis* unlike in chromatographic techniques

2.4 Instrumentation

- A very important aspect of CE is sample introduction
- There are two main methods of sample introduction: *Electrokinetic injection and pressure injection*
- *Electrokinetic injection:* One end of the capillary column and its electrode is removed from the buffer and placed in the sample holder. High voltage is applied for an amount of time. This makes the sample to enter the column by ionic migration and electroosmotic flow. The capillary end and its electrode is then returned to the buffer for separation. This method introduces larger amount of mobile ions than slower ions
- *Pressure injection:* sample introduction end of the capillary column in the sample container and a pressure difference drives the sample solution into the capillary. The pressure difference is created by applying a vacuum at the detector end or elevating the sample end (hydrodynamic injection or pressurizing the sample. This method does not discriminate ion based on mobility but it is not suitable for gel electrophoresis
- Typical injection volume is about 5-50 nL
- *Detection*: Detectors applied to CE are similar to that of HPLC.

Common detection methods includes: Absorption, fluorescence, electrochemical and mass spectrometry, diode array, fid etc

2.5 Basis for Elecrophoretic Separations

The migration rate of the sample ions in an electric field, *v*, is

$$v = \mu_e E \qquad (1)$$

where μ_e = the Elecrophoretic mobility ($cm^{2/}Vs$) and Electric field (V/cm) which acts only on ions.

μ_e is directly proportional to the charge on the ion and inversely to the frictional retarding factors. The *frictional retarding forces depend on the size, shape of ions, and the viscosity of the migration medium.*

Ions experience different frictional forces are have different charges; hence, they are easily separated from each other.

2. 6 Capillary Electrophoresis (CE)

Electrophoresis performed with a capillary is faster, gives good resolution, needs a small sample size (0.1-10 nL range) and can be detected (using the same detectors used in chromatography) and quantified. The *migration rate of sample ions(v) depends on the electric field strength. The electric field is directly proportional to the applied voltage (V) and inversely to the length over which the electric field is applied.* Therefore,

$$v = \mu_e \text{ x } V/L \quad (2)$$

Equation 2 indicates that high voltage and short capillary (slab) length is desirable for quick separations in CE.

2.7 Plates Height in CE

- CE is not a chromatographic process but CE separation process is treated in the same manner like chromatography. In chromatography, longitudinal and mass transfer processed contributes largely to band broadening. This is not so with CE. Only longitudinal, contribution of joule heat (from the voltage source) and the injection process contributes to band broadening in CE. The plate count is obtained with equation 3
- $N = \mu_e V/2D = 16(t_m/W)^2$ (3) where D is the diffusion coefficient of the solute (cm^2/s) and W is the base peak width
- Equation 3 shows that plate count and resolution increases with high V and not with the length of the capillary column
- With slab electrophoresis, joule heating limits the size of the voltage that can be applied to 500V but not so with CE because of the following reasons: (i) the capillary is long with small X-sectional area (ii) the solution resistance is very high (iii) Power dissipation is inversely proportional to resistance (P=I2/R) hence, CE solution resistance that is high results into little heat dissipation and higher voltage can be applied to CE than slab electrophoresis (iv) capillaries have high surface/volume ratio which enhances cooling.
- Because of reason (iii) and (iv) convective mixing due to heat does not occur with CE, therefore, band broadening is lessened
- Typical applied voltages for SE and CE: 100-400 V/cm
- Typical power source voltage: 10-25 kV
- Peak width with CE is limited by theoretical limits set by the longitudinal process
- Typical value of N for CE: 100,000-200,000 HPLC: 5000- 20000
- N = 3 million has been reported for capillary zone electrophoresis (CZE) and 10 million for polynucleotide's.

2.8 Contribution of Electroosmotic Flow

- Electroosmotic flow is the bulk movement of the buffer solution to the cathode when high voltage power is applied to the capillary tube.
- The rate of migration is substantial. A 50mM pH 8 buffer flows through a 50cm column at 5cm/min with 25kV voltage
- ***Process:*** At pH ≥ 3, the inside surface of silica capillary wall is negatively charged (surface silanol (Si-OH) is ionized)
- This attracts all the buffer cations and a double layer is formed
- All the outer cations in the double layer are solvated and are attracted to the cathode and therefore, drags along the bulk buffer solution to produce Electroosmotic flow
- The end result of this is a *laminar flow (flat profile)* across the tube instead of the parabolic flow typical of high pressure pump induce flow of HPLC
- The flat profile does not contribute to band broadening
- Electroosmotic flow is > than electrophoretic flow of the individual analyte ions and hence it is considered at the pump device for CE
- The strength of electroosmotic flow is high enough such that it drags along all ± and neutral ions towards the same end of the capillary for detection
- An electropherogram is obtained for these ions but with narrow peaks compared to that of a HPLC chromatogram
- The order of separation of CE ions is: fastest cation> slower cations> neutrals in a single zone> slowest anions> fastest anion
-

2.9 Variations of CE

- There are other variations of CE such as () capillary zone electrophoresis (CZE): applied field separate ions into zone according to their mobility (ii) Capillary gel electrophoresis (CGE): uses porous gel polymer matrix with a buffer for separation (iii) Capillary Isotachophoresis: all bands migrate at the same velocity. Sample is injected between two buffers with the leading one containing a fast moving ion (chloride) and the terminating one containing a slow moving ions (heptanoate) (iv) capillary isoelectric focusing: used to separate amphiprotic species: amino acids and proteins (v) Micellar Electrokinetic chromatography
-

Chapter3 Ion Exchange Chromatography

3. 1. Ion-exchange chromatography (or *ion chromatography*) is a process that allows the separation of ions and polar molecules based on their charge. It can be used for almost any kind of charged molecule including large proteins, small nucleotides and amino acids. It is often used in protein purification, water analysis, and quality control.

Ion exchangers have macromolecular base (polymer or a resin). Resins can be natural (Zeolite) or synthetic. The resin can ionize to either positive or negative ions (sodium aluminosilicate, Zeolite) and a counter ion (could be group I or II ions). This type of chromatography is subdivided into cation exchange and anion exchange chromatography depending on the charge of the counter ion. Cation exchange chromatography retains positively charged cations because the stationary phase displays a negatively charged functional group:

$$\mathrm{R\text{-}X^-C^+ + M^+B^- \rightleftarrows R\text{-}X^-M^+ + C^+ + B^-}$$

Anion exchange chromatography retains anions using positively charged functional group:

$$\mathrm{R\text{-}X^+A^- + M^+B^- \rightleftarrows R\text{-}X^+B^- + M^+ + A^-}$$

The resin's charge is fixed while the counter ions are mobile or can be exchanged with other ions. When water containing other ions (Ca^{2+}, Fe^{3+}, Mg^{2+} called the analyte) is passed through the ion-exchanger, the counter ions (Na^+, K^+ etc) will be replaced by these ions and washed out of the system. Ion-exchange chromatography retains analyte molecules on the column based on coulombic (ionic) interactions. The resin can be regenerated by washing the resin again with appropriate solutions containing counter ions of interest; therefore, the undesired counter ion will be removed from the column. (Z- natural resin, Use R= synthetic resin)

$MgZ + NaCl_{aq} \rightarrow NaZ + Mg^{2+}_{aq}$ – regeneration

$MR + 2H^+ \rightarrow H_2R + M^{2+}$ - regeneration

$H_2R + Mg^{2+} \rightarrow MgR + 2H^+_{aq}$ – ion exchange process (2 moles of H^+ ion is exchanged for each mole of the group 2 cation)

The ion chromatogram shown if Figure 6.1 is obtained with an anion exchange column.

