Signal Area Measurements in EPR

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I. THE IMPORTANCE OF SIGNAL AREA MEASUREMENTS IN EPR

Although the sensitivity and dynamic range of EPR are high, the precision of concentration measurements has often been low. Consequently, EPR spectroscopy has a reputation as a rather nonquantitative method. Interlaboratory comparisons of signal area measurements many years ago yielded very discouraging results (1). Since then there have been important improvements in instrumentation and useful quantitative studies of the spectrometer-sample interaction. It is now realistic to aspire to much greater accuracy in EPR signal area measurements.

In many research problems EPR is the only technique that can provide desired information. Of the five fundamental types of information that can be obtained by EPR (*g*-value, electron-electron and electron-nuclear spin-spin coupling, line shape, intensity, and relaxation times), this review is concerned only with intensity. Often it is necessary to answer the question: "How many spins are present?" In metalloenzyme studies, for example, it could be important to know whether all of the copper and iron atoms in a particular system yield EPR signals. The relative concentrations of metal species and organic free-radical species in a system could also be crucial to interpretation of a mechanism. Although it seems almost too trivial to mention, it is important to know whether the EPR signal being displayed represents all of the species in the sample or only an impurity or decomposition product. Lack of attention to order-of-magnitude quantitation can lead to publication of impurity or decomposition spectra even in cases in which the bulk of the sample has an EPR spectrum but with greater line width, or to publication of background spectra as spectra of the sample. These egregious errors can be avoided, and

indeed it is possible to attain quantitative accuracy to within a few percent in many cases if attention is paid to the details of the literature results, which are summarized in this review.

The assumption is made throughout that spectra are obtained under adiabatic slow-passage conditions.

II. THE SPECTROMETER

In EPR spectroscopy it is more important than in most other instrumental techniques to understand details of the way the spectrometer works and to calibrate separate functions of the spectrometer. The general principles in the following discussions apply to most spectrometers that use a reflection cavity and magnetic field modulation, but the details relate specifically to the Varian E-3 and E-line spectrometers since most relevant data in the literature are for these spectrometers. Other than improvements in the electronics, stability, ease of use, and so on, the major change in the E-line series relative to the V-4500 series was the use of the "reference arm" bridge. The reference arm bridge was a major contribution towards making routine quantitative measurements of signal area possible. The new Varian "Century" series spectrometers provide new capabilities such as saturation transfer spectroscopy and further improvements in electronics, but use the same cavity as the E-line and are essentially identical to the E-lines series with respect to the signal area measurements discussed in this review. A detailed consideration of linearity in Varian V-4500, E-3, and E-line spectrometers was published recently by Goldberg (2).

A. System Linearity

Modern spectrometers are manufactured with high-quality, well-matched components so that a change in a dial setting from 1 to 2 usually results in doubling the parameter to within a percent or so. However, to achieve tight specifications there are usually several adjustable resistors, which might need adjusting.

1. Magnetic Field

Field calibration is needed both for g-value measurements and area measurements. The most accurate way to calibrate the magnetic field is with an NMR gaussmeter. When magnetic field measurements are examined in detail, it is found that the field at the position of the sample can be significantly different from the field at the position of a gaussmeter probe outside the cavity and that the reproducibility of this difference can be the limiting accuracy in very careful g-value measurements (3, 4). The area of a first-derivative EPR signal is proportional to the width squared. The accuracy is more dependent on the linearity of the magnetic field scan than on the precise magnitude of the field strength. For signal area measurements it is usually adequate to ensure that the magnetic field is linear to within ca 0.1% of the field scan width. If a gaussmeter is not available, several published studies of readily available radicals can be used as the basis for calibration. Wertz and Bolton (5) tabulate the major lines in Würster's blue perchlorate. Other samples which have been suggested include Fremy's radical (6), a manganese-containing mineral (7),* and $[Cr(NH_3)_5Cl]Cl_2$ diluted in $[Co(NH_3)_5Cl]Cl_2$ (8). Even though the lines are broad for some of these samples the field positions can be identified with sufficient accuracy for the field linearity calibration required for area measurements. If the magnetic field is found to be significantly nonlinear, careful mathematical treatment of line width and line position data would be necessary. We have found our Varian E-9 to be linear to within recorder-arm settability over the full range of the instrument. Magnetic field linearity is probably not a major source of error with modern spectrometers.

