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High-Performance Liquid Chromatography Analysis of Caffeine and Trigonelline in Instant Coffee: A Malaysian Market Study

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Abstract: Caffeine and trigonelline are key alkaloids in coffee that significantly influence its quality and effects on human well-being. However, exceeding the recommended intake can produce negative effects. Yet, instant coffee products often lack their content labelling. This study addresses this gap by quantifying these compounds in 15 instant coffee products marketed in Malaysia. A reverse-phase high-performance liquid chromatography method was developed and validated for the simultaneous analysis of black, two-in-1, and three-in-1 instant coffee products. Separation was achieved on a C18 column with gradient elution using water and methanol, with heptafluorobutyric acid as the ion pairing agent. Caffeine content ranged from 2.02 to 47.54 mg/g, and trigonelline from 0.37 to 11.00 mg/g. Per serving, this corresponded to 36.43–183.33 mg of caffeine and 6.70–41.41 mg of trigonelline. Caffeine levels per serving met safety recommendations, but multiple servings would exceed the advised daily limits of 400 mg for adults and 200 mg for pregnant women. Seven products exceeded the 100 mg/day limit for adolescents in just one serving. Additionally, one product contained only 50.5% of its declared caffeine content. In conclusion, this study underscores the need for accurate labelling and informed consumer decisions regarding instant coffee consumption.

Keywords: *Caffeine, trigonelline, instant coffee, high-performance liquid chromatography.*

1. Introduction

Coffee, a globally embraced beverage, has attracted significant scientific interest due to its potential health benefits. Notably, Malaysia, traditionally a tea-drinking country, has seen a remarkable increase in coffee consumption in recent years (Ali & Ramanathan, 2021). This shift is important, given the association between coffee consumption and reduced risks of fatty liver disease, type 2 diabetes, cardiovascular diseases, and various metabolic and neurological disorders (Kolb et al., 2021; Safe et al., 2023). These health benefits can be linked to specific bioactive compounds in coffee, with caffeine and trigonelline being the most notable. Besides their health benefits, trigonelline and caffeine also play a key role in determining coffee flavour quality (Ogutu et al., 2022). Figure 1 presents their chemical structures.

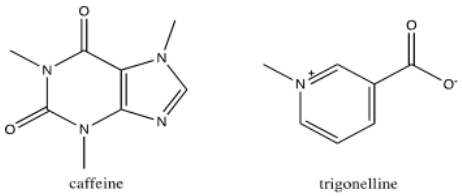

Figure 1. Chemical structures of caffeine and trigonelline analyzed in this study.

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Caffeine is the primary alkaloid in coffee and contributes to the signature bitterness of the beverage (Seninde & Chambers, 2020). It is recognized for its stimulant effects and potential health benefits, including enhanced cognitive function, mood regulation, and reduced risk of certain chronic diseases (Açıkalın & Sanlier, 2021; Fiani et al., 2021; Rodak et al., 2021). However, excessive intake can pose risks, including elevated risk of miscarriage and adverse effects on the gastrointestinal, liver, cardiovascular, renal, bone, and reproductive systems (Cornelis, 2019; Depaula & Farah, 2019). Chronic consumption may also influence mood, sleep, and behavior (van Dam et al., 2020). Hence, current guidelines recommend limiting daily caffeine intake to 400 mg for adults (European Food Safety Authority (EFSA), 2015).

Trigonelline is the second most abundant coffee alkaloid. It is found in green coffee beans (Saud & Salamatullah, 2021), with levels up to 34.2 g/kg. It contributes to the appealing aroma of coffee through roasting-induced formation of alkylpyridiniums. At the same time, its strong correlation with pH suggests its significant role as a major flavor precursor, influencing taste and aroma. Beyond its sensory contributions, trigonelline offers various health benefits, including potential blood glucose reduction, support for liver autophagy, and decreased risk of neurological diseases and heart conditions. It also exhibits sedative, anticarcinogenic, antimigraine, antidiabetic, antibacterial, and antiviral properties (Mohamadi et al., 2018). Recent findings suggest it helps reduce dental caries by inhibiting *Streptococcus mutans* (de Almeida et al., 2021; Narayan Biswal et

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al., 2020). Despite some concerns regarding its potential health effects, a 2023 risk assessment concluded that both acute and chronic exposure to trigonelline is safe for human health (Konstantinidis et al., 2023), resulting in no established safe consumption limit.

Given the rise in coffee consumption in Malaysia, there is an urgent need for accurate quantification of these compounds, not only for consumer knowledge but also for ensuring the quality and consistency of coffee products. Despite the extensive consumption of instant coffee, there is a notable lack of comprehensive studies that specifically quantify and compare the caffeine and trigonelline content across different brands available in the Malaysian market. Hence, this work aims to develop and validate a high-performance liquid chromatography (HPLC) method for the simultaneous measurement of these compounds in instant coffee products sold in the country. HPLC, recognized for its high precision, offers a broad dynamic linear range, selective separation, and superior sensitivity, making it suitable for this purpose.

We hypothesize that the caffeine and trigonelline content in these products per serving do not exceed the recommended guidelines. Moreover, we hypothesize variability in the caffeine and trigonelline content among different instant coffee products in Malaysia, which could result from differences in coffee bean origin, processing methods, and formulation techniques used by various manufacturers (Olechno et al., 2021).

2. Materials and Methods

Chemicals and Reagents

Caffeine (Sigma-Aldrich, Merck Peruana, Lima), trigonelline (Sigma-Aldrich, Merck Peruana, Lima), HPLC-grade methanol (Merck KGaA, Germany), and heptafluorobutyric acid (Alfa Aesar, Thermo Fisher Scientific, Great Britain) were used in this study. Ultrapure water (UPW) was prepared using a Sartorius AG model H2OPRO-VF-T system and utilized for all experimental procedures.