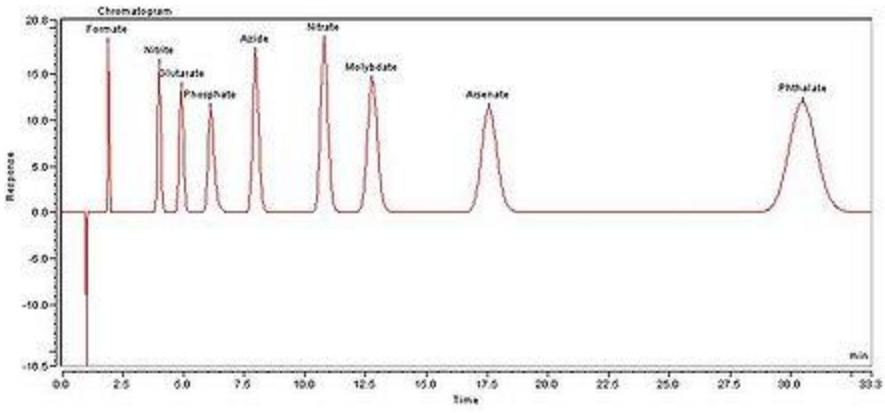


Figure 6.1 Anion exchange Chromatogram

3.2 Typical technique

A sample is introduced, either manually or with an autosampler, into a sample loop of known volume. A buffered aqueous solution known as the mobile phase carries the sample from the loop onto a column that contains some form of stationary phase material. This is typically a resin or gel matrix consisting of Agarose or cellulose beads with covalently bonded charged functional groups. The target analytes (anions or cations) are retained on the stationary phase but can be eluted by increasing the concentration of a similarly charged species that will displace the analyte ions from the stationary phase. For example, in cation exchange chromatography, the positively charged analyte could be displaced by the addition of positively charged sodium ions. The analytes of interest must then be detected by some means, typically by conductivity or UV/Visible light absorbance.

In order to control an IC system, a chromatography data system (CDS) is usually needed. In addition to IC systems, some of these CDSs can also control gas chromatography (GC) and HPLC. A typical ion exchange column is shown in Figure 6.2 below

3.3 Separating protein

Figure 3.2 Preparative-scale ion exchange column used for protein purification

Proteins have numerous functional groups that can have both positive and negative charges. Ion exchange chromatography separates proteins according to their net charge, which is dependent on the composition of the mobile phase. By adjusting the pH or the ionic concentration of the mobile phase, various protein molecules can be separated. For example, if a protein has a net positive charge at pH 7, then it will bind to a column of negatively charged beads, whereas a

negatively charged protein would not. By changing the pH so that the net charge on the protein is negative, it too will be eluted.

Elution by changing the ionic strength of the mobile phase is a more subtle effect - it works as ions from the mobile phase will interact with the immobilized ions in preference over those on the stationary phase. This "shields" the stationary phase from the protein, (and vice versa) and allows the protein to elute.

Separation can be achieved based on the natural isoelectric point of the protein. Alternatively a peptide tag can be genetically added to the protein to give the protein an isoelectric point away from most natural proteins (e.g. 6 argentines for binding to cation-exchange resin such as DEAE-Sepharose or 6 glutamates for binding to anion-exchange resin).

Elution from ion-exchange columns can be sensitive to changes of a single charge-chromato-focusing. Ion-exchange chromatography is also useful in the isolation of specific multimeric protein assemblies, allowing purification of specific complexes according to both the number and the position of charged peptide tags

3.4 Clinical utility

IC is used in measurement of HbA1c, porphyrin & water purification.

Assignment: After being passed through a column containing ion exchange resin H_2R, a 100.0 mL sample of hard water requires 15.17 mL 0f 0.0265M NaOH for its titration. What is the hardness of water, expressed as ppm of Ca^{2+}?

Answer

Step 1. When the water is passed through the resin, the following reaction happens

$H_2R + Mg^{2+} \rightarrow MgR + 2H^+_{aq}$ then the $2H^+_{aq}$ ion are titrated as shown below

Titration Reaction: $H^+ + OH^- \rightarrow H_2O$

# of mmoles H^+ = 0.01517L NaOH x 0.0265 mmoleNaOH/mLNaOH x mmoleH/mmoleNaOH = 4.02×10^{-4} mmole H^+

Step 2. Determine the mole of Ca2+

For every mmole of Ca^{2+} 2 mmoles of H^+ is exchanged- ($H_2R + Mg^{2+} \rightarrow MgR + 2H^+_{aq}$)

2 mmoles of H^+ = 1 mmole of Ca^{2+}

0.402×10^{-4} mmol H^+ = 4.02×10^{-4}/2mmol Ca^{2+} = 2.01×10^{-4}mmole Ca^{2+} is in 0.1L of water

Step 3: Determine the gram of Ca^{2+} in 100 mL of water sample (At. Weight Ca = 40.08 g/mol)

# of mg of Ca^{2+} = 2.01 x 10^{-4} mmole x 40.08 mg/mmol Ca^{2+}

= 8.06 x $^{10-3}$mg

Step 4. Express grams of Ca^{2+} in parts per million (ppm)

8.06 x $^{10-3}$ mg of Ca^{2+} is in 100 mL of water = 8.06 x $^{10-3}$ mg of Ca^{2+} in 0.1L of water

In 1L of water, the # of mg of Ca^{2+} ion is = (8.06x10^{-3} x 1x10^{4} mg) = 80.6 mg Ca^{2+} /L = 80.6 ppm

3.5 Affinity Chromatography

Affinity chromatography is a method of separating biochemical mixtures based on a highly specific interaction such as that between antigen and antibody, enzyme and substrate, or receptor and ligand. Affinity chromatography can be used to:

- Purify and concentrate a substance from a mixture into a buffering solution
- Reduce the amount of a substance in a mixture
- Discern what biological compounds bind to a particular substance
- Purify and concentrate an enzyme solution.

3.6 Principle

The stationary phase is typically a gel matrix, often of Agarose; a linear sugar molecule derived from algae. Usually the starting point is an undefined heterogeneous group of molecules in solution, such as a cell lysate, growth medium or blood serum. The molecule of interest will have a well known and defined property, and can be exploited during the affinity purification process. The process itself can be thought of as an entrapment, with the target molecule becoming trapped on a solid or stationary phase or medium. The other molecules in the mobile phase will not become trapped as they do not possess this property. The stationary phase can then be removed from the mixture, washed and the target molecule released from the entrapment in a process known as elution. Possibly the most common use of affinity chromatography is for the purification of recombinant proteins.

3.7 Batch and column setup: Batch, hybrid and expanded bed adsorption methods

Binding to the solid phase may be achieved by column chromatography whereby the solid medium is packed onto a column, the initial mixture run through the column to allow setting, a wash buffer run through the column and the elution buffer subsequently applied to the column and collected. These steps are usually done at ambient pressure. Alternatively, binding may be achieved using ***a batch treatment***, for example, by adding the initial mixture to the solid phase in a vessel, mixing, separating the solid phase, removing the liquid phase, washing, re-centrifuging, adding the elution buffer, re-centrifuging and removing the eluate.

Sometimes a ***hybrid method*** is employed such that the binding is done by the batch method, but the solid phase with the target molecule bound is packed onto a column and washing and elution are done on the column.

A third method, ***expanded bed adsorption***, which combines the advantages of the two methods mentioned above, has also been developed. The solid phase particles are placed in a column where liquid phase is pumped in from the bottom and exits at the top. The gravity of the particles ensures that the solid phase does not exit the column with the liquid phase.

Affinity columns can be eluted by changing salt concentrations, pH, pI, charge and ionic strength directly or through a gradient to resolve the particles of interest.

3.8 Specific Uses: Affinity chromatography can be used in a number of applications, including nucleic acid purification, protein purification from cell free extracts, and purification from blood.

Immunoaffinity

Another use for the procedure is the affinity purification of antibodies from blood serum. If serum is known to contain antibodies against a specific antigen (for example if the serum comes from an organism immunized against the antigen concerned) then it can be used for the affinity purification of that antigen. This is also known as Immunoaffinity Chromatography. For example if an organism is immunized against a GST-fusion protein it will produce antibodies against the fusion-protein, and possibly antibodies against the GST tag as well. The protein can then be covalently coupled to a solid support such as agarose and used as an affinity ligand in purifications of antibody from immune serum.

For thoroughness the GST protein and the GST-fusion protein can each be coupled separately. The serum is initially allowed to bind to the GST affinity matrix. This will remove antibodies against the GST part of the fusion protein. The serum is then separated from the solid support and allowed to bind to the GST-fusion protein matrix. This allows any antibodies that recognize the antigen to be captured on the solid support. Elution of the antibodies of interest is most often achieved using a low pH buffer such as glycine pH 2.8. The eluate is collected into a neutral tris or phosphate buffer, to neutralize the low pH elution buffer and halt any degradation of the antibody's activity. This is a nice example as affinity purification is used to purify the initial GST-fusion protein, to remove the undesirable anti-GST antibodies from the serum and to purify the target antibody.

A simplified strategy is often employed to purify antibodies generated against peptide antigens. When the peptide antigens are produced synthetically, a terminal cysteine residue is added at either the N- or C-terminus of the peptide. This cysteine residue contains a sulfhydryl functional group which allows the peptide to be easily conjugated to a carrier protein (e.g. Keyhole Limpet Hemocyanin (KLH)). The same cysteine-containing peptide is also immobilized onto an agarose resin through the cysteine residue and is then used to purify the antibody.

