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Determination of CaffeineIn Beverages: A Review

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ABSTRACT :*Caffeine is a well-known stimulant which is added as an ingredient to various carbonated soft drinks. Caffeine has drawn more attention due to its physiological effects beyond that of its stimulatory effect. Consumers are interested in knowing the exact amounts of caffeine existing in beverages. However, limited data exist, especially for store brand beverages. Therefore, it is pertinent to review the various methods that will effectively determine the caffeine contents in different carbonated drinks. HPLC, UV-Visible Spectrometry and Gas Chromatography are among the popular used methods.*

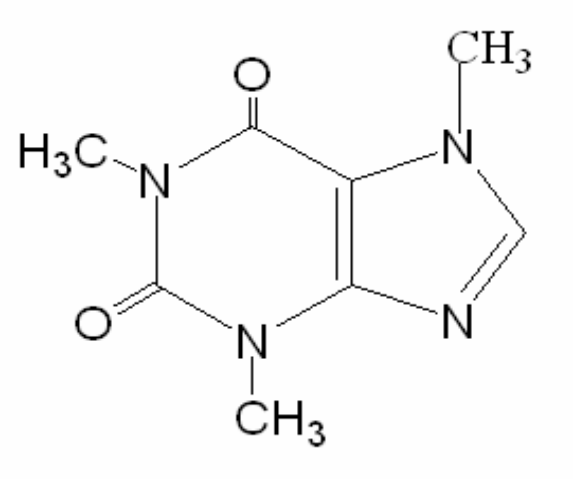
KEYWORDS;*Carbonated-drinks, Analysis, Extraction, Additive, Determination, Caffeine.*

I. INTRODUCTION

Caffeine is a naturally occurring alkaloid which is found in the leaves, seeds and fruits of over 63 plants species worldwide (Abdul Mumin*et al*., 2006; NourVioleta*et al*.,2008; Wanyika*et al*., 2010; VioletaNour*et al*., 2010) It is an alkaloid of methylxanthine family (Wanyika*et al*.,2010; Marcia *et al*., 2002). The methylxanthines caffeine (1,3,7-trimethyxanthine), theobromine (3,7- dimethylxanthine), and theophylline (1,3-dimethylxanthine) can be normally found in cola nuts, coffee beans, cocoa beans, tea leaves, mate leaves and other kinds of plants (Paradkar and Irudayaraj, 2002). While coffee and tea beverages naturally contain caffeine and other methylxanthines, caffeine serves as an ingredient in many carbonated soft drinks including colas, pepper-type beverages, and citrus beverages. Pure caffeine occurs as odorless, white, fleecy masses, glistening needles of powder. Its molecular weight is 194.19g, melting point is 236°C, point at which caffeine sublimes is 178°C at atmospheric pressure, pH is 6.9 (1% solution), specific gravity is 1.2, volatility is 0.5%, vapor pressure is 760mmHg at 178°C, solubility in water is 2.17%, vapor density 6.7 (Komes*et al*., 2009; NourVioleta*et al*., 2008; Hiroshi Ashihara*et al*., 1996; Abdul Mumin*et al*., 2006).Caffeine has drawn more attention in the past decades due to its physiological effects beyond that of its stimulatory effect. The Food and Drug Administration (FDA) defines caffeine as a generally recognized as safe (GRAS) substance. However, FDA specifies that the maximum amount in carbonated beverages is limited to 0.02% (FDA 2006). Therefore, the highest legal amount of caffeine allowed in a 355 mL (12oz) can of soft drink is about 71mg. Caffeine has attracted the interest of consumers and health professionals alike due to its wide consumption in the diet by a large percentage of the population and its pharmacological effects in humans (Mandel 2002). The human's saliva caffeine level, which demonstrates the extent of absorption, peaks around 40 minutes after caffeine consumption (Liguori*et al* 1997). Its physiological effects on many body systems have been reported by researchers, including the central nervous, cardiovascular, gastrointestinal, respiratory, and renal systems (Nehlig*et al* 1992). The International Olympic Committee (IOC) defined caffeine as a drug and abuse is indicated when athletes have urine caffeine concentrations higher than 12µg/mL (de Aragao*et al* 2005).

II. CAFFEINE CHEMISTRY AND GENERAL INFORMATION

Caffeine (1,3,7-trimethyxanthine), theophylline (3,7- dimethylxanthine), and theobromine (1,3-dimethylxanthine) are in the family of alkaloid methylxanthines.



Caffeine (1, 3, 7-trimethylxanthine)

Caffeine is an odorless, white solid that has the form of needles or powder. Caffeine has a bitter taste. The molar mass of caffeine is 194.19 g/mol. Caffeine is slightly soluble in water due to its moderate polarity. Caffeine is a natural central nervous system stimulant, having the effects of reducing drowsiness and recovering alertness. Since it is widely consumed by humans, caffeine is considered the most frequently used psychoactive substance in the world (Ligouri*et al* 1997).

Physiological effects of caffeine to human

Caffeine has numerous physiological effects on major organ systems, including the nervous system, cardiovascular system, digestive system, and respiratory system. Renal function and skeletal muscles are also affected by caffeine. Numerous studies have proven caffeine to be a stimulant to human's central nervous system (Spiller, 1998).It is also increase heartbeat rate, dilate blood vessels and elevate levels of free fatty acids and glucose in plasma. 1 g of caffeine leads to insomnia, nervousness, nausea, ear ringing, flashing of light derillum and tremulosness. In cases of overdosing and in combination with alcohol, narcotics and some other drugs, these compounds produce a toxic effect, sometimes with lethal outcome (Mamina and Pershin, 2002; Ben Yuhas, 2002; Wanyika*et al*., 2010; James *et al*., 1990; Tavallali and Sheikhaei, 2009). Caffeine facilitates the conduction velocity in the heart and directly affects the contractility of the heart and blood vessels. Nevertheless, caffeine may significantly reduce cerebral blood flow by constricting of cerebral blood vessels. Caffeine provides a diuretic effect due to elevating the blood flow and glomerular filtration rate of the kidneys. Heartburn is an issue for some subjects' gastrointestinal system after consuming caffeine. The effects of caffeine to skeletal muscles are mainly the increasing occurrence of tremors (James 1991; Spiller 1998).

