



APPLICATION OF RESPONSE SURFACE METHODOLOGY ON THE EXTRACTION OF CAFFEINE IN TEA AND COFFEE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TECHNIQUE

(Aplikasi Kaedah Gerak Balas Permukaan terhadap Analisis Kafein dalam Teh dan Kopi Menggunakan Teknik Kromatografi Cecair Berprestasi Tinggi)

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Abstract

A rapid and simple extraction method was developed for the analysis of caffeine in tea and coffee using high performance liquid chromatography (HPLC). Independent factors such as extraction method, temperature and residing time were optimized using response surface methodology (RSM) followed by Box-Behnken Design (BBD). All samples were analyzed using HPLC and separated on C₁₈ (150 mm × 4.6 mm, 5µm particle) column. The mobile phases were: (A) water and (B) acetonitrile at the flow rate of 1 mL min⁻¹ under ultraviolet (UV) detection at 273 nm. Good linearity was obtained in the range of 1–60 mg L⁻¹ (regression coefficient (R²) > 0.995). The limit of quantification (LOQ) and limit of detection (LOD) were 1.0 and 0.4 mg L⁻¹, respectively. Relative standard deviation (RSD) at the range between 3.11 and 11.37 is deemed as highly precise and it is 92% to 98% accurate. Based on the RSM-BDD analysis, extraction of caffeine from tea was directly proportional to temperature, time and method extraction, which exhibited all independent factors of this study were significant. The most optimal condition of caffeine extraction was heat treatment at 100 °C in 16 min and free from any chemical treatment. The highest concentration of caffeine was 366 mg L⁻¹ in Mahmood's green tea, while it was 334 mg L⁻¹ in Mahmood's coffee. The proposed method exhibited good results and easier quantification of caffeine in different samples using HPLC, and free from possible interferents.

Keywords: caffeine, tea, coffee, Box-Behnken design, high performance liquid chromatography

Abstrak

Kaedah pengekstrakan cepat dan mudah telah dibangunkan untuk penganalisaan kafein dalam sampel teh dan kopi dengan menggunakan kromatografi cecair berprestasi tinggi (HPLC). Faktor bebas yang penting seperti kaedah pengekstrakan, suhu dan masa tinggal telah dioptimumkan menggunakan kaedah gerak balas permukaan (RSM) diikuti oleh reka bentuk Kotak-Behnken (BBD). Semua sampel telah dianalisis menggunakan HPLC dan diasingkan pada turus C₁₈ (150 mm × 4.6 mm, 5µm). Fasa gerak alih yang digunakan ialah (A) air dan (B) asetonitril pada kadar aliran 1 mL min⁻¹ di bawah pengesanan ultra ungu (UV) pada 273 nm. Kelinearan yang baik diperoleh dalam julat 1–60 mg L⁻¹ (pekali regresi (R²) > 0.995). Had kuantifikasi (LOQ) dan had pengesanan (LOD) masing-masing ialah pada 1.0 dan 0.4 mg L⁻¹. Kejutuan sisihan piawai relatif (RSD) pada julat antara 3.11 dan 11.37 didapati hampir tepat dan mempunyai ketepatan antara 92% hingga 98%. Berdasarkan analisis RSM-BDD, pengekstrakan kafein daripada teh adalah berkadar terus dengan suhu, masa dan kaedah pengekstrakan yang menunjukkan bahawa semua faktor bebas tersebut adalah signifikan. Keadaan pengekstrakan kafein paling optimum ialah pada suhu 100 °C selama 16 minit dan bebas daripada sebarang rawatan kimia. Kepekatan kafein tertinggi sebanyak 366 mg L⁻¹ diperoleh dalam teh hijau Mahmood manakala 334 mg L⁻¹ dalam kopi Mahmood. Kaedah yang dicadangkan mempamerkan keputusan yang baik dan mudah untuk mengukur kafein dalam sampel yang berbeza menggunakan HPLC.

Kata kunci: kafein, teh, kopi, reka bentuk Kotak-Behnken, kromatografi cecair berprestasi tinggi

Introduction

Caffeine is an alkaloid, which is a naturally occurring compound with a wide range of pharmacological properties [1]. Caffeine is used as stimulant drug to reduce headache and other treatment purposes. It is commonly found in popular beverages worldwide consumed during breakfast, tea and dinner. It was reported that caffeine as an antioxidant plays an important role to reduce risk of coronary heart disease [2,3].

Caffeine belongs to methylxanthine group, which is a derivative of xanthine that occurs naturally in plants with no obvious odor and slightly bitter. The empirical formula of caffeine is C₈H₁₀N₄O₂ and its molecular structure is shown in Figure 1. Caffeine is not easily biodegradable nor absorbed into the human system as they can be continuously present in the aquatic environment through human excretion and its bioaccumulation into the wastewater, domestic wastewater and hospital effluents [15,16,17]. Furthermore, it can further affect the marine ecosystem.

