The Cambodia Journal of Basic and Applied Research 7(Spl) 2025

Print ISSN: 2790-3508 Online ISSN: 2790-3516

The Cambodia Journal of Basic and Applied Research

journal homepage: https://cjbar.rupp.edu.kh

Optimization and Validation of HPLC Method for Quantification of Caffeine Content in Energy Drinks Available in Cambodia

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ARTICLE INFO

Editorial responsibility: Prof. CHEY Chan Oeurn
Received: 10 December 2024
Revised: 27 February 2025
Accepted: 14 March 2025
Published online: 13 May 2025

Keywords:
HPLC, Optimization, Caffeine, Energy drink, Cambodia.

សង្ខិត្តន័យ

កាហ្វេអ៊ីនតែងតែត្រូវបានបញ្ចូលជាសារធាតុផ្សំចំបងនៅក្នុងភេសជ្ជៈប៉ូវកម្លាំង ដើម្បីជំរុញនិងបង្កើនថាមពលក្នុងរាងកាយបានយ៉ាងឆាប់រហ័ស ប៉ុន្តែវាក៏ផ្តល់នូវផលប៉ះពាល់អវិជ្ជមានដល់សុខភាពផងដែរ ជាពិសេស ចំពោះកុមារ ស្ត្រីមានផ្ទៃពោះ និងមនុស្សចាស់។ ភេសជ្ជៈប៉ូវកម្លាំងជាច្រើនមិនបានបង្ហាញពត៌មានឱ្យបានត្រឹមត្រូវអំពីបរិមាណកាហ្វេអ៊ីនឡើយ។ អវត្តមាននៃរបាយការណ៍ដែលគួរឱ្យជឿទុកចិត្តបានស្តីពីកម្រិតជាតិកាហ្វេអ៊ីននៅក្នុងភេសជ្ជៈប៉ូវកម្លាំងដែលកំពុងធ្វើចរាចរណ៍នៅក្នុងប្រទេសកម្ពុជា បង្ហាញពីតម្រូវការវិធីសាស្ត្រវិភាគដ៏ត្រឹមត្រូវសម្រាប់ផ្តល់ពត៌មានយល់ដឹងដល់អ្នកប្រើប្រាស់ និងការត្រួតពិនិត្យការប្រើប្រាស់ដោយផ្អែកលើបទប្បញ្ញត្តិ។ ដូច្នេះ ការប្រើប្រាស់ម៉ាស៊ីនវិភាគបែបទំនើប HPLC ក្នុងទំរង់ជាផាសចល័តដែលជាល្បាយនៃទឹកនិងអាសេតូនីទ្រីល ហើយដែលល្បាយនេះមានសមាមាត្រថេរ ត្រូវបានរៀបចំធ្វើយ៉ាងល្អបំផុត និងបានបង្ហាញពីភាពត្រឹមត្រូវក្នុងការកំណត់រកបរិមាណសារធាតុកាហ្វេអ៊ីនក្នុងភេសជ្ជៈប៉ូវកម្លាំងផ្សេងៗ។ វិធីសាស្ត្រនេះមានការចំណាយតិច មានភាពរហ័ស ហើយប្រើប្រាស់ជាតុំលាយសរីរាង្គតិចបំផុត ដែលមិនប៉ះពាល់ដល់បរិស្ថាន។ ការញែកសារធាតុកាហ្វេអ៊ីនចេញពីភាគសំណាកភេសជ្ជៈប៉ូវកម្លាំង ដែលទទួលបានលទ្ធផលល្អបំផុត គឺនៅពេលប្រើផាសចល័ត (ល្បាយនៃទឹកនិងអាសេតូនីទ្រីល) តាមសមាមាត្រ (87:13 v/v) ដោយប្រើកូឡោនញែក C18 (30) នៅល្បឿនលំហូរថេរ 1 mL/min និងជំហានរលក 273 nm ។ កម្រិតដែនកំណត់ទាបបំផុត (LOD) នៃការរកឃើញវត្តមានកាហ្វេអ៊ីនគឺ 14.34 g/L និងកម្រិតដែនកំណត់ទាបបំផុតនៃការកំណត់បរិមាណ (LOQ) កាហ្វេអ៊ីន គឺ 43.45 g/L។ កម្រិតលីនេអ៊ែរដែលទទួលបាននៅក្នុងវិធីសាស្ត្រនេះគឺ $R^2 \geq^3$ 0.9994 នៅពេលដែលប្រើ

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DOI: https://www.doi.org/10.61945/cjbar.2025.7.spl.03.

សូលុយស្យុងស្តង់ដាកាហ្វេអ៊ីន និងភាគសំណាកភេសជ្ជៈប៉ូវកម្លាំង។ ជាងនេះទៅទៀត ការធ្វើតេស្តសារជាថ្មីដើម្បីផ្ទៀងផ្ទាត់ គឺទទួលបានភាគរយគម្លាតស្តង់ដា (RSD) តូច 0.11% ដែលបង្ហាញពីភាពត្រឹមត្រូវកម្រិតខ្ពស់។ ការធ្វើតេស្តនេះ បានរកឃើញកំហាប់នៅក្នុងចន្លោះ 99.58-99.79%។ លទ្ធផលនេះបញ្ជាក់ថា វិធីសាស្ត្រនេះមានកម្រិតត្រឹមត្រូវខ្ពស់ក្នុងការរកឃើញកំហាប់ពិត។ ការធ្វើតេស្តដើម្បីវាយតំលៃវិធីសាស្ត្រ Robustness ទៅលើការប្រែប្រួលតូចតាចនៃសីតុណ្ហភាពនៅត្រង់តំបន់បំពង់ញែក ល្បឿនលំហូរដោសចល័ត (ល្បាយនៃទឹក និងអាសេតូនីទ្រីល) ឬផលធៀបនៃដោសចល័តនេះពុំបានបង្ហាញឥទ្ធិពលគួរឱ្យកត់សម្គាល់ទៅលើលទ្ធផលនេះទេ ដែលឆ្លុះបញ្ចាំងពីប្រសិទ្ធភាពនិងភាពត្រឹមត្រូវកម្រិតខ្ពស់នៃវិធីសាស្ត្រនេះក្នុងការរកកំហាប់ពិត ក្នុងចន្លោះ 97.54-97.74% និងភាពត្រឹមត្រូវក្នុងចន្លោះពី 0.07-0.45% ។ ទោះជាយ៉ាងណាក៏ដោយ ការផ្លាស់ប្តូរបន្តិចបន្តួចនៃលក្ខខណ្ឌទាំងនេះបានជះឥទ្ធិពលយ៉ាងខ្លាំងទៅលើរយៈពេលនៃការញែកកាហ្វេអ៊ីន ដោយក្នុងលក្ខខណ្ឌល្អបំផុតនៃវិធីសាស្ត្រនេះ បានរកឃើញនៅនាទីទី 7.02 ខណៈពេលដែលការផ្លាស់ប្តូរសីតុណ្ហភាពនៃកូឡោនញែក (25) អត្រាលំហូរ (0.8 mL/min) និងសមាមាត្រដោសចល័ត (85:15 v/v) ត្រូវបានសង្កេតឃើញនៅនាទីទី 7.36 8.86 និង 5.70 រៀងគ្នា។ វិធីសាស្ត្រក្នុងលក្ខខណ្ឌល្អបំផុតនេះ ត្រូវបានប្រើសម្រាប់កំណត់រកបរិមាណកាហ្វេអ៊ីនក្នុងភេសជ្ជៈប៉ូវកម្លាំងចំនួន ២៥ ម៉ាកយីហោផ្សេងគ្នាដែលប្រមូលបានពីទីផ្សារក្នុងប្រទេសកម្ពុជា។ បរិមាណកាហ្វេអ៊ីនដែលបានរកឃើញជាអប្បបរមាគឺ 82.4 mg/L និងអតិបរមាគឺ 424.4 mg/L ។ ភាគច្រើននៃភេសជ្ជៈប៉ូវកម្លាំង ដែលមានបិទស្លាកសញ្ញាបរិមាណកាហ្វេអ៊ីនត្រឹមត្រូវ បានបង្ហាញពីកម្រិតទទួលយកបាននៃការដាក់បរិមាណកាហ្វេអ៊ីនក្នុងចន្លោះ 90-110% (ឬ ±10%)។