Relevant Literatures

Many methods exist for determining the methylxanthine contents of food and beverages. Some of these methods include UV-Visible spectrophotometry, potentiometry, high performance liquid chromatography (HPLC), ion chromatography, high performance thin layer chromatography (HPTLC), capillary electrophoresis, micellar capillary electrophoresis, gas chromatography, and solid-phase microextraction gas chromatography (Armenta *et al.*, 2005). Of the above methods, HPLC has become one of the most commonly used analytical methods.One study demonstrated using an HPLC method with an octadecylsilyl (ODS) column and a water-acetonitrile-phosphoric acid mobile phase to analyze eight catechins and caffeine. Within 20 min, the catechins (epicatechin, epigallocatechin, epicatechingallate, epigallocatechingallate, catechin, catechingallate, gallocatechin and gallocatechingallate) and caffeine were separated by an acetonitrile gradient. Two different types of Japanese green teas, Matcha and Sencha, both high and low grades for each tea, had their catechins and caffeine contents determined. The researchers found the caffeine contents were higher in Matcha tea than in Sencha tea (Goto*et al.*, 1996). Wang *et al.*, (2000) applied an isocratic elution system to determine the contents of catechins, caffeine, and gallic acid in green and black tea. The separation system included a C18 reverse-phase column, a mobile phase of methanol/water/orthophosphoric acid (20/79.9/0.1), and an UV detector. The flow rate was set at 1.0 mL/min. The wavelength of detection was 210 nm. The validation of this method was confirmed by all analytes exhibiting good linearity within the range tested and correlation coefficients ranging from 0.988 to 1.000. The amounts of caffeine in Gunpower, roasted green tea (RGT), Sencha, Keemun, and Sri Lanka were found to be 23.9, 30.3, 28.9, 38.2, and 22.9 mg/100 mL, respectively (Wang *et al.*, 2000).

Mashkouri*et al.*, (2003) quantitated the caffeine existing in black tea leaves by Fourier transform infrared (FTIR) spectrometry. The caffeine of tea samples was extracted using $CHCl_3$ after wetting with an aqueous NH_3 solution. The spectrometric data were collected over the wave number range of 1800-1300 cm^{-1}. This 11 method had a detection limit of 35µg/mL, a sampling frequency of 6 h^{-1}, and a coefficient of variation of 0.8%. A black tea sample contained 3.68% w/w caffeine. The authors obtained similar results for the caffeine content from FTIR (3.68 ± 0.03% w/w) and a reference HPLC technique (3.60 ± 0.07% w/w). The advantages of the FTIR method for determining caffeine in tea leaves includes its quickness, precision, and accuracy, enabling it to be a possible alternative to the HPLC method (Mashkouri*et al.*, 2003). However, one potential shortcoming of this method is the fairly high detection limit.

Nishitani and Sagesaka (2004) developed an improved HPLC analytical method for simultaneously determining caffeine and the eight catechins as well as other phenolic compounds in tea. The proposed method provided additional ability to analyze phenolic compounds when compared with former HPLC methods. This procedure was based on an improved reverse-phase ODS column operated at 4°C, a binary gradient elution system of water-methanol-ethylacetate-phosphoric acid, and a photodiode array detector. The quantitative measurement of eight catechins and caffeine confirmed the validity of this proposed method. The detection limits of these analytes ranged from 1.4-3.5 ng per injection volume. The recovery rates of the analyses were in the range of 96-103%. The caffeine contents of Sencha, Matcha, Gunpowder, Tie Kuan yin, and Darjeeling determined in this study were 2.94±0.007, 3.62±0.005, 2.61±0.059, 2.51±0.019, and 3.24±0.016% (dry weight), respectively (Nishitani and Sagesaka 2004). Caudle *et al.*, (2001) tried to improve the Association of Official Analytical Chemists (AOAC) official analytical method for analyzing methylxanthines in cocoa-based food products. Theobromine and caffeine contents could be obtained by 12 reverse-phase HPLC. The AOAC method's degree of accuracy and precision was not reliable, especially for caffeine. In this study, the AOAC analytical method only showed recoveries of theobromine and caffeine to be 89.3 and 74.5%, respectively. The authors successfully changed from an organic extraction to an aqueous extraction and analyzed the samples via reverse-phase HPLC to improve the recoveries of theobrominethe samples via reverse-phase HPLC to improve the recoveries of theobromine and caffeine to 99.6 and 103.4%, respectively (Caudle *et al.*,2001).

Zuo*et al.*, (2002) analyzed various substances in several green, Oolong, black and pu-erh teas by HPLC. They used a methanol-acetate-water buffer gradient elution system and a C-18 column; detection utilized a photodiode array detector. After multiple extractions with aqueous methanol and acidic methanol solutions, four major catechins, gallic acid and caffeine could be simultaneously determined within 20 min. This improved the previous studies' problem of catechins and caffeine remaining in tea residues after a single extraction. The results demonstrated that green teas contain higher amounts of catechins than Oolong, pu-erh, and black teas due to their fermentation processes reducing the levels of catechins significantly. An interesting finding was a lower caffeine content in Oolong teas, especially in Fujian Oolong tea (Zuo*et al.*, 2002).

Horie*et al.*, (1997) adapted capillary zone electrophoresis (CZE) in order to simultaneously determine the major compounds in green tea. Separation occurred in a fused-silica capillary column. The borax buffer was set at pH 8.0, and UV detection was at 200 nm. The major compounds in green tea were epicatechin, epigallocatechin, epicatechingallate, epigallocatechingallate, catechin, caffeine, theanine, and ascorbic acid. The authors found the concentration of each compound was significantly different among each tea sample. One interesting finding was relatively lower caffeine contents in canned tea drinks. The authors concluded CZE is more appropriate for analyzing the properties and contents of green tea than HPLC due to its shorter analysis time and ability to separate more compounds (Horie*et al.*, 1997). Schulz *et al.*, (1999) investigated the accuracy of rapidly predicting the amounts of polyphenol and alkaloid compounds in the leaves of green tea by a near-infrared reflectance spectroscopic (NIRS) method. The pretreatment of the NIR spectra with weighted multiple scatter correction effectively eliminated interferences of scatter and improved the final calibration results. The results were compared with those from analysis by HPLC. The potential of this NIRS method is demonstrated by the high correlation between its prediction and HPLC values for caffeine and major catechins. The authors claimed that the NIRS method may be an alternative technique to HPLC due to its high degree of accuracy for prediction and analysis time of less than 1 min per measurement (Schulz *et al.*, 1999). Farah *et al.*, (2006) investigated the relationship between the Arabica coffee cup quality and the contents of sucrose, caffeine, trigonelline, and chlorogenic acids. The researchers applied reverse-phase HPLC analysis to determine each compound. Sucrose was analyzed by using 80% acetonitrile and 20% water as the mobile phase and a refractive index detector. For analyzing caffeine, the UV detector was set at 272 nm. The mobile phase was composed of

60% water and 40% methanol. The results demonstrated that the caffeine content was the highest in the highest quality sample and the lowest content was found in the poorest quality sample. However, trigonelline and 3,4-dicaffeoylquinic acid gave a better indication of high quality coffee (Farah *et al.,* 2006). Huck *et al.,* (2005) compared the contents of caffeine, theobromine, and theophylline in 83 liquid coffee extracts determined by a NIRS method and HPLC coupled to mass spectrometry method. In the NIRS method, the spectra were recorded over a wave number range of 4008 to 9996 cm^{-1} with a resolution of 12 cm^{-1} in the reflectance mode. The authors obtained high robustness and reproducibility of the NIRS model for quantification of caffeine and theobromine. The lower limit of detection made it difficult for theophylline to fit the NIRS model and correctly be determined. Nevertheless, NIRS provides the coffee industry with an alternative method to quickly determine caffeine and theobromine (Huck *et al.,* 2005).

