EWVoigt and EWVoigtn:
Inhomogeneous line shape simulation and fitting programs

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1. DISCLAIMERS AND THE USER'S AGREEMENT:

2. INTRODUCTION

The program is undergoing a constant testing and further improvement in our laboratory. If you find any errors or you have a suggestion or an idea how to improve our program please let us know - we are always happy to hear from you. The authors, Dr. Alex I. Smirnov can be reached at: Alex_Smirnov@ncsu.edu.

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This package contains two programs, EWVOIGT and EWVOIGTN. EWVOIGTN is an extended version of EWVOIGT and is suitable for least squares simulations of two overlapping spin label nitroxide spectra. Some of EWVOIGTN extended features include:
- second order perturbation approximation for calculating nitroxide spectra;
- simulation of two nitroxides with different inhomogeneous line width parameters, for example, different width of the Gaussian envelope;
- explicit accounting for ^{13}C satellite lines in the simulation;

- extended equality constraints for fitting parameters.

We shall start with description of EWVOIGT in this manual.

2.1 Introduction to Convolution-based Fitting of Continuous Wave EPR Spectra

EWVOIGT is designed for simulation/optimization of inhomogeneously broadened EPR spectra, which are very common in EPR spectroscopy (*e.g.*, anisotropy broadening, hyperfine interaction, inhomogeneities in the magnetic field and others). With the help of this program one could accurately and quickly extract spectral parameters from one class of continuous wave EPR spectra $I(B)$ - those that are uniformly broadened along the magnetic field coordinate and can be described by a convolution integral:

$$I(B) = \int p(B - B')f(B')dB' = p(B) \otimes f(B) \qquad (1)$$

where $\otimes$ is the convolution symbol, $p(B)$ is the envelope function (or an unbroadened spectrum), and $f(B)$ is the line shape of an individual spin packet (or broadening function).

The model given by Eq.1 is more common than it first appears. One type of data that is well modeled by a convolution integral is an isotropic inhomogeneously broadened EPR spectrum. Normally, electron spin packets are distributed under an envelope whose shape is determined by details of the inhomogeneous broadening mechanisms. The model describing the inhomogeneous line shape requires that the broadening must come from interactions outside the spin system and must be adiabatic during the time of the spin transition (Portis, 1953). Examples of inhomogeneous broadening include anisotropy broadening, hyperfine interaction, inhomogeneities in the magnetic field, and others. Ultimately, all EPR spectra are inhomogeneously broadened at least to some extent. However, Eq.1 works well only for the spectra that are broadened uniformly such as, for example, individual nitrogen hyperfine components of spin label EPR spectra in the fast motional limit. Then the envelope $p(B)$ can be assigned to the proton hyperfine structure. This envelope can be depicted by a stick diagram and directly used in the simulations or approximated by a Gaussian envelope (Bales, 1989). The resonance line profile of an individual spin packet is given by a Lorentzian function with the center at B_0 and the width $\Delta B_{1/2}$ at half of the line height:

$$f(B) = \frac{2A}{\pi} \frac{\Delta B_{1/2}^2}{\Delta B_{1/2}^2 + 4(B_o - B)^2} \qquad (2)$$

where A is the value of the double integral which is proportional to the number of spins and $\Delta B_{1/2}$ is the line width that is determined by the rotational relaxation and spin-spin interactions. For spin labels in fast motional limit the individual line widths, $\Delta B_{1/2}(m_I)$ should be assigned to each of the m_I nitrogen hyperfine transitions.

Slow-motion EPR spectra require more sophisticated simulation analysis, which generally involves a larger number of parameters than the fast limit spectra (Budil, Lee, Saxena, and Freed 1996). However, even these spectra may contain isotropically broadened components. For example, if some hyperfine interaction (*e.g.*, with protons of methyl groups) can be considered as "pre-averaged" by a fast (on the EPR time scale) intramolecular rotation, then those interactions will contribute to an anisotropic slow-motional EPR spectra as a uniform broadening. This kind of

broadening can be again modeled by Eq.1 in which the slow-motional spectrum is treated as an envelope *p(B)* and *f(B)* is assigned to the additional uniform broadening.

The second and, perhaps, one of the most practically important examples is uniform broadening of the EPR spin label spectra by magnetic interactions with similar or dissimilar electron spins. These include Heisenberg spin exchange (this is the dominant mechanism of broadening of spin label EPR spectra by molecular oxygen) and isotropic dynamic dipolar interaction. The latter dominate the magnetic interactions of spin-labels with some paramagnetic metal ions such as Gd^{3+} in non-viscous liquids (Hyde, Swartz, and Antholine 1979). Spin exchange between the nitroxides in solutions at concentrations corresponding to slow exchange also leads to line broadening, but the shape remains Lorentzian only to a first approximation (Molin, Salikhov, Zamaraev 1980). Another case of uniform spectral broadening is caused magnetic interactions between two nitroxide labels introduced into a protein by site-directed spin-labeling methods. Under certain conditions, *e.g.*, if the spins in such a pair are separated by *ca*. >10-12 Å and the rotational correlation time of the interspin vector are short enough, the static dipolar interaction is averaged out and the broadening function is again a Lorentzian and can be considered to be uniform across the nitroxide spectrum (*e.g.*, Mchaourab, Oh, Fang, and Hubbell 1997). In all these cases, the "envelope" spectrum can be assigned to that taken in absence of magnetic interactions. For example, in EPR oximetry experiments, the envelope is measured in absence of oxygen or at a lower oxygen concentration while in spin-label pair distance measurements the envelope is the sum of double-integral-normalized EPR spectra from single-labeled protein mutants.

In 1995 Rabenstein and Shin suggested that even when the static part of dipolar interaction is not averaged out, under certain conditions the dipolar broadening effects on the EPR spectra can be approximated by a convolution integral (although more rigorous treatment shows that, generally, these effects are not uniform across the nitroxide spectra). In their experiments, Rabenstein and Shin (1995) measured EPR spectra of single- and double-labeled mutants at low temperatures (*ca*. 77 K) to eliminate dynamic averaging of dipolar interactions. Then these authors followed the original paper by Pake (1948) and treated polarization of dipoles as static, so only shifts in the resonance frequency are produced. The EPR spectrum in presence of the dipolar interaction was approximated by a convolution of an unbroadened EPR spectrum (which we can consider as an envelope in Eq. 1) with the Pake's dipolar pattern (Pake, 1948). A somewhat similar approach to that of Rabenstein and Shin was independently developed by Steinhoff (Steinhoff, Dombrowsky, Karim, and Schneiderhahn 1991; Steinhoff, Radzwill, Thevis, Lenz, Brandenburg, Antson, Dodson, and Wollmer 1997). In their studies of frozen spin-label samples, Steinhoff and coworkers also treated the dipolar interaction as static and approximated the EPR spectrum from spin-label pairs as a convolution integral of an unbroadened spectrum and the dipolar broadening function; however, the broadening function was constructed digitally under the assumption of a Gaussian distribution for the interspin distances.

The third example is inhomogeneous broadening due to local heterogeneity of biological samples. In some cases this heterogeneity could be manifested in a strain broadening - a distribution in hyperfine coupling constants and *g*-factors due to local variations in electrostatic and hydrogen bonding environment of spin labels. Heterogeneity of biological membranes could also result in a distribution of line widths due to different mobilities of spin probes located in different membrane domains. Superposition of EPR spectra with different widths could be treated as an additional inhomogeneous broadening (Sankaraman and Marsch 1999). Characteristic parameters of such a spectrum such as, for example, an intensity ratio (see *ibid*.), can be used to deduce statistical distribution of the spin labels.

Overall, the examples above demonstrate that in many cases EPR spin-label spectra can either be fully described by a convolution integral or have a spectral contribution given by Eq.1. The purpose of this Manual is (*i*) to provide a practical guide to least-squares simulations of such spin-label spectra and (*ii*) to discuss the range of problems to which this convolution-based simulation approach can be applied. We will show that our approach is flexible enough to accommodate a variety of experiments and is very accurate (*e.g.*, Gaussian-Lorentzian convolution often being calculated with accuracy better than 10^{-6}). The main advantage of the method is that it allows one to extract the amount of the uniform broadening from the spectra directly and with fewer parameters than the simulations of the full spectra. The algorithm is combined with an efficient Levenberg-Marquart optimization algorithm, which converges rapidly and allows for estimates of parameter uncertainties. Applications of the technique are demonstrated with selected examples ranging from studies of rotational diffusion to EPR oximetry in membranes and structural protein studies.

2.2 Brief Description of EWVOIGT Capabilities

The main application of EWVOIGT/EWVOIGTN is least-squares simulation of solution spectra in regime of fast motion; however, it can be also successfully used for extracting of oxygen and other homogeneous broadening from EPR spectra of nitroxides undergoing slow and restricted motion. The program is very advantageous in studies of microviscosity using the nitroxide spin label/probe method; it drastically improves the accuracy in EPR oximetry measurements (including low-frequency EPR oximetry with a significant phase distortion of the spectra) and spin-label bio-reduction studies.

In brief, EWVOIGT and EWVOIGTN offer:
- the most accurate way of computing Voigt functions using a precision Fast Convolution algorithm;
- speed, optimization of spectral shapes is completed in a matter of a few seconds;
- optimization of simulated parameters to fit experimental data is carried out by a modified Levenberg-Marquart algorithm which usually converges in 4-7 iterations;
- estimates of parameter uncertainties;
- sequential mode of processing of similar data - automatic fit to a sequence of up to 1000 data files.

The current version of EWVOIGT is capable of:
- reading any sequential ASCII or EW (EPR data acquisition program supplied by Scientific Software Services) data-file of up to 2048 data points;
- accounting for up to 3 different types of paramagnetic centers with a common envelope function;
- accounting for 2 types of non-equivalent nuclei with 1/2 spins (such as protons) and optimization of corresponding hyperfine   structure;
- optimizing spectra in "first integral" or "first derivative" mode;
- optimizing a dispersion contribution to the experimental spectra;
- accounting for a "fixed" hyperfine pattern which can be entered as any function digitized in a sequential file;
- saving all fitting parameters, status of fitting parameters, borders of variable fitting intervals, and other program parameters under a separate file available for easy retrieval by user;
- saving experimental spectrum, best-fit spectrum, and residual in a comma-separated table format for easy importing into other graphical programs;
- saving fitting parameters after sequential run as a separate file in a comma-separated table format for easy importing into other graphical programs.

2.3 Installing and Running EWVOIGT/EWVOIGTN Programs on a PC Computer

While initially EWVoigt and EWVoigtn were developed for a MS DOS environment, one could run these programs under MS Windows. In order to install EWVOIGT and/or EWVOIGTN go to the local disk C: and create a new folder (directory) named EWVOIGT. Copy all the files into EWVOIGT folder and create Windows shortcuts for EWVOIGT.EXE and EWVOIGNT.EXE by right clicking on these files and choosing "Create Shortcut". Copy shortcut(s) into Windows desktop:

Figure 1. Windows shortcut for the EWVOIGT program.

If your applications require using versions of EWVOIGT and/or EWVOIGTN compiled for maximum 2048 data points in your spectra, you may encounter an error message while running these programs under Windows. Typically, an error message is generated when insufficient conventional memory is allocated by Windows to run these DOS-designed programs. Depending on configuration of your computer, these error messages can be also generated when running EWVOIGT and/or EWVOIGTN compiled for 1024 data points (these versions require less allocation of conventional memory).

