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Optimization of Micelle-Mediated Extraction and Cloud Point Pre-Concentration for the Simultaneous Determination of Caffeine, Theophylline and Theobromine in Green Tea Leaves by High Performance Thin Layer Chromatography

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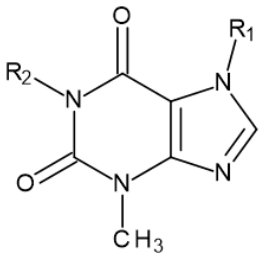
Abstract

In this paper, a micelle-mediated extraction and cloud point pre-concentration method was developed for the determination of the purine alkaloids caffeine, theophylline and theobromine in unfermented *Camellia sinensis* leaves (Green tea) by high performance thin liquid chromatography. The non-ionic surfactants Genapol X-080 and Triton X-114 were tested as extraction solvents and no back-extraction or liquid chromatographic steps were used to remove the targeted purine alkaloids from the surfactant-rich extractant phase. A uniform experimental design approach was demonstrated using the Boxe-Behnken design for the optimization of experimental factors involved in the micelle-mediated extraction process. The separation of the purine alkaloids was achieved on silica gel 60F 254 HPTLC plates using methylene chloride-ethyl acetate-methanol- ammonia (10:10:1:0.05, v/v/v/v) as the mobile phase. The quantitation of caffeine, theophylline and theobromine was carried out using the densitometric reflection/absorption mode at 275 nm. The method was validated for peak purity, precision, accuracy, and robustness. The proposed technique is a low-cost, simple, and sensitive method with high cleanup effect. Finally, the method was successfully applied to separate and determine purine alkaloids in Green tea extracts even at very low concentration levels compared to conventional solvents like methanol.

Keywords: Green tea, caffeine, theobromine, theophylline, micelle-mediated extraction, HPTLC.

1. Introduction

Tea is the second most consumed beverage in the world after water **(Ratnani & Malik, 2022)**. In recent years, the health benefits of consuming *Camellia sinensis* (green tea), including the prevention of cancer **(Huynh et al., 2014)** and cardiovascular diseases **(Ohishi et al., 2022)**, the anti-inflammatory **(Bae et al., 2022)**, antiarthritic **(Singha et al., 2023)**, antibacterial **(Park et al., 2022)**, antiangiogenic **(Luz et al., 2023)**, and cholesterol-lowering effects **(Huang et al., 2022)** of green tea and isolated green tea constituents are under investigation. It is believed that drinking Green tea is advantageous to human health, because it contains polyphenols, which are known to have high antioxidant activity **(Huiling Xu et al., 2022)**, but green tea also contains the purine alkaloids caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine) and theophylline (1,3dimethylxanthyne) (Figure 1).



$R_1=R_2=CH_3$	Caffeine
$R_1=CH_3$, $R_2=H$	Theobromine
$R_1=H$, $R_2=CH_3$	Theophylline

Figure 1: Structures of caffeine, theobromine, and theophylline.

Caffeine, theobromine and theophylline show various physiological effects on various body systems, including the central nervous, cardiovascular, gastrointestinal, respiratory, and renal systems. Theophylline is also a widely used broncho-dilating agent with a narrow serum therapeutic range **(Palai et al., 2023)**.

Different analytical techniques have been developed for the simultaneous determination of these three methylxanthines. For routine analysis of caffeine, theobromine, and theophylline mostly liquid chromatography (LC) in the reversed-phase (RP) mode using porous alkylated (C18) silica phases combined with UV-detection are used **(Ali et al., 2022)(Samanidou et al., 2003)(H Horie & Kohata, 2000)**. An alternative separation can also be achieved by ion chromatography **(Q.-C. Chen & Wang, 2001)**. Separation based on electrophoresis comprise capillary zone electrophoresis (CZE) with cyclodextrins **(H Horie & Kohata, 2000)**, carbon nanotubes **(Mekassa et al., 2017)**, buffers like borax **(Hideki Horie et al., 1997)** added to the electrolyte, or micellar electro-kinetic chromatography **(Ciura et al., 2021)**, or capillary electrochromatography (CEC) **(Lai & Dabek-Zlotorzynska, 1999)**. An HPTLC method has been reported for the determination of methylxanthines in tea using image analysis, however theophylline was not detected in most samples as its level was lower than the LOQ for the method **(Kaltbach et al., 2020).**

