

4. Simulations of EPR Spectra

To gain an understanding of how EPR spectra are built up and how they have to be interpreted, the creation of such spectra via simulations is very instructive.

4.1 Simulation Software

For the simulations we will be using the software package that comes together with the Biomolecular EPR spectroscopy book by Wilfred Hagen (www.bt.tudelft.nl/biomolecularEPRspectroscopy). The minimal system requirements to run these programs are: off-the-shelf PC with Windows XP and a monitor with at least 1280 pixels resolution in the x-dimension. The used file format (input and output) is: bare Ascii files with 1024 amplitude entries (program EPR File Converter can be used to convert from other file formats to this standard).

As a general philosophy this set of eight programs is intended to cover the most common problems in numerical analysis (simulation and manipulation) of CW biomolecular EPR powder spectra. The design of the programs is towards maximization of “ease of use”: each program is set up around a standardized single-window user interface that should be intuitive and (nearly) self-explanatory. Choices of mathematical and numerical approaches are automatic and thus completely screened off from the user.

There are eight programs which can be divided into four categories: an introductory, practicing tool, three number crunchers for a variety of anisotropic spectra, two programs to modify experimental and/or simulated spectra, and two programs for the special cases of radicals in solution and for integer-spin systems with strong zero-field interactions.



The program ‘Simple Spectrum’ is mainly intended as an introductory, hands-on learning tool for those who are inexperienced in EPR spectral simulation.



The program ‘Hyperfine Spectrum’ expands the options available for the spectral simulation. First of all it allows the simulation of two species at the same time. Each species can have central (metal) hyperfine splittings and also ligand superhyperfine splittings. Also, for each component a ‘checkbox’ can be ticked for the program to use the natural isotope distribution for one of the elements Cu, Mo, or W.



The program ‘GeeStrain-5’ can generate sum spectra of up to five different components subject to broadening by g -strain. This situation is typically encountered in respiratory-chain complexes and in complex enzymes with multiple electron-transfer prosthetic groups. In a g -strained spectrum the linewidth is no longer described by three components W_x , W_y , W_z , along the molecular axes, but by six components of a symmetrical g -strain tensor. Each component can be positive or negative. g -Strain results in characteristic patterns of skewed and asymmetric features in the powder spectrum. g -Strain is a reflection of protein conformational distributions.



The program ‘Visual Rhombo’ is for half-integer high-spin systems with strong zero-field

interaction, which frequently occur in a variety of iron proteins and in high-spin cobalt proteins. It can be used in two ways. Firstly, entering a value for the rhombicity $h = E/D$ will generate a complete set of effective g -values, which may be traced back in experimental spectra. Secondly, spectra (sums of all intradoublet sub spectra) can be generated, where realistic simulation of experimental data frequently requires the rhombicity to be distributed (' D -strain'), and one has to specify the width and the number of digital steps of the simulation.



The program 'EPR File Converter' is a tool to convert experimental data files to the standard (1024 ascii amplitude values) used by the programs in this package. As an extra option 'EPR File Converter' can also produce the first integral (the EPR absorption spectrum) or the second derivative of an experimental or a simulated spectrum.



The program 'EPR Editor' can do several things. When difference spectra have to be produced it is important that the two source spectra have the same frequency. The program normalizes two experimental spectra to the microwave frequency of one of them. The program also provides the numerical value of the second integral of a spectrum (or a difference spectrum) calculated between two limits defined by the user. The second integral is required for quantification (or: spin counting). 'EPR Editor' can also read-out g -values from experimental data which can help with the spectral simulations.



The program 'Isotropic Radicals' is for radicals in solution that tumble so fast as to completely average out any anisotropy in the g - and A -values.



The program 'Single Integer Signal' is for integer spin systems subject to strong zero-field interaction, e.g. Fe(II) complexes, reduced 3Fe-4S clusters or reduced 2Fe oxo clusters, which typically exhibit only a single transition within their highest non-Kramers doublet detectable in parallel-mode EPR and, with increased linewidth and reduced intensity, in normal-mode EPR. 'Single Integer Signal' generates these two spectra simultaneously (parallel-mode in bright red, normal mode in dark red). The shape is a function of three parameters only: an effective g -value and two linewidth parameters that model rhombic D -strain.

Note that the following type of problems are not covered by any of the here provided programs: (i) systems with non-colinear tensors (except for g -strain broadening); (ii) intermediate-field high-spin systems (i.e. systems with electronic Zeeman and zero-field interactions of comparable strength); (iii) data from pulsed techniques. It is not easy to design universally applicable simulators for these problems, whose solution is anyway sufficiently complex as to call for either teaming up with a specialist or, alternatively, to become a specialist oneself and to develop dedicated code for specific cases.

4.2 Simulations

Most EPR spectra from metalloproteins can be satisfactorily simulated on basis of only 6 parameters: three g -values and three linewidths (W_i in Gauss; It is **defined differently in the literature, either as the distance between the inflection points or linewidth at half height, and will therefore have different values**). If there is hyperfine interaction, then three additional parameters have to be provided: the hyperfine splitting constants (A_i in Gauss: distance between the hyperfine lines). The cause of the hyperfine interaction, interaction with a nuclear spin of the ion in question (hyperfine interaction) or with the nucleus of a ligand (superhyperfine interaction), is of no concern for the simulation, since they are treated in the same way.

If we are dealing with a single crystal, then there is only one EPR line (all molecules have the same orientation), which might be split up by (super)hyperfine interaction. A single line is also obtained for a radical in solution or for a small metal complex in solution (rapid tumbling).

If we are dealing with a powder or with a frozen solution of a paramagnetic species, then it may be somewhat more complicated. The spectrum is then dependent on the symmetry of the magnetic environment of the paramagnet.

For all simulations below, we must choose a 'spectral window': an approximation of the field range that would be required when actually measuring the sample on the EPR spectrometer. We first take a window for the $g = 2$ region and use a microwave frequency of 9500 MHz.

