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Analysis of caffeine in energy drinks by ultra-fast liquid chromatography

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Abstract. The objective of this work was to standardize the method of analysis of caffeine and determine its content in three energy drinks (Vive 100, Peak and Red Bull) consumed in the Colombian market, which were selected based on the results of a significant survey conducted to 384 people in the metropolitan area of “San José de Cúcuta, Colombia”, from each energy drink two production lots were taken and in turn from each lot three drinks were taken for a total of 18, each one was analyzed in triplicate; caffeine concentration was determined on a Shimadzu Prominence ultra-fast liquid chromatograph. The average concentrations in mg of caffeine/L, for lot 1 were 256.96, 218.10 and 235.73 for drinks Vive 100, Peak and Red Bull, respectively and for lot 2, the respective values were 229.30, 233.86 and 238.33. As established in Resolution 4150 of 2009, the maximum limit allowed is 320 mg of caffeine/L, it was observed that the concentrations in all the energy drinks analyzed are lower, therefore, they are complying with the provisions of the law.

1. Introduction

Energy drinks initially arose for athletes because of the energy burden they generate, they were mainly created to provide certain benefits such as increasing physical endurance, achieving a higher level of concentration, providing a sense of well-being, avoiding sleep and eliminating harmful substances for the organism. It is estimated that these drinks began to be marketed in Colombia in 2000, with Red Bull being the pioneer. When these drinks appeared, in Colombia there was no legislation to regulate their composition, so they demanded the amounts of ingredients recommended by the Food and Drug Administration (FDA) of the United States [1]. Today in Colombia, the consumption of energy drinks has shown a special increase by the general population; according to figures from [2] the retail energy drinks market, increased by volume 25.8% in 2016, this year sold 61.46 million liters of energy drinks. The highest growth in recent years was in 2012, with a rate of 142.8%.

In 2021, it is predicted that the energy drinks market will sell 146.2 million liters in Colombia, this leads to think about the possible consequences that would occur in the body of a person due to excessive consumption of caffeine, one of its main components. Caffeine is a mild psychoactive stimulant, it has a diuretic effect, it is known for its ability to provide energy and increase alertness, in addition to a long list of benefits such as reducing cardiovascular diseases, cerebrovascular diseases and raising pressure (ideal for hypotensive). However, the problem with caffeine is that it can cause symptoms such as insomnia, irritability, anxiety, nervousness, digestive problems (heartburn, esophageal reflux and diarrhea, among others), dehydration (because it causes diuresis) and tachycardia. Therefore, it is important to determine the amount of caffeine present in energy drinks according to current regulations,



in order to verify if the maximum permitted levels are being met and in turn to inform the community of the consequences of excessive consumption [3]. Resolution 4150 of 2009 [4] of Colombian legislation establishes the requirements that energy drinks must meet for human consumption and in article 6 of chapter IV, the maximum level of caffeine that these drinks must contain is established. On the other hand, manufacturers must comply with the provisions of resolution 5109 of 2005 [5], in which they determine the labeling and labeling requirements of packaged foods, taking into account that the maximum caffeine content in an energy drink is 32 mg/100mL.

The use of energy drinks by the population in general is becoming more frequent, due to the easy access that consumers have to them and the little control in their consumption; however, most consumers are unaware that its excessive use and improper handling is detrimental to the health because of the caffeine content present in them, for this reason, the method for the determination of caffeine in energy drinks consumed in “San José de Cúcuta, Colombia”, by means of ultra-fast liquid chromatography (UFLC). The investigation was carried out in two phases, the first one was to adapt the parameters for the standardization of the method and the second, in determining the caffeine content in some energy drinks marketed in the metropolitan area of “San José de Cúcuta, Colombia”. Three commercial brands of energy drinks were analyzed, in order to verify if the concentration of caffeine present in them was consistent with its labeling and if they were complying with current regulations.

The literature reports some related works such as [6] in which they determined the caffeine content present in energy drinks marketed in the metropolitan area of “San Salvador”, by high performance liquid chromatography (HPLC) analysis [7]. In another work, was analyzed caffeine in energy drinks by ultraviolet-visible (UV/VIS) spectroscopy at a wavelength of 270 nm, caffeine was extracted by liquid-liquid extraction using carbon tetrachloride (CCl₄) as solvent, generated a calibration curve with concentrations of 10 mg to 50 mg of caffeine/L, with which they determined the concentration of caffeine in energy drinks, and concluded that among the analyzed beverages, Red Bull had the highest concentration (58.31 mg of caffeine/L) [8].

