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A REVIEW ON 2D NMR SPECTROSCOPY

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Abstract:
Two-dimensional nuclear magnetic resonance spectroscopy (2D NMR) is a set of nuclear magnetic resonance spectroscopy (NMR) methods which give data plotted in a space defined by two frequency axes rather than one. Types of 2D NMR include correlation spectroscopy (COSY), J spectroscopy, exchange spectroscopy (EXSY), and nuclear Overhauser effect spectroscopy (NOESY). Two-dimensional NMR spectra provide more information about a molecule than one-dimensional NMR spectra and are especially useful in determining the structure of a molecule, particularly for molecules that are too complicated to work with using one-dimensional NMR. The first two-dimensional experiment, COSY, was proposed by Jean Jeener, a professor at the University Libre de Bruxelles, in 1971. This experiment was later implemented by Walter P. Aue, Enrico Bartholdi and Richard R. Ernst, who published their work in 1976.

KEY WORDS: *2D NMR, homonuclear correlations, heteronuclear correlations, J-resolved, structural determination.*

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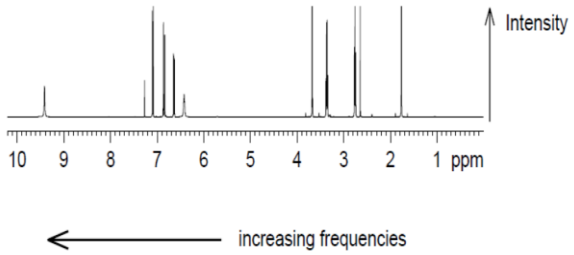
INTRODUCTION:

Definition: The horizontal axis is defined as F2 (direct dimension) and the vertical axis as F1 (indirect dimension). This definition is valid for Bruker spectrometers; Varian actually uses it the other way around. If both dimensions contain chemical shifts, the experiment is called shift-correlated 2D NMR, if one dimension denotes scalar couplings, the spectra are called J-resolved. (1)

COMPARISON OF 1D WITH 2D

A one a dimensional NMR spectrum has two dimensions: The X axis corresponds to the frequency axis (the chemical shifts in ppm) and the Y axis corresponds to the intensity. (2)



In contrast, a 2D NMR spectrum contains two frequency axes. Intensities present the third axis and therefore usually displayed as contour plots. (3)

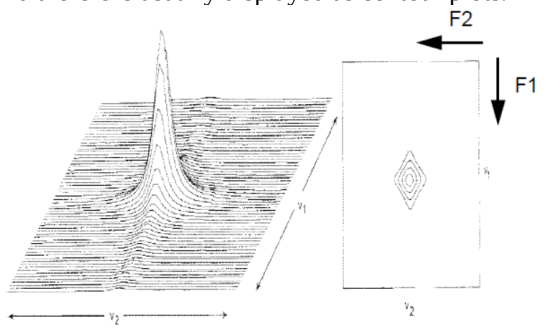


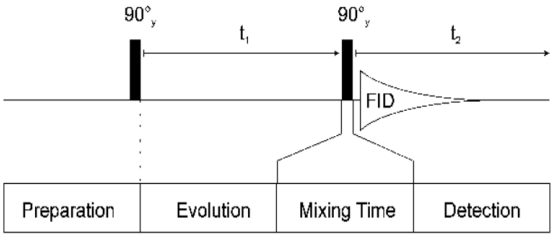
Figure: Two different presentations of a 2D spectrum: Stacked plot (left), contour plot (Right) [Taken from: Derome, A.E., Modern NMR Techniques for Chemistry Research]

INTRODUCTION: Two-dimensional NMR spectra provide more information about a molecule than one dimensional NMR spectra and are especially useful in determining the structure of molecule. Each experiment consists of a sequence of radio frequency RF pulses with delay periods in between them. It is timing, frequencies, and intensities of theses pulses that distinguish different NMR experiments from one another. (4)

PRINCIPLE: 2D NMR SPECTROSCOPY

The construction of a 2D experiment is simple: In addition to preparation and detection which are already known from 1D experiments the 2D experiment has an indirect evolution time t_1 and a mixing sequence. This scheme can be viewed as: (5)

- Do something with the nuclei (preparation),
- let them process freely (evolution),
- do something else (mixing),
- Detect the result (detection, of course).



$$Int = f(t_1, t_2)$$

After preparation the spins can process freely for a given time t_1. During this time the magnetization is labelled with the chemical shift of the first nucleus. During the mixing time magnetization is then transferred from the first nucleus to a second one. Mixing sequences utilize two mechanisms for magnetization transfer: scalar coupling or dipolar interaction (NOE). Data are acquired at the end of the experiment (detection, often called direct evolution time); during this time the magnetization is labelled with the chemical shift of the second nucleus. (6)

How many 1D experiments need to be recorded for the complete 2D spectrum?

