

GPAT Online Class for B.Pharm Students



Ananthapuramu Local Branch

Pharmaceutical Analysis – Part 3 (3rd July 2020)

By

Dr P Ramlingam, *M.Pharm., PhD*
Director – Research

Raghavendra Institute of Pharmaceutical Education and Research – Autonomous
Affil: **JNT University Anantapur**, Anantapur, Andhra Pradesh, India

GPAT Online Class for B.Pharm Students

RIPER
AUTONOMOUS
NAAC &
NBA (UG)
SIRO- DSIR

Pharmaceutical Analysis – Part 3 NMR Spectroscopy & Mass Spectroscopy: Fundamentals

By

Dr P Ramlingam, *M.Pharm., PhD*

Director – Research

Raghavendra Institute of Pharmaceutical Education and Research – Autonomous

Affil: **JNT University Anantapur**, Anantapur, Andhra Pradesh, India



Raghavendra Institute of Pharmaceutical Education and Research - Autonomous
K.R.Palli Cross, Chiyyedu, Anantapuramu, A. P- 515721

Disclaimer

“ The contents in this presentation are compiled from different sources, this presentation is delivered at the interest of students and not involved any commercial benefits. Thus the presenter shall not claim any copyright for this presentation, and not responsible for any copyright issues if arise”

NMR Spectroscopy

NMR spectroscopy is based on the absorption of radiofrequency (R_f) by the spinning nuclei (Spin quantum number $I > 0$).

Note: if, $I = 0$ it indicate the nuclei does not possess spin, hence they do not absorb radiofrequency Example: C^{12} , O^{16}).

In general, for a nuclei if both atomic number and atomic weight are even, then the spin quantum number become zero.

Example: ${}_6C^{12}$ do not absorb radio frequency but C^{13} absorbs radiofrequency.



H¹-NMR Spectroscopy

RIPER
AUTONOMOUS
NAAC &
NBA (UG)
SIRO- DSIR

Proton NMR (also called as **Hydrogen-1 NMR** or **H¹ NMR**) is a type of nuclear magnetic resonance (**NMR**) spectroscopy represents the hydrogen (H¹ isotope - Proton) within the **molecule** of a substance, where Proton absorbs radio-frequency.

As per isotope abundance, 99.99 % will be proton (H¹) isotope remaining will be deuterium (H²) and tritium (H³).

C¹³-NMR – C¹³ absorbs radiofrequency and gives information about carbon in the chemical structure.



How to predict the nuclei spin quantum number ??

Mass number	Atomic number	Nuclear spin quantum number (I)	Examples
Odd	Odd/ Even	1/2, 3/2, 5/2..	$^1\text{H}_1$, ^{13}C , $^{15}\text{N}_7$, ($I=1/2$) $^{17}\text{O}_8$ ($I=5/2$)
Even	Odd	1, 2, 3, 4,.....	$^2\text{H}_1$, $^{14}\text{N}_7$, ($I=1$) $^{10}\text{B}_5$, ($I=3$)
Even	Even	0	$^{12}\text{C}_6$, $^{16}\text{O}_8$, $^{32}\text{S}_{16}$ NMR inactive

NMR inactive: They don't absorb radiofrequency

Principle of Radiofrequency absorption by Nuclei

RIPER
AUTONOMOUS
NAAC &
NBA (UG)
SIRO- DSIR

Any spinning nuclei ($I > 0$) will have different energy level under external field, and the number of energy level is depends on spin quantum number.

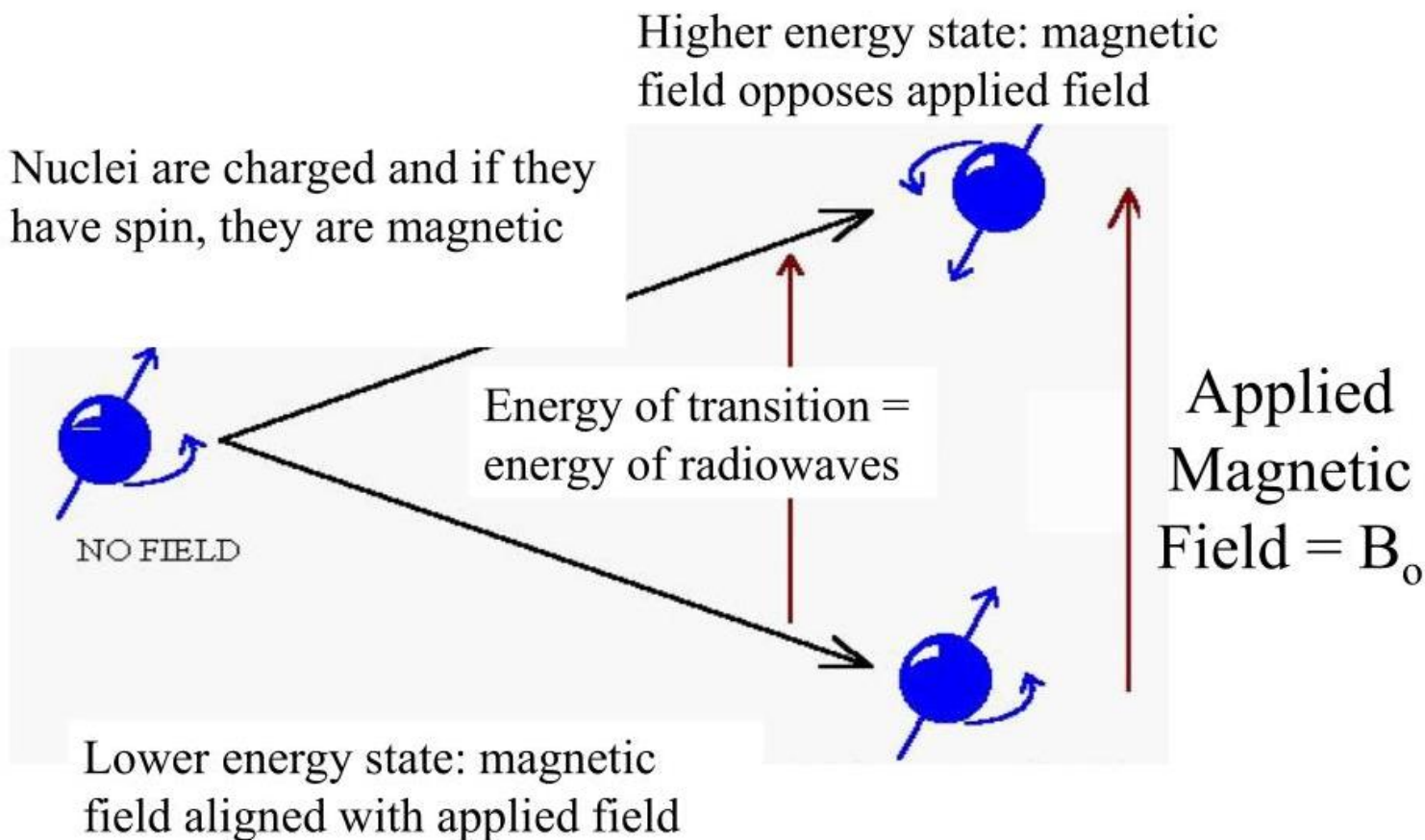
For example both H^1 and C^{13} possess spin quantum number of half integral ($I = \frac{1}{2}$), where H^2 possess integral ($I = 1$).

Hence the number of energy level for C^{13} and H^1 under applied magnetic field (B) will be,

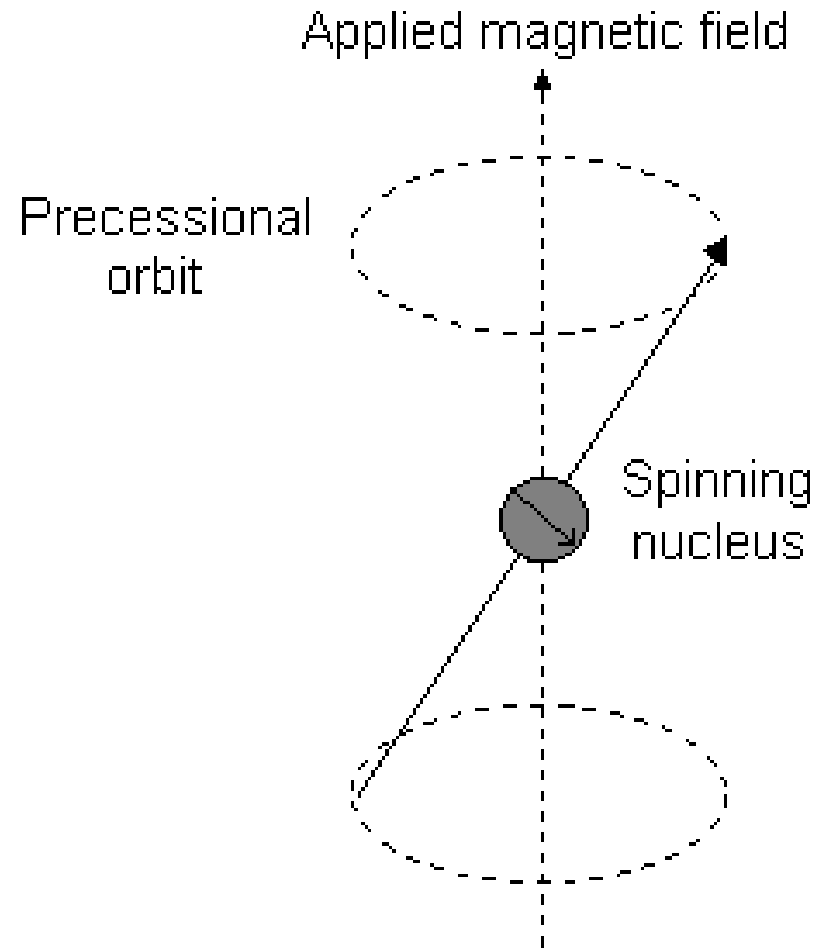
$$= (2I + 1) = 2 \times \frac{1}{2} + 1 = 2 \text{ (+1/2 , -1/2)}$$



+1/2 and -1/2 respectively for higher energy (beta) and lower energy (alpha) orientations under the applied magnetic field (B_0 or H_0).



