

Heteronuclear Multiple Bond Correlation Spectroscopy- An Overview

Y.Vasavi¹, N.Parthiban¹, D.Sathis Kumar*¹, David Banji¹, N.Srisutherson¹,
Somsubhara Ghosh¹, M. Vinay Kumar Chakravarthy².

¹Department of Pharmaceutical Analysis, Nalanda College of Pharmacy,
Nalgonda, Andhra Pradesh -508001, India.

²JNT University, Hyderabad, India.

*Corres. author: satmpdina@yahoo.co.in
Mobile: +919966796051

Abstract: HMBC is one of the two dimensional experiments. Two-dimensional nuclear magnetic resonance (2D NMR) provides one of the foremost contemporary tools available for the elucidation of molecular structure, function, and dynamics. HMBC is a long range correlation experiment which provides information about carbons bonded to proton which are 2-3 bonds away. HMBC has applications in every field for identifying and characterising the structure of compound. In the present review, we discussed the following, the HMBC process, its advantages over 1D NMR techniques and comparisons with HETCOR and HMQC.

Key words: Heteronuclear, Multiple Bond Correlation Spectroscopy.

Introduction:

Two dimensional NMR spectroscopy can be defined as a spectral method in which the data are collected in two different time domains: acquisition of the free induction decay, FID (t_2), and a successively incremented delay (t_1) to elapse before detection, then during this time interval (the evolution period) the nuclei can be made to interact with each other in various ways, depending on pulse sequences applied.^[1,2] The resulting FID is accordingly subjected to two successive sets of Fourier transformations to furnish a two-dimensional NMR spectrum in two frequency axes. The major difference between one- and two- dimensional NMR methods is therefore the insertion of an evolution time, t_1 .^[1] For 20 years centre stage has been occupied by two-dimensional (and now

three and four-dimensional) NMR techniques. 2D NMR offers distinct advantages like relief from overcrowding of resonance lines, as the spectral information is spread out in a plane or a cube rather than along a single frequency dimension, opportunity to correlate pairs of resonances, has features in common with various double resonance methods and is far more efficient and versatile^[3]. A convenient way to understand the modern 2D NMR experiment is in terms of magnetisation vectors. A $\pi/2$ pulse is that which rotates the magnetisation vector by 90° . That is the net magnetisation vector rotating about the z-axis is now rotating in xy plane. A 90° pulse gives the strongest signal, as the signal y-axis component is only detected. The figure 1 below explains this.

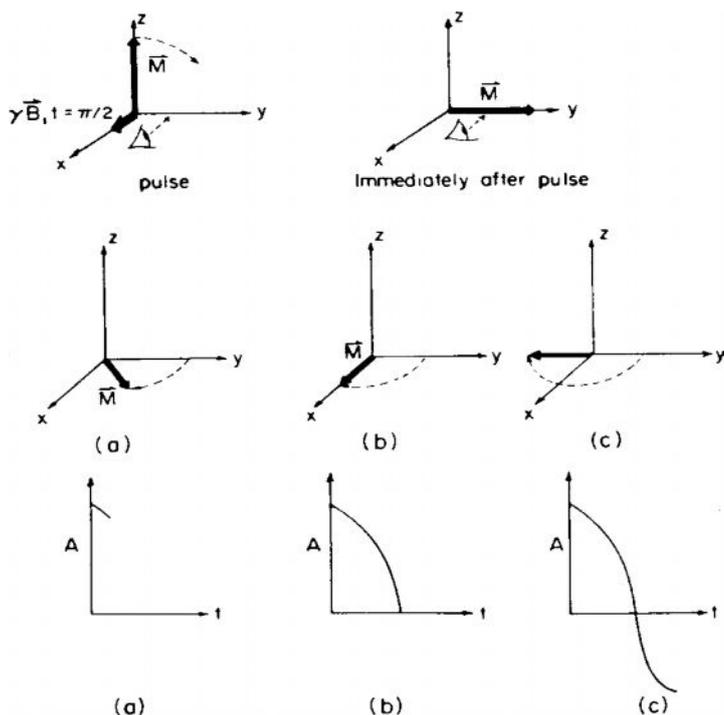


Figure 1: Classical picture of a pulsed NMR experiment. Relation between precessing moment (top and centre) and the observed transverse component of the magnetization as a function of time (bottom) [ref: Affa-ur-rahman, Muhammad chaudary, Solving problems with NMR spectroscopy, Pakistan, 1991, Academic Press.]

Data are taken as a function of a single time parameter, and the relation between these data and the frequency spectrum is Fourier transform relation.^[2] The resulting time domain signal is changed to frequency domain signal by some mathematical calculations (Fourier transformations). This is represented in the following figure 2.^[4]

