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Original Article

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF CAFFEINE IN GREEN COFFEE BEANS (*COFFEA ARABICA* L.) FROM THREE DISTRICTS OF WEST JAVA, INDONESIA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Objective: To develop and validate a simple, accurate, and precise HPLC method for the determination of caffeine in green coffee beans (*Coffea arabica* L.) from three districts of West Java, Indonesia.

Methods: The analytical method was conducted using Enduro C-18 (250 x 4.6 mm) column with methanol: water (37: 63) as a mobile phase, the flow rate was 1.0 ml/min, and the detector wavelength was set at 274 nm. The selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, and system suitability testing were evaluated as the parameters of validation.

Results: The retention time of caffeine was 6.36 min. % RSD for precision was 0.192. The linearity of the method was obtained using a concentration range of 1-200 ppm with the correlation coefficient of 0.998. The limit of detection was 9 ppm and the limit of quantitation was 28 ppm. The accuracy was in between 90.723%-102.853%. Caffeine levels from Garut, Pangalengan, and Tasikmalaya were 1.454 ± 0.004%, 1.574 ± 0.082%, and 2.280 ± 0.004%.

Conclusion: The proposed HPLC method meets the acceptance criteria of validation parameters and can be applied for routine analysis.

Keywords: Analytical Method Development, Caffeine, HPLC, Validation

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INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is a central nervous system stimulant that prevents drowsiness, improves short-term memory, influences human circadian timing, and improves the effectiveness of particular drugs. It is an alkaloid which naturally found in the seeds, leaves or fruits of more than 63 plants species worldwide [1–3]. Caffeine is widely consumed by humans for many years as foods and beverages containing caffeine including coffee beverage [4-6]. The world's major source of caffeine is the coffee bean, that is the seed of the coffee plant [2].

Indonesia produced at least 748 thousand tons or 6.6% of world coffee production in 2012. In West Java, Indonesia, the production of coffee beans is mostly produced from the districts of Pangalengan, Garut, and Tasikmalaya. Every year, consumption of processed coffee products in Indonesia has grown to reach an average of 7.5%. Indonesia produces robusta coffee 700 kg of beans/ha/year and arabica coffee of 800 kg beans/ha/year [7]. Robusta coffee contains caffeine almost twice (1.7-4.0%) compared to arabica coffee (0.8-1.4%) [8, 9].

The agricultural practices, processes, storages, and the agro-climatic conditions such as temperature, air/wind changes, humidity, sunlight are the factors that can create variations in the chemical composition of green coffee [1, 10]. Caffeine levels indicate the quality of coffee, and it becomes advantageous information in the selection of raw materials of foods and beverages containing caffeine. The objective of this study was to develop a simple analytical method in order to characterize and identify the amount of caffeine in coffee beans from three districts of Indonesia.

MATERIALS AND METHODS

Materials

The standard substance of caffeine was produced by Merck, USA. Methanol for HPLC was obtained from Merck, USA and water was obtained from PT. Ikapharmindo Putramas, Indonesia. Ethanol, iron (III) chloride, 1% gelatin solution, ammonia aqueous, chloroform, hydrochloric acid, Mayer reagent, Dragendorff reagent, magnesium, amyl alcohol, ether, vanillin-sulfuric acid reagent, sodium hydroxide solution, Liebermann-Buchard reagent were also purchased from Merck, USA.

Apparatus

The method development was performed with an ultraviolet-visible spectrophotometer (Analytik Jena Specord 200®) and Dionex Ultimate 3000 HPLC with an ultraviolet-visible detector (Ultimate 3000 wavelength detector).

Preparation of caffeine standard solution

Caffeine standard (50 mg) was weighed and then dissolved with water until 25 ml in order to obtain a concentration of 2000 ppm.

Determination of maximum wavelength

Wavelength measurement performed by ultraviolet-visible spectrophotometer. Standard solution of caffeine taken as much as 50 µl and then diluted with 10 ml of water. Then maximum wavelength was analyzed by ultraviolet-visible spectrophotometer and the absorbance was read at the maximum wavelength of caffeine.

Determination of molar extinction of caffeine

Caffeine standard solution was pipetted and diluted with water up to 10 ml, in order to obtain the final concentration of 12.87 µM, 25.75 µM, and 51.50 µM. Then it was analyzed with a spectrophotometer and the absorbance was read at the maximum wavelength and the value of molar extinction was determined.

Optimization of the condition of analysis

Caffeine standard solution with a concentration of 0.1 ppm was optimized using Enduro C18G column (diameter 4.6 mm and length 250 mm) with a flow rate 1.0 ml/min and the injection volume of 20 µl. Methanol and water were used as mobile phase with composition of 37:63. Detection was done using UV detector at 274 nm.