Instant Coffee Products

Instant coffee products in powder form were obtained from supermarkets in Malaysia. Products placed at the eye level of the shelves were selected for analysis in this study. Coffee samples were classified into black coffee (BC), two-in-one coffee (2I1C), and three-in-one coffee (3I1C), with five different brands chosen for each group.

Preparation of Stock and Working Standard Solutions

Stock standard solutions of caffeine and trigonelline (200 µg/mL) were prepared in UPW and sonicated for 15 minutes using an ultrasonic bath (Model FB 15061, Fisher Scientific, UK). Working standard solutions were prepared by diluting the stock solutions with UPW. All solutions were filtered through a 0.22 µm nylon syringe filter (Thermoline) prior to HPLC injection.

Preparation of Coffee Samples

BC coffee powder samples were prepared by dissolving 0.3 g of coffee powder in 100 mL UPW, stirring at 100°C until fully dissolved, cooling to room temperature, and filtering through qualitative filter paper. For 2I1C and 3I1C samples, 0.5 g of coffee powder was dissolved in the same way. Each filtrate was filtered using a 0.22 µm nylon syringe filter and stored at 4°C until analysis.

Determination of Maximum Wavelength Absorbance (λ_{max})

UV absorbance spectra of 10 µg/mL caffeine and trigonelline standard solutions were recorded between 200 and 400 nm using a Shimadzu UV-1800 spectrophotometer, with UPW as the blank solution. The λmax was identified as the wavelength at which the maximum absorption occurs.

Chromatographic Conditions

Chromatographic analysis was performed using an Agilent 1260 Infinity HPLC system, including a quaternary pump (G1311C), an autosampler (G1329B), and a diode array detector (DAD) (G1315D). Data processing was performed using Agilent OpenLAB CDS ChemStation software. A GraceSmart reverse phase C18 analytical column (150 mm x 4.6 mm, 5 µm) was utilized for chromatographic separation at room temperature. The mobile phase consisted of UPW with 5 mM HFBA (mobile phase A) and methanol with 5 mM HFBA (mobile phase B). A gradient method was applied, starting with 5% B and increasing to 70% B over 8 minutes. The mobile phase composition returned to 5% B within 2 minutes, followed by a 6-minute equilibration period. The flow rate was 0.8 mL/min, with an injection volume of 20 µL. Caffeine and trigonelline were detected at wavelengths of 273 nm and 264 nm, respectively. The autosampler temperature was held at 4°C.

System Suitability Test

A system suitability test was performed to ensure the HPLC system and method could generate valid results. The parameters evaluated were repeatability, plate number, resolution, and symmetry.

Repeatability

Repeatability assesses the HPLC system's performance consistency when the same sample or standard is injected multiple times under the same conditions. It was assessed by determining the peak area and retention time consistency of 7 replicate injections of a mixed working standard solution containing 20 µg/mL each of caffeine and trigonelline. Percentage relative standard deviation (% RSD) values under 2% were considered acceptable, calculated using Equation (1).

$$\% \text{ RSD} = \frac{\text{Standard deviation of peak area}}{\text{Mean of peak area}} \text{ x } 100\% \qquad (1)$$

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Plate Number

Plate number, N, indicates the efficiency of the column. A higher plate number corresponds to improved separation efficiency. Injection of a mixed working standard solution containing 20 µg/mL each of caffeine and trigonelline was conducted. The plate number was calculated using Equation (2). Plate number values greater than 2000 were considered acceptable.

$$N = 5.54 \left(\frac{t_R}{w_{0.5}}\right)^2 \quad (2)$$

where t_R is the retention time of caffeine and trigonelline peaks and $w_{0.5}$ the width of their respective peak at half of the height.

Resolution

Resolution, Rs measures the degree of separation between the caffeine and trigonelline peaks. A resolution value of 1.5 or above indicates baseline resolution between the two peaks. Resolution was evaluated by injection of a mixed working standard solution containing 20 µg/mL each of caffeine and trigonelline. The resolution was calculated using Equation (3). Resolution values of 1.5 or above were considered acceptable.

$$Rs = 1.18 \text{ x } \frac{(t_2 - t_1)}{(w_{0.5}1 + w_{0.5}2)} \quad (3)$$

where t_2 and t_1 are the retention times for caffeine and trigonelline, respectively, while $w_{0.5}1$ and $w_{0.5}2$ are the peak widths measured at half the peak height for caffeine and trigonelline, respectively.

Symmetry Factor

The symmetry factor is a coefficient that indicates the degree of peak symmetry. It was determined with a mixed working standard solution containing 20 µg/mL each of caffeine and trigonelline. Symmetry values of no more than two were considered acceptable (Ravisankar et al., 2015).

HPLC Method Validation

The optimised HPLC method underwent validation according to the International Council for Harmonisation (ICH) guidelines, focusing on linearity and range, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ) (ICH, 2005).

Linearity and Range

The linearity curve for caffeine and trigonelline was assessed at five concentration levels ranging from 1 to 200 µg/mL. Each solution underwent triplicate injections into the HPLC system, and a calibration curve was constructed using the mean values. The acceptance criterion for the linearity test was a correlation coefficient (R^2) value equal to or greater than 0.9999.

Precision

Precision was determined as % RSD for intra-day precision (repeatability) and inter-day precision (intermediate precision). Intra-day precision was evaluated by performing seven determinations of mixed working standard solutions at 20 µg/mL on the same day. Inter-day precision was assessed by repeating the intra-day precision procedure over three days. The acceptance criterion for precision was % RSD of the peak area not exceeding 2%, calculated using Equation (1).

LOD and LOQ

The LOD, the minimum analyte concentration detectable by the analytical method, was established by identifying the lowest concentration at which the analytes could be detected. This involved measuring individual working standard solutions of the analytes until the concentration reached a level distinguishable from background noise. The LOQ, defined as the lowest analyte concentration reliably quantified by the analytical method, was determined by establishing the lowest analyte concentration at which the analytes can be consistently and reliably quantified with acceptable precision (% RSD of peak area < 20%) and accuracy (% Recovery ± 20%).