In most cases, however, these methods involve tedious and laborious pre-treatment steps before the chromatographic determination, require a large sample volume and are time-consuming. In particular, the traditional liquid–liquid extraction method is also dangerous to analysts because of the large amounts of toxic and volatile organic solvents required. So, a simple and environmentally friendly method should be established. The micelle-mediated extraction and cloud-point pre-concentration (CPE) method offers a convenient alternative to the conventional extraction systems **(Bamdad & Raziani, 2020)**. A plethora of reports have shown application of CPE in extracting and of enriching inorganic, organic and biological analytes prior to their analysis by spectroscopy, capillary electrophoresis, or liquid chromatography **(Carabias-Martínez et al., 2000)**. There have been few reports on the use of CPE for extraction and pre-concentration of chemical constituents from solid materials, especially plants or herbal materials **(Giovanoudis et al., 2023)**; Fang **(Q. Fang et al., 2000)** and Choi **(Choi et al., 2003)** have reported the extraction of ginsenosides from Chinese herbal medicine with Triton X-100 as extractant. Another report studied the feasibility of employing non-ionic surfactant solution as an alternative and effective solvent for the extraction of tanshinones from *Salvia miltiorrhiza* **(Bi et al., 2011)** and isoflavone daidzein from *Puerariae radix* **(Qu et al., 2020)**. Moreover, Motikar and his team have successfully applied CPE on pomegranate peels, as a source of polyphenols **(Motikar et al., 2021)**. No previous reports have been published about how to extract caffeine, theophylline, and theobromine from green tea leaves by CPE.

The micelle-mediated extraction process can be divided into two parts: the first step is to solubilize and purify analytes into the aqueous surfactant solution; the second step is to pre-concentrate analytes based on phase separation by the cloud-point methodology. The small volume of the surfactant rich phase allows for pre-concentration of the analytes **(Doronin et al., 2020)(Mazzola et al., 2008)**. This methodology offers the advantages of safety, low cost, ability to concentrate solutes, easy disposal of surfactant, and low toxicity compared with classical organic solvents, etc. **(Arya et al., 2019)**.

In this paper, the application potential of the micelle-mediated extraction and cloud point pre-concentration method has been further evaluated by employing non-ionic surfactant Genapol X-080 and triton X-114 for the extraction and pre-concentration of the purine alkaloids from commercial green tea leaves.

To optimize the micelle-mediated solubilization and purification process, an experimental approach (uniform design) was used to characterize the various experimental factors that affect the extraction process. The major advantage of uniform design (UD) is that when compared to commonly known methods such as factorial design, the number of experiments can be significantly reduced to produce reliable results even when the number of levels for each experimental variable is large

(Aoyagi et al., 2019; K. Fang & Hickernell, 2008; X. Yang et al., 2020).

The pre-concentrated purine alkaloids were then analyzed by high-performance thin layer chromatography (HPTLC). Planar chromatography, and its high-performance version (HPTLC), coupled with densitometric detection, is among the various methods reported for the quality control of pharmaceutical products containing caffeine **(Abourashed & Mossa, 2004).** It has the advantages of simplicity, speed, reproducibility and cost effectiveness and can thus provide an affordable and reliable alternative to other analytical techniques, such as HPLC or GC **(Ivanović et al., 2023)**. In this report, a developed HPTLC method was validated for specificity, linearity of calibration, recovery, accuracy and precision (repeatability) for the simultaneous determination of the three purine alkaloids using densitometry.

2. Materials and methods

2.1. Chemicals and samples

Triton X-114 (polyethylene glycol mono [4-(1,1,3,3-tetramethylbutyl)-phenyl] ether) with an average ethylene oxide (EO) chain length of n = 7.5; TX-114, $C_{30}H_{54}O_9$ (MW 537 g/mol) 223 (max, nm), 0.20–0.35 (CMC, mM) and Genapol X-080 (isotridecyl poly ethylene glycol ether), GP-80, $C_{30}H_{62}O_{10}$ (MW 553 g/mol) 210 (max, nm), 0.6–0.15 (CMC, mM) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and were used without further purification.

All other solvents used for extraction and chromatographic analysis were of analytical or HPLC grade (E. Merck Ltd., Mumbai, India).

HPTLC analyses were performed on Merck 20 X 10 cm HPTLC silica gel 60F254 (0.25 mm) plates.

Caffeine, theophylline and theobromine were supplied by Sigma, Aldrich, Germany. A stock solution (0.2 mg/mL) was prepared by dissolving an appropriate amount of each of caffeine, theobromine and theophylline in methanol and stored under dark conditions at 4 °C.

Dried leaves of *Camellia sinensis* (green tea) were purchased from a local supermarket (Alexandria, Egypt). The tea samples were powdered, sieved, and then stored in a sealed brown bottle at room temperature. For quantitative determination of caffeine, theobromine and theophylline in green tea samples, 100 mg of powder were extracted using 5 ml of 20% Genapol X-080 concentration, 42°C ultrasonic extraction temperature and 60 min of ultrasonic extraction time.

2.2. Instrumentation

Sample solutions for HPTLC analyses were applied by means of a CAMAG Wilmington, NC, USA Linomat IV automated spray-on band applicator. Zones were quantified by linear scanning at 275 nm with a CAMAG TLC scanner 3 with a deuterium source in the reflection mode, slit dimension settings of length 6 and width 0.1, a monochromator bandwidth 20 nm and a scanning rate of 15 mm s^{-1}. The peak areas of chromatograms were determined using the WINCATS TLC software (version 4.X).

