

Performance of Caffeine Content Analysis in *Robusta sp* and *Theobroma cacao L* using Iodometry, UV-Vis Spectrophotometry and High Performance Liquid Chromatography

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Received 4 May 2020; Accepted 25 December 2020

ABSTRACT

Caffeine is a xanthine alkaloid compound found in *Robusta sp* and *Theobroma cacao L*. The caffeine extract was obtained by evaporating CHCl_3 using a rotary evaporator and determine its content using iodometry, UV-Vis spectrophotometry and high performance liquid chromatography, then validated by measurement methods. The iodometric quantify the caffeine content in *Theobroma cacao L* is 900 mg/kg while UV-Vis spectrophotometric is 4,000 mg/kg, and for HPLC in *Robusta sp* is 19,475 mg/kg. The iodometry performance gives the linearity value, R^2 of 0.9791. The precision values at 5.0 mg/L and 15.0 mg/L are 4.6% and 8.3%. Accuracy for 5.0; 10.0; and 15.0 mg/L are 106%, 100% and 94%, respectively. The performance of UV-Vis spectrophotometry gives linearity value, R^2 of 0.9948. The precision value at 1.0 mg/L and 6.0 mg/L gives the variance coefficient value of 4.2% and 1.2%, while the accuracy at 1.0 mg/L and 6.0 mg/L are 99% and 101%.

Key word: caffeine, HPLC, iodometry, *Robusta sp*, spectrophotometry, *Theobroma cacao L*

INTRODUCTION

Coffee (*Robusta sp*) and Cocoa (*Theobroma cacao L*) are natural plants which are grown and cultivated in several regions in Indonesia. Both plants contain crystalline alkaloid compound, namely caffeine or 1,3,7-trimethylxanthine, where the main constituent compounds are protein derivatives in the form of xanthine purines (Figure 1) [1-3].

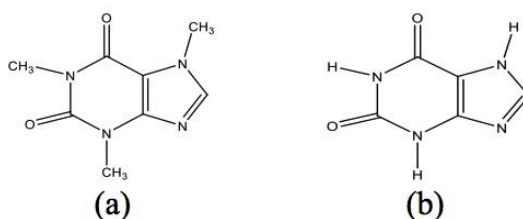


Figure 1. Structure (a) Caffeine; (b) xanthine purines

Caffeine has odorless and bitter property, soluble in chloroform and dichloromethane [4]. In normal body condition, caffeine is easily absorbed by the body and is efficacious as an

The journal homepage www.jpacr.ub.ac.id
p-ISSN : 2302 – 4690 | e-ISSN : 2541 – 0733

analgesic drug which can reduce pain and fever, stimulate the nervous and respiratory system, relax the smooth muscle, and stimulate the cardiac muscle [5-7]. Caffeine usually is utilized as a drug and food supplement which can be used as a light psychoactive stimulant [8-9].

Quantitative identification of caffeine in coffee beans by iodometry, UV-Vis spectrophotometry and high performance liquid chromatography (HPLC) has been widely carried out [3,5,10]. Beside quantitative, Solid Phase Extraction (SPE), TLC Scanner dan Gas Chromatography-Flame Ionisation Detector (GC-FID), Fourier Transform Infrared (FTIR), NIR reflectance spectrometry, and capillary electrophoresis have also been used qualitatively to analyze caffeine [10-12].

Iodometry is a practical, simple and efficient analytical method. This method is a redox titration based on the sample color changing after titration [13-15]. Meanwhile, the UV-Vis spectrophotometry is a relatively quick and easy method on determining the content in various sample, and while high performance liquid chromatography is rapid analytical method which can separate the mixture into a single component and has a good validity [16-18]. Iodometry and UV-Vis spectrophotometry are analytical methods which are often validated. Validation is an assesment technique to prove whether an analytical method can be used based on the linearity, precision and accuracy parameters. The analytical method can be clasified having a good performance if the linearity value R^2 close to 1, accuracy (recovery %) of 98-102 % and precision is expressed by variance coefficient $\leq 3\%$ [11,14,19].

