Original Article

Identification Of Caffeine In Extracts Of Camellia Sinensis By Liquid Chromatography

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Abstract

One of the most popular drinks in the world, tea is a member of the family Theaceae, obtained from the plant *Camellia Sinensis*, found explicitly in humid and warm regions, attracts interest for its flavour and scent as well as its advantageous health properties. It is divided into three main categories: unfermented green tea and white tea, partially fermented oolong tea, and black tea that has undergone fermentation. Green tea is currently the psychostimulant most often used worldwide, as it has been shown to enhance cognitive thinking, improve neuromuscular coordination, and reduce tension and anxiety. However, it also contains caffeine members of methylxanthines which, in high amounts, more than its moderate dose, i.e., 400mg/day, can cause significant toxicity and even lethality showing symptoms such as involuntary contraction of the muscles, failure of the respiration, and heart, coma, migraine, or even death of the person in adverse cases. This study's objective is to determine the qualitative content of caffeine released in green tea after boiling over time. Liquid chromatography is used for the detection of caffeine. To establish a more exact relation between the amounts of caffeine taken and its physiological effects, it is crucial to create more accurate, easy, and quick procedures for the detection of caffeine. Caffeine was successfully eluted using this technique in three minutes, with a few additional peaks. The standard calibration curve showed exemplary linearity, and the correlation coefficient was above 0.9983.

Keywords: Green tea, HPLC, Caffeine dose, Toxic effects, Chromatography,

1. INTRODUCTION

Camellia sinensis, a Theaceae tea plant, is used to make green tea by inactivating the oxidative enzymes present in the leaves, then rolling and drying them (Chacko *et al*., 2010). Green tea drink is made by boiling tea leaves at extremely high temperatures (Cabrera *et al*., 2006). This drink includes polyphenols, alkaloids, flavonoids, phenolic acid, and flavanols like catechins named epigallocatechin (EGC) and epigallocatechin gallate (EGCG) (Musial *et al*., 2020 & Namita *et al*., 2012). The reason to analyze green tea is due to its protective properties against cancer, diabetes, hypertension, and cardiovascular diseases. The beneficial anti-oxidative properties of tea are a huge selling point for it, which is contributed by the presence of EGC and EGCG (Robb *et al*., 2002). Along with that, green tea is a complex blend of various biomolecules. Tea contains proteins, enzymes, amino acids such as glutamic acid, tryptophan, valine, serine, and aspartic acid; carbohydrates such as cellulose, glucose, sucrose, and fructose; vitamins such as B2, B3, C, E, and K; and lipids such as sterol and linoleic acid. Protein accounts for 15-50 percent of the dry weight, whereas carbohydrates account for 5-7 percent. It includes minor elements such as zinc, calcium, copper, iron, magnesium, nickel, and aluminum, as well as macro elements such as iodine, phosphorous, and fluorine. Green tea extracts include important pigments including chlorophyll and carotenoids, as well as aldehydes such as alcohols, lactones, esters, and hydrocarbons. Flavonoids coexist with phenolic acids such as proanthocyanins and gallic acid. Catechins such as epigallocatechin gallate (EGCG), epigallocatechin (ECG), epicatechin (EC), and catechin are typical green tea flavonoids (Cabrera *et al*., 2006 & Musial *et al*., 2020).

Green tea also contains caffeine which if present in excess affects consumers. Caffeine, theobromine, and theophylline are the major types of alkaloids present as a constituent of green tea. Purine 2,6-diol in its trimethyl form is used to make the caffeine in tea. It is colorless and powdery at room temperature and is soluble in different solvents, such as water, alcohol, chloroform, and acetone. It may be found in a range of beverages, including tea, coffee, and soft drinks. It is made from the nucleotide adenosine in tea leaves. Caffeine dissolves the fastest among the constituents of green tea, hence its rate of diffusion is important. It is a stimulant that is found in 2-5 percent of the dry weight of 20-40gms of green tea (Vuong *et al*., 2014). It is currently the psychostimulant that is most often used worldwide as it has been shown to enhance cognitive thinking, improve neuromuscular coordination, and reduce tension and anxiety.