2.3. Procedures

2.3.1. Ultrasonic assisted micelle-mediated extraction and

Powdered leaves of *Camellia sinensis* (0.1 g each) were accurately weighed and placed in 10 ml centrifuge tubes; 5 ml non-ionic surfactants (Genapol X-080 and Triton X114) solution (0.5-40%, v/v) were separately added to these centrifuge tubes. The tubes were capped and blended adequately and then placed in an ultrasonic cleaning bath (29-55 °C) for ultrasonic extraction (30-100 min). During the extraction, mixing was provided by an ultrasonic bath. After ultrasonic-assisted extraction, the extract of *Camellia sinensis* leaves was centrifuged at 3000 rpm for 10 min, and then the supernatant was collected and optimized for pre-concentration of the three purine alkaloids.

2.3.2. Cloud-point pre-concentration procedure

The supernatant was transferred into a 10 mL centrifuge tube. The cloud-point extraction procedure was processed at equilibration temperature of the surfactant, if the temperature is lower than the cloud-point, two phases cannot be formed. The optimal equilibration temperature of the extraction occurs when the equilibration temperature is 15–20 °C greater than the cloud-point temperature of surfactant. Therefore, 60 °C was selected as the working equilibration temperature.

The sample solution was then kept in a thermostatic water bath at 60 °C for 30 min until the solution completely separated into two distinct phases—the upper phase was the small volume of surfactant-rich phase, and the lower phase was the large volume of aqueous phase. The aqueous phase was separated from the sticky surfactant-rich phase. No salting out agent was used as complete phase separation occurred. The volume of the surfactant rich phase was adjusted to 5ml using methanol which was added to lower the viscosity of the surfactant-rich phase. The pre-concentrated purine alkaloids were then analyzed by applying the surfactant-rich phase on HPTLC plates.

2.3.3. Optimization of UAMME parameters using BoxeBehnken design

For experiments involving the use of the uniformity design method, to find the best set of parameters for extraction, a sample amount of 100 mg of *C. sinesis* leaves powder was used. Experimental conditions

for the various extraction variables are listed in Table 1. After extraction, the extracts, which contained various purine alkaloids in aqueous surfactant solutions, were subjected to HPTLC analysis.

BoxeBehnken design is a class of nearly rotatable second-order designs based on three level incomplete factorial design, which is 3-level second-order design introduced by Box and Behnken for fitting the second-order response surface model **(Ferreira et al., 2007; S. Yang et al., 2017)**. The UAMME variables were optimized by varying the operating parameters according to the BoxeBehnken design. The three independent factors used in the UAMME were ultrasonic extraction temperature (X_1), non-ionic surfactant concentration (X_2) and ultrasonication extraction time (X_3), while the responses were the yield of caffeine, theobromine and theophylline in mg/g from *C. sinensis* leaves powder. Experiments were randomized, which obtained the maximum effects of unexplained variability in the observed responses, due to extraneous factors.

For predicting the optimal point, a second-order model was fitted to correlate the relationship between independent variables and responses. For the three factors, the equation is:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$$

where Y is the predicted response; b_0 is model constant; X_1, X_2 and X_3 are independent variables; b_1, b_2 and b_3 are linear coefficients; b_{12}, b_{13} and b_{23} are cross-product coefficients; and b_{11}, b_{22} and b_{33} are the quadratic coefficients.

Experimental design, data analysis, and quadratic model building were conducted using the Design Expert software (Trial Version 7.1.3, Stat-Ease Inc., Minneapolis, MN, USA).

2.3.4. Chromatographic procedure

Standard solutions were applied in the form of bands on pre-coated HPTLC silica gel plates 60F254 (20 X 10 cm with 250 mm thickness) by means of a Linomat IV automated spray-on band applicator operated with the following settings: band length 6 mm, application rate 15 s/mL, distance between each 2 bands 4 mm, distance from the plate side edge 1 cm and distance from the bottom of the plate 1.5 cm. Twenty milliliters of mobile-phase methylene chloride: ethyl acetate: methanol: ammonium hydroxide (10:10:1:0.05, v/v/v/v) were used per development. Ascending development of the plates was carried out in a 20X20 cm CAMAG HPTLC double twin trough chamber saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 20 min at room temperature. Plates were developed to 8 cm beyond the origin. The development time was 8 min. After development, the plates were air-dried for 5 min. Densitometric scanning was performed on a CAMAG TLC scanner 3 in the reflectance/absorbance mode at 275 nm. The source of radiation utilized was a deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. The slit dimension was kept at 6 X 0.1 mm. Concentrations of the standards chromatographed were determined from the intensity of diffusely reflected light. Evaluation was by peak area measurement with polynomial regression.

2.3.5. Method validation

Method validation was performed according to the ICH (The International Conference on Harmonisation) **(Dixon, 1999)** guidelines. All the data were evaluated using standard statistical packages for Windows and XLSTAT (Trial version). Statistical significance was considered at 95% probability level (P, 0.05).

The stock standard solutions of caffeine, theophylline and theobromine were serially diluted to six standard solutions. A volume of 2.0 µL of each solution was applied on the HPTLC plate to deliver 0.2–1.2 ug of each of caffeine, theophylline and theobromine per spot. This was done in triplicate and repeated for 3 days. For each concentration, the applied spot bands were uniformly distributed throughout the plate to minimize possible variation along the silica layer. Six microliter aliquots of the prepared sample solutions, for the analysis of theobromine and theophylline, and one microliter aliquots of the prepared sample solutions, for the analysis of caffeine, were subjected to HPTLC analysis as designated in "Chromatographic procedure" section.

