Original Article

Simplifying quantitative measurement of free radical species using an X-band EPR spectrometer

Yusuke Makino,[1,2] Megumi Ueno,[2] Yoshimi Shoji,[3] Minako Nyui,[2] Ikuo Nakanishi,[3] Koji Fukui,[1] and Ken-ichiro Matsumoto[2,*]

[1]Molecular Cell Biology Laboratory, Systems Engineering and Science, Graduate School of Engineering and Science, Shibaura Institute of Technology, Fukasaku 307, Minuma-ku, Saitama 337-8570, Japan
[2]Quantitative RedOx Sensing Group, Department of Radiation Regulatory Science Research, National Institute of Radiological Sciences, Quantum Life and Medical Science Directorate, National Institutes for Quantum Science and Technology, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan
[3]Quantum RedOx Chemistry Group, Institute for Quantum Life Science, Quantum Life and Medical Science Directorate, National Institutes for Quantum Science and Technology, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan

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The quantitative measurement of free radicals in liquid using an X-band electron paramagnetic resonance (EPR) was systematized. Quantification of free radicals by EPR requires a standard sample that contains a known spin amount/concentration. When satisfactory reproducibility of the sample material, volume, shape, and positioning in the cavity for EPR measurements can be guaranteed, a sample tested and a standard can be directly compared and the process of quantification can be simplified. The purpose of this study was to simplify manual quantitative EPR measurement. A suitable sample volume for achieving a stable EPR intensity was estimated. The effects of different solvents on the EPR sensitivity were compared. The stability and reproducibility of the EPR intensity of standard nitroxyl radical solutions were compared among different types of sample tubes. When the sample tubes, sample volumes, and/or solvents were the same, the EPR intensity was reproduced with an error of 2% or less for μM samples. The quantified sample and the standard sample in the same solvent and the same volume drawn into the same sample tube was able to be directly compared. The standard sample for quantification should be measured just before or after every daily experiment.

***Key Words*:** **electron paramagnetic resonance, quantitative measurement, reproducibility, sensitivity, sample tube**

Electron paramagnetic resonance (EPR), one magnetic resonance modality, can detect paramagnetic species, i.e., unpaired electron(s) on the molecule such as free radicals and/or free electrons on transition metal complexes. However, the quantitative ability of EPR spectrometers is principally poor because it is difficult to maintain the same resonance conditions for each sample. When performing a quantitative EPR measurement, an external and/or internal standard sample with a known spin concentration is required.[1,2]

An internal Mn^{2+} standard has been conventionally used for quantitative comparison of the EPR signal intensity (peak area) measured in several discrete samples. The signal intensity of an external standard sample with a known spin concentration is compared with that of the internal Mn^{2+} standard to obtain a comparative ratio of signal intensities when both external and internal standard samples are measured simultaneously. The EPR signal intensity of a sample with an unknown spin concentration can be converted to the spin concentration of the sample using the previously obtained comparative signal intensity ratio.

Comparison among samples having slightly different volumes, shapes, positions in the cavity/resonator, and/or dielectric nature of solvents may be possible using an internal Mn^{2+} standard under the assumption that the ratio of EPR sensitivities at the locations of the Mn^{2+} standard (near the wall of the cavity) and the sample (center of the cavity) is constant. In other words, the three-dimensional distribution of the comparative sensitivity in the EPR cavity/resonator must be constant even if the absolute sensitivity in the EPR cavity/resonator varies. Indeed, the absolute sensitivity at the location of the Mn^{2+} standard and at the center of the cavity differs, mainly due to slight differences in modulation field amplitudes. Although the Mn^{2+} standard itself cannot be quantified, comparison of EPR signal intensity based on a Mn^{2+} standard is an advantage for the accurate quantification of free radicals.

The compared sample must be dissolved in the same solvent and held in the same volume to quantitatively compare the EPR signal intensities in discrete samples. The sensitivity in the EPR cavity/resonator can vary by volume, shape, position, and/or material of the sample. When satisfactory reproducibility of the sample volume, shape, and positioning in the cavity for all EPR measurements can be guaranteed, the external standard and the tested sample dissolved in the same solvent can be directly compared and the process of comparison for quantification can be simplified. Such a method has been used in several previous papers,[3–9] but it has not been described in technical detail.

The purpose of this study was to systematize and simplify quantitative EPR measurement. The distribution of sensitivity in the EPR cavity was measured and a suitable sample volume was discussed. Suitable sample tubes for quantitative measurement were adopted. The effects of the solvent, sample tubes, and daily condition of the spectrometer on the EPR sensitivity were investigated. A political method of quantitative EPR measurement for samples dissolved in an aqueous or organic solvent was manualized.

Materials and Methods

Chemicals. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from FUJIFILM Wako Pure Chemical Corporation

*To whom correspondence should be addressed.
E-mail: matsumoto.kenichiro@qst.go.jp

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(Osaka, Japan). 4-Hydroxy-2,2,6,6-tetramethylpiperidin-*N*-oxyl (TEMPOL) was purchased from Sigma-Aldrich Co. (St. Louis, MO). DMPO was purchased from Dojindo Laboratories, Ltd. (Kumamoto, Japan). Ultrapure water deionized by the Milli-Q system (Merck Millipore, Billerica, MA) was used for preparing the standard TEMPOL solutions. Other chemicals were of analytical grade.

Sample tubes and holders. The reusable flat quartz cuvette (capacity: 130 µl, length: 50 mm, width: 10 mm, thickness: 2.01 mm) purchased from LABOTEC Co., Ltd. (Tokyo, Japan) is shown in Fig. 1A. Liquid samples were drawn into the cuvette and held inside by placing the end cap. Polytetrafluoroethylene (PTFE) tubing (i.d.: 0.81 ± 0.025 mm, wall thickness: 0.051 ± 0.013 mm) purchased from ZEUS (Orangeburg, SC) is shown in Fig. 1B. A 24–25-cm piece of PTFE tubing was used as a disposable sample tube. The liquid sample was held in the tubing and the tubing was folded at the center. A disposable glass capillary (capacity: 100 µl, length: 116 mm, i.d.: 1.05 mm, o.d.: 1.44 mm) purchased from Drummond Scientific Co. (Broomall, PA) is shown in the left panel of Fig. 1C. The liquid sample was drawn into the glass capillary and held inside by stuffing clay at one end. The PTFE tubing or glass capillary bearing the liquid sample was put into a glass sample holder (Fig. 1C, right panel) for placement in the X-band TE-mode cavity. In addition, the combination of a glass capillary and quartz sample holder equipped with a silicon cap to vertically fix the glass capillary (Fig. 1D) was tested.

Distribution of sensitivity in the X-band TE-mode cavity. A small crystal of DPPH was stuck to the outer surface of a glass tube (o.d. 5 mm) with adhesive cement, which has no EPR signal. The glass tube mounted DPPH was set in the TE-mode cavity of the X-band EPR spectrometer. The vertical position of the glass tube was carefully moved in 1.0-mm steps and the single-line EPR signal of solid DPPH was measured at each position.

