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Method Development and Validation for the Determination of Purine Alkaloid Caffeine from *Camellia sinensis* by RP-HPLC Method

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Abstract

Objective: This study was done with the objective of developing a validated method for routine analysis of caffeine content in tea by reversed phase high performance liquid chromatography method. Caffeine a purine alkaloid a xanthine derivative obtained from tea leaves *Camellia sinensis*.

Methods: Caffeine crystals were isolated from tea leaves separated through a reversed-phase C18 Chromatopak (250 mm X 4.6 X 5 micron) column using a mobile phase composed of water: Acetonitrile (60:40) at a flow rate of 1.0 ml/min. The peak response time for caffeine was observed at 1.275 minutes using UV detector set at 273 nm. The developed method was validated according to the International Conference on Harmonisation (ICH) guidelines, which includes specificity, linearity, precision, accuracy, robustness, limit of detection and limit of quantitation.

Results: The developed method validates good linearity with excellent correlation coefficient (R^2>0.999). In repeatability and intermediate precision, the percentage relative standard deviation (% RSD) of results was less than 1% shows high precision of the method. The recovery rate for caffeine was within 97.4-98.6% indicates high accuracy of the method. The low limit of detection and limit of quantitation of caffeine enable the detection and quantitation of caffeine from *Camellia sinensis* at low concentrations.

Conclusion: The developed RP-HPLC method is a simple, rapid, precise, accurate can be widely accepted and can be recommended for efficient assays. The run time was 30 mins the Rt was 1.275, the run time can be decreased to less than 10 mins, the mobile phase and run time can be saved.

Keywords: Caffeine; *Camellia sinensis*; Method development; Validation; RP-HPLC

Abbreviations: RP-HPLC: Reverse Phase High performance Liquid Chromatography; HPLC: High Performance Liquid Chromatography; GC-MS-Gas: Chromatography Mass Spectroscopy; LC-MS: Liquid Chromatography Mass Spectroscopy; LC: Liquid Chromatography; RSD: Relative Standard Deviation; SD: Standard Deviation; RT: Retention Time; UV: Ultraviolet Spectroscopy; T: Tailing factor; N: Theoretical Plates; nm: Nanometer; ppm: Parts Per Million; LOD: Limits of detection; LOQ: Limits of Quantification; R2: Correlation Coefficient.

Introduction

Green tea (*Camellia sinensis*) is a popular herbal plant having multiple beneficial health applications in humans. Owing to the emerging antibiotic resistance, nowadays researchers are searching for alternatives of antibiotics to be used for safeguarding human health, efficiently acting against the pathogenic microorganisms, enhancing immunity and as growth promoters and improving efficacy in humans. Tea is the second most-consumed beverage throughout the world [1]. Green tea (*Camellia sinensis*), a type of tea, is used as an antioxidant with abundant health benefits and also considered as one of the most well-known beverages in the world, mostly due to its prospective health welfares [2-4] **(Figure 1)**.

Tea leaf contains carbohydrates, which add to the sweetness of tea. Polyphenols referred as flavonoidal compounds such as the alkaloid caffeine and two similar compounds theobromine and theophylline, which gives the bitterness of tea. The other active constituents present are tannins which are responsible for the health claims of tea. The major flavanols in tea are catechin, epicatechin, epicatechin gallate, gallocatechin, epigallocatechin, and epigallocatechin gallate. The amino acid L-theanine found in tea [5]. Over the last 40 years, the qualitative and quantitative analyses of high volatile compounds were determined by GC and GC/MS. The main components responsible for aroma of green tea were revealed, and the low volatile compounds basically were determined by HPLC and LC/MS methods. Most studies focusing on the determination of catechins and caffeine in various teas involved HPLC analysis.

Leaves of green tea contain antioxidative catechins. Tea catechins have many health benefits such as anti-inflammatory, antiarthritic, anticarcinogenic, anti-cancerous, antimutagenic, antibacterial, antiviral, antifungal, anticoccidial, antiprotozoal, antiparasitic, anti-infective, hypocholesterolemic, resistant to capillary blood congestion and hypolipidemic effects [6-17].

