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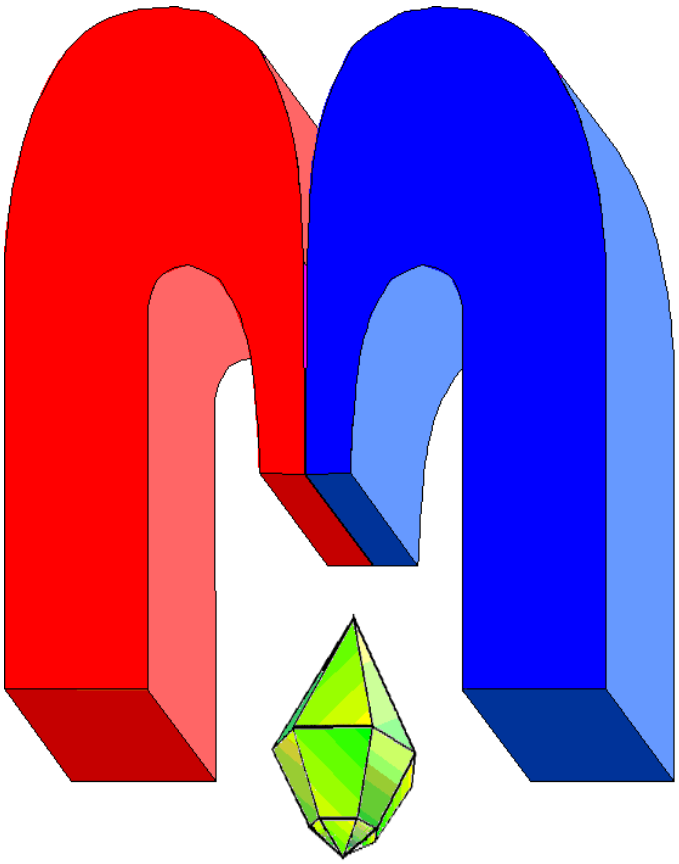

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† In Kazan University the Electron Paramagnetic Resonance (EPR) was discovered by Zavoisky E.K. in 1944.

Two-dimensional normal distribution of zero field splitting parameters for EPR spectra of human serum transferrin

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An approximation of a distribution of zero field splitting (ZFS) parameters by using two-dimensional normal distributions in discrete representation is proposed. The results of modelling the X-band EPR spectra of human serum transferrin are in a good accordance with experiment. For a number of studied EPR spectra, it was found that the desired distribution consists of two components with various rhombicity parameters. The first component is characterized by a relative high degree of correlation between the parameters E and D. For the second component of ZFS, which we associate with iron bound to the C-lobe iron site of human serum transferrin, a high dispersion of the correlation coefficient is observed.

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Keywords: electron paramagnetic resonance, X-band, human serum trasferrin, zero field splitting, two-dimensional normal distribution.

1. Introduction

Continuous-wave electron-paramagnetic-resonance (cw EPR) spectroscopy of transition metal ions is a powerful tool for studying the structure and structural-functional relationships in the studied substance [1, 2]. This concerns, in particular, high-spin EPR of metalloproteins, which are characterized by a substantial splitting of the magnetic sublevels in zero field [3]. The parameters of the spin Hamiltonian of the active centers of transition ions in frozen proteins reflect the distributions in different conformations. Such conformational heterogeneity of transition ions leads to an inhomogeneous and non-isotropic distribution of zero field splitting parameters, called ZFS strain.

A complete understanding of experimental EPR spectra often requires numerical modeling. In cases where the broad distribution of parameters is unknown, modeling is a challenging task. Widely used software package EasySpin makes it possible to take the strains into account when calculating EPR spectra [4]. This software package uses a normal distribution to approximate ZFS parameters, but this approach only gives good results when the width of the distribution is significantly smaller than the size of the parameter itself.

A numerical procedure called "grid-of-errors" was proposed in [5] to extract the magnetic interaction distribution from cw EPR spectra. The procedure is illustrated for the Fe^{3+}-EDTA (ethylenediaminetetraacetic acid) complex. This method involves creating a library of spectra using the EasySpin EPR simulation package [4] to analyse the powder spectrum with an isotropic g-tensor and a small isotropic linewidth for each point on the grid. Later, these authors in [6] applied a modified numerical "grid-of-errors" procedure to model the EPR line shape and determine broad distributions of ZFS parameters of high-spin Fe^{3+} ions ($S = 5/2$) in a metalloprotein, namely transferrin (Tf). Transferrin is a blood plasma protein that transports iron ions. This protein is of interest both from a fundamental and a medical-practical point of view, as evidenced, in particular, by the 2021 review by Silva et al. [7].