Evaluation of a suitable sample volume for quantitative measurement. Exactly 172 mg of TEMPOL was weighed into a 10-ml measuring flask filled with Milli-Q water to obtain a 100 mM stock solution. The 100 mM stock solution was 1,000-fold diluted to get 0.10 mM standard solution for use. Several different volumes (0.7–52 µl) of 0.10 mM water solution of TEMPOL was loaded into a glass capillary. The capillary-loaded TEMPOL solution was placed in the X-band TE-mode cavity such that the center position of the TEMPOL solution matched the center of the sensitivity area in the cavity. The EPR signal intensities of different volumes of TEMPOL solution were measured. One of the triplet lines of TEMPOL observed at the lowest field was recorded. The glass capillary tube has an inner diameter of 0.95 mm, which was measured by calipers with 0.01-mm accuracy. The length (meniscus to meniscus) occupied by the liquid held inside the capillary was also measured using the same calipers. The volume of the liquid loaded into the capillary was calculated as follows: volume (µl) = $(0.95\ \text{mm}/2)^2 \times \pi \times$ length (mm).

Effects of solvent on the distribution of EPR sensitivity in the cavity. A small dry DPPH crystal was put into a 5-mm piece of PTFE tubing, which was folded to hold the DPPH crystal. The PTFE tubing holding the DPPH crystal was stuck on the outer surface of a glass sample holder (o.d. 4.8 mm) using adhesive tape. The glass sample holder-mounted DPPH crystal was set in the TE-mode cavity of the X-band EPR spectrometer, and the position of the DPPH crystal was adjusted to the center of the sensitivity distribution and fixed. An aliquot (100 µl) of one solvent, i.e., milli-Q water, 100 mM phosphate buffer (PB), DMSO, ethanol, 1-pentatol, or soybean oil, was loaded into a 24-cm peace of PTFE tubing. Neat DMPO (99%) liquid was also tested with the other solvent samples. The PTFE tubing-loaded solvent was put into the glass sample holder set in the TE-mode cavity. The resonance condition was adjusted to be optimal for each sample. The signal intensities of the DPPH crystal and the third line of the 6-line signal of the Mn^{2+} standard were

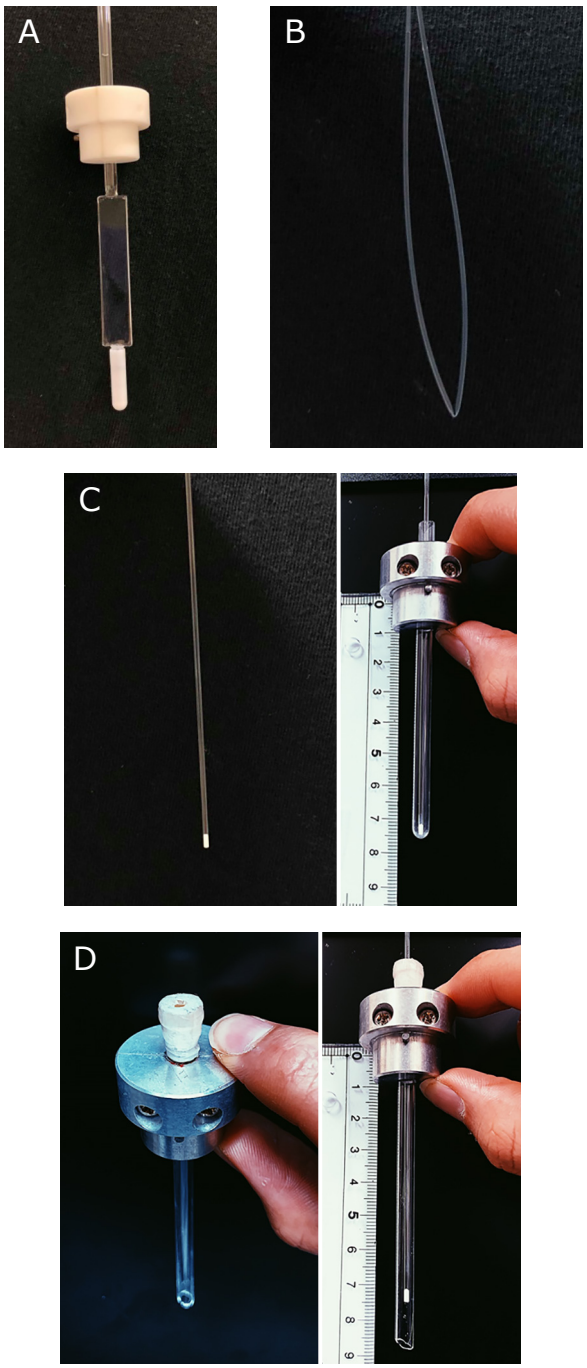

Fig. 1. Appearances of sample tubes and holders for setting liquid samples in an X-band EPR cavity. (A) The flat quartz cuvette was reused after cleaning for each measurement. Liquid samples were drawn into the cuvette and held inside by placing the end cap. (B) A 24–25-cm piece of PTFE tubing was used as a disposable sample tube. Liquid samples were held in the tubing, which was folded at the center. (C) A glass capillary was used as a disposable sample tube. Liquid samples were drawn into the glass capillary and held inside by stuffing clay at one end (left panel). The glass capillary requires a glass sample holder (right panel) to set it in the X-band TE-mode cavity. The PTFE tubing also requires the glass sample holder. (D) An original quartz sample holder (left panel) was equipped with a silicon cap to vertically fix the glass capillary inside (right panel).

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measured. The ratio of the signal intensity of the DPPH crystal to the Mn^{2+} signals was noted.

Comparison of signal intensities obtained from different sample tubes. PTFE tubing, a glass capillary, or flat quartz cuvette was filled with 0.10 mM water solution of TEMPOL. The sample volume was approximately 100 µl for PTFE tubing, 80 µl for the capillary, and 130 µl for the flat cuvette. The sample holder supporting the PTFE tubing, the glass capillary inside, or the flat cuvette with the attachment bracket was set in the TE-mode X-band EPR cavity, and the sample position was adjusted to the center of the sensitive area in the cavity. EPR measurements were repeated 16 times. The flat quartz cuvette was reused for 16 measurements, but the sample TEMPOL solution was replaced for each measurement. The PTFE tubing and glass capillaries were disposed after each measurement. All measurements were carried out during the same day.

Variation of EPR intensities during a 3-month period. Accurate 0.10 mM standard TEMPOL water solution was prepared and stocked in a sealed plastic tube at 4°C. An aliquot of the sample TEMPOL solution was loaded into PTFE tubing, a glass capillary, or a flat quartz cuvette, and measured by an X-band EPR spectrometer 3 times. The measurements were performed 16 times during a 3-month period. All measurements were carried out on the same EPR spectrometer.

Variation of EPR intensities during a 2-year period. Exactly 2.86 mg of TEMPOL was weighed into a 10 ml measuring flask filled with Milli-Q water to obtain 1,660 µM standard TEMPOL solution, and stocked in a sealed plastic tube at 4°C. A concentration series (1.66, 2.28, 3.24, 4.84, 7.69, 13.3, 25.9, 61.5, 106, 208, 338, 492, 756, 1,250, and 1,660 µM) of TEMPOL water solution was prepared by diluting the standard solution before each measurement. A 100-µl aliquot of the sample TEMPOL solution was loaded into PTFE tubing and measured by an X-band EPR spectrometer 3 times for each concentration. In total, 19, 18, or 8 measurements were performed for 1.66–25.9 µM, 61.5–1,250 µM, or 1,660 µM samples during a 2-year period. The stock 1,660 µM solution was prepared twice during a 2-year period. The JES-RE1X spectrometer (JEOL, Tokyo, Japan) was used for only this experiment and the center peak of TEMPOL was noted.

