RESEARCH ARTICLE

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Analytical Method Development and Investigation of Caffeine Content from *Coffea arabica* and Anti-Obesity Formulation as per ICH Q2 (R1) Protocol of Validation

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Abstract: Precise and sophisticated HPTLC and HPLC methods were developed for determination of caffeine from *coffee bean extract*. HPTLC analysis was performed on silica gel 60 F_{254} plate using ethyl acetate: methanol: water in the proportion of 10: 1.4: 1 *(v/v/v)* as mobile phase. A clear band of caffeine was observed at Rf value 0.55 (±0.1). Caffeine shows linearity within the concentration range of 200-600 ppm with regression coefficient of 0.9987, at 254 nm. HPLC analysis was performed by using mobile phase of methanol and water (40:60) with Zorbax SB C-18 column. Quantification was carried out by using PDA detector at 272nm. The method was validated in terms of specificity, precision, linearity, robustness, accuracy, limit of detection (LOD) and limit of quantification (LOQ) as per ICH Q2 (R1) guidelines. The % RSD for validation parameters including accuracy, method precision, intermediate precision, robustness was found to be less than 2.0 %. In the present study, accurate and sophisticated HPTLC and HPLC methods were validated for determination of caffeine in *coffee bean extract.* Also qualitative analysis was performed for the estimation of caffeine from prepared capsule formulation using HPTLC method.

INTRODUCTION

Obesity is a serious health problem in the world whose prevalence increases day by day in developed and developing country because of change in human lifestyle and intake of high energy diets. It is a chronic disease associated with increased risk of insulin resistance, cardiovascular disease, type 2 diabetes, cancer, osteoarthritis, oxidative stress, gallstones and fatty liver diseases. [1-5] Obesity is a condition in which imbalance between the energy intake and expenditure occurs. It is caused due to altered lipid metabolic processes including lipogenesis and lipolysis. Lipogenesis is the process in which free fatty acids are stored in the form of triglycerides. To overcome or reduce those abnormalities some plants constituents are helpful. *Coffea arabica* is one of the most widely consumed beverages in the world. Seeds of this plant contain carbohydrates, protein, lipids, minerals and vitamins. Caffeine is obtained from seeds of *Coffea arabica* belonging to family Rubiaceae, shows potent anti-obesity activity due to its chemical constituents. It is reported that daily dose of caffeine below 250 mg is beneficial. Excessive consumption may cause increased heart rate, anxiety, nervousness and insomnia. Caffeine (1, 3, 7-trimethyl-xanthine), a purine alkaloids compound having molecular formula $C_8H_{10}N_4O_2$, has good anti-obesity activity. [6-10] Caffeine, an inhibitor of adipogenesis, has thermogenesis effect which can reduce the size and number of adipose cells so that the accumulation of adipose tissue is reduced. Long term use of caffeine reduces adipose pad size and the number of cells of adipocytes. Thus caffeine helps in reducing obesity by the suppression of lipogenesis and the acceleration of lipolysis. [11-15]

The aim of this study was to develop precise and sophisticated HPTLC as well as HPLC methods for the analysis of caffeine from *coffee bean* extract. The HPLC method has been validated as per the ICH guidelines. [16]

MATERIALS AND METHODS

Chemicals

AR grade solvents such as ethyl acetate, methanol were obtained from Merck Ltd. Bangalore India. Standard caffeine was purchased from SDFCL Ltd. Mumbai and sample of *coffee bean extract* was purchased from K. Patel Phyto extraction Pvt. Ltd, Mumbai India. HPLC grade solvent such as methanol was purchased from Merck Life Science Private Limited, Vikhroli (East), Mumbai, India.

Instruments

The instruments used for the analysis are mentioned in the Table 1.

HPTLC Evaluation

1. Preparation of Standard Solutions

The standard solution was prepared by accurately weighing 10.0 mg caffeine and dissolving in a 25.0 ml methanol. For homogeneous mixing the solution was sonicated for 30 minutes in ultrasonic water bath. The solution was filtered using Whatman No. 41 filter paper. The resulting solution was used as standard solution.

2. Preparation of the Sample Solution

For sample solution preparation, 100.0 mg of coffee *bean* extract was weighed and dissolved in 25.0 ml methanol. The resultant solution was sonicated in ultrasonic water bath for 30 min for homogeneous mixing. The solution was filtered by using Whatman No. 41 filter paper. The resulting solution was used as sample solution.

