

## Technical Treatments of Antioxidant Test with DPPH Method Affecting IC50 Value

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### ABSTRACT

Antioxidant tests are widely applied to foods and beverages, even at the nanoscale, because antioxidant activity is a crucial indicator of a substance's ability to neutralize free radicals. A common technique is the DPPH method, which utilizes the DPPH reagent, making its application according to SNI standards interesting to study. However, obtaining valid results requires identifying and mitigating potential errors throughout the testing procedure. Many studies present uncorrected **IC50 values** due to inconsistencies in treatment and methodology. Errors can arise at any stage, from sample preparation to data analysis, and may stem from the technician, equipment, materials, or environmental conditions. Ultimately, these procedural flaws can lead to inaccurate or invalid IC50 values, evidenced by a poor **linear regression curve**. Therefore, this study was conducted to examine all sources of error that affect the determination of IC50 values.

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**Received:** September 26, 2026; **Accepted:** October 05, 2026; **Published:** October 10, 2026

**Keywords:** Antioxidant Test, DPPH, Error, Laboratory Technique

### Introduction

Free radicals in the human body can be a source of disease. Chemical reactions between free radicals and target substances are very dangerous, because they can trigger the growth of carcinogenic substances [1]. To suppress free radical activity, antioxidant substances have been proposed to counteract free radicals by changing free radicals into neutral substances [2].

Antioxidants are a topic of health needs today. The data is important and much needed in the manufacture/production of healthy food ingredients with various varieties [3]. In fact, all food ingredients have different antioxidant activities, because food ingredients contain many different substances. Recently, there have been many antioxidant tests for nanomaterial compounds [4,5].

In addition, antioxidant analysis can also produce less/inaccurate data due to the lack of accuracy of the measurement method, and one of these is the antioxidant test using the DPPH method [6]. The antioxidant test method with DPPH has become a standard in various laboratory tests. Antioxidant tests can be performed for various materials, such as plant extracts, animals, and food. The trend of many antioxidant tests is to explore unstudied materials, such as duku [7]. The advantage of this method is fast

enough result. The disadvantage of this method is that the DPPH reagent must be made fresh when analyzing. When analyzing, it is important to pay attention to accuracy and precision. Many studies published in journals reported uncorrected results of IC50 values based on the method used because of error treatments [8]. Additionally, error treatments will affect the IC50 value calculation. Previous studies have studied the errors and inconsistencies of reaction stoichiometry and IC50 values [9]. In detail. The study has deepened the errors in terms of concepts and data interpretation analysis. However, the study has not deepened the possibilities of technical errors procedurally.

Therefore, this study pragmatically observes the technical treatments in antioxidant testing with the DPPH method. These treatments may cause applicative errors that are easy to occur so that IC50 values were disrupted dynamically. Furthermore, this study represents the effect of error treatments to IC50 values change. Some errors of technical treatments found by the author can be a reference for information to apply the antioxidant test method with the DPPH method.

### Materials and Methods

Chemicals used in this study were DPPH (p.a., Smartlab), Ascorbic acid (p.a., smartlab), and methanol (p.a., Smartlab). Samples (ready to use) used as model applied in this study were

local leaf, local coffee, and local oxide. The testing used UV-Vis Spectrophotometer (Thermo Scientific Evolution 201 UV-Visible Spectrophotometer).

### Antioxidant Testing using DPPH Method

The antioxidant test procedure using DPPH generally consisted of: (1) solution preparation, (2) mixture preparation, (3) incubation, and (4) absorbance measurement using UV-V is spectroscopy. All of the aspects were evaluated as technical treatment. The procedure in this study applied the SNI 8623:2018. Firstly, the sample solution preparation was carried out using 1000 ppm, namely: sample (10 mg) dissolved in 10 mL methanol until homogeneous. Next, the sample solution was varied between 0 - 1000 ppm with a minimum of 5 concentration variations. On the other hand, the positive control solution (ascorbic acid) was prepared with a concentration of 100 ppm, namely: ascorbic acid (1 mg) dissolved in 10 mL methanol.

