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Research Article

Optimization and Validation of Caffeine in Robusta Coffee (*Coffea canephora*), Black Tea (*Camellia sinensis*), and Cocoa (*Theobroma cacao* L.) using RP-HPLC Liquid-Liquid Extraction

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ABSTRACT

Caffeine, an alkaloid compound naturally found in various consumer products, has become the focus of increasing research in the analysis of products such as black tea, cocoa, and coffee. The chemicals found in coffee, cocoa, and black tea have pharmacological actions including antiviral, antihypertensive, antidiabetic, antioxidant, and hepatoprotective effects against hepatitis B. The chemical components include caffeine, theobromine, theophylline, tannins, adenine, vitamins (including vitamin C), flavonoids, methylxanthine, naringenin, and polyphenols. The resultant viscous extract was mixed with 25 milliliters of chloroform and 1.5 grams of $CaCO_3$ in a separatory funnel and stirred. Three times, chloroform solution was added. The HPLC analytical method requires a wavelength of 272 nm, a methanol: redistilled water (70:30) mobile phase, and a flow rate of 1.0 mL/min to be validated. Results show resolutions of 1.49 and 1.57 for selectivity values, linearity of r = 0.9994, LOD 8.511 ppm and LOQ 28.370 ppm, accuracy of 99–101% for average recovery value, and precision of %The RSD level is 0.0095%. The results of the caffeine content determination test showed that the ethanol extract of robusta coffee beans obtained from the caffeine content ± SD obtained was 9.682 ± 1.254, black tea 34.525 ± 0.064, and cacao 6.890 ± 0.488.

INTRODUCTION

Caffeine, an alkaloid compound naturally found in various consumer products, has become the focus of increasing research in the analysis of products such as black tea, cocoa, and coffee. Its presence in these matrices plays a crucial role in determining their organoleptic characteristics and physiological effects. Analytical research on the amount of caffeine in different products offers important information on their qualities and attributes, as well as how they relate to the comfort and health of consumers. Coffee, cocoa beans, and tea leaves are common sources of caffeine, an alkaloid belonging to the methylxanthine group (Wanyika *et al*., 2010). Caffeine can boost alertness and replenish wasted energy in people. Basically, the central nervous system is stimulated, which results in this effect. The most prevalent metabolites with secondary nitrogen atoms in plant and animal tissues are called alkaloids. The majority of alkaloid chemicals come from plants. Plant components that contain alkaloids include flowers, seeds, leaves, stems, roots, and bark (Juliantari *et al*., 2018).

In Indonesia, some consumable goods that could be produced as herbal plants include cocoa, black tea, and robusta coffee. There is strategic potential for growth in Indonesian coffee production. As an alternative to pharmaceuticals, coffee contains chemicals that have pharmacological benefits, including as an antiviral treatment for hepatitis B, with antihypertensive, antidiabetic, antioxidant, and hepatoprotective properties (Farhaty & Muchtaridi, 2016). The active components of robusta coffee (*Coffea canephora*) are essential to coffee's health benefits. Coffee's active ingredients include polyphenols, alkaloids, tannins, and saponins (Chairgulprasert & Kongsuwankeeree, 2017; Sabarni & Nurhayati, 2019).

Black tea is produced by fermentation, or the oxidation of polyphenols in fresh leaves using the catalyst polyphenol oxidase. The dark-colored, sharp-tasting tea is the result of this fermenting process (Fazel *et al.*, 2010). There are numerous active chemicals in black tea that can be used to treat and prevent both non-chronic and chronic symptoms. Theobromine, theophylline, caffeine, adenine, tannins, polysaccharides, essential oils, lipids, amino acids, vitamins (including vitamin C), quercetin, naringenin, and polyphenols are some of these chemical components (Sangi *et al.*, 2019).

One of the plants that is important to Indonesia's economy is cocoa (*Theobroma cacao* L.). With an annual yield of 240,000 tons, Indonesia is the world's third-largest cocoa producer, behind Ghana and the Ivory Coast in West Africa (Aksara *et al.*, 2013). Cocoa beans can be processed into various products with higher added value. Cocoa beans contain various contents including protein, fat, carbohydrates, polyphenolic compounds, flavonoids, and methylxanthines (Rodriguez-Campos *et al.*, 2012). Theobromine and caffeine are the two primary alkaloids found in cocoa beans. Both are well-known methylxanthine chemicals that are present in cocoa and consumable goods made from it (Bartella *et al.*, 2019; Rojo-Poveda *et al.*, 2021; Viana *et al.*, 2018).