3. Chromatographic Conditions for HPTLC

a. High performance thin layer chromatography (HPTLC) model: CAMAG HPTLC with Linomat 5 applicator,

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Table 1: List of Instruments

S. No.	Instruments	Make	Model
1	Balance	Sartorious BT-224S	SBT 224S CW
2	Sonicator	LabMan	13L300H
3	HPTLC with winCATS software	Camag	Linomat 5 applicator TLC visualizer TLC scanner
4	HPLC with PDA Waters Empower chromatography software	Waters	Waters 2998 PDA Detector
5	HPLC with UV detector Waters Empower Chromatography software	Waters	Waters 2489 UV/Vis Detector
6	UV Spectrophotometer	SHIMADZU	Shimadzu UV-1800

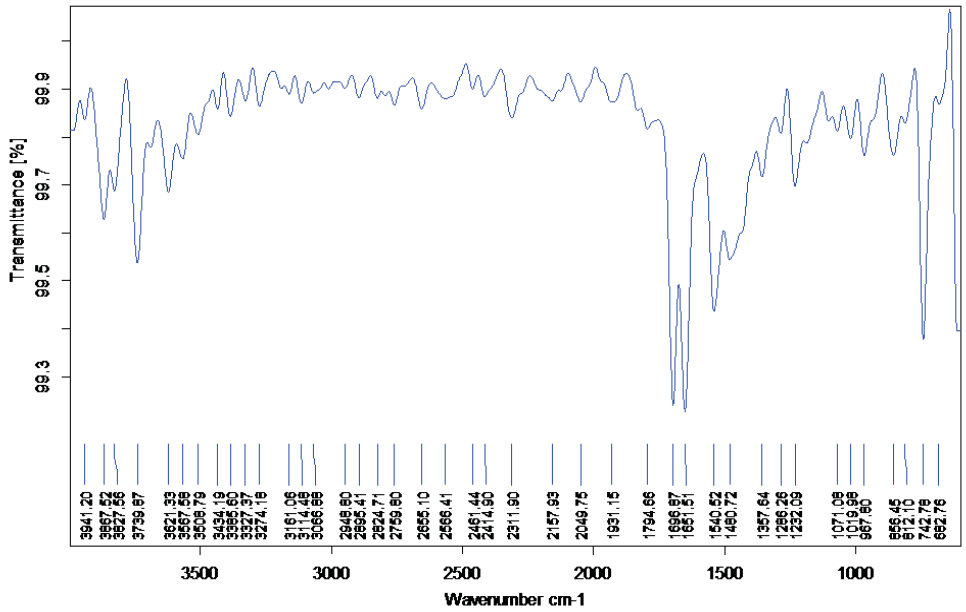

Figure 1: IR spectra of Caffeine

Scanner 4, CAMAG TLC visualizer, CAMAG - 108540DC0084-6038 sprayer and Win CATS software.

b. Silica plate: Pre-coated silica 60 F_{254} (10 cm × 10 cm) plates
c. Mobile Phase: ethyl acetate: methanol: water (10:1.4:1 *v/v/v*)
d. Chamber: Twin through chamber
e. Saturation time: 20 min
f. Activation of plate: at 105°C for 5 min in oven
g. Scanning wavelength: 254 nm.

HPLC Evaluation

1. Preparation of the Standard Solution

10 mg of caffeine was weighed and transferred into 100 ml volumetric flask and sonicated in ultrasonic water bath for 10 minutes. Then the solution was filtered using 0.45µ syringe filter. Resulting solution was used as standard solution.

2. Preparation of Sample Solution

100 mg of coffee bean extract was weighed and transferred into 25 ml volumetric flask and methanol was added and sonicated in ultrasonic water bath for 10 minutes. Then the solution was filtered using 0.45µ syringe filter. Resulting solution was used as sample solution.

3. HPLC conditions

a. HPLC model: Water e2695 Alliance system with 2998 photo diode array detector (PDA)
b. Column: Zorbax SB C 18 Column, pore size 5µ, internal diameter 4.6nm and length 250mm
c. Flow rate: 1 ml/min (constant)
d. Column temperature: at 30°C
e. Detection wavelength: 272 nm
f. Mobile phase: Water: Methanol (60:40) both are HPLC grade
g. Sample injection volume: 10 µl.

Preparation of Sample Solution for Formulation

The average weight of capsule formulation was 645.0 mg. Hence by calculating equivalent %of coffee bean extract 161.25 mg was dissolved in 100 ml methanol.

UV Evaluation

1. Preparation of Standard Solution for UV

The standard solution was prepared by dissolving 50.0 mg caffeine in 100.0 ml of methanol in a volumetric flask. The resultant solution was sonicated in ultrasonic water bath for 30 min. From above solution 5.0 ml was transferred into a volumetric flask and diluted up to 50.0 ml with methanol. The resulting solution was used as standard solution.

Table 2: Formula for the Anti-Obesity Capsule

S. No.	Name of Ingredients	Quantity (mg/cap)	Role of Ingredients
1	Drug (Extract)	400.00 mg	Active pharmaceutical ingredients
2	Microcrystalline cellulose 101	130.00 mg	Diluent
3	Ethyl cellulose	12.00 mg	Binder
4	Isopropyl alcohol	q.s.	Binder
5	Magnesium stearate	3.00 mg	Lubricant
6	Dibasic calcium phosphate (Granular)	52.00 mg	Diluent