Most monoclonal antibodies have been purified using affinity chromatography based on immunoglobulin-specific Protein A or Protein G, derived form bacteria.

3.9 Immobilized metal ion affinity chromatography

Immobilized metal ion affinity chromatography (IMAC) is based on the specific coordinate covalent bond of amino acids, particularly histidine, to metals. This technique works by allowing proteins with an affinity for metal ions to be retained in a column containing immobilized metal ions, such as cobalt, nickel, copper for the purification of histidine containing proteins or peptides, iron, zinc or gallium for the purification of phosphorylated proteins or peptides. Many naturally occurring proteins do not have an affinity for metal ions, therefore recombinant DNA technology can be used to introduce such a protein tag into the relevant gene. Methods used to elute the protein of interest include changing the pH, or adding a competitive molecule, such as imidazole.

A chromatography column containing nickel-agarose beads used for purification of proteins with histidine tags

3. 10 Recombinant proteins

Possibly the most common use of affinity chromatography is for the purification of recombinant proteins. Proteins with a known affinity are protein tagged in order to aid their purification. The protein may have been genetically modified so as to allow it to be selected for affinity binding; this is known as a fusion protein. Tags include glutathione-S-transferase (GST), hexahistidine (His), and maltose binding protein (MBP). Histidine tags have an affinity for nickel or cobalt ions which have been immobilized by forming coordinate covalent bonds with a chelator incorporated in the stationary phase. For elution, an excess amount of a compound able to act as a metal ion ligand, such as imidazole, is used. GST has an affinity for glutathione which is commercially available immobilized as glutathione agarose. During elution, excess glutathione is used to displace the tagged protein.

3.11 Lectins

Lectin affinity chromatography is a form of affinity chromatography where lectins are used to separate components within the sample. Lectins, such as Concanavalin A are proteins which can bind specific carbohydrate (sugar) molecules. The most common application is to separate glycoproteins from non-glycosylated proteins, or one glycoform from another glycoform.

4. 0. Size Exclusion Chromatography (SEC) or Gel Filtration

Introduction: Size-exclusion chromatography (SEC) is a chromatographic method in which molecules in solution are separated by their size, and in some cases molecular weight. It is usually applied to large molecules or macromolecular complexes such as proteins and industrial polymers. Typically, when an aqueous solution is used to transport the sample through the column, the technique is known as **gel-filtration chromatography**, versus the name **gel permeation chromatography**, which is used when an organic solvent is used as a mobile phase. SEC is a widely used polymer characterization method because of its ability to provide good molar mass distribution (Mw) results for polymers.

4.1 Applications

The main application of gel-filtration chromatography is the fractionation of proteins and other water-soluble polymers, while gel permeation chromatography is used to analyze the molecular weight distribution of organic-soluble polymers. Either technique should not be confused with gel electrophoresis, where an electric field is used to "pull" or "push" molecules through the gel depending on their electrical charges.

4.2 Theory and method

One requirement for SEC is that the *analyte does not interact with the surface of the stationary phases*, with differences in elution time between analytes ideally being based solely on the volume the analytes. Thus, a small molecule that can penetrate every region of the stationary phase pore system "sees" a total volume equal to the sum of the entire pore volume and the inter-particle volume. This small molecule will elute late (when the pore- and inter-particle volume has passed through the column ~80% of the column volume). On the other extreme, a very large molecule that cannot penetrate any region of the pore system "sees" only the inter-particle volume (~35% of the column volume) and will elute earlier when this volume of mobile phase has passed through the column. The underlying principle of SEC is that particles of different sizes will elute (filter) through a stationary phase at different rates. This results in the separation of a solution of particles based on size. Provided that all the particles are loaded simultaneously or near-simultaneously, particles of the same size should elute together.

However, as there are various measures of the size of a macromolecule (for instance, the radius of gyration and the hydrodynamic radius), a fundamental problem in the theory of SEC has been the choice of a proper molecular size parameter by which molecules of different kinds are separated. The observed correlation based on the hydrodynamic volume became accepted as the basis of universal SEC calibration.

Each size exclusion column has a range of molecular weights that can be separated. The exclusion limit defines the molecular weight at the upper end of the column 'working' range and is where molecules are too large to be trapped in the stationary phase. The lower end of the range

is defined by the permeation limit, which defines the molecular weight of molecules that is small to penetrate all pores of the stationary phase. All molecules below this molecular mass are so small that they elute as a single band

Figure 4.1 A size exclusion column

This is usually achieved with an apparatus called a column, which consists of a hollow tube tightly packed with extremely small porous polymer beads designed to have pores of different sizes. These pores may be depressions on the surface or channels through the bead. As the solution travels down the column some particles enter into the pores. Larger particles cannot enter into as many pores; the larger the particles, the faster the elution. The filtered solution that is collected at the end is known as the **eluate**. The **void volume** includes any particles too large to enter the medium, and the solvent volume is known as the **column volume**.

4.3 Factors affecting filtration

In real-life situations, particles in solution do not have a fixed size, resulting in the probability that a particle that could be hampered by a pore passing right by it. Also, the stationary-phase particles are not ideally defined; both particles and pores may vary in size. Elution curves, therefore, resemble Gaussian distributions. The stationary phase may also interact in undesirable ways with a particle and influence retention times, though great care is taken by column manufacturers to use stationary phases that are inert and minimize this issue.

Like other forms of chromatography, increasing the column length will enhance the resolution, and increasing the column diameter increases the capacity of the column. Proper column packing is important to maximize resolution: An *over-packed* column can collapse the pores in the beads, resulting in a loss of resolution. An *under-packed* column can reduce the relative surface area of the stationary phase accessible to smaller species, resulting in those species spending less time trapped in pores. Unlike affinity chromatography techniques, a solvent head at the top of the column can drastically diminish resolution as the sample diffuses prior to loading, broadening the downstream elution.

4.4 Analysis

In simple manual columns, the eluent is collected in constant volumes, known as fractions. The more similar the particles are in size the more likely they will be in the same fraction and not detected separately. More advanced columns overcome this problem by constantly monitoring the eluent.

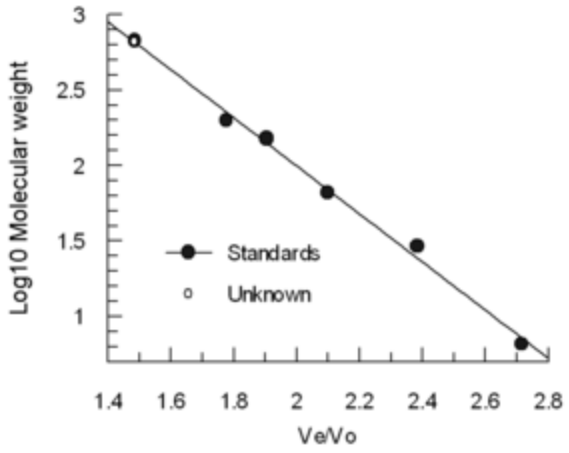


Figure 4.2 Standardization of a size exclusion column.

The collected fractions are often examined by spectroscopic techniques to determine the concentration of the particles eluted. Common spectroscopy detection techniques are refractive index (RI) and ultraviolet (UV). When eluting spectroscopically similar species (such as during biological purification), other techniques may be necessary to identify the contents of each fraction. It is also possible to analyze the eluent flow continuously with RI, Multi-Angle Laser Light Scattering MALS, UV, and/or viscosity measurements.

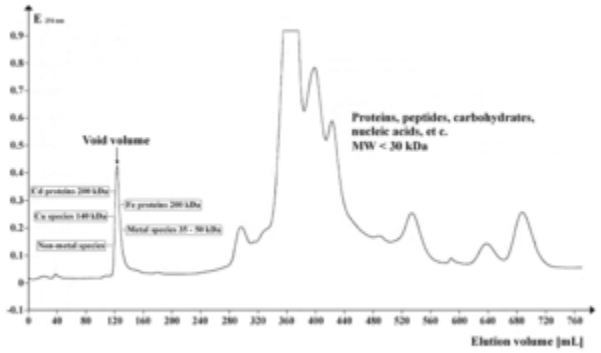


Figure 4.3 SEC Chromatogram of a biological sample

The elution volume (Ve) decreases roughly linear with the logarithm of the molecular hydrodynamic volume. Columns are often calibrated using 4-5 standard samples (e.g., folded proteins of known molecular weight), and a sample containing a very large molecule such as thyroglobulin to determine the void volume. (Blue dextran is not recommended for Vo determination because it is heterogeneous and may give variable results) The elution volumes of the standards are divided by the elution volume of the thyroglobulin (Ve/Vo) and plotted against the log of the standards' molecular weights.

