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Rapid High-Performance Liquide ChromatographicMethod for Quantitative Determination of Caffeine in Different Soft and Energy Drinks Available in Bangladesh

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ABSTRACT

Caffeine is one of the commonly used food additives, which has unique flavor characteristics and bitter taste and used in soft drinks as flavor enhancer. An experimental study was designed to determine the concentration of caffeine in different brands of soft drinks and energy drinks available in Bangladesh by using HPLC. For chromatographic analysis, A Luna 5μ C18 (2) 100A column (250×4.6 mm) was used at 37°C temperature at the wavelength of 272nm.Chromatographic separation was determined using buffer of sodium acetate and acetic acid with acetonitrile at a ratio of 80:20 (pH=4.0; flow rate of 1.0 ml/min).The results of this study showed that caffeine content in soft drinks ranged from 19.63 to 101.73 mg/100ml and highest concentration of caffeine found in brand 3 samples while lowest concentration found in brand 2 samples. Significantly higher concentration of caffeine ($p<0.05$) found in six soft drinks sample when compared to BSTI and FDA reference value except brand 2 sample ($p>0.05$). Quantification of caffeine in different brands of energy drink sample revealed that, four brand sample contained caffeine; among them brand 3 sample showed the highest levels of caffeine 295.86 mg/100ml and lowest amount found in brand 1 sample (101.74 mg/100ml). Concentration of Caffeine in soft and energy drinks exceeded the national and international standard recommended range hence this proposed HPLC method can be used for routine determination and control of caffeine content in different drinks.

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Introduction

Caffeine (1,3,7-trimethylxanthine) is an alkaloid naturally present in the seeds, leaves and fruits of cocoa, coffee and tea and more than sixty other natural plants.[1,2] The availability in different plants gave caffeine and caffeine-containing products a long-standing popularity all over the world.[3,4] Besides, natural occurrence, caffeine is the most widely consumed legal psychostimulants nowadays and is present in many and diverse kinds of foods and beverages.[5,6] Pure caffeine is white crystalline powder, odorless and bitter in taste.[7] Caffeine exhibits a number of biological effects, feeling of heightened alertness, decrease fatigue, boost of energy, enhance motor performance diuretic and stimulant properties at low dose level but may be swapped to common psychological problem with high dose level.[8] Excessive caffeine intake by those with psychological problems may exaggerate symptoms like insomnia, headaches, irritability and nervousness.[9] Regular high doses of caffeine consumption are associated with cardiovascular disease, reduce cerebral blood flow, nervous system disorders, poor liver function[10] and stimulates gastric secretion[11] as well as produce toxic effect in combination with narcotics, alcohol and some other drugs.[12] Researchers found a significant correlation between high intake of caffeine and the risk of miscarriage and also observe irritability and wakefulness in a breast- fed baby during lactation.[13] Availability and chemical nature of caffeine made it ideal ingredient for soft drinks and beverage.[14] Caffeine has a unique taste and added to soft drinks as a flavoring agent to enhance the overall quality of soft drinks. Although caffeine is used as a flavor enhancer but the health safety issue to the consumer should are now the prime concerns. The Food and Drug Administration (FDA) specify the maximum limit for safe uses in carbonated beverages is 0.02% means 71mg of caffeine in 355 mL soft drinks.[15] Caffeine are using in different product and extensively in beverage at developing country like Bangladesh without following any rules and regulation. Hence, it is important to develop an appropriate analytical method to separate and determine concentration levels of caffeine in soft and energy drinksfor quality assurance purposes as well as consumer protection. Therefore, the aim in this study is to optimize, validate sample preparation procedures, identification and quantification of caffeine from different brands of soft and energy drinks brands available from the local market in Bangladesh by applying HPLC technique.

Material and Methods

Chemicals and Reagents

Sodium acetate (97%), HPLC grade acetonitrile, Glacial acetic acid and commercial standards of anhydrous caffeine were procured from Sigma Chemical Co. (Darmstadt, Germany). Deionized water (18.2 MΩ) was obtained from a Barnstead Nano pure water purification system (Barnstead, USA).

Sample Collection

A total of 90 (ninety) samples from ten brands of soft drinks and 54 samples from six brands of energy drinks were purchased from Bangladeshi market after getting verbal consent from shopkeepers. The expiry dates of all collected samples were within the study period.

