HPTLC Method

Quantification of caffeine in coffee by HPTLC.

Method Creator	Validated by	Final Authoriser	History	Date
Anchrom Enterprises (I) Pvt. Ltd. Mumbai.	**NA**			**07-02-2019**

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Method created at: **India - Specific HPTLC Application Research Lab**

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Objective: Quantification of caffeine in coffee by HPTLC.

Introduction: Caffeine is a chemical compound found in coffee, tea and colas. It is a Central nervous system stimulant. A method has been developed to quantify caffeine n coffee.

Method suitable for: Food Industry

Analysis method

Reagents and chemicals required: GR grade Methanol, Toluene, Acetone

1.5% potassium permanganate solution: Dissolve 1.5 gm $KMnO_4$ in water and dilute to 100ml.

Dilute phosphoric acid solution: To 15 ml of phosphoric acid, add 70 ml of water.

Reducing solution: Dissolve 5 gm of sodium sulphite (Na_2SO_3) and 5 gm of ammonium thiocyanate (NH_4CNS) in water and dilute to 100 ml.

Sodium hydroxide solution: Dissolve 2.5 gm of NaOH in 7.5 ml of water.

TLC Al Si Gel 60 F_{254}, 20x10cm (Merck Catalogue No. 1.05554.0001). If no. of samples and standards to be applied is less than 10, then use a 10 x 10 cm sized plate.

HPTLC system requirements:
HPTLC system software – VisionCATS
Sample Band Applicator – Camag Linomat 5 or ATS-4

Development Chamber – Automatic Developing Chamber with humidity control or Twin Trough Chamber, 20x10 cm and a desiccator filled with $Mgcl_2.6H_2O$ saturated solution to keep 20x10 cm or 10x10cm sized plates 45 mins exposed to dessicator.
Chromatogram Visualisation – Camag UV cabinet
Image documentation – Camag Visualiser
Scanning Densitometry – Camag Scanner 4
Plate Cutter- Camag Smart Cut
Solvent Front Detector- Camag Smart Alert

Standard Preparation: i) Caffeine- 0.1mg/ml Chloroform

SST Standard: i) Methyl Paraben- (1mg/ml) methanol

ii) Coumarin- (1mg/ml) methanol

Samples Preparation: Weigh 75 mg of powdered coffee sample in a 250 ml conical flask. Add 3.75ml of $KMnO_4$ solution. Add 7.5 ml of reducing solution. Add 0.75ml of phosphoric acid solution Add 0.75ml of sodium hydroxide (NaOH) solution. Mix well after addition of each solution. Transfer the solution to 500ml separating funnel. Add 100ml chloroform and shake for 5 minutes. Allow the layers to separate and drain the chloroform layer into a 500ml conical flask after passing it through anhydrous sodium sulphate. Re-extract aqueous solution with 50ml of chloroform and drain as before. Repeat this 50 ml extraction. Collect the chloroform layers and concentrate to a final volume of 25ml in a water bath at 60ºC. This is *Test Solution.* [conc. 3µg/µl].

1. **CHROMATOGRAM LAYER:**

20x10 cm TLC Al Silica gel $60F_{254}$ on plate (Merck 5554). If you have less than 10 tracks to apply (standard + sample), use a 10x10 cm silica gel $60F_{254}$ plate. Write ID on each plate. Mark with pencil at 70 mm, if no Automatic Development Chamber (ADC2) is available.

Mobile phase: Toluene: Acetone (7:3) v/v (Pipette out each volume separately in a 25ml stoppered conical flask, shake well.)

2. **Camag visionCATS HPTLC software.**
   a) Switch on and select instruments that will be used (Linomat or ATS for sample application, ADC-2, if available, Camag Visualiser, Camag Scanner etc.
   b) Sample Applicator parameters:

Parameters for Linomat 5	Parameters for ATS 4
No of bands – 15 Band length – 8mm Track distance – 11.4mm Distance from lower edge – 8mm Distance from left side edge – 20mm Application speed- Ethyl Acetate (200 nl/s) Application volumes – i. Standard: 2,4,6,8,10,12,14,16 µl ii. Sample: 2µl, 4µl	No of bands – 15 Band length – 8mm Track distance – 11.4mm Distance from lower edge – 8mm Distance from left side edge – 20mm Application speed- Ethyl Acetate (200 nl/s) Application volumes – i. Standard: 2,4,6,8,10,12,14,16 µl ii. Sample: 2 µl, 4 µl

