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Validation and Determination of Caffeine Contents in Energy Drinks Available on the Iraqi Market by Using High Performance Liquid Chromatography (HPLC)

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ABSTRACT

Caffeine was determined by Reversed phase High Performance Liquid Chromatography (RP-HPLC). The main advantages of this method are: simple, sensitive and rapid. The separation was achieved by using HAISIL100A-C_{18} (150 mm x 4.6mm, 5µm particle) analytical column at 25^{o}C; the mobile phase water - methanol (40:60 v/v); respectively; flow rate was applied for 1mL/min (in 2.50min) using UV detection at 273nm. The retention time of Caffeine was found to be (1.999) min. The validity of the proposed method was evaluated by determining the value of linearity, accuracy, recovery, precision, LOD and LOQ. It was found that the values of linearity and correlation coefficient of the method was (1-200) µg/mL (r^2 = 0.9999). The percentage recovery for Caffeine was (98.091-100.500) %. LOD and LOQ were found to be (0.579) µg/mL and (1.809) µg/mL; respectively. The effect of pH, volume injection and flow rate were also determined. The results of this study show that the proposed method was successfully applied to estimate the Caffeine in energy drinks. In conclusion, the present method is appropriate to determine the percentage of Caffeine in the samples under study.

INTRODUCTION

Naturally, Caffeine can be found in many leaves and seeds of many plants, there is about more than 63 plant species around the world contain Caffeine. Its chemical name is 1, 3, 5-trimethylxanthine and its chemical formula is $C_8H_{10}N_4O_2$. Caffeine in pure form can be appears as white, woollen mass, sparkling needles of powder. Caffeine molecular mass is 194.19 g/mole, and its melting point is 236°C. Sublimation degree and is 178°C. Figure (1) showed the structural form of Caffeine. The spread of the presence of caffeine in a variety of plants played a major role in the long-term popularity of caffeinated products. The major sources of caffeine are coffee, tea, guarana, cola nuts and cocoa. The measure of caffeine found in these items differs, the most noteworthy sums are found in guarana (4-7%), tea leaves (3.5%), coffee beans (1.1-2.2%), cola nuts (1.5%) and cocoa beans (0.03%). A fatal dose of caffeine has been calculated to be more than 10g (about 170mg kg^{-1} body weight). The reported caffeine content in the main dietary sources varies significantly: 93.0–163.5mg per cup in ground coffee, 46.7– 67.6mg per cup in instant coffee, 30.2 – 67.4mg per cup in bag tea and 0.32–0.54mg/g in dark sweet chocolate. A previous study has provided that there is no difficult health effect can cause by acceptable level of individuals, namely (400– 450mg/day), (300mg/day), and (45mg/day) for healthy adults, women contemplating pregnancy and young children age 4–6years, respectively[1].

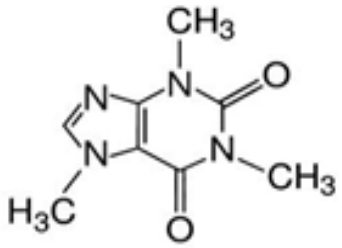

Fig.(1) : showed the structural form of Caffeine

It was found that caffeine "1,3,7-trimethyl-1H-purine-2,6(3H,7H)-dione" has also been recognised to play a significant role in terms the pharmaceutical industries has pharmaceutically important due to it's chemical properties. For example, Caffeine is a weak base , cannot loss a proton from or act as a cid at pH value under 14. Furthermore, Caffeine act as electrophile in position 1,2 and 3 . In blood, there in no highly amount of protein bound in caffeine. Moreover, caffeine found in brain with high concentration due to the lipophilic properties [2]. The main

techniques can use to determine the levels of caffeine are LC-Spectrophotometric [3], Spectrophotometric method[4,5,6], and HPLC[7,8,9].

MATERIALS AND METHODS

Experimental part

Materials and Chemical

The caffeine samples were collected from the different drug stores in Kerbala, Iraq. In addition, ultra-high-purity grade reagents were used for the dilution, preparation and analysis of samples so as to avoid contamination at trace element levels. Double beam UV-Visible –Spectrophotometer -1800, Shimadzu, (Japan). Equipped with quartz cell (1cm), High-Performance Liquid Chromatography (HPLC), UFLC -Shimadzu, CBM 20A, (Japan), Equipped with HAISIL100A -C_{18} (150mm x 4.6mm, 5µm particle) analytical column, UV-Visible detector and Column oven CTO-20A (4- 85)^{o}C, Shimadzu (Japan), Digital Balance, Denver –TP-214, (Germany), FT-IR, Bruker, TENSOR 27, (Germany), pH-meter, Hanna-pH211, (Romania) and Ultrasonic cleaner, KQ200E, (Chain) were employed for the estimation**.**

Preparation of standard stock solution

Standard stock solutions of caffeine were prepared by weighing 0.01g from drug and the solutions were then diluted to 50 ml with mobile phase using a polyethylene volumetric flask. On each occasion the mixture was sonicated (15 minutes, 35 MHz). Standard stock solution was further diluted with mobile phase to prepare serial solutions for standard curve.