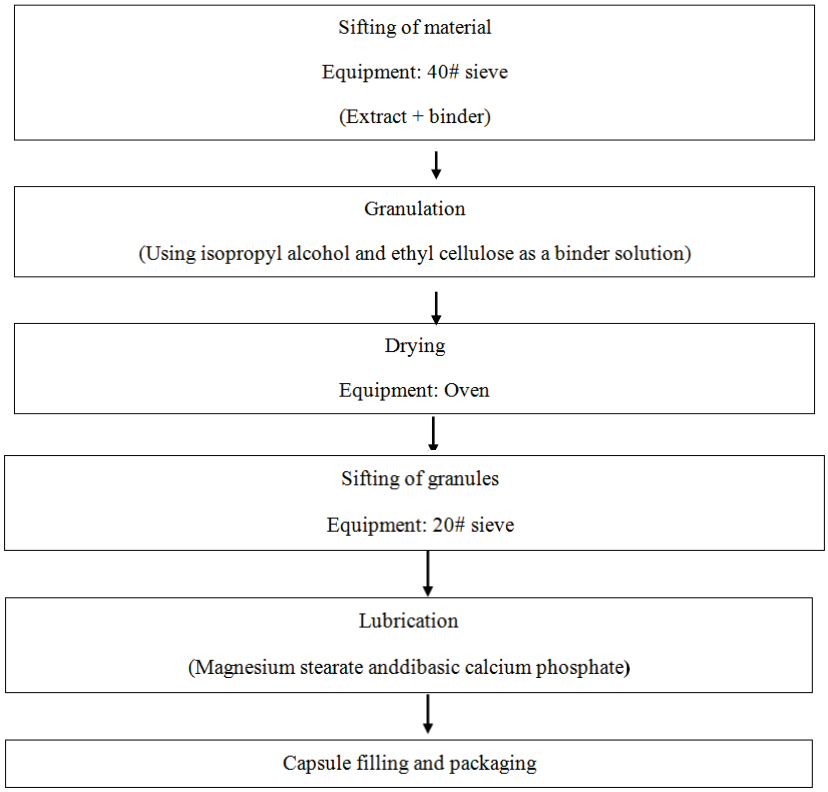

Figure 2: Manufacturing process for anti-obesity capsule formulation

IR Evaluation

IR spectra for standard caffeine were obtained in between 400-4000 cm^{-1} as shown in Figure 1.

Validation Parameters

The proposed HPTLC and HPLC method was validation in terms of specificity, precision, accuracy, limit of detection (LOD) and limit of Quantification (LOQ), standard solution stability, sample solution stability and robustness as per the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guidelines. [16]

1. Specificity

The specificity of the method was determined by using of STD, blank and sample solutions in duplicate. The densitogram of STD, blank and sample solution were obtained and compared amongst them.

2. System Precision

System precision was performed by using STD solution in replicates of five. The peak area was obtained and the results were expressed in terms of % RSD.

3. Method precision

Method precision was determined by performing six repetitive analysis of sample solutions of 400 ppm and 100ppm concentration for HPTLC and HPLC respectively. The % assay of caffeine in sample was determined and results were expressed in terms of % RSD.

4. Intermediate precision

The intermediate precision was determined by comparing analysis on different days and the results were expressed in terms of % RSD.

5. Linearity

The linearity of caffeine was determined using five different concentrations (50-150%) of STD stock solution. The regression coefficient (R^2) and the equation of line were determined.

6. Robustness

The influences of deliberate changed parameters on the chromatographic conditions were tested according to ICH guidelines to demonstrate sufficient robustness of the method. The tests for HPTLC were carried out by varying