Sample Application in ATS 4

Track	Vial ID	Description	Volume	Position	Type
1	RC1819201-01	Methyl paraben (1mg/ml)	1.0 µl	N/A	Reference
+	RD1819201-01	Coumarin (1mg/ml)	2.0 µl	N/A	Reference
2	SA1819201-01	75 mg coffee	2.0 µl	N/A	Sample
3	SA1819201-01	75 mg coffee	2.0 µl	N/A	Sample
4	SA1819201-01	75 mg coffee	2.0 µl	N/A	Sample
5	RA1819201-01	Caffeine (0.1mg/ml) in Chloroform	2.0 µl	N/A	Reference
6	RA1819201-01	Caffeine (0.1mg/ml) in Chloroform	4.0 µl	N/A	Reference
7	RA1819201-01	Caffeine (0.1mg/ml) in Chloroform	6.0 µl	N/A	Reference
8	RA1819201-01	Caffeine (0.1mg/ml) in Chloroform	8.0 µl	N/A	Reference
9	RA1819201-01	Caffeine (0.1mg/ml) in Chloroform	10.0 µl	N/A	Reference
10	RA1819201-01	Caffeine (0.1mg/ml) in Chloroform	12.0 µl	N/A	Reference
11	RA1819201-01	Caffeine (0.1mg/ml) in Chloroform	14.0 µl	N/A	Reference
12	RA1819201-01	Caffeine (0.1mg/ml) in Chloroform	16.0 µl	N/A	Reference
13	SA1819201-01	75 mg coffee	4.0 µl	N/A	Sample
14	SA1819201-01	75 mg coffee	4.0 µl	N/A	Sample
15	SA1819201-01	75 mg coffee	4.0 µl	N/A	Sample
Sequence table notes					

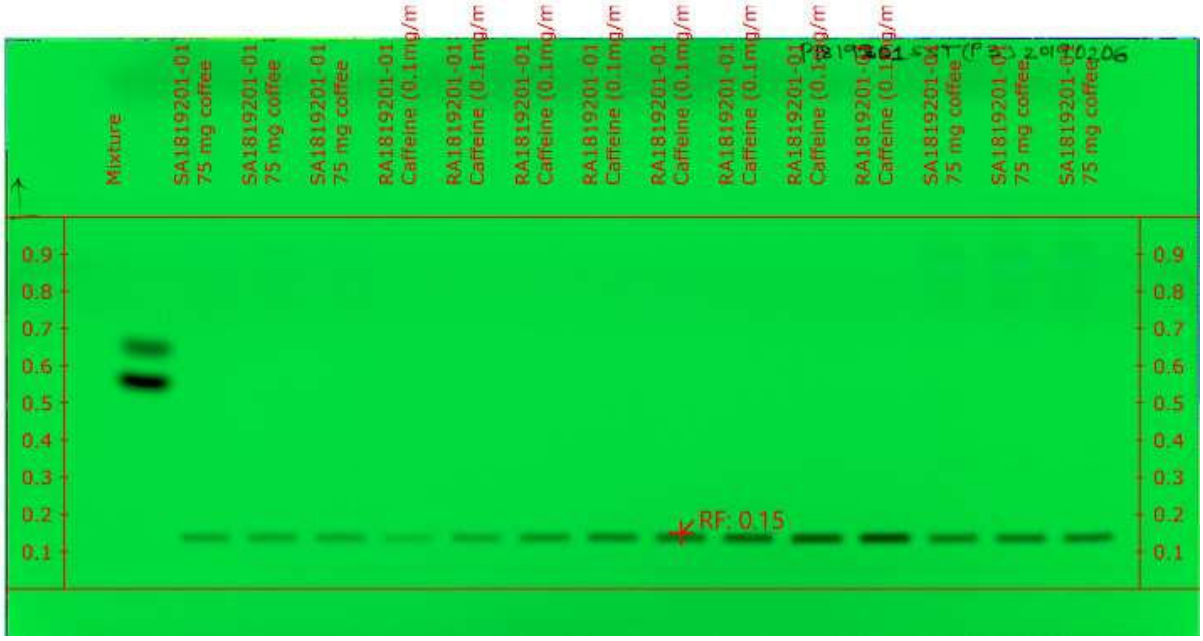
3. **Chromatogram development:** If Auto development chamber is not available after sample application, keep the plate in a desiccator over saturated $Mgcl_2.6H_2O$ solution for 45 min, then quickly transfer to Twin trough chamber.