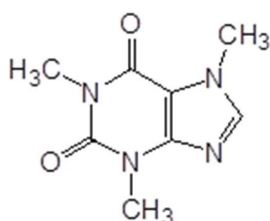


Figure 1. Structural formula of caffeine

In order to collect more information regarding the direct consumption of caffeine in consumer's daily diet, a comprehensive study of analysis of caffeine in tea and coffee samples was performed. The application of response surface methodology is vital in this study to determine the amount of consumed caffeine per cup, as there is no previous data regarding this study in Iraq. Caffeine has been analyzed in different samples such as biological fluids, foods, plants and water samples using different analytical instruments such as high-performance liquid chromatography (HPLC) [4], gas chromatography–mass spectrometry–flame ionization detection (GC–MS–FID) [5], Fourier transform-infrared spectrophotometry (FT–IR) [6], near-infrared spectroscopy (NIR) [7], Ultraviolet–Visible spectroscopy (UV–Vis) [8], FT-Raman spectrometry [9], and capillary electrophoresis (CE) [10]. Among the listed analytical techniques, HPLC offers advantages, such as simplicity and selectivity coupled with ultraviolet-visible (UV–Vis) detector, was the most preferred analysis system for the analysis of caffeine [8,11,12].

The issue of extracting caffeine from tea without using chemical reagents has led to the development of various extraction techniques. Conventional liquid–liquid extraction (LLE) and solid-phase extraction (SPE) have been used for pre-concentration and clean-up before analysis. Liquid-liquid extraction is time-consuming and hazardous to health due to the high volume of toxic solvents used [13]. Solid-phase extraction requires less

solvent, but is still time consuming, and often requires a concentration stage that presents disadvantages such as losses in the evaporation step [14]. To resolve time consumption of the proposed extraction techniques, response surface methodology has been utilized to determine the significant factors of this study.

The aims of this study are: (i) to optimize different extraction method, temperature and residing time using response surface methodology; (ii) to identify and confirm presence of caffeine in tea using LC-TOF/MS as a very accurate instrument based on mass-to-charge ratio; (iii) to determine the concentration of caffeine in real samples using HPLC-UV.

Materials and Methods

Reagents and chemicals

Caffeine (purity 99%) was purchased from Sigma-Aldrich (USA). Acetonitrile (ACN), methanol (MeOH) and ethyl acetate were HPLC-grade and provided by Sigma-Aldrich (USA). Other reagents such as hydrochloric acid and sodium carbonate were purchased from Sigma-Aldrich (USA). Double distilled water was prepared using the distillation apparatus (Manufacturer, Country of Manufacturer) in the laboratory setting.

Sample Collection

Samples are Mahmood green tea, Mahmood red tea, Al Ghazaleen red tea, Brazilian coffee and Mahmood coffee samples were collected from the local market in

Babylon City, Iraq as presented in Table 1. All samples were stored at 4 °C refrigerator throughout the analysis to avoid further enzymatic or denaturation effect on the samples. Laboratory glassware and glass apparatuses were properly cleaned with tap water and rinsed with double distilled water.

Sample preparation

Each sample was treated with three different extraction methods (extraction method 1, extraction method 2 and extraction method 3), three different temperatures (40, 70 and 100 °C) and three different residing times (4, 10 and 16 min). Each independent factor was optimized using RSM to select the significant output.

Experimental design

Response surface methodology has been used to investigate each variable on the effectiveness of caffeine extraction through: extraction method (A), temperature (B), and residing time (C). For each of the three studied variables, high and low set points were selected to construct an orthogonal design (Table 2). Box-Behnken Design (BBD) was used to optimize the values of these three factors and reach the best response. Design included 30 treatments in three levels for three factors and consisted of six center points. The experimental design matrix and data analysis were carried out using the software package Minitab 19 statistical software. The peak area and/or concentration of caffeine was used as the HPLC response to evaluate the extraction efficiency.

Table 1. Description of tea and coffee samples

Brand Name	Origin	Expiry Date
Mahmood red tea	Produced and packed in Sri Lanka	06/2022
Mahmoud green tea	Produced and packed in Sri Lanka	02/2023
Al Ghazaleen Tea	Produced in Sri Lanka- Ceylon	04/2022
Brazilian coffee	Produced in India	09/2022
Mahmood coffee	Produced in Poland	11/2021

Table 2. The experimental range and levels of the variables in the BBD

Factor	Symbol	Range and Levels		
		Low	Centre	High
Extraction Method	A	1	2	3
Temperature (°C)	B	40	70	100
Residing time (min)	C	4	10	16

