**One and Two dimensional NMR Spectroscopy: Concepts and Spectral Analysis**
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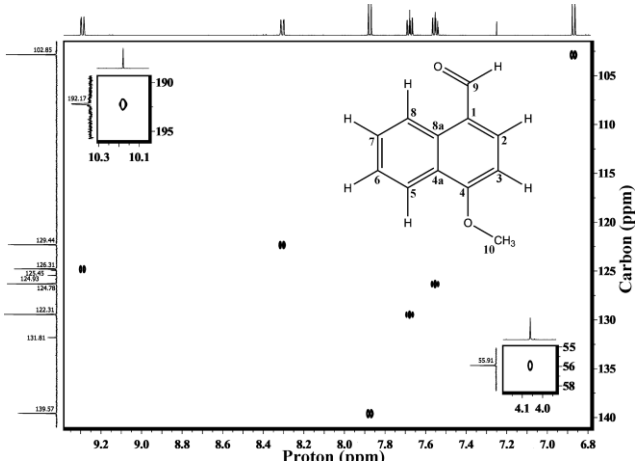
**Lecture 46: HSQC and HMBC**

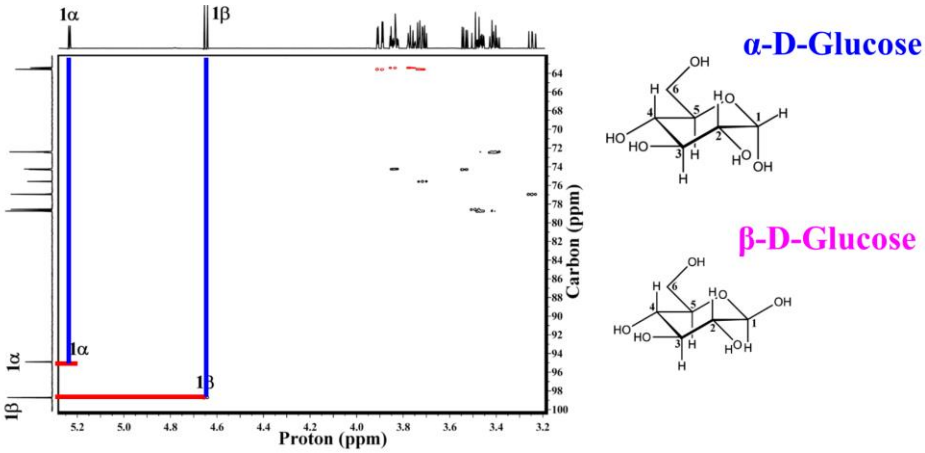
Welcom all of you. In the last one or two classes, we have been discussing more about heteronuclear correlation experiments. Extensively, we discuss direct correlation experiment, inverse correlation experiment like HSQC and how we do the experiment, how does the pulse sequence works, etc. We understood, that the stransfer the magnetization from proton to carbon takes and then back from carbon to proton. Of course, I took the example of carbon, it could be any dilute spin to any abundant spin, does not matter any of the two abundant and dilute spins you can consider for HSQC experiment. And of course, we also understood how to interpret the HSQC experiment. Varieties of experiments are possible, proton coupled, proton decoupled in either dimensions.

We took the example of how to interpret the coupled and decoupled HSQC spectrum of some hypothetical molecule where we could get one bond CH couplings and also long range proton-proton couplings. So, if you take the carbon coupled proton spectrum, proton carbon coupling appear as satellites. From the satellite spectrum, you get HH coupling. Identically, in the direct dimension, in the proton dimension, you get proton chemical shifts plus one bond CH and also HH couplings that was the coupled HSQC. In the decoupled version, CH coupling will be removed and we get only HH couplings. So, many such examples are available to interpret the spectrum, We took a few examples to show how we can interpret in a realistic example of the molecules. But of course, in a crowded region, what we can do is that we can identify CH carbon, CH2 carbon, CH3 peaks based on the number of protons attached to each carbon. How we do that? In the carbon 13 number when we understood, we also discuss about DEPT sequences. The DEPT- 45, DEPT-90, DEPT-135 are the three experiments used to identify different carbons. Like carbon 13 editing we can do to identify different carbons. DEPT-135 sequence if you join for this HSQC, that is called multiplicity edited HSQC, wherein you can identify all the CH2 carbons, carbons attached to two protons, that is even number of protons, they become negative in intensity, whereas CH and CH3 carbons are positive in intensity. Thus we can find out from looking at this signs. And we also got a cross section to see they are really negative in peaks in the intensity version and then positive for CH and CH3. And of course, examples of the 3 heptanone, methyl heptanone and ethyl acetate we took to see how we can interpret the HSQC spectrum. So, we will

