ORIGINAL ARTICLE

# Study of extraction and HPTLC - UV method for estimation of caffeine in marketed tea (*Camellia sinensis*) granules

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A simple, precise and accurate high-performance thin-layer chromatographic (HPTLC) method has been established and validated for screening and quantitative estimation of caffeine in different extracts of tea samples (*Camellia sinenesis*). Separation was performed on silica gel 60 $F_{254}$ HPTLC plates with ethyl acetate:methanol in the proportion of 27: 3 (*v/v*), as a mobile phase. The determination was carried out in the ultraviolet (UV) region using the densitometric remission-absorbance mode at 274 nm. Maximum recovery of caffeine was achieved when extracted with 5% diethyl amine in DM water (*v/v*). The maximum concentration of caffeine in tea samples was found to be 2.145%, dry weight basis. Caffeine response was found to be linear over the range of 2-14 µg per zone. Limits of detection and quantitation were found to be 40 and 120 ng/spot, respectively. The HPTLC method was validated in terms of precision, accuracy, sensitivity and robustness. Some rare parameters for the HPTLC method like calculation of flow constant (k) and plate efficiency (N) are included specially.

**Key words:** Caffeine, *Camellia sinensis*, HPTLC - UV method, theaceae, tea granules

## INTRODUCTION

Caffeine [Figure 1] occurs naturally in over 60 plant species including tea, coffee and cola. It is produced commercially both by extraction from natural sources and synthetic procedures. The majority of caffeine produced is used in the beverage industry. It is also used as a flavour enhancer in foods and as a flavouring agent in baked goods, frozen dairy desserts, gelatins, puddings, fillings and soft candy. Caffeine is also used therapeutically. The average daily caffeine intake in the United States is ~200 mg per individual.[1] Caffeine is regulated by the Food and Drug Administration (FDA).

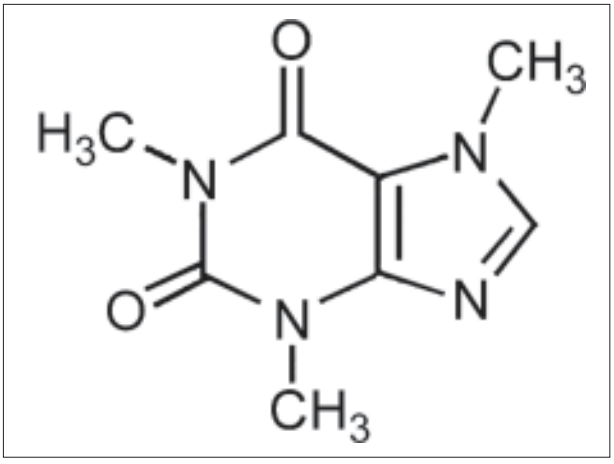


**Figure 1:** Structure of caffeine

Data collected from a number of epidemiological studies on the long-term health effects of caffeine have indicated no marked elevations in risk for most diseases, including cardiovascular disease, ulcers, breast disease and for effects of various target organs. Absorption of caffeine from the gastrointestinal tract is pH-dependant, rapid and virtually complete. After absorption, it is distributed rapidly into the body fluids. It binds to the plasma proteins, mainly albumins. Metabolism is performed by hepatic microsomal enzymes and does not appear to occur significantly in other organs. In humans, caffeine is metabolized into more than 25 metabolites, primarily paraxanthine, theobromine and theophylline. In humans, acute exposure to caffeine can cause gastric symptoms, insomnia, diuresis, restlessness, headache and tremors. At concentrations of up to 10 nmol/mL in blood, caffeine stimulates the central nervous system (CNS).[1] Previous studies[2-5] have shown that green tea and black tea have antioxidant effects and chemopreventive activity against chronic diseases including some forms of cancer.[6] The biological effect of an aqueous extract of black tea against $CCl_4$-induced lipid peroxidation (LPO) is determined by the formation of thiobarbituric acid. Reactive substances in the liver, kidneys, and testes of rats have been studied, and the

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results suggest that the protective effects of black tea against $CCl_4$-induced LPO in the liver, kidneys and testes is at least partly due to its antioxidant properties, scavenging $CCl_4$-associated free radicals.[7]

These days caffeine is being utilized as flavour-enhancer by most of the beverage (soft drinks), coffee and tea companies. So, its analytical estimation/determination methods become more important for its regulation. High Performance Liquid Chromatography (HPLC),[8,9] *Thin layer chromatography* (TLC),[10] high-performance thin-layer chromatography (HPTLC),[11] reversed-phase thin layer chromatography (RPTLC),[12] isotachophoresis[13] and UV-spectrophotometric[14] methods for determination of caffeine either in bulk, dosage forms, herbal products, energy drinks or in biological fluids have been described previously. Herein, we have developed and validated the HPTLC - UV method, for the quick determination of caffeine from marketed granules of tea. Validation was performed with special reference to theoretical plate counts and flow constant. The method not only finds importance in the quality control analysis of caffeine, but also shows the importance of screening marketed tea granule samples for the caffeine content.

