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Development of Chemometric Methods for Determining Caffeine Content in Robusta Coffee as Alternative Standardization Techniques

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ABSTRACT

There is a necessity for standardization to uphold coffee quality due to its frequent production and consumption. High-performance liquid chromatography (HPLC) serves as the benchmark for caffeine analysis in coffee in Indonesia. However, its high cost has prompted the exploration of alternative methods. This study aims to obtain a suitable alternative method for determining the caffeine content in robusta coffee as an effort toward standardization. Employing statistical and mathematical techniques in chemistry, chemometrics emerges as a promising alternative in analyzing caffeine in coffee. The approach was integrated with an ultraviolet (UV) spectrophotometer capable of analyzing substances across wavelengths from 200 to 400 nm. From the five replications, the HPLC method showed a caffeine content of 1.435 ± 0.011% (w/w), while the spectrophotometer showed a content of 1.723 ± 0.003% (w/w). Through the partial least squares regression (PLSR) and principal component regression (PCR) methods in RStudio, the results obtained were 1.432 ± 0.003% and 1.430 ± 0.002% (w/w), respectively. These results indicate good REP values of 0.022% for PLSR and 0.155% for PCR. With these findings, it is hoped that chemometrics can become an alternative method for analyzing caffeine in coffee.

INTRODUCTION

Coffee is a popular beverage around the world including Indonesia, and also one of Indonesia's export commodities. From October 2021 to January 2022, Indonesia experienced an 11.6% increase in coffee exports, totaling 2.76 million bags (International Coffee Organization, 2022). Production estimates for coffee in Indonesia from 2019 to 2020 reached 10.7 million bags. Coffee consumption in Indonesia is expected to continue increasing to 4.9 million bags due to rising consumer demand (Global Agricultural Information Network, 2022). One of the commonly cultivated and produced types of coffee in Indonesia is robusta coffee (Prastowo *et al*., 2010).

Caffeine is a key component of coffee and has been found to enhance physical performance by increasing endurance and muscle contraction. Additionally, it improves alertness, alleviates drowsiness, and enhances mood (Lantunra, 2021). Studies on caffeine in powdered coffee and tea using Ultraviolet-Visible (UV-Vis) spectrophotometry have been conducted (Ulfa and Novita, 2018), as well as caffeine determination using high-performance liquid chromatography (HPLC) and gas chromatography (Wanyika *et al*., 2010; McCrusker *et al*., 2003). The UV-Vis spectrophotometric method has also been used to analyze differences in the presence of caffeine in seed extracts, fruit peels, and coffee leaves (Dewi, Fajaryanti and Masruriati, 2017). Additionally, studies regarding caffeine content in traditional Gayo and Lombok coffee have been conducted using HPLC and UV-Vis spectrophotometric methods (Aprilia *et al*., 2017). Analysis of caffeine content in coffee brewed in coffee shops has also been carried out (Elfariyanti, Silviana and Santika, 2020). Recent research related to the analysis of caffeine levels in organic coffee has also been done using the *et*

*al*HPLC method (Decevi *et al*., 2023). Studies comparing HPLC and UV spectrophotometry methods have also been conducted, and showed that the UV spectrophotometry method is more precise in determining caffeine levels in coffee (Susanti *et al*., 2019).

In Indonesia, according to SNI Number 01-3542-2004, the determination of caffeine content in powdered coffee can be performed using the HPLC method (BSN, 2002), which is an instrumental method used for compound analysis, offering the advantage of compound separation. However, it presents challenges such as difficulty in accurately identifying peaks in chromatograms and achieving good resolution for highly complex samples (Hirjani *et al.*, 2018). Moreover, its lengthy analysis time is a challenge. HPLC analysis is also considered relatively costly (Nurhayati and Saputri, 2016). On the other hand, a spectrophotometer can analyze compounds within a wavelength range of 200 to 800 nm in a relatively short measurement time, but it cannot separate compounds. Hence, there is a need to achieve similar results with more cost-efficient methods.