The precision of the method was determined by the measurement of inter- and intra-day precision. The repeatability or intra-day precision was experimented by examining the standard solutions repetitively, in the same laboratory and on the same day, at three concentrations. Intermediate precision incorporated the analysis of the standards three times each day over a period of 3 days by different analysts. The results of repeatability and intermediate precision are conveyed as relative standard deviation (RSD) (%).

The accuracy of the methods was established by standard addition techniques. Known quantities of standard caffeine, theophylline, and theobromine in a range of low, medium, and high concentrations were added to pre-analyzed samples and investigated under optimized conditions. Addition experiments for every concentration were performed in triplicate and the accuracy was calculated as the % of analyte recovered. Three analyses per concentration were performed and mean +SD was determined.

Table 1: BoxeBehnken design and the corresponding response for the yield of caffeine, theophylline and theobromine (mg/g).

Experiment	Ultrasonication Temperature C	Concentration of surfactant (%)	Ultrasonication time (min)	Yield of caffeine (mg/g)	Yield of theobromine (mg/g)	Yield of theophylline (mg/g)
1	55.8786 (1)	20.25 (0)	98.7167(1)	19.8	1.77	0.38
2	42.5 (0)	20.25 (0)	67.5 (0)	19.6	1.71	0.39
3	29.1214 (-1)	20.25 (0)	36.2833 (-1)	18.6	1.66	0.35
4	42.5 (0)	20.25 (0)	67.5 (0)	18.4	1.62	0.35
5	55.8786 (1)	20.25(0)	36.2833 (-1)	19.6	1.67	0.38
6	29.121 (-1)	31.9934(1)	67.5(0)	17.2	1.48	0.33
7	55.8786 (1)	8.50658 (-1)	67.5(0)	8.1	0.71	0.14
8	42.5 (0)	20.25(0)	67.5(0)	19.6	1.74	0.38
9	42.5 (0)	31.9934(1)	36.2833 (-1)	17.6	1.75	0.4
10	42.5(0)	20.25(0)	67.5(0)	19.6	1.69	0.37
11	55.8786(1)	31.9934(1)	67.5(0)	17.4	1.58	0.34
12	42.5(0)	8.50658 (-1)	98.7167(1)	9.6	0.83	0.16
13	42.5(0)	8.50658 (-1)	36.2833 (-1)	9.2	0.79	0.13
14	42.5(0)	20.25(0)	67.5(0)	19.6	1.78	0.39
15	29.1214	8.50658 (-1)	67.5(0)	7.3	0.51	0.12
16	42.5(0)	31.9934(1)	98.7167(1)	17.4	1.47	0.34
17	29.1214 (-1)	20.25(0)	98.7167(1)	19.1	1.52	0.37

3. Results and Discussion

3.1. Optimization of the micelle-mediated extraction conditions

3.1.1. Selection of the surfactant

At the beginning of this work, Triton X-114 and Genapol X-080 were both tried as extraction solvents. However, the Triton X-114 showed high UV absorbance and gave very broad peaks in the HPTLC chromatogram, and thus, interfered with the determination of the purine alkaloids. Genapol X-080 is a $C_{13}E_8$ surfactant that has eight oxyethylene units and tridecyl alkyl moieties (critical micellar concentration (CMC) = 0.05mmolL−1 (0.028%, v/v), cloud-point 42 °C (in pure water)). Several research groups have successfully used Genapol X-080 in the extraction procedures **(Y. Chen et al., 2018; Giovanoudis et al., 2023; Sun & Liu, 2008)**. Because it possesses no aromatic moiety, Genapol X-080 does not absorb above 210 nm, and thus will not interfere with the determination of the target analytes. So, Genapol X-080 was chosen as the CPE surfactant in this study. The ability of the aqueous non-ionic Genapol X-080 solution in extracting the alkaloids may be related to the solubility-enhancement effect of the surfactant micelles.

3.1.2 Optimization of micelle-mediated extraction using experimental design

The use of experimental design methods, which would allow for more efficient optimization of the various experimental variables in micelle-mediated extraction, has not been previously reported for the micelle-mediated extraction of purine alkaloids. It includes systematic investigation of the large number of experimental variables (e.g., pH, temperature, surfactant concentration, ionic strength, analyte concentration, equilibration, ultrasonication time, etc.) that affect performances in micelle-mediated extraction. The most efficient way of setting the optimal region is to apply one of the available response surface designs. For this purpose, three level designs should be used and the most widely used are central composite design and Box-Behnken design. Three level designs are recommendable as they enable determination of a critical point (maximum, minimum, or saddle) in the response surface. In this study, Box-Behnken design was selected for the estimation of some selected parameters. It is based on three-level incomplete factorial designs. This design consists of three parts of four runs and replications at the central point. Within each part, two factors are arranged in a full two-level design, while the level of the third factor is set at zero **(J. Chen et al., 1993; Hongquan Xu, 2005)**.