Behaviour of Nuclei in Applied magnetic field



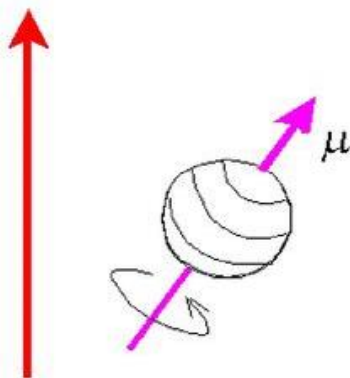
frequency of the incoming radiation that will cause a transition

$$\nu = \left(\frac{\gamma}{2\pi} \right) B_o$$

gyromagnetic ratio γ

strength of the magnetic field

The above equation is called LARMOR equation represent the Fundamental NMR equation.



The magnetic moment, μ is proportional to the spin quantum number I

$$\mu = \gamma I (h/2\pi)$$

where γ = Magnetogyric ratio = $\frac{\text{magnetic moment}}{\text{angular momentum}}$

γ is different for each nucleus (a constant)

I is allowed only certain values and these values can range from $m_I = -I$ to $+I$ in integer steps ONLY

Our proton is simple as it has $I = \frac{1}{2}$, so only two values of m_I are permitted, $+1/2$ and $-1/2$

Larmor frequency (Precessional frequency)

Larmor frequency (Precessional frequency) of the nuclei is depends on the applied magnetic field and the environment of the nuclei.

$$\nu = \frac{\gamma B_0}{2\pi} \quad \text{for } ^1\text{H, } \nu \text{ is 60 MHz for } B_0 = 14,092 \text{ gauss}$$

$$\gamma = \text{magnetogyric ratio} = 26,753 \text{ radians/gauss for } ^1\text{H}$$

$$\gamma = \frac{2\pi\mu}{hI} \quad \mu = \text{magnetic moment}$$



Gyromagnetic ratio & Nuclei's Larmor frequency

The gyromagnetic ratio differs from nuclei to nuclei and depending on the environment.

Example: CH_3OH –
Environment of CH_3 proton and OH proton are different. Hence they are magnetically not equivalent and absorb R_f at different energy and give different signal

Nuclear spin

$$\mu = \gamma I \hbar$$

μ - magnetic moment

γ - gyromagnetic ratio

I - spin quantum number

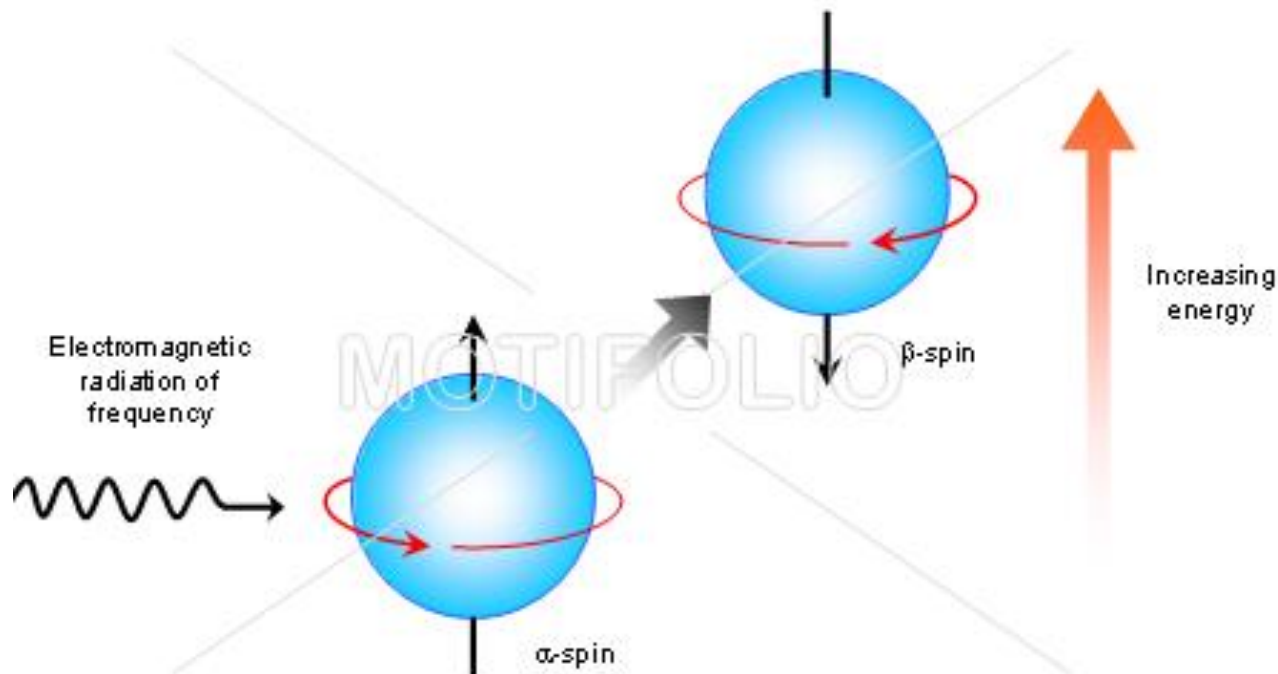
\hbar - Planck's constant



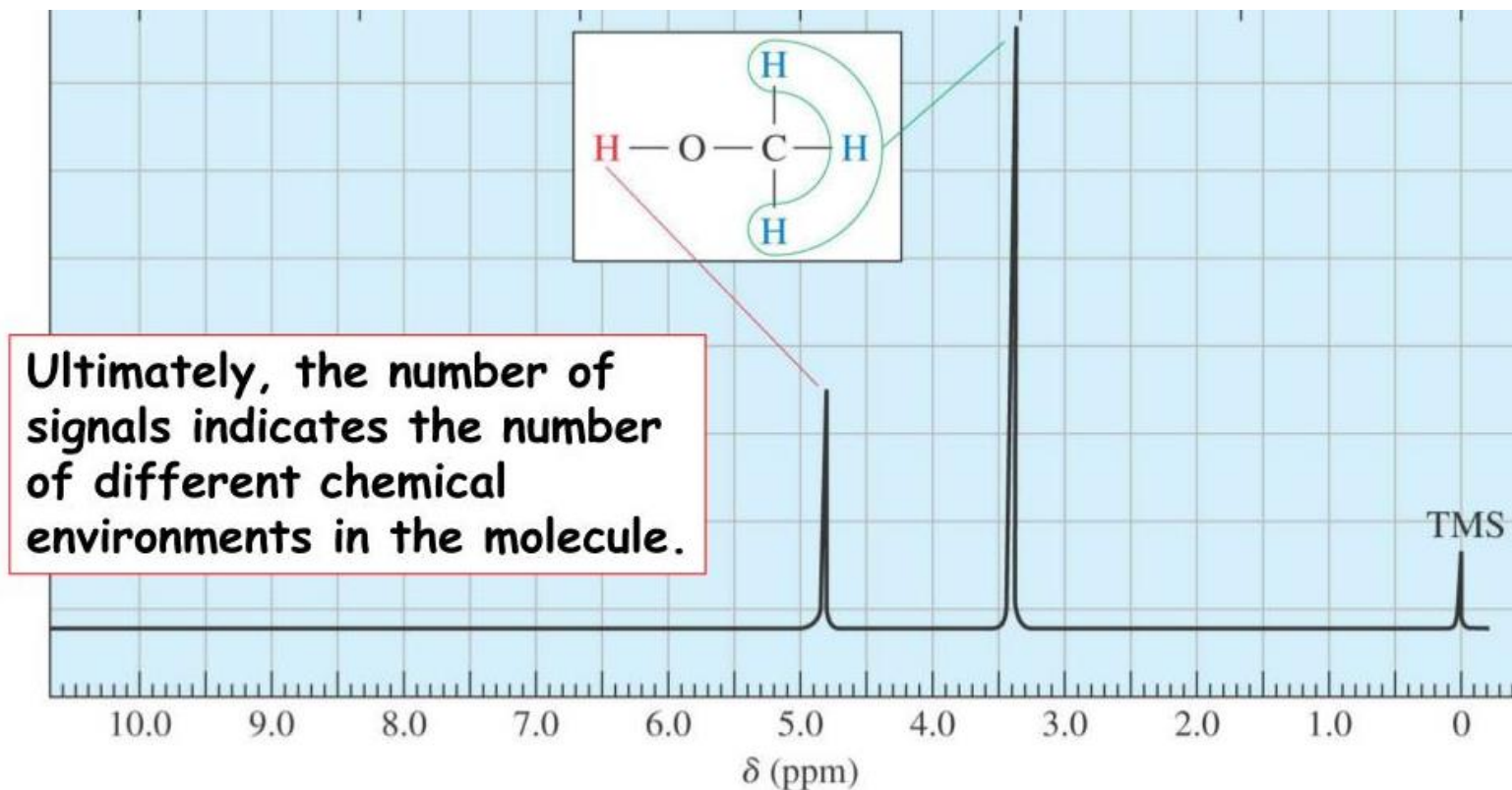
Rf absorption by Nuclei

The radiofrequency used will be 60, 90, 100, 300, 600 MHz, as the frequency increases, the resolution increases. Radiofrequency is absorbed by a molecule only when,