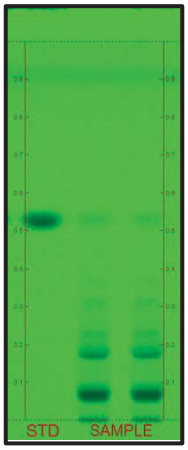

Figure 3: Developed HPTLC plate image at 254 nm

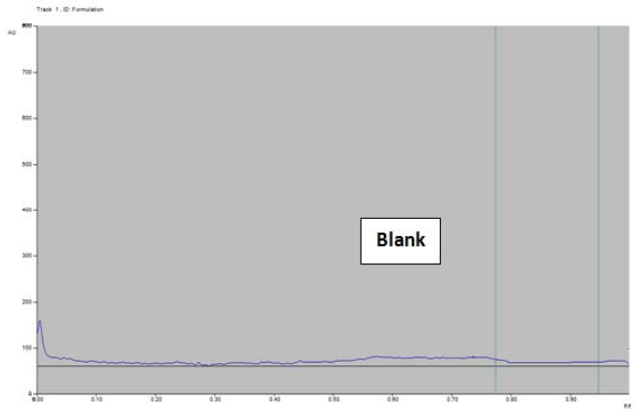

Figure 4: HPTLC densitogram of blank solution

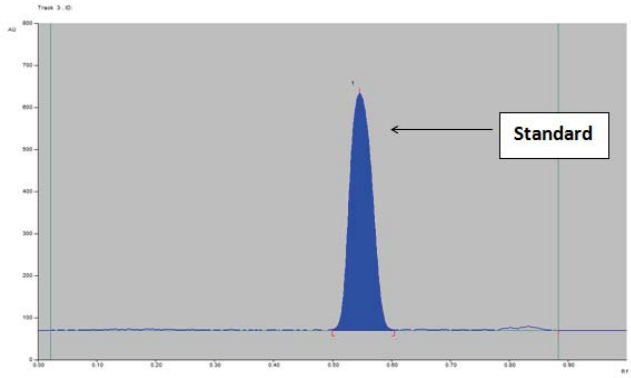

Figure 5: HPTLC densitogram of standard solution

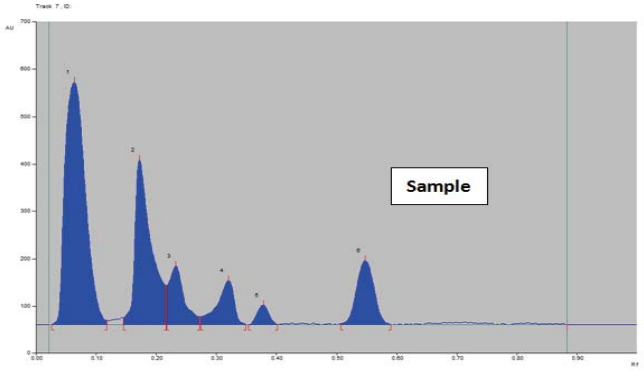

Figure 6: HPTLC densitogram of sample solution

the chromatographic parameters such as saturation time, mobile phase volume and volume of polar solvent and for HPLC flow rate, wavelength and temperature.

7. Accuracy

The accuracy was determined from recovery studies, where a known, varying amount of STD was spiked into sample solution at 80%, 100% and 120% levels of working concentration in triplicate. The spiked sample solutions were analyzed and results were calculated in terms of % recovery.

8. Stability of Standard and Sample Solution

The standard and sample solutions were prepared and kept at room temperature. The standard and sample solutions were analyzed at initial and at different time (2 hours) intervals till 24 hours.

9. Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limit of detection (LOD) and limit of quantification (LOQ) were determined based on the standard deviation of the response and the slope of the calibration curve at all concentration levels according to ICH guidelines.

Formulation

The oral solid capsule formulation of coffee bean extract was formulated having anti-obesity activity. Table 2 describes the formula for anti-obesity capsule formulation. The anti-obesity capsule formulation was formulated by using the process described in the Figure 2.

RESULTS AND DISCUSSION

Analysis by HPTLC

Using the optimized extraction method and chromatographic conditions, the HPTLC method developed was validated in terms of linearity, limit of detection, limit of quantitation, precision, accuracy and specificity and the results are mentioned as follows. Developed HPTLC plate for coffee bean extract has been shown in Figure 3.

1. Specificity

The densitogram of blank, STD and sample solutions, as shown in Figure 4-6, depicts that no band was observed at Rf value 0.55 (± 0.1), as shown in Table 3, indicating specificity of the method. The spectra of caffeine from STD and sample for HPTLC were shown in Figure 7.

2. System Precision

The peak area of STD caffeine was determined, as mentioned in Table 4 and the results were expressed in % RSD, which was found to be less than 2.0%.

3. Method Precision

The % assay of caffeine was determined, as mentioned in Table 5 and the results for method precision in terms of relative standard deviation (% RSD), were found to be less than 2.0%.

Table 3: Specificity for HPTLC

S. No.	Sample Name	Rf
1	Caffeine STD	0.55 (±0.1)
2	Blank	No band observed
3	Sample Solution	Band for Caffeine 0.55 (±0.1)

Table 4: System Precision for HPTLC

Band Number	Area of STD Caffeine
1	3661
2	3578
3	3745
4	3667
5	3575
Mean	3645
SD	70.938
% RSD	1.95

Table 5: Method Precision for HPTLC

Sample No.	% Assay of Caffeine in Extract
1	9.38
2	9.44
3	9.50
4	9.32
5	9.51
6	9.64
Mean	9.47
SD	0.11088
% RSD	1.17

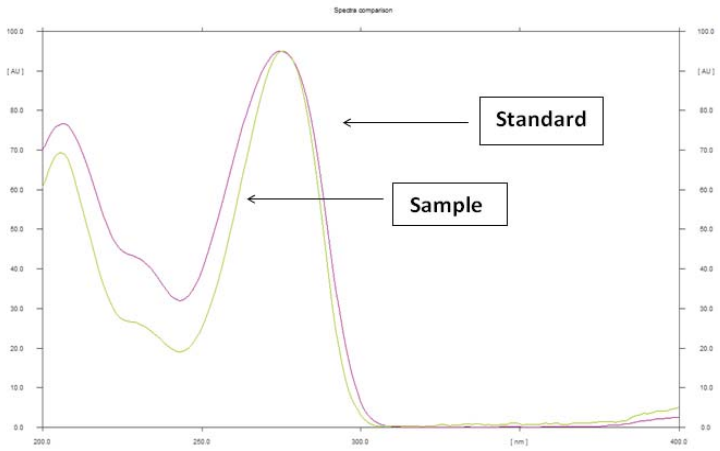

Figure 7: Spectra of caffeine from STD and sample for HPTLC

4. Intermediate Precision

The overall % RSD for both analysis on different days (analysis 1 and 2) was found to be less than 2.0%, as mentioned in Table 6.