## MATERIALS AND METHODS

### Plant Material

Tea (*Camellia sinensis*) granules were purchased from the local market. These tea granules were not pulverized to powder and extracted as such using different solvents.

### Chemicals and Reagents

All chemicals used in this study were of analytical grade. Sulphuric acid (98%) used in the study was purchased from s. d. fine-chem. Limited, Mumbai. Standard caffeine was purchased from Merck, Germany.

### Apparatus

HPTLC - UV analysis was performed on a computerized densitometer scanner 3, controlled by *winCATS* planar chromatography manager *version 1.4.2.* (CAMAG, Switzerland), having the facility of multiwavelength Scanning. Drying and concentration steps were performed using a rotatory evaporator (Buchi, Switzerland) equipped with an auto vacuum controller device. The ultrasonic bath (Enertech, Mumbai, India) was used for homogenizing test and standard solutions.

### Extraction and Test Sample Preparation

Extraction of 1 g tea granules was performed with 30 mL of the corresponding solvent for 5 hours. After completion of 5 hours. The extract was filtered via filter paper and the residual tea granules washed with (5 mL × 3 times) the corresponding solvent. The organic layer thus obtained was concentrated *in vacuo* via the rotatory evaporator to dryness and redissolved in 20 mL of chloroform:methanol (1:1, *v/v*). This solution was taken as a test sample for quantification purposes. In case of the aqueous solvent, the procedure was the same up to the clean up step, after that caffeine was extracted in 20 mL of chloroform (20 mL × 3 times) and total volume of this layer passed through anhydrous sodium sulphate. The drying step was performed as described above for the organic layer.

### Standard Sample Preparation and Calibration Graph

A stock solution of caffeine was prepared by dissolving 100 mg of caffeine in 100 mL mixture of chloroform:methanol (1:1, v/v) in a 100 mL volumetric flask. This solution was sonicated for 10 minutes over an ultrasonic bath, to obtain a homogenous solution. A linear calibration curve was obtained on spotting the increasing concentration of caffeine (2-14 μg $spot^{-1}$).

### Thin-Layer Chromatography

Thin-Layer Chromatography (TLC) was performed on 20 cm × 10 cm aluminium-backed HPTLC plates coated with 200 μm thick layers of silica gel 60 $F_{254}$ (E. Merck, Darmstadt, Germany). Before use the plates were prewashed with methanol and activated at 60°C for 5 minutes. The samples were applied at 9.0 mm from the base of HPTLC by the spray-on technique, along with nitrogen gas supply, for simultaneous drying of bands, by means of a Camag (Switzerland) Linomat V sample applicator using a 100 μL syringe (Hamilton, Bonaduz, Switzerland). Plates were developed to a distance of 9.8 cm, in the dark, with ethyl acetate: methanol, 27:3 (*v/v*), as a mobile phase. The volume of the mobile phase was 30 mL. Before development the chamber was saturated with a mobile phase for 10 minutes at room temperature (25 ± 2°C). Chromatography was performed in the Camag's twin-trough thin-layer chromatography chamber. A chromatogram [Figure 2]

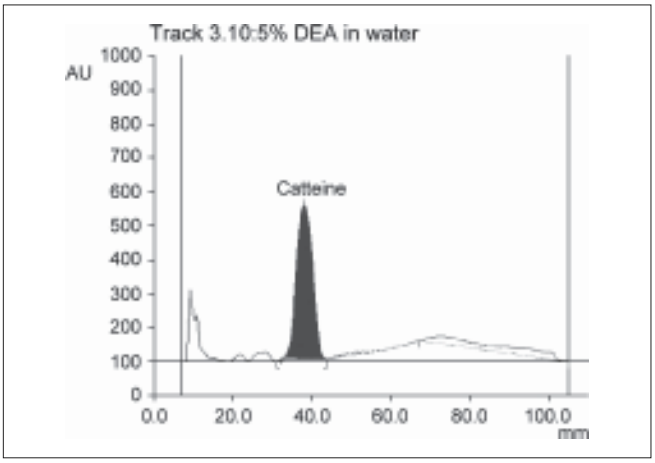


**Figure 2:** HPTLC chromatogram of test caffeine (Rf 0.39).

was obtained after densitometric scanning was performed with a Camag TLC scanner 3 in remission-absorbance mode at 274 nm, under control of Camag *winCATS* planar chromatography manager software. The slit dimensions were 5 mm × 0.45 mm and the sample track and spot spectrum scanning speeds were 20 mm/second and 100 nm/second, respectively. Data resolution of the sample track and spot spectrum were performed online at 100 µm/step and 1 nm/step, respectively.