Transferrin consists of two homologous lobes, termed the N- and C-lobe, each capable of binding strongly and reversibly an Fe^{3+} ion. A significant contribution to analysis of the cw

EPR spectra of human serum Tf (h-Tf) were the works of a group of authors [6, 8]. First, EPR measurements were carried out at various frequencies ranging from ∼ 9.7 (X-band) up to 275 GHz (J-band). At 275 GHz, the quantum energy is sufficient to cause transitions between the three Kramers doublets ($S = 5/2$), which allow to determine the parameters of ZFS. Second, the authors related the double peak characteristic of transferrin in the X-band EPR spectrum to the fourth order terms in the spin Hamiltonian. Further, to find the distribution of ZFS parameters, a method "grid-of-errors" was applied, this made it possible to visualize the distributions of the ZFS parameters extracted from the J-band spectra [6]. At the same time, it should be noted that all measurements at a frequency of 275 GHz in [6] were carried out on specially prepared bilobal, but monoferric human serum transferrin prepared by disabling iron binding in one of the lobes through mutations.

In this paper, we propose the development of the "grid-of-errors" method [5, 6], the results of which are used for a preliminary analysis of the distribution of splitting parameters in a zero field. In the following stages, the main emphasis is placed on approximating the desired distribution with a two-dimensional normal distribution (TDND). By analogy with the "grid-of-errors" method, we applied a discrete representation: a library of spectra calculated at all grid points is used to calculate the spectrum. This eliminates the distribution width limitation inherent in the continuous distribution in the Easyspin package. In addition, the problem of spectral redescription and non-uniqueness of the solution, characteristic of the "grid-of-errors" method, is removed. The proposed technique was used to model the X-band EPR spectra of high-spin Fe^{3+} ions in h-Tf, recorded at liquid nitrogen temperature. It is shown that in most cases two TDND are sufficient to describe the experimental spectra, one of which indicates a strong correlation of ZFS parameters.

2. Experimental details

Human venous blood collection and serum isolation were performed in a clinical medical laboratory according to accepted standard protocols. Whole blood samples were collected using Vacutainer venous blood collection tubes. Serum was obtained by centrifugation of whole blood tubes for 10 min at 2000 g. Within two hours after preparation of serum the same amount of sample for all measurements was placed in quartz ampoules and frozen in liquid nitrogen.

EPR spectra were recorded from samples of equal volume (0.15 ml) on a Bruker EMX Plus spectrometer at a frequency of ∼ 9.38 GHz with a modulation frequency of 100 kHz, modulation amplitude of 5 G, microwave power of 20 mW.

3. Simulation of EPR spectra

The EPR spectra of high-spin Fe^{3+} sites were interpreted using the spin Hamiltonian, which includes fine structure terms up to the fourth order [6, 9]:

$$H = \mu_B g\, \mathbf{B}_0\, \mathbf{S} + \mathbf{SDS} + \sum_q B_4^q O_4^q(\mathbf{S}), \tag{1}$$

where $S = 5/2$, $g = 2$ is the electron g-factor, μ_B is the Bohr magneton. The first term of the Hamiltonian describes the Zeeman splitting in an external magnetic field. The second term describes the second-order zero-field splitting of six magnetic sublevels into the three Kramers doublets. The tensor $\mathbf{D}$ is symmetric and traceless, so the three main values of the $\mathbf{D}$-tensor are expressed by two parameters, namely D and E. It is assumed that $D = \frac{3}{2}D_z$, and $E = \frac{1}{2}(D_x - D_y)$. D is the axiality parameter of the system, and E is parameter of asymmetry

or parameter of rhombic distortion of the structure. The ratio E/D is an indicator of the rhombicity of the **D**-tensor. For $\mid D_x \mid < \mid D_y \mid < \mid D_z \mid$ and $0 \le E/D \le 1/3$, there is a transition from axial to rhombic symmetry. In the third term O_4^q is the extended Stevens operator, where q varies from -4 to $+4$. At $q = -3$ this term violates axial symmetry and appears in low-symmetry crystals (e.g. trigonal, monoclinic). It describes the contribution to the crystal field from structural distortions, such as incorrect coordination of ligands around a metal ion, or asymmetric arrangement of charges in protein complexes (e.g. in transferrin when binding anions).