Suitability system test

Caffeine standard solution with a concentration of 0.1 ppm was injected into the HPLC with the flow rate of 1 ml/min and varies of mobile phase composition. The plate number (N), height equivalent theoretical plate (HETP), tailing factor, and the capacity factor was determined.

Analytical method validation

Linearity

Linearity was measured by varies the concentration of caffeine standards, 1, 5, 10, 25, 50, 100, and 200 ppm were injected into the HPLC system (optimized) and repeated 3 times. A calibration curve was made by plotting the average peak area vs. concentration standard. The result of the plot was the linear curve, y = bx+a with R^2 as determinant linearity [11].

Accuracy

Recovery performed by the sample with the addition of a standard concentration of 80%, 100%, and 120% caffeine then each was measured 3 times (triplo). The area under curves was entered into the regression equation of the curve calibration. Recovery (%CV) should be between 80-120% [12].

Precision

Standard solution of caffeine was 6 times injected and analyzed using HPLC on the same day. Precision values were expressed by the relative standard deviation (RSD) ≤ 2.0% [12].

Limit of quantification and limit of detection

The limit of detection and limit of quantification were calculated statistically through regression equation of the calibration curve; the measurement value was calculated from the value of a in the regression equation y = ax+b [12].

Selectivity

Specificity or degree of deviation (selectivity) is the ability of a method to measure the analyte closely to the other components in the sample matrix. Specificity performed by optimizing to obtain the desired compound separated perfectly with other compounds, good resolution value is>1.5 [13].

Preparation of the extract

Plant material used was Arabica coffee (*Coffea arabica* L.) beans crude obtained from Garut (-7.104543899999999, 107.89615370000001), Pangalengan (-7.112753799999999, 107.60525580000001), and Tasikmalaya (-7.381957799999999, 108.32376550000004) with voucher specimen number: 19/HB/10/2014. All the plant materials were ground into powder in the different containers. 20 grams of each coffee beans powder was extracted with digestion method using 250 ml water at a temperature of 40-50 °C for 30 min using a magnetic stirrer [14, 15].

Phytochemical screening

Phytochemical screening was performed to determine phytochemical compounds contained in the extract as alkaloid, flavonoid, tannin, polyphenol, saponin, monoterpenoid, sesquiterpenoid, steroid, triterpenoid, and quinone. Phytochemical screening performed based on the Farnsworth method [16].

Determination of caffeine concentration

The sample solutions were prepared and filtered using mini pork 0.45 µl then the filtered samples were injected into the HPLC system. The resulting chromatogram was then used to calculate the concentration of the caffeine in the samples.

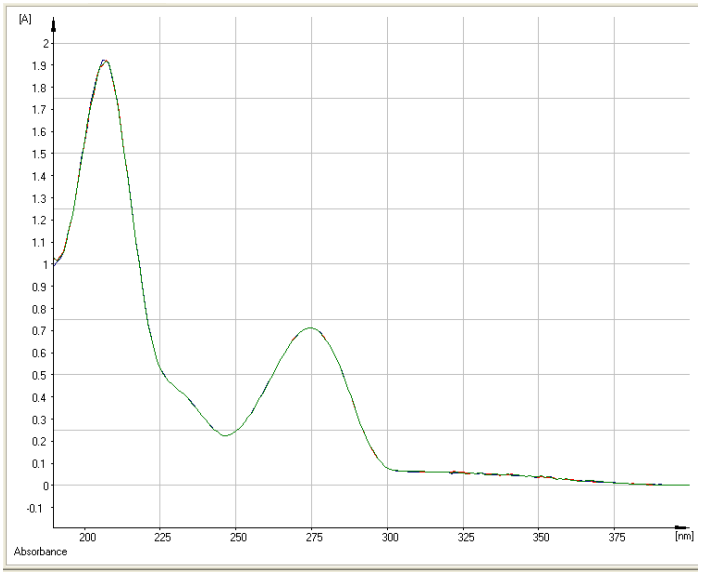

Fig. 1: Maximum wavelength of caffeine

RESULTS AND DISCUSSION

Determination of wavelength maximum

According to the British Pharmacopoeia 2013, the identification of caffeine performed at a wavelength of 275 nm, the maximum wavelength from the optimization result was not much different, which was 274 nm (fig. 1) [17]. Ali *et al.* (2012) showed that the maximum wavelength of caffeine was 270 nm [18]. The differences of the maximum wavelength can be caused by different conditions between the analyses used. Because the maximum wavelength of caffeine was in the range of 200-400 nm, the caffeine can be analyzed by using the ultraviolet detector.