One could ask a very common question: "My PC has 512 Mb of memory. Why this is insufficient for running EWVOIGT". The answer is that the DOS platform of EWVOIGT and/or EWVOIGTN loads both the program and the data into conventional memory that cannot exceed *ca*. 640 kb for 32-bit CPU. The amount of conventional memory is fixed regardless how much of additional memory is installed in a PC.

If EWVoigt/EWVoigtn would not run under Windows after the simple installation as described above, the following installation procedure is recommended. Scientific Software Services recommends Windows 2000 Professional or Windows XP Professional as operation systems. The following instructions are given for Windows 2000. Instructions for Windows XP are essentially the same and the only difference is slight changes in appearances of Shortcut menus. Please note while EWVOIGT/EWVOIGTN could be installed the same way for other earlier versions of Windows, no extensive testing has been carried out. Installation on PC equipped with Celeron processors should proceed with caution.

1. Install EWVOIGT/EWVOIGTN as described by creating a Windows shortcut.
2. Start the program by double-clicking on a shortcut. If a memory-related error messages are generated after starting the program, modify the shortcut as below.
3. Start with locating C:\WINNT\SYSTEM32 in your computer. This folder contains AUTOEXEC.NT and CONFIG.NT files which are used to specify environment for MS DOS programs.

4. While keeping original AUTOEXEC.NT and CONFIG.NT files in the C:\WINNT\SYSTEM32 folder, make copies of these files under different names, such as AUTOEXEC-EW.NT and CONFIG-EW.NT, and place them into the same folder.

5. Edit AUTOEXEC-EW.NT (*e.g.*, with a Notepad) by adding REM at the beginning of each line except the first one containing *@echo off* and saving the file after that. An example of such an edited file is given here:

> *@echo off*
> *REM AUTOEXEC.BAT is not used to initialize the MS-DOS environment.*
> *REM AUTOEXEC.NT is used to initialize the MS-DOS environment unless a*
> *REM different startup file is specified in an application's PIF.*
> *REM Install CD ROM extensions*
> *REM lh %SystemRoot%\system32\mscdexnt.exe*
> *REM Install network redirector (load before dosx.exe)*
> *REM lh %SystemRoot%\system32\redir*
> *REM Install DPMI support*
> *REM lh %SystemRoot%\system32\dosx*

6. Typically, your CONFIG-EW.NT file does not have to be modified. In many cases, it contains many comment lines (those start with REM and are not executed) and also specifies memory/file configuration as follows (this is found at the very end of the file):

> *dos=high, umb*
> *device=%SystemRoot%\system32\himem.sys*
> *files=40*

If other lines are present, modify them by typing REM at the start of each such line. The reason for these modifications is to minimize the number of other programs and drivers loaded into conventional memory under DOS prompt when EWVOIGT and/or EWVOIGTN are running. Note, these new AUTOEXEC-EW.NT and CONFIG-EW.NT files will affect only the DOS environment of EWVOIGT and/or EWVOIGTN program but not the other programs installed in your computer.

7. Now, the EWVOIGT and EWVOIGTN shortcuts have to be modified to instruct the Windows to utilize specific environment for these programsIn order to do this, right-click on the shortcut and open "Properties":

Figure 2. Opening shortcut dialog for EWVOIGT.
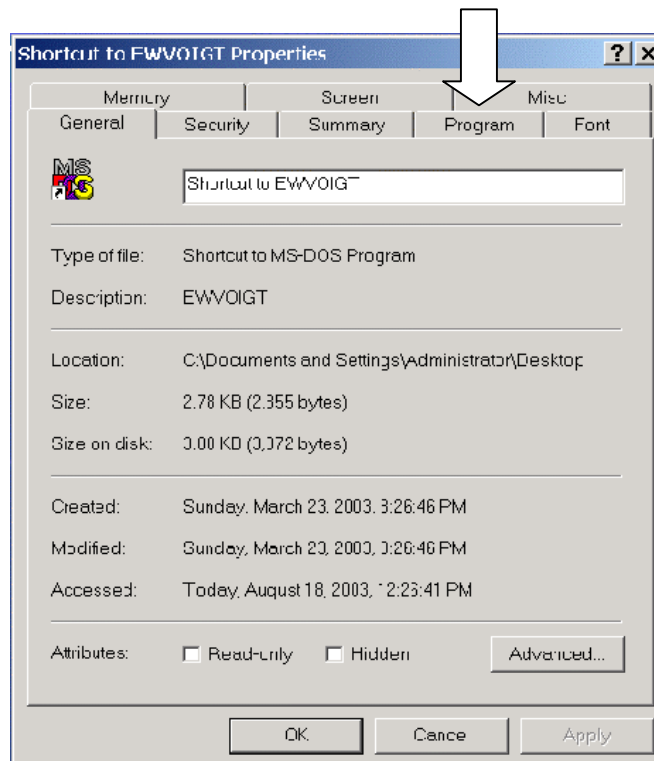
8. In "Properties" window click on the "Program":

Figure 3. Opening Program dialog menu. The "Program" button is pointed to by an arrow.

9. In the "Program" window, shown below, click on "Advanced...":
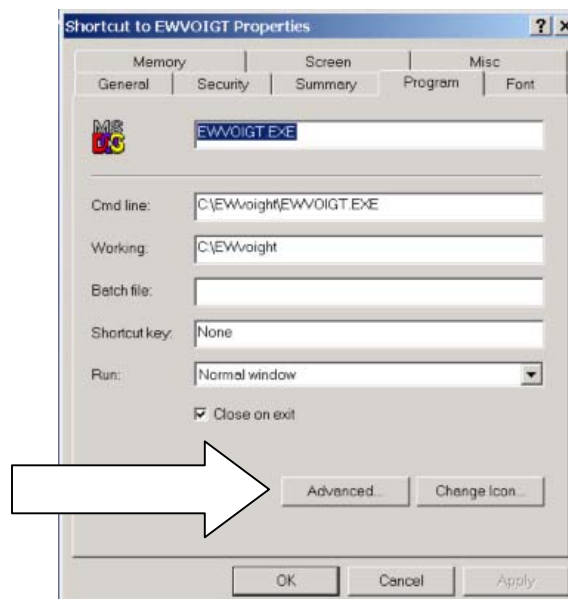

Figure 4. Opening PIF/advance setting dialog menu. "Advanced..." button is pointed to by an arrow.

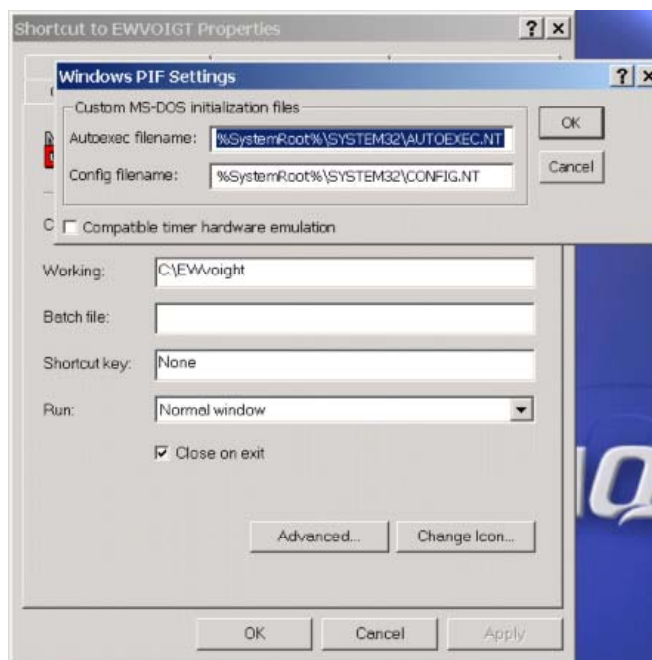10. A Windows PIF Settings dialogue will open:

Figure 5. Editing the names of MS-DOS initiation files using PIF/advance setting dialog menu.

11. Using this dialog menu change the Autoexec and Config filenames to AUTOEXEC-EW.NT and CONFIG-EW.NT respectively.
12. Close the all the windows by clicking OK buttons. You are now ready to run EWVOIGTN and/or EWVOIGTN on your Windows PC.

    It is worthwhile to emphasize here once again that EWVOIGT and EWVOIGTN are DOS programs. Therefore you should follow the DOS convention for file names and the folder (directory) structure. Specifically, all the file and the folder names should not be longer than 8 characters and the file name extension should be no longer than three characters. DOS does not differentiate between low and capital characters. Colon, semicolon, period, comma, backslashes, and "wildcard" characters should not be used in file/folder names.
    Running DOS programs under Windows also has some peculiarities. One could suspend the DOS program without quitting it by pressing a "Windows" button. While this is a useful feature that could be used for searching for the location of experimental data files and plotting intermediate results, it should not be noted that EWVOIGT and EWVOIGTN would not run in a background when these other tasks are performed. Both programs utilize the entire CPU resources and therefore will not run when other programs are executed. Thus, once suspended, these programs will immediately stop optimization/simulation till the user maximize these programs.

2.4 Customizing Your Version Of EWVOIGT and EWVOIGTN

    Every time you run EWVOIGT and/or EWVOIGTN, you can customize parameters and settings that are loaded into your program upon startup. These include line width simulation

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parameters, settings for least-squares optimization, path for reading/writing data, *i.e.,* virtually all the settings except the experimental data set. When a user quits the program in a normal way all these defaults will be saved in a special separate file in the folder where your directory from which you have started the program. For EWVOIGT the name of this file is EWVOIGT1.SET while for EWVOIGTN the name is EWVOIGTN.SET. You can also save the defaults under user-specified file for reading any time later (see section X.X of this manual). When either of the programs starts up from a directory in which a "SET" file is missing, it automatically creates such a file and sets all the parameters to the default build-in values.

3. USING EWVOIGT AND EWVOIGTN – A QUICK START

Here we describe the simple uses of EWVOIGT* programs by providing step-by-step instructions and illustrations. We suggest starting with EWVOIGT first because it is a simpler program. Once the user becomes familiar with EWVOIGT the use of EWVOIGTN will be straightforward.

3.1 Convolution Algorithm with Levenberg-Marquardt Optimization for Fitting Inhomogeneous EPR Spectra

Briefly, the general function for fitting inhomogeneously broadened EPR lines is an extension of a convolution integral given by Eq.1 with addition of a polynomial baseline term of the order N (typically, $N=1$ or $=2$ is sufficient):

$$I(B) = f(B) \otimes p_1(B) \otimes p_2(B) \otimes \ldots \otimes p_k(B) + \sum_{i=0}^{N} l_i B^i \qquad (3)$$

EWVOIGT employs a polynomial with $N=1$ while $N=2$ in EWVOIGTN. The Lorentzian line shape is given by $f(B)$, and the $p_j(B)$'s are optional envelope functions. Typically, one of the envelope functions is a Gaussian, which is a very common model of inhomogeneous broadening. Other envelopes are assigned to isotropic hyperfine interactions with magnetic nuclei and also may include a non-adjustable envelope function, which is read as a file. The latter could be, for example, an experimental spin-label EPR spectrum in absence of oxygen and/or some other broadening function due to isotropic dipolar interactions.

Continuous-wave EPR spectra are usually detected in the form of a first derivative, $I'(B)=\partial I(B)/\partial B$. Since

$$I'(B) = p'(B) \otimes f(B) = p(B) \otimes f'(B) \qquad (4)$$

only one of the two functions in the right-hand part of Eq.4 is differentiated in order to describe the experimental first-derivative spectrum. This property of the convolution integral is also very useful for efficient calculations of partial derivatives required for the Levenberg-Marquardt optimization.