Chemometrics can be a powerful instrument to enhance spectrophotometric analysis of coffee. Chemometrics applies statistical and mathematical methods to chemical analysis. This method helps us develop relationships between the quantitative results and the evaluation of the chemical properties (Otto, 2016). One of the chemometric methods that can be utilized is multivariate analysis. Previous research on coffee quality has shown that multivariate analysis using partial least squares regression (PLSR) can produce accurate predictive models for acidity, cleanliness, overall quality, bitterness, body, and flavor of coffee (Ribeiro *et al*., 2012).

Research on coffee using spectrophotometry in chemometrics has been conducted as a method for detecting adulteration in civet coffee. Several wavelengths are considered contributors to determining coffee content, such as the 276 nm wavelength associated with caffeine absorbance (Suhandy and Yulia, 2017). Spectrophotometry in chemometrics has also been developed as a method for identifying fresh and expired roasted robusta coffee (Suhandy and Yulia, 2018). Coffee adulteration studies have also been conducted using UV spectrophotometer and near-infrared spectroscopy methods followed by principal component analysis (Adnan *et al*., 2020).

Considering the various strengths and weaknesses of the aforementioned methods, this study aims to develop a more efficient and suitable method for determining the caffeine content in robusta coffee as an effort toward standardization. The method development that the researchers attempted involved a chemometrics approach.

METHODS

Materials

This study utilized caffeine powder (PT Brataco) and robusta coffee from Temanggung, Central Java, Indonesia. Determination was made using a certificate from Materia Medica Batu number 000.9.3/092/102.20/2024, methanol L.C. Grade (Merck), Ethanol p.a. (Merck), Chloroform a.r. grade (SmartLab), Lead Oxide (Merck) and Lead Acetate (Sigma).

Instrumentation and Software

The study employed the following instruments and software: Shimadzu LC-20AD HPLC with PDA detector, Hypersil ODS C18, 250 x 4,6mm, Hitachi UH5300 UV-vis spectrophotometer, personal computer (Lenovo LOQ, 12th Gen Intel(R) Core (TM) i5-12450H (12 CPUs), 16384MB RAM and Windows 11 Home Single Language 64-bit), Microsoft Excel 2021, and RStudio 4.3.3 with pls, readxl, and car packages.

Lead Acetate Solution Preparation

Lead acetate solution was prepared by weighing 115g of lead acetate and 60g of lead oxide. The two compounds were then dissolved in 500 mL of distilled water in a volumetric flask until a white mist formed.

Standard Solution Preparation

A total of 0.125g of caffeine powder was dissolved in absolute ethanol: distilled water, filtered in a ratio of 1:4 (v/v) into a 250 mL volumetric flask. The standard solution was subsequently prepared at several concentration levels ranging from 20 to 80 ppm.

Sample Preparation

Approximately 1g of coffee was weighed and transferred into a 100 mL Erlenmeyer flask. It was then dissolved in approximately 40mL of distilled water with an addition of 1mL of lead acetate. After being heated at 100°C for 15 minutes, it was cooled to room temperature. Subsequently, it was transferred into a 100mL volumetric flask using a funnel, and rinsed with distilled water three times. Distilled water was then added up to the mark. The solution was filtered with Whatman No.1 filter paper into a

100mL beaker. Next, 10 mL of the filtrate was subjected to liquid-liquid extraction using chloroform. The chloroform was then evaporated in a porcelain dish, and the remaining coffee extract was dissolved in 5 mL of distilled water. The filtrate was filtered using a syringe and a Millipore membrane filter (0.45 µm).

Analytical Method Validation

Specificity testing was carried out by analyzing standard solutions and samples. These solutions were injected into a set of HPLC instruments, and the peak similarity were observed. Linearity was established by creating seven different concentrations. Concentrations were prepared incrementally at 20, 30, 40, 50, 60, 70, 80 ppm. Range testing was done by analyzing standard solutions with five concentration levels in a standard curve. All the prepared standard solutions were injected into a set of HPLC instruments and a UV spectrophotometer and a linear regression curve was then created.

Limit of detection (LOD) and limit of quantification (LOQ) were calculated based on data obtained from the standard curve. LOD indicates the method's ability to detect an analyte in the sample, while LOQ indicates the method's ability to quantify the amount of analyte in the sample. Accuracy and precision were performed intra-day and inter-day. Analysis was conducted by selecting three concentrations representing low, medium, and high concentrations. Accuracy and precision testing was carried out using the addition method due to the unavailability of the sample matrix.