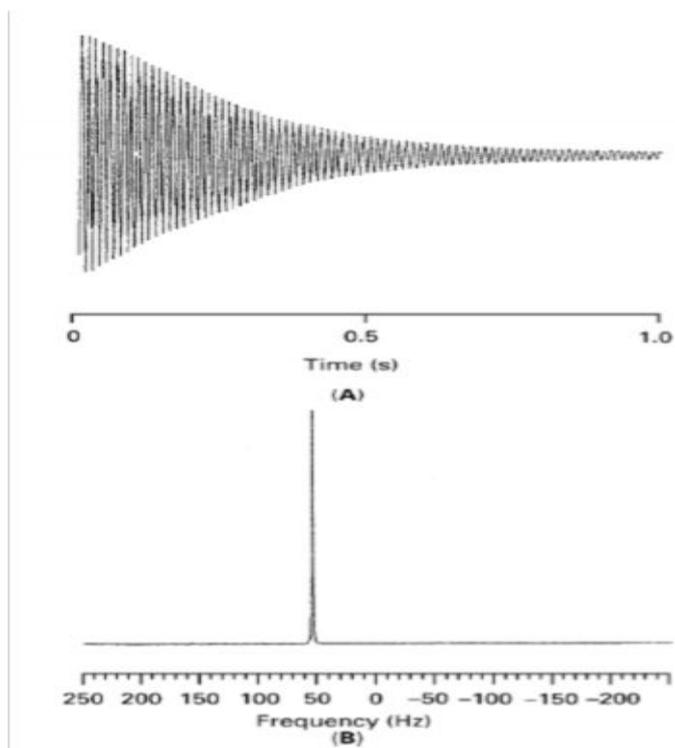


Figure 2: The FID time signal (A) and resultant ¹H frequency spectrum (B) for a single off-resonance peak. [ref: John C.Lindon, 2000.]

Basic principle:

2D NMR essentially allows us to irradiate all of the chemical shifts in one experiment and gives us a matrix or two dimensional maps of all of the affected nuclei. There are four steps to any 2D experiment^[5]. Preparation, evolution, mixing and detection.^[6, 7] Preparation is usually just a 90° pulse which excites all of the sample nuclei simultaneously. During preparation nucleus, A (when two nuclei A and B are considered) gets excited creating magnetisation in x-y plane. During evolution, chemical shift of the nucleus A is measured.^[8] During mixing, transfer of magnetisation from nucleus A to the nucleus B via J or NOE. Detection is simply recording an FID and finding the frequency of nucleus B by Fourier transformation. All possible pairs of nuclei in the sample go through this process at the same time. To get a second dimension, the chemical shift of nucleus A have to be measured before it passes its

magnetization to nucleus, B. This is accomplished by simply waiting a period of time (called t_1 , the evolution period) and letting the nucleus, A magnetization rotate in the x-y plane. The experiment is repeated many times over (for example, 512 times), recording the FID each time with the delay time t_1 incremented by a fixed amount. The time course of the nucleus, A magnetization as a function of t_1 is used to define how fast it rotates and thus its chemical shift. Mixing is a combination of RF pulses and/or delay periods which induce the magnetization to jump from A to B as a result of either a J coupling or an NOE interaction. Different 2D experiments (e.g., NOESY, COSY, HETCOR, etc.) differ primarily in the mixing sequence, since in each one we are trying to define the relationship between A and B within the molecule in a different way.^[9]

The general pulse sequence for the two dimensional experiment is as follows:^[1]

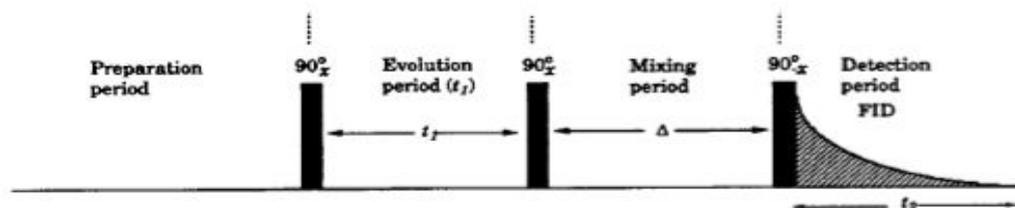


Figure 3: 2D NMR pulse experiment. Time axis is divided into preparation and an evolution period, t_1 . [ref: Affa-ur-rahman, University of Karachi, Karachi, Pakistan and Muhammad chaudary, 1991.]

The actual view of 2D NMR plot can be seen below in figure 4.^[3]

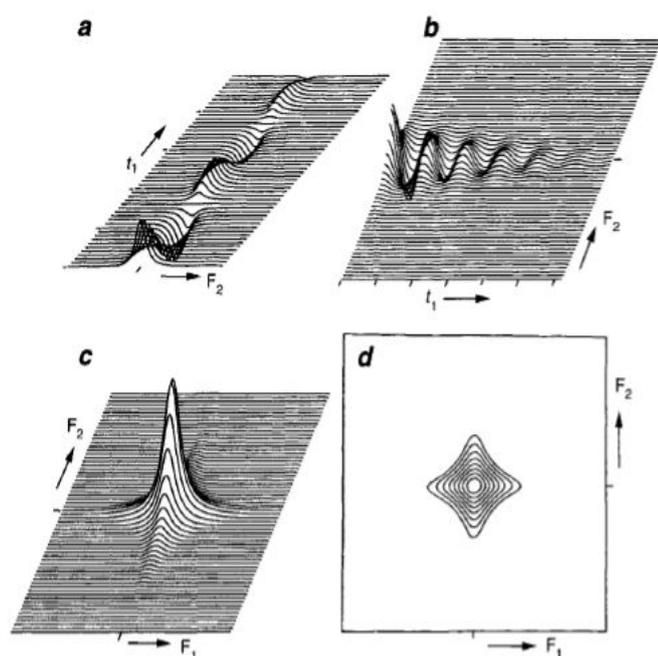


Figure 4: Data from 2D pulse sequence. Spectra resulting from a) Fourier transformation with respect to t_2 axis at various t_1 , (b) Rotation of axes in (a) to show modulation in signal amplitude as a function of t_1 . (c) Signal after second Fourier transform with respect to t_1 showing a single peak at coordinates, (d) Contour plot, showing the peak from (c) [ref: Edwin D. Becker,2000]