Therefore, to study the possible interaction between the parameters, a 17-run BoxeBehnken design with the three factors ultrasonic extraction temperature, non-ionic surfactant concentration and ultrasonication extraction time and three levels, including five replicates at the center point, was used for fitting a second-order response surface. The experimental design is shown in Table 1. The analytical results of the BoxeBehnken design are

shown in Table 2. In general, exploration and optimization of a fitted response surface may produce poor or misleading results unless the model exhibits a good fit (Wang et al., 2008). The P-values of the model for the yield of caffeine, theophylline, and theobromine (in mg/g) were 0.0001, which means significant. Meanwhile, the lack of fit values were 0.363, 0.428 and 0.636, respectively, which mean not significant. These two values confirmed that the model fitness was good. Coefficient of determination (R^2) is also a measurement of the degree of fitness and defined to be the ratio of the explained variation to the total variation **(Ozer, 1985)**. By analysis of variance, the R^2 values were 0.9927, 0.991 and 0.9901 for caffeine, theobromine, and theophylline, respectively.

From Table 1, Table 2, and Fig. 2, we can find that the non-ionic surfactant concentration and ultrasonic extraction temperature have a significant effect on the yield of the three alkaloids. It means that the higher non-ionic surfactant concentration and ultrasonication temperature are suitable for the UAMME. The ultrasonication extraction time has no significant effect on the yield of the three alkaloids. It can be seen from Fig. 2 that the yield of the three alkaloids increases when the surfactant concentration increases from 8% to 20% (w/v) and then tends to decrease in the surfactant concentration range of 25-35%. When the surfactant concentration rises to 35%, the solution will become too sticky to be dealt with. The best UAMME conditions for yield of the three alkaloids were: 20% of non-ionic surfactant Genapol X-080 concentration, 42 °C ultrasonic extraction temperature and 60 min of ultrasonication extraction time. The Final equations for the prediction of the yield of caffeine, theobromine and theohphylline with the three factors; ultrasonication temperature (X_1), concentration of surfactant (X_2) and ultrasonication time (X_3) are shown in Table 3.

3.2. Method validation

Method validation was performed on the parameters such as linearity, limit of sensitivities, specificity, precision, accuracy, recovery, and robustness as per ICH guidelines **(Dixon, 1999)**. All the data were evaluated using standard Microsoft office 10 Excel statistical packages for Windows and XLSTAT premium (Trial version). Statistical significance was considered at 95% probability level ($p<0.05$). Pearson's coefficient (r^2) was used to evaluate the significance of correlations. Computing and comparing HPTLC standard curves with reference compounds and spiked samples curves were in compliance with the ICH validation requirements **(Dixon, 1999)**.

3.2.1. Calibration curves, limits of detection/quantification (LOD/LOQ)

The calibration curves were prepared by the least-squares method using absolute amount (ug/band) as independent variable (X) and the peak area of standards as dependent variable (Y). The selection of working concentration range of respective purine alkaloids was based on their response. Polynomial regression of the data points for standard caffeine, theobromine and theophylline resulted in a calibration curve with the equations $y = -3630.7x^2 + 8305.4x + 1313.6$ for caffeine, $y = -3636.1x^2 + 9268x + 3810.8$ for theobromine and $y = -2839.4x^2 + 7735.2x + 777.86.86$ for theophylline with regression coefficient (R^2) = 0.9925, 0.9941and 0.9961, respectively. Most assays usually have a linear operating region where the intensity response linearly varies as the spike-in concentration of the target analyte varies. In fact, determining this linear region is one of the goals of running the response curve. The linearity range for caffeine, theophylline and theobromine was 0.2-0.8 mg/ml with the calibration curves equations and R^2 values of $4818x + 1981.7$, $R^2=0.9929$, $y = 5759.1x + 4489.1$, $R^2=0.9926$, and $y=4818x + 1981.7$, $R^2=0.9945$ for caffeine, theobromine, and theophylline, respectively. However, due to the great variation in the amount of the three alkaloids in Green tea leaves, it was not possible to prepare a sample within the linear range of the three alkaloids simultaneously as theobromine and theophylline are present in Green tea leaves in significantly lower amounts than caffeine. Attempts to concentrate the sample showed very high caffeine levels outside the linearity range. Therefore, the quadratic polynomial model is considered the best choice for the simultaneous determination of the three alkaloids in Green tea leaves. Meanwhile, the linear regression equations were used for determination of LOD and LOQ using: LOD = 3Sy, x/b and LOQ = 10Sy, x/b, where Sy, x is the standard deviation of the Y-value distribution around the regression line and b is the slope of the calibration curve. Results are shown in Table 4. LODs for caffeine, theobromine and theophylline were 0.062, 0.063 and 0.055 mg/ml, respectively and LOQs determined in the same way were 0.20, 0.21 and 0.18 ml, respectively.

