

# Quantitative EPR

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## Chapter 2

# Why Should Measurements Be Quantitative?

Even if the question is simply “is there a radical present?” it is important to know, e.g., whether <1 or 100% of the species are in the radical form or in a particular metal oxidation state. There are many examples in the literature in which an impurity or a slight dissociation resulted in the EPR signal observed. A goal of this book is to provide guidance on issues that the EPR spectroscopist should consider when designing experiments to obtain quantitative results. The focus of this book is on radicals in condensed phases.

Among the common type of measurements in which intensity quantitation is essential are:

- How many spins are there in a biological sample?
- What is the spin state of a metal complex as a function of temperature?
- What is the age of an archeological artifact?
- What is the radiation dose?
- What will be the shelf life of foods and beverages?
- How much free radical is produced in certain industrial materials?

Line width quantitation is essential for:

- Oxymetry
- Molecular motion
- Relating line width to relaxation times and hyperfine couplings

Magnetic field quantitation is essential for:

- Measurement of g factors
- Measurement of hyperfine splittings
- Comparison of g or hyperfine with computation of these parameters

## 2.1 Examples of Applications of Quantitative EPR

Burns and Flockhart (1990) reviewed several applications of quantitative EPR, including assays for drugs in body fluids (free radical assay technique, FRAT), radiation dosimetry, molybdenum in sea water, Fe, Mn, and even assays for diamagnetic metals by use of spin-labeled ligands. When the goal is to measure the paramagnetic component of a complicated mixture, EPR may be more selective than other common analytical techniques. An advantage of EPR comes from the fact that it can be applied to samples with scattering properties or opacity that prevent the use of quantitative optical techniques. EPR often does not require any chromatography or other separations techniques before the analysis. For example, samples with a mixture of metals can create a time consuming separations exercise that must be performed before colorimetric, gravimetric or elemental analysis techniques can even be started. EPR can also be applied nondestructively to species such as radiation defects that would not persist through the sample preparation phase for many other types of analytical procedures. Thus, it is not surprising that many reviews of EPR target the analytical community (Saraceno et al. 1961; Molin et al. 1966; Alger 1968; Randolph 1972a, b; Goldberg and Bard 1983; Burns and Flockhart 1990; Eaton and Eaton 1990, 1997; Blakley et al. 2001).

Among the many tools the analyst has available, EPR has some special advantages. For example, one can use several methods to detect the presence of a particular metal, but the EPR  $g$  values and hyperfine couplings described briefly in Chap. 1 and in more detail in Chap. 3 provide information about the metal species, not just its presence, thereby permitting speciation as well as quantitation. EPR is very specific to unpaired electrons, and thus is a very good “needle in the haystack” method. It detects only the species with unpaired electrons, ignoring essentially everything else in the sample matrix.

Transition metals with  $S = 1/2$  in common oxidation states include V(IV) (especially  $\text{VO}^{2+}$ ), Cr(V), Mo(V), and Cu(II). All of these are easily studied in solids or in solution at room temperature. Although most of the detail in this book is specific to  $S = 1/2$  spin systems, most transition metals with odd numbers of unpaired electrons and  $S > 1/2$  exhibit readily-observed EPR transitions between  $+1/2$  and  $-1/2$  spin states. Such common metal ions include  $S = 3/2$  Cr(III),  $S = 5/2$  Mn(II),  $S = 5/2$  Fe(III), and  $S = 3/2$  high-spin Co(II). Of the +3 lanthanides,  $S = 7/2$  Gd(III) has relaxation times long enough to be readily studied by EPR. The study of even-spin systems, such as  $S = 1$  Ni(II), and  $S = 2$  high-spin Fe(II), require special EPR techniques and are mentioned only briefly in this book. For more details about even-spin and high-spin species, see Grinberg and Berliner (2004), Möbius and Savitsky (2009), and Weil and Bolton (2007).