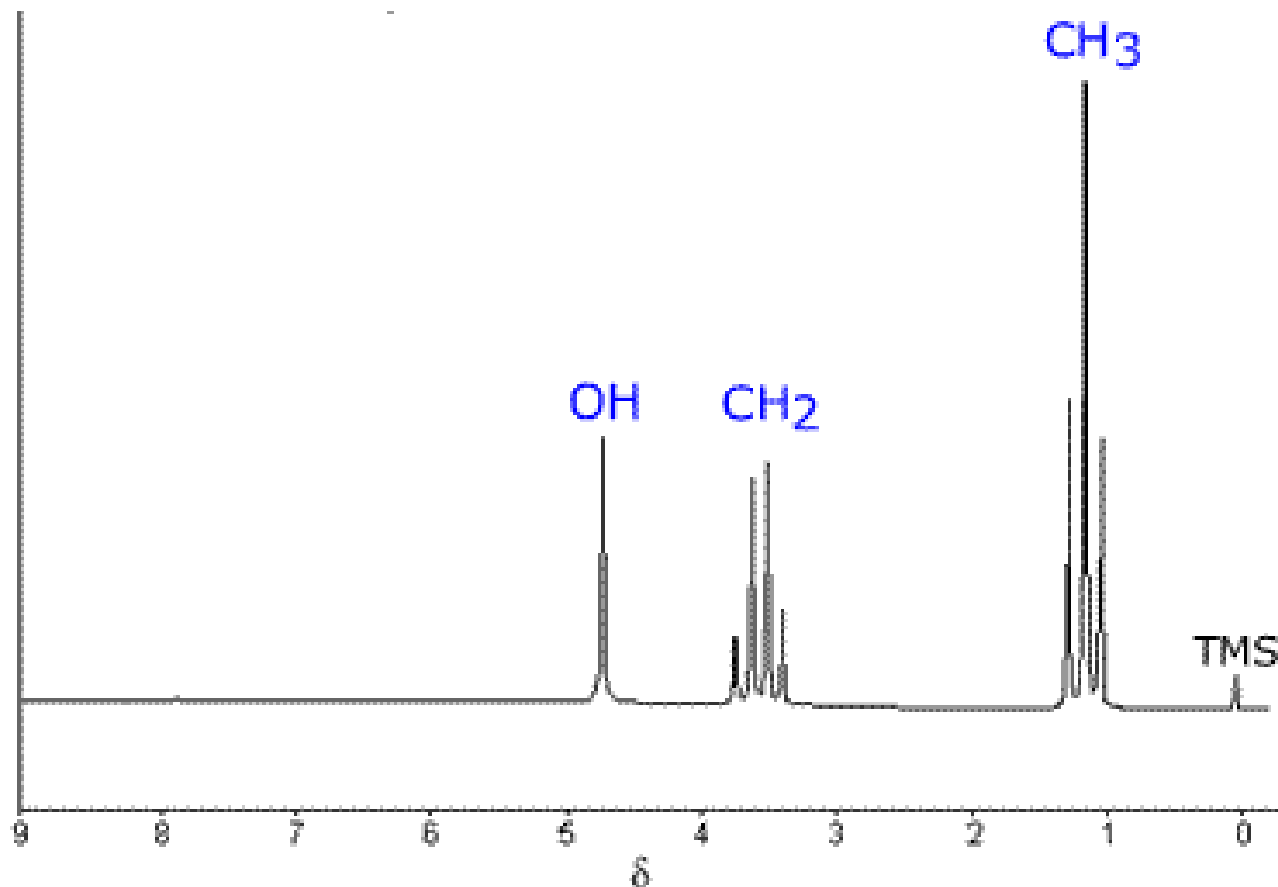
Precessional Frequency (Larmor) = Applied radiofrequency (Rf)



Angular momentum differ based on environment,
so change in Gyromagnetic ratio and energy levels



Same way, in the example of Ethanol, there are three type of protons, CH_3 , CH_2 and OH , they resonate with the applied R_f at different field strength



CHEMICAL SHIFT

- The number and position of signals in NMR spectrum signifies the number and nature of protons in the molecule.
- Each of these protons will have different electronic environments and thus they absorb at different applied field strengths.
- When a molecule is placed in a magnetic field, its electrons are caused to circulate and thus they produce secondary magnetic field i.e., induced magnetic field.
- The induced magnetic field can either oppose or reinforce the applied field.



A **chemical shift** is defined as the difference in parts per million (ppm) between the resonance frequency of the observed proton and tetramethylsilane (TMS) hydrogens.

TMS is the most common reference compound in NMR, it is set at $\delta=0$ ppm

$$\text{Chemical shift, } \delta = \frac{\text{frequency of signal} - \text{frequency of reference}}{\text{spectrometer frequency}} \times 10^6$$



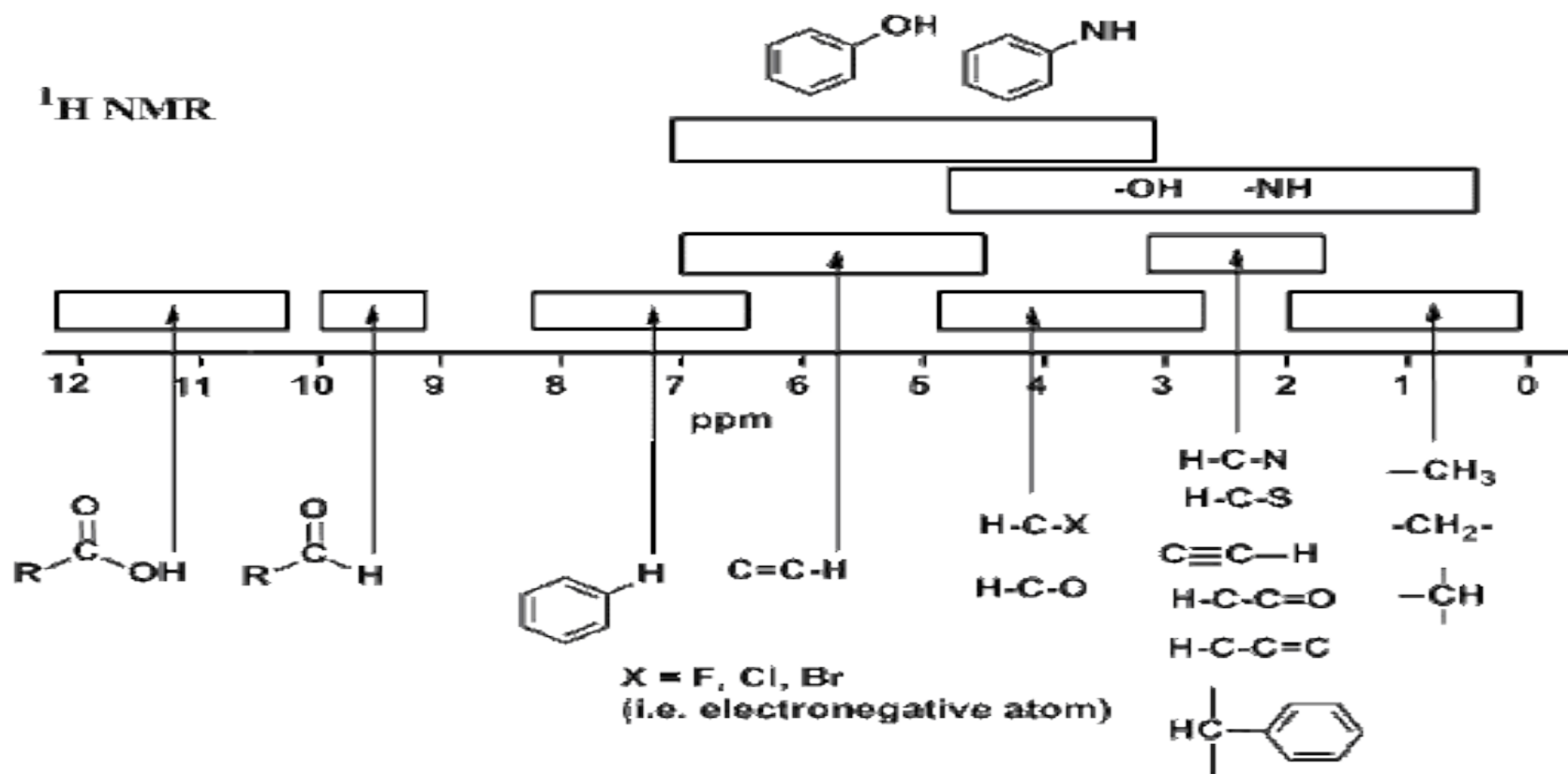
Formula for chemical Shift

$$\delta_{\text{sample}} = \left(\frac{\nu_{\text{sample}} - \nu_{\text{reference}}}{\nu_{\text{reference}}} \right) \times 10^6$$

Other formula are

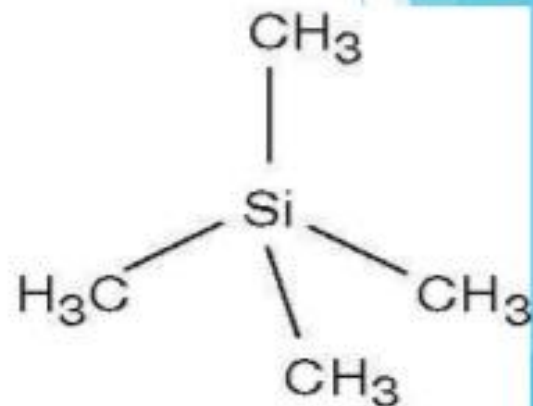
- Chemical shift =
Chemical shift of Test proton - Chemical shift of TMS
- Chemical shift in Tau value = 10 – delta value (ppm)
- Chemical shift (delta in ppm)
= Shift of proton in HZ / Instrument frequency in MHZ

Chemical shift values

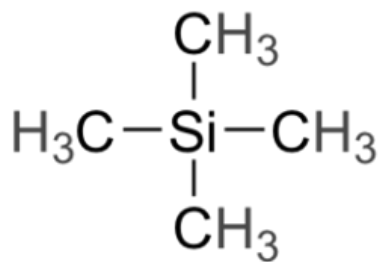


Measurement of Chemical Shift

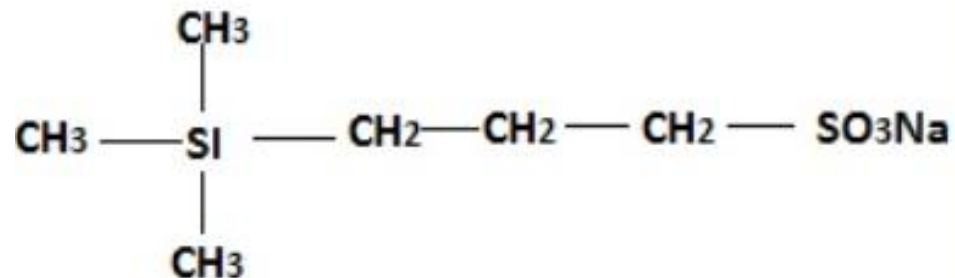
- ▶ In order to measure the magnitude of chemical shifts of different kinds of protons,
- ▶ There must be some standard signal .
- ▶ 0.5% **Tetra methylsilane (TMS)** $(\text{CH}_3)_4\text{Si}$ is used as reference or standard compound.



- ▶ Chemical shift is represented by δ
- ▶ Δ scale: 0 to -10 scale
- ▶ TMS as zero markers.
- ▶ Dimensionless expression; negative for most protons.