For a typical 2D [^{1}H, ^{1}H]-COSY spectrum usually a series of 512 1D spectra is recorded. The 1D spectra contain resonances at identical frequency, but the amplitudes (intensities) of the signals are modulated (vary) from experiment to experiment.

A Fourier transformation along the direct frequency dimension F2 results in a set of 1D spectra containing all chemical shifts and couplings, which are active during the acquisition period t_2. Because the signals physically give rise to a signal in the detection coil this dimension is called the direct dimension. Only so-called single-quantum frequencies can be recorded, because only these will result in a signal in the coil.

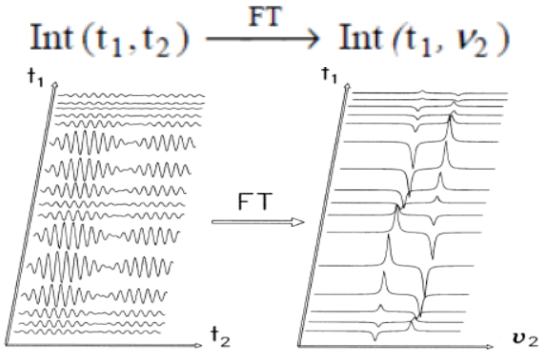


Figure: Schematic representation of a set of free induction decays (FIDs) (left) subject to the first Fourier transformation. [Taken from: van de Ven, F.J.M., Multidimensional NMR in Liquids]

The modulation of the amplitude of the signals in the different 1D spectrum is due to evolution of chemical shifts and scalar couplings during the evolution time t_1. A second Fourier transformation is performed in the orthogonal dimension (along t_1), and data points correspond to different FIDs.

$$\text{Int}(t_1, \nu_2) \xrightarrow{\text{FT}} \text{Int}(\nu_1, \nu_2)$$

Since the frequencies are derived from the amplitude modulation of the signals indirectly, the F1 frequency dimension is called the indirect dimension. The second FT therefore yields the full spectrum with two frequency dimensions.

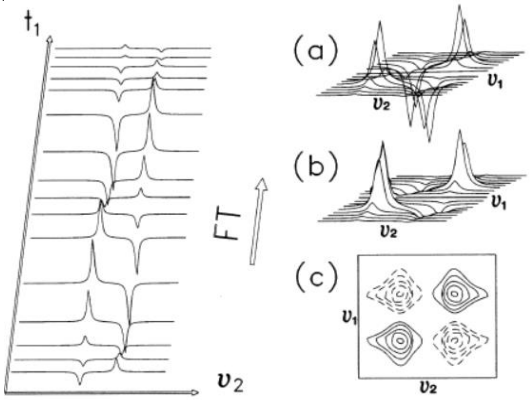


Figure: FT along t1 will yield the full 2D spectrum. Cross peaks may be displayed either as cross peaks with a contour plot (c) or a stacked plot (a, b) [taken from: van de Ven, F.J.M., and Multi-dimensional NMR in Liquids]

Depending on whether only scalar couplings and chemical shifts were active during t_1, a J-resolved or a shift-correlated spectrum will result. In a COSY experiment, chemical shifts are active during t_1 and t_2,
and coherence transfer takes place via scalar couplings. [7]

TYPES:

(1) Homonuclear through-bond correlation methods

- Correlation spectroscopy (COSY)
- Exclusive correlation spectroscopy (ECOSY)
- Total correlation spectroscopy (TOCSY)
- Incredible natural-abundance double-quantum transfer experiment (INADEQUATE)

(2) Heteronuclear through-bond correlation methods

- Heteronuclear single-quantum correlation spectroscopy (HSQC)
- Heteronuclear multiple-bond correlation spectroscopy (HMBC)

(3) Through-space correlation methods

- Nuclear Overhauser effect spectroscopy (NOESY)
- Rotating frame nuclear Overhauser effect spectroscopy (ROESY)

2D EXPERIMENTS

The [^{1}H, ^{1}H]-COSY-experiment

The first and most popular two-dimension NMR experiment is the homonuclear correlation spectroscopy (COSY) sequence, which is used to identify spins which are coupled to each other. It consists of a single RF pulse (p1) followed by the specific evolution time (t1) followed by a second pulse (p2) followed by a measurement period (t2). [8]

The COSY (correlated spectroscopy) experiment correlates nuclei via their scalar couplings. Chemical shifts are displayed along both frequency dimensions. In contrast to the TOCSY experiment, correlations will only appear between protons that possess a resolved coupling to each other.