EXPERIMENT

Chemicals and instrumentation

Chemicals used for research include caffeine standard, chloroform (Merck), calcium carbonate (Merck), starch, ammonia (Merck), sulfuric acid (Merck), potassium iodide (Merck), sodium thiosulfate (Merck), potassium iodate (Merck), sodium chloride (Merck), iodine (Merck), methanol p.a, lead acetate (Merck), and aquades.

Sample of *Robusta sp* dan *Theobroma cacao L* were taken from Seram Island, Maluku Province and specimen was identified by Maluku Agriculture Technology Assessment Center (Maluku BPTP).

Instruments and tools used for analysis include UV-Vis spectrophotometer (Rayleigh UV-9200), high performance liquid chromatograph (HPLC, Shimadzu LC-2010C), hot plate, analytical balance (Ohaus AR 2140), rotary evaporator, oven, separating funnel, burette, buchner funnel, dan glassware (Pyrex).

Coffee and cocoa bean preparation

Fresh sample of coffee (*Robusta sp.*) and cocoa (*Theobroma cacao L*) already skin-pilled was sorted. Then, it was dried for 3 hours at 105 °C and roasted until blackish brown were resulted. While cocoa seeds were fermented for 5 days and dried for 5 hours at 105 °C.

Caffeine extraction

The sample was cooled, mashed and dissolved with 100 mL of hot distilled water. The filtrate was extracted for 15 minutes using 100 mL of CHCl_3 with the addition of 1.0 g of CaCO_3 and 10 mL of 3% ammonia solution. The extract was evaporated using rotary evaporator.

Iodometry analysis of caffeine

Standardization of Na₂S₂O₃ solution

A 10 mL of solution KIO₃ 0.1N was placed into 100 mL erlenmeyer and 5.0 mL of KI solution 10% and 2 mL of 10% sulfuric acid solution were added into the solution. The solution the homogenized and titrated with Na₂S₂O₃ 0.1 N until colorless.

Determination of caffeine content

A 5.0 mL of caffeine extract was added with 2.5 mL of H₂SO₄ 2N, 25 mL of I₂ 0.1 N, and 10 mL of saturated NaCl. Then, the solution mixture was homogenized and titrated with Na₂S₂O₃ 0.1 N.

$$\text{Caffeine Content (\%)} = \frac{(V_b - V_s) \frac{N(\text{Na}_2\text{S}_2\text{O}_3)}{0.1\text{N}} \times 4.85}{\text{sample weight (mg)}} \times 100\%$$

V_b is blank titrant volume (mL), V_s is sample titrant volume (mL), N(Na₂S₂O₃) is normality or concentration (in Normality) for solution of Na₂S₂O₃, and 1.0 mL of Na₂S₂O₃ solution equals to 4.85 mg of caffeine.

UV-Vis spectrophotometric analysis of caffeine

Caffeine standard solution

A 10 mg of caffeine is dissolved and homogenized with distilled water in 100 mL volumetric flask.

Standard curve and caffeine analysis

A 10 mL of 2.0-20.0 mg/L standard solution were made from 100 mg/L caffeine standard solution. And then, the absorbance of each solution was measured at λ_{max}. The caffeine analysis was conducted by dissolving the sample in volumetric flask, homogenized and determined the absorbance.

HPLC analysis of caffeine

A 1.0 g of sample was dissolved in 40 mL of distilled water and 1 mL of Pb-acetate was added to the solution, heated at 100 °C for 15 minutes and cooled. This mixture is transferred to 100 mL volumetric flask and homogenized. A 10 mL of filtrate was placed to 50 mL volumetric flask, distilled water was added until the boundary markers. The solution, then, was filtered and measured.

$$\text{Caffeine content (mg/kg)} = \frac{C_{\text{reg}} \text{ (mg/L)} \times \text{sample volume (L)} \times \text{dilution factor}}{\text{sample weight (kg)}}$$

Analytical method performances

Iodometry

Linearity

Standard solution of 5.0-30.0 mg/L were made from caffeine standard solution 100 mg/L in 10 mL volumetric flask. Then each was diluted. A 5.0 mL of the solution were placed in different 100 mL erlenmeyers and added with 2.5 mL H₂SO₄ 2 N, 25 mL of I₂ 0.1 N, and 10 mL of saturated NaCl. Each solution, then, was titrated with solution of Na₂S₂O₃ 0.1 N.