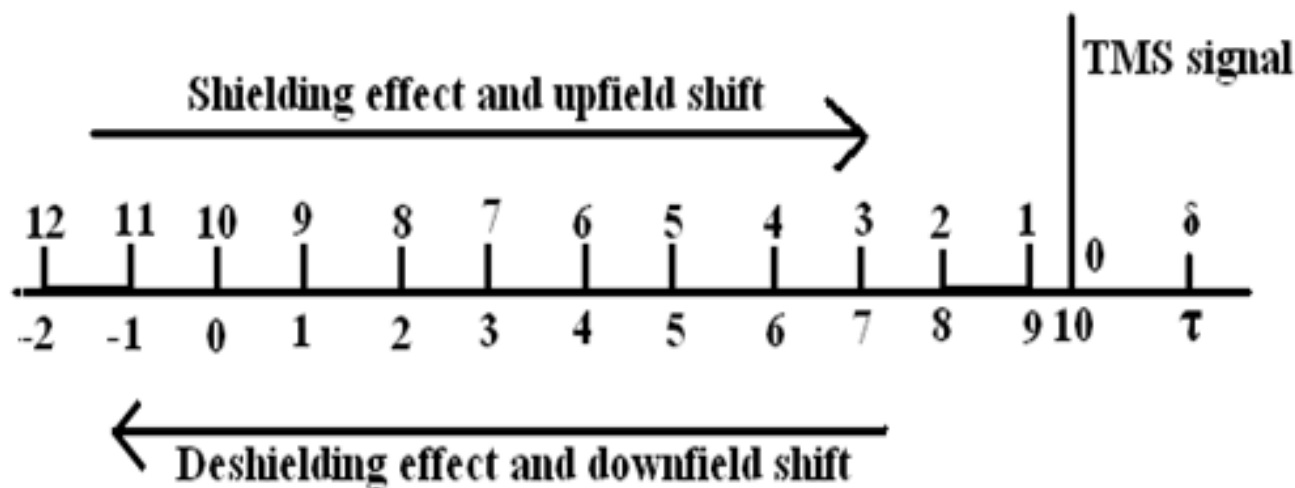
Tetramethyl saline sulfonate (TMS):
Water insoluble



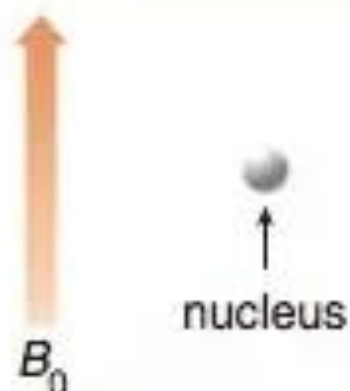
Sodium, 3-(Trimethyl silyl) propane sulfonate: water soluble

NOTE:

Maleic acid,
Dimethyl sulfones,
1,4-BTM-d₄
DSS-d₆
are other reference
compounds used
in Quantitative
NMR

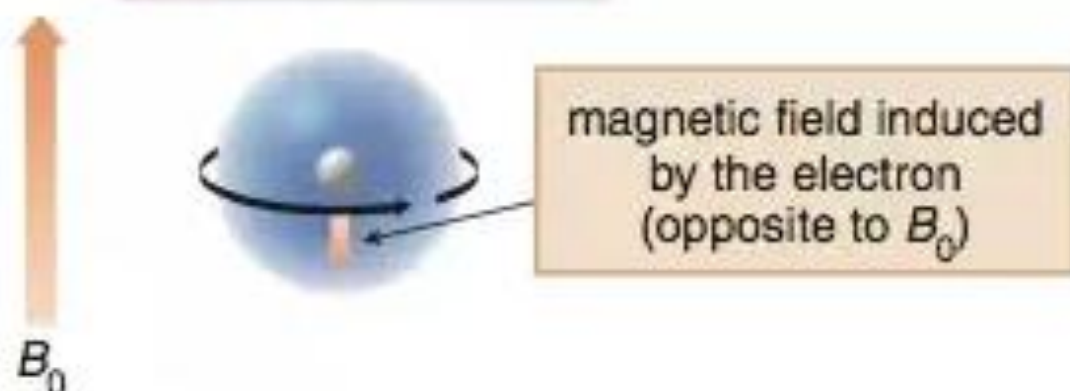


An isolated proton



The nucleus "feels" B_0 only.

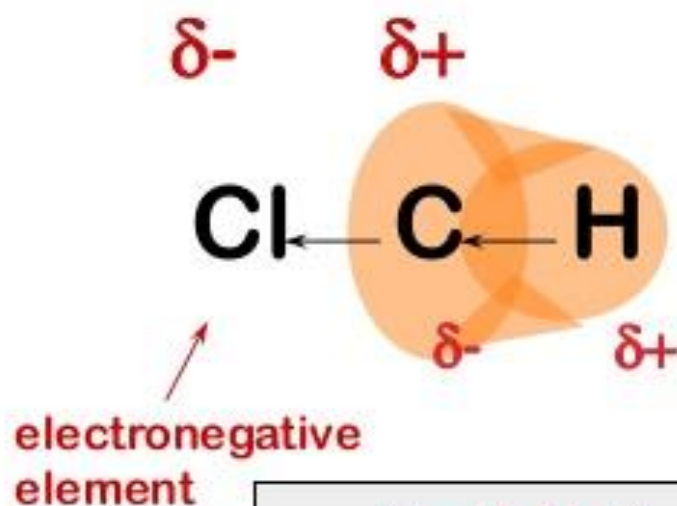
A proton surrounded by electron density



The induced field *decreases* the strength of the magnetic field "felt" by the nucleus.

This nucleus is shielded.

Electronegativity and Inductive effect



Chlorine “deshields” the proton, that is, it takes valence electron density away from carbon, which in turn takes more density from hydrogen deshielding the proton.

NMR CHART

“deshielded”
protons appear
at downfield
(higher δ)

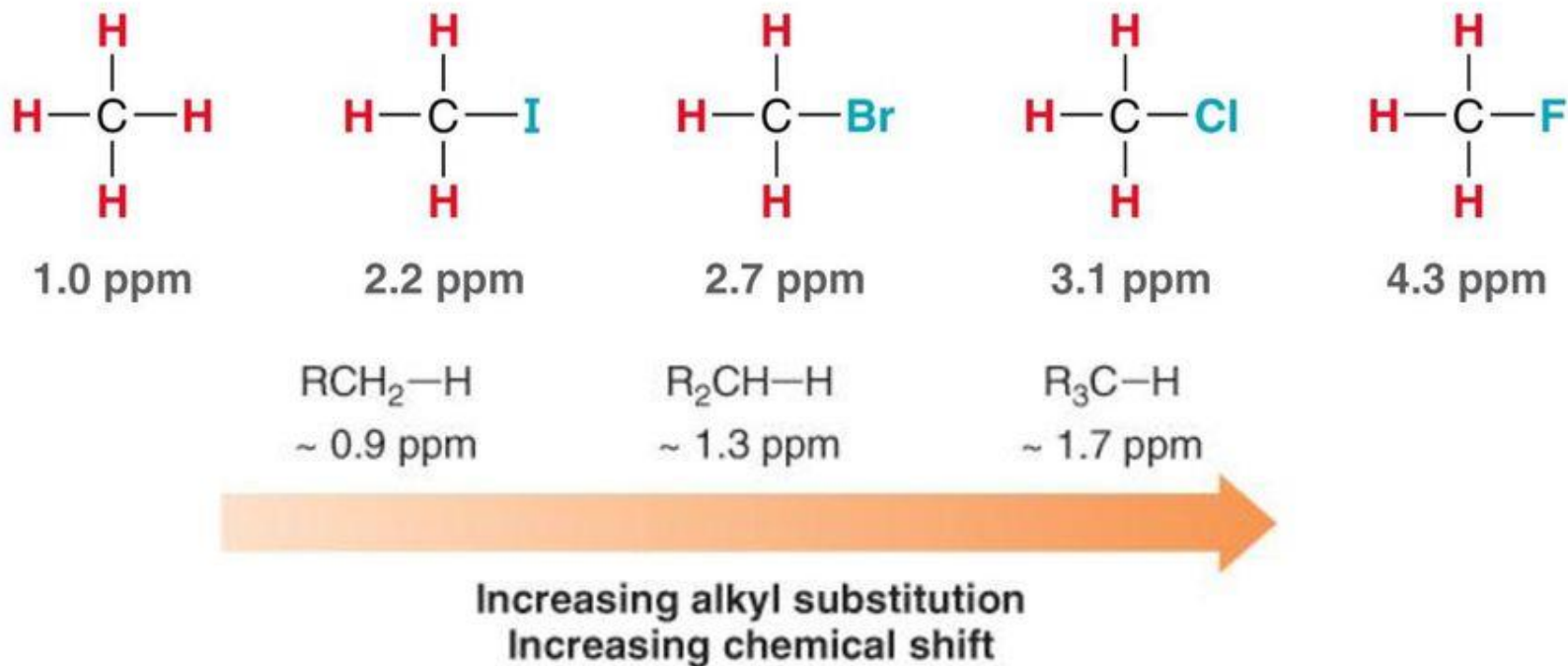
“highly shielded”
protons appear
at upfield
(lower δ)