The regression equation in uncoded units was presented below:

$$\text{CONC.} = -217.7 + 55.8 \text{ A} + 4.199 \text{ B} + 1.89 \text{ C} - 32.27 \text{ A}^2 - 0.04961 \text{ B}^2 - 1.049 \text{ C}^2 + 1.217 \text{ AB} + 4.854 \text{ AC} + 0.2958 \text{ BC}$$

Three different extraction methods used in this research were:

Extraction Method 1

One gram of each tea leaves and coffee beans were soaked in 100 mL of boiled water (100 °C) separately and continuously, for 10 minutes. After cooling each extract, 5 mL aliquot was mixed with 0.1 M hydrochloric acid (2.5 mL) and saturated basic acetate solution (2 mL). The solution was centrifuged at 8000 rpm for 15 minutes. NaHCO₃ solution (0.1 g NaHCO₃ to 10 mL of water) was added to the supernatant then shaking using stir device. The solution was centrifuged again at 10000 rpm for 15 minutes, after that the extract was diluted with water into a 50 mL volumetric flask. 20 µL of final extract was filtered through a 0.45 mm membrane (Millipore) then injected into the HPLC column.

Extraction Method 2

One gram of tea and/or coffee sample has been soaked in 100 mL of hot water (100 °C) and stirring for 10 minutes. The aqueous phase was extracted with ethyl acetate using separation funnel, which is categorized as liquid-liquid extraction. Each extract was injected into the HPLC instrument.

Extraction Method 3

One gram of tea and/or coffee sample was soaked in 100 mL of hot water, separately for 10 minutes. This method does not require any treatment with reagents and/or chemicals, which is categorized as normal extraction

method. After that each extract was cooled and filtered by a sterile filter (0.45 µm). Before injection into HPLC instrument, each extract was diluted in 10% double distilled water then 20 µL of extract was injected directly into the HPLC.

Preparation of stock and working solution

Caffeine stock solution of 1000 mg L⁻¹ was prepared by accurately weighing 0.01 gram of standard caffeine, then dissolved in 10 mL methanol. Working standard caffeine solutions of 1, 10, 30 and 60 mg L⁻¹ were prepared by serial dilution of the stock solution with double distilled water. The calibration curve of peak areas versus concentration of the standards was plotted. The caffeine content (mg L⁻¹) of the various samples was calculated by interpolation within the regression equation of the best line of fit.

Chromatographic separation

All selected samples in the present study were analyzed using high performance liquid chromatography (HPLC, Knauer Germany system) to identify and quantify caffeine compound. A binary high pressure pump and diode array detector were involved in the instrument. The separation was provided on C₁₈ column (150 mm × 4.6 mm I.D., 5 µm particle size; Knauer, Germany). The mobile phase is consisted of water (A) and acetonitrile (B). The initial gradient composition (5% B) was maintained for 4 min and then linearly increased to 95% B over 9 min and maintained for 2.5 min before being decreased to 2% B over 0.1 min. Then, equilibration was performed for 2.9 min. The injection volume was 20 µL

and the flow rate was set to 0.3 mL min⁻¹. The column temperature was fixed at 35°C. All samples were subjected to 73 nm wavelength.

Mass spectrometry analysis (MS) was carried out using a time-of-flight (ToF) instrument (Bruker/Germany) equipped with a Z-spray electrospray interface. The results were obtained with the following settings: MS capillary voltages, 4000 (PI); collision energy for diclofenac, 10 eV; drying gas flow rate, 8.0 L min⁻¹; drying gas temperature, 190 °C; set capillary, 4000 V; set end plate offset, -500 V; set collision cell RF, 250 Vpp; and nebulizer pressure, 4.0 bar. The ToF results were collected between *m/z* 50 and 600. All analytes were acquired using an independent reference spray via the LockSpray interference to ensure accuracy and reproducibility; a mixture of sodium hydroxide and FA was used as the lock mass at *m/z* 90.9766–974.8132. The accurate mass was calculated using Daltonic Analysis software incorporated into the instrument.

Method validation

Analytical method was validated for linearity, limit of detection (LOD), limit of quantification (LOQ), intra day precision and accuracy. Calibration curve was performed in the range of 1 to 60 mg L⁻¹. The linearity was assessed by correlation coefficient (*R*²). The LOQ and LOD values are calculated with the following equation (1):

$$\frac{S}{N} = \frac{2H}{h} \quad (1)$$

Where “S” is the signal of target peak. “N” is the noise adjacent to target peak. “H” is the height of target analyte peak so it refers to the signal “S”. “h” is the height of noise.