While caffeine is typically considered safe in everyday doses i.e., 400 mg per day in healthy adults (Nawrot *et al*., 2003), it can cause significant toxicity and even lethality if caffeine is consumed in excess. A few sensitive people might also become toxic or fatal at or below 400 mg per day dose levels. Caffeine intoxication which in simple terms means the poisoning caused by consuming too many caffeine-containing items is characterized by a variety of clinical symptoms, such as gastrointestinal symptoms including nausea, vomiting, and diarrhoea, cardiovascular indications including hypertension, atrioventricular block, hypotension, and neurological symptoms including anxiety and seizures. Additionally, it can cause metabolic symptoms such as hypokalaemia, hyponatremia, hypocalcemia, metabolic acidosis, or musculoskeletal symptoms like weakness, rigidity, or tremor. It can also cause pulmonary indications like hyperventilation, acute respiratory distress, dizziness, or even the person's death (Wilson., 2003). Hence, there is a significant need for more evidence despite our overall understanding of the hazardous and deadly levels of caffeine, and its ill effects, particularly regarding establishing methods to estimate the amounts present in different variants of tea regarding establishing acceptable amounts in vulnerable populations.

Caffeine estimation is most commonly performed via HPLC (High-Performance Liquid Chromatography). Caffeine concentration can also be estimated using rapid techniques such as spectrophotometry with less accuracy because of the presence of additional compounds. However, it is necessary to consider the concentration of caffeine released over time. As opposed to black tea, the available expertise on caffeine extraction from green tea is quite restricted. HPLC (Ravindran *et al*., 2011; Kulkarni *et al* 2017) is an efficient technology for characterizing, separating, and identifying components in green tea samples. It is a technology for the separation of molecules in columns that uses high-performance pumps to provide the appropriate solvent at a set rate to the detector (Vuong *et al*., 2014). The substance to be examined must be soluble in the solvent. HPLC enables quantitative and qualitative examination of the substance of interest (Shivgami *et al*., 2019). The solvent used to separate the liquid sample is referred to as the mobile phase, and the column filled with polar or nonpolar material is referred to as the stationary phase. The sample is injected into the solvent, which is subsequently transported onto the column to be separated. Following separation, the samples are detected by a detector, which also assists in sample quantification. The pump keeps the flow rate of the mobile phase constant. The mobile phase flows through the column once the degassing procedure is complete. The temperature of the column is controlled by the substance to be examined. The detector identifies the eluted substance, producing a chromatogram (Saito *et al*., 2006). The retention time of a chemical acts as its identifier. Is defined as the period elapsed between sample injection and the peak reaching its maximum point. The graph of the substance studied is shown between intensity (on the Y-axis) and time (on X-axis). The area beneath the peak is a quantitative measure of the concentration of the substance present.

The present work was designed to offer a unique technique for extracting caffeine from green tea and estimating the concentration of caffeine released over time. This procedure is better than traditional extraction methods since it is simple to use with maximum output. In the present study, the procedure was standardized by comparing the results to established reference concentrations.