Furthermore, the DPPH solution was prepared with a concentration of 0.76 mM or 300 ppm, namely: DPPH powder (3 mg) dissolved in 10 mL methanol. Subsequently, the mixing preparation consisted of: negative control solution, sample control solution, and sample/positive control solution. The mixed negative control was carried out between 0.75 ml DPPH solution and 3 mL methanol. The mixed sample control was carried out between 0.75 ml methanol and 3 mL sample. The mixed sample/positive control was carried out between 0.75 ml DPPH solution and 3 mL sample/positive control. After being vortexed, incubation was carried out at room temperature in an incubator for 30 minutes. Next, UV-V is measurements were carried out to obtain the absorbance values of methanol blank and the all mixing tube). The calculating the percentage of inhibition was using Equation 1. Next, the IC50 value was calculated using linear regression equation.

$$\% \text{ Inhibition} = \frac{(A_{\text{negative control}} - A_{\text{blank}}) - (A_{\text{sample or positive control}} - A_{\text{sample control}})}{(A_{\text{negative control}} - A_{\text{blank}})} \times 100\% \quad (1)$$

Note: A: absorbance value

## Results and Discussions

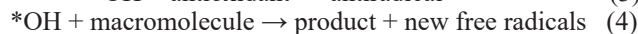
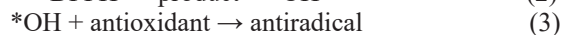
### Sample Preparation

Sample preparation is important to be done consistently and correctly. Accuracy of weighing materials is important. Accuracy of methanol volume added in preparing solutions is also important to be concerned. In addition, pipetting accuracy is very important to ensure the accuracy of the solution concentration, especially when dilution is carried out to prepare various concentrations. So, the accuracy of weighing and volume can later have an impact on the reaction results [10].

Many test sample solutions are made in 1000 ppm [11]. The variation of the sample solution concentration is made until it can detect % inhibition about 50%. If the concentration value does not produce a range of 50 % inhibition, then extrapolation and calculation of the linear regression equation cannot produce a true calculation. On the other hand, the concentration of DPPH is considered very important. Many studies do not pay attention to the value of DPPH concentration, for example, a 0.1 mM DPPH solution [12]. This affects the maximum absorbance value. The reaction of compounds between DPPH and antioxidants is certainly influenced by each concentration [13].

### Possible Reaction of DPPH and Antioxidant

The reaction of DPPH with antioxidants is a chemical reaction without a catalyst. The conditions required are without a light, at room temperature (25 °C), and within 30 minutes. DPPH as a free radical model reacts with antioxidants which will produce a safe and neutral product [14]. The reaction equation can be described as Equations 2 and 3, and the reaction in the absence of antioxidants can be seen at Equation 4.



The new free radical will start the same reaction with the molecules around it [15]. On the other hand, the ratio of the volume of the mixture between DPPH and the sample also affects the reaction that occurs. In the SNI standard, the mixture of DPPH: sample is 1: 4. Of course, the influence is the moles available in the reaction.

### Analyzing Technical Treatments After UV-Vis Measurement Affecting IC50 Value

Data analysis for antioxidant testing is of two acceptable types. In SNI, a linear curve is made between log10 concentration (x-axis) and probit value (y-axis) where the probit value can be searched from the probit value table or with the excel formula (=NORMINV ((percent value)/100,5,1 or =NORMINV ((percent value)/100;5;1) (Figure 1.a). However, in many article discussions, a linear curve is made between concentration (x-axis) and % inhibition (y-axis) (like Figure 1.b) [16,17]. It is important to get 50% inhibition in the testing in order to obtain more accurate IC50 values, so IC50 value in Figure 1.b is less accurate value. The concentration unit is also important to note regarding the ppm unit. This ppm unit explains the unit of  $\mu\text{g/mL}$ . Mathematically, a linear curve between Ln concentration and % inhibition is unacceptable (Figure 1.c), because the results of the linear regression equation make IC50 much different from other calculations.