2D NMR classification:

2D NMR studies can be classified into Homonuclear Correlation studies and hetero nuclear correlation studies. Homonuclear correlation studies are done between similar nuclei (like proton-proton correlation, etc). This can be further classified depending on whether the correlation (magnetisation transfer) is through bond or through space into two types. 2D COSY and 2D TOCSY are through bond and NOESY and ROESY are through space. Coming to heteronuclear correlation studies, magnetisation transfer is between two dissimilar nuclei, may be between C^{13} and H^1 or N^{15} and H^1 , etc. This is again classified based on detection methods. Determination is direct in HETCOR and LR-HETCOR, while the determination is indirect/ inverse in HMBC, HMQC, and HSQC. Other two types are J-Resolved correlation where correlation studies are based on coupling constants (may be homo-J or hetero-J) and multiple quantum correlation which includes INADEQUATE, DQFCOSY.

COSY (Correlation Spectroscopy) is good for determining basic connectivity via J-couplings (through bond). TOCSY (Total Correlation Spectroscopy) same as COSY, but is also able to generate cross peaks via intermediate spins. This uses a spin lock that produces RF heating of the sample and hence requires many steady state scans (ss). This is also called as HOHAHA (HOMonuclear-HARTmann-HAhn spectroscopy). NOESY (Nuclear Overhauser Effect Spectroscopy) allows one to see through-space effects, and is useful for investigating conformation and for determining proximity of adjacent spin systems. This is not so useful for MWs in the 1 kDa (kilo Dalton) range due to problems arising from the NMR correlation time. Here longitudinal exchange of magnetisation between nuclei takes place. ROESY (Rotational Overhauser Effect Spectroscopy) is same as NOESY, but works for all molecular weights. Here transverse exchange of magnetisation between nuclei takes place^[10]. HMQC (Heteronuclear Multiple Quantum Correlation) allows one to pair NH or CH resonances. It often uses X-nucleus decoupling and hence gives rise to RF heating, requires reasonably well calibrated pulses and many steady state scans. Only protons directly bonded to C^{13} nuclei produce cross peaks. HSQC (Heteronuclear Single Quantum Correlation) provides the same information as HMQC, but gives narrower resonances for 1H - ^{13}C correlations. This also requires X-decoupling and hence a large number of steady state scans and is also more sensitive to pulse imperfections. HMBC (Heteronuclear Multiple Bond Correlation) is a variant of the HMQC pulse sequence that allows one to correlate X-nucleus

shifts that are typically 2-4 bonds away from a proton.^[9,11] This is Popular among the NMR spectroscopists in organic labs/ Parma industries. For small molecules generally homonuclear 2D techniques are sufficient for structure elucidation. However, when there is extensive overlap even in the 2D spectrum it helps to do heteronuclear correlation experiments. It is a technique which helps to determine which 1H of a molecule is bonded to which X nucleus in the molecule. Transfer of magnetization takes place between nuclei of different types. The two axes show the chemical shift of the respective type of nucleus. If a transfer has taken place (due to coupling), a signal appears at the intersection of the two frequencies. The first step in processing a 2D dataset is to Fourier transform each of the FIDs in the array. The resulting spectra are loaded into a matrix with the rows representing individual spectra. The horizontal axis is labelled F2 and the vertical axis is labelled F_1 ^[7]. Table 1 expressed different types of correlation spectroscopy and its differentiation.

Table1: Types of correlation spectroscopy

Name	F1 nucleus	Mixing	F2 nucleus
COSY	1H	J	1H
TOCSY	1H	J_2, J_3, \dots	1H
NOESY	1H	NOE	1H
HETCOR	1H	$^1J_{CH}$	^{13}C
HMQC	^{13}C	$^1J_{CH}$	1H
HMBC	^{13}C	$2,3J_{CH}$	1H

HMBC:

HMBC (Heteronuclear Multiple Bond Correlation) spectroscopy is a modified version of HMQC suitable for determining long-range 1H - ^{13}C connectivities. Since it is a long-range chemical shift correlation experiment, HMBC provides the information about the chemical shift of carbon atoms that are about 2-3 bonds away from the proton to which they correlate. Hence also quaternary carbon atoms will be detected.^[8] 1D protein spectra are far too complex for interpretation as most of the signals overlap heavily. By the introduction of additional spectral dimensions these spectra are simplified and some extra information is obtained. The invention of multidimensional spectra was the major leap in NMR spectroscopy apart from the introduction of FT-NMR. Consequently, both techniques were acknowledged by a Nobel Prize.

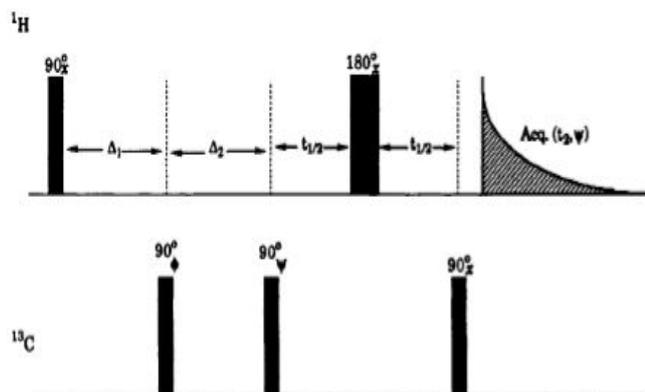


Figure 5: The pulse sequence for the HMBC experiment. [ref: Affa-ur-rahman, Muhammad chaudary, 1991].

