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Quantification of some additives in energy drinks using high-performance liquid chromatography

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ABSTRACT

Introduction: Energy drinks (EDs) are products in the form of a beverage or concentrated liquid designed to increase both mental and physical stimulations. Their popularity has grown tremendously, especially among children and adolescents, regardless of the growing number of undesirable health consequences associated with their consumption. This study aimed to evaluate the content of additives in EDs available in the Bosnian and Herzegovinian (B&H) markets.

Methods: Twenty-two EDs from 15 brands were analyzed. The contents of quinine (QUIN), caffeine (CAF), benzoic acid (BZA), and sorbic acid (SA) were determined by high-performance liquid chromatography.

Results: The median value of QUIN, CAF, SA, and BZA was 0.15 ppm, 309.05 ppm, 75.35 ppm, and 90.80 ppm, respectively. The highest CAF content variation was found in EDs of brand 4, and the lowest was in brand 6. A statistically significant difference was found between the obtained values in relation to the recommended daily intake of CAF for adolescents by the Centers for Disease Control and Prevention and the American Academy of Pediatrics ($p < 0.001$).

Conclusion: The CAF content in EDs deviates by 10% from the content stated in the product declaration. All EDs on the B&H market should carry a clear warning: "High CAF content must not be mixed with alcohol and is not recommended for children, pregnant and/or lactating women, and CAF-sensitive individuals." Given the behavioral trends associated with the potential risks of excessive CAF consumption, particularly among youth, national agencies in B&H should recognize areas of intervention such as responsible marketing and advertising, and education and awareness-raising. Further research and monitoring would be needed to determine the effectiveness of the various aspects of the proposed risk management approach.

Keywords: Energy drinks; additives; caffeine; high-performance liquid chromatography; Bosnia and Herzegovina

INTRODUCTION

Energy drinks (EDs) are products in the form of a drink or concentrated liquid designed to increase mental and physical stimulation (1). Although there are differences in composition and form, they are classified as "products in food form," which are intermediate between food and natural health products. EDs are subjects of the regulatory framework for food, the Food and Drug Regulations (2). They contain high stimulants, especially caffeine (CAF), taurine, ginseng, guarana, sugar, B vitamins, and others (1,3). The primary active component is CAF as the most widely taken legal stimulant in the world. Its benefits on energy and focus are frequently complemented by additional chemicals that contribute to the beverages' stimulating impact (2). The content of 0.02% is generally considered safe CAF although it varies from 47 to 207 ppm in different EDs (4,5). However, not all of its consequences are beneficial. Daily CAF use in excess can result in symptoms of CAF toxicity ranging from nausea and vomiting to convulsions and significant cardiac issues, regardless of age or medical condition (6). Children and adolescents, as the most frequent consumers of ED, are more prone to suffer CAF's negative effects (7). Leading health authorities, including the Centers for Disease Control and Prevention (CDCP) and the American Academy of Pediatrics (AAP), recommend that daily CAF intake should not exceed 100 mg/day for adolescents aged 12–18 years (8). The Canadian government advises intake based on 2.5 mg/kg body weight, while recommendations for children ages 4–12 range from 45 to 85 mg/day (9). Besides CAF, EDs also contain chemical preservatives to prevent spoilage or to improve the