2. Materials and methods

2.1. Selection of samples

The population was constituted with the 10 brands of energy drinks that are marketed in the Metropolitan Area of “San José de Cúcuta, Colombia”, the samples that were taken to perform the analysis were, Vive 100, Peak and Red Bull, which were selected based in the results of a significant survey of 384 people. The survey was carried out in different points of the metropolitan area of “San José de Cúcuta, Colombia”, in order to identify which were the three drinks most consumed by the population, to determine the commercial brands to analyze. Sampling was done by simple random sampling. Two batches were analyzed for each brand, three beverages for each batch and triplicate analysis was performed for each drink.

2.2. Standardization of the method

The standardization of this method was based on the technical standard NTC-ISO 20481: 2008 [9], which is the reference method for the determination of caffeine by HPLC, in which the aspects to take into account for the determination of caffeine by this technique are shown.

2.2.1. Equipment used. A Shimadzu Prominence series UFLC was used, which has a DGU-20A5R solvent degassing module, two-channel LC-20AT pump, SIL-20AHT autosampler, CTO-20A column heater, CBM-20A control module and SPD-M20A diode array detector. The equipment is equipped with Shimadzu LabSolutions software. For degassing of the solvents used as a mobile phase, a Sharperter brand ultrasound with a capacity of 25 L was used.

2.2.2. Preparation of the standard solutions. The working range was established with the elaboration of a Ringbom curve [10], obtained by analyzing standard solutions of different concentration, in the

range of 0.5 mg - 90.0 mg of caffeine/L by UV/VIS spectroscopy, the solutions were prepared at from a stock solution of 100 mg of caffeine/L. The calibration curve for UFLC analysis was developed using the external standard method. To determine the calibration sensitivity, the standard solutions prepared for the calibration curve (between 0.025 mg/L and 30.0 mg/L) were taken into account. To determine the limit of detection (LOD) and the limit of quantification (LOQ) of the equipment, several solutions of low concentration were prepared: 0.0125 mg, 0.025 mg, 0.050 mg, 0.100 mg and 0.500 mg of caffeine/L, each of the solutions was analyzed in triplicate.

2.2.3. Chromatography conditions. As recommended by NTC-ISO 20481 [9], the analysis of caffeine by UFLC was carried out in reverse phase, with mobile phase flow of 1.00 mL/min, the oven temperature of 40 °C, the run of the standards of calibration and sample was performed by isocratic elution, with HPLC methanol: water type I (24:76). The maximum caffeine absorption wavelength was set at 272 nm in the photodiode array detector (PDA). A C18 column (4.6 mm x 50 mm x 2.2 µm) was used. The injection volume, both for standards and for energy drink samples, was 10 µL.

2.2.4. Preparation of samples for ultra-fast liquid chromatography analysis. Initially the energy drinks to be analyzed were degassed by magnetic stirring for 20 minutes, with a subsequent rest of 15 minutes. Energy drinks report a content of 320 mg of caffeine/L and are classified as mixed drinks, therefore, caffeine must be extracted with subsequent injection of the extract into UFLC. The extraction was carried out in a diluted solution of the energy drink, which was intended to remain with a theoretical concentration of 16.0 mg of caffeine/L, for this 1.25 mL of the bottle 2 of the Vive 100 drink Lot 2 was taken, and placed in a 25.00mL volumetric balloon (1:20 dilution). 6.25 g of high purity MgO and approximately 15.0 mL of type I water were added to the solution, then it was heated until the solution reached 90 °C, left for 20 minutes with occasional stirring, then allowed to cool and type I water was added until capacity is completed. The solids were allowed to decant, an amount of the supernatant was filtered using a 0.45 µm regenerated cellulose syringe filter [9].

Through the review of other bibliographic references, it was found that in the work of [11], another way to prepare energy drinks for analysis by HPLC. In this, the degassing was performed by magnetic stirring and subsequent dilution of the drinks, then the diluted solution was filtered and injected into the chromatograph. Taking into account the above, the dilution of the same drink was made, also using a dilution factor of 1 to 20, so that the concentration of the diluted solution would be within the working range of the calibration curve. Subsequently, the diluted solution of the energy drink was passed through a 0.45 µm regenerated cellulose syringe filter. The solutions obtained through the two preparation methods were analyzed by UFLC under the established chromatographic conditions, to compare and select the best method, to subsequently prepare all the samples.