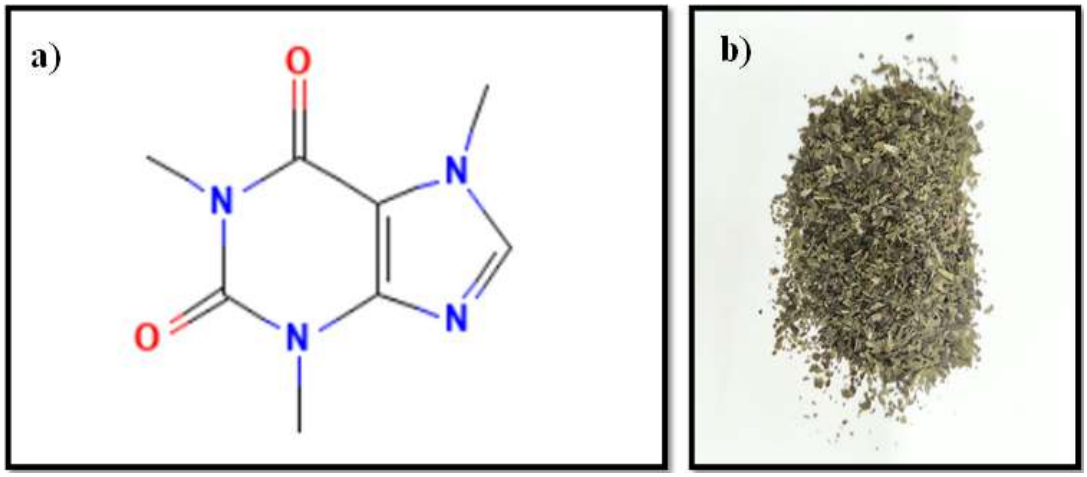

Figure 1: a) 2D chemical structure of caffeine b) Commercially available green tea leaves

2. EXPERIMENTAL SECTION

2.1 Chemicals

Commercially available instant green tea was purchased from a departmental store in Pune, Maharashtra, Standard Caffeine was obtained from Sigma Aldrich, and Acetonitrile (HPLC grade) and Trifluoracetic acid (analytical grade) were purchased from Merck. A Milli-Q system was used to purify the water (Millipore).

2.2 Instrumentation

Shimadzu Prominence Liquid Chromatography, Kyoto, Japan, was the HPLC instrument used for the investigation. The column used was thermo hypersil gold (C18 column). The instrument is outfitted with a DGU-20AS mobile phase degasser and a SIL-20AC injector valve with a temperature control oven. SPD-M20A photodiode array detector for detection at various wavelengths, LC20AD pump, and HPLC column utilized was a C18 column with dimensions of 150 mm x 5mm and filled with 5-micron particle size. The peaks were detected and characterized using the Shimadzu HPLC and the LC-Quant software. The mobile phase conditions were optimized (Suthar *et al*., 2017, Ravindran *et al*., 2019).

2.3 Preparation of standard solution and samples

The stock solution of standard caffeine; 5mg/ml was prepared by dissolving in the mobile phase. Then dilutions (less concentrated solutions) were prepared as follows: 0.05mg/ml, 0.075mg/ml, 0.1mg/ml, 0.5mgml, 1mg/ml, and 1.5mg/ml in the same mobile phase (Khambadkar *et al.,* 2020).

For the preparation of the sample, the study utilizes commercially accessible green tea bags. In an electric kettle, 100 mL of distilled water was boiled. The green tea bag (1.3g) was dunked in boiling water, and 200µL of samples were collected in HPLC vials every 10 minutes for a total of 60 minutes.

Table 1: Concentrations of standard Caffeine used in the study

Serial No	Caffeine Concentration (mg/mL)
1	0.05
2	0.075
3	0.1
4	0.5
5	1
6	1.5

3 RESULTS:

Chromatographic (Kulkarni *et al*., 2017), spectrophotometric, or electrochemical techniques have been the main focus of most research on *Camellia sinensis*. To establish a more accurate link between the amounts of caffeine taken and its physiological effects, it is crucial to create more trustworthy, easier-to-use, and quicker methods for determining the presence of caffeine from various sources. In this method, various experiments were performed with different ratios of solvent, flow rate, and temperature to assess the retention time and peak shape before choosing chromatographic conditions. It was determined that an acetonitrile/water mobile phase ratio of 90:10 was the most efficient as mobile phase A, while an acetonitrile/water mobile phase ratio of 10:90 was the most efficient as mobile phase B. The injection volume of the sample was 10uL, the column temperature was kept at 35 degrees, and the run time was 10 minutes for each sample from different periods (Ravindran *et al*., 2019). The HPLC results obtained are as follows.

