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Development of The Scanometric Method for Caffeine Determination in Pharmaceutical Preparations

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Abstract

The caffeine assay can be developed by using sensitive reagents for caffeine detection, such as sodium periodate ($NaIO_4$), acetic acid (CH_3COOH), and 3-methyl-2-benzothiazolinone hydrazone (MBTH) inside 96-microwell plate. The addition of caffeine solution could change the color of the reagent from white to deep blue which can be then scanned and quantified by a flatbed scanner and the color image analysis program, known as the scanometric method. Analytical characterization such as linearity, LOD, LOQ, precision, accuracy, and stability of the developed method was established by using caffeine standard. The result of caffeine analysis using the developed method agreed with that of the spectrophotometric method, suggesting that the developed method can be used as an alternative method for caffeine assay in pharmaceutical preparations.

Keywords: *$NaIO_4$; CH_3COOH; MBTH; caffeine; scanometric method*

Introduction

Caffeine, the main alkaloid found in coffee beans, is known to have various pharmacological effects, such as central nervous system and heart muscle stimulant, bronchial smooth muscle relaxant, and diuretic. Caffeine is formulated into intravenous (IV) injection preparations or oral caffeine citrate solutions for the treatment of apnea in neonates [1]. Caffeine is often formulated with analgesic and anti-inflammatory drugs such as paracetamol, acetylsalicylic acid, and ibuprofen for headache medicine [2–4] and formulated with ergotamine for the treatment of migraines. It is also formulated in influenza drugs, as it can reduce the sedative effects of antihistamine drugs which are typically formulated in influenza drugs [5]. Currently, there are 96 drugs with caffeine content registered with the National Agency for Food and Drug Control (*Badan POM*)[6]. Therefore, developing a method for determining caffeine levels in pharmaceutical preparations is needed, in part of assuring the quality of the products in the pharmaceutical industry.

Various analytical methods employing chromatographic and spectroscopic techniques have been developed to detect caffeine in various pharmaceutical preparations. The high-performance liquid chromatography (HPLC) method is a standard method for determining caffeine levels in injection or tablet preparations with caffeine content in the latest United States Pharmacopoeia [7] and the Indonesian Pharmacopoeia [8]. This chromatographic method was also used for caffeine determination in tablet and capsule preparations [4,9–11]. Other chromatographic methods like thin layer chromatography (TLC) or high-performance TLC (HPTLC) with a densitometer were used to determine the caffeine level in the pharmaceutical preparations [3,5]. Additionally, the spectroscopic method which employs a UV-Vis spectrophotometer was used for determining caffeine content in various pharmaceutical tablets [2,4,11–13]. Despite the

versatility of chromatographic and spectroscopic methods, they suffered from various drawbacks like expensive instruments, time-consuming, large amounts of reagents and samples, and demanding people with proper knowledge and skill in chemical analysis. Thus, an alternative method for caffeine determination which was accurate, fast, cheap, and easy to operate is demanding.

The development of an analytical method for caffeine determination can be pursued by employing sensitive reagents for caffeine detection like $NaIO_4$, CH_3COOH, and MBTH in the colorimetric setting. In slightly acidic conditions (CH_3COOH addition), $NaIO_4$ oxidized caffeine to its oxide form. This caffeine oxide can be further reacted with a chromogen like MBTH and gently heated to generate a caffeine oxide-MBTH complex with an intense blue color that is proportional to the initial caffeine [14,15]. This blue color intensity can be quantified by measuring its absorbance at 630 nm in the UV-Vis spectrophotometer. Fortunately, the blue color intensity can also be scanned and quantified by using a flatbed scanner and color image analysis program like Adobe Photoshop or ImageJ, which is known as the scanometric method [16–18]. The current study aimed to develop the scanometric method for caffeine determination in pharmaceutical preparations. The method employed a 96 microwell plate as a chemical reaction medium, which uses a smaller reagent volume than that used in a cuvette in the spectrophotometric method. Furthermore, since the method uses a flatbed scanner that is much cheaper than a UV-Vis spectrophotometer, it can be applied to a laboratory with limited facilities.