3. Results

3.1. Standardization of the method

3.1.1. Calibration curve. Taking into account the established chromatographic parameters, the calibration curve was prepared for the analysis of caffeine by the external standard method; for this, a stock solution of 200 mg of caffeine/L was prepared and from this standard solutions were prepared with concentrations of 0.025 mg, 2.0 mg, 5.0 mg, 10.0 mg, 15.0 mg, 20.0 mg and 30.0 mg of caffeine/L. For the chromatographic analysis a volume was injected, in triplicate of 10 µL of each of the standard solutions and the retention time (t_R) of the caffeine was established under the established conditions, it was around 2.050 minutes. Taking into account the average chromatographic areas of standard caffeine solutions, the calibration curve of the method shown in Figure 1 was developed.

3.1.2. Calibration sensitivity. The calibration sensitivity is constant with respect to the analyte concentration, it is defined as the slope of the calibration curve (m), since it establishes the ratio of

change of the measured property (area) per unit of concentration, the recorded value for this method it was 37705.64 as shown in Figure 1. The parameters to be defined when the sensitivity of a method is evaluated are the limits of detection and quantification, which can be estimated from the regression curve, provided that low concentrations of analyte have been considered, by extrapolation to zero concentration [12].

To determine the LOD and LOQ, another calibration curve was developed, with standard solutions of low concentration (0.0125 mg, 0.025 mg, 0.050 mg, 0.100 mg and 0.500 mg of caffeine/L), these were measured in triplicate under chromatographic conditions were established, the data obtained are shown in Table 1. The pattern with a concentration of 0.0125 mg/L was not integrated by the software since it did not report an area signal, therefore, when finding the LOD and the LOQ, this concentration was not taken into account. Figure 2 shows the equation of the line generated by the data in Table 1, in which the response at zero concentration was extrapolated and an estimate of the blank response was obtained ($Y_{bl} = 80.43$), which corresponds to the cut with the y-axis.

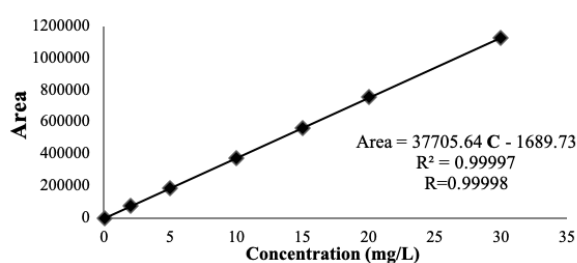


Figure 1. Calibration curve for UFLC caffeine analysis.

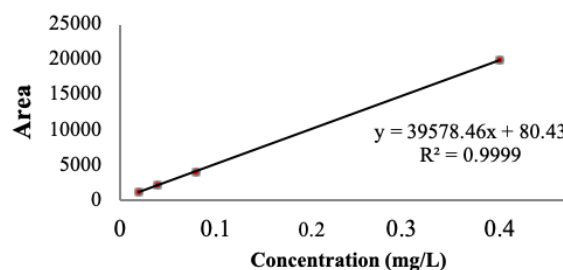


Figure 2. Calibration curve to determine the LOD and LOQ of caffeine by UFLC.

Table 1. Data to estimate the LOD and LOQ of caffeine by UFLC.

Concentration (mg of caffeine/L)	Area (accounts)			Average area (accounts)	Std dev (accounts)	%CV
0.025	1060.0	1059.0	1065.5	1061.5	3.50	0.333
0.050	2075.0	2106.0	2131.0	2104.0	28.05	1.333
0.100	4007.0	3971.0	4016.0	3998.0	23.81	0.596
0.500	19844	19877	19900	19873.7	28.15	0.142

Table 1 shows the data of the standard deviation (Std dev) of the areas corresponding to each concentration, these were used to create a curve plotting the standard deviation (y) vs. concentration (x), in order to estimate the standard deviation of the target (S_{bl}), which corresponds to the cut with the y axis. When performing the regression analysis, the equation of the calculated line was $y = 25.826x + 16.519$, taking the value of the intercept of this curve as the standard deviation of the target ($S_{bl} = 16.519$). Detection and quantification limits are calculated taking into account Equations (1) and Equations (2), respectively [12].

$$\text{LOD} = \frac{Y_{bl} + 3S_{bl}}{m}, \quad (1) \quad \text{LOQ} = \frac{Y_{bl} + 10S_{bl}}{m}. \quad (2)$$

With the values calculated above (Y_{bl} , S_{bl} and m) and using Equations (1) and Equation (2), the LOD = 0.0034 mg caffeine/L and the LOQ = 0.0065 mg caffeine/L of the method for the analysis of caffeine by UFLC, the determined values were established according to the sensitivity of the equipment, as observed are relatively low.

