HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

DR. DARSHANA MEHTA

SCHOOL OF STUDIES IN CHEMISTRY AND BIOCHEMISTRY,

VIKRAM UNIVERSITY, UJJAIN – 456 010,

MADHYA PRADESH, INDIA

HPTLC- High Performance Thin Layer Chromatography

HPTLC is a modified and an advanced version of TLC technique (Daniel, 1991). HPTLC- High Performance Thin Layer Chromatography is a sophisticated, a powerful, reliable, efficient and automated form of TLC having the latest technical developments for quality assessment and evaluation of botanical materials.

The advancements include:

- **Enhancement to the basic method of TLC to automate the different steps (Automation in HPTLC is useful to avoid uncertainty in application size and position, when the sample is applied manually to the TLC plate)**
- **Increase the resolution,**
- **High sample throughput together with better analytical precision**
- **Accurate quantitative measurements with reduced consumption of mobile phase per sample**

VALUE OF HPTLC

- HPTLC today is more than just plates and instruments. It is also a concept, including a scientific basis, standardized methodology, and validated methods.
- In HPTLC separations, normal phase silica gel is most frequently employed, using adsorption chromatography, which benefits from the ability to separate substances according to the type, number and position of functional groups.
- There are also other modern stationary phases include reversed, amino-, diol-, and cyano-bonded phases available for HPTLC. This makes setting up two orthogonal separations possible.
- In HPTLC, the plates are precoated with a stationary phase with a typical mean particle size of 5 µm. The plates give better separations and reproducibility than normal precoated TLC plates (mean particle size 12 µm) and they also allow more sensitive detection. Shorter developing distances are required. The number of theoretical plates is in the 5000 range, while for HPLC the range is 6–10000.

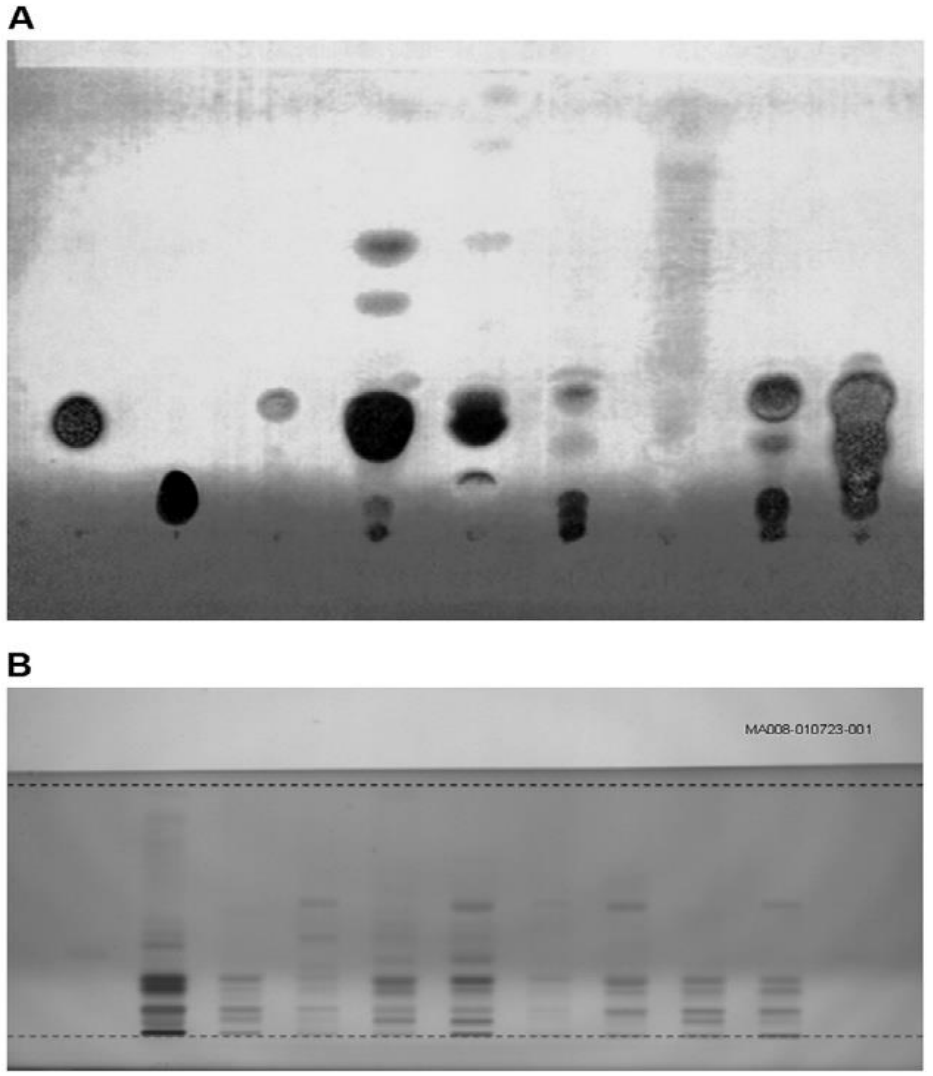
Parameters	TLC	HPTLC
Chromatographic plate used	Hand made	Precoated
Layer thickness	250μm	100-200μm
Prewashing of plates	Not followed	Must
Application of sample	Manual	Automatic
Shape	Spot	Spot/band
Sample volume	1-10 μL	0.2-5 μL
Efficiency	Low	High
Analysis time	Slow	Greatle reduced
Development Chamber	More amount of solvent	Less amount of solvent
Spots size	2-4 mm	0.5-1 mm
Scanner	Not available	Use of UV/Visible/Fluorescence scanner (Densitometer)

Comparison of manual TLC (A) and instrumental HPTLC (B):

- "TLC" is commonly used for mostly manually performed analyses on TLC plates,

- "HPTLC" implies the use of instruments for all steps of the chromatographic process on HPTLC plates

Separation of berberin containing plants, visualization with Dragendorff's Reagent.



USE OF PLANAR CHROMATOGRAPHY IN PLANT DRUG STANDARDIZATION

- Here we have used High-Performance Thin-Layer Chromatography coupled with computerized densitometry detection of plant constituents separated on to thin layer.
- Planar chromatographic applications enabled us to identify in terms of color and intensity of separated band.
- A unique identity of zone can be established by scanning spectrum. This spectrum acts as a unique identity of particular zone.
- Further a zone purity analysis can be performed indicating purity of separated zone on to HPTLC.
- Using planar chromatography a unique Chemical Fingerprints / Profiles may be generated and can also be documents to be used as future reference in the performance of Quality Control Studies (QCS) or Quality Monitoring Studies (QMS) of medicinal plants.

Different steps of HPTLC fingerprinting

A. Chamber saturation: Chromatographic chamber is filled with the solvent system 30 minutes prior to development of plate, to get uniform distribution of solvent vapors in the chamber.

B. Application of sample and standard: Sample is spotted on the TLC plate with automatic applicator Linomat IV attached with the compressed nitrogen gas cylinder and operated with software winCATS.

- To take full advantage of the separation power and reproducibility of HPTLC, precise automated positioning and volume dosage is mandatory.
- At the high end, autosamplers/applicators are available, e.g., from CAMAG, that require no operator presence and can apply sample as spots by contact transfer or as a rectangular band by a spray-on technique, essentially using technology similar to that used in ink-jet printers.
- The spray-on technique permits sample application as bands or rectangles with volumes as little as 0.5 µL to >50 µL. Prior to chromatography, these rectangles are focused into narrow bands with a solvent of high elution strength.