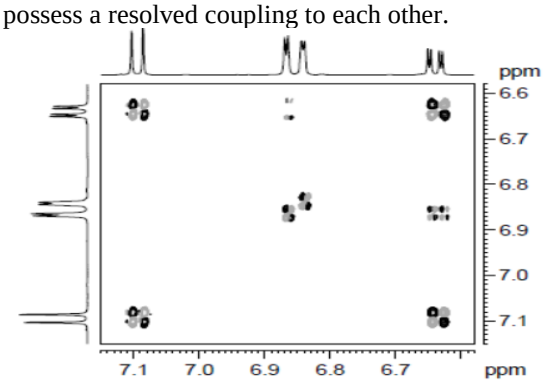
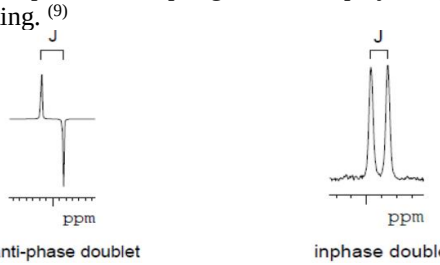


Fig: Expansion of the region displaying correlations between aromatic protons in the 2D [^{1}H, ^{1}H]-COSY spectrum of Melatonin

Active vs. Passive Couplings

Cross peaks in the COSY spectra display a characteristic fine structure, this reflects the scalar couplings. Active couplings are those that give rise to the cross peaks, if the cross peak is observed at the frequencies (ν_a, ν_b) then the $J_{A,B}$ coupling is the active coupling. Active couplings are in anti-phase, the corresponding peak components display opposite phase. Couplings to all other nuclei are called passive couplings and display in-phase splitting. [9]



The cross peak pattern, shown in the figure above, arises only for correlations between nuclei that possess no further scalar couplings. The separation of the multiple components is given by $J_{A,B}$. For the following spin system consisting of a linear chain of three protons, in which C is coupled to A and B coupled to A

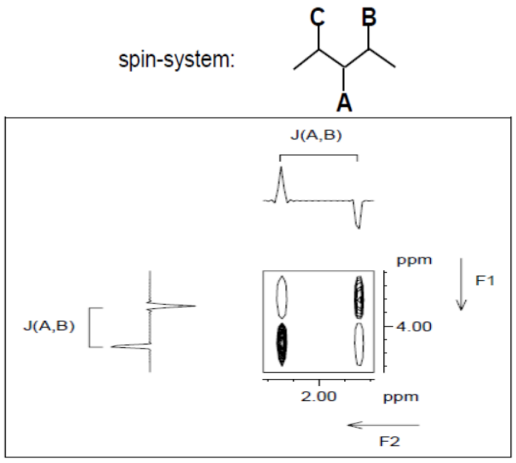


Fig: Cross peak of a 2-spin system in the COSY-spectrum

The cross peak (ν_A, ν_B) would be as illustrated in the following figure:

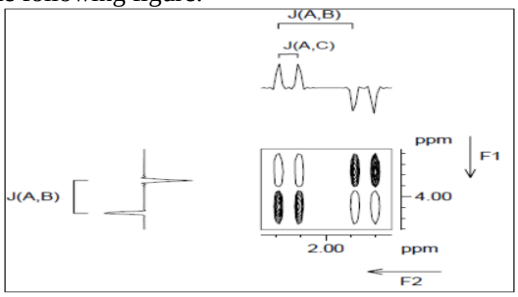


Fig: Cross peak of the three-spin system in the COSY

The active coupling $J_{A,\,B}$ leads to the anti-phase splitting. Due to the passive coupling $J_{A,\,C}$ an additional in-phase splitting occurs. The distance separation of the in-phase components therefore allows, in principle, to extract the passive coupling $J_{A,\,C}$ (however, partial signal cancellation leads to wrong values for small couplings; these are better extracted from ECOSY spectra). [10]

Artifacts in COSY spectra:

T_1-Noise is noise strips running parallel to the frequency axes. They mostly originate from instrumental instabilities, with temperature instabilities are being the most serious source. Since the noise is proportional to the signal height, they are most prominent for strong signals, e.g. singlet methyl groups or other sharp lines. T_1-noise always degrades spectrum quality but becomes particularly annoying when cross peaks with small intensities should be interpreted.