## RESULTS AND DISCUSSION

### Screening of Extraction Solvents and Conditions

Different organic solvents and aqueous mixtures of varying nature were used for the screening of caffeine extraction from tea granules. Order of recovery of caffeine with different organic solvents and aqueous mixtures was: *n*-hexane < ethyl acetate < methylene dichloride < chloroform < methanol < DM water < 5% sulphuric acid in water < 5% diethyl amine in DM water. Thus, mixture of DM water: DEA (95:5, *v*/*v*) was selected for furtherstandardizations towards maximum recovery of caffeine.

### Optimization of Best Extraction Conditions

As DM water: DEA (95:5, *v*/*v*) gave the highest recovery of caffeine (2.145±0.032%, dry weight basis), it needs optimization of the diethyl amine (DEA) concentration, so as to recover the maximum possible caffeine from the tea granules. Different DEA concentrations of 5%, 10% and 15% in DM water (*v*/*v*) were used for this purpose. Five per cent aqueous DEA was found to extract the maximum possible caffeine from tea granules.

### Effect of Acidic and Basic Extraction

The effects of acid and base with DM water used for extraction of caffeine, and results of the extraction have been summarized in the Table 1. Addition of sulphuric acid in DM water had almost no effect on the recovery of caffeine as compared to DM water itself. While addition of base (DEA) with DM water increased recovery of caffeine drastically. It is probably due to '*like dissolves like*'.

## METHOD VALIDATION

### Linearity

The linearity of the caffeine calibration plot [Figure 3] was evaluated by spotting increasing amounts of the caffeine working standard solution, starting from 2 to 14 µg/spot. The method showed good linearity in the given range [Table 2].

### Precision

Precision of the method was determined by three replications of each sample. The precision (%RSD) of the replications was found to be less than two, which is indicative of a precise method. Peaks of caffeine eluted on to the HPTLC plate were found to be pure [Table 2].

### Limit of Detection and Quantitation

Limit of detection and quantitation (LOD and LOQ) was determined by spotting increasing amounts (20 - 140 ng; n = 2) of standard caffeine solution of concentration 10 µg/mL until the average responses were 3 and 10 times of the

**Table 1: Screening based extraction of caffeine from tea granules for optimization of extraction**

| Extraction solvent | % Caffeine content, dry weight basis, (n = 3) | Mean % caffeine content | Standard deviation | %RSD |
|---|---|---|---|---|
| *n*-Hexane | 0.017 | 0.021 | 0.004 | 19.048 |
| | 0.021 | | | |
| | 0.025 | | | |
| Chloroform | 0.280 | 0.281 | 0.009 | 3.203 |
| | 0.291 | | | |
| | 0.273 | | | |
| Methylene chloride | 0.221 | 0.215 | 0.007 | 3.256 |
| | 0.218 | | | |
| | 0.207 | | | |
| Ethyl acetate | 0.173 | 0.173 | 0.004 | 2.312 |
| | 0.177 | | | |
| | 0.169 | | | |
| Methanol | 1.590 | 1.500 | 0.078 | 5.200 |
| | 1.449 | | | |
| | 1.460 | | | |
| Tetrahydrofuran | 0.459 | 0.460 | 0.001 | 0.217 |
| | 0.461 | | | |
| | 0.460 | | | |
| DM[1] water | 1.549 | 1.553 | 0.004 | 0.258 |
| | 1.555 | | | |
| | 1.556 | | | |
| 5% DEA[2] in DM water[†] | 2.122 | 2.145 | 0.032 | 1.492 |
| | 2.131 | | | |
| | 2.182 | | | |
| 5% $H_2SO_4$ in DM water | 1.590 | 1.560 | 0.032 | 2.051 |
| | 1.564 | | | |
| | 1.527 | | | |

[1]De-mineralized water; [2] Diethyl amine; [†] Best optimize condition for extraction