C. Selection of mobile phase:

- **The solvent system is selected considering the nature of the components to be separated like polar or non-polar and also solubility, affinity and resolution, as the compound will follow the rule of 'like dissolves like'.**
- **The desired mobile phase would provide the greatest solubility, while providing affinity for the sample on the stationary phase.**
- **Highly polar solvents are water, methanol, ethanol, acetone, diethyl ether, ethyl acetate, etc. while non-polar solvents are dichloromethane, toluene, chloroform, cyclohexane, petroleum ether, hexane etc.**
- **The developing solvent must be of high purity.**
- **The presence of small amounts of water or other impurities can produce irreproducible chromatograms.**

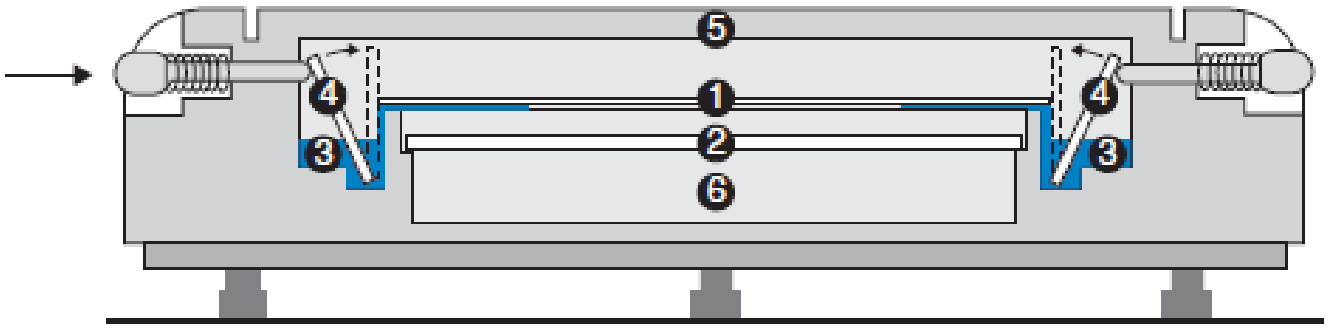
Solvent	Refractive Index[a]	Viscosity, cP[b]	Boiling Point, °C	Polarity Index, P'	Eluent Strength,[c] ε^0
Fluoroalkanes[d]	1.27–1.29	0.4–2.6	50–174	<−2	−0.25
Cyclohexane	1.423	0.90	81	0.04	−0.2
n-Hexane	1.372	0.30	69	0.1	0.01
1-Chlorobutane	1.400	0.42	78	1.0	0.26
Carbon tetrachloride	1.457	0.90	77	1.6	0.18
i-Propyl ether	1.365	0.38	68	2.4	0.28
Toluene	1.494	0.55	110	2.4	0.29
Diethyl ether	1.350	0.24	35	2.8	0.38
Tetrahydrofuran	1.405	0.46	66	4.0	0.57
Chloroform	1.443	0.53	61	4.1	0.40
Ethanol	1.359	1.08	78	4.3	0.88
Ethyl acetate	1.370	0.43	77	4.4	0.58
Dioxane	1.420	1.2	101	4.8	0.56
Methanol	1.326	0.54	65	5.1	0.95
Acetonitrile	1.341	0.34	82	5.8	0.65
Nitromethane	1.380	0.61	101	6.0	0.64
Ethylene glycol	1.431	16.5	182	6.9	1.11
Water	1.333	0.89	100	10.2	Large

Different steps of HPTLC fingerprinting

D. Chromatographic development and drying:

Sorbent layer thickness of TLC plate is 100µm and due to this smaller particle size, separations are achieved at low distance route viz. at 3 to 5 cm. Because of this shorter migration distance, less amount of mobile phase is required and the analysis time is greatly reduced. After development, remove the plate and Dry in vacuum desiccator.

More sophisticated development chambers are frequently used with HPTLC. The horizontal developing chamber is a versatile development tank that provides reproducible results. In this device, it is possible to spot the sample(s) on opposite ends of a plate. The chamber contains a solvent trough at both ends. Development and sample application can be from one end or both ends. In the latter case, after sample spotting at both ends, the plate is laid face down on edges of the solvent troughs, and development begun simultaneously from both ends. This doubles sample throughput, provided the migration distance in double-ended development is enough for the intended separation. The horizontal chamber is suitable for developments in unsaturated, saturated, and sandwich modes and can be used for preconditioning of plates.

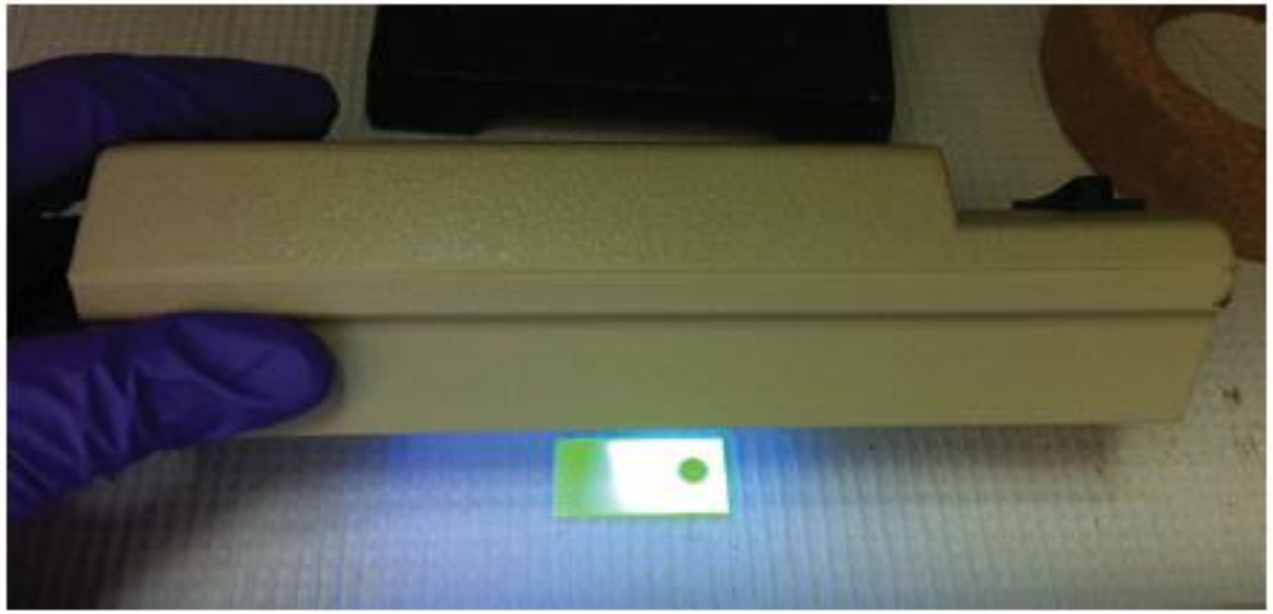


A horizontal developing chamber.
(1) An HPTLC plate (face down),
(2) glass plate (location for second plate for “sandwich” mode operation),
(3) solvent reservoir,
(4) glass strip (provides wicking),
(5) cover plate,
(6) conditioning tray (solvent-soaked pad can be put here).