2. Amplifier Gain

The linearity of the amplifier is easily checked with any stable sample merely by measuring chart displays of spectra run with different amplifier-gain settings. It should be noted that some nonlinearities may be introduced at very high gain settings. Consequently it is important to determine the range of linearity even in modern spectrometers (e.g., the Varian manual for the Century series spectrometer cautions against operation with the "receiver level" much beyond midscale). The signal area is linearly dependent on the true amplifier gain. Goldberg found substantial nonlinearity in older spectrometers (2).

3. Modulation Amplitude

The EPR signal area is linearly dependent on the amplitude of the magnetic field modulation, even when the sample is "overmodulated" (i.e., when the modulation amplitude is greater than 1/10 of the line width and peak distortion occurs) (9). This feature can be helpful since spectra that would be too noisy for integration if obtained with a nondistorting modulation amplitude can be overmodulated to obtain a better signal-to-noise ratio. Similarly, very narrow spectra can be broadened

*The authors of ref. (7) claim that the mineral used was forsterite, but the spectrum is not the same as that previously reported for forsterite by A. Chatelain and R.A. Weeks, *J. Chem. Phys.* **52,** 5682 (1970). We thank Dr. Ira B. Goldberg for bringing this to our attention.

to improve spectral fidelity in digitized spectra. The mathematical aspects of field modulation with phase-sensitive detection are too long to reproduce here (10-13), but some results are worthy of consideration. With modulation at frequency ω and amplitude A, the output of a phase-sensitive detector operating at ω is the first derivative. The 2ω component is the second derivative, with signal amplitude proportional to the *square* of the modulation amplitude. If I_n represents the area under the n th derivative display and the actual signal intensities are identical, the results of numerical integration (first derivative integrated twice, second derivative integrated three times) would be (5):

$$I_2 = \frac{I_1 A}{4}$$

Since second derivatives are routinely accessible on new instruments (second-harmonic "in phase" detection), these features of first- and second-derivative spectra will become increasingly important in quantitative EPR spectroscopy.

Since the amplitude of the chart display of first-derivative spectra will be linearly proportional to modulation amplitude only for small modulation amplitudes (modulation amplitude <0.1 times line width), it is necessary to calibrate large modulation amplitude settings either by double integration using a standard sample or by measuring the modulation amplitude via the distortion of the signal as outlined by Poole (11). Goldberg suggests using a sample of anhydrous $MnSO_4$, which has a line so broad (380 G) that even high modulation amplitudes will not broaden the spectrum much (2).

4. Crystal Detector Response

Modern EPR spectrometers are designed so that the crystal detector in the microwave bridge operates in the region in which the signal amplitude should increase as the square root of the power (P) incident on the cavity. Thus a plot of signal amplitude vs $P^{1/2}$ should be linear if the signal is not saturated. It is, of course, important to verify that signals whose areas are to be determined are not significantly saturated. Some workers interested in quantitative chemical analysis via EPR have performed least-squares fits of log (signal amplitude) vs log P. Instead of 0.5, the exponents of P were found to have values from 0.48 to 0.55 (14). It has recently been pointed out that these results were not obtained with a critically matched cavity (2). Goldberg obtained an exponent of 0.5000 ± 0.0010 on an E-3 spectrometer (2). For a sample that does not saturate at high power, such as a transition-metal complex at room temperature, the validity of the expected $P^{1/2}$ dependence should be checked for each spectrometer.

It should also be noted that the output of the crystal detector depends on the magnitude of the bias current to the detector. If the detector current drifts, significant errors in signal area can result.