Radicals

A free radical, e.g. an unpaired electron in a π -orbital system of a benzene ring, has no fixed environment. Any local asymmetry experienced by the electron will be average out in time: the electron has a totally symmetric environment and shows an *isotropic* spectrum

Open the program **isotropicradicals**. Set the frequency to 9500 MHz, B-min to 3300 gauss, and B-max to 3500 gauss.

- 1) Take “g-Value” = 2 and “Linewidth” = 3 G (so $g_x = g_y = g_z$ and $W_x = W_y = W_z$). Note that in this example there are no (0) interacting nuclei.

Spectrum: _____ .dat

- 2) Now take this radical and put it on a nitrogen atom. The electron is now fixed in position, but we assume a rapid tumbling of the molecule in solution, which likewise results in an ‘averaging out’ of any asymmetry in the environment of the unpaired spin. Natural nitrogen (^{14}N) has a nuclear spin of 1. Take several values for the hyperfine interaction and make four spectra:

A-value = 25 G

Spectrum: _____ .dat

A-value = 15 G

Spectrum: _____ .dat

A-value = 5 G (That looks funny. Why?)

Spectrum: _____ .dat

A-value = 2 G

Spectrum: _____ .dat

- 3) Now give the radical an interaction with three equivalent protons (nuclear spin $\frac{1}{2}$). Take A-value = 40 G for all of them. Explain the result by drawing a ‘stick’ spectrum’.

Spectrum: _____ .dat

- 4) As (3), but now all protons have been exchanged by deuterons. These have a different nuclear spin ($I=1$) which is about 0.31 times as strong, so take A-value = 12.4 G. This exercise is sometimes useful to predict whether an actual experiment (exchange of H by D in a compound) is worthwhile in order to verify one’s interpretation.

Spectrum: _____ .dat

- 5) The radical now interacts with three non-equivalent protons;

For proton 1: A-value = 5 G

For proton 2: A-value = 10 G

For proton 3: A-value = 20 G

Predict the spectrum by making a ‘stick spectrum’ by hand. Why does the simulated spectrum looks so funny?

Spectrum: _____ .dat

- 6) Now a somewhat more complicated case: a radical having interaction with 5 non-equivalent protons and 1 nitrogen nucleus. Take;

For proton 1: A-value = 5 G
For proton 2: A-value = 10 G
For proton 3: A-value = 10 G
For proton 4: A-value = 10 G
For proton 5: A-value = 20 G

For nitrogen: A-value = 20 G

Try to make a stick spectrum!

Spectrum: _____ .dat

- 7) The last example involves a cobalt radical ($I = 7/2$) with superhyperfine interactions of 1 N and 3 H nuclei. Take now:

B-min: 3100 gauss
B-max: 3700 gauss.

For Co: A-value = 50 G
For nitrogen: A-value = 20 G
For hydrogen: A-value = 10 G

This spectrum looks misleading! Just try to pinpoint the cobalt splitting!

Spectrum: _____ .dat

To understand this, also perform the simulation where the interaction of the nitrogens and hydrogens are removed.

Spectrum: _____ .dat

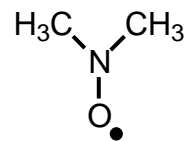
Can you now explain the result of (7)?

- 8) Simulation of the dimethyl nitroxyl radical.

Start by loading the experimental spectrum, "dimethylnitroxyl.dat".

Adjust the parameters:

Frequency: 9766.13 MHz
B-min: 3380 gauss
B-max: 3580 gauss.



First try to find the central g -value (This should be a position in the middle of the whole spectrum). Then introduce the nitrogen and hydrogen hyperfine splittings. First use an equal value of A for both nuclei and try to match the overall splitting pattern. After that, play around with the A -values.

$g =$ _____, $W =$ _____, $A_N =$ _____, $A_H =$ _____, Spectrum: _____ .dat

Powder Spectra

9) *Axial symmetry* (so $g_x = g_y \neq g_z$). Use the program **simplespectrum**.

As an example we take the line shape of an Fe-S cluster.

Frequency: 9500 MHz
B-min: 3000 gauss
B-max: 3800 gauss.
 g_{zyx} : 2.054, 1.925, 1.925
 W_{zyx} : 10, 16, 16

Now we have to take a finite value for dim-1 (cos Theta), since we are no longer dealing with a total symmetric environment. Playing with the parameter dim-1 gives a good impression of how a powder spectrum is actually built up. We therefore take a number of dim-1 values (dim-2 (Phi) is 1 in all cases)

Dim-1: 1	Spectrum: _____ .dat
Dim-1: 2	Spectrum: _____ .dat
Dim-1: 5	Spectrum: _____ .dat
Dim-1: 15	Spectrum: _____ .dat
Dim-1: 50	Spectrum: _____ .dat
Dim-1: 100	Spectrum: _____ .dat
Dim-1: 150	Spectrum: _____ .dat

Then make a spectrum with $W_{zyx} = 2, 4, 4$ and dim-1 = 50. Compare this with the previous one with dim-1 = 50. What is causing the difference and why?

Spectrum: _____ .dat

Compare the spectra with dim-1 = 50, 100 and 150 to see when the number of spatial orientations was large enough (i.e. when the powder was 'fine' enough). For this you have to import the spectra in the for example Origin and make an overlay plot of the three spectra.

Note: to obtain the best simulation, one always has to increase the number of space orientations until the spectrum doesn't change anymore (infinitely fine powder).

10) *Rhombic symmetry (so $g_x \neq g_y \neq g_z$).*

We take the line shape of a rhombic Fe-S cluster with

Frequency: 9500 MHz
 B-min: 3100 gauss
 B-max: 3900 gauss.
 g_{zyx} : 2.02, 1.91, 1.85
 W_{zyx} : 10, 13, 18

Now also dim-2 needs to be larger than 1, since the x- and y-directions are no longer equivalent. 'Play' again with the parameter dim-2 and see what the effect is. Take:

<u>dim-1</u>	<u>dim-2</u>	
1	1	Spectrum: _____ .dat
80	1	Spectrum: _____ .dat
80	2	Spectrum: _____ .dat
80	5	Spectrum: _____ .dat
80	15	Spectrum: _____ .dat
150	100	Spectrum: _____ .dat

(On older slower computers you might notice a slight increase in computing time. In the last spectrum 15000 first derivative Gaussian lines had to be calculated.)