Instrumentation and Operating Condition

The HPLC (Shimadzu) analysis operate with isocratic pump (10Avp), column, UV-Vis detector, degasser, oven and a LC Workstation Class-VP were used for data acquisition. A Luna 5 µ C18 (2) 100Å column (250×4.6 mm) at 37°C was used for the separation process. About 20µl sample was injected into the injector. The caffeine analysis was done using buffer of sodium acetate and acetic acid with acetonitrile at a ratio of 80:20 (pH=4.0; flow rate of 1.0 ml/min). The chromatograms were detected at the wavelength of 272 nm.[16]

Mobile Phase Preparation

The mobile phase prepared with buffer (80%) and acetonitrile (20) following slightly modified method of Pylypiw and Grether.[17] About 1 g of sodium acetate and 1 ml of glacial acetic acid mixed with 50 ml de-ionized water in a 1 L volumetric flask with proper shake and then added deionized water to make 1000 ml. After that 800 ml of the prepared acetate buffer were mixed with 200 ml of acetonitrile in another volumetric flask. The mixture was then filtered with a nylon-66 (pore size 0.2 µm) filter membrane.

Preparation of Standard Solution

About 50 mg of anhydrous caffeine and 20 ml 50%aqueous acetonitrile were taken in a 50 ml volumetric flask and shaking well and then added 50% aqueous acetonitrile up to 50 ml mark. The

prepare solution was then filteredwith a syringe filter and labeled as standard stock solution-1 (Concentration 1.0 mg/ml). Then 1ml of stock solution-1 was taken in 50ml volumetric flask and mobile phase were added up to mark and labeled as standard stock solution-2 (Concentration 20µg/ml). Similarly standard solutions 1.7, 3.12, 6.25, 12.5 and 25.0 µg/ml were also prepared by dilution of aliquots and filtered through sample filters (pore size 0.2 µm) prior to inject into the column.

Preparation of Sample Solution

Approximately 1ml of sample (soft drinks or energy drinks) was taken in 50 ml volumetric flask and 25% acetonitrile and 75% water were added up to the 50ml mark. After a proper mixing, 5ml of the sample solution was filtered with sample filter (pore size 0.2µm) and 20 µl was injected onto the HPLC column.

Caffeine Identification and Quantification

The wavelength of the stock caffeine was analyzed using UV-spectrophotometer at 272 nm. The retention time was set 4.62±0.2 minutes by running standard several times. To plot calibration curve, 20 µl of each standard solution (1.7, 3.12, 6.25, 12.5 and 25 µg/ml) were injected. Then peak area (y) was then plotted against the concentration (µg/ml) of the caffeine(x). Least square linear regression analysis was done to calculate the slope, correlation coefficients and y-intercept of the standards plots. Limit of quantification (LOQ) and Limit of detection (LOQ) were determined by considering 10 and 3 time signal to noise ratios respectively estimated by the regression lines mention in the previous report.[18] The correlation coefficient for standard curve was 0.9995.

Recovery Studies

Recovery studies were also carried out to verify the precision and accuracy by spiking some samples (2.0mg/l, 4.0 mg/l, and 6.0mg/l) from a known standard. About 2.0 ml of 25.0 mg/L standard were mixed with 2.0 ml of sample to make the concentration 12.5 mg/L and injected. To obtain the percentage of recoveries, observed concentrations were divided by known concentration and then multiply by 100. The spiked samples were measured in three replicates

Statistical Analysis

All the sample analyses were performed in triplicate and descriptive statistics were analyzed by using SPSS software package version 16.0 (SPSS Inc., Chicago, IL, USA) for all variables.The significance of the differences between the means of the two groups was determined by independent sample Student's t-test. Differences were considered to be significant at $p< 0.05$.