Abstract

Caffeine is often included as a valuable ingredient in energy drinks to provide a quick boost of energy and increase mental alertness, but it led to increased adverse health effects for children, pregnant women, and old people. Many energy drinks lack proper labeling of their caffeine content. The absence of reliable reports on caffeine levels in energy drinks in Cambodia highlights the need for accurate analysis for consumer awareness and regulatory monitoring. Therefore, high-performance liquid chromatography (HPLC) in isocratic mode was optimized and validated for quantifying caffeine content in various energy drinks, which is inexpensive, fast, and utilizes the least quantity of organic solvent, making the method eco-friendly. The optimum caffeine separation from the matrix sample was obtained when the water/acetonitrile (87:13 v/v) mobile phase operated in the C18 column (30℃) at a fixed flow rate of 1-mL/min and 273 nm wavelength. The Limit of Detection (LOD) of 14.34 µg/L and the Limit of Quantitation (LOQ) of 43.45 µg/L were obtained. The linearity was achieved with $R^2 \geq 0.9994$ when caffeine standard solution and matrix energy drink were employed. Additionally, the repeatability test yielded a small RSD of 0.11%, indicating high precision. The recoveries were between 99.58 to 99.79%, reflecting an exceptionally high recovery rate. The robustness test on small variations of column temperature, flow rate, or mobile phase ratio exhibited insignificant influent on the results, reflecting the high recovery efficiency of 97.54 to 97.74% and precision of 0.07 to 0.45%. However, these slight changes led to a significant effect on caffeine retention to observe at 7.02 min for the optimum conditions, while the change of column temperature (25℃), flow rate (0.8 mL/min), and mobile phase ratio (85:15 v/v) were observed at 7.36, 8.86, and 5.70 min, respectively. This optimized method was employed for quantifying caffeine content in 25 different brand names of energy drinks collected from Cambodia's market. The minimum caffeine content of 82.4 mg/L and the maximum of 424.4 mg/L were observed. For those energy drinks that existed the proper company label of caffeine content mostly exhibited the specific tolerance range for caffeine content within 90 to 110% (or ±10%).

Research highlights

- Optimization and validation of HPLC method for quantification of caffeine contents.
- Applying the optimized method for analyzing 25 different brand names of energy drinks in Cambodia's market.

Introduction

Energy drinks have gained immense popularity worldwide, particularly among adolescents and young adults seeking to enhance their physical and mental performance (Abbood & Aldiab, 2017; Presson, 2022; Richards & Smith, 2016). These drinks containing stimulant compounds, usually caffeine, perform as stimulants of the central nervous system (Ahmad Sharoni, Surib, Shahrul, & Zawani, 2021; Alsunni, 2015; Nadeem et al., 2021). Caffeine is valuable due to its ability to momentarily prevent sleepiness and increase alertness, making it a key ingredient in many energy drinks (Mirza et al., 2021). However, excessive consumption of energy drinks can have serious health effects resulting from high caffeine, particularly in children, pregnant women, and old people (Nawrot et al., 2003). High levels of caffeine consumption can cause unpleasant and dangerous effects, i.e., anxiety, insomnia, digestive issues, muscle breakdown, addiction, high blood pressure, rapid heart rate, fatigue, and frequent urination and urgency (De Sanctis et al., 2017; Ehlers, Marakis, Lampen, & Hirsch-Ernst, 2019).

Energy drinks are widely used in Cambodia, which began the first start of flow to the markets in the year 1993(Fuel the Energy Levels with Cambodia's Refreshing

and Revitalizing Energy Drinks, 2024). Currently, the manufacturing of energy drinks has significantly risen in Cambodia due to several socio-economic factors that have shaped their popularity, such as the young generation, marketing strategy, economic growth, urbanization, and health trends (Fuel the Energy Levels with Cambodia's Refreshing and Revitalizing Energy Drinks, 2024). More than 55% of Cambodian consumers, according to a study, are now more inclined to buy energy drinks with extra vitamins and minerals for health benefits(Fuel the Energy Levels with Cambodia's Refreshing and Revitalizing Energy Drinks, 2024). However, some of these drinks lack adequate labeling regarding their caffeine content. Additionally, there is a lack of scientific studies and reports that provide reliable caffeine content in energy drinks in Cambodia, while the caffeine analysis is important for several reasons. First, it ensures that manufacturers comply with regulatory standards and accurately label the caffeine content, which is essential for consumer safety and informed choice (Attipoe, Leggit, & Deuster, 2016; Chen, Liu, Jaenicke, & Rabinowitz, 2019). Regulatory bodies, such as FDA and EFSA, have established guidelines for the maximum allowable caffeine content in energy drinks(EFSA Panel on Dietetic Products & Allergies, 2015; Rosenfeld, Mihalov, Carlson, & Mattia, 2014). Second, precise caffeine quantification helps in assessing the potential health risks associated with excessive caffeine consumption (Ehlers et al., 2019; Khouja et al., 2022; Sankararaman, Syed, Medici, & Sferra, 2018; van Dam, Hu, & Willett, 2020). According to FDA and EFSA guidelines, the caffeine intake recommended for an adult is 400 mg per day (Fajara, 2017; Reyes & Cornelis, 2018). The UK Food Standards Agency has recommended that pregnant women should limit their caffeine intake, out of prudence, to less than 200 mg per day (Ruiz & Scherr, 2019; Wierzejska, Jarosz, & Wojda, 2019).