To obtain caffeine compounds, a separation method is required by adopting the process of alkaloid extraction from various mixtures. One of them is the liquid-liquid extraction method using specific solvents. In this investigation, 1.5 grams of $CaCO_3$ are combined with chloroform, the solvent, in a separating funnel, and the mixture is swirled until two layers develop. The solution of chloroform is added three times. This extraction is performed three times (Wardani & Fernanda, 2016), and a high-performance liquid chromatography (HPLC) technique for caffeine was developed using the Central Composite Design (CCD) approach (Najmi *et al.*, 2024). Additionally, methods for extracting caffeine components have been developed, including solid-phase extraction (SPE) and microextraction based on deep eutectic solvent (Santanatoglia *et al.*, 2024; Shishov *et al.*, 2019).

The extract obtained from the extraction process has been analyzed and the caffeine content determined using various analytical methods, including high-performance liquid chromatography – mass spectrometry (HPLC-MS) (Bartella *et al.*, 2019; Sabarni & Nurhayati, 2019), HPLC-diode array detection (DAD) and ultrahigh (UH)PLC-MS/MS (Viana *et al.*, 2018), Fourier transform infrared (FTIR)-derivative (Nugrahani *et al.*, 2019), and voltammetry (Stoytcheva *et al.*, 2023). Each of these methods has clear advantages and disadvantages, such as requiring long analysis times and being more complex. Therefore, this research adopts an experimental design approach by utilizing liquid-liquid extraction to develop an analysis method for caffeine in black tea, cocoa, and coffee, which will be analyzed using the HPLC- photodiode array (PDA) method. The applied analytical method is expected to provide accuracy and reliability in quantifying caffeine, thus contributing to a deeper understanding of these products in the context of health and industry. All three samples can be effectively separated when measured using the same method, thus enabling continuous use of this method for routine analysis of caffeine samples.

METHODS

Material

Robusta coffee, black tea, and cocoa extracts, chloroform (Merck), ethanol (brataco), $CaCO_3$ (Merck), standard of caffeine (Sigma Aldrich), methanol (Merck), filter (Whatman), acetonitrile (Merck), and redistilled water.

Tools

HPLC (Agilent), Beaker, Büchner funnel, Vacuum pump, Ultrasonicator, Volumetric flask, Fume hood, Separatory funnel, Filter holder, HPLC syringe injector, Erlenmeyer flask, Watch glass, Bunsen burner, and tripod stand.

Methods

The maceration extraction method is used to make robusta coffee, black tea, and cocoa extracts. Each extract is prepared using a 1:10 ratio, which consists of 2000 milliliters of 96% ethanol and 200 grams of coffee bean powder. Over the course of seven days, the mixture is mixed for five minutes every day. Chloroform is one of the organic solvents used in liquid-liquid extraction (Sholeha *et al*., 2019). In a separating funnel, the resultant concentrated extract is mixed with 25 milliliters of chloroform and 1.5 grams of $CaCO_3$, and the mixture is agitated until two layers form. Finally, three additions of the chloroform solution are made., and this extraction is performed three times (Wardani & Fernanda, 2016). This is one of the liquid–liquid extraction methods that separates compounds based on their difference in solubility in two immiscible solvents.

A 500 ppm concentration of standard caffeine stock solution is made. Standards for caffeine are made with concentrations of 10, 50, 100, 200, and 300 parts per million. The standard 500 ppm caffeine solution is utilized at 0.2, 1.0, 2.0, and 4.0 concentrations. Next, as much as 6.0 mL are dissolved in 10 mL of solvent in a volumetric flask. The solvent used in this study is a mixture of methanol: distilled water with a ratio of 70:30. The solution is then filtered using a 0.45 µm Whatman filter in a Buchner funnel assisted with a vacuum pump, then allowed to air-dry using an ultrasonicator for 5 minutes. 25 mg of caffeine standard is weighed and dissolved with solvent in a 50 mL volumetric flask, resulting in a caffeine stock solution with a concentration of 500 µg/mL (Saputri & Muchtaridi, 2018).