Using Camag Automatic Developing chamber 2	Using Camag Twin Trough Chamber 20x10 cm
Mobile phase saturation- 25ml MP for development - 10ml Paper lining – yes Layer preconditioning with 33% RH – Yes Development distance – 70mm	Mobile phase for saturation -5ml MP for development- 5ml Paper lining – yes After application, keep the plate in a desiccator over saturated $Mgcl_2.6H_2O$ solution for 45 min. Use Smart Alert to track the development of plate (Suitable for glass plates). Development distance – 70mm
Plate drying – 5 min	Plate drying – Hold plate vertically and the hair drier away for 10 mins. Use cool air only.

4. **Camag UV Cabinet – Inspect the plate under 366 nm for 30 seconds and then at 254nm to make sure that chromatography is performed well.**

5. **Image documentation by industrial camera with no image recording variables or user settings.**
   Document images at

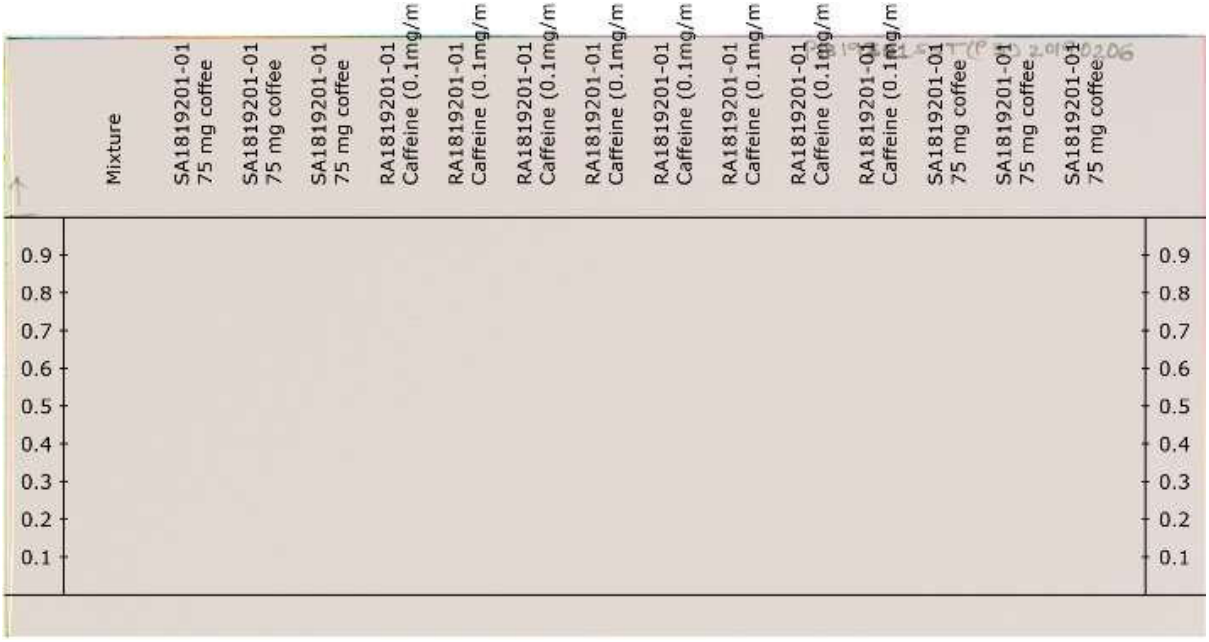
UV 254 nm



In the above image caffeine is observed at Rf 0.15.