Determination of Content

Sample solution was injected into the HPLC system with a C18 stationary phase, distilled water: Methanol (70:30), and 0.75 ml/min as the conditions. Analysis was done at 272 nm both in the UV spectrophotometer and HPLC.

Preparation of Calibration and Validation Solution Set

Preparation of the calibration and validation solution set involved diluting the stock solution of caffeine to achieve 31 different concentrations. Each concentration was scanned using a UV spectrophotometer in the range of 200 nm to 400 nm. The absorbance for each composition was recorded at 1 nm intervals. The absorbance data obtained for each wavelength was used to generate calibration and validation models. The model was developed by plotting the caffeine concentration at each specified composition using HPLC as the actual value against the chemometrics predicted value.

Data Analysis

After obtaining the caffeine content from HPLC, the results were processed and compared with the absorbance at wavelengths ranging from 200 to 400 nm with 1 nm intervals on a UV spectrophotometer using the RStudio 4.3.3 "Angel Food Cake" application with PLSR and principal component regression (PCR) programs. PLSR and PCR were run using the “pls” package. Calibration and validation were conducted using the PLSR method through RStudio. In calibration, parameters were evaluated using the coefficient of determination (R2), root mean square error of calibration (RMSEC), and root mean square error of cross-validation (RMSECV), while in validation, R2 and root mean square error of prediction (RMSEP) were utilized. A comparison was made between the actual concentration data and the prediction. Actual concentrations were obtained through measurement using the HPLC method, while predictions were obtained through readings with the spectrophotometer. Additionally, to observe any differences, an ANOVA test was conducted on the UV spectrophotometer, chemometrics and HPLC. The ANOVA test was run using “readxl” and “car” packages in RStudio.

RESULTS AND DISCUSSION

This research is classified under analytical procedures for quantifying the main components of bulk drug substances or active ingredients (including preservatives) in pharmaceutical products, falling into category 1 in the Validation of Compendial Procedures in the USP. When analyzing caffeine using HPLC, method validation is necessary, which should include accuracy, precision, specificity, linearity, and range (USP, 2023).

In this study, robusta coffee from Temanggung was used. The coffee was roasted at 205°C for 10 minutes and ground to a fine grind level. To prepare the sample solution, approximately 1g of coffee was transferred into a 100 mL Erlenmeyer flask and then dissolved in approximately 40mL of distilled water with the addition of 1mL of lead acetate. Lead acetate was used to precipitate impurity compounds that might interfere with the analysis. The sample solution was heated at 100°C for 15 minutes, and then cooled to room temperature. It was subsequently transferred into a 100mL volumetric flask using a funnel and rinsed with

distilled water three times. Distilled water was then added up to the mark. The solution was subsequently filtered with Whatman No.1 filter paper into a 100mL beaker. Approximately 10 mL of the filtrate was subjected to liquid-liquid extraction using chloroform. The selection of the solvent in liquid-liquid extraction was based on the physicochemical properties of caffeine to ensure a more optimal extraction process. The chloroform was evaporated in a porcelain dish, and the remaining coffee extract was dissolved in 5 mL of distilled water. The solution was then filtered using a syringe and a Millipore membrane filter 0.45 µm.

In this study, the selected wavelength used is 272 nm, and the mobile phase components are water and methanol (70:30), with a flow rate of 0.75 mL/minute. The analysis using HPLC indicated that caffeine has a retention time in the range of 10.646 minutes (see Figure 1). The selected wavelength according to the Indonesian National Standard (SNI) related to determining the caffeine content in powdered coffee using HPLC is 272 nm (BSN, 2002).

In HPLC and UV spectrophotometric validation, linearity was determined by reading solutions with concentrations ranging from 20 to 80 ppm. The linearity curve was obtained by performing regression on the concentration and area. Range testing in this study was performed with concentrations covered in linearity. The range testing aimed to ensure the range of analyte amounts were accurately measured using the tested method. LOD and LOQ were also measured to determine the method's capability to detect and quantitatively analyze the caffeine content in the sample.

In HPLC, specificity testing was conducted using peak identity by registering the peak of the standard caffeine chromatogram in the HPLC library, then comparing it with the peak of the sample having the closest retention time. The results of the peak identity show a similarity of 0.999 indicating that the compound analyzed in the sample is caffeine.