Accuracy

Accuracy was evaluated through nine determinations across three concentration levels, each including three replicates. Decaffeinated coffee samples were spiked with low (10 µg/mL), medium (100 µg/mL), and high (190 µg/mL) concentrations of the analytes. The % Recovery values were calculated by dividing the experimentally measured concentration of each analyte spiked into the blank by its theoretical amount (as per Equation (4)). Acceptable accuracy was defined as % Recovery falling within the 90% to 110% range.

$$\% \text{ Recovery} = \frac{\text{Experimental concentration of analyte}}{\text{Theoretical concentration of analyte}} \text{ x } 100\% \quad (4)$$

Robustness

The method's robustness was evaluated by calculating the % RSD of peak areas for caffeine and trigonelline in a 100 µg/mL mixed standard solution, following intentional minor variations in flow rate (± 0.1 mL/min) and detection wavelength (± 2 nm).

Stability

The stability of caffeine and trigonelline working standard solutions at 100 µg/mL was assessed at ambient temperature and 4 °C for 24, 48, and 72 hours to confirm stability for autosampler and storage conditions. Stability assessment involved comparing the concentration of each analyte in a freshly prepared solution ($Conc_{theoretical}$) with its concentration after specified time points ($Conc_{measured}$), as per Eq. (5).

$$\% \text{ Relative error (\% RE)} = \frac{Conc_{measured} - Conc_{theoretical}}{Conc_{theoretical}} \text{ x } 100\% \quad (5)$$

Caffeine and Trigonelline in Coffee Samples

A calibration curve (peak areas versus concentrations) was established for each analyte within 1–200 µg/mL. The regression equation derived from these calibration curves was used to

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calculate the caffeine and trigonelline amounts in the coffee samples based on their peak areas. Each coffee sample was analyzed in triplicate, and the mean concentration of each analyte was reported.

3. Results and Discussion

HPLC Method Development

An analytical technique for measuring caffeine and trigonelline simultaneously in instant coffee products was developed using HPLC-DAD. The DAD wavelength was set to the maximum absorbance peaks of trigonelline (264 nm) and caffeine (273 nm). The mobile phase consisted of methanol and water with HFBA as the ion pairing reagent. The optimized method involved gradient elution with a runtime of 10 minutes and a flow rate of 0.8 mL/min. Under these conditions, caffeine and trigonelline standards showed distinct retention times of 3.332 and 6.369 minutes, respectively (Figures 2(A) and 2(B)). Complete separation was confirmed by injecting a mixed standard solution of caffeine and trigonelline, resulting in baseline separation (Figure 2(C)).

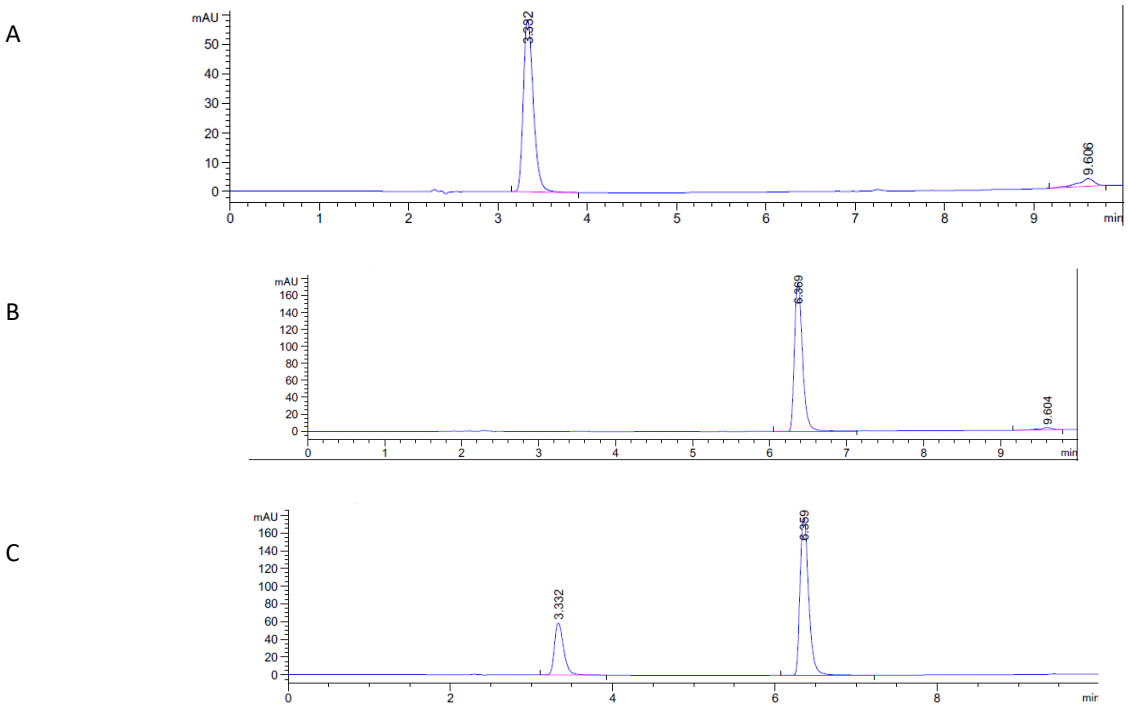

Figure 2. HPLC chromatograms illustrating (A) standard trigonelline at concentration 20 µg/mL, (B) standard caffeine at concentration 20 µg/mL, and (C) a mixed standard solution (20 µg/mL), displaying baseline-resolved peaks for both trigonelline and caffeine.