4.5 Applications

(i) Biochemical applications

In general, SEC is considered a low resolution chromatography as it does not discern similar species very well, and is therefore often reserved for the final "polishing" step of purification. The technique can determine the quaternary structure of purified proteins that have slow exchange times, since it can be carried out under native solution conditions, preserving macromolecular interactions. SEC can also assay protein tertiary structure, as it measures the hydrodynamic volume (not molecular weight), allowing folded and unfolded versions of the same protein to be distinguished. For example, the apparent hydrodynamic radius of a typical protein domain might be 14 Å and 36 Å for the folded and unfolded forms, respectively. SEC allows the separation of these two forms, as the folded form will elute much later due to its smaller size.

(ii) Polymer synthesis

SEC can be used as a measure of both the size and the polydispersity of a synthesized polymer, that is, the ability to be able to find the distribution of the sizes of polymer molecules. If standards of a known size are run previously, then a calibration curve can be created to determine the sizes of polymer molecules of interest in the solvent chosen for analysis (often THF). In alternative fashion, techniques such as light scattering and/or viscometry4 can be used online with SEC to yield absolute molecular weights that do not rely on calibration with standards of known molecular weight. Due to the difference in size of two polymers with identical molecular weights, the absolute determination methods are, in general, more desirable. A typical SEC system can quickly (in about half an hour) give polymer chemists information on the size and polydispersity of the sample. The preparative SEC can be used for polymer fractionation on an analytical scale. .

4.6 Drawback

In SEC, mass is not measured so much as the hydrodynamic volume of the polymer molecules, that is; how much spaces a particular polymer molecule takes up when it is in solution. However, the approximate molecular weight can be calculated from SEC data because the exact relationship between molecular weight and hydrodynamic volume for polystyrene can be found. For this, polystyrene is used as a standard. But the relationship between hydrodynamic volume and molecular weight is not the same for all polymers, so only an approximate measurement can be obtained. Another drawback is the possibility of interaction between the stationary phase and the analyte. Any interaction leads to a later elution time and thus mimics a smaller analyte size

Chapter 5 Distillation

5.1 Distillation is a process of separating the component substances from a liquid mixture by selective evaporation and condensation. Distillation may result in essentially complete separation (nearly pure components), or it may be a partial separation that increases the concentration of selected components of the mixture. In either case the process exploits differences in the volatility of mixture's components. In industrial chemistry, distillation is a unit operation of practically universal importance, but it is a physical separation process and not a chemical reaction.

Commercially, distillation has many applications. For example:

- In the fossil fuel industry distillation is a major class of operation in obtaining materials from crude oil for fuels and for chemical feed stocks.
- Distillation permits separation of air into its components — notably oxygen, nitrogen, and argon — for industrial use.
- In the field of industrial chemistry, large ranges of crude liquid products of chemical synthesis are distilled to separate them, either from other products, or from impurities, or from unreacted starting materials.
- Distillation of fermented products produces distilled beverages with a high alcohol content, or separates out other fermentation products of commercial value.
- The application of distillation can roughly be divided in four groups: laboratory scale, industrial distillation, distillation of herbs for perfumery and medicinal (herbal distillate), and food processing. The latter two are distinctively different from the former two in that in the processing of beverages, the distillation is not used as a true purification method but more to transfer all volatiles from the source materials to the distillate.
- The main difference between laboratory scale distillation and industrial distillation is that laboratory scale distillation is often performed batch-wise, whereas industrial distillation often occurs continuously. In batch distillation, the composition of the source material, the vapors of the distilling compounds and the distillate change during the distillation. In batch distillation, a still is charged (supplied) with a batch of feed mixture, which is then separated into its component fractions which are collected sequentially from most volatile to less volatile, with the bottoms (remaining least or non-volatile fraction) removed at the end. The still can then be recharged and the process repeated.
- In continuous distillation, the source materials, vapors, and distillate are kept at a constant composition by carefully replenishing the source material and removing fractions from both vapor and liquid in the system. This results in a better control of the separation process

5.2 Idealized distillation model

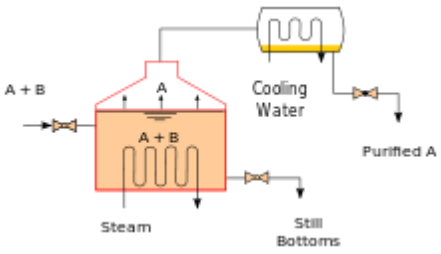
- The boiling point of a liquid is the temperature at which the vapor pressure of the liquid equals the pressure around the liquid, enabling bubbles to form without being crushed. A special case is the normal boiling point, where the vapor pressure of the liquid equals the ambient atmospheric pressure.
- It is a common misconception that in a liquid mixture at a given pressure, each component boils at the boiling point corresponding to the given pressure and the vapors of each component will collect separately and purely. This, however, does not occur even

in an idealized system. Idealized models of distillation are essentially governed by Raoult's law and Dalton's law, and assume that vapor–liquid equilibria are attained.

- Raoult's law states that the vapor pressure of a solution is dependent on 1) the vapor pressure of each chemical component in the solution and 2) the fraction of solution each component makes up aka the mole fraction. This law applies to ideal solutions, or solutions that have different components but whose molecular interactions are the same as or very similar to pure solutions.
- Dalton's law states that the total vapor pressure is the sum of the vapor pressures of each individual component in the mixture. When a multi-component liquid is heated, the vapor pressure of each component will rise, thus causing the total vapor pressure to rise. When the total vapor pressure reaches the pressure surrounding the liquid, boiling occurs and liquid turns to gas throughout the bulk of the liquid. Note that a mixture with a given composition has one boiling point at a given pressure, when the components are mutually soluble.
- An implication of one boiling point is that lighter components never cleanly "boil first". At boiling point, all volatile components boil, but for a component, its percentage in the vapor is the same as its percentage of the total vapor pressure. Lighter components have a higher partial pressure and thus are concentrated in the vapor, but heavier volatile components also have a (smaller) partial pressure and necessarily evaporate also, albeit being less concentrated in the vapor. Indeed, batch distillation and fractionation succeed by varying the composition of the mixture. In batch distillation, the batch evaporates, which changes its composition; in fractionation, liquid higher in the fractionation column contains more lights and boils at lower temperatures.
- The idealized model is accurate in the case of chemically similar liquids, such as benzene and toluene. In other cases, severe deviations from Raoult's law and Dalton's law are observed, most famously in the mixture of ethanol and water. These compounds, when heated together, form an azeotrope, which is a composition with a boiling point higher or lower than the boiling point of each separate liquid. Virtually all liquids, when mixed and heated, will display azeotropic behavior. Although there are computational methods that can be used to estimate the behavior of a mixture of arbitrary components, the only way to obtain accurate vapor–liquid equilibrium data is by measurement.
- It is not possible to *completely* purify a mixture of components by distillation, as this would require each component in the mixture to have a zero partial pressure. If ultra-pure products are the goal, then further chemical separation must be applied. When a binary mixture is evaporated and the other component, e.g. a salt, has zero partial pressure for practical purposes, the process is simpler and is called evaporation in engineering.

5.3 Batch distillation

Main article: Batch distillation



A batch still showing the separation of A and B.

Heating an ideal mixture of two volatile substances A and B (with A having the higher volatility, or lower boiling point) in a batch distillation setup (such as in an apparatus depicted in the opening figure) until the mixture is boiling results in a vapor above the liquid which contains a mixture of A and B. The ratio between A and B in the vapor will be different from the ratio in the liquid: the ratio in the liquid will be determined by how the original mixture was prepared, while the ratio in the vapor will be enriched in the more volatile compound, A (due to Raoult's Law, see above). The vapor goes through the condenser and is removed from the system. This in turn means that the ratio of compounds in the remaining liquid is now different from the initial ratio (i.e., more enriched in B than the starting liquid).

The result is that the ratio in the liquid mixture is changing, becoming richer in component B. This causes the boiling point of the mixture to rise, which in turn results in a rise in the temperature in the vapor, which results in a changing ratio of A : B in the gas phase (as distillation continues, there is an increasing proportion of B in the gas phase). This results in a slowly changing ratio A : B in the distillate.