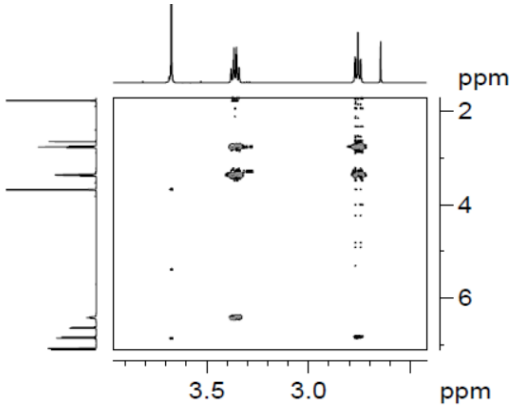


Fig: Example for t_1-noise in a COSY-Spectrum

If the chosen relaxation delay is too short, so-called rapid-scanning artifacts are observed. They occur at the double-quantum frequencies (the sum of the frequencies of the coupled nuclei) and lead to a second diagonal, twice as steep. They can (and should!) be easily recognized by the fact that occur at position at which no signals are found in the 1D spectrum.

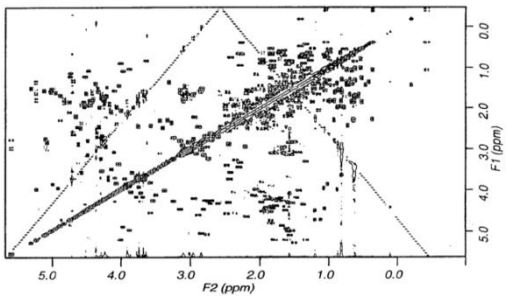


Fig: taken from: Cavanagh, J. et al. Protein Spectroscopy

The anti-phase character of COSY cross peaks leads to cancellation of signal intensities for small couplings. It is important to note that the resolution in the two frequency dimensions is usually very different. Therefore, the two symmetry-related peaks may not both be observed, but only one of them may occur:

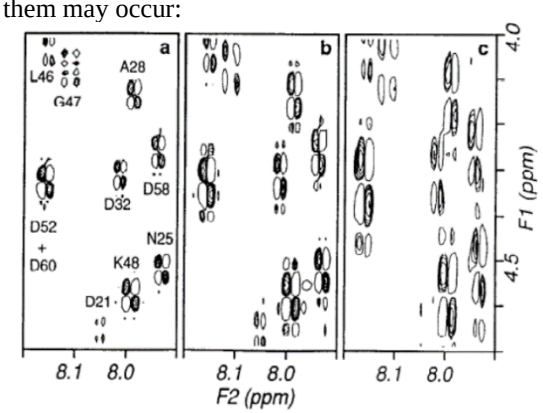


Fig: [taken from: Cavanagh, J. et al. Protein Spectroscopy]

The [X, X]-EXEY-experiment

The EXSY experiment is a homonuclear, shift correlated experiment, in which coherence transfer takes place through chemical or conformational exchange. In fact, the pulse sequence is the same as the one used for the NOESY. Because exchange is usually faster than the NOE buildup, shorter mixing times may be used for the EXSY. From recording a series of EXSY spectra with different mixing times, exchange kinetics may be deduced.

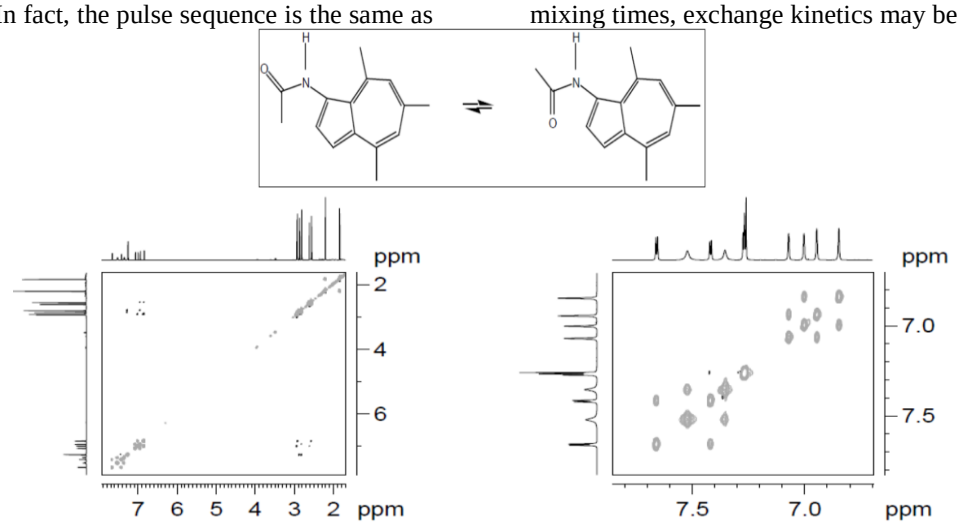


Fig: [^{1}H, ^{1}H]-EXSY-spectrum displaying exchange between the two rotamers in the figure on top.