Given the widespread use of energy drinks and the potential health risks associated with excessive caffeine consumption, it is crucial to develop accurate and reliable methods for analyzing caffeine (Pereira, Rodríguez-Cordero, López, Robles, & Aller, 2021). Several analytical methods have been reported for the determination of caffeine in different energy drinks including UV-visible spectrophotometry (Ahmad Bhawani, Fong, & Mohamad Ibrahim, 2015; Khalid et al., 2016), HPLC (Aşçı, Dinç Zor, & Aksu Dönmez, 2016; Mirza et al., 2021), Ultra-HPLC (UHPLC) (Aqel et al., 2019; Gaibor, Morales, & Carrillo Terán, 2020), Ultra-Performance Liquid Chromatography (UPLC) (Ahmad et al., 2022; Turak, Güzel, & Dinç, 2017), and Gas Chromatography-Mass Spectroscopy (GC-MS) (Al-Bratty et al., 2020; Amini & Hashemi, 2018). However, most of these methods involved complicated extraction procedures, while the usage of organic solvents either in liquid extraction or in the mobile phase is relatively large (Ali, 2022; Kakade & Kandekar, 2022). These techniques are most frequently considered high-cost and time-consuming methods due to sample preparation steps and instrumental analysis, including solvent consumption, column deterioration, and operational service (Al-Bratty et al., 2020; Amini & Hashemi, 2018). These disadvantages can be eliminated via method modification and optimization. For instance, HPLC is one of the most commonly used due to its high sensitivity, specificity, and ability to separate caffeine from other components in complex matrices with a better, cost-effective procedure (Ali, 2022; Aşçı et al., 2016; Kakade & Kandekar, 2022).

The objective of this study is to optimize and validate the HPLC procedure to provide a simple, quick, reliable, accurate, and cost-effective method with the least amount of solvent consumption for the determination of caffeine content in matrix samples. This enhanced procedure was employed to quantify 25 different brand names of energy drinks that are available in Cambodia without the need for further assisted solvent extraction.

Materials and Method

Materials

Deionized water (DI) for HPLC grade is produced by HHitech (18.2 MΩ cm) with a pore size of filter 0.22 µm. Acetonitrile (100%) was supplied by VWR International S.A.S/France. The caffeine reference standard (99.6%) was supplied from origin USA. Energy drinks (25 brand names) were purchased from local supermarkets, which were coded as Sample 01 to Sample 25. All the chemical was used without further treatment.

HPLC ArcTM Water (Waters Corporation, USA) with software Empower equipped with a PDA detector was utilized to optimize and validate the method for the quantitative detection of caffeine in energy drinks. HPLC Agilent 1100 (Agilent Technologies, USA) with ChemStation software adapted with a UV detector was utilized to assess the intermediate precision. A semi-microbalance Sartorius (Cubis MSA225S-100-DU) was employed to weigh samples of energy drinks as well as the reference standard of caffeine. Ultrasonic Cleaner (UCP-10) was used to homogenize and degas the reference standards of caffeine solution and energy drinks. Three HPLC columns were employed in this research: (1) Green Mall®GU-C18, 4.6 x 250 mm, 5µm was denoted as Green Mall®GU-C18, (2) ZORBAX Eclipse XDB-C18, 4.6 x 250 mm, 5µm was denoted as ZORBAX C18, (3) InertSustain C18 4.6 x 250 mm, 5µm was denoted as InertSustain C18.

Standard Curve Preparation

A caffeine stock solution of 1000 ppm was prepared by weighting 50 mg of caffeine RS and was transferred into a 50 mL volumetric flask. DI water (30 mL) was added and shaken (1 min) followed by sonicating (5 min), then

filled with DI water to reach a total volume of 50 mL. The concentrations of 0.5, 1.0, 5.0, 10.0, 15.0, 20.0, and 25.0 mg/L were prepared from the standard stock solution.

Sample Preparation

All samples of energy drinks were degassed for 15 minutes using an ultrasonic machine. The degassed sample (3 mL) was transferred into a 50 mL volumetric flask and then 30 mL of DI water, mechanically shaken (1 min), and sonicated (5 minutes). Next, DI water was filled to a total volume of 50 mL. Finally, each sample was filtered via a 0.45 µm diameter membrane into HPLC's vial and labeled with the sample code (Sample 01 to Sample 25).

Optimization of HPLC method

The mobile phase (water/acetonitrile) ratios were optimized to enhance caffeine separation from the matrix energy drinks by employing HPLC Arc™ Water equipped with a Green Mall®GU-C18 column. The HPLC's condition was fixed with mobile phase flow rate (1.0 mL/min) and column temperature (30°C) at 273 nm wavelength of caffeine. The diluted Sample 01, Sample 07, and standard caffeine solution were injected into HPLC at various mobile phase compositions in an isocratic mode as follows: Mobile Phase 1 (MP1) is Water/Acetonitrile (92:8, v/v), Mobile Phase 2 (MP2) is Water/Acetonitrile (90:10, v/v), Mobile Phase 3 (MP3) is Water/Acetonitrile (87:13, v/v), and Mobile Phase 4 (MP4) is Water/Acetonitrile (85:15, v/v).

Validation of HPLC Method

The optimized conditions of HPLC i.e., a mobile phase of water:acetonitrile (87:13 v/v), flow rate (1.0 ml/min), column temperature (30°C), and wavelength (273 nm) was used for validation of caffeine analysis following International Conference on Harmonization (ICH guidelines) (ICH, 1994). The validation was conducted by observing LOD, LOQ, precision (repeatability and intermediate precision), accuracy/recovery, specificity, linearity and range, and robustness (column temperature, flow rate, and ratio of mobile phase) (Borman & Elder, 2017; ICH, 1994, 2022). The HPLC Arc™ Water and Green Mall®GU-C18 column was used for LOD, LOQ, intra-day precision, accuracy, specificity, linearity, range, and robustness.

The LOD and LOQ were evaluated by measuring a series of standard caffeine solutions ranging from 10 to 1000 µg/L (n = 19). The following equations were employed for assessing LOD and LOQ, where SD is the standard deviation of the intercept and S is the slope of the calibration curve:

$$LOD = 3.3\ (SD/S) \quad (1)$$

$$LOQ = 10\ (SD/S) \quad (2)$$

Table 1: Parameters variation for robustness test

Robustness	*Initial*	*First*	*Second*	*Third*
Column Temperature (°C)	30	25	30	30
Flow rate (mL/min)	1.0	1.0	0.8	1.0
Mobile phase (v/v)	87:13	87:13	87:13	85:15

The specificity of the optimized method was observed to ensure that the method accurately measures the target analyte of interest in the presence of other interfering substances (Borman & Elder, 2017; ICH, 1994, 2022). In this study, specificity was conducted by employing HPLC Arc™ Water equipped with Green Mall®GU-C18 column at a fixed flow rate of 1.0 mL/min, column temperature (30°C), and mobile phase ratio (MP3). The matrix Sample 01 with a caffeine content of 15 mg/L were injected in the presence of other ingredients for observation of the specificity. The standard caffeine with concentrations ranging between 0.5 and 25 mg/L was also injected, then the retention time was compared with the matrix samples.