The model of inhomogeneous line shape we describe here is general and allows for different line shapes $f(B)$. One practically important example is a dispersion shape or a mixture of dispersion and absorption. Dispersion and absorption signals are related by Kramers-Kronig equations. If the contribution from dispersion is measured as a phase shift $\Delta\varphi$ ($\Delta\varphi=0$ corresponds to absorption line), then the first-derivative Lorentzian EPR line is given by:

$$f(B) = \frac{2A}{\pi} \frac{8\Delta B_{1/2}^L(B_0 - B)\cos\Delta\varphi + \left[4(B_0 - B)^2 - (B_{1/2}^L)^2\right]\sin\Delta\varphi}{\left[(\Delta B_{1/2}^L)^2 + 4(B_0 - B)^2\right]^2} \tag{5}$$

where $\Delta B_{1/2}^L$ is the Lorentzian width measured at half height of an absorption signal, A is the area under the resonance absorption curve or (double-integral) intensity of the signal, B_0 is the resonance field. Then the inhomogeneous EPR spectrum for an arbitrary $\Delta\varphi$ is given by a convolution of $f(B, B_0, \Delta B_{1/2}^L, \Delta\varphi)$ with an inhomogeneous envelope. Thus, only one function in the convolution integral has to be corrected for the dispersive component. Although the dispersion line shape can be computed for both Gaussian and Lorentzian functions, expression for the dispersive Lorentzian shape is much simpler while calculations of the dispersive Gaussian shape require evaluations of the error function integral.

Accounting for the dispersive contribution in least-squares fitting of spin-label EPR spectra is especially important because those spectra are often collected from aqueous samples, which have high dielectric constants. For these samples the EPR signal always has some dispersive component because the microwave phase varies along the sample. However, even in this case the observed EPR signal $I(B)$ appears as a mixture of absorption, $A(B)$, and dispersion, $D(B)$, signals with an effective phase shift $\Delta\varphi_e$. Indeed, if $\Delta\varphi(\vec{r})$ is the microwave phase expressed as a function of a spatial vector $\vec{r}$, then:

$$
\begin{aligned}
I(B) &= \int_V \left[A(B)\cos(\Delta\varphi(\vec{r})) + D(B)\sin(\Delta\varphi(\vec{r}))\right]d\vec{r} = \\
&= A(B)\langle\cos(\Delta\varphi(\vec{r}))\rangle + D(B)\langle\sin(\Delta\varphi(\vec{r}))\rangle = \\
&= R\left[A(B)\cos(\Delta\varphi_e) + D(B)\sin(\Delta\varphi_e)\right]
\end{aligned}
\tag{6}
$$

where

$$R^2 = \langle\cos(\Delta\varphi(\vec{r}))\rangle^2 + \langle\sin(\Delta\varphi(\vec{r}))\rangle^2 \tag{7}$$

$$\Delta\varphi_e = \tan^{-1}\frac{\langle\sin(\Delta\varphi(\vec{r}))\rangle}{\langle\cos(\Delta\varphi(\vec{r}))\rangle}$$

Thus, the shift observed in such an experiment cannot be always experimentally compensated by adjusting the spectrometer reference arm. The phase shift problem becomes much more severe in high frequency (95-250 GHz) EPR spectrometers and also can be a problem in certain low-frequency (0.5-2.0 GHz) EPR experiments when a single channel is used for the signal detection. These phase shifts can significantly distort line shapes and lead to data misinterpretation if they are not accounted for. This can be overcome by using Eq.5 in the simulations or by correcting experimental spectra by mixing in an out-of-phase component calculated using Kramers-Kronig equations.

For simulations, initial values of the parameters in fitting function given by Eq.3 are determined by a search for crude local extrema in the experimental spectrum $E(B)$. The initial microwave phase $\Delta\varphi$ is usually set to 0 or π. Both values correspond to pure absorption spectra at different modulation phases. Baseline coefficients are set to zero. A multiparameter line shape function, $f(B, \alpha_i)$, is derived analytically and then, if desired, is digitally convoluted with optional envelope functions $p_i(B)$'s using the FFT convolution algorithm. Envelope functions are derived analytically or read as a file. Some of the envelopes, such as Gaussian and modulation and/or time

constant broadening should be centered over the spectral window and are easily simulated in the frequency domain. The use of FFT requires that the simulated spectrum $I(B_i)$ has n data points, where n is a power of 2. If the experimental spectrum was collected with a different number of data points, EWVOIGT program automatically generates additional data points via linear extrapolation to make the appropriate length of the data file.

The simulated function $I(B_i, \vec{a})$ is compared with the experimentally measured spectrum $E(B_i)$ by a square norm χ^2 test:

$$\chi^2(\vec{a}) = \sum_{i=1}^{N} \left[E(B_i) - I(B_i, \vec{a}) \right]^2 \qquad (8)$$

where $\vec{a}$ is a parameter vector and the standard deviations at each experimental data points are assumed to be the same. The parameter vector $\vec{a}$ is then adjusted to minimize the value of $\chi^2(\vec{a})$.

For the minimization, we have utilized the Levenberg-Marquardt algorithm (*e.g.*, Press, Teukolsky, Vetterling, and Flannery 1986; Ball, Kuester, and Mize (eds.) 1973). Many modifications of this algorithm require a user-supplied subroutine which, for the input value B_i, returns the value of the model function $I_i = I(B_i, a_j)$ and the vector of the derivatives $\partial I(B_i, a_j)/\partial a_j$ (*e.g.*, see Press et al. 1986). In order to take the advantage of the FFT convolution algorithm, we modified this procedure as follows. Instead of calculating one value of I_i and the vector $\partial I(B_i, a_j)/\partial a_j$ at each value of B_i at a time, we calculate these functions for the whole set of B_i; $i=1,...,n$. Then the calculation procedure is organized as follows:

(1) The functions $p_k(B_i)$, $f(B_i, a_j)$, and $\partial f(B_i, a_j)/\partial a_j$ are generated from analytically derived expressions for all values of B_i; $i=1,...,n$. Alternatively, a non-adjustable envelope function is read as a file.

(2) The convolution $p(B)$ of functions $p_1(B)$, ... $p_k(B)$ ($p(B)=p_1(B) \otimes p_2(B) \otimes ... \otimes p_k(B)$) is computed digitally by the FFT convolution algorithm and all intermediate Fourier images of envelopes and their products are stored in the computer memory.

(3) All functions generated in step (1) are convoluted with $p(B)$; $f(\omega, a)$ is stored. Since $p(\omega)$ was previously stored during step (2), each convolution requires only two Fourier transforms instead of three. Convolution of $p(B)$ with $\partial f(B_i, a_j)/\partial a$ gives a matrix of partial derivatives $\partial I(B_i, a_j)/\partial a_j$ for all values of B_i, since:

$$\frac{\partial I(B)}{\partial a_j} = p_1(B) \otimes p_2(B) \otimes \ldots \otimes p_k(B) \otimes \frac{\partial f(B, a_j)}{\partial a_j} \qquad (9)$$

The vector of the simulated function $I(B_i)$ is computed by adding a baseline term (Eq.3).

(4) If envelopes contain adjustable parameters σ_j, the vectors $\partial p_k(B_i, \sigma_j)/\partial \sigma_j$ are computed from analytical expressions and then are convoluted digitally with other envelopes $p_m(B)$ and $f(B, \vec{a})$. The number of required Fourier transforms can be decreased by using vectors $p_m(\omega_i)$ and $f(\omega_i, \vec{a})$ stored during steps (2) and (3).

(5) Partial derivatives with respect to the baseline parameters and with respect to the amplitude parameter are computed without additional convolutions. A complete matrix of partial derivatives $\partial I(B_i, a_j)/\partial a_j$ is formed and then supplied for Levenberg-Marquardt optimization. The iterations are stopped after the value of $\chi^2(\vec{a})$ is improved insignificantly (*i.e.*, by 10^{-3} of the previous value) for a second time. The estimates of parameter uncertainties are calculated in a standard manner (Press et al. 1986).

The advantages of the described algorithm are such that it does not require digital differentiation of simulated functions with respect to the simulation parameters. Most functions required for calculations are derived in analytical forms. The final step involves digital convolution of the generated functions by FFT. Saving the intermediate results can be used to reduce the number of Fourier transforms. For convolutions with Gaussian and/or modulation and time constant broadening functions, simulating these functions directly in the frequency domain from the analytical expressions, and then digitally transforming the product back to the field domain can further decrease the computational time. In EWVOIGTN the fitting function (3) is extended by summation over non-equivalent line shape functions $f_j(B,a_i)$ and/or non-equivalent paramagnetic centers.

The basis of the computation algorithms has been initially described by Smirnov and Belford (1995). A comprehensive review of the use of convolution-based fitting in EPR has been also published (Smirnov and Smirnova, 2004).

3.2 Basic Options: a Short Tour Of EWVOIGT

Figure 6. Menu structure of EWVOIGT.

EWVOIGT is navigated by a series of MENUS. These MENUS consist of a series of options prefaced by letters and a cursor shaped like a box. You can move the cursor box using the arrow keys. Pressing the ENTER key with the cursor box over that option will select that option. A shorter way to start up a menu (or an option) is to press an appropriate letter. For example, pressing "A" will start FILE MENU no matter where the cursor currently is. The same menu system is used throughout both EWVOIGT and EWVOIGTN. The basic outline of menu structure of EWVOIGT is shown in Fig. 6.

3.2.1 Starting the Program

If you start EWVOIGT for the first time from a folder that does not contain EWVOIGT1.SET file you will get an error message as shown below:

```
No designated files on this disk   Press Enter to continue
```

Figure 7. Start up message.

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Press enter to proceed. EWVOIGT will automatically generate the default file for you and save it in the folder from the program was started.

You will now be presented with the greeting screen containing the version number and release data. Press any key to continue.

3.2.2 Main Menu

You will now be presented with the MAIN MENU (Fig. 8).

There are twelve options in this menu. The purpose of the main menu is to allow you to read and write files and to access the simulation/fitting menu. The top line of the main menu shows the current path for reading and writing files (C:\EWVOIGT).

```
MAIN MENU - ESC to quit.     READ/WRITE PATH=C:\EWVOIGT

D Read File                E Length= 1024      F <Experiment>
G Simulation/Fitting       H Lines to skip= 2  I Extension=<FLS>
J Write as EW              K Write for PLOT41   L Change drive/path
M Read defaults            N Save defaults      O Show directory
```

Figure 8. EWVOIGT Main menu

All menus in EWVOIGT are navigated using keys on keyboard and mouse or other pointing devices are not supported. The current menu selection is shown as a box. For example, the current selection of the Main Menu is "D Read File". The box could be moved around using arrow keys. Pressing the "Enter" key would activate any current menu selection highlighted by the box. For example, pressing "Enter" when the box is "highlighting" the "D Read File" option will activate reading the file. There is also a shortcut: by pressing a capital letter key shown in front of the option one would activate that option immediately (and also move the cursor box to the new selection).

The program is able to read any sequential data files with any DOS extension. In brief, according to DOS the file name should be no longer than 8 characters and the extension should be no longer than 3 characters. Note, that DOS does not distinguish between low case and capital characters. In addition, no spaces and other characters such as punctuation characters as period, comma, semicolon, colon, paragraph, backslash, wild character "*" *etc.* are not allowed.

The current file extension is shown in the menu selection "I Extension=<FLS>." Extension "FLS" is reserved for experimental data files generated by EW program supplied by Scientific Software Services. You can change this extension by choosing the option :I" and entering any three letter sequence. If you have entered any extension except the DOS wildcard character *, only the files with this extension will be displayed (option "O") or can be read (option "D"). If the DOS wildcard character * is entered, then all the files will be displayed (option "O") or can be read (option "D").