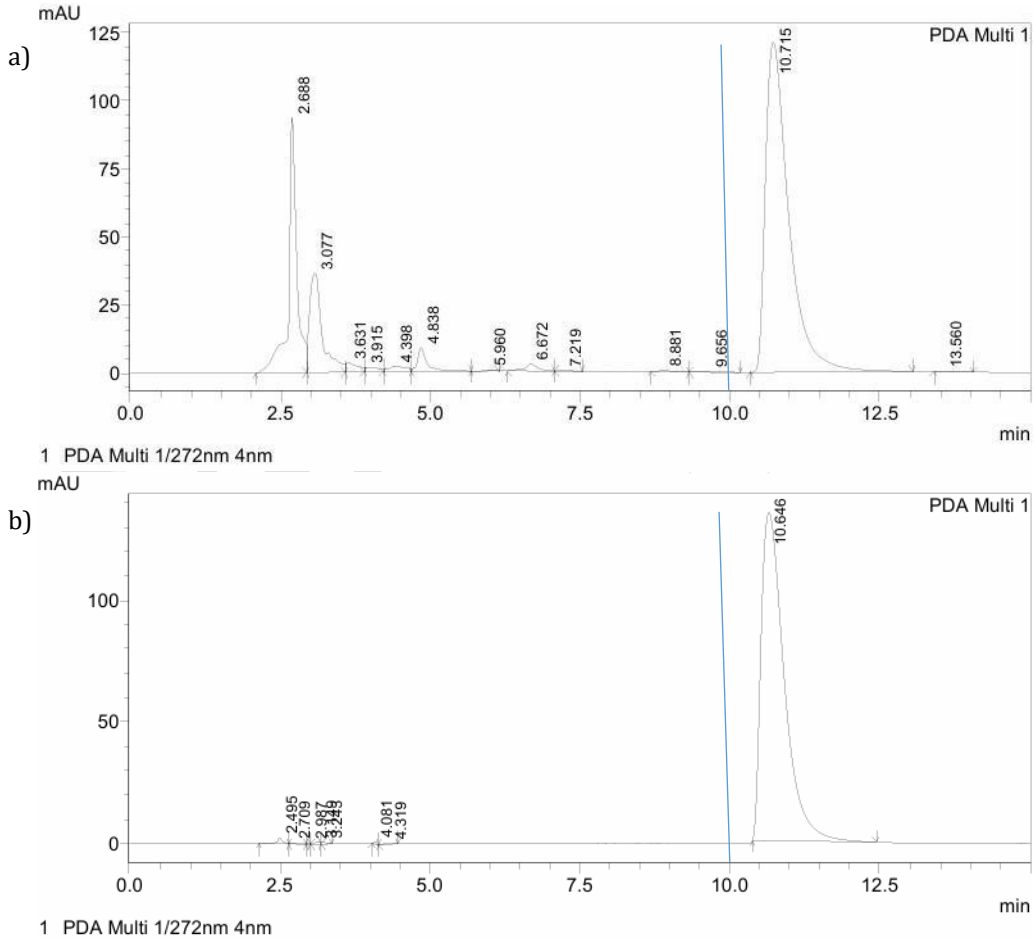


Figure 1. Sample (A) and Caffeine Standard (B) Chromatogram.

Table 1. Performance of HPLC and UV spectrophotometric validation

Validation	Method	
	HPLC	UV Spectrophotometric
Linearity	y=72645x – 22359	y=0.0362+0,4244
	r= 0.999	r= 0.999
Range	y = 72892x – 93888	y= 0.0353x+0.4476
	r= 0.999	r= 0.999
Specificity	0.999	
LOD	2.016	1.743
LOQ	6.721 ppm	5.809 ppm
Accuracy	100.788%	102.099%
Precision	0.976%	1.164%

HPLC, High-performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; UV, ultraviolet.