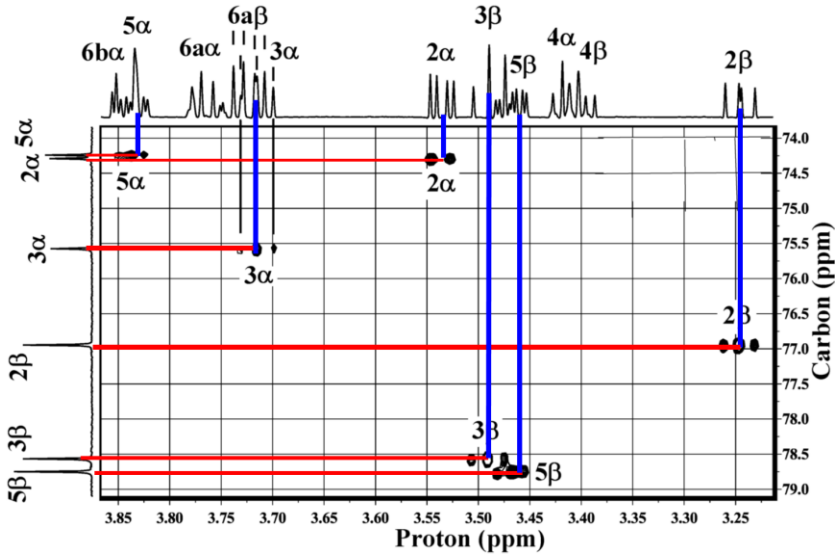
continue now with another example, one or two examples, or few more examples and then we will see what are the other things we can do. And few more details about HSQC we will try to understand. Of course, this we started also. I discussed this, this is the molecule which we analyzed in the proton spectrum.




The phenyl ring has only two protons, two carbons attached to two protons. From the proton spectrum which we analyzed, these are the two protons and the carbon chemical shifts we can get. And then from this you get carbon chemical shifts and proton chemical shifts we get like this. Of course, for the other phenyl group there are four protons and I wrote already two of them. Once you know the analysis of proton spectrum of the molecule containing phenyl groups and using that you can also assign HSQC spectrum and get carbon 13 satellites. But only thing you should see here in this molecule there are different carbons, 1, 2, 3 and 4 carbons which cannot give rise to any peaks in the HSQC because HSQC gives peaks only for directly bonded carbon with protons; one bond JCH should be there. But how do you make sure, how do you assign this type of carbons that is the thing which we are going to discuss today, may be after couple of slides.



We will continue further we also discuss alpha and beta glucose, the mixture of two glucose are there. In the D-glucose you have alpha isomer and beta isomer in the ratio of 36 : 64 or 34 :66, that we saw. Aand of course, multiplicity edited we can see here, we can get these are all red which are CH2s, rest are all CHs. We can easily identify by doing this and of course, anomeric protons we all know these are the two things which we already know. We use this to start the analysis of the COSY spectrum also. So, starting with this of course, this anomeric proton for the beta glucose, and of course, this is the carbon chemical shift and this is the proton chemical shift. Similarly, for anomeric proton of alpha and the expanded region of this one is taken here. When you expand that basically what if you understand is proton 6 in this has CH2 that is what we see red color here, even number of protons. But you see interesting thing is these two protons are non-equivalent, they are non-equivalent protons although they are attached a single carbon. So, if you see that very easily you can identify. In fact if you get a difficulty of which proton is which, whether 6A or 6B or whether it is alpha isomer or beta isomer you can identify here. Simply go horizontal along this axis, this corresponds to alpha isomer and there are two protons here. This is the carbon chemical shift, these are two proton chemical shifts from the center. In the crowded region just from the center we can identify the chemical shifts here also. So, there is no confusion at all. Here also if you see this is a carbon chemical shift, exactly from the center you get proton chemical shift for both 6A and 6B of the beta isomer. This is the advantage, the same carbon with two different protons are attached, but with non-equivalent different chemical shifts, that also easily you can assign by using HSQC like this. This is an advantage of that. Of course, same thing when you expand it you can clearly see for the alpha, the proton 4 again alpha beta. When there is a crowded region very easily you can identify which is which.