Experimental

Equipment

The 96-microwell plates (Nunc, USA) were used as a chemical reaction medium. The ultrasonicator bath (Elma S 30H) was used for caffeine extraction. The flatbed scanner (Epson V850 Pro, Japan) and UV-Vis Spectrophotometer (U-1800, Hitachi, Japan) were used for caffeine analysis.

Material

The chemicals used in the current study like $NaIO_4$, MBTH, and CH_3COOH (Sigma Aldrich, USA), as well as dichloromethane (Merck, Germany) were in analytical grade. Water for injection (PT. Widatra Bhakti, Indonesia) is employed for dissolving the chemicals. The pharmaceutical preparations (injection and tablet) were purchased from the local pharmacy, Jember Indonesia.

Methods

Reagent preparation

The caffeine reagent was prepared in a water solution and made at different concentrations as follows: $NaIO_4$ (0.001, 0.01, and 0.1 M), MBTH (0.001, 0.01, and 0.1 M), and CH_3COOH (0.01, 0.1, and 1 M).

The procedure of the scanometric method for caffeine analysis

Caffeine analysis was initiated by reacting 25 µl of caffeine standard solution with 50 µl $NaIO_4$ and 25 µl CH_3COOH into a microwell and heated for a few minutes in the oven. Afterward, the mixed solution was cooled at room temperature, added with 100 µL MBTH, and stood for a few minutes. The microwell plate was properly placed at the center of the tabletop of the flatbed scanner. The lid of the scanner was closed to secure the microwell plate from the surrounding lights. The scanning process was done by transmittance mode and 1200 dpi resolution to obtain the digital image of each microwell. Then, the color intensity of the microwell was measured by ellipse selection and RGB measure using the ImageJ program, generating the numeric data of different color values as red (R), green (G), blue (B), and red-green-blue (RGB) color values, respectively. The color intensity was corrected against the blank, expressed as Δ mean R, G, B, and RGB, respectively.

Optimization of analysis condition

The analytical condition such as reagent composition and concentration, heating temperature and time, as well as color value

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selection was optimized by using the standard caffeine solution at 50, 150, 300, 450, and 600 ppm concentration.

Characterization of caffeine analysis

The analytical parameters like response time, linearity, detection limit (LOD), quantification limit (LOQ), precision, and accuracy were characterized by using the standard caffeine solution.

The application of the scanometric method in real sample

Before the caffeine analysis, the pharmaceutical samples were first prepared as follows. The caffeine injection sample was diluted 100 times and used without any modification. The caffeine-containing tablets were ground, extracted with dichloromethane for 15 minutes at the ultrasonicator bath, filtered, and adjusted with dichloromethane in a 1:10 w/v ratio. The pharmaceutical sample solutions were then analyzed using the scanometric method. The caffeine concentration was calculated by using the calibration curve using the scanometric method. The results were then compared with those obtained by the spectrophotometric method [14] using an independent *t*-test.

Results and Disscusion

Reagent optimization

Reagent optimization was scrutinized by using different formulas as seen in Table 1. The caffeine serial solution (50, 150, 300, 450, and 600 ppm) was added (25 µl) to a microwell that contained 50 µl $NaIO_4$ and 25 µl CH_3COOH and heated for 2 minutes at 40° C. The mixed solution was cooled at room temperature, added with 100 µL MBTH, and allowed to stand for 15 minutes. By using the scanometric method, the caffeine calibration curve (caffeine concentration vs blue color value) was constructed for each formula. The result showed that formula 2 not only posed the most significant color change (Figure 1) but also gave the highest correlation coefficient (*r*) among other formulas (Table 1). Hence, the reagent composition of Formula 2 was used for the next experiments.

Table 1. The calibration curve of caffeine (50-600 ppm) with different reagent compositions which individually constructed by the scanometric method ($n = 3$).