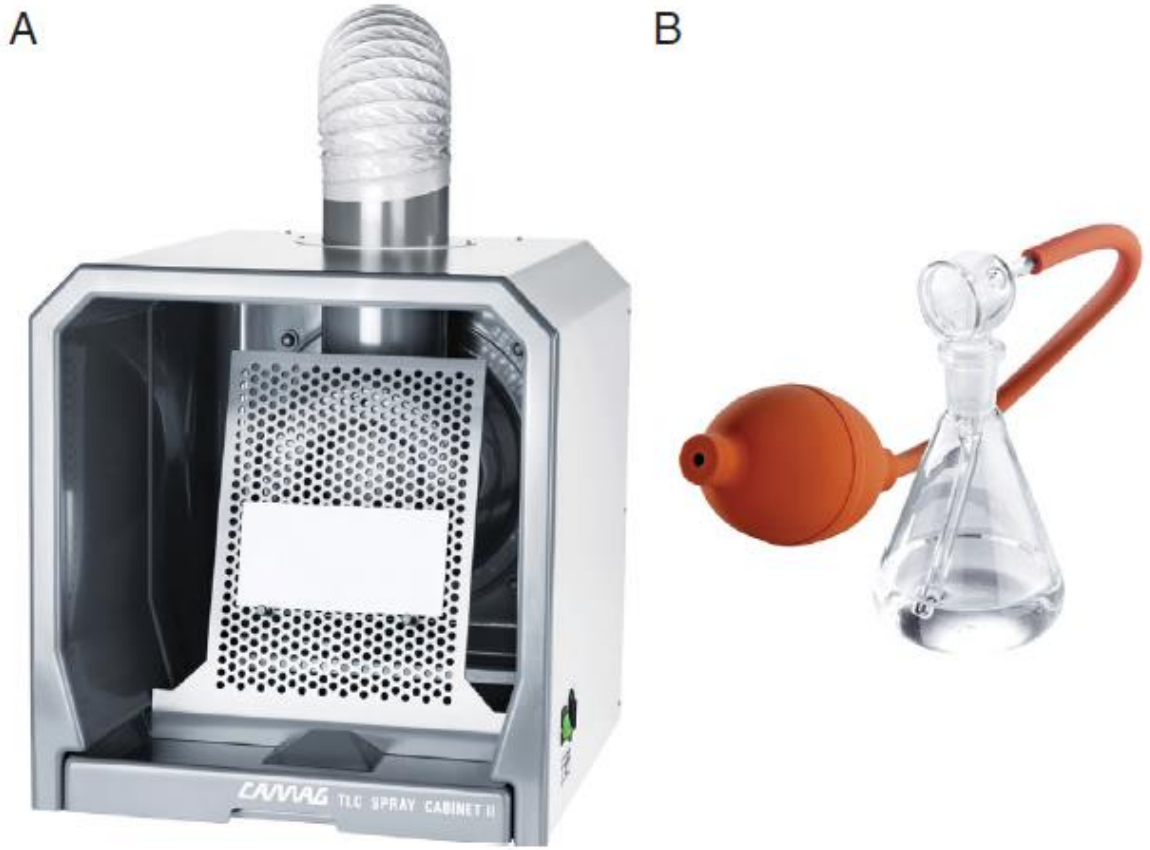
E. Detection and visualization:

- **Different compounds are distinguished by their retention factor, or Rf values generated.**
- **The Rf value determines the distance travelled of each individual compound within a mixture.**
- **Rf is the ratio of the distance travelled by the compound to the distance travelled by the solvent front.**
- **Detection under UV light is first choice non destructive method. Spots of fluorescent compounds can be seen at 254 nm (short wave length) or at 366 nm (long wave length).**
- **When individual component does not respond to UV - derivatisation required for detection.**

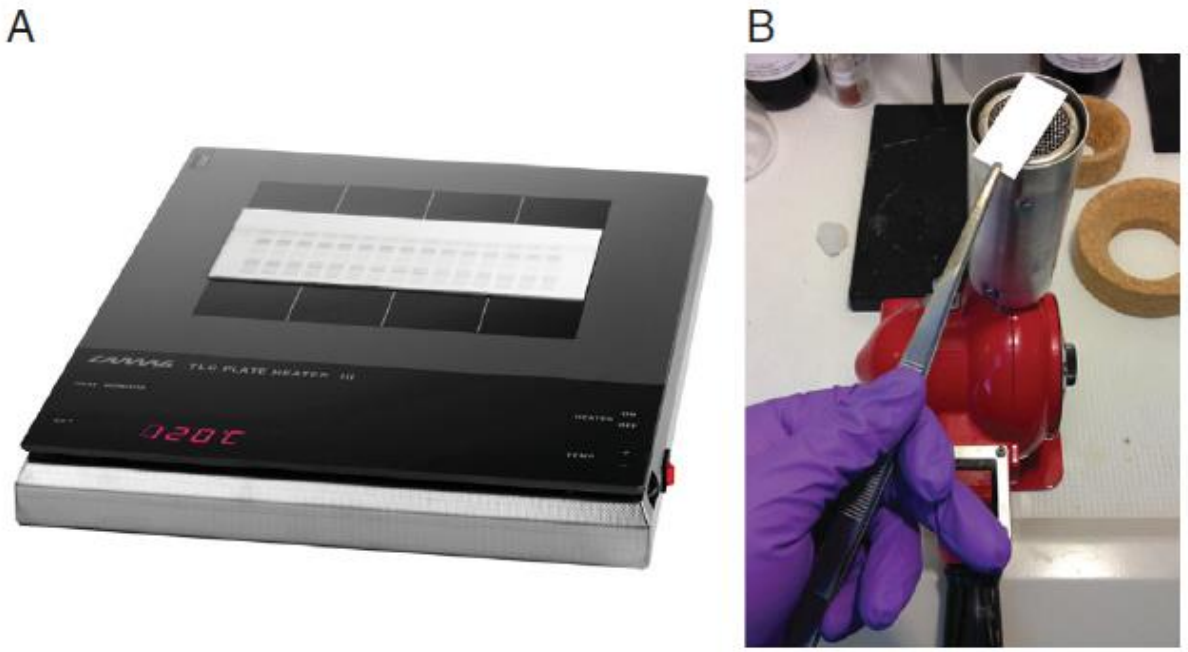
- **Photographic imaging and software based densitometry is straightforward and has the advantage that the entire plate can be imaged at once.**
- **Variable wavelength reflectance-based densitometric scanners are available commercially and covers the entire 190–900 nm range. Broadband light source(s) are used with a monochromator; a slit of adjustable length and width then controls the spatial resolution of the scan. Each chromatographic track is scanned at a time, measuring the diffusely reflected light.**
- **Background corrected absorption spectra for any desired spot can also be acquired for identification of the analyte, as well as selecting the best measurement wavelength.**
- **In principle, diode-array-based TLC scanners as well as fluorescence scanners should be feasible.**
- **Similarly, bioluminescence is now a popular assay to determine, for example, toxicity of substances, detection systems specifically to measure bioluminescence on TLC plates are also available.**



Visualization under a UV lamp (UVP 4-W compact UV lamp from UVP).



(A) CAMAG TLC Spray Cabinet equipped with a blower and
(B) Glass Reagent Sprayer.



(A) CAMAG TLC Plate Heater. Figure courtesy of CAMAG Scientific Inc. (B) Heating TLC plate with a heat gun.

Different steps of HPTLC fingerprinting

F. Quantification:

- After development of the chromatogram, it is scanned in Camag TLC scanner III having UV/ visible/ fluorescence scanning facility.
- The scanner converts band into peak and peak height or area is related to the concentration of substance on spot/band.
- The peak height and area under spot are measured by instrument and are recorded.

G. Documentation:

- The chromatogram is automatically recorded during photo documentation.
- It is important because labelling every single chromatogram can avoid mistake in respect of order of application.
- Type of plate, chamber system, composition of mobile phase, running time and detection method should be recorded.

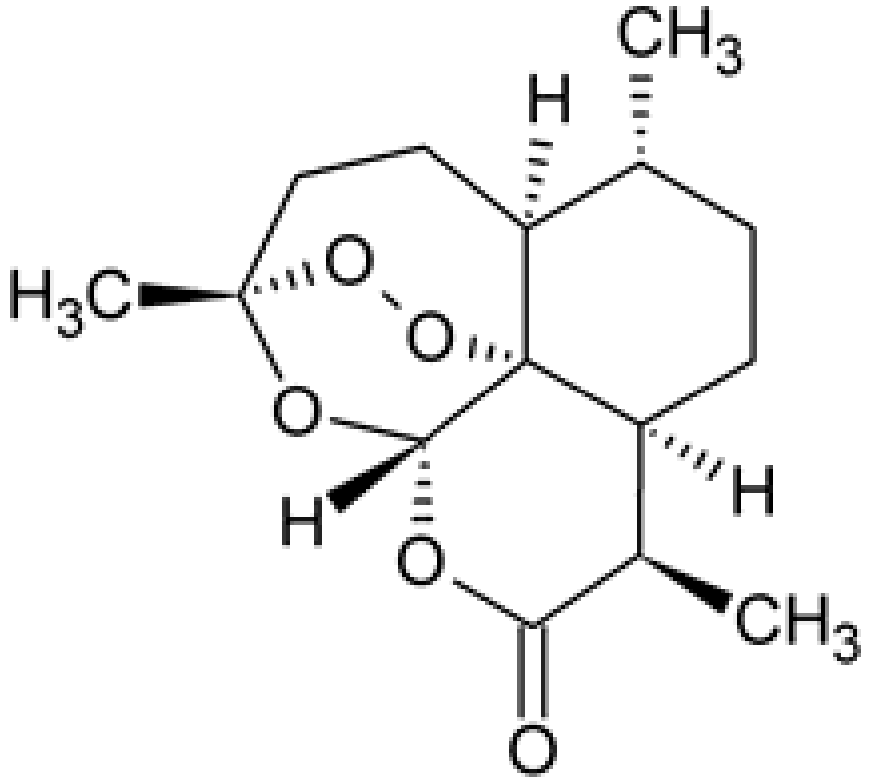
QUALITY MONITORING STUDIES (QMS) ON *ARTEMISIA ANNUA & STEVIA REBAUDIANA*