Chen and Wang (2001) analyzed the level of artificial sweeteners (sodium saccharin, aspartame, acesulfame-K), preservatives (benzoic acid, sorbic acid), caffeine, theobromine, and theophylline in carbonated cola drinks, fruit juice drink, fermented milk drink, preserved fruit, and one pharmaceutical preparation by an ion exchange chromatography method. Analytes were separated using an anion-exchange analytical column maintained at 40°C and detected by wavelength-switching ultraviolet absorption. The detection limits ranged from 4-30 ng/mL for all analytes. The average recoveries for samples ranged from 85 to 104%. In addition, the data obtained from this method were in good agreement with those determined by reference HPLC procedures. Two carbonated cola drinks were found to contain around 36 mg caffeine/12 oz (Chen and Wang 2001). Chen *et al.* (2006) investigated the feasibility of using near infrared (NIR) spectroscopy as a fast method which is non-destructive and less time consuming thanother frequently used analytical methods for estimating the content of caffeine and total polyphenols in green tea. The calibration was performed by a partial least squares (PLS) algorithm. The result indicated that correlation coefficients of the prediction models were approximately 0.97 for the caffeine and 0.93 for total polyphenols. This method's potential to rapidly determine the caffeine and polyphenols of tea to control industrial processes has been proven by this study (Chen *et al.,* 2006).

Yao *et al.* (2006) examined 20 leaf tea and 36 teabag samples obtained from Australian supermarkets. Each sample was prepared as a diluted tea solution, which was treated with lead acetate and hydrochloric acid solutions. After filtering and treating with a sulfuric acid solution, the measurement of caffeine was completed by using a UV/Visible spectrophotometer at 570 nm. The results showed that caffeine contents of black leaf tea and teabags were 3.89 and 3.87%, respectively. Similar results were found in the green leaf tea and teabags, 3.71 and 3.83%, respectively. These contents are generally higher than that claimed by the manufacturers (i.e., < 3%). This study revealed a need to establish quality control for both imported and Australian-made teas (Yao *et al.,* 2006). Brunetto*et al.* (2007) developed a reversed-phase HPLC method with an on-line sample cleanup to determine theobromine, theophylline, and caffeine in cocoa samples. The cocoa samples were prepared by an on-line solid-phase extraction of analytes and loaded into a home-made dry-packed precolumn with ODS-C18 in a column-switching system. The mobile phase consisted of 20% of methanol in water, under isocratic conditions, at a flow-rate of 1.4 mL/min. Chromatographic separation was performed on a NOVA-PAK C18 column (150 mm x 3.9 mm, 4 μm). The procedure demonstrated a recovery of over 95% with coefficients of variation less than 3.2%. The precolumn proved its long average life span by showing no signs of deterioration after approximately 1000 injections of sample cocoa extracts (Brunetto*et al.,* 2007).

Pura (2001) modified a HPLC method for determining caffeine and theobromine contents in aqueous cocoa extracts. Instead of directly injecting the extracts on the column, the improved method can successfully remove the interfering cocoa pigments by passing them through a Sep-pak C18 cartridge which was also used to separate the theobromine and caffeine. This method enhanced the efficiency of the column and prolonged its life. After this treatment, the recoveries of caffeine and theobromine were 98.0-100.1 and 97.8-100%, respectively. The modified method displayed good resolution and sharp peaks on chromatograms that favored correct determination of theobromine and caffeine (Pura, 2001). Thomas *et al.* (2004) measured the contents of caffeine, theobromine, and theophylline in a food-matrix standard reference material (SRM) 2384, Baking Chocolate by a reverse-phase HPLC method. The stationary phase was composed of an inactive silica support to which C-18 was bonded. The mobile phase consisted of 10% acetronitrile90% water (pH acidified to 2.5 with acetic acid). The flow rate was at 1.5 mL/min and UV detection was at 274 nm. The results of each sample could be obtained within 15 min. The results showed the reproducibility for caffeine, theobromine, and theophylline determinations was 5.1, 2.3, and 1.9%, respectively. This method had a limit of determination for all analytes at levels less than 100 ng/mL or 0.1μg/mL. The measurements of caffeine, theobromine, and theophylline of SRM 2384

BakingChocolate were comparable with those from National Institute of Standard and Technology (Thomas *et al.*, 2004). Abourashed and Mossa (2004) applied HPTLC densitometric analysis to determine the level of caffeine in several herbal products and energy drinks. The HPTLC plates were made of pre-coated silica gel. The solvent system contained 85% ethyl acetate and 15% methanol. The wavelength for detecting caffeine was set at 275 nm. The proposed method had a mean recovery of 98.9 ± 3.5% with a coefficient of variation less than 5%. The caffeine ranges of herbal products and energy drinks in this study were found at 4.76-13.29% (w/w) and 0.011-0.032% (w/w), respectively. The HPTLC method demonstrated effective determination of caffeine for stimulant herbal products and carbonated energy drinks (Abourashed and Mossa 2004). Armenta *et al.* (2005) applied a solid-phase Fourier transform-Raman (SP-FT-Raman) spectrometry-based method to determine caffeine contents in commercial energy drinks. The caffeine content of each sample was obtained from setting Raman intensity between 573 and 542 cm^{-1} with a two points corrected baseline between 580 and 540 cm^{-1}. The limit of detection of SP-FT-Raman method was 18 µg/mL. The combination of FT-Raman and solid-phase increased the sensitivity of detecting caffeine by a factor of 31 times when compared with using direct Raman measurement alone. The results of caffeine contents obtained from SP-FT-Raman method and liquid chromatography (LC) found no significant differences between the two methods. The SP-FT-Raman method displayed higher sampling frequency than the LC method. However, the LC method had a lower detection limit (0.05 µg/mL). The reduced reagent consumption and waste generation are also benefits of this method as compared to the LC method (Armenta *et al.*, 2005).

