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**Research Paper / Araştırma Makalesi**

# Determination of Caffeine Contents in Soft Drinks by High Performance Liquid Chromatography (HPLC) Method

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## ABSTRACT

Soft drinks have evolved from traditional herbal tinctures into scientifically formulated beverages designed to enhance physical and mental performance. In this study, caffeine, the primary active ingredient in soft drinks, was analyzed using the HPLC-UV method across 15 different commercial products in Türkiye. The caffeine content of most soft drinks was below the legal limit of 150 µg/mL, and caffeine was not detected in some products, and a single drink contained caffeine slightly above the legal limit. For example, Brands 1, 4 and 7 had the caffeine contents of 131.72, 154.38 and 14.64 µg/mL, respectively. The ability of caffeine to block adenosine receptors and enhance intracellular cAMP levels boosts its performance-enhancing effects, including improved endurance, increased alertness, and accelerated fat metabolism. However, risks associated with high-dose consumption, such as elevated blood pressure and reduced ergogenic benefits at doses above 9 mg/kg, require the need for moderated use. This study aims to determine the caffeine content of a variety of energy drinks sold in Türkiye and compare these levels against legal thresholds. In addition, it presents validation data for the HPLC method to confirm analytical reliability.

**Keywords:** Caffeine, Ergogenic effect, Health risks, HPLC, Soft drinks

## Yüksek Performanslı Sıvı Kromatografi (HPLC) Yöntemi ile Meşrubatlarda Kafein İçeriğinin Belirlenmesi

## ÖZ

Gazlı içecekler (meşrubatlar), geleneksel bitkisel tentürlerden fiziksel ve zihinsel performansı artırmak için tasarlanmış bilimsel olarak formüle edilmiş içeceklere dönüşmüştür. Bu çalışmada, gazlı içeceklerin birincil aktif bileşeni olan kafein, Türkiye'deki 15 farklı ticari ürün üzerinden HPLC-UV yöntemiyle analiz edilmiştir. Çoğu gazlı içeceğin kafein içeriği, yasal sınır olan 150 µg/mL'nin altında bulunmuş, bazı ürünlerde ise kafein tespit edilmemiştir ve tek bir içecekte kafein seviyesi yasal sınırın biraz üzerinde çıkmıştır. Örneğin, 1., 4. ve 7. markaların kafein içerikleri sırasıyla 131,72, 154,38 ve 714,64 µg/mL olarak belirlenmiştir. Kafeinin adenozin reseptörlerini bloke etme ve hücre içi cAMP seviyelerini artırma yeteneği, dayanıklılığın artması, uyanıklığın artması ve yağ metabolizmasının hızlanması gibi performans artırıcı etkilerinin temelini oluşturmaktadır. Ancak, 9 mg/kg'ın üzerindeki dozlarda kan basıncının yükselmesi ve ergojenik faydaların azalması gibi yüksek doz tüketimiyle ilişkili riskler, ölçülü kullanım ihtiyacını gerektirmektedir. Bu çalışma, Türkiye'de satılan çeşitli gazlı içeceklerin kafein içeriğini belirlemeyi ve bu seviyeleri yasal sınırlarla karşılaştırmayı amaçlamaktadır. Ayrıca, analitik güvenilirliği doğrulamak için HPLC yöntemiyle ilgili doğrulama verilerini sunmaktadır.

**Anahtar Kelimeler:** Kafein, Ergojenik etki, Sağlık riskleri, HPLC, Meşrubatlar

## INTRODUCTION

Energy is a cornerstone of biological processes and is vital for the survival of living organisms. Each cell within the human body depends on energy to sustain its essential functions. The energy generated through nutrient oxidation and adenosine triphosphate (ATP) production is indispensable for muscle contractions, nerve transmission, cellular renewal, and the fortification of the immune system [1]. The origins of soft drinks are deeply rooted in human history, reflecting a timeless quest to fulfill energy demands. This pursuit is exemplified by the widespread use of plant-based tinctures throughout antiquity. Remarkably, the active components in contemporary soft drink formulations often parallel with the botanical remedies of the past. For centuries, diverse mixtures and plant extracts considered precursors to modern soft drinks have served various functional purposes [2]. The enduring human endeavor to harness energy, from traditional herbal solutions to scientifically advanced formulations, underscores a rich legacy that continues to evolve. This progression highlights the intricate interplay between tradition, scientific innovation, and the growing consumer demand for effective energy solutions [3].

