



## EPR

# Identification and Quantification of Radicals and Transition Metals with Bruker's Quantification Package

Innovation with Integrity

### Abstract

Identification and quantification of specific species in scientific research and process/quality control using analytical techniques can be challenging. One technique is Electron Paramagnetic Resonance (EPR) spectroscopy, which is used to identify and quantify radicals and transition metal ions in liquid or solid samples. Bruker has brought ease and convenience to EPR with the patented quantification package including SpinCount and SpinFit modules. Quantification package comes standard with all Bruker EPR spectrometers using Xenon and Xepr software and is optional with ESRStudio for Magnettech ESR5000. Using the quantification package, samples are accurately characterized without comparison to standard samples or separately measured calibration curves. With this, Bruker provides a simple and reliable solution applicable to a wide variety of scientific studies or process/quality control.

### Introduction

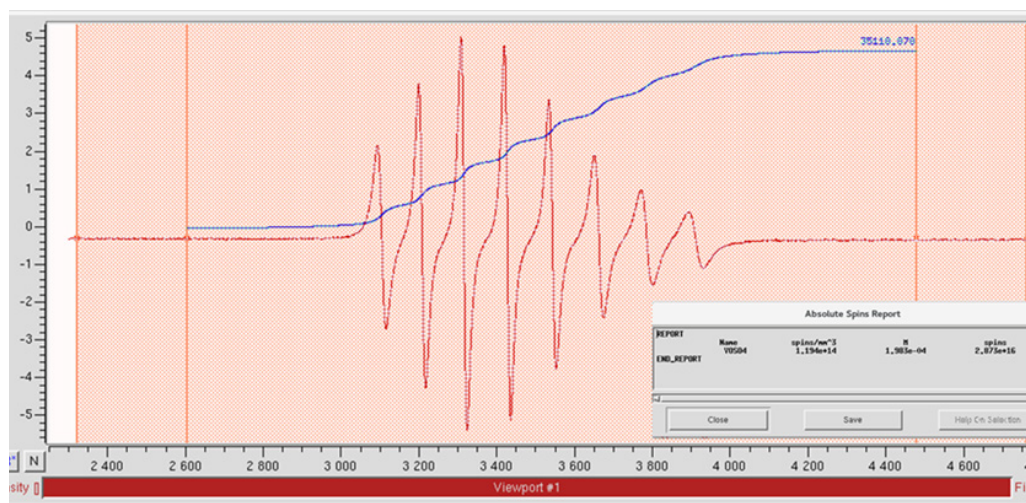
Using spectroscopy to identify or measure the concentrations of specific species in samples is a cornerstone of scientific research and process/quality control. With Electron Paramagnetic Resonance (EPR) spectroscopy researchers can identify and quantify radicals and transition metal ions in samples. Unlike most optical spectroscopies, EPR has no limitations with regards to degree of opacity or turbidity and quantitative information does not depend on the accuracy of extinction coefficients. Identification of paramagnetic species with EPR depends only on the properties of the species themselves. In contrast to other magnetic resonance techniques like Nuclear Magnetic Resonance (NMR) spectroscopy, EPR does not have restrictions due to molecular mass or size. Quantification requires only double integration of the EPR response which originates from the radical or transition metal ion of interest and does not suffer from interference from non-paramagnetic species. However, even with EPR, identification can depend on prior knowledge and quantification often requires standard curves that can be time consuming to construct.

To overcome the common limitations of optical spectroscopies and the challenges of EPR, Bruker has created the patented SpinFit and SpinCount package which offers reference-free quantification and unambiguous identification of paramagnetic species in a wide range of samples. With SpinCount, researchers can access quantitative information from raw data without the need for comparison to standard samples or the use of calibration curves. With SpinFit modules and the included library of spectra for common radicals and transition metal ions, researchers can identify species even with little prior knowledge. Depending on the software up to three members of SpinFit family are available: Isotropic SpinFit, SpinFit Liquids and Aniso SpinFit. SpinFit makes identification easy by fitting the raw data to a narrow set of parameters with minimal input from the user. Isotropic SpinFit and SpinFit Liquids are spectral simulation and fitting programs for CW-EPR spectra of radicals and transition metals in solution. Isotropic SpinFit simulates and fits isotropic spectra of no viscous solutions. SpinFit Liquids extends this functionality to include and determine the tumbling rate (rotational correlation time) of radicals in solution. Simulations of spectra in different dynamic regimes require different theoretical descriptions that are implemented in SpinFit Liquids with an automatic determination of the dynamic regime. Anisotropic SpinFit is designed to simulate spectra from frozen solutions and powders.

