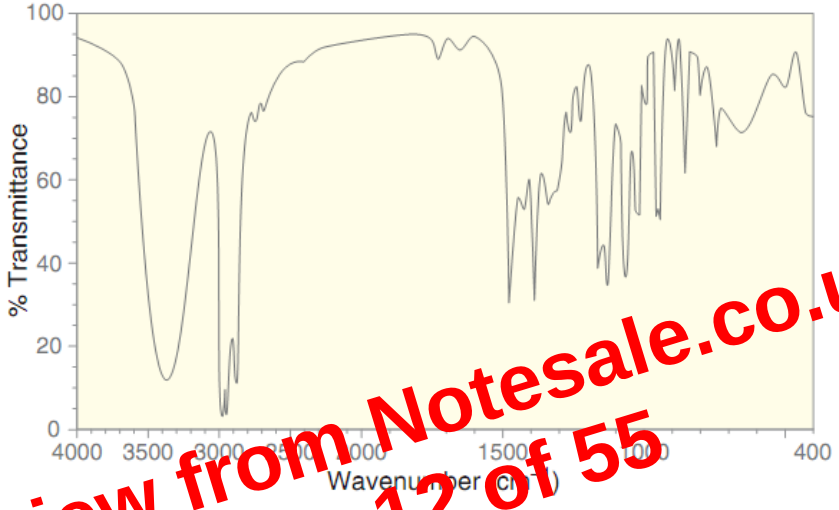
IR spectrometer: In an IR spectrometer, a sample is irradiated with frequencies of IR radiation, and the frequencies than pass through (that are not absorbed by the sample) are detected. A plot is then constructed showing which frequencies were absorbed by the sample. The most commonly used type of spectrometer, called a Fourier transform (FT-IR) spectrometer, irradiates the sample with all frequencies simultaneously and then utilized a mathematical operation called a Fourier transform to determine which frequencies passed through the sample.

Several techniques are used for preparing a sample for IR spectroscopy. The most common method involves the use of salt plates. These expensive plates are made of NaCl and are used because they are transparent to IR radiation. If the compound under investigation is a liquid at room temperature, a drop of the sample is sandwiched in between two salt plates and is called a neat sample. If the compound is a solid at room temperature, it can be dissolved in a suitable solvent and placed in between two salt plates. Alternatively, insoluble compounds can be mixed with powdered KBr and the passed into a thin, transparent film, called a KBr pellet. All of these sampling techniques are commonly used for IR spectroscopy.

The general shape of an IR absorbance spectrum: An IR spectrometer measures the percent transmittance as a function of frequency. This plot is called an absorption spectrum (figure below).



All the signals, called absorption bands, reported on an IR spectrum. The location of each signal on the spectrum can be specified either by the corresponding wavelength or by the corresponding frequency of radiation that was absorbed. Several decades ago, signals were reported by their wavelengths (measured in micrometers or microns). currently, the location of each signal is more often reported in terms of a frequency-related unit, called wavenumber ($\tilde{\nu}$). The wavenumber is simply the frequency of light divided by a constant (the speed of light, c):

$$\tilde{\nu} = \frac{\nu}{c}$$

The units of wavenumber are inverse centimeters (cm^{-1}), and the values range from 400 to 4,000 cm^{-1}. Wavenumber is proportional to frequency, and therefore, a large wavenumber represents higher energy. Signals that appear on the left side on spectrum correspond with higher energy radiation, while signals on the right side of the spectrum correspond with lower energy radiation. Every signal in an IR spectrum has three characteristics: wavenumber, intensity and shape.

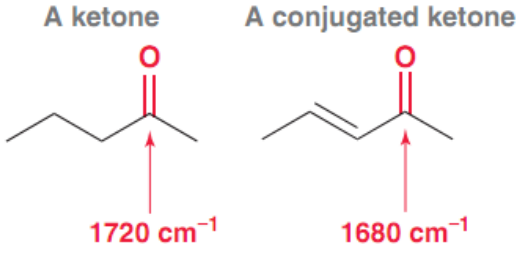
Signal characteristics

Wavenumber

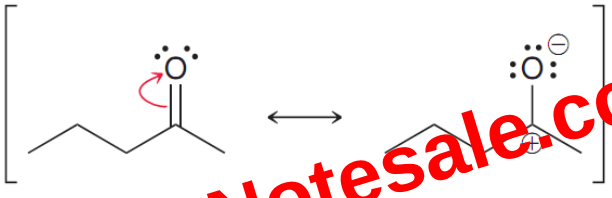
Hooke's law: For every bond, the wavenumber of absorption associated with bond stretching is dependent on two factors: (1) bond strength and (2) masses of the atoms sharing the bond. The impact of these two factors can be rationalized when we treat a bond if it were a vibrating spring connecting two weights:

(<1500 cm^{-1}) contains signals resulting from the vibrational excitation of most single bonds (stretching and blending). This region generally contains many signals and is more difficult to analyze.