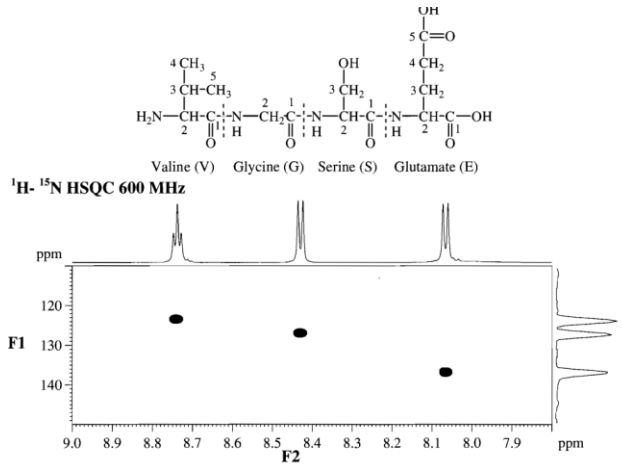


This is the extension of those things for alpha and beta isomers. All of them very easily you can start doing that. This is the beta isomer proton 2, this is carbon 13. Similarly, this is for 5, this is 2 alpha, all beta and alpha carbons and protons we can get chemical shift also. Of course, from HSQC also you can get proton chemical shifts. Since you have already analyzed the COSY spectrum very easily we can go and continue with this thing. With this of course, HSQC spectrum is fairly easy to interpret like HETCOR experiment there is no difference at all. One thing we may have to answer the question in a molecule you have long range couplings 2 bond CH, 3 bond CH also is there. But we observe only one bond correlation in HSQC, one JCH correlation. The question comes why two JCH and three JCH correlation peaks cross peaks are absent in HSQC. If you have to answer that first of all we have to find out the cross peak intensity. I do not have to go into the mathematics of that and discuss everything. It is lengthy mathematics, and one can find out what is the cross peak intensity. The cross peak intensity is simply given by an expression like this.

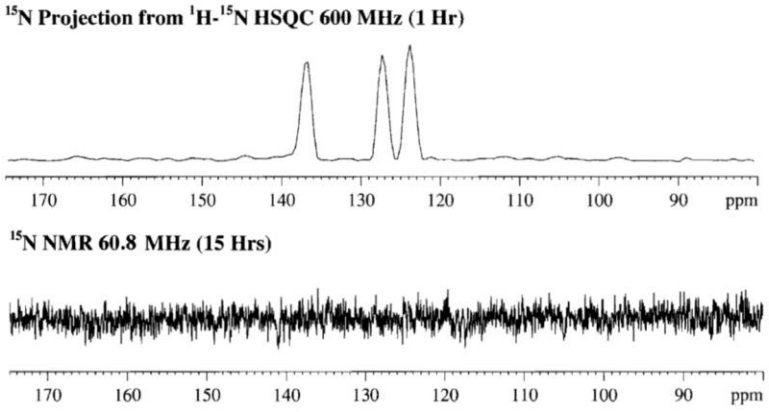
$$\alpha \sin^2[(\pi/2)^* J_{obs}/J_{filt}]$$

alpha into sine square pi by 2 star into J observed over J filtered. What is J observed is actual JCH value. J filtered is the value used for calculation of the mixing time. What is J filtered here? In our case J filter is one bond is 1JCH, observed as usual. So, J filter is set for using 1JCH as 135. But if the long range coupling I have to see what do? What is JCH long range coupling? it is of the order of 5 to 10 hertz. Sometimes 2 bond, 3 bond if you want to see, 4 Hertz or 5 Hertz will be there. What is the intensity of the cross peak when the long range coupling is of the order 4 Hz? Just plug this value into this equation. There is not a big mathematics. We have sine square pi by 2, the J observed is the value which we are looking for, we are observing long range coupling, and this is a filter we have set for 1JCH, 135 Hz,that is some value I have chosen. It can be anything approximately around 140 to 150 hertz is what is normally chosen. Let us calculate the intensity of the cross peaks due to long range coupling. First J filter I use 135, J observed I use 4 Hz. I have plugged in the values, the sine square pi by 2 into 4 divided by 135 this is the equation. See equation here J observed over J filtered. So, J observed is 4 Hz, I want to see why I am not seeing the cross peak if there is a long range coupling of 4 hertz in HSQC spectrum. 1 JCH is 135. Just use simple calculator, calculate this value, calculate what is this value of sine square for this particular value. This value you can find out it is 2.6666 and sine square value of that is 0.0022. What does it mean? The cross peak intensity is 0.0022 percentage with respect to JCH, the filter what you have used, That is it is 0.2 percent intensity if JCH peak is 1 bond coupling is of some intensity one, let us say. Compared to that the relative intensity of the observed peak for long range of 4 Hz is 0.2 percent. Hardly you can see that. That is the reason why you will not be able to see that. You understand why 1 bond correlations only are seen in HSQC why