←
deshielding moves proton
resonance to lower field and higher δ

Chemical Shift

Proton NMR shifts normally range from 0 ppm (TMS) to 10 ppm

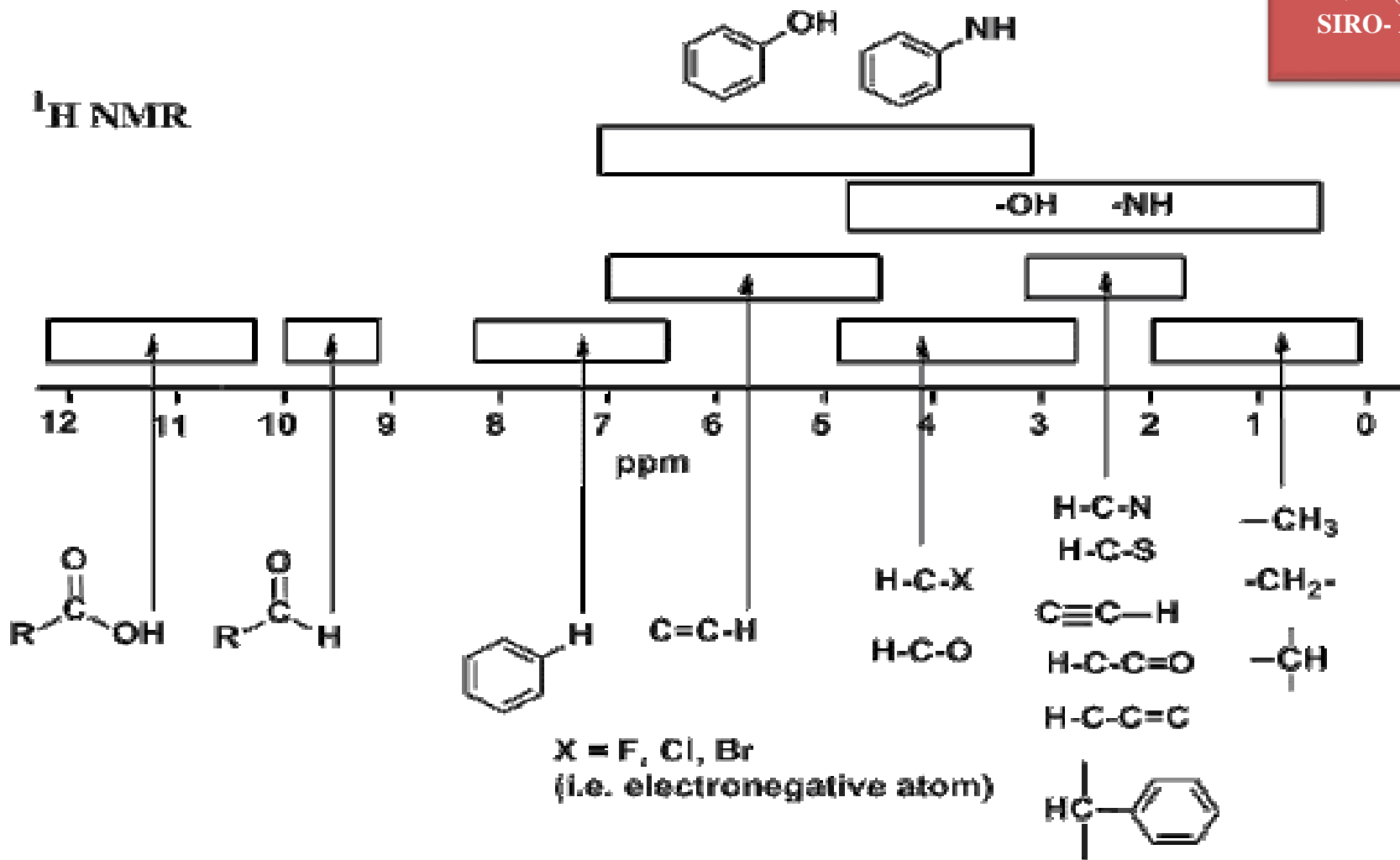
Inductive effects explain a lot:

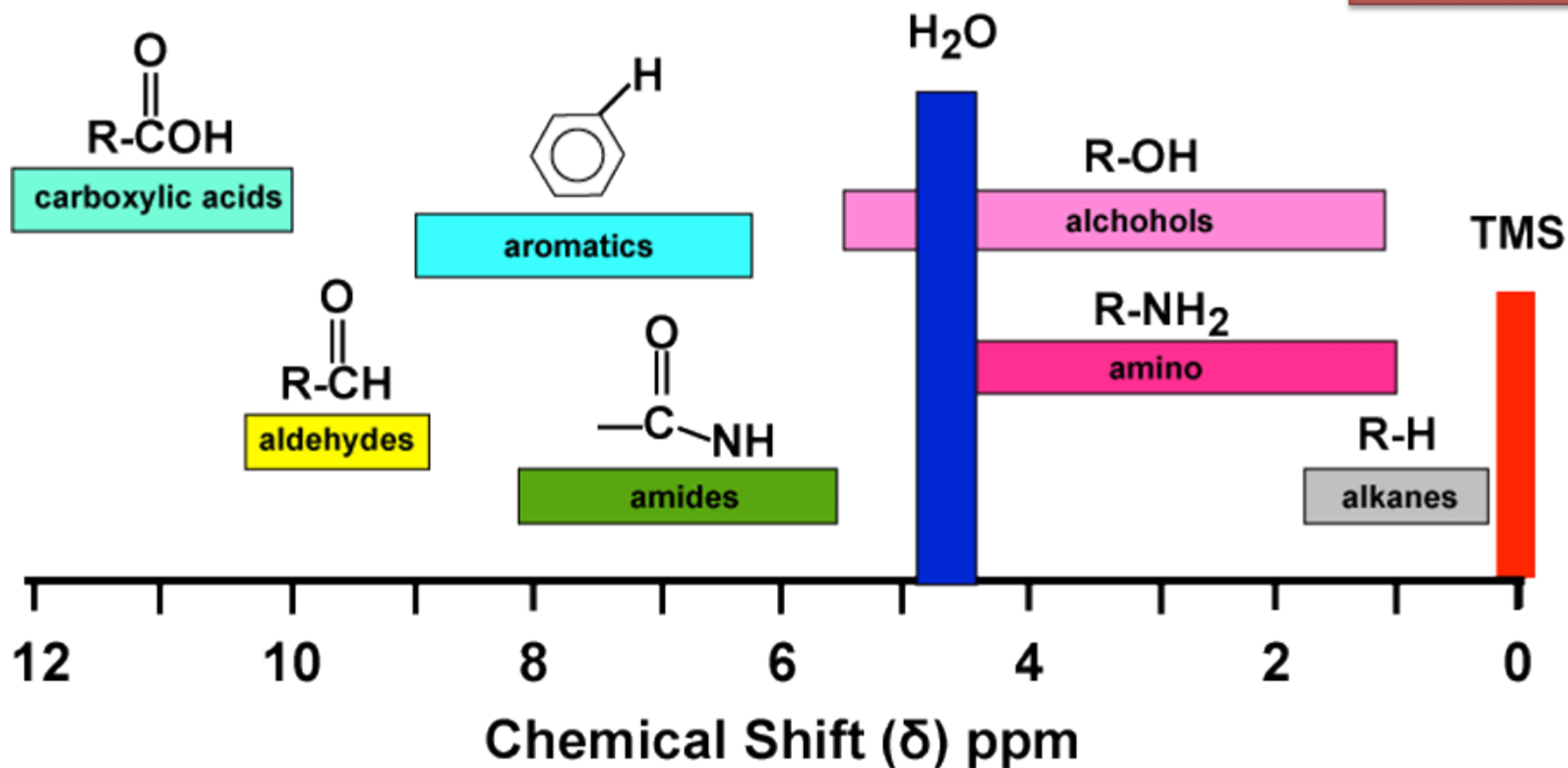


CHCl_3 in CDCl_3 = 7.26 ppm

H_2O in CDCl_3 = 1.55 ppm

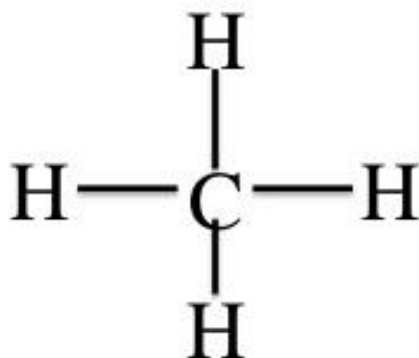
Chemical shift values





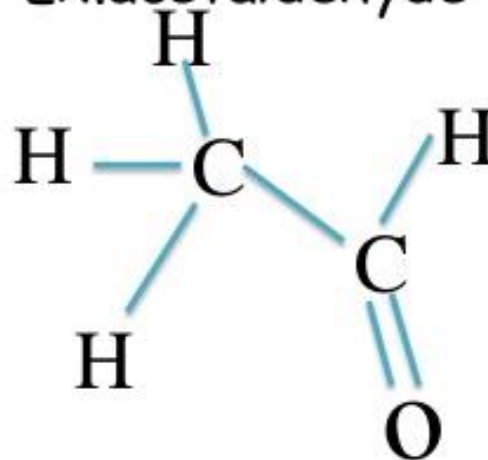
Magnetically equivalent (same environment) protons gives same signals

- Equivalent Protons
Ex. Methane

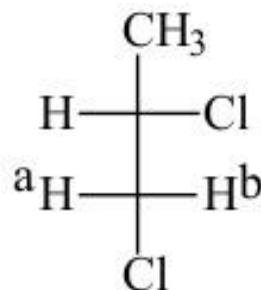
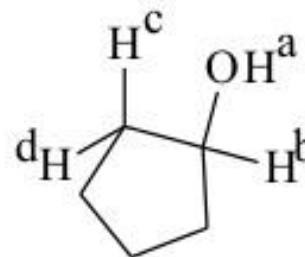
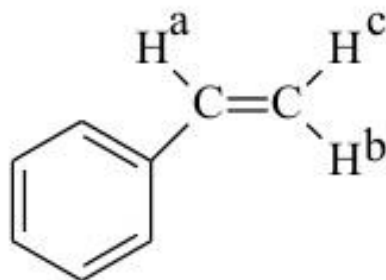


- Non-Equivalent protons

Ex. acetaldehyde



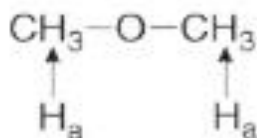
Some Nonequivalent Protons



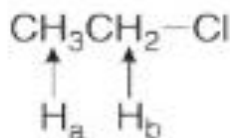
\Rightarrow

^1H NMR—Number of Signals

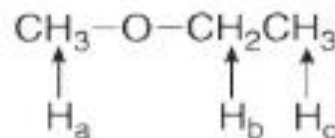
- The number of NMR signals equals the number of different types of protons in a compound.
- Protons in different environments give different NMR signals.
- Equivalent protons give the same NMR signal.



All equivalent H's
1 NMR signal

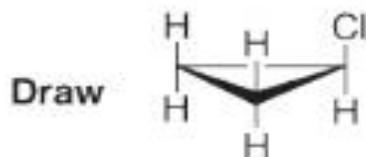


2 types of H's
2 NMR signals



3 types of H's
3 NMR signals

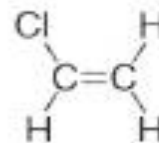
- To determine equivalent protons in cycloalkanes and alkenes, always draw all bonds to hydrogen.



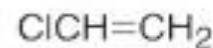
NOT



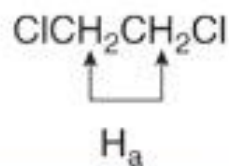
Draw



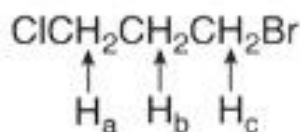
NOT



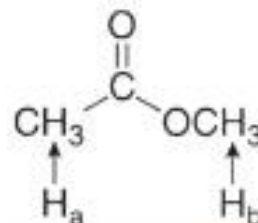
^1H NMR—Number of Signals



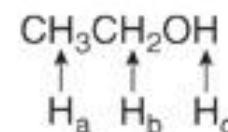
1 type of H
1 NMR signal



3 types of H's
3 NMR signals

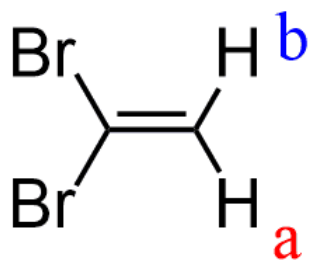


2 types of H's
2 NMR signals



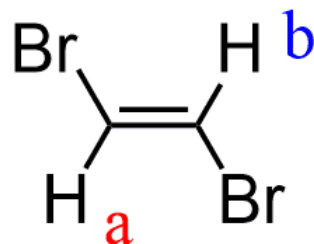
3 types of H's
3 NMR signals

Equivalent protons in alkenes must be cis to the same group



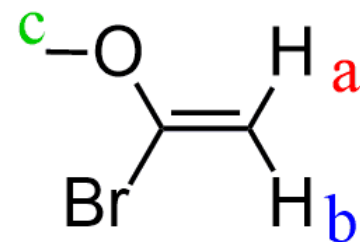
Ha and Hb are **both**
cis (trans) to the Br
They are **equivalent**.