Formula	Reagent composition	*r*	Calibration curve equation
1	$NaIO_4$ 0.001 M, CH_3COOH 0.01 M MBTH 0.001 M	0.946	y = 0.0183x + 25.573
2	$NaIO_4$ 0.01 M, CH_3COOH 0.1 M MBTH 0.01 M	0.992	y = 0.0077x + 67.078
3	$NaIO_4$ 0.1 M, CH_3COOH 1 M MBTH 0.1 M	0.955	y = 0.0217x + 196.64

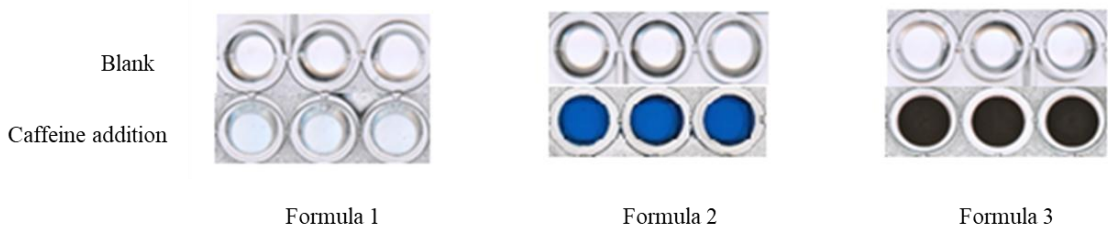

Figure 1. The observed color change in the mixed solution of different reagent formulas after it was individually added with 300 ppm caffeine

Table 2. The calibration curve of caffeine (50-600 ppm) of different heating temperatures which individually constructed by the scanometric method (n = 3).

Heating temperature	r	Calibration curve equation
50°C	0.995	y = 0.0080x + 67.682
60°C	0.991	y = 0.0114x + 72.063
70°C	0.989	y = 0.0088x + 81.036

Table 3. The calibration curve of caffeine (50-600 ppm) with different heating times which individually constructed by the scanometric method (n = 3).

Heating time (minutes)	r	Calibration curve equation
2	0.995	y = 0.0080x + 67.682
6	0.957	y = 0.0131x + 75.547
10	0.956	y = 0.0168x + 99.949

Temperature optimization

The optimum heating temperature for caffeine detection was determined by analyzing the caffeine serial solution with the optimum reagent composition at different heating temperatures (50, 60, and 70°C) using the scanometric method. Here, the mixed solution (caffeine, $NaIO_4$, and CH_3COOH) was heated for 2 minutes at those individual temperatures. After 15 minutes, the MBTH solution was added to the mixed solution and analyzed using the scanometric method, resulting in the different calibration curves (caffeine concentration vs blue color value) as seen in Table 2. The result showed that the heating temperature at 50°C gave the highest correlation coefficient. Hence, this heating temperature was used for the next experiments.

Heating time optimization

The optimum heating time for caffeine determination was done by analyzing the caffeine serial solution with the optimum reagent composition at different heating times (2, 6, and 10 minutes) using the scanometric method. Here, the mixed solution (caffeine, $NaIO_4$, and CH_3COOH) was heated at 50°C with individual heating times. After 15 minutes, the MBTH solution was added to the mixed solution and analyzed using the scanometric method, resulting in the different calibration curves (caffeine concentration vs blue color value) as seen in Table 3. The result showed that heating time at 2 minutes gave the highest correlation coefficient. Hence, this heating time was used for the next experiments.

Color value optimization

Color value optimization was done using the previous optimum parameters, described as follows. The mixed solution (caffeine, $NaIO_4$, and CH_3COOH) was heated at 50°C for 2 minutes. After 15 minutes, the MBTH solution was added to the mixed solution and analyzed using the scanometric method, resulting in the different calibration curves as seen in Table 4. The result showed that the blue color value gave the highest correlation coefficient (Table 4). Hence, this color value was used for the next experiments.

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Table 4. The calibration curve of caffeine (50-600 ppm) with different color values which individually constructed by the scanometric method (n = 3).