B. Microwave Magnetic Field

As discussed above, the area of an EPR peak is expected to be proportional to $P^{1/2}$ for a nonsaturating sample. The microwave magnetic field, H_1, is linearly related to $P^{1/2}$. The precise proportionality has been examined in detail by various workers (15-32) with slightly differing results. Varian states that the *peak* H_1 field in the rectangular TE_{102} E-231 cavity in the absence of sample, sample holder, or Dewar is given by

$$2H_{1\,max} = 2.95 \times 10^{-2} (QP)^{1/2} \text{ gauss}$$

where P is the incident power in watts and the cavity Q factor is defined as

$$Q = \frac{2\pi \text{ (maximum microwave energy stored in the cavity)}}{\text{energy dissipated per cycle}}$$

Varian states that the cavity Q is 3500 when the cavity is matched (33). Clearly, changes in cavity dimension due to temperature variation and anything that changes Q will change the proportionality between H_1 and $P^{1/2}$. Since everything the experimentalist inserts into the cavity changes Q, it is very difficult to obtain an estimate of H_1. This is probably the largest source of uncertainty in EPR peak area measurements. The following paragraphs delineate some of the major influences various researchers have investigated.

1. Normal Distribution in the Cavity

Most commercial EPR cavities have a cosine distribution of H_1 along the length of the cavity, with a peak at the center:

$$H_1 \propto \cos\left(\pi \frac{x}{L}\right)$$

where L is the cavity length along the sample and x is the distance from the cavity's center. The deviations from the theoretical cosine distribution, presumably caused by the finite size of the opening through which the sample is inserted, have been probed by the method of perturbing spheres (27). H_1 decreases less rapidly with increasing x/L than the cosine function predicts (see Figure 2 in reference 27).

Thus not all portions of a finite sample see the same H_1 field. Consequently it is important that samples whose signal areas are to be compared have the same linear dimensions unless they are small enough to be

"point" samples or extend beyond the active volume of the cavity. Treatment of intermediate sized samples is considered by Mailer et al (34) and Goldberg et al (35). It is worth noting that if H_1 is large enough to cause saturation, different parts of the sample will be saturated to different extents, yielding inaccuracies in the results of continuous wave relaxation measurements (27, 30, 34).

2. Distortion by Variable Temperature Equipment

Insertion of any material into the EPR cavity changes both the resonant frequency of the cavity and the distribution of electric and magnetic fields in the cavity. This can be a major source of error if it is not recognized, but it can also be exploited to improve signal-to-noise ratios of nonsaturating samples.

Introduction of metallic material shifts the resonant frequency to larger values, while introduction of dielectric material shifts it to lower values. The shift in frequency can be a significant fraction of the resonant frequency. For example the resonant frequency of the E-231 cavity shifts from ca 9.5 GHz (empty) to ca 9.1-9.3 GHz upon introduction of the standard variable temperature (VT) quartz Dewar. The magnitude of the shift depends on the diameter and wall thickness of the Dewar. The cylindrical quartz Dewar concentrates the H_1 field within the Dewar. Several publications (36-38) provide experimental data on the H_1 concentration effect of quartz inserts. With a nonsaturating copper complex we found that the signal area with a VT insert was 1.73 times the area without the VT insert. Since the area is proportional to $P^{1/2}$ this result implies that the effective power density at the sample with the VT insert was three times higher than without the insert. This would be equivalent to 600 mW (maximum) instead of the 200 mW (maximum) indicated by the spectrometer dial settings. Different results could be anticipated for other VT inserts, or even for different positioning of the same VT insert, due to variations in wall thickness, camber, and so on. Each experimental arrangement must be calibrated.

If the sample does not saturate at high power, a quartz sleeve around the sample can be used to concentrate H_1 in the sample to obtain a higher signal-to-noise ratio and hence to improve the precision of area determinations.

3. Distortion by the Sample

It is evident that the considerations discussed above for the VT insert also apply to the sample. A tube containing a sample will concentrate H_1 in the sample, and the sample itself could affect the distribution of H_1. It is obvious that one needs to know the internal dimensions of a tube in order to calculate the amount of sample per unit length. The sample tube should therefore be calibrated like any other volumetric glassware. The wall thickness of the tube is also important because of the H_1 concentration effect. For careful work the same tube, or tubes calibrated relative to one another, should be used for signal area determinations.