In many journal references, % inhibition is calculated using a formula such as Equation 5 [16,17]. This blank absorbance is prepared from DPPH solution. Then the sample absorbance contains a mixture of sample solution and DPPH solution that has been incubated. The lacks of Equation 5 are the absorbance of the sample control (in SNI) (sample solution + methanol). It is important treatment to prepare the sample control because the sample control in a case maybe has a higher absorbance value than the blank (methanol). Therefore, the requirements since using Equation 5 are (1) the sample must look clear, no suspension, and no absorbance value, and (2) the blank is DPPH solution must not degraded.

On the other hand, the formula for calculating % inhibition in SNI considers the effect of the absorbance of the sample control as the formula Inhibition in Equation 1. For example, the absorbance of the sample control from green leaf extract can have an absorbance value > 0.020. This value can affect the % inhibition value. Figure 1 shows the analyzing results from antioxidant testing.

$$\% \text{ inhibition} = \frac{(\text{Absorbance of Blank} - \text{Absorbance of Sample})}{\text{Absorbance of Blank}} \quad (5)$$

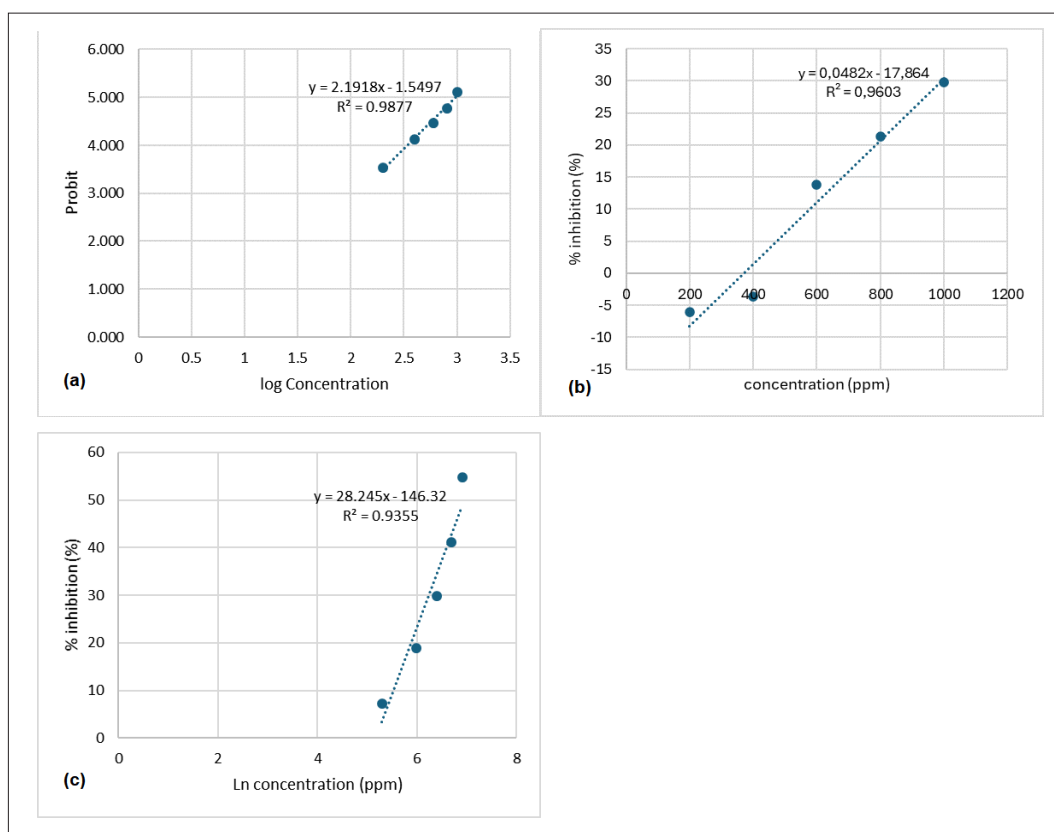


**Figure 1:** (a) Example of Log C vs Probit Value Graph Results (IC50 (anti log result) = 973.362), (b) Example of Results Not Finding the Concentration Range Where there is 50% Inhibition, (c) Example of x-axis Error Results (IC50 (Anti Ln Result) = 1043.787),