The primary purpose of consuming soft drinks is to enhance physical and mental performance, albeit temporarily, under prevailing circumstances [4]. These drinks, widely consumed, especially among young people, typically contain caffeine, taurine, sugar, B vitamins, and plant extracts [5]. While each component is safe at specific doses, long-term usage may pose health risks, necessitating conscious and informed consumption. To comprehend their properties, a detailed analysis of each ingredient is essential [6].

Caffeine ($C_8H_{10}N_4O_2$) is a naturally occurring compound in plants and belongs to the alkaloid class, recognized as one of the most potent stimulants [7]. Prominent sources of caffeine include tea, coffee, and cocoa [8]. Due to its effects on endurance enhancement and fat oxidation, caffeine has garnered significant interest among young individuals and athletes. These attributes have solidified its reputation as a preferred supplement for improving both physical performance and energy levels. The efficacy of caffeine in these domains is wellsupported by extensive documentation [9;10]. In this study, caffeine is analyzed as the target compound for determining the legal compliance of soft drinks, and the chemical structure and physical properties are described briefly to support the analytical method. Figure 1 shows us the chemical structure of caffeine.

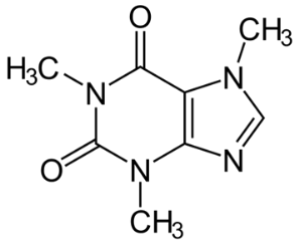


Figure 1. Chemical structure of caffeine

Caffeine is a compound belonging to the purine class and has two methyl groups, an imidazole ring, and a pyrimidine ring. This structural feature differentiates caffeine from other methylxanthines, such as theobromine and theophylline. Caffeine contains methyl groups at positions 1,3 and 7, which is the primary structural feature that distinguishes it from other methylxanthines [11]. Caffeine is considered a strong organic base and has a pKa of 10.4, which allows it to undergo a variety of chemical reactions. One of its most common reactions is that it can behave as a base or acid, taking or giving protons. However, it is also prone to degradation in oxidative media. By hydrolysis, theophylline can be separated into methylurea and other byproducts [11]. Caffeine is soluble in water, alcohol, and ether, but its solubility in water is low. Its solubility increases with increasing temperature, and while it is stable at room temperature, it can undergo degradation reactions at elevated temperatures [12]. Caffeine is not an optically active compound, i.e., it cannot rotate polarized light. It can be analysed spectroscopically by physical techniques (such as UV-Vis, FTIR, HPLC) [13].

Caffeine's primary mechanism of action involves its effects on the central nervous system (CNS), where it functions by blocking adenosine receptors. This action leads to a reduction in perceived exertion and pain, enabling athletes to sustain higher levels of exertion for extended durations [14]. This reduction in perceived exertion is particularly advantageous during high-intensity exercise, a phase during which athletes frequently experience fatigue. Studies have demonstrated that caffeine can enhance motor unit recruitment and rate coding, which are essential for optimizing muscle contraction and strength [15]. Caffeine increases intracellular cAMP levels by inhibiting the enzyme phosphodiesterase (PDE), which in turn promotes the release of free fatty acids by initiating the process of lipolysis through protein kinase A (PKA) activation [16].

The performance-enhancing effects of caffeine are well documented in both endurance and high-intensity activities, as evidenced by numerous studies. By lowering the perceived level of exertion, it allows athletes to work longer and at a higher intensity. A meta-analysis showed that caffeine intake (3-6 mg/kg body weight) significantly increased muscular endurance, strength, and anaerobic performance [17]. In terms of endurance performance, caffeine was found to delay fatigue, allowing athletes to work longer during exercise [18].

Caffeine, a widely consumed ergogenic supplement, can exert adverse effects on the cardiovascular system when consumed in high doses (over 400 mg/day), including heart rhythm disturbances and elevated blood pressure [19]. Although the effects are usually temporary and reversible when consumed up to 600 mg/day, the risks may increase above this level [20]. High doses can markedly increase blood pressure and may increase the risk of hypertension, particularly in vulnerable individuals. Furthermore, it has been demonstrated that caffeine may begin to reduce its ergogenic effects at doses above 9 mg/kg [21]. It is therefore recommended that caffeine

consumption be managed by considering individual dose adjustments and long-term effects. Caffeine shows physiological effects thanks to its complex chemical structure and physical properties and is a widely used ergogenic supplement with these effects. Benefits such as increasing alertness, accelerating fat metabolism, and increasing endurance have made caffeine an indispensable supplement for athletes [22]. However, it is imperative to note that individual dose adjustments and long-term effects should be considered during use.