The precision was evaluated by %RSD in terms of intra-day (repeatability) and inter-day (intermediate) precisions by conducting six replicate measurements of an energy drink (Sample 01) with a known concentration of 15 mg/L. The inter-day precision was conducted by using two different HPLCs on two different days, where HPLC Arc™ Water was equipped with ZORBAX C18 and HPLC Agilent 1100 was equipped with InertSustain C18 column used. The equation (3) was applied to calculate %RSD, where SD is the standard deviation:

$$\%RSD = \frac{SD\ 100}{Mean\ Value} \quad (3)$$

The accuracy of the method was examined by the percentage (%) of recovery. The study was conducted by spiking the diluted Sample 01 (15 mg/L) with a standard caffeine stock solution (1000 mg/L). The final concentration of spike solution contained 0.15 mg/L of caffeine from Sample 01 and the spiked concentration of standard caffeine 8, 10, and 12 mg/L. Each spike concentration was prepared with three replicates. The following equations were applied:

$$\%Recovery = \frac{(Conc\ of\ spike\ sample - Conc\ of\ unspiked\ sample)\ 100}{Added\ conc\ of\ spike} \quad (4)$$

The linearity was studied using known concentrations of standard caffeine solutions and the Sample 01 with a vary concentration ranging from 8 to 12 mg/L (n=5). The peak areas were plotted against the known concentrations, where the value of R^2 was used to confirm the linearity with the acceptable range greater than 0.99.

Robustness tests were conducted on the parameters as shown in Table 1 to assess the satisfaction of the optimized method, in which the RSD (%) value was calculated and compared with the acceptance criteria RSD ≤ 2% (Chapter, 2020; Le, Phung, & Le, 2019; Sivagami et al., 2019). The first robustness test was conducted by changing the column temperature from optimal at 30℃ to 25℃, while the flow rate and water/acetonitrile mobile phase were maintained at 1.0 mL/min and 87:13 v/v, respectively. The second robustness test was observed by changing the flow rate from 1.0 to 0.8 mL/min, while the temperature and water/acetonitrile mobile phase were fixed at 30℃ and 87:13 v/v, respectively. The third robustness test was examined by changing the water/acetonitrile mobile phase from 87:13 to 85:15 v/v at constant temperature (30℃) and flow rate (1.0 mL/min).

Results and Discussion

Optimization of HPLC method

The mobile phase (Water/Acetonitrile) ratios were optimized for separating caffeine from the matrix energy drink samples. The different mobile phase ratios were observed for producing a sharp peak, a symmetry band, a good tailing factor (<1.4), and a suitable retention time. The result in Figure 1 indicated that the mobile phase MP1 and MP2 exhibited excellent caffeine extraction from the matrix solution, which was observed at retention times of 25.62 min and 16.29 min, respectively. However, the consumption of acetonitrile solvent is relatively large volume compared with mobile phase MP3 and MP4. The mobile phase MP4 exhibited the shortest retention time of caffeine at 7.59 min. However, this peak is fairly close to the interference bands produced by other ingredients in the matrix sample of energy drinks. In addition, the mobile phase MP3 was considered the optimal condition, where the band of caffeine at a retention time of 9.75 min was completely separated from the interference bands.

The estimation of the solvent consumption volume (mL) was tabulated in Table 2.

Validation of HPLC Method

LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) serve as indicators of sensitivity, with lower LOD and LOQ values indicating a higher sensitivity of the method. Various concentrations of caffeine standard solution ranging from 10 to 1000 µg/L (n = 19) were measured progressively from low to high concentration as shown in overlay chromatogram Figure 2 (right). The band of caffeine standard was interpreted and plotted the peak area against each concentration as shown in Figure 2 (left). Then, LOD and LOQ of caffeine were calculated according to equations (1) and (2). The result indicated that the LOD of 14.34 µg/L and LOQ of 43.45 µg/L were obtained, respectively.

Specificity

The specificity of this optimized method was observed by measuring various concentrations of Sample 01 and standard caffeine while the consistency of caffeine's retention time was compared. The overlay peaks from the chromatogram in Figure 3 indicated that the matrix samples

Table 2: Variation of mobile phase ratio and solvent consumption

Mobile phase	*Ratio*	*Retention time*	*Solvent consumption*	
	Water/acetonitrile, v/v	*min*	*Water (mL)*	*ACN (mL)*
MP1	92:8	25.62	23.57	2.05
MP2	90:10	16.29	14.66	1.63
MP3	87:13	9.75	8.47	1.27
MP4	85:15	7.59	6.45	1.14

*Note: Estimated solvent consumption (mL) = Flow rate (mL/min) × retention time (min) × Solvent (%v/v)

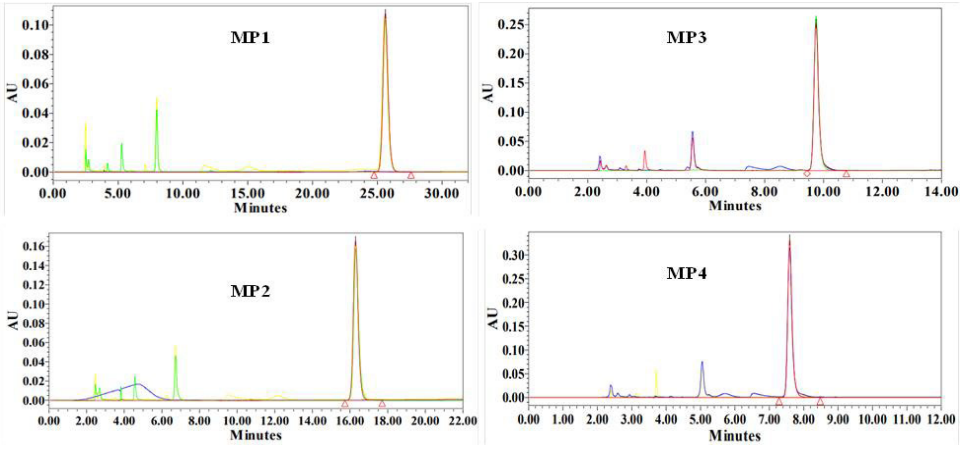

Figure 1: Overlay chromatogram of blank, standard caffeine, Sample 01, and Sample 07 respected to mobile phase composition

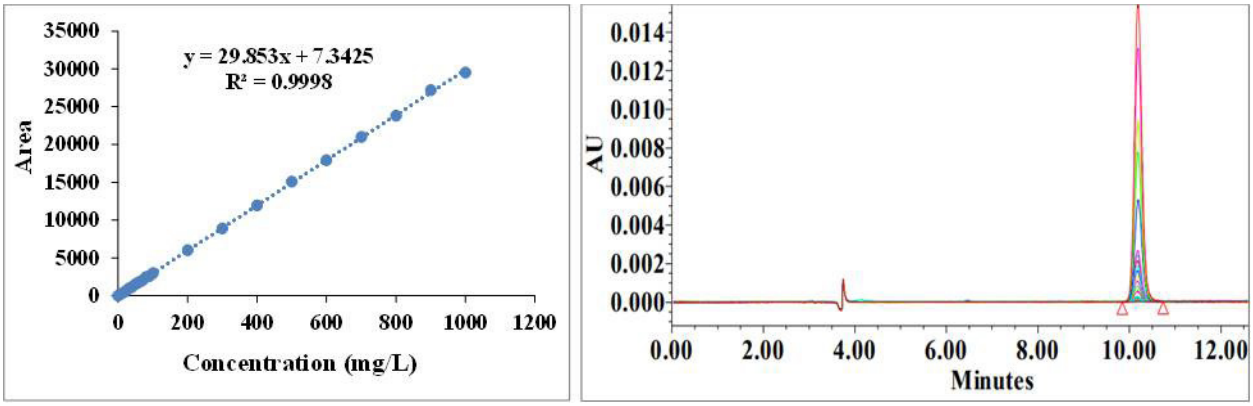

Figure 2: Calibration curve of standard caffeine at concentrations between 10 µg/L to 1000 µg/L (n = 19) and their overlay chromatogram

