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COMPARATIVE ANALYSIS OF CAFFEINE CONTENT IN COLD AND HOT BREWED ROBUSTA COFFEE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

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ABSTRACT

Coffee is one of the most popular beverages globally, cherished for its unique taste, aroma, and the stimulating effects of its caffeine content. The proliferation of creative coffee shops has introduced various new methods for enjoying coffee, including cold and hot brew techniques. These processing techniques can significantly influence the physicochemical characteristics of coffee, particularly its caffeine content. This study compares the caffeine content in Robusta coffee using cold and hot brewing techniques. The hot brew coffee was prepared using water at approximately 96°C with a French press for six minutes. In contrast, the cold brew method involved brewing with water at room temperature (20-25°C) using a French press, followed by storage for 12 hours in a refrigerator (2-8°C). Qualitative analysis involved examining the color reaction, while quantitative analysis was conducted using High-Performance Liquid Chromatography (HPLC). HPLC is a highly accurate method that is extensively used in the food and pharmaceutical industries. The results indicated that the caffeine content in cold-brewed Robusta coffee was significantly higher, with a concentration of 44.63 µg/mL ± 0.199% and a Relative Standard Deviation (RSD) of 0.4459%. Conversely, hot-brewed coffee showed a caffeine concentration of 23.96 µg/mL ± 0.278%, with an RSD of 1.1602%. The parametric Analysis of Variance (ANOVA) revealed a significance value of 0.000 ($p < 0.05$), indicating a significant difference in caffeine levels between hot-brewed and cold-brewed coffee. These findings suggest that the choice of Robusta coffee processing technique can be crucial for individuals with specific health conditions seeking to manage their caffeine intake.

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INTRODUCTION

Coffee is one of the most widely consumed beverages worldwide, including in Indonesia. It represents one of the largest global industries, with international consumption for the 2020/2021 period reaching 166.3 million bags (60 kg each), marking a 1% increase from the previous year [1]. In Indonesia, coffee production totals 11.85 million bags (60 kg), with *Coffea canephora* var. *Robusta* exceeds *Coffea arabica* L. *Robusta* coffee production stands at 10.5 million bags, while *Arabica* production is at 1.3 million bags. This ranks Indonesia as the third-largest coffee-producing country in the world for the 2022/2023 period [2]. Given Indonesia's significant role as a leading producer, particularly of *Robusta* coffee, research within this domain is vital for understanding the global impact of coffee processing.

Coffee belongs to the Rubiaceae family and features two main commercial species: *Coffea arabica* and *Coffea canephora* (including the Conilon and *Robusta* varieties) [3], [4]. Numerous studies have highlighted the health benefits of coffee, which include mitigating conditions such as Alzheimer's disease, Parkinson's disease, depression, various nervous and digestive system disorders, obesity, type II diabetes, carcinogenesis, and cancers of the large intestine, breast, ovary, and endometrium [3]-[5]. Coffee beans contain nonvolatile components like water, carbohydrates, fiber, free amino acids, lipids, and minerals [8]. They also comprise complex chemical compounds and thousands of natural chemicals, including carbohydrates, lipids, nitrogen compounds, vitamins, minerals, alkaloids, and phenolic compounds, with chlorogenic acid and caffeine being the most abundant [9]. Given the rich chemical content of coffee, with caffeine as a primary component, there is a compelling need to explore the influence of caffeine on coffee quality and its health benefits.

Caffeine's impact varies depending on age, gender, source, and dosage. Low doses enhance cognitive performance, memory, and brain function. The beneficial effects of caffeine have been noted in various conditions, including Alzheimer's disease, Parkinson's disease, asthma, cirrhosis, fibrogenesis, and kidney stones [10]. However, at high doses, coffee is not advisable for individuals suffering from gastroesophageal reflux, peptic ulcer disease, or acute gastritis. Pregnant and breastfeeding women are also advised to limit their coffee intake [11]. Caffeine can induce nervousness and anxiety, negatively affecting individuals with hypertension, children, adolescents, and the elderly [10]. Therefore, examining the caffeine content in coffee presents a significant area of research aimed at developing coffee processing strategies suitable for consumers with specific health conditions. According to SNI 01-7152-2006, the maximum limit for caffeine in food and beverages is 150 mg/day and 50 mg/serving [12], whereas FDA regulations permit caffeine levels of less than 400 mg/day [13].

