HPLC Analysis of Caffeine in Coffee:

How consistent is the concentration of caffeine in coffee, and is it worth the price?

Kennedi Bose

Dr. Diane Nutbrown

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ABSTRACT

Caffeine is the most commonly consumed stimulant in the United States with 64% of the adult population drinking coffee (1). Acknowledging that a lot of people drink coffee, we were curious about the consistency of the caffeine content in popular coffee brands; specifically Starbucks and McDonalds. The questions we will attempt to answer are: is the amount of caffeine consistent between brews on different days from the same source, is caffeine consistent from the same sources in different states, and does the amount of caffeine match the reported value given from the franchise. High-performance liquid chromatography (HPLC) is an established method for measuring the amount of caffeine in beverages and was the technique adopted for this study. Data was collected on our behalf from both Regis University and the University of Omaha - Nebraska. It was determined that the caffeine content was fairly consistent when compared on different days. However, the content of caffeine in coffee between states from the same source, was not consistent. The reported caffeine content and the experimentally determined amount, showed that caffeine content was not accurate. However, the McDonald's purchased coffee, was very close to it's reported amount. Based on the results, for people who drink coffee for the caffeine boost, it would be more cost effective to brew your own coffee at home. A small coffee from McDonalds cost $1, while a small coffee from Starbucks costs $1.85 (2, 3). Keurig pods, however, cost $15.18 and includes 32 pods! That's roughly $0.47 per cup of coffee (4).

INTRODUCTION

Many people drink coffee as a way to perform better at work or to help energize themselves in the morning. According to CBSN Minnesota, seven of 10 Americans drink coffee every week, and 62% drink it every day (5). In 2020-21, about 166.63 million 60 kilogram bags of coffee were consumed worldwide, a slight increase from 164 million bags in the year before (6). A recent study done by the National Coffee Association shows that coffee consumption has gone up 5 percent in the last five years. With the amount of coffee consumption on the rise, it is important to know how much caffeine is in a cup of coffee, how it affects your body, and if the coffee is worth the price. Determining the amount of caffeine in a cup of coffee can be accomplished through high-performance liquid chromatography. By using an HPLC analysis, we will compare and contrast home-brewed coffee samples and franchise bought coffee.

What we commonly refer to as caffeine is known as 1,3,7-trimethylpurine-2,6-dione and has the chemical formula $C_8H_{10}N_4O_2$ (Figure 1) (7). Caffeine is naturally found in the leaves and fruits of various plants. The most common among these plants are cocoa beans, tea leaves, and coffee beans.

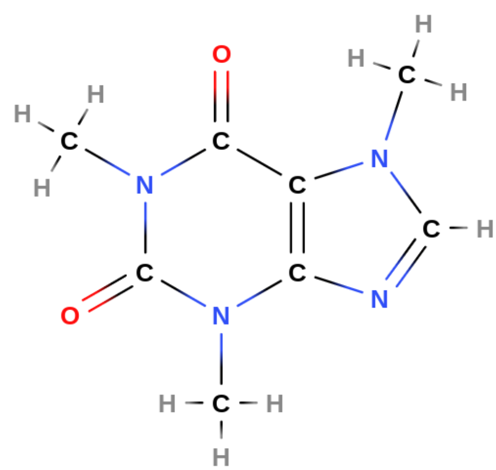

Figure 1. Lewis structure of caffeine (7).

Caffeine is used in a variety of drinks and foods which makes it a common component of people's daily diets and is one of the most commonly used stimulants. Various energy drinks, chocolate bars, teas, coffees, cough syrups and other food items include caffeine in its ingredients (8). The average caffeine content varies from different drinks depending on volume and product (Figure 2).

Figure 2. Average amount of caffeine in common drinks (9).

Caffeine is one of the more popular stimulant drugs. A stimulant drug is a term used to describe drugs that increase activity of the central nervous system and the body (10). Unlike other stimulants, such as nicotine, adderall, or risperdal, caffeine is not an inherently dangerous substance (11). It helps speed up the signals being sent to the brain, causing better productivity and awareness. However, just like any other stimulant, overconsumption can have negative effects on your body. These effects include jitters, seizures, nausea, insomnia, dysphoria, or fast heart rate (9). According to the FDA, 400 mg of caffeine is considered to be a healthy, daily amount of caffeine for healthy adults. Consuming more than 400 mg can lead to negative effects on the body (9).

