Determination of Caffeine and Sodium Benzoate in Both Imported and Locally Manufactured Energy Drinks Using HPLC and Spectrophotometer

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Abstract

Three groups of energy drinks have been examined for sodium benzoate and caffeine using HPLC and spectrophotometer methods. Those brands include original UK brand which coded (RE), another two brands of (RL and B) which are imported from Austria to local companies, and the rest produced locally which are coded (T, FB, S and FE). The results showed that all brands contain sodium benzoate at different concentrations. FB brand comes out on top by containing around 416mg/l which was far above the permitted level by FAO/WHO, on the other hand the RE brand contained the lowest amount of sodium benzoate when analysed using HPLC. Results of caffeine examination showed that all samples contained lower levels of caffeine as it is claimed in their label to contain 300mg/l, in contrary to sodium benzoate the lowest concentration of caffeine found in the FB brand which was 13.78mg/l and the highest level of caffeine was found in FE brand which was 208.95mg/l. From the obtained results it is clear that none of the energy drink brands declared the actual content of both caffeine and sodium benzoate which usually required by food legislation and law. Therefore, it is considered as mislabelling which is recommended that the health authorities in our country to subject the local producers and the importers to present the required information on the labels without misleading consumers. It is also recommended to educate the population and put restriction on the local shops and supermarkets not to sell energy drinks to individuals under 16 years old.

Keywords: Energy drinks, Sodium benzoate, Caffeine, HPLC, Spectrophotometer.

1. Introduction

Beverages, especially, energy-drinks is becoming a part of the daily life among most of the people particularly between teenagers due to the producers claims of their role in enhancing body performance and boosting energy [1]. They are non-alcoholic beverages, usually but not necessarily carbonated, with some added sweeteners, acids, and flavouring agents. Different types of energy drinks are available in local markets. However, due to their content of caffeine and synthetic preservatives it may pose a health risk to consumers particularly young individuals who consume it daily solely or mixed with other drinks, Energy drinks generally contain stimulants, chiefly caffeine, taurine, guarana, and ginseng [2]. Due to the pleasant taste and availability at a low price, the population demand and sales of commercial energy drinks are increasing all over the world [3].

Different compounds are added to these beverages, these include different types of food additives, especially preservatives and antioxidants to enhance and maintain the drink quality and its characteristics as well as promoting food safety [1]. Food additives are also widely used to improve organoleptic properties of the energy drink and to prolong the shelf life [4]. The main and important ingredient in any energy drink is usually caffeine which is a xanthine alkaloid, and it acts as a diuretic and a stimulant on the central nervous system [5]. It is absorbed and transferred throughout the body by blood to the brain [6]. Although the consumption of caffeine allowable in certain amounts, the overconsumption of these ingredients could potentially be harmful to the human body [7]. This may be the cause of different sicknesses such as nausea, vomiting, restlessness, anxiety, depression, tremors, and difficulty sleeping. The acceptable range of caffeine for the average adult is 250 mg per day [8]. Caffeine added as a flavouring agent and to make the drinks acceptable among people and as addictive chemical [5]. Pure caffeine is an odourless, white solid powder and has a bitter taste. The molecular weight of caffeine is 194.19g/mol, and its melting point is 236°C. Caffeine is soluble in water with the maximum solubility of 21.7g/L at room temperature due to its moderate polarity [2].
Figure 1. Caffeine structure.

Caffeine is widely used in foods and beverages as a stimulant. It is a white crystalline powder with a chemical formula of $\text{C}_8\text{H}_{10}\text{N}_4\text{O}_2$. It has a bitter taste and occurs in many plants such as coffee beans, tea leaves and cocoa nuts [9]. Due to the presence of Caffeine, manufacturers always proclaim that energy drinks can relieve fatigue, restore energy, and promote alertness. Numerous investigational reports have indicated that intake of Caffeine in large amounts is associated with many clinical diseases such as coronary heart disease, myocardial infarction, cancers (urinary tract, kidney, and pancreas), anxiety, and fibrocystic breast disease, nervous system, digestive system, and respiratory system. It also increases heartbeat rate, dilates blood vessels and elevates the level of free fatty acids and glucose in plasma [10,11].