3.2.2. Specificity

The method specificity was assessed by comparing the R_f (caffeine 0.44, theobromine 0.39, and theophylline 0.32) and absorption spectra of the

reference compounds in sample and standard tracks. On comparison of spectra at peak start, peak apex and peak end positions of the band, an acceptable correlation (r^2 = 0.95–0.99) was obtained between standards and sample overlay spectra which confirms the purity of caffeine, theobromine, and theophylline peaks in the sample tracks.

3.2.3. Precision

The repeatability of measurement (n = 6) for peak area of standards caffeine, theobromine and theophylline has been expressed in terms of percent coefficient of variation (RSD %). The intra- and inter-day variations were also evaluated at 0.6 ug/band for the three purine alkaloids. The results are shown in Table 4, which depicts that the method is accurate and precise for the analysis of test markers in Green tea leaves.

3.2.4. Recovery

The mean percentage recovery for each compound was calculated at each concentration level and reported with its standard deviation. The mean results obtained for caffeine, theobromine, and theophylline at the 80%, 100% and 120% concentration levels are shown in Table 4.

Table 2: Analysis of variance for the response surface quadratic model of the yield of caffeine (caf.), theobromine (theob.) and theophylline (theop.) against the three factors, ultrasonication temperature (X_1), concentration of surfactant (X_2) and ultrasonication time (X_3).

Source	Degree of freedom	Sum of Squares			Mean Square			F Value			p-value Prob > F			
		Caf.	Theob.	Theop.	Caf.	Theob.	Theop.	Caf.	Theob.	Theop.	Caf.	Theob.	Theop.	
Model	9	330.31	2.87	0.17	36.70	0.32	0.018	108.42	83.71	78.63	< 0.0001	< 0.0001	< 0.0001	Significant
X_1	1	0.91	0.039	6.125E-004	0.91	0.039	6.125E-004	2.69	10.28	2.61	0.1449	0.0149	0.1505	
X_2	1	156.65	1.48	0.092	156.65	1.48	0.092	462.76	388.10	393.40	< 0.0001	< 0.0001	< 0.0001	
X_3	1	0.10	9.800E-003	1.250E-005	0.10	9.800E-003	1.250E-005	0.30	2.57	0.053	0.6014	0.1529	0.8242	
X_1X_2	1	0.090	2.500E-003	2.500E-005	0.090	2.500E-003	2.500E-005	0.27	0.66	0.11	0.6220	0.4447	0.7538	
X_1X_3	1	0.022	0.014	1.000E-004	0.022	0.014	1.000E-004	0.066	3.78	0.43	0.8040	0.0930	0.5350	
X_2X_3	1	0.090	0.026	2.025E-003	0.090	0.026	2.025E-003	0.27	6.72	8.62	0.6220	0.0359	0.0219	
X_1^2	1	1.13	0.039	1.012E-003	1.13	0.039	1.012E-003	3.33	10.29	4.30	0.1107	0.0149	0.0767	
X_2^2	1	169.38	1.23	0.069	169.38	1.23	0.069	500.38	323.93	293.55	< 0.0001	< 0.0001	< 0.0001	
X_3^2	1	0.79	7.967E-003	3.800E-004	0.79	7.967E-003	3.800E-004	2.33	2.09	1.62	0.1710	0.1915	0.2441	
Residual	7	2.37	0.027	1.645E-003	0.34	3.811E-003	2.350E-004							
Lack of Fit	3	1.22	0.012	5.250E-004	0.41	4.133E-003	1.750E-004	1.41	1.16	0.63	0.363	0.4288	0.6356	not significant
Pure Error	4	1.15	0.014	1.120E-003	0.29	3.570E-003	2.800E-004							
Total	16	332.68	2.90	0.17										

Table 3: Final equations for the prediction of the yield of caffeine, theobromine and theohphylline with, the three factors ultrasonication temperature (X_1), concentration of surfactant (X_2) and ultrasonication time (X_3):

	Caffeine	Theobromine	Theophylline
Intercept	+19.36	+94.40	+0.38
X_1	+0.34	+3.25	+8.750E-003
X_2	+4.43	+23.00	+0.11
X_3	+0.11	-1.50	-1.250E-003
X_1X_2	-0.15	-1.25	-2.500E-003
X_1X_3	-0.075	-0.25	-5.000E-003
X_2X_3	-0.15	-2.25	-0.022
X_1^2	-0.52	-3.83	-0.015
X_2^2	-6.34	-31.33	-0.13
X_3^2	+0.43	+2.17	+9.500E-003

Table 4: Analytical characteristics of the validated HPTLC method for the simultaneous quantitation of caffeine, theobromine and theophylline in Green tea leaves.