System Suitability Test

Before validating the method, a system suitability test was conducted to evaluate the HPLC system's performance for analyzing caffeine and trigonelline. A system suitability test is a series of checks performed to ensure that the HPLC system and method can produce accurate results. These tests are essential in confirming that the chromatographic system is functioning correctly before the analysis of samples begins. The main objective is to verify that the system performance satisfies the necessary criteria for the analysis to be dependable. Using a mixed working standard solution (20 µg/mL), the test assessed column efficiency, peak resolution, symmetry, and repeatability (Table 1). All measured parameters complied with the predefined acceptance criteria outlined in the methodology chapter (Section 2.7). This confirmed that the HPLC system is functioning correctly and is suitable for the intended analysis.

Table 1. Results of system suitability.

Parameter	Caffeine	Trigonelline	Acceptance limit
Repeatability of retention time (% RSD)[a]	0.05	0.23	< 2.0%
Repeatability of peak area (% RSD)[a]	0.04	0.06	< 2.0%
Plate number, N	18157	4466	> 2000
Resolution, Rs	15.53	15.53	≥ 1.5
Symmetry, S	0.74	0.68	< 2.0

[a]Repeatability was reported as the % RSD of seven injections.

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Method Validation

Linearity and Range

The linearity of the HPLC method was evaluated across a concentration range of 1-200 µg/mL for both caffeine and trigonelline. This parameter assesses the method's ability to generate responses directly proportional to the analyte concentration within this specified range. Figure 3 illustrates a strong linear relationship between peak area and concentration, with correlation coefficients exceeding 0.999. The regression equations were y = 61.023x + 37.257 for caffeine and y = 28.05x + 3.9441 for trigonelline. These results demonstrate the precision of the method in quantifying these compounds within the defined concentration range.

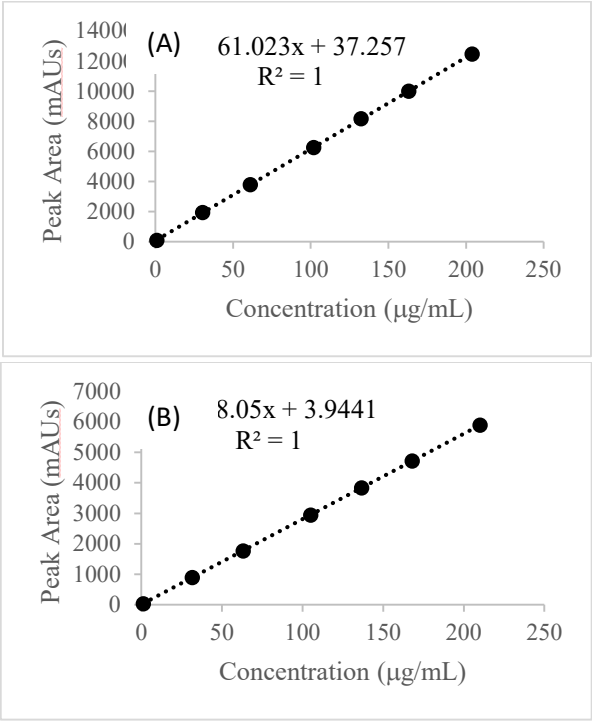

Figure 3. Calibration curves for (A) caffeine and (B) trigonelline. Each data point represents the mean ± standard deviation (SD) of three injections.

LOD and LOQ

LOD and LOQ were determined for both caffeine and trigonelline to establish method sensitivity. Individual standard solutions at estimated LOD levels indicated LOD values of 0.1 µg/mL for caffeine and 0.2 µg/mL for trigonelline (Figure 4). The LOQ values were defined as 0.2 µg/mL for caffeine and 0.3 µg/mL for trigonelline. To confirm the validity of the established LOQ values, accuracy and precision assessments were conducted at LOQ concentrations. At the LOQ, the % Recovery values for caffeine and trigonelline were 97.00% and 113.87%, respectively, while the % RSD peak area (n=3) for caffeine and trigonelline were 8.19% and 3.48%, respectively. Therefore, the accuracy and precision assessments at LOQ confirmed the method's validity, meeting the predefined acceptance criteria of % Recovery within ±20%, and % RSD below 20%.

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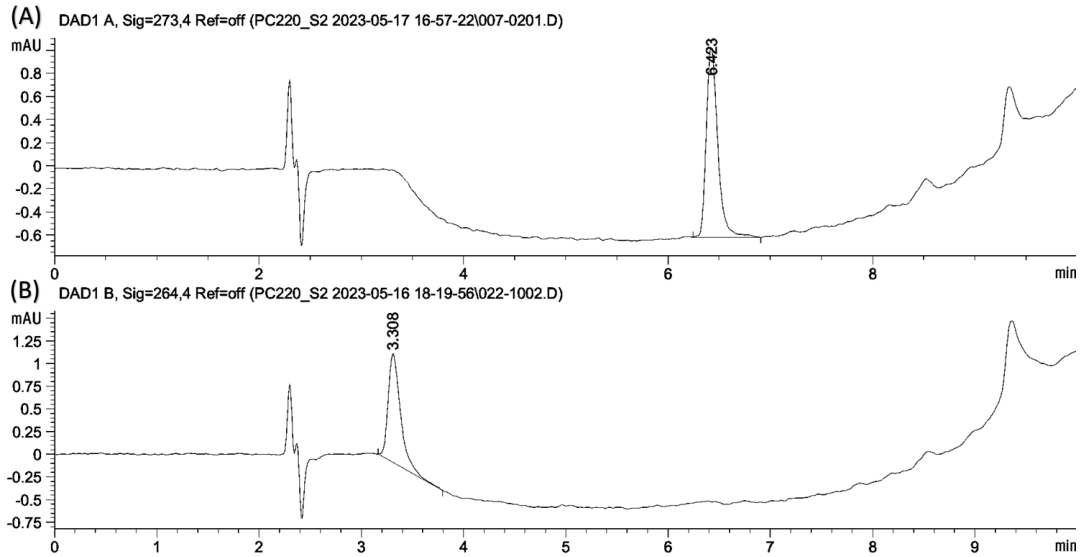

Figure 4. HPLC chromatograms of (A) standard caffeine (0.1 µg/mL) with elution at 6.423 mins and (B) standard trigonelline (0.2 µg/mL) with elution at 3.308 mins.

