EXTRACTION AND ESTIMATION OF THEOBROMINE IN MARKETED TEA BY HPTLC AND UV METHOD

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ABSTRACT: A rapid, simple and sensitive chromatographic method (RP-HPTLC) has been developed for the extraction and quantitative estimation of Theobromine in different extract tea samples (Camellia sinensis). Separation was performed on Silica gel 60 f_{254} HPTLC plates with ethyl acetate: methanol (27:3 v/v), as mobile phase. The determination was carried out in the UV region, using densitometric absorbance at 274 nm. The maximum concentration of Theobromine in tea samples was found to be 2.313 %. The theobromine response was found to be linear over range 3-15 µg per zone. Limit of detection and quantitation were found to be 30 and 140 ng/ spot respectively. The HPTLC method was validated in terms of precision, accuracy, sensitivity and robustness. The proposed method provided precise and accurate analysis for extraction and estimation of theobromine by HPTLC-UV.

Keywords: Theobromine, Camellia sinensis, Theobroma cacao, Tea, HPTLC-UV.

INTRODUCTION: Theobromine (Figure: 1) belongs to a class of alkaloid molecules known as methylxanthines. Methylxanthines naturally occur in as many as sixty different plant species and include caffeine (the primary methlyxanthine in coffee) and theophylline (the primary methylxanthine in tea). Theobromine is the primary methylxanthine found in products of the cocoa tree, *theobroma cacao*.
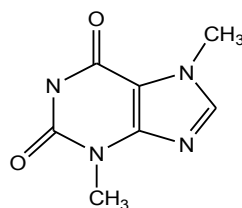
Theobromine
3,7-dihydro-3,7-dimethyl-1H-purine-2,6-dione

Figure: 1

Theobromine affects humans similarly to caffeine, but on a much smaller scale. Theobromine is mildly diuretic (increases urine production), is a mild stimulant, and relaxes the smooth muscles of the bronchi in the lungs. In the human body, theobromine levels are halved between 6-10 hours after consumption. It has been used as a drug for its diuretic effect, particularly in cases where cardiac failure has resulted in an accumulation of body fluid. It has been administered with digitalis in order to relieve dilatation. Because of its ability to dilate blood vessels, theobromine also has been used to treat high blood pressure[1].

These days theobromine is being utilized as flavour-enhancer by most of the chocolate manufacture industries. So, its analytical estimation/determination methods become more important for its regulation. High Performance Liquid Chromatography (HPLC)[2,3], *Thin layer chromatography* (TLC)[4], high-performance thin-layer chromatography (HPTLC)[5],reversed-phase thin layer chromatography (RPTLC)[6], isotachophoresis[7] and UV-spectrophotometric [8] methods for determination of caffeine have been described previously. Herein, we have developed and validated the HPTLC - UV method, for the quick determination of theobromine 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 theobromine, but also shows the importance of screening marketed tea granule samples for the theobromine 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.

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 1g tea granules was performed with 30mL 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 (5mL × 3 times) the corresponding solvent. The organic layer thus obtained was concentrated *in vacuum* via the rotatory evaporator to dryness and redissolved in 20mL 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 20mL of chloroform (20mL × 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 theobromine was prepared by dissolving 100mg of caffeine in 100 mL mixture of chloroform: methanol (1:1, v/v) in a 100mL volumetric flask. This solution was sonicated for 10minutes over an ultrasonic bath, to obtain a homogenous solution. A linear calibration curve was obtained on spotting the increasing concentration of theobromine (2-14 µg spot^{-1}).

Thin-Layer Chromatography:

Thin-Layer Chromatography (TLC) was performed on 20cm × 10cm aluminium-backed HPTLC plates coated with 200μm 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 9.0mm 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 100μL syringe (Hamilton, Bonaduz, Switzerland). Plates were developed to a distance of 9.8cm, in the dark, with ethyl acetate: methanol, 27:3 (*v/v*), as a mobile phase. The volume of the mobile phase was 30mL. 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 was obtained after densitometric scanning was performed with a Camag TLC scanner 3 in remission-absorbance mode at 274nm, under control of Camag *winCATS* planar chromatography manager software. The slit dimensions were 5mm × 0.45mm 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 1nm/step, respectively.

Result and Discussion:

Screening of Extraction Solvents and Conditions:

Different organic solvents and aqueous mixtures of varying nature were used for the screening of theobromine extraction from tea granules. Order of recovery of theobromine 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 further standardizations towardsmaximumrecoveryoftheobromine.