3.1.3. Preparation of samples for ultra-fast liquid chromatography analysis. The two forms of sample preparation described in the methodology were performed, for this purpose the same drink was taken (bottle 2 of the Vive 100 lot 2 energy drink). The extracts obtained and the dilutions of the drink were analyzed in triplicate under the established chromatographic conditions. The respective data of the

areas obtained, the average areas, the standard deviations and the coefficients of variation, are shown in Table 2. By means of the statistical software R Project version 3.2.2, a T test was performed, to determine if there was a significant difference between the two forms of sample preparation, the probability value obtained was 2.2×10^{-16} (less than 0.05), therefore it is established that there are significant differences between the forms of sample preparation with respect to the areas obtained.

In Table 2, the data obtained by the two procedures for preparing the sample of the energy drink for analysis by UFLC are observed. When performing the process of extracting caffeine using magnesium oxide (MgO), the average areas are smaller, compared to those in the dilution process of energy drinks. With the above, it is established that when performing the extraction of caffeine, it is possibly not fully recovered, this being one of the disadvantages of this form of preparation, the others are, the large amount of MgO used for each sample, the high cost of high purity MgO, in addition the time required to prepare the sample is much greater than the sample preparation by dilution. Therefore, the preparation of the energy drink samples was carried out by degassing and dilution.

Table 2. Comparison of results of the caffeine analysis in bottle 2 of the Vive 100 lot 2 energy drinks by the extraction and dilution methods.

	Area (accounts)					
	MgO extraction			Dilution		
	(1)	(2)	(3)	(1)	(2)	(3)
Replica 1	400218	391204	395779	436680	431258	429562
Replica 2	392864	390145	391027	431820	421007	421457
Replica 3	390815	390542	390527	430820	420327	421851
Average	394632	390630	392444	433107	424197	424290
Std dev	4944.6	535.0	2898.7	3134.7	6124.2	4569.9
%CV	1.253	0.137	0.739	0.724	1.444	1.077

3.2. Sample analysis

The solutions of the Vive 100, Peak and Red Bull energy drinks, prepared by degassing and dilution of each beverage by a factor of 1 to 20 (0.50 mL of energy drink at 10.00 mL with type I water) were analyzed according to chromatographic conditions established. Two batches were taken from the three brands selected to perform the analysis, three drinks from each lot and each triplicate analysis was performed for a total of 54 chromatographic runs.

Table 3 shows the data of the chromatographic analysis of the dilutions of the Vive 100 lot 1 energy drinks, the observed values allow to establish that the measurements have good precision, since the coefficients of variation do not exceed 2%. Figure 3 shows the chromatographic profiles of the replica 2 of the three Vive 100 lot 1 energy drinks, which have a great similarity for the three drinks of the same batch.

Table 3. Data from the chromatographic analysis of dilutions of the Vive 100 lot 1 energy drinks.

	Area (accounts)		
	Bottle 1	Bottle 2	Bottle 3
Replica 1	497278	480159	479628
Replica 2	484379	479001	480131
Replica 3	486085	477675	480709
Average	489247.3	478945.0	480156.0
Std dev	7006.9	1242.9	540.9
%CV	1.432	0.260	0.113

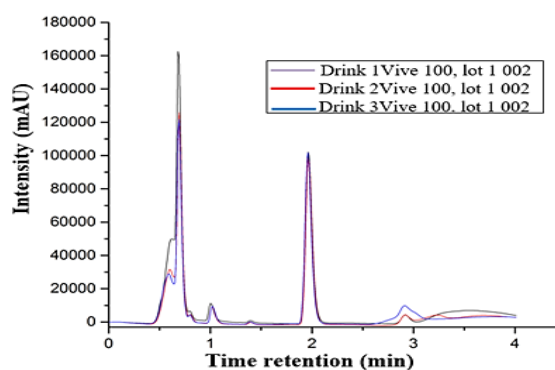


Figure 3. Comparison of the chromatographic profiles of the Vive 100 lot 1 energy drinks.