Reversed-phase high-performance liquid chromatography (RP-HPLC) involves the separation of molecules on the basis of hydrophobicity. Extensive literature survey revealed that few methods were reported for the estimation of caffeine by RP-HPLC. Hence, an attempt has been made to rapid development and validates a new, simple, accurate, precise, and economically feasible RP-HPLC method for the quantification of caffeine in Camellia sinensis [18-26] **(Figure 2)**.

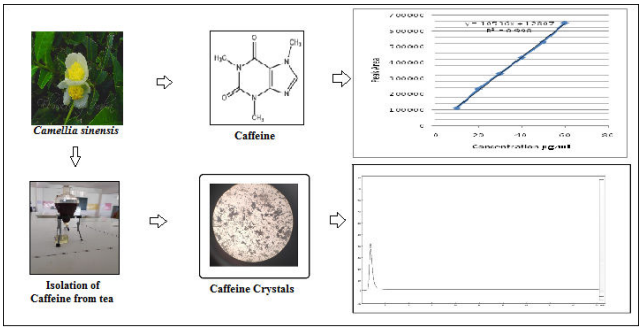

Figure 1 Graphical abstract of HPLC chromatogram.

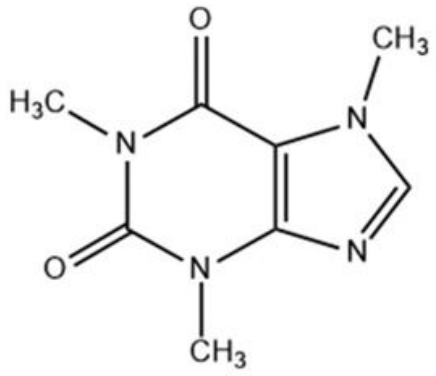

Figure 2 Chemical structure of caffeine.

Materials and Methods

Tea leaf granules were procured from departmental stores, Tirupati, Chittoor Andhra Pradesh India. Caffeine Standard was obtained from Sigma Aldrich. Reagents and solvents were analytical grade and HPLC grade procured from S.D. Fine Chem Pvt Ltd.

Instrumentation and chromatographic conditions

Instrument: HPLC was performed on an Analytical Technology system equipped with a quaternary low-pressure gradient solvent delivery P3000A HPLC pump.

Sonicator: Model: 1.5L 50 degasser units.

Detector: A high-sensitivity UV 3000 ultraviolet (UV) detector.

Software: The system controlled and data analyzed by HPLC workstation software.

Column: A separation was carried out in C18 Chromatopak (250 mm × 4.6 × 5 micron).

Mobile phase: The mobile phase consists of isocratic elution with a low-pressure gradient using double-distilled HPLC grade Water: HPLC grade acetonitrile (A:B): (60:40)

Flow rate: Flow rate of 1.0 ml/min and the injection volume of 20 µl.

Degassing: All solutions were degassed and filtered through 0.2 µm pore size filter.

Column Temperature: The column was maintained at 40°C throughout analysis.

Detector (nm): UV detector was set at 273 nm.

Run time: The total LC run time was 30 min.

Before choosing the chromatographic condition, a number of trials were carried out with different ratios of solvents, flow rate, and temperatures to check the retention time (RT), peak shape, tailing factor (peak symmetry), and theoretical plates of the analyte. In addition, the system suitability parameters such as tailing factor (T) and theoretical plates (N) are calculated to the caffeine standard solution (25 ppm) **(Table 1)**.

Table 1 Chromatographic trial.