Precision

Standard solution of 5.0 mg/L dan 15.0 mg/L were made from caffeine standard solution 100 mg/L in 25 mL volumetric flask was diluted. A 5.0 mL of each solutions were placed in different 100 mL erlenmeyers, and was added with 2.5 mL of H₂SO₄ 2 N, 25 mL of I₂ 0.1 N and 10 mL of saturated NaCl. Each solution was titrated with Na₂S₂O₃ 0.1 N (5 times for each concentration).

Accuracy (Recovery %)

A 5.0 mL samples without spikes were placed into 250 mL erlenmeyer, then it was added with 2.5 mL H₂SO₄ 2 N, 25 mL of I₂ 0.1 N and 10 mL of saturated NaCl. The solution, then, was homogenized and titrated with 0.1 N of Na₂S₂O₃ solution until colorless, and the caffeine content was calculated. Then, a 1.25, 2.50 and 3.75 mL of the caffeine standard solution 100 mg/L were taken into each 25 mL volumetric flask and homogenized with the sample. Each 5.0 mL of the solution were taken and placed to the erlenmeyer, then added with 2.5 mL of H₂SO₄ 2 N, 25 mL of I₂ 0.1 N, and 10 mL of saturated NaCl. They were homogenized and titrated with solution of Na₂S₂O₃ 0.1 N.

UV-Vis spectrophotometry

Linearity

A 10 mL of 1.0-12.0 mg/L of standar solution were made from caffeine standard solution 100 mg/L, and each solutions was measured their absorbance at λ_{maks} .

Precision

The absorbance of caffeine standard solution 1.0 mg/L and 6.0 mg/L were measured 5 times each. The precision value was indicates by % of coefficient of variation (CV). The SD is deviation standard was measured from the value of each measurement.

$$SD = \sqrt{\frac{\sum(x_i - \bar{x})^2}{n-1}} \quad CV = \frac{SD}{\bar{x}} \times 100\%$$

Accuracy (Recovery %)

A 0.1 mL and 0.6 mL of caffeine 100 mg/L solution were placed into each 10 mL of volumetric flask. Each was diluted until the boundary marker. The resulted solutions were compared with sample solutions without standard addition and standard solutions with spikes.

$$\text{Recovery \%} = \frac{CF - CA}{C^*A} \times 100\%$$

Where CF is concentration of the measurement sample, CA is an actual sample concentration, and C*A is concentration of the analyte added.

RESULT AND DISCUSSION

Caffeine (1,3,7-trimethylxanthine) (Figure 1) is an alkaloid compound with the chemical formula C₈H₁₀N₄O₂. This compound has a molecular weight of 194.19 g.mol⁻¹, melting point 237 °C, and density of 1.05 g.cm⁻¹ [10,20]. Iodometry, UV-Vis spectrophotometry and high performance liquid chromatography are quantitative methods which can analyze caffeine and examine the method performance.

Content analysis by iodometry

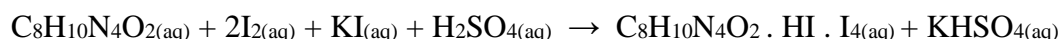
The solution of sodium tiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) has unstable properties due to the influence of low pH, light and particularly the presence of bacteria which will utilize sulfur during storage. Hence, it is necessary to determine the actual concentration of $\text{Na}_2\text{S}_2\text{O}_3$ before using it in the analysis. The thiosulfate standard applied for analysis is 0.1040 N (Table 1).

Table 1. Standardization of $\text{Na}_2\text{S}_2\text{O}_3$ 0.1 N with KIO_3 0.1N

KIO₃ volume (mL)	Na₂S₂O₃ Normality (N)	Na₂S₂O₃ titration volume (mL)	Na₂S₂O₃ Normality (N)	Na₂S₂O₃ Actual concentration (N)
10.0	0.1	9.6	0.1041	
10.0	0.1	9.6	0.1041	0.1040
10.0	0.1	9.6	0.1041	

Determination of caffeine content

The sample caffeine analysed for this step was isolated from *Theobroma cacao* L. Caffeine molecule is an easily reduced by an iodide in acidic condition. Dissolution of I_2 using KI is conducted since I_2 is a solid substance which is difficult to dissolve in water. After the addition of I_2 0.1 N, the color of the solution changes to yellowish brown with the reaction occurs as below.