Artifacts in EXSY spectra:

- NOESY-Peaks

Exclusive correlation spectroscopy (ECOSY):

ECOSY was developed for the accurate measurement of small J-couplings. It uses a system of three active nuclei (SXI spin system) to measure an unresolved coupling with the help of a larger coupling which is resolved in a dimension orthogonal to the small coupling. (11)

The [^{1}H, ^{1}H]-TOCSY-experiment

TOCSY is sometimes called "homonuclear Hartmann–Hahn spectroscopy" (HOHAHA). The TOCSY experiment is similar to the COSY experiment, in that cross peaks of coupled protons are observed. However, cross peaks are observed not only for nuclei which are directly coupled, but also between nuclei which are connected by a chain of couplings. This makes it useful for identifying the larger interconnected networks of spin couplings. This ability is achieved by inserting a repetitive series of pulses which cause isotropic mixing during the mixing period. Longer isotropic mixing times cause the polarization to spread out through an increasing number of bonds. (12)

Similar to the COSY, the TOCSY is a homonuclear, shift-correlated 2D NMR experiment, in which coherence transfer takes place via scalar couplings. Cross-peaks contain both passive and active couplings in-phase. In contrast to the COSY, correlation between a spin and all other spins from the same spin system[3] may be observed. For example, correlations from the amide proton will, under favorable conditions, include all side-chain protons from the same amino acid (e.g. for lysine). (13)

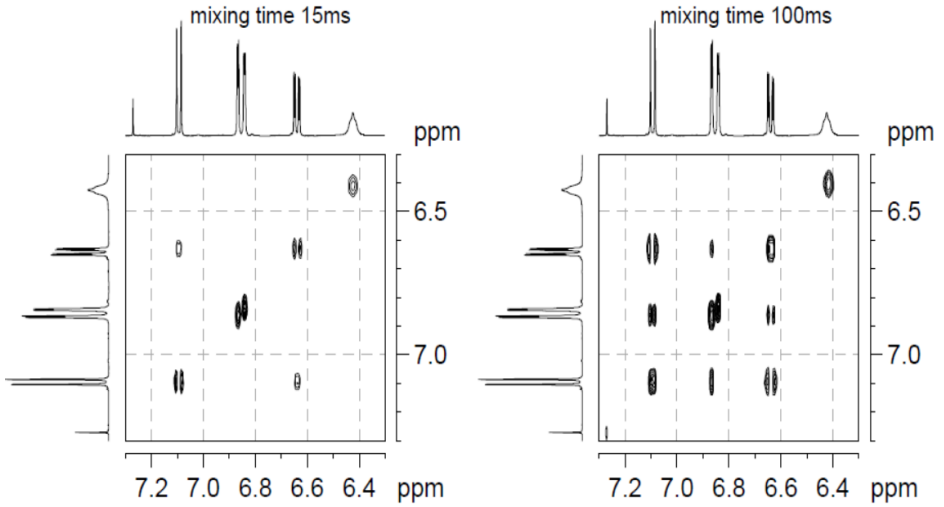


Fig: Expansion of the region displaying aromatic correlations of Melatonin, for different settings of the mixing time.

The [^{13}C, ^{13}C]-INADEQUATE-experiment

INADEQUATE is a method often used to find ^{13}C couplings between adjacent carbon atoms. Because the natural abundance of ^{13}C is only about 1%, only about .01% of molecules being studied will have the two nearby ^{13}C atoms needed for a signal in this experiment. Each coupled pair of nuclei gives a pair of peaks on the INADEQUATE spectrum which both have the same vertical coordinate, which is the sum of the chemical shifts of the nuclei; the horizontal coordinate of each peak is the chemical shift for each of the nuclei separately. (14)

In F1 the double quantum frequencies are recorded, and hence the cross peaks have the following coordinates: F2: ν(a), F1: ν(a) + ν(b). Because ^{13}C, ^{13}C isotopomers are very rare (0.01*0.01=0.0001) in ^{13}C natural abundance molecules, extremely concentrated samples are required. The experiment is very powerful, and very useful for highly substituted compounds, in which proton density is low. The following figure displays an expansion of an INADEQUATE experiment recorded on melatonin:

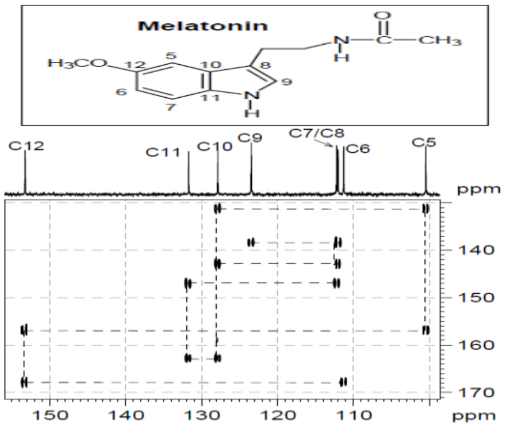


Fig: Expansion of a 2D INADEQUATE spectrum recorded on melatonin

Cross peaks are spilt into doublets by the one-bond C-C coupling. The two coupled resonances can be recognized as two separate peaks at a common frequency in F1 (on a horizontal line). Sometimes one of the two peaks is missing due to low signal-to-noise. The coupled partner can be easily calculated, because its frequency plus the frequency of the coupling partner must add up to the F1 frequency.