X-band EPR measurement. The EPR spectrometer JES-RE2X (JEOL, Tokyo, Japan) was used. The microwave frequency was 9.4 GHz. The main magnetic field was 330.9–336.2 mT, which was adjusted depending on the sample and sample holder settings. The microwave power was 4 mW. The field modulation frequency was 100 kHz. The field modulation amplitude was 0.0063 mT for DPPH crystal and 0.004 or 0.125 mT for TEMPOL water solution. The sweep width was ±4.0 mT for DPPH crystal and ±1.5 mT for TEMPOL water solutions. The sweep rate was 2.0 mT/min for DPPH crystal and 1.2 mT/min for TEMPOL water solution. The time constant was 0.3 s. The field sweep resolution was 8192 points for a full sweep. Individual single lines at the lowest field of a 3-line spectrum of TEMPOL was measured and saved. Digitalized EPR spectral data were acquired by WIN-RAD ESR Data Analyzer (Gigatec Co., Ltd., Sagamihara, Japan) equipped on the X-band EPR spectrometer.

EPR data handling. The single-line EPR spectrum of DPPH crystal or individual single line of a triple-line spectrum of TEMPOL water solution was analyzed using an in-house line-fitting program. A Lorentzian line shape, L(i), was calculated by Eq. 1, and fitted to Mn^{2+} and DPPH signals (Fig. 2). A Gaussian line shape, G(i), was calculated by Eq. 2, and fitted to TEMPOL signal (Fig. 3). H_i is the magnetic field increment with i mT.

$$L(i) = I_{pp}/2 \times e^{1/2} \times (H_0 - H_i)/(H_{pp}/2) \times \mathrm{EXP}(-1/2 \times ((H_0 - H_i)/(H_{pp}/2))^2) \quad [1]$$

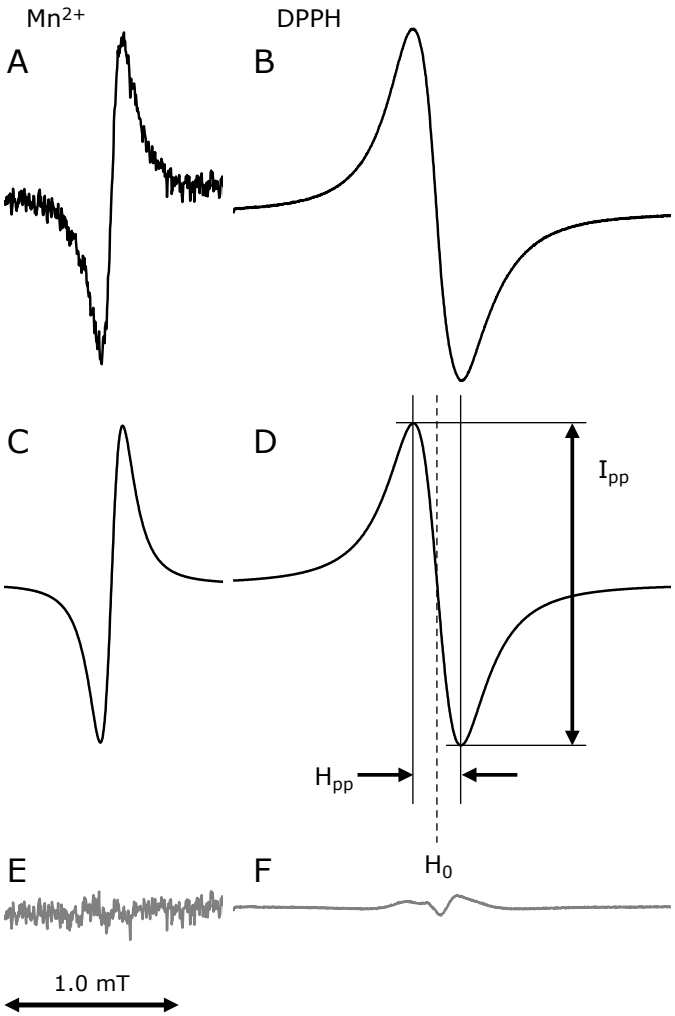

Fig. 2. Examples of line fitting. (A) The third line from the lower field of the 6-line EPR spectrum of Mn^{2+} and (B) DPPH crystal was experimentally observed. The gain was 200 for Mn^{2+} and 5 for DPPH. The signal of Mn^{2+} and DPPH was obtained with 512 and 1,024 data points, which corresponded to a field sweep width of 1.25 and 2.5 mT, respectively. (C), (D) Best fitted Lorentzian lines calculated for (A) and (B), respectively. I_{pp} is the peak-to-peak signal height. H_{pp} is the peak-to-peak linewidth. H_0 is the center magnetic field of the resonance line. (E), (F) Residues after subtracting the calculated Lorentzian line from the experimental spectrum.

$$G(i) = 16 \times I_{pp}/2 \times (H_0 - H_i)/(H_{pp}/2)/((3 + ((H_0 - H_i)/(H_{pp}/2))^2)^2) \quad [2]$$

The signal height and the line width of the fitted line shape were recorded, and the area of the single peak was calculated by Eq. 3 for Lorentzian or Eq. 4 for Gaussian lines.

$$\text{Lorentzian Peak Area} = I_{pp} \times {H_{pp}}^2 \times 3.63 \quad [3]$$

$$\text{Gaussian Peak Area} = I_{pp} \times {H_{pp}}^2 \times 1.03 \quad [4]$$

The area was multiplied by the number of splitting lines, such as multiplying by 3 for TEMPOL. The calculated peak area was divided by the gain used and the absolute EPR signal intensity was noted.

Results and Discussion

The distribution of the sensitive area in the X-band TE-mode

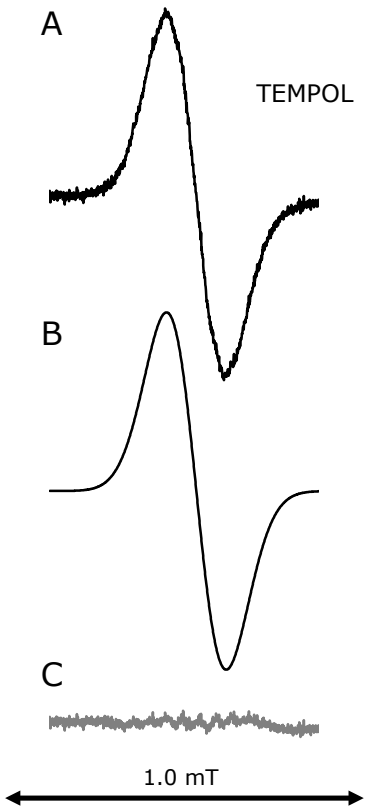

Fig. 3. Examples of line fitting. (A) The center line of the triplet EPR spectrum of 26 μM TEMPOL water solution. The EPR signal was obtained with 1,024 data points, which corresponds to a field sweep width of 0.75 mT, and the gain was 125. (B) Best fitted Gaussian lines calculated for (A). (C) Residues after subtracting the calculated Gaussian line from the experimental spectrum.