The aim of this study is to determine the caffeine content of various soft drinks available on the Turkish market, compare these contents with legal limits, and discuss the potential effects of caffeine consumption on young individuals. Furthermore, the study provides validation data for the HPLC analysis to ensure methodological reliability and evaluates the results from a public health perspective.

## MATERIALS and METHODS

### Materials

In this study, soft drinks were obtained from local markets and chain stores during December 2024. Care was taken to obtain samples from different lot numbers. The analyses were performed in triplicate. Three samples were taken from each commercial beverage sample, each belonging to a different batch number, and each sample was analyzed in triplicate. Thus, a total of nine measurements were performed for each brand. The results obtained in the statistical evaluation are presented as mean ± standard deviation (SD). The caffeine contents of these drinks were quantitatively analysed by HPLC-UV. The main objective of the study was to determine whether the caffeine content of the drinks was within the legal limit. A precise measurement was achieved with the HPLC-UV device, which offers a highly efficient separation and accuracy. In this study, a new method for the determination of caffeine in HPLC-UV was developed and validated. This method was used because it is a reliable analysis technique especially for the separation of caffeine from complex matrices.

Acetonitrile (HPLC grade, ISOLAB, Germany) and potassium dihydrogen phosphate ($KH_2PO_4$, ≥99%, Sigma-Aldrich, USA) were used as mobile phase components, while ultrapure water (resistivity ≥18.2 MΩ·cm, Milli-Q system) was used for buffer preparation. All reagents were of analytical or HPLC grade, and the mobile phase components were filtered through a 0.45 µm membrane filter and degassed before use.

### Methods

### Extraction of Caffein from Soft Drinks

In this study on the determination of caffeine contents in various brands of commercially available soft drinks, to ensure repeatability, samples with different values from each brand were analyzed and each extraction was performed three times. Samples were diluted 1:1 (v/v) with ultrapure water as extraction solvent. Extraction of the samples was carried out in an ultrasonic bath for 10 minutes, followed by vortexing for 1 minute to ensure homogeneity**.** The extracts were then filtered. Subsequently centrifuged at 2500 rpm for 5 min. Aliquots taken from the supernatant were filtered through a 0.45 µm hydrophilic ptfe filter [23]. All experimental steps were carried out under controlled laboratory conditions (22-25°C, light-protected environment).

### Caffeine Analysis by HPLC-UV

A method was developed for the analysis of caffeine in soft drinks using a HPLC-UV device. In order to determine the amount of caffeine in different brands of commercial soft drinks, the samples were diluted 1:1 (v/v) with ultrapure water as extraction solvent, and the extraction processes of the samples were carried out in an ultrasonic bath for 10 min. After vortexing, the extracts were filtered. Then centrifuged at 2500 rpm for 5 min. Aliquots taken from the supernatant were filtered through a 0.45 µm hydrophilic PTFE filter. Caffeine contents were determined quantitatively by HPLC-UV. Analyses were performed with HPLC-UV (Shimadzu, LC-20A). InertSustain C18 (4.6 x 250mm, 5µm particle size) was used as a column and performed at 25°C. The injection volume was 20µL. The flow rate of the gradient method was 1.0 mL/min and the method time was 20 minutes. Mobile Phase A: 99.9% Acetonitrile (ISOLAB) and Mobile Phase B: 25 mM potassium dihydrogen phosphate (Sigma-Aldirch) (pH: 3.1) buffer. The detection wavelength was 254 nm. Table 1 shows the details of the gradient method.

Table 1. Gradient system of caffeine analysis by HPLC-UV

| Time (minute) | Mobile Phase A: Acetonitrile (%) | Mobile Phase B: 25 mM Potassium Dihydrogen Phosphate ($KH_2PO_4$) (%) |
|---|---|---|
| 0.0 | 0 | 100 |
| 4.0 | 0 | 100 |
| 14.0 | 35 | 65 |
| 14.5 | 80 | 20 |
| 15.0 | 80 | 20 |
| 19.0 | 80 | 20 |
| 19.5 | 0 | 100 |
| 20.0 | 0 | 100 |

### Method Validation

The parameters evaluated in the method validation study conducted in this study were specificity, linearity, limits of detection and determination, accuracy, and reproducibility. All method validation studies were carried out with standard solutions of different concentrations obtained by diluting the stock standard solution with methanol solution. Specificity is defined as the observation of only analyte peaks at the retention time of the target analyte in the presence of other components (impurities, matrix components, and degradation products) that may be present in the matrix. The evaluation of specificity is achieved through two distinct approaches: the analysis of the new matrix formed by introducing external components to the existing matrix,

and the generation of degraded products by subjecting the matrix to various stress conditions, such as heat treatment, exposure to an acidic or basic environment, and illumination.The analysis of specificity involves the labeling of peaks in the HPLC chromatogram of the target analyte and other components, followed by a comprehensive examination of their solubility. All extractions and analyses were performed in triplicate for each sample to ensure reproducibility.