## Effect of Acidic and Basic Extraction:

The effects of acid and base with DM water used for extraction of theobromine 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 theobromine as compared to DM water itself. While addition of base (DEA) with DM water increased recovery of theobromine drastically.

Table-1: Extraction of theobromine from tea:

Extraction solvent	Mean theobromine content	Standard deviation	% RSD
chloroform	0.280	0.009	3.20
Ethyl acetate	0.221	0.007	3.25
methanol	0.069	0.078	5.20
tetrahydrofuran	0.45	0.001	0.21
DM water	1.54	0.004	0.25

Method Validation:
Linearity:

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

Figure-2: Calibration curve of theobromine
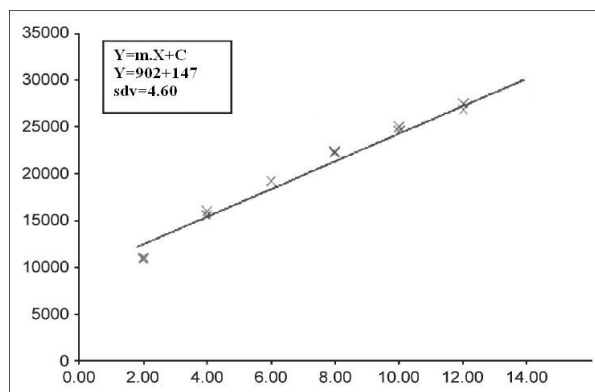
Precision:

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 theobromine eluted on to the HPTLC plate were found to be pure.

Limit of Detection and Quantitation

Limit of detection and quantitation (LOD and LOQ) was determined by spotting increasing amounts (20 - 140ng; n = 2) of standard theobromine solution of concentration 10 μg/ mL until the average responses were 3 and 10 times of the 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 120ng/spot, respectively.
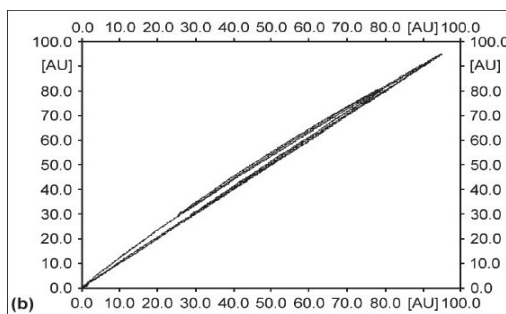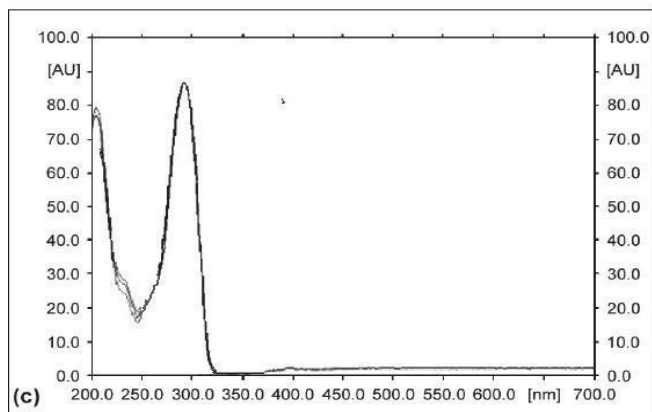
Specificity

The developed HPTLC - UV method was found to be specific as no interfering peak was detected during the detection of theobromine, as is also evidenced by the peak purity data (Figure 3,4and5).

Figure-3: UV spectra of test Theobromine
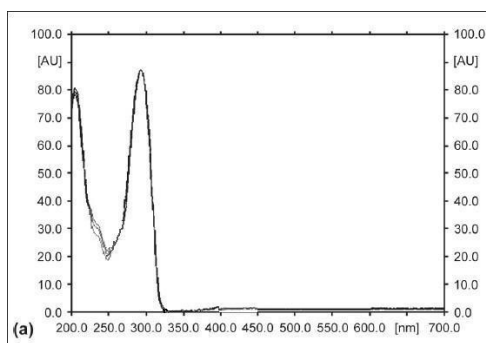
Figure-4: Correlation of center and slopes spectra of test theobromine

Figure-5: UV spectra of standard Theobromine

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 indicative of a robust method.

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