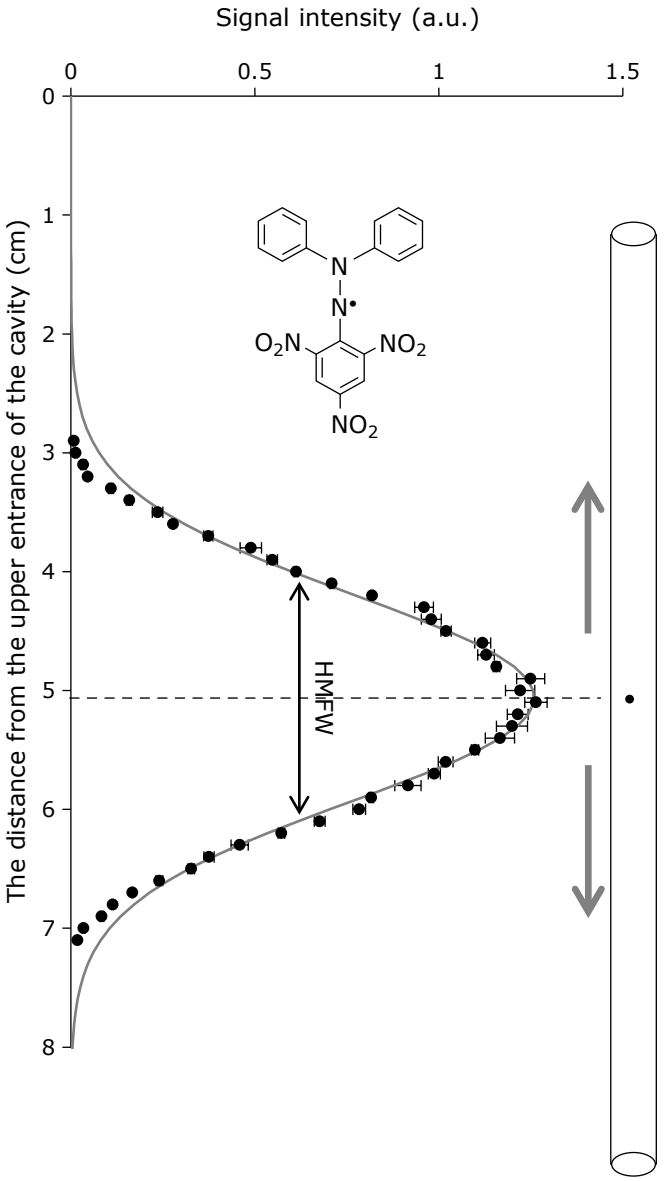

Fig. 4. Vertical distribution of sensitivity in an X-band TE-mode cavity. The position of the glass tube with a DPPH crystal mounted on its surface was moved vertically in the cavity in 1-mm steps. The signal intensity of singlet EPR signal of the DPPH crystal was measured. The EPR signal intensity detected (horizontal axis) was plotted vs distance from the cavity entrance (vertical axis). Marks and error bars indicate the average value and SD of 3 measurements, respectively. The center of the sensitive area (dotted line) was 51 mm from the entrance of the cavity. The half maximum full-width of the sensitive area distribution was 20.5 mm (double-headed arrow). The insertion at the right side of the figure is a schematic drawing of the glass tube with a DPPH crystal mounted on the surface.

cavity is shown in Fig. 4. The gray solid line in Fig. 4 indicates a Gaussian line shape fitted on the distribution of the EPR sensitivity, and the center and half maximum full-width was observed from the fitting parameter. The center of the sensitive area (dotted line) was 50.6 mm from the entrance of the cavity, which was almost the center of the TE-mode cavity. The half maximum full-width of the sensitive area distribution was 20.5 mm (double-headed arrow in Fig. 4). The sample volume should always occupy the constant sensitive area when turning over the sample in order to not vary resonance conditions. This can be achieved by using a sufficiently large sample whose volume path through the sensitive area is always placed at the same position in the cavity.

Next, the sample volume required to give constant sensitivity was estimated using aqueous 0.1 mM solution of TEMPOL. The relationship between the detected EPR signal intensity and sample volumes (length occupied in a capillary) is shown in Fig. 5. The EPR signal intensity increased with an increase in sample volume, and plateaued when the sample occupied more than 3 cm in the capillary. Therefore, a liquid sample drawn into a capillary, tubing, or flat cell should occupy more than 3 cm of the vertical length to maintain the same resonance conditions.

The effects of solvent samples on the EPR sensitivity in the cavity are shown in Fig. 6. The EPR signal intensity of an identical DPPH crystal and identical Mn^{2+} standard at a fixed location was measured under optimal resonance conditions, except that a different solvent at an identical volume was inserted into the cavity. The EPR sensitivity decreased when the solvent in the cavity had a high dielectric constant such as water (Fig. 6A and B). However, the ratio of signal intensities of DPPH to Mn^{2+} was almost constant (Fig. 6C). Therefore, comparison of radical amounts in different solvents is possible by comparison with the Mn^{2+} standard. This suggests however that an external standard sample prepared at a known concentration for quantification should be prepared in the same solvent as the sample when the external standard and the quantified sample are to be directly compared.

The effects of whole blood, blood plasma, and 5-fold diluted liver homogenate on the EPR sensitivity estimated with the same

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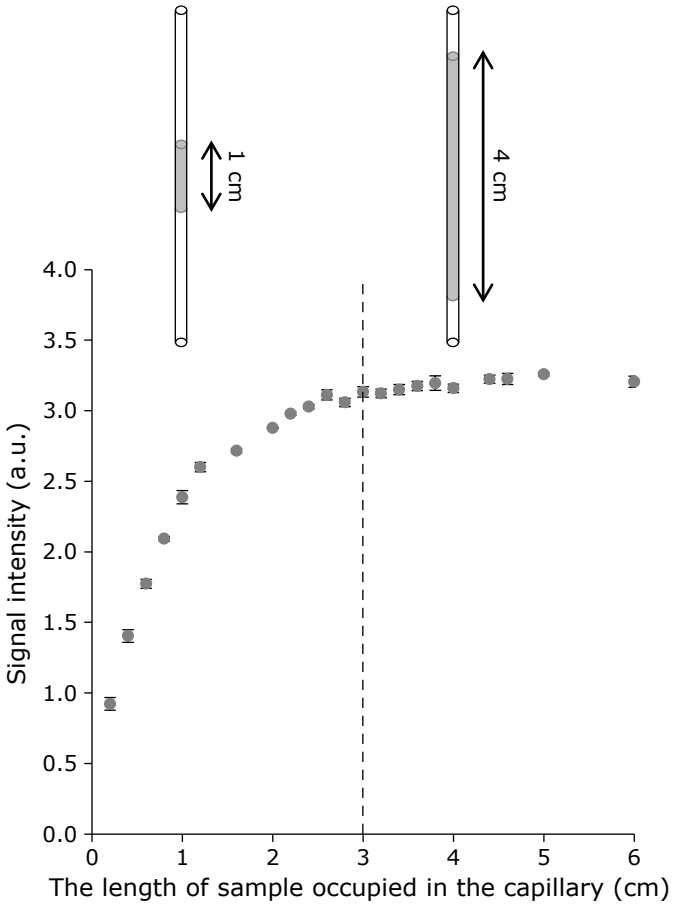