Sample preparation

Different kinds of energy drinks were purchased from different Iraqi local supermarkets and 10 samples were analyzed using the indicated HPLC method. When test containers were opened, the beverages were degassed and homogenized , then every sample was filtered by using 0.45µm filter paper, filtered beverage tests of 2 mL were diluted to 20 times with mobile phase.

Wavelength selection

Caffeine solutions at concentration of 10µg/mL in diluent were scanned by UV-Visible spectrophotometer in the range of (200-400) nm. From the UV spectra, suitable wavelength considered for monitoring the drug were 273nm on the basis of higher response.

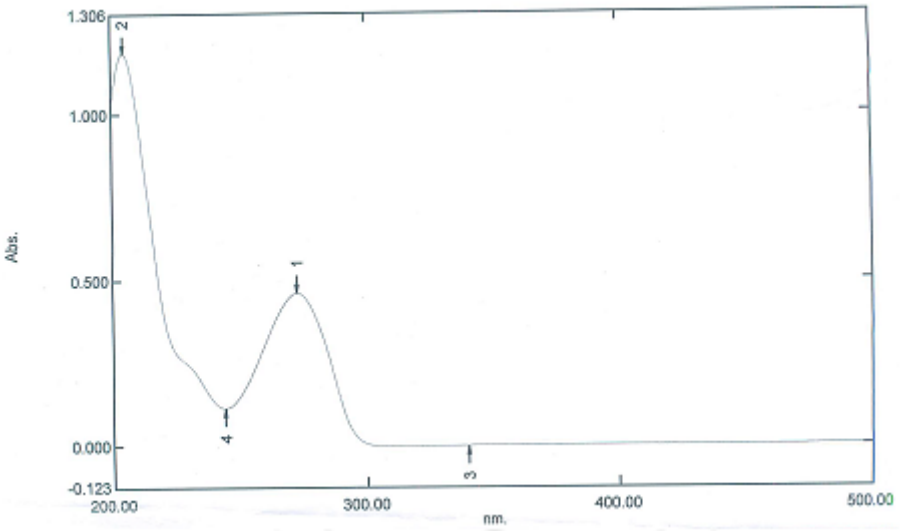

Fig.(2): UV-Visible spectrum for standard solution of Caffeine.

FT-IR spectrum of Caffeine

FT-IR spectrum was recorded for Caffeine. The spectrum was compared with standard spectrum in order to identify this compound.

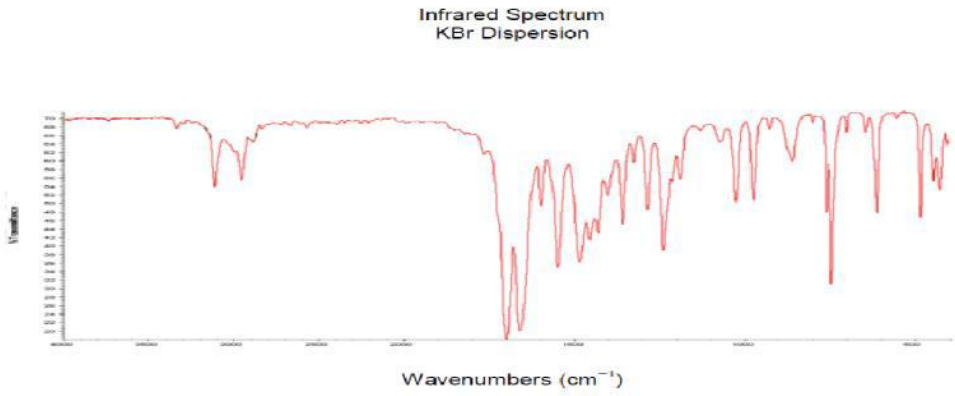

Fig.(3): FT-IR spectra for standard Caffeine (Sigma –Aldrich)