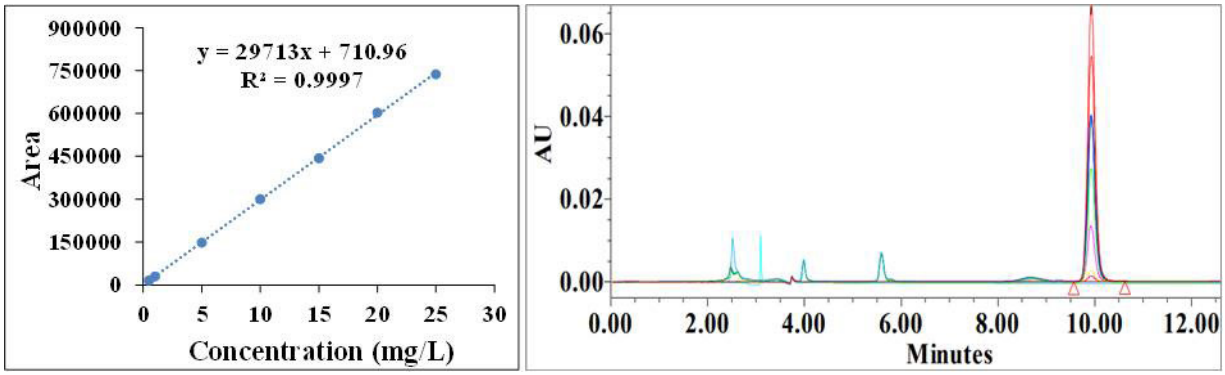

Figure 3: Calibration curve of standard caffeine at concentrations between 0.5 to 25.0 mg/L (n = 7) and overlay chromatogram with Sample 01

exhibited the band of caffeine appearing at the same retention time as standard caffeine, reflecting sensitive method separation. The other bands corresponding to various ingredients presented in the matrix samples completely separated from the caffeine peak. This result indicated a good specificity of the optimized method, which was essentially used for qualitative and quantitative analysis of caffeine in various matrix samples.

Precision

The lower RSD values indicate higher precision, where the RSD ≤ 2% was considered an accepted criterion (ICH, 1994). The intra-day precision (repeatability) was assessed by conducting 6 replicate measurements of caffeine at a known concentration of 15 mg/L in an energy drink (Sample 01). The standard linear curve in Figure 3 was used for the calculation of caffeine concentration in energy drinks. The result in Table 3 showed an RSD of 0.11% was obtained within the acceptable range, indicating high precision.

The inter-day (intermediate) precision was determined by measuring six replicates of known concentration (15 mg/L) caffeine content in Sample 01 using two different HPLCs equipped with two varieties of columns. The chromatograph in Figure 4a and 4b showed that the retention time of standard caffeine obtained from ZORBAX C18 and InertSustain C18 columns was observed at 6.996 min and 9.813 min, respectively. The caffeine concentration from Sample 01 was calculated from the calibration curve, as shown in Figure 4c for the ZORBAX C18 column and Figure 4d for the InertSustain C18 column. The result, as shown in Table 4 indicated that the use of

Table 3: Summary of intra-day precision using a known concentration of Sample 01

Replication	*Injected concentration**	*Found Concentration*	*Percentage*
	mg/L	*mg/L*	*%*
1	15.013	14.727	98.09
2	15.013	14.684	97.81
3	15.009	14.691	97.88
4	15.039	14.739	98.01
5	15.039	14.738	98.00
6	15.011	14.686	97.83
Average repeatability			97.94
RSD			0.11

*Note: The injected concentration was calculated by weighting and diluting the known caffeine concentration of Sample 01.

Table 4: Summary of inter-day precision using a known concentration of Sample 01. Flow rate 1.0 mL/min, 30℃, mobile phase MP3

Replication	*HPLC Arc™ Water, ZORBAX C18 column*			*HPLC Agilent 1100, InertSustain C18 column*		
	*Injected Conc.**	*Found Conc.*	*Percentage*	*Injected Conc.**	*Found Conc.*	*Percentage*
	mg/L	***mg/L***	***%***	***mg/L***	***mg/L***	***%***
1	15.047	14.523	96.52	15.000	14.285	95.23
2	15.034	14.791	98.38	15.002	14.328	95.51
3	15.037	14.741	98.03	15.000	14.263	95.09
4	15.038	14.773	98.24	15.001	14.334	95.55
5	15.045	14.813	98.46	15.007	14.354	95.65
6	15.013	14.706	97.96	15.024	14.750	98.18
Repeatability			97.93			95.87
RSD			0.73			1.20
Average Repeatability			96.92%			
RSD			1.44%			

*Note: The injected concentration was calculated by weighting and diluting the known caffeine concentration of Sample 01.

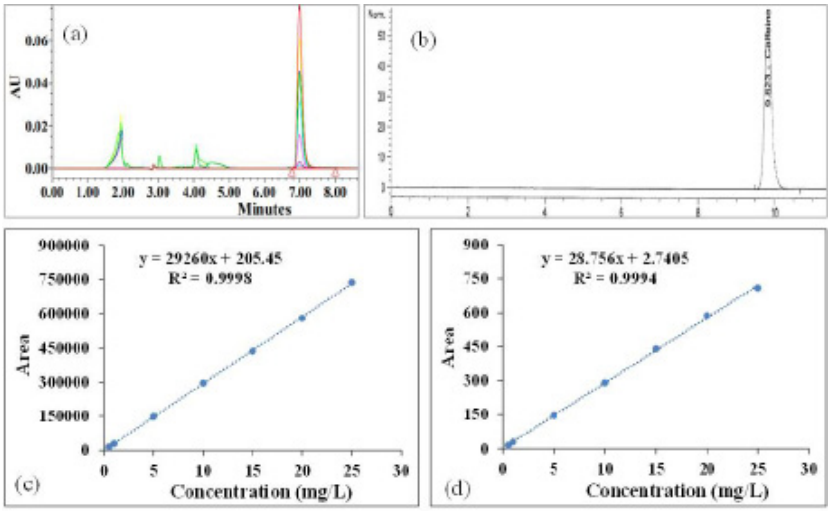

Figure 4: Chromatogram and a calibration curve of caffeine observed by (a)&(c) HPLC Arc™ Water equipped with ZORBAX C18 column and (b)&(d) HPLC Agilent 1100 equipped with InertSustain C18 column

the ZORBAX C18 column provided repeatability as high as 97.93% with a high precision (RSD) of 0.73%. A similar result was observed for the InertSustain C18 column, in which the repeatability of 95.87% and precision of 1.20% were obtained. Overall, the average repeatability of both HPCLs was obtained as high as 96.92%, in which the precision (RSD) of 1.44% was achieved in the acceptable range.