Fig. 5. The relationship between the length of the sample in the capillary and the EPR signal intensity detected. Different volumes of 0.1 mM water solution of TEMPOL were drawn into a capillary and placed vertically in the TE-mode cavity. The center of the length occupied by TEMPOL solution in the capillary was adjusted to the center of the sensitive area in the cavity. Then, the EPR signal intensity was measured and plotted vs the length occupied by TEMPOL solution in the capillary. Marks and error bars indicate the average value and SD of 3 measurements, respectively. The insertion at the upper side of the figure is a schematic drawing of a capillary holding TEMPOL solution.

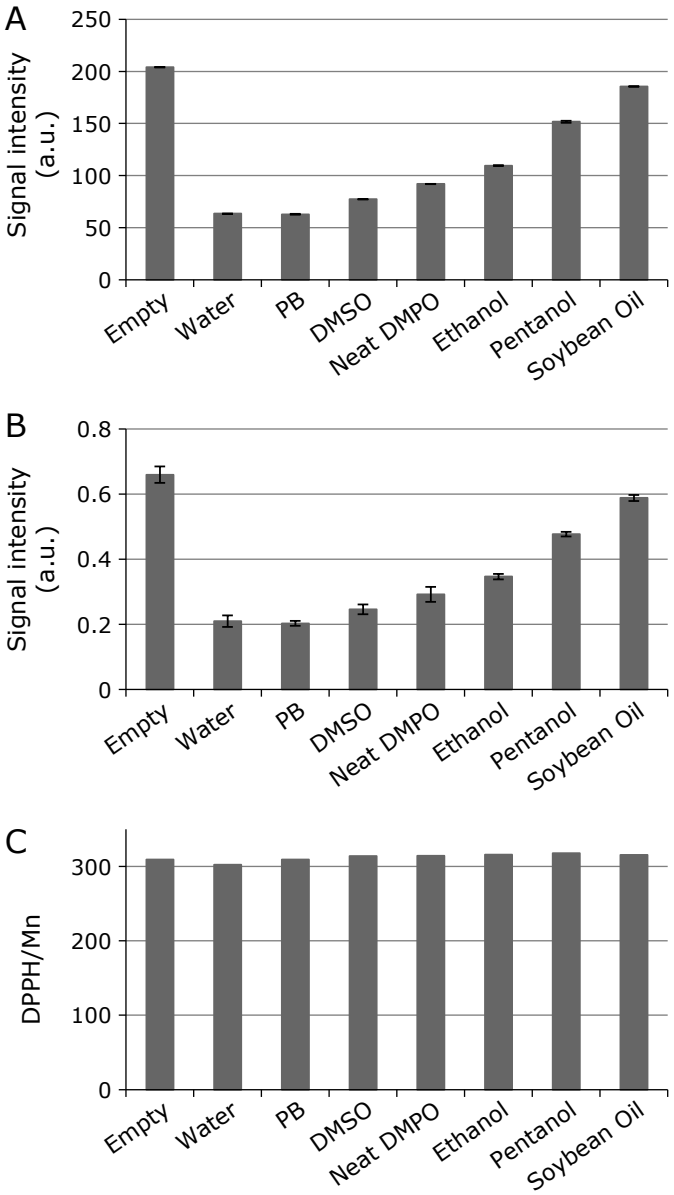

Fig. 6. Effects of solvents on the sensitivity in the cavity. (A) Comparison of sensitivity at the center of the cavity (location of the sample, i.e., the DPPH crystal and the solvent). (B) Comparison of sensitivity near the wall of the cavity (location of Mn^{2+} standard). Columns and error bars in (A) and (B) indicate the average value and SD of 3 measurements, respectively. (C) Comparison of the ratio of signal intensity of DPPH to the Mn^{2+} standard. Columns in (C) indicate the ratio of values in (A) to values in (B), i.e., DPPH/Mn of corresponding solvent.

procedure as in the experiment shown in Fig. 6 are shown in Supplemental Fig. 1*. The width of Q-dip observed by setting 100 µl of water, PB, whole blood, plasma, or tissue homogenate in the cavity was almost the same; however, the EPR intensities were slightly different. Blood plasma and 5-fold diluted tissue (liver) homogenate yielded a similar EPR intensity to Milli-Q water under the optimum resonance condition. When aqueous free radicals in blood plasma and/or 5-fold diluted tissue homogenate samples were quantified, water solution of stable radicals, such as TEMPOL, can be used as the standard sample. Whole blood yielded a slightly large EPR intensity, whereas PB yielded a slightly small EPR intensity. This study demonstrated that the dielectricity of Milli-Q water, blood plasma, and 5-fold diluted homogenate is the same, but whole blood has slightly lower dielectricity and the PB has slightly higher dielectricity. Electrolytes increase the dielectricity and organic substances, such as cell lipids, reduce it. When free radicals in a biological sample, such as cell culture, tissue homogenate, or secretory fluid, are to be quantified, the standard sample solution should ideally be prepared from the same biological fluid.

Considering the inaccuracy of the concentration of a standard sample prepared using blood, tissue homogenate, and/or other viscous biological fluids, the differences in sensitivity observed in Supplemental Fig. 1* among aqueous solvents tested, even between PB and whole blood, were probably smaller than the experimental/technical error in the sample preparation. Water solution of a stable radical with an accurate concentration may be better as the standard solution for such biological aqueous samples. The investigator must carefully watch the width of the Q-dip observed with the sample inserted in the cavity and decide the solvent for the standard sample.

Lipophilic free radical compounds incorporated in cells or bound on cellular components may exhibit slight line-broadening due to anisotropy, which may be caused by distribution to the