Trials	Results
Trial 1	Was performed on LC, Chromotopak C18 (100 mm × 4.6 mm i.d; 3.5 µm particle size) column using mobile phase of water: Acetonitrile in the ratio of 60:40v/v at U.V detection at 273 nm.
	A Broad peak appeared so this was not satisfactory.
Trial 2	Was performed on LC, Chromotopak C18 (100 mm × 4.6 mm i.d; 3.5 µm particle size) column using mobile phase of water: Acetonitrile: in the ratio of 60:40 v/v at U.V detection at 273 nm.
	Fronting peaks and some extra peaks appeared so this was not satisfactory.
Trial 3	Mobile phase of Water : Acetonitrile 60:40 v/v at U.V detectection at 273 nm
	A sharp peak with a retention time of 1.275.
	The trail 3 was selected for the further work in HPLC as the result was satisfactory with a sharp peak with the retention time of 1.275 min

Isolation of caffeine from tea

About 200 g of tea powder was weighed accurately, transferred into a 1000 ml beaker; 800 ml of water was added heated for half an hour. The hot decoction was filtered using tea filter the filtrate was transferred into a separating funnel. The decoction was extracted with 30 ml of chloroform consecutively for three times, all the extracts were mixed together. The extract was filtered (Watt Mann No 1 filter paper) with charcoal, the filtered extract was concentrated up to 20 ml on a water bath for half an hour and 10 ml chloroform was added to the extract until the caffeine crystals were formed the yield obtained was around 0.28 g.

Identification test for caffeine (Murexide test)

The (solid caffeine crystals) sample was first treated with conc. nitric acid, which was slowly evaporated to dryness with subsequent addition of ammonia solution (NH_4OH) a purple color was obtained with addition of Murexide (ammonium purpurate) **(Figures 3 and 4)**.

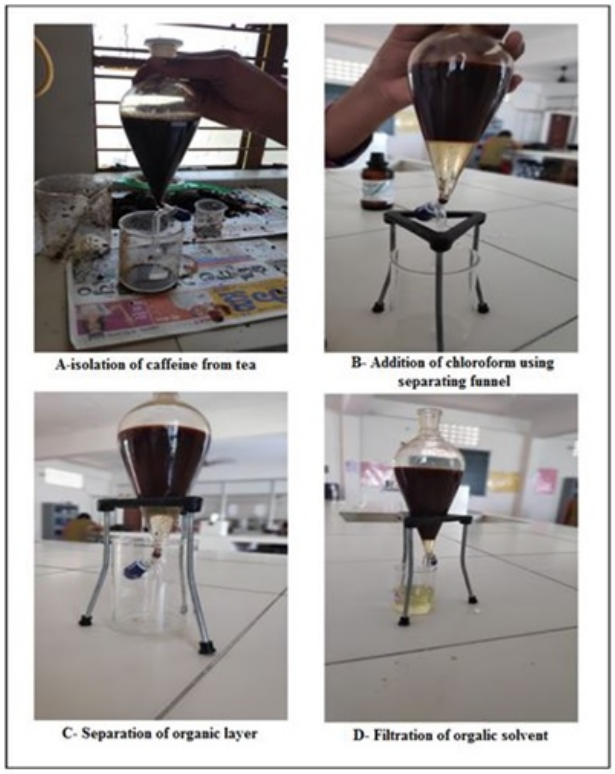

Figure 3 Pictorial representation of isolation of caffeine from tea.

Preparation of standard solution (25 ppm)

50 mg of caffeine was weighed accurately into a 100 ml volumetric flask dissolved and diluted to the volume with mobile phase. Further 5 ml of the above solution was diluted to 100 ml with mobile phase.

Preparation of sample solution

50 mg of caffeine (sample) was weighed accurately into a 100 ml volumetric flask dissolved and diluted to the volume with mobile phase. Further 5 ml of the above solution was diluted to 100 ml with mobile phase.

Preparation of spiked sample solution

The accuracy for the method was determined by spiking the blank samples at standard concentrations (10, 50 and 100 ppm) and analyzing their recoveries.