UV 366 nm

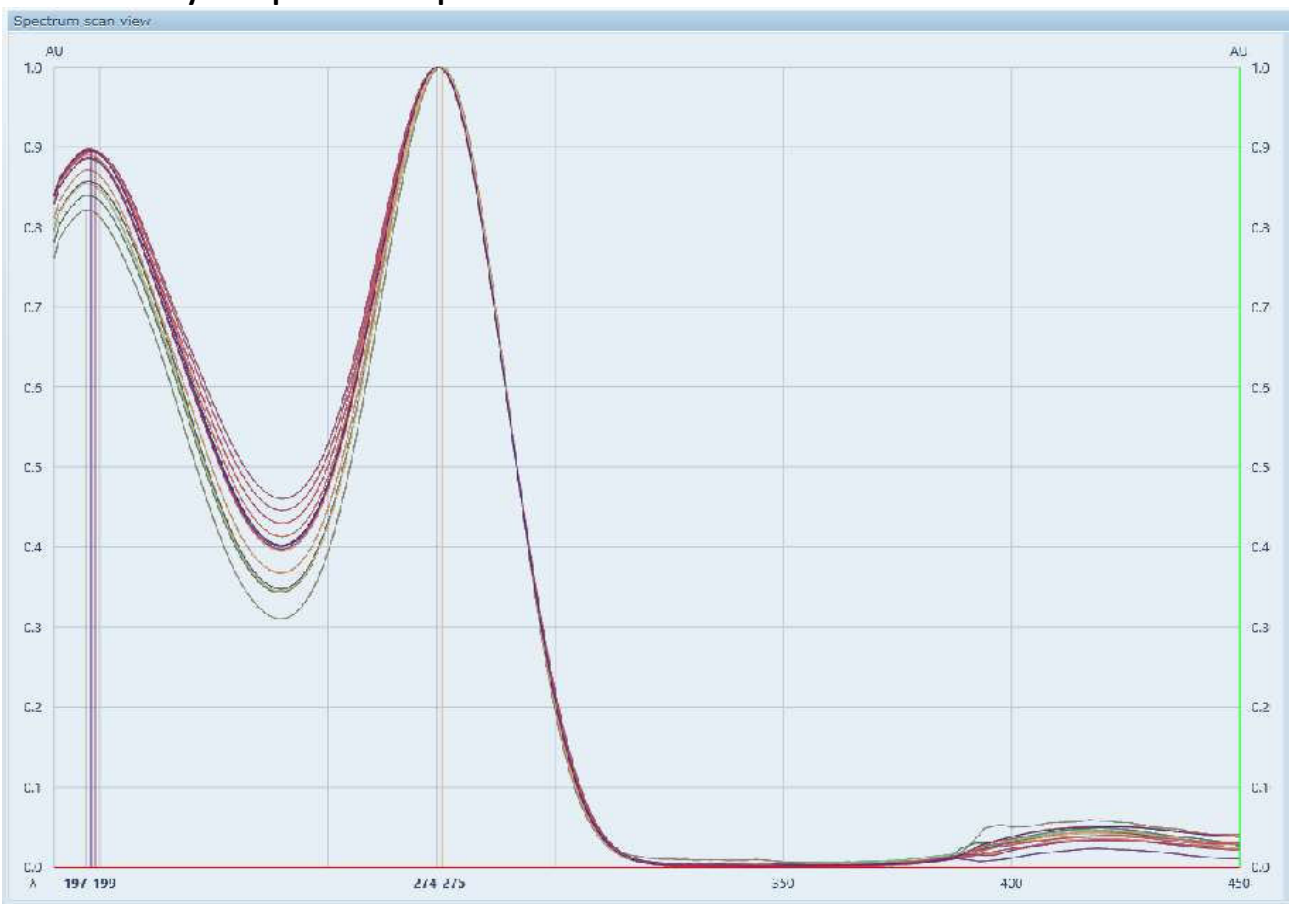
White light, from top

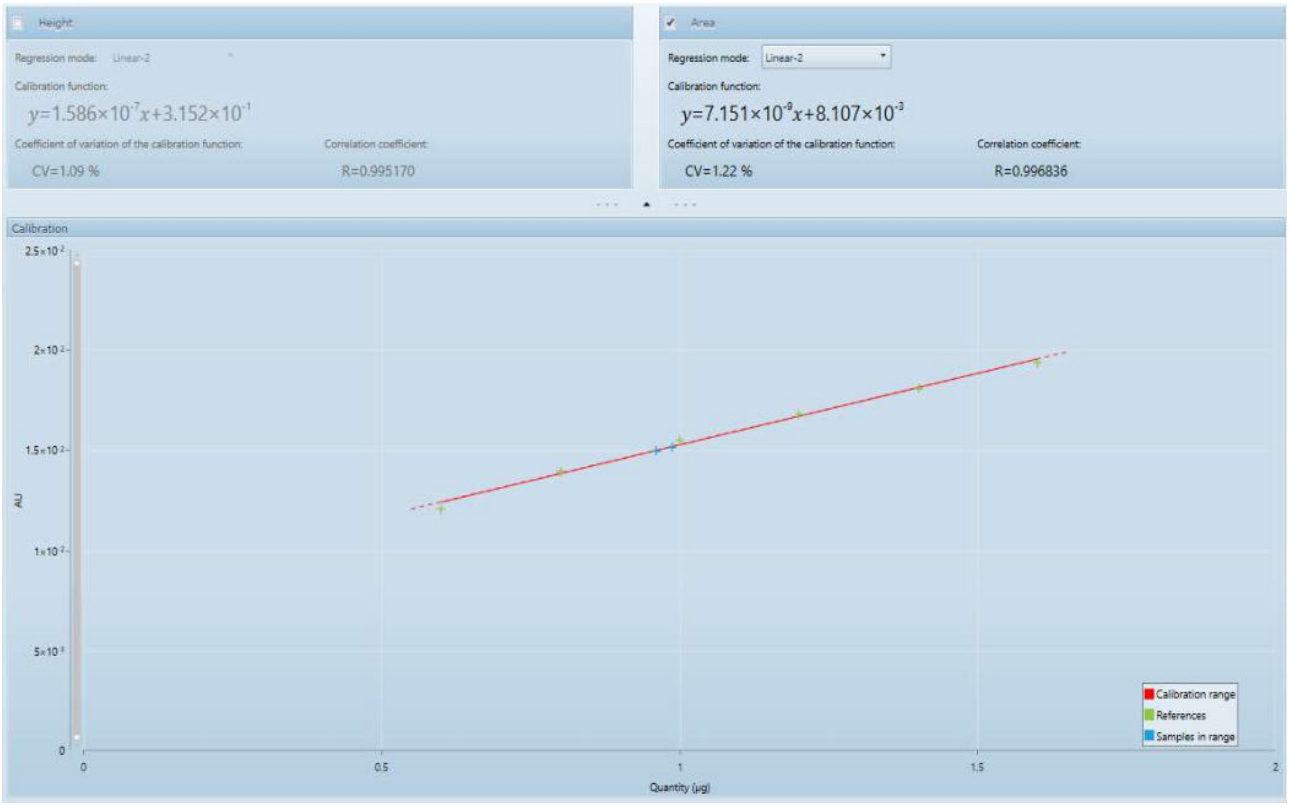


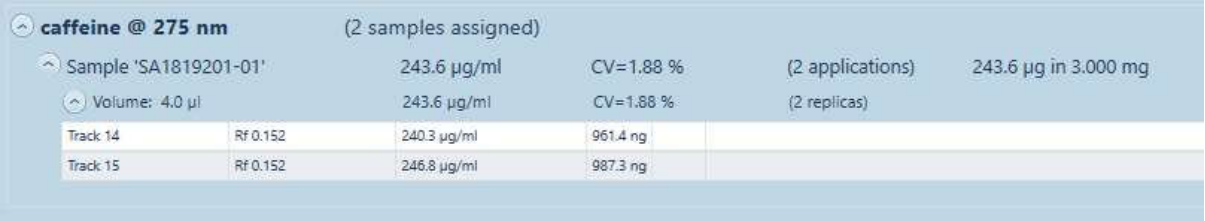
6. **Scanning densitometry:**

   Scanner Model – Camag Scanner 4
   Scan Slit dimension: 6 x 0.45mm
   Scan speed – 20 mm/sec
   Spectrum recording UV (190-400 nm); Band Width -5nm (spectrum)
   Scanning positions – Automatic
   Scan wavelength – 254nm, 275nm

7. Overlay the spectra of all peaks at Rf 0.15 of Caffeine







caffeine @ 275 nm	(2 samples assigned)				
Sample 'SA1819201-01'		243.6 µg/ml	CV=1.88 %	(2 applications)	243.6 µg in 3.000 mg
Volume: 4.0 µl		243.6 µg/ml	CV=1.88 %	(2 replicas)	
Track 14	Rf 0.152	240.3 µg/ml	961.4 ng		
Track 15	Rf 0.152	246.8 µg/ml	987.3 ng		

A HPTLC method was established to quantify caffeine in coffee.

Results

Acceptance Criteria	Sample ID	Value Found	Pass	Fail
Band of caffeine is observed at Rf 0.15	SA1819201-01	NA	YES	-

Analysis Done:
By Ankita Sarnath
On 07-02-2019

Approved:
By Dr. Saikat Mallick
On 07-02-2019