Lucena*et al.* (2005) manipulated a continuous flow auto analyzer for sequential determination of total sugars, class IV caramel and caffeine contents in 20 different soft drink samples. This apparatus consisted of on-line coupling of a continuous solid-phase extraction unit and two detectors which were UV-visible and evaporative light scattering (ELSD) detectors. The caffeine has the property of being retained on the sorbent column and other compounds can be preferentially determined due to their low affinity to the sorbent column. The caffeine can be detected later by the ELSD after it has been eluted with acetonitrile and the signal registered in the ELSD. In order to evaluate the performance of this analyzer, the authors carried out a recovery test. The results ranged from 90 to 102%. Unspecified colas were found to contain caffeine ranging from 14.9 mg/12 oz to 49.7 mg/12 oz (Lucena*et al.*, 2005). Walker *et al.* (1997) utilized capillary electrophoresis (CE) to simultaneously analyze the aspartame, benzoic acid, and caffeine contents of carbonated beverages in 2 min with 20 mM glycine buffer at pH 9.0 and detection at 215 nm. Good reproducibility for both peak area and migration times were observed (2.0-3.8% and 0.13-0.37%, respectively). The spiked recovery of the analytes ranged from 98 to 114%. The results of soft drinks samples in this study were comparable with those data evaluated by HPLC, but slightly higher in some cases using CE. The main advantages of CE over HPLC are relatively simpler operation, lower cost, no organic mobile solvents, and a shorter analysis time (Walker *et al.*, 1997).

Types of drinks : Non-alcoholic soft drink beverage can be divided into fruit drinks and soft drinks. Soft drinks can be divided into carbonated and non-carbonated drinks. Examples of carbonated drinks are Cola, lemon and oranges and non-carbonated drinks include mango drinks. Soft drinks can also be divided into cola products and non-cola products. Cola products like Pepsi, Coca-Cola, Thumps Up, and Diet Coke, Diet Pepsi etc. account for nearly 61-62% of the total soft drinks market. Non-Cola products constitute 36%, and based on the types of flavors available can be divided into Orange, Cloudy Lime, Clear Lime and Mango (India Infoline Sector Report, 2002).Below are highlight of some work which employed the various methods in analyzing the level of caffeine from different beverages.In this review, I will attempt to go in details into some of the experiments carried out in determining caffeine content and also publishing of some of their results as shown below;

The quantitative determination of caffeine in beverages and soft drinks using UV wavelength spectroscopy

JENWAY**,** producersof instrumentations for Chemistry related practical, sampled five beverages and soft drink. Samples were chosen they include instant coffee (Nescafe), brewed tea (PG Pyramid Tea Bags), Coca Cola, Pepsi Cola and Red Bull. The analysis is performed on a Jenway 7305 spectrophotometer controlled using the free-of charge PC software, supplied with each model in the 73 series. The software allows the user to emulate all measurement tasks normally performed on the instrument with the additional benefit of allowing data to be seamlessly transferred to external Microsoft office applications. TheReagents used are Caffeine,Dichloromethane and Purified water

Standard Preparation: A 1000 ppm stock standard of caffeine was prepared by dissolving 198.2mg of caffeine in 200.0ml purified water. Working standards were prepared by pipetting 25, 12.5, 10, 7.5, 5 and 2.5ml aliquots of the stock standard solution into separate 50.0ml volumetric flasks and diluting to volume with purified water.
Sample Preparation: 200ml aliquots of boiling purified water was added to each of two 250ml beakers containing 2g of instant coffee and a single PG pyramid tea bag (3.2g of dried tea leaves) respectively. The coffee and tea preparations were stirred for 30 seconds using a magnetic stirrer (500rpm) and allowed to cool to room temperature.

Caffeine Extraction Procedure:A 50ml aliquot was taken from each working standard or sample solution. This aliquot was placed into a separating funnel and 25ml of dichloromethane was added. The caffeine was extracted by inverting the funnel at least three times, venting the funnel after each inversion. The dichloromethane layer was removed to a clean flask and the extraction procedure was repeated twice more and the solvent layers combined.

Sample Measurement: Aliquots of the extracted standards were placed into quartz cuvettes (part code 035 028) and analysed using a 7305 spectrophotometer. The Photometrics mode was accessed by selecting the Photometrics icon from the main menu screen in the PC software and measurements were performed according to the procedure described in the instrument and PC software operating manuals. Microsoft Excel was used to tabulate the measurement data and perform a linear regression analysis. This allowed a concentration factor to be calculated which was then input into the settings in the Concentration mode of the 7305 PC software. The concentration mode was then used to quantify the caffeine concentration of the sample solutions with measurements performed against a dichloromethane blank.

III. RESULT

The absorbance values of the six working standard solutions were measured;
A linear regression of absorbance versus standard concentration, forced through the origin, gave equation 1.

$$y = 0.0181x \quad \text{[1]}$$

A linear regression of concentration vs absorbance allowed the factor of 55.358, included in equation 2, to be determined.
Equation 2 was then used to calculate the concentration of caffeine in the extracted sample solution, from the solutions measured absorbance value.

$$\text{Conc (ppm)} = 55.358 \times \text{Abs} \quad \text{[2]}$$

The final caffeine content of the beverage under test is then calculated from the extracted sample solution's concentration using equation 3. Dividing this value by the volume of the drink gives the caffeine content per ml.

$$\text{Caffeine content mg} = \text{Conc (ppm)} \times \frac{(\text{Total Sample Vol [ml]})^2}{(\text{Measured Sample Vol[ml]}) \times 1000} \quad \text{[3]}$$

Of the five samples tested the sample of Red Bull had the highest total volume per ml content of caffeine. When ranking the drinks in terms of caffeine content per serving the order was Red Bull ⇒ Pepsi Cola ⇒ Instant Coffee ⇒ Coca Cola ⇒ PG Tea. Instant coffee and PG Tea rise from third and fifth places to second and third respectively when the drinks are ranked in order of the caffeine content per ml.

IV. CONCLUSIONS

When the results obtained in this application note are compared to data published in previous reports, or by the drinks manufacturer, it can be seen that the results obtained using the 7305 spectrophotometer are broadly in line with expectations with the exception of the two cola drink samples. The higher than expected values obtained for the cola drink samples may be the result ofadditional compounds, that absorb light at thewavelength used in this application note, beingextracted into the dichloromethane samplesolution. The resulting solution would give ahigher than expected absorbance reading andcalculated caffeine content. Alternativeextraction procedures could be investigated tosee of the interfering compounds could beexcluded from the extraction solution.Also using UV spectrophotometric procedures Ahmad H, *et al* 2005 determined the content levels of some food additives in 29 different beverage samples commercially available in Riyadh local markets. These analytical measurements were undertaken primarily to assess the compliance of content levels of the investigated food additives and their daily intake doses with the permissible levels. They observed that the results obtained from this study indicated that the average quantity level of caffeine in the analyzed beverages is 18.99 ppm.