Option "O" will display files beneath the menu. The path from which files are read or to which files are written can be change with the option "L". Upon activating this option, the cursor appears on the top line of the screen and the path could be typed in (Fig.9):

```
MAIN MENU - ESC to quit.     READ/WRITE PATH=C:\?  C:\EPR\DATA █


D Read File                  E Length= 1024      F <Experiment>
```
Figure 9. Changing directory (folder) in EWVOIGT.

If the drive is not ready or the path does not exist, an error informational message will appear on the second line of the screen and EWVOIGT will attempt to return to the previous path (Fig. 10).. If that path is also not ready, EWVOIGT will return to the path from which it was started.

```
MAIN MENU - ESC to quit.     READ/WRITE PATH=C:\?  C:\EPR\DATA
Drive not ready              Hit enter to continue
D Read File                  E Length= 1024      F <Experiment>
```
Figure 10. Error message generated by EWVOIGT for non-existing or not available device or folder.

       EWVOIGT automatically reads the EPR data files saved in the "FLS" format of EW data acquisition program supplied by Scientific Software Services. In the example illustrated in Fig. 10 the file EWVOIGT1.FLS is being read. Note, that once extension is set there is no need to type in the extension: only the file name is needed. For "FLS" files the program automatically determines the number of experimental data points (independent of the value of "Length" in the option "E"). If the number of data points is not equal to an integer power of 2, EWVOIGT automatically adds data points at the high field end of the spectrum via linear extrapolation. For example, if the original file contains 1000 data points, EWVOIGT will automatically add 24 data points starting with the high field end of the spectrum so the resulting file would contain 1024 data points. After such an interpolation the option "E" of the MAIN MENU will indicate an updated length of the experimental data file.

```
Enter the name of the file or RETURM to exit.
 ?   EWVOIGT1 █
```
Figure 11. Reading experimental file EWVOIGT1.FLS with "I Extension" set to "FLS".

If the file is not in the "FLS" format (even if it has the extension FLS), EWVOIGT will read only the number of data points set by the option "E Length" after skipping the number of lines shown in the option "H Lines to skip". This allows for reading of any file by skipping unnecessary information in the file header and also for reading only a portion of the file (by skipping lines selected in the option "H Lines to skip").

       If the file is in the "FLS" format, the user still will be able to read only a portion of that file by setting the file extension to the DOS wildcard character *. For the latter extension selection, both the file name and the extension should be entered. For files in the "FLS" format, one should skip the first 5 lines that contain a record of spectrometer parameters and choose the desired number of data points.

       After reading the file the spectrum is displayed on the screen in dark blue. The spectrum is scaled automatically so that the data occupies half the height of the data window. In order to proceed with least-squares simulations of this spectrum the user should proceed to the SIMULATION/FITTING MENU that is activated by the "G Simulation/Fitting" option.

EWVOIGT also offers an option is to read an experimental spectrum or any other file as an envelope function $p_1(B)$ in Eq.1. This is achieved by toggling the option F that displays current selection. Detailed use of the envelope function in simulations of EPR spectra is described below.

3.2.3 Fitting/Simulation Menu

```
FITTING/SIMULATION MENU - ESC to MAIN MENU
A Start Optimization      B Iterations = 3      C Re-enter Parameters
D Save Parameters         E Change Interval     F Simulate
G Number of Centers= 1    H Parameter Status    I Equality Constraints
J Mode=1-st Der.          K Fit Sequence        L Exp. File=ewvoigt1
M Number of Prot. A= 0    N Number of Prot. B=0 O Invert Interval=off
```

Figure 12. Fitting/Simulation menu.

This is the main menu for controlling the simulation and fitting of spectra. In current version of EWVOIGT the number of centers can be varied between 1 and 3. Inhomogeneous line width of all centers is assumed to be the same. The latter includes proton hyperfine, Gaussian inhomogeneous line width, and the envelope function (if entered). Note, that once the envelope function was read into a program, it cannot be unloaded. To unload the envelope function, simply exit and restart the program. Each of the centers assigned individual Lorentzian line width, magnetic field positions, and double integrated intensity. Note, that double integrated intensities and field positions of different centers could be set the same using the "I Equality Constraints" option (see below). All other parameters are always equivalent in the current version. The spectra could be simulated either in first derivative or first integral mode: the option "J Mode" toggles between the first derivative and first integral modes.

The following menu is activated by the "C Re-enter Parameters" option of the main menu:

```
INPUT PARAMETERS FOR SIMULATION; Esc - to go to SIMULATION/FIT menu
Center number=1
D Phase= 0                E Field from= 1       F Field to= 1024
G Gaussian= 14.14214      H Position=250        I Lorentzian=17.32051
J Linear=0                K Offset=0            L Intensity= 1
M Mode=<Data points.      N Hyperfine A=0       O Hyperfine B=0
```

Figure 13. Reentering simulation parameters

All parameters can be entered by activating the corresponding option with exception of the magnetic field values. Note, the EWVOIGT keeps track of the experimental magnetic field values for files in EW format. If the experimental data file was extrapolated by the program to make, for example, a 1024 data point file instead of the original 1000, the field values will be adjusted automatically.

The simulation parameters can be entered in units of data points or magnetic field: the option "M mode" toggles between these two options. The center number is displayed in the upper left corner. The simulation parameters are:

1) Option "D Phase" – a phase shift in radians corresponding to an admixture of a dispersive componentto an absorption signal. For example, 0 corresponds to the pure absorption, $\pi/2 \approx 1.5707963$ is the pure dispersion, and $\pi \approx 3.1415926$ is the negative absorption signal.

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2) Option "G Gaussian" – a peak-to-peak line width of the Gaussian component that is common to all centers. When Gaussian width is set to zero it will not be used in the calculations and it cannot be adjusted during least-squares optimization. Note, that slightly overmodulated EPR spectra can be modeled by adding a Gaussian component.

3) Option "H Position" – a position of the center of the line in the spectrum. This value is specific for each center.

4) Option "I Lorentzian" – a peak-to-peak line width of the Lorentzian component. This parameter is also specific for each center.

5) Option "J Linear" – a baseline slope. It iw recommended to use zero for the initial parameter for the simulations.

6) Option "K Offset" – a vertical offset of the baseline. Some of the files converted from Bruker Biospin programs contain a large numerical value of the offset signal. It is recommended to find out that value first and enter an approximated value as a starting parameter.

7) Option "L Intensity" – a double integrated intensity corresponding to the Lorentzian contribution to the line shape. This parameter is specific for each center.

Note: when an inhomogeneous broadening is present the values of the "Intensity" provided by the program should be appropriately corrected. For example, if a Gaussian inhomogeneous broadening is present, then the intensity values should be renormalized by dividing by the value of Gaussian line width.   This operation is unnecessary when all spectra are simulated with the same Gaussian line width.

8) Option "M Mode" toggles between data points and magnetic field units for all relevant parameters. The "magnetic Field" mode is activated only for the experimental files recorded in EW format.

9) Option "N Hyperfine" – an isotropic hyperfine constant for the group of protons (nuclear spin ½) A (common to all centers).

10) Option "O Hyperfine" – an isotropic hyperfine constant for the group of protons (nuclear spin ½) A (common to all centers).

Note that options "N" and "O" are inaccessible unless the number of protons is set using the options "M" and "N" of the preceding menu (FITTING/SIMULATION MENU).

For trial parameters please enter the following values into the parameter menu:

```
 INPUT PARAMETERS FOR SIMULATION; Esc – to go to SIMULATION/FIT menu
 Center number=1
 D Phase= 0                    E Field from= 1        F Field to= 1024
 G Gaussian= 0                 H Position=530         I Lorentzian=90
 J Linear=0                    K Offset=0             L Intensity= 9
 M Mode=<Data points>          N Hyperfine A=0        O Hyperfine B=0
```

Figure 14. An example of parameters for the initial simulation.

Now, in order to return to the FITTING/SIMULATION MENU, please press Esc key and then activate option "F Simulate". The results are shown below in Fig.15. The original data will be shown in light blue, the simulation will be shown in green and the difference between the experimental and the simulated spectra (*i.e.*, the residual) shown in red with an offset toward the bottom of the graphics window.

```
FITTING/SIMULATION MENU - ESC to MAIN MENU
 A Start Optimization     B Iterations= 3        C Re-enter parameters
 D Save parameters        E Change Interval       F Simulate
 G Number of centers= 1   H Parameter Status      I Equality Constraints
 J Mode=1-st Der.         K Fit sequence          L Exp. file=ewvoigt1
 M Number of prot. A= 0   N Number of prot. B= 0  O Invert interval=off
```
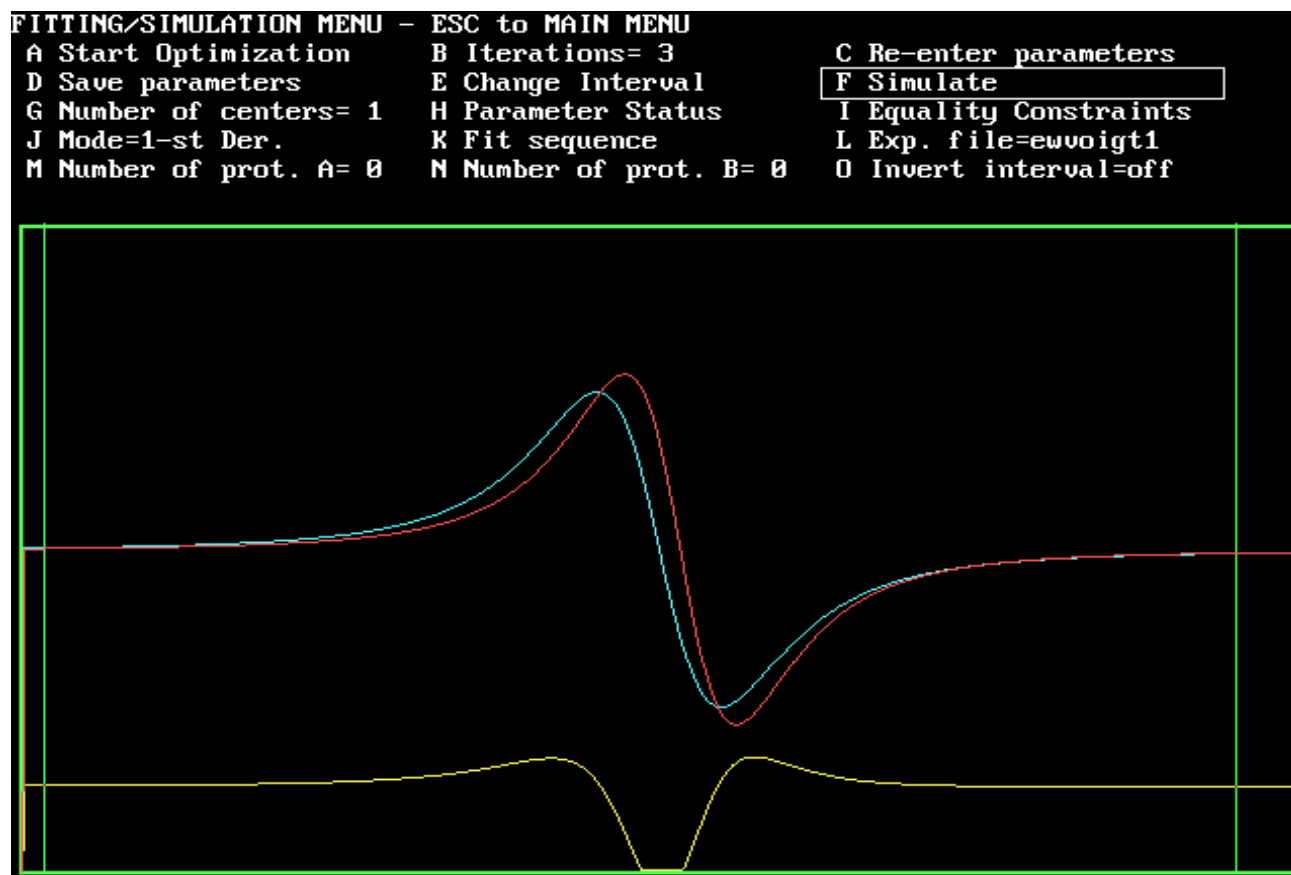
Figure 15. Results of an initial simulation of experimental file EWVOIGT1.FLS with parameters as given in Fig. 14.