Color value	r	Calibration curve equation
R	0.909	y = 0.0039x + 235.95
G	0.983	y = 0.0065x + 135.56
B	0.996	y = 0.0087x + 67.037
RGB	0.985	y = 0.0258x + 146.19

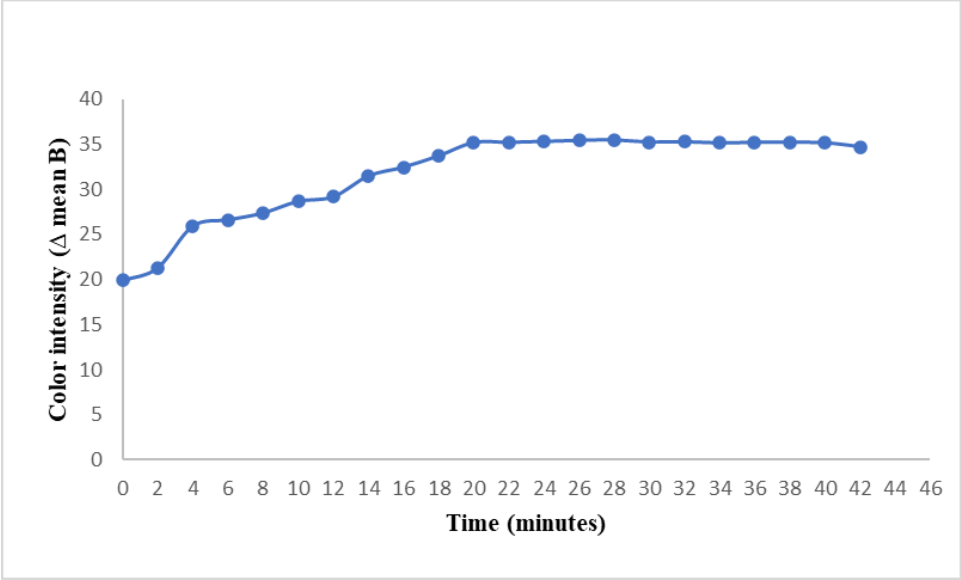

Figure 2. The observed color intensity profile of 300 ppm caffeine using the developed method at 0-42 minutes.

Response time

Determination of response time was done by monitoring the color intensity of caffeine 300 ppm every 2 minutes for 42 minutes using the developed scanometric method. As depicted in Figure 2, the color intensity stabilized at 20-40 minutes. Hence, the response time was selected at 20 minutes, while the next measurement was done at that time.

Linearity

As described previously in the color value optimization section, the linearity of the developed scanometry method was carried out by plotting caffeine serial concentration (50-900 ppm) against the blue color value. Figure 3 shows a linear profile of caffeine concentration, with a coefficient correlation of 0.999.

Detection limit and quantification limit

The detection limit (LOD) and quantification limit (LOQ), defined as the lowest concentration of analyte that can be detected and quantified or can give a significant color change against blank [19] were calculated using the previous caffeine linearity equation. The result showed that LOD and LOQ of the caffeine were found at 46.04 and 139.51 ppm, respectively.