One of the most famous early applications of EPR for chemical analysis was the study by Saraceno et al. (1961) of vanadium in petroleum oils. A vanadium porphyrin was used as a standard. Analytical applications of EPR, with a focus on metal ions in solutions, are discussed by Goldberg and Bard (1983). Warren and Fitzgerald (1977) used EPR to quantitate Cu(II), Fe(III) and Co(II) ions bound to ion

exchange resins. Molybdenum in seawater was determined by converting the Mo to the  $\text{Mo}(\text{SCN})_5$  complex and extracting into isoamyl alcohol (Hanson et al. (1977)). The comparison standard was 2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylic acid.

Because of g and hyperfine anisotropies, some species have short relaxation times and broad EPR spectra at room temperature. Sometimes, changing the coordination environment can dramatically sharpen the spectrum. For example, Fe(III) exhibits broad spectra in most coordination environments, but sharpens in the presence of excess  $\text{F}^-$ . However, in spite of several reports that the EPR of the  $\text{FeF}_6^{3-}$  complex can be used to analyze for total iron in an aqueous sample (e.g., Burns and Flockhart 1990; Bryson et al. 1975), the equilibrium constant for  $\text{FeF}_6^{3-}$  formation is not high enough for quantitative analysis. A reproducible analysis could be performed under well-defined and controlled conditions, but a correction for percent complex formation would be necessary for quantitative analysis. This is an example of a case in which the EPR measurement is straight-forward, but the sample preparation is challenging.

Measurement of peroxy radical concentration permitted the determination of absolute rate constants for the autoxidation of polyolefins (Chien and Wang 1975).

Quantitative EPR has been used to determine concentrations of a very complicated spin system – a mixture of  $\text{Cr}_2\text{O}_3$  and  $\text{NaCrO}_2$  (Goldberg et al. 1977). Cr is present as Cr(III) in both species, and both exhibit antiferromagnetic interactions between Cr(III) ions. However, transition to a paramagnetic state occurs at different temperatures in these species, so double integrals of EPR spectra obtained over a range of temperatures revealed relative concentrations after taking into account sample size, temperature, etc.

Concentrations of even diamagnetic ions such as Zn(II) can be determined by EPR if they can be selectively complexed in the sampled matrix with a paramagnetic ligand, or with a ligand that can subsequently be converted to a free radical. The latter case is illustrated by complexing Zn(II) in an aqueous solution with a hydroquinone-containing ligand, which after extraction into an organic solvent subsequently was oxidized to the free radical (Burns and Flockhart 1990).

A very common problem is to determine the extent to which a spin labeling protocol was successful. Did the experiment succeed in adding one and only one nitroxide spin label to a protein or nucleic acid? What is the concentration of spin-labeled lipids in a micelle? Since such answers usually have to be obtained in aqueous samples, obtaining quantitative results implements everything discussed in this book. These problems stimulated studies such as that by Molin et al. (1966).

Whether one is measuring cigarette smoke, protein redox, or beer stability, the answers provided by EPR can be accurate within a few percent, or wrong by more than a factor of two, depending on how carefully one follows good practices as outlined in this book. A few examples of issues that should be considered in designing quantitation experiments are given below.

## 2.2 Measuring Unstable Radicals by Spin Trapping: Effect of Resonator Q

The steady state concentration of unstable radicals often is too low for direct measurement. Reaction with a spin trap converts these species to more stable trapped radicals. Common spin traps include the pyrroline derivatives DMPO, POBN, and PBN. Blakley et al. (2001) showed that there were large differences in apparent free radical concentration in identical samples depending on the resonator used in the analysis, because the standard and the sample had different effects on resonator Q (see Chap. 7). Adding 0.1 M PBN (*n*-tert-butyl- $\pm$ -phenylnitron) to a benzene solution of tempo decreased the resonator Q from 4,400 to 2,600. Only after comparing samples in which radicals were trapped by PBN with standards containing the same concentration of PBN was radical concentration agreement achieved on all three spectrometers tested. Similarly it is also possible to scale the intensity or double integration results by the ratio of the resonator Q-values that is observed from two samples.