The selection of flow rates at 1.0; 1.2; 1.4 per minute in the analyte separation has been studied. According to research conducted by Rachmawaty *et al*. (2018), it is noted that the retention time decreases with increasing flow rate and produces good peaks. The mobile phase optimization is conducted to obtain the best chromatogram comparison using variations of the mobile phase methanol: distilled water (30:70), methanol: distilled water (30:70), acetonitrile: methanol: distilled water (80:5:15). The determination of the maximum wavelength of caffeine is done at concentrations of 100, 300, and 500 µg/mL obtained from the intermediate caffeine solution of 1000 µg/mL. Then each solution is read for its absorbance at wavelengths ranging from 200-800 nm using a PDA. The maximum observation wavelength is determined based on the detector (Gonzales-Yépez *et al.*, 2023; Widhyani *et al.*., 2021).

Subsequently, the method was validated utilizing three samples with specific measurement parameters, linearity, accuracy, precision, Limit of Detection (LOD), Limit of Quantification (LOQ), and others. Selectivity testing is performed by pipetting a certain amount of samples and then dissolving them in solvent to obtain caffeine concentrations according to the results of concentration optimization experiments. The prepared solution is filtered with a 0.22 µm Whatman filter membrane and then injected into the HPLC instrument using the selected method. Resolution (Rs) refers to the separation of two adjacent compounds or peaks in the chromatogram. It is considered selective if the Rs value > 1.5 (Yuwono & Indrayanto, 2005). Linearity data are obtained by creating eight concentration points of standard caffeine solution with a concentration range of 50-120% of the sample concentration. The standard caffeine solution is then filtered and injected into the HPLC system. The Area Under the Curve (AUC) value for each concentration series is obtained from the chromatogram. The AUC values are then plotted against each series of standard caffeine concentrations to calculate the regression line equation $y = bx + a$ and the r value.

The LOD and LOQ are obtained from a series of the six smallest concentration points of standard caffeine solution that still provide a response when injected under certain HPLC conditions. The LOD and LOQ values are calculated based on the standard deviation (SD) and slope data (Wardani, 2022). A standard caffeine solution with concentration series ranging from 50% - 120% of the test concentration is prepared in six different solvent concentrations. Then, the standard caffeine solution is injected into the selected HPLC system for six repetitions, and the coefficient of variation (RSD), which should be less than 2%, is calculated. Accuracy testing is conducted using the standard addition method or with standard addition. Sample additions are prepared with a concentration of 100 ppm in cocoa bean samples and added with standard caffeine solution to achieve concentrations of 30%, 45%, and 60%. Each sample addition is replicated three times. The accuracy test samples are then filtered and injected into the HPLC under the selected conditions. The accuracy parameter values are calculated from the scan data by comparing the sample concentration results with the analytical standard concentration at each concentration series and matching the results with the accuracy value requirements. The resulting data are processed to obtain the % recovery value (AOAC, 2019; Yuwono & Indrayanto, 2005).

RESULTS AND DISCUSSION

Optimization of condition

Flow rate

By examining flow rate parameters, mobile phase composition, and wavelength, the best

Table 1. The outcomes of the flow rate development in high performance liquid chromatography

Flow rate	Symmetry	N > 2000	tR	Rs
1.0	0.615	2124	3.708	1.55
1.2	0.619	1002	2.944	1.45
1.4	0.612	645	2.706	1.28

Table 2. Results optimization of mobile phase

Mobile phase	*symmetry*	N > 2000	tR
Methanol: *redistilled water* (30:70)	0.60	698	3.700
Methanol: *redistilled water* (70:30)	0.65	1013	1.657
Asetonitril:ethanol: *redistilled water* (80:5:15)	0.92	3192	1.454

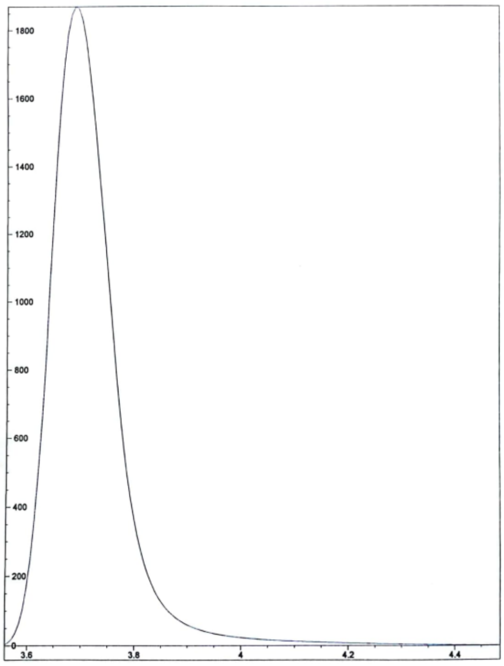


Figure 1. Isoplot 2D wavelength of caffeine standard.