## Reference-free quantification with SpinCount

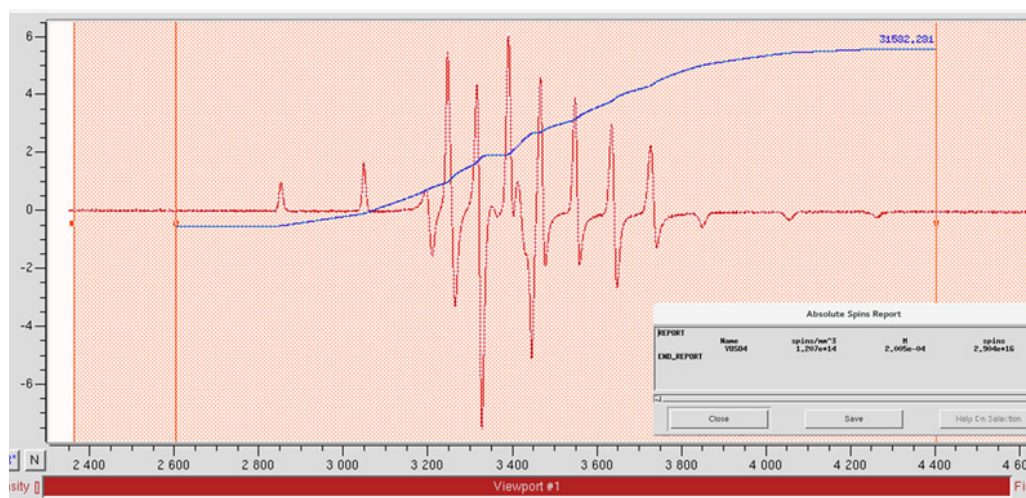
- High precision, reference-free calculation of concentration directly from raw data via double integration
- Incorporation of instrument parameters in the SpinCount calculation
- Incorporated into the spectrometer control software so researchers can quickly and easily quantify radicals immediately after data collection

Figure 1 demonstrates SpinCount applied to an aqueous Vanadyl Sulfate ( $\text{VO}_2\text{SO}_4$ ) solution studied at 290 K. Within SpinCount the user simply selects the area for double integration with a qualifier region and the double integral of this region is converted to the total number of spins as well as the molarity and spins/ $\text{mm}^3$  in the sample.



**Figure 1:** The experimental spectrum is shown in red. From the double integral (blue) of the experimental spectrum, SpinCount determines the number of spins, molarity, and spins/ $\text{mm}^3$  as shown in the Absolute Spins Report.

One important consideration is varying sample conditions. For example, the observed EPR spectrum will change as a function of temperature which will lead to a different double integral for the same sample studied at different temperatures. Figure 2 illustrates this with the spectrum for the  $\text{VO}_2\text{SO}_4$  sample from Figure 1 in the frozen state at 150 K. SpinCount considers the sample temperature in the calculation to provide consistent quantification regardless of the change in the double integral. This also applies to changes in physical state without associated temperature changes as liquid to solid transitions alter the spectrum in a similar manner.