For transferrin in the X band, the weak-field limit $\mu_B g\mathbf{B}_0\mathbf{S} \ll \mathbf{SDS}$ is realized.

The EasySpin software package was used to simulate X-band EPR spectra from h-Tf samples, in particular, the "pepper" utility, designed to simulate EPR spectra from powdered solid samples.

The problem of finding the distribution of ZFS parameters was solved in two stages: 1) finding the initial values of the parameters; 2) finding the optimal values of the parameters.

At the first stage, the "grid-of-errors" method described in [5, 6] is applied. To implement this method, the EPR spectra $f_{i,j}(H)$ are calculated at each point *(i, j)* of the rectangular grid of parameters D and E (step 50 MHz). The resulting model EPR spectrum is obtained by multiplying such EPR spectra by the corresponding weight coefficients (of the desired distribution) $w_{i,j}$ and summing over all points of a rectangular grid:

$$F(H) = \sum_{i}^{N_D} \sum_{j}^{N_E} w_{i,j} f_{i,j}(H). \tag{2}$$

Next, the standard deviation between the model and experimental spectrum is calculated. By changing the weight coefficients of the distribution at each point of the grid *(i, j)* by some constant value, the change in the standard deviation is found – the error $\delta_{i,j}$, which forms the "grid-of-errors". If the standard deviation decreased, then a new, improved coefficient was placed in the grid. The procedure was repeated until a good agreement was reached between the simulated and experimental spectra.

Furthermore, the distribution of the splitting parameters in the zero field, obtained by the "grid-of-errors" method, was approximated by TDNDs (usually two), each of which had the form:

$$\begin{aligned} f(D,E) = A \exp\Big[& \frac{-((D-D_0)\cos\alpha + (E-E_0)\sin\alpha)^2}{2\sigma_D^2} \\ & - \frac{(-(D-D_0)\sin\alpha + (E-E_0)\cos\alpha)^2}{2\sigma_E^2} \Big], \end{aligned} \tag{3}$$

where D_0, E_0 are coordinates of distribution centers, σ_D and σ_E are the parameters characterizing the width of the distribution, α is the angle describing the orientation of the distribution in the plane $E - D$, A is the amplitude. The parameters of these functions were selected from the point of view of maximum similarity to the distribution obtained by the "grid-of-errors" method.

At the second (final) stage, a direct search for the optimal parameters of TDND was carried out, based on the maximum similarity between the experimental and model EPR spectra. The results of the previous stage were taken as starting parameters.

It should be noted that equation (3) is equivalent to the formula describing the TDND [10]. The probability density for two random variables (in our case E and D) is described by the

formula:

$$f(D,E) = \frac{1}{2\pi\sigma_D\sigma_E\sqrt{1-\rho^2}} \exp\Big\{\frac{1}{2(1-\rho^2)}\Big[\frac{(D-m_D)^2}{\sigma_D^2} - 2\rho\frac{(D-m_D)(E-m_E)}{\sigma_D\sigma_E} + \frac{(E-m_E)^2}{\sigma_E^2}\Big]\Big\}, \tag{4}$$

where m_D and m_E are mathematical expectations, σ_D and σ_E are standard deviations of random variables D and E, ρ is correlation coefficient. The angle α and correlation coefficient are related by a simple relationship:

$$\alpha = \frac{1}{2}\arctan\left(\frac{2\rho\sigma_D\sigma_E}{\sigma_D^2 - \sigma_E^2}\right). \tag{5}$$

To test the proposed method, a model spectrum was constructed (Fig. 1b) corresponding to the distribution of D and E and the sum of two TDND (see Fig. 1a). The distribution parameters are given in Table 1. White normal noise was additionally superimposed on the EPR spectra. The results for 30 dB noise are shown in Table 1. The distribution centers were determined with an accuracy of about 1%, the widths are about of 10-20%. The values of the correlation parameter close to zero are determined least accurately (50%).