Parameters	Caffeine	Theobromine	Theophylline
R_f	0.44	0.39	0.32
Regression equation	$-3630.7x^2 + 8305.4x + 1313.6$	$-3636.1x^2 + 9268x + 3810.8$	$-2839.4x^2 + 7735.2x + 777.86.86$
Correlation coefficient r^2	0.9925	0.9941	0.9961
Non -linear working concentration range (mg/ml)	0.2-1.2	0.2-1.2	0.2-1.2
linear working concentration range (mg/ml)	0.2-0.8	0.2-0.8	0.2-0.8
Precision			
Inter-day (RSD%) n=6	1.24	1.19	1.48
Intra-day(RSD%) n=6	0.977	0.987	0.941
Accuracy (Recovery %)	99.76%±1.3	98.3%±1.2	98.3%±1.7
Alkaloids amount quantified in green tea leaves powder (% w/w)	1.9	0.17	0.04

The % recoveries for caffeine at the three concentration levels were 98.6%±1.8, 99.5%±1.1 and 99.7%±0.9, while the mean recovery for all the concentration levels was 99.76%±1.3. For theobromine, the % recoveries at the same concentration levels were 97.6±1.2%, 99.8%±0.8 and 98.5%±1.5. The mean value covering all concentration levels was 98.3%±1.2. The % recovery values for theophylline were 98.2%±1.1, 97.1%±1.4 and 99.5%±1.6, respectively and the overall mean was found to be 98.3%±1.7. In conclusion, the method was considered to have an acceptable recovery and trueness.

3.2.5. Robustness

To test robustness of the method, small changes in the chromatographic parameters were deliberately made, which may affect the performance of the method such as mobile phase composition, chamber saturation time, delay between spotting and plate development and delay in digital scanning after derivatization, but only negligible changes in the peak areas were found. Quantitation was not significantly affected by changing scanning wavelength ±5 nm.

3.3. Quantitative evaluation of Caffeine, Theobromine and Theophylline

Quantification of caffeine, theobromine and theophylline was performed according to the procedure described in the "Chromatographic procedure" section. The analyses were performed in triplicate and the results are summarized in Table 4. The amounts of the three alkaloids in Green tea leaves were determined from the calibration graphs and were found to be 1.9 % caffeine, 0.17% theobromine and 0.04 % theophylline. These results are in accordance with the previous reports **(Huck et al., 2005)**. It is evident from Fig. 3 that the purine alkaloids in green tea leaves samples shows characteristic UV-spectra after densitometry of HPTLC plates. The same figure shows that acceptable separation was achieved without any interference of the nearby components under the specified conditions.

3.4. Comparison of the extraction efficiency of Genapol X-080 with conventional extraction solvent-methanol

Compared with methanol, 20% GenapolX-080 has higher extraction efficiency under identical experimental conditions (0.1 g powder mixed with 10 ml methanol followed by ultrasonication at 42 °C for 60 min). With methanol as extractant, determined content caffeine and theobromine are 1.82%, 0.13%, respectively while theophylline was not detected. The reason why the extraction efficiency of 20%GenapolX-080 is higher than that of methanol may be due to the more complete diffusion of surfactant solution into the particles of the herbal material and the solubility-enhancing effect of the surfactant micelles.

4. Conclusions

In this study the results obtained indicate that non-ionic surfactant Genapol X-080 solution is an effective alternative for the extraction of purine bases, from *C. sinensis*. From the analytical point of view, it provides the possibility of extracting and pre-concentrating analytes of different polarities in a simple procedure. With the aid of an experimental design method such as uniform design like Boxe-Behnken, efficient and rapid extraction of hydrophobic ingredients is possible without needing

to use expensive and potentially toxic organic solvents. The present work further demonstrates that micelle-mediated extraction is a potentially powerful tool in the large-scale extraction and purification of active ingredients from herbal materials.

Furthermore, the standardized HPTLC procedure may be used effectively for the simultaneous screening analysis as well as quality evaluation of the green tea, extracts, fractions, or its derived herbal products even at very low concentrations of the target analytes.

Compliance with Ethical Standards.