Determination of molar extinction of caffeine

The results of the determination of molar extension (ε) value from three different concentrations of caffeine, which were 12.87 µM, 25.75 µM, and 51.50 µM can be seen in table 1. It was 26398.049 ± 5541.642 M-1 cm-1 which was greater than 10000 M-1 cm-1, so it can be concluded that caffeine can be detected using a UV detector as a chromophore.

Table 1: Calculation of molar extinction (ε) of caffeine

No	Molar concentration (μM)	Absorbance ± SD (n=3)	Molar Extinction ± SD (n=3)
1	12.87	0.544 ± 0.013	43916.058 ± 2112.283
2	25.75	0.275 ± 0.004	10839.952 ± 455.714
3	51.50	0.125 ± 0.017	24438.137 ± 4200.922
Total			79194.148 ± 16624.925
$\bar{X}$			26398.049 ± 5541.642

Optimization of the condition of analysis

Determination of caffeine in the extract performed by reversed-phase HPLC method, the column used was Enduro C-18G (25 cm x 4.6 mm), a flow rate of 1 ml/min, the injection volume of 20μl, UV detector at wavelength 274 nm, and the mobile phase used was methanol and water. Use of octadecyl silica has several advantages including octadecyl silica capability to separate the compounds with low polarity, moderate, or high [13].

The mobile phase used was methanol and water with ratio 37:63 obtained from the results of optimization. Water and methanol as mobile phase performed because of the economic value and ease of preparation of the solution. In the previous study, this solvent was used with a different composition. Pokhrel *et al.* used methanol: water 40:60 and produced recovery which was greater than 97% [19]. Methanol: water 30:70 was used as a mobile phase by Camargo *et al.* to determine caffeine from chocolate [20].

Table 2: Results of the optimization composition of the mobile phase

Composition of mobile phase (methanol: water)	Retention time (min)	N	HETP (L/N)	Tailing factor	K'
30:70	9.187	4156.40	0.060	1.5	3.835
33:67	7.707	4124.85	0.0606	1.1	2.987
35:65	6.960	3364	0.0743	1.1	2.756
37:63	6.360	2393.46	0.104	1.05	2.419

System suitability test

In table 2, it can be concluded that the composition ratio of methanol: water (37:63) is the most efficient mobile phase composition because it meets all the criteria of the optimum condition of HPLC and it has the shortest retention time and capacity factor which shows that this composition of the mobile phase gives the most efficient system for the analysis of caffeine among others. In another study, caffeine levels are determined by using more methanol than water (95:5) [21]. This becomes an oddity due to caffeine is more difficult to dissolve in water thus it is possible to expect the tailings at the peak of caffeine.

Analytical method validation

Linearity

The linear regression equation was y = 0.642x+3024 with R^2 = 0, 9985 (fig. 2). Coefficient correlation value obtained was very good, because according to UNODC, 2009 the value of the coefficient should be ≥ 0.990 [22].

Accuracy

Recovery performed by measuring the levels of caffeine in the sample with the addition of 80%, 100%, and 120% of the standard concentration of caffeine by then each was measured 3 times. The values of recovery meet the acceptance of accuracy, which was between 90.723%-102.853%.

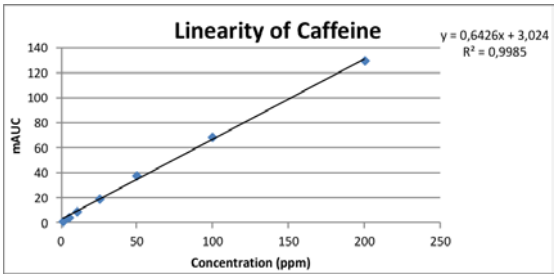

Fig. 2: Linearity of caffeine

Precision

Table 3: Precision results

ppm	AUC	Concentration
100	70.542	105.069
100	70.989	105.766
100	70.549	105.081
100	70.513	105.026
100	70.949	105.704
100	70.863	105.570
	Total	632.216
	Average	180.633
	Standard deviation	0.347
	%RSD	0.192

Note: Number of experiments: 6

Precision values expressed by the relative standard deviation (RSD) ≤ 2.0%.

Based on the results obtained in table 3, % RSD ≤ 2.0%, which was 0.192% so it can be concluded that the analytical method of caffeine meets the acceptance of precision

Limit of quantification and limits of detection: Limit of detection obtained based on the peak area was 9 ppm. Limit of quantification is defined as the smallest quantity of analyte in a sample that can be quantified and me*et al.* l the acceptance of the parameters [12]. Limit of quantification value obtained based on the peak area was 28 ppm.