In addition to caffeine, energy drinks also contain chemical preservatives to prevent the spoilage, improve the microbiological stability and any alterations in taste. Benzoic acid and/or benzoate are commonly used as preservatives in many foods and beverages [12]. Benzoic acid is a simple aromatic carboxylic acid, it can prevent the internal proton pumping level to cross the plasma membrane of microbial cells; as a result, it has been used to inhibit microbial growth and food spoilage, in non-alcoholic beverages [13]. Due to the electrolytic nature of the sodium benzoate, it is 200 folds more soluble than benzoic acid in water. Therefore, it has been used more widely in soft drinks, particularly for carbonated and non-alcoholic beverages [13]. Sodium benzoate allowed to be added to beverage on the limited range if it will use more than range will be toxicity and has potential effects on human health. The acceptable level of Sodium benzoate is 0-5 mg per kg daily [14].

Figure 2. Benzoic acid and Sodium benzoate structure.

This study aimed to determine the caffeine and sodium benzoate content of different energy drinks consumed in local markets. Moreover, comparing the two common approaches used to determine Sodium benzoate and caffeine content in energy drinks.

2. Materials and Methods

2.1 Materials

All solvents and chemicals used were of analytical grade. In the study, high purity standards of (Benzoic acid(purity-99.9%), Caffeine standard > 99%, Acetic acid glacial (100%), Ammonium acetate(98%), lead acetate, either sodium benzoate, Sodium salicylate and petroleum benzine were purchased from Merk (Darmstadt, Germany). Methanol (100%) and acetonitrile HPLC grade were purchased from Caledon Company (Canada). Potassium hexacyanoferrate(II)99.95%, Zinc sulfate, sulfuric acid, sodium hydroxide and hydrochloric acid were purchased from Scharlau Co. (Spain). Deionised water was prepared by a Branstead Easypure II system (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.2 Methods

2.2.1 Sample collection

The samples of energy drinks were composed of seven brands, purchased from the grocery shops and supermarkets of Sulaimani city from December 2019 to January 2020 and each brand coded as following original UK brand coded
(RE), imported from Austria to local company which coded (RL and B), and the rest produced locally which are coded (T, FB, S and FB). Five of them - were in packed in aluminum cans and two of them were packed in glass bottles. All samples were stored at 4°C in the refrigerator until the analysis is performed. All experiments were completed within their shelf lives period. For each brand a triplicate determination of each parameter was performed for the reliability of the results.

2.2.2 pH determination

Energy drinks acidity was determined using the Sartorius pH meter (model Jenway 3510) calibrated with buffer solutions of 4.0 and 7.0 according to the AOAC (2000) [15].

2.2.3 Determination of benzoic acid using High-performance liquid chromatography (HPLC)

The HPLC method performed according to ISO No. 22855 (2008) [16] with some modifications. In this study internal standards used to improve accuracy and precision in determining the parameters

2.2.3.1 The preparation of the mobile Phase

The mobile phase consisted of 80% of ammonium acetate buffer (pH4.2) and %20 HPLC-grade. After the preparation of the mobile phase, it was mixed, degassed, filtered through a 47 mm×0.45μm membrane filter, and used for sample dilution, standard dilution and the HPLC mobile phase.

2.2.3.2 The preparation of the standards

Exactly 100.0 mg of sodium benzoate stock solution (100.0 mg/ml] were added to a 100.0 ml volumetric flask and brought to volume with HPLC-grade water., Dilution of the stock solution (mobile phase) was made separately in to HPLC-grade water to obtain 500, 250, 125, 75, 50 and 25 mg/L of sodium benzoate solution. Sodium salicylate was used as internal standard.

200 microliter of internal standard (1,000 mg/L) and 1,000 μL of working solutions of sodium benzoate was diluted 1:10 with mobile phase in a 10 mL volumetric flask to yield 100, 50, 25, 15, 10, and 5 mg/L calibration standards, respectively.

2.2.3.3 Preparation of the HPLC samples

The samples were prepared by mixing 10ml of the liquid samples with 75 ml of extraction solution (mobile phase) in a 100ml volumetric flask, after that the flask was put to the ultrasonic bath, mix the content for at least 10 min and then make up to the mark with extraction solution at 20°C, were filtered through a 25 mm×0.45 μm nylon filter to remove particulate matter from the samples.

2.2.3.4 HPLC Apparatus

The analysis was carried out by Knauer k-1001 HPLC system with K-2600 UV detector, Knauer Degasser, K-1001 pumps.

The HPLC operating mode was isocratic, the injection volume was 10μL, and the column temperature was 20°C. The chromatography column was a C8, 250 mm×4.6mm, particle size 5μm, The flow rate was 1-2 ml/min and UV detection was performed at 225 nm.