In addition, the concentrations of these food additives have been converted into the daily intake doses based on beverages consumption. It was estimated that the mean daily intakes of aspartame, caffeine and

sodium benzoate by the adult population of Riyadh city through the consumption of the analyzed beverages were 92.5 mg, 6.3 mg and 6.46 mg, respectively. None of the analyzed beverage samples was found to violate the current legal limits practiced in the Saudi food regulations.In order to establish the spectrophotometric determination of caffeine, they studied thewavelength within the interval 220–320 nm using a 3.88 ppm caffeine standard solution. The obtained results gave an absorption spectrum, which was characterized by a single intensive absorption band located in the UV range at λmax = 276nm.

It was observed that as the concentration of caffeine was varied over the range from 2.5×10^{-6} to 3.6×10^{-4}mol l^{-1} (0.5-70 ppm), it was accompanied by a proportionalenhancement in the monitored absorption intensity over such wide concentration range.
The calibration equation was calculated by least-squares method from nine measurements and it has the form:

$A = -0.032 + 1.01 \times 10^{4}$ C (mol l^{-1}) r = 0.999, n = 9

In addition, the analytical utility of the employed quantitative method was also investigated in similar manner to that previously discussed for the spectrometric analysis of aspartame artificial sweetener. The recovery of the used procedure, which reflects the accuracy of the analytical method, was evaluated by analyzing caffeine-free drink sample spiked with 9.7 ppm caffeine. The mean recovery of five measurements obtained by standard addition approach was found to be 97.66% with standard deviation of ±0.3%. The mean of the obtained results was found to be not significantly different from the value of added caffeine concentration, since the calculated t-test value (2.6) was less than the tabulated t-test value (4.6) at 99% confidence level. The analytical precision of the spectrophotometric method was assessed from the reproducibility of 10 determinations of 10 ppm caffeine solution and a relative standard deviation of 0.1 RSD% was calculated.

Practical determination of the studied food additive in commercial drink samples : Different kinds of beverages brands, including regular and diet cola, carbonated refreshment drinks, beverages with added fruit juices, energy drinks and preservatives free canned fruit juices were purchased from different local supermarkets and 29 samples were analyzed in quintuplicate (*n* = 5) using the indicated spectrophotometric method. Once sample bottles were open, the drinks were degassed, homogenized andfiltered. In all cases, five aliquots of each drink sample were placed in the spectrophotometric cell after adequate dilution. In order to reduce the interference effectparticularly that expected with fruit juices, all analytical determinations were carried outby the standard addition approach.

They discovered that the concentrations of caffeine food additive (flavor enhancer) in what so called energy drinks collected from local supermarkets are noticeably higher than their counterpart concentration levels in the refreshment soft drinks. The calculated analytical results in Table 3 and Table 4 demonstrate the caffeine content levels in energy drinks and carbonated soft drinks, respectively. The caffeine contents in energy drink samples ranged from 22.64 ppm to 34.96 ppm. The minimum caffeine content level was observed in Drink 4 sample, while Drink 2 sample showed the highest caffeine content. The mean of caffeine quantity in the analyzed energy drinks was found to be in the level of 28.23 ppm. However, the analyzed carbonated soft drink samples contained much lower caffeine contents since its mean concentration level of 9.76 ppm is virtually one third the average caffeine content observed in energy drinks. The analyzed samples in the carbonated soft drink group showed caffeine content in the range of 2.8 - 12.76 ppm.

Table 1. Caffeine content levels in the carbonated soft drink

Soft drinks	Caffeine content (ppm)	Daily intakes (mg)
Regular Cola	1 8.58 ± 0.04	3.25
Regular Cola 2	11.89 ±0.07	4.5
Regular Cola 3	8.84±0.02	3.38
Regular Cola 4	2.84±0.02	1.07
Diet Cola 1	12.76±0.03	4.79
Diet Cola 2	11.77±0.08	4.41
Lemon Cola 11	62±0.07	4.38

All over the world, the caffeine contents in soft drinks varies according to the type of the brand, yet its average content in soft drinks is approximately 18 mg per six ounce (i.e. 100 ppm) (Barone and Roberts, 1984.). In fact, the US Food and Drug administration (FDA) limits the maximum caffeine amount in carbonated beverages to 6 mg/oz (72 mg/355 ml). Therefore, caffeine content level allowed in soft drinks is up to 200 ppm.

Clearly, the caffeine mean content level in the analyzed beverage samples manufactured and marketed in Riyadh city, is well below the above food industry guidelines.

Quantitative Determination of Caffeine and Alcohol in Energy Drinks and the Potential to Produce Positive Transdermal Alcohol Concentrations in Human Subjects

According to the Journal of Analytical Toxicology, Vol. 33, January/February 2009 Quantitative analysis of caffeine was performed by gas chromatography–mass spectrometry (GC–MS) using an Agilent 5975 MSD based upon a previously published procedure by "S. Kerrigan and T. Lindsey. Fatal caffeine overdose: two case reports.*Forensic Sci. Int.* **153(1):** 67–69 (2005)". Briefly, energy drinks were diluted 1:100 with 100mM pH 6.0 phosphate buffer prior to analysis. Energy drinks, calibrators and controls (1 mL) were fortified with 50 µL 0.1 mg/mL caffeine-d10 in methanol. Because of the large dilution of energy drinks (1:100), calibrators and controls were prepared directly in phosphate buffer. A methanolic working standard was used to prepare caffeine calibrators in the range 1–10 mg/L (0.001–0.010 mg/mL). Samples were transferred to SPE columns and drawn through the column under vacuum. Columns were then successively rinsed using 1 mL deionized water, 1 mL acetic acid (1 M) and dried under full vacuum for 5 min. Ethyl acetate (1 mL) was added to the column and the eluate collected. Columns were rinsed once again using methanol (1 mL). A secondeluent consisting of ethyl acetate with 2% concentrated ammonium hydroxide (1 mL) was added and the eluate collected. The two fractions were combined, evaporated to dryness under nitrogen at room temperature, and reconstituted in 25 µL of ethyl acetate.

Samples were analyzed by GC–MS using an Agilent 6890 GC with a 5975 MSD. The injector and interface were set at 250 and 280°C, respectively. Separation of components in each 2-µL injection was achieved using a 30-m DB-5 capillary column.Injections were made in split mode with a 10:1 split ratio. Following an initial oven temperature of 160°C and hold time of 0.5 min, the temperature was increased at 30°C/min to 290°C. The final hold time was 7.17 min, and the total run time was 12 min. Helium was used as the carrier gas at a flow rate of 1.3 mL/min. Caffeine (*m/z* **204**, 115, 70) was used as the internal standard for the quantitative determination of caffeine (*m/z* **194**, 109, 67). Acquisition was in selected ion monitoring mode, and quantitation ions are shown in bold. The limit of quantitation (LOQ), defined as the concentration of caffeine that produced a signal-to-noise ratio of at least 10:1 with a calculated concentration within 20% of the expected value, was < 1 mg/L. The linear range of the assay was 1–25 mg/L, accuracy was 102%, and intra-assay CV was < 3% (n =2) at 1 mg/L.