HPLC method is developed. Standard caffeine is injected in order to optimize the procedure. Determination of the flow rate is performed at three flow rate variations, namely 1.0, 1.2, and 1.4 ml/minute.

Mobile phase

The mobile phase used is a combination of several solvents: methanol: redistilled water (30:70), methanol:distilled water (70:30), and acetonitrile:methanol: redistilled water (80:5:15). Methanol: redistilled water (70:30) has the best flow rate and mobile phase composition, with a flow rate of 1.0 ml/minute.

Wavelength

The optimization of the HPLC method was done by analyzing parameters such as flow rate, mobile phase composition, and wavelength. Standard caffeine was injected for the optimization process. Flow rate determination was performed at three flow rate variations: 1.0; 1.2; and 1.4 ml/min. The mobile phase consisted of a combination of solvents including methanol:redistilled water (30:70), methanol:redistilled water (70:30), and acetonitrile:methanol:redistilled water (80:5:15). The most optimal flow rate and mobile phase composition was methanol:redistilled water (70:30) with a flow rate of 1.0 ml/min.

Wavelength determination was conducted by injecting a standard caffeine solution and then viewing the 2D isoplot to determine the maximum wavelength. The maximum wavelength obtained was 272 nm. A previous study conducted by Fajriana and Fajriati (2018) also reported the maximum absorption wavelength of caffeine compounds at 272 nm.

Validation of the analysis method

These values have satisfied the criteria for a good chromatogram resolution. The standard resolution values with other analytes and samples with other analytes in this investigation are 1.49 and 1.57, respectively. These values satisfy the criteria for a high resolution chromatogram.

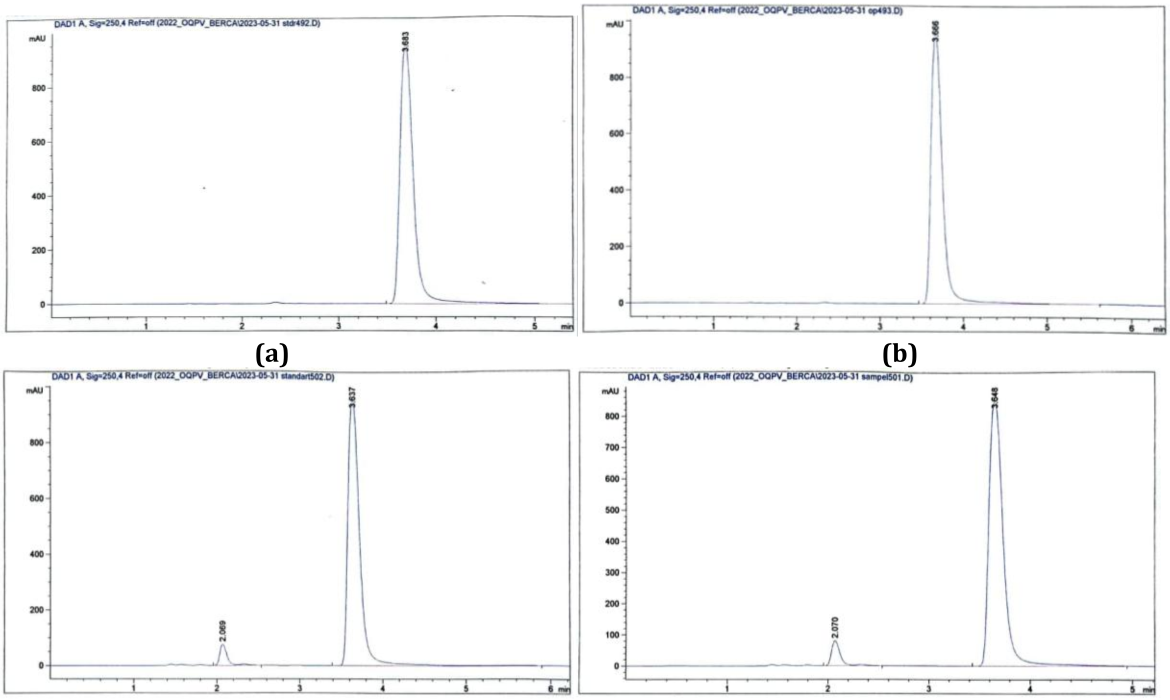
The purity factor values indicate the purity factor appearing in the chromatogram, where from 4 repetitions of injections into the instrument, the match factor value is indicated. The values obtained are 999.620; 999.645; 999.683; and 999.689. A sample is considered pure if the match factor value is close to 1000, impure if the match factor value is 950-990, and contaminated if the match factor value is <950 (AOAC, 2019). It can be concluded from these parameters that the chromatogram in this investigation satisfies the criterion for selectivity. The chromatogram results for each treatment can be seen in the Figure 2.