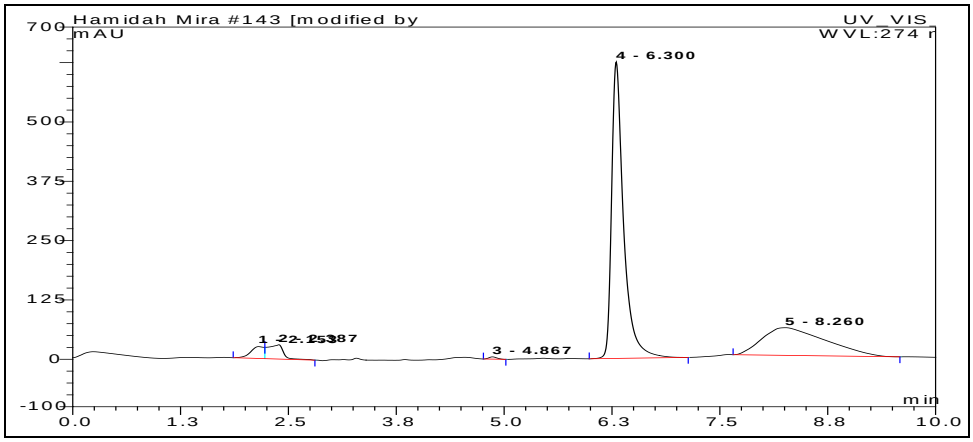

Fig. 3: Chromatogram of caffeine in the sample

Selectivity

As mention in fig. 3, can be seen the retention time of caffeine, which was 6.3 min, apart from other peaks from the solvent with a retention time of 2.153 min with a resolution value 1.508. Specificity performed by the optimization to obtain the desired compound separated perfectly with other compounds of resolution values>1.5 [13]. This resolution value indicates that the HPLC method can be used to analyze caffeine.

Preparation of the extract

The extracts obtained were liquid extracts with a characteristic smell of coffee and the colors were green until dark green. The volume of the liquid extract from Pangalengan was 160 ml and the color was light green and characteristic smell of green coffee, the extract from Garut was 150 ml and the color was pale green with a characteristic smell of green coffee, and the extract from Tasikmalaya was 160 ml and the color was dark green and characteristic smell of green coffee with a characteristic smell of green coffee.

Phytochemical screening results

Phytochemical screening results derived from three different regions showed similar results, which contained an alkaloid, flavonoid, tannin, polyphenol, monoterpene, sesquiterpene and triterpenoid in the extract (table 4).

Determination of caffeine concentration

Sample solution that has been filtered by using minipore 0.45 µm then injected into HPLC system. It is intended that the sample injected into the HPLC has a smaller size than 0.45 µm so it will not clog the column on HPLC. The area under curve was generated and then inserted into the linear regression equation and calculate the concentration of the sample used.

Table 4: Phytochemical screening results

Secondary metabolites	Garut	Pangalengan	Tasikmalaya
Alkaloid	+	+	+
Flavonoid	+	+	+
Tannin	-	-	-
Polyphenol	+	+	+
Saponin	-	-	-
Monoterpene and Sesquiterpene	+	+	+
Triterpenoid	+	+	+

Description: (+): Detected (-): Not detected

Table 5: Determination of caffeine in the samples from three districts West Java

Sample	Average AUC ± SD	Concentration ± SD (ppm)	% Level ± SD
Garut	77.794 ± 0.249	1163.561 ± 3.724	1.454 ± 0.004
Pangalengan	83.924 ± 4.373	1258.950 ± 65.599	1.574 ± 0.082
Tasikmalaya	120.244 ± 0.243	1824.160 ± 3.686	2.280 ± 0.004

Note: Number of experiments: 3

Caffeine concentration in the samples from Garut, Pangalengan, and Tasikmalaya were 1.454 ± 0.004%, 1.574 ± 0.082%, and 2.280 ± 0.004%, respectively (table 5). The concentrations of caffeine from three districts were different. The chemical composition of the caffeine within the same species may vary depending on the geography and season of collection [23].

CONCLUSION

The optimum condition for the analytical method of caffeine by HPLC (High-Performance Liquid Chromatography) was carried out by using Enduro C-18 column, mobile phase methanol: water (37:63) at a wavelength of 274 nm, and the flow rate of 1 ml/min. This method meets the acceptance parameters of validation. Caffeine concentration in the samples from Garut, Pangalengan, and Tasikmalaya was 1.454 ± 0.004%, 1.574 ± 0.082%, and 2.280 ± 0.004%, respectively.

AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

Declared none

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