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Research Article

NATURAL PRODUCTS

Development and Validation of Ultra Performance Liquid Chromatography (UPLC) Method for the Quantitative Estimation of Caffeine in Non-Alcoholic Soft and Energy Drinks

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Abstract

Background: The popularity of beverages such as cold coffee, iced tea, and energy drinks has risen tremendously among athletes and youths. Energy drinks are used to enhance performance due to the presence of a high amount of caffeine (CFN) and sugars, as well as other constituents such as vitamins, amino acids, taurine, extracts of *Ginkgo biloba*, ginseng, guarana, and other herbal products. Commercial drinks are promoted as being beneficial to health; thus it is an important concern regarding adverse effects linked with these drinks or products.
Objective: The aim of the study is to develop and validate the ultra performance liquid chromatography-photodiode array detector (UPLC-PDA) method for the estimation of CFN in eight marketed non-alcoholic drinks including two soft drinks and six energy drinks.
Method: The chromatographic separation of CFN was achieved on Acquity UPLC® CSH™ C_{18} 1.7 μm, 2.1 × 100 mm column, using isocratic mode, mobile phase comprising acetonitrile and water (30:70, v/v) at a flow rate 0.3 mL/min using injection volume 1 μL.
Results: The elution of CFN occurred at 1.06 min, and the calibration curve of the CFN was computed from the peak area ratio detected at 273 nm. All the validation parameters were found within the assay variability limits as per ICH guidelines. The obtained results revealed that the soft drinks SD1 and SD2 have 87.21 ± 1.28 and 101.81 ± 1.52% (w/w), whereas CFN concentration in energy drink brands ED1, ED2, ED3, ED4, ED5, and ED6 was 95.90 ± 1.62, 64.0 ± 1.07, 76.68 ± 1.95, 74.97 ± 2.33, 82.09 ± 2.43, and 88.04 ± 2.94% (w/w) of labeled claims, respectively.
Conclusions: The developed UPLC method was found suitable for the quality control of commercial soft and energy drinks containing CFN.
Highlights: The developed chromatographic method is very simple, cost effective and could be utilized for the routine analysis of caffeine in the soft and energy drinks.

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The popularity of drinks such as soft and energy drinks has tremendously rise among athletes and youths. Energy drinks are used to enhance performance. Commercial energy drinks contain high amounts of CFN and sugars, and also other constituents such as vitamins, amino acids, taurine, extracts of *Ginkgo biloba*, ginseng, guarana, and other herbal products (1–4). Commercial drinks are promoted as being beneficial to health; thus it is an important concern regarding adverse effects linked with these drinks or products. CFN (1,3,7 trimethylxanthine) (Figure 1) acts as a mild CNS stimulant throughout the world and is promptly absorbed into the systemic circulation (5). CFN has extensively been used in chocolates and commercial drinks, as well as in pharmaceutical preparations. Recently, the use of CFN in commercial drinks has risen considerably owing to its enhancing performance and restoring mental alertness.

Globally, around 80% of the people use caffeine (CFN) daily, and incessant investigation is being conducted to estimate beneficial and harmful effects associated with it. The quantity of CFN used every day in foods and drinks fluctuates broadly. A mug of coffee has nearly 100 milligrams of CFN, and green tea has 20 to 30 mg of CFN. CFN consumption from all sources reaches around 210 to 238 mg in the United States and Canada, and also 400 mg per person every day in Sweden and Finland (6, 7).

The primary and most potent sources of CFN in the foods of people in the U.S. are coffee, tea, soft drinks, and energy drinks (8, 9); other than this, many OTC drugs such as painkillers, cold medicine, and allergy medicine also contain CFN but a relatively small amount (10).

CFN has been considered the chief ingredient of energy drinks to improve performance and restore mental alertness. Many researchers have suggested that various disorders are linked with the high consumption of CFN, including anxiety, heart disease, carcinogenesis, kidney malfunction, diuresis, sleep disturbance, insomnia, headache, fatigue, and depression (11–13).