The [^{1}H, ^{13}C]-HSQC-experiment

HSQC detects correlations between nuclei of two different types which are separated by one bond. This method gives one peak per pair of coupled nuclei, whose two coordinates are the chemical shifts of the two coupled atoms. (15)

Nuclei, usually separated by one bond are correlated via their scalar couplings. In [^{13}C, ^{1}H]-HSQC spectra, no correlations to quaternary carbons are observed. Since the one-bond proton-carbon or proton-nitrogen couplings are large and rather uniform, these spectra are quite sensitive.

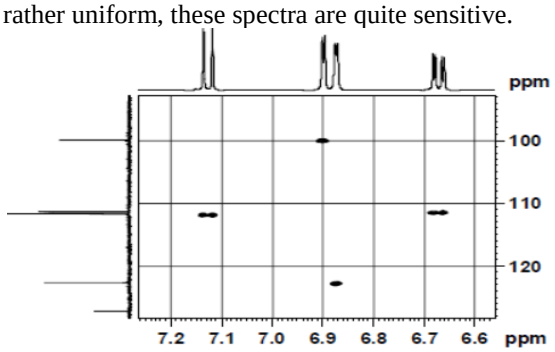


Fig: Expansion of a [^{13}C, ^{1}H]-HSQC recorded on Melatonin

Artifacts in the HSQC

- Folding in F1

If the spectral width of the indirect dimension is chosen too small (this is sometimes done on purpose) folding of signals occurs. Whereas folded signals in the direct dimension are usually strongly attenuated by audio filters folding in F1 gives signals with full intensity at erroneous positions in the spectrum. Depending on the quadrature detection mode in F1signals may be folded about the near or the distant edge:

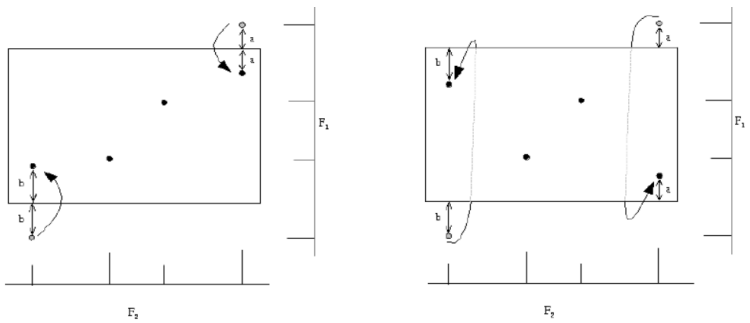


Fig: Folding in data sets with real (left) or complex (right) acquisition.

The [^{1}H, ^{13}C]-HMBC-experiment

HMBC detects heteronuclear correlations over longer ranges of about 2–4 bonds. The difficulty of detecting multiple-bond correlations is that the HSQC and HMQC sequences contain a specific delay time between pulses which allows detection only of a range around a specific coupling constant. The ^{1}H, ^{13}C ^{3}J coupling, for example, displays a Karplus-type dependence on the dihedral angle. Therefore, some couplings may be close to zero, and such correlations will then of course be absent from the spectrum. Depending on the system under study, the ^{2}J or the ^{3}J coupling may be larger, so that these spectra contain much ambiguity. Nevertheless, the HMBC is a very useful experiment, because it contains correlations to quaternary carbons. (16)

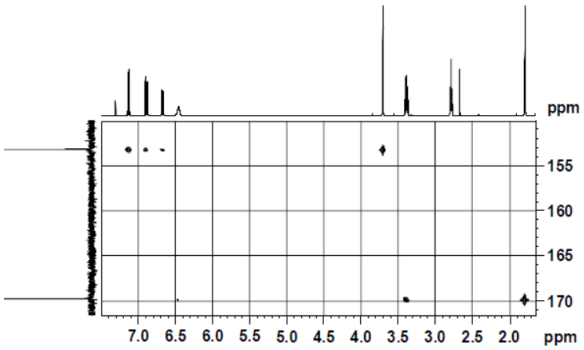


Fig: Expansion of the [^{13}C, ^{1}H] - HMBC-spectrum of Melatonin

Artifacts

- HMBC spectra often contain correlations due to ^{1}Jc, H-couplings. Since HMBC spectra are not usually decoupled during acquisition, these couplings will show up as rather larger (e.g. 200 Hz) doublets. The HMBC contains a filter for such correlations, which however fails to work when the one-bond couplings differ significantly from standard values (e.g. from 140Hz, aromatic carbons).