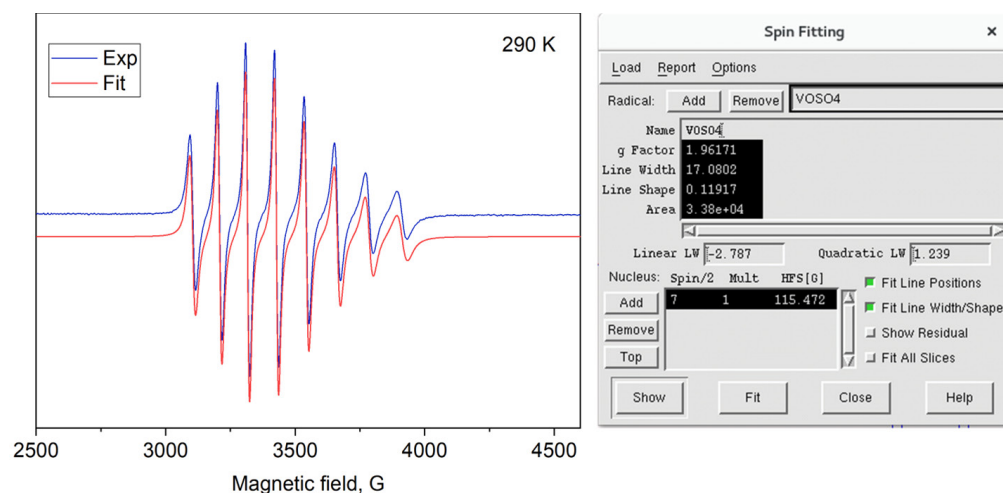


**Figure 2:** The same  $\text{VO}_2\text{SO}_4$  complex from Figure 1 studied frozen at 150 K. SpinCount uses the same double integral procedure to obtain the number of spins, molarity, and spins/ $\text{mm}^3$  from the experimental spectrum.

## Quick and easy identification of radical species with SpinFit modules

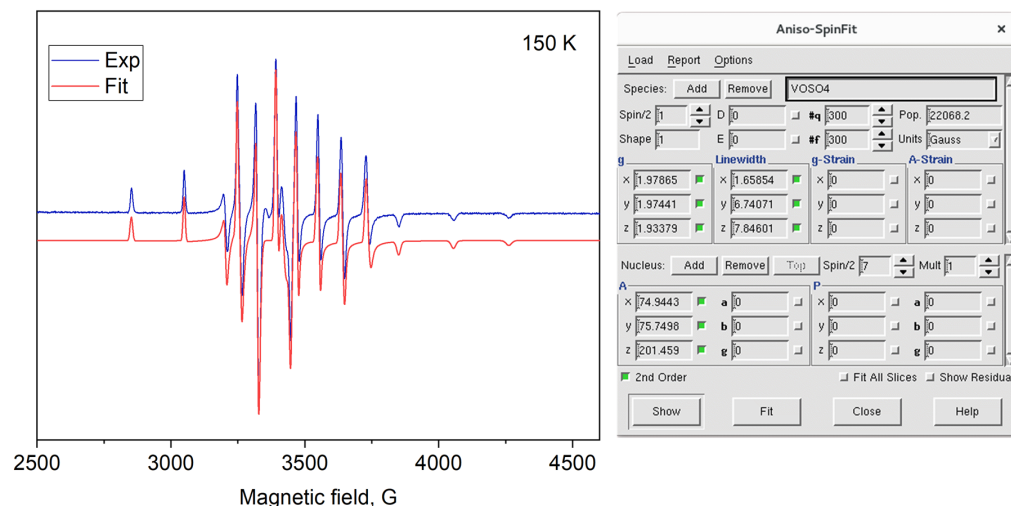
- Designed to handle radicals and transition metal species in both solid (Aniso SpinFit) and liquid (SpinFit Isotropic and SpinFit Liquids) samples
- Comes with a spectra library of common species to use for identification
- Identification and resolution of multiple species
- Conveniently interfaced with SpinCount
- Automatic choice of dynamic regime (SpinFit Liquids)
- Incorporated into the spectrometer control software so researchers can quickly and easily identify paramagnetic species immediately after data collection

Unpaired electrons of paramagnetic species interact with their local environment which results in characteristic EPR spectra and can be used for identification of the species. SpinFit makes identification easy by fitting the raw data to a narrow set of parameters with minimal input from the user. In cases where the paramagnetic species is unknown, SpinFit comes with an extensive library of common radical and transition metal spectra with corresponding fit parameters to allow for quick and easy identification. Because samples can be studied in both liquid and solid states, SpinFit offers a variety of parameter options to handle the unique considerations needed for each. Figure 3 demonstrates the application of SpinFit Isotropic to the spectrum for  $\text{VOSO}_4$  studied at 290 K. The user only needs to input or load a starting set of parameters and SpinFit Isotropic automatically finds the correct set of parameters to accurately fit the spectrum.



**Figure 3:** The experimental spectrum (blue) for the Vanadyl Sulfate ( $\text{VOSO}_4$ ) complex in liquid solution shows a characteristic splitting pattern consisting of eight lines due to hyperfine coupling with the  $M_I = 7/2$  Vanadium nucleus. The parameters for fitting the spectrum are shown in the Spin Fitting window and the resulting fit is shown in red.