## 2.3 Measuring Weak Signals in the Presence of Strong Ones: Dynamic Range Issues

Distances between unpaired electrons in the range of ca. 4–12 Å can be measured by comparing the relative intensity of the half-field transition to the intensity of the transitions in the  $g \approx 2$  region (Eaton and Eaton 1982; Eaton et al. 1983). The relevant formula is

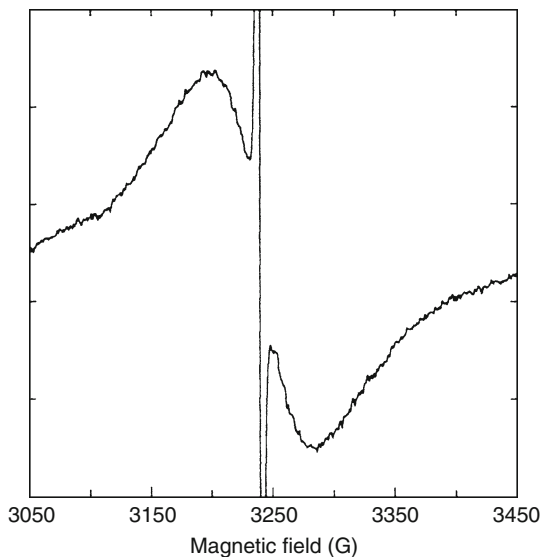
$$\text{relative intensity} = \frac{(19.5 \pm 0.5)(9.1)^2}{r^6 \nu^2}, \quad (2.1)$$

where  $r$  is the distance in Å and  $\nu$  is the microwave frequency in GHz.

The relative intensity of the half-field transition is very small. For example, at 8 Å the relative intensity is about  $7 \times 10^{-5}$ . This would be very demanding of the S/N and dynamic range of the spectrometer. However, since the area, and not the line shape, is desired, larger than normal modulation amplitude can be used to improve the S/N. Further, the half-field transition is less easily saturated than is the  $g \approx 2$  signal, so higher microwave power can be used for the half-field transition. Integration of these signals requires care about background corrections. Paying close attention to these matters, each of which is discussed in more detail later in this book, provides accurate results for an important and useful measurement.

Another aspect of dynamic range in EPR is illustrated by the spectra of radical species derived from  $C_{60}$ . After many studies it has been learned that there is a radical impurity in most preparations of  $C_{60}$ . In the early days, the spectrum of this radical impurity was incorrectly identified as the spectrum of  $C_{60}^-$ . The problem

**Fig. 2.1** CW EPR spectrum of the  $C_{60}$  anion radical with a sharp impurity signal superimposed. From Schell-Sorokin et al. (1992)



was that this spectrum is very narrow, so when it was full scale, the spectrum of the anion was weak enough that it was possible to miss it. Figure 2.1 shows the broad spectrum of  $C_{60}^-$  when the narrow spectrum is off-scale (Schell-Sorokin et al. 1992).

## 2.4 Signals in Mixtures

Not all samples to which one might want to apply quantitative EPR are “clean”. One interesting problem is to correlate the EPR spectrum in Fig. 2.2 to the age of a blood stain (Fujita et al. 2005). The ratio of the signal labeled H to that labeled g4 provided a linear log–log plot up to 432 days, as shown in Fig. 2.3 (Fujita et al. 2005).

## 2.5 Radiation Dosimetry

Almost anything used in a hospital operating room is sterilized either with  $\gamma$  rays or e-beam. Many other items, such as food and mail are also treated with ionizing radiation. To ensure sterility (and avoid the legal liability if goods are not sterile) and to run a cost-effective business, the radiation dose measurement must be reliable, reproducible, accurate, and often performed by unskilled laborers. Also the results must be easily transferred to a LIMS (Laboratory Information Management System) for auditing purposes. The preferred method of measuring the radiation dose used for sterilization is EPR radiation dosimetry using alanine dosimeters.