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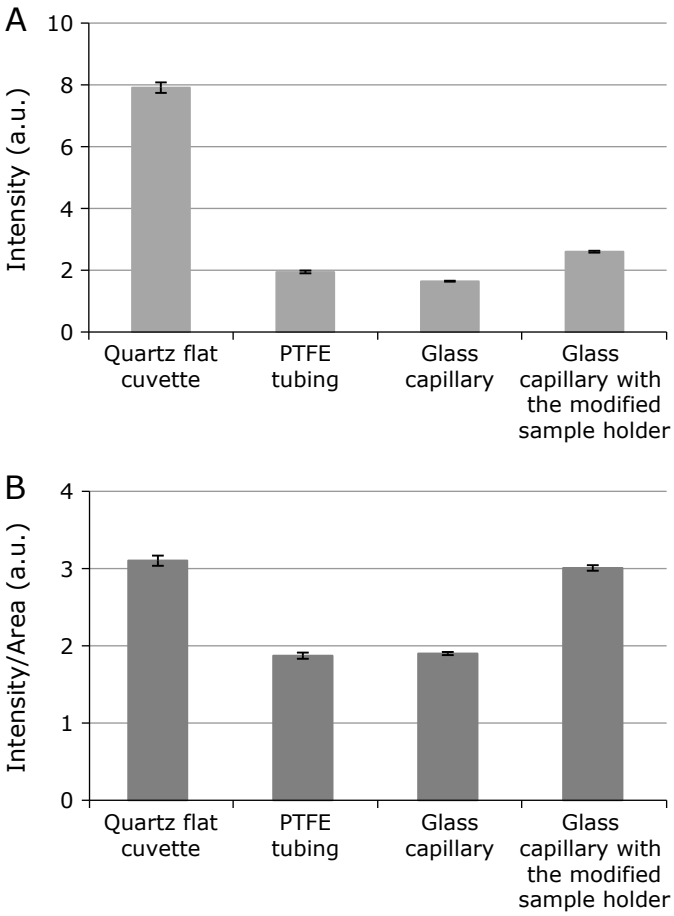

Fig. 7. Comparison of available signal intensities of 0.1 mM TEMPOL water solution using the flat quartz cuvette, PTFE tubing, or glass capillary filled with the sample solution. (A) The EPR signal intensities observed were simply compared. (B) The EPR signal intensities were standardized by the cross-sectional area of the sample tubes. Columns and error bars indicate the average ± SD of 16 measurements.

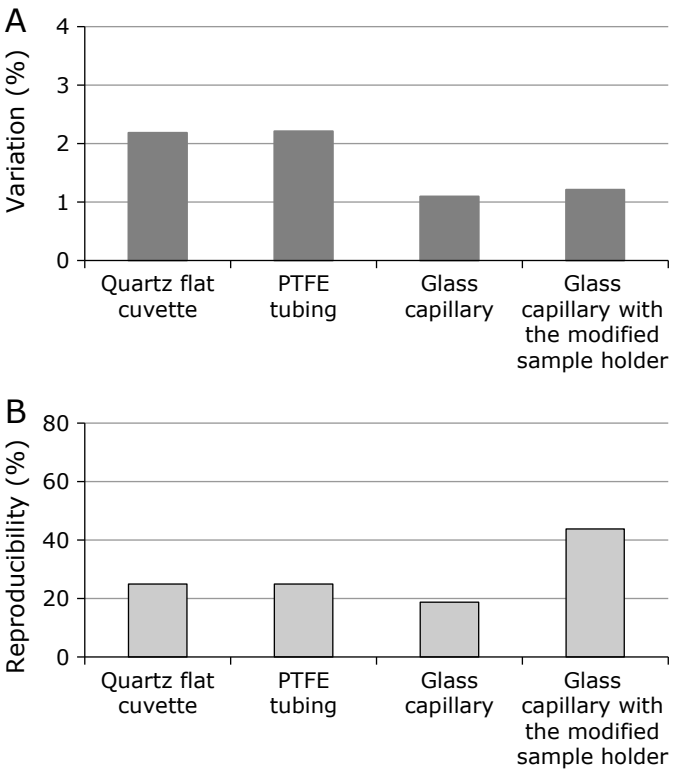

Fig. 8. Comparison of levels of variation and reproducibility of EPR signal intensities during 16 individual measurements. (A) The variation, defined as the ratio of the SD to the average EPR signal intensity, was compared among sample tubes and holder settings. (B) Comparison of the reproducibility of EPR signal intensities among sample tubes and holder settings. Columns indicate estimated values for 16 measurements. The 0.5%-reproducibility was the ratio of the values within ±0.5% of the average.

lipid membrane and/or by adsorption to large protein molecules. However, the difference in EPR line shapes did not affect the quantification based on the EPR peak area. Quantification of free radicals in a lipidic sample may require sufficient experience.

The comparison of available signal intensity using the flat quartz cuvette, PTFE tubing, and glass capillary filled with an identical preparation of 0.1 mM TEMPOL water solution is shown in Fig. 7A. The vertical length of the sample in the flat cuvette, folded PTFE tubing, and glass capillary was approximately 50, 96, and 92 mm, respectively. The flat quartz cuvette resulted in a markedly larger EPR signal due to its large sample volume than the other sample tubes tested. The EPR signal intensity of the TEMPOL solution increases according to the spin concentration in the sample and sample volume occupying the sensitive area, but is simultaneously suppressed by the volume of water, i.e., dielectric solvent, and volume and material of the sample holder according to its dielectric nature. The rectangle cross-sectional area of the sample solution in the flat quartz cuvette was 0.25 mm × 10 mm = 2.5 mm^2. The cross-sectional area of sample solution in the folded PTFE tubing (as shown in Fig. 1B) was 0.52 mm^2 × 2 = 1.04 mm^2. The cross-sectional area of sample solution in the glass capillary was 0.87 mm^2. The comparison of corrected signal intensity, which is the signal intensity divided by the cross-sectional area of tubes, is shown in Fig. 7B. The flat quartz cuvette and glass capillary with a modified sample holder had similar higher intensity/area values than the PTFE tubing and glass capillary with a normal glass holder, which had similar lower values. The glass sample holder may suppress the sensitivity more than the quartz sample holder. The glass capillary itself may have no effect on EPR sensitivity.

A comparison of degrees of variation of EPR signal intensity during 16 individual measurements when the sample tubes were replaced each time is shown in Fig. 8A. The "variation" was defined as the ratio of the SD to the average EPR signal intensity. When the variation values were compared among sample tubes and holder settings (Fig. 8A), the experiments using a glass capillary resulted in smaller values than those using the flat quartz cuvette or PTFE tubing. A comparison of the reproducibility of EPR signal intensities is shown in Fig. 8B. The "0.5%-reproducibility" was calculated as the ratio of the values coming within ±0.5% of the average of 16 measurements. The glass capillary with a modified quartz sample holder had the highest 0.5%-reproducibility, but that used with a normal glass sample holder had the smallest 0.5%-reproducibility even though the variation in values observed with this setting was the smallest (Fig. 8A). As the "variation" and "reproducibility" intrinsically contradict each other, the distribution of signal intensity values obtained using a glass capillary with a normal glass sample holder may not be a typical normal distribution. The glass capillary in the normal glass sample holder was not fixed and leaned on the wall of the sample holder. The unfixed glass capillary in the sample holder was leaned on the wall of the sample holder and slightly slanted. The EPR sensitivity was have varied due to the direction of the capillary, as shown in Supple-

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mental Fig. 2*. The histogram of the intensity variation caused by the leaning direction of the capillary had a rectangular shape, which produced a similar pattern from the lowest to the highest values. The intrinsic experimental variation of signal intensity may result in a Gaussian histogram when the capillary is fixed at an identical position. The values observed in the experiment shown in Fig. 8 were mainly affected by the leaning direction of the capillary rather than the intrinsic variation. The flat quartz cuvette and PTFE tubing had similar values for both the variation and 0.5%-reproducibility. The 1–2% variation in EPR intensity may have been caused by changing samples. This can be minimized by repeating measurements and accumulating data. The values observed with other sample tube settings, i.e., the flat quartz cuvette, PTFE tubing, and the fixed capillary, may have been affected only by the intrinsic experimental variation.