Currently, the manufacturing of soft and energy drinks for the development of the food industry has risen enormously. From the safety perspective, a quality control study of commercial drinks has very vital significance for human health and quality of life. Several chromatographic methods have been reported for the determination of CFN in different tea, coffee, soft drink, energy drink, and beverage samples including UV-visible spectrophotometry (14, 15), HPTLC (16–18), HPLC (19–21), UPLC (22, 23), gas chromatography-mass spectroscopy (GC-MS) (1, 24–27), electrophoresis (28), capillary electrophoresis (29), solid phase-raman spectroscopy (30), and microemulsion electrokinetic chromatography (31). It is important to establish new strong analytical cost-effective methods to giving rapid, reliable, precise, and robust results for the estimation of CFN content in commercial drinks. The current investigation was carried out to develop a simple, highly sensitive, reliable, precise, fast, and robust UPLC-PDA method for the estimation of CFN in non-alcoholic soft and energy drinks.

Experimental

Chemicals

Standard (STD) CFN (HPLC ≥99.0%) pure was acquired from Sigma Aldrich, HPLC gradient grade acetonitrile and water were procures from Chromasolv (Germany). The soft and energy drinks were bought from a hypermarket in Rakkah, Al-Khobar, Saudi Arabia.

Chromatographic Condition

CFN was eluted on an an Acquity H-Class UPLC photo diode array detector (PDA) (Waters, Milford, MA), and Empower software was used for the chromatographic separation and identification using Acquity UPLC CSH^{TM} C_{18} 1.7 μm, 2.1 × 100 mm column. Column oven temperature was maintained at 35 ± 5°C. Mobile phase comprising water and acetonitrile in the 70:30 (v/v) ratio on isocratic mode at 0.3 mL/min flow rate, and injection volume was 1 μL. The chromatographic method total run time was 2.0 min.

Stock Solutions

A stock solution of standard CFN 1.0 mg/mL was prepared by weighing 50 mg of CFN in 50 mL of volumetric flask dissolved in water to make up the volume 50 mL. From the working solution of CFN, a serial dilution was prepared for the calibration curve using water and acetonitrile (70:30, v/v). All the sequential dilutions of the CFN were filtered through 0.22 μm membrane filters before injecting into UPLC used for the calibration curve.

Sample Preparation

Eight samples of soft and energy drinks (two soft and six energy drinks) were bought from a hypermarket in Rakkah, Al-Khobar. The brand names of soft drinks were coded as SD1 and SD2, whereas the energy drinks were ED1, ED2, ED3, ED4, ED5, and ED6. The amounts of CFN in specific soft and energy drinks are illustrated in Table 1. Before analysis, all the soft and energy

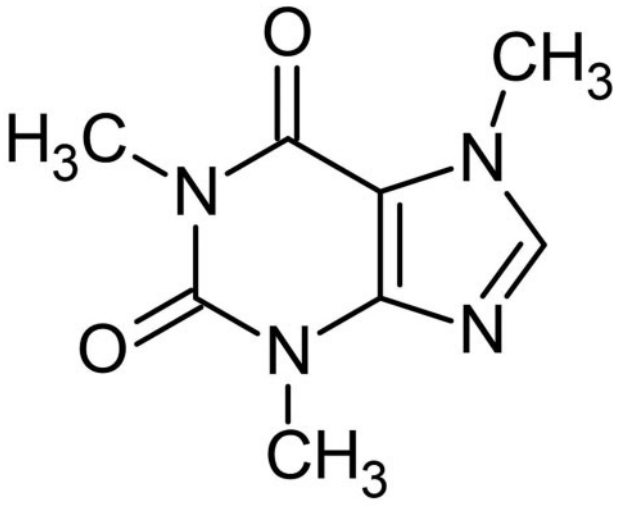

Figure 1. Chemical structure of CFN.