5. Linearity

Linearity of caffeine was determined by plotting graph of concentration (as x-value) versus area (as y-value) as shown in Table 7. Calibration curve for caffeine was found to be linear from 200 ppm to 600 ppm as shown in Figure 8. The linearity graph of area versus concentration of STD caffeine was plotted and the correlation coefficient (R^2) was found to be 0.9987 with equation of line as 9.875x-255.2.

6. Robustness

The % RSD after deliberate change in saturation time, mobile phase volume and volume of polar solvent was found to be less than 2.0 % as shown in Table 8. Thus, method was robust.

7. Accuracy

The % recovery of caffeine at three levels 80%, 100% and 120% were found to be within the range of 98-102% indicating the accuracy of the method, as shown in Table 9.

8. Stability of Standard and Sample Solution

% RSD for stability of standard and sample solutions were found to be less than 2.0% for all validation parameters,

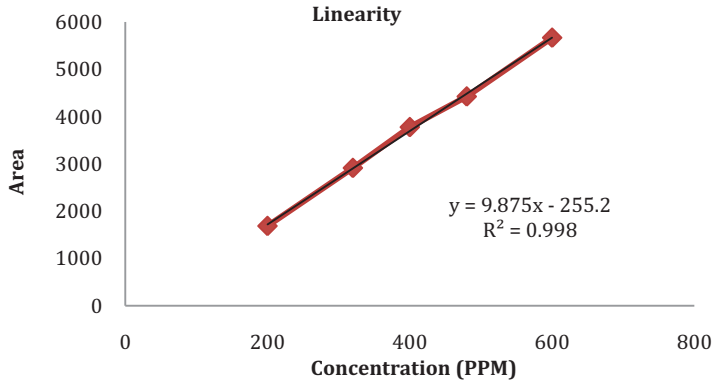

Figure 8: Linearity for HPTLC (concentration vs. area)

Table 6: Intermediate Precision for HPTLC

Name of Analyte	S. No.	Assay (% w/w, Analysis-1)	Assay (% w/w, Analysis-2)
Caffeine	1	9.38	9.82
	2	9.44	9.63
	3	9.50	9.85
	4	9.32	9.67
	5	9.51	9.38
	6	9.64	9.67
	Average	9.47	9.67
	% RSD	1.17	1.72
	Overall % RSD	1.44	

Table 7: Linearity for HPTLC

Conc. Caffeine (PPM)	Average Area of Band of Caffeine
200	1689
320	2915
400	3778
480	4425
600	5667

Table 8(a): Robustness Parameter for HPTLC

S. No.	Parameters	Working Parameter	- Changes	+ Changes
1	Saturation Time (minute)	20	19	21
2	Polar Solvent Volume (Water)	1.0	0.9	1.1
3	Mobile Phase Volume	12	11	13

Table 8(b): Robustness for HPTLC

Robustness Parameter		% RSD	Rf 55 Rf (±0.1)
Saturation Time (minute)	19	1.18	0.55
	20	1.21	0.55
	21	1.20	0.55
Polar Solvent Volume (Water)	0.9	1.02	0.55
	1.0	1.25	0.55
	1.1	1.22	0.55
Mobile Phase Volume	11	1.07	0.55
	12	1.35	0.55
	13	1.30	0.55

thus, the standard and sample solutions were stable up to 24 hours at room temperature.

9. Limit of Detection (LOD)

LOD for Caffeine was found to be 50 ppm which was the lowest concentration where the analyte was detectable but below that detection was impossible.

10. Limit of Quantification (LOQ)

LOQ for Caffeine was found to be 150 ppm which was the lowest concentration where the analyte can be quantified with % RSD less than 2 but below that concentration quantification was not precisely possible.

11. Assay of Extract

Table 9: Recovery for HPTLC

Band	Sample	Recovery Levels (%)	Std Wt. (Spiked)	Amount Recovery	% Recovery	Avg % Recovery
1	Sample 1	80	8.03	8.09	100.76	
2	Sample 2	80	8.05	8.06	100.17	100.27
3	Sample 3	80	8.02	8.01	99.88	
4	Sample 1	100	10.05	10.07	100.17	
5	Sample 2	100	10.08	10.04	99.60	99.90
6	Sample 3	100	10.10	10.09	99.94	
7	Sample 1	120	12.08	12.20	101.02	
8	Sample 2	120	12.00	12.23	101.91	100.99
9	Sample 3	120	12.01	12.02	100.05	

Table 10: % Assay of Caffeine from Coffee Bean Extract by HPTLC

Sample No.	% Assay of Caffeine
1	9.61
2	9.89
3	9.80

Table 11: Specificity for HPLC

S. No.	Sample Name	Analyte Name	Purity Flag	Specificity	Purity Angle	Threshold
1	Sample	Caffeine	No	Specific	-	-
2	Standard	Caffeine	No	Specific	-	-
3	Blank	No Peak	-	-	-	-