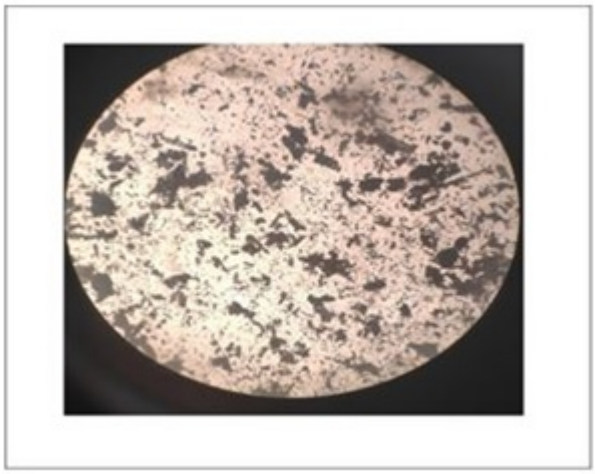

Figure 4 Microscopical pictogram of caffeine crystals.

Validation of the method

The validation of the developed analytical method was done according to the International Conference on Harmonisation (ICH) guidelines [27,28]. The method is validated for specificity, linearity, recovery, precision, and the limits of detection (LOD) and limits of quantification (LOQ).

Linearity

Linearity was determined by different known concentrations of caffeine standard solution in triplicate by diluting the standard stock solution. For the determination of linearity, five aliquots were pipetted out from standard stock solution (i.e., 60 ppm). 2-10 ml of standard stock solution was pipetted out in to a series of 10 ml volumetric flasks and volume was made up with the solvent to obtain concentration ranging from 10 ppm to 60 ppm of caffeine. The calibration curve was constructed by plotting the peak areas against concentration, and the linear regression equations were calculated. The correlation coefficient was also computed **(Table 2 and Figure 5)**.

Table 2 Linearity data for caffeine.

Concentration	Peak Area
10	112000
20	233380
30	330677
40	431778
50	530553
60	650988

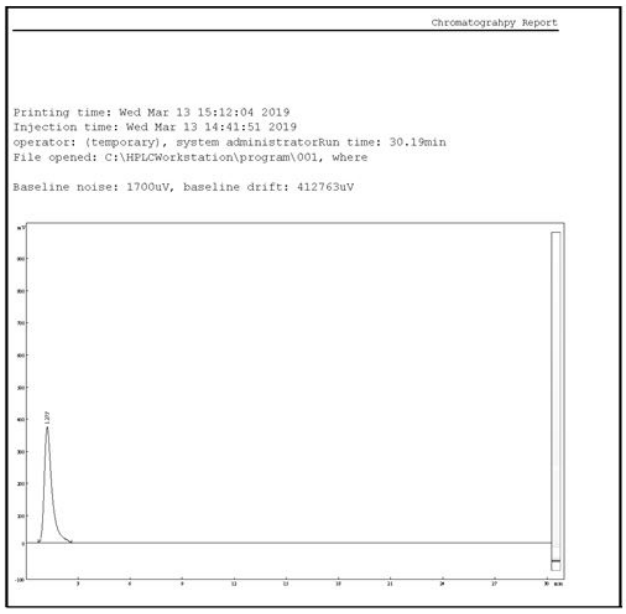

Figure 5 Optimized chromatogram.

Precision

Precision was determined by studying the repeatability (intraday) and intermediate (inter day) precision. The repeatability and intermediate precision of the method was determined by calculating the percentage relative standard deviation (% RSD). The repeatability was examined on the same day, whereas intermediate precision examined on different days by the different analyst **(Table 3)**.

Table 3 Repeatability data for caffeine (25 ppm).

Number	Concentration of Sample (ppm)	Retention time (min)	Area	Result %
1	25	1.276	235470	99.2
2	25	1.275	233380	99.4
3	25	1.277	245310	99.3
4	25	1.27	240120	99.7
5	25	1.273	247110	99.8
6	25	1.276	234150	99.9
	Average	1.274	239256	99.55
	Std. Dev.	0.002588	5899.49	1.87
	% RSD	0.20%	2.47%	0.19%

Accuracy

The accuracy for the method was determined by spiking the blank samples at standard concentrations (10, 50 and 100 ppm) and analyzing their recoveries **(Table 4)**.