Titration is carried out until the color of the solution turns to aqueous yellow. This indicate that $\text{Na}_2\text{S}_2\text{O}_3$ has been oxidized. Starch 1% addition will ensure the end of titration marked by color changes.

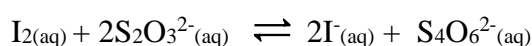


Table 2. Caffeine content in the sample of *Theobroma cacao* L

Sample volume (mL)	Blank volume (mL)	Caffeine content (%)	Average caffeine content (%)	Caffeine content (mg/kg)
11.2	11.4	0.09		
11.2	11.4	0.09	0.09	900 mg/kg
12.0	12.2	0.09		

The result for iodometric analyzed caffeine in the sample extracted from *Theobroma cacao* L as tabulated in Table 2. Caffeine content in analysis 0.09% or caffeine content in the extract 900 mg/kg of sample.

Content analysis using UV-Vis spectrophotometry

Content analysis of caffeine using UV-Vis spectrophotometry is based on the determination on the maximum wavelength (nm) of caffeine. The UV-Vis spectrum of caffeine

is depicted in Figure 2, and the λ_{\max} of caffeine is indicated at the maximum absorbance at 277 nm. Further analysis of caffeine content is determined under this λ_{\max} value.

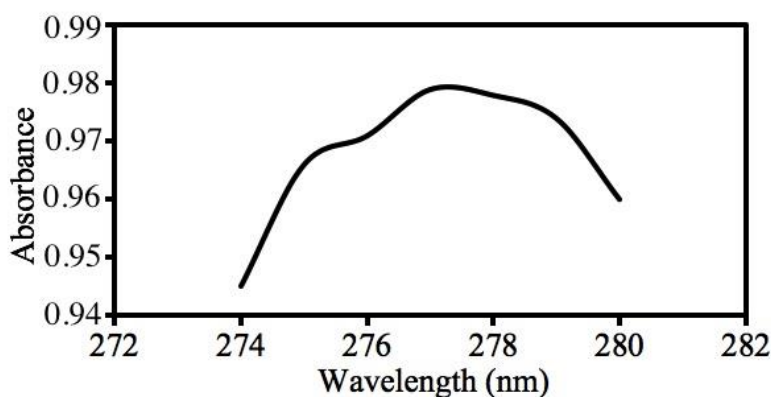


Figure 2. The UV wavelength of caffeine

Standard curve and content of caffeine

The absorbance values of caffeine standard with several concentrations is plotted into a graph, as displayed in Figure 3. The straight line is resulted, with correlation coefficient (R^2 0.9990). The concentration of caffeine contained in the sample of *Theobroma cacao* L was determined by extrapolation of the absorbance value (0.325 absorption unit) resulted from analysis into the concentration the x-axis from the graph (Figure 3). It was found 4,000 mg/kg of sample contained in the sample.