For repeatability measurement, samples were analyzed with three replicates in the same run. The results are represented as the percentage coefficient of variation values of the concentration of the analyte. All results were treated statistically using Microsoft Excel. Accuracy, however, was calculated after adding known

amount of caffeine 1 mL of 10, 30 and 60 mg L⁻¹ into 9 mL of tea sample then subtracting the peak area from the origin sample to find recovery% according to equation (2).

$$\text{Accuracy \%} = \frac{A_{\text{spike}} - A_{\text{unspike}}}{A_{\text{standard}}} \times 100\% \quad (2)$$

Where *A*_{spike} is adding known amount of caffeine to tea sample. *A*_{unspike} is only tea sample. *A*_{standard} is the standard concentration of caffeine.

Evaluation of selectivity

Selectivity was evaluated by comparing the chromatograms of three different samples: real sample, spiked real sample, and double distilled water. The results revealed that no interfering peaks visible at the same retention time of caffeine.

Results and Discussion

Method validation

The linearity of analytical method of caffeine was assessed through five concentrations of caffeine: 1, 10, 30 and 60 mg L⁻¹. All working solutions were eluted out of the column at the same retention time (10.6 min) as presented in Figure 2.

Calibration curve was obtained by plotting the peak area of caffeine against the working standard concentration. Calibration curve was fitted to the least-square linear regression. The results showed good linearity for caffeine, in which (*R*²) was 0.995. It was observed that limit of quantification (LOQ) was calculated according to the injection of samples reaching up to low concentrations then calculated using equation (1), which is equivalent to 1.0 mg L⁻¹. It was observed that at 0.2 and 0.5 mg L⁻¹ of caffeine, the signal was very small compared to noise signal, hence, both concentrations have signal-to-noise ratio less than 10. However, 1 mg L⁻¹ of caffeine was analyzed, and it could be confirmed that the concentration has signal-to-noise ratio 10.4 means it represent LOQ.

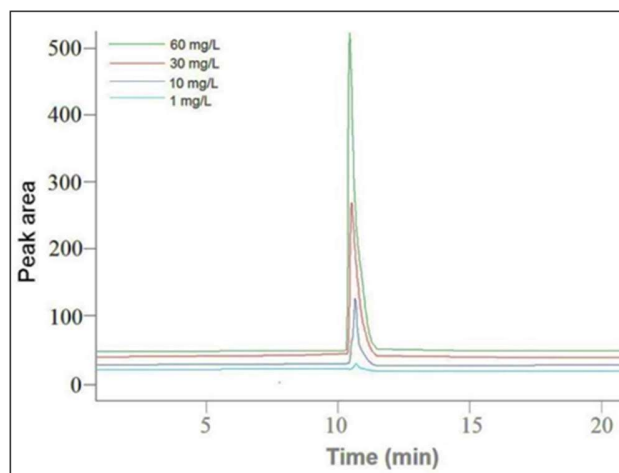


Figure 2. Chromatograms of caffeine at different concentrations (1, 10, 30 and 60 mg L⁻¹)

The precision of liquid chromatography method was evaluated through analysis of three samples replicates performed within the same day (refer to Table 3). The precision was determined by analyzing three different concentrations of caffeine (10, 30, and 60 mg L⁻¹). The results were presented as RSD%, ranging from 3.11 to

11.37%, which is considered as small and precise. Accuracy was determined by adding known amounts of caffeine to the sample (10, 30 and 60 mg L⁻¹). The results are in good agreement with the acceptable values for the validation of an analytical procedure, with the accuracy ranging from 92% to 98% as presented in Table 3.

Table 3. Precision data for the analysis of caffeine

Compound	Concentration (mg L ⁻¹)	Accuracy %	Precision (RSD%)	LOQ (mg L ⁻¹)	LOD (mg L ⁻¹)	Regression Linear Equation
Caffeine	10	92	11.37	1.0	0.4	$y = 63.96x + 83.54$
	30	96	8.31			
	60	98	3.11			

Selectivity of method has been evaluated based on the comparison of standard caffeine, spiked caffeine in tea and blank sample. It was observed that no peaks interfered at the retention times of caffeine at 10.6 minute.

Box-Behnken design-based optimization

It is well known that extraction process is important to make caffeine ready for analysis, when it is present in complex samples like plants and beverages. Tea and coffee were extracted by three different methods as described previously. In comparison to second and third extraction methods, the first extraction method has produced the lowest peak area. This may be caused by the frequent sample transfers that resulted with some

caffeine loss. Caffeine was dispersed between two layers in the second extraction method, which uses two immiscible solvents, but the peak area was still moderate in comparison to the third extraction method. However, the third extraction method without chemical reagents provides the most optimal caffeine recovery and less lost of caffeine compared to other extraction methods. Table 4 represents the ANOVA and regression analysis to assess the significance of independent factors, which are extraction method (A), temperature (B) and residing time (C). The model presented a high coefficient of determination ($R^2 = 0.9928$). This value indicated a good accordance with the experimental data and a good fitting ability for the model. Thus, the procedure demonstrated the ability of the model to work as a

predictive tool. The value of the adjusted determination coefficient (adjusted $R^2 = 0.9895$) is also sufficiently high to support a high significance for the model.