A point to note is that the EPR signal intensity of the Mn^{2+} internal standard had a similar variation of several % depending on the amount of Mn^{2+} inserted into the cavity. The EPR signal from Mn^{2+} is usually not high. Therefore, comparison of EPR signal intensity mediated by the Mn^{2+} standard may increase the inherent variation values, being of concern.

The flat quartz cuvette was reused after washing and/or rinsing inside to turn over the sample. This washing process took a slightly longer time than the use of other disposable sample tubes. The time interval required from finishing a measurement to starting the next measurement was 1 min or longer. The time interval required from finishing a measurement to starting the next measurement was approximately 30 s when the disposable PTFE tubing or glass capillaries were used with a normal glass sample holder. When the glass capillaries were used with the special quartz sample holder equipped with a silicon cap, the time interval required from finishing a measurement to starting the next measurement was approximately 45–60 s or longer due to adjusting the sample position.

Daily EPR signal intensities of the standard 0.1 mM TEMPOL water solution measured over 3 months are shown in Fig. 9A. The measurements were performed for different sample tube settings. The variation in each daily experiment was relatively small, similar to the variation shown in Fig. 8A, which was 2 or 1%; however, the variation over 3 months was 8.5–15.8% (Fig. 9B), which was larger than the daily variation. As the daily up-down patterns in intensity of 0.1 mM TEMPOL solutions were similar using different sample tube settings (Fig. 9A), EPR intensity may be unstable due to the daily condition of the EPR instrument such as electric noise from the power supply. The relative intensity ratio among the sample tube settings was similar to that shown in Fig. 7A and was almost constant. The 5%-reproducibility of signal intensity among 16 separate experiments (Fig. 9C) was highest for the glass capillary in a modified quartz sample holder, which was similar to that shown in Fig. 8B, but it was lowest for the flat quartz cuvette. Although reproducibility of signal intensity may be improved by reproducing and fixing the position of the sample, daily EPR conditions can cause an 8.5–15.8% variation in EPR intensity. The "variation" and "reproducibility" contradicted each other in this 3-month follow-up experiment.

Overall, PTFE tubing may be preferable as the sample tube due to its reproducibility and quick use. The variation in EPR signal intensity of several different concentrations of TEMPOL solution over a 2-year period was measured using PTFE tubing. The R^2 values between the absolute EPR signal intensities and the concentrations of TEMPOL measured each day were almost constant (Fig. 10B) and exhibited a highly linear relationship ($R^2 \geq 0.9884$). The variation in each daily experiment was relatively small, being less than 2% for 7.7 μM or higher solutions, and less than 4% for those less than 7.7 μM (Fig. 10C). However, variations in EPR signal intensities over a 2-year period were relatively larger at 7–20% (Fig. 10D). As the variations in signal intensity on a different day only slightly depend on the concentration of TEMPOL, daily variation in the signal to noise ratio may have a minor influence on the daily variation in signal intensities. The plotted up-down patterns of intensities of different concentrations of TEMPOL solutions were similar; therefore,

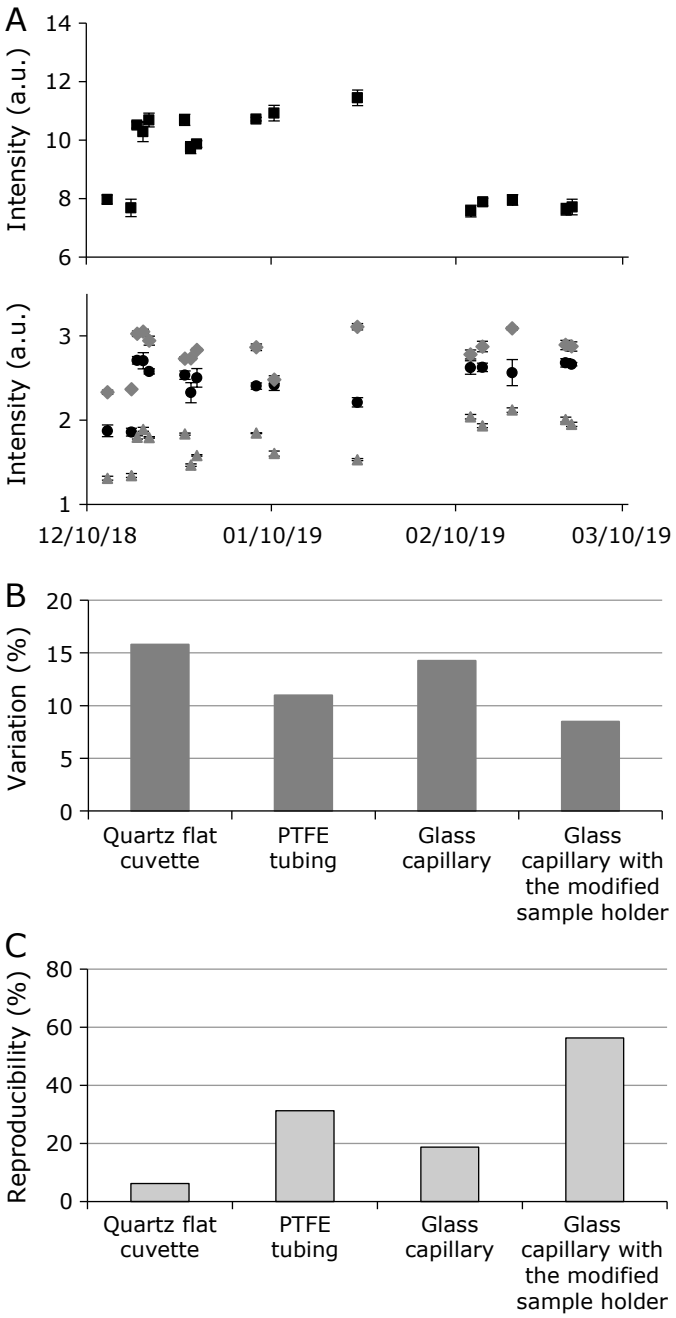

Fig. 9. Variation in EPR signal intensities during a 3-month period. (A) Plots of EPR signal intensities of 0.1 mM standard TEMPOL water solutions observed in 16 experiments during 3 months. The marks and error bars indicate the average and SD of 3 measurements, respectively. Squares, circles, triangles, and diamonds indicate values observed with a flat quartz cuvette, PTFE tubing, glass capillary, or glass capillary with modified quartz sample holder, respectively. (B) Variations, i.e., SD divided by average, of 16 measurements during 3 months. (C) 5%-Reproducibility of EPR signal intensities among sample tubes and holder settings. Columns indicate estimated values for 16 measurements. The 5%-reproducibility was the ratio of the values within ±5% of the average.