Figure 1 shows an example of the results of linear regression curve calculations. The application of antioxidant testing cannot be done only once, at least twice to ensure the results of the curve. The curve results are obtained from UV-V is spectrophotometer measurements. If the instrument is not calibrated, it can affect the absorbance value.

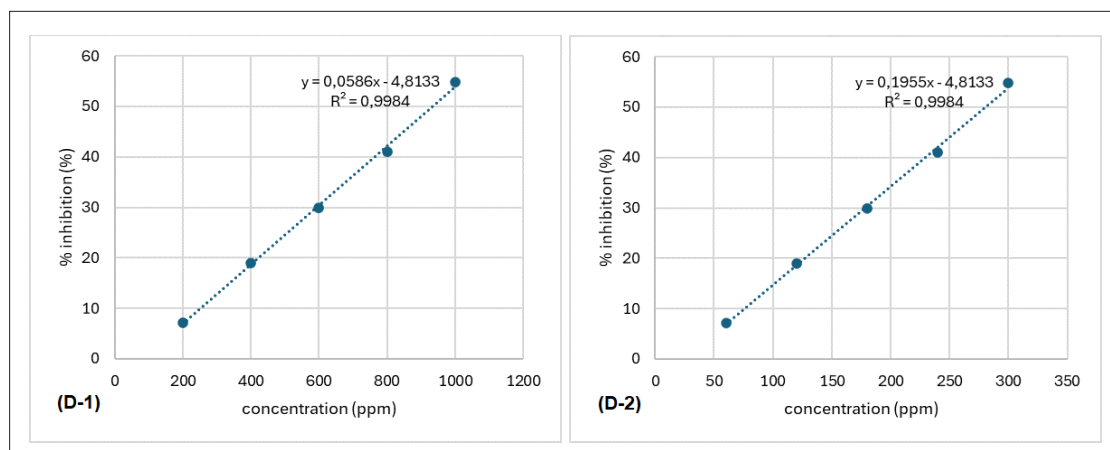


**Figure 2:** (d-1) Example of Error in Calculating the Concentration of the Test Solution (IC50 = 935.4 ppm), (d-2) Example of True in Calculating the Concentration of the Test Solution (IC50 = 280.4 ppm), (e-1) Example of Error in Incubation Temperature of 20 °C (IC50 = 280.4 ppm),

Figures 1.d-1 to 1.d-2 show the importance of a corrected concentration of the sample solution. The uncorrected concentration of the sample will give linear regression equation differentiating, so the IC50 will be incorrect.