3.3. Determination of caffeine content in samples

Using the equation of the calibration curve for caffeine analysis obtained by the method of the external standard of Figure 1, (Area = 37705.64C – 1689.73), the concentration of each energy drink was calculated. The concentrations obtained for the energy drinks of the Vive 100, Peak and Red Bull brands are found in Table 4 and Table 5, in which it is observed that the coefficients of variation do not exceed 2%, indicating good measurement accuracy.

Table 4. Caffeine concentration in Vive 100 and Peak energy drinks.

	Concentration (mg of caffeine/L)											
	Vive 100						Peak					
	Lot 1			Lot 2			Lot 1			Lot 2		
	B.1	B.2	B.3	B.1	B.2	B.3	B.1	B.2	B.3	B.1	B.2	B.3
Replica 1	264.7	255.6	255.3	218.0	227.5	240.4	209.3	211.9	230.0	230.2	232.9	238.9
Replica 2	257.8	255.0	255.6	220.4	228.1	241.2	209.5	212.3	230.3	229.7	233.0	239.4
Replica 3	258.7	254.3	255.9	217.6	228.5	241.8	209.3	216.3	233.8	227.6	233.5	239.5
Average	260.4	254.9	255.6	218.7	228.0	241.2	209.4	213.5	231.4	229.2	233.1	239.3
Std dev	3.717	0.659	0.287	1.502	0.500	0.713	0.151	2.437	2.117	1.367	0.300	0.293
%CV	1.427	0.259	0.112	0.687	0.219	0.296	0.072	1.141	0.915	0.597	0.129	0.123

The caffeine concentration values calculated by the external standard method, shown in Table 4 and Table 5, are lower than the value reported by the manufacturers of energy drinks (320 mg caffeine/L). It is observed that the energy drinks with the highest caffeine content are those that make up lot 1 of the Vive 100 energy drink (260.4 mg, 254.9 mg, and 255.6 mg of caffeine/L), also that between the two batches of the same drink there is a significant difference between concentrations (Lot 1: 260.4 mg, 254.9 mg, and 255.6 mg of caffeine/L; and Lot 2: 218.7 mg, 228.0 mg, and 241.2 mg of caffeine/L).

Table 5. Caffeine concentration in Red Bull energy drink.

	Concentration (mg of caffeine/L)					
	Lot 1			Lot 2		
	B.1	B.2	B.3	B.1	B.2	B.3
Replica 1	233.1	237.0	237.0	238.5	240.0	232.7
Replica 2	232.7	236.3	237.2	240.0	241.4	239.4
Replica 3	232.4	237.5	238.5	239.9	240.1	232.9
Average	232.7	236.9	237.6	239.5	240.5	235.0
Std dev	0.342	0.612	0.842	0.831	0.805	3.820
%CV	0.147	0.258	0.355	0.347	0.335	1.626

4. Conclusions

The method for the determination of caffeine in energy drinks was standardized by UFLC, based on the technical standard NTC-ISO 20481: 2008. It was established that the most suitable sample preparation method to perform the analysis of the energy drinks by UFLC, is by degassing by magnetic stirring and dilution with type I water, since when extracting the caffeine from the samples, the chromatographic areas of the caffeine peak, and therefore their respective concentrations, were lower compared to those obtained at dilution, stating that caffeine was possibly not extracted in its entirety; in addition, a large amount of high purity MgO is required for each extraction, which is very expensive, and the time required for extraction is much longer than when preparing the sample by dilution. Caffeine concentrations in Vive 100, Peak, and Red Bull energy drinks were determined by UFLC based on NTC-ISO 20481, using the external standard method, observing that the average concentrations of caffeine in these drinks were 243.1 mg/L, 226.0 mg/L and 237.1 mg of caffeine/L, respectively, is lower than that reported by the manufacturers (320 mg caffeine/L), as well as stipulated by Resolution 4150 of 2009, indicating that they are complying with the provisions of the law. According to the values of the coefficients of variation reported for the replicas between the standard solutions used for the calibration curve by the external standard method, as well as those reported for each replica of the analyzed energy

drinks, the method can be considered It has a good precision presenting values lower than 3%, for this reason this method is suitable for the determination of caffeine in these drinks.

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