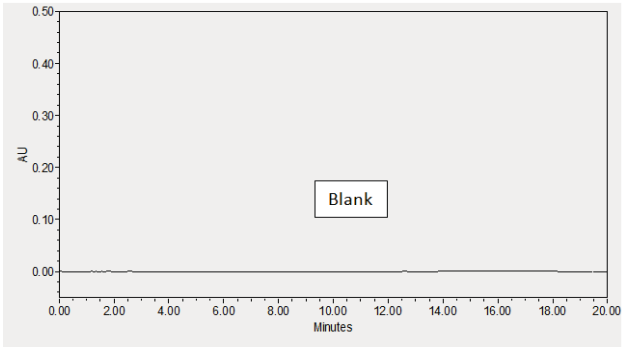

Figure 9: HPLC chromatogram of blank solution

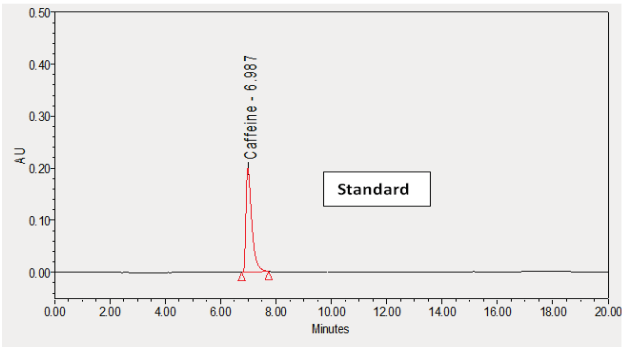

Figure 10: HPLC chromatogram of standard solution

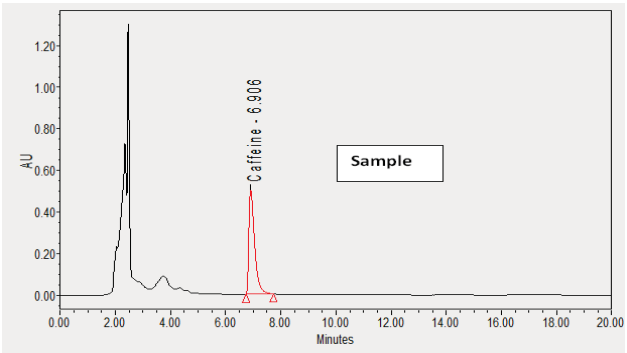

Figure 11: HPLC chromatogram of sample solution

The % assay of caffeine was determined in extract, as mentioned in Table 10 and the results in terms of relative standard deviation (% RSD), were found to be less than 2.0%.

Analysis by HPLC

The HPLC method for estimation caffeine from coffee bean extract was validated in terms of specificity, precision, linearity, robustness, accuracy, stability, limit of detection, limit of quantitation in standard as well as sample solution and results are mentioned as follows.

1. Specificity

The chromatograph of blank, STD and sample solutions are shown in Figure 9-11 respectively. The spectra of standard caffeine and purity of caffeine showed in Figure 12-13 respectively and the method was found to be specific as mentioned in Table 11.

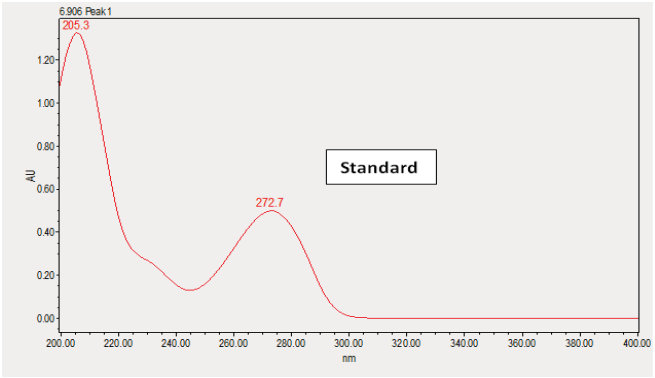

Figure 12: Spectra of caffeine for HPLC

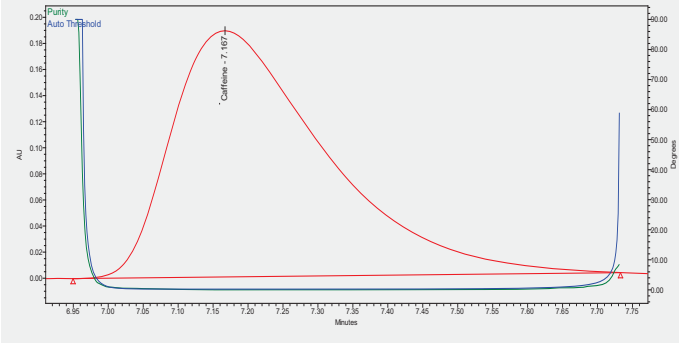

Figure 13: Purity plot of caffeine by HPLC

Table 12: System Precision for HPLC

Injection No.	Peak Area of Caffeine
1	3073796
2	3097051
3	3019658
4	3013164
5	3055303
6	3033232
Mean	3048700
% RSD	1.17