For some people, caffeine is necessary to boost their energy and productivity in the morning. Knowing the caffeine content and its consistency in a drink is very important. Not only will it help you decide if it's worth the price, it can help you control your daily caffeine intake. A recent study from December 2020 reported that Americans between the ages of 25-34 spent an average of $2,008 on coffee, while people ages 35-44 spent $1,410 per year (12). By understanding which brand of cafe-bought or home-brewed coffee produces the most amount of caffeine, people can make a cost friendly choice.

There are established methods for determining the concentration of caffeine in a variety of substances, and several of these methods have been described for use in college level instructional labs. To ensure that our experiment could be done at the high school level, we focused our search on papers in the *Journal of Chemical Education* that describe successful experiments for the determination of caffeine. The most popular methods in determining the amount of caffeine in coffee are capillary electrophoresis

(CE), high performance liquid chromatography (HPLC), and solid phase microextraction gas chromatography-mass spectrometry method (SPME-GC/MS). Ultra violet visible spectroscopy (Uv-vis) was also used in some experiments; because some components in caffeinated drinks absorb UV light, this technique can produce inaccurate results without using any method of first separating the caffeine.

A detailed experiment using SPME-GC/MS to determine the amount of caffeine in caffeinated beverages (teas, soft drinks, regular coffee, decaffeinated coffee), was reported by Min J. Yang, Maureen L. Orton, and Janusz Pawliszyn. Two 5-mg/mL stock solutions of the caffeine were created, separately prepared in methanol and added to each beverage as an internal standard. It was concluded that the SPME-GC/MS method was an accurate and simple way to determine the caffeine amount in drinks (13). Solid-Phase Microextraction (SPME) is the extraction process of compounds in preparation of a gas chromatography (GC) or high performance liquid chromatography (HPLC) analysis (14). However, a drawback of using SPME-GC/MS is the small number of commercially available stationary phases, which limits the choice of selectivity (15).

In a study done in the Netherlands, a group of students performed a series of tests to find the amount of caffeine in store-bought coffee. They first diluted the coffee and used a stock solution of 0.100 M caffeine (16). HPLC, CE, and UV-vis techniques were used to find the amount of caffeine in the coffee. The caffeine concentration in the UV-vis technique was significantly higher than the concentration reported during the HPLC and CE run. Because of this, the UV-vis method does not seem to be as accurate as the others. This will be the paper we will most closely follow during the experiment, adopting the HPLC method to test caffeine.

HPLC Theory

Chromatography is a technique used to separate the components of a mixture. Liquid chromatography is a variation of this technique featuring a liquid mobile phase which moves through the solid stationary phase in a single direction of flow, bringing the separated components along with it. The 'high performance' part of the acronym HPLC, describes the accuracy of the technique and means that the process will be more precise, efficient, and fast. Figure 3 shows the components of an HPLC system. In HPLC the mixture is dissolved in the mobile phase and then transported through the stationary phase.

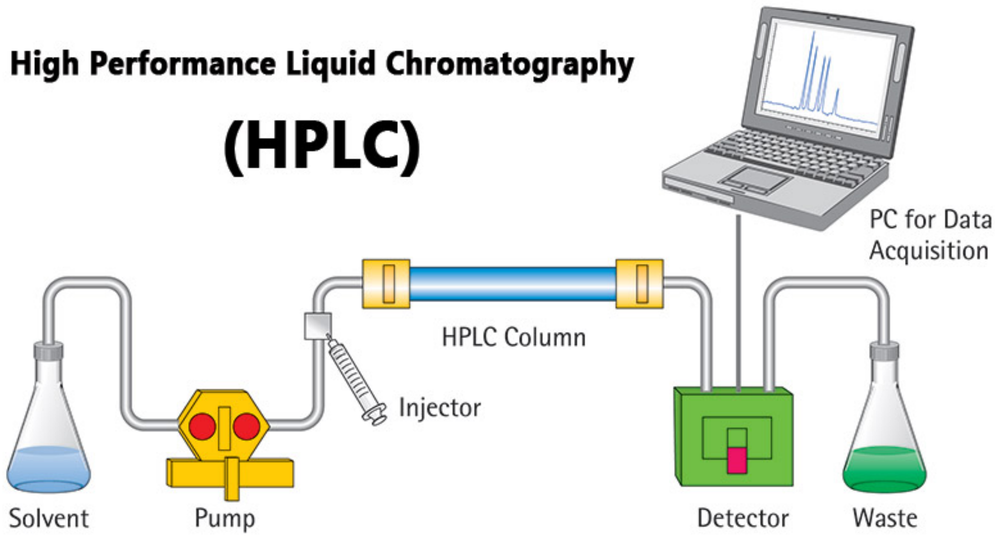

Figure 3. Schematic diagram of an HPLC system (17).