Placement of the sample tube in the cavity is also of concern, and it has been suggested that the sample tube should be left in the cavity and the sample changed by flow or syringe techniques or that a rigid mounting should be used (39, 40). The effect of sample tube variations will be less if the VT insert is in the cavity than if the cavity is otherwise empty (30). No comparison of these sample tube considerations has been published. With the standard Varian collets and sample supports and commercially available standard quartz tubes, we find that sample placement contributes less than one percent uncertainty to area determinations for line samples such as liquid solutions extending the length of the cavity. Sample placement is more important for small "point" samples and for liquid samples in a flat cell than for line samples. Nonuniform packing of solids or heterogeneity of samples can make sample placement more critical for solid samples than for liquid samples in the same type of sample holder.

When a dual cavity is used to obtain EPR spectra, particular attention must be given to the effect of the samples on the H_1 distribution. When a sample is introduced in one half of a dual cavity, there is a shift in the position of the maximum H_1 field in the other half of the cavity (41). The shift (as a fraction of wavelength) is of the order of the relative change in cavity resonant frequency (41). Thus for most small samples the shift in H_1 distribution is of the order of the sample positioning uncertainty. However, if a large dielectric, such as the VT insert, is introduced in one half of a dual cavity the position of H_{1max} can shift by 1-2 mm toward the Dewar (41). Rataiczak and Jones (30) observed a 0.8 mm shift. Clearly, great caution is required even in the case in which a reference sample is kept in one half of a dual cavity while "unknowns" are substituted in the other half.

C. Modulation Amplitude Distribution in the Cavity

The details of magnetic field modulation vary for different cavity types. Some early cavities used an internal loop of wire to generate the modulation field. The E-231 and V-4231 cavities use circular modulation coils mounted in the sides of the cavity. The following discussion applies to the E-231 cavity except where specific reference to other cavities is made.

It has been found experimentally (34) that the amplitude of the modulation field along the central axis of the

E-231 cavity is approximately proportional to $\cos^2(\pi x/L)$. Comparing this result with the cosine distribution of H_1, it is evident that most of the signal intensity results from sample close to the center of the cavity. This helps to decrease the impact of the H_1 distribution on power saturation studies described in Subsection II.B.1, but errors of as much as a factor of 1.8 in $P_{1/2}$ can still result (34). $P_{1/2}$ is the power at which the signal amplitude is half the value expected in the absence of saturation. With a solid sample this sharp-peaked function of H_1 and modulation amplitude requires uniform packing in the sample tube and careful placement of "point" samples.

Varian has recently marketed a TM_{110} E-238 cavity designed for use with lossy samples. The distribution of the modulation field in this cavity deserves special mention. The cavity walls on which the modulation coils are mounted are farther apart in the E-238 cavity than in the E-231 cavity. The amplitude of the modulation field is 59% greater at the sidewall than at the center of the cavity. Hence the dimension of the sample in this direction is also important in signal area determinations, but no analysis of the sample geometry effect has been published beyond the curves in the Varian manual (42).

D. Temperature at the Sample

The EPR signal intensity depends strongly on temperature because of the Boltzmann population of spin states. The lower the temperature the higher the signal intensity for a normal doublet state molecule. If singlet-triplet equilibria or other spin state changes are involved, the temperature dependence is more complicated. Molecular conformational dependence on temperature with resulting changes in zero-field splittings, exchange interactions, and so on, can also have a strong effect on signal area. For molecules in solution, changes in molecular tumbling rate and ligand-exchange dynamics that attend temperature changes can result in changes in the measured intensity (43). A related effect was seen for protein-bound Mn^{2+}, which showed only 0.26 times the signal area of an equal concentration of Mn^{2+} free in aqueous solution, because only $M = \frac{1}{2}$ components were observable in the former case (44).

The assumption usually made is that the spin susceptibility follows the Curie law unless one of the features mentioned above obtains. This has been shown to be an oversimplification in some cases since the Weiss constant, θ, in the Curie-Weiss law is not negligible (45-48). The Varian pitch sample was found to have $\theta = 5$ K (47), but for diphenylpicrylhydrazyl (DPPH), values of θ between -10 and -55 K have been reported (47). These workers conclude that a determination of the number of unpaired spins in a sample requires use of $T-\theta$ instead of T, so that even if the standard and the unknown are at the same temperature, the comparison is not valid unless the temperature dependence of the spectral area of both samples is known (45-48). This appears to be a potentially large source of error overlooked by most other workers.