On the other hand, Aniso SpinFit is capable of addressing the additional parameters needed to study the solid-state spectrum. Figure 4 demonstrates Aniso SpinFit applied to the frozen  $\text{VOSO}_4$  sample from Figure 2. As with the liquid state spectrum, the full range of parameters can be input by the user or simply loaded from the spectral library.

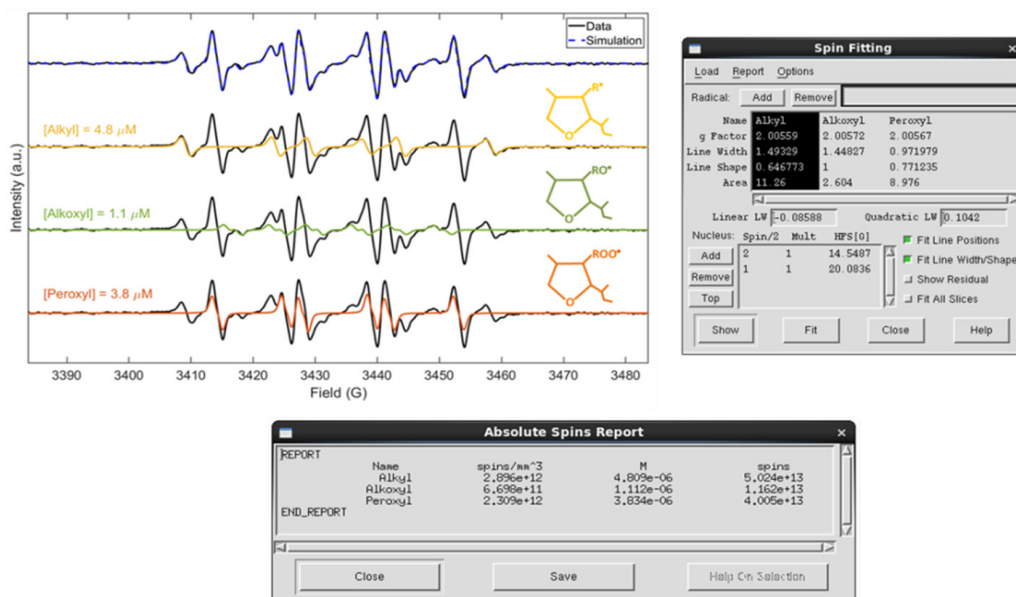


**Figure 4:** When the  $\text{VOSO}_4$  sample from Figures 1 and 3 is studied at 150 K, the spectrum is consistent with a solid-state sample. When fitting, Aniso SpinFit considers additional parameters necessary to fully describe a spectrum for a solid sample.

## Integration of SpinCount with SpinFit modules

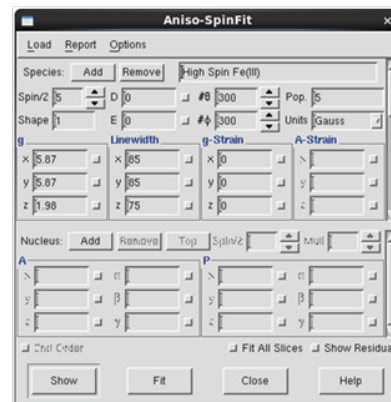
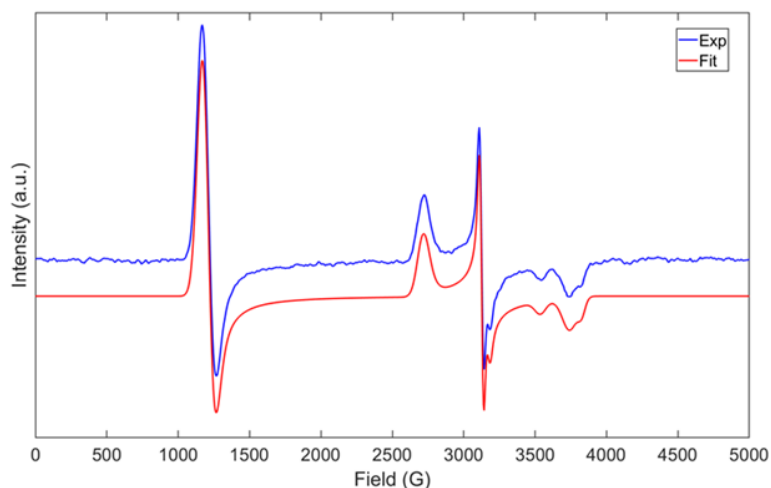
- Quantify with high-precision even with low signal-to-noise ratio
- Resolve multiple overlapping spectra to measure their individual concentrations
- Same quantitative information contained in raw data is also accessible with SpinFit modules