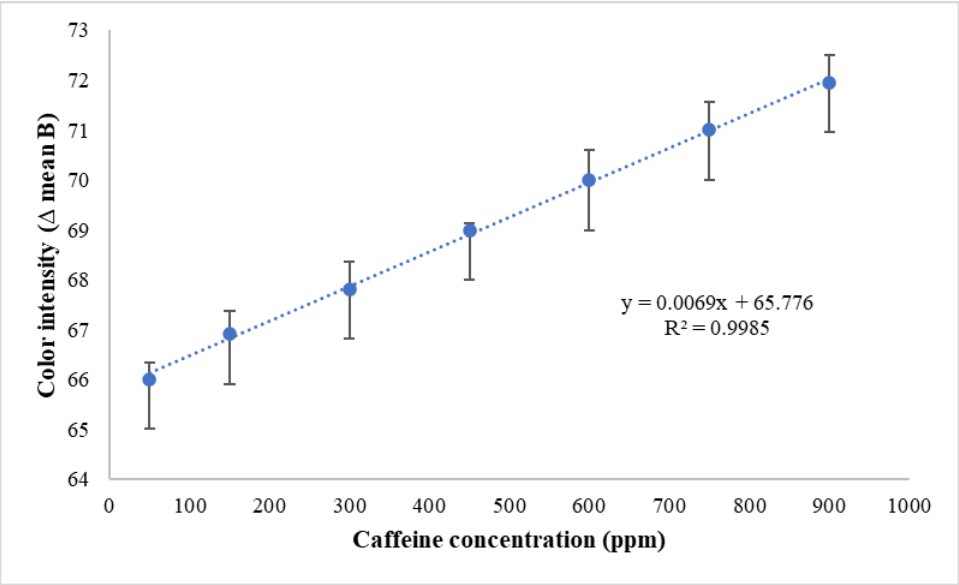

Figure 3. The calibration plot of caffeine (50-900 ppm), constructed by the developed scanometric method at 20 minutes, shows a linear profile at optimum condition (n = 3

Table 5. The results of caffeine determination in simulated samples using the scanometric method (n = 6).

Sample	Color intensity (Δ *mean B*)	Caffeine mass (mg)
1	25.692	50.681
2	25.648	50.043
3	25.681	50.522
4	25.669	50.348
5	25.637	49.884
6	25.633	49.826
	Average	50.217
	RSD (%)	0.640

Precision

The precision study of the scanometric method was performed by six measurements of simulated samples (containing 50 mg caffeine). As seen in Table 5, the coefficient variation (RSD) of six measurements using the developed method was calculated at less than 2%, suggesting the analytical procedure of the developed method was repeatable [8,19].

Accuracy

The accuracy of the scanometric method was determined by measuring the caffeine concentration of the simulated sample after 30, 45, and 60% caffeine addition of its initial caffeine concentration, following the method suggested by Huber [20]. The recovery of caffeine was found at 99%, as depicted in Table 6, suggesting that caffeine analysis using the developed method was accurate [20].

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Table 6. The caffeine recovery in the simulated sample after 30, 45, and 60% addition of initial caffeine concentration at 500 ppm (n = 3).

Sample addition (%)	Caffeine recovery (ppm)	Caffeine recovery (%)
30	655.853 ± 4.688	100.897 ± 0.038
45	712.625 ± 30.769	98.293 ± 3.465
60	804.375 ± 15.020	100.547 ± 0.123
		99.912 ±1.143

Table 7. The result of caffeine determination using the developed scanometric and the spectrophotometric method (n = 3).

Pharmaceutical sample	Scanometric method (mg)	Spectrophotometric method (mg)	p
Injection	19.629 ± 0.435	19.705 ± 0.100	0,784
Tablet A	50.020 ± 0.163	51.286 ± 0.870	0,113
Tablet B	49.487 ± 0.094	50.157 ± 1.059	0,423
Tablet C	49.953 ± 0.249	50.047 ± 0.712	0,869

Application in pharmaceutical preparations

The caffeine concentration in real pharmaceutical preparations independently determined by the scanometric and the spectrophotometric methods are presented in Table 7. It was known that the p-value of all samples was higher than the α value (0.05) of statistical t-test calculation, suggesting that the result of both methods was significantly comparable [14]. Therefore, it opened the feasibility of the use of the developed method as an alternative method for caffeine determination in pharmaceutical preparations.

Conclusions

Development of the scanometric method for caffeine determination in pharmaceutical samples was done by employing $NaIO_4$, CH_3COOH, and MBTH and a microwell plate as a reagent and chemical reaction medium, respectively. The use of a flatbed scanner as an analytical instrument made the method more economical than the standard spectrophotometric method. Analytical parameters like linearity, LOD, LOQ, precision, and accuracy were characterized by using caffeine, while the result of caffeine determination using the developed method was agreed with the standard method, opening the feasibility of its use as an alternative method for caffeine determination.

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