Coffee consumption in Indonesia has recently become a popular trend, reflecting changes in people's lifestyles and the rise in consumerism. The coffee industry is experiencing rapid growth, with numerous creative coffee shops introducing innovative brewing techniques and new ways to enjoy coffee [14], [15]. Traditionally, coffee was prepared using the hot brew method and served hot [4]. However, with the increasing demand for high-quality coffee, cold brew is gaining popularity [16], [17]. In the hot brew method, coffee is briefly brewed with hot water (approximately 92°C), typically no more than 5 minutes [11]. In contrast, the cold brew technique involves brewing at room temperature (20-25°C or colder) and steeping for 8 to 24 hours, significantly longer than the traditional hot brew method [17]. The processing techniques, such as grinding, extraction time, and temperature, influence coffee's physicochemical characteristics and flavor, including its caffeine content [18]. However, comprehensive research comparing caffeine content between hot and cold brew methods is limited.

One of the most effective and popular methods for determining caffeine content in

beverages is high-performance liquid chromatography (HPLC). This method is highly accurate and widely utilized in the food and pharmaceutical industries [19]. The HPLC method allows for directly separating and identifying caffeine from other substances. Another technique is UV-Vis spectroscopy, which measures light absorption by caffeine. This method is quick and straightforward but less precise than HPLC [20]. By employing HPLC to analyze caffeine in *Robusta* coffee, this research aims to enhance understanding of coffee processing techniques that align with public health interests.

This research aims to compare the caffeine content in *Robusta* coffee processed using both hot and cold brew techniques with the HPLC method. The findings could inform the selection of *Robusta* coffee processing techniques considering public health implications.

METHODS

1. Materials and Instrumentation

The materials utilized in this research included *Robusta* coffee from the Ciwidey area, Bandung, West Java, Indonesia; standard caffeine 99.0% (Sigma-Aldrich); methanol 99.0% (Merck); distilled water; calcium carbonate (Merck); Parry reagent (Nitra Kimia); chloroform (Merck); HPLC-grade methanol 99.0% (Merck); and aqueous ammonia 10% (Brataco). The instrumentation employed comprised an HPLC (Shimadzu-AT20), a VP-ODS column (250 mm x 4.6 mm; 4.6 µm) (Shim-pack), beaker glass (IWAKI), funnel, analytical balance (Mettler Toledo), French press (Fackelmann), test tube (IWAKI), 100 mL volumetric flask (IWAKI), and a 0.2 µm filter (Minisart).

2. Research Methods

This study will employ an experimental method to compare the caffeine levels in *Robusta* coffee processed using cold and hot brew techniques. The raw material, *Robusta* coffee, was sourced from the Ciwidey area, Bandung, West Java, Indonesia, renowned as one of the country's premier coffee-producing regions. The coffee was ground, packaged, and acquired from coffee shops in the Bandung City area. This approach aims to ensure the consistency and quality of the coffee used in the experimental procedures.

a. Sample Preparation

Hot brew coffee is prepared by adding 200 mL of water heated to approximately 96°C to 20 g of ground coffee in a French press. After steeping for 6 minutes, the coffee is separated by depressing the plunger. Cold brew coffee is prepared similarly by adding 20 g of ground coffee to 200 mL of room temperature water in a French press, which is left to steep for 12 hours. Following this period, the coffee solution is separated by pressing the plunger [17].

b. Caffeine Identification

Caffeine identification in the samples was performed using the Parry method. Both hot and cold brewed coffee samples were dissolved in 99% methanol, Parry's reagent, and 10% aqueous ammonia. A color change to dark blue or green indicates the presence of caffeine [21]. The Parry method was selected for its specific ability to identify caffeine.

c. Caffeine Extraction

Approximately 1.5 g of calcium carbonate ($CaCO_3$) was added to 150 mL of each coffee sample, hot and cold brew. The mixture was then placed into a separating funnel and extracted four times with 25 mL of chloroform each time. The chloroform and bottom layers were collected, and the solvent was evaporated using a rotary evaporator. The concentrated caffeine extract was then transferred to a 100 mL volumetric flask, diluted with distilled water to the mark, and thoroughly mixed [22]. The final solution was filtered into an HPLC vial through a 0.2 µm filter. The caffeine concentration was determined using the HPLC method at a wavelength of 272 nm [23].