Precision

The precision of the HPLC method was assessed by evaluating repeatability (intra-day) and reproducibility (inter-day) of the peak area and retention time for both compounds. Seven replicate injections of a 20 µg/mL mixed working standard solution were analyzed on the same day and over three consecutive days. The calculated % RSD values for peak area and retention time remained consistently below 2.0% (Table 2). Therefore, the method is demonstrated to be repeatable and capable of producing consistent results within and across multiple days.

Accuracy

Accuracy measures how closely the measured concentration reflects the true value of the analyte in the sample. To evaluate accuracy, we determined the % Recovery at low quality control (LQC), mid quality control (MQC), and high quality control (HQC) concentration levels. The % Recovery values ranging from 93.92% to 109.09% were within the acceptable range (Table 2), confirming the method's reliable quantification of caffeine and trigonelline in instant coffee samples. However, the % Recovery values for caffeine were lower than those for trigonelline. This difference may be due to several factors related to the chemical properties of the compounds. The lower solubility and stability of caffeine in the extraction solvent compared with those of trigonelline may have influenced the efficiency of its extraction and subsequent quantification. Additionally, interactions between caffeine and the coffee matrix components might have caused variations in recovery rates. For instance, a study by other researchers using hot water extraction of instant coffee also reported lower % Recovery of caffeine compared to trigonelline when spiked at concentrations between 0.02-0.08 g/100g (Liu et al., 2012).

Table 2. Precision and accuracy assessment of the HPLC method.

Analyte	Accuracy		Intra-day precision		Inter-day precision	
	Spike level (µg/mL)	% Recovery[a]	% RSD of retention time (n=7)	% RSD of peak area (n = 7)	% RSD of retention time (n=7)	% RSD of peak area (n = 7)
Caffeine	10	93.92 ± 2.14	0.05	0.04	0.21	1.41
	100	96.09 ± 0.08				
	190	97.18 ± 0.14				
Trigonelline	10	101.20 ± 0.18	0.23	0.06	0.55	0.19
	100	108.37 ± 0.01				
	190	109.09 ± 0.12				

[a]Recovery values are expressed as the mean ± SD of triplicate injections.

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Robustness

In ICH Q2 (R1), "the robustness of an analytical procedure is a measure of its ability to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage." ICH Q2 (R1) also states that the robustness assessment "should demonstrate the reliability of an analysis with respect to deliberate variations in method parameters." If measurements are susceptible to variations in analytical conditions, the analytical conditions should be properly controlled, or a precautionary statement should be included in the procedure. We validated the robustness of our method by varying the mobile phase flow rate and detector wavelength. These parameters are frequently studied in robustness tests of HPLC methods (Epshtein et al., 2018). We varied the flow rate by ±0.1 mL/min and the wavelength by ±2 nm. These variations are common in the literature for HPLC robustness experiments (Epshtein et al., 2018).

A % RSD of less than 2% for peak area was established as the acceptable limit. This implies the analysis remains reliable if, after deliberate changes in the method parameters, the % RSD of the peak areas remains below 2%. As shown in Table 3, the % RSD for all parameters examined was found to be less than 2%. Other reported HPLC methods used to quantify caffeine and trigonelline in instant coffee products have not evaluated robustness with respect to changes in flow rate and wavelength (Arai et al., 2015; Gant et al., 2015; Syamimi et al., 2022).

Table 3. Results of the robustness study were determined using a 100 µg/mL mixed working standard solution.

Parameter	% RSD of peak area	
	Caffeine	Trigonelline
Flow rate (mL/min)		
0.70	0.02	0.04
0.80*	0.03	0.03
0.90	0.03	0.05
Wavelength (nm)		
271	0.04	-
273*	0.04	-
275	0.04	-
Wavelength (nm)		
262	-	0.05
264*	-	0.06
266	-	0.06

*normal chromatography condition

Stability of Caffeine and Trigonelline Solution

The stability of caffeine and trigonelline solutions was assessed over three days at room temperature and under refrigeration (4–8°C). These conditions were selected because the solutions were prepared at room temperature, while storage and autosampler temperatures were maintained at 4–8°C during the study. Evaluating stability under these conditions ensures the compounds remain stable throughout the experimental processes. The % RE was employed to monitor any concentration fluctuations. Trigonelline solutions exhibited excellent stability under both conditions, with % RE values between 0.00% and 0.11% (Table 4). In contrast, caffeine solutions showed higher % RE values, especially at room temperature (0.35%–4.19%) compared to refrigerated storage (0.02%–1.48%).

Caffeine, an alkaloid, is thermally stable during coffee bean roasting (Wei et al., 2012). In aqueous solution, caffeine is generally stable at moderate temperatures and pH. However, its degradation in the environment is affected by factors such as UV radiation (Edwards et al., 2015). In this study, caffeine solutions stored at room temperature were kept in clear vials, unprotected from light, potentially leading to faster degradation due to exposure to environmental factors such as light and oxygen. Conversely, caffeine solutions stored in the refrigerator were less exposed to light, as the refrigerator lacks built-in light. Thus, the stability of caffeine solutions was better maintained under refrigerated conditions. Nevertheless, the stability of the caffeine stock and working solutions in this study was preserved because these solutions were promptly stored at refrigerated temperatures shortly after their preparation at the bench. For future routine analysis, it is advisable to store caffeine solutions under refrigeration and protect them from light to maintain accurate quantification and reduce stability-related issues.

Table 4. Stability assessment of 100 µg/mL mixed working standard solution at room temperature and 4°C after 72 hours.