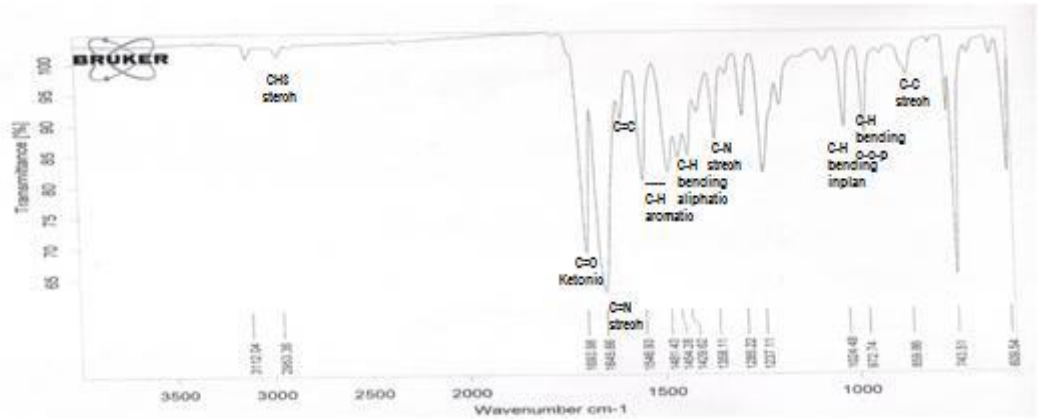

Fig.(4): FT-IR spectra for standard Caffeine.

Chromatographic conditions of isocratic elution system

HPLC analysis was performed by isocratic elution. The flow rate was 1mL/min. The mobile phase composition was water: methanol (40:60 v/v), adjusting pH to 5 by diluted (HCl / NaOH). All injected solvent were filtered through 0.45µm filter paper and degassed by using ultrasonic water bath , the volume of injected solvent was 20µL and the wavelength 273 nm. Running time for overall analysis less than 3 minute as shown in figure(5).

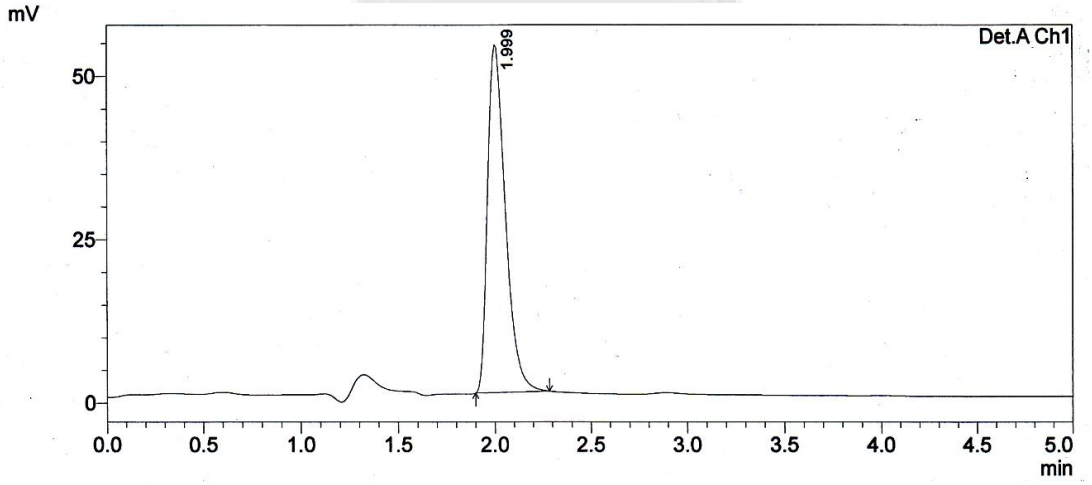

Fig.(5): Chromatogram of standard solution of Caffeine.

Calibration curve

Standard solutions containing Caffeine (1-200) µg/mL were prepared in the mobile phase. To study the reproducibility of detector response at different level of concentration, 20µL of standard solution was injected; calibration curve was obtained by plotting peak area against concentration to calculate the calibration equation and correlation coefficients.

Optimization of HPLC method

Separation of caffeine was carried out by using by using a mixture of water- methanol with the volume ratio (v/v) of (40:60), adjusting pH to 5 by diluted NaOH, at flow rate of 1mL/min with isocratic program and gave acceptable retention time of (1.999) min.

Validation of the method

Optimization of the HPLC instrument was performed daily and the instrument operating parameters were made in order to achieve the maximum sensitivity as shown below:

Linearity and range

The linearity range was evaluated by using the standard solution prepared in the mobile phase. The calibration range for caffeine was found to be 1 – 200 µg/mL. The calibration graph was automatically drawn by plotting the value of peak area against the concentration of caffeine.

Specificity

The specificity of the proposed method was evaluated by contrasting chromatogram acquired from standards Caffeine and that from marketed solutions [10].