d. Determination of Optimum Conditions for HPLC Instruments

The HPLC instrumentation was optimized at a wavelength of 272 nm, and a pump pressure of 150 kg/cm², and the column used was a VP-ODS with dimensions of 250 mm x 4.6 mm and 4.6 µm. To determine the optimal mobile phase composition, a standard caffeine solution was injected, amounting to 10 µL into the HPLC injector with methanol: water ratios varying from 100:0 to 50:50. Furthermore, the optimum flow rate was determined at various rates, including 0.5, 0.8, 1, and 1.2 mL/minute [24]. A calibration curve was then established by varying the concentration of the caffeine standard solution at 25, 50, 75, 100, 125, and 150 ppm. Following this, 10 µL of each concentration was injected into the HPLC column under optimal conditions. The chromatogram was recorded, and a calibration curve was created from the peak area, facilitating the calculation of the regression equation and correlation coefficient [25]. These optimal chromatography conditions also allowed for the confirmation of the retention time of caffeine by separately injecting the corresponding standard [26].

e. Caffeine Content Determination

An amount of 10 µL of the sample was injected into the column and replicated thrice under the selected conditions. The retention time (tR) data was obtained to calculate the selectivity (α) of the peaks resulting from the chromatogram profile. The area obtained was observed and then applied to the caffeine regression equation to determine the caffeine content in the sample [27]. According to the Indonesian Standard Agency (SNI 01-7152-2006), the maximum limit for caffeine in food and beverages is set at 150 mg/day and 50 mg/serving [28].

f. Statistical Data Analysis

The caffeine content data were statistically analyzed using the Shapiro-Wilk test to determine the normality of the data. Analysis of variance was conducted using one-way ANOVA. Results with $p<0.05$ were considered statistically significant. One-way ANOVA was utilized to identify statistical differences between the means of two or more groups [29].

RESULTS AND DISCUSSION

1. Identification of Caffeine

Caffeine identification in *Robusta* coffee samples using hot and cold brew

methods with a French press was conducted using the Parry test method, as shown in Table 1. These results indicate that all samples tested positive for caffeine.

Table 1. Identification of caffeine by the Parry method

Sample*	Parry Method		Result
	Before	After	
1	White precipitate	Green	+
2	Brown	Green	+
3	Brown	Green	+

* Sample 1 is a caffeine standard, Sample 2 is Hot Brew Coffee, and Sample 3 is Cold Brew Coffee

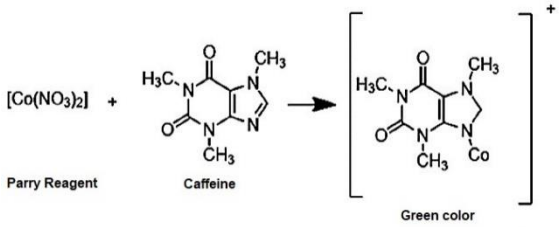

Figure 1. Caffeine reaction by Parry method.

The Parry method is known for its specificity in identifying caffeine. This technique revealed that the cold and hot brew coffee samples exhibited a green color change, consistent with the caffeine standard. In using the Parry method, a positive shift to green was observed, indicative of the formation of a cobalt-caffeine complex compound (Figure 1) [30][31]. The cobalt ion in the Parry reagent is positively charged, enabling it to bond with the nitrogen group found in the caffeine molecule [32]. The outcomes of this preliminary qualitative analysis served as a baseline for subsequent quantitative analyses using the High-Performance Liquid Chromatography (HPLC) method to determine the caffeine content in the samples.

2. Optimum Conditions of HPLC Instruments

The chromatographic conditions were meticulously varied to achieve optimal results in measuring caffeine content in *Arabica* coffee processed using hot and cold brew methods. Key variables adjusted included the wavelength, the mobile phase ratio of methanol to water, and the flow rate, which were determined in stages. Effective separation in HPLC is influenced by the composition of the mobile phase, which must allow for a retention time within the desired range, providing adequate resolution to separate compounds [33]. The optimization results, illustrated in Figure 2, show that methanol to water ratio 90:10 yields the highest peak area and good resolution without significant peak broadening, facilitating maximal caffeine detection.

The employed ODS (Octadecylsilane) column is nonpolar. Hence, a polar mobile phase transports the sample through the column without damaging the silica. The chosen mobile phase of methanol and water is crucial for this separation because caffeine dissolves completely in this mixture. The methanol in the mobile phase reduces polarity, enabling a quicker elution process and shorter retention time [24].