we are not seeing the long range correlations, it is because of this. Intensity if you calculate for 4 Hz it turns out to be 0.2 percent. You may say 4 Hz is not a realistic value. Let us take some other value 8 or 10 hertz 15 Hz, does not matter we will see for 8 hertz. Let us say double the long range coupling instead of 4 hertz I look 8 hertz. Why I do not see that. We will see, plug in this value sine square pi by 2 star 8 over 135 and this is 5.333 and sine square of that is 0.0084. What does it mean? Intensity is 0.86 percent. Even then you do not see. We are looking at carbon 13. Remember HSQC we are seeing carbon 13 long range correlation with proton. And this is such a small intensity of the cross peak compared to the cross peak you get for 1JCH of 135 Hz, you will not see that. If cross peak for 1JCH is of this value and other one is this much. So, relatively it is so weak you do not see it. That is the reason why long range couplings are not seen in the HSQC experiment. So, the cross peaks are too difficult to see, and usually not seen because of this very low intensity. Of course, in some example I want to warn you, you can get long range correlation, where JCH are are quite large. The JCH in this molecule like this for example, a phenyl group with C double bond CH you know 2 JCH here is nearly 50 hertz it is not 8 hertz. Now plug in this value here and for in this molecule this carbon to this proton coupling is about 26 Hz that is too large. See 49.5 almost more than one third of 135 hertz as a consequence they will give rise to peaks. In such examples long range correlations are also seen in HSQC, but with weak intensity. You can calculate what should be intensity of that peak compared to 1JCH. It is reasonable value. The intensity in this case is comparable to previous example. So, these are all specific examples where you may get long range correlations also. One may ask a question am I doing HSQC only for carbon-13? No. Most of the times, people when they study proteins, peptides, etcetera, they have molecule like this for example, in the case of a tetra peptide we have NH. I told you this correlations each amino acid has an NH group we can get the nitrogen 15 proton, nitrogen 15 proton HSQC. Then you can get the chemical shift of nitrogen. You correlate those NH peaks to corresponding proton chemical shifts. And if you have a bigger molecule like protein there will be N number of protons, huge complex HSQC spectrum you are going to get and we can start interpreting each of them.

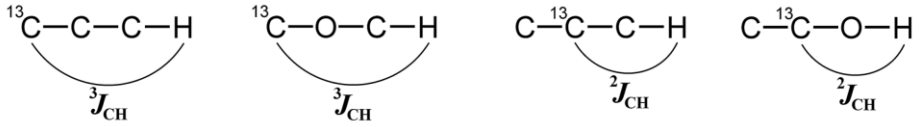


And this is a simple example. In this case we are going to see 3 peaks here and 3 NH peaks we are going to see here. This NH2 is there, this, this, this, this 3 NHS are there and of course, this is also there one more, but we are seeing of course, may be overlapped here. These are two 3 doublet, these two doublets are overlapped here. And if you take the projection of that you will see that and this is the projection of HSQC for the previous molecule. The spectrum was obtained in just 1 hour, rememberi in just 1 hour and we are going to get the signal. The same spectrum without HSQC conventional way for 15 hours no signal nothing is in because nitrogen 15 is inherently very, very low sensitivity, very insensitive nuclei.




So, you see you are not seeing any peak at all. See the advantage of HSQC. So, about HSQC what I want to summarize here is you are not only seeing carbon 13 proton HSQC, you can get Nitrogen-15 proton HSQC also and especially in the biomolecules like peptides and proteins Nitrogen-15 HSQC is routinely used and you can see a reasonable intensity of the peaks, in the cross peaks. And if you take the projection we will see very good peaks with very good signal to ratio compared to the direct observation of Nitogen- 15. So, this is what I just want to tell you about carbon 13 proton, nitrogen 15 HSQC you can take any other heteronuclei also no problem. So, basically we understood how to interpret the HSQC spectrum, why we do not see the cross peak in HSQC especially due to long range carbon proton or nitrogen proton couplings. So, we understood that and took lot of examples to interpret everything.

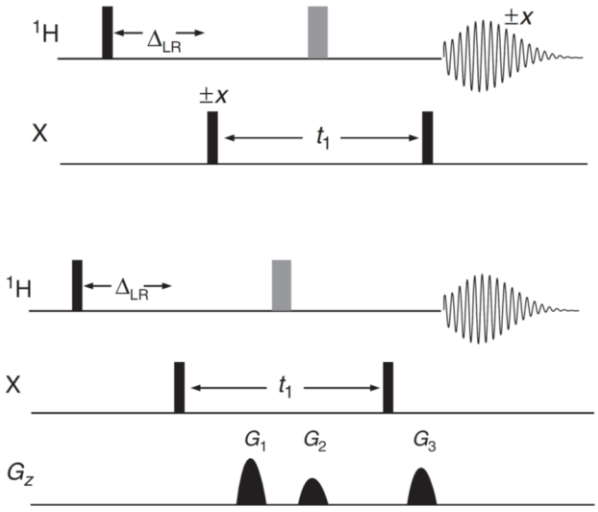
And we need to understand one another experiment called HMBC, heteronuclear multiple bond correlation. Multiple bond means not have several at a time, two nucleus separated by multiple bonds that is what the conventional meaning. So, multiple bond correlation we can use that. The advantage here is it is not only directly bonded long range coupling, tha is remotely bonded, that we can get. You see this is a very immediate advantage.



For example, directly one bonded carbon proton can be seen, but there is a let us say O is here in between. This 1, 2, 3 bond separated 3 JCH is there, quite reasonable and we can see that. And of course, there is a carbonyl carbon which is 2 bond separated and coupled to another proton somewhere let us say. Can we see? There are long range couplings very well known in the carbon 13 NMR. We can observe long range CH coupling these are all reasonable value. And then how do you detect such carbons? First of all the advantage of that is non-protonated carbon which you do not see in HSQC can also be utilized because of the long range coupling to different protons. So, here are salient points of HMBC what do we do? We want to do HMBC.