The mobile phase consists of the solvent being used in the experiment. As the mobile phase travels down the stationary phase, it picks up the components that are attracted to the solvent. The stationary phase is a fixed material that acts as a constraint for many components of the solvent when it passes through. The solubility of certain components in the mobile phase and the strength of their affinities in the stationary phase will cause some components to move faster than others, allowing the components in that mixture to be separated. Another factor that determines which phase the components are attracted to is solubility. Typically, the more polar elements will be attracted to the mobile phase and the nonpolar elements will be attracted to the stationary phase. In connection to polarity, there are four intermolecular forces that determine the relationship between polar and nonpolar elements. These intermolecular forces include dispersion, dipole dipole, hydrogen bonding, and ion dipole.

Columns in an HPLC system are very important since that is where the separation of the sample components takes place. Usually it is filled with silica gel which does not react with the mobile phase. The material filled columns are known as the stationary phase. There are four different types of columns: normal phase, reverse phase, ion exchange phase, and size exclusion phase. For this experiment, we'll be focusing on reverse phase columns. Opposite to the normal phase, it consists of a non polar stationary phase and a polar mobile phase. Non-hydrocarbons, such as C18, are used in the stationary phase while aqueous solutions, such as water-methanol/acetonitrile, are used in the mobile phase. The majority of the studies I've read used a reversed phase HPLC (RP-HPLC). The columns used were solid and usually nonpolar, while the mobile phase was polar. The most important intermolecular force for this experiment is H-bonding because this will ultimately determine the retention time. Retention time is the time the solute spends in the column (15). A stronger attraction will be present between the polar solvent and the polar molecules while it is passing through the column. There will not be a great attraction between the polar molecules and the hydrocarbon chains. This means that the retention time will be shorter (quicker). Typically, nonpolar molecules will be attracted to the hydrocarbons in the column, causing the retention time to be greater (slower). However, there is potential for h-bonding in caffeine.

Undoubtedly, there have been many undergraduate laboratory experiments designed to measure the concentration of caffeine in coffee and other caffeinated drinks. However, none have been published that test the amount of caffeine in food chain coffees, nor have they critiqued the consistency of brewed coffee caffeine content. In our study, standard black coffee from McDonald's and Starbucks retail locations, as well as Keurig cups (K-cups) of those two brands, will be analyzed via HPLC. The concentration of caffeine will be determined for each sample and comparisons will be made within each brand for coffee brewed on two different dates in order to assess the consistency of the caffeine content. An RP-HPLC from *The Determination of Caffeine in Coffee* will be adopted, using the same solute in the mobile phase; 50:50 water and methanol (16).

EXPERIMENTAL

Data was collected on our behalf by Dr. Lynetta Mier at Regis University and Dr. Andrew Miller at the University of Nebraska-Omaha (UNO). The experimental details are summarized for each.

Materials (Regis)

A small black coffee was purchased from McDonald's (12 oz.) and Starbucks (12 oz.) on March 9th and 19th. Home brewed coffee was made using a Starbucks K-Cup and a McDonalds K-Cup on March 10th and 19th; the setting on the Keurig Coffee Maker was set to medium. Deionized water was degassed by the instrument at Regis University. HPLC Grade Methanol was purchased from VWR Chemicals and caffeine was purchased from Sigma Chemical Company (purity unknown). Deionized water and methanol were used as solvents.

Materials (UNO)

A coffee from McDonald's, Starbucks, Burger King, and Scooter's were purchased in order to complete the experiment. However, only the data from McDonald's and Starbucks were necessary for this experiment.

Preparation of Standards (Regis U.)