Linear regression analysis of calibrators in the range 0–10 mg/L yielded an $R2$ value of 1.000 and the control sample fortified with 1.0 mg/L caffeine produced a calculated concentration of 1.02 mg/L (102%). Quantitative caffeine determinations in diluted samples yielded concentrations ranging from 2.74 to 5.31 mg/L. These correspond with caffeine doses of 65–126 mg per 8-oz serving. In another study where caffeine content of energy drinks was quantitatively determined, doses of 33–77 mg were reported per 8-oz serving (16). Although caffeine content in the beverages tested in this study were considerably higher (65–126 mg) for equivalent serving sizes, results for the one energy drink (Red Bull) that was included in both studies were in excellent agreement: 67 mg and 69 mg, respectively. Caffeine (1,3,7-trimethylxanthine, guaranine) is a plant-derived alkaloid and psycho stimulant that is present in tea leaves, coffee, cocoa beans, and kola nuts. Individuals may be exposed to caffeine via beverages, food, over-the-counter drugs, prescription drugs, dietary supplements, and cosmetic treatments. An average cup of coffee is reported to contain 100 mg caffeine, although much higher doses have been reported, particularly among specialty coffees (17). Caffeine is also available in numerous dietary supplements, over-the-counter drugs, and in prescription drug mixtures at doses ranging from 32 to 200 mg (18). A dose of 50–200 mg is generally consistent with mild stimulation.

Ken-Hong, (2005) analyzed the caffeine content of 56 types of national and 75 types of store brand carbonated beverages. The caffeine determination was accomplished by utilizing high performance liquid chromatography (HPLC) equipped with a UV/Visible detector. The mobile phase consisted of 20%:80% (v/v) acetonitrile and deionized water. The chromatographic separation occurred on two C-18 columns. Each beverage sample was diluted 3-fold with deionized water. Duplicate analyses of multiple lots were performed on all beverage samples.

Chemicals and reagents

Anhydrous caffeine used for preparation of the standard solutions was purchased from Sigma (St. Louis, MO, USA). The acetonitrile for the mobile phase was HPLC grade (Fisher Scientific, Pittsburgh, PA,

USA). Deionized water was obtained from a water purification system. Sodium phosphate monobasic and HPLC- grade 85% phosphoric acid was obtained from Fisher Scientific (Pittsburgh, PA, USA).

Preparation of standard solution

Caffeine (about 25 mg) was weighed with an electric balance and transferred into a 250 mL volumetric flask. Deionized water was added to get a 250 mL bulk standard solution. Sonication was applied to completely dissolve the caffeine. One vial was filled and labeled with the bulk standard. The 2nd, 3rd, and 4th vials were obtained through consecutive 2-fold dilution with deionized water by pipetting (Precision Pipette, Atlanta, GA, USA). A second bulk solution was prepared using about 15 mg caffeine/250 mL water. The second bulk was diluted in the same manner as described above. The eight standard solutions were stored at 4°C in the refrigerator. These eight standard solutions were analyzed during each day's analysis to prepare the appropriate standard curve.

Preparation of mobile phase

Volumetric flasks were used to measure 250 mL of acetonitrile and 1000 mL of deionized water to achieve 20 % acetonitrile concentration (v/v). Sodium phosphate monobasic (1 g) was dissolved into the solution. The purpose of adding sodium phosphate monobasic was to increase the mobile phase's resistance to pH change. Phosphoric acid was added to acidify the solution to pH 3. The solution was vacuum filtered through a 0.45µm nylon filter. The solution was poured into a storage bottle and degassed by sonication.

Samples and sample preparation

The national-brand prepackaged (e.g., cans, bottles) carbonated beverages were collected across the southeastern United States. The samples were stored at room temperature until analysis. The store-brand beverages were acquired from Bruno's, Food Lion, Dollar General, IGA, Winn-Dixie, Kroger, Ingle's, Piggy Wiggly, Publix, Save-a-lot, 7-Eleven, Rite-Aid, Walgreens, Supervalu, and Wal-Mart. The cola, citrus, and pepper-type carbonated beverages as well as their diet varieties were analyzed in the present study. Average caffeine contents of each carbonated beverage were determined from a minimum of two different lots. The beverages analyzed in this study were purchased from June 2005 to July 2006.

Each beverage (50 mL) was poured into an Erlenmeyer flask and degassed in a sonicator. Each sample was diluted 3-fold with deionized water (1 mL sample + 2 mL water). Duplicate dilutions were performed on all samples. An aliquot of these diluted samples was injected into the HPLC system to quantitate the caffeine concentration.

Apparatus

The caffeine content was determined by isocratic reverse-phase high performance liquid chromatography (HPLC) equipped with a UV/Visible detector adapted from that used by Grand and Bell (1997). The injector with a 20 µL loop introduced a known sample volume into the system. The chromatographic separation occurred on a Prodigy 150-mm x 4.6-mm C-18 column (Phenomenex, Torrance, CA, USA) in series with a Novapak C-18 150-mm x 3.9-mm C-18 column (Water, Eatontown, NJ, USA). The mobile phase consisted of 20%:80% (v/v) acetonitrile and deionized water, acidified to pH 3 with phosphoric acid. The combination of these two analytical columns was designed to eliminate the interference of caffeine separation caused by other components in some samples, such as colors, artificial sweeteners, flavors, and preservatives. The wavelength of detection was set at 254 nm and flow rate was set at 1 mL/min. Caffeine eluted around 4.1 min. Data were recorded by a Hewlett Packard HP3395 integrator (Palo Alto, CA, USA). Sample chromatograms for Diet Coke and Dr. Pepper are shown in Figure 3.1 and 3.2, respectively.