Table 1. CFN content in soft drinks and energy drinks

Drink name	Volume, mL	Labeled quantity, mg/100 mL	Obtained quantity, mg/100 mL	% w/v
SD1	360	11	9.59	87.21 ± 1.28
SD2	330	10	10.16	101.81 ± 1.52
ED1	250	15	14.39	95.90 ± 1.62
ED2	250	36	23.04	64.0 ± 1.07
ED3	250	29	22.23	76.68 ± 1.95
ED4	250	32	23.98	74.97 ± 2.33
ED5	250	30	24.62	82.09 ± 2.43
ED6	250	32	28.17	88.04 ± 2.94

drinks were sonicated in a sonicator bath for 20 min to eliminate entrapped air, and 10 mL of samples were withdrawn and diluted in a 100 mL volumetric flask using mobile phase. The final solutions were filtered using 0.22 μm membrane filters prior to injecting into UPLC.

Method Validation

The proposed UPLC procedure for the estimation of CFN was validated according to the International Conference on Harmonization (ICH) guidelines (32, 33). The linearity of the CFN was estimated by using varying concentrations of the STD CFN and calibration curve plotted for the regression analysis. Specificity of the UPLC method was confirmed by using Rt and spectra of the peak compared in samples with that of the standard CFN.

Accuracy, as recovery, was estimated by standard addition procedure. A known quantity of STD CFN was added in preanalyzed samples with additional CFN (50, 100, and 150%) and reanalyzed. Percent recovery and RSD were calculated for all different concentration levels. Repeatability and intermediate precision of the samples were assessed as interday and intraday precision were determined by using three varying amounts in triplicate, and obtained results were expressed as RSD, %.

Robustness of the proposed UPLC method was ascertained by the influence of minor deliberate changes in the chromatographic conditions during estimation of CFN. Robustness was evaluated by changing flow rate and detection wavelength. The limit of detection (LOD) and limit of quantitation (LOQ) were measured based on the regression analysis data of slope and standard error.

Results and Discussion

Optimization of UPLC Conditions

To obtain the best peak separation of the selected compound, it was necessary to select column type, size, oven temperature, flow rate, and composition of mobile phase for optimization of the chromatographic method. Various mobile phases in different proportions of solvents such as acetonitrile, methanol, buffers, and waters tried for the best separation of CFN. A mobile phase comprising water and acetonitrile (70:30, v/v) using Acquity UPLC CSH C_{18} column and 0.3 mL/min flow rate with an oven temperature of 35 °C was found to be suitable for the separation of CFN at retention time (Rt) observed at 1.06 min (Figure 2), within a total run time of 2.0 min in soft drinks and energy drinks. Sample injection volume was 1.0 μL, and 273 nm detection wavelength was chosen for the quantitative analysis of CFN (Figures 3 and 4).

Method Validation

(a) *Linearity*.—Linearity of the CFN was evaluated over a varying concentration range of 40 to 200 ng/mL. Linear coefficient regression analysis (r^2 0.9994) was achieved by least-squares linear regression model using the peak area of the chromatogram (Figure 5). The LOD and LOQ were found as 7.34 and 22.24 ng/mL, respectively.

(b) *Precision*.—Precision is an assessment of the reproducibility of the analytical process under standard working conditions. The results of precision are illustrated in Table 2, in terms of RSD, which were found in acceptable range.

(c) *Accuracy*.—Accuracy expressed the closeness of understanding among the values, which is demonstrated as an ordinary genuine value or established reference value and the obtained value. The recovery results were 97.80–103.28%; these results showed accuracy of the method (Table 3).

(d) *Robustness*.—Results of robustness are illustrated in Table 4. Low value (0.34 to 1.13) of RSD indicated the robustness of the UPLC method by deliberate change into the flow rate and detection wavelength in the chromatographic condition.