11) Simulation of the [2Fe-2S] ($S = \frac{1}{2}$) signal detected in ferredoxin from *Clostridium pasteurianum*. First load the spectrum in 'WinEPR' and read out the g -values. Then load the experimental spectrum 'Cp2Fe_spec' into the 'simplespectrum' simulation program. Adjust the parameters:

Frequency: 9630 MHz
 B-min: 3005 gauss
 B-max: 4005 gauss.

$g_{xyz} =$ _____, _____, and _____
 $W_{xyz} =$ _____, _____, and _____

Spectrum: _____ .dat

Spectra of Simple Metalloproteins

(The treatment of organo-metallic complexes is completely analogous)

As an example we take the EPR spectra of a copper protein (or complex). We will be using the program **hyperfinespectrum**.

Effect of hyperfine interaction on the EPR powder line shape – Hyperfine interactions can have a highly deceiving effect on the appearance of powder-type spectra. This is illustrated here with copper ($I = 3/2$).

12) To start, we first produce an axial spectrum of a metalloprotein without a nuclear magnetic moment:

Frequency: 9500 MHz
B-min: 1900 gauss
B-max: 3900 gauss
Dim-1: 150
Dim-2: 1
 g_{zyx} : 2.90, 2.07, 2.07
 W_{zyx} : 20, 20, 20

Spectrum: _____ .dat

It should be easy enough to recognize the parameters in the spectrum.

13) We now include the hyperfine interaction of a copper nucleus as it usually appears in the so-called ‘type II’ (non-blue) copper proteins:

Metal A_{zyx} : 150, 15, 15

Spectrum: _____ .dat

The simulation now takes 4 times as long. This is due to the fact that our unpaired electron in reality ‘feels’ 4 different magnetic fields. The real spectrum essentially is an overlap of 4 different spectra.

14) We then shift the g_z -value slowly into the direction of the g_{xy} . All other parameters, also the A values, remain unchanged. Make 4 spectra, with:

g_z : 2.4
 g_z : 2.3
 g_z : 2.2
 g_z : 2.1

Spectrum: _____ .dat

Spectrum: _____ .dat

Spectrum: _____ .dat

Spectrum: _____ .dat

Note the changes in the x,y -direction. The extra line appearing at the high-field side of the g_{xy} -line is called an **overshoot** line. This occurs rather often in X-band EPR spectroscopy and not only for Cu proteins. Interpretation of such spectra is often impossible, especially so when we also deal with very rhombic spectra. To analyze such spectra, measurement of the sample at a higher microwave frequency is desired.

15) Simulation of an experimental copper spectrum. For the next exercise we take the spectrum of the 'dirty copper' in the preparation of cytochrome *c* oxidase. First we have to determine the line shape of this dirty copper. Two spectra have been recorded for the enzyme. The first one ('CoxCu20') was measured at low power. Under these conditions the spectra of Cu_A from the enzyme and the dirty copper are both non-saturated. The second one ('CoxCu10') was measured at high power. The Cu_A is not saturated, but the dirty copper saturates to a great extent at high power and 77 K. So the spectrum represents mainly the one from the intrinsic Cu_A.

The two spectra represent two equations with two unknowns, so one can determine the line shape of both individual spectra by subtraction. Use the program **epredictor**. Read in both spectra and adjust the 'B-amplitude' value until you got the correct settings (This will need some prior knowledge of what Cu_A looks like, just ask the teacher for help). Note that the spectra were normalized and the amplitude cannot be directly compared.

Hint: You are subtracting too much when you get negative peaks.

Cu_A- Spectrum: _____ .dat
 Dirty Cu - Spectrum: _____ .dat

You can use the same program to obtain the parameters required for the simulation from the thus obtained experimental spectrum of the dirty copper. For this you have to fill in the measurement parameters:

Frequency: 9140 MHz
 B-min: 2475 gauss
 B-max: 3500 gauss

Estimated values:

$g_{xyz} =$ _____, _____, and _____
 $(W_{xyz} =$ _____, _____, and _____)
 $(A_{xyz}^{Cu} =$ _____, _____, and _____)

Hint: the spectrum has axial symmetry. The A_{xy} splitting is not resolved and can be taken as $0.1 \times A_z$. Take the linewidths the same in all directions

$g_{xyz} =$ _____, _____, and _____
 $W_{xyz} =$ _____, _____, and _____
 $A_{xyz}^{Cu} =$ _____, _____, and _____

Spectrum: _____ .dat

In comparing the simulation with the experimental spectrum extra splitting might be noticed in the x,y -direction in the experimental spectrum. These are due to superhyperfine interaction of nitrogen nuclei in the coordination of the dirty copper (the copper sticks to the protein mainly via binding to peptide bonds.) How many N nuclei might be involved?