If the difference in vapor pressure between the two components A and B is large (generally expressed as the difference in boiling points), the mixture in the beginning of the distillation is highly enriched in component A, and when component A has distilled off, the boiling liquid is enriched in component B.

5.4 Continuous Distillation

Continuous distillation is an ongoing distillation in which a liquid mixture is continuously (without interruption) fed into the process and separated fractions are removed continuously as output streams occur over time during the operation. Continuous distillation produces a minimum of two output fractions, including at least one volatile distillate fraction, which has boiled and been separately captured as a vapor, and then condensed to a liquid. There is always a bottoms (or residue) fraction, which is the least volatile residue that has not been separately captured as a condensed vapor.

Continuous distillation differs from batch distillation in the respect that concentrations should not change over time. Continuous distillation can be run at a steady state for an arbitrary amount of time. For any source material of specific composition, the main variables that affect the purity of products in continuous distillation are the reflux ratio and the number of theoretical equilibrium stages, in practice determined by the number of trays or the height of packing. Reflux is a flow from the condenser back to the column, which generates a recycle that allows a better separation with a given number of trays. Equilibrium stages are ideal steps where compositions achieve vapor–liquid equilibrium, repeating the separation process and allowing better separation given a reflux ratio. A column with a high reflux ratio may have fewer stages, but it refluxes a large amount of liquid, giving a wide column with a large holdup. Conversely, a column with a low reflux ratio must have a large number of stages, thus requiring a taller column.

5.5 General Improvements

Both batch and continuous distillations can be improved by making use of a fractionating column on top of the distillation flask. The column improves separation by providing a larger surface area for the vapor and condensate to come into contact. This helps it remain at equilibrium for as long as possible. The column can even consist of small subsystems ('trays' or 'dishes') which all contain an enriched, boiling liquid mixture, all with their own vapor–liquid equilibrium.

There are differences between laboratory-scale and industrial-scale fractionating columns, but the principles are the same. Examples of laboratory-scale fractionating columns (in increasing efficiency) include

- Air condenser
- Vigreux column (usually laboratory scale only)
- Packed column (packed with glass beads, metal pieces, or other chemically inert material)
- Spinning band distillation system.
- Laboratory scale distillations are almost exclusively run as batch distillations. The device used in distillation, sometimes referred to as a *still*, consists at a minimum of a **reboiler** or *pot* in which the source material is heated, a **condenser** in which the heated vapor is cooled back to the liquid state, and a **receiver** in which the concentrated or purified liquid, called the **distillate**, is collected. Several laboratory scale techniques for distillation exist (see also distillation types).

5.6 Simple distillation

- In **simple distillation**, the vapor is immediately channeled into a condenser. Consequently, the distillate is not pure but rather its composition is identical to the composition of the vapors at the given temperature and pressure. That concentration follows Raoult's law.
- As a result, simple distillation is effective only when the liquid boiling points differ greatly (rule of thumb is 25 °C) or when separating liquids from non-volatile solids or oils. For these cases, the vapor pressures of the components are usually different enough that the distillate may be sufficiently pure for its intended purpose.

5.7 Fractional Distillation

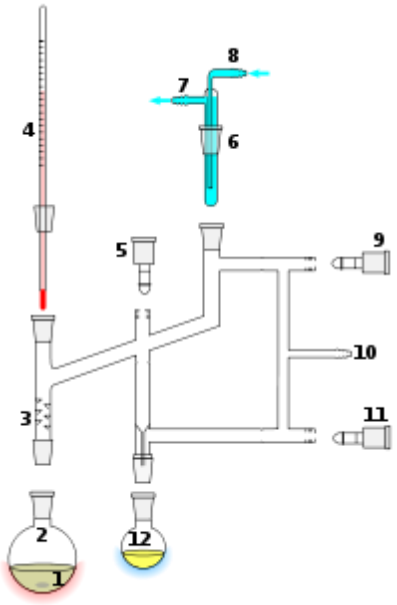
- For many cases, the boiling points of the components in the mixture will be sufficiently close that Raoult's law must be taken into consideration. Therefore, **fractional distillation** must be used in order to separate the components by repeated vaporization-condensation cycles within a packed fractionating column. This separation, by successive distillations, is also referred to as **rectification**.
- As the solution to be purified is heated, its vapors rise to the fractionating column. As it rises, it cools, condensing on the condenser walls and the surfaces of the packing material. Here, the condensate continues to be heated by the rising hot vapors; it vaporizes once more. However, the composition of the fresh vapors is determined once

again by Raoult's law. Each vaporization-condensation cycle (called a *theoretical plate*) will yield a purer solution of the more volatile component.[16] In reality, each cycle at a given temperature does not occur at exactly the same position in the fractionating column; *theoretical plate* is thus a concept rather than an accurate description.

- More theoretical plates lead to better separations. A spinning band distillation system uses a spinning band of Teflon or metal to force the rising vapors into close contact with the descending condensate, increasing the number of theoretical plates.

5.8 Steam Distillation

- Like vacuum distillation, **steam distillation** is a method for distilling compounds which are heat-sensitive. The temperature of the steam is easier to control than the surface of a heating element, and allows a high rate of heat transfer without heating at a very high temperature. This process involves bubbling steam through a heated mixture of the raw material. By Raoult's law, some of the target compound will vaporize (in accordance with its partial pressure). The vapor mixture is cooled and condensed, usually yielding a layer of oil and a layer of water.
- Steam distillation of various aromatic herbs and flowers can result in two products; an essential oil as well as a watery herbal distillate. The essential oils are often used in perfumery and aromatherapy while the watery distillates have many applications in aromatherapy, food processing and skin care.
- 
- Dimethyl sulfoxide usually boils at 189 °C. Under a vacuum, it distills off into the receiver at only 70 °C.



- **Perkin triangle distillation setup**
  1: Stirrer bar/anti-bumping granules **2:** Still pot **3:** Fractionating column **4:** Thermometer/Boiling point temperature **5:** Teflon tap 1 **6:** Cold finger **7:** Cooling water out **8:** Cooling water in **9:** Teflon tap 2 **10:** Vacuum/gas inlet **11:** Teflon tap 3 **12:** Still receiver

5.9 Vacuum Distillation

- *Main article: Vacuum distillation*

- Some compounds have very high boiling points. To boil such compounds, it is often better to lower the pressure at which such compounds are boiled instead of increasing the temperature. Once the pressure is lowered to the vapor pressure of the compound (at the given temperature), boiling and the rest of the distillation process can commence. This technique is referred to as **vacuum distillation** and it is commonly found in the laboratory in the form of the rotary evaporator.
- This technique is also very useful for compounds which boil beyond their decomposition temperature at atmospheric pressure and which would therefore be decomposed by any attempt to boil them under atmospheric pressure.
- **Molecular distillation** is vacuum distillation below the pressure of 0.01 torr.[19] 0.01 torr is one order of magnitude above high vacuum, where fluids are in the free molecular flow regime, i.e. the mean free path of molecules is comparable to the size of the equipment. The gaseous phase no longer exerts significant pressure on the substance to be evaporated, and consequently, rate of evaporation no longer depends on pressure. That is, because the continuum assumptions of fluid dynamics no longer apply, mass transport is governed by molecular dynamics rather than fluid dynamics. Thus, a short path between the hot surface and the cold surface is necessary, typically by suspending a hot plate covered with a film of feed next to a cold plate with a line of sight in between. Molecular distillation is used industrially for purification of oils

Chapter 6 Ultra Violet-Visible Spectroscopy

6.1 UV-visible spectroscopy is widely used to identify and determine many different inorganic and organic compounds. Usually, molar absorptivities that range from zero to a maximum on the order of 10^5 L/mol cm occur in the UV-Visible region. For any absorption maxima, the magnitude of ε depends on the capture cross section of the species and the probability for an energy absorbing transition to occur. A typical cross –section of an organic compound has been estimated by diffraction and X-ray to be around 10^{-15} cm^2/molecule and all transition probabilities to be between 0 and 1. Typical strong absorptions have ε = 10^4 to 10^5 l mol^{-1} cm^{-1}. Any absorption maxima with ε equal to or less than 10^3 is said to be of low intensity. Usually, this occurs when the transition is forbidden.

6.2 Absorbing Species

The UV-Visible absorption process occurs in two steps represented below:

$$M + h\nu = M^* \quad (1)$$

$$M^* \rightarrow M + \text{heat} \quad (2)$$

The first step involves the specie absorbing UV-visible radiation to become and excited specie and the second step involves the excited specie been deactivated by many process, like heat, photochemical decomposition to a new specie, or fluorescence or phosphorescence emission. The life time of the excited specie is very short (10^6 – 10-9 ^{s}) that its concentration might be very small. The thermal energy released during relaxation is also very small such that absorption measurement does not change the organic specie except when there is decomposition.