Effect of resonance on wavenumber of absorption: We will now consider the effects of resonance on the wavenumber of absorption. As an illustration, compare the carbonyl groups (C=O bonds) in the following two compounds:



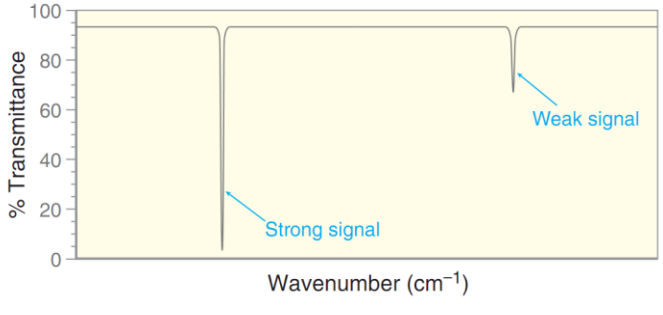
The second compound is called an unsaturated, conjugated ketone. It is unsaturated because of the presence of a C=C bond and it is conjugated because the π bonds are separated from each other by exactly one σ bond. As shown, the carbonyl group of an unsaturated, conjugated ketone produces a signal at lower wavenumber (1680 cm^{-1}) than the carbonyl group of a saturated ketone (1720 cm^{-1}). In order to understand why, we must draw resonance structures for each compound. Let's begin with the ketone:



Ketones have two significant resonance structures. The carbonyl group is drawn as a double bond in the first resonance structure, and it is drawn as a single bond in the second resonance structure. This means that the carbonyl group has some double-bond character and some single-bond character. In order to determine the nature of this bond, we must consider the contribution from each resonance structure. In other words, does the carbonyl group have more double-bond character or more single-bond character? The second resonance structure exhibits charge separation as well as a carbon atom (C+) that has less than an octet of electrons. For both of these reasons, the second resonance structure contributes only a small amount of character to the overall resonance hybrid. Therefore, the carbonyl group of a ketone has mostly double-bond character.

Intensity

In an IR spectrum, some signals will be very strong in comparison with other signals on the same spectrum (figure below). That is, some bonds absorb IR radiation very efficiently, while other bonds are less efficient at absorbing IR radiation.



3. Chromatographic Techniques

Chromatography: Chromatography is a technique or method for separating the components of a mixture on the basis of the relative amounts of each solute distributed between a moving fluid stream (mobile phase) and stationary phase. The mobile phase may be either a liquid or a gas, while the stationary phase is either a solid or a liquid.

Kinetic molecular motion continuously exchanges solute molecules between the two phases.

If, for a particular solute, the distribution favours the moving fluid, the molecules will spend most of their time migrating with the stream and will be transported away from other species whose molecules are retained longer by the stationary phase.

The driving force for solute migration is the moving fluid, and the resistive force is the solute affinity for the stationary phase; the combination of these forces, as manipulated by the analyst, produces the separation.

Types:

A. **Techniques by chromatographic bed shape:**
    1. **Column chromatography:** The stationary bed is within a tube.
    2. **Planar chromatography:** The stationary phase is present as or on a plane.
        - Paper chromatography
        - Thin layer chromatography
    3. **Displacement chromatography:** A sample is placed onto the head of the column and is then displaced by a solute that is more strongly sorbed than the components of the original mixture.
B. **Techniques by physical state or mobile phase:**
    1. **Gas chromatography:** The mobile phase is a gas.
    2. **Liquid chromatography:** The mobile phase is a liquid.
C. **Affinity chromatography:**
    1. Supercritical fluid chromatography
D. **Techniques by separation mechanism:**
    1. Ion exchange chromatography
    2. Size exclusion chromatography
    3. Expanded bed adsorption chromatographic separation
E. **Special techniques:**
    1. Reversed phase chromatography
    2. Hydrophobic interaction chromatography
    3. Two dimensional chromatography
    4. Simulated moving bed chromatography
    5. Pyrolysis gas chromatography
    6. Fast protein liquid chromatography
    7. Countercurrent chromatography
    8. Periodic counter current chromatography
    9. Chiral chromatography
    10. Aqueous normal phase chromatography

Applications of chromatography:

1. The purification of reaction mixtures in chemical synthesis.
2. The purification of biomolecules such as proteins for pharmaceutical research.
3. The analysis of complex sample mixtures such as those obtained in forensics (body fluids, paints etc.) and
4. The analysis of environmental samples.