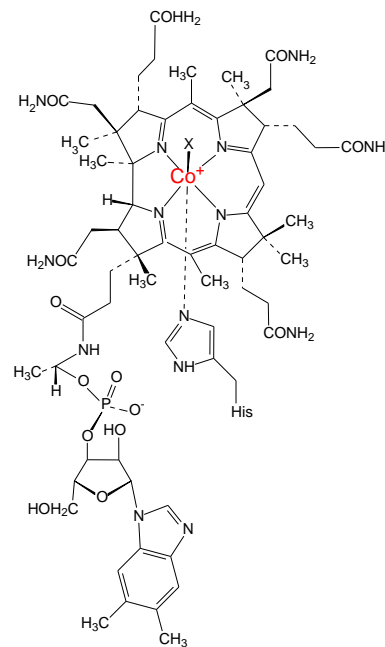
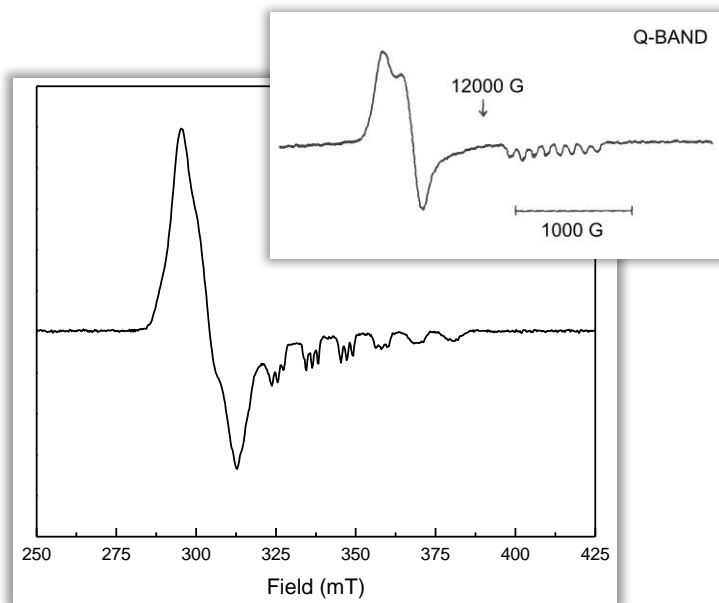
Hint 1: Disregard the unresolved interaction of N in the z -direction. Only estimate A_{xy}^N .

Hint 2: Note that in the experimental spectrum, the N hyperfine splitting in the x,y -direction is partly resolved. This means that this splitting is greater than the linewidth there, so adjust this linewidth.

$$\begin{aligned}
 g_{xyz} &= \underline{\hspace{2cm}}, \underline{\hspace{2cm}}, \text{ and } \underline{\hspace{2cm}} \\
 W_{xyz} &= \underline{\hspace{2cm}}, \underline{\hspace{2cm}}, \text{ and } \underline{\hspace{2cm}} \\
 A_{xyz}^{Cu} &= \underline{\hspace{2cm}}, \underline{\hspace{2cm}}, \text{ and } \underline{\hspace{2cm}} \\
 \underline{\hspace{1cm}} \times N: A_{xyz}^N &= \underline{\hspace{2cm}}, \underline{\hspace{2cm}}, \text{ and } \underline{\hspace{2cm}}
 \end{aligned}$$

Spectrum: .dat

16) In the next example we will simulate a spectrum for the cobalt-containing vitamin B₁₂ cofactor that can be found in several different types of enzymes.



Before we start a small description of the origin of this spectrum is needed. It is due to a Co(II) ion ($3d^7$) with the unpaired electron in the d_{z^2} orbital. That means that the electron is not interacting with the four nitrogen ligands in the ring. It is interacting, however, with an axial nitrogen ligand from a histidine residue. The 8 hyperfine lines due to the interaction with the Co nucleus can be easily detected in the Q-band spectrum. The low-field lines are more difficult to detect in the X-band spectrum. Also not from the Q-band spectrum that the spectrum is not completely axial. For convenience the g -values for the

Again we will be using the program **hyperfinespectrum** with the spectrum 'Co_spec'.

Hint 1: Use a relative large linewidths for the g_x - and g_y -peaks with a small Co splitting. Assume there is no N splitting for these peaks.

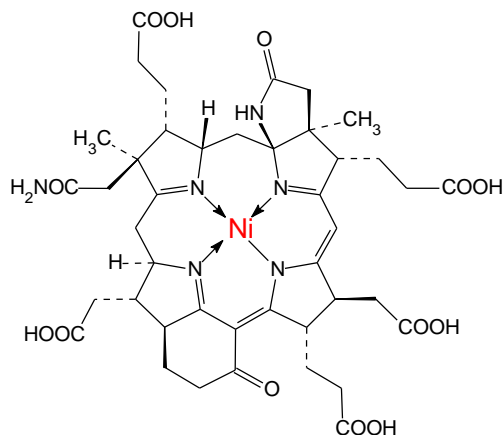
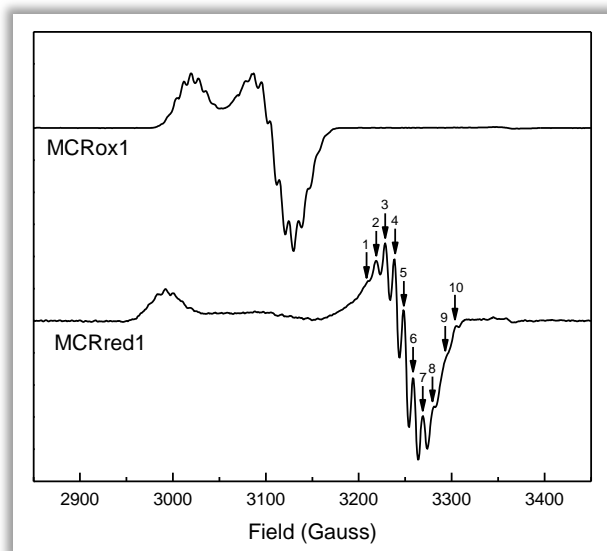
Frequency:	9596.06 MHz	$g_{xyz} =$	<u>2.275</u>	,	<u>2.220</u>	,	and	<u>2.006</u>
B-min:	2000 gauss	$W_{xyz} =$	_____	,	_____	,	and	_____
B-max:	5000 gauss	$A_{xyz}^{Co} =$	_____	,	_____	,	and	_____
		$A_{xyz}^N =$	_____	,	_____	,	and	_____

Spectrum: _____ .dat

Repeat the simulation assuming all N in the sample has been replaced with the ¹⁵N isotope ($I = \frac{1}{2}$)

Spectrum: _____ .dat

17) The nickel in methyl-coenzyme M reductase is also present in a tetrapyrrole ring. The spectra however are very different. In this case two redox states are accessible: Ni^{3+} ($3d^7$) and Ni^{1+} ($3d^9$). The figure shows the spectra for the MCRox1 form (Ni^{3+}) and the MCRred1 form (Ni^{1+}). In both cases the free electron is in the $d_{x^2-y^2}$ orbital and interacts with all four of the tetrapyrrole nitrogen atoms. Note that this interaction should result in 9 hyperfine lines. Instead 10 hyperfine lines are detected on the g_{xy} -peak. Since there are no other interactions with the nickel this means that the g_x and g_y have to be slightly different (which was proven by doing measurements at higher frequency). Try to simulate either (or both) of the MCR spectra, 'MCRox1_spec' or 'MCRred1_spec'.