First of all HMBC is usually acquired in magnitude mode. HSQC as I said is always acquired in phase sensitive mode, very easy. And of course, HMBC because is in magnitude mode, of course you know what is the magnitude mode, the real and imaginary we will take sum of the squares, it is the square of this thing and the square of that and get the intensity and no question of phase correction at all. In the HMBC spectra there is no need to do the phase correction because always it is recorded in the magnitude mode. This is one thing. Secondly, usually HMBC is acquired without decoupling there is no need to do the decoupling here because long range coupling is there it is very weak. And of course, there is an advantage of this, that is there is no restriction the acquisition time. You can acquire for a long time, that is an advantage. So, it is always recorded without decoupling. Of course, you can do the decoupling, nobody can stop you, but generally it is done without decoupling. And here cross peaks are always split into doublets in the F2 dimension. Why? We are not doing decoupling. There is a long range 2j, 3j CH couplings will be there, which is of the order of 8 to 10 hertz. So, it will be seen. Sometimes what happens the long range coupling is very weak and it will broaden the signal. And for example, when a peak is a triplet in 1D NMR the cross peaks appear as doublets of triplets because 1J coupling is here, in the 1H NMR it is a triplet and the cross peak will be triplet like this, doublets of a triplet. And another thing is remember HMBC is very very less sensitive than HSQC. Why? Very easily you can understand. Remember in the long range I told you even during the TOCSY polarization transfer told you, the polarization transfer efficiency is better if the coupling strength is larger. If the coupling strength become very very weak and and very weak coupling, the magnetization transfer efficiency is less. Obviously, if you go to 2 bond coupling carbon proton 3 bond coupling carbon proton their strengths are so small. As a consequence the magnetization transfer takes enormous amount of time. It takes more time not very fast. During that process what happens spins also start relaxing there is a signal loss. When the spins relax they go back to magnetization go back to z axis. As a consequence when we are detecting the signal in the x y plane it is less signal is there. As a consequence, HMBC is less sensitive compared to HSQC because of the relaxation during long delays. HMBC usually requires twice as many scans as possible on the same sample. That means, it is number of times signal averaging have to do. You have to acquire for more

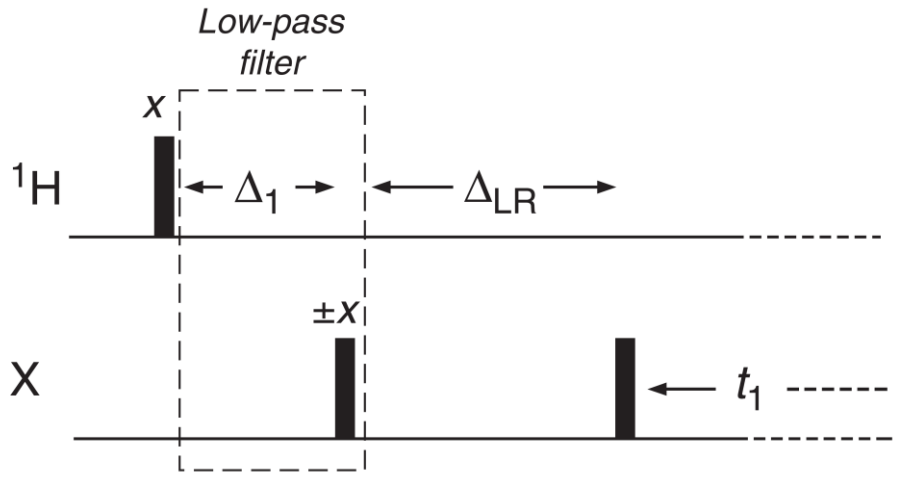
number of scans because signal and sensitivity is very very low. And generally this is the HMBC sequence used. I told you in HSQC sequence, do not forget that we have in HSQC, sequence INEPT, T1 period the T1 period you can apply a 180 pulse for doing breaking the carbon proton coupling reverse INEPT, detect the proton and decouple the carbon. You do not forget that, that is a sequence for INEPT. The HMBC sequence is a different one. Look at it, it is a simple proton carbon here we have a filter called long range filter which is given as symbol delta Lr this delta Lr is tuned to detect correlation via small couplings. Here deliberately we have to suppress the one bond couplings. Remember we have a long range correlation to be detected. So, one bond correlation will be of huge intensity. Let us subtract that. That is a important challenge here. So, we have a filter which is tuned to detect long range coupling and the rest of the thing is very simplest possible way it can be done. This is simple sequence and we detect the proton, as I told you no decoupling, always observed in a coupled manner. And of course, the same thing you can do by using what is called the pulse field gradients there is an advantage of that pulse field gradients. So, I will apply the pulse field gradients like this. Remember I also told you this when we wanted to subtract the carbon 12 attached proton signal or nitrogen 14 attached proton signal in the HSQC sequence. We can do that with two ways one is by phase cycling other by field gradients. That is what I told you. The same way you can also do with the pulse field gradients. We can apply that. This is the experiment with a pulse field gradient version of that.