Gas chromatography: Gas chromatography is a type of chromatography used for separating and analyzing based on boiling point (vapor pressure) differences of volatile compounds that can be vaporized without decomposition.

- **Pump:** The pump suctions the mobile phase from solvent reservoir and forces it to column and then passes to detector. Maximum 250 kgfcm^2 is the operating pressure of the pump. This operating pressure depends on column dimensions, particle size, flow rate and composition of mobile phase.
- **Sample injector:** The injector can be solitary infusion or a computerized infusion framework. An injector for a HPLC framework should give infusion of the fluid specimen inside the scope of 0.1 mL to 100 ml of volume with high reproducibility and under high pressure.
- **Columns:** Columns are typically made of cleaned stainless steel, are somewhere around 50 nm and 300 nm long and have an inward distance across of somewhere around 2 and 5 nm. They are generally loaded with a stationary phase with a molecule size of 3 µm to 10 µm. Columns with inner diameters varied from application to application (2 – 20 mm are used). The temperature of the mobile phase and the column should be kept consistent during investigation to keep precise retention time.
- **Detector:** The HPLC detector, suited toward the end of the column distinguishes the analytes as they elute from the chromatographic column. Regularly utilized detectors are UV spectroscopy, fluorescence, mass spectrometric and electrochemical.
- **Data collection devices or integrator:** Signals from the detector might be gathered on graph recorders or electronic integrators that fluctuate in many-sided quality and in their capacity to process, store and reprocess chromatographic information. The PC coordinates the reaction of the indicator to every part and places it into a chromatograph that is anything but difficult to interpret.

Chromatography theory: Chromatography is a separation method that defines the differences in partitioning behavior between a mobile phase and a stationary phase to separate the components in a mixture. Components of a mixture may be interacting with the stationary phase based on charge, relative solubility or adsorption. There are two theories of chromatography, the plate and rate theories.

Retention time: The retention is a measure of the speed at which a substance moves in a chromatographic system. The retention volume of a solute is that volume of mobile phase that passes through the column between the injection point and the peak maximum. In HPLC or GC, the retention is usually measured as the retention time, R_t or t_R, the time between injection and detection.

The retention of a compound can differ between experiments and laboratories due to variations of the eluent, the stationary phase, temperature and the setup. RT is used to identify a compound in a test sample.

Number of theoretical plates: Column efficiency can be calculated by the four methods:

- Tangent line method
- Half peak height method
- Area height method
- EMG method

Theoretical plate calculation:

- **Tangent line method:** N, the number of theoretical plates, is one index used to determine the performance and effectiveness of columns, and is calculated using equation below:

$$N = 16\left(\frac{tr}{W}\right)^2$$

Where,

tr = retention time

W = peak width. This peak width, W is based on the baseline intercepts of tangent lines to a Gaussian peak, which is equivalent to the peak width at 13.4% of the peak height.

- **Half peak height method:** Width is calculated from the width at half the peak height ($W_{0.5}$). Since, width can be calculated easily by hand, it is the most widely used method. This is the method used by the DAB (German Pharmacopoeia), BP (British Pharmacopoeia) and EP (European Pharmacopoeia)

$$N = 5.54\left(\frac{tr}{w_{0.5}}\right)^2$$

1. **In large scale mapping of chromosomes:** Because this technique facilitates analysis of DNA fragments on the scale of individual chromosomes, it has found extensive uses in large scale mapping chromosomes.
2. **In bacterial taxonomy:** It has particular importance in bacterial taxonomy allowing the identification of relationships between existing and novel strains of bacteria.
3. **To know polymorphisms:** In eukaryotes, pulsed field gel electrophoresis of yeast chromosome has revealed widespread length polymorphisms.
4. **Study on strand breaks:** This technique can also be applied to studies on strand breaks in human chromosomes as a result of exposure to toxic chemicals.

Isotope: The masses of atoms of the same element may be different. For example, the masses of oxygen atoms in nature is 16, 17 and 18 units. These are known as isotopes. The atoms, which have the same number of protons, but whose masses are different, are called isotopes of each other. Isotopes are atoms of same element. They have same position in the periodic table. Hence, this name isotope is attributed to them. Iso means same and topos means position.