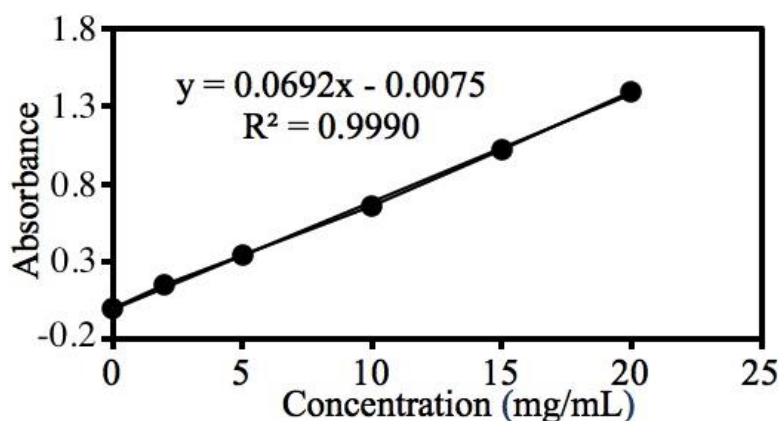


Figure 3. Curve standard of caffeine determined from UV-Vis spectrophotometric

Content analysis using HPLC

In total 7 solutions of caffeine standard were prepared in different concentration. The analysis of each using HPLC provided chromatograms, and detect caffeine peak at retention time in about 8.46 minutes. Two example of chromatograms are depicted in Figure 4(a-b). Caffeine standard at concentration 0.5 mg/L (Figure 4a) and 100 mg/L (Figure 4b). The increase of caffeine concentration inline to the increase of area under curve of chromatograms. That means, increase the caffeine content in the solution of sample. The linearity of trend is represented as a high value of coefficient correlation (R^2 0.9994).

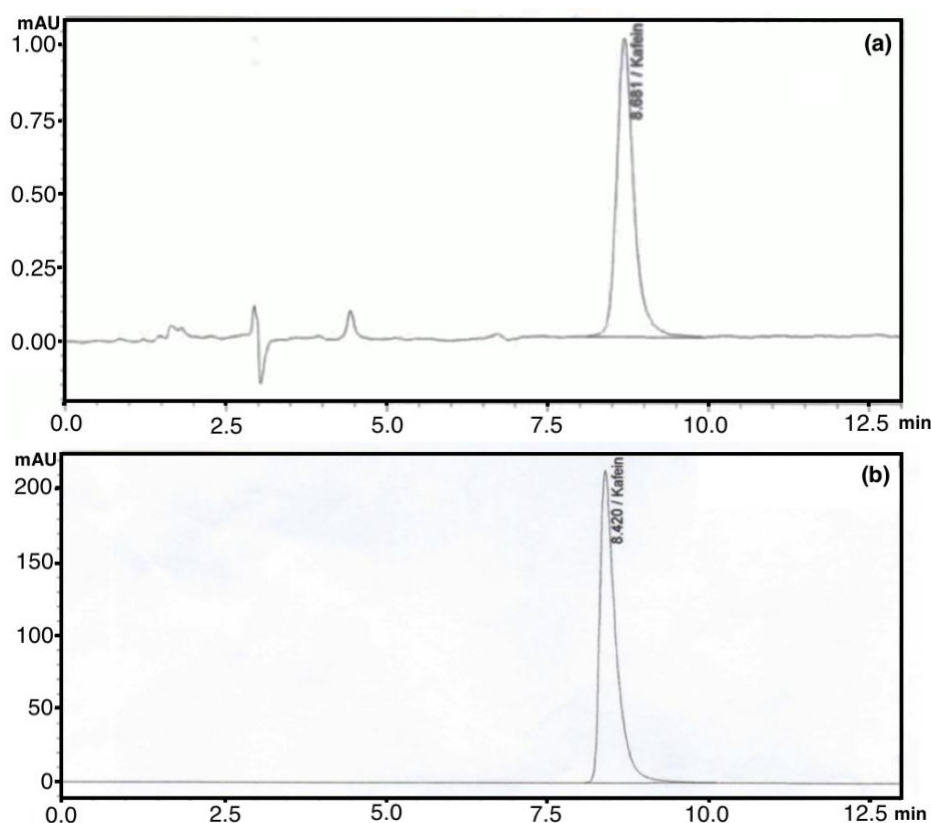


Figure 4. The chromatogram from analysis with HPLC of caffeine standard with concentration 0.5 mg/L (a) and 100 mg/L (b) (Condition operation: C18 column, mobile phase methanol/aquades (1:2) isocratic mode, flow rate 0.75 mL/min and detected at wavelength 272 nm).