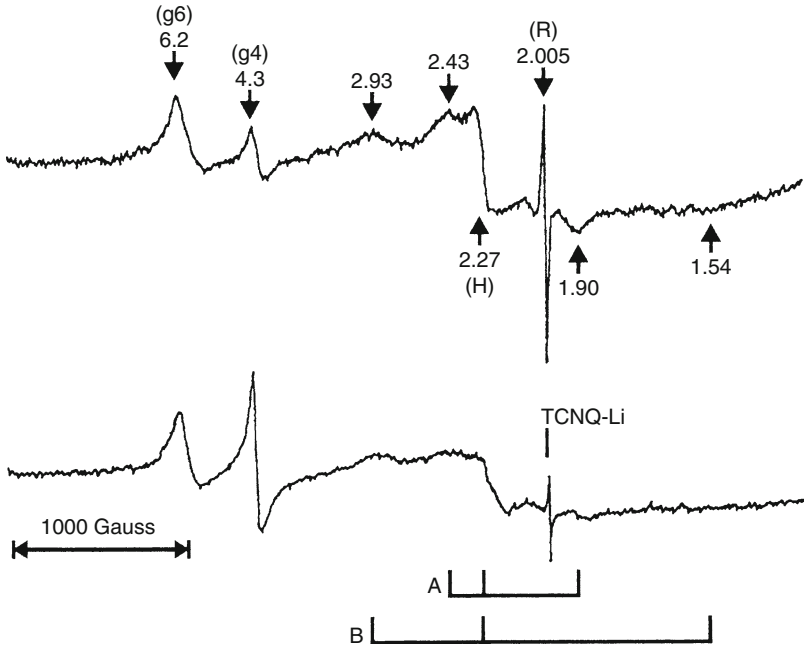


Fig. 2.2 EPR of a blood stain. From Fujita et al. (2005)

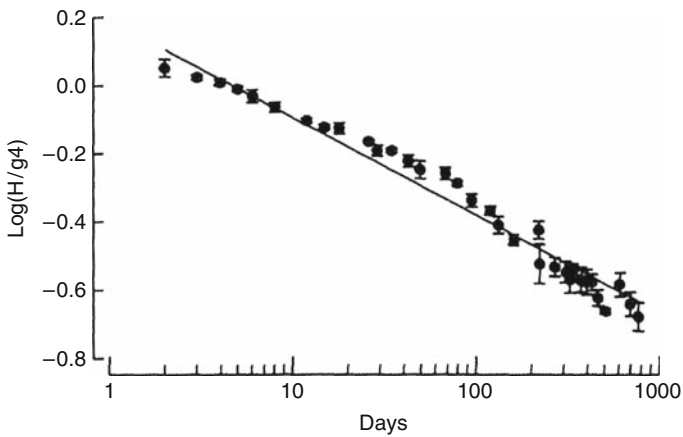


Fig. 2.3 EPR intensity vs. age of dried human blood stains. From Fujita et al. (2005)

The Bruker e-scan benchtop EPR spectrometer has been specifically designed for such applications. The resonator incorporates a special sample holder that reproducibly positions an alanine film or pellet dosimeter in the resonator. The sample holder also has a relative intensity reference standard so that variations in instrument response can be used to normalize the intensity. A bar-code reader is