Absorption of UV-visible radiation results from excitation of bonding electrons; hence the absorption wavelength can be correlated with the types of bond in the specie. Therefore, UV-visible absorptions can be used to identify functional groups in a molecule. It can also be used to do quantitative determinations. Absorption of UV-visible radiations by species occurs in one or more electronic absorption bands. Each electronic band is made up of closely packed vibrational and rotational energy states and transitions occur from the ground state to any of these vibrational and rotational energy states, hence each typical electronic band is made up of many space lines.

The appearance of the lines in an electronic band is affected by the state of the species under study. If the specie is in the gas phase, the vibrational and rotational energy states may be well separated from each other such that they can freely vibrate and rotate to show different lines in the electronic band. When the specie is in a liquid or solid state, the vibrational and rotational energy states may not be well separated and cannot freely vibrate and rotate therefore; will give a single broad peak.

6.3 Absorption by Organic Compounds

Organic compounds can absorbs emr because they contain valence electrons that can be excited to higher energy levels. The excitation energies associated with electrons of single bonds are sufficiently adequate that absorption can occur in the vacuum UV region ($\lambda < 185$ nm) where components of the atmosphere also absorbs. Such electronic transitions involve excitation of nonbonding *n* electrons to *σ* orbitals*. The molar absorptivities of $n \rightarrow \sigma^*$ transitions vary from low to intermediate and usually range from 100 to 3000 L/mol cm. Mostly, organic compound transition have been found to be longer than 185 nm.

Most transitions involving organic compounds are based transitions for *n or Π* electrons to Π* excited states because the energies required for these visible region (200-70nm) processes bring about absorption bands into the UV-visible region(200-700nm). Both n--.Π* and Π--.Π* group require the presence of unsaturated functional group to provide the Π orbitals. Molecules containing such functional groups can absorb UV-visible radiation and are called ***chromophores***

The electronic spectra of organic compounds are very complex because the vibrational transition overlaps the electronic transitions. The result is very broad band of absorptions that appears continuous. This makes theoretical analysis difficult but qualitative or semi-qualitative statements concerning the type of electronic transitions that occur possible from molecular orbital considerations are feasible. Table 3.1 shows the common organic chromophores and their approximate wavelengths at which they occur (λ_{max}). The table includes the peak intensities ε_{max} which can serve as a rough guide at identifying them. Conjugation of two chromophores cause shift in position to longer wave lengths and other factors such as solvents also affects the band position.

Table 3.1 Absorption Characteristics of Some Common Chromophores

Chromophore	**Example**	**Solvent**	**λ_{max}**	**ε_{max}**	**Transition Type**
Alkene	$C_6H_{13}CH=CH_2$	n-heptane	177	13000	Π-Π*
Alkyne	$C_5H_{11}C\equiv C-CH_3$	n-Heptane	178	10000	Π-Π*
			196	2000	
			225	160	
Carbonyl	$CH_3C(O)CH3$	n-Hexane	186	1000	n-σ*
			280	16	n-Π*
	$CH_3C(O)H$	n-Hexane	180	large	n-σ*
			293	12	n-Π*
Carboxyl	CH_3COOH	Ethanol	204	41	n-Π*
Amido	$CH_3C(O)NH_2$	Water	214	60	n-Π*
Azo	$CH_3N=NCH_3$	Ethanol	339	5	n-Π*
Nitro	CH_3NO_2	Isooctane	280	22	n-Π*
Nitroso	C_4H_9NO	Ethyl Ether	300	100	
			665	20	n-Π*
Nitrate	$C_2H_5ONO_2$	Dioxane	270	12	n-Π*

Vibrational transition broadens peaks in UV-visible regions makes the determination of absorption maximum difficult. The molar absorptivities of n- Π* transitions are low and in the range of 10 – 100 L/mol cm whereas for Π-Π* transitions it is large and in the range of 1000-15000 L/mol cm.

Saturated organic compounds sometimes containing heteroatom's like Oxygen, nitrogen, sulfur, halogens has nonbonding electrons that can be excite with radiation in the range of 170- 250 nm. Absorption in this region is sometimes used to identify sulfur and halogen containing substances.

6.4 Absorption by Inorganic Species

A number of inorganic ions show some UV absorption bands that are a result of exiting nonbonding electrons. Examples are Nitrate (313nm), carbonates (217), nitrite (360, 280 nm), azido (230nm),thiocarbonate (500nm) ions. In general, the ions and complexes of elements in the first two transition series absorb broad bands of UV radiation in at least one of their oxidation states. Here absorption involves transitions between filled and unfilled d-orbitals with energies that depend on the type of ligands bonded to them. The energy of these differences between the orbital (and thus the position of the absorption maxima) and on the position of the element in the periodic table, its oxidation state, and the nature of the ligand bonded to it.

Absorption of spectra of lanthanides and actinides are different the absorption of these ions or metals. The electrons responsible for their transitions (4f and 5f) are shielded from any external influences by electrons that occupy orbitals with larger principal quantum numbers; hence, their bands are narrow and relatively unaffected by the species bonded by the outer electrons.

6.5 Charge transfer Absorption

A charge-transfer complex reaction consists of an electron-donor group bonded to an electron acceptor. When the complex absorbs radiation, an electron from the donor is transferred to an orbital that is associated with the acceptor. The excited state is therefore the product of a kind of internal oxidation-reduction process. This process is different from that of organic chromophores in which the excited electron is in a molecular orbital shared by two or more atoms. For quantitative purposes, charge-transfer absorption is important because their molar absorptivities are unusually large ($\varepsilon > 10{,}000$) which leads to high sensitivity.

Common examples are phenolic complex of iron(iii), the 1,10 Phenanthroline complex of iron(iii), hexacyanoferrate(II), the iodide complex of molecular iodine and hexacyanoferrate(III) complex of Prussian Blue. The red color of iron (III) thiocyannate is absorption of a photon that transfer an electron from the thiocyannate ion to the iron (III). Occasionally, the excited complex may dissociate to oxidation –reduction products. In most charge-transfer complexes, the metal is

the acceptor. Exceptions are the 1,10 Phenanthr**ol**ine iron(ii) complex and Cu(I) complex where the ligand is the acceptor and the metal the donor.

6.6 Qualitative Applications of UV-Visible Spectroscopy

UV-Visible spectroscopies are useful in detecting chromophores groups such as those shown in table 3.1. Large parts of organic complex absorbs in the region between 200-400nm and is clear indication of the presence on unsaturation or atoms such as sulfur or halogens. Usually UV spectra do not have enough fine structure to permit analyte to be identified unambiguously. Thus its data must be supplemented with other physical or chemical evidences such as IR, NMR or ms

Width slit has an effect on peak heights and separation. Spectra for qualitative applications should be measured with minimum slit widths.

UV spectra are often obtained with dilute solutions of the analyte. For volatile compounds, gas-phase spectra are often more useful than the liquid phase or solution spectra. In choosing a solvent, consideration should be given to its transparency and its effect on the absorbing system. In general polar solvents like water alcohols, esters and ketones tend to obliterate spectra fine structure arising from vibrational effects. Spectral similar to gas phase spectra are usually obtained with non-polar solvents. Also, the position on absorption maximum is affected the nature of the solvents. As a rule the same solvent must be used when comparing spectra for identification purposes. Table 3.2 show some solvents with the wavelength below which they cannot be used (i.e. their cutoff wavelengths). Common solvents used in UV spectroscopy are water, 95% ethanol, cyclohexane and 1,4 –dioxane. For the visible region any colorless solvent is suitable.

Table 3.2 Solvents for UV-Visible Regions

Solvent	**Lower λ (nm)**	**Solvent**	**Lower λ (nm)**
Water	180	Diethyl ether	210
Ethanol	220	Acetone	330
Hexane	200	Dioxane	320
Cyclohexane	200	Cellosolve	320
Carbon tetrachloride	260		

6.7 Detection of Functional Groups

UV –visible spectrometry is used in detecting the presence of functional groups in a compound. The presence at 280-290 nm which is displaced towards the shorter wavelength in with increasing solvent polarity indicates the presence of a carbonyl bond. Such shift is called ***hypsochromic or blue shift***. The UV spectra of aromatic hydrocarbons are characterized by three sets of bands that are Π-Π* transitions. Benzene has strong peak at 184 nm ($\varepsilon_{max=}$ 60000), a

weaker band called E_2 bandbatb204 nm (ε_{max} = 7900) and a still weaker band called B band at 256 nm(ε_{max} = 200). Table 3.3 show common absorption wavelengths for organic compounds.