Limits of detection and Limit of quantitation

Sensitivity of the proposed method was assessed as far as (LOD) and (LOQ)[11]. "LOD = 3 SD/S and LOQ = 10 SD /S, where S.D. is the standard deviation of y-intercept and S is the slope of the line"[12].

Effect of pH

The effect of pH variation of mobile phase on the selectivity and retention times was determined by using mobile phase with pH from (3.00 to 6.00) under optimum condition.

Effect of variation of flow rate

Different flow rate were examined to determine its effect on separation of Caffeine under optimum condition. HPLC system was injected with a standard solution prepared according to the method of testing using flow rate (0.5, 0.7, 1.0 and 1.3) mL/min.

Analysis of a marketed energy drink

To determine the content of Caffeine in commercial energy drinks illustrated below in table (1):

Table (1): Marketed energy drink assay.

Brand name	Manufactured	Labeled claim (µg/mL)
rip it (TRIBUTE)	United States Of America	416.666
rip it (BOMB)	United States Of America	416.666
rip it (C.Y.P-X)	United States Of America	333.333
POWER HORSE	AUSTRIA	320
STING (GRAPE)	IRAQ	300
ONE TIGER	JORDAN	300
Red bull	AUSTRIA	300
BOM BOM	Saudi Arabia	290
STING (GOLD)	IRAQ	200
300 POWER	JORDAN	135

HPLC system was injected with 20µL sample solution under chromatographic conditions of the method. Peak areas were determined at wavelength 273nm and concentration of samples were determined by using calibration curve obtained on the same HPLC system with same condition by utilizing linear regression equation.

RESULTS AND DISCUSSION

Validation of the method

Validation of the optimized HPLC method was carried out with respect to the following parameter:

Calibration curve and linearity study

Caffeine showed good correlation coefficient in concentration range of (1-200) µg/ml. The detector response over wide range of concentrations of analyte were plotted to obtain the calibration curve figure (6). The square of the correlation coefficient and equation for the curve is shown in table (2).

Table (2): Linearity and regression characteristics of standard Caffeine.

Parameters	Linearity range µg/mL	Regression equation	Correlation coefficient (r^2)
Linearity range µg/mL	1-200	Y = 33191x + 98839	r^2 = 0.9999

(r^2) value is greater than 0.9997. From this result, it is acceptable to use a single point calibration in analysis of actual samples [13].

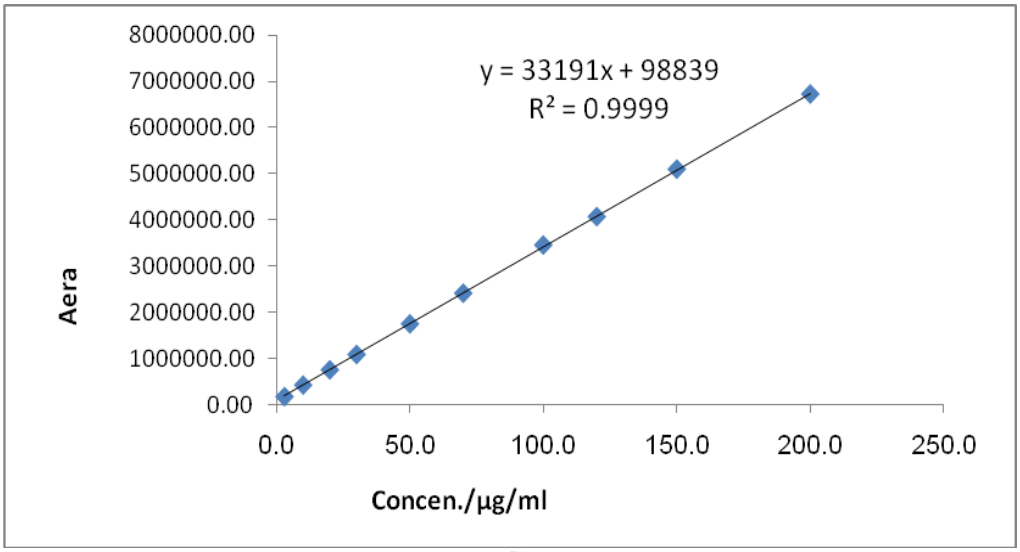

Fig.(6): Calibration curve of standard solution of Caffeine.

Precision

Precision can be determined by using the replicate analysis of caffeine sample. The values of relative standard deviation (RSD %) was calculated and found not more than 1, and E% less than 2 which indicates that the developed method is precise and reproducible [14].Results were illustrated in table (3).

Table (3): Precision for standard Caffeine.