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

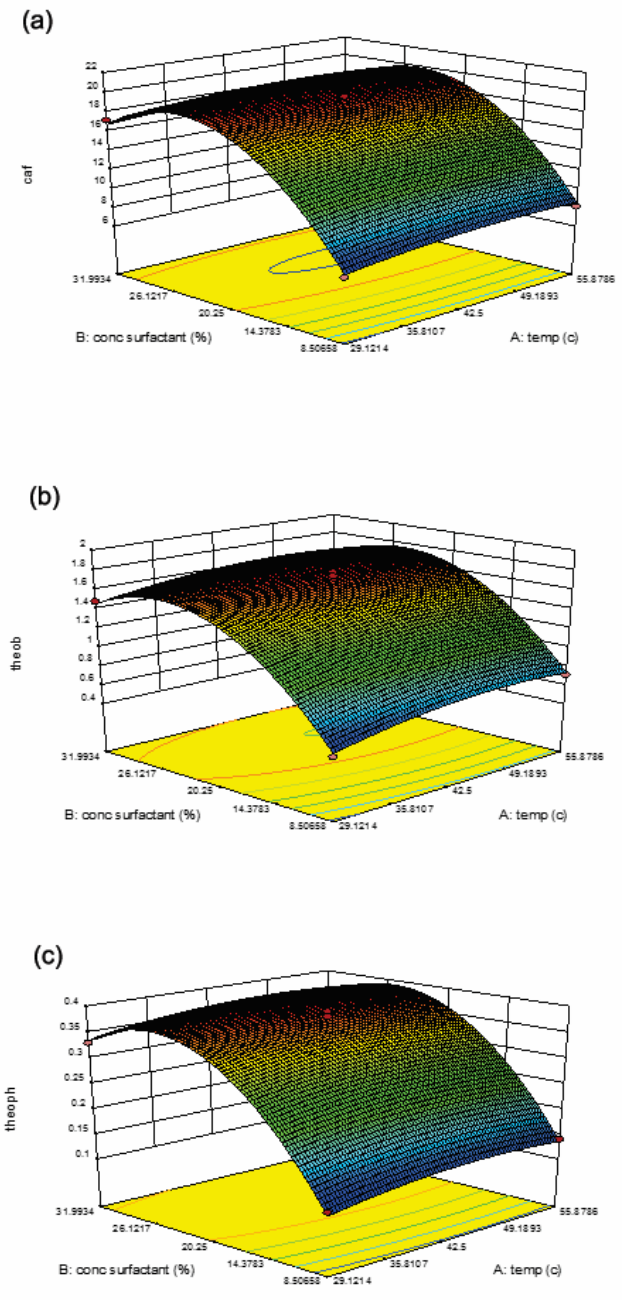


Figure 2: 3D response surface plots showing effect of non-ionic surfactant concentration and ultrasonic extraction temperature on amount of Caffeine (a), theobromine (b), theophylline (C) extracted.

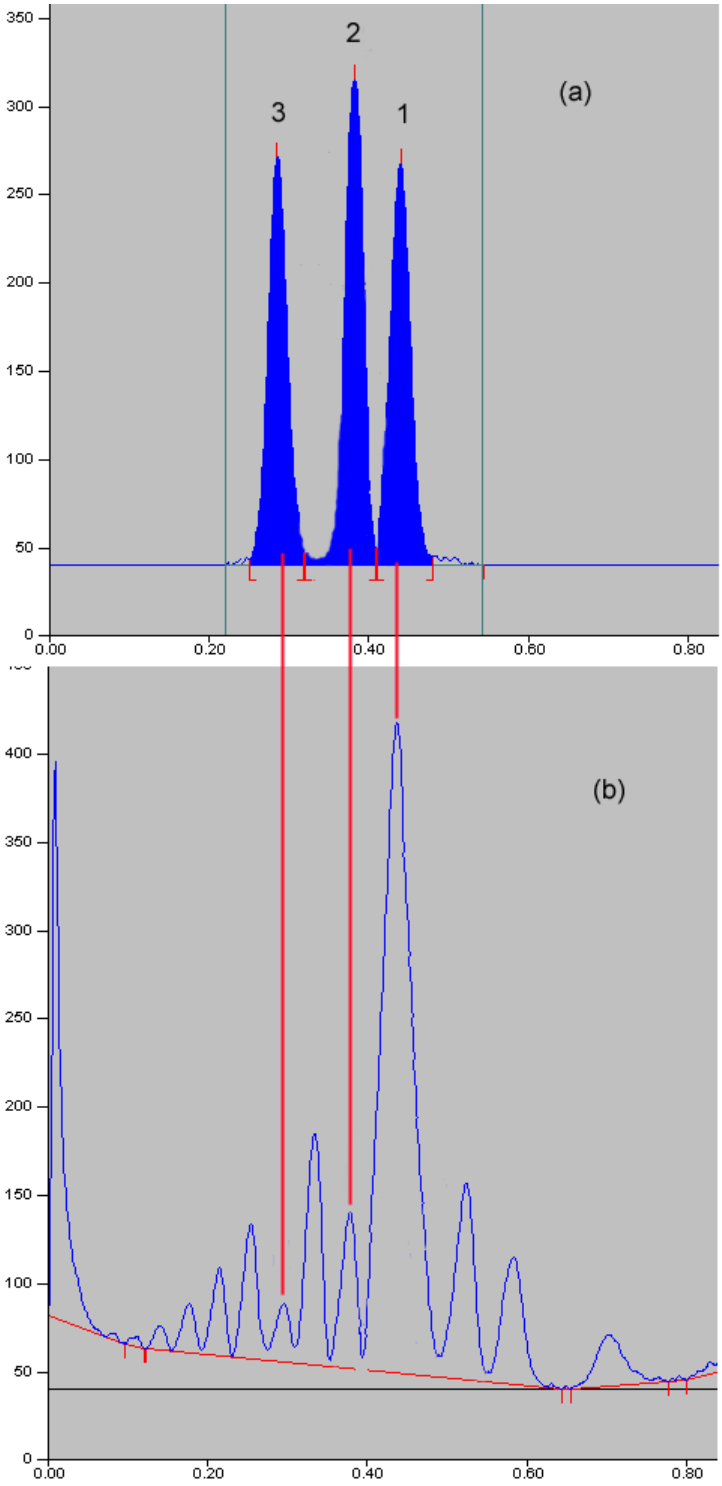


Figure 3: shows characteristic UV-spectra of caffeine, theobromine, and theophylline after densitometry of HPTLC plates.

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