Caffeine standard

The retention time for the standard caffeine sample was found to be 2.3 minutes as shown in figure 2 (a). To calculate the caffeine in prepared samples of varying quantities, a calibration curve was plotted taking area obtained from retention time 2.3, on Y-axis and known concentrations on X-axis as shown in figure 2 (b). The obtained correlation coefficient is 0.9983 indicating linearity in the prepared calibration curve. The area obtained for caffeine by HPLC analysis is shown in table 2.

Table 2: HPLC area for concentrations of standard Caffeine

Serial No.	Concentration (mg/L)	Area
1	0.05	2604291
2	0.075	3951763
3	0.1	5159194
4	0.5	18429371
5	1	34446150
6	1.5	54227882

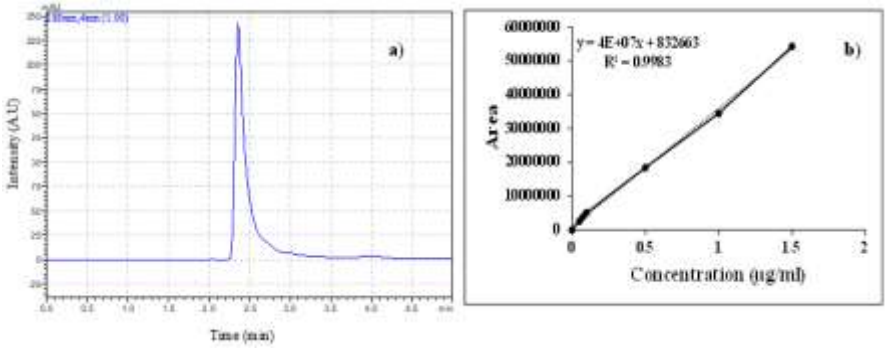

Figure 2: a) HPLC chromatogram showing retention time of standard caffeine. b) Calibration curve was obtained with a concentration of caffeine on the X-axis and an area from HPLC on the Y-axis

Green tea analysis-

Green tea samples were taken every 10 minutes and analyzed by HPLC at a run time of 10 minutes and a wavelength of 254nm. At a retention time of 2.3 minutes, there was a consistent peak area indicating the presence and quantity of caffeine in green tea extract confirmed and validated by comparative examination with the standard caffeine retention time. The peak areas obtained are shown in table 3 and HPLC chromatograms obtained are shown in figure 3 and the area obtained is mentioned.

Chromatogram shown indicates the additional peaks between 1.5 to 2.5 minute reflect the presence of catechins or other phenolic acids which are known to be present in green tea.

Further, the amount of caffeine released in mg was calculated using the equation:
Y= mx+c where,
y= Area obtained from HPLC, m = Slope, x= (Calculated for determining the amount of caffeine released), c= Intercept. Calculated amounts are shown in table 3 for samples obtained at different time points and the results are shown in Table 4, indicating the amount released in between the range of 0.12 - 0.20 mg/ml. This method can be utilized to calculate the amount of unknown caffeine present in different tea samples.

Serial no.	Retention time (min)	Sampling Time (mins)	Area
1	2.3	0	2254255
2	2.3	5	6928751
3	2.3	10	5567302
4	2.3	20	8402457
5	2.3	30	8670266
6	2.3	40	8187843
7	2.3	50	8538074
8	2.3	60	8668941

Table 3: HPLC area obtained at retention time 2.3 for prepared green tea extract at different sampling times

Table 4: Amount of caffeine released in mg from green tea extract at different time

Time (mins)	Area	Caffeine released (mg)
0	2254255	0.036
5	6928751	0.15
10	5567302	0.12
20	8402457	0.19
30	8670266	0.20
40	8187843	0.18
50	8538074	0.19
60	8668941	0.20