Test for HPLC recovery and variability

Specific amounts (12.6 mg and 43.1 mg) of caffeine were measured and put into different 250 mL volumetric flasks. Degassed caffeine-free diet coke (250 mL) was added to each volumetric flask to obtain two spiked samples. A 1 mL aliquot of the first spiked sample was transferred to 5 vials and diluted 3-fold with deionized water. The same method was used to treat the second spiked sample to obtain another 5 diluted solutions. Samples were analyzed using the HPLC method described previously; using the standard

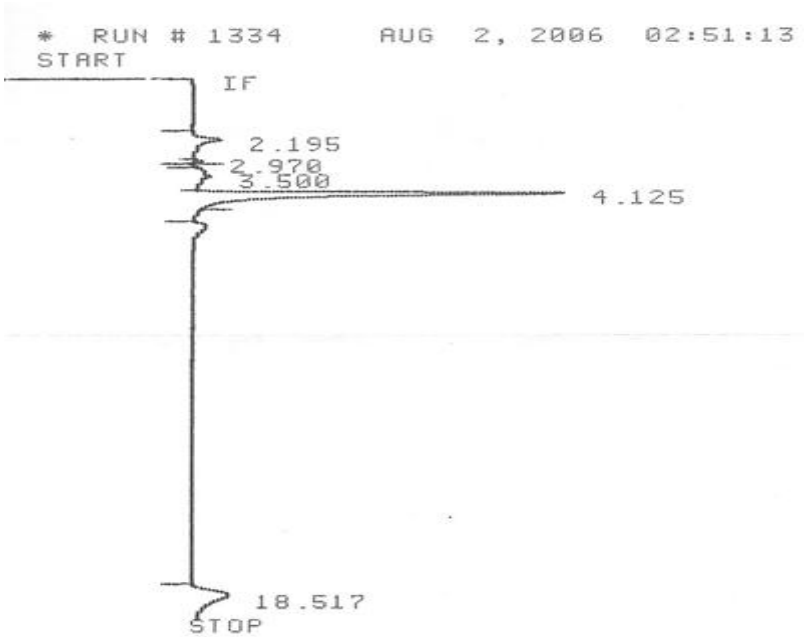


Figure showing Chromatogram of Diet Coke HPLC analysis by two C-18 columns using 20%/80% (v/v) acetonitrile and deionized water as mobile phase.

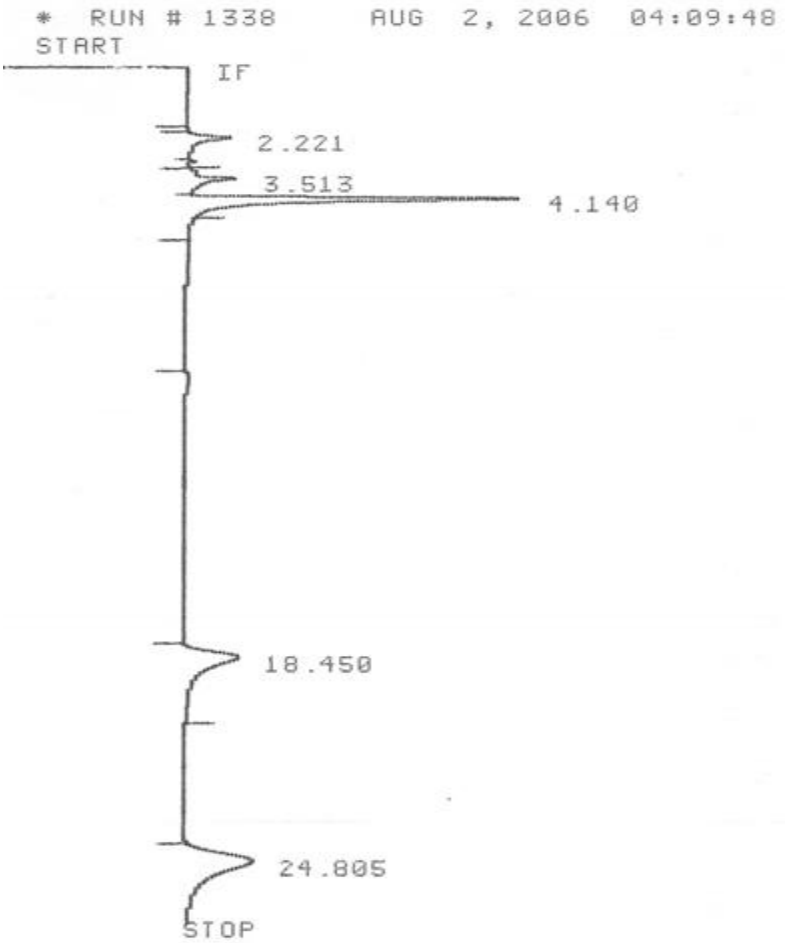


Figure showing Chromatogram of Dr. Pepper HPLC analysis by two C-18 columns using 20%/80% (v/v) acetonitrile and deionized water as mobile phase

calibration curve, the concentration of each sample was calculated. The coefficients of variation were determined from the standard deviation of the measurements divided by the sample's average. The percent recovery was calculated by the average of the measurements divided by the original concentration. The percent recovery and coefficients of variation were 96.7 to 100.8 % and 0.6%, respectively for this analytical method. These values are similar to that found by Grand and Bell (1997).

V. DATA ANALYSIS

The caffeine contents of the samples were calculated using the peak areas reported by the integrator and the standard curve. The caffeine content per 12 oz can was calculated. Every type of beverage had duplicate measurements per lot, which were averaged to give the mean caffeine content for the lot. Data from these duplicate dilutions were typically found to vary by less than two percent. The caffeine contents for the various lots were then averaged to give the mean caffeine content for the beverages.Within the national brand beverage category, the lowest caffeine content was determined to be in Ritz Cola, which contained 10 mg caffeine per 12 oz. The highest caffeine values of the national brand beverages were observed among Vault Citrus, Vault Zero, and Diet SunDrop at 70-74 mg caffeine per 12 oz. There is approximately a 6-fold difference between the highest and lowest caffeine values within national brand beverages. Most colas and pepper-type products contained around 40 mg caffeine per 12 oz. A more robust extent of quality control appeared to exist in the national brand carbonated beverages as demonstrated by less lot to lot variability. Within the store brand beverage category, the IGA Cola contained the lowest caffeine value (5 mg per 12 oz). The highest caffeine contents (around 60 mg per 12 oz) were found in Big Fizz Cola and Dr. IGA. The caffeine contents within beverage types were quite disperse for the store brand beverages. In addition, variability between lots was greater than for the national brand beverages. The caffeine data of the current study may be used as a more extensive database to replace that removed from the American Beverage Association website and improve the vague classification of beverages by the USDA. The caffeine data determined in the present study suggests that consumers concerned about limiting daily caffeine ingestion from carbonated beverages may select the lower caffeine-containing store brand beverages. Consumers desiring caffeine may likewise select from the higher caffeineproducts. However, broad generalizations about the caffeine contents of carbonated beverages are difficult to make. The varied contents should be either accounted for in databases or caffeine values placed on food labels so consumers can be better informed.

Mei Musa *et al.*, (2012) in his study of determining caffeine in some Sudanese beverages by high performance liquid chromatography validate simple revered-phase HPLC.