Box-Behnken design of response surface methodology was used for the analysis of the effect of temperature, residing time and extraction method on the extraction of caffeine and optimization of the independent variables. The linearity and quadratic effect of the independent parameters, their interaction and coefficients on the

response variables were obtained by analysis of variance (ANOVA) (Table 4). The significance of the lack-of-fit, regression model and coefficient of determination (R^2) were used to judge the validity of the Box-Behnken design model. The analysis of the P-values of temperature, residing time, and extraction method, as well as the double interaction between these factors, show that those independent factors had a statistically significant effect on caffeine extraction from tea samples, with a p-value less than 0.05, which is 0.001.

Table 4. Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	301901	33545	305.96	0.000
Linear	3	233097	77699	708.70	0.000
A	1	58443	58443	533.07	0.000
B	1	100806	100806	919.47	0.000
C	1	73848	73848	673.58	0.000
Square	3	28675	9558	87.18	0.000
A*A	1	7690	7690	70.15	0.000
B*B	1	14719	14719	134.26	0.000
C*C	1	10535	10535	96.09	0.000
2-Way Interaction	3	40129	13376	122.01	0.000
AB	1	10658	10658	97.21	0.000
AC	1	6786	6786	61.90	0.000
BC	1	22684	22684	206.91	0.000
Error	20	2193	110		
Lack-of-Fit	3	1295	432	8.18	0.001
Pure Error	17	897	53		
Total	29	304094			

Figure 3a represents normal distribution of the data towards the straight line (red color) in normal probability plot, means good significance of the results and no evidence of any deviation. Accumulating the datasets represented by blue dots along the straight line refers to a perfectly fitting model with no possibility of deviation. Figure 3b represents the Pareto chart, which is an indication of the individual interaction effect between the independent variables. Student's t-test was used to confirm that some factors are higher than zero point, which are significant. The vertical line (2.09) in Pareto chart refers to all factors that are statistically significant (confidence level > 95%) either individually or overlapped as they lied on the right-hand dashed line.

Therefore, extraction of caffeine from tea was directly proportional to temperature, time and method of extraction. Consequently, increasing of time and temperature or development in extraction method, yield high amount of caffeine in the same sample of tea. Figure 3c shows the normal probability plot of the standardized effects with $p \leq 0.05$, to evaluate the significance of each independent factor and its interactions on extraction of caffeine from tea. Normal probability plot could be separated into two regions, right and left. Right region includes positive coefficients (from B to AC), while left region includes negative coefficients (AA to BB). It can be concluded through normal distribution of the data in normal probability plot

and perfect fitting model; Pareto chart with confidence level $>95\%$ and $p \leq 0.05$ of the combined factors such as Extraction Method with Temperature (AB),

Extraction Method with Time (AC), Temperature with Time (BC), all independent factors are highly significant.