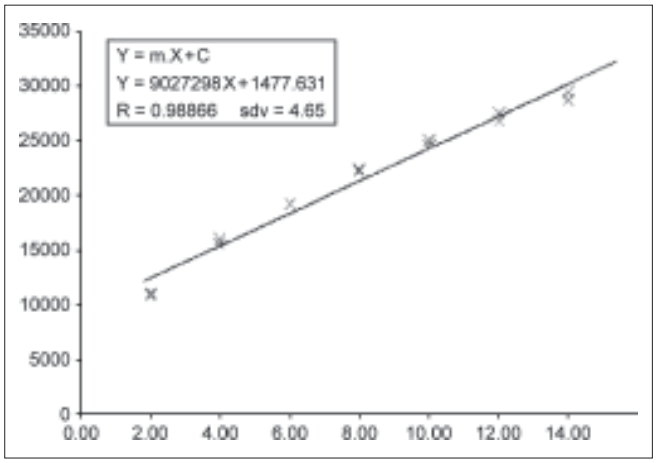


**Figure 3:** Calibration curve of caffeine

noise (or 3 and 10 times of the standard deviation of the responses for three replicate determinations) for LOD and LOQ, respectively. LOD and LOQ were found to be 40 and 120 ng/spot, respectively.

**Table 2: Summary of method validation parameters of caffeine**

| Parameters | Results |
|---|---|
| Linearity range (µg/spot) | 2-14 |
| Linear regression equation | Y = m X + C |
| Slope (m) | 1478 |
| Intercept (C) | 9027 |
| Correlation coefficient (r) | 0.98866 |
| Standard deviation of calibration curve | 4.65 |
| Peak purity of eluted test caffeine spot (n = 3) | |
| Correlation coefficient, r (s, m) | 0.999864 |
| Correlation coefficient, r (m, e) | 0.998843 |
| Precision (%RSD) | |
| Repeatability of sample peak area (n = 3) | 1.159 |
| Repeatability of standard peak area (n = 2) | 0.553 |
| Repeatability of retardation factor, Rf (n = 14) | 1.852 |
| Limit of detection (LOD) | 40.0 ng/spot |
| Limit of quantification (LOQ) [%RSD < 2] | 120.0 ng/spot |
| Specificity | specific |
| Plate efficiency (N) | 947.5 |
| Flow constant (k) | 8.631 $mm^2/s$ |

**Specificity**

The developed HPTLC - UV method was found to be specific as no interfering peak was detected during the detection of caffeine, as is also evidenced by the peak purity data [Table 3 and Figure 4].

**Robustness**

Robustness of the method was determined by performing small variations in the mobile phase ratio, height of plate development and TLC tank saturation time. The results indicated insignificant differences in the assay results, thus

**Table 3: Peak purity correlations of test and standard caffeine samples at peak start, middle and end**

| Sample | No. of replications (n) | Correlation of centre and slope spectra of caffeine | | Mean correlation of centre and slope spectra of caffeine | |
|---|---|---|---|---|---|
| | | r (s, m) | r (m, e) | r (s, m) | r (m, e) |
| Test caffeine | 03 | 0.999923 | 0.998792 | 0.999864 | 0.998843 |
| | | 0.999821 | 0.998734 | | |
| | | 0.999849 | 0.999002 | | |
| Standard caffeine | 02 | 0.999833 | 0.999052 | 0.999847 | 0.999155 |
| | | 0.999860 | 0.999257 | | |