Table 13: Method Precision for HPLC

S. No.	Assay of Caffeine (% w/w)
1	9.61
2	9.53
3	9.64
4	9.56
5	9.55
6	9.89
Mean	9.63
% RSD	1.40

Table 14: Intermediate Precision for HPLC

S. No.	HPLC System 1	HPLC System 2
1	9.61	9.78
2	9.53	9.76
3	9.64	9.68
4	9.56	9.77
5	9.55	9.50
6	9.89	9.56
Average	9.63	9.68
% RSD	1.40	1.23
Overall % RSD	1.31	

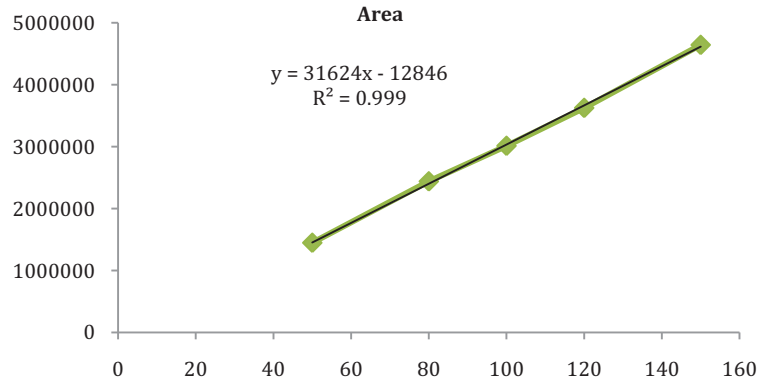

Figure 14: Linearity for HPLC (concentration vs. area)

Table 15: Linearity for HPLC

Conc. of Caffeine (PPM)	Average Peak Area of Caffeine
50	1448522
80	2439780
100	3013164
120	3625446
150	4642600

Table 16(a): Robustness for HPLC

S. No.	Parameters	Working Parameter	- Changes	+ Changes
1	Flow	1 ml/minute	0.9 ml/minute	1.1 ml/minute
2	Temperature	25°C	20°C	30°C
3	Wavelength	272 nm	267 nm	277 nm

Table 16(b): Robustness for HPLC

Robustness Parameter		% RSD	Peak Tailing	Theoretical Plates	Remark
Wavelength (nm)	267	1.17	1.75	2356	Pass
	272	1.15	1.85	2541	Pass
	277	1.12	1.92	2865	Pass
Temperature (°C)	20	1.32	1.85	2358	Pass
	25	1.38	1.87	2315	Pass
	30	1.25	1.81	2366	Pass
Flow (ml/min)	0.9	1.39	1.86	2425	Pass
	1.0	1.34	1.79	2569	Pass
	1.1	1.32	1.81	2475	Pass

Table 17: Recovery for HPLC

Band	Sample	Recovery Levels (%)	Std Wt. (Spiked)	Amount Recovery	% Recovery	Avg % Recovery
1	Sample 1	80	8.03	8.06	100.39	
2	Sample 2	80	8.05	8.20	101.89	101.28
3	Sample 3	80	8.02	8.14	101.56	
4	Sample 1	100	10.05	10.18	101.31	
5	Sample 2	100	10.08	10.20	101.23	101.37
6	Sample 3	100	10.10	10.26	101.56	
7	Sample 1	120	12.08	12.01	99.44	
8	Sample 2	120	12.00	12.08	100.69	100.31
9	Sample 3	120	12.01	12.11	100.81	

Table 18: IR Functional Groups of STD Caffeine

	Wavelength	Functional group
Caffeine	1750	C=O
	1651	C=N
	1540	C=C
	1266	C-N

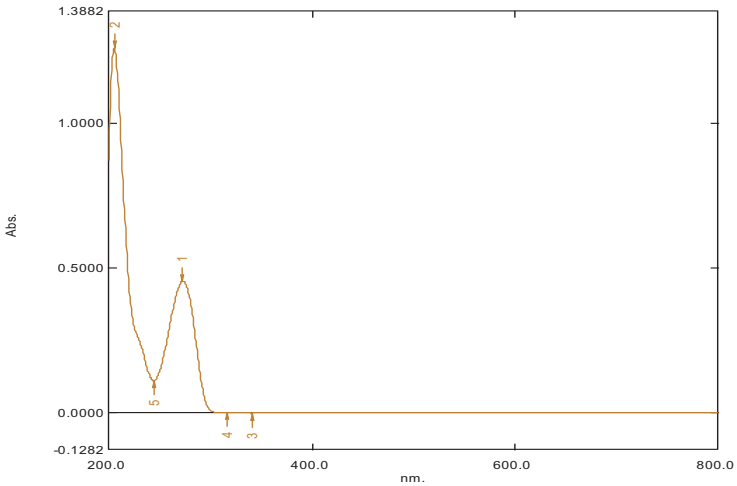

Figure 15: UV spectra of caffeine

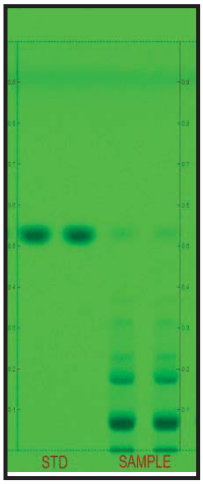

Figure 16: HPTLC plate of formulation

Table 19 (a): Incompatibility Study

S. No.	Name of Excipients	Ratio of Extract: Excipient	Storage condition
1	Micro crystalline cellulose 101	1:1	40°C/75%RH
2	Ethyl cellulose	1:1	40°C/75%RH
3	Magnesium stearate	1:1	40°C/75%RH
4	Dibasic calcium phosphate	1:1	40°C/75%RH