Process and pulse sequence:

If the delays Δ_1 , Δ_2 are adjusted so they correspond to J_{CH} of 130-150 Hz, then only the direct one bond couplings between sp^2 and sp^3 hybridised C^{13} - H^1 bonds will be seen. If the value of delay is adjusted to correspond to J_{CH} of 5-10 Hz, long range couplings can be seen. These cross peaks will then correspond to coupling interactions between H^1 and C^{13} nuclei separated by 2, 3 even 4 bonds. In such a long range heteroscopy experiment, the one bond magnetisation transfer is suppressed and long range heteronuclear couplings observed.^[1] The figure 5 explains the pulse sequence for HMBC experiment for both proton and carbon irradiation. First 90° pulse of C^{13} nucleus occurs at $1/(2 \text{ } 1J_{XH})$ after the first 1H 90° pulse, serves as a low-pass J-filter to suppress one-bond correlations in the 2D spectrum. It does this by creating heteronuclear multiple quantum coherence for 1H 's directly coupled to a ^{13}C nucleus, and this unwanted coherence is removed from the 2D spectrum by phase cycling the first ^{13}C 90° pulse with respect to the receiver.^[12] Hence cross peaks due to direct connectivities do not appear, allowing long range C^{13} - H^1 connectivities to be recorded. Second 90° C^{13} pulse creates the desired zero and double quantum coherences for 1H 's J-coupled to a ^{13}C nucleus 2 or 3 bonds away. This is followed by evolution time. Due to the 180° H^1 pulse, placed halfway through t_1 , the above created zero and double quantum coherences are interchanged (removes the effect of 1H chemical shift from the t_1 modulation frequency). The final ^{13}C 90° pulse occurs directly after the evolution period, and is followed immediately by the detection period t_2 . After the last 90° C^{13} pulse, the H^1 signals resulting from C^{13} - H^1 multiple-quantum coherence are modulated by C^{13} chemical shifts and homonuclear proton couplings.^[1,13] Because of phase modulation, the final spectrum has peaks which are a combination of

absorption and dispersion line shapes. It is not possible to phase correct the spectrum so that the peaks are purely absorptive, and so the spectrum must be presented in magnitude mode. If more than one long-range 1H - ^{13}C connectivity is detected for one particular proton, the relative intensities of the corresponding resonances are directly related to the magnitude of the coupling constant^[12]. The difference between the two hetero nuclear correlation experiments, HETCOR and HMBC is that in HETCOR F_1 is proton domain and F_2 is C^{13} domain. This can be explained as follows. It is easier to obtain a higher digital resolution in 2D experiments in the F_2 domain than in the F_1 domain, since doubling the acquisition time t_2 results in little overall increase in the experiment time. This is so because the increase can be compensated by decreasing the relaxation delay between successive experiments. However, if the maximum time reached during t_1 , is doubled, the number of t_1 experiments should be doubled, there by not only increasing the total time taken for the experiment but also suffering a loss of signal due to relaxation during t_1 . This problem is avoided in inverse experiment, because C-H correlation is detected via the proton spectrum and the crowded proton region now lies in the F_2 dimension, which has a high digital resolution, where as the better dispersed C^{13} spectrum, lies in F_1 dimension, which requires a lower digital resolution.^[13]

The HMBC experiment, like the 2D COLOC (correlation spectroscopy for long range coupling) experiment, is particularly useful for locating the quaternary carbons by identifying various protons interacting with them through two-bond, three-bond occasionally four-bond coupling interactions.^[1] However some common pitfalls and artefacts that may lead to unsatisfactory results. For 2D experiments, spinning should always be turned off, as the spinning may introduce artefacts leading to criss-cross noise in

the spectra. Note the adjusting of the z and eventually also the z^2 shim, if sample rotation is stopped. Therefore it is best to run already the preparatory 1D experiment without spinning. If the acquisition time is not long enough or the number of increments is too small, the echo signal will be clipped. This may lead to unreliable peaks in the spectrum, while the real long range peaks may be very weak or missing. The number of scans and dummy scans needs to be a multiple of the minimum given in the pulse program. If that is not the case, the phase cycle is not completed which lead to auxiliary diagonals may parallel to the real diagonal.^[8]

Acquisition and processing:^[11]

The preliminary steps include the following. Insert the sample in the magnet. Lock the spectrometer. Re-adjust the Z and Z^2 shims until the lock level is optimized. Tune and match the probe head for ^1H observation ^{13}C decoupling. Reference spectrum for both H^1 and C^{13} should be run separately.^[14]

H^1 reference spectrum: Since HMBC is a ^1H -observe experiment, the first step is to obtain a reference ^1H

spectrum of the sample. This reference spectrum will be used to determine the correct parameters for ^1H and for the F_2 dimension, and can also be used as the F_2 projection of the HMBC spectrum.^[11]