Table 3.3 Absorption Characteristics Of Aromatic Compounds

		E_2bAND		**B BAND**	
COMPOUND		**λmax**	**λmin**	**λmax**	**λmin**
Benzene	C_6H_6	204	7900	256	200
Toluene	$C_6H_5CH_3$	207	7000	261	300
m-Xylene	$C_6H_4(CH_3)_2$			263	300
Chlorobenzene	C_6H_5Cl	210	7600	265	240
Phenol	C_6H_5OH	211	6200	270	1450
Phenolate ion	$C_6H_5O^-$	235	9400	287	2600
Aniline	$C_6H_5NH_2$	230	8600	280	1430
Aniliniun ion	$C_6H_5NH_3^+$	203	7500	254	160
Thiophenol	C_6H_5SH	236	10000	269	700
Naphthalene	$C_{10}H_8$	286	9300	312	289
Styrene	$C_6H_5CH=CH_2$	244	12000	282	450

An ***auxochrome*** is a functional that by itself absorb UV light but can shift chromophores peaks to longer wavelengths as well as increasing their intensities. Such a shift is called a ***bathochromic or red shift***.

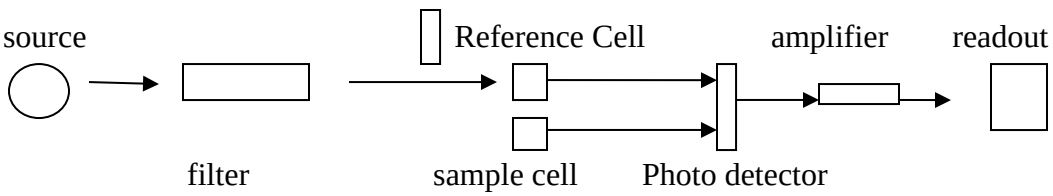
An auxochrome has at least alone pair of electrons which can interact with the Π electrons of the ring and stabilize the Π^* state hereby lowering its energy and increasing the wavelength of the corresponding band.

6.8 Types of instruments

There are 4 general types of instruments use in UV-Visible spectroscopy viz: (1) single bean (2) Double beam in space (3) double beam in time and (4) multichannel

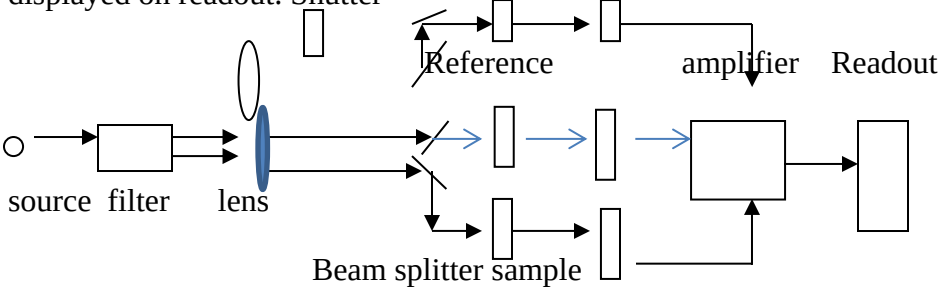
6.8.1 Single Beam Instruments

The figure below shows a single bean configuration for absorption measurements, It consist of a tungsten or deuterium lamp, a filter or a monochromator for wavelength selection, matched cells for placing alternately the reference and sample in the radiation beam, traducers, an amplifier, and a readout device.



6.8.2 Double Beam in Space

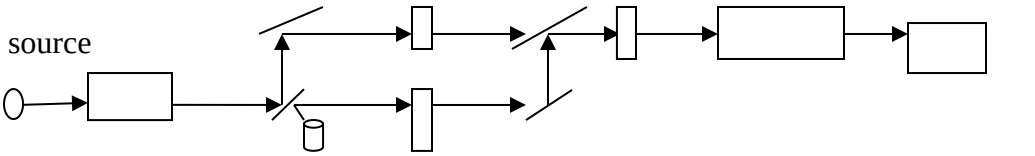
The figure below shows a double beam in-space instrument configuration in which two beams are split by a V-shaped mirror called abeam splitter. One beam passes through the reference cell to a photo detector and the second simultaneously to a sample and to a matched detector. The two outputs are amplified and their ratio is determined electronically of by a computer and displayed on readout. Shutter



Photon detector

6.8.3 Double Beam in Time

The configuration of a beam splitter in time is shown in the figure below. Here the beam are separated in time by a rotating sector mirror that directs the entire beam from the monochromator first through the reference cell and then through the sample cell. The pulses of radiation are recombined by another sector mirror which transmits one pulse and reflects the other to the transducer. The sector mirror is motor driven and is made up of pie-shaped segments, half of which are mirrored and half of which are transparent. The mirrored section and held together by blacked metal frames that periodically interrupt the beam and prevent it reaching the traducer. The detection circuit is programmed to these periods to perform dark current adjustment. The double in time is preferred because of the difficulty in matching the two detectors.



6.8.4 Multichannel Instruments

It is a new type of spectrophotometer based on the array detectors (photodiode array or linear charge couple device). This instrument is usually of single beam design. The dispersive system is a grating spectrograph paced after the sample or reference cell. The array detector is placed at the focal plane of the spectrograph. With single beam designs, the array dark current is measured and stored; next, the spectrum of the source is obtained and stored in the memory after dark current subtraction. Finally the raw spectrum of the sample is obtained and after dark current

subtraction the sample values are divided by source values at each wavelength to give absorbance's. Multichannel instruments can also be configured as a double beam in time instrument. They are capable of acquiring spectrum in a few milliseconds. They are good for transient intermediates studies in moderately fast reactions, for kinetic studies and for qualitative a d quantitative liquid chromatography or capillary electrophoresis column. Also good for general purpose scanning experiments

6.9 Transmittance

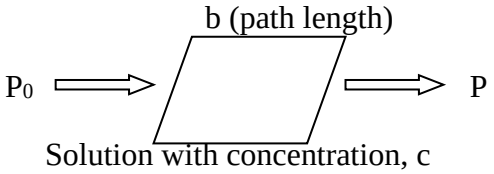


Figure 1 Attenuation of a beam of light by an absorbing solution

6.9.1 Terms Used in Absorption Spectroscopy

Table 2 shows the common terms used in absorption spectroscopy. The first two columns are terms used by ASTM (American standard of Testing Materials). The other columns can be found in older literatures.

Table 3.4 Important Terms and Symbols Used in Absorption Spectroscopy

Term and Symbol*	**Definition**	**Alternative Name and Symbol**
Radiant Power, P, P_0	Energy of radiation(in ergs) impinging on a 1-cm^2 area of a detector per second	Radiation Intensity, I, I_0
Absorbance, A	Log P_0/P	Optical density, D, extinction, E
Transmittance, T	P/P_0	Transmission, T
Path length of radiation,**b		l, d
Absortivity, **a	A/bc	Extinction coefficient, k
Molar absortivity, !, Ɛ	A/bc	Molar extinction coefficient

**Terminology recommended by the American Chemical Society. ** c = Concentration which may be expressed in g/L, b = Path length of the cell which may be expressed in cm or in units of length, c is expressed in units of mol/L, b = expressed in cm*

A monochromatic beam of light with intensity P_0 passes perpendicularly through a cell of length b containing a solution with concentration c. The intensity of the beam will be attenuated to P by the absorbing specie. The *transmittance*, T, of the solution is defined as

$T = P/P_0$ which often expressed as a percentage.

6.10Absorbance

Absorption spectroscopy based on UV-Visible radiation is a useful tools available to the scientist for qualitative analysis. Important characteristics of spectrophotometric and photometric methods are: (i) wide applicability to organic and inorganic systems (ii) typical detection limits of 10^{-4} to 10^{-5} M or lower (iii) moderate to high selectivity (iv) good accuracy (typical uncertainties ar 1-3% or lower in some cases) (v) ease and convenience of use

The amount of light absorbed by the solution, A is defined as

$$A = -\log_{10} T = \log P_0/P$$

6.10.1 Applications to Absorbing and Non-absorbing Species

Compound listed in Tables 1-3 or any other compound containing one or more chromophores listed in the table has a potential to absorb UV-Visible radiation. A number of inorganic species also absorbs UV-Visible radiation. Numerous reagents react selectively with non-absorbing species to give products that absorbs strongly in UV-Visible region. If the amount of the product is limited by the analyte, the absorbance of the product is proportional to the analyte concentration. Color forming reagents are often used for determination of absorbing or non-absorbing species. The molar absorptivity of the product is usually orders of magnitude greater than that of the species before reaction. A host of complexing agents are used to analyze inorganic species. For example, thiocyannate ion for iron, cobalt and molybdenum, hydrogen peroxide for titanium, vanadium and chromium, iodide for bismuth, palladium and tellurium.