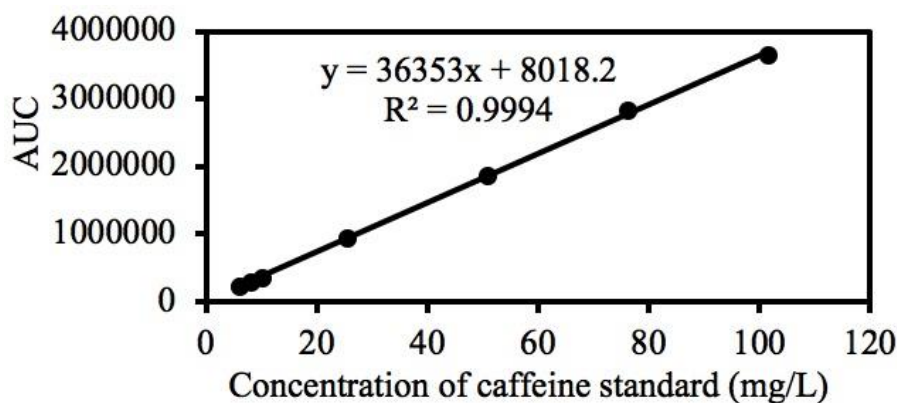


Figure 5. Regression graph correlation of caffeine standard in different concentration with their area under curve (AUC) of chromatograms.

The analysis of sample contained caffeine from the extract of *Robusta sp* following HPLC analysis give the area under curve (AUC) of chromatogram 1553983.49, and calculation following the equation of linear regression (Figure 5) give the caffeine content in the sample 19,475 mg/kg.

Performance of iodometry and UV-Vis spectrophotometry

Linearity

Linearity test using UV-Vis spectrophotometry is determined based on the relation of standard concentration (x) to absorbance (y) through the regression equation in order to get the R^2 correlation coefficient value. As for iodometry, R^2 value got from the relation of titrate volume (y) to standard concentration (x). A good linearity value is $R^2 > 0.99$ [21], and iodometry give R^2 value 0.9791 (Figure 6b). Meanwhile, based on the linearity test using UV-Vis spectrophotometry, the value of R^2 is 0.9948 (Figure 6a).

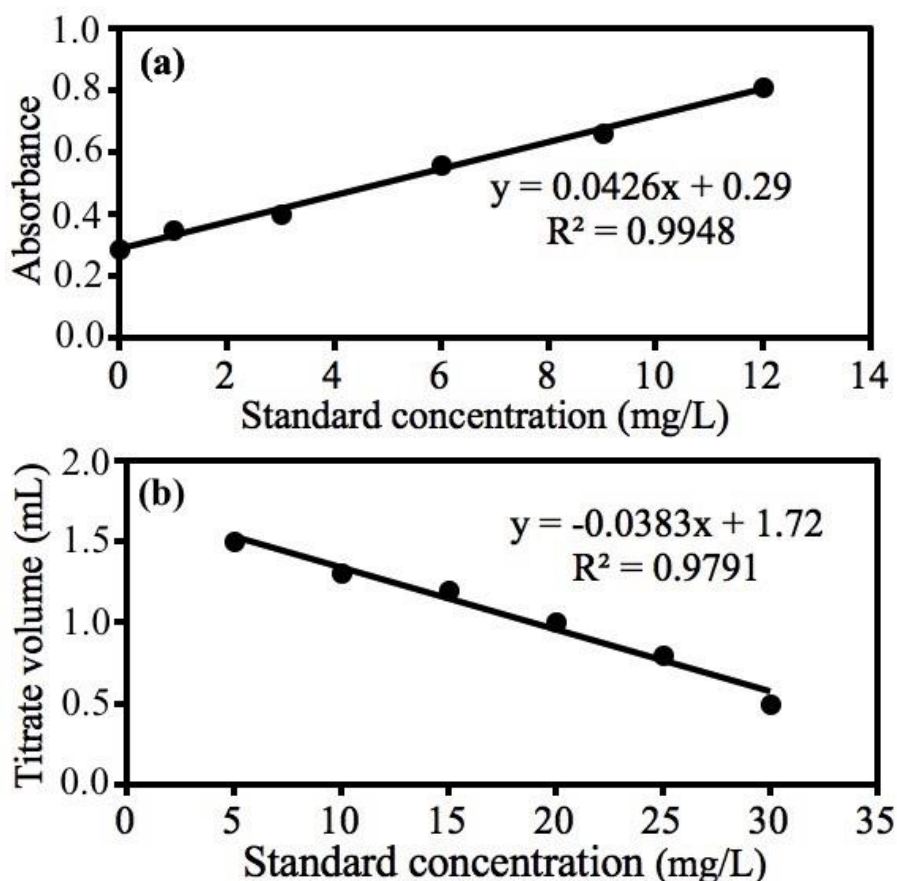


Figure 6. Linearity graph of caffeine concentration which was determined following UV-Vis spectrophotometry (a) and iodometry (b).