Linearity

A method's linearity shows how well it can calculate the standard curve's proportionality. Linearity is performed by injecting of 10-100 ppm caffeine standard. The regression equation obtained is y = 947x + 1272.3 with a correlation coefficient (r) value of 0.9994. A good linearity value is indicated when the correlation coefficient (r) approaches 1 (>1). It may be inferred from the linearity data analysis results that the calibration curve is linear (Naveen *et al.*, 2018).

Table 3. Resolution of the selectivity test and the standard and sample's purity factor

Test	Notes	Rs	tR	Purity Factor
1	Standard without matrix interference	-	3.683	999.620
2	Sample without matrix interference	-	3.664	999.645
3	Standard with other value	1.49	3.684	999.683
4	Sample with other value	1.57	3.637	999.689



(a) (b)

(c) (d)

Figure 2. Selectivity chromatogram results: (a) distraction-free standard; (b) distraction-free sample; (c) distraction-free standard with different value; (d) distraction-free sample.

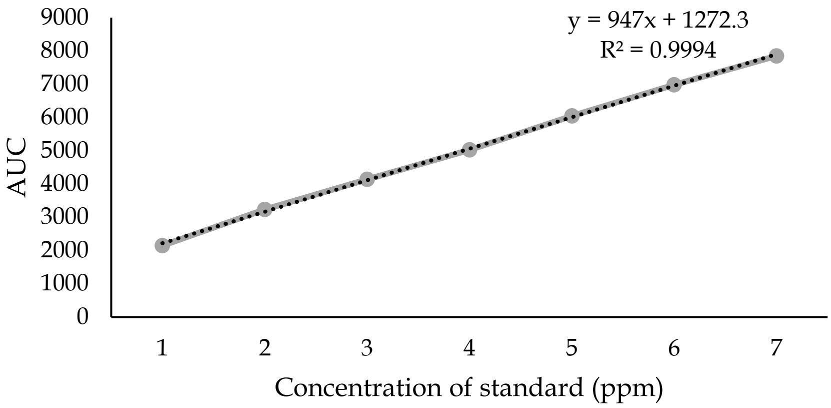


Figure 3. Standard linearity of caffeine.

Table 4. Accuracy results

Conc (n=3)	AUC	Concentration (ppm)	The Rate concentration of caffeine (ppm)	% *Recovery*	Rate of % *Recovery*	SD	% RSD
80%	5926.61475	4.914	4.778	101.148%	100.684 %	0.139	0.0142
	5803.66375	4.784		99.945%			
	5663.28467	4.636		100.960%			
100%	6250.80713	5.257	5.276	100.155%	100.342 %	0.017	0.0174
	6278.28613	5.286		99.583%			
	6280.02686	5.287		101.290%			
120%	7118.47217	6.173	6.249	100.016%	99.726 %	0.123	0.0126
	7129.40088	6.184		96.702%			
	7325.62549	6.392		102.460%			

RSD, relative standard deviation.

Linearity shows the ability of a method to obtain how proportional the standard curve is. Linearity was carried out by injecting 7 series of standardized standards. The regression equation obtained is y = 947x + 1272.3 with a correlation coefficient (r) value of 0.9994.

Limit of Detection and Quantification

The Limit of Detection (LOD) and Limit of Quantification (LOQ) are determined by creating 9 series of standard concentrations from the lowest point. The LOD and LOQ parameters aim to determine the detection limit and quantification limit that can be achieved by the instrument. In determining the LOD and LOQ, a calibration curve is utilized with the condition that the correlation coefficient (r) approaches 1 (>1). The LOD can be calculated using the formula LOD = 3 x SD / slope, and the LOQ is calculated using the formula LOQ = 10 x SD / slope, where SD is the residual standard deviation from the calibration curve, and the slope is the sensitivity value of the analysis method. A higher slope value indicates greater sensitivity of the method (Hacıoğlu *et al.*, 2015). In this study, the obtained LOD value is 8.511 ppm, and the LOQ value is 28.370 ppm.