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microbiological stability and any alterations in their taste of soft drinks (10). The types of chemical preservatives can be used depending on the chemical and physical properties of both the preservative and the beverage. BZA is commonly added to many foods and beverages as preservatives. When dissolved in water, it partially dissociates into its conjugate base, benzoate. One negative aspect about using benzoates as preservatives is that benzoates react with ascorbic acid in soft drinks to form benzene, which is classified as a human carcinogen. This reaction diminishes the value of soft drinks, especially when they are stored for extended periods at high temperatures. Therefore, it is very important to monitor the storage conditions and concentration of benzoates in soft drinks over time (11). BZA inhibits bacterial development and SA is an antifungal preservative. The pH at which they have effective antibacterial activity is different. BZA is mainly used for acidic food products, while SA is used for food products with higher pH (12). Their presence at levels higher than permitted safety levels can be harmful to human health. Some adverse effects, such as metabolic acidosis, convulsions, hyperpnoea, and allergic reactions in experimental animals and in humans, are described (13). Quinine (QUIN) is a white crystalline alkaloid occurring naturally in the bark of the cinchona tree, which grows in South America. In medicine, it has many applications due to its fever reducing, pain killing, and anti-inflammatory properties. Another generally known property is its bitter taste. Nowadays, it is widely used as a favored ingredient for bitter drinks and food products, yet in limited concentration. Consumption of higher amounts can lead to a health problem for certain consumer groups (14).

Since the introduction of EDs in the 1960s in Europe and Asia, their popularity has grown tremendously (1). The energy market is forecast to reach $108.40 billion by 2031, up from an estimated $45.80 billion in 2020. Asia-Pacific and North America provided around 56.6% of the worldwide market share for ED, while European countries are in the third place (3). According to the National Institutes of Health, EDs and multivitamins are the most popular supplements for teens and young adults in the United States. Men between the ages of 18 and 34 consume the most ED, and nearly one-third of adolescents between the ages of 12 and 17 drink them regularly (15). One of the reasons for the success of these beverages is the companies' targeted and youth-oriented advertising efforts (2). According to the available scientific research, the effects of consuming EDs are mainly related to their CAF and sugar content. The levels of other substances in EDs appear to have little negative short-term impacts (2). An additional problem lately has been identified among college-aged individuals who combine EDs and alcohol consumption. At present, the consequences of these mixtures on the still developing adolescent brain are the subject of numerous researches (16). Recognizing the significance of potential health consequences and the lack of similar research in our country, this study aimed to evaluate the content of additives in EDs available in the Bosnian and Herzegovinian (B&H) markets.

METHODS

The Ethical Board of the University of Sarajevo – Faculty of Health Studies approved an experimental, cross-sectional study. In the Federal Institute of Public Health laboratories, the analytical part of the study was conducted in May 2020. The experimental phase included an analysis of additives (QUIN, CAF, SA, and BZA's) by high-performance liquid chromatography (HPLC) in 22 EDs from 15 different brands chosen by the method of random sampling. The samples were divided by brand into seven groups and coded as brands 1–6 and others. In the first five groups, two samples from each brand were analyzed, the sixth group consisted of three samples of one brand and the seventh group contained individual samples from other manufacturers.

Standards were purchased from Sigma-Aldrich (Germany). All solvents were of an analytical grade. The chemicals and reagents used were ammonium acetate ($C_2H_7NO_2$) p.a., ≥98%, acetonitrile HPLC grade p.a. ≥99,9%, acetic acid (CH_3COOH) p.a. ≥99,8%, ethanol C_2H_6O p.a. (all purchased from Sigma-Aldrich, Germany), and purified water for HPLC.

Analysis was performed using HPLC system (Agilent Technologies 1220 Infinity LC, with autoinjector, UV/VIS detector, system for degas, one pump, oven with thermostat, and program for data analysis OpenLAB) and column Zorbax Eclipse XDB C-18, 25 cm × 4.6 mm, 5 μm from Agilent (USA). Separation was performed using gradient chromatography. The used mobile phases were solution A (water +0.1% acetic acid) and solution B (acetonitrile +1% acetic acid). A 214 nm wavelength was used.

Primary reference materials for CAF, QUIN, sodium benzoate, and potassium sorbate were prepared by taking 100 g of standard, transferred to a 20 mL volumetric flask, dissolved in purified water, quantitatively transferred to a 100 mL measuring cup, and filled up to the mark.

Standards for the calibration curve were 5 ppm, 10 ppm, 25 ppm, 50 ppm, 75 ppm, and 100 ppm.