2.2.4 Determination of caffeine by High-performance liquid chromatography (HPLC)

The HPLC method used according to [17] and [18], with some modifications. Internal standards used to improve the accuracy and precision in the current study.High-Performance Liquid Chromatography (HPLC), Equipped with HAISIL100A-C18 (150mm x 4.6mm, 5μm particle) analytical column, UV-Visible detector.

2.2.4.1 Preparation of standard stock solution

Standard stock solutions of caffeine were prepared by weighing 10ml from the energy drink 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). A standard stock solution was further diluted with mobile phase to prepare serial solutions for the standard curve.
2.2.4.2 Sample preparation

The drinks from sample containers were transferred into beakers then degassed and homogenized, then each sample was filtered using 0.45μm filter paper. Then 2ml of filtrate of each sample diluted 20 folds with mobile phase solution.

2.2.4.3 Chromatographic conditions of isocratic elution system

HPLC analysis was performed by isocratic elution (mobile phase). The flow rate was 1mL/min. The mobile phase composition was deionised water: methanol (40:60 v/v), adjusting pH to 5 using diluted (HCl / NaOH). The injected solvent was filtered through 0.45µm filter paper and degassed by using an ultrasonic water bath, the volume of injected solvent was 20µL and the wavelength 227 nm, running time for overall analysis was less than 3 minutes as shown in figure3.

2.2.4.4 Calibration curve

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

2.2.4.5 Optimization of HPLC method

Separation of caffeine was carried out by using a mixture of water-methanol of (40:60v/v), adjusting pH to 5 using diluted NaOH, at a flow rate of 1ml/min with the isocratic program and retention was (7.232) min.

2.2.5 Determination of sodium benzoate using spectrophotometer

The UV method used according to ISO No. 5518 and 6560 procedure (ISO) 1978:1983[19].

2.2.5.1 The preparation of the standards

Exactly 100.0 mg of either sodium benzoate dissolved in a 100ml volumetric flask. Dilution of the stock solutions were prepared using distilled water to obtain different concentrations of 60, 50, 40, 30, 20, 10 mg/L of sodium benzoate standard solutions. Then, hydrochloric acid (0.4mL, 6M) was added to the 5 ml of standard solution and then extracted with 45 ml petroleum benzine. The absorbance of the sodium benzoate standard samples was detected at 227 nm.

2.2.5.2 Sample Preparation

10 g of the samples with 90 ml of deionised water for 2 min, the blended sample was filtered, and 0.4 ml of hydrochloric acid (6M) was added to 5.0 ml of the filtered liquid. It was extracted by 45 ml of petroleum benzine.

2.2.5.3 UV Apparatus

The determination of sodium benzoate was carried out using Shimadzu UV-visible spectrophotometer system (UV-1601-PC) add country of origin. with UV detection at 227 nm.

2.2.6 Determination of caffeine by UV/ Vis spectrophotometer

The caffeine content of energy drinks detected by UV/ Vis spectrophotometer according to [20] as following:

2.2.6.1 Preparation of standard solutions of Caffeine

Caffeine stock solutions (1000 ppm) was prepared by dissolving 100mg of pure caffeine in 100ml of distilled water. Then a serial of caffeine working solutions of 0, 10, 20, 40, 60 and 80 ppm or (mg/l) were prepared by dilution of the stock solution in 25 ml volumetric flasks with addition of 1.0 ml hydrochloric acid.

2.2.6.2 Sample preparation and analytic determination

20 ml of sample solutions were pipetted into 250 ml flask then 10ml of 0.01mol/ L hydrochloric acid and 2ml basic lead acetate solution were added and filled the flask with distilled water, shaken up and filtered to clarify. 50 ml of the filtrate was
pipetted and added into 100 ml flask. 0.2ml 4.5M Sulfuric acid was added and the volume completed with distilled water, shaken up and filtered. The absorbance of the standard solutions and samples were measured on a UV/Vis spectrophotometer (Shimadzu) at 274 nm using 10 mm quartz cuvette. The caffeine levels of the samples were calculated from the regression equation of the best line of fit of the standards [21].
The following equation obtained to determine the caffeine content in ppm:
\[
\text{Regression equation (R.equ) } y (\text{Conc.mg/ml}) = (-0.002+0.018) \times \text{(absorbance)}
\]
\[
\text{Caffeine concentration (ppm)} = (\text{Caffeine concentration from R.equ.) (DF) (1000, 000)}
\]

2.2.7 Experimental design and statistical analysis
Analysis of variance (ANOVA) was performed to evaluate the caffeine and sodium benzoate content of the different energy drinks. The data were statistically analyzed through the method of analysis of variance as a general test of the treatments while the comparisons among means, the reversed least significant different test Duncan at P< 0.05 iss used.