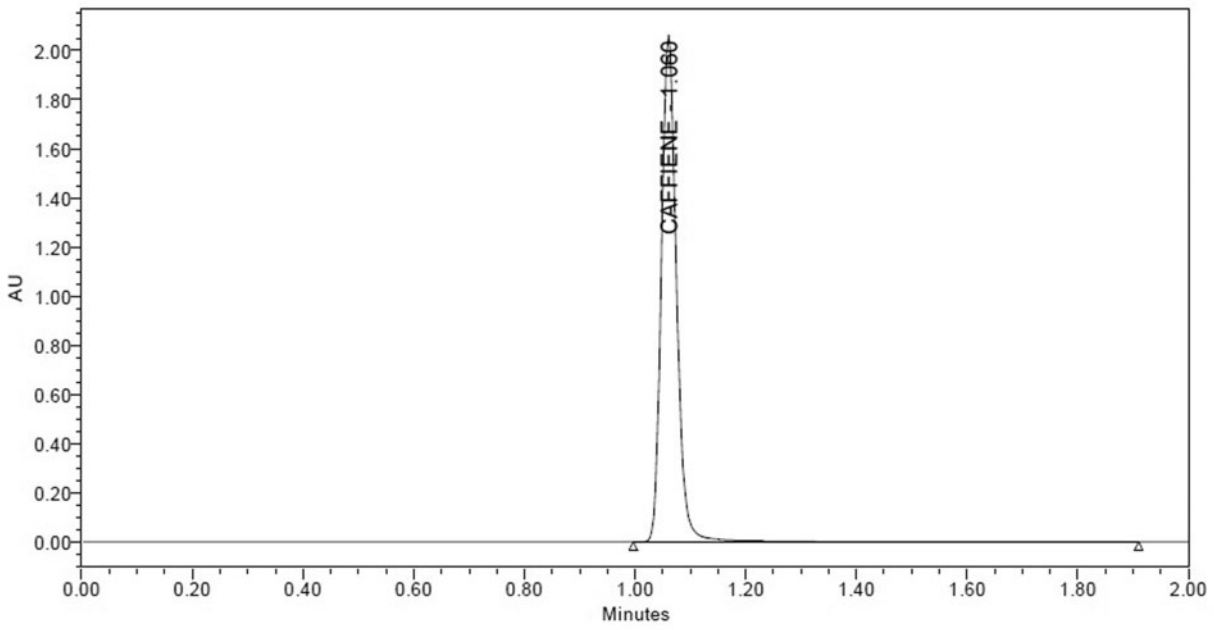

Figure 2. UPLC chromatogram of standard CFN at 273 nm.

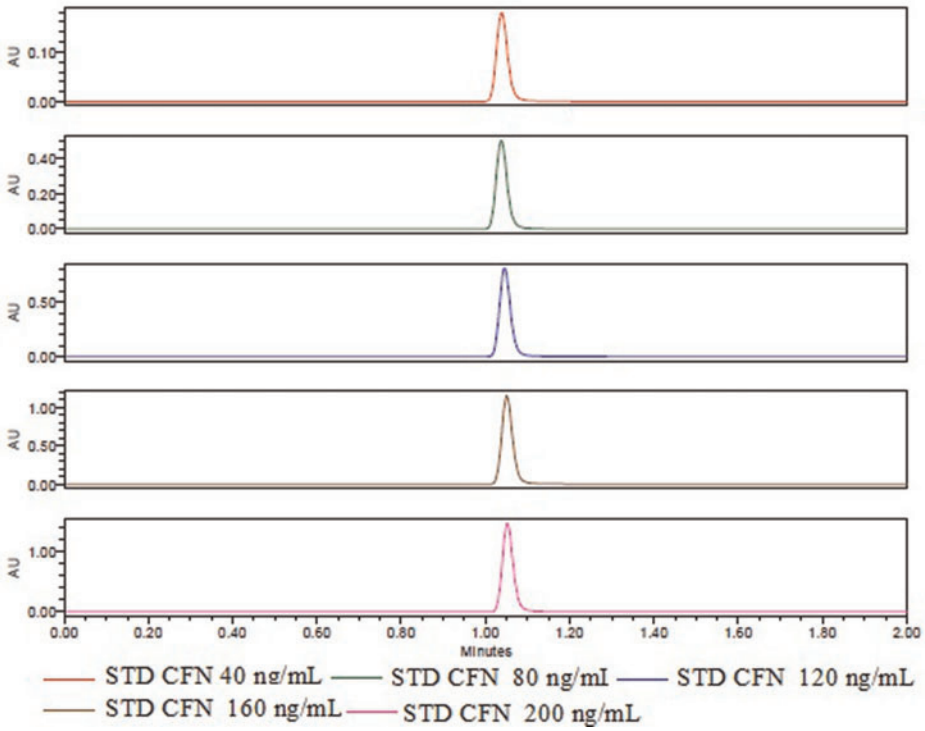

Figure 3. UPLC stacked chromatograms of standard CFN of varying concentrations.

