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Method Validation and Estimation of Measurement Uncertainty in The Determination of Total Polyphenols Content in Land Spinach by Uv-Vis Spectrophotometry

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Abstract

Land spinach contains polyphenols which can be determined by the spectrophotometry method. Data on method validation and estimation of uncertainty measurement of polyphenols in land spinach are not yet available, so method validation and estimation of uncertainty measurement of polyphenol content determination were carried out using a UV-Vis spectrophotometer. The validation parameters of the tested methods include linearity, precision, accuracy, and LOD and LOQ. Analysis of total polyphenol content was carried out using the Folin-Ciocalteu method with the gallic acid standard. Based on the results of method validation, the equation of the line y = 0.7213x-0.0096 with a coefficient of determination (R^2) is 0.9994. These results indicate that the correlation between standard concentrations of gallic acid and absorbance shows good linearity. Repeated measurements showed the average total polyphenol content and the estimated measurement uncertainty value was (3.0501±0.2886) mg/Kg GAE. The RSD percentage was obtained at 1.79% were the result entered the acceptance condition because it was ≤ 2%. Percent recovery was obtained at 81.50% and 85.66%, and the terms of acceptance range between 80-110%. The LOD and LOQ values obtained were 0.1024 mg/L and 0.3412 mg/L. Based on the data obtained, it can be said that the method used has good validity.

Keywords: Folin-ciocalteu, method validation, phenolic content, UV-Vis, and uncertainty

Introduction

Land spinach (*Ipomoea reptans poir*) is one of the horticulture that is often found in Indonesia. Swamp cabbage is widely used as a source of food that is often consumed by the community. Land spinach contains chemical compounds of polyphenols, flavonoids, and quinones. Phenolic compounds or polyphenols are secondary metabolites in plants (Rajauria, 2018; Gomez et al., 2019; Pereira et al., 2017). Polyphenols can be found in vegetables, cereal, fruit, spices, and herbs (Martinez et al., 2019); (Akomeng & Adusei, 2021). Polyphenol compounds have antioxidant activity (Alfaris et al., 2021); (Rojas-Ocampo et al., 2021). Polyphenols are widely used in diet programs and the medical field (Caldas et al., 2018). These compounds have the potential to impact positively human health because of their antioxidant and antiradical properties (Lohani & Muthukumarappan, 2021).

Extraction is an important step in the isolation and identification of phenolic compounds. Solid-liquid extraction (SLE) followed by a stage of concentration and purification is the most widely used method to make a selective extraction of phenolic compounds from various matrices. A considerable increase in the mean content of extracted polyphenols can be observed when methanol or ethanol were used instead of water (Bajkacz et al., 2018).

Several analytical techniques have been proposed for the quantification of polyphenols in plant extracts due to their structural complexity and diversity (Zhong et al., 2019). UV-visible spectrophotometry is an adequate method to determine the total phenol content (Ivanova et al., 2010). The tests were carried out using the Folin-Ciocalteu (FC) method with gallic acid standards.

The general technique of the FC method for the quantification of phenols is based on the spectrophotometric determination of the phosphotungstate-molybdate complex (Folin & Denis, 1912). This complex in the presence of sodium carbonate to form a blue-colored complex (Kupina et al., 2018). In this oxidation-reduction reaction, the phenolic ion is oxidized while the heteropoly-phosphotungstate-phosphomolybdate

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complex is reduced to form a blue chromophore. Folin-Ciocaltue chemical reactions highlight a redox reaction in which the elements tungsten (W) and molybdenum (Mo) are reduced to the +5 ionic form, and the phenolic ring is oxidized (Ford et al., 2019). The intensity of the blue color is proportional to the amount of reactive phenolic compounds in the sample. The phenolic content is determined by measuring the absorbance of the sample solution at 765 nm and comparing it with a calibration curve using gallic acid as a standard.