3. Results and Discussion
3.1 The Sodium benzoate content of different energy drinks
3.1.1 Determination of sodium benzoate using HPLC method
Table 1 shows that the highest level of Sodium benzoate (416.194mg/l) was obtained in the FB brand, while the lowest level was observed in the RE brand (14.124mg/l). Similarly, sodium benzoate concentrations in the FE brand was 209.200 mg/l, and it was 208.290 mg/l. The content of Sodium benzoate in the S brand was 179 mg/l, which is three folds higher than the value in the T sample. These results are consistent with those of [21], who found that the Sodium benzoate concentrations in Iranian brands were (147.72 and 11.54ppm, respectively) was roughly similar to that of B and RE brands. Also, the maximum amount of sodium benzoate in international brands was 251.50 ppm, which is comparable to the concentrations in the RL and FE brands.

| Brands | Sodium benzoate (mg/l) ± SD |
|--------|----------------------------|
|        | HPLC                      |
|        | spectrophotometer          |
| FB     | 416.19 ± 1.08 *            |
|        | 160.66 ± 0.29d             |
| FE     | 209.20 ± 0.08b             |
|        | 669.66 ± 0.41a             |
| RL     | 208.29 ± 0.34b             |
|        | 425.10 ± 0.1c              |
| S      | 179.00 ± 0.45c             |
|        | 533.50 ± 0.5b              |
| B      | 122.09 ± 0.07d             |
|        | 113.46 ± 0.05e             |
| T      | 57.18 ± 0.19e              |
|        | ND                         |
| RE     | 14.41 ± 0.4f               |
|        | 112.80 ±0.3f               |

Different letters indicate significant differences between different energy drink brands (p<0.05).

3.1.2 Determination of sodium benzoate using spectrophotometer
On the other hand, when a spectrophotometer was used to determine the Sodium benzoate contents of different energy drinks different results to HPLC data were obtained as shown in table 1. A higher level of sodium benzoate was obtained for FE (669.667 mg/l) which was 400 mg/l higher than that obtained from the HPLC method. T sample had a lower level which was (zero or not detectable) while it was 57.18 mg/l where HPLC was used. The same trend is observed for the rest of the samples, S and RL were (553.5and 425.1mg/l) respectively, therefore, these brands had a higher level than a permitted level by the European Commission (150mg/l) [22].Moreover, B, RE brands had a compatible level with permitted levels were obtained (113.467 and 112.8mg/l) respectively. According to FAO/WHO Expert Committee on food, additives, the daily intake of benzoate is 0-5 mg/kg body weight [23]. The concern is that sodium benzoate can convert to benzene when vitamin C is available in the drinks, which may be harmful and carcinogen for human even at low levels if consumed regularly. It is also found that inflammation, Attention Deficit Hyperactivity (ADHD) and many other health problems [24]. Surprisingly, despite the presence of high concentrations of sodium benzoate in some brands such as B, RE, RL and FE their manufacturers did not mention it on the label. On the other hand, other companies such as S, FB and T have admitted the existence of this substance without mentioning its concentration in their products.
3.2 The caffeine content of different energy drinks

Other experiments were performed to determine the caffeine content of different energy drink samples using both HPLC and spectrophotometer.

3.2.1 The caffeine content of different energy drinks using HPLC

It is observed that caffeine contents of FE and RL brands were (208.95, 208.77mg/l, respectively) which were higher than the content of caffeine compared to the other samples as shown in table 2. In contrast, the amounts in T and FB samples were lower (57.189 and 13.783 mg/l, respectively), which were consistent with [25] who determined the caffeine content in soft beverages, and they found that they contained between 8.43 to 50.63 ppm. While the RE, B and S brands contain lower concentrations of caffeine 185.077,182.762 and 156.553 mg/l, respectively. These findings are higher than 150-200 mg/l of caffeine, which is approved by European Union legislation. Products that contain more than 150 mg/l of caffeine must the advisory statement high caffeine content followed by the amount of caffeine contained in mg/100ml. However, according to the claims on their labels, the caffeine content of all samples was (0.03%) which is equivalent to 300 mg/l that is higher than the determined caffeine content in all brands.