Frequency: 9596.06 MHz (ox1)
 B-min: 2000 gauss
 B-max: 5000 gauss

$g_{xyz} =$ _____, _____, and _____
 $W_{xyz} =$ _____, _____, and _____
 $A_{xyz}^N =$ _____, _____, and _____

Spectrum: _____ .dat

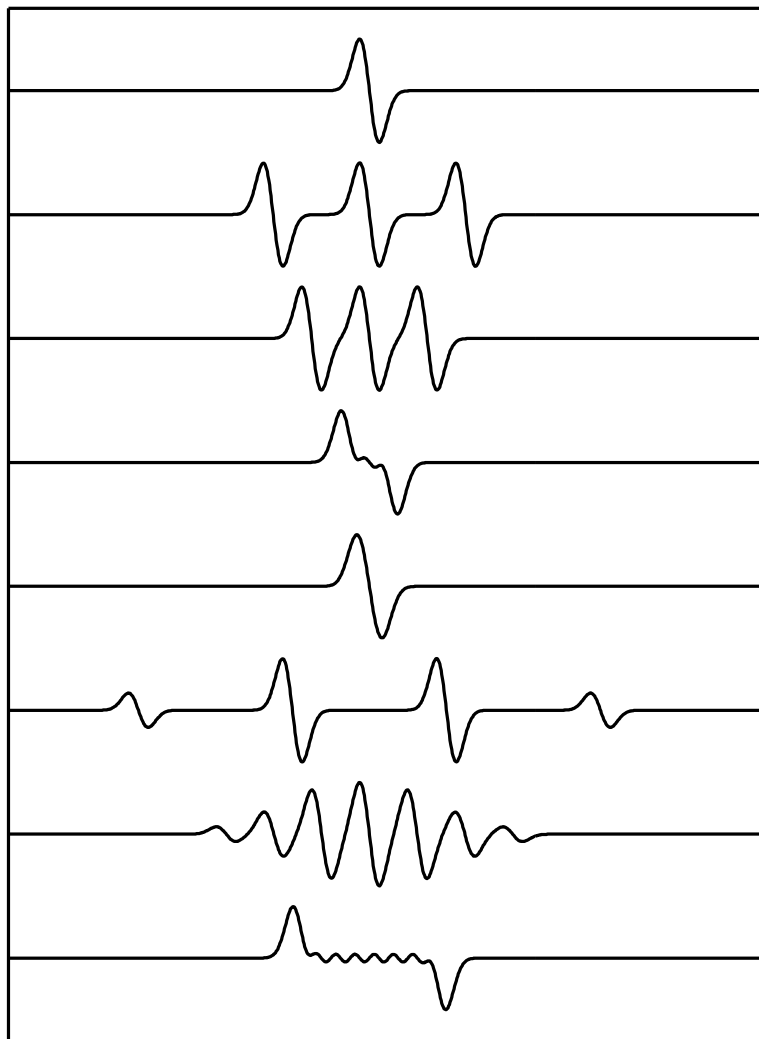
Frequency: 9431.35 MHz (red1)
 B-min: 2000 gauss
 B-max: 5000 gauss

$g_{xyz} =$ _____, _____, and _____
 $W_{xyz} =$ _____, _____, and _____
 $A_{xyz}^N =$ _____, _____, and _____