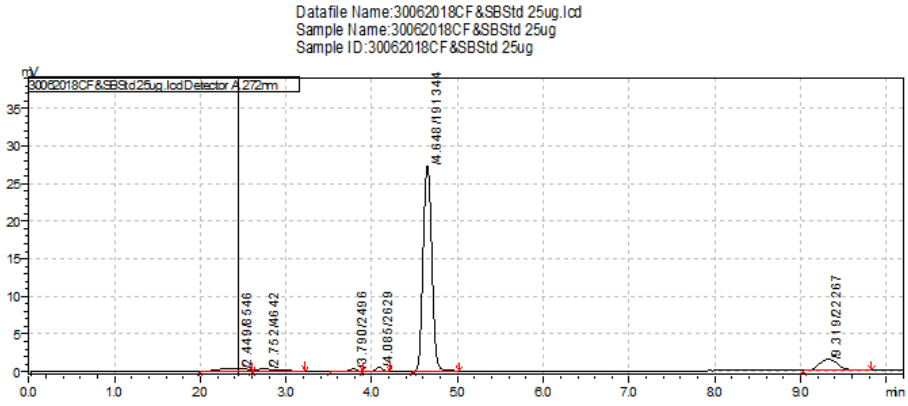

Fig.1: HPLC chromatogram of 20 µg/L caffeine standard solution

Results and Discussion

Analysis Of Chromatogram

Caffeine is used as a flavor enhancer in soft drinks due to its unique taste which helps to enhance the overall quality of soft drinks. But there are standard limit to use caffeine in soft drinks and other beverages products. This is the major concern to check whether the right amount is used or not. In this study Caffeine in different brands of soft and energy drinks was analyzed using HPLC.

The optimum flow-rate of eluent was set at 1.0 ml/min to identify caffeine standard and fixed the rate for all determinations. The overlaid UV spectrum showed good response at 272 nm for the caffeine. In optimized conditions, caffeine was separated with a resolution of more than 4 and the retention time was found to be 4.648 minutes (Figure 1). The calibration curve for caffeine was plotted on the basis of peak areas of chromatograms and a very good linearity was found as presented in figure with an excellent regression factor (0.9998).Linear regression line was obtained y=6820.6x-966.35 (Figure 3).

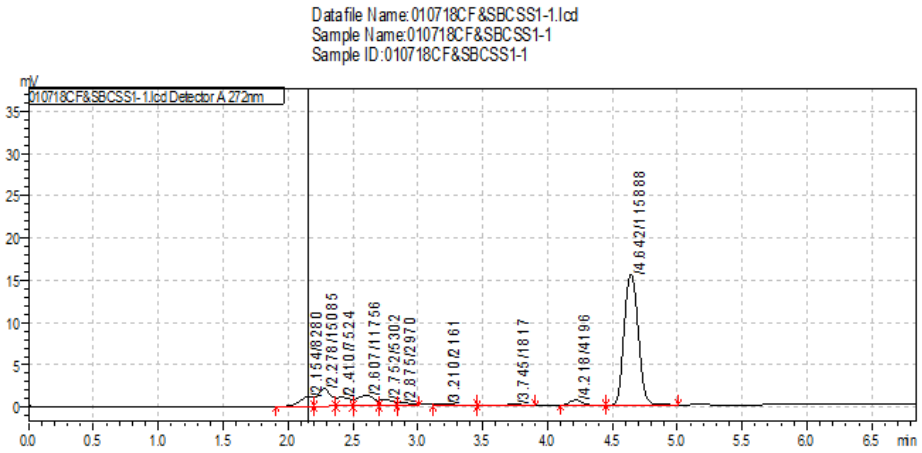

Fig. 2: Chromatogram of Brand -1 soft drinks obtained from shop-1

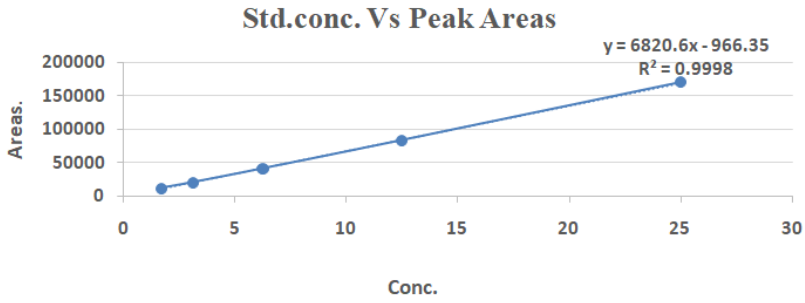

Fig. 3: Calibration curve for the caffeine standard

Table 1: Analytical characteristics of HPLC method

Parameter	Value
Accuracy	101.39±4.61
Slope	6820.6
Intercept	966.35
Linearity range	1.8µg/ml to 25.08µg/ml
Correlation coefficient	0.9998
SE of intercept	0.324
SD of intercept	0.0000475
LOD	0.00015mg/100ml
LOQ	0.00047mg/100ml

Table 1 revealed the analytical characteristics of HPLC method.A linear relationship was observed between the concentration of caffeine and the peak area in HPLC at 272 nm. The correlation coefficient for standard curve was 0.9998. Standard error of intercept and standard deviation of the intercept was obtained from the Microsoft Excel function as 0.324 and 0.0000475 respectfully. The quantification limit (LOQ) and detection limit (LOD) were calculated using ICH[19] method and found 0.000471mg/100ml and 0.00015mg/100ml respectively.