With the standard commercial variable-temperature accessories, the temperature at the sample is not known accurately. The Varian manual provides data illustrating a 5°C gradient along a one-inch sample with an average temperature of 300°C (49). A smaller gradient (ca 0.4°C) occurs at -160°C (49). Since the thermocouple used to monitor the sample temperature must be located in a nonperturbing position, the difference in temperature between its location and the center of the cavity must be calibrated. It should be noted that this difference will depend on the rate of flow of the heated or cooled gas. Thus it is necessary to calibrate the reproducibility both of the thermocouple positioning and of the temperature control system. Clearly, errors of greater than a percent can be introduced in peak area comparisons for non-ambient measurements unless care is taken to ensure accurate calibration and reproducibility of conditions. The variation of temperature over the length of the sample can be important in kinetic studies, determination of melting curves, and so on.

E. Background Signals

Perfectly flat background spectra seldom occur in EPR. The importance of correcting for background signals, whether due to impurities, thermal drift, or magnetostriction (which causes magnetic field dependence of the cavity response), depends on the relative intensity of the signal whose area is to be estimated. Since the area is proportional to $(\text{width})^2 \times (\text{height})$, a very broad signal that is not visually distinguishable from noise in the first-derivative spectrum can dominate the second integral. Unless the signal of interest is strong relative to the background, subtraction of the background prior to integration is essential. To permit background subtraction the AFC gain setting must be the same for the two spectra.

III. THE SAMPLE

Because of the intimate interaction between the sample and the EPR spectrometer, many aspects of the effect of the nature of the sample on signal-area estimation have already been discussed in II, particularly, the effects of sample and sample-holder size, shape, and dielectric properties (II.B.3, II.C).

A. Sample Size

The use of samples with very intense EPR signals does not improve the accuracy of signal quantitation because of the impact of the sample power absorption on the spectrometer response. It has been demonstrated that unless the power absorbed by the sample at resonance is a negligible fraction of the incident power, the cavity Q will change significantly across the signal (50). Detailed examinations of the properties of cavities containing large samples have been published (51, 52). Very intense EPR signals are broadened and have smaller calculated areas than one expects from experience with more dilute samples (52).

B. The Sample Matrix

1. "Lossy" Solvents

Of particular importance in EPR is the value of the complex dielectric constant ($\epsilon - i \tan \delta$) of the sample container and sample matrix at the frequency of the experiment. The dielectric loss factor (often called the loss tangent), tan δ, is of particular importance in the microwave region. Background and some data are given in references (53, 54). Unfortunately data are not available for many solvents at normal EPR frequencies. The loss tangent is temperature dependent (48, 53) and sometimes shows a maximum. For example, CH_2Cl_2 is very lossy below room temperature. The loss tangent becomes very small when the solvent is frozen. The loss tangent affects the EPR signal-area determination via its effect on the cavity Q: a lossy sample lowers the Q and hence H_1 at a given power, yielding a lower apparent spin concentration (for a nonsaturated sample). Few quantitative comparisons have been found in the literature (see, e.g. (48, 55)). The dielectric properties of the solvent also have a "lens" effect on H_1, just as does the sample tube (38, 48).

2. Conductivity

Even though the EPR spectrometer may be tuned to observe the absorption signal, it is possible for the observed line shape to be a mixture of absorption and dispersion. There seems not to have been any systematic study that would indicate those conditions under which dispersion contributions are important. Asymmetry of EPR lines of conducting aqueous solutions has been attributed to mixing of the real and imaginary parts of the susceptibility (43, 56). Methods of finding the relative absorption and dispersion contributions and for correcting the center and line width have been published (43, 57).

The line-shape asymmetry introduced by mixing dispersion and absorption components clearly will cause problems in signal-area determinations by approximate methods such as (width)^2 × (height). Errors result even when a full double integration is performed, because concomitant with a mixing in of the dispersion signal there is a loss of the absorption signal, and the second integral of the derivative dispersion signal is zero.