Analyte	Storage Conditions (°C)	% RE[a] Day 1	Day 2	Day 3
Caffeine	Room temperature	0.35 ± 0.05	4.43 ± 0.02	4.19 ± 0.02
	4–8	0.02 ± 0.50	1.31 ± 0.08	1.48 ± 0.04
Trigonelline	Room temperature	0.00 ± 0.04	0.08 ± 0.02	0.09 ± 0.03
	4–8	0.06 ± 0.09	0.11 ± 0.02	0.04 ± 0.03

[a] % RE values are expressed as the mean ± SD of triplicate injections.

Caffeine Content in Instant Coffee Products

Fifteen commercially available instant coffee products from various brands sold in Malaysia were analyzed for caffeine and trigonelline content. Using the validated HPLC-DAD method, simultaneous quantification of these compounds was achieved, and the results are presented in Table 5 with manufacturer-declared values. Chromatograms of BC, 2I1, and 3I1 products are shown in Figure 5.

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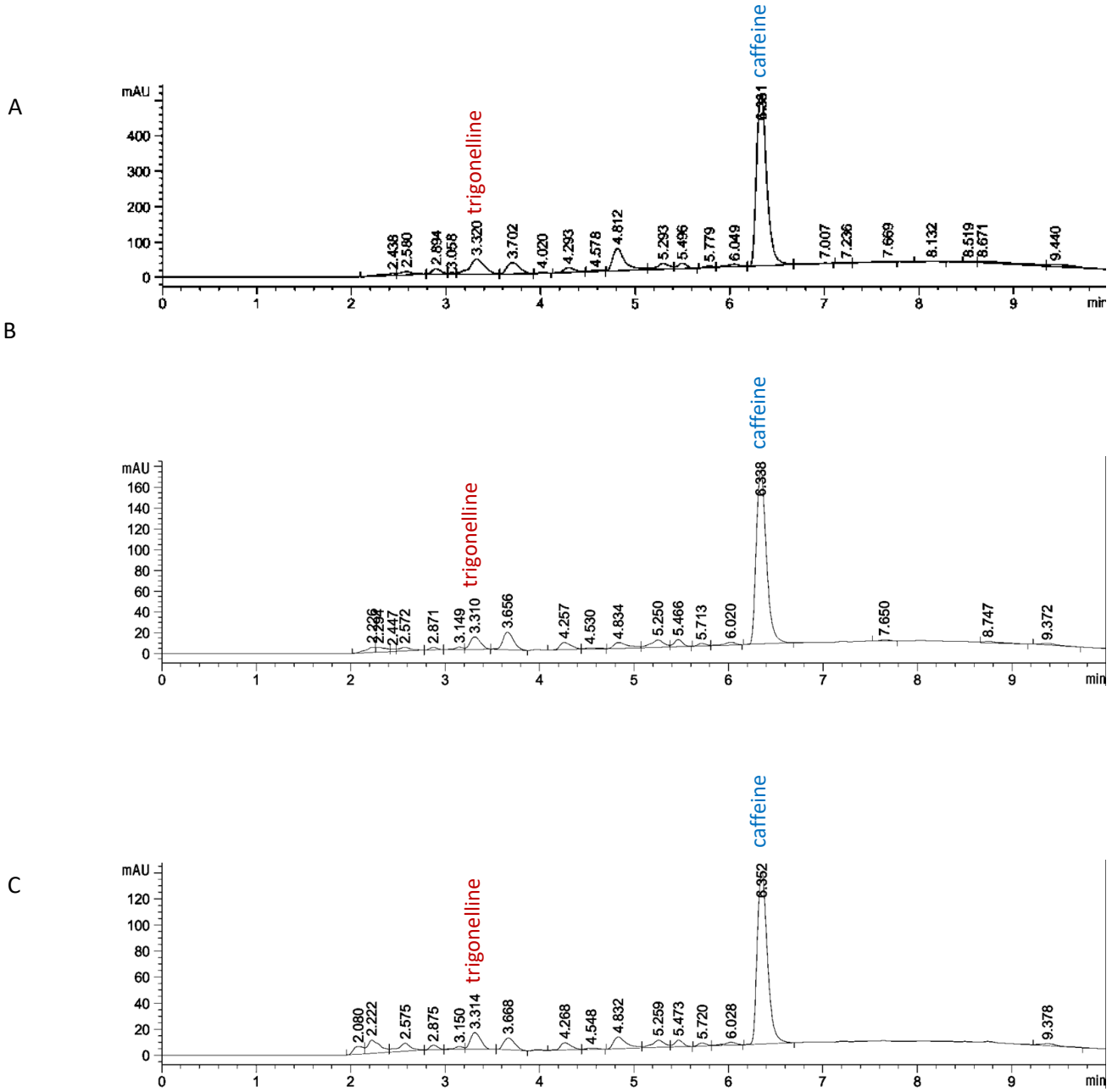

Figure 5. HPLC chromatograms of instant coffee products analyzed by the validated HPLC method showing caffeine and trigonelline peaks: (A) BC-3, (B) 2I1C-3, and (C) 3I1C-1.

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Table 5. Quantification of caffeine and trigonelline in instant coffee products using the validated HPLC-DAD method.