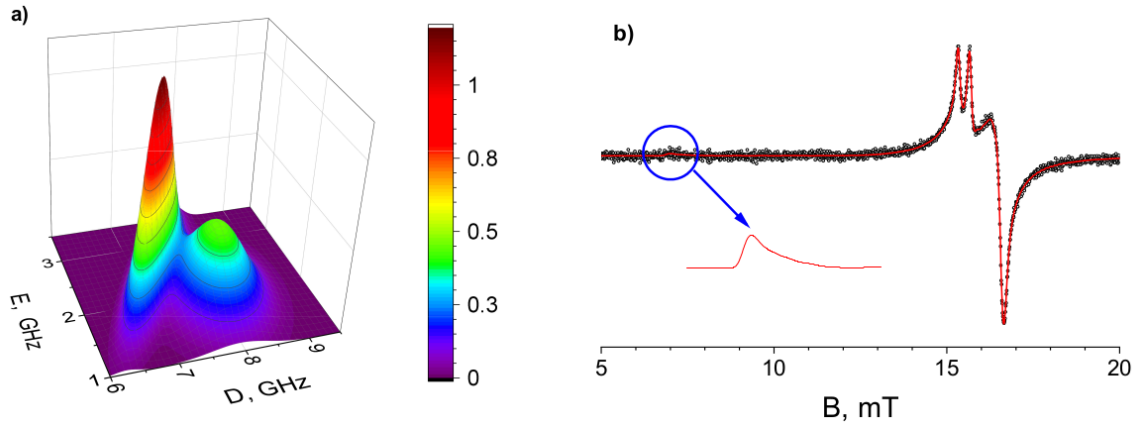

Figure 1. The distribution of ZFS parameters (panel a), used to construct the model spectrum (panel b), is shown by the red line. The black dots are the same spectrum after adding of 30 dB white normal noise

Table 1. Parameters used to construct the two-dimensional distribution (Fig. 1a) and determined as a result of applying the proposed method, as well as the difference between them is Δ

	Model		Determined		Δ(%)	
	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2
D, MHz	7242	8006	7224	7937	18 (0.2%)	69 (0.8%)
E, MHz	2391	1956	2373	1931	18 (0.9%)	25 (1%)
ΔD	403	435	411	408	8 (2%)	3 (1%)
ΔE	500	471	499	480	1 (0.2%)	9 (10%)
ρ	0.90	0.11	0.91	0.21	0.01 (1%)	0.1 (50%)

As can be seen from the table, this method gives quite satisfactory results. Since the problem being solved is a typical problem of finding the extremum of a function, the problem of finding its global value arises (in this case it is the minimum). In this regard, the search was conducted with

various initial conditions. The Parameter Space Investigation (PSI) method is used for choice starting parameters [11]. This method is similar to the Monte Carlo method, but instead of a random number generator it uses a Sobol sequence of points. Such sequences give significantly better results, especially in a multi-dimensional space search. The details of algorithm will be described elsewhere.

4. Results and discussion

Fig. 2 shows the X -band EPR experimental spectrum of human serum transferrin, recorded at liquid nitrogen temperature. The spectrum has a characteristic double peak and the so-called "shoulder" in the region with effective $g' \approx 4.3$. A weak line is also found in the region with effective $g' \approx 9.0$. The exact shape of the spectrum (splitting of the peaks, height of the shoulder) depends on the organism and differs slightly for the N- and C-lobes of the protein [6, 8].

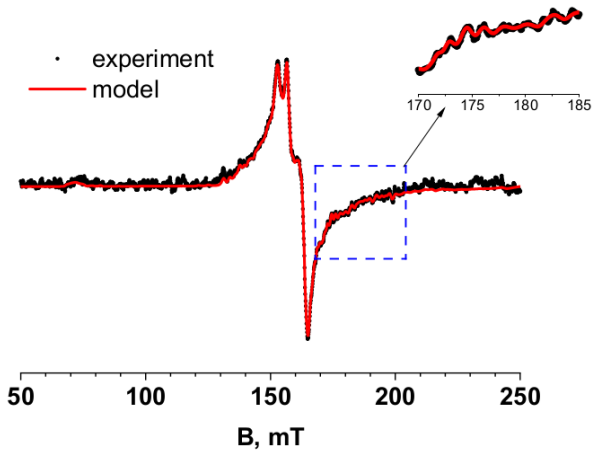

Figure 2. Experimental EPR spectrum of Fe^{3+} in h-Tf, recorded in the X -band at 80 K and the result of computer simulation using the "grid-of-errors" method