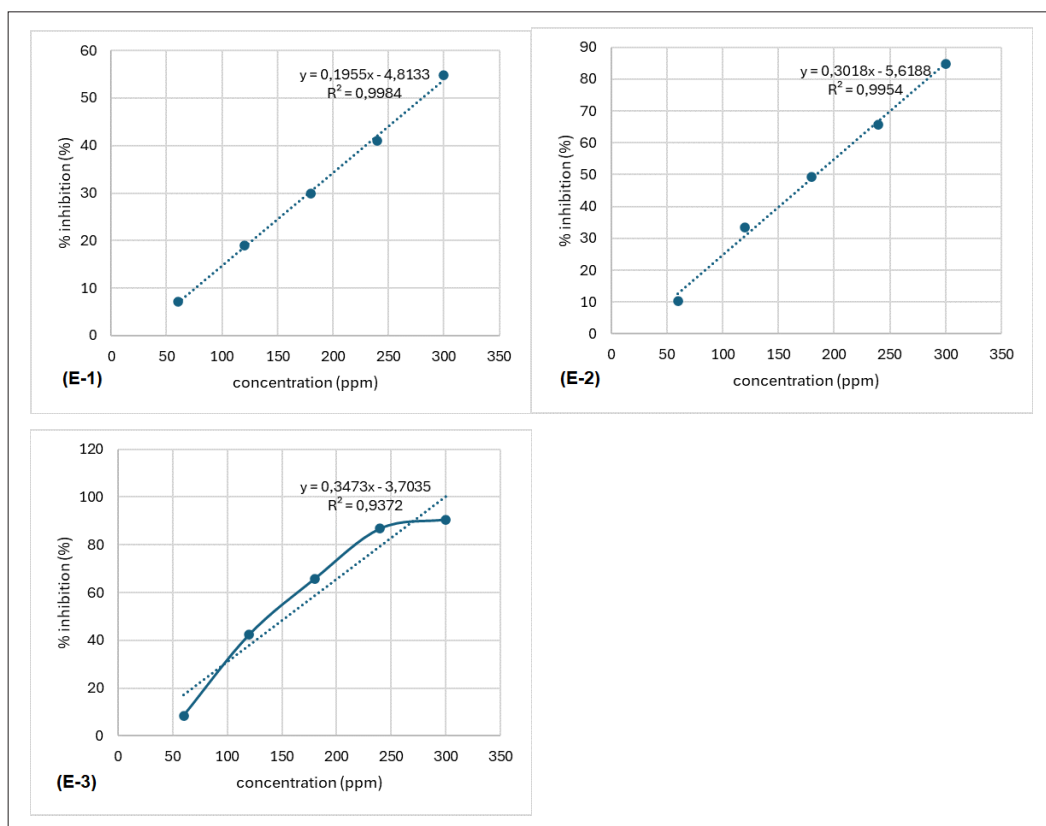


**Figure 3:** (e-1) Example of Error in Incubation Temperature of 20°C (IC50 = 280.4 ppm), (e-2) Procedural True of Incubation Temperature of 25 °C (IC50 = 184.3 ppm), (e-3) Over Incubation Until Empty Methanol (IC50 = 154.6 ppm)

Figures 1.e-1 to 1.e-3 show the importance of a corrected temperature of the incubation. Low temperature will inhibit the reaction. High temperature will evaporate the methanol content. Furthermore, over incubation (methanol empty) will cause a side reaction, so the antioxidant activity will be uncontrollable and form complex products.

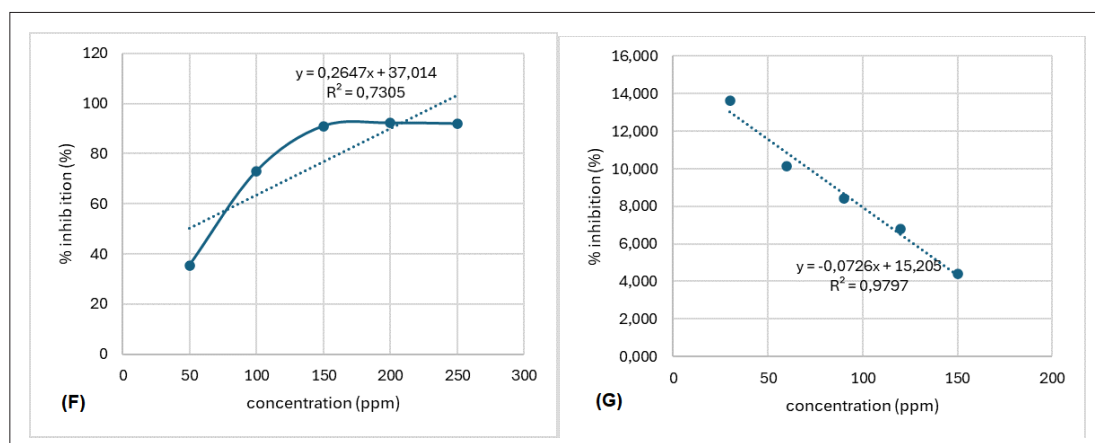


**Figure 4:** (f) Example of Graph Results that can no Longer be Linear, (g) Example of Flat Graph Results (no inhibitory activity)