The previous examples demonstrate the ability of SpinCount to convert an EPR spectrum to a number of spins in a sample, as well as the ability of SpinFit modules to model EPR spectra. One common problem that researchers face is to access quantitative information from samples containing multiple paramagnetic species, resulting in overlapping spectra. In these cases, double integration of the spectrum cannot be used to give quantitative information for the individual species. By linking the capabilities of SpinFit with SpinCount, quantitative information is not limited to samples with single species. SpinFit fits the experimental spectrum with a model that includes multiple species and separates the individual spectra. From this, SpinCount quantifies through calculation rather than through double integration. In Figure 5, for example, the spectrum arises from the overlap of three spectra, each from one of three different products of the chemical oxidation of polysorbate. The identification of three polysorbate radicals by SpinFit Isotropic translates to quantification of each of the radicals separately in SpinCount. With this, SpinFit Isotropic makes it simple to identify and quantify individual species even when they are part of a sample that contains multiple species.



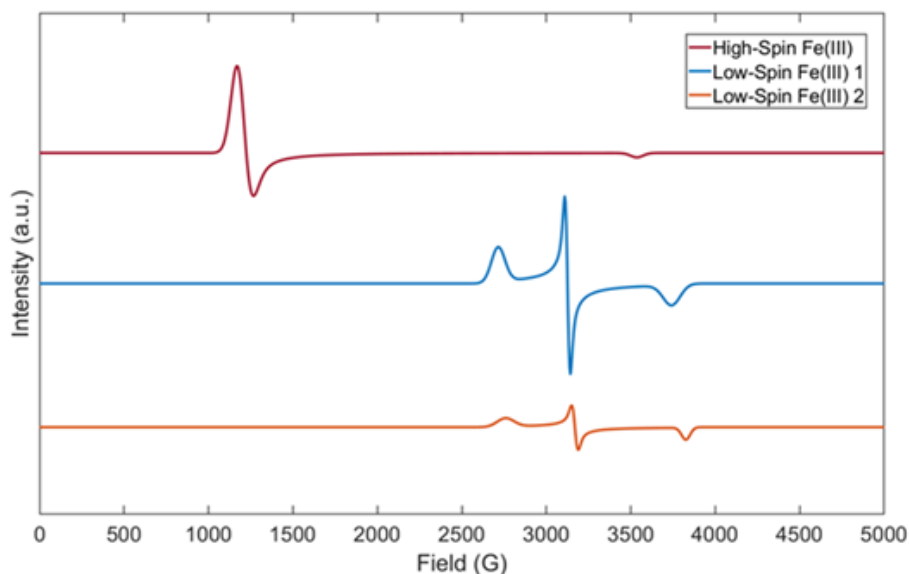
**Figure 5:** The experimental spectrum (black) is original data collected for a mixture of polysorbate radicals. The simulation (blue dashed) is the sum of individual spectra shown in yellow (alkyl), green (alkoxy), and orange (peroxy). The simulation results from the weighted sum of the three individual simulated spectra according to the concentrations shown for each. SpinFit Isotropic calculates each of the three individual spectra and the sum in parallel so that each are isolated and analyzed separately. SpinCount uses this information to provide individual concentrations despite the spectral overlap.

Figures 6 and 7 demonstrate how Aniso SpinFit is applied to identify and quantify a mixture. In this example, the spectrum arises from three transition metal complexes that differ in their coordination environments. Starting with the Anisotropic SpinFit library, both low-spin and high-spin Fe(III) signals are identified in the spectrum. Aniso SpinFit then automatically finds the correct parameters for each species to accurately fit the overall spectrum.



**Figure 6:** The experimental spectrum (blue) arises from a mixture of three heme complexes that differ in their coordination environment. This data is adapted from (Ruetz, M. et al., A Distal Ligand Mutes the Interaction of Hydrogen Sulfide with Human Neuroglobin, *J. Biol. Chem.*, (2017) 292, 6512-6528). Using the Aniso SpinFit spectra library to provide a convenient starting point, the experimental spectrum is fit (red) to a mixture of three different Fe(III) spectra corresponding to one high-spin and two low-spin complexes. The individual spectra are shown in Figure 7.