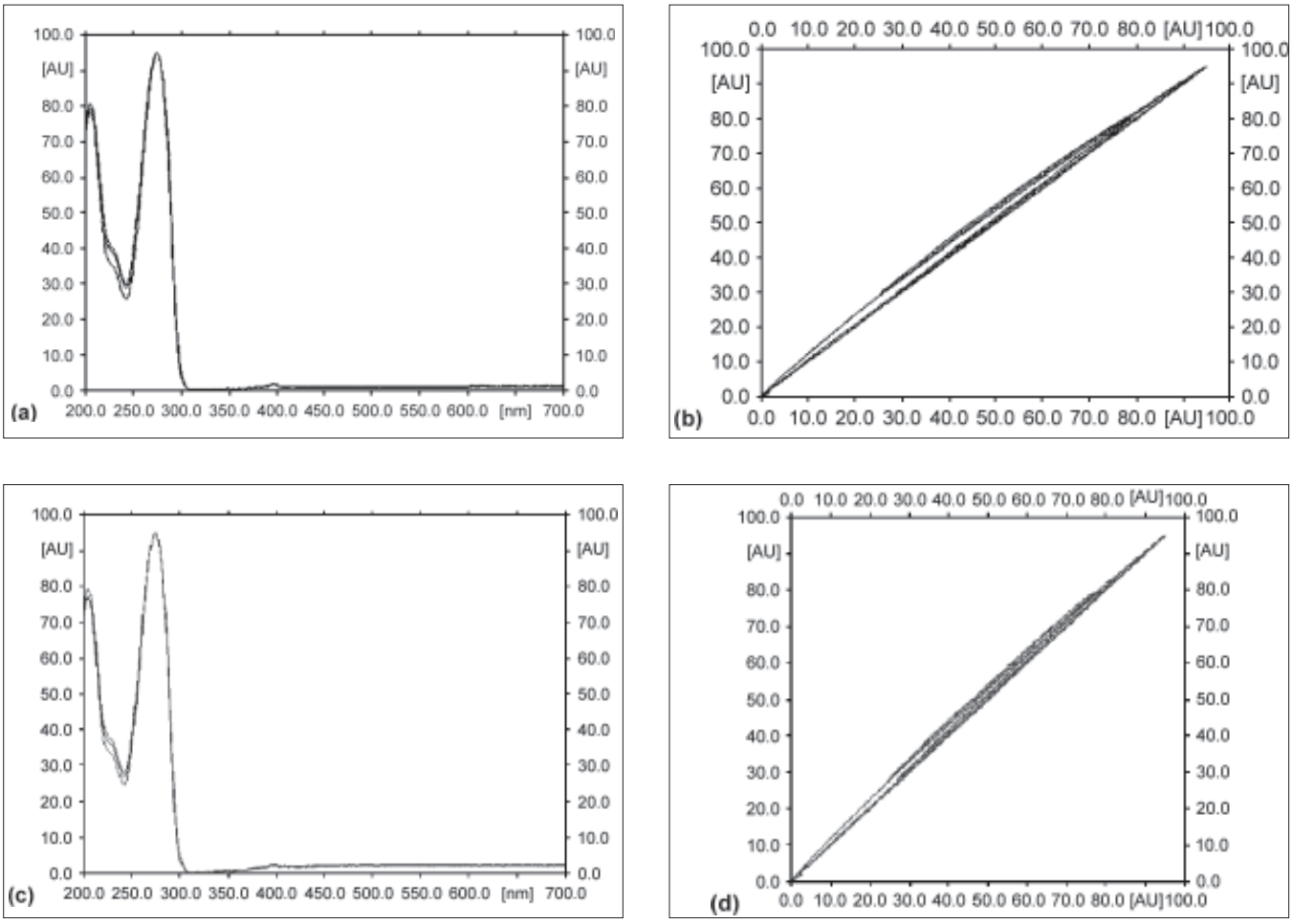


**Figure 4:** Complete spectrum of caffeine and peak purity; (a) Center and slopes spectra of test caffeine; (b) Correlation of center and slopes spectra of test caffeine; (c) Center and slopes spectra of standard caffeine; (d) Correlation of center and slopes spectra of standard caffeine

indicative of a robust method.

**Calculation of Flow Constant[15,18]**

The flow constant or velocity constant (k) is a measure of the migration rate of the solvent front. It is an important parameter for TLC users and can be used to calculate, for example, development times with different separation distances, provided that the sorbent, solvent system, chamber type and temperature remain constant. The flow constant is given by the following equation:

$$k = \frac{Z_F^2}{t}$$

where, k is the flow constant [$mm^2/s$], $Z_F$ is distance between the solvent front and the solvent level [mm] and $t$ is the development time [s]. The flow constant as calculated by this method was found to be 8.631 $mm^2/s$.

**Calculation of Plate Efficiency (N)**

Plate efficiency, also known as the number of theoretical plates was calculated for the described method by the following equation:[16-18]

$$N = \frac{16 \times l \times z}{w^2}$$

where, $l$ is the distance (in mm) travelled by the solvent front, $z$ is the distance (in mm) travelled by the target spot from the application point and $w$ is the width of the spot (in mm), in the direction of the ascending mobile phase. The plate efficiency was calculated to be 947.5 for caffeine.

## CONCLUSION

This new method for caffeine represents an improved approach for its determination, taking into consideration the 'number of theoretical plates' as well as 'flow constant,' as parts of the validation. It offers the advantages of speed, simplicity and selectivity. Moreover, now-a-days, computer controlled densitometers are readily available and the cost of analysis per sample is very nominal as compared to HPLC. The above-described method may be useful in the process of the analytical method for caffeine determination as well as for screening purposes.

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