- To examplify QMS, we have selected to very important industrial medicinal plants:
  - *Artemisia annua* (Compositae)
  - *Stevia rebaudiana* (Compositae)

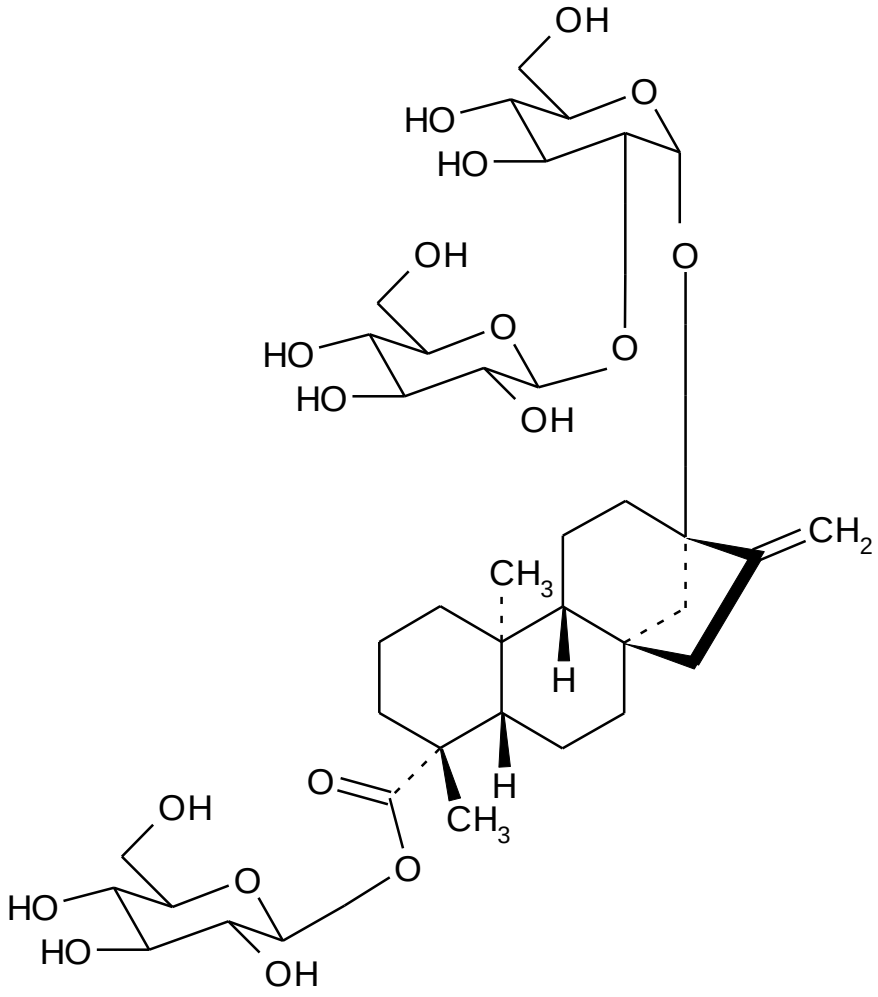
- ***Artemisia annua*** is bitter plant having **Artemisinin** as principal active constituent. **Artemisinin** is the priority molecule of WHO and is used in the form of ACTs for the treatment of Complicated malaria.

- ***Stevia rebaudiana*** is a natural sweetener. Principal active constituent is **Stevioside**.

QUALITY MONITORING STUDIES (QMS) ON *ARTEMISIA ANNUA* & *STEVIA REBAUDIANA*



Artemisinin



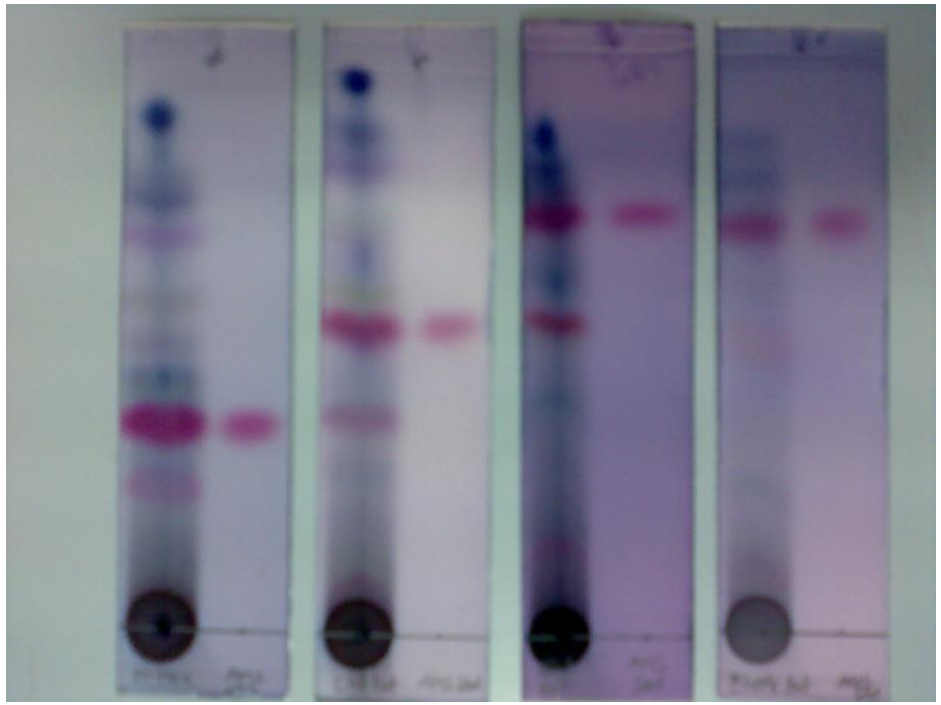
Stevioside

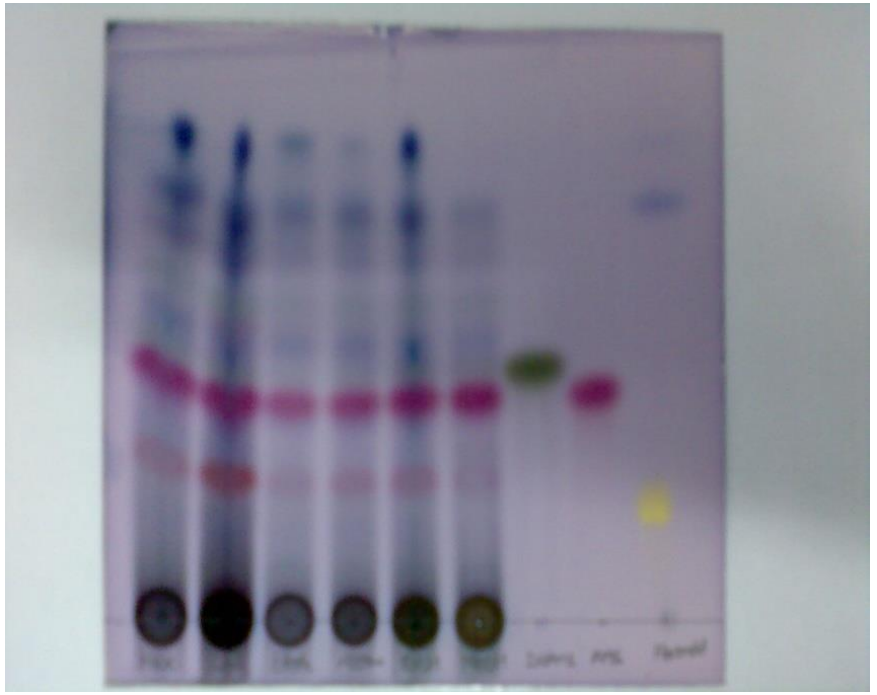
PHYSICAL APPEARANCE

Artemisia annua L.