All samples were degassed in an ultrasonic bath. After degassing, samples were filtered with the help of a syringe through a "syringe" disk filter into a beaker (about 5 ml of the sample for repetition/dilution) and directly into the vial from which the injection is made (first 1 mL was discarded during filtration). Dilution of samples is done depending on the obtained or expected content of individual components (dilution 2, 4, 5, or 10 times).

The result for the BZA and SA is expressed as the free acid concentration in ppm. The result read from the line must be converted into free acid as follows:

Concentration of sodium benzoate (ppm) × 0.847 = concentration of BZA (ppm)

(F = M acids/M salts = 0.847)

Concentration of potassium sorbate (ppm) × 0.746 = concentration SA (ppm)

(F = M acids/M salts = 0.746)

The result for the content of CAF and QUIN is expressed directly.

If it was necessary to dilute the sample, then the obtained result is multiplied by the number of dilutions.

SPSS computer program version 26.0 and MedCalc Version 15.3 were used for statistical analysis. MS Office package 2019 was used for data processing. Categorical

variables are represented by frequency as an absolute number. Numerical values were analyzed to standardize the data with the Shapiro–Wilk test, and the deviation from the normal distribution of the data was determined. Nonparametric tests, Kolmogorov–Smirnov test for one sample, and Kruskal–Wallis H test for analysis of within-sample difference were used. The accepted level of significance was $p < 0.05$, with the results presented in tabular or graphical form.

RESULTS

After chemical analysis, the values were tested for data standardization and data distribution. Given the sample size (<50), the Shapiro–Wilk test was used. The obtained value of $p < 0.05$ for three substances indicates an improper distribution of data (Table 1).

The obtained values in 22 samples were compared with the maximum allowed concentrations prescribed by the Rulebooks (17,18). The median value and interquartile range (IQR) of QUIN, CAF, SA, and BZA were 0.15 ppm (0.11–0.20), 309.05 ppm (302.10–313.40), 75.35 ppm (71.50–85.70), and 90.80 ppm (80.5–95.5), respectively (Table 2).

Brand 1 had the highest median QUIN value of 0.33 ppm (IQR 0.15–0.50) followed by brand 4 with 0.28 ppm (IQR 0.25–0.30). Other brands had slightly lower values: Brand 2 median 0.20 ppm (IQR 0.12–0.25), brand 3 median 0.13 ppm (IQR 0.10–0.16), brand 5 median 0.15 ppm (IQR 0.10–0.20), and brand 6 with median 0.10 ppm (IQR 0.08–0.12). In other brands, the median value was 0.14 ppm with IQR 0.11–0.15. The highest variation in the content of QUIN was found in EDs of brand 1. Based on the Kruskal–Wallis test, the values between the brands were analyzed and it was determined that there is no significant difference in QUIN values, $p = 0.239$ (Figure 1).

Based on the results shown in Figure 2, the highest median concentration of CAF (312.35 ppm) was found in brand 5 with an IQR of 305.80–318.90 then follows brand 1 with 311.20 ppm (IQR 309.00–311.40), brand 3 with 309.55 ppm (IQR 309.10–310.00), brand 2 with 307.30 ppm (300.10–315.00), brand 6 with 306.75 ppm (IQR 302.10–311.40), and brand 4 with 297.85 ppm (IQR 280.50–315.20). In the seventh group, the median value of CAF was 306.75 ppm with an IQR of 300.50–311.20. The highest CAF content variation was found in EDs of brand 4 and the lowest in brand 6. The Kruskal–Wallis test did not determine the existence of statistically significant differences in CAF content between brands ($p = 0.985$).