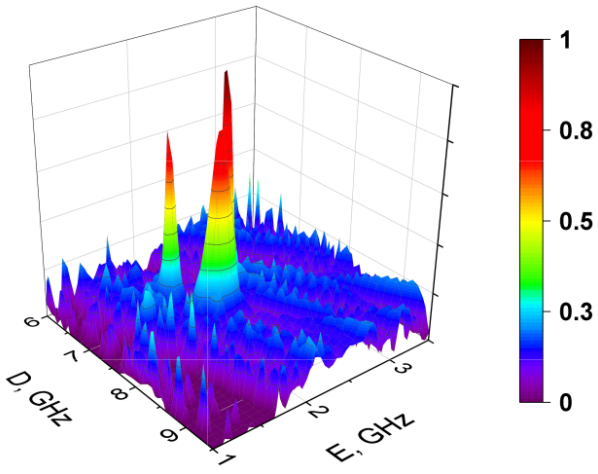

Figure 3. Distribution of ZFS parameters for the EPR spectrum of h-Tf, obtained by the "grid-of-errors" method

The result of applying the "grid-of-errors" method, both for numerical simulation of the experimental spectrum and for determining the distribution of ZFS parameters, is presented in Fig. 2 and Fig. 3. Unlike [6], where Fibonacci numbers were used, in our case, a rectangular

grid was used. The range of variation of parameters D and E was determined during the distribution search and amounted to 6000 – 9500 and 1000 – 3500 MHz, respectively. The step for both parameters was chosen the same equal to 50 MHz. Thus, to apply the "grid-of-errors" method, a libraryof 3500 spectra was created for each spectrum. The fourth-order fine structure terms in equation (1) were taken to be zero, except for $B_4^{-3}O_4^{-3}(\mathbf{S})$ with a value of approximately 70 MHz (similar to [6]).

As seen from Fig. 2, the "grid-of-errors" method makes it possible to describe the EPR spectrum with good accuracy. However, there are some serious difficulties in using this method. Firstly, the resulting distribution of ZFS parameters has a noise-like character, which makes interpretation difficult (Fig. 3). Further, there is an obvious "redescription" of the spectra, namely, in some spectral regions such an algorithm leads to a description of fluctuations in the noise track (see the inset in Fig. 2). The next serious problem is the ambiguity of the solution. Calculations show that the "grid-of-errors" method makes it possible to obtain calculated spectra corresponding to different distributions of the ZFS parameters and the resulting distribution depends on the choice of initial conditions.

The authors of [6] at the final stage of modeling the EPR spectra approximate the distribution obtained by the "grid-of-errors" method with one or two Gaussian functions. We have made two changes to the algorithm proposed by Azarch et al. [6]. First, TDND was used taking into account the correlation coefficient. It should be noted that the Easyspin package takes into account the correlation between the parameters of the zero field splitting. However, Easyspin has a significant limitation, namely, that the width of the distribution must be much smaller than the values of the coordinates of the distribution centers.

Secondly, the final solution is not the result of the approximation of the distribution obtained by the "grid-of-errors" procedure, but the result of direct fitting of the normal distribution. According to our method, the parameters of the normal distribution are sought directly in the fitting process: amplitude, center coordinates (D_0, E_0), widths in D and E, and the correlation coefficient. The distribution parameters obtained as a result of fitting by two different methods, namely the "grid-of-errors" method and directly from the EPR spectra, differ significantly.

The result of our approach to the description of EPR spectra is presented in Fig. 4, and the corresponding parameter distributions are shown in Fig. 5 – Fig. 7. Fig. 4 shows that the desired distribution cannot be described by one function. Calculations have shown that the desired distribution of ZFS parameters consists of two components (Fig. 5). The first one is characterized by a ratio of $\lambda_0 = E_0/D_0 = 0.3 \pm 0.006$ and describes the central part of the EPR spectrum (Fig. 6), including the double peak and shoulder. For the second component $\lambda_0 = 0.23 \pm 0.007$ (Fig. 7) and it describes the edges of the spectrum. The mean values and standard deviations of parameters of distribution components were determined for 15 EPR spectra (see Table 2). As can be seen from the table, the coordinates of the distribution centers remain virtually unchanged for the entire set of blood serum samples studied (the change does not exceed 3%). Changes in other parameters from sample to sample are also not arbitrary. Quite curious in this respect is the behavior of the correlation coefficient. For the first component, there is a strong correlation between the parameters E and D (about 0.8), and for the second component, there is a strong scatter in the values of this parameter for different samples.