The advantages of this method are that it is simple and fast, but it is less selective because it can react with ascorbic acid, sugar, and aromatic amines (Sánchez-Rangel et al., 2013). In the absence of method validation data and estimation uncertainty of the measurement of total phenol determination in land spinach, it is necessary to validate the method for determining total polyphenol in land spinach. Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. The purpose of method validation is to provide results that are following already-established acceptance criteria. The method validation parameters include linearity, the limit of detection (LOD), the limit of quantification (LOQ), precision, and accuracy. The uncertainty is the best estimate of how far an experimental quantity might be from the true value. The uncertainty of measurement is the key indicator of the quality of any experimental result.

Therefore, this study aims to validate the method for determining total polyphenols content in land spinach using the spectrometric method with the parameters tested including linearity, LOD, LOQ, precision, and accuracy. In this study, the estimation of measurement uncertainty is also presented.

Methods

The equipment used is a UV-Vis spectrophotometer (Hitachi UH5300), 250 mL volumetric flask, 25 mL volumetric flask, spatula, 100 mL beaker, 500 mL beaker, vial, 1 mL measuring pipette, 10 mL measuring pipette, analytical balance (Ohaus), ultrasonic (GT sonic), Buchner, rotary evaporator, desiccator, mortar, pestle, and micropipette.

The materials used in this study were standard gallic acid, Na_2CO_3, sample, distilled water, 96% ethanol, filter paper, and Folin Ciocalteu (Methan Tirta Kimia, Merck KGaA, 64271 Darmstadt Germany).

Sample preparation

A total of 6 kg of land spinach are picked from the leaves, then cleaned, cut the land spinach leaves to small sizes. Land spinach is oven-dried at 60°C for 8 hours. Dried land spinach leaves are made into powder with a grinding machine. A total of 500 g of land spinach powder was weighed, then macerated by mixing 5 L of 96% ethanol (1:10). The mixed land spinach was filtered using a Buchner and filter paper. The extract was concentrated with a rotary evaporator at a temperature of 60°C at a speed of 60 rpm and stored in a desiccator.

Linearity

A total of 0.05 g of the gallic acid standard was weighed and put in a 250 mL volumetric flask. The standard was added with 30 mL of distilled water and ultrasonicated for 5 minutes. The solution was diluted. The solution was prepared with a concentration of 0.2; 0.4; 0.6; 0.8; and 1 mg/L as much as 25 mL. The diluted solution was pipetted 1 mL and 5 mL of Folin-Ciocalteu and 4 mL of Na_2CO_3 solution were added. The solution was homogenized and allowed to stand for 2 hours in a dark place.

The Folin-Ciocalteu reagent is very stable if it is protected from reducing agents and light during the dissolution process, the basic mechanism of the Folin-Ciocalteu method is measuring polyphenols in natural materials with phenolic groups being oxidized and metal ions being reduced (Agbor et al., 2014). The solution was then measured absorbance at the optimum wavelength.

Limit of detection dan limit of quantification

The limit of detection (LOD) is the smallest concentration of analyte that can be detected under approved experimental conditions. The limit of quantitation (LOQ) is a parameter that shows the lowest concentration of analyte detected with precision and accuracy. The limit of detection and limit of quantification is determined using equations (1) and (2):

$$LOD = 3\,x\,\frac{Sy/x}{Slope} \quad (1)$$

$$LOQ = 10\,x\,\frac{Sy/x}{slope} \quad (2)$$

Where Sy/x is calculated using equation (3)

$$Sy/x = \sqrt{\frac{\Sigma\,(y-y1)^2}{n-2}} \quad (3)$$

Precision

Precision is a measure of the closeness of an analysis result obtained from a series of repeated measurements of the same size. Precision testing was carried out by weighing 0.2 g of the sample and adding a 250 mL volumetric flask. 50 mL of distilled water was added, ultrasonicated for 5 minutes, and adjusted using distilled water to the 5 mL mark. The sample solution was diluted to 25 mL. 1 mL of the solution was pipetted, then 5 mL of Folin-Ciocalteu solution and 4 mL of Na_2CO_3 were added. The solution was allowed to stand for 2 hours in the dark place and the absorbance was measured at the optimum wavelength. The determination of the precision test was carried out for 7 repetitions. Precision is expressed in % RSD which is calculated by equations (4) and (5):