^{13}C reference spectrum

It can be assumed that the sample used for an inverse experiment such as HMBC has too small a ^{13}C signal to make it practical to obtain a ^{13}C reference spectrum. Thus, the user will need to make an educated guess as to the appropriate values of parameters for the F_1 dimension. Note that, because HMBC is a multiple bond correlation experiment, one can expect to detect signals from ^1H 's coupled to quaternary ^{13}C 's, in addition to primary, secondary and tertiary ^{13}C 's. Thus, the ^{13}C spectral width should be larger than that used for HMQC. Then a series of steps should be followed like create a new file directory for the 2D data set, change to 2D parameter mode, Set up the acquisition parameters, Acquire the 2D data set, Set up the processing parameters, Process the 2D data set, Adjust the contour levels, Phase correct the spectrum, Plot the spectrum, and finally interpretation.^[11]

Interpretation of HMBC spectra: Let us take a simple spectrum and understand its interpretation,

HMQC and HMBC of

1. Ipsenol interpretation^[15]

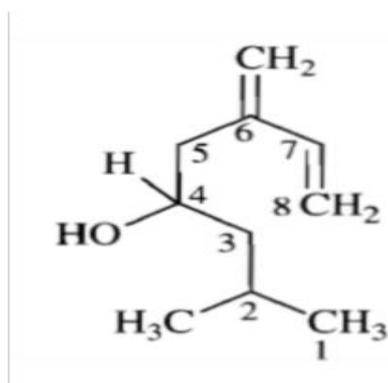


Figure6: structure of ipsenol. (Ref: Robert M. Silverstein, Francis X. Webster, David J.Kiemle, 2005).

DQF COSY	HETCOR	HMQC	HMBC
A homo nuclear correlation spectroscopy where plot is between two protons molecules which are attached directly or a little away.	A heteronuclear correlation spectroscopy where plot is between two dissimilar nuclei which are directly attached to each other.	A highly resolved inverse detected heteronuclear correlation spectroscopy where plot is between two dissimilar nuclei attached to each other (single bond coupling).	A highly resolved inverse detected heteronuclear correlation spectroscopy where plot is between two dissimilar nuclei attached at longer distance(2 or 3 bonds couplings).
Proton of 3 rd carbon shows 3 cross peaks, two for the protons attached to 3 rd carbon and one for proton attached to 5 th carbon.	3 rd carbon shows two cross peaks which represent protons attached to 3 rd carbon directly.	3 rd carbon shows two cross peaks which represent protons directly coupled to 3 rd carbon through single bond.	3 rd carbon shows five cross peaks which represent 2 or 3 bond couplings of 3 rd carbon with various protons. 2JCH – cross peak for coupling of 3 rd carbon with proton of 2 nd and proton of 4 th carbon. 3JCH – cross peak for coupling of 3 rd carbon with proton of 1 st and proton of 4 th carbon.
Proton of 4 th carbon shows five cross peaks, for the protons attached to 3 rd and 5 th carbons (doublets), 4 th carbon (singlet).	4 th carbon shows a single cross peak which represents correlation between 4 th carbon and its protons.	4 th carbon shows a single peak which are directly bonded to it.	4 th carbon shows five cross peaks, 2JCH- cross peaks for coupling with proton of 3 rd and 5 th carbon. 3JCH – cross peak for coupling with proton of 2 nd carbon.

In HMBC spectrum of ipsenol, the ellipse and diamond shaped area represents absence of direct bond coupling of 3rd and 5th carbon with corresponding protons.

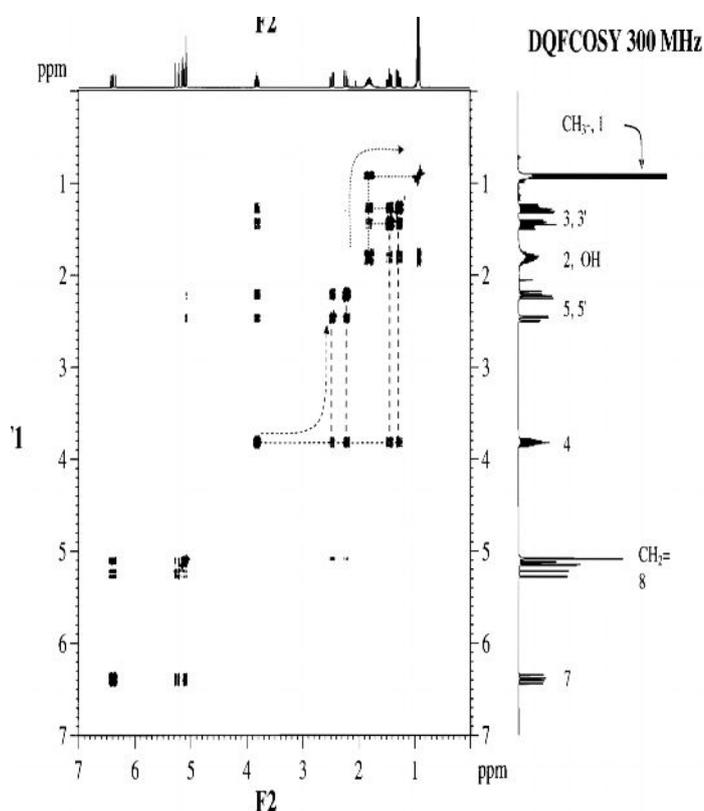


Figure 7: DQFCOSY spectrum of ipsenol. (Ref: Robert M. Silverstein, Francis X. Webster, David J.Kiemle, 2005).