The presence of the listed patterns allows us to hope that our hypothesis about the two-dimensional normal distribution of ZFS parameters is correct.

As can be seen from Fig. 6, the first component of the ZFS distribution cannot be described

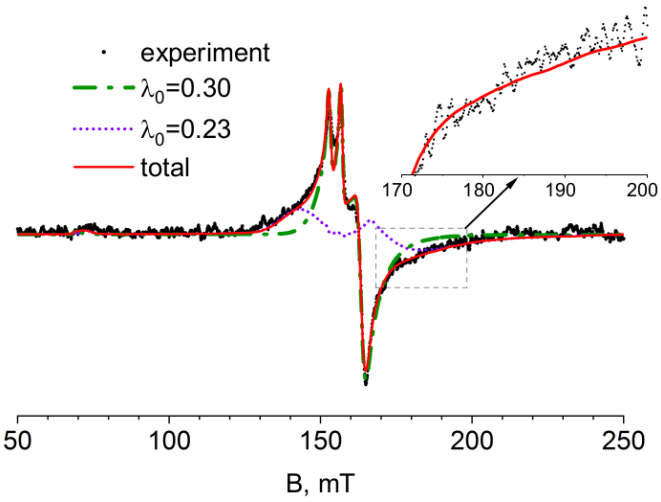

Figure 4. Experimental EPR spectrum of Fe^{3+} in h-Tf recorded in the X -band at 80 K and the result of computer simulation under the assumption of a normal distribution of the ZFS parameters

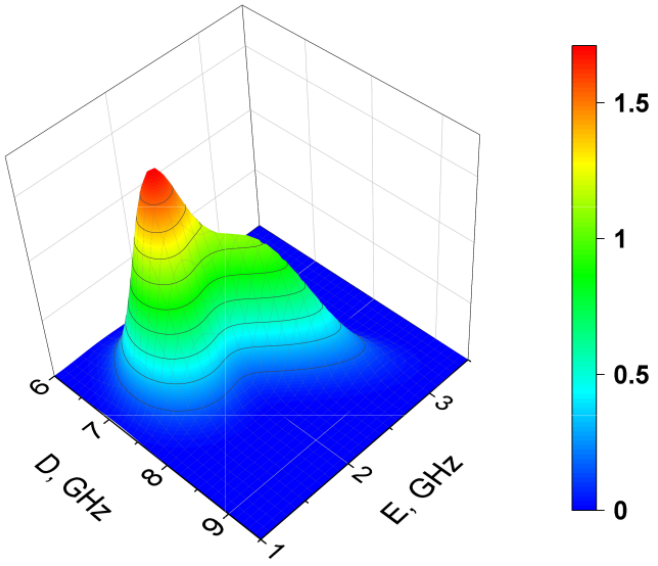

Figure 5. Two-dimensional normal distribution of ZFS parameters corresponding to model EPR spectra in Fig. 4

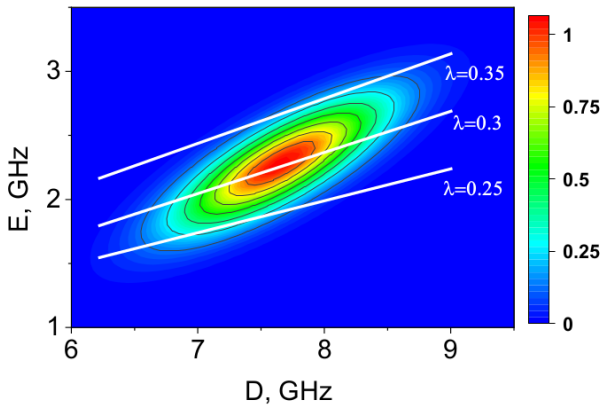

Figure 6. First component of distribution of ZFS parameters of the EPR spectrum of h-Tf: $\lambda_0 = 0.30$. White lines correspond to: λ_0; $\lambda_0 + 0.05$ and $\lambda_0 - 0.05$