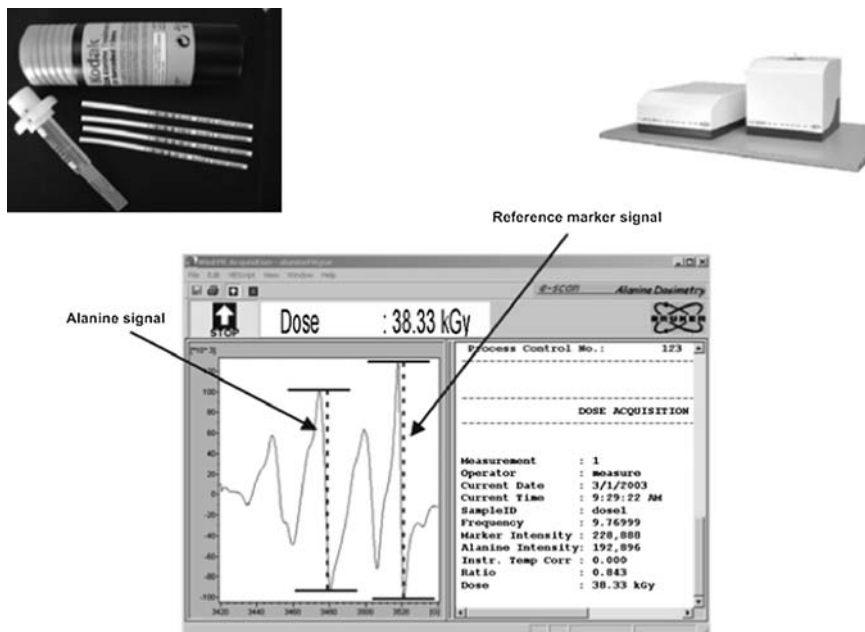


Fig. 2.4 Bruker e-scan with film holder and bar-coded film

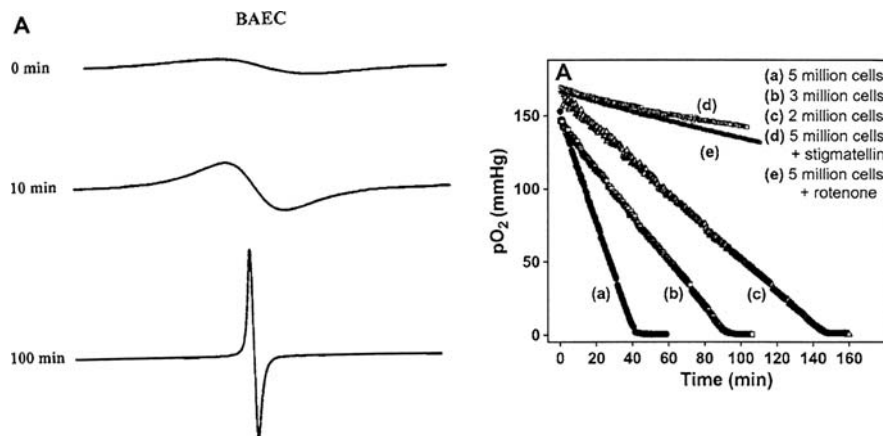
incorporated so that the individual dosimeters are identified and the results logged properly into the quality control database.

Through careful control of sample positioning, size, and properties, the instrument provides reproducibly better than 0.5% (Fig. 2.4).

In vivo radiation dosimetry is also an important application of quantitative EPR. For a review of current efforts, see Swartz et al. (2006).

## 2.6 Use of Accurate Line Width Information

Current effort in many laboratories (refer to the 2004 Workshop on EPR Imaging available on the Bruker BioSpin website) seeks to use quantitative EPR to measure  $O_2$  concentration in vivo. This is a case in which accuracy of line width information is more important than amplitude or g value. Some papers since the 2004 Workshop illustrate successful steps in this field. Halpern and coworkers have carefully calibrated the EPR oximetry method against the standard (more invasive) fluorometric probe method that requires inserting a fiber optic probe into the animal. There is good agreement, and the EPR method has the advantage that it provides three-dimensional information as opposed to just a spot measurement. In addition,



**Fig. 2.5** The line width of the X-band EPR spectrum of LiPc microcrystals in a suspension of bovine aortic endothelial cells (BAEC) decreased with time as the cells consumed the oxygen that broadened the EPR signal of the LiPc. The EPR measurements were made with sufficient accuracy to define three phases of cellular respiration. From Presley et al. (2006)

EPR does not damage the tissue that it measures (Elas et al. 2006). Presley et al. (2006) used EPR oximetry to measure cellular respiration (Fig. 2.5).

## 2.7 Catalysis and Mineralogy

Many applications of quantitative EPR to catalysis and mineralogy were summarized by Dyrek et al. (1994, 2003). Catalytic systems often involve metals in multiple oxidation states and multiple coordination environments. When one of more of these states is paramagnetic, EPR can provide considerable information about the changes in the materials. For example, Sojka and Che (1995) studied species generated during the oxidation of methanol on Mo/SiO<sub>2</sub> catalysts. EPR distinguishes tetra-, penta- and hexa-coordinated Mo(V) species on the SiO<sub>2</sub> substrate, and shows the presence of CH<sub>2</sub>OH radical on the surface. When the catalyst was reacted with N<sub>2</sub>O, the EPR signal of O<sup>-</sup> radicals was observed. The full catalytic cycle was mapped out with the aid of EPR spectra obtained as a function of reagent concentrations and of time at low and room temperature.