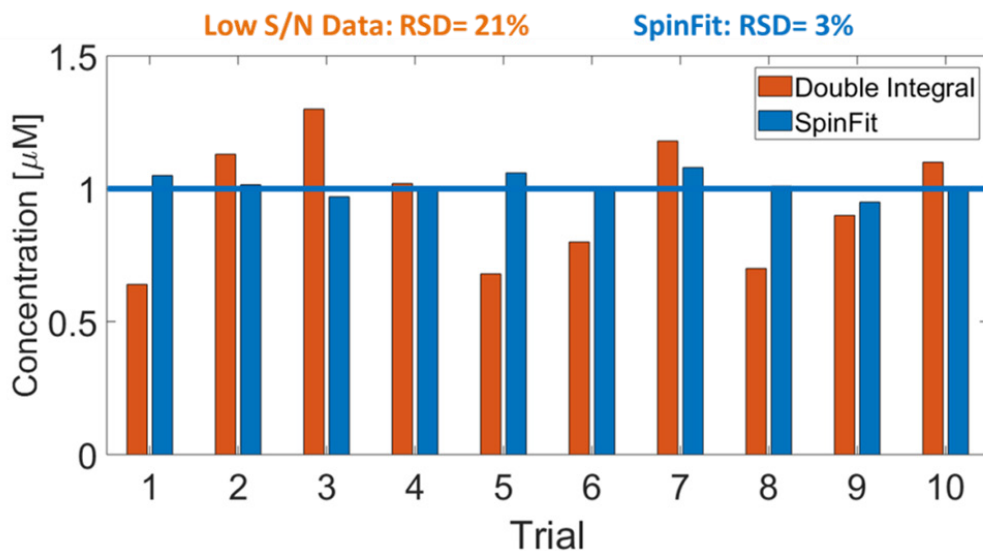
The spectra in Figure 7 are the individual spectra that Aniso SpinFit identified from the experimental spectrum in Figure 6. Aniso SpinFit uses the relative contributions of each to the total spectrum to calculate the concentrations of each individual species.



**Figure 7:** Aniso SpinFit identifies the three Fe(III) species and SpinCount quantifies each. From the Absolute Spins Report it is determined that the sample is 2.3  $\mu\text{M}$  in a high-spin Fe(III) species (red spectrum), 47  $\mu\text{M}$  in a low-spin Fe(III) species (blue spectrum), and 12  $\mu\text{M}$  in a second low-spin Fe(III) species (orange spectrum).

REPORT	Name	spins/ $\text{nm}^3$	H	spIns
	High Spin Fe(III)	1.404e+12	2.332e-06	7.796e+12
	Low Spin Fe(III)	2.808e+13	4.663e-05	1.589e+14
	Low Spin Fe(III) 2	7.021e+12	1.166e-05	3.898e+13
END_REPORT				

In addition to overlapping spectra, researchers are often challenged by low radical concentration resulting in data with low signal-to-noise ratios. Low concentrations may be an intrinsic limitation imposed by sample size restrictions or poor yield in a reaction of interest. While signal averaging could be used, this can be time consuming or may be limited by fast reaction kinetics. In these cases, the same cooperativity between SpinFit and SpinCount is used to good advantage. The noisy experimental spectrum is fit and the concentration calculated from the noiseless simulated spectrum. Figure 8 shows a bar graph representing this comparison for repeated measurements of a sample with 1  $\mu\text{M}$  radical concentration. Determining the concentrations by taking the double integrals results in a low accuracy of the SpinCount results. If the noisy spectra are first fit with SpinFit and the concentrations are directly calculated, the accuracy is dramatically improved.