Sometimes it is useful to limit the interval over which the spectrum is fitted to the theoretical model. This can be achieved by using the option "E Change Intervals" by entering the left and right borders in data points. The current values of the borders are shown in the dialog menu and new values can be entered. When simulating extrapolated data files, for example, when a 1000 data point file was extrapolated to 1024 data points, it is desirable not to include extrapolated data points into the fitting interval.

EWVOIGT also offers an additional flexibility of setting fitting intervals: one could exclude a range of data points from the spectrum by toggling the option "O Invert Interval" to the "on" state. In other words, when the invert interval option is "off" all the data points of an EPR spectrum that are contained between the two vertical green lines on the screen are used in least-squares optimization. When the invert interval option is toggled "on", the data points between the vertical green lines are excluded from optimization and all the data points laying outside this interval are used instead. This feature is useful when there is extraneous data or when the goal is to fit a broad baseline and to extract a narrow signal from the baseline signal (in this case, the baseline is simulated and the narrow signal appears as the residual).

The status of parameters for the optimization procedure could be accessed by choosing the option "H Parameter Status". This option would activate STATUS OF OPTIMIZATION PARAMETERS menu that is shown in Fig. 16. This menu shows the status of the parameters that are employed in the fitting procedure. The status of parameters can be toggled between <Fixed> and <Adjust.> by choosing the corresponding option. Field values cannot be adjusted. Note that in

course of least-squares simulations, only the adjustable parameters are varied to obtain the best possible fit while <Fixed> fixed parameters are kept at initial values set in the INPUT PARAMETERS menu. The parameter status for fitting an experimental spectrum to a Lorentzian function with no phase shift and at a fixed filed position is shown below.

```
STATUS OF OPTIMIZATION PARAMETERS; Esc - to go to SIMULATION/FIT menu
Center number=1
D Phase= <Fixed>          E Field from=<Fixed>  F Field to=<Fixed>
G Gaussian=<Fixed>        H Position=<Fixed>    I Lorentzian=<Adjust.>
J Linear=<Adjust.>        K Offset=<Adjust.>    L Intensity=<Adjust.>
M Hyperfine A=<Fixed>     N Hyperfine B=<Fixed>
```

Figure 16. Status of optimization parameters menu.

Before starting optimizing simulation parameters chosen in the INPUT PARAMETERS menu, one should set the maximum number of iterations using the option "B Iterations" (Fig. 12). This option sets the maximum number of successful iterations EWVOIGT would attempt to find the best fit. Typical values are 3 to 6 depending on how close the initial iterations are to the best fit of the actual data. Experimental data with a low signal-to-noise ratio or ill-defined problems would require more iterations. The program will always attempt the maximum number of iterations set by the option "B Iterations"; however, the optimization procedure would stop automatically if the chi square of the residual spectrum (i.e., the difference spectrum between the experimental and the simulated one) is not improving by more than 0.01% for the second consecutive iteration. The least-squares fitting cycle can be also interrupted by pressing CTRL-X simultaneously; however, the program will abort iterations not immediately but after completing the current iteration.

Although the least-squares optimization process does not occur instantaneously, typically, the best fit is found within a few seconds. During the fitting the program may attempt several unsuccessful iterations before it finds the best approach. These attempts are displayed as white lines on top of the light blue data so that the user can follow the progress of the fit. These unsuccessful attempts are not counted as iterations. As with any derivative-based optimization procedures, it might happen (although not very often) that the program will find a local but not the global minimum. This situation may be realized by viewing the residual. If the residual contains "something else rather than noise", it is advised to try other parameters or change the fitting model. For fitting a Voigt function (a line shape that is given by a convolution integral of a Lorentzian and a Gaussian functions) the fitting may converge slowly if the initial parameters are far from the optimal ones. It this case it might be useful to swap the parameters for Gaussian and Lorentzian widths and start the optimization over. In order to save time it is always recommended to start with simulation parameters that reproduce characteristic features of the experimental spectrum.

Upon initiating iterative optimization, EWVOIGT displays initial chi square and then reports the new chi square after each successful iterations. The program also reports a "vector" parameter of the Levenberg-Marquardt optimization (see Press et al. 1986). When the optimization converges normally, the vector coefficient is decreased tenfold with each successful iteration. If iteration is unsuccessful, the program will increase the vector coefficient tenfold and attempt another iteration. In general, there are three main reasons for unsuccessful iterations: *i*) optimization already reached the minimum, *ii*) the problem is ill defined, and *iii*) the fitting model is inappropriate.

After the iterations are completed, the last simulated spectrum is displayed in light red together with the experimental spectrum (light blue) and the residual (yellow). All the simulation

parameters are updated in the INPUT PARAMETER menu. The best-fit parameters can be saved as a text file using the "D Save Parameters" option.   The parameter file automatically receives extension "FIT". In addition to all parameters, the file also contains parameter uncertainties that are calculated using covariance matrix method (Press et al. 1986) and correspond to 68% confidence intervals. To obtain 92% confidence intervals, these values should be multiplied by 2. Additional considerations on parameter uncertainties are discussed by Press et al. 1986.

Note that the simulation parameters can be in a "FIT" in either data points or magnetic field units. If the the option "M Mode" in the INPUT PARAMETER MENU is set to "Data points" then all parameters will be displayed and saved in data points. Selecting magnetic field by toggling the option "M Mode" will convert all parameters to magnetic field units and the "FIT" will be recorder in the latter format. For experimental data in the "FLS" format, the program also reads the time when the file was created, converts the value to seconds from the midnight, and saves at the bginning of the corresponding "FIT" file. This is convenient for sequential processing of kinetric data collected by EW data acquisition software from Scientific Software Services.

3.2.4 Initial Examples

Let us now demonstrate the use of the program on specific examples using test files provided with EWVOIGT.

1) Simple Lorentzian function; one center with noise (filename SAMPLE1.FLS). In order to proceed with simulations:

- From the MAIN MENU, set extension to FLS (option "I"), then read SAMPLE1 (option "D").   The spectrum will be displayed.
- Select the option "M Read Defaults" and enter the name of the parameter file, SAMPLE1. This will load the initial parameters and parameter status suitable for fitting this Lorentzian line.
- Select the option "G Simulation/Fitting Menu".
- Select the option "C Re-enter parameters" to view the values of the parameters.
- Select the option "H Parameter Status" to view the parameter status.
- Select the option "F Simulate" to ensure that the initial trial parameters generate a simulation that is close in shape and amplitude to the experimental spectrum.
- Select the option "A Start Optimization".
The results of the simulation are shown in Fig. 17.

```
FITTING/SIMULATION MENU - ESC to MAIN MENU
A Start Optimization     B Iterations= 6      C Re-enter parameters
D Save parameters        E Change Interval    F Simulate
G Number of centers= 1   H Parameter Status   I Equality Constraints
J Mode=1-st Der.         K Fit sequence       L Exp. file=sample1
M Number of prot. A= 0   N Number of prot. B= 0   O Invert interval=off
 Chi square= 9.782312E-03  Amplitude= 3.201128
```
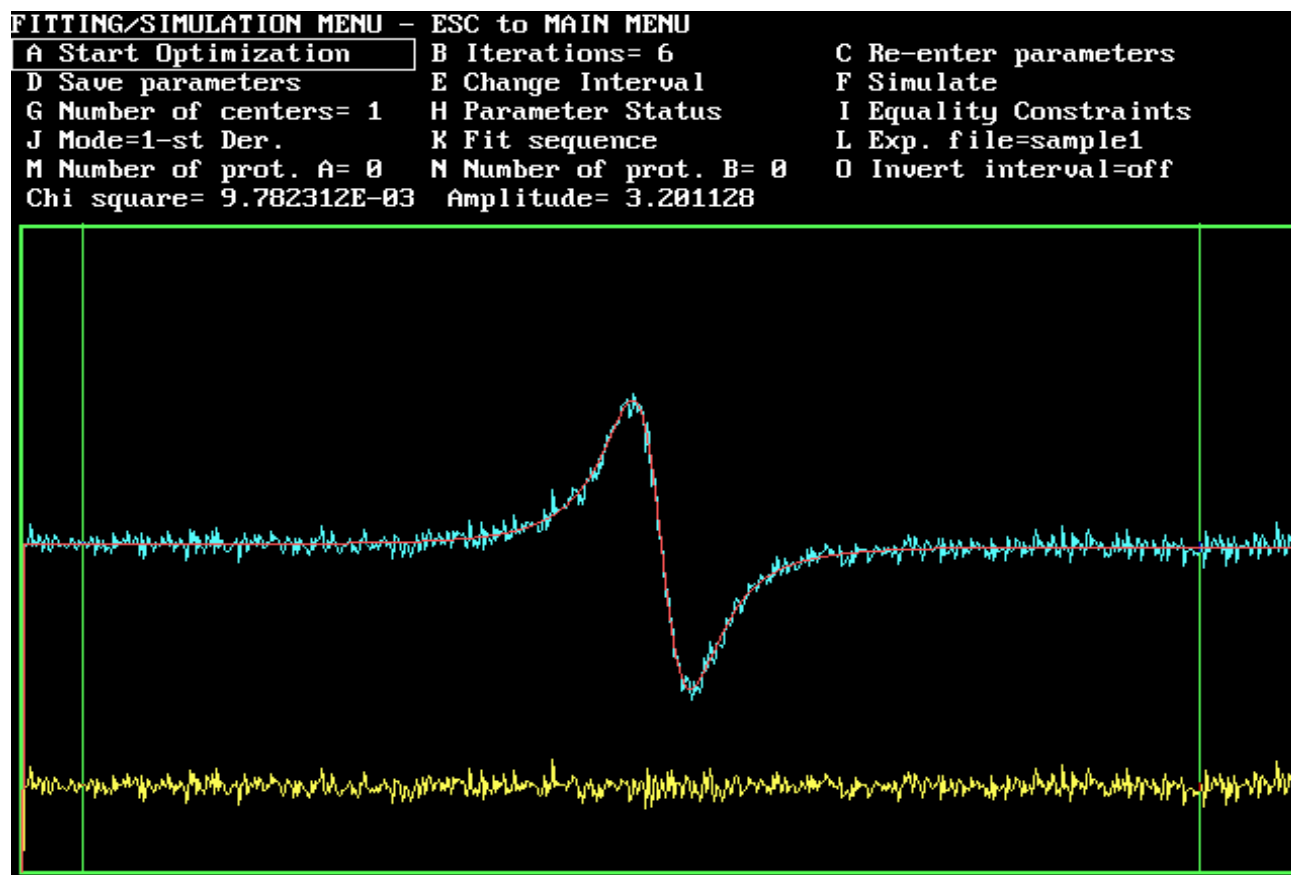
Figure 17. Results of least-squares simulations of a model spectrum SAMPLE1.FLS.