$$SD = \sqrt{\frac{\Sigma (xi-x)^2}{n-1}} \quad (4)$$

$$\%\text{RSD} = \frac{SD}{x} x100\% \quad (5)$$

Accuracy

Accuracy is a measure that shows the degree of closeness of the analysis results to the true value. Accuracy testing was carried out by adding 5 mL of the sample solution with 0.1 mL of 200 mg/L gallic acids adjusted to 25 mL. The solution was taken 1 mL and added 5 mL of Folin-Ciocalteu and 4 mL of Na_2CO_3 solution. The solution was allowed to stand for 2 hours in a dark place and the absorbance was measured at the optimum wavelength. Determination of the accuracy test was carried out 7 times. Accuracy is expressed in percent recovery and expressed in equation (6):

$$\%\ Recovery = \frac{A-B}{C} x100\% \quad (6)$$

Where A is the sample and standard concentration (mg/L), B is the sample concentration (mg/L) and C is the standard concentration (mg/L).

Measurement uncertainty

Measurement uncertainty is defined as the probability or level of confidence. Each measurement made will have some associated uncertainty and the quoted uncertainty interval will be a range in the true value that lies at a certain level of confidence. According to Eurachem (2012), the steps in determining measurement uncertainty are: creating a test model, determining the quantities that have been measured and expressed in formulas, identifying sources of uncertainty, simplifying by grouping sources of uncertainty into type a and type b, measuring components that have been grouped, calculate the remaining components, convert the components to standard deviations, calculate the combined uncertainty, review if re-evaluation is needed and calculate the expanded uncertainty.

Results and Discussion

Linearity

Linearity is the ability of an analytical method to provide a proportional response to the analyte concentration in the sample. Determination of linearity was carried out using standard gallic acid with a concentration of 0; 0.2; 0.4; 0.6; 0.8; 1 mg/L. The results of the absorbance measurement of the standard solution series are shown in **Table 1**.

Table 1. Result of measurement of absorbance of a standard solution of gallic acid

Concentration (mg/L)	Absorbance			Mean
	1	2	3	
0.0	0.000	0.000	0.000	0.000
0.2	0.123	0.124	0.124	0.124
0.4	0.280	0.278	0.278	0.279
0.6	0.421	0.421	0.422	0.421
0.8	0.569	0.569	0.568	0.569
1.0	0.714	0.715	0.714	0.714

Based on **Table 1**, there is a positive correlation between the analyzed gallic acid concentration and the absorbance value. This is under Lambert Beer's law which states a linear relationship between the analyte concentration and the measured absorbance. The linearity test was carried out by making a curve by plotting the concentration data as the x value and the absorbance value as the y value as shown in **Figure 1**. Based on the results of the calibration curve on the gallic acid standard, the equation of the line y=0.7213x-0.0096 obtained the slope value. 0.7213, the intercept value is -0.0096 and the coefficient of determination (R^2) is 0.9994.

The analytical method is said to be linear in a certain concentration range if the correlation coefficient obtained is at least 0.997 (Chan, 2004). Based on this statement, it can be said that the linearity test carried out meets the acceptance requirements because the correlation coefficient (R) obtained is 0.9997.

The slope value indicates the sensitivity of the method, the greater the slope value, the higher the sensitivity or the stronger the instrument response to changes in analyte concentration. An intercept value close to zero indicates a better measurement because the blank or analyte-free solution should not respond when the measurement is taken.

Intercept values that are not equal to zero can be caused by interference, contamination, and other sources of bias. A negative intercept value indicates the presence of positive contaminants, causing the measured sample concentration to be greater than the actual value. In this research, positive contaminants can be sourced from chemical compounds in land spinach such as flavonoids and quinones. The regression equation for determining the total polyphenol content before the study of y = 1.3595x – 0.003 with R^2 value 0.9946 (Bastola et al., 2017).