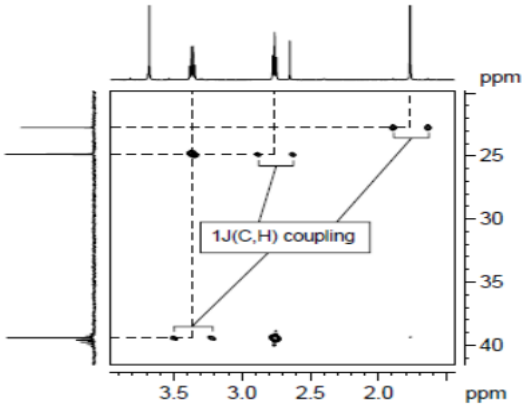


Fig: HMBC-spectrum displaying correlations due to the ^{1}Jc, H-couplings

- Axial peaks (artifacts which can be found on a horizontal line along the center frequency).
- Folded signal similar to the situation encountered for HSQC spectra.

The [^{1}H, ^{1}H]-NOESY-experiment

In NOESY, the nuclear Overhauser cross relaxation between nuclear spins during the mixing period is used to establish the correlations. The spectrum obtained is similar to COSY, with diagonal peaks and cross peaks, however the cross peaks connect resonances from nuclei that are spatially close rather than those that are through-bond coupled to each other. NOESY spectra also contain extra axial peaks which do not provide extra information and can be eliminated through a different experiment by reversing the phase of the first pulse. (17)

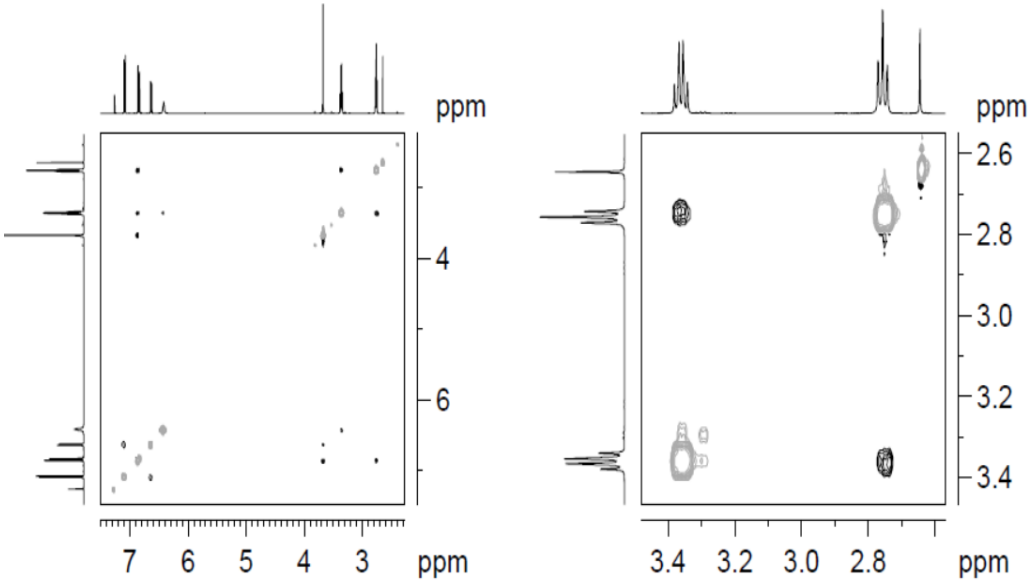


Fig: NOESY-spectrum of Melatonin with expansion. Gray peaks: positive signals, black peaks negative phase.

The underlying effect of the NOESY is the nuclear Overhauser effect. The NOE describes a phenomenon whereby a non-equilibrium population of α- and β-states relaxes back to its equilibrium value, such that populations of energy levels of other spins (and hence their signal intensities) are changed. The sign of the NOE (increase or decrease of signal intensity) depends on the tumbling properties and is positive for small and negative for large molecules. For intermediate-size molecules the NOE may actually be small or close to zero:

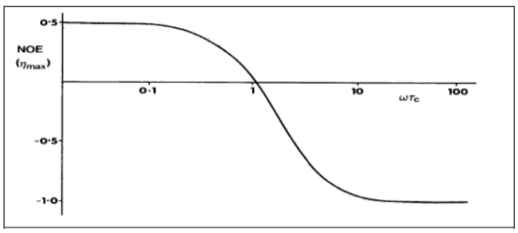


Fig: Dependency of the proton, proton NOE on the molecular reorientation time τ_c [taken from: Neuhaus, D., Williamson, M., the NOE in Structural and Conformational Analysis]