The highest content of SA was found in brand 1 with 93.55 ppm and IQR 77.10–110.00, and brand 2 with 90.30 ppm and IQR 75.20–100.20 (Figure 3). Lower values were determined in brands 3 to 6, namely, 78.25 ppm (IQR 70.80–85.70), 77.75 ppm (IQR 75.50–80.00), 70.55 ppm (IQR 70.20–70.90), and 77.65 ppm (IQR 77.20–78.10), respectively. In Group 7, the median value was 73.80 ppm with IQR 71.50–75.20. The highest variation in the content of SA was found in EDs of brand 1 and the lowest among brand 3. The Kruskal–Wallis test did not determine the existence of statistically significant differences in SA content between brands ($p = 0.177$).

Among the examined samples shown in Figure 4, the highest content of BZA was determined in EDs of brand 6 (median 94.95 ppm, IQR 94.20–95.70). Similar values were found in drinks brands 5 and 7 (91.85 ppm and 91.80 ppm), with greater variation in IQR (88.20–95.50 and 85.90–100.00). This is followed by brand 3 EDs with a median of 87.30 ppm (IQR 84.20–90.4), brand 4 (median 87.30 ppm, IQR 80.10–94.50), brand 2 (median 84.30 ppm, IQR 75.20–101.80), and brand 1 (median

TABLE 1. Data distribution test

Variable	Shapiro–Wilk test		
	Statistic	df	p
Quinine	0.823	21	0.001
Caffeine	0.864	21	0.009
Sorbic acid	0.822	21	0.001
Benzoic acid	0.958	21	0.550

TABLE 2. Values of additives in a total sample

Variable	Median	IQR	MAC
Quinine (ppm)	0.15	0.11–0.20	100
Caffeine (ppm)	309.05	302.10–313.40	320
Sorbic acid (ppm)	75.35	71.50–85.70	300
Benzoic acid (ppm)	90.80	80.50–95.50	150

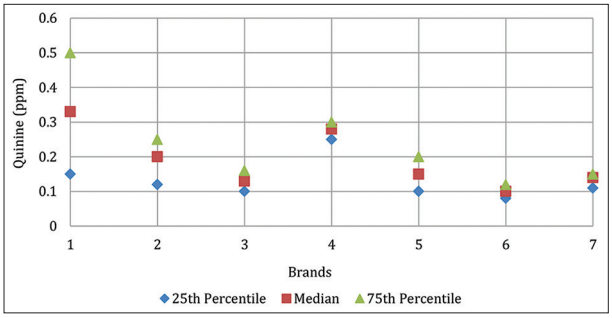

FIGURE 1: Quinine content in energy drinks brands.

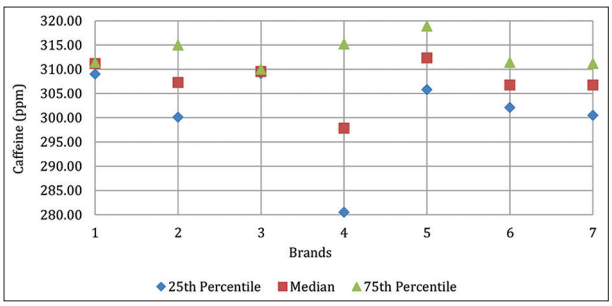

FIGURE 2: The caffeine content in energy drinks brands.

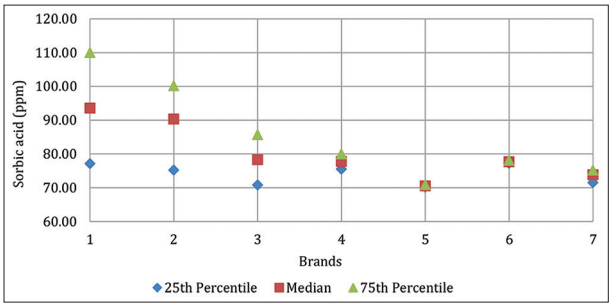

FIGURE 3: The sorbic acid content in energy drinks brands.

80.35 ppm, IQR 80.20–80.50). The highest variation in the content of BZA was found in EDs of brand 2 and the lowest among brand 1. No statistically significant differences in the content of BZA between brands were found ($p = 0.668$).