The LOD and the LOQ for caffeine were found to be 2.12 ppm and 7.24 ppm, respectively. Low LOD and LOQ of caffeine enable the detection and quantitation of this alkaloid in *C. sinensis* at low concentrations.

Table 4 Recovery study of caffeine.

Concentration in the blank (ppm)	Amount of spiked caffeine (mg)	Amount of caffeine found (mg)	Recovery %
10	10 mg	9.86	98.6
25	25 mg	24.4	97.8
50	50 mg	48.7	97.4

Results and Discussion

Experimental trails

Before choosing the chromatographic condition, a number of trials were carried out with different ratios of solvents, flow rate, and temperatures to check the retention time (RT), peak shape, tailing factor (peak symmetry), and theoretical plates of the analyte **(Figure 5)**.

Acceptance criteria: Correlation coefficient should not be less than 0.998.

Robustness: The Robustness of the method was determined by making slight changes in the experimental conditions such as change in the flow rate, mobile phase and wave length **(Table 5)**.

Table 5 Summary of robustness.

Parameters	Condition	Rt
Flow rate	0.9 ml/min	1.276
Actual flow rate	1 ml/min	1.275
Flow rate	1.1 ml/min	1.27
Wavelength	271 nm	1.221
Wavelength	273 nm	1.275
Wavelength	275 nm	1.274

Discussion

Results obtained from method validation study can be used to judge the quality, reliability, and consistency of analytical results. An RP-HPLC method was developed and validated for the determination of caffeine in *C. sinensis*. Several mobile phase compositions were tried, and a satisfactory separation was obtained using the mobile phase composition water: Acetonitrile (A:B): (60:40 v/v). An optimized chromatogram of blank, standard, and sample was shown in **Figures 6-8**. The Rt of standard and sample peak was found to be at 1.275 and 1.278 min, respectively.

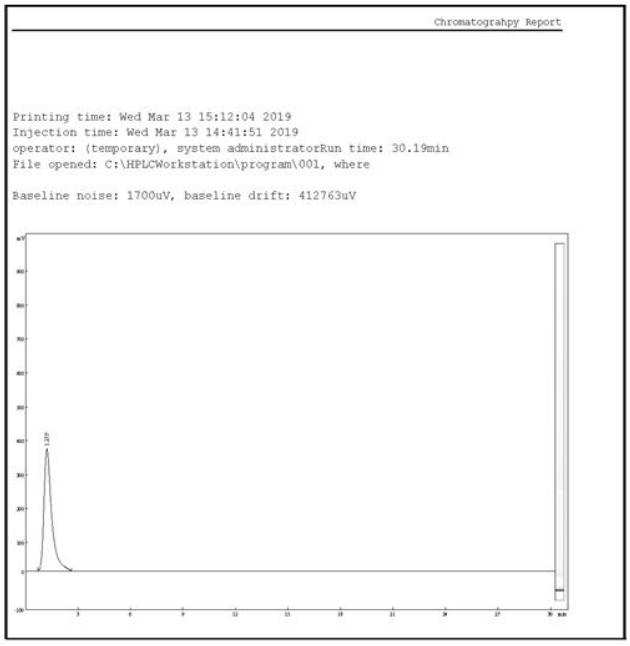

Figure 6 HPLC chromatogram of caffeine standard.

The linearity of the analytical method, calibration curves were plotted by peak area versus concentration of caffeine standard in the range 10-60 ppm. This indicated that good fitting of the curve. The correlation coefficient (R^2) of caffeine standard solution was 0.998. The acceptance criteria for linearity are that the correlation coefficient (R^2) should not be less than 0.999. This indicates that the method showing good linearity.