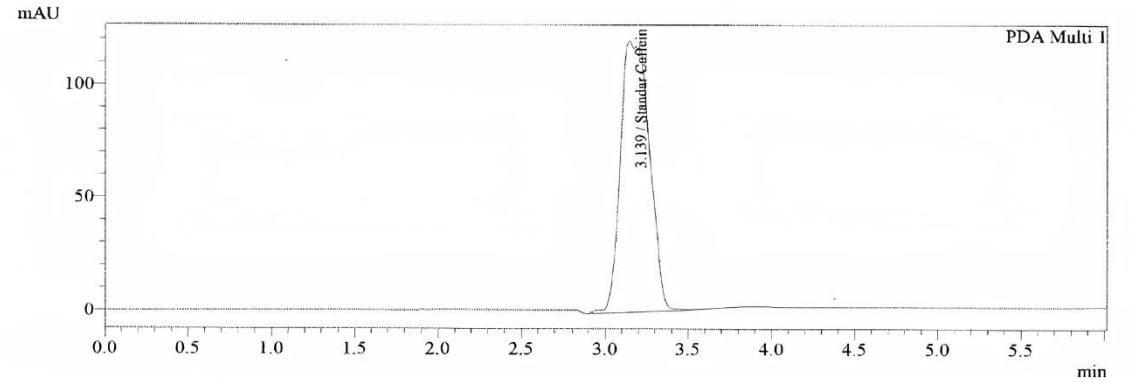

Figure 2. Mobile phase optimization results, mobile phase composition 90:10 (methanol: water).

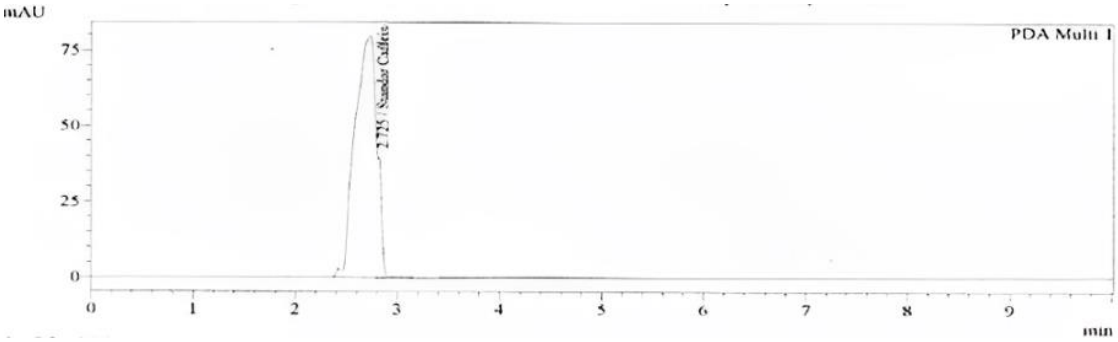

Figure 3. Results of flow rate optimization at 1.2 mL/min.

The flow rate of the mobile phase plays a pivotal role in the analytical performance. As shown in Figure 3, a flow rate of 1.2 mL/minute is optimal, producing the highest peak area and the fastest retention time of approximately 2.7 minutes, indicative of satisfactory column efficiency. The requirements for an adequate chromatogram include a symmetric peak shape, a retention time of less than 10 minutes, and a resolution of at least 1.5 [33]. Analysis of the chromatogram data from various flow rate variations reveals that higher flow rates decrease the peak area and reduce retention times. Consistent with previous research, slower flow rates prolong the compound's retention time [33].

The calibration curve in Figure 4 follows the linear regression equation y = 284547x + 3317.9, with an R^2 value of 1. This coefficient of determination signifies a perfect positive linear relationship between the concentration and response variables. Acceptance criteria for linearity dictate that the correlation coefficient (R^2) should not be less than 0.990 [26]. A higher degree of linearity in the standard curve suggests superior accuracy [33], confirming the method's robust linearity.

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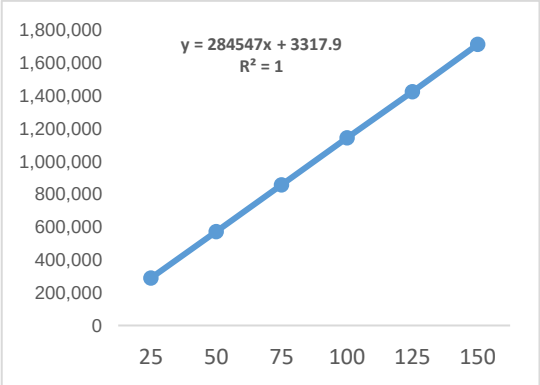

Figure 4. Calibration curve of caffeine.