Stevia rebaudiana Bert.

HPTLC PHOTOGRAPHS OF EXTRACT PROFILING OF *ARTEMISIA ANNUA*



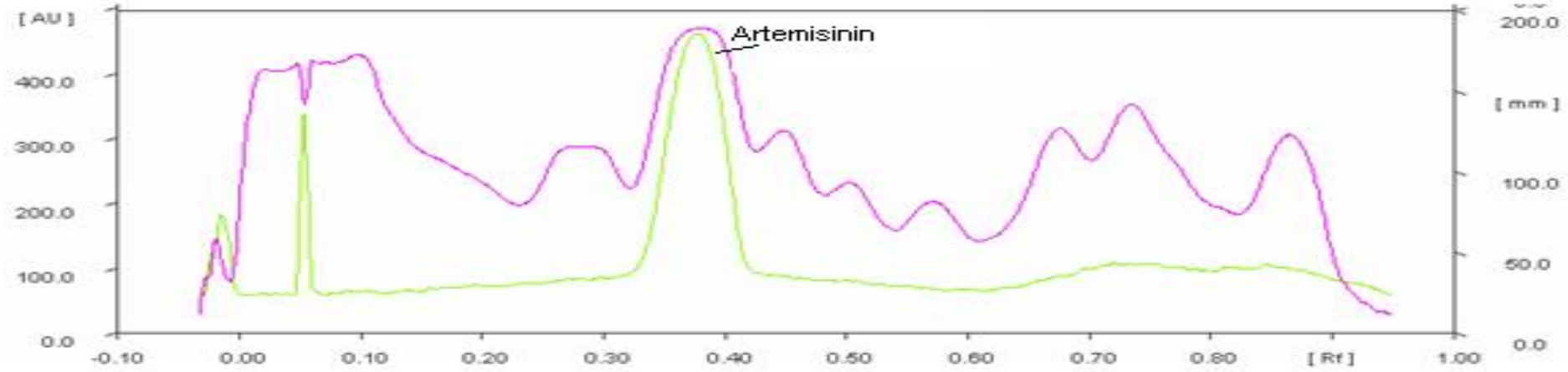


Hexane Chloroform Ethyl acetate

Markers: Green zone = Deoxyartemisinin; Pink is Artemisinin and Yellow is isolated Flavone

CHEMICAL FINGERPRINTS OF DIFFERENT EXTRACTS USING REFERENCE MARKER CHEMICAL ARTEMISININ

***n*-Hexane Extract:**

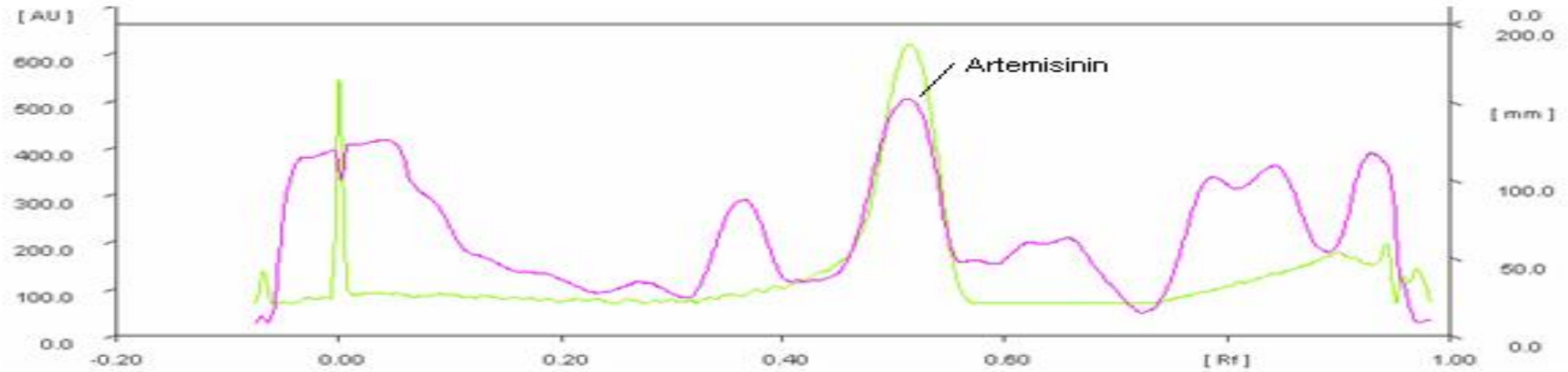


Track 1, ID: Hexane Extract

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	-0.03 Rf	55.6 AU	-0.02 Rf	62.9 AU	5.40 %	-0.01 Rf	50.8 AU	461.1 AU	1.14 %	unknown *
2	-0.01 Rf	51.8 AU	0.02 Rf	151.5 AU	13.02 %	0.03 Rf	74.2 AU	3322.5 AU	8.22 %	unknown *
3	0.03 Rf	374.3 AU	0.05 Rf	49.8 AU	4.28 %	0.05 Rf	23.2 AU	452.7 AU	1.12 %	unknown *
4	0.05 Rf	323.2 AU	0.06 Rf	34.1 AU	2.93 %	0.07 Rf	36.2 AU	193.3 AU	0.48 %	unknown *
5m	0.08 Rf	386.9 AU	0.10 Rf	52.3 AU	4.50 %	0.12 Rf	10.8 AU	1251.7 AU	3.09 %	unknown *
6m	0.16 Rf	243.3 AU	0.16 Rf	6.0 AU	0.51 %	0.22 Rf	76.3 AU	203.9 AU	0.50 %	unknown *
7	0.23 Rf	168.3 AU	0.27 Rf	26.7 AU	2.30 %	0.28 Rf	57.1 AU	496.2 AU	1.23 %	unknown *
8	0.28 Rf	257.1 AU	0.28 Rf	26.7 AU	2.30 %	0.32 Rf	33.9 AU	527.0 AU	1.30 %	unknown *
9	0.32 Rf	194.0 AU	0.38 Rf	217.1 AU	18.66 %	0.43 Rf	51.2 AU	13002.7 AU	32.15 %	Artemisinin
10	0.43 Rf	251.9 AU	0.45 Rf	62.9 AU	5.41 %	0.48 Rf	33.5 AU	1655.3 AU	4.09 %	unknown *
11	0.48 Rf	183.6 AU	0.50 Rf	40.3 AU	3.47 %	0.54 Rf	28.9 AU	1031.9 AU	2.55 %	unknown *
12	0.54 Rf	129.2 AU	0.57 Rf	51.8 AU	4.45 %	0.61 Rf	12.3 AU	1707.3 AU	4.22 %	unknown *
13	0.61 Rf	112.4 AU	0.68 Rf	89.3 AU	7.67 %	0.70 Rf	37.0 AU	2976.7 AU	7.36 %	unknown *
14	0.70 Rf	237.5 AU	0.73 Rf	110.3 AU	9.48 %	0.82 Rf	53.6 AU	4893.9 AU	12.10 %	unknown *
15	0.82 Rf	154.1 AU	0.86 Rf	181.5 AU	15.61 %	0.94 Rf	3.9 AU	8268.0 AU	20.44 %	unknown *

Chemical Fingerprints of Different Extracts using Reference Marker Chemical Artemisinin

Chloroform Extract:

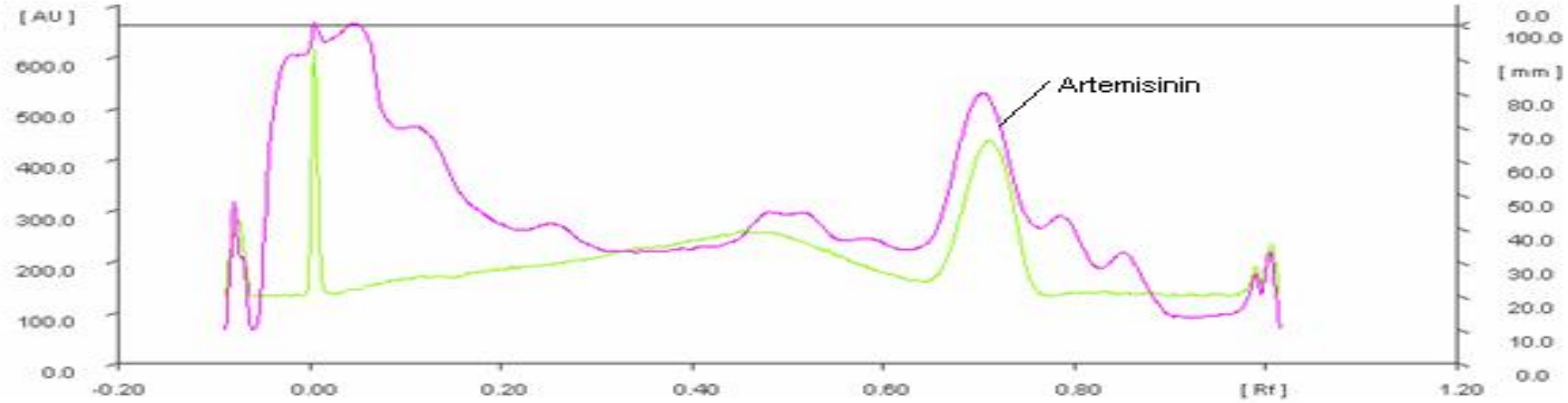


Track 1, ID: Chloroform Extract

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	-0.06 Rf	1.2 AU	-0.00 Rf	222.2 AU	14.87 %	0.00 Rf	33.7 AU	7855.4 AU	13.35 %	AutoGenerated4
2m	0.01 Rf	376.5 AU	0.04 Rf	64.6 AU	4.32 %	0.07 Rf	34.3 AU	1765.8 AU	3.00 %	AutoGenerated8
3m	0.07 Rf	281.7 AU	0.07 Rf	21.3 AU	1.43 %	0.11 Rf	52.9 AU	385.2 AU	0.65 %	AutoGenerated11
4m	0.12 Rf	144.4 AU	0.12 Rf	3.9 AU	0.26 %	0.16 Rf	36.3 AU	60.4 AU	0.10 %	AutoGenerated15
5m	0.16 Rf	106.3 AU	0.17 Rf	12.3 AU	0.82 %	0.23 Rf	34.9 AU	367.7 AU	0.62 %	AutoGenerated13
6	0.23 Rf	62.7 AU	0.27 Rf	28.4 AU	1.90 %	0.31 Rf	51.9 AU	995.8 AU	1.69 %	AutoGenerated10
7	0.31 Rf	51.5 AU	0.36 Rf	189.0 AU	12.64 %	0.41 Rf	36.7 AU	7877.1 AU	13.38 %	AutoGenerated5
8	0.43 Rf	87.8 AU	0.51 Rf	363.2 AU	24.30 %	0.56 Rf	27.3 AU	19433.1 AU	33.02 %	Artemisinin
9m	0.56 Rf	127.8 AU	0.57 Rf	5.0 AU	0.33 %	0.59 Rf	23.8 AU	69.9 AU	0.12 %	AutoGenerated14
10	0.59 Rf	123.5 AU	0.62 Rf	20.6 AU	1.38 %	0.64 Rf	35.8 AU	395.2 AU	0.67 %	AutoGenerated12
11	0.64 Rf	166.0 AU	0.66 Rf	52.2 AU	3.50 %	0.72 Rf	20.4 AU	1715.3 AU	2.91 %	AutoGenerated9
12	0.72 Rf	20.5 AU	0.79 Rf	112.9 AU	7.56 %	0.81 Rf	33.1 AU	3482.0 AU	5.92 %	AutoGenerated6
13	0.81 Rf	283.2 AU	0.84 Rf	111.6 AU	7.47 %	0.89 Rf	48.9 AU	3810.8 AU	6.47 %	AutoGenerated7
14	0.89 Rf	149.1 AU	0.93 Rf	287.1 AU	19.21 %	0.97 Rf	0.6 AU	10642.9 AU	18.08 %	AutoGenerated3

CHEMICAL FINGERPRINTS OF DIFFERENT EXTRACTS USING REFERENCE MARKER CHEMICAL ARTEMISININ

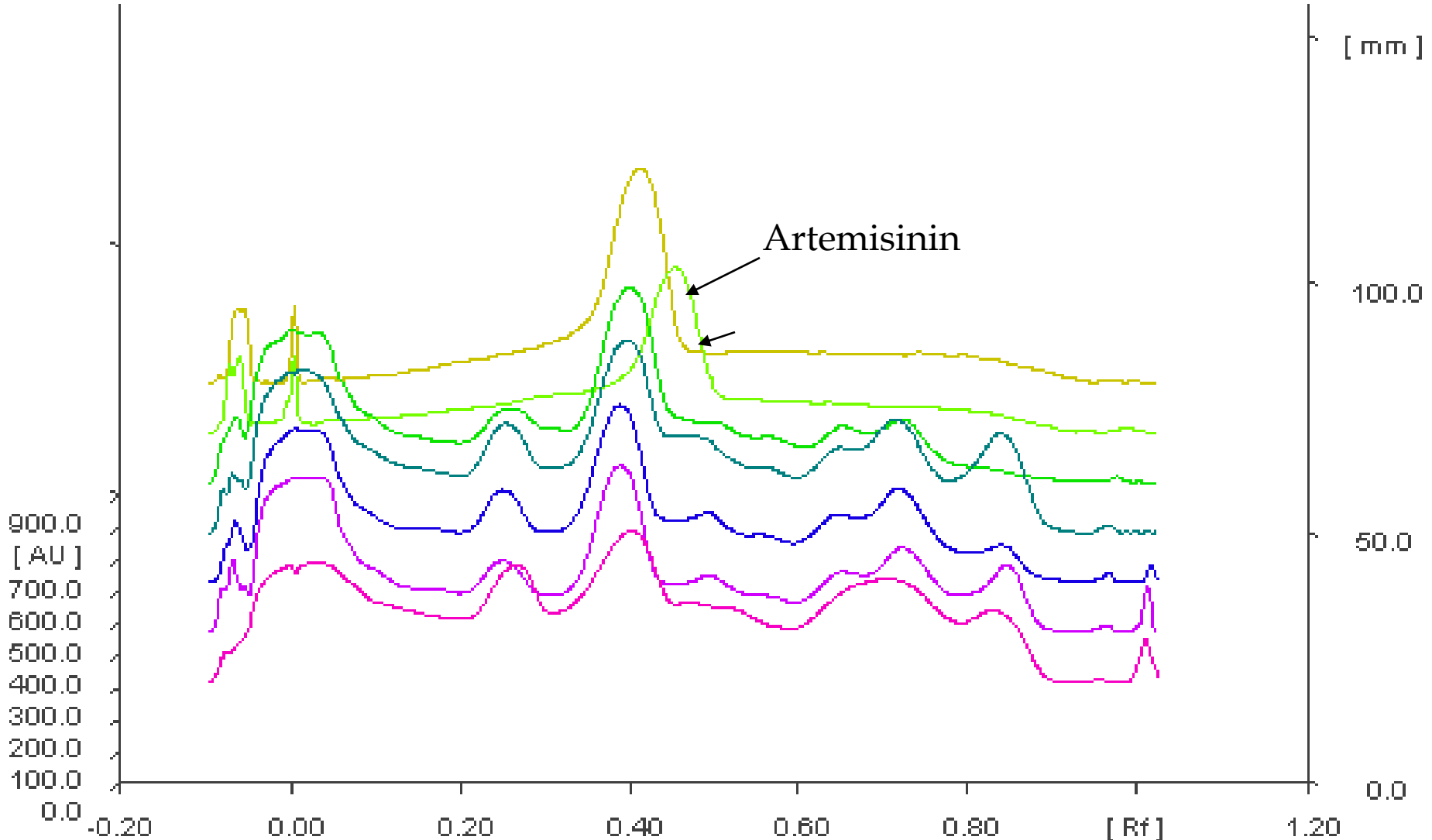
Ethyl acetate Extract:



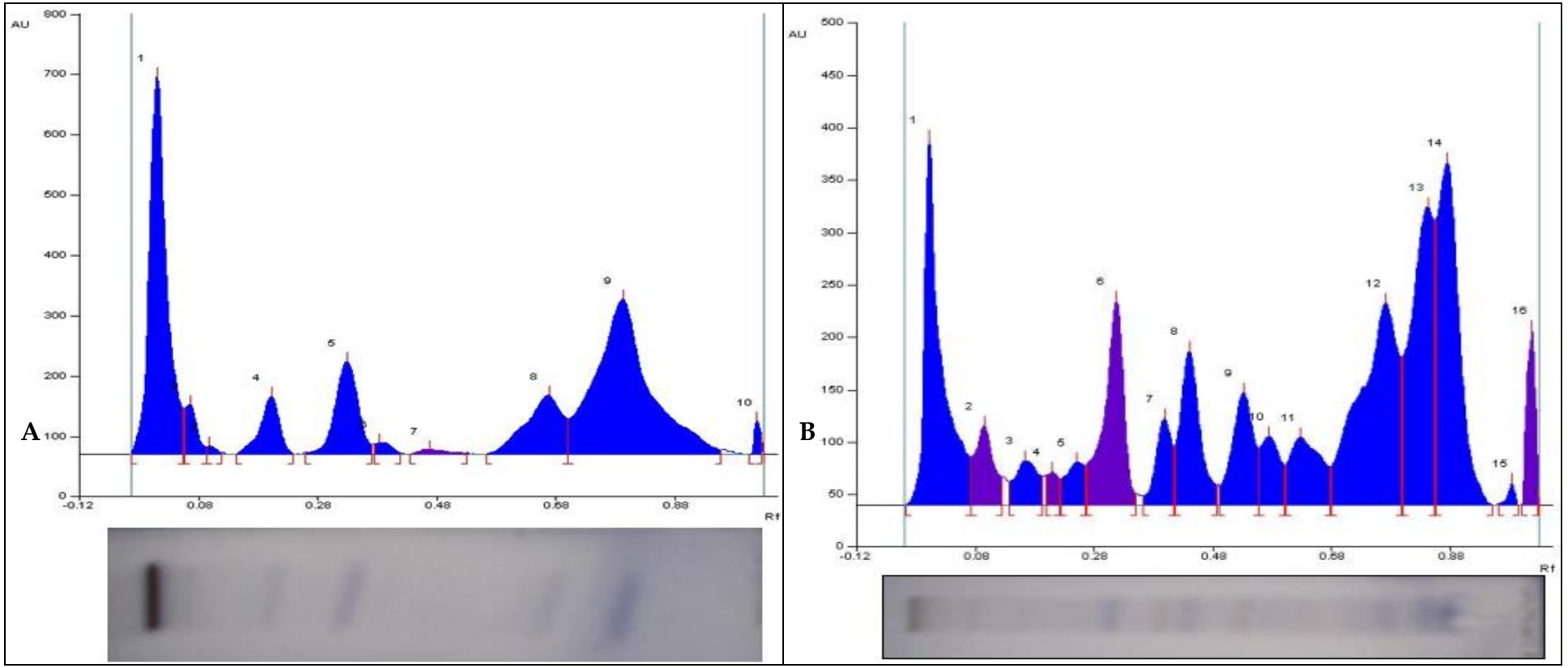
Track 1, ID: Ethyl acetate extract

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	-0.09 Rf	20.8 AU	-0.08 Rf	235.5 AU	19.56 %	-0.06 Rf	2.0 AU	2561.6 AU	6.70 %	unknown *
2	-0.06 Rf	2.3 AU	0.01 Rf	308.3 AU	25.60 %	0.02 Rf	32.7 AU	10766.0 AU	28.16 %	Artemisinin
3	0.02 Rf	563.4 AU	0.05 Rf	118.9 AU	9.87 %	0.09 Rf	32.7 AU	3510.7 AU	9.18 %	unknown *
4	0.10 Rf	392.8 AU	0.11 Rf	51.3 AU	4.26 %	0.22 Rf	34.5 AU	1627.4 AU	4.26 %	unknown *
5	0.22 Rf	194.5 AU	0.25 Rf	27.2 AU	2.26 %	0.31 Rf	53.0 AU	900.6 AU	2.36 %	unknown *
6	0.42 Rf	162.9 AU	0.48 Rf	26.6 AU	2.21 %	0.50 Rf	24.9 AU	646.6 AU	1.69 %	unknown *
7	0.50 Rf	224.7 AU	0.52 Rf	22.3 AU	1.85 %	0.56 Rf	73.4 AU	494.9 AU	1.29 %	unknown *
8	0.56 Rf	173.4 AU	0.59 Rf	12.3 AU	1.02 %	0.62 Rf	56.0 AU	306.0 AU	0.80 %	unknown *
9	0.63 Rf	156.1 AU	0.70 Rf	283.4 AU	23.53 %	0.76 Rf	38.6 AU	14123.2 AU	36.95 %	unknown *
10	0.77 Rf	198.6 AU	0.79 Rf	53.6 AU	4.45 %	0.83 Rf	19.9 AU	1400.9 AU	3.66 %	unknown *
11	0.83 Rf	120.5 AU	0.85 Rf	64.9 AU	5.39 %	0.90 Rf	24.9 AU	1887.4 AU	4.94 %	unknown *

CHEMICAL FINGERPRINTS OF DIFFERENT EXTRACTS USING REFERENCE MARKER CHEMICAL ARTEMISININ



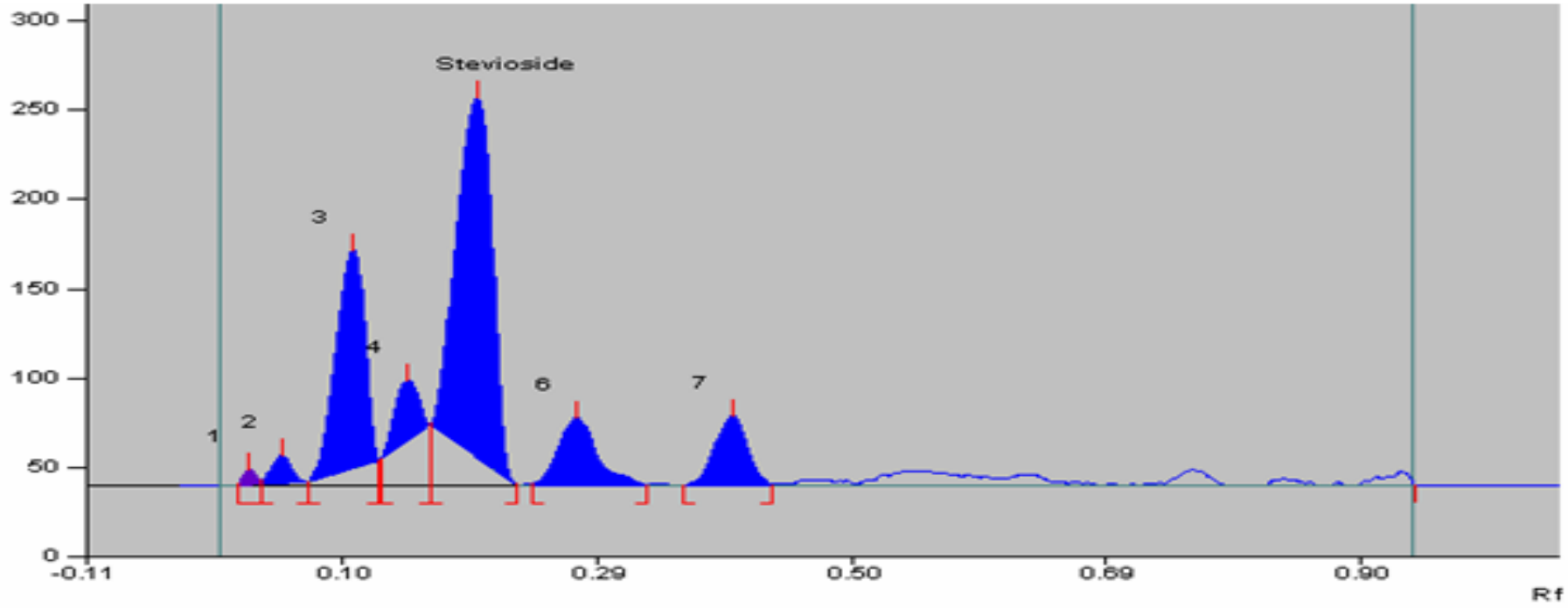
CHEMICAL FINGERPRINTS OF NON-POLAR AND MEDIUM-POLAR EXTRACTS OF *STEVIA REBAUDIANA*