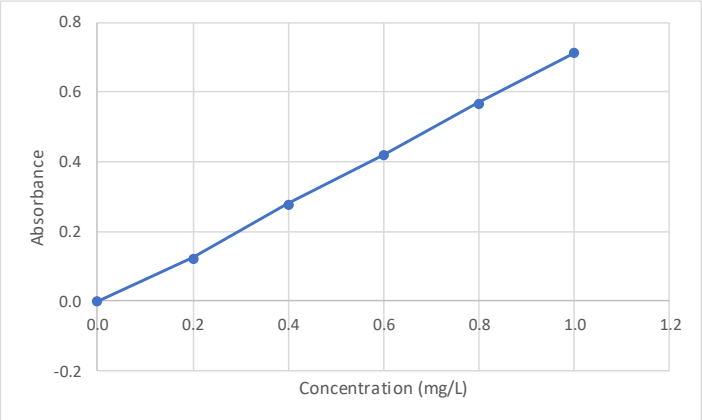

Figure 1. Calibration curves of a standard solution of gallic acid

Limit of detection dan limit of quantification

The limit of detection and limit of quantification in this study was calculated based on the standard curve of gallic acid. The LOD and LOQ values obtained indicate the sensitivity value of the test results using this method. Sensitivity is a measure of the quality of the method that describes the ability of the method to detect a sample. Based on Table 2, the calculation results show that the LOQ has a smaller value than the average sample concentration, which is 0.4856 mg/L. This means that the measurement results are obtained with accuracy and precision.

Table 2. Limit of detection and limit of quantification

The concentration of the standard (mg/L)	y	y_i	y-y_i	$(y-yi)^2$
0.0	0.0000	0.0096	-0.0096	0.000092
0.2	0.1237	0.1539	-0.0302	0.000912
0.4	0.2787	0.2981	-0.0195	0.000378
0.6	0.4213	0.4424	-0.0210	0.000443
0.8	0.5687	0.5866	-0.0180	0.000323
1.0	0.7143	0.7309	-0.0166	0.000274
		0.3703	Total	0.0024
			Sy/x	0.0246
			LOD	0.1024 mg/L
			LOQ	0.3412 mg/L

Accuracy

Accuracy testing is important because in the analysis method the sample is not directly measured by instruments but there are stages of preparation. The stages of preparation in this research are maceration, filtration, and distillation. The efficiency of these steps must be ensured so that no addition or loss of analyte occurs during sample preparation. Therefore, in this study, an accuracy test was carried out which was expressed in the value of % recovery.

Testing the accuracy of determining polyphenols in a sample using the spiking technique. The addition of the standard volume of gallic acid is 2% of the total spike volume. The added standard solution also has a high concentration of 200 mg/L. The addition of a standard solution with a high concentration is intended to avoid changes in the sample matrix. The spike concentration was 2.42 times the sample concentration before being added to the standard. This is done so that the accuracy test gives a real influence on the evaluation of the accuracy test. The limit acceptance of the % recovery value with the analyte concentration in the 1 mg/L sample is 80%-110%, so it can be concluded that the accuracy test (Table 3) is included in the terms of acceptance.

Table 3. Results of recovery

Identity	Absorbance	Concentration(mg/L)	% Recovery
Spike 1	0.858	1.1762	81.50
Spike 2	0.862	1.1818	85.66

Precision

The precision of the measurement is to be done to determine whether the response of the instrument to an analyte is repeatable from time to time. In this study, the precision of the analytical method is expressed in repeatability. Repeatability is the precision value obtained if all measurements are made by one analyst within a certain period, using the same sample, the same reagents, and equipment in the same laboratory. The results of the precision test in this study are presented in Table 3. Based on Table 4. the polyphenol content in the sample can be reported as (3.0510 ± 0.0545) mg/Kg GAE with the % RSD value obtained at 1.79%. RSD values and error percentages lower than 10% are quite satisfactory (Pereira et al., 2018). This value indicates that the measurement results have good precision.