### Quantification for Caffein Content in Soft Drinks

The caffeine standards were prepared as six points with ultrapure water at concentrations of 31.25, 62.50, 125, 250, 500, and 1000 µg/mL. Calibration curve graphs were plotted in each of these standards in two replicates according to guidelines [24]. The calibration curve graph was plotted in Excel (Microsoft Office Professional Plus 2016) using the area values of the solutions, and regression analysis was performed. The linearity of the method was calculated using the regression formula (y=14638x-20740.0) and the correlation coefficient value $R^2$= 0.9998 was obtained.

The limit of detection (LOD) and limit of determination (LOQ) values were calculated using the slope of the caffeine concentration calibration curve and the standard deviation of the y-intercepts in the regression line. These values were determined using equations (1) and (2), yielding LOD= 24.79 and LOQ=75.13. [25]

$$LOD = 3.3 * \frac{SD}{Slope} \quad (1)$$

$$LOQ = 10 * \frac{SD}{Slope} \quad (2)$$

The solubilities of the peaks of the target analyte and other components were calculated and analyzed according to formula (3). In the resolution formula, Rs is the separation power, $t_1$ and $t_2$ are the retention times of the target analyte and the peak closest to the target analyte, $w_1$ and $w_2$ are the peak widths. The accepted resolution value between the target analyte peak and the peak closest to the target analyte is Rs ≥ 2, and in this study, the average value of caffeine standards was calculated as Rs = 4.81 [25].

$$Rs = 2 * \frac{(t_2 - t_1)}{(w_1 + w_2)} \quad (3)$$

$$Recovery\ (\%) = 100 * \frac{C_1}{C_2} \quad (4)$$

The recovery values of the method were calculated using the formula outlined in equation (4). Concentration $C_1$, expressed in µg/mL, refers to the concentration of caffeine obtained from the calibration curve, while $C_2$ denotes the prepared concentration value. The recovery rates are shown in detail in Table 3. [24].

$$\%RSDr = 100 * \frac{SD}{C_{avg}} \quad (5)$$

The precision parameter is indicative of the proximity of the measurement results to each other. SD is calculated from the calibration curve and represents the variability of the concentration values. Cort is the mean of the concentration values calculated from the calibration curve. In this study, the percentage of relative standard deviation (RSD) was calculated as according to equation. Table 2 shows numerical results obtained from caffeine analysis using HPLC.

Table 2. Quantification parameters for caffeine contents in soft drinks using the HPLC-UV method

| Parameter | Value |
|---|---|
| Retention Time (min) | 13.68 |
| Detection Wavelength (nm) | 254 |
| Calibration Range (µg/mL) | 31.25-1000 |
| Linearity Equation | y=14638x-20740.0 |
| LOD (µg/mL) | 24.79 |
| LOQ (µg/mL) | 75.13 |
| $R^2$ | 0.9998 |

As shown in Table 3, as part of method validation, recovery studies were conducted for caffeine at three different concentration levels (% spike). Samples spiked with 30, 100 and 1000 µg/mL caffeine were analyzed in triplicate. The average caffeine amounts obtained were found to be 30.523 µg/mL, 100.343 µg/mL and 1003.713 µg/mL, respectively. These results demonstrate that the HPLC-UV method used comfortably meets international validation criteria in terms of recovery (recovery 80-120%, %RSD <2%) and that the method provides high accuracy and reproducibility.

The standard deviations and %RSD values calculated from these values were 0.175 µg/mL (%0.57) for 30 µg/mL, 0.116 µg/mL (0.12%) for 100 µg/mL, and 1.017 µg/mL (0.10%) for 1000 µg/mL. The recovery percentages based on nominal concentrations were 101.74%, 100.34% and 100.37%, respectively. In the reanalysis of caffeine standard retention times, the intra-day average was 13.691 min (SD: 0.0147 min; %RSD: 0.11) and the inter-day average was found to be 13.685 min (SD: 0.0076 min; %RSD: 0.056). These values demonstrate that the HPLC method exhibits high repeatability and stability in terms of retention time.