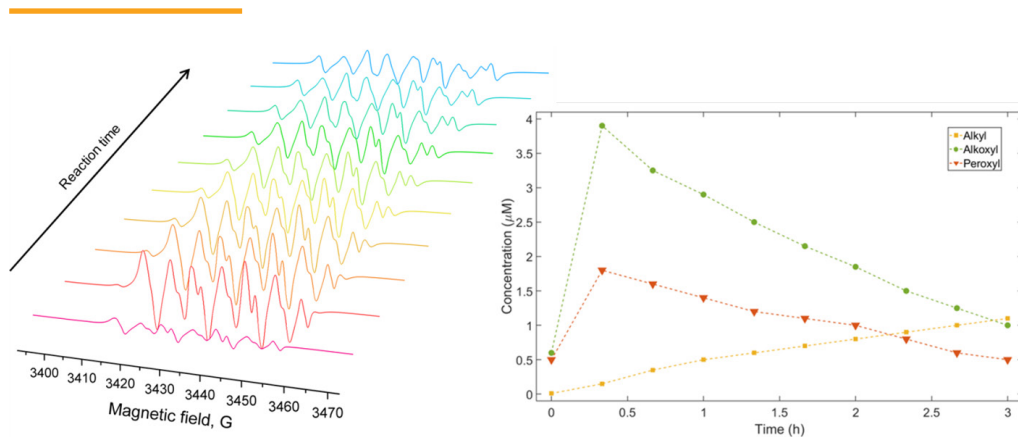
**Figure 8:** The SpinCount results obtained from the double integrals (orange) of EPR spectra from a low concentration sample result in an RSD of 21%. If the noisy spectra are first fit and the concentrations are directly calculated (blue) the accuracy is improved, resulting in an RSD of 3%.

### Analyses of two-dimensional data made easy with SpinCount and SpinFit

- Studying the effects of external stimuli on the EPR spectrum to build two-dimensional datasets
- Identification and quantification of species as a function of external stimuli

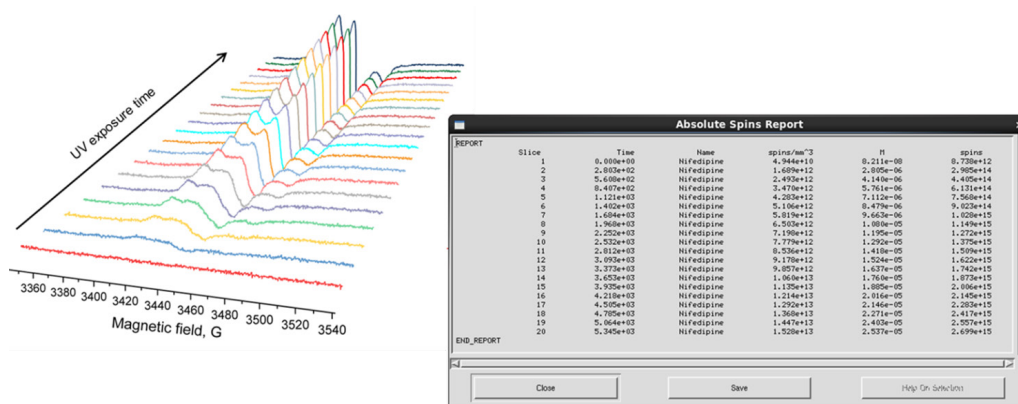
Bruker EPR spectrometers are designed to enable many types of EPR studies with an extensive range of available accessories. EPR spectra are often monitored as a function of common external stimuli such as sample temperature or UV irradiation *in situ*. The resulting time evolution of the EPR spectrum under the influence of external stimuli can provide insight into reaction mechanisms and kinetics. To match this experimental flexibility, Bruker EPR spectrometers offer several one- and two-dimensional experiments. However, with multi-dimensional EPR data, analysis procedures can be time consuming. Bruker has addressed this issue by designing the SpinCount and SpinFit package to analyze each slice of two-dimensional data sets.

Figure 9 demonstrates how Isotropic SpinFit is used in tandem with SpinCount to quantify time varying concentrations even in mixtures like the polysorbate example given in Figure 5. While the single spectrum in Figure 5 is a snapshot of the process that takes place during the chemical oxidation of polysorbate, the reaction occurs over time with a buildup and decay of each species. The evolving spectrum reflects the varying concentrations of each species as a function of reaction time. The SpinCount results demonstrate the simultaneous calculation of concentration for each species. With this, SpinFit and SpinCount offer insights into reaction mechanisms or kinetics with respect to each species individually, even when the sample consists of multiple species.



**Figure 9:** The evolution of the EPR spectrum during the chemical oxidation of polysorbate is observed by collecting spectra as a function of reaction time. The concentration vs. time plot demonstrates how SpinCount, with the assistance of SpinFit, simultaneously tracks the concentrations of each of the species shown in Figure 5, even when their spectra overlap.

Figure 10 gives an example of quantification of the Nifedipine radical using SpinCount. In this example, the Nifedipine radical is generated by UV light exposure over the course of 1.5 hrs. With increased exposure time, the radical concentration increases, which is observed through the increasing signal amplitude of the spectrum. SpinCount calculates a double integral of each time point individually to provide concentration as a function of time.