Analysis of Caffeine In Soft Drinks Sample

After HPLC analysis majority of the soft drinks (six out of ten) contained caffeine at different

level. No peak in the chromatogram of the sample brand 7, 8, 9 and 10 was matched to the peak of caffeine standard. The concentration of caffeine in soft drink samples ranged from 19.63 mg/100ml to 101.73 mg/100ml (Table 2). Highest concentration of caffeine found in brand 3 sample whereas lowest concentration of caffeine found in brand 2 sample. The mean concentration of caffeine found 75.17 mg/100ml which was higher than the mean concentrations 40.01 mg/L recorded by Hillary *et al*.,[20] in Coca-Cola drinksand 9.79 –14.38 mg/100 mL found by Nour, Trandafir and Ionică.[21]

Table 2: Concentration of caffeine different brand of soft drinks

Sample	Concentration of caffeine (mg/100ml)				Mean±SD (mg/100ml)
	Shop	Sample 1	Sample 2	Sample 3	
Brand 1	1	93.20	89.54	86.72	87.35±3.25
	2	91.27	86.64	88.62	88.84±2.32
	3	77.26	88.55	84.38	83.40±5.71
Brand 2	1	18.65	21.23	20.64	20.17±1.35
	2	20.82	21.56	21.47	21.28±0.40
	3	21.55	18.09	19.26	19.63±1.76
Brand 3	1	101.58	101.80	101.81	101.73±0.13
	2	99.77	95.89	98.19	97.95±1.95
	3	101.27	100.46	101.67	101.14±0.61
Brand4	1	70.95	70.29	71.05	70.76±0.41
	2	68.28	66.26	68.60	67.72±1.27
	3	66.59	64.80	72.20	67.86±3.86
Brand 5	1	98.41	90.65	99.70	96.25±4.89
	2	97.40	91.69	82.59	90.56±7.47
	3	95.10	92.93	88.35	92.13±3.45
Brand 6	1	79.85	78.64	76.25	78.25±1.83
	2	81.74	82.54	80.36	81.55±1.10
	3	83.34	83.89	84.80	84.01±0.74
Brand7	1	Nd	Nd	Nd	Nd
	2	Nd	Nd	Nd	Nd
	3	Nd	Nd	Nd	Nd
Brand 8	1	Nd	Nd	Nd	Nd
	2	Nd	Nd	Nd	Nd
	3	Nd	Nd	Nd	Nd
Brand 9	1	Nd	Nd	Nd	Nd
	2	Nd	Nd	Nd	Nd
	3	Nd	Nd	Nd	Nd
Brand 10	1	Nd	Nd	Nd	Nd
	2	Nd	Nd	Nd	Nd
	3	Nd	Nd	Nd	Nd

Nd= not detected; Data are expressed as Mean ± Standard Deviation

According to the US FDA and BSTI, the acceptable range for caffeine in soft drinks is 20mg and 14.5mg/100ml respectively.[15] Brand-3 soft drinks contained almost five times higher caffeine than the standard limit set by FDA and BSTI (Table 3). There was a significantly higher concentration of caffeine ($p<0.05$) found in six soft drinks sample when compared to BSTI and FDA reference value except brand 2 sample which is not statistically significant ($p>0.05$).

Table 3: Concentration of caffeine different brand of soft drinks

Sample	Concentration of caffeine (mg/100ml)			Mean±SD (mg/100ml)	BSTI (mg/100ml)	FDA (mg/ 100ml)	p-value
	Sample 1	Sample 2	Sample 3				
Brand 1	89.82	88.84	83.39	87.35±3.46	14.5	20	P^a=0.000; P^b=0.000
Brand 2	20.17	21.28	19.63	20.36±0.84			P^a=0.000; P^b=0.500
Brand 3	101.73	97.95	101.13	100.27±2.03			P^a=0.000; P^b=0.000
Brand4	70.76	67.71	67.86	68.77±1.71			P^a=0.000; P^b=0.000
Brand 5	96.25	90.56	92.13	92.98±2.94			P^a=0.000; P^b=0.000
Brand 6	78.25	81.55	84.01	81.27±2.89			P^a=0.000; P^b=0.000
Brand7	Nd	Nd	Nd	Nd			
Brand 8	Nd	Nd	Nd	Nd			
Brand 9	Nd	Nd	Nd	Nd			
Brand 10	Nd	Nd	Nd	Nd			

Nd= not detected; Data are expressed as Mean ± Standard Deviation; P^a value compared with BSTI; P^b value compared with FDA.