You can now experiment with changing the values of the trial parameters and the status (fixed or adjustable) of the parameters. After making the changes, go back to the Simulation/Fitting Menu and simulate using the trial parameters (option "F"). See how the line changes shape and size, then start the optimization procedure (option "A"). If your trial simulation is very far away in shape and amplitude from the experimental data, the optimization procedure may not converge. The algorithm is quite robust, however, and the initial simulation can be amazingly bad and convergence will still take place given sufficient number of iterations.

2) Nitroxide spin label with unresolved superhyperfine structure (fast motion, filename SAMPLE2.FLS)

Follow the same steps as in 1) above, but this time use SAMPLE2 as the data and the default parameter file. In this example, inhomogeneous broadening is modeled by a Gaussian envelope function which width is adjusted during the optimization. Be sure to note the typical parameter values in the input parameters menu and the parameter status (what is allowed to vary during the optimization).

Additionally, for nitroxide radicals, the double integrated intensity of each of the three nitrogen hyperfine lines must remain the same. Therefore, only one intensity parameter is required for all three lines. This setting could be provided by the EQUALITY CONSTRAINTS menu that is accessible using the option "I". Equality constraints can be toggled between <Equal> and <Non-equal>. For nitroxides, the Equality constraint for the "Intensity" should be set to <Equal>. If so then the intensity parameter is forced to be equal for all three centers. Therefore, changing this

parameter in the input parameters menu for any one center will change the value for the other two centers automatically. When the equality constraint is non-equal, the intensities and status of the intensities can be changed independently for each center. Results of the simulations are shown in Fig. 18.


```
FITTING/SIMULATION MENU - ESC to MAIN MENU
 A Start Optimization      B Iterations= 5        C Re-enter parameters
 D Save parameters         E Change Interval       F Simulate
 G Number of centers= 3    H Parameter Status      I Equality Constraints
 J Mode=1-st Der.          K Fit sequence          L Exp. file=sample2
 M Number of prot. A= 0    N Number of prot. B= 0  O Invert interval=off
 Chi square= 88932.78        Amplitude= 15528.43   Max.-Min.= 26 (estimate)
```
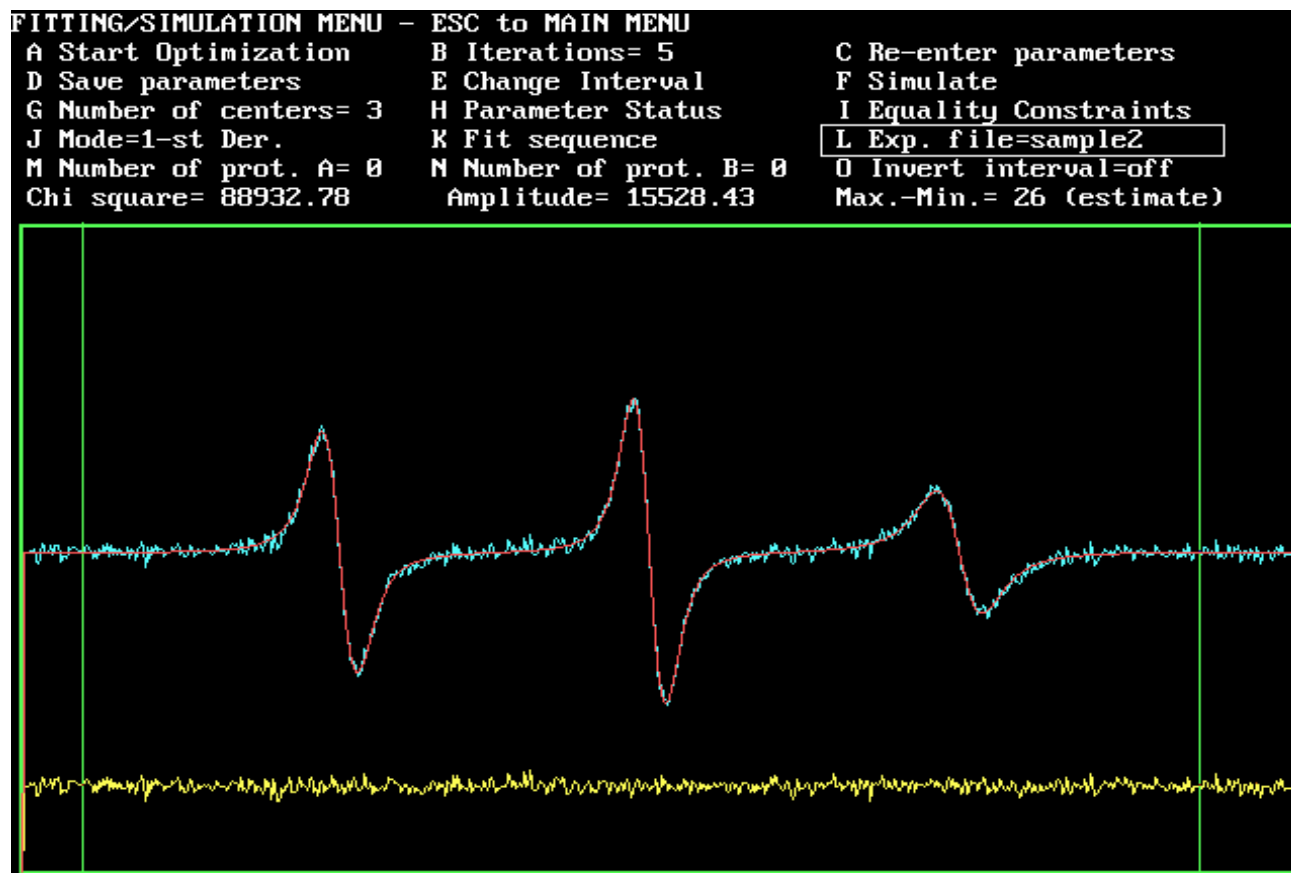
Figure 18. Results of least-squares simulations of a model EPR spectrum SAMPLE1.FLS.

The simulated spectrum can be saved in a format suitable for a variety of graphics programs as well as in the Scientific Software Services "FLS" format. To save the simulated data file in the "FLS format, return to the MAIN MENU and select the option "J Write as EW". The option "K Write for PLOT41" saves the spectra as comma-separated columns. The first column is the magnetic field (in gauss or data points depending on the "Mode" setting), the second column is the best-fit simulation, the third column is the experimental spectrum, and the fourth column is the residual (with the same offset downward as in the program screen). An example of a beginning of a PLOT41 file generated by EWVOIGT is shown in Fig. 19. This file automatically receives the extension "PRN".

```
3284.146 ,-1.379014 , 0 ,-11896.77
 3284.244 ,-1.221481 , 0 ,-11896.92
 3284.342 ,-1.062808 , 0 ,-11897.08
 3284.439 ,-.9025684 , 0 ,-11897.24
 3284.537 ,-.7408685 , 0 ,-11897.41
 3284.635 ,-.5774043 , 151.332 ,-11746.24
 3284.732 ,-.4121701 ,-224.341 ,-12122.08
 3284.83 ,-.2455169 , 350.4611 ,-11547.44
 3284.928 ,-.077332 , 225.0672 ,-11673
 3285.025 , 9.239496E-02 ,-111.2018 ,-12009.44
```

Figure 19. Beginning portion of a simulation data file in PLOT41 format generated by EWVOIGT.

3) Least-squares simulations of one hyperfine component of X-band EPR spectrum from 0.2 mM aqueous solution of nitroxide CTPO (3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrodine-1-yloxyl). At room temperature, the EPR spectrum from CTPO in aqueous solution has three nitrogen hyperfine components. Under conditions of a low (<0.5 mM) CTPO concentration, absence of oxygen, and a fast tumbling rate (*e.g.*, aqueous solution at room temperature), additional splittings of the components are observed because of the hyperfine interactions with 12 equivalent and 1 different protons (Hyde and Subczynski, 1984). Fig. 20 shows a typical spectrum of the central (m_I =0) nitrogen hyperfine component of the CTPO EPR spectrum after purging the 0.2 mM aqueous solution of this nitroxide with nitrogen for a half-hour.
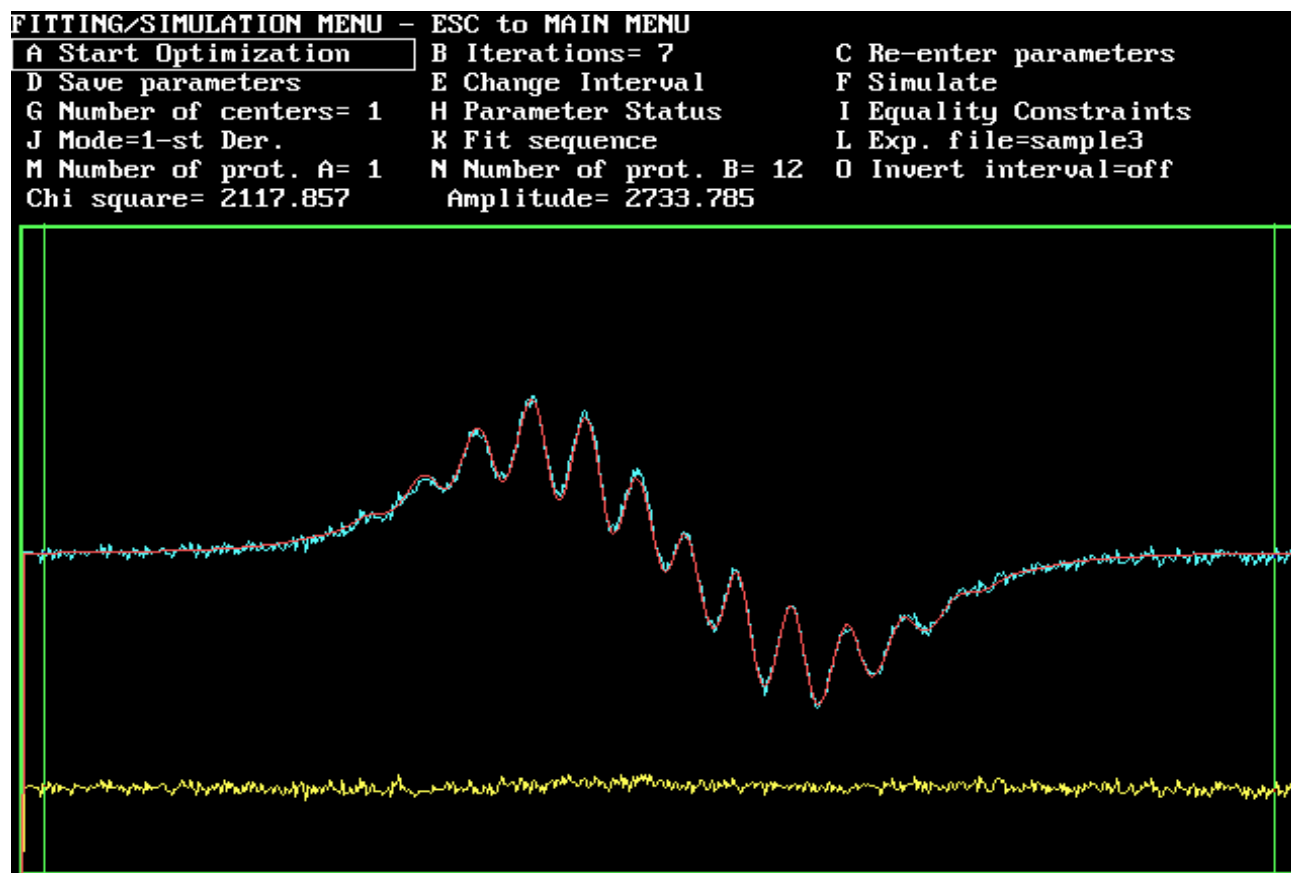

Figure 20. Results of least-squares simulations of the central (m_I =0) nitrogen hyperfine component of the 0.2 mM nitrogen-equilibrated CTPO EPR X-band spectrum (file SAMPLE2.FLS).