The conductivity of the sample also affects the depth the microwaves can penetrate into the sample. The skin depth, δ, is defined as the distance from the surface of a plane conductor at which the electric and magnetic fields have decreased to e^{-1} of their values at the surface (58). For a highly conducting sample only a very thin layer δ contributes to the EPR signal:

$$\delta = 5.03 \times 10^3 \sqrt{\frac{\rho}{f}}$$

where ρ is the resistivity in ohm-cm and f is the frequency in Hz. Hence there is an optimum sample size from a signal-to-noise ratio standpoint. If the sample size is a significant fraction of the skin depth, substantial errors can be introduced by small changes in sample size. Slangen suggests that the conductivity not exceed 0.01 ohm^{-1}m^{-1} (48).

C. Relaxation Times

Quantitation of EPR signals is affected by extreme electron spin relaxation times, i.e., very short and very long relaxation times. If the relaxation time is very short the EPR signal could be so broad that a proper baseline for the integration cannot be achieved. In extreme cases the signal may be so broad as to be unobservable (e.g., most paramagnetic Ni(II) species). A broad signal in the presence of a narrow signal constitutes a major problem because of the limited amplitude and field resolution of current computer-interfaced spectrometers. If relaxation times are very long it may be difficult to achieve reasonable signal-to-noise ratios and spectrometer stability without saturating the spectrum.

D. Line Shape

Since most EPR signals are not pure, homogeneously broadened, single Lorentzian lines, any attempt to compare areas of signals other than by full double integration can lead to serious errors unless the line shapes happen to be the same. Some indication of the potential for error is given by the fact that when a Lorentzian and a Gaussian line have the same derivative amplitude and the same peak-to-peak width, the Lorentzian line corresponds to 3.51 times as many spins as the Gaussian line (59). Even a full double integration can lead to errors because of the significant area under the wings of the spectrum (2).

E. *g*-Value

In a very important recent paper Aasa and Vanngard (60) demonstrated that the integrated intensity of a field-swept EPR signal is proportional to its *g*-value and not to g^2 as had been concluded previously. The use of g^2 is correct for frequency-swept spectra. All prior work was in error. This correction must be kept in mind when taking formulae from standard texts and review articles. Although this correction is not very important when comparing species with *g*-values very close to 2, it could change published conclusions concerning the relative concentrations of species with very different *g*-values (e.g., $g = 4$ or 6 vs 2).

F. Spin Multiplicity

In solutions of low viscosity the integrated signal intensity of species with two or more unpaired electrons is proportional to $S(S + 1)$, where S is the total spin. However, the width of the individual transitions depends on the symmetry of the immediate environment of the ion and the rate at which it is tumbling (61). Thus, in many cases more extensive study is necessary to determine which transitions are being observed (one case involving Mn^{2+} was cited above) before a conclusion on the significance of the area can be reached.

IV. RECORDING THE SPECTRUM

In quantitative work it is especially important to ensure that the spectral display is not distorted by scanning too quickly relative to spectrometer time constants, and to ensure that the spectrum is not saturated. But as described above, it is acceptable to overmodulate the spectrum when the area is to be determined by direct double integration. Particular attention must be given to the significant fraction of the total intensity that is in the "wings" of a Lorentzian line. Some examples for calculated spectra have been published (2, 46, 48, 62-64). Often, signal-to-noise and baseline-flatness problems will limit the scan width over which the integration can be performed before errors from these sources make matters worse.

There is no question that the labor involved in manually integrating spectra makes direct digital recording of spectra a practical necessity. The use of a computer introduces new constraints that have to be considered. Of particular concern is the number of data points used to define the spectrum and the resolution of the analog-to-digital conversion of the amplitude. It is possible to get erroneous results, especially for the case of narrow lines in the presence of broad lines, if there are not sufficient points to define the narrow lines. Six to ten points for each peak-to-peak line width seem to be adequate.

V. ESTIMATING THE AREA

Prior to the availability of computer-interfaced spectrometers, various approximate methods were developed to facilitate double integration of EPR spectra (1, 63, 65). The simplest method for first derivative spectra is to make use of the proportionality.

$$\text{area} \propto (\text{width})^2 \times (\text{height})$$

This relationship is validly applied to any line shape (66), but it is critical that the line shapes of the spectra being compared be identical or differ in a mathematically defined way so that a correction can be applied. In practice it is probably not reasonable to use (width)^2 × (height) unless the line shapes are confirmed identical. If the line shapes are identical and the widths are identical, then subtracting the spectra is a very convenient way to compare concentrations. This is a very special situation, but one that is frequently of interest in the case of overlapping spectra (67).