## 2.8 Free Radical Content in Commercial Materials

Free radicals are produced in many industrial processes and can have an adverse effect on the quality of a commercial material. Oxidative reactions caused by light, heat, transition metal catalysis (or any combination of the three) are often unavoidable in a large scale manufacturing method. EPR provides a rapid and simple

analytical technique for evaluating the effect that industrial process changes have on the free radical content of the material. The manufacturer can actually use the EPR instrument to optimize their processing methods; then also use EPR as a quality control technique once the optimized method has been put into routine use. In these cases, the operator is not usually concerned with the exact type of free radical that is produced, but merely how much is produced. Thus, relative EPR intensity quantitation of the signal of interest is employed. Relative intensity standards (discussed in Appendix E) help to reduce the uncertainty of the measurements which can often be as low as 1–2%. Some examples of industrial applications that use relative intensity measurements for process and quality control are listed below.

- Shelf life stability in beer
- Rancidity control in vegetable oil based foods
- Irradiation of polymers for use in orthopedic implants
- Oxidation of automotive paint
- Oxidation of pharmaceutical drug excipients
- Free radical production in cigarette smoke

## 2.9 Feasibility of Quantitative EPR

In this chapter a few of the many applications of quantitative EPR are illustrated. Unfortunately, EPR has previously been judged as inadequately quantitative due to some early inter-laboratory comparisons. Amongst the very practical guidance on EPR techniques in his 1968 book, Alger reported (pp. 202–206) comparative measurements by seven leading laboratories of the number of spins per gram in samples of irradiated sucrose that were circulated among the laboratories and compared with local standards. The range of results between laboratories was as large as 1:1.6. Goldberg (1978) documented that some early EPR spectrometers were not constructed for the degree of precision now desired. In 1994 Yordanov and Ivanova reported comparison by 12 laboratories of samples of Mn(II) and pyrolyzed sucrose. The results “are characterized by a big variance”. These studies should not discourage readers of this book – it is possible, even fairly easy, to obtain good quantitative EPR results. The rest of this book explains how. EPR is based on straight-forward physical principles, and as such, must be quantitatively accurate if the relevant experiment parameters are properly set and controlled. Stimulated by this belief, Dalal et al. (1981a, b) and More et al. (1984) documented the effect of lossy solvents on resonator Q and the effect of solvents, sample tubes, and variable temperature Dewars on  $B_1$  at the sample. The key issue in quantitative EPR is sampling and sample preparation, as is the case for any other analytical method. Beyond that, the interaction of the sample with the spectrometer is important in EPR, so this book provides extensive guidance about that aspect. One should not overemphasize the “uniqueness” of this interaction in EPR. Often, introductions to

EPR and other types of spectroscopy invoke the presumably easily understood concept of transmission visible spectroscopy, so an example from visible spectroscopy is presented here. It is very easy to obtain non-quantitative results in visible absorption spectroscopy with elementary errors of technique, such as non-perpendicular orientation of the sample cell, a small sample that is not in the center of the beam, dirty sample cells, concentration outside the linear range of the spectrometer, and so on. It is the EPR analogs of these elementary mistakes from visible spectroscopy that this book teaches one to avoid.

Unlike early spectrometers, modern commercial EPR spectrometers are designed to provide high levels of linearity and precision in the relevant parameters. Modulation amplitude can be accurately calibrated, and power sensors are incorporated so that the microwave power is known accurately. In the latest spectrometers even the slight variations over the frequency range of the spectrometer are calibrated and stored in the spectrometer. All of the spectrometer settings that this book recommends for quantitative EPR experiments can be included in the software of a spectrometer that is totally computer controlled and calibrated. Thus, the newest spectrometers can include an almost automatic “spin-count”. The operator is still responsible for sample preparation and for making sure that all of the assumptions are met and spectrometer parameters are selected appropriately.

To be concise, and precise, the guidance from this book uses specific Bruker hardware/software examples, but the lessons, themselves, are general. Users of other types of EPR spectrometers, or users of earlier or later versions of Bruker hardware and/or software should have little difficulty translating the specifics given in this book to their local hardware/software environment.

## 2.10 Further Reading

Bolton, Borg, and Swartz (Bolton et al. 1972) summarized selection of experimental parameters, and Randolph discussed quantitative aspects, for the study of biological samples (Randolph 1972a, b).