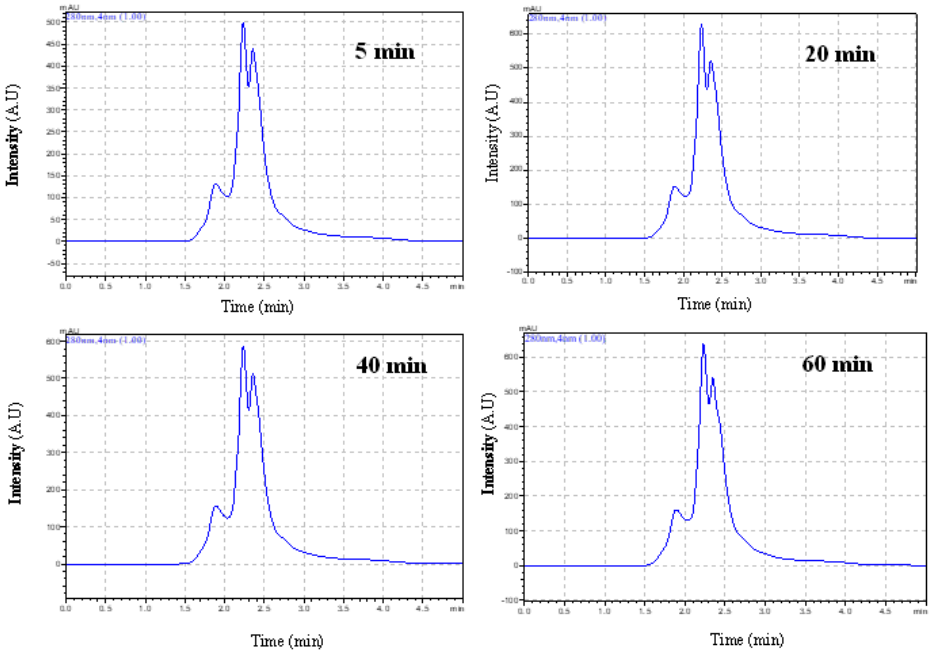

Figure 3: HPLC chromatograms for green tea extract obtained at different time

4. DISCUSSION

Due to its numerous health advantages, green tea is one of the most popular beverages (Chacko *et al*., 2010). It contains polyphenols like catechins that have health benefits, but caffeine is another ingredient that might have adverse effects if consumed more than a moderate amount (Oliveira *et al*., 2012). This investigation aims to quantify the quantity of caffeine that green tea releases after prolonged boiling. An efficient method for testing caffeine was developed using liquid chromatography. A caffeine standard was tested at various concentrations to compare and quantify the amount of caffeine released and determine how the length of boiling affected the caffeine concentration. The peak was observed at a retention time of 2.3 minute with typical caffeine concentrations. Two more peaks were also identified from the HPLC analysis of the boiled green tea extract samples. In contrast to caffeine present in the sample, which was confirmed after comparison to the retention time of standard caffeine, the peak at 1.4 retention time may correlate to catechins (flavonoids), which are the primary component of green tea. The peak observed at 2.3 minutes corresponds to the caffeine present in the sample. Additionally, the amount of caffeine present was determined to range from 0.12 to 0.20 mg/ml for different samples boiled for various time points, indicating that the amount of caffeine released from green tea is not greatly affected by the length of time of boiling, as shown in Table 4. However, green tea does contain caffeine, which, if consumed in large quantities, can have negative health effects. To conclude we can say that this established method was measuring caffeine using HPLC, is a rapid and accurate method. LC-MS/MS analysis of the peaks is required to validate the other peaks identified, LC-MS/MS will also assist in determining all the constituents and their bioavailability.

5. ACKNOWLEDGEMENT

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6. CONFLICT OF INTEREST

None declared

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