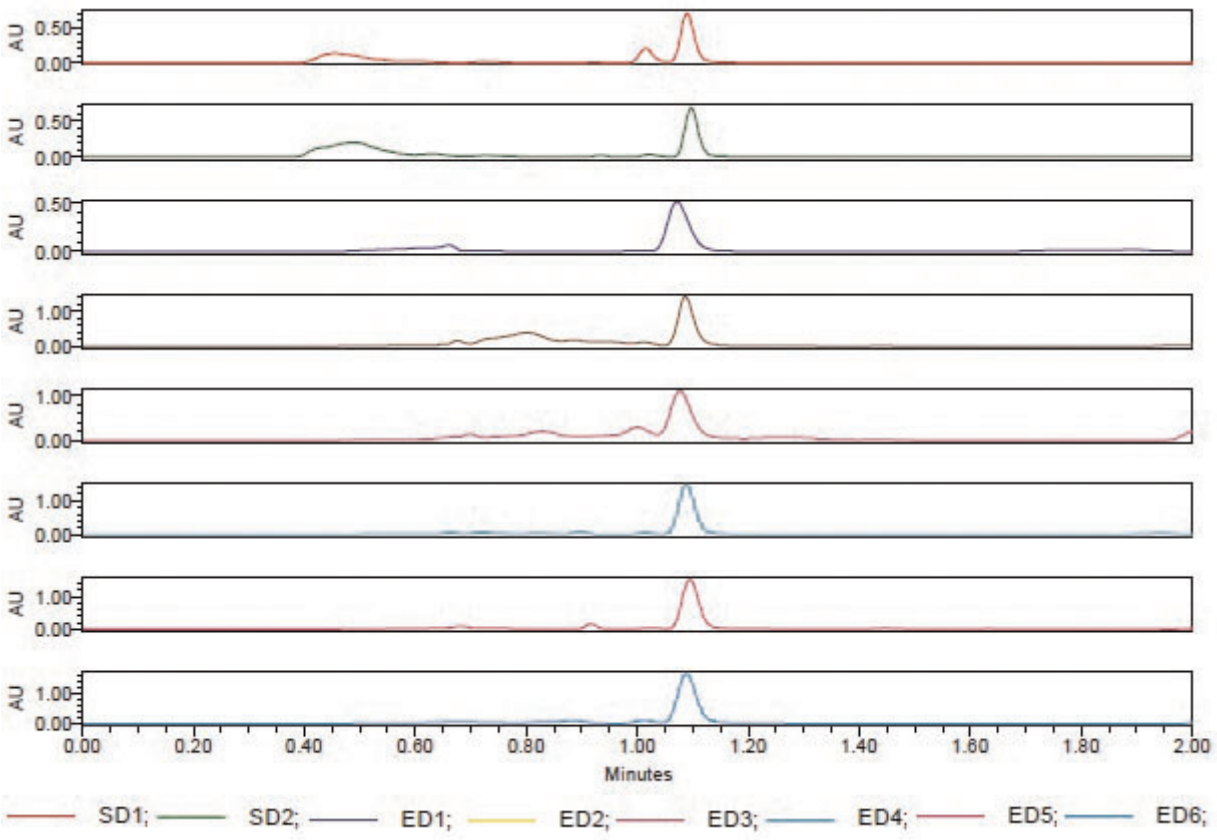

Figure 4. Stacked chromatograms for samples containing CFN.