All analyzed samples had CAF values above 280 ppm (Figure 5). A statistically significant difference was found between the obtained values in relation to the recommended daily intake of CAF for adolescents by the CDCP and AAP ($p < 0.001$).

DISCUSSION

Based on the analysis of 22 samples of EDs of different brands, it was found that the content and concentration of additives are within the reference ranges and that the concentrations of the tested substances are consistent or very similar to the concentrations indicated on the product declaration. Eight samples contained up to 300 ppm of CAF according to the declaration, which means, given a tolerance of ± 10 mg per volume of 0.4–1 l, that eight EDs had a value of more than 310 ppm of CAF. A study conducted by Al-Bratty et al. showed that CAF concentrations in the beverages tested were within 100 ± 10% of label claims (19). Based on these results, we recommended that the manufacturers of these beverages determine the content of added ingredients more precisely. Lage-Yusty et al. analyzed the presence of certain substances in EDs using liquid chromatography with photodiodes and fluorescence detection. In their study, CAF levels varied from 252 to 304 mg, which is consistent with our results (20). Attipoe Selesi studied the composition of the best-selling EDs in Maryland and Michigan. Of the 14 samples analyzed, five did not contain the correct amount of CAF and taurine. A deviation of ± 15% was found in nine drinks that had the specified values (21). The average value of CAF in 75 commercial EDs examined in the study by Jagim et al.

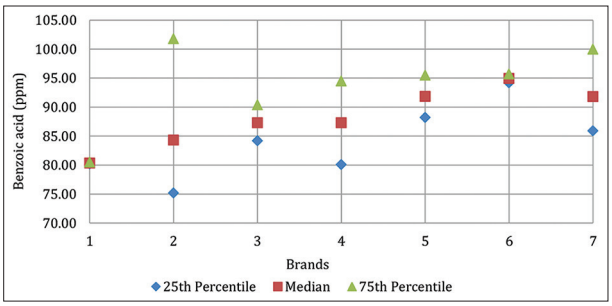

FIGURE 4: The benzoic acid content in energy drinks brands.

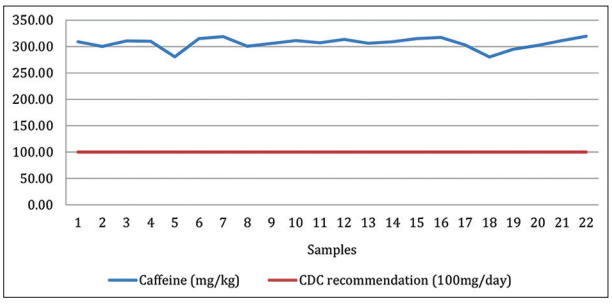

FIGURE 5: Comparison of obtained values with cdc and aap recommendations for caffeine daily uptake for adolescents aged 12–18.