The repeatability of the method was investigated by performing 6 repeated analysis of 1 standard solution (25 ppm) on the same day (for intra-day repeatability) and different day for inter day precision. The results showed that the % RSD for retention time and area were satisfactory for further analysis.

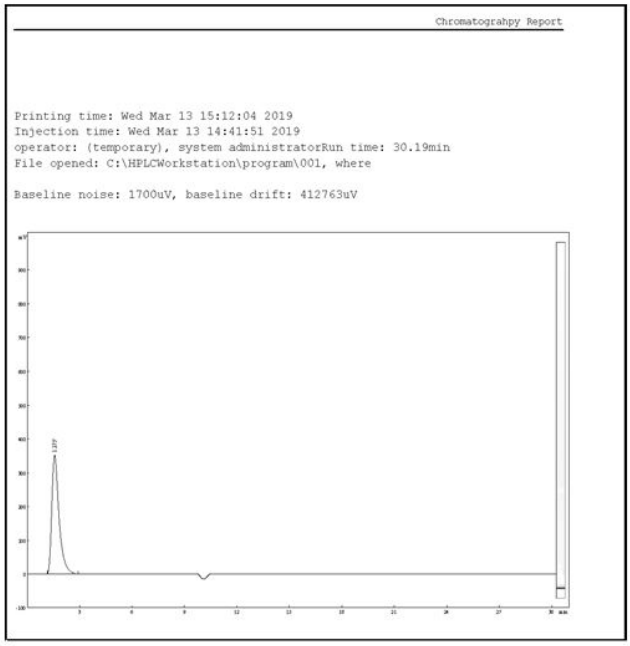

Figure 7 HPLC chromatogram of caffeine sample.

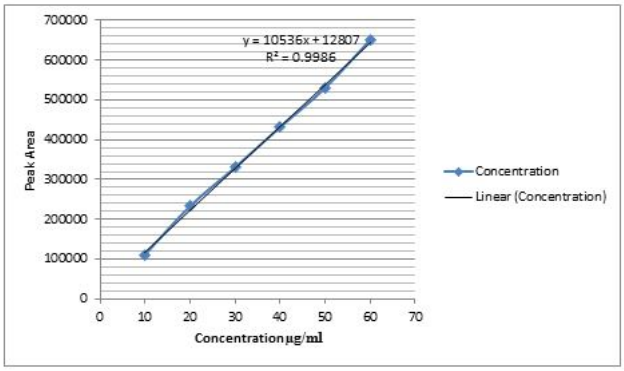

Figure 8 Linearity of caffeine.

The accuracy for the method was determined by spiking the blank samples at standard concentrations (10, 50 and 100 ppm) and analyzing their recoveries. Sensitivity of the method was measured in terms of Limit of detection (LOD) and limit of Quantification (LOQ).

Conclusion

A simple reverse phase HPLC method was developed for the determination of Caffeine present in tea leaves. An LC Chromatopak C18 (250 mm × 4.6 × 5 micron) column with UV Detector in isocratic mode, with mobile phase water: Acetonitrile (60:40 v/v) was used. The flow rate was 1.0 ml/min and effluent was monitored at 273 nm. The retention time was 1.275 min for Caffeine. The linearity range was found to be 10-60 µg/ml. The proposed method was also validated. The proposed study describes a new RP-HPLC method for the Estimation of Caffeine. A new RP-HPLC method was developed for the estimation of Caffeine using simple mobile phase–Water: Acetonitrile: (60:40 v/v) was used. The method gave good results within a short analysis time. The developed method was validated in accordance with ICH guidelines and all of the results were within the limits. The developed HPLC method is a simple, rapid, precise, accurate and can be widely accepted and can recommended for efficient assays in routine work. Since the run time was 30 min the Rt was 1.275, the run time can be reduced to 10 min, the time and mobile phase can be saved.

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Conflict of Interest

The authors do not have any conflict of interest.

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