Isotopes of an element differ in masses, due to the presence of different number of neutrons in the nucleus. For example, ${}^{16}_{8}O$ isotope has 8 neutrons, ${}^{17}_{8}O$ has 9 neutrons and ${}^{18}_{8}O$ has 10 neutrons. All of them have 8 protons in the nucleus and 8 electrons outside nucleus.

Isotopes of any element have same chemical and physical properties, only slight variation in melting and boiling points may occur. In nature, all elements except a very few like Na, Au have more than one isotope. Moreover, many isotopes of all elements have been synthesized.

Radioisotope: A radioactive isotope of an radioactive element having the same atomic number (protons) but with different number of neutrons is called a radioisotope. **Example:** I-125, I-131. These radioactive elements undergo radioactive decay by emitting α, β or γ radiation until they reached the stable form of elements. These may occur naturally as in the case of radium or uranium or may be created artificially.

Properties of radioisotopes:

1. Emits radioactive radiation which
    - Have different penetrating ability with materials of different thickness and densities
    - Kills cells
    - Cause cell mutation
    - Ionize molecules
2. Have the same chemical properties as non-radioactive isotopes of the same element.
3. Its activity decreases with time.

Creation of radioisotope: Scientists create artificial isotopes by bombarding stable atoms of an element with subatomic particles in a nuclear reactor or in an atom smasher or cyclotron.

Labeled or tagged atom: When the nucleus of a stable atom is charged by bombarding particles, the atoms usually become unstable or radioactive, and is said to be labeled or tagged.

Radiopharmaceutical: A radiopharmaceutical is a molecule that consists of a radioisotope tracer attached to a pharmaceutical. After entering the body, the radiolabeled pharmaceutical will accumulate in a specific organ or tumor tissue. The radioisotope attached to the targeting pharmaceutical will undergo decay and produce specific amounts of radiation that can be used to diagnose or treat human diseases and injuries. The amount of radiopharmaceutical administered is carefully selected to ensure each patient's safety. Radioisotopes are an essential part of radiopharmaceuticals.

A selected radioactive source is an encapsulated quantity of a radioisotope which is used to produce a beam of ionizing radiation. Industrial sources usually contain radioisotopes that emit γ or X rays.

Ionizing radiation is a radiation which can knock electrons to out of atoms, either by direct interaction with the atoms or by other methods. α and β particles, neutrons, X rays and γ rays are examples of ionizing radiation.

Uses of radioisotopes:

A. **In medicine**
    1. Uses radiation to provide information about the function of the specific organs of a patient or to treat disease.
    2. A radioisotope is taken by a patient.
    3. The radiation emitted enables organs to be easily imaged by imaging equipment.
    4. Disorders can be detected and treated.
        - **Thrombosis:** Sodium-24 is injected into the bloodstream to detect the position of blood clots or thrombosis in the blood vessels.
        - **Brain tumor:** Can be detected and treated by using phosphorous-32.
    5. **Sterilization:** Radioisotope cobalt-60 is used to sterilize medical equipment.
B. **Agriculture:**
    1. Study the effectiveness of fertilizers:

Autoradiograph: An autoradiograph is an image on X-ray film or nuclear emulsion produced by the pattern of decay emissions (e.g. beta particles or gamma rays) from a distribution of a radioactive substances. Alternatively, the autoradiograph is also available as a digital image (digital autoradiography), due to the recent development of scintillation gas detectors or rare earth phosphor imaging systems. The film or emulsion is apposed to the labeled tissue section to obtain the autoradiograph (also called an autoradiogram).

The auto prefix indicates that the radioactive substance is within the sample, as distinguished from the case of histo-radiography or micro-radiography, in which the sample is X-rayed using an external source.

Method:

Living cells are briefly exposed to a pulse of a specific radioactive compound
↓
Samples are taken, fixed and processed for light or electron microscopy
↓
Left in the dark for days or weeks (while the radioisotope decays)
↓
The photographic emulsion is developed (as for conventional photography)
↓
Counter stained
↓
Alternatively, pre-staining of the entire block of tissue can be done
↓
It is not necessary to coverslip these slides
↓
The position of the silver grains in the sample is observed by light or electron microscopy
↓
These autoradiographs provide a permanent record
↓
Full details on the batch of emulsion used (dates, exposure time and conditions) should be kept for each experiment.

Micro-autoradiography: Some autoradiographs can be examined microscopically for localization of silver grains (such as on the interiors of cells or organelles) in which the process is termed micro-autoradiography. For example, micro-autoradiography was used to examine whether atrazine was being metabolized by the hornwort plant or by epiphytic microorganisms in the biofilm layer surrounding the plant.