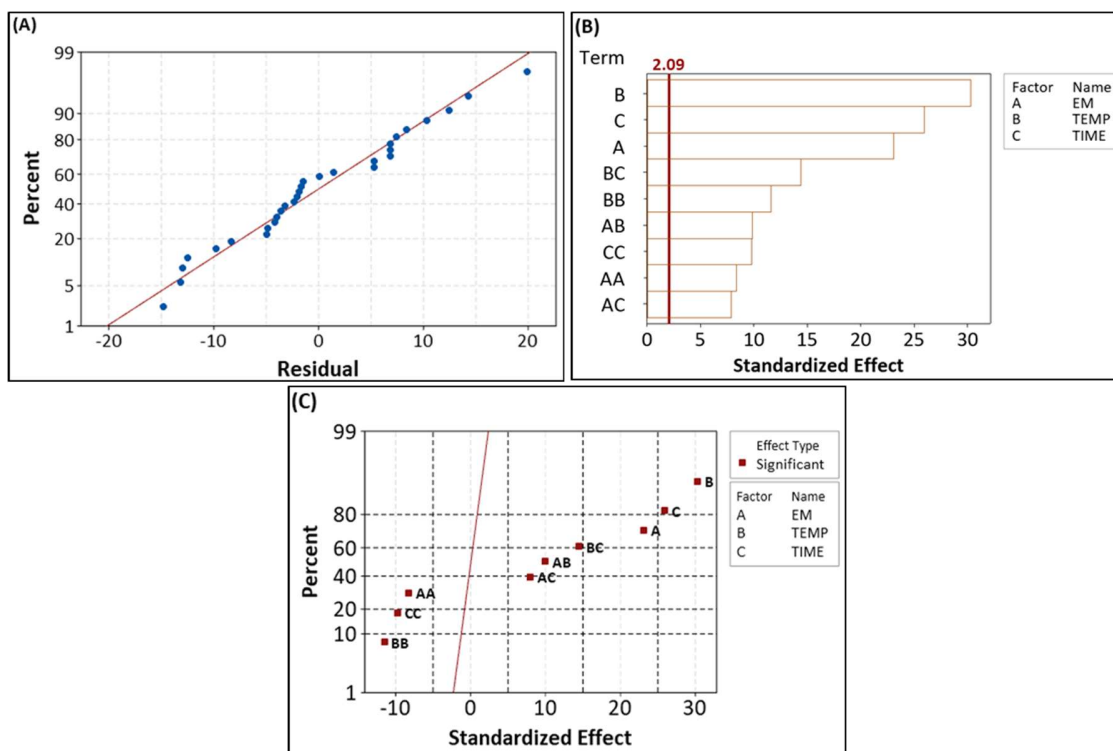


Figure 3. (a) The predicted response vs the actual response, (b) Pareto chart of standardized effects on the extraction efficiency of caffeine at $p = 0.05$, and (c) Normal probability plot of the standardized effects on the extraction efficiency of caffeine at $p = 0.05$.

The result is highly correlating that caffeine content increases considerably with heating time and specific method of extraction 3. In other words, following normal extraction without adding any chemicals is preferred. Furthermore, increasing of time reflect high positive impact on enhancement of caffeine extraction from tea leaves. These results are supported by the contour plot given in Figure 4a as can be seen, whereby tea boiled in 16 min at $100\text{ }^{\circ}\text{C}$ resulted to caffeine concentration reaching up to 400 mg L^{-1} . Figure 4b showed the efficiency of caffeine extraction is improved with increasing time and temperature. However, it could be observed that the extraction of caffeine decreases at short time and low temperature when extraction method

3 was applied. It could be attributed to the role of heating, which accelerates the extraction of caffeine even at a shorter time as supported by the contour plot. The contour plot presented in Figure 4c, exhibited the major role of temperature regardless the type of extraction. It could be observed that all extraction methods performed in this study resulted to reasonable caffeine content. Caffeine concentration is in the range between $300\text{--}400\text{ mg L}^{-1}$ at 16 min and $100\text{ }^{\circ}\text{C}$ by using extraction method 3. Similarly, extraction method 2 exhibited caffeine concentration in the range between 200 and 300 at mg L^{-1} in the same time and temperature as extraction method 3.