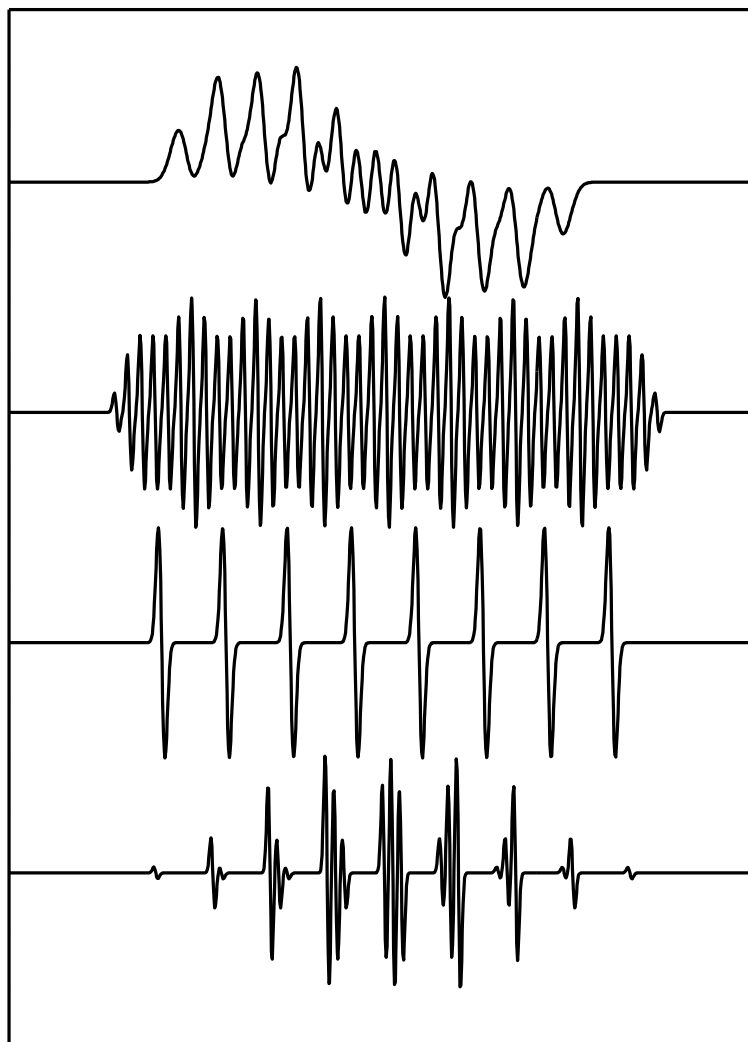
Spectrum: _____ .dat

Spectra Obtained in the Different Exercises

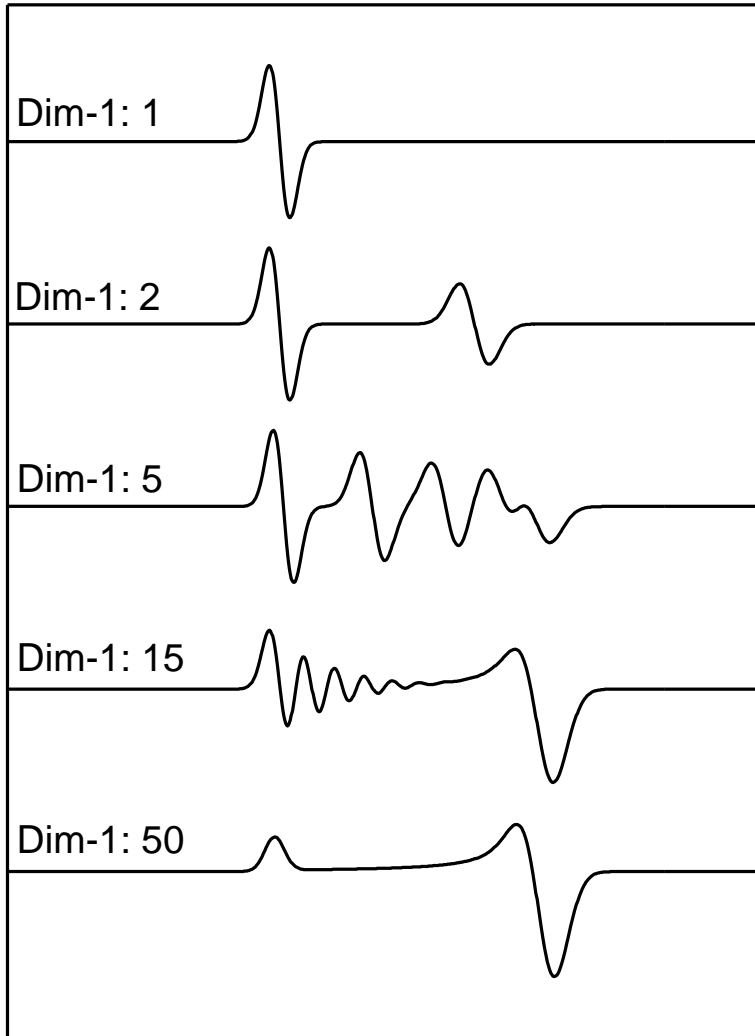
Radicals: 1-5



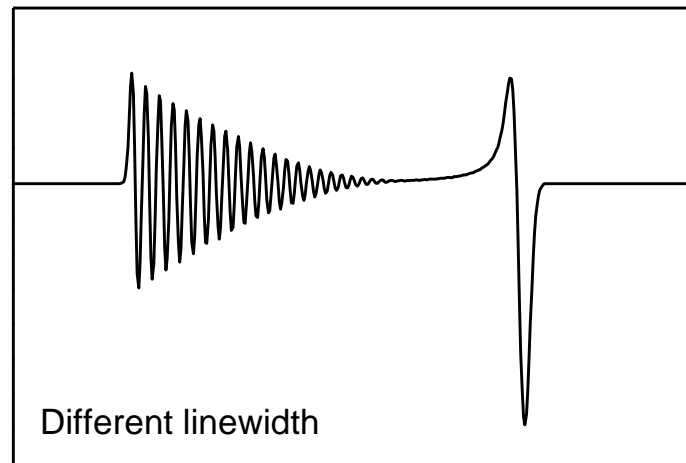
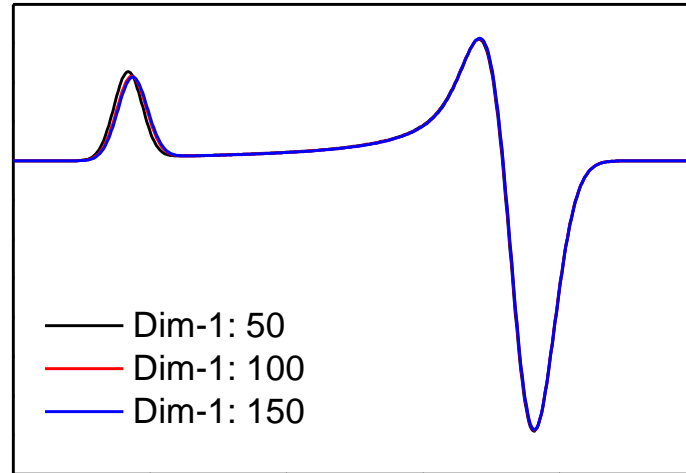
Radicals: 6-8