Table 4. Results of precision

Repetition	Sample concentration (mg/L)	Sample level (mg/Kg)	xi-x	$(xi-x)^2$
1	0.4733	2.9755	-0.0755	0.0057
2	0.4789	3.0102	-0.0408	0.0017
3	0.4816	3.0189	-0.0322	0.0010
4	0.4844	3.0448	-0.0062	0.0000
5	0.4899	3.0795	0.0285	0.0008
6	0.4927	3.0968	0.0458	0.0021
7	0.4983	3.1315	0.0805	0.0065
mean	0.4856	3.0510	Total $(xi-x)^2$	0.0178
			SD	0.0545
			%RSD	1.79

Measurement uncertainty

Every measurement has a deviation. Reliable measurement results must meet the requirements of being traceable and having a good track record. The traceability of a test can be related to the value of uncertainty. The traceability of the test results relates to the correct value derived from the reference material. Uncertainty states the distribution of quantitative values obtained from test results based on the information used. Uncertainty is traced to determine the correct value of the validation of the polyphenol determination method so that it is expected to provide a method with valid results. Estimated measurement uncertainty is a parameter related to the accuracy of the measurement results. The measurement uncertainty value can be used to evaluate a laboratory in conducting a proficiency test (Kusumaningtyas et al., 2016).

The estimation of measurement uncertainty was determined according to Eurachem (2012), so the value of the estimation results in this study can be presented in Table 5. Based on Table 5, the uncertainty value of polyphenol determination can be reported as (3.0501±0.2886) mg/Kg GAE. Determination of the uncertainty estimate using a 95% confidence interval so that the coverage factor has a value of 2 so that the expanded uncertainty value of 0.2886 mg/Kg GAE.

Table 5. Estimation of measurement uncertainty

Parameters	μx	x	Unit	μx/x	$(\mu x/x)^2$
1 mL measuring pipette	0.006	1	mL	0.006	3.60×10^{-5}
5 mL measuring pipette	0.03	5	mL	0.006	3.60×10^{-5}
25 mL volumetric flask	0.04	25	mL	0.0016	2.56×10^{-6}
250 mL volumetric flask	0.15	250	mL	0.0006	3.60×10^{-7}
Precision	0.02	1	-	0.0178652	3.19 x 10-4
Mass of sample	0.00006	0.2	g	0.0003	9.00×10^{-8}
Calibration curves	0.020958	0.4881662	mg/L	0.042932	1.84×10^{-3}
Total					0.0022373
Combined uncertainty (mg/Kg)					0.1443137
Expanded uncertainty (mg/Kg)					0.2886273

The biggest contributor to the uncertainty value is on the calibration curve, which is 57.02% (Table 6). This is because the uncertainty of the calibration curve is influenced by many factors namely slope, residual deviation, repetition of measurement, number of standards used, standard concentration, and sample concentration.

Table 6. Contribution of uncertainty sources

No	Source of uncertainty	Contribution (%)
1	1 mL measuring pipette	7.97
2	5 mL measuring pipette	7.97
3	25 mL volumetric flask	2.12
4	250 mL volumetric flask	0.80
5	Precision	23.73
6	Mass of sample	0.40
7	Calibration curves	57.02

Conclusions

Validation method of the determination of polyphenol levels in the ethanol extract of land spinach (*Ipomoea reptans. Poir*) by UV-Vis spectrophotometer. it can be concluded that the polyphenol content of land spinach was (3.0501±0.2886) mg/Kg GAE. The results of the linear equation y = 0.7213x-0.0096 with a coefficient of determination (R^2) 0.9994 indicates the resulting linearity is good. The LOD value was obtained at 0.1024 mg/L while the LOQ value was obtained at 0.3412 mg/L. The precision value obtained by the RSD percentage is 1.79%. where the results enter the acceptance requirements (< 2%). Accuracy values obtained a % recovery of 81.50% and 85.66% with an acceptability range between 80-110%. Based on the data obtained. it can be concluded that the analytical method for determining polyphenols in land spinach was declared to have sufficient evidence to be declared valid.

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