One NMR signal



Ha and Hb are **both**
cis (trans) to the Br
They are **equivalent**.

One NMR signal



Ha is **cis** to **methoxy**,
while Hb is **cis** to the **Br**
They are **NOT equivalent**.

Two NMR signals (a, b)
and one signal from CH₃

SPIN-SPIN SPLITTING

Often a group of hydrogens will appear as a multiplet rather than as a single peak.

Multiplets are named as follows:

Singlet	Quintet
Doublet	Septet
Triplet	Octet
Quartet	Nonet

This happens because of interaction with neighboring hydrogens and is called **SPIN-SPIN SPLITTING**.

SPIN-SPIN Coupling (Splitting)

Sometime signal will appear without split (singlet)
sometime split as two (doublet), three (triplet) etc.
It due to SPIN-SPIN relaxation process.

The spit intensity will be based on the following
Pascal's triangle.

The following is the Pascal triangle where “n” is
number neighboring protons.



Relaxation

- ◆ **Spin-lattice relaxation** converts the excess energy into translational, rotational, and vibrational energy of the surrounding atoms and molecules (the lattice).
- ◆ **Spin-spin relaxation** transfers the excess energy to other magnetic nuclei in the sample.

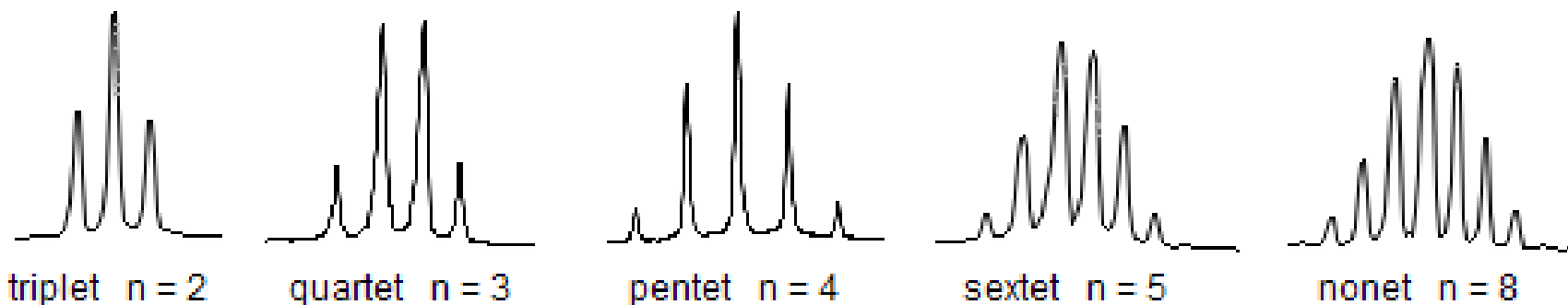
n	(n + 1) multiplicity	relative intensities	multiplet name	abbreviation
0	1	1	singlet	(s)
1	2	1:1	doublet	(d)
2	3	1:2:1	triplet	(t)
3	4	1:3:3:1	quartet	(q)
4	5	1:4:6:4:1	quintet	(quint)
5	6	1:5:10:10:5:1	sextet	(sext)
6	7	1:6:15:20:15:6:1	septet	(sept)
7	8	1:7:21:35:35:21:7:1	octet	(oct)
8	9	1:8:28:56:70:56:28:8:1	nonet	(non)

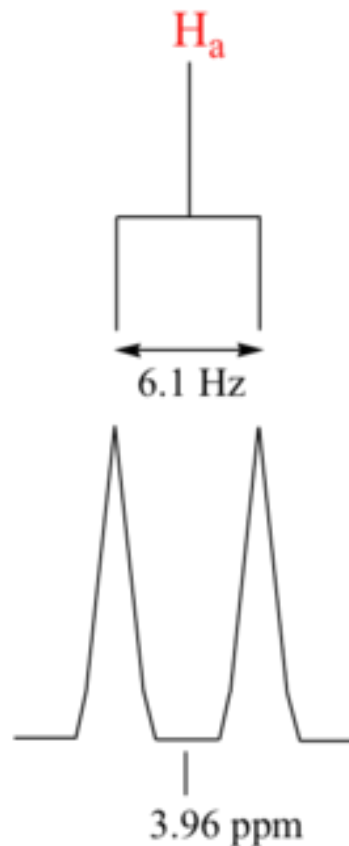
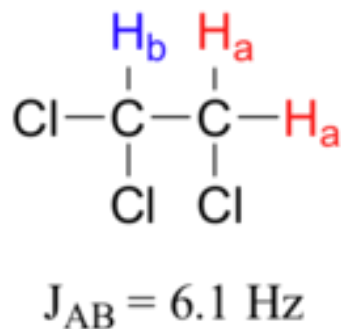
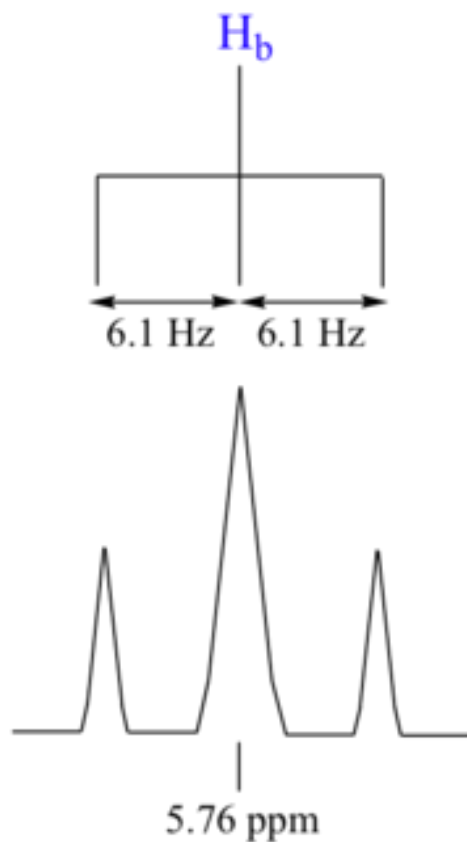


The split of signal is due coupling of proton with neighbor proton. The value between split is measured as Hz (**coupling constant**) which is important factor in assigning conformations and configuration.

N+1 Rule

Type of splitting **follows** the " $n+1$ rule": a proton with ' n ' neighbour appears as a cluster of $n+1$ **split** peaks. Based on the number of split they named as doublet, triplet etc.

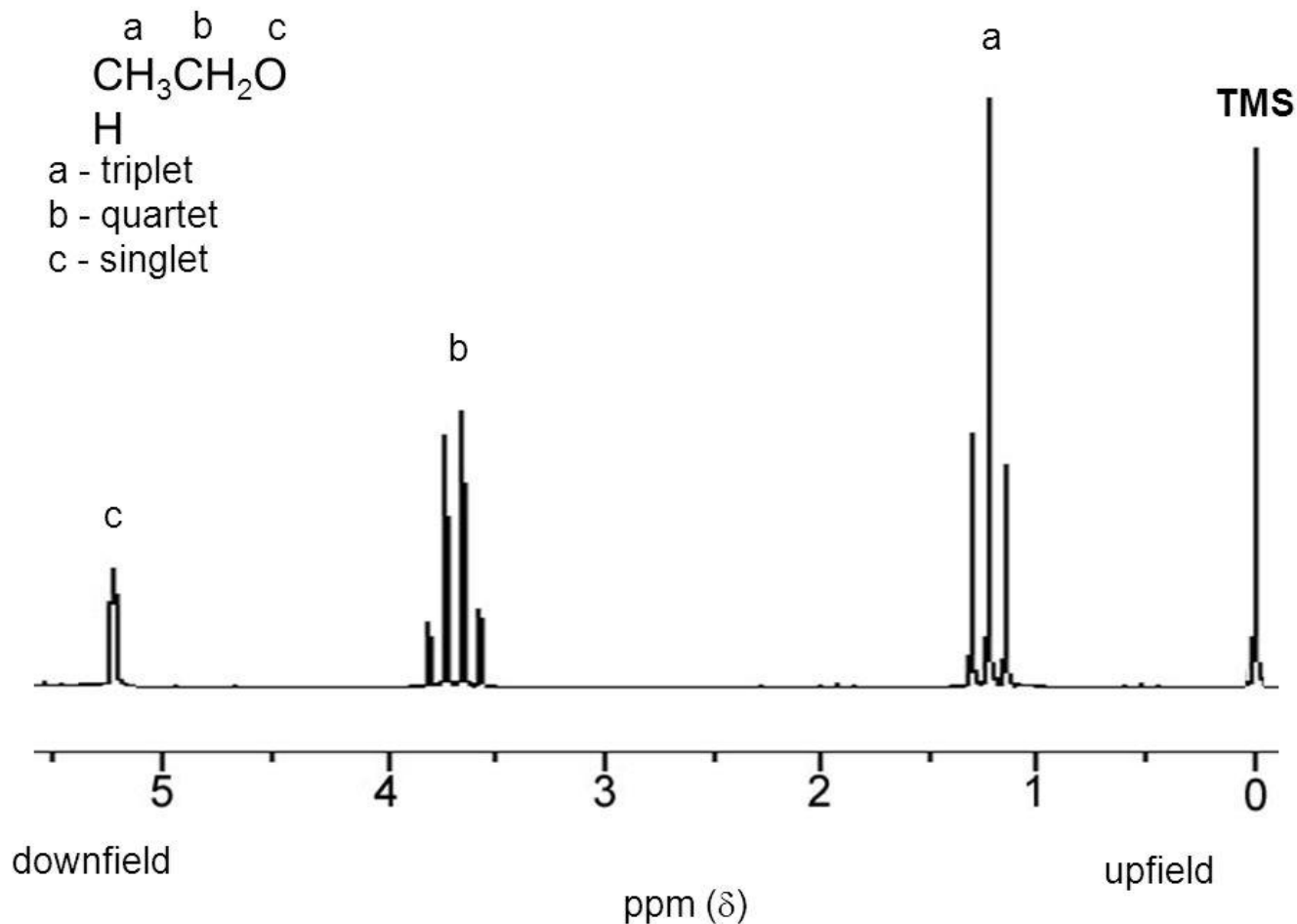




6.1 Hz = Coupling Constant

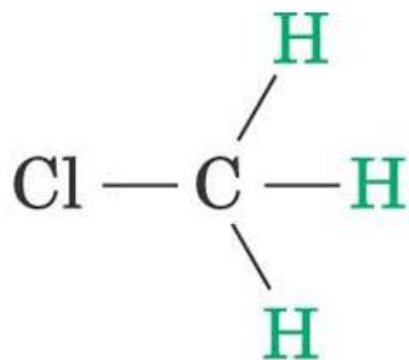
^1H NMR Spectrum of Ethanol: Spin-Spin Splitting