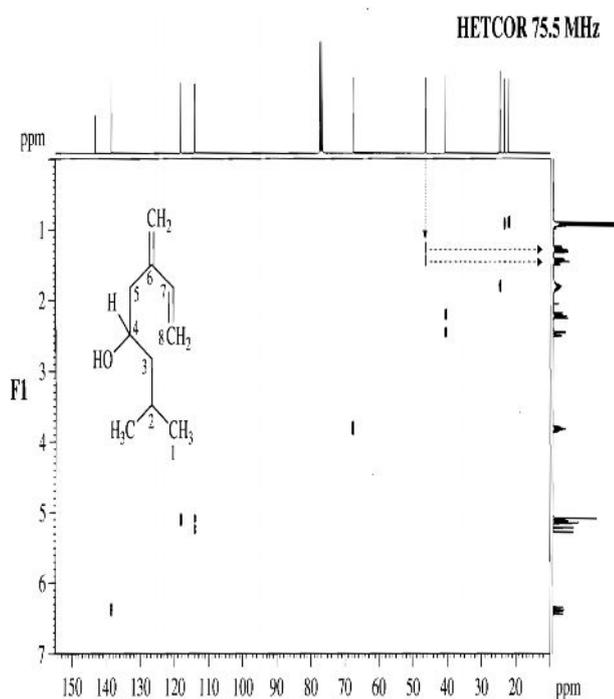


Figure 8: HETCOR spectrum of ipsenol. (Ref: Robert M. Silverstein, Francis X. Webster, David J.Kiemle, 2005).

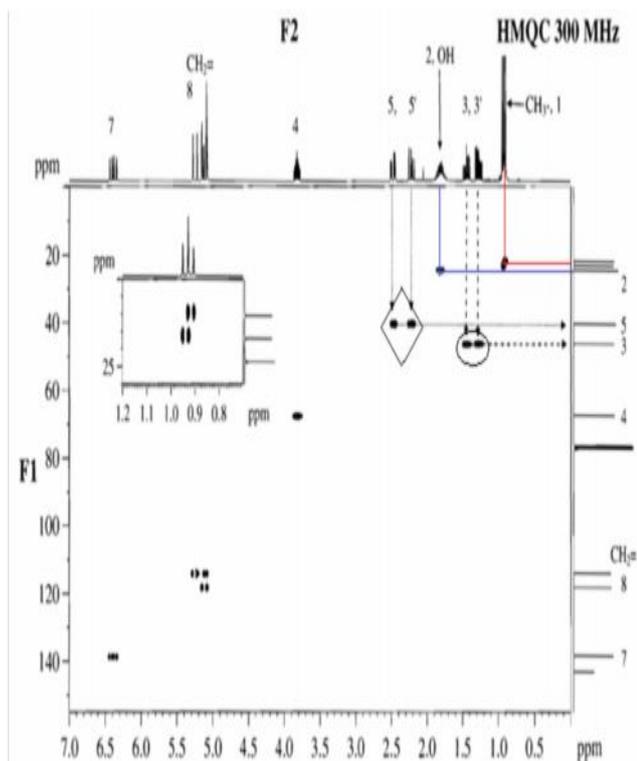


Figure 9: HMQC spectrum of Ipsenol (Ref: Robert M. Silverstein, Francis X. Webster, David J.Kiemle, 2005).