An initial stock solution of caffeine was prepared by dissolving 0.125 g of caffeine in 100 mL. Dilutions of 10 mL of the initial stock (1.25×10^{-3} g/mL) to 100 mL produced a 1.25×10^{-4} g/mL working solution. The standards were prepared in the range of 5 x 10^{-6} g/mL - 1 x 10^{-4} g/mL by diluting 1-10 mL of working solution to 25 mL.

Preparation of Standards (UNO)

Four standards (0.001 M, 0.002 M, 0.0002 M, 0.0006 M) were prepared by dissolving caffeine in 20 mL of 95% ethanol and then diluting to 100 mL with distilled water. The solvent was approximately 19% ethanol, 4% acetic acid, and 75% water.

Preparation of Samples (Regis U.)

Black coffee was purchased over two consecutive days from McDonald's and Starbucks to test the consistency of their caffeine content. K-Cups for each brand were also used to determine the consistency of caffeine over a period of two days. Each coffee sample was diluted 50-fold.

Preparation of Samples (UNO)

Coffee from McDonald's, Starbucks, Burger King, and Scooter's was purchased to test the amount of caffeine present. Each coffee sample was diluted 10 to 25 mL before analysis.

HPLC Analysis (Regis U.)

An Agilent 1100 HPLC with autosampler was operated via ChemStation software to run the HPLC test. The machine includes a multi-wavelength UV-Vis detector and fluorescence detection (DAD) with a kinetex 5 µm EVO C-18 100 Å LC Column (150 x 4.6 mm). The isocratic mobile phase consisted of HPLC grade methanol and deionized water (50:50 v/v). The flow rate was 0.8 mL/min. The wavelength of detection was 272 nm with 4 nm bandwidth and reference at 360 nm with 100 nm bandwidth. Finally, the injection volume was 5 uL.

HPLC Analysis (UNO)

An Agilent 1220 LC System was used to determine the amount of caffeine in the coffee. An Agilent Eclipse Plus C18 column with a 5 micron particle size that was 4.6 mm diameter and 150 mm long was used. The mobile phase was 20:4:76 v/v ethanol:acetic acid:water. The software that was used during the experiment was LC Open Lab. The injection volume was 20 microliters. A flow rate of 1.00 mL/minute and a wavelength detection of 275 nm were also used. The solvent for the mobile phase was 50:50 methanol:water.

RESULTS & DISCUSSION

The standard curve for caffeine is given in figures 5 and 6. Figure 5 shows the standard curve from Regis University and Figure 6 shows the standard curve from University of Nebraska-Omaha. The equation of the line of best fit was used to calculate the concentration of the injected samples by substituting the area of the caffeine peak for y and solving for x. The original samples have been diluted by a factor of 5/10/20, therefore the injected samples were multiplied by 5/10/20 to determine the concentration in a cup of coffee. To calculate the mass of caffeine, first a conversion from ounces to liters was made. After the conversion, the concentration of the original sample of caffeine was multiplied by the product of the conversion to get the mass of the caffeine. Table 1 summarizes the results for each trial.