3. Caffeine Content in Robusta Coffee Hot and Cold Brew Techniques

The analysis of caffeine levels, as presented in Table 2, indicates that the caffeine content in *Robusta* coffee prepared using the cold brew technique is higher than that prepared using the hot brew technique. The data show that the average caffeine content from three replications in *Robusta* coffee using the hot brewing technique was 23.96 µg/mL ± 0.278% with an RSD (Relative Standard Deviation) value of 1.1602%. In contrast, the caffeine content in *Robusta* coffee using the cold brewing technique was 44.63 µg/mL ± 0.199% with an RSD value of 0.4459%. The RSD values, less than 1.0%, demonstrate that this method is precise and reproducible [26].

The variation in caffeine content between hot and cold brew *Robusta* coffee can be attributed to the differences in brewing temperatures and times. Temperature significantly influences the solubility of caffeine, resulting in markedly different compositions in coffee when brewed hot or cold [34]. The higher caffeine content observed in cold brew coffee is due to the brewing process involving water at a temperature of approximately 20-25°C, which is then stored in a refrigerator at 2-8°C for 12 hours. Conversely, hot brew coffee, which involves brewing with water at approximately 96°C for 6 minutes, lowers caffeine content.

Table 2. Caffeine content in Robusta Coffee Hot and Cold Brew Technique

R*	Area (*UAC*)		Concentration (µg/mL)	
	Hot Brew	Cold Brew	Sample 1	Sample 2
1	6,910,994	12,675,512	24.28	44.53
2	6,785,163	12,663,027	23.83	44.50
3	6,768,362	12,767,744	23.77	44.86
Rata-rata			23.96 µg/mL	44.63 µg/mL
SD			0.278%	0.199%
RSD%			1.1602%	0.4459%

* R for replication of analysis

If the roasting temperature increases, the caffeine content in the coffee will decrease. This reduction occurs because, during the roasting process, a small portion of the caffeine evaporates, forming volatile components such as aldehydes, furfural, ketones, alcohol, esters, formic acid, and acetic acid. Consequently, the higher the roasting temperature, the more readily caffeine evaporates and decreases levels [35]. Caffeine, an alkaloid in the pseudoalkaloid group with four nitrogen atoms, exhibits chemical properties that make it susceptible to decomposition by heat and light in the presence of oxygen [36]. In the hot brew coffee technique, high water temperatures can increase intragranular diffusion and reduce the concentration of extractable compounds in coffee compared to the cold brew coffee technique [3]. Moreover, the extended brewing time in the cold brew method results in higher caffeine

content because compound diffusion through the grinding matrix can limit the kinetic extraction [17].

Statistically, the caffeine content of *Robusta* coffee prepared using hot and cold brew techniques shows significantly different levels ($p < 0.05$). According to SNI 01-7152-2006, the maximum limit for caffeine in food and beverages is 150 mg/day and 50 mg/serving [28], while FDA regulations allow caffeine levels less than 400 mg/day [13]. The caffeine content in both cold and hot brew *Robusta* coffee adheres to these maximum caffeine limit requirements. These findings have practical implications for consumers and producers in the creative coffee industry, potentially influencing recommendations for brewing techniques based on consumer preferences or health requirements. For individuals with caffeine intake restrictions, it is advisable to opt for a processing technique that yields lower caffeine content; in this case, the hot brewing technique emerges as the preferable option to the cold brewing technique. Overall, the outcomes of this study will have implications for the development of coffee processing techniques, aiming to maximize health benefits for the community.

CONCLUSION

This study conclusively demonstrates that *Robusta* coffee, when brewed using both hot and cold methods via a French press, contains detectable levels of caffeine. Importantly, the caffeine content in the cold brew coffee is significantly higher than in the hot brew counterpart. Statistical analyses confirm this variation, as evidenced by a p-value of less than 0.05, indicating a statistically significant difference in caffeine levels between the two brewing methods. These findings highlight the influence of brewing temperature and duration on the extraction of caffeine, suggesting that the brewing method can substantially affect the caffeine dosage received from coffee. This research provides valuable insights for consumers and professionals within the coffee industry regarding optimal brewing practices tailored to caffeine content preferences.

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