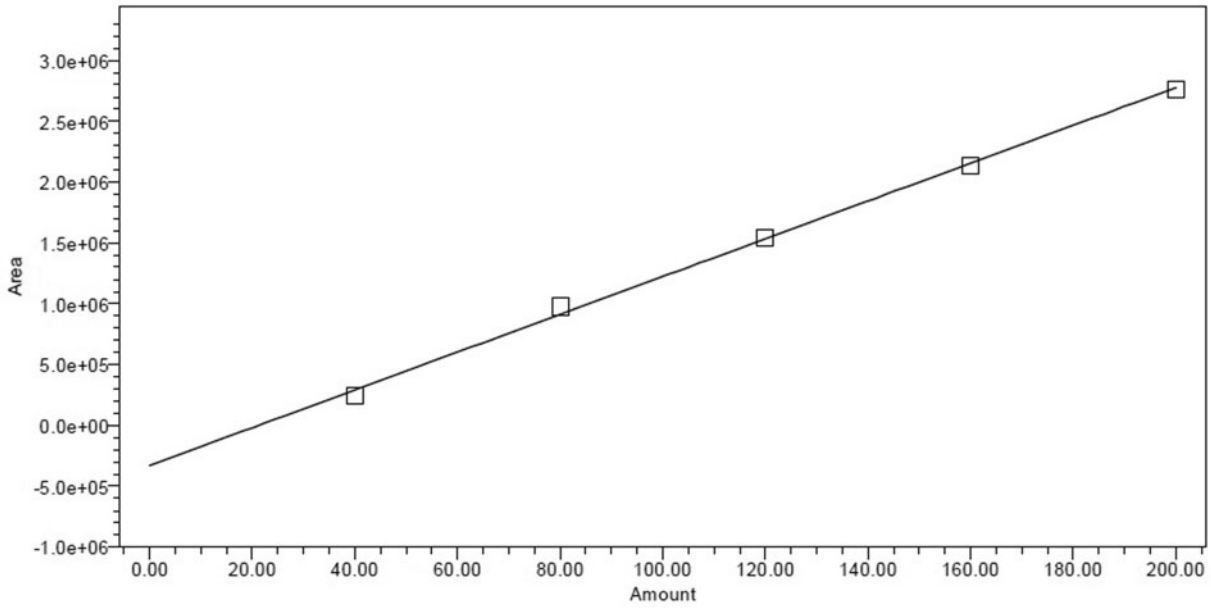

Figure 5. Calibration curve of standard CFN.

Table 2. Precision of the UPLC method of CFN

Amount ng/mL	Interday precision		Intraday precision	
	Mean peak area ± SD	RSD, %	Mean peak area ± SD	RSD, %
80	905 891.40 ± 10 726.10	1.18	979 522.55 ± 3211.59	0.32
120	1 613 401.09 ± 11 435.86	0.70	1 542 131.36 ± 16 672.71	1.08
160	2 195 709.38 ± 26 387.61	1.20	2 135 372.72 ± 32 031.35	1.5

Table 3. Accuracy of CFN content

Excess spike concentration	% Recovery of CFN
50%	97.80 ± 0.97
100%	100.63 ± 0.06
150%	103.28 ± 0.32

Analysis of Commercial Non-Alcoholic Soft Drinks and Energy Drinks

Two commercial non-alcoholic soft drinks (coded as SD1 and SD2) and six energy drinks (ED1, ED2, ED3, ED4, ED5, and ED6) samples were bought from a hypermarket of Rakkah, Al-Khobar and analyzed for CFN content and compared with their labeled claims in Table 1. The concentration of CFN in ED3 was below 20%, ED4 was below 25%, and ED2 was well below 30% of their labeled claims. Alam et al. reported the CFN content in SD1, SD2, ED1, ED2, ED3, and ED4: 88.8, 110.7, 76.9, 65.6, 88.1, and 89.1% w/v in soft and energy drinks, respectively, by using the UPLC-ESI-MS method (23). For the safety point of view, the content of CFN was found to be within the acceptable range in the tested soft drinks and energy drinks.