VI. MATERIALS AND METHODS

Experimental: All reagents used in this study were of analytical or HPLC grade and all solutions were prepared by using distilled water.

Standard solution preparation: Caffeine stock standard solution of 1000 ppm was prepared by dissolving 0.1000 gram of caffeine standard (Sigma- Aldrich) in 80 mL distilled water and sonicated for 10 min. Then solution was transferred to 100 mL volumetric flask and the volume was completed to mark by distilled water. The stock solution was stored in dark places at +4°C. Working standard solutions were prepared by suitable dilution for stock solution and they were prepared freshly.

Sample preparation: Different kinds of beverages brands including regular and diet cola, energy drinks, tea and coffee were purchased from different local supermarkets and 15 samples were analyzed using the HPLC method. All measurements were performed in triplicate were opened, the drinks were degassed by sonication, homogenized and filtered. Then each sample was filtered through a 0.45 µm syringe filter with a 5 mL syringe. Filtered drink sample of 2 mL were 5 times diluted in distilled water. 20 µl of each diluted sample was injected into the HPLC column. The relative peak areas were determined for three replicates of each dilute sample. Then the concentration of each dilute sample and finally the concentration of caffeine in soft drinks samples were calculated from calibration curve. One sample of Pepsi cola, Coca cola, energy drink (Red Bull) was spiked with 20 ppm caffeine standard for recovery determination.

Tea and coffee samples preparation: 2.00 g of tea and coffee samples were weighed and put into 250 mL beakers.100 mL of boiling distilled water was added and let to stand for five minutes with stirring, the solution was cooled and filtered into conical flasks. 5 mL of the filtrate were pipetted into clean 50 mL volumetric flasks

and made to the mark with the distilled water. The samples were filtered through 0.45 µm syringe filter and run in the HPLC system according to experimental conditions.
Tea and coffee samples were spiked with 20 ppm caffeine standard for recovery determination.
Instrumentation: The HPLC system used in this study was isocratic Waters HPLC, which consisted of a model 1515 isocratic pump, vacuum degasser and 2996 PDA detector (USA). The injector was a model 7725i Rheodyne injector with injection loop 20 µL. The analytical column used was Shim-pack VP-ODS with internal diameter 4.6 mm and length 250 mm (Shimadzu Corporation, Kyoto, Japan). All chromatographic results were acquired and processed by Empower software (Waters Corporation).

VII. METHOD VALIDATION

Precision: The analytical precision of the method was assessed from the reproducibility of 6 determinations of40 ppm caffeine solution and a relative standard deviation of 1.25% was calculated for peak area.
The retention time of caffeine was 7,347 min, with a relative standard deviation RSD = 0.5% therefore, instandard solutions, the HPLC method provides stable retention times.

Detection and quantification limits: Table 2 summarizes the method detection limit (MDL) andMethod Quantification Limit (MQL). MDL was estimated as Standard Deviation (SD) of the peak area of seveninjections multiplied by 3.14 (at n = 7). MQL was calculated by multiplying SD by 10 (Dionex, 2007).

Linearity: The calibration graph was generated using 20µl injection loop. Six different concentrations of caffeinefrom 10 ppm to 100 ppm were analyzed according to experimental conditions. Then the calibration curve wasestablished according to the obtained response (peak area) and the concentrations of caffeine in standardsolutions. The results show a good linear relationship.

Rachel *et al.* while studying the caffeine content o specialty of coffees using samples of Starbucks Breakfast Blend purchased over the course of six consecutive days from Starbuck shop in Gainesville, FL, used a method that can also be used in determining caffeine content in carbonated drinks. The caffeine was extracted from coffee using a liquid-liquid extraction procedure. To 0.1 mL of coffee, 10Ul of mepivacaine and 10uL of 10M of NaOH was added. All samples were vortex mixed for 5s, and 4.0mL of chloroform (Fischer, certified ACS) was added. All samples were place on a rotator for 15 min and then centrifuged for 10 min 3000rpm. The organic layer was then transferred to auto sampler vials, and analysed by gas chromatography (GC).
1.0mg/Ml caffeine (Alltech-Applied science labs) stock standard solution was prepared in methanol (Fischer Scientific, certified ACS). Caffeine stock control solution were prepared in methanol from two separate sources (Alltech-Applied Science Labs and Sigma-Aldrich Company), both at concentrations of 1.0mg/mL. A 1.0-mg/mL solution of mepivacaine was prepared in methanol for use as an internal standard.Quantization of caffeine was based on a calibration curve prepared in a concentration range of 50-500mg/L with mepivacaine as the internal standard. Control samples, prepared at concentrations of 75 and 250mg/L (three each), were included in each batch.An Agilent 6890 series GC system with nitrogen-phosphorus detector was utilized. The GC was fitted with a cross-linked methyl siloxane capillary column (HP-5, 30m x 0.32-mm i.d.,0.25-mm film thickness) with ultra-high-purity helium as the carrier gas (constant flow rate, 1.0mL/min). Injections (0.5mL) were made in the splitless mode. The GC temperature settings were as follows: injection port, 250^{o} C; detector, 300^{o} C; initial column temperature, 90^{o} C; hold time, 0.50 min. The total run time was 11.70 min.

VIII. CONCLUSION

The validated HPLC method for the quantification caffeine in beverages was found to be simple, precise, sensitive and accurate and allowed the obtaining of good results. In spite of the number of drink samples analyzed is small, the data presented in this study gave a preliminarily outline about the content levels in tea, coffee, soft and energy beverages frequently.Shim-pack VP-ODS column was used with methanol: water (30:70)% (v/v) eluent. The detector wavelength was set at 270 nm. Linearity of the method was check from 10-100 ppm and the correlation coefficient was 0.9999. The method detection limit was 0.023 ppm and the precision was 1.25% at 40 ppm caffeine concentration. The spiked recoveries for caffeine were 99%, 105%, 99.2%, 102% and 102% in Pepsi Cola, Coca Cola, Red Bull, Gazaltain black tea and Coffee samples respectively. The caffeine contents in tea samples ranged from 440 ppm to 473 ppm with average concentration of 458.6 ppm. The caffeine concentrations in energy drinks samples ranged from 170.6 ppm to 324 ppm with average concentration of 255.6 ppm. The coffee sample contains 252.4 ppm. The carbonated soft drinks showed caffeine content in the range of 32.4 ppm to 133.3 ppm with average concentration of 96 ppm. In addition, the concentrations of caffeine have been converted into the daily intake doses based on beveragesconsumption. The mean values of caffeine daily intakes were 183 mg, 101 mg, 64 mg and 38 mg throughthe ingestion of tea, coffee, energy drinks and soft drinks, respectively.

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