In order to proceed with simulation of this spectra, repeat the steps as in example 1) but use SAMPLE3 as the name for the data file and default parameter file. The number of iterations is increased to 7. In this case, the fitting model includes 12 protons with one hyperfine coupling and 1 proton with a second hyperfine coupling (both coupling are constants, field position of the spectra, Lorentzian line width and a few other parameters are adjusted during the fit). This optimization is quite sensitive to changes in the initial parameters because of a complicated minimization surface. In general, the initial hyperfine pattern should match the experimental spectrium in order for the

EWVOIGT to converge to the true minimum. This is expected for a gradient minimization method – Levenberg-Marquardt algorithm that is employed in EWVOIGT.

Note that th simulations above did not include any Gaussian component. Now, read SAMPLE3A as a new default parameter file. By reviewing parameters, you will see that the Gaussian selection is now adjustable and that the values of the Gaussian, Lorentzian, and intensity parameters, have changed. Run the optimization again. The chi square should decrease by only about 5% and examination of the "FIT" file would indicate that the error in the Gaussian parameter is rather large. For more accurate evaluation of the Gaussian component less noisy data would be required.

4) Finding phase shift for a spectrum distorted by a dispersion contribution (file SAMPLE4.FLS)

On occasion, EPR spectra may have contributions from both absorption and dispersion. This is typically a result of a phase shift of the microwaves across the sample and/or non-optimal tuning. Use the data file SAMPLE4 and the default parameter file SAMPLE4 to optimize spectral parameters for this spectrum that has both component. Signal-to-noise ratio is rather poor for this spectrum and this is characteristic of, for example, samples of oxygen0sensitive chars employed for EPR low-frequency *in vivo* oxymetry. The results of this optimization are shown in Fig. 21.
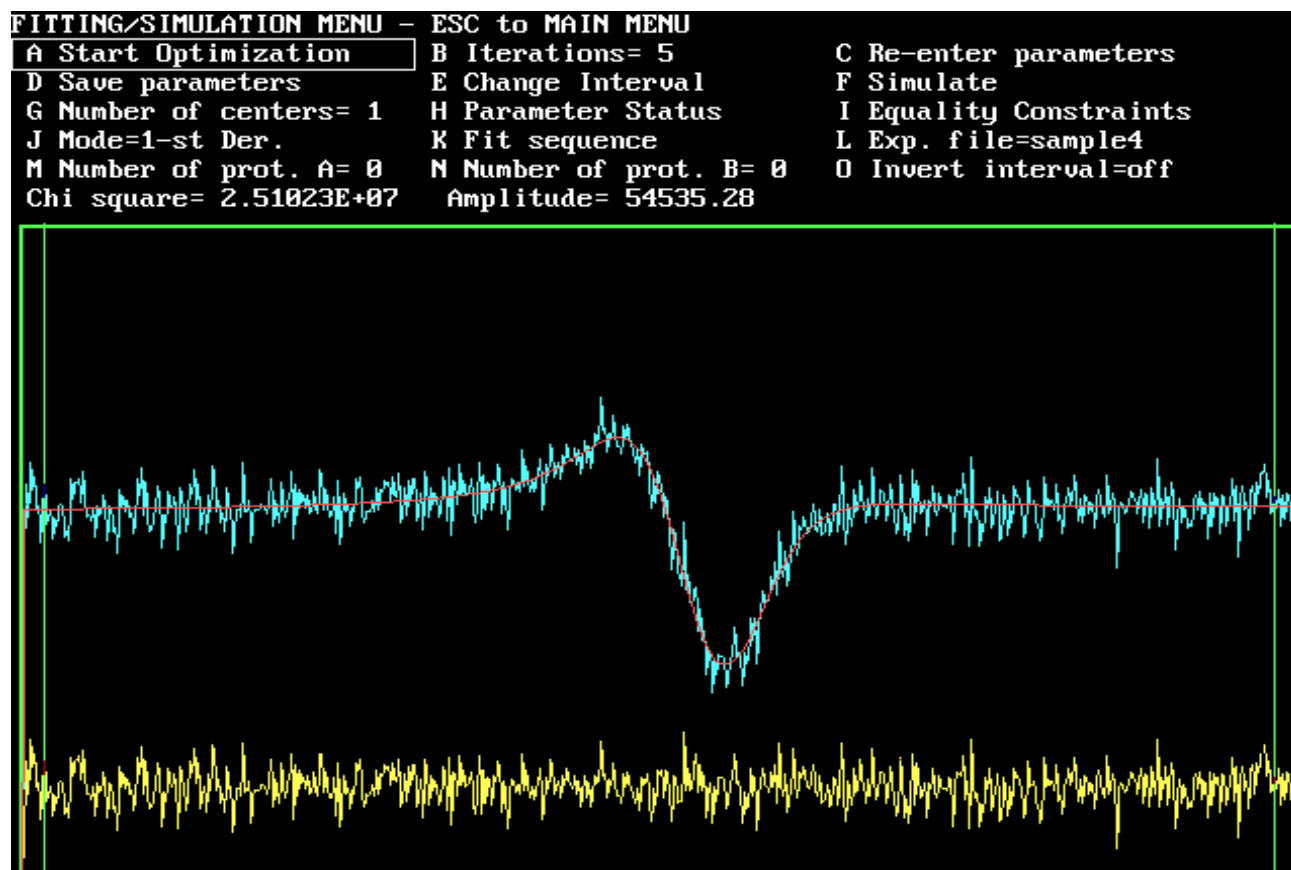

Figure 21. Results of least-squares simulations of a model EPR spectrum SAMPLE4.FLS.

If some line width or other spectral parameters are known from other sources or from other experiments, these parameters could be fixed and not optimized during iterative fitting. This would improve the accuracy of the remaining adjustable parameters. To illustrate this, re-read the SAMPLE4 parameter file, change the width of the Gaussian component to 50 data points, fix this parameter using PARAMETER STATUS menu, and then carry out least-squares simulations. These simulations will produce an exceptional fit. Examination of the parameter file saved using "D Save Parameters" option will demonstrate about fourfold improvement in the predicted uncertainty for the Lorentzian line width. Another approach to minimize uncertainty in inhomogeneous broadening is to apply an experimental spectrum as a non-adjustable envelope (see example 6) below).

5) Automatic fitting of a sequence of experimental EPR spectra for extracting kinetic parameters.

This is an example of an EPR experiment carried out by Morse and Smirnov (1995) on a bioreduction of a perdeuterated nitroxide Tempone (4-oxo-2,2,6,6-tetramethylpiperidine-N-oxyl) by baby Hamster Kidney (BHK). EPR spectra from the central (m_I =0) nitrogen hyperfine component of this nitroxide were collected sequentially in equal time intervals using EW data acquisition program from Scientific Software Services and stored under the names SAMPL500.FLS through SAMPL516.FLS. During the experiment both oxygen and nitroxide concentrations were observed to decrease (Morse and Smirnov 1995).

In order to start with simulations, please read in the SAMPL500.FLS file as an experimental file (MAIN MENU, option "D") and then the file SAMPLE5.VFP as a parameter file (MAIN MENU, option "M"). Check that these parameters are appropriate for an initial simulation and optimize this simulation in about 4 iterations. It is recommended to check how well the initial spectrum can be fitted with the model before processing the whole sequence of the spectra.

Then chose the option "K Fit sequence". An example dialogue is shown in Fig.22.

```
Enter the name of the file or RETURN to exit.
? sample5
Enter the first number of <Experiment> spectrum in the sequence=? 0
Enter the last  number of <Experiment> spectrum in the sequence=? 16
Quit the program after the fitting <Y>es-N
Center pre-adjustment? <Y>es-Y
```

Figure 22. Entering parameters for an automatic fitting of sequential EPR files.

For this sequence the sequence name is SAMPL5. The file mode (MAIN MENU) is set to "Experiment" and therefore the spectra SAMPL500.FLS through SAMPL516.FLS will be read as experimental data. Note that if the file mode is toggled to <Envelope>, the current spectrum could be fitted to a sequence of envelope functions. The spectra could also be read in reverse by entering, for example, 16 as the first number and 0 as the last number. You may also fit any smaller portion of the sequence by entering specific numbers. For example, entering 3 and 8 will fit the spectra from SAMPL503.FLS through SAMPL508.FLS. The current version of the program is capable to handle up to 1000 data files if numbered sequentially. For example, if files EXP3003 through EXP3129 were collected in an EPR experiment, then for fitting the entire sequence of the data one should enter "EXP3" as the sequence name, "3" as the first file number of the sequence, and "129" as the last number.

6) Fitting EPR spectra with a fixed envelope function: extracting oxygen broadening from X-band CW EPR spectra from 5-doxyl stearic acid in phospholipid bilayers (file SAMPLE6.FLS).

This example closely follows the least-squares simulation method for extracting small oxygen broadening from continuous wave EPR spectra of nitroxides in slow and/or intermediate motion regime as reported by Smirnov, Clarkson, and Belford (1996). This method was utilized for measuring oxygen permeability profiles of model membranes composed of 1,2-dimyristoyl-sn-glycero-3-phospho-choline (DMPC) above and below the main phase transition (*ibid.*). Refer to the abovementioned paper for additional details.

To carry out the fitting, start with reading in the SAMPLE6 file as an experimental file. Then, toggle the option "F" of the MAIN MENU to <Envelope> and read in the envelope file SAMPLE6A. The experimental file was measured from a spin labeled DMPC sample at 27.7° C in the presence of oxygen. The envelope file is from the same but deoxygenated sample. Read SAMPLE6.VFP as a parameter file (MAIN MENU, option M).

When a file is read as an envelope function, the rest of the spectral shapes generated by EWVOIGT will be convoled with such an envelope (see Eq.3). Hence, for extracting oxygen-induced broadening, it is important that no other factors than the one under evaluation are changing. For example, spectra used as envelope functions should be taken at the same temperature so that changes in motional contributions to the spectrum are minimized. Further details on extracting oxygen broadening from nitroxide spectra lipid bilayer membrane probes were provided by Smirnov and Belford (1995), Smirnov, Clarkson, and Belford (1996), and Smirnov and Smirnova (2004). .

Note that when both the envelope and the experimental spectrum are recorded as first derivative spectra, the mode for simulation of homogeneous broadening should be set to the first integral (option "J" in the FITTING/SIMULATION menu).