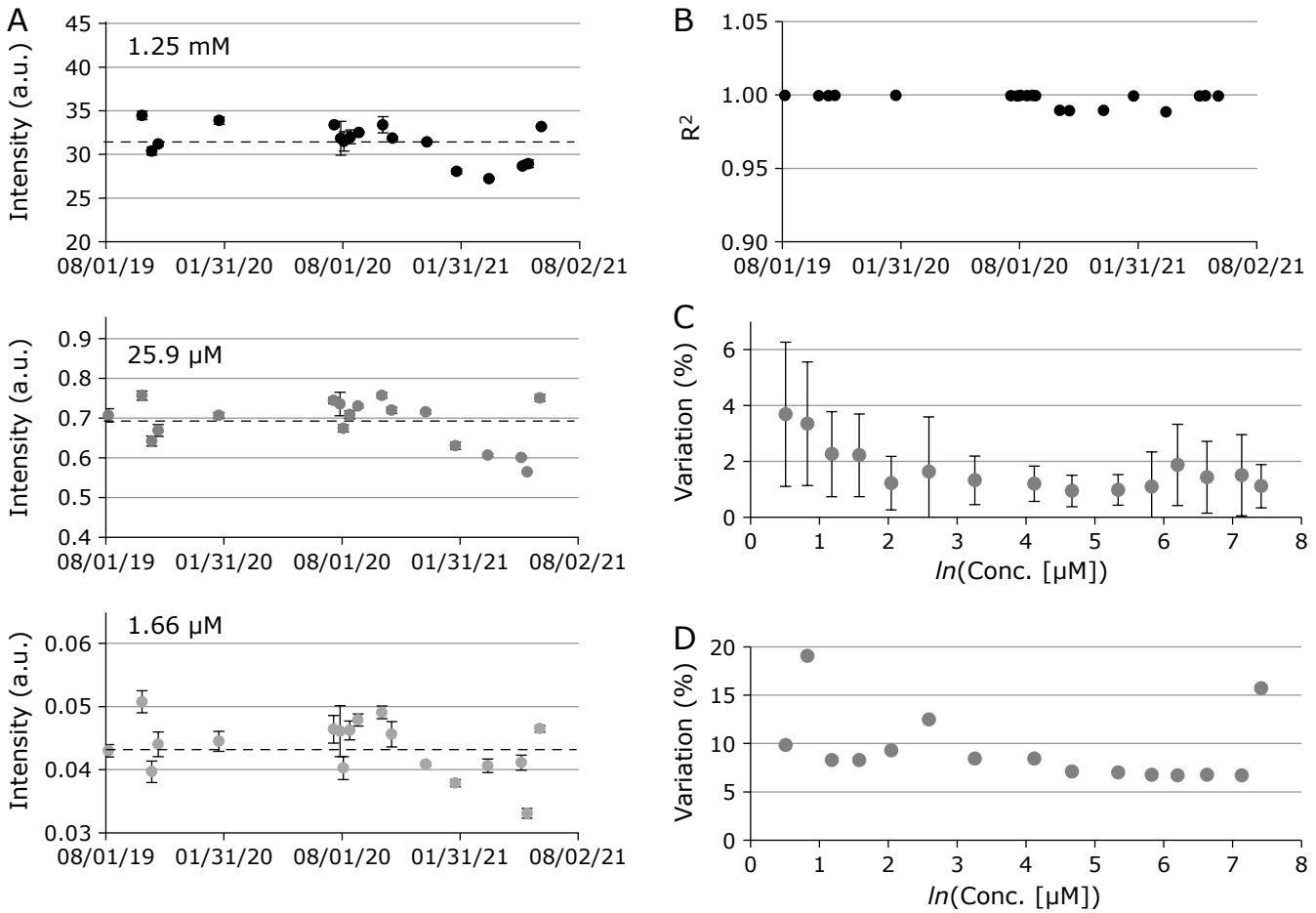

Fig. 10. Variation in EPR signal intensities during a 2-year period. (A) Plots of EPR signal intensities of 1.25 mM (upper panel), 25.9 μM (middle panel), and 1.66 μM (lower panel) TEMPOL water solutions observed in 18 or 19 experiments during 2 years. The marks and error bars indicate the average and SD of 3 measurements, respectively. The horizontal dotted line indicates the average value in each panel. (B) R^2 values of standard curves obtained by daily experiments. (C) Variations, i.e., SD divided by average, in EPR signal intensities observed by 3 measurements were averaged among 18 or 19 experiments during the 2-year period. Marks and error bars indicate the average and SD. (D) Variation in EPR signal intensities observed in 18 or 19 experiments over 2 years.

EPR intensity may be unstable due to the daily condition of the EPR instrument. The variation increased and the reproducibility decreased when the sample concentration decreased, but the variation and reproducibility depending on the sample tubes and holder settings were similar. A standard sample for quantification should be measured just before each daily experiment.

Comparison of the EPR signal intensities of multiple samples mediated by Mn^{2+} internal standard is not preferable; therefore, a simplified EPR quantification procedure is introduced below.

1. Prepare a standard sample solution of an arbitrary concertation using the same solvent of the sample being quantified.
2. Load the standard sample and samples being quantified into a sample tube with the same volume, shape, and material.
3. Measure the X-band EPR signal of the sample solutions under exactly the same parameter settings except for gain (amplitude).
4. The whole spectrum does not have to be scanned if the EPR spectrum of the subjected radical has a well-separated line. A well-separated single peak is preferable to an entire spectrum.
5. Digital data acquisition and the line fitting procedure are not necessary to observe the peak height and peak width. The peak height and peak width can be measured using calipers or ruler from a spectrum chart.
6. Calculate the single peak area by (signal height) × (linewidth)^2 × 3.63 for a Lorentzian line shape or × 1.03 for a Gaussian line shape, and then multiply by the number of splitting peaks, where the signal height and linewidth are the peak-to-peak signal height and peak-to-peak linewidth of the deviation spectrum, respectively. If no separated peak is found, the peak area can obtained by double integrating the entire EPR spectrum. However, digital data are required. Integration of a spectrum with noise may cause baseline distortions. The area of the integrated spectrum is the signal intensity necessary for quantification.
7. Divide the signal intensity by the gain (amplitude) to obtain the absolute signal intensity for the experimental setting and parameters used at the time.
8. Compare the absolute signal intensities of the standard and the sample to be quantified, and simply calculate the concentration or amount of spins, which is directly proportional to the absolute signal intensity.

When the volumes, positions, and solvent were the same between 2 samples, the EPR signal intensities of those 2 samples were able to be directly compared under identical EPR parameter settings. The procedure for quantification of free radicals can be simplified by suitable sample preparation.

When the sample tubes, sample volumes, and/or solvents were the same, the EPR signal intensity was reproduced with an error

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of 2% or less. Therefore, using the standard sample in the same solvent and the same volume drawn into the same sample tube, the EPR signal intensity of the sample can be directly compared with that of the standard sample. Thus, comparison mediated by a Mn^{2+} internal standard is required when the sample tubes, sample volumes, and/or solvent differ between the standard sample and the sample to be quantified. Using PTFE tubing or a glass capillary as a sample tube is an easy-to-use approach to quantify free radicals in liquid by X-band EPR. To detect free radicals at low concentration, a flat quartz cuvette may yield a better signal intensity. For accuracy, fixing a glass capillary vertically and tightly at the center of the quartz sample holder may improve EPR sensitivity and reproducibility. To avoid daily shifts in the condition of the EPR instrument, the standard sample for quantification should be measured just before or after each daily experiment.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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