Figure 1.f shows the importance of a corrected range of the concentration of the sample solution. Range of the concentration was adjusted in order to get 50% of % inhibition. So, the range of the concentration may be higher or lower for obtaining the good linear regression equation. Figure 1.g shows the importance of a corrected the sample preparation. The fault of the preparation will give the concentration value error, so the absorbance will be a poor value. From the explanation above, we can present the categories of errors that can appear during the analysis as in Table 1.

As mentioned in Table 1, uncertainty calculation can contribute to the error analysis. Many factors of uncertainty to ensure the truth of the antioxidant activity value [18,19]. Figure 2 shows the uncertainties in the antioxidant testing. In the testing laboratory, all errors were prevented by regulation of ISO 17025:2017. Therefore, this study can contribute to the regulation.

**Table 1: Types of Errors Affecting IC50 Value Results**

| Error source            | Possibility Error                                                                                                                                                                                                                                                                                           |
|-------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| user                    | <ul style="list-style-type: none"> <li>Not using personal protective equipment (PPE)</li> <li>Conceptual errors</li> <li>Calculation errors</li> <li>False preparation</li> <li>False setting on the instrument</li> <li>Inconsistencies in research procedures</li> <li>Uncertainty calculation</li> </ul> |
| instrument              | <ul style="list-style-type: none"> <li>expired instrument calibration</li> <li>The instrument is old</li> <li>Error setting the test process</li> <li>Power supply error</li> <li>A measuring instrument is not calibrated</li> <li>The instrument is not clean</li> </ul>                                  |
| chemicals               | <ul style="list-style-type: none"> <li>Material contains contaminants</li> <li>Less accurate concentration of solution</li> <li>Expired material</li> </ul>                                                                                                                                                 |
| Testing room conditions | <ul style="list-style-type: none"> <li>Unstable room temperature or above normal temperature</li> <li>Too high humidity (&gt;80%)</li> <li>Unstable air pressure (rare)</li> </ul>                                                                                                                          |

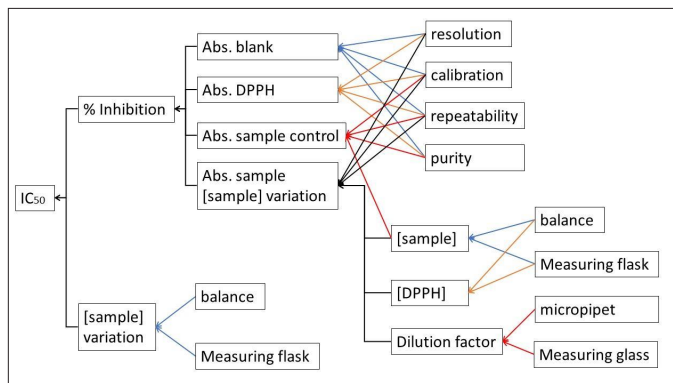


**Figure 2: Mapping Uncertainties of the Antioxidant Activity Testing**

## Conclusion

This study can provide the key to minimize the fault of antioxidant analysis. This study has conveyed the errors that may occur during the antioxidant test. The procedures that often occur incorrectly are concentration preparation, calculation, and spectrophotometer reading errors. However, each sample needs to know the concentration range which can produce % inhibition of 50% and  $R^2$  of the regression equation value close to 1. Thus, the IC50 value can be trusted validly. More specific errors can be studied in further studies.

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