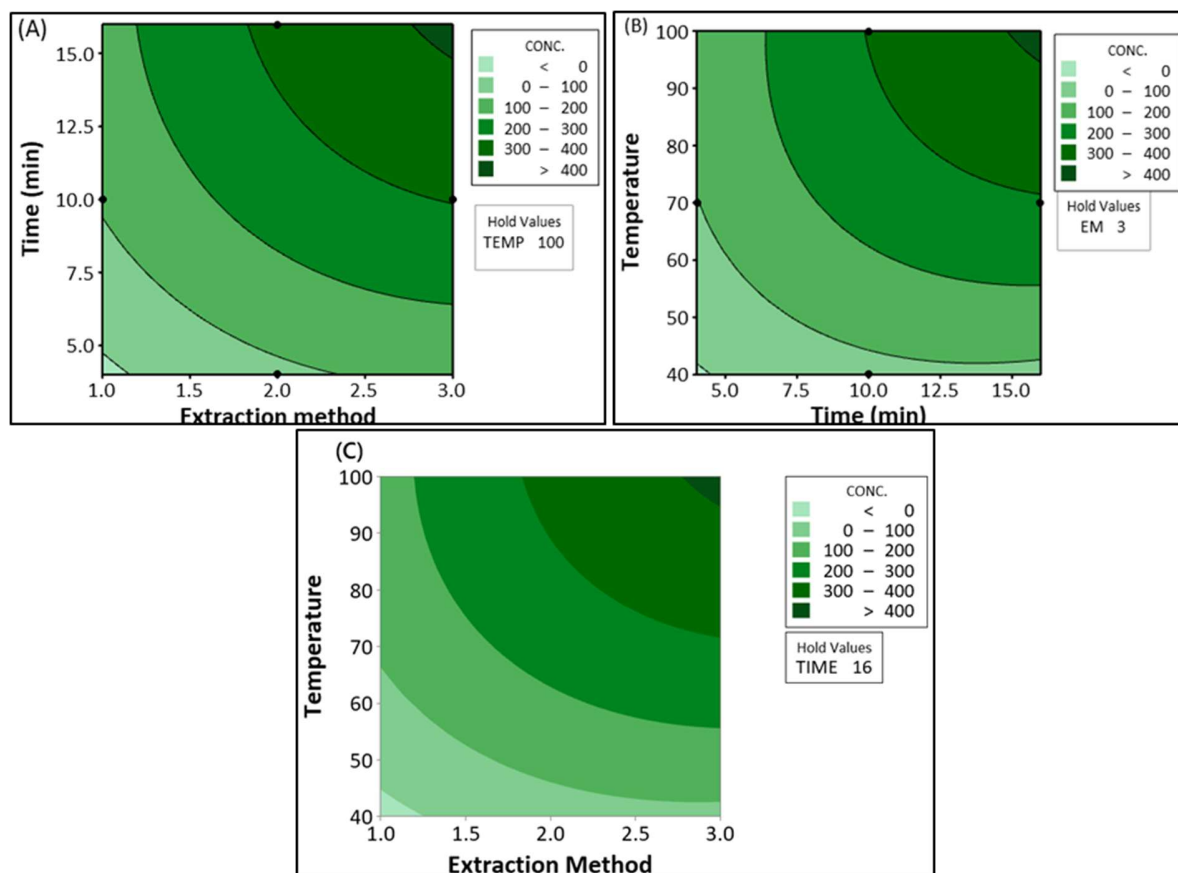


Figure 4. Response using the Box-Behnken Design obtained by contour plotting: (a) time vs. extraction method at 100°C, (b) temperature vs. time using extraction method 3, (c) temperature vs. extraction method in 16 minutes.

Analysis of caffeine in tea, and coffee

Caffeine was identified based on the mass value (m/z) in regards to mass spectrometry and retention times in regards to HPLC. Confirmation of presence of caffeine was carried out in the ToF/MS mode by extracting the narrow window extracted ion chromatogram (nwXIC) of the protonated molecular ion $[M+H]^+$. The nwXIC was typically extracted using a 20 mDa window. Positive identification for caffeine was based on (a) an accurate mass measurement of the base ion with an error of $\leq \pm 0.5$ ppm; and (b) LC retention time of the analyte compared with that of a standard [18]. The base peak at m/z 195.0884 for caffeine represented protonated molecular ion $[M+H]^+$ as shown in Figure 5. A small daughter peak at m/z 138.0655 appeared after the fragmentation of caffeine. This small, intense peak

refers to the loss of C_2H_3NO from caffeine to form $C_6H_7N_3O$. The results agreed with previous study reported by Choi et al. [19] on the formation of metabolite of m/z 138.12 from caffeine after losing C_2H_3NO moiety.

For analysis of caffeine in tea and coffee samples, 100 mL of double distilled water was boiled in a glass beaker. After that heating was stopped and 1.0 gram of tea leaves or coffee beans was soaked in the boiled water. During this stage, the beaker was covered with a watch glass and stirring continued at a constant speed. Each sample was withdrawn at 1.0 mL after 10 min and filtered with 0.45 μ m syringe filter. Caffeine was quantified by the developed reversed phase high pressure liquid chromatography method. All six samples

of tea leaves and coffee beans were analyzed and quantified using HPLC as shown in Table 5 and Figure

6. It was observed that caffeine has same retention time (10.6 min) in all selected samples.

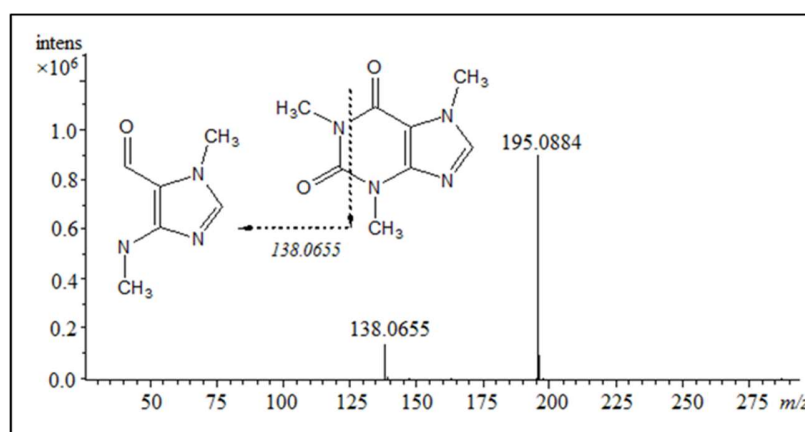


Figure 5. Mass spectrum of caffeine

Table 5. Result of caffeine content in coffee and tea samples using HPLC by extraction method without chemicals after 10% dilution

Sample Name	Caffeine concentration (mg L ⁻¹), M $\pm$ SD
Mahmood red tea	311 $\pm$ 6.9
Mahmoud green tea	366 $\pm$ 10.3
Al Ghazaleen Tea	290 $\pm$ 8.1
Brazilian coffee	278 $\pm$ 10.9
Mahmood coffee	334 $\pm$ 5.7