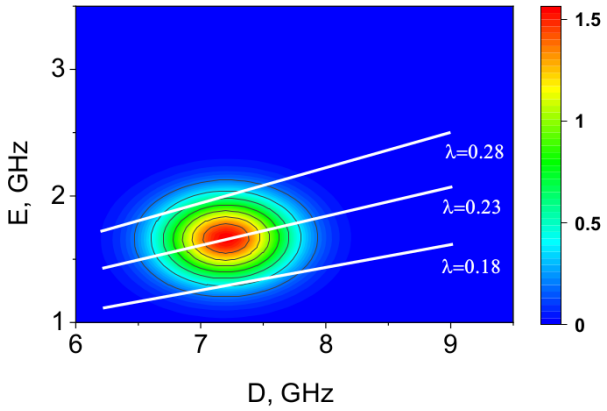

Figure 7. Second component of distribution of ZFS parameters of the EPR spectrum of h-Tf: $\lambda_0 = 0.23$. White lines correspond to: λ_0; $\lambda_0 + 0.05$ and $\lambda_0 - 0.05$

Table 2. Mean values and standard deviation of normal distribution parameters determined from 15 EPR spectra

	1^{st} component			2^{nd} component		
	Mean	sd	sd/Mean (%)	Mean	sd	sd/Mean (%)
Amplitude	0.56	0.16	29	0.45	0.19	42
D_0, MHz	7705	62	**0.8**	7184	15	**0.2**
E_0, MHz	2251	26	**1.2**	1637	49	**3**
σ_D, MHz	592	64	11	390	70	18
σ_E, MHz	374	31	8	232	36	16
ρ	**0.82**	**0.10**	**12**	**0.25**	**0.28**	**112**
λ_0	**0.30**	**0.006**	**2**	**0.23**	**0.007**	**3**

by TDND with $\rho = 0$, since it rotates at a significant angle relative to the axes. The presence of such a rotation is also clearly demonstrated by the preliminary analysis using the "grid-of-errors" method, namely, the observed fringes are rotated with respect to the coordinate axes (see Fig. 3). Therefore, it is really necessary to introduce a rotation of the function in the plane, or, what is the same, to introduce a correlation coefficient.

It should be noted that in the case of approximation of the desired distribution by normal ones, the "redescription" effect (see the inset in Fig. 4) characteristic of the "grid-of-errors" method, is absent.

The listed problems are signs of inverse problems related to ill posed [12]. As is known, these include problems that do not satisfy one of the three criteria: the existence of a solution, uniqueness, and stability. The ill posed problems include a large number of practically important tasks, for which, in particular, regularization methods are applicable. Quite a lot of attention is paid to these methods, and they are being intensively developed at the present time [13–15].

According to Tikhonov [12], the possibility of determining approximate solutions to ill posed problems is based on the use of additional information about the solution. This information can be quantitative in nature, which makes it possible to narrow down the class of possible solutions. On the other hand, only qualitative information about the solution (for example, information about the nature of its smoothness) can be used.

In our case, such information can be the assumption that the distribution of the splitting

parameters in the zero fields obeys a TDND: the problem is reduced to finding the parameters of the corresponding functions. As starting conditions for such a search, it turns out to be convenient to use the approximation by TDND functions, previously obtained by the "grid-of-errors" method. It should be noted that finding a solution exclusively within the normal distribution is difficult due to the large number of local extrema of the function being minimized. A preliminary analysis is necessary, for example, using the "grid-of-errors" method. Of course, additional a priori information could be provided by quantum chemistry and molecular dynamics calculations.

As can be seen from Table 2, the most striking difference between the components of the normal distribution is the mean value and the dispersion of the correlation coefficient. If for the first component the dispersion of the correlation coefficient is about 12%, then for the second component it is an order of magnitude higher and is about 112%. Undoubtedly, the differences in the dispersion of the correlation coefficients between the first and second components of the distribution indicate a significant difference in their electronic structure.