Accuracy

The recovery was conducted to evaluate the accuracy of this optimized method by measuring the percentage recovery of added analyte (spike) into the known concentration of the sample matrix. The acceptant criteria for recovery are within 98.0 to 102.0% (Le et al., 2019). First, Sample 01 was prepared with three replicates separately, where a caffeine concentration of 14.65 ± 0.02 mg/L was calculated by using the calibration curve in Figure 3. Then, these known concentration samples were spiked with standard caffeine stock solution to achieve the target concentrations. The final concentrations of standard caffeine in spike samples were varied (8, 10, and 12 mg/L), while the caffeine in Sample 01 was 0.1465 ± 0.0002 mg/L calculated from the calibration curve in Figure 5. Each spike sample was prepared with three replicates. The result in Table 5 indicated that the high recovery between 99.58 to 99.79% was achieved with the precision (RSD) of 0.08 to 0.89%. The recovery stayed in the acceptable range, indicating the high accuracy of the method.

Linearity

The linearity is used to evaluate the relationship between analyte concentration and analytical response over a certain range. Linearity can be assessed by constructing calibration curves and determining the coefficient of

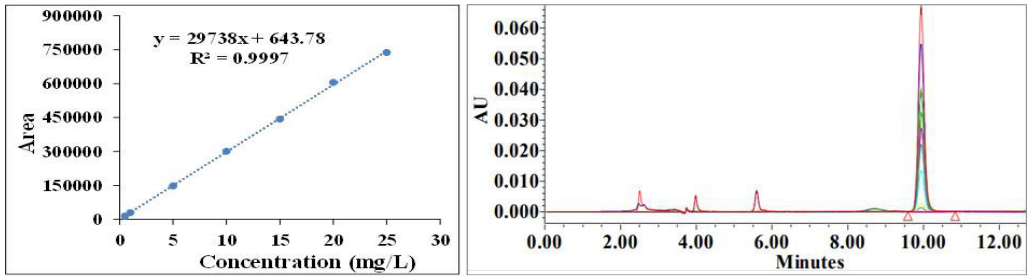

Figure 5: Calibration curve of standard caffeine at concentrations between 0.5 to 25.0 mg/L (n = 7) and overlay chromatogram with Sample 01

Table 5: Caffeine in un-spike and spiked

Analyte	*Added concentration (mg/L)*	*Found concentration (mg/L)*	*Recovery (%)*	*%RSD*
Caffeine	0	0.1465±0.0002	-	-
	8	8.1149±0.0709	99.61	0.89
	10	10.1040±0.0691	99.58	0.69
	12	12.1215±0.0691	99.79	0.08

determination (R^2). According to the United Nations Office on Drugs and Crime (UNODC, 2009), the value of correlation coefficient $R^2 \geq 0.990$ is considered an acceptant criterion for the regression line of the correlation (Naveen, Lingaraju, Deepak, Medhini, & Prasad, 2018; Saputri & Muchtaridi, 2018). In this study, HPLC Arc™ Water equipped with ZORBAX C18 column was employed, in which the separation condition was fixed at a flow rate (1.0 mL/min), column temperature (30°C), and mobile phase ratio (MP3). The standard caffeine and Sample 01 were prepared by varying the concentration from 8 to 12 mg/L, which were used to construct the linear regression line of peak area and known concentration. The result in Figure 6 indicated that both standard caffeine and Sample 01 have good linearity, with the evidence of $R^2 \geq 0.9994$.

Robustness

Sample 01 was prepared and injected into HPLC with a slight change in column temperature, mobile phase flow rate, and mobile phase composition from the optimal condition to observe the robustness of the method. The injected concentration (15 mg/L) of Sample 01 was prepared with three replicates, in which the actual concentration was calculated from a calibration curve of standard caffeine in Figure 8, regarding slight changes in parameters. The result, as shown in Figure 7, exhibited the overlay band of standard caffeine and Sample 01 after separation with a minor change of separation conditions. In all cases, caffeine peaks were symmetric shapes (tailing factor < 0.4). In Table 6, the minor change in column temperature from normal 30 to 25°C insignificantly changed the detection ability, in which the recovery was as high as 97.63% and good precision (RSD) of 0.41%. Similar good results were achieved when

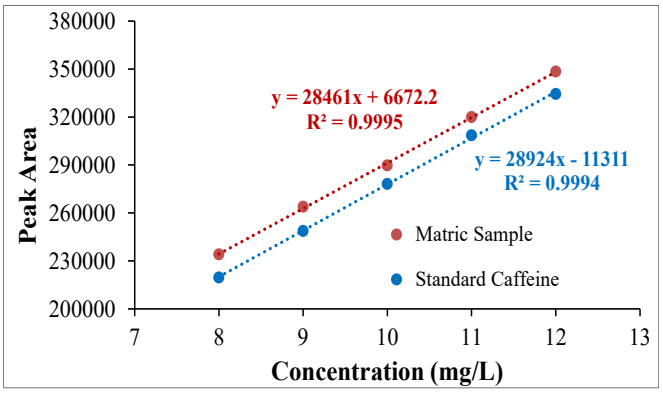

Figure 6: Linear regression lines prepared from standard caffeine and Sample 01

the minor change in mobile phase ratio (switch from MP3-MP4) and flow rate (switch from 1-0.8 mL/L). This evidence eased the conclusion that no significant changes were detected upon applying small variations to the chromatographic conditions, reflecting the method is robust to small, deliberate changes.

Overall, this optimized method is comparable to the previous findings but uses different amounts of solvents. This research study significantly minimized the amount of solvent to be used to separate caffeine from the matrix energy drink samples. As tabulated in Table 7, the method validation exhibited better precision, accuracy, sensitivity (LOD and LOQ), recovery, and robustness regarding ICH guidelines. This result reflects that the current study is fully parameterized, which is considered a simple, quick, reliable, accurate, and cost-effective method for separating and quantifying caffeine from the matrix samples.