Comparison With Reported Analytical Methods

The UPLC assay for the quantitative determination of CFN was compared with reported analytical methods. The results for comparison are illustrated in the Table 5. Some parameters including linearity, run time, mobile phase, and retention time of the UPLC method were compared with reported methods. The linearity range, run time, and retention time of CFN in the literature of HPLC methods have been reported as 0.151–250 μg/mL, 9–17 min, and 3.5 to 12 min, respectively, which were not better than the current UPLC method (linearity range = 40–200 ng/mL, run time = 2 min, and retention time = 1.06 min) (34–39). However, the UPLC method reported by Fatma et al. showed CFN linearity range 4–44 μg/mL, 14 min runtime, and 3 min retention time using buffer and acetonitrile as mobile phase (40), whereas CFN linearity range was 30–440 PPM, 7 min runtime, and 1.78 min retention time using UPLC as reported by Jena et al. (41). Overall, the UPLC method for CFN analysis was found to be superior over all reported literature analytical methods, in terms of cost-effective, less time-consuming, precise, and robust for the analysis of CFN commercial drinks/products.

Conclusions

A UPLC method with PDA detector has been developed for the quantification of CFN in non-alcoholic soft drinks and energy drinks. The method is fast, simple, less time-consuming, and economic, and short duration of run time is appropriate for the quality control of CFN in non-alcoholic soft and energy drinks. Two soft and six energy drinks were evaluated for the CFN content by using the developed method. Out of six energy drinks, in

Table 4. Robustness of the developed method

		Mean peak area ± SD	Mean Rt area ± SD	RSD, % of area	RSD, % of Rt
Flow rate, mL/min	0.2	955,871.88 ± 8058.24	1.24 ± 0.01	0.84	1.0
	0.3	977,008.92 ± 3353.29	1.06 ± 0.00	0.34	0.38
	0.4	960,298.81 ± 7595.86	1.10 ± 0.01	0.79	1.12
Change in wavelength (nm)	271	957,394.31 ± 8652.11	1.10 ± 0.012	0.90	1.13
	273	977 406.20 ± 5012.(0).40	1.05 ± 0.02	0.51	0.19
	275	963 584.22 ± 5309.26	1.09 ± 0.08	0.55	0.74

Table 5. Comparative evaluation of the present UPLC assay with reported analytical assays for the quantitative estimation of CFN

Serial number	Analytical method	Column	Run time	Linearity, μg/mL	Mobile phase	Retention time	Ref.
1	HPLC	C18	9	C: 0.151–200 μg/mL	0.01 M KH_2PO_4–methanol–acetonitrile–isopropyl alcohol (420:20:30:30)	5.84	(34)
2	HPLC	C18	10	C: 2.5–50 μg/mL	Methanol–water 40:60	3.5	(35)
3	HPLC	C18	10	C: 0.1–30 μg/mL	Methanol–water 40:60	5.3	(36)
4	HPLC	NR[a]	13	C: 0.1–40 μg/mL	10 mM phosphate buffer, pH 6.8, and acetonitrile	12	(37)
5	HPLC	C8	9	NR[a]	Mobile phase was water–THF (0.1% THF in water adjusted to pH 8 with 0.1 M NaOH)–acetonitrile (90:10)	7.5	(38)
6	HPLC	C18	17	C: 0.4–250 μg/mL	Methanol–phosphate 40:60	5.3	(39)
7	UPLC	C18	14	C: 4–44 μg/mL	Acetonitrile and 0.2 M H_3PO_4 (11:89, v/v)	3.0	(40)
8	UPLC	C18	7	C: 30–240 PPM	A methanol–acetonitrile (50:50) B water–acetonitrile–H_3PO_4 (80:20:0.1)	1.78	(41)
9	UPLC	C18	2	40–200 ng/mL	Acetonitrile and water (30:70, v/v)	1.06	CW[b]

[a] NR = Not reported.
[b] CW = Current work.

one energy drink the CFN was found to be a significantly smaller amount (64.0%) in the labeled claim. The proposed UPLC method can be useful for the routine analysis of beverages containing CFN as an ingredient.

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Conflict of Interest

The authors declare no conflict of interest.

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