Instant coffee products[a]	Serving size (g)[b]	Amount detected (mg/g)[c]		Manufacturer declared amount (mg/g)		Calculated amount per serving (mg)[c]		Calculated amount in 2.38 servings (mg)[c]	
		Caffeine	Trigonelline	Caffeine	Trigonelline	Caffeine	Trigonelline	Caffeine	Trigonelline
BC-1 (Arabica and Robusta mixture)	02.0	37.76 ± 0.04	11.00 ± 0.04	25–45	Not declared (n.d)	75.52 ± 0.07	21.99 ± 0.07	179.73 ± 0.17	52.35 ± 0.17
BC-2 (Arabica)	02.0	31.78 ± 0.08	9.67 ± 0.00	n.d	n.d	63.56 ± 0.16	19.35 ± 0.01	151.28 ± 0.37	46.04 ± 0.02
BC-3 (Arabica)	01.8	24.50 ± 0.01	5.43 ± 0.02	n.d	n.d	44.11 ± 0.02	9.77 ± 0.03	104.97 ± 0.05	23.26 ± 0.07
BC-4 (Arabica and Robusta mixture)	02.0	47.54 ± 0.01	10.38 ± 0.00	n.d	n.d	95.09 ± 0.02	20.75 ± 0.01	226.31 ± 0.04	49.39 ± 0.02
BC-5 (Arabica)	01.6	26.48 ± 0.01	9.68 ± 0.04	n.d	n.d	42.37 ± 0.02	15.49 ± 0.06	100.83 ± 0.05	36.86 ± 0.15
2I1C-1	25.0	5.23 ± 0.00	1.05 ± 0.00	n.d	n.d	130.87 ± 0.05	26.16 ± 0.05	311.48 ± 0.12	62.27 ± 0.13
2I1C-2	30.0	6.11 ± 0.01	1.38 ± 0.00	n.d	n.d	183.33 ± 0.20	41.41 ± 0.03	436.31 ± 0.46	98.55 ± 0.07
2I1C-3	30.0	4.90 ± 0.00	0.62 ± 0.00	5	n.d	146.89 ± 0.10	18.63 ± 0.04	349.59 ± 0.23	44.34 ± 0.09
2I1C-4	16.0	3.85 ± 0.00	0.68 ± 0.00	n.d	n.d	61.63 ± 0.02	10.88 ± 0.03	146.68 ± 0.04	25.88 ± 0.07
2I1C-5	25.0	6.35 ± 0.00	0.77 ± 0.00	n.d	n.d	158.63 ± 0.08	19.30 ± 0.03	377.53 ± 0.18	45.93 ± 0.06
3I1C-1	40.0	3.22 ± 0.00	0.70 ± 0.00	n.d	n.d	128.68 ± 0.15	28.02 ± 0.03	306.27 ± 0.35	66.68 ± 0.07
3I1C-2	38.0	3.47 ± 0.00	0.69 ± 0.00	n.d	n.d	131.70 ± 0.03	26.25 ± 0.05	313.45 ± 0.06	62.49 ± 0.11
3I1C-3	18.0	2.67 ± 0.00	0.38 ± 0.00	n.d	n.d	47.99 ± 0.06	6.88 ± 0.01	114.21 ± 0.14	16.38 ± 0.03
3I1C-4	18.0	2.02 ± 0.00	0.37 ± 0.00	4	n.d	36.43 ± 0.04	6.70 ± 0.02	86.70 ± 0.09	15.95 ± 0.04
3I1C-5	38.0	4.17 ± 0.00	0.91 ± 0.00	n.d	n.d	158.65 ± 0.08	34.46 ± 0.04	377.58 ± 0.19	82.00 ± 0.09

[a]BC: black coffee; 2I1C: two-in-one coffee; 3I1C: three-in-one coffee.

[b]Values indicated on the product label.

[c]Values are expressed as the mean ± SD of triplicate injection

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Caffeine concentrations varied among different types of instant coffee products, with BC containing the highest amount per gram (average 33.61 mg/g), followed by 2I1C products (average 5.29 mg/g), and the lowest in 3I1C products (average 3.11 mg/g). Instant BC products typically consist solely of instant coffee powder and roasted ground coffee, which likely explains their greater caffeine content. In contrast, 2I1C and 3I1C products include additional components such as creamer, sugar, and additives, which reduce the concentration of caffeine.

Moreover, variability within similar product types was also observed. In BC products, caffeine content ranged from 24.50 to 47.54 mg/g, while for 2I1C and 3I1C products, it varied between 3.85 and 6.35 mg/g and 2.02 to 4.17 mg/g, respectively. These differences within product types can be attributed to factors such as coffee bean variety, origin, degree of roasting, and processing methods (Olechno et al., 2021). For example, in our study, BC samples containing a blend of Arabica and Robusta coffee (BC-4 and BC-1) exhibited higher caffeine concentrations than samples consisting solely of Arabica coffee. This finding corresponds with previous research indicating that Robusta coffee generally contains more caffeine than Arabica coffee (Nyoro et al., 2018).

Only three of the 15 coffee products tested (BC-1, 2I1C-3, and 3I1C-4) provided information on their caffeine content (Table 5). Product 2I1C-3 closely matched its declared caffeine content, meeting 98.0% of the specified amount on the label. Similarly, the caffeine content in product BC-1 (37.76 mg/g) was within the manufacturer's claim of 50–90 mg/2 g as published on its website. However, product 3I1C-4 met only 50.5% of its label claim, highlighting potential inconsistencies and misinformation in food labelling practices (da Costa et al., 2022; Duffy et al., 2021).

According to the Malaysian Standard MS1360 (1994), it is assumed that all coffee species contain an average of 0.9% (w/w) caffeine, equivalent to 9 mg/g (Standards & Industrial Research Institute of Malaysia, 1994). In our study, the measured caffeine levels in BC (24.50 to 47.54 mg/g) generally exceeded this standard. The measured caffeine content in our study is generally consistent with other findings in the literature (Table 6). The closest study to ours is by Nyoro et al., who quantified caffeine in seven locally available coffee products in Malaysia using chloroform extraction and ATR-FTIR analysis (Nyoro et al., 2018). The average caffeine content reported was 0.55% (5.5 mg/g), with Arabica coffee exhibiting lower caffeine levels compared to Robusta coffee. The lower caffeine content reported by Nyoro compared to our study could be ascribed to differences in solvent type, extraction method, and analytical techniques employed in their study.

Table 6. Comparison of caffeine content in instant coffee powder determined in this study and the past 10 years of literature.