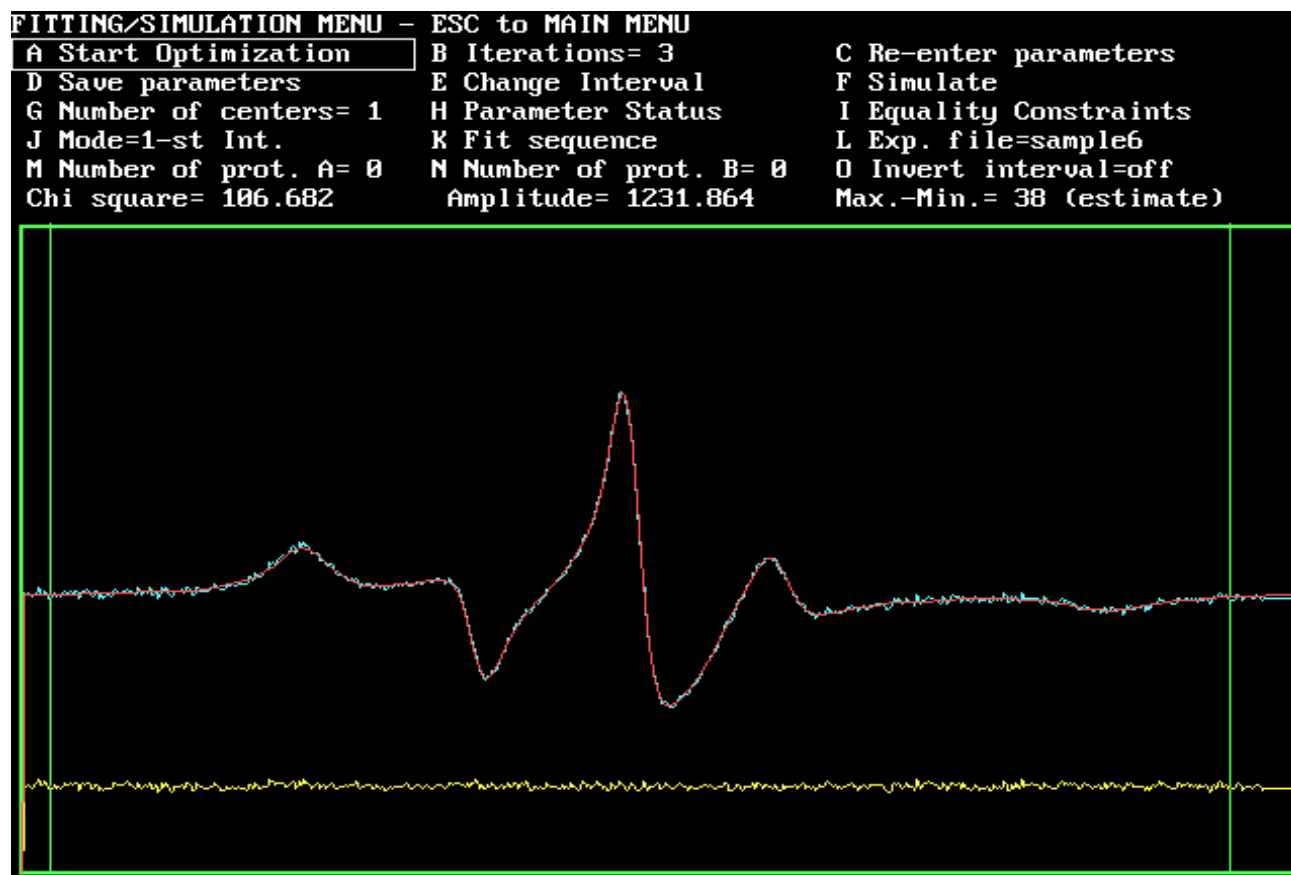The results of the optimization are shown in Fig. 23.

Figure 23. Results of least-squares simulations of a model EPR spectrum SAMPLE6.FLS with an experimental envelope given by the file SAMPLE6.FLS.

4. USING EWVOIGTN

EWVOIGTN is an extended version of EWVOIGT that was designed to fit two overlapping spectra of nitroxides in fast motion regime. Such spectra may arise from nitroxides residing in different environments (an example is a nitroxide that partitions between the aqueous and hydrocarbon phases of a lipid bilayer). While the MAIN MENU remains essentially unchanged, other menus were extended to accommodate new options and parameters. Essential changes are outlined below.

4.1 SIMULATION/FITTING MENU

```
FITTING/SIMULATION MENU – ESC to MAIN MENU
A Start Optimization      B Iterations = 3      C Re-enter Parameters
D Save Parameters         E Change Interval      F Simulate
G Number of Centers= 2    H Parameter Status     I Equality Constraints
J Mode=1-st Der.          K Fit Sequence         L Exp. File=510n09
M Save separately         N Custom interv.=off   O Chi square=1.12345
```
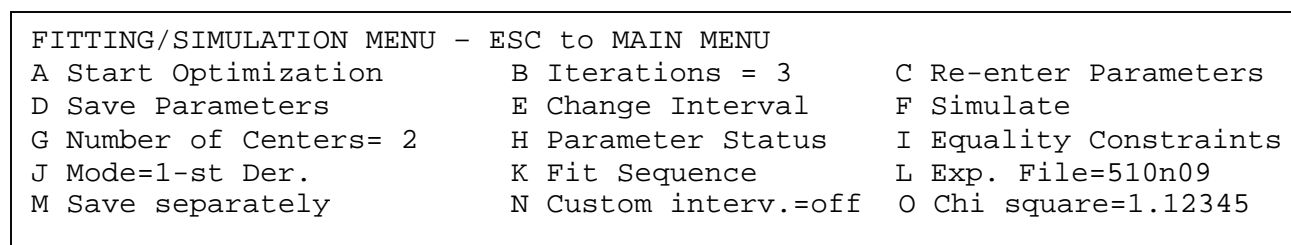
Figure 24. FITTING/SIMULATION MENU of EWVOIGTN.

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FITTING/SIMULATION MENU of EWVOIGTN is shown in Fig. 24. .

The order of the options in the FITTING/SIMULATION menu has changed. More options have been added for fitting and manipulating the data:

a) Option "M - Save Separately"
After finding a fit for a spectrum, the individual component spectra as well as the fit residual (a difference between the experimental and the simulated spectrum) can be saved separately in the FLS format. The first 8 characters of the file name will automatically remain the same but the extensions will change reflecting which component is saved. Specifically, the first spectrum (from center #1) is saved with an extension "001" while the second spectrum (from center #2) receives the extension "002", and the residual – "003". In the case of only one simulated center, the file extensions are "001" for the center #1 and "003" for the residual. If you are simulating a series of spectra, the spectra will be saved using the filename of the original data.

b) Option "N - Custom Intervals"

```
Weight start=? 100
Weight ends=? 331
Weight=? 10
Weight start=? 331
Weight ends=? 900
Weight=? 0.3
```

Figure 25. Assigning weight to the fitting intervals in the dialog triggered by the option "N - Custom Intervals"

In course of least-squares optimization, chi square is evaluated after each of iteration. In EWVOIGT the weights of each data point within the fitting intervals are assumed to be the same. EWVOIGTN offers a more flexible option to assign different weights to up to three different intervals of the spectrum. When "Custom Intervals" is "off", then a weight of 1 is assigned to all data points within fitting interval defined by the vertical cursors, and a weight of 0 is assigned to the remainder of the spectrum. When "Custom Intervals" is "on", you may specify different intervals and assign different weights to each of the intervals. The example in Fig.25 shows assignment of a weight of 10 to the first interval between the data points 100 and 331 and a weight of 0.3 to the second interval between 331 and 900 data points. Responding to "Weight starts" and "Weight ends" with RETURN will terminate the weighing entry procedure. All weight intervals are specified in data points, not Gauss. Such selective weighting is useful when it is desired to emphasize certain parts of the spectrum for simulation. For example, if an impurity line is present in a spectrum, its presence can be "ignored" by assigning zero weight to the spectral interval of the impurity line..

c) Option "G - Number of centers"

EWVOIGTN provides an option to simulate EPR spectra from either one or two nitroxides by selecting the "G" option. If "1" is entered, EWVOIGTN behaves similarly to EWVOIGT. If "2" is entered, then there are several additional options change. These are described below.

d) Option "C - Re-enter parameters"

```
FITTING/SIMULATION MENU – ESC to MAIN MENU
A Start Optimization     B Iterations = 3      C Center number=? █
D Save Parameters        E Change Interval     F Simulate
G Number of Centers= 2   H Parameter Status    I Equality Constraints
J Mode=1-st Der.         K Fit Sequence        L Exp. File=510n09
M Save separately        N Custom interv.=off  O Chi square=1.12345
```

Figure 26. A new dialog triggered by activating the "C Re-enter parameters" option is shown inside the cursor box.

If the number of centers is 1, then the option "C Re-enter parameters" will immediately activate the INPUT PARAMETER MENU. If the number of centers is 2, then the C option will first activate a short dialog about which center you wish to enter parameters for (Fig. 26). Entering "0" will activate a new menu for parameters common for both centers (Fig. 27). Note, that combination of "G Number of Centers= 2" and "C Center number=0" is the only way to access these common parameters. The latter include the linear (option "G Linear") and the quadratic ("I Quadratic") terms of the baseline, "H Offset" of the spectrum from zero, and a dispersion angle which is set by the "D Disper." Option. It is suggested to exercise caution when changing these parameters; it should be rare that you enter this menu. All variables displayed in this menu could be adjusted during the least-square optimization procedure.

```
INPUT COMMON PARAMETERS FOR SIMULATION; – ESC go to the SIMULATION/FIT menu


                                        F Dispersion=-7.170157E-03
G Linear=-5.394447E-03    H Offset= 0   I Quadratic=-2.274113E-07
```

Figure 27. INPUT COMMON PARAMETERS FOR SIMULATION of EWVOIGTN.

While parameter entry for centers 1 or 2 these is the same as for EWVOIGT, the menu itself is different (see Fig. 28). Note, that all the parameters appearing in the menu are specific and unique for that center. The units for parameters may be shuttled between data points and Gauss (assuming the experimental file has values for field center and field sweep) by toggling the "C" option.

```
INPUT PARAMETERS FOR Center=1              ESC go to the SIMULATION/FIT menu
  Field from=2950.05    Field to=3053.35         C Mode=<Magn. Field>
D Position  =3038.268   E Gaussian    =1.559419  F Nitr. Hyp.= 15.58782
G Lor. (+1) =.3804871   H Lor. (0)    =.2714435  I Lor. (-1) = .4386149
J Protons A = 0         K Hyperfine A =1.209834  L Amplitude =1.45
M Protons B = 0         N Hyperfine B =0         O C13 Lines =<On>
```

Figure 28. INPUT PARAMETERS MENU in EWVOIGTN.

E) Option " O - C13 lines"

```
PARAMETERS FOR C(13) SATELLITES; – ESC go to the SIMULATION/FIT menu

D Probability= .044          E Hyperfine= 6.062345
```

Figure 29. Entering parameters for the ^{13}C satellite lines.

When the option "O" is turned "ON", then the ^{13}C lines are included in the simulations. When "O" is off, these lines are not included. Toggling on this option will activate an additional menu to reset ^{13}C lines' parameters (Fig. 29). The intensity of the ^{13}C satellite lines can be set by using the option "D Probability" (note, that abundance of the ^{13}C isotope is 1.11%) while the splitting between these lines is set by the option "E Hyperfine".

F) Option " I Equality constraints"

```
Equality constraints; – ESC go to the SIMULATION/FIT menu

D Position =<Non-Equal>      E Gaussian =<Non-Equal>  E Nitr.hyp=<Non-Equal>
F Lor. (+1)=<Non-Equal>      G Lor.  (0)=<Non-Equal>  H Lor.(-1)=<Non-Equal>
I Hyperf. A=<Non-Equal>      J Hyperf. B=<Non-Equal>  K Amplitude=<Non-Equal>
```

Figure 30. EQUALITY CONSTRAINTS MENU of EWVOIGTN.

EWVOIGTN provides an option of setting equality constraints between the two centers. If the parameters are non-equal, the program considers the variables to be independently defined for each center. When the equality constraint is set to equal, the variable is considered to be the same for each center. For example, the magnitude of Gaussian broadening for two centers could be set equal. Then the same value of the Gaussian width entered in for the center #1 will be also used for the simulation of the center #2.

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