Precision

Precision shows the measurements repeatability with very little variation. It is stated as standard deviation (SD) and coefficient of variation (CV). Quality of precision can be accepted if the $CV \leq 1\%$ (very high), $\leq 2\%$ (high), $\leq 5\%$ (moderate) dan $\geq 5\%$ (low). In addition, the SD value must be smaller than the CV value [19]. Based on the calculation of SD and CV value of both UV-Vis spectrophotometry and iodometry (Table 3), precision of analysis of caffeine has a very high repeatability. However, iodometry indicate, give a higher standard deviation than that analysis under UV-Vis spectrophotometry. Caffeine analysis for 1.0 mg/L has deviation standard 0.002 and correlation variation 4.2% under UV-Vis spectrophotometry, meanwhile

iodometry measured caffeine in 5.0 mg/L with deviation standard 0.17 and coefficient variation 4.6%.

Table 3. Precision of analysis caffeine based on standard deviation (SD) and coefficient variation (CV) using UV-Vis spectrophotometry and iodometry

UV-Vis Spectrophotometry			Iodometry		
Concentration	SD	CV	Concentration	SD	CV
1.0 mg/L	0.002	4.2 %	5.0 mg/L	0.17	4.6%
6.0 mg/L	0.003	1.2 %	15.0 mg/L	0.15	8.3%

Accuracy

Accuracy describes the proximity of analyte resulted to the actual concentration. Accuracy test is done by analyzing of the caffeine extract, then analyzing the standard solution which has been added to the caffeine extract. Accuracy is accepted if the range of recovery % is around 80-120% [22-23]. The accuracy test of both method iodometry and UV-Vis spectrophotometry is summarized in Table 4 and Table 5, respectively. Accuracy test on Iodometry shows that the higher spike concentration give the lower recovery %, meanwhile using the UV-Vis spectrophotometry, at the higher spikes concentration also give a higher percentage of recovery. In overall, UV-Vis spectrophotometry give a better accuracy than the iodometry does.

Table 4. Iodometry recovery %

Spike content	Sample content	Calculated content	Measurable content	Recovery %
5.0 mg/L		22.48 mg/L	2.98 mg/L	106%
10.0 mg/L	18.40 mg/L	26.56 mg/L	8.00 mg/L	100%
15.0 mg/L		30.64 mg/L	13.26 mg/L	94%

Table 5. UV-Vis Spectrophotometry recovery %

Spike content	Sample content	Calculated content	Measurable content	Recovery %
1.0 mg/L	2.98 mg/L	3.9 mg/L	3.95 mg/L	99%
6.0 mg/L		8.9 mg/L	8.80 mg/L	101%

CONCLUSION

The analytical result of caffeine content on *Theobroma cacao L* by iodometry is 900 mg/kg, while by UV-Vis spectrophotometry is 4000 mg/kg and for HPLC on *Robusta sp.* is 19,475 mg/kg. The iodometry performance gives the linearity value, R^2 of 0.9791. The precision values at 5.0 mg/L and 15.0 mg/L are 4.6% and 8.3%. Accuracy for 5.0; 10.0; and 15.0 mg/L are 106%, 100% and 94% respectively. The performance of UV-Vis spectrophotometry gives linearity value, R^2 of 0.9948. The precision value for the variance

coefficient at 1.0 mg/L and 6.0 mg/L is 4.2% and 1.2% and the accuracy at 1.0 mg/L and 6.0 mg/L of 99% and 101%, respectively.

CONFLICT OF INTEREST

Authors declare no competing interest with publishing the manuscript.

ACKNOWLEDGMENT

The researchers would like to thank the Head of Chemistry Laboratory at Pattimura University for facilitating the research process. Also to PT. Saraswanti Indo Genetech (SIG Bogor) and Maluku Agricultural Technology Assessment Center (BPTP Maluku) for assisting the analysis and identification process.

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