Table 2. Caffeine content of energy drinks analysed by HPLC and UV-spectrophotometer at 272nm.

| Brands | Caffeine (mg/l) ± SD | HPLC       | spectrophotometer |
|--------|----------------------|------------|-------------------|
| FE     | 208.95 ± 1.6 a       | 516.00 ± 2.6 a |
| RL     | 208.77 ± 1.41 a      | 322.00 ± 1.8 c |
| RE     | 185.07 ± 1.19 b      | 343.06 ± 16.3 d |
| B      | 182.76 ± 4.44 b      | 298.53 ± 4.4 e |
| S      | 156.55 ± 0.37 c      | 418.53 ± 5.3 b |
| T      | 57.18 ±0.33 d        | 388.00 ±16.4 c |
| FB     | 13.78 ±1.22 e        | 246.66 ± 21.7 f |

Different letters indicate significant differences between different energy drink brands (p<0.05).

3.2.2 The caffeine content of different energy drinks using spectrophotometer

On the other hand, the caffeine content of different energy drinks was analysed using UV-spectrophotometer and the results are as shown in table 2.

3.3 A comparison between HPLC and UV-spectrophotometer in determining sodium benzoate in energy drinks

Figure 3 emphasizes the differences between different analytical methods used to determine the sodium benzoate content in different samples in the current study. The highest concentration of sodium benzoate determined by spectrophotometer was 669.6 mg/l in the FE brand when spectrophotometer is used, while it was 209.20 mg/l when HPLC was used which was more than three folds lower. Sodium benzoate in the T brand was not detectable while a concentration of 57.18 mg/l was detected by HPLC. Generally, as shown in figure 1 the obtained concentration of sodium benzoate in all samples were higher when the spectrophotometer was used except for the T brand. Therefore, it is more reliable to use HPLC instead of the spectrophotometer may be due to the Limit of Detection LoD of the spectrophotometer at lower concentration or less sensibility. As observed the higher levels in both methods for FE and FB brands were above the European Commission’s permitted level (150 mg/l) [26].
Figure 3. The comparison between HPLC and spectrophotometer in determining sodium benzoate in different energy drink brands.

Overall, the results demonstrate that there are significant differences between the RL sample by spectrophotometer and the RL sample by HPLC, with the benzoic content being two folds than the RL brand found by spectrophotometer (425.1 mg/l). FE sample, on the other hand, was detected by HPLC at a higher level than the spectrophotometer level.

3.4 A comparison between HPLC and UV-spectrophotometer in determining caffeine content of different energy drinks

Figure 4 illustrates the levels of caffeine content in different energy drinks determined by spectrophotometer and HPLC. Generally, as demonstrated in sodium benzoate determination using both methods it can be observed that the same trend is repeated in caffeine determination for all samples in which the concentration of caffeine determined by spectrophotometer was generally greater than those measured by HPLC. As a result, it is clear that the highest value was obtained in FE brand 516mg/L, which was contrary to FDA approved caffeine, which limited the maximum caffeine content of cola-type soft drinks to 0.02% caffeine, or 71 mg/12ounce beverage[26], and that higher caffeine energy drinks were similar to results reported by [26]. While the lowest caffeine content was detected by HPLC in FB and T samples were (13.7 mg/l) and (57.1 mg/l) respectively, these values corresponded to [26] approved caffeine and caffeine-limited energy beverages, as well as lower caffeine energy drinks[22]. Furthermore, the RL sample result (322 mg/l) is similar to the sample published by [28] who obtained caffeine content in an energy drink sample (322.8 mg/l) using the spectrophotometer method. Furthermore, there is a correlation between T brand and a sample published by [18] with a caffeine concentration of (388 mg/l) comparable to 160 mg/16 ounces. Caffeine values were found to be very identical by the HPLC technique for two different brands, FE and RL (208 mg/l) for both samples. And the results of the two samples were found to be nearly the same using the same technique HPLC for RE and B brands were (185 and 182 mg/l, respectively).
Conclusion

Nowadays, different energy drinks are available in local markets all over the world. The consumption of energy drinks increased in the last decades, especially among teenagers. The results of the current study showed that there is an unconformity between the concentrations of both caffeine and benzoate acid content on the label and the actual contents in the samples. HPLC method was more accurate than spectrophotometer regarding the obtained results.

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