RIPER
AUTONOMOUS
NAAC &
NBA (UG)
SIRO- DSIR



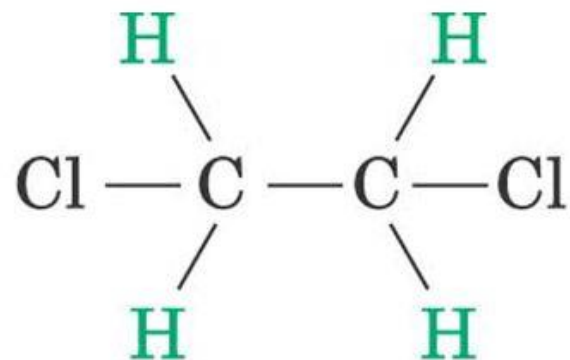
Raghavendra Institute of Pharmaceutical Education and Research - Autonomous
K.R.Palli Cross, Chiyyedu, Anantapuramu, A. P- 515721

Equivalent Protons do not Couple



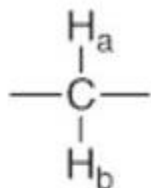
Three C–H protons are chemically equivalent; no splitting occurs.

©2004 Thomson - Brooks/Cole

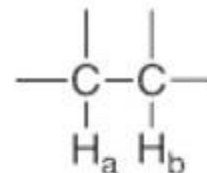
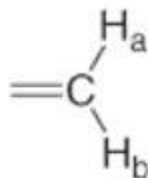


Four C–H protons are chemically equivalent; no splitting occurs.

If H_a and H_b are **not equivalent**, splitting is observed when:

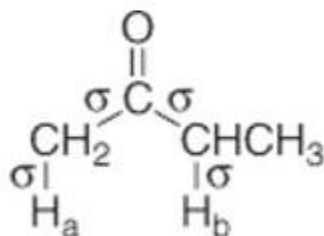


H_a and H_b are on the **same** carbon.



H_a and H_b are on **adjacent** carbons.

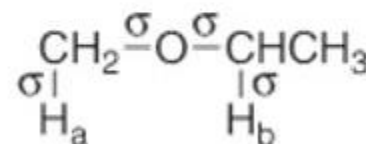
Splitting is not generally observed between protons separated by more than three σ bonds.



2-butanone

H_a and H_b are separated by four σ bonds.

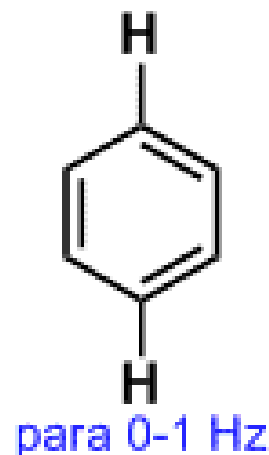
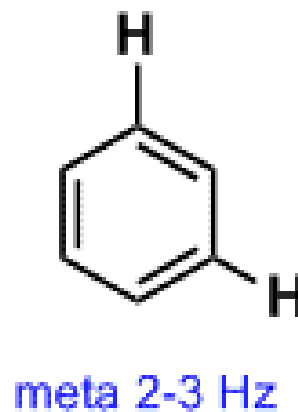
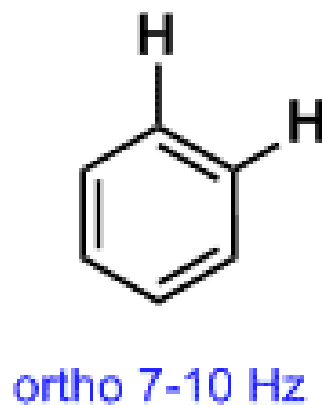
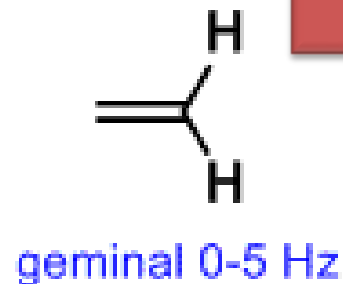
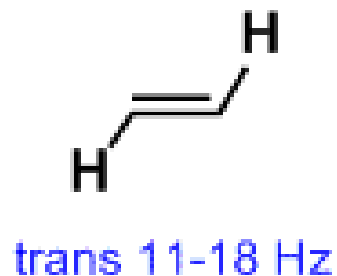
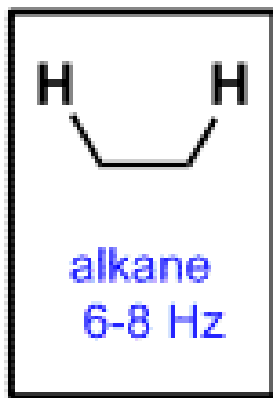
no splitting between H_a and H_b



ethyl methyl ether

H_a and H_b are separated by four σ bonds.

no splitting between H_a and H_b



MASS Spectroscopy



Raghavendra Institute of Pharmaceutical Education and Research - Autonomous
K.R.Palli Cross, Chiyyedu, Anantapuramu, A. P- 515721

Mass spectrometry is a most important analytical technique used to

- find out the molecular weight,
- identify unknown compounds within a sample,
- determine the isotope ratio
- Elucidate the structure and
Study of different isomers

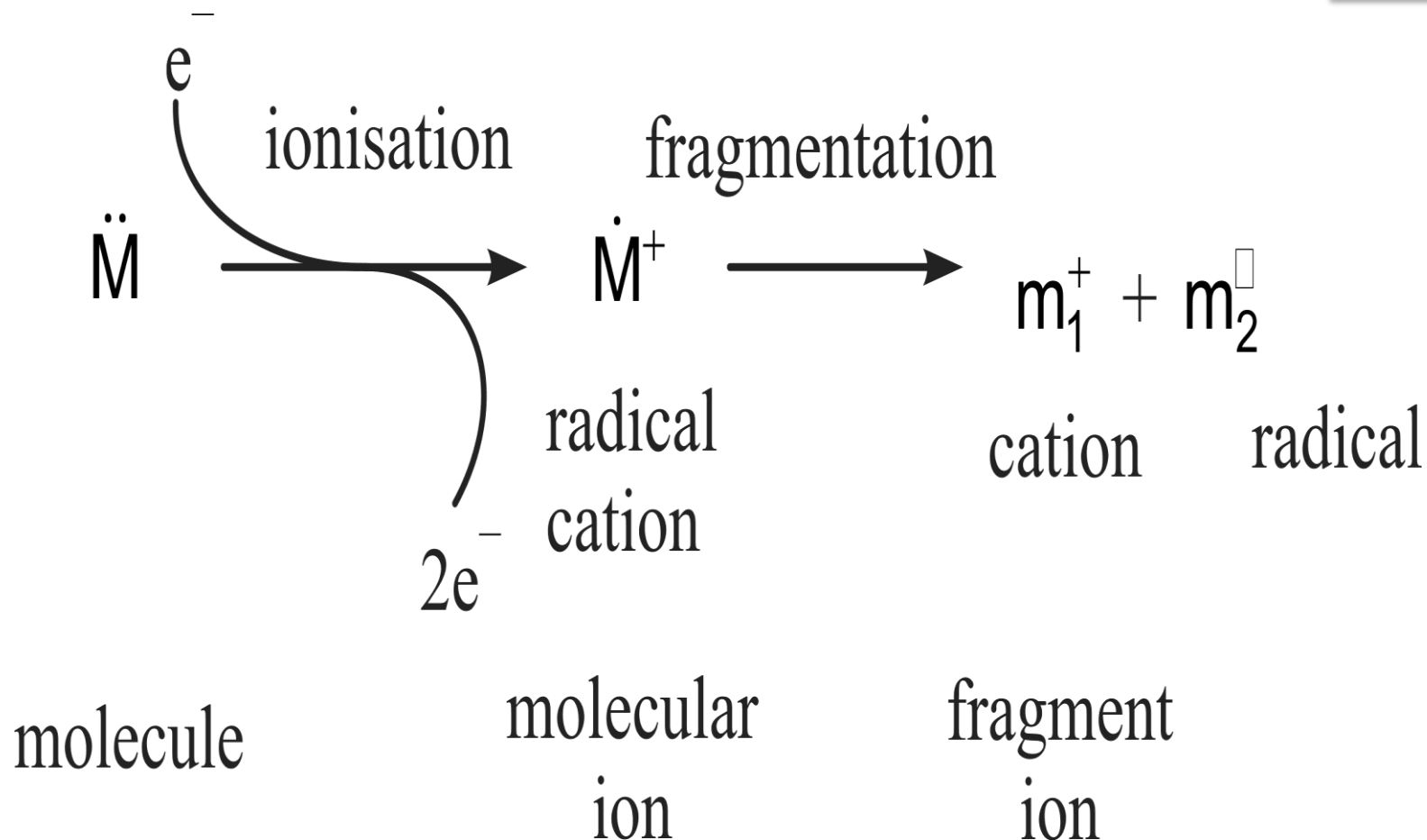


The mass spectrometry involves the conversion of the **neutral** molecule into gaseous ions, with or without fragmentation,

which are then characterized by their mass to charge ratios (m/z) and % relative abundances.

The conversion of neutral gas molecule to positively charged molecule is **called ionization process** it is attained by different ionization techniques.