What is the delta Lr the long range delay that we have to use to see the correlation of the long range coupling. Remember long range coupling NJCH is very very small value. Set this delta Lr equal to 1 over 2 times the long range coupling, the delay approximately 100 millisecond will be the one for that is a small long range proton-proton coupling to evolve about 5 hertz to 10 hertz. The 100 millisecond is the long range delay, the filter

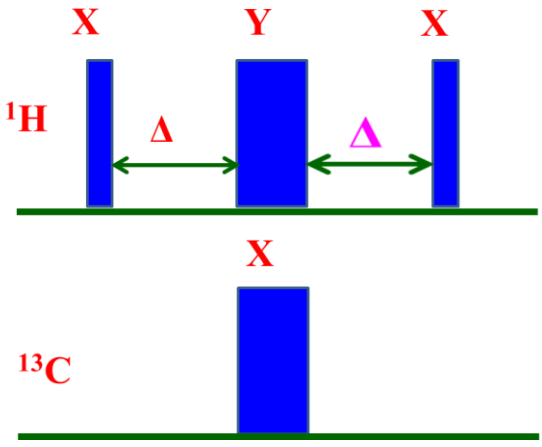
delay, we have to apply in the beginning of the pulse sequence. This delay is essential, then this magnetization, that the spins due to long range coupling, they will start evolving. And during the Lr period because it is a huge value remember 100 milliseconds. What is going to happen there are also proton-proton couplings in the molecule, that will also evolve because the strength of the proton-proton couplings is almost equal to that of the long range couplings. The language JCH is about 8 to 10 hertz. What is the proton-proton couplings? around 10 to 15 Hz we saw, a similar magnitude. As a consequence, in the filter period Lr, in the HMBC in addition to long range CH couplings we also have proton-proton coupling coming into the picture. As a consequence the phase of the peaks get little bit distorted. So the HMBC spectra because of this reason we always record in the magnitude mode. Please remember HSQC is always recorded in the phase sensitive mode, HMBC spectra are represented in the magnitude mode. the phase information is lost. And what is this low pass filter used for? the low pass filter it is going to pass the magnetization corresponding to long range coupling but prevent the evolution of one bond coupling, the magnetization due to one bond coupling. So it removes one bond correlation and allows only long range correlation. And during Lr this what happened the multiples of a long range couplings also can evolve. Remember one more thing because if I say my Lr I said is 8 hertz, if I take 80 hertz 80 hertz is 10 times 8. As a consequence what will happen? during Lr period multiples of long range coupling can also evolve. So that is also possible and we have to suppress them by using low pass filter. Then this filter allow only those couplings that are smaller than the cutoff value. Anything above that is going to be prevented, you understand this. Remember in the long filter that you are going to use in HMBC, the multiples of long range couplings can also evolve. For example long range coupling is let us say of 8 hertz, if you take 80 hertz coupling is there, that correlation also can evolve. Wo we have to suppress that by using low pass filter and then that low pass filter advantage is, it allows only those couplings that are smaller than the cutoff value set anything above that it is going to be removed and this is why HMBC sequence generally have two filters. One is a filter for the long range coupling for evolution other one to prevent the long evolution of this one bond couplings. So that means we have two filters one is called a J accept filter and the J reject filter. What is that J accept J accept is set to very small value 8 to 10 hertz this what we want to detect long range correlation. J reject is set to one bond CH coupling this is which we want to reject or stop it, do not allow. You see if you know physics you would have understood low pass, band pass filters, etc. And you know in the reject filter something will be allowed something will be stopped here, you have two filters one to pass the coupling correlations coming because of long range correlations, one to stop the correlation coming because of directly bonded one bond proton correlation. That coupling is for the order 135 hertz and the J reject is used to remove correlations of 1JCH. These are all called HSQC cross peaks. You know that HSQC cross peak is because of 1JCH. That HSQC cross peaks are to be suppressed so J

reject will suppress the HSQC cross peaks, will allow only long range correlations. And this is a basic low pass filter that we use.