	(µg/mL)	R.S.D %	E%
Caffeine	20.00	0.022	1.930
	50.00	0.128	1.411
	150.00	0.022	0.472

Accuracy

Accuracy was examined by using three different concentrations for all samples. In this study, the degree of agreement between a measured value and a true value was determined by using the values of recoveries (%R, replicate analysis n = 3).

Table (4): Percentage recovery data for standard drug

	Amount added (µg/mL)	Amount found (µg/mL)	Recovery [%]
Caffeine	20.00	19.620	98.091
	50.00	50.710	101.501
	150.00	150.500	100.500

From these acceptable limit of percentage recovery, Estimation of caffeine can be accurate quantitatively [15].

Specificity

The retention time of the standard Caffeine was illustrated table (5).

Table (5): Retention time of ten energy drinks assayed and standard drug solution

	t_R (min)
Standard Caffeine solution	1.999
BOM BOM	2.009
300 POWER	2.021
rip it (TRIBUTE)	2.006
rip it (C.Y.P-X)	2.030
rip it	2.011
STING (GOLD)	2.000
STING	2.026
ONE TIGER	2.034
Red bull	2.019
POWER HORSE	2.036

Result in table (5) showed that the retention time of the standard Caffeine and Caffeine in energy drink solutions were very close, almost same, so the method was specific.

Limit of detection (LOD) and Limit of quantitation (LOQ)

The results of limit of detection and limit of quantification were illustrated in table (6). The values indicate that the method is sensitive.

Table (6): Limit of detection and Limit of Quantification

Caffeine	Regression equation	Slope	LOD µg/mL	LOQ µg/mL
	Y = 33191x + 98839	33191	0.597	1.809

Influence of factors

Effect of pH

The change of pH was tested by decreasing the buffer pH from (6) to strong acidic (3) which lead to protonation of Caffeine. The high polarity of the resulted compound decreased its (t_R) and sensitivity to the UV detector [16]. Successfully applied a mobile phase with a pH 5.0 Adjustment was performed with the use of 0.1M NaOH. The result in table (7) showed that pH had only a slight effect on retention time of Caffeine. Resolution was improved when the pH decreased to 5; also the shape of Caffeine peak was improved in this pH.

Table (7): Effect of pH on separation of standard Caffeine.

pH	Caffeine	
	Area	R_t (min)
3.0	244050	1.862
3.5	147722	1.866
4.0	216489	1.976
4.5	84048	1.979
5.0	288587	1.997
5.5	208197	1.976
6.0	282848	1.853

Effect of Flow rate

For the present study, flow rate 1mL/min was selected on the basis of less retention time, good peak shape, Acceptable back pressure, good resolution and better separation of the drug. The results were presented in table (8).

Table (8): Effect of Flow rate on separation of Caffeine

Flow rate (mL/min)	t_R (min)
	Caffeine
0.5	3.969
0.7	2.862
1.0	1.999
1.3	1.547

Assay of energy drink

The chromatographic method was applied to the determination of Caffeine in energy drink. Analysis was carried out using optimized mobile phase and HPLC conditions. Results for Caffeine practically identical with its comparing labeled amount and R.S.D % are illustrated in table (9).

Table (9): Percentage recovery data for marketed energy drinks.

Caffeine		
Name of Energy drink	**Labeled amount (µg/mL)**	**Amount found (µg/mL)**
rip it (TRIBUTE)	416.666	445.429
rip it (BOMB)	416.666	396.292
rip it (C.Y.P-X)	333.333	230.040
POWER HORSE	320.000	347.100
STING (GRAPE)	300.000	295.165
ONE TIGER	300.000	310.733
Red bull	300.000	290.523
BOM BOM	290.000	275.344
STING (GOLD)	200.000	190.776
300 POWER	135.000	130.234

Results in table (9) showed that the estimation of energy drink was accurate within the acceptable level.

CONCLUSION

A reversed-phase HPLC method was developed and validated with UV detection for the determination of Caffeine and proved to be more convenient and effective for the quality control of Caffeine in energy drinks. The method gave good resolution for Caffeine with a short analysis time below 2.5 minutes. The technique used in this methods was observed to be simple, conservative and helpful with acceptable LOD and LOQ . Rapidity and ability of evaluating low concentration of Caffeine, made this method helpful for variety of analyses, including pure medication investigation. The proposed methods did not use any extraction process for recovering the Caffeine from the formulation excipients matrices thereby, decreased the error, time for estimation of Caffeine and the general expense of the investigation. The mobile phase used was simple with isocratic elution and low buffer solution contrasted with the reported methods. The method is suitable for the determination of Caffeine in energy drink without interference from commonly used excipients, and could be used in a quality control laboratory for routine sample analysis.

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