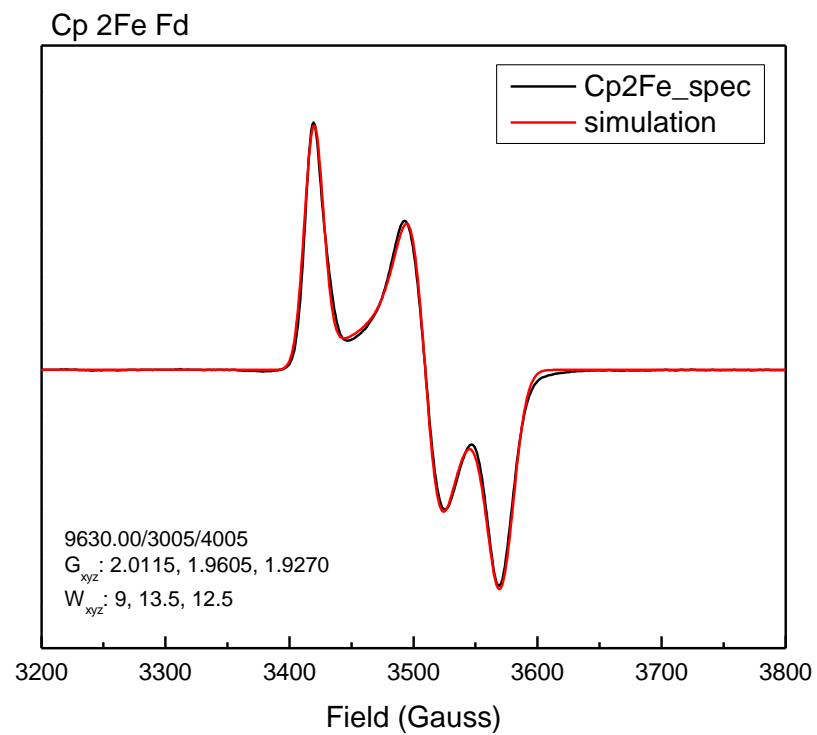
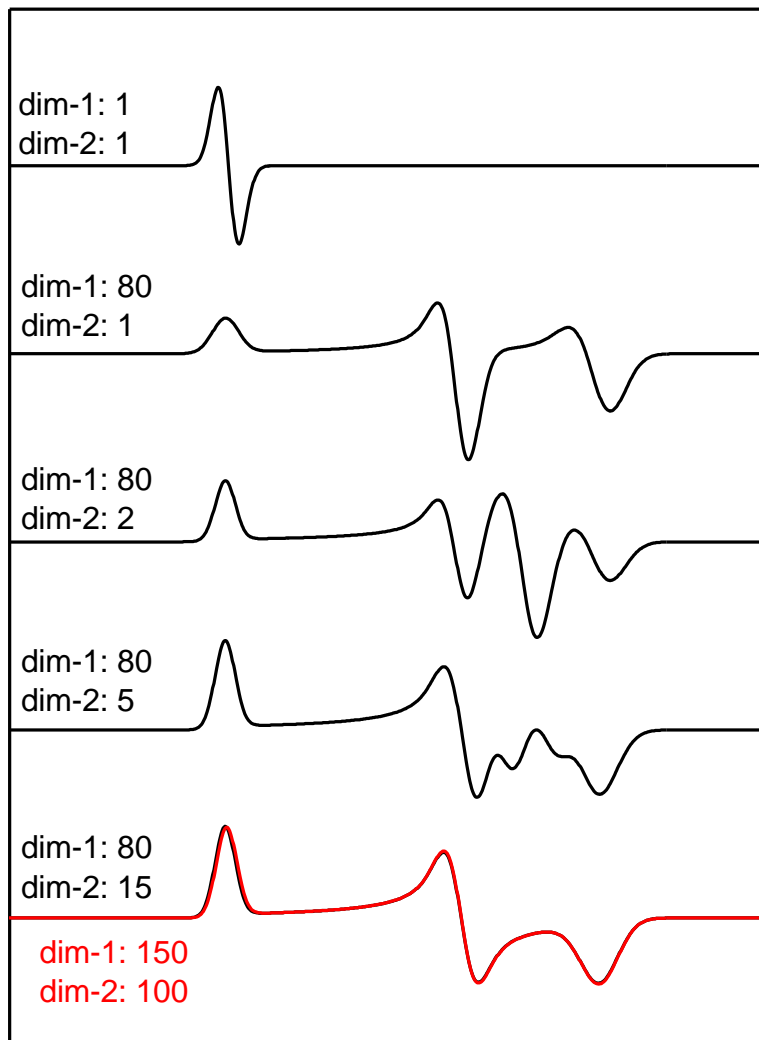
Axial spectrum: 9