Table 3. Recovery study results for caffeine solutions with three different concentrations

| Spiked Level (µg/mL) | Mean (µg/mL) | SD (µg/mL) | RSD % (n=3) | Recovery % |
|---|---|---|---|---|
| 30 | 30.523 | 0.175 | 0.57 | 101.74 |
| 100 | 100.343 | 0.116 | 0.12 | 100.34 |
| 1000 | 1003.713 | 1.017 | 0.10 | 100.37 |

Table 4. Caffeine study results on the same day and different days regarding the time of consumption

| Parameter | Repeat type | |
|---|---|---|
| | Intra-day (n=6) | Inter-day (n=6) |
| Mean retention time (min) | 13.691 | 13.685 |
| SD (min) | 0.015 | 0.008 |
| RSD % | 0.110 | 0.056 |

Table 4 shows that in the reanalysis of the retention time for the caffeine standard, the intra-day average was 13.691 min (SD: 0.0147 min; %RSD: 0.11) and the inter-day average was found to be 13.685 min (SD: 0.0076 min; %RSD: 0.056). These values

demonstrate that the HPLC method exhibits high repeatability and stability in terms of retention time.

## RESULTS and DISCUSSION

The method of HPLC-UV is frequently employed to identify caffeine in soft drinks. Studies show that HPLC is a dependable and efficient method for analyzing caffeine and other ingredients in soft drinks. For example, Gliszczyńska-Świgło and Rybicka [26] used HPLC to successfully identify water-soluble vitamins and caffeine in soft drinks. Furthermore, Prasad et al. [27] used HPLC-UV to test the amounts of taurine and caffeine in soft drinks sold in Nepal, proving the method's wide range of applications. In recent years, an association has been observed between caffeine use among young people and significant health consequences. Studies conducted in Türkiye and other countries have indicated that caffeine intoxication, a condition more prevalent among young people, may be attributable to excessive consumption of soft drinks [28]. The deleterious effects of high caffeine concentrations in soft drinks on young people have been highlighted in studies by Breda et al. [29]. Rocha et al. [30] have developed a caffeine content table for the Brazilian population, providing standard values for soft drink analysis and serving as a valuable research instrument in academic and medical domains. Table 5 displays the caffeine concentrations measured in soft drink samples from various brands and markets, indicating variability in caffeine levels across products. There is not much inconsistency in the results obtained. Caffeine was not detected in some brands although it appeared on the label. Regulations on the amount of caffeine contained in soft drinks vary from country to country. In general, the caffeine content of soft drinks varies between 150 and 320 µg/mL. For example, in Türkiye, the caffeine content of soft drinks cannot exceed 150 µg/mL. The European Food Safety Authority (EFSA) has set the maximum daily caffeine intake for a healthy individual at 400 mg. This amount is equivalent to 4-5 cups of coffee. A comparison of caffeine and the sample is presented in Figure 2.

Table 5. Caffeine concentrations measured in soft drink samples

| Commercial Product | Product Type | Concentration (µg/mL) | RSD (%) (n=3) |
|---|---|---|---|
| Brand 1 | Carbonated drink | 131.72 | 1.17 |
| Brand 2 | Carbonated drink | 131.45 | 1.23 |
| Brand 3 | Still drink | below the detection limit | |
| Brand 4 | Carbonated drink | 154.38 | 2.34 |
| Brand 5 | Still drink | below the detection limit | |
| Brand 6 | Carbonated drink | 138.02 | 1.19 |
| Brand 7 | Carbonated drink | 14.64 | 0.41 |
| Brand 8 | Carbonated drink | 121.95 | 0.83 |
| Brand 9 | Carbonated drink | 46.87 | 1.05 |
| Brand 10 | Carbonated drink | 129.04 | 1.93 |
| Brand 11 | Carbonated drink | 94.38 | 2.42 |
| Brand 12 | Still drink | below the detection limit | |
| Brand 13 | Still drink | below the detection limit | |
| Brand 14 | Still drink | below the detection limit | |
| Brand 15 | Carbonated drink | 90.92 | 1.06 |

Caffeine concentrations in commercial products ranged from below the detection limit to ≤154 µg/mL. The legal limit in Türkiye is ≤150 µg/mL. The highest measured value, 154.38 µg/mL, was close to this limit. This situation highlighted the need for control in terms of product labeling and shelf life. Regulations on the amount of caffeine contained in soft drinks may vary from country to country. In general, the caffeine content of soft drinks varied between 150 and 320 µg/mL. For example, in Türkiye, the caffeine content of soft drinks cannot exceed 150 µg/mL. The European Food Safety Authority (EFSA) has set the maximum daily caffeine intake for a healthy individual at 400 mg. This amount is equivalent to 4-5 cups of coffee.