Quantitative Analysis Caffeine in Energy Drinks

The optimized method was employed for quantifying caffeine contents in various energy drinks as listed in Table 8 by using HPLC Arc™ Water equipped with ZORBAX C18 column at conditions flow rate of 1.0 mL/min, column temperature (30°C), and mobile phase ratio (MP3). Energy drinks (code sample 01-11) existed the caffeine label issued by the manufacturer, indicating the caffeine content per net bottle. Whereas, the energy drinks (code sample 12 to sample 25) were not mentioned

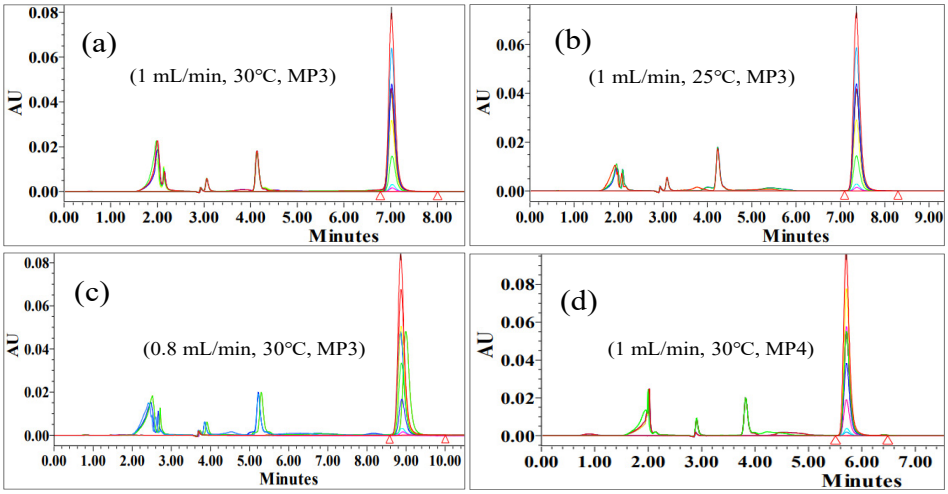

Figure 7: Overlay chromatogram of standard caffeine and Sample 01 after minor change on HPLC Arc™ Water parameters: (a) initial, (b) change column temperature, (c) change flow rate, and (d) change mobile phase.

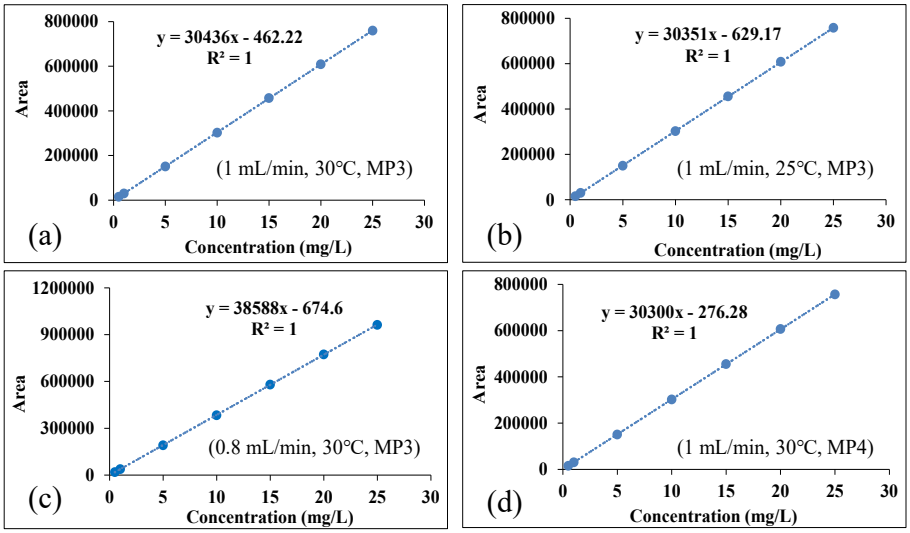

Figure 8: Calibration curve of standard caffeine observed by HPLC Arc™ Water parameters: (a) initial, (b) change column temperature, (c) change flow rate, and (d) change mobile phase.

Table 6: Summary of robustness observation by using Sample 01 and HPLC Arc™ Water

Robustness	*Injected Conc.**	*Found Conc.*	*Percentage*	*RSD*
	mg/L	*mg/L*	*%*	*%*
Initial	15.0184±0.0032	14.6483±0.0352	97.54	0.26
Changed column temperature	15.0184±0.0032	14.6618±0.0577	97.63	0.41
Changed flow rate	15.0184±0.0032	14.6587±0.0684	97.60	0.45
Changed mobile phase	15.0184±0.0032	14.6789±0.0074	97.74	0.07

*Note: The injected concentration was calculated by weighting and diluting the known caffeine concentration of Sample 01.

the specific concentration of caffeine. The caffeine standard and energy drinks spectra were illustrated as overlay chromatograms, as shown in Figure 9 (right). The calibration curve of standard caffeine at a linear concentration range of 0.5 to 25.0 mg/L was constructed versus the peak areas plotted in Figure 9 (left). The regression line with correlation coefficient R^2 = 1 was obtained, indicating a good linear relationship within the caffeine concentration range.

The result of caffeine content from various energy drinks was tabulated in Table 8. According to Industry standards, many companies adhere to the specific tolerance range

Table 7: Comparison between this research study and previous findings

Mobile phase	*Methanol:Water (80:20)*	*Acetonitrile:Water (80:20)*	*Sodium acetate:Acetic acid:Acetonitrile 80:20 (pH = 4.0)*	*95% acetate buffer, pH = 6.0*	*Water:Acetonitrile (87:13)*
Diluent	Mobile phase	Methanol	Acetonitrile	Water	Water
Column	C18, 4.6 x 250mm, 5µm	C18, 4.6 x 150mm, 5µm	C18, 4.6 x 250mm, 5µm	C18, 4.6 x 250mm, 5µm	C18, 4.6 x 250mm, 5µm
Column temperature (°C)	35	14 ± 2	37	N/A	30
Flow rate (mL/min)	1	1	1	1	1
Retention time (minutes)	7.77	10	4.64	N/A	9.75
LOD & LOQ	0.5 & 1.5 µg/mL	0.02 & 2 µg/mL	47.1 & 15 µg/mL	0.10-0.19 µg/mL & 0.33-0.63 µg/mL	0.014 & 0.43 µg/mL
Intra-day & inter-day precision	1.9 and 1.93%	< 4%	N/A	≤ 1.923 & ≤ 1.950%	0.11 &1.44%
Accuracy & recovery	> 90%	99.9-120%	95.41-97.08%	≥ 95.75%	99.58-99.79%
Specificity	N/A	N/A	N/A	N/A	Yes
Linearity	0.9998	> 0.999	0.9998	> 0.9962	≥ 0.9994
Robustness	No	≤ 5%	No	No	≤ 0.45%
Guideline	ICH	ICH	ICH	ICH	ICH
Reference	(Pandey, Yadav, Dutta, & Tyagi, 2022)	(Moura-Silva et al., 2023)	(Mirza et al., 2021)	(Aşçı et al., 2016)	Our research

Table 8: Summary of caffeine content in various energy drinks per net bottle. HPLC Arc™ Water and ZORBAX C18 column (flow rate 1.0 mL/min, 30°C, mobile phase MP3)