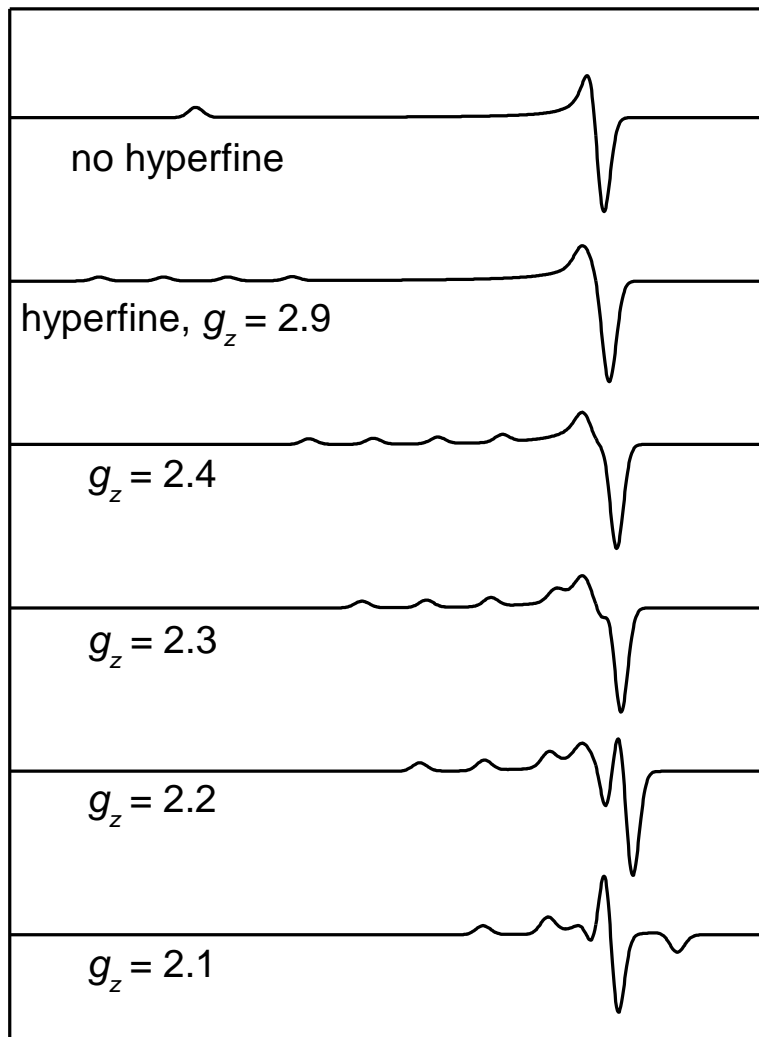
Axial spectrum: 9



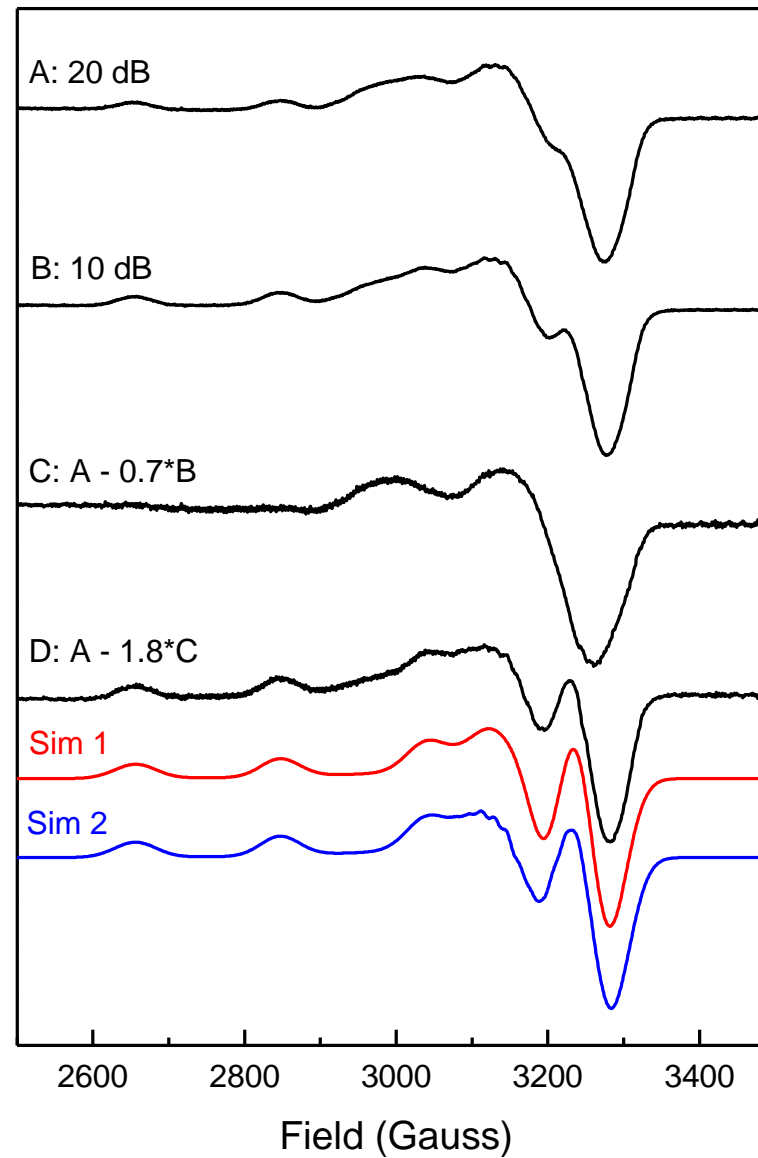
Rhombic spectrum: 10



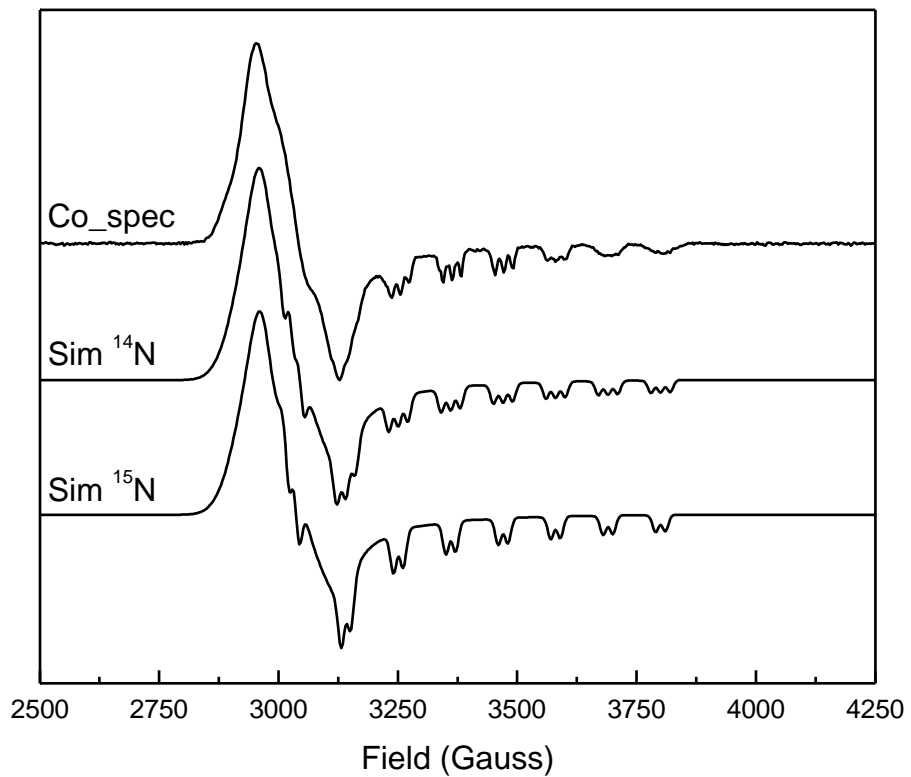
Cu: 12-14



Cu: 15



Co: 16



Ni in MCR: 17