Accuracy testing was conducted by determining the percent recovery. In this study, accuracy and precision testing were performed using the standard addition method due to the unavailability of a sample matrix. Accuracy and precision testing were conducted using three concentrations representing low, medium, and high concentrations in this analysis, and the testing was performed over three days. From the analysis, a recovery value of 100.788% was obtained. This value is acceptable according to AOAC standards, which state that for concentrations between 10 and 100 ppm, the recovery value should fall within the range of 85 to 110%. Precision testing was conducted by examining the coefficient of variation. From the testing, a coefficient of variation value of 0.976% was obtained. The precision value is also considered satisfactory as it is below 4% (AOAC, 2019). The validation results from the HPLC and UV spectrophotometric methods are presented in Table 1.

The determination of caffeine content using HPLC was conducted by analyzing the sample using a validated method and conditions. Five replications were performed for sample readings. From the analysis, the caffeine content in robusta coffee samples was 1.435 ± 0.011% (w/w). In addition, the determination of caffeine content using a UV spectrophotometer shows us the robusta coffee sample containing 1.723± 0.003% (w/w) caffeine. This result is consistent with another study, which stated that robusta coffee has a caffeine content ranging from 0.69% to 2.15% (Aryadi *et al*., 2020). Additionally, the result also meets the requirements of the Indonesian National Standard (SNI) for powdered coffee. The SNI specifies acceptable caffeine levels between 0.45% and 2% (w/w) (BSN, 2002).

Chemometrics is the application of statistical and mathematical methods in chemistry to design or select optimal measurement procedures and experiments, as well as providing chemical information based on data analysis. In this study, PLSR and PCR were used (see Tables 2 and 3). Sample and standard spectra are demonstrated in Figure 3. In the chemometric analysis using RStudio in this study, in the calibration of the PLSR method, the RMSECV value obtained was 0.9229, and the RMSEC value obtained was 0.348, with an R2 of 0.997. The validation results of the PLSR method show an RMSEP value of 0.730 with an R2 of 0.998.

The PCR method was also used in this study. In PCR analysis, the RMSEC value of 0.229 was obtained, and the RMSECV value of 0.9214 was obtained, with an R2 of 0.997. Validation conducted on the PCR method yielded an RMSEP value of 0.730 with an R2 of 0.998.

The R2 value indicates how well the PLSR and PCR models explain the variation in response data using predictor variables. In this study, the predictor variable is the standard caffeine concentration, and the response variable is the spectrophotometer absorbance. In both the PLSR and PCR models, the coefficient of determination values are close to 1, indicating that models created using both methods sufficiently explain the variation in the data. RMSECV is a common evaluation metric used in PLS analysis to assess the performance of regression models on testing data obtained through cross-validation techniques. In this study, the leave-one-out technique was used for cross-validation. In addition, RMSEC is a common evaluation metric used in PLS analysis to evaluate the performance of regression models on calibration data. Both RMSECV and RMSEC can describe the accuracy of

the selected calibration method (Short *et al.*, 2007).

Table 2. Performance of PLSR and PCR models in calibration set

Compound	Model	Calibration			
		Number of Component	R^2	RMSECV	RMSEC
Caffeine	PLSR	21	0.997	0.9229	0.384
Caffeine	PCR	21	0.997	0.9214	0.229

PLSR, partial least squares regression; PCR, principal component regression; RMSEC, root mean square error of calibration; RMSECV, root mean square error of cross-validation.

Table 3. Performance of PLSR and PCR models in validation set

Compound	Model	Validation		
		Number of Component	R^2	RMSEP
Caffeine	PLSR	10	0.998	0.730
Caffeine	PCR	10	0.998	0.729

PLSR, partial least squares regression; PCR, principal component regression; RMSEP, root mean square error of prediction.