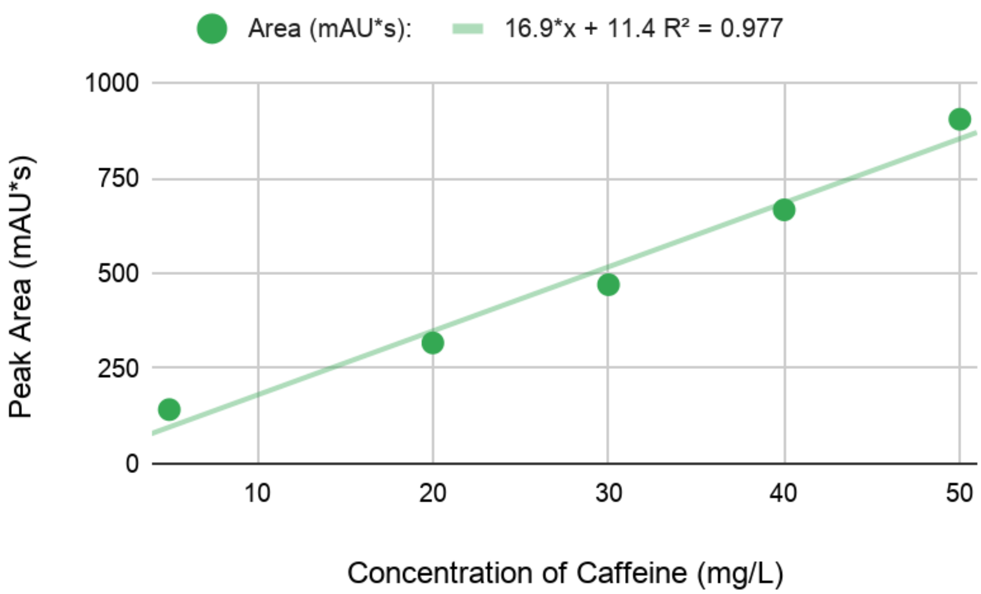

Figure 5. Standard curve for caffeine; includes the equation for the line of best fit. The line of best fit can be used to calculate the concentration of the caffeine in a coffee sample.

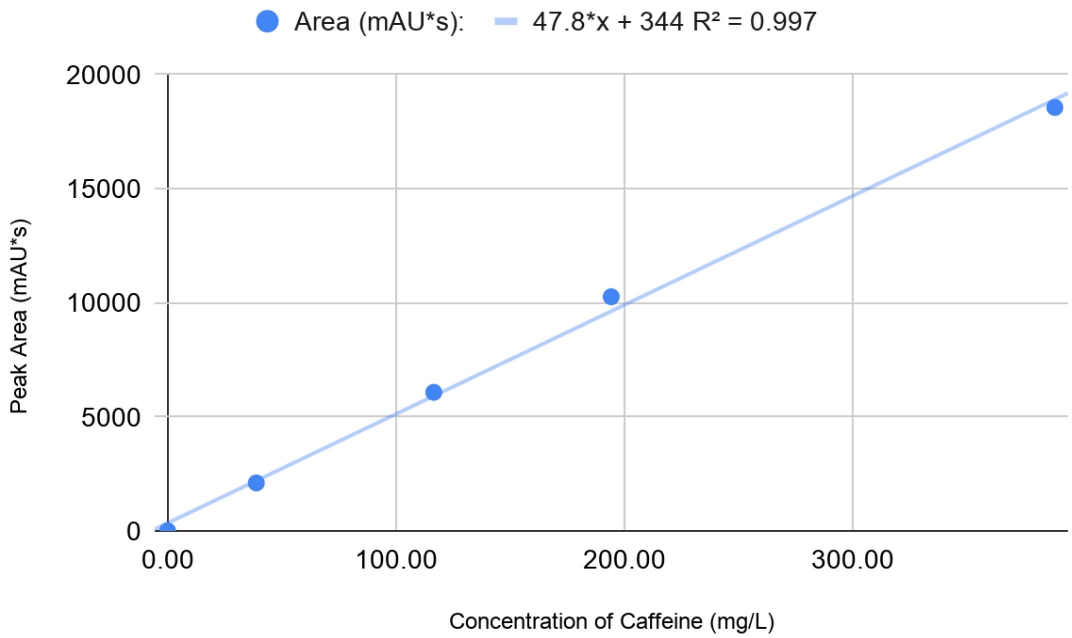

Figure 6. The standard curve for caffeine is shown, including the equation of the graph, which can be used to calculate the concentration of caffeine. The line of best fit can be used to calculate the concentration of the caffeine in a coffee sample.

Table 1a. HPLC results for coffee tested at the Regis University (purchased 3/9/2021, 3/10/21, 3/19/21)

Brand	Date	Trial	Peak Area (mAU*s)	Conc. of Caff. in Injected Sample	Conc. in Original Sample (mg/L)	Mass
McDonald's purchased from franchise	3/9/21	1	582.69	33.80	338.0	120
		2	581.37	33.73	337.3	120
		3	992.51	58.05	290.3	100
		4	990.19	57.92	289.9	100
	3/19/21	1	357.12	20.46	204.6	73
		2	535.75	31.03	310.3	110
		3	979.77	57.30	286.5	100
		4	967.83	56.59	282.9687781	100
McDonald's Keurig K-cup	3/10/21	1	801.51	46.75	467.5	170
		2	705.58	41.08	410.8	150
		3	1652.4	97.10	485.5	170
		4	1395.9	81.92	409.6	140
	3/19/21	1	773.11	45.07	450.7	160

		2	775.58	45.22	452.2	160
		3	1688.2	99.22	496.1	180
		4	1689.2	99.28	496.4	180

Table 1b. HPLC results for coffee tested at the Regis University (purchased 3/9/2021, 3/10/21, 3/19/21) (cont.)