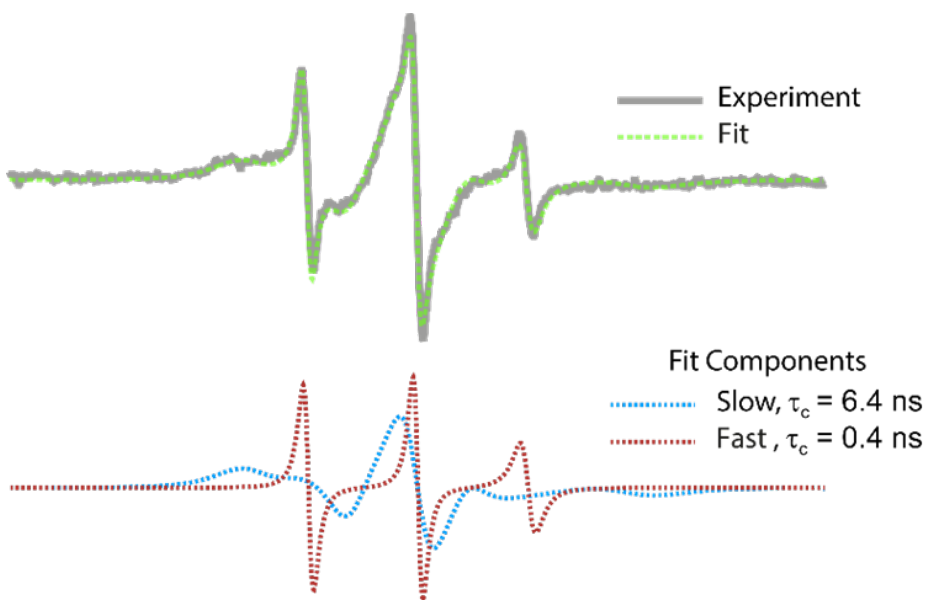


**Figure 10:** The Nifedipine radical is produced with UV light exposure. This plot demonstrates the growth of spectral amplitude with exposure time. The Absolute Spins Report demonstrates how SpinCount determines the concentration for each time slice to provide concentration as a function of UV exposure time.

## Molecular motion analysis with SpinFit Liquids

- Automatic determination of dynamic regime
- Calculating rotation correlation times

CW EPR signals can be classified in different dynamic regimes. (1) The *isotropic limit* observed for rapidly tumbling molecules in non-viscous solutions. (2) The *intermediate regime*, which is further differentiated into fast- and slow-motion regimes for samples in viscous solutions or molecules with large molecular mass limiting their motion. (3) The *rigid-limit observed* for solid samples such as powders, glasses, and frozen solutions. The SpinFit Liquids simulation module addresses spectra of all liquid samples covering both the intermediate regime and the isotropic limit. Simulation of multicomponent EPR spectra can help in studying dynamics of complex biological systems. Figure 11 shows an EPR spectrum of a spin labeled analogue of ADP (ADP-NO) binding to a protein from the histidine kinase family. As seen in the figure, the spectrum is a superposition of two components: the three narrow lines correspond to the unbound ADP-NO in solution while the broad component is the signal from ADP-NO bound to the protein. The molecular tumbling rate of a molecule is described by the rotational correlation time,  $\tau_c$ , and often expressed in ns. The smaller the rotational correlation time, the higher the tumbling rate. SpinFit Liquids allows for the simultaneous multicomponent simulation resulting in the molecular dynamics parameters for the two components and their relative fractions. Such studies can provide important insight into the function of ATP-dependent proteins and biopolymers in general.



**Figure 11:** Unravelling two components of a spin-labelled protein with SpinFit Liquids.

### Summary

EPR is a convenient and robust analytical method for identification and quantification of specific species in research and process/quality control. Using EPR, researchers can resolve reaction kinetics, study samples as a function of external stimuli or changing environmental conditions, and even resolve multiple species in a single sample. With the patented SpinCount and SpinFit package that is available with all Bruker EPR spectrometers, researchers can have quick and convenient access to all relevant information within EPR data immediately after the data is collected. The extensive library of common EPR spectra that comes standard with SpinFit ensures researchers have a good starting point for analysis of data, even when the sample contains unknown species. With the unique capabilities afforded by the SpinCount and SpinFit package, EPR can be applied to its full potential.

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