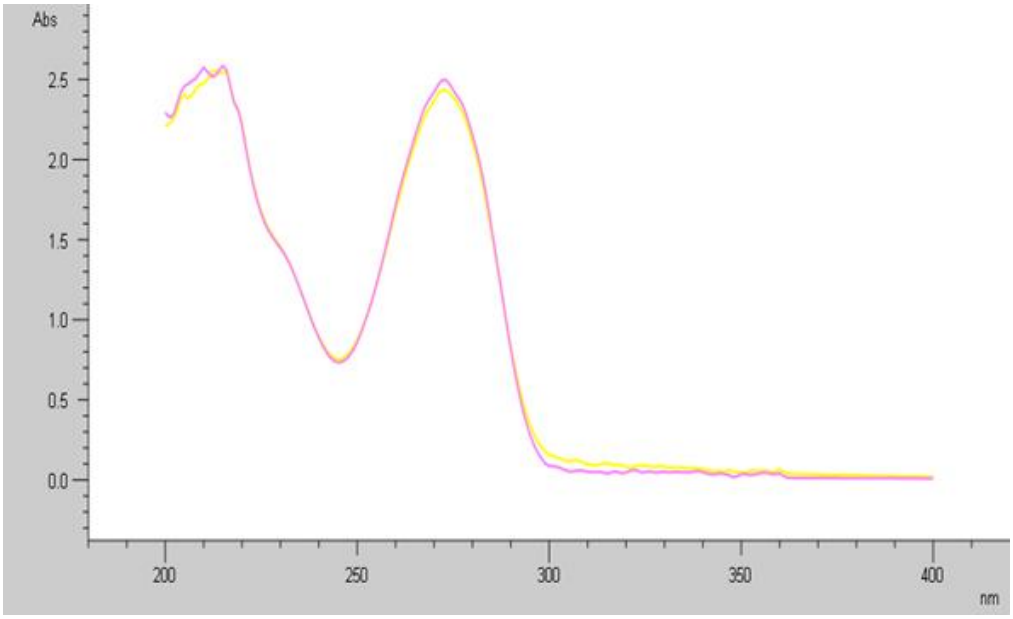


Figure 3. UV Spectra of Caffeine Standard and Sample (Yellow: caffeine standard, Red: Sample).

Table 4. Pairwise test results

	HPLC	PLSR	PCR
PLSR	0.64		
PCR	0.43	0.64	
Spectrophotometric	$< 2 \times 10^{-16}$	$< 2 \times 10^{-16}$	$< 2 \times 10^{-16}$

HPLC, High-performance liquid chromatography; PLSR, partial least squares regression; PCR, principal component regression.

From the research data, PLSR has a potential inaccuracy of 0.348% and a PCR of 0.229%. RMSEP is an analytical method used to measure the predictive capacity of a model. It compares the predicted values with the observed values (PLSR and PCR in this study) (RProject, 2024).

The analysis results from the PLSR and PCR models were subsequently used to predict the caffeine content in robusta coffee. The results obtained from the PLSR model were 1.432 ± 0.003% (w/w), and the analysis using PCR also showed a result of 1.430 ± 0.002% (w/w). These results have REP values for PLSR and PCR of 0.022% and 0.155%, respectively, when compared to HPLC as the gold-standard method.

After obtaining prediction results using both methods, a one-way ANOVA test was conducted to determine whether there were significant differences between HPLC, UV spectrophotometry and chemometrics. The one-way ANOVA followed by pairwise test results, performed using RStudio, showed significant differences among the four tested methods, indicated by a p-value of less than 0.05 in the analysis results. Since there were significant differences, a pairwise test was conducted to identify which methods had significant differences. The pairwise test results indicated that the HPLC method did not differ significantly from the chemometric methods, neither PLSR nor PCR, shown by p-values greater than 0.05 (see Table 4).

Based on the study results, the chemometric method does not show significant differences compared to HPLC. This is demonstrated by the one-way ANOVA test. This result can be achieved due to the capability of chemometric methods in enhancing data quality by using response variables, such as absorbance across multiple wavelengths, to develop a model that can predict the content more accurately. Furthermore, this method still provides satisfactory results, as seen from the REP values of 0.022% for PLSR, 0.155% for PCR, and 20.256% for the spectrophotometer when compared to HPLC.

CONCLUSIONS

Based on the research results, the chemometric method shows no significant difference compared to HPLC and provides good results, as seen from the REP values of 0.022% for PLSR and 0.155% for PCR when compared to HPLC. Therefore, the chemometric method can be used as an alternative method for determining the caffeine content in robusta coffee.

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CONFLICT OF INTEREST

The authors would like to declare that there are no financial or non-financial conflicts of interest related to this research. The first author is a master's student in the Faculty of Pharmacy at University A. The second and third authors are faculty members who assisted during the research process. All authors have no financial or professional relationships with any organizations or companies that could influence the results of this research.

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