Brand	Date	Trial	Peak Area (mAU*s)	Conc. of Caff. in Injected Sample	Conc. in Original Sample (mg/L)	Mass
Starbucks purchased from franchise	3/9/21	1	1023.5	59.89	589.9	210
		2	1022.6	59.83	598.3	210
		3	532.83	30.85	617.1	220
		4	534.59	30.96	619.2	220
	3/19/21	1	1323.5	77.64	776.4	280
		2	1321.4	77.51	775.1	280
		3	2016.9	118.7	593.3	210
		4	2028.4	119.4	596.8	210
Starbucks Keurig	3/10/21	1	985.87	57.66	576.6	200

K-Cup		2	948.95	55.48	554.8	200
		3	1527.6	89.72	448.6	160
		4	2209.1	130.0	650.2	230
	3/19/21	1	900.44	52.61	526.1	190
		2	901.31	52.66	526.6	190
		3	1831.7	107.7	538.6	190
		4	1840.0	108.2	541.0	190

The results of the HPLC test are listed above for all four samples tested on various days. An average mass was calculated for each sample per trial. As shown above, the average mass of caffeine in coffee varies from day to day, even though it's from the same source. However, the changes in mass are miniscule.

Table 2. Reported Amount VS. Experimentally Determined Amount (Regis University & UNO)

Source:	Reported Amount of Caffeine (mg):	Experimentally Determined Avg Mass (mg) -Regis:	Experimentally Determined Avg Mass (mg) -UNO:
McDonald's Small Coffee	109	100	210
Starbucks Small Coffee	155	230	310
McDonald's K-Cup Pod, medium	100-140	170	N/A
Starbucks K-Cup Pod, medium	100-140	190	N/A

The average mass of caffeine was calculated for each sample to compare our experimentally determined mass, to the reported mass of caffeine. The calculated mass and the reported mass are noticeably different. There is a significant difference in the Starbucks reported and determined mass. However, it's not clear whether or not the determined mass for the Keurig Cups are within the range of the reported mass, due to an unclear reported amount of caffeine. During the procedure at University of Omaha Nebraska, only one trial for each sample was done. Due to this, it is hard to come to a definitive conclusion about the consistency of caffeine compared to the reported amount given by the franchise, and the experimental amount that was found. However, there is a clear difference between the two values.

Table 3. HPLC results for coffee tested at the University of Omaha - Nebraska (purchased 2/11/2021)

Brand	Peak Area (mAU*s)	Concentration of Caffeine (mg/L)		Mass of caffeine in 12 oz. serving
		in injected sample	in coffee cup (mg/L)	
McDonald's small coffee	11821	240.1	600.3	210
Starbucks small coffee	17151	351.6	879.0	310

The University of Omaha Nebraska, is located in Nebraska, while Regis University is located in Colorado. An interesting question we had for this project was, is the amount of caffeine consistent from state to state. The table above shows the results of the HPLC test from UNO. Table 1a and 1b show the results from Regis University. Based on the data shown, it is clear that the reported amount of caffeine given by the franchise, is not consistent from state to state. The coffee samples in Nebraska were significantly greater in caffeine compared to the samples from Colorado.

Table 4. Statistical Analysis to Determine Consistency of Brews (Regis University)

Coffee	average mass of caffeine in a small serving (mg)	standard deviation	t-test p-value
Purchased Coffee McDonald’s	109	13.714692775	0.1620534368
McDonalds K-Cup	100	11.54700538	0.240279854
Purchased Coffee Starbucks	180	23.094010766	0.1924816913
Starbucks K-Cup	100	28.72281323	0.579655134

The table above shows the statistical analysis for the dataset provided by Regis University. Typically, 0.05 is the numerical value used as the cutoff for significance. If the p-value is less than 0.05, we can reject the null hypothesis and determine that a significant difference does exist. However, if the p-value is greater than 0.05, a conclusion cannot be made. As shown in the table, the p-value for all of the samples was greater than 0.05. This means that a conclusion about significant differences does not exist. Standard deviation tells us how close a value is to its average. The lower the number, the closer it is to the mean. An observation made from the standard deviations for the samples, is that the Starbuck brand samples are close to each other, while the McDonald brand samples are close to each other as well.

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