Accuracy

The percentage relative standard deviation (RSD) values of 0.0142% at 80% concentration, 0.0174% at 100% concentration, and 0.0126% at 120% concentration were determined by the accuracy test analysis. The percentage RSD values at these three addition concentrations meet the acceptance criteria, where a good RSD value is <2%. The average percent recovery obtained in this study in 80%, 100%, and 120% addition concentrations were 100.684%, 100.342%, and 99.726%, respectively.

Precision

Precision indicates that an analysis method can provide the same or similar results when repeated. In this study, the precision test of repeatability is conducted by injecting the standard six times of 100% concentration. Precision is determined through the analysis of the percentage of RSD, which indicates the level of variation in research results. The percentage RSD value obtained in this study is 0.0095%. This value meets the acceptance criteria, where a good RSD value is < 2 (ICH Guideline, 2022). Precision shows that an analytical method can give the same or similar results when repeated. In this study, the repeatability precision test was done by injecting 6x standards.

Determination of caffeine content

After all validation parameters meet the requirements, it can be said that the method used in this study is valid and can be used to determine of caffeine content in robusta coffee, black tea, and cocoa samples. The determination of the content is done by injecting the sample solution three times.

Research on the injection of robusta coffee sample solution has shown that the area produced is 2845.926 ppm, 3160.003 ppm, and 3312.350 ppm. The level of caffeine compounds obtained was 9.682% (%b/b). The average caffeine content ± SD obtained was 9.682 ± 1.254. According to studies on the injection of black tea sample solutions, the area produced was 6576.504 ppm, 8010.486 ppm, and 8847.513 ppm. The level of caffeine compounds obtained was 34.525% (%b/b). The mean caffeine content ± standard deviation was 34.525% ± 0.064. According to the findings of the investigation into the injection of brown sample solution, the area generated was 2486.776 ppm, 2671.205 ppm, and 2574.422 ppm. The obtained amount of caffeine compounds was 6.890% (%b/b). The mean caffeine concentration ± standard deviation was 6.890% ± 0.488. Acceptability according to SNI 2014 requires a minimum caffeine content of 2.5%. Caffeine is relatively soluble in certain organic solvents, so if an organic solvent is chosen that has a greater affinity for caffeine than the air phase, most of the caffeine is transferred to the organic phase so that high recovery can be achieved. This is the basic principle that explains why the choice of solvent is very important in determining the results of liquid-liquid extraction (Pramanik *et al.*, 2024; Vandeponseele *et al.*, 2021).

CONCLUSIONS

The optimal settings for HPLC analysis include a wavelength of 272 nm, a mobile phase composed of methanol:redistilled water (70:30), and a flow rate of 1.0 mL/min. Selectivity, linearity, accuracy, precision, LOD, and LOQ are all met by the analytical method's validation, which yields good results. There are resolutions of 1.49 and 1.57 for the selectivity values, r = 0.9994 for linearity, 8.511 for LOD, and 28.370 for LOQ. Average recovery values of 99–101% are used to obtain accuracy, while the RSD for precision is 0.0095% for concentration. According to the caffeine analysis results, the ethanol extract of robusta coffee beans derived from the caffeine content is ± SD 9.682% ± 1.254, whereas the cocoa is 6.89%0 ± 0.488, and the black tea is 34.525% ± 0.064.

Table 5. Results of determination

Replication	AUC	Concentration (ppm)	The Rate concentration of caffeine (ppm)	The Rate concentration of caffeine (x̄) (ppm)	SD	% RSD
Robusta coffee	2845.926	1.662	8.310	9.682	1.254	0.132
	3160.003	1.993	9.965			
	3312.350	2.154	10.770			
	x̄ caffeine rates ± SD				9.682 ± 1.254	
Black tea	6576.504	5.601	28.005	34.525	0.064	0.007
	8010.486	7.115	35.575			
	8847.513	7.999	39.995			
	x̄ caffeine rates ± SD				34.525 ± 0.064	
Cocoa	2486.776	1.282	6.410	6.890	0.488	0.051
	2671.205	1.477	7.385			
	2574.422	1.375	6.875			
	x̄ caffeine rates ± SD				6.890 ± 0.488	

RSD, relative standard deviation; SD, standard deviation.

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CONFLICT OF INTEREST

There is no conflict of interest.

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