Surprisingly, the highest concentration of caffeine was present in Mahmood green tea, with the concentration reaching 366 mg L⁻¹ compared to Mahmood red tea, which is slightly lower at 311 mg L⁻¹. Hence, it signifies both green tea and red tea cannot be categorized as caffeine-free. Meanwhile, in coffee, the caffeine concentration was 278 and 334 mg L⁻¹ in Brazilian coffee and Mahmood coffee, respectively; which is almost identical to Al Ghazaleen tea and Mahmood red tea. In comparison to other tea samples and coffee, the concentration of caffeine is high in the present study compared with that caffeine detected in BOH tea (138.3 $\pm$ 4.5 mg/L), Milo (30.4 $\pm$ 2.5 mg/L) and white coffee (82.7 $\pm$ 2.9 mg/L) as presented in our previous

study [18]. Similar study conducted using liquid chromatography has successfully proven that most Malaysian produced coffee and tea products are less in caffeine compared to the imported coffee and tea, which are proven to be highly concentrated with caffeine. The mean caffeine content of tea, green tea, per se, is higher than that of coffee. The fate of caffeine will remain in the body of the consumer throughout the digestion that may cause increased anxiety, increased heart rate and blood pressure, disorientation, hallucinations, acid reflux and sleep disturbance, and being excreted to the environment causing further pollution and bioaccumulation into the wastewater, aquatic and marine environment, and animals [20, 21].

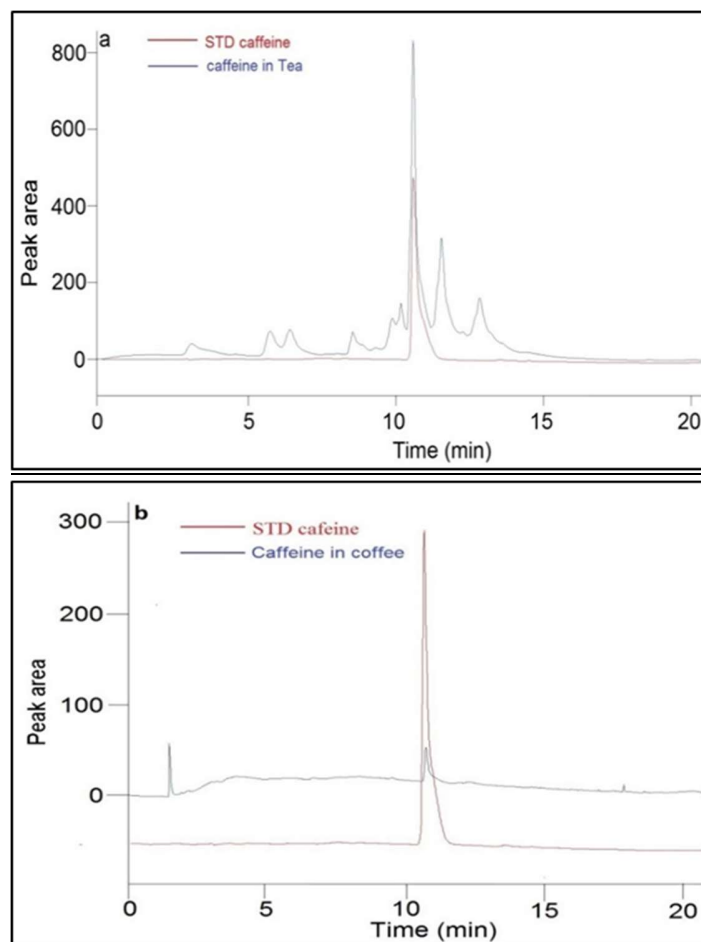


Figure 6. Chromatograms of caffeine in real samples: (a) tea leaves and (b) coffee sample

Conclusion

HPLC is a good technique and instrument to be used in identifying and quantifying caffeine in all selected samples namely tea and coffee. It was observed that HPLC reduces the labor work procedure in which requires less extensive usage of chemicals as proven by extraction method 3 with zero chemical used, but at boiling point and less than 20 minutes extraction time. Good linearity ($R^2 = 0.995$), a specific and sensitive detection system, low quantification limit 1 mg L^{-1} and fast analysis method are the main advantages of the proposed method. Furthermore, no interferences can be detected through this complex matrix and zero chemical compound. Highest concentration of caffeine was

detected in tea sample 366 mg L^{-1} while the lowest concentration was 278 mg L^{-1} as detected in Brazilian coffee sample. This study has significantly exhibited that tea has more caffeine than coffee, which would deter the myth that tea is lighter than coffee, however, tea releases caffeine in a smaller amount but yet more long-lasting than coffee and leaves smaller carbon footprint.

Acknowledgments

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