Thus, quantitative analysis of all X-band cw EPR spectra of the h-Tf recorded at 80 K using our method, allowed us to determine D, E space distribution of ZFS parameters. It was established that there are two separate regions with a different electronic structure of the iron binding site with average ZFS parameters equal to $E_0/D_0 = 0.3$ (the first component of the distribution) and $E_0/D_0 = 0.23$ (the second component of the distribution). Note that for both conformations, the dispersion of E_0/D_0 does not exceed 3%. The conformational heterogeneity reflected in the ZFS strain (see, Fig. 6, Fig. 7) is the same for them and equal to 0.05: $\lambda = E/D = 0.3 \pm 0.05$ for the first component of the distribution and $\lambda = 0.23 \pm 0.05$ for the second.

The first component of the distribution of ZFS parameters in the X-band EPR spectrum of the h-Tf has near rhombic symmetry with $E_0/D_0 = 0.3$. This is quite consistent with the generally accepted approach to processing X-band EPR spectra given in the works of R. Assa [16] and A. Jang, J. Gaffney [17], according to which at least one of the iron sites in Tf is characterized by the ratio of zero-field splitting parameters E/D equal $0.31 - 0.32$. Moreover, from the rhombograms for systems with $S = 5/2$ given in [6], it follows that in the X frequency band for an absorption line with an $g' \sim 4.3$, the value of E/D should be close to 1/3. It can be assumed with high probability that this component of the ZFS distribution arises due to the iron ion in the N-lobe site (Fe^N) of human serum Tf. However, the distribution of ZFS parameters extracted from the J-band spectrum [6, 8] for the main conformation of bilobal but monoferric Tf-Fe^N (obtained by disabling the C-lobe) is $E_0/D_0 = 0.19$, and this is completely inconsistent with our data. The most probable reason for such discrepancy is the inclusion of the correlation between E and D. In addition, the taking into account the parameters correlation leads to the fact that the distribution is no longer described by a single value of $\lambda = E/D$. This is especially relevant for cases where correlation coefficient is close to 1.

For the second component of the distribution of the ZFS parameters, the value of E_0/D_0 is equal to 0.23. The presence of such a component of the distribution of ZFS parameters is consistent with the data of A. Jang, J. Gaffney [17], according to which the second site of iron in the Tf is characterized by $E_0/D_0 = 0.22$. We associate this component with iron bound to the C-lobe iron site of h-Tf. Moreover, the average ratio $E_0/D_0 = 0.25$ of the iron binding site in the C-lobe (monoferric transferrin was prepared by disabling N-lobe), extracted by authors [6] from analysis of the J-band spectrum, is quite close to the value determined by us.

5. Summary

To determine the distribution of splitting parameters in a zero field, an approximation of such a distribution by two TDND is proposed. The results of modeling the EPR spectra of the h-Tf show the legitimacy of this approach. The resulting distributions yield calculated spectra that satisfactorily describe the experimental ones. The solutions are free of "redescription" effects and are probably unique. At the same time, the "grid-of-errors" method is necessary for preliminary analysis, determination of the solution area, and trends.

Also, the distribution of ZFS parameters can be expressed both by formula, containing the angle α (describe the orientation of the distribution in the plane $E-D$) and by formula, expressed through the correlation coefficient ρ. Both representations are equivalent. The functional expressing the standard deviation of the model spectrum from the experimental one converges to a minimum faster when using the formula with α, but it is more convenient to interpret the modeling results using the concept of the correlation coefficient.

For a number of studied X-band EPR spectra of h-Tf, it was found that the sought distribution of ZFS parameters consists of two regions with various rhombicity parameters: $\lambda_0 \approx 0.3 \pm 0.006$ and $\lambda_0 \approx 0.23 \pm 0.007$. The first component of ZFS distribution is characterized by a relative high degree of correlation between the parameters E and D and is attributed to iron loaded to N-lobe iron site of human serum Tf. For the second component of ZFS distribution, which we associate with iron bound to the C-lobe iron site of h-Tf, a high dispersion of the correlation coefficient is observed. To clarify the reasons for such a difference in the distribution of ZFS parameters obtained from the EPR spectra of human serum Tf, further systematic analysis is necessary, including the use of quantum chemistry and molecular dynamics methods.

The proposed method for describing the distribution of splitting parameters in the zero field can apply to any EPR spectra with ZFS. The discrete representation eliminates the limitation on the width of TDND that inherent in Easyspin. The program code optimization has cut the spectrum library's calculation time to just a few hours. Fitting model spectra to experiments takes tens of minutes on a computer like the Intel Core i5-3340 at 3.10 GHz.

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