Sample code	*Appearance*	*Net bottle*	*Found Concentration*		*Matching with label*
		mg/net volume	*mg/net volume*	*mg/L*	*%*
Sample 01	Dark blue can and yellow liquid	80 mg/250 mL	79.6 mg/250 mL	318.4	99.5
Sample 02	Light blue can and yellow liquid	80 mg/250 mL	78.9 mg/250 mL	315.6	98.6
Sample 03	Red/white can and yellow liquid	80 mg/250 mL	77.3 mg/250 mL	309.2	96.6
Sample 04	Light blue/white can and light blue liquid	100 mg/250 mL	33.2 mg/250 mL	132.8	33.2
Sample 05	Yellow can and brown liquid	80 mg/250 mL	80.4 mg/250 mL	321.6	100.5
Sample 06	Yellow can and dark yellow liquid	80 mg/250 mL	106.1 mg/250 mL	424.4	132.6
Sample 07	Yellow/white can and dark yellow liquid	80 mg/250 mL	83.4 mg/250 mL	333.6	104.3
Sample 08	Dark blue/white can and yellow liquid	80 mg/250 mL	75.4 mg/250 mL	301.6	94.2
Sample 09	Gold can and yellow liquid	80 mg/250 mL	79.5 mg/250 mL	318.0	99.4
Sample 10	Green can and green liquid	150 mg/500mL	137.1 mg/500 mL	274.2	91.4
Sample 11	Pink can and yellow liquid	160 mg/500mL	160.1 mg/500 mL	320.2	100.3
Sample 12	Green can and dark yellow liquid	Caffeine/250 mL	80.0 mg/250 mL	320.0	
Sample 13	Green bottle and dark yellow liquid	Caffeine/150 mL	49.4 mg/150 mL	329.3	
Sample 14	Yellow bottle and dark yellow liquid	Caffeine/150 mL	49.4 mg/150 mL	329.3	
Sample 15	Blue/white can and blue liquid	Caffeine/250 mL	33.9 mg/250 mL	135.6	
Sample 16	Pink/white can and light brown liquid	Caffeine/250 mL	34.3 mg/250 mL	137.2	
Sample 17	Gold can and yellow liquid	Caffeine/330 mL	67.5 mg/330 mL	204.5	

Cont...

Sample 18	Yellow cap bottle and yellow liquid	Caffeine/500 mL	105.6 mg/500 mL	211.2
Sample 19	Red cap bottle and red liquid	Caffeine/500 mL	108.5 mg/500 mL	217.0
Sample 20	Red can and red liquid	Caffeine/250 mL	23.2 mg/250 mL	92.8
Sample 21	Blue can and brown liquid	Caffeine/330 mL	31.8 mg/330 mL	96.4
Sample 22	Blue can and brown liquid	Caffeine/330 mL	28.3 mg/330 mL	85.8
Sample 23	Red text/white can and brown liquid	Caffeine/330 mL	38.8 mg/330 mL	117.6
Sample 24	Black text/red can and brow liquid	Caffeine/330 mL	27.2 mg/330 mL	82.4
Sample 25	White text/red can and brow liquid	Caffeine/330 mL	28.7 mg/330 mL	87.0

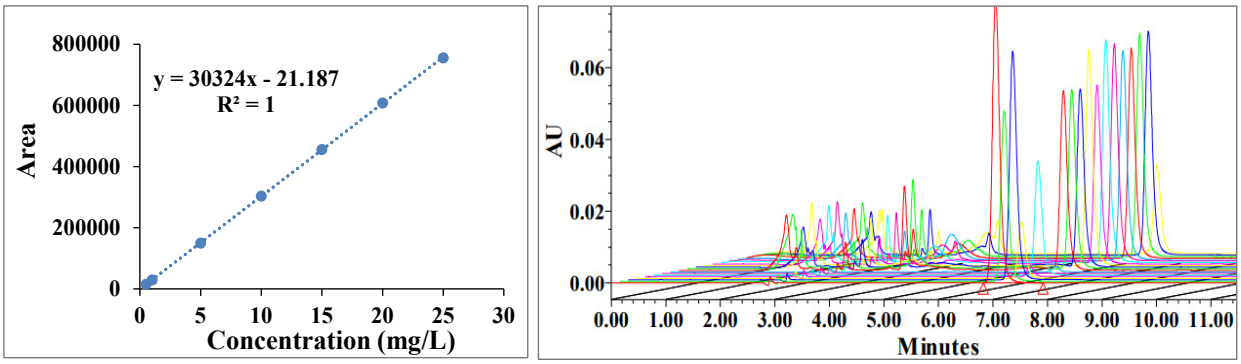

Figure 9: Calibration curve of standard caffeine at concentrations between 0.5 mg/L to 25.0 mg/L (n = 7). HPLC Arc™ Water and ZORBAX C18 column (flow rate 1.0 mL/min, 30℃, mobile phase MP3).

for caffeine content in energy drinks within the 90 to 110% (or ±10%) range as a standard practice to ensure consistency and quality control (USP, 2024). For energy drinks (Sample 01–11), the result indicated that most of these energy drinks achieved the specific tolerance range within the standard. The exceptions were observed for Sample 04 (33.2%) and Sample 06 (132.6%), which were below and above the limit range, respectively. In the case of energy drinks (Sample 12–25), the caffeine contents were most frequently observed between 2 to 3 times lower than those of labeled energy drinks. The exceptions were observed for the Samples 12, 13, and 14.

Conclusion

The research optimized and validated a reliable HPLC method for caffeine quantification, which potentially differentiates between labeled and actual caffeine levels in energy drinks in Cambodia. The method was introduced to analyze caffeine content in 25 different brands of energy drinks collected from the Cambodian market. The water/acetonitrile (87:13, v/v) was considered as the optimal mobile phase ratio, which was conducted using isocratic mode in the C18 column (30°C) at a fixed flow rate of 1 mL/min with a wavelength of 273 nm. The detection values were obtained at 14.34 µg/L for LOD and 43.45 µg/L for LOQ. The linearity (R^2) of the standard caffeine and matrix sample (energy drink) was observed to be greater than 0.9994. High repeatability and intermediate precision were achieved, as shown by an RSD of 0.11% for intra-day precision and 1.44% for inter-day precision. Additionally, the recovery test exhibited recovery values between 99.58 and 99.79%, indicating high accuracy. Finally, the robustness test on varying the column temperature, flow rate, or mobile phase ratio was revealed with insignificant influent on recovery efficiency and precision. The caffeine content in various energy drinks analyzed from this optimized method exhibited a minimum content of 82.4 mg/L and a maximum of 424.4 mg/L. Those energy drinks, without mentioning the specific caffeine content on their label, most frequently exhibited contents 2 to 3 times lower than those of labeled energy drinks. Significantly, this method is suitable for quantifying caffeine in energy drinks without interference from other ingredients, which is beneficial to employ as a quality control laboratory for routine matrix sample analysis.

Acknowledgment

The authors gratefully acknowledge the support from the National Health Products Quality Control Center (NHQC) funded through the Ministry of Health (MoH), Cambodia.

Declaration of Competing Interest

The authors declare that none of the work described in this publication may have been influenced by known competing financial interests or personal ties.

Data availability

The authors declare in this study that the data supporting the findings are available within the paper. If any alternative format is required for the raw data files, they can be obtained with a reasonable request.

Credit authorship contribution statement

Dr. Bunthoeun Nim: Conceptualization of the research, investigation and supervision, review-comment, and editing of the manuscript. Mr. Leab Ly: Research design, experimentation, data collection, data analysis, writing-original draft of the article. Dr. Chorney Eang and Dr. Sereilakhena Phal: Reviewing and editing the manuscript.

Data availability statement

The authors declare in this study that the data supporting the findings are available within the paper. If any alternative format is required for the raw data files, they can be obtained with a reasonable request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Funding declaration

No funding was received to assist with the preparation of this manuscript.

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