Table 19(b): Incompatibility Study

S. No.	Name of Sample	Change in Color	Lump Formation	% LOD
1	Micro crystalline cellulose 101	**No**	**No**	**3.8**
2	Ethyl cellulose	**No**	**No**	**4.2**
3	Magnesium stearate	**No**	**No**	**3.9**
4	Dibasic calcium phosphate	**No**	**No**	**4.1**

Table 20: % Assay of Caffeine from Coffee Bean Extract by HPLC

Sample No.	% Assay of Caffeine
1	9.94
2	9.91
3	9.79

Table 21: % Assay of Caffeine from Capsule Formulation of Coffee Bean Extract by HPTLC and HPLC

Sample No.	% Assay of Caffeine by HPTLC	% Assay of Caffeine by HPLC
1	9.78	9.82
2	9.82	9.65
3	9.56	9.45

2. System Precision

The results for system precision are mentioned in Table 12. The relative standard deviation (% RSD) for caffeine was found to be less than 2.0%.

3. Method Precision

The average % assay and % RSD for caffeine was calculated and mentioned in Table 13.

And the relative standard deviation was found to be less than 2.0%.

4. Intermediate precision

The intermediate precision for both HPLC system and different analyst are mentioned in Table 14 and % RSD was found to be less than 2.0%.

5. Linearity

The linearity of peak area response for caffeine was determined from 50 to 150 % level of working concentration. The stock solutions of caffeine were diluted to five different known concentrations. A graph was plotted of concentration (as x-value) versus peak area (as y-value) as shown in Figure 14. The correlation coefficient, y-intercept and slope of the regression were calculated and tabulated in Table 15.

6. Robustness

The % RSD after deliberate change in flow rate, temperature and wavelength was found to be less than 2.0%, as mentioned in Table 16. Hence, the method was found to be robust.

7. Accuracy

The % recovery of caffeine at three levels 80%, 100% and 120% was found to be within the acceptance criteria of 98-102% indicating the accuracy of the method, as shown in Table 17.

8. Stability of Standard and Sample Solution

% RSD for stability of standard and sample solution was found to be less than 2.0% for all validation parameters, thus, the standard and sample solutions were stable up to 24 hours at room temperature.

9. Limit of Detection (LOD)

LOD for Caffeine was found to be 0.0039 ppm which was the lowest concentration where the analyte was detectable but below that detection was impossible.

10. Limit of Quantitation (LOQ)

LOQ for Caffeine was found to be 0.0120 ppm which was the lowest concentration where the analyte can be quantified with % RSD less than 2 but below that concentration quantification was not precisely possible.

Analysis by UV

A UV spectrum of standard caffeine was observed at wavelength 272 nm as shown in Figure 15.

Analysis by IR

IR spectra for standard caffeine were obtained and the functional groups were observed which were mentioned in Table 18.

Formulation

The oral solid capsule formulation of coffee bean extract was formulated having anti-obesity activity. The analysis of formulation was carried out using incompatibility study, assay for the quantification of caffeine from coffee bean extract.

1. Incompatibility Study

Extract-excipient compatibility was studied for 1 month and the ratio of extract: excipient taken for analysis was, as mentioned in Table 19(a).

The physicochemical tests were performed like change in color, lump formation and % loss on drying (LOD) to study the extract-excipient compatibility and the results mentioned in Table 19(b).

2. Assay

The HPTLC % assay of caffeine from coffee bean extract and formulation was found to be within the range of 9.3-10.0 (Table 20-21) and formulation plate for HPTLC as shown in Figure 16.

CONCLUSION

Precise, accurate and sophisticated HPTLC and HPLC methods have been developed for identification and quantification of caffeine from *coffee bean extract.* HPLC method for quantification of caffeine from *coffee bean extract* was validated as per ICH guidelines. Also identification of caffeine from *coffee bean extract* using UV spectrophotometer and IR was done. Since caffeine is known for anti-obesity activity, a formulation in the form of capsule was prepared using *coffee bean extract*. The extract-excipient compatibility was studied for 1 month and the best suited excipients were used to prepare capsule. Prepared capsule was passed on all the physical parameters and was stable. Caffeine from prepared capsule was identified using HPTLC method. Thus, a standardized formulation in the form of capsule was prepared using standardized *coffee bean extract* to be used for the management of obesity.

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