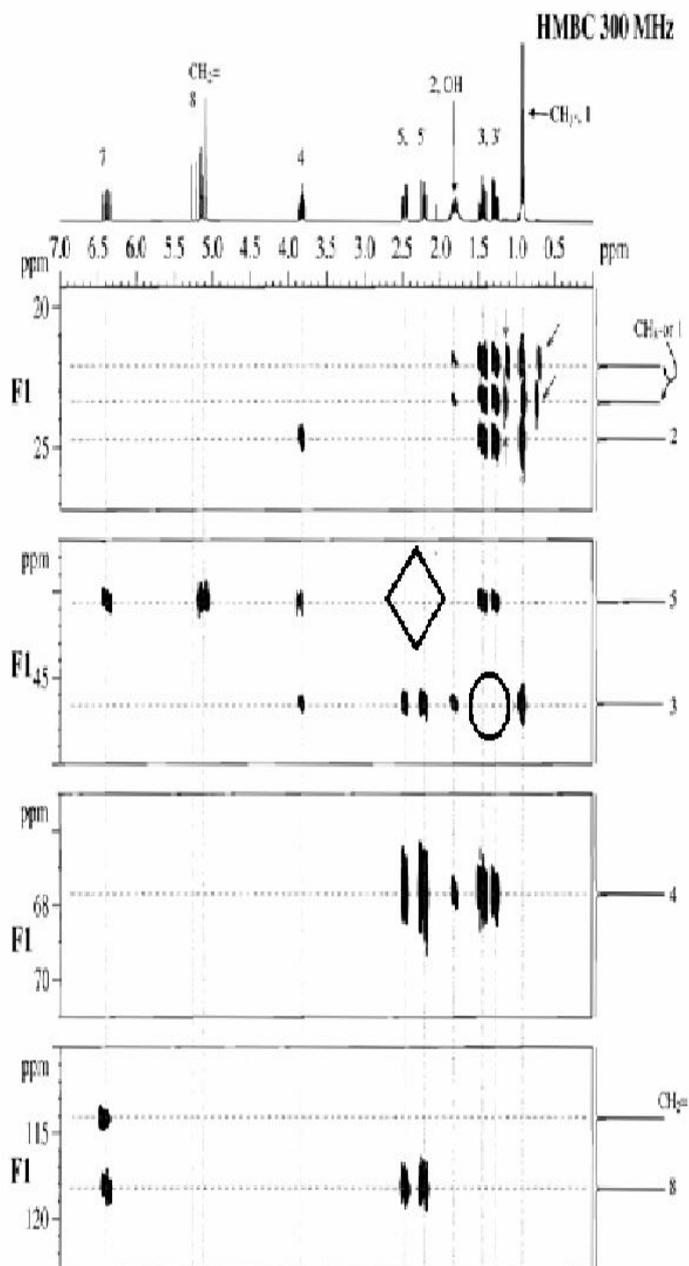


Figure 10: HMBC spectrum of Ipsenol (Ref: Robert M. Silverstein, Francis X. Webster, David J.Kiemle, 2005).

Thus the **spectral differences** for a compound are that there cannot be direct coupling peaks in HMBC spectrum which can only be seen in HMQC spectrum. And also for a quaternary carbon there occurs no cross peaks in HMQC spectrum while through long range

coupling cross peaks can be seen in HMBC spectrum. This is shown in spectrum of Ipsenol by different markings.

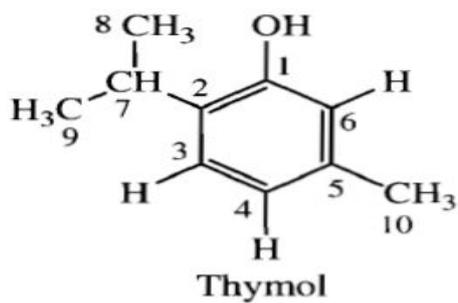
2. Thymol interpretation: ^[15]

Figure 11: structure of Thymol (Ref: Robert M. Silverstein, Francis X. Webster, David J.Kiemle, 2005).

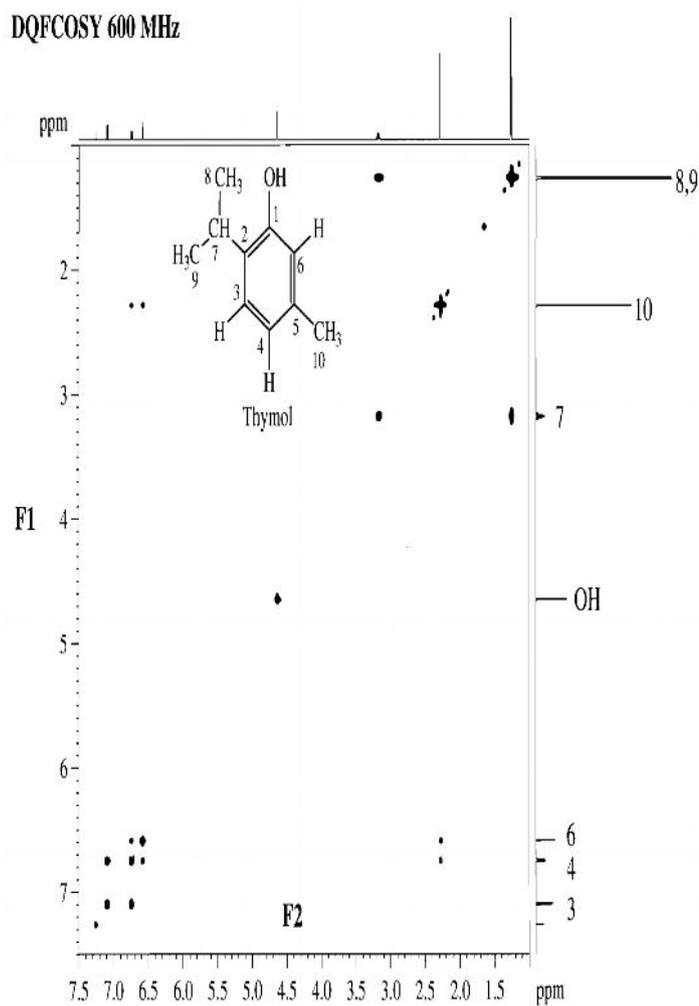


Figure 12: DQF COSY spectrum of Thymol compound. (Ref: Robert M. Silverstein, Francis X. Webster, David J.Kiemle, 2005).

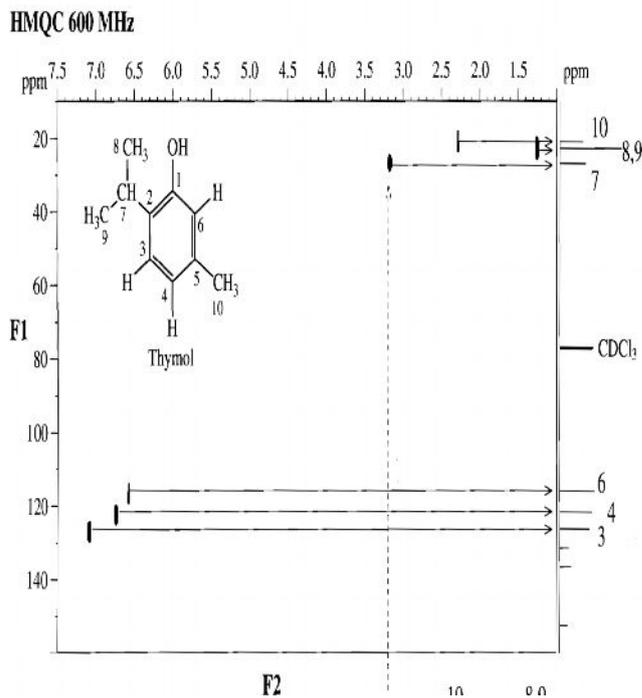


Figure 13: HMQC spectrum of Thymol. (Ref: Robert M. Silverstein, Francis X. Webster, David J.Kiemle, 2005).