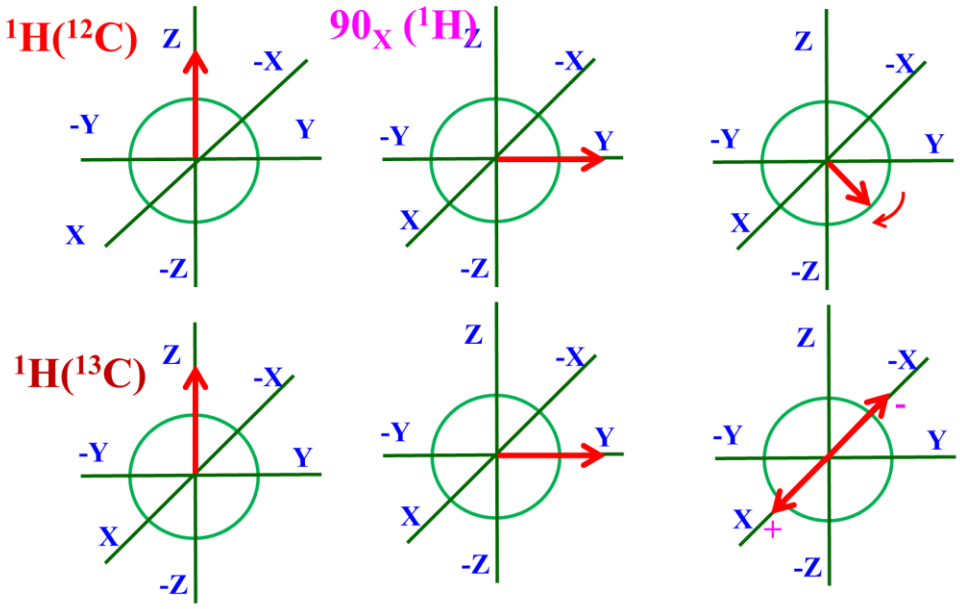


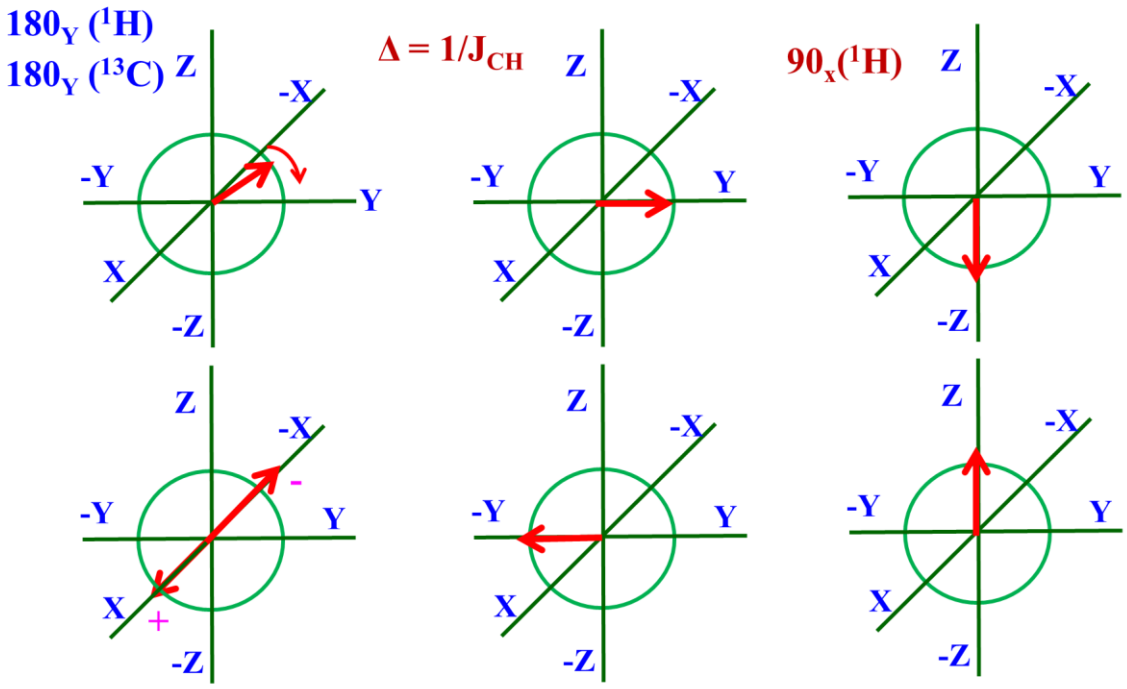

Okay, and this is a usual sequence and we have in between the low pass filter is put in such a way, the first carbon 13 pulse creates multiple quantum coherence, that is for 1JCH only. The delta 1 is set for 1JCH this filter LR is for long range, the delta 1 is for one bond. So this one delta 1 is set for 1 JCH as a low pass filter and what will happen and this creates multiple quantum only for 1 bond CH, because this is calibrated for that value. And this delay is very short compared to long range. Remember delta LR when we calculated, it was in the order of 100 millisecond, this is much smaller, because it is a large coupling. As a consequence in this short delay the NJCH will not evolve. That is how low pass filter will filter this thing. Okay the filter element is placed at the start of the HMBC sequence prior to the transfer of polarization and to carbon that is a filter that is what we saw in the sequence. Then we do two experiments one experiment with the same as the first carbon 13 plus x and other one is with minus x, and sign of the receiver phase is always kept unchanged. This is an experiment we do and then what we do is the addition of both the data. Then you remove that one bond correlations. They are suppressed. Remember how we do the suppression of one bond JCH correlation in HMBC. In this we have a filter here you set the value for the filter for long range coupling and do two experiments and with the first carbon -13 pulse, here a carbon 13 pulse plus x is there, and minus x is there. First you do with plus x, second time you do with minus x and then do the two experiments, keep the receiver phase constant both the times. After both the experiments do the co-addition of the data. When you do the co-addition of the data, the one bond JCH correlations are suppressed. That will be removed and then we are going to detect only long range correlations. This is what normally done, but of course there are always difficulties in the experiment when you do. There will be always some leakage some signals will not be very good, some distortions will be there, imperfections will be there. As a consequence there are certain difficulties, certain