Table 4: Concentration of caffeine different brand of energy drinks

Sample	Concentration of caffeine (mg/100ml)				Mean±SD (mg/100ml)
	Shop	Sample 1	Sample 2	Sample 3	
Brand 1	1	96.93	98.75	97.64	97.77±0.92
	2	95.59	100.45	98.82	98.28±2.48
	3	108.96	107.83	110.74	109.18±1.47
Brand 2	1	105.48	106.98	110.21	107.56±2.42
	2	102.66	101.58	99.75	101.33±1.47
	3	119.20	113.10	115.05	115.78±3.12
Brand 3	1	296.48	301.07	305.72	301.09±2.62
	2	292.85	286.45	291.09	290.13±3.30
	3	300.94	295.19	293.01	296.38±4.10
Brand4	1	109.07	111.97	110.82	110.62±1.46
	2	111.57	112.00	110.64	111.40±0.70
	3	115.24	114.33	112.40	113.99±1.45
Brand 5	1	Nd	Nd	Nd	Nd
	2	Nd	Nd	Nd	Nd
	3	Nd	Nd	Nd	Nd
Brand 6	1	Nd	Nd	Nd	Nd
	2	Nd	Nd	Nd	Nd
	3	Nd	Nd	Nd	Nd

zNd= not detected; Data are expressed as Mean ± Standard Deviation

Determination of Caffeine in Energy Drinks Sample

Table 4 showed the concentration of caffeine (mg/100ml) in energy drinks sample. Identification of caffeine reveled that most of the branded sample contained caffeine higher that the recommended valued of BSTI and FDA. Highest concentration of caffeine found in brand3 energy drink sample whereas lowest concentration of caffeine found in brand 1 sample while in brand 5 and brand 6 samples no concentration of caffeine was detected (Table 25). Brand-3 energy drinks sample recorded the highest concentration of caffeine, which is 295.86 mg/100ml followed by brand-4 and brand 2 which is 112 mg and 108.[22] mg/100ml respectively. The caffeine concentration of the energy drink samples ranged from 101.74 mg/100ml to 295.86 mg/100ml which was below the concentration (969-3079 mg/100 ml) recorded by Nevena Grujic-Letic *et al*;22and higher the concentration (39.48 mg/100mL) found by Nour, Trandafir and Ionică.[21]

Table 5: Concentration of caffeine different brand of energy drinks

Sample	Concentration of caffeine (mg/100ml)			Mean±SD (mg/100ml)	BSTI (mg/100ml)	FDA (mg/ 100ml)	p-value
	Sample 1	Sample 2	Sample 3				
Brand 1	97.77	98.28	109.18	101.74±4.44	20	14.5	Pa=0.000; Pb=0.000
Brand 2	107.55	101.32	115.78	108.22±3.25			Pa=0.000; Pb=0.000
Brand 3	301.08	290.12	296.37	295.86±2.50			Pa=0.000; Pb=0.000
Brand4	110.62	111.40	113.98	112.00±1.76			Pa=0.000; Pb=0.000
Brand 5	Nd	Nd	Nd	Nd			
Brand 6	Nd	Nd	Nd	Nd			

Nd= not detected; Data are expressed as Mean ± Standard Deviation; Pa value compared with BSTI; Pb value compared with FDA.

Recommended value for caffeine in energy drinks according to FDA and BSTI is 20mg and 14.5mg/100ml respectively. Identification of caffeine in different brand energy drink sample reveled that, there was a significantly higher concentration of caffeine ($p<0.05$) found in four brand sample when compared to BSTI and FDA reference value. There was no caffeine was detected in other two brand sample (Table 5).

Recovery Studies

The known amount of caffeine was added to Brand-1 soft drinks at three different levels of concentration considered as: low (2.0µg/ml), medium (4.0 µg/ml) and high (8.0µg/ml). Percentage of recovery of three concentrations was 97.08±1.18, 96.35±1.48 and 95.41±0.92.

Conclusion

The outcome of the present study revealed that, some brands of soft (six out of ten) and energy drinks (four out of six) contained caffeine exceed the permitted range that is set by international body FDA and national authority BSTI. So government authorized agency such as BSTI should take control and regular monitoring to check the level of caffeine in all brands of soft and energy drinks.

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Conflict of Interest

Authors have declared that no competing interests exist.

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