was 174.4 ± 81.1 mg (22). The declarations of several analyzed EDs do not state the levels of QUIN, SA, and BZA. In its 2008 Food Information Directive, the European Commission regulated the same issue, and EDs sold on the European Union market also carried a warning about the high concentration of CAF in the drink (23). The results of scientific studies are worrying, as they show numerous harmful consequences of EDs consumption, ranging from mental disorders and risky behavior to increased blood pressure, obesity, diabetes, kidney damage, etc. (24). Alford et al. found that the EDs Red Bull caused significant changes in mental performance, including decision time, concentration, and memory, during three phases of monitoring in 36 volunteers (25). The Canadian Food and Drug Administration conducted a study on the adverse effects of EDs and found 61 adverse reactions. Of these, 32 were classified as serious and 15 were cardiovascular related (arrhythmias, rapid pulse, palpitations, and chest pain). In addition, six of these 15 adverse events occurred among teenagers between 13 and 17 years. Almost all of the reported adverse reactions occurred in healthy, young individuals. However, other causes such as underlying diseases or interactions with other drugs were not identified. In four deaths, a direct association with EDs leading to cardiac arrest was found (26). Several studies have shown an increase in heart rate and arterial blood pressure after EDs consumption. These results have been attributed to the ergogenic effects of the CAF. In addition, significant cardiac manifestations such as ventricular arrhythmias, ST-segment elevations, and QT prolongations have been documented after excessive consumption of EDs (27). A study of adolescents aged 15–16 years showed a strong association between CAF consumption and violent behavior and conduct disorders. Several reports suggest that EDs may contribute to ischemic strokes and trigger epileptic seizures (28). Doherty and Smith studied the synergistic effects of ingredients in EDs on the nervous system. They discovered the occurrence of extreme euphoria followed by complete depression. They also defined the phenomenon of modern addiction resulting from the consumption of these drinks (29). Manufacturers have recently shifted their target audience from athletes to young people, so EDs are aggressively advertised in places popular with teenagers and young adults (30). Their consumption by children has increased concern about the harmfulness of these drinks; a particular risk has been observed in schoolchildren (16). An additional problem that has occurred in the last decade is the drastic increase in the consumption of EDs with alcohol, especially among the younger population (31). This combination of drinks reduces the feeling of drunkenness and promotes a higher consumption of the EDs than when consumed without alcohol. Research shows that the presence of an ED alters the consumer's usual response to alcohol and that this combination represents a risky scenario for the consumer due to the increased stimulant effect and high level of impulsivity (27).

The Committee on Food Additives and Nutrient Sources Added to Food of the European Food Safety Authority has submitted a scientific opinion on the re-evaluation of SA (E 200), potassium sorbate (E 202), and calcium sorbate (E 203) as feed additives. There was no evidence of

genotoxic effects of SA or potassium sorbate, it was determined (32). A systematic review of the potential risks of preservatives, benzoates, and sorbates concluded that these substances have no toxic effects on mammals when used alone. However, when they interact with other compounds in the gastric environment, such as nitrite and ascorbic acid, carcinogenic substances can be formed. In addition, animal studies reveal the possibility of teratogenic effects and liver damage, as well as adverse effects on neuron development and growth retardation, hematological abnormalities, and organ damage. *In vitro* studies indicate increased oxidative stress, damage to genetic material, inhibition of leptin release in adipocytes, and damage to mitochondria, according to the review's authors (33). Sodium benzoate and potassium sorbate are highly effective preservatives for food and beverages, but their effect on health, particularly in children, is uncertain. Benzoate can react with ascorbic acid in beverages to produce carcinogenic benzene, and allergic reactions have been reported. In addition, benzoate can affect neurotransmission and cognitive function. There is no solid evidence that it is a Type 2 diabetes risk factor (34). A Nigerian study revealed the presence of sodium benzoate and potassium sorbate in certain soft drinks and fruit juices, with manufacturers favoring sodium benzoate. Preservative concentrations in the majority of samples tested were within acceptable limits. About 70% of the samples may pose a health risk to children weighing <30 kg, despite the fact that the majority of the brands analyzed can be labeled as safe based on the permitted levels of preservatives. In light of the fact that soft drinks are among the most consumed products in the world, it is strongly advised that the additive content of soft drinks and similar beverages be analyzed frequently (35).

CONCLUSION

The tested substances QUIN, CAF, BZA, and SA correspond to the reference values. The CAF content in EDs deviates 10% from the content stated in the product declaration. All EDs on the B&H market should carry a clear warning: "High CAF content must not be mixed with alcohol and is not recommended for children, pregnant and/or lactating women, and CAF-sensitive individuals." Given the behavioral trends associated with the potential risks of excessive CAF consumption, particularly among youth, national agencies in B&H should recognize areas of intervention such as responsible marketing and advertising, and education and awareness-raising. Further research and monitoring would be needed to determine the effectiveness of the various aspects of the proposed risk management approach.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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