As stated in the literature, doses above 9 mg/kg may reduce ergogenic benefits while increasing the risk of hypertension and arrhythmia. The caffeine content of different brands on the Turkish market complies with legal limits. The developed HPLC method provides sufficient sensitivity and repeatability for such controls and regulatory checks. At spiked levels of 30, 100, and 1000 µg/mL, it provides recovery around 100-102% and <1% RSD, meeting the expected range of 80-120% and <2% RSD criterion in international guidelines (ICH Q2, Eurachem). Retention time values show %RSD <0.2 both intra-day and inter-day, indicating the stability of the instrument and method.

## CONCLUSION

The HPLC analysis part of the study determined whether commercially available products indicated the caffeine values specified on the labels. It showed that most commercial soft drinks comply with legal caffeine content limits, but there are some differences between brands. However, the legal limit of not exceeding 150 mg/L was stated in soft drinks sold in Türkiye. However, the exact amount in the content is not specified. While the values of other active ingredients in soft drinks were specified exactly; caffeine content is expressed as “max 150 µg/mL”. Although it is far below the daily dose that should not be exceeded, the individual does not know the exact amount of consumption in a box of soft drinks.

The HPLC method developed that caffeine was not detected in some of the fifteen products analysed in this study. It was even found that the value of one product was slightly above the legal limit for Türkiye. This situation is considered to be deterioration due to shelf life.

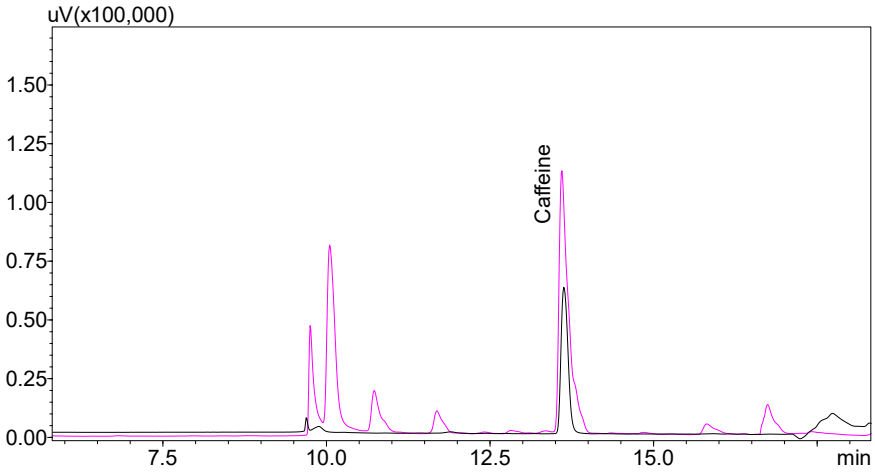


Figure 2. Comparison of caffeine standard (black) and brand 4 chromatogram

Based on the literature, it is known that high caffeine consumption may pose potential risks such as increased blood pressure and heart rhythm disorders, and that these risks may be more pronounced in particularly sensitive individuals. Considering that long-term consumption may increase these risks, it should be emphasized that individuals who consume caffeine regularly should be cautious. The results of this study emphasized the need for standardised guidelines in soft drink formulations and the importance of regulations to ensure consumer safety and product consistency. Differences in ingredient concentrations between brands require consumers to choose carefully. In conclusion, while soft drinks can be a valuable short-term performance enhancement tool, their consumption requires a balanced approach between benefits and potential health risks. Future research should focus on the long-term effects of soft drink consumption and innovative formulations that minimise risks while maximising benefits.

## AUTHOR CONTRIBUTIONS

ESD: Designed the experiments, performed data curation, and wrote the paper; PK: Performed the literature review and data curation; BK: Conducted formal analysis, methodology, investigation, software, and validation.

## DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## DECLARATIONS

Conflict of interest: The author declared no conflict of interest. Ethical approval: N/A. Consent to participate: N/A. Consent for publication: N/A.

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