complications will be there in any experiment. This one can improve. We can keep on improving the sequences so that we can get better and better correlations. One of the improved sequence is what is called a BIRD sequence. The BIRD sequence is bilinear rotation decoupling. The BIRD means bilinear rotation decoupling pulse.




It is alternate to low pass filter. Instead of doing a low pass filter we can use a bird sequence. What is the bird sequence does? I will explain to you in a short and very simple way. This is a BIRD element. In the BIRD element we have a proton pulse 90 pulse with a delay, proton 180, delay proton 90, and then at the same time on the carbon 13 channel when the proton 180 is applied carbon 180 is also applied. And the delay in both the cases is set to 1/JCH. This is how the bird element is used, But how does this bird work?






How does it do the filtering we will see these things by a vector picture. We will understand the BIRD element. For example there are two types of carbons in any molecule. One carbon 12 attached attached to protons. The other is carbon 13 attached to proton. When you put the both sample in a magnetic field in both the cases magnetization will be along z axis. Both carbon 12 attached to proton carbon 13 attached to proton are along z axis, as soon as I put the sample in a magnetic field. This is what happens. Apply a 90 degree x pulse on proton then what happens? the protons attached the carbon 12 will come to this. Similarly proton attached the carbon 13 also will come to 90 degree because 90 degree pulse brings to y axis. Give a delay of 1/JCH. Here the trick comes. What happens this magnetization moves, start moving by a certain angle. We know how much it moves we know how to calculate also. Theta is equal to how much it moves. How much it precess is given by theta is equal to PI into delay. In that we know what is J that we know. So, we apply this and if you see that this starts moving like this. It is single component vector there is no J coupling. Carbon 12 will not coupled\ to proton, carbon 12 spin is 0. So it is still a singlet. Only keeps precessing, evolves on its chemical shift. Whereas carbon 13 attached to proton is a doublet. The anti-phase doublet will be created. After a certain delay it continues further. Immediately when we apply a 180 pulse, the 180 pulse takes the magnetization which was here to this axis and then continue to process in the same direction like here. i\It was going like this it continues to process. But whereas this antiphase remains same. Nothing will happen. And what will happen is you give another delay after 180 pulse. This carbon 12 magnetization gets refocused along y axis, completely gets refocused. There is no chemical shift evolution. Similarly, for carbon 13 vector what will happen? If you give a delay, these vectors you know start moving in the opposite directions and they refocus along minus y. See the

difference, the carbon-12 magnetization will refocus along plus y. whereas the ecarbon 13 attached refocuses along minus y. And if you now we apply 90 degree pulse this will come to minus z axis, whereas this will go to plus z axis. What is going to happen now? Now the advantage is the effect of the bird element is the selective inversion of the carbon 12 signal. See the carbon 12 signal attached to proton is selectively inverted. Carbon 13 we did not invert, but we inverted only selectively attached carbon 12 proton. So, proton chemical shift evolution is refocused. The heteronuclear one bond coupling evolves throughout. This is what happens and for carbon 13 attached to proton chemical shift will refocus and after a total two delay the doublet vector come back to z axis, by the final proton pulse we can start detecting. And for carbon 13 spin BIRD has no effect, only carbon 12 attached proton gets inverted. So, with this I told you how the BIRD works and everything. I am going to stop here and we will continue with the analysis of the BIRD sequence and few more HMBC sectra and everything in the next class. But in this class we understood something about what is the HMBC pulse sequence after completing the HSQC. Why the signal intensity is very weak everything we understood, Especially for the long range correlation why they do not evolve, they will not be seen because of intensity is very small, so we do not see it. Whereas in the case of HMBC we understood the pulse sequence is there we suppress the one bond and detect the long range correlations. For that we have two filters J accept and J reject. The J accept will allow correlation coming because the long range couplings, J reject will reject the one bond correlations. How do we reject that? there are several ways we can do the experiment plus X minus X phase cycling, or with the pulse field gradient experiment. And also we can use different filters for doing this, one of them is a BIRD sequence. I explained to you what is a BIRD sequence. The BIRD will have a selective inversion for the protons attached to carbon 12, but for protons attached to carbon 13 it has no effect. And how we can use this in HMBC, how it can be utilized we will discuss in the next class. Thank you very much.