Clearly, double integration is the "best" way to estimate the area under the EPR signal when the signal of interest can be separated from background, overlapping signals, and so on. Examples are given for on-line analysis (67) and for off-line analysis (68).

VI. STANDARDS

It is more common to attempt to estimate the relative spin concentrations of two samples than to estimate the absolute number of spins in a sample based on detailed calibration of the spectrometer response (69). Thus, even a "total spin concentration measurement" is in practice a measurement relative to a standard sample. In view of the many cautions outlined above it is evident that the "best" standard is the one that can be most accurately compared with the sample being studied. Commonly this will be a sample that is similar to the sample being studied although the trade-off between accuracy of sample preparation and the accuracy of corrections for differences between samples must be taken into consideration.

The National Bureau of Standards (70) has issued a synthetic ruby ($Al_2O_3:Cr^{3+}$) sample (SRM 2601) as an EPR intensity standard, which could be useful for some cases. However if the interest were in nitroxyl free radicals it would be better to prepare gravimetrically a sample of a highly purified nitroxyl radical as the standard.

Other species that have found use as concentration standards include DPPH (1, 5), $MnSO_4 \cdot H_2O$ (5, 52), Mn in

MgO, Mn in ZnS (71), $K_3Mo(CN)_8$ (72, 73), $Cu(NH_3)_4^{2+}$ (74), $CuSO_4 \cdot 5H_2O$ (1, 5), $(C_6H_5 \cdot C_6H_5)_2$ $CrOC_6H_5 \cdot 4H_2O$ (46), $CuCl_2 \cdot 2H_2O$ (46), 4-benzoyl-2,2,6,6-tetramethylpiperidino-1-yloxy (46), $CrCl_3$ diluted with KI (75), di-*p*-anisylnitroxide (75), Fremy's salt $[NO(SO_3)_2]^{2-}$ (5, 75, 76), $CuCs_2(SO_4)_2 \cdot 6H_2O$ (75), carbon samples formed at 500°C (77), powdered coal (38, 48), 4-chloro-2,2,6,6-tetramethylpiperidino-1-yloxy (76), 2,2,5,5-tetramethyl-3-pyroline-1-oxyl-3-carboxylic acid (78), di(1-cyanocyclohexyl) nitroxide (48), $MnCl_2$ in gelatin (34), vanadyl etioporphyrin (79), Mn^{2+} in forsterite (7), 4-keto-2,2,6,6-tetramethylpiperidino-1-yloxy (80), 2,2,6,6-tetramethylpiperidino-1-yloxy (5), and silicon doped with phosphorus (81).

VII. OTHER SOURCES OF INFORMATION

Sources that provide amplified discussion of some of the items discussed in this review include (1, 2, 63, 82-84). The considerations involved in the use of EPR for quantitative determination of metal ions have been summarized by Warren and Fitzgerald (73, 85). A review of analytical applications of EPR has been prepared (45). The details of the analysis of some Cr samples by EPR have been described (71). The use of EPR for quantitative determinations in the gas phase was summarized by Westenberg (86). Error analyses of EPR experiments have been discussed (38, 46, 48, 63). Use of a transmission EPR spectrometer for determination of spin concentration was described by Slangen (48). Special features of quantitation of heme EPR signals have been discussed (87).

VIII. CONCLUDING COMMENTS

The above discussion has treated the spectrometer as if it were a well-defined species, but in fact an EPR spectrometer is a collection of modules (irrespective of the cosmetic aspects of commercial packages). Changing cavities, changing modulation frequencies, or "tuning up" some part will usually change the signal output. One must be careful to calibrate after each change to enable comparison of signal areas with spectra obtained prior to the change.

Everything in this review is "well known" to those whose research involving EPR spectroscopy has led them to examine quantitatively the spectrometer-sample interaction. However the researcher whose emphasis is in another area, such as biochemistry, could be misled by some results, since some of the peculiarities of EPR spectroscopy are "obvious" only to those enlightened by much study. If this review makes quantitative EPR accessible to a broader community of researchers it will have achieved its purpose.

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