Because the sign of the NOE depends on the molecular reorientation time τ_c of the molecule, peaks in the NOESY may be positive (large molecules) or negative (small molecules). The reorientation time is largely influenced by the viscosity of the solvent. Even smaller molecules therefore tend to behave like large molecules when measured in DMSO. A dramatic influence on motional properties is also seen by the temperature: As a rule of thumb, changing the temperature by 20 degrees corresponds to the same change in motional properties, as would be observed upon doubling the molecular weight. The following table describes the behavior of molecules of different size in NOESY experiments: [18]

Artifacts in the NOESY

- EXSY-(exchange)-peaks: They often display large intensities, possess the same phase as the diagonal peaks, and are often also observed for very short mixing times. Typical examples are exchange peaks between amide protons, or sugar hydroxyl protons, and the water signal.

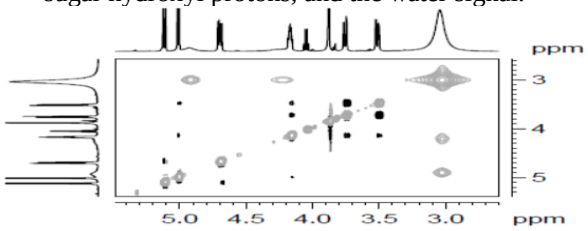


Fig: NOESY-spectrum containing exchange peaks.

- COSY-peaks (anti-Phase COSY-type peaks; zero-quantum interference peaks): They are observed between protons that display both dipolar and scalar couplings (e.g. for germinal protons), and for shorter mixing times. Due to the different relaxation properties of protons in larger molecules, they disappear in NOESY spectra of proteins. These peaks are manifested by their COSY-type typical anti-phase peak pattern. When overlapped with genuine NOESY signals they lead to partial cancellation of NOE cross peak intensity resulting in titled peaks.

The [^{1}H, ^{1}H]-ROESY-experiment

ROESY is similar to NOESY, except that the initial state is different. Instead of observing cross relaxation from an initial state of *z*-magnetization, the equilibrium magnetization is rotated onto the *x* axis and then spin-locked by an external magnetic field so that it cannot process. This method is useful for certain molecules whose rotational correlation time falls in a range where the Nuclear Overhauser effect is too weak to be detectable, usually molecules with a molecular weight around 1000 daltons, because ROESY has a different dependence between the correlation time and the cross-relaxation rate constant. In NOESY the cross-relaxation rate constant goes from positive to negative as the correlation time increases, giving a range where it is near zero, whereas in ROESY the cross-relaxation rate constant is always positive. ROESY is sometimes called "cross relaxation appropriate for mini molecules emulated by locked spins" (CAMELSPIN). [19]

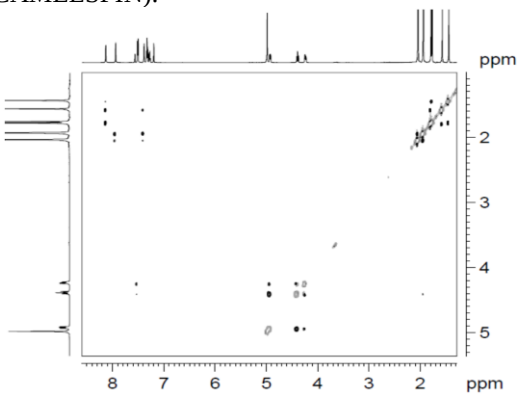


Fig: ROESY-spectrum of a peptide; grey peaks = positive signals, black peaks = negative signals

Artifacts in the ROESY

- TOCSY-Peaks, (in-phase, positive), observed for germinal protons, whose chemical shift difference is small
- Spin-diffusion peaks (ROE-ROE relay peaks) (in-phase, positive)
- TOCSY-ROESY transfer Peaks (in-phase, negative)
- Exchange peaks (positive)

- It can be seen that almost all artifacts can be readily recognized from the different sign of the peaks. [20]

CONCLUSION:

It's commonly accepted by users (biologists, pharmacologists, healthcare professionals) that the recent introduction of 2D-NMR methods represents a huge qualitative gap for metabolic investigations, for them, it's obvious and natural that more information and more power. But for the moment, no statistical study proved this clearly.

So, we are trying to fill this lack. We are working to show in a encouraging way that 2D-NMR tools are statistically robust tools, and more that 2D-COSY experiments seems to be more repeatable and reliable than corresponding 1D methods.

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