Applications of autoradiography:

1. To determine the tissue (or cell) localization of a radioactive substance.
2. To determine the date of DNA replication.
3. To find and investigate the various properties of DNA.
4. To detect protein phosphorylation.
5. To find the site and performance of targeted drugs.
6. To locate the metabolic activity site in the cell and
7. To inspect aircraft components for small defects by Krypton-85.

Introduction to the therapeutic gene to correct gene defect.

↓

Selection of the genetically corrected cells (stable transformation) and growth.

↓

Transplantation of the modified cells to the patient.

The procedure basically involves the use of the patient's own cells for culture and genetic correction, and then their return back to the patient. This technique, is therefore, not associated with adverse immunological responses after transplanting the cells. Ex vivo gene therapy is efficient, only if the therapeutic gene (remedial gene) is stably incorporated and continuously expressed. This can be achieved by using vectors.

Selected examples of ex vivo gene therapy:

1. Therapy for adenosine deaminase (ADA) deficiency
2. Therapy for familial hypercholesterolemia
3. Therapy for Lesch-Nyhan syndrome

ii. **In vivo gene therapy:** The direct delivery of genes into the cells of a particular tissue is referred to as in vivo gene therapy.

Process:

Copies of the therapeutic gene are inserted into viral DNA, liposome or in the form of plasmid DNA

↓

Genetically altered DNA is inserted into patient's body by cell specific direct tissue injection.

↓

Inside the body, the inserted DNA is incorporated into the cells of the specific tissue it was injected into. These cells now encode and produce the needed protein encoded by the inserted gene.

Parameters to success: The success of in vivo gene therapy mostly depends on the following parameters:

1. The efficiency of the uptake of the remedial (therapeutic) gene by the target cells.
2. Intracellular degradation of the gene and its uptake by nucleus.
3. The expression capability of the gene.

Types of vectors or delivery methods:

A. Viral vectors and
B. Non-viral vectors

A. Viral vectors:

1. Adenovirus
2. Retrovirus
3. Lentivirus
4. Herpes virus
5. Parvovirus AAV

B. **Non-viral vectors:** It may be chemical or physical.

a. Chemical:

- Nanoparticles
- Polyplexes
- Polymeric micelles
- Lipoplexes

b. Physical:

- Gene gun
- Electroporation
- Magnetofection

Advantages of viral vectors over physical and chemical gene delivery system:

1. Gene transfer is more efficient and specific than physical and chemical methods.
2. Multiple and repeated doses are required in case of physical and chemical method, whereas in case of viral vector even a single dose is sufficient.

Limitations of viral vectors:

1. Acute immune response to viral vectors.
2. Repeated treatments are needed.
3. Genes lost when the cell goes through mitosis cell division.
4. Viral vectors could become pathogenic.
5. Genes spliced at random into the genome could upset other genes.
6. Multigene disorders are too complex to treat.

Applications of gene therapy:

1. Curing genetic diseases.
2. Correcting cancer genes.
3. Inducing cancerous cells to make toxins so that they kill themselves.
4. Harnessing the immune response.
5. Gene therapy to enhance cancer treatment.
6. Pro-drug gene therapy.
7. For the treatment of neurological disorder.
8. Blocking viral genes e.g. HIV.
9. Creating stem cells from somatic cells.

Progress and prospects:

Progress:

1. Gene therapy clinical trials for cancer are moving rapidly from phase I to III and the first anti-cancer gene therapy drug has been licensed in China.
2. Some serious adverse events following gene therapy have been occurred, but progress has been made in understanding and overcoming these problems.
3. Clinical trials involving genetic modification of stem cells other than bone marrow derived (for example skin precursor cells) have been carried out.
4. Ex vivo transduction of stem cells with integrating retroviruses ensures high and persistent level of gene transfer and is promising strategy for several diseases.

Prospects:

1. Lentiviral vectors will move from preclinical safety assessment studies to clinical trials.
2. Studies to evaluate the genotoxicity of any gene transfer vector will become a fundamental feature of gene therapy research.
3. Stem cells from a range of different tissues or organs will become the target cells for many gene therapy applications.

Limitations and ethical issues:

1. **Unwanted immune system reaction:** Body's immune system may see the newly introduced viruses as introducers and attack them. This may cause inflammation and in severe cases, organ failure.
2. **Targeting the wrong cells:** Because viruses can affect more than one type of cells, it is possible that the altered viruses may infect additional cells – not just the targeted cells containing mutated genes. If this happens, healthy cells may be damaged, causing other illness or diseases such as cancer.
3. **Infection caused by the virus:** It is possible that once introduced into the body, the viruses may recover their original ability to cause disease.