Type of instant coffee powder	Caffeine content	Analytical method used	Reference (Past 10 years)
Black coffee (This study)	24.50 – 47.54 mg/g	HPLC	-
2I1 coffee (This study)	3.85 – 6.35 mg/g	HPLC	-
3I1 coffee (This study)	2.02 – 4.17 mg/g	HPLC	-
Commercial regular coffee	1.7 – 9.8 mg/g	FTIR	(Nyoro et al., 2018)
Commercial regular coffee	0.49 – 9.64 mg/g	HPLC	(Gonzales-Yépez et al., 2023)
Commercial regular coffee	28.8 – 35.0 mg/g	HPLC	(Arai et al., 2015)
Seven different types	65 – 1503 (µg, w/w%) in 2.5 mg/ml of coffee	HPLC	(Syamimi et al., 2022)

To assess consumer caffeine intake per serving, the caffeine content per serving was calculated based on the specified serving size on the product packaging (Table 5). Among different coffee types, 2I1C exhibited the highest caffeine content per serving (average 136.27 mg/serving), followed by 3I1C (average 100.69 mg/serving), with the lowest in BC (average 64.13 mg/serving). Within the 15 tested products, 2I1C-2 contained the greatest caffeine amount per serving (183.33 mg).

Considering the average daily coffee consumption of 2.38 cups among Malaysians (UKEssays, 2018), 2.38 servings of 2I1C-2 would exceed the FDA recommended daily allowance of 400 mg for adults. This scenario highlights the alarming ease with which recommended daily intake limits can be surpassed, particularly for frequent coffee consumers. Exceeding these limits has been associated with a twofold higher risk of anxiogenic effects (Jahrami et al., 2020). Adolescents and children, who are especially vulnerable to even moderate caffeine doses, should not exceed 100 mg/day and 2.5 mg/kg per day, respectively (Seifert et al., 2011). Pregnant women should also avoid consuming multiple servings of instant coffee daily to remain within the recommended limit of 200 mg/day (European Food Safety Authority (EFSA), 2015). According to our analysis, 8 out of 15 products contained more than 200 mg of caffeine in 2.38 servings (products BC-4, 2I1C-1, 2I1C-2, 2I1C-3, 2I1C-5, 3I1C-1, 3I1C-2, and 3I1C-5). This demonstrates the critical importance of monitoring caffeine intake, especially among specific demographic groups, to reduce potential health risks.

Trigonelline Content in Instant Coffee Products

The concentration of trigonelline in instant coffee products showed a strong correlation with caffeine concentrations. Similar to caffeine, BC exhibited the highest trigonelline content (average value of 9.23 mg/g), followed by 2I1C (average value of 0.9 mg/g) and 3I1BC (average value of 0.61 mg/g). These values are consistent with existing literature (Arai et al., 2015). In our study, the highest trigonelline content per gram of product was

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recorded in BC-1 (11.00 mg/g), while 2I1C-2 contained the highest trigonelline content per serving (41.41 mg/serving).

Despite its potential health benefits, toxicological data on trigonelline remain limited, and no recommended limits have been identified. However, an oral LD50 of 5000 mg/kg bw from rat studies provides a threshold for acute oral toxicity (Konstantinidis et al., 2023). Extrapolating these data to the Benchmark Dose Lower Confidence Limit (BMDL10) suggests a daily intake of 34.3 g for an individual weighing 70 kg. Considering Malaysia's average daily coffee consumption of 2.38 cups, reaching this amount would require consuming approximately 828 servings of product 2I1C-2 in a single day, exceeding any reasonable consumption pattern. Therefore, instant coffee beverages are a safe source of dietary trigonelline, and no toxic or adverse effects are expected upon acute oral exposure to trigonelline when consuming multiple servings of the instant coffee products tested in this study.

4. Conclusion

This study quantified the caffeine and trigonelline content in 15 instant coffee products sold in Malaysia using HPLC-DAD. The developed HPLC method was validated and exhibited precision, accuracy, reliability, and linearity within the specified concentration range. On average, the highest concentration (per gram) of caffeine was observed in BC (33.61 mg/g), followed by 2I1C (5.29 mg/g), and the lowest in 3I1BC (3.11 mg/g). However, when considering content per serving, the order changed to 2I1C (136.27 mg/serving), 3I1C (100.69 mg/serving), and BC (64.13 mg/serving). Consuming one serving per day of any tested product complies with the FDA's recommended daily caffeine limit of 400 mg for adults. However, consuming more than 2.38 cups per day (the average cups of coffee consumed by Malaysians) of product 2I1C-2 would surpass this limit, emphasizing the need for moderation. For pregnant women, 2.38 cups of products BC-4, 2I1C-1, 2I1C-2, 2I1C-3, 2I1C-5, 3I1C-1, 3I1C-2, and 3I1C-3 would exceed the recommended daily caffeine limit of 200 mg. Regarding label accuracy, one product failed to meet its declared caffeine content by 50.5%, highlighting discrepancies that could mislead consumers. Trigonelline content per gram and per serving varied similarly with caffeine, with the highest in BC (9.23 mg/g), followed by 2I1C (0.90 mg/g), and the lowest in 3I1BC (0.61 mg/g). Per serving, the amount was 23.28 mg/serving in 2I1C, 20.46 mg/serving in 3I1C, and 17.47 mg/serving in BC. Unlike caffeine, trigonelline poses no overdose risk even with multiple daily servings. In conclusion, the findings in this study emphasize the importance of accurate labelling and transparent information for consumers to make informed decisions about their coffee consumption. Adults are advised to limit instant coffee intake to no more than two servings daily, especially for 2I1C and 3I1C products, to avoid caffeine overdose. Adolescents should avoid consuming instant coffee, as one serving daily would exceed the recommended limit of 100 mg/day in seven out of the 15 products tested.

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