TYPES of Ionization process in Mass spectrometry

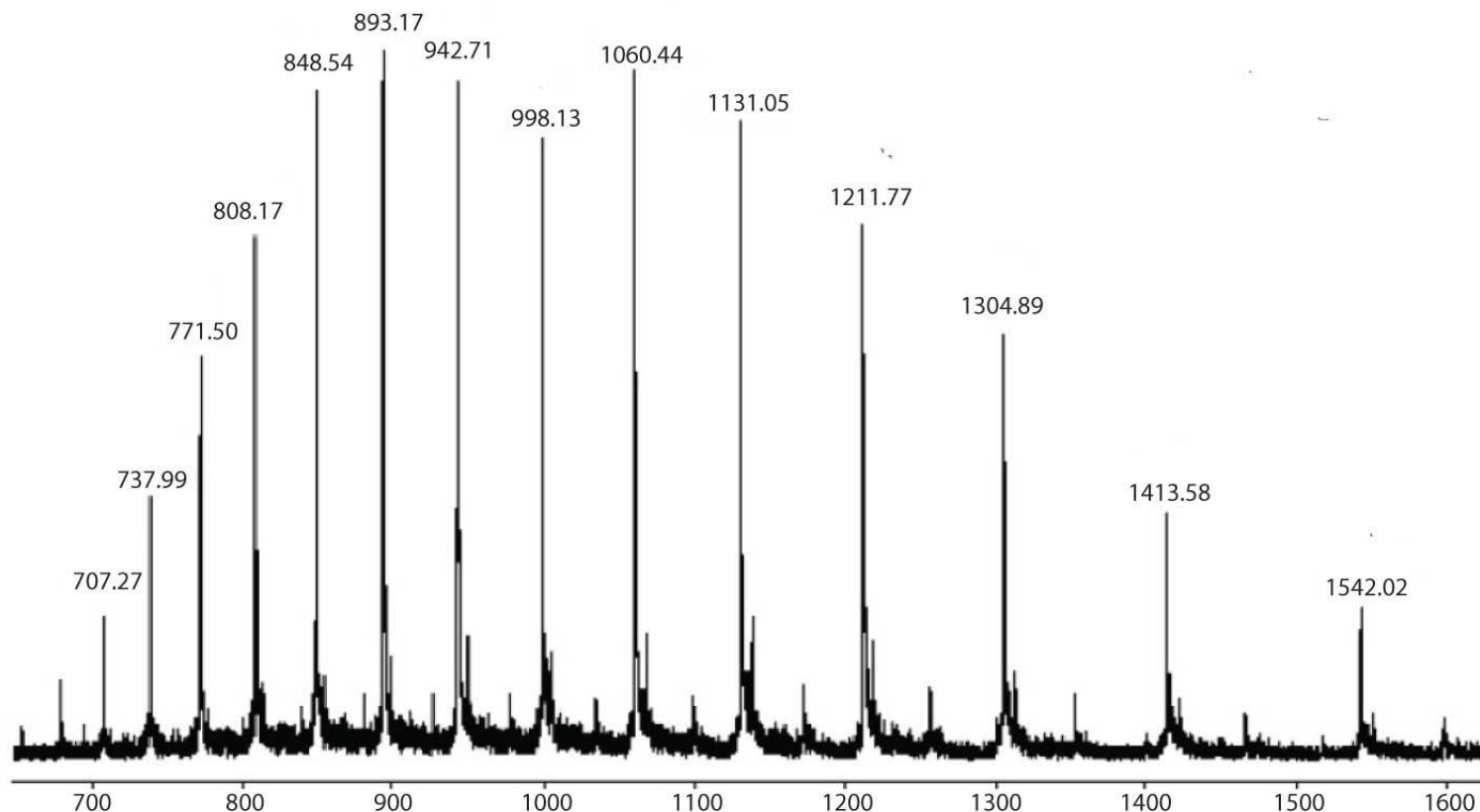
- Electron impact (EI) – 70 eV used to ionize (Hard ionization Process).
- Chemical ionization (CI)- Reagent gas (methane, ammonia etc)
- Fast atom bombardment ionization (FAB) – For soft molecule based on the collision between high velocity Xe or Ar gaseous molecule.
- Electro-spray Ionization (ESI)– Based on voltage at spray nozzles used for polar molecule in LC-MS



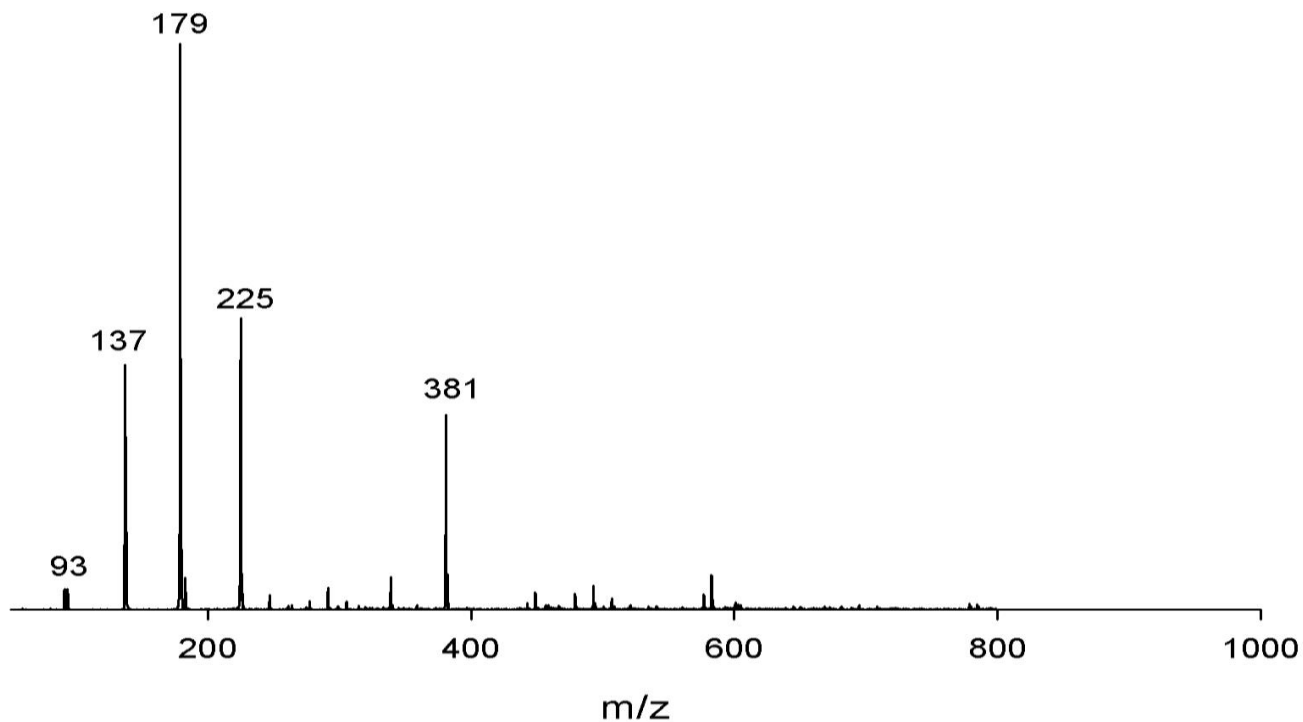
- Atomic pressure chemical ionization (APCI)- for Non –polar molecule in LC-MS
- Matrix assisted Laser desorption ionization (MALDI)- based on the sample-matrix (sample mixed with urea, nicotinic acid etc.) which was ionized by the irradiation of LASER.

Above all, except EI, all other ionizations are soft ionization process. $M+1$ (quasi molecular ions) ion is more common in all techniques except Electron impact ionization

Mass Spectrum



Bar Graph of Mass Spectrum (Noise are removed)



Different Peaks in Mass Spectrum

Molecular ion (parent peak – highest m/z)

Base peak (100 %)

Fragment peaks (lower m/e value than molecular ion peak)

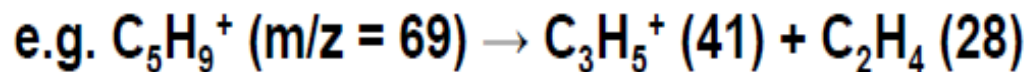
Isotope peak ($M+1$, $M+2$) – should not be confused with quasi-molecular ion peak.

Meta-stable ion peak



- Meta-stable ion peak (m/z value with decimal value.. example 36.4, its due to abnormal translation al energy of ions which has ionized at analyzer instead at ionization chamber).
- If M^* is metastable peak, the following formula will be used for calculation

$$M^* = M_2^2/M_1$$



Calculated $m^* = (41)^2/69 = 24.36$, observed $m^* = 24.4$

Nitrogen Rule

'The nitrogen rule states that organic molecules that contain hydrogen, carbon, nitrogen, oxygen, silicon, phosphorus, sulfur, and the halogens have an odd nominal mass if they have an odd number of nitrogen atoms or an even mass if they have an even number of nitrogen atoms are present'.

Even molecular weight	Nitrogen may be present or may not be present	Presence of Nitrogen in even number or Zero
Odd molecular weight	Molecule contain nitrogen	Presence odd nitrogen only .

Ring equivalence plus double bond equivalence or Index of Hydrogen deficiency

The following formula from a molecular formula will give the information of number of ring / double bond in the structure.

$$\text{Rings} + \pi \text{ Bonds} = u = C - \frac{H}{2} - \frac{X}{2} + \frac{N}{2} + 1$$

Formula	u = value	Justification
CO ₂ (O=C=O)	2	The presence of two double bond in the structure
H ₂ O (H-O-H)	0	No double bond or Ring
C ₆ H ₆	4	the three double bond and one ring in Benzene

Even electron rule

The even electron rule states that ions with an even number of electrons (cations but not radical ions) tend to form even-electron fragment ions and whereas the odd-electron ions (radical ions) form odd-electron ions or even-electron ions.

Even-electron species tend to fragment to another even-electron cation and a neutral molecule rather than two odd-electron species

Rule of 13

- The **Rule of 13** is used for tabulating possible chemical formula for a given molecular mass.
- It is based on the assumption that only carbon and hydrogen are present in the molecule, so the molecule comprises some number of CH "units" (has a nominal mass of $12+1 = 13$).
- If the molecular weight of the molecule in question is M , the number of possible CH units is n and if r is the remainder

$$\frac{M}{13} = n + \frac{r}{13}$$

The base formula for the molecule is **C_nH_{n+r}**

Stevenson's Rule

“According to **Stevenson's rule**, if two fragments are in competition to produce a cation, the fragment with the lowest ionization energy will be formed more frequently”. It means longest chain will be cleaved as radical.