6.10.2 Procedural Details

The first step in any photometric or spectrophotometric determinations is to develop a condition that yields a reproducible relationship (usually linear) between the absorbance and analyte concentration. For highest sensitivity, determination is done at wavelength corresponding to absorption maximum because the change in absorbance per unit of concentration is greatest at this point. Also, absorbance is nearly constant at this wavelength which leads to close adherence to Beer's law and errors due to failing to reproduce precisely the wavelength setting of the instrument have less influence at absorption maximum. Accurate determinations are made when good quality matched cells are used. Cells should be calibrated against each other regularly to detect changes due to scratches, etching, and wear.

6.11 Relationship between Absorption and Concentration

The method of external standards is often used to establish the absorbance-concentration relationship. After deciding the conditions of analysis, a calibration curve is prepared form a series of standard that bracket the concentration of the analyte. It is not a good idea to base results of analysis on literature value.

Beers Law: Absorption, A = abc

where a = absorptivity constant, b = path length of the of the radiation passing through absorbing specie (which is equal to the length of the cell) and c = concentration of the specie
Absorbance has no unit
If c = moles/L and b is in cm, a = molar absorptivity, Ɛ, hence,

$$A = \text{Ɛ}bc$$

Ɛ = L/cm mol

Hence, $A = \log P_o/P = \text{Ɛ}bc$

Ideally, a calibration should approximate the concentrations of the sample and also the concentration of other species in the sample matrix (matrix effect). This will minimize the effect of matrix on the measured absorbance. To counter matrix effect the ***standard addition*** method can be used for analysis.

6.12 Experimental Measurement of Transmittance and Absorption

The equation $A = abc = \text{Ɛ}bc$ are useful in chemical analysis. P_0 and P cannot be directly measured in the laboratory because sample to be measured will have to be held in a container Interaction between the beam and the walls of the container, solution, solvent, glass/air, air/glass interfaces, pH of solution, and temperature of analyte, high concentration of analyte and presence of impurities will occur and affect the absorption of the analyte.
To compensate for these interactions, the power of the beam after passing through the solvent and as well as the solution (solute + solvent) are measured separately and compared to each other:
$A = \log P_{solvent}/P_{solution}$ will approximate the true absorbance of the solution

6.13 Application of Beer's Law to Mixtures

For a mixture where the species do not interact with each other, each specie will have its own absorbance. The absorbance for the solution will be the sum of the absorbance's for each of the specie contained in the solution
$A = \text{Ɛ}_1bc_1 + \text{Ɛ}_2bc_2 + \text{Ɛ}_3bc_3 + \text{Ɛ}_4bc_4$
To analyze the mixture molar absorptivities of the standard are determined at two different wavelengths at which the absorptivities of the two differs greatly. Then, the mixture is analyzed at the same wavelengths. From the known molar absorptivities and path length the following equations hold:
$A_1 = \varepsilon_1bc_1 + \varepsilon_1bc_2$ at λ_1
$A_2 = \varepsilon_2bc_1 + \varepsilon_2bc_2$ at λ_2
The relationship is valid if Beer's law holds at both wavelengths and the components behave independently of with more components can be similarly each other. The greatest accuracy is obtain if the λ chosen is one at which the components molar absorptivities are largely different. Sample analyzed but the degree of uncertainties increase as the number of components increases. Some array detectors can be used to reduce these uncertainties by

using data points that are more than the unknown components. It will effectively match the spectra of the unknown as closely as possible by least square methods. However, the spectra of standard
of each component is required for this analysis. Also computer techniques have been developed based on principal component or factorial analysis to determine the number of components, their concentration and absorptivities. These methods are only applied to data obtained with array detectors.

6.14 Limitations of Beers Law

Absorbance is linear with the path length (b) when concentration is constant
But there is a deviation for all cases of constant path length (b) when concentrations vary
Some of the deviations are fundamental and forms real limitations to Beer's law: for example,
Other deviations are instrumental (depending on the way the absorbance is measured) or chemical (changes that occur to the analyte such as concentration changes)
Beer's law is only applicable to dilute solutions (10^{-2})
For concentrations > 0.01 M, the analyte may not absorb a certain wavelength of radiation and therefore, causes deviation from the linearity between absorbance and concentration
This deviation can also occur if the analyte concentration is low while that of an interfering ion is very high
Ɛ is dependent on the refractive index (n) of the solvent
Alterations in the refractive index affect the value of Ɛ

6.15 Chemical Deviations

Association, dissociation, or reaction of analyte with the solvent can cause deviations from the Beer's law
The deviation is more obvious than real and is due to shifts in chemical equilibrium and not from changes in molar absorptivity, Ɛ, of the analyte
Deviations from the Beer's law can be predicted from the equilibrium constants and the molar absorptivities of the solutes. If the molar absorptivities are different at different wavelengths, then, there will be a deviation from the Beer's law

6.16 Instrumental Deviations with Polychromatic Radiation

Beers law is strictly obeyed if the source of radiation is monochromatic
In practice, using a source of radiation that is monochromatic is almost impossible
Devices that isolate part of the output from radiation sources produce a symmetrical band of λ's around the desired λ
For example, if a source of radiation has two λ's: λ_1 and λ_2, then,

$$A_1 = Ɛ_1 bc = \log P^1{}_0/P^1 \text{ or } P^1{}_0/P^1 = 10^{\,Ɛ_1 bc} \quad (1)$$

$A_2 = \varepsilon_2 bc = \log P^2_0/P^2$ or $P^2_0/P^2 = 10^{\varepsilon_2 bc}$ (2)

If absorbance is made with the radiation (composing of the 2 wavelength's), the power of the beam coming out of the solution will be $(P^1 + P^2)$ and the one coming out of the solution will be $(P^1_0 + P^2_0)$

The measured absorbance $A_M = \log (P^1_0 + P^2_0)/ (P^1 + P^2)$ (3)

From equations 1 and 2,

$P^1_0/P^1 = 10^{\varepsilon_1 bc}$ then

$P^1 = P^1_0\, 10^{-\varepsilon_1 bc}$ and $P^2 = P^2_0\, 10^{-\varepsilon_2 bc}$ (4)

Then $A_M = \log (P^1_0 + P^2_0)/ (P^1_0\, 10^{-\varepsilon_1 bc} + P^2_0\, 10^{-\varepsilon_2 bc})$

If $\varepsilon_1 = \varepsilon_2$ then

$A_M = \varepsilon_1 bc$ or $A_M = \varepsilon_2 bc$ and Beer's law will be followed

If $\varepsilon_1 \neq \varepsilon_2$, then there will be deviations from Beer's law. The deviations will increase as the differences increases

If the wavelength band of polychromatic light used titration curves n performing an experiment does not include spectral regions where changes in ε is large, then deviations from Beer's law will not be noticed. The converse is true. Polychromatic light with stray radiations also causes deviation from Beer's law

6.17 Spectrophotometric Applications

UV-visible spectrometers can be used in any field that requires quantitative determinations. For example it can be applied to photometric titrations and kinetic methods.

6.17.1 Applications of Photometric Titrations

Photometric titrations often prove more accurate results than direct photometric analysis because the end point is gathered from many measurements. Again the presence of absorbing species may not interfere because only a change in absorbance is measured. The advantage of end points determined fro linear segment photometric is that the experimental data is collected away from the equivalence point where the absorbance changes gradually. Therefore the equilibrium constant might not be as large at that determined from a sigmoid titration curve that depends on observation near the equivalence point. For the same reason more dilute solutions may be used in photometric detection.

Photometric end points have been applied to many types of reactions. For example, most standard oxidizing agents have characteristic absorption spectra and produce photometrically detectable end points. Although standard acids and bases do not absorb, the introduction of acid-base indicators permits photometric neutralization titrations. Photometric determinations have been used in many EDTA titrations and other complexing agents. For example, it has been used in hardness of water determinations with Eriochrome Black T indicator. The absorbance of the indicator is monitored at 610 nm.

6.17.2 Kinetic Methods