As described in the previous example, lines were drawn parallel to proton axis and interpreted. But now unlike previous example, spectrum is studied with reference to proton spectrum here. The proton attached to seventh carbon shows a single cross peak in HMQC

spectrum, as that is the only possibility. Similarly single cross peaks were observed for 3, 4, 6 and 10 carbons. The following figure 11 shows HMBC spectrum of the same compound.

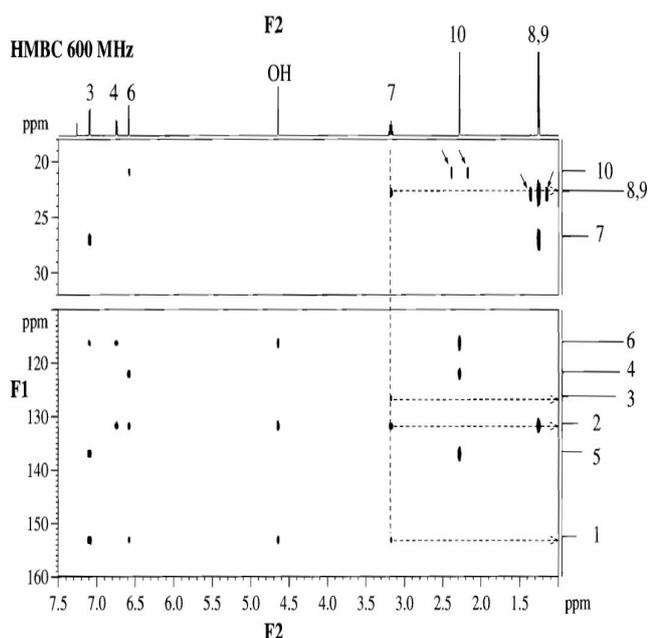


Figure 14: HMBC spectrum of Thymol. (Ref: Robert M. Silverstein, Francis X. Webster, David J.Kiemle, 2005).

Considering the same seventh proton, in HMBC spectrum, this shows four cross peaks of which none is showing directly coupled carbon. This proton has three carbons in alpha position and two carbons in beta position. As 8, 9 carbons are equivalent they show single cross peak in the spectrum, thus this proton has two non equivalent carbons in alpha position (8, 9, and 2) and two in beta position (1, 3). HMBC spectrum has no cross peak in seventh position. Similar studies should be done for every proton to understand its environment and its correlation with remaining carbon atoms. Consider the quaternary carbons 1, 2, 5; they have no cross peaks in HMQC while many cross peaks can be seen in HMBC spectrum. By comparing these two spectrum, one can conclude that 1, 2, 5 carbons have no attached protons (or quaternary carbons). For elucidating the structure of a compound completely all the spectrum needed is 1D proton spectrum, 1D C^{13} spectrum, COSY, DQF-COSY, HETCOR, HMQC, and HMBC.

Applications:

1. Microstructure determination of poly (acrylonitrile-co-methyl methacrylate-co-methyl acrylate) terpolymers by 2D HMBC.^[16]
2. Recent progress in heteronuclear long-range NMR of complex carbohydrates: 3D H2BC and *clean* HMBC.^[17]
3. Use of heteronuclear multiple bond coherence NMR spectroscopy to monitor nitrogen metabolism in a transformed root culture of *Datura stramonium*.^[18]

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4. Valproic acid intoxication is identified by $H^1 - C^{13}$ correlated NMR spectroscopy from urine samples.^[19]
5. Using HMBC studies on cholesteryl acetate, cyclosporine and isopropylidene glycerol, structures have been elucidated.^[20]
6. $^1H-^{15}N$ HMBC as a valuable tool for the identification and characterization of nitrones.^[21]
7. Use of ^{15}N -HMBC NMR techniques to determine the orientation of the steroidal units in ritterazine A^[22]
8. Determination of a symmetrical dimer structure in benzo[c]phenanthridine alkaloids by pulsed-field-gradient HMBC^[23]
9. Differentiation of HMBC two- and three-bond correlations: A method to simplify the structure determination of natural products^[24]
10. Variable temperature gradient $^1H, ^1H-^{13}C$ GE-HSQC and GE-HMBC NMR studies of flavonols and flavones in organic and aqueous mixtures^[25]
11. Structural investigation of poly (methyl acrylate) by 2D HMBC NMR.^[26]

Conclusion:

For the structural elucidation of a compound, a simple 1D NMR (H^1 and C^{13}) is not sufficient. Advanced techniques like correlation spectroscopic methods, when understood properly give the perfect way to determine the structure for a given formula. The combination of all the spectra DQFCOSY, DEPT, HETCOR, HMQC, HMBC are all very useful in identification and characterization of the structure, and orientation of bonds in a molecule. By using HMBC studies, large molecules can also be elucidated easily.

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