Non- polar profile

Medium-polar profile

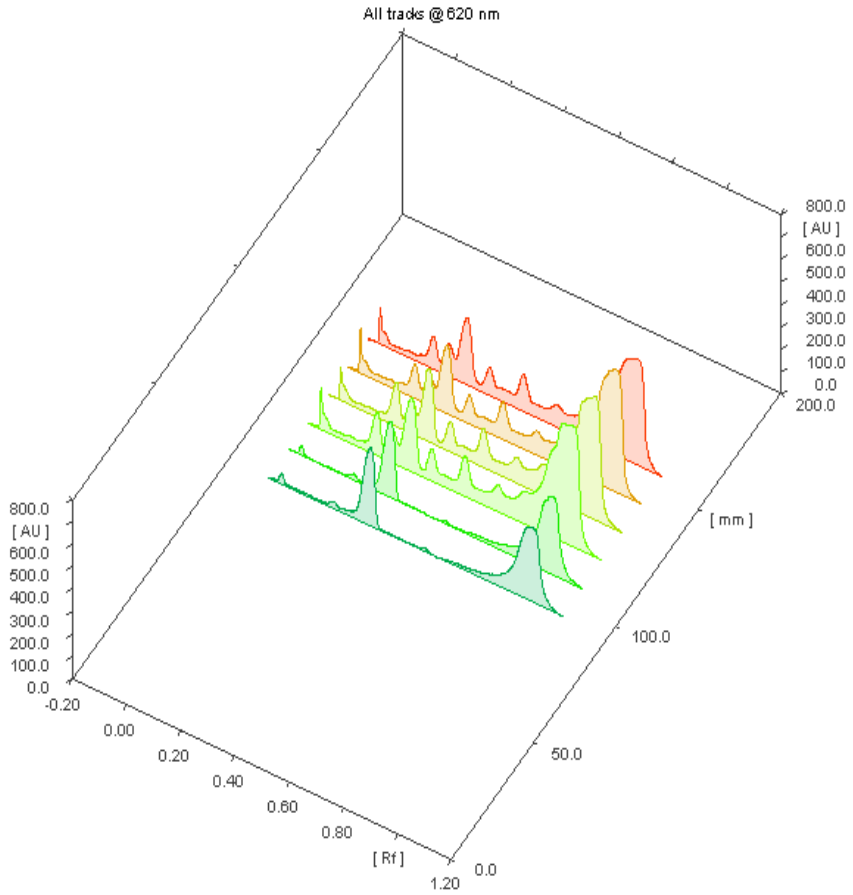
CHEMICAL FINGERPRINTS OF POLAR (METHANOLIC) EXTRACT OF *STEVIA REBAUDIANA USING* MARKER STEVIOSIDE

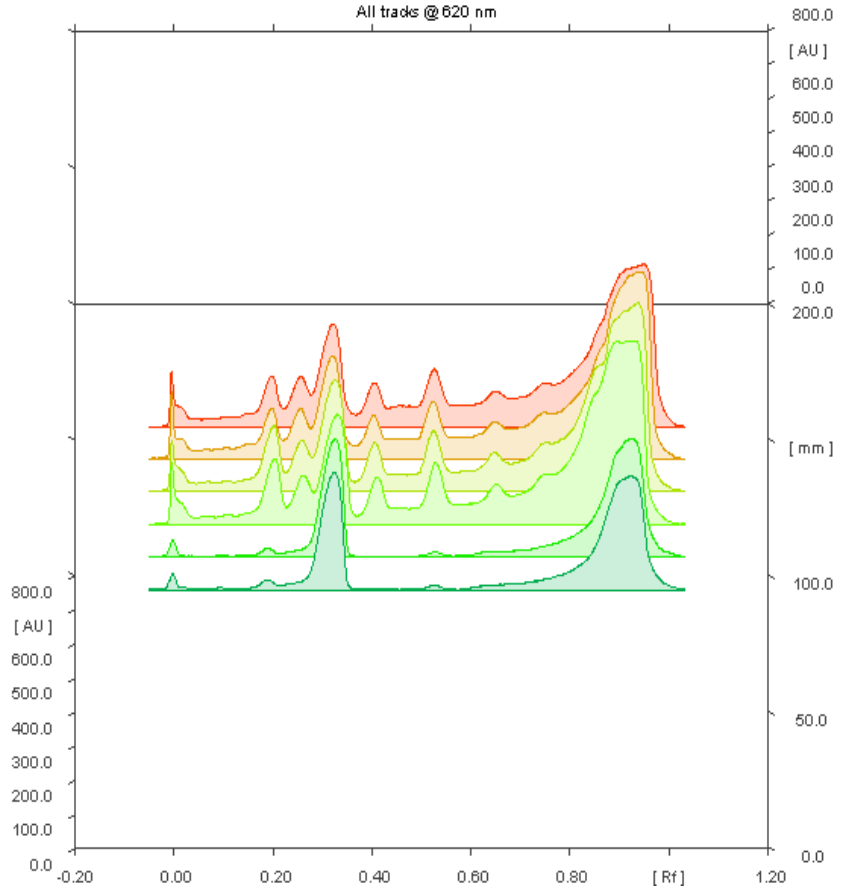


Track 1, ID: Soxhlet/MeOH/1g/100mL (5uL)

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1m	0.01 Rf	1.1 AU	0.02 Rf	8.3 AU	1.82 %	0.03 Rf	3.4 AU	95.8 AU	0.77 %	unknown *
2	0.03 Rf	3.3 AU	0.05 Rf	15.4 AU	3.38 %	0.07 Rf	2.1 AU	258.9 AU	2.09 %	unknown *
3	0.07 Rf	2.4 AU	0.10 Rf	121.8 AU	26.66 %	0.12 Rf	14.1 AU	2955.0 AU	23.85 %	unknown *
4	0.13 Rf	14.6 AU	0.15 Rf	33.8 AU	7.39 %	0.16 Rf	34.4 AU	655.8 AU	5.29 %	unknown *
5	0.17 Rf	34.6 AU	0.20 Rf	200.8 AU	43.96 %	0.23 Rf	0.6 AU	6082.3 AU	49.10 %	Stevioside
6	0.25 Rf	1.2 AU	0.28 Rf	37.7 AU	8.25 %	0.33 Rf	0.2 AU	1255.2 AU	10.13 %	unknown *
7	0.36 Rf	0.2 AU	0.40 Rf	39.0 AU	8.54 %	0.43 Rf	0.5 AU	1084.9 AU	8.76 %	unknown *

CHEMICAL FINGERPRINTS OF BENZENE-ACETONE AND ACETONE EXTRACT OF *STEVIA REBAUDIANA* USING MARKER STEVIOSIDE





Nanomat 4 and Capillary Dispenser
Manual sample application with disposable capillary pipettes

Twin Trough Chamber and Horizontal Developing Chamber smartALERT
Developing chamber for improved chromatography

TLC/HPTLC Sprayer

Easy derivatization by spraying

UV Cabinet with UV Lamp 254/366 nm
UV inspection, easy observation of results

ADVANTAGES OF HPTLC

- Relatively inexpensive (equipment & materials needed).
- Efficiency of time (multiple samples in single run).
- Small amounts of solvents used.
- Provides characteristic fingerprint of the plant with or with out reference standards.
- Appropriate for small or large companies.
- Multiple applications (verification of species, identification of adulterants, basic qualitative assessment, detection of adulterants, product characterization, quantitative evaluation, raw material or end product screening).
- Minimum amount of training needed to use as an effective qualitative assessment tool.

Thank You for your attention !