Chromatography

Chromatography is a physical method of separation, in which the components to be separated are distributed between two phases:

1. Stationary phase
2. Mobile phase

Types of chromatography

Based on the technique being used, chromatography is broadly classified:

1. Adsorptive Chromatography: the chemical mixture in question need to be passed over an adsorbent bed
   a. Adsorptive column chromatography
   b. Thin-layer chromatography (TLC)

2. Partition Chromatography: a mixture is separated by making use of partition of a solute between two solvents
   a. Partition column chromatography
   b. Paper chromatography
   c. Gas-liquid chromatography
   d. HPLC

3. Ion-exchange Chromatography: Affinity Chromatography

4. Molecular sieve Chromatography: Gel Filtration Chromatography

Stationary phase

Plates used in TLC & HPTLC

a. Hand made plates
   - CELLULOSE (native): 15 g of cellulose dissolved in 100 ml of water, Plate thickness: 250 µm
   - CELLULOSE WITH STARCH: 20 g acid washed cellulose, 0.4 gm starch in 60 ml of water, Plate thickness: 200 µm
   - CELLULOSE MICROCRYSTALLINE: 25 g of cellulose in 45 ml of CH₂OH + 30 ml of water, Plate thickness: 200 µm

b. Precoated plates
   - Types of Precoted Plates:
     a. Glass support
     b. Polyester sheets
     c. Aluminum sheets
   - Glass support
     Thickness: 1.3 mm
     Advantages: 1. Superior smooth flat surface 2. Chemical & heat resistant
     Disadvantages: 1. Heavy weight 2. High production cost

Polyester (plastic sheet)

Advantages:
1. Unbreakable
2. Less packing material
3. Less space (in roll form)
4. Spots can be cut & eluted
Disadvantages:
1. Charity reaction above 120°C

Aluminum sheet

Thickness: 0.1 mm
Advantages:
1. Same as polyester
2. Heat resistant, compatible with organic solvents and acids
Disadvantages:
1. Mineral acids & conc. NH₄OH can attack

Commonly available precoated plates with their applications

- SILICA GEL 60 (Unmodified):
  - Used in more than 85% of analyses
  - ALUMINUM OXIDE:
    - Used for basic substances e.g. alkaloids & steroids
  - HIGH PURITY SILICA GEL 60:
    - Used for vitamins
  - CELLULOSE MICROCRYSTALLINE:
    - Used for amino acids, peptides, sugars, antibiotics & other compounds
    - Cannot be chromatographed on active silica gel layer

- PEI IMPREGNATED CELLULOSE:
  - Used for mono and oligosaccharides, co-enzymes, sugar phosphates

- POLYAMIDE/MICROPOLYAMIDE:
  - Used for dansyl-amino acids, antibiotics, antioxidants, antipyretics, optical brighteners, pesticides, dyes stuff, steroids, hormones, vitamins, sulfonamides & sugars
Silica gel chemically modified plates:
- NH₂ (Aminos)
- Carbamoyl groups, phenols, nucleotides, vitamins (B₁, B₂, B₃), uric acid, santonines derivatives
- CN (cyanos)
  - Pharmaceuticals preservatives
- CH₂-CH₂
  - Resolution of enantionic substances for optical purity such as amino acids, dipeptides & lactones
- 20% DOLS
  - Hormones and steroids

Impregnated plates:
- Lipid peroxide, buffers, silver nitrate, ion exchange resins, acids, bases or dyes

Hybrid plates (RP-18 W F 254s)
- HPTLC grade silica gel 60 (6 μm pore size)
- Advantages:
  - Controlled reaction condition ensure that certain silanol groups are chemically bonded with octadecyl chain while remaining silanol groups remain unbounded thus giving hybrid (hydrophilic and hydrophobic) characters to plates
  - Suitable for reversed phase and normal phase as well as mixtures of aqueous-organic solvents
  - Development time is significantly reduced

Prescreen TLC plates
- RPTLC C₁₈ PLATES
  - Plate Size: 5 x 7.5 cm
  - Advantages:
    - For inexpensive preview of separation trait prior to HPLC analysis
    - Helpful in optimization of sample preparation parameters for use in HPLC

Dual phase TLC plate
- Durasil 25 UV 254 and Nonano-Durasil 20 UV 254
  - Base: Aluminium or polyester base
  - Part of plate is coated with reversed phase and remaining with normal phase coating
  - Hard, water proof but still wet-able with hybrid characters

Preparatory plates
- Plate Thickness: 1-2 mm
- Advantages:
  - Large sample volume applied at streak volume
  - Available at precoated soft layer
  - Easy to recover the separated compounds
  - Preferred as compared to column chromatography (easy in recovery)
- Disadvantages:
  - Give less sample resolution than analytical plates
  - Sample volume must be moderate

Silica gel chemically modified plates:
- NH₂ (Aminos)
  - Carbamoyl groups, phenols, nucleotides, vitamins (B₁, B₂, B₃), uric acid, santonines derivatives
- CN (cyanos)
  - Pharmaceuticals preservatives
- CH₂-CH₂
  - Resolution of enantionic substances for optical purity such as amino acids, dipeptides & lactones
- 20% DOLS
  - Hormones and steroids

Impregnated plates:
- Lipid peroxide, buffers, silver nitrate, ion exchange resins, acids, bases or dyes

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Pre-washing of pre-coated plates
- Pre-washing is necessary for the removal of water vapor, volatile substances, elutable components & binders
- Method used for Pre-washing:
  - Ascending (superior but longer time, 10-20% improvement)
  - Dipping (quicker and uniform, 10ml/chamber, A.B.C)
  - Continuous mode (Excellent results)
- Use method most commonly used
- Advantages:
  - Reduction in signal to noise ratio
  - Improve the limit of detection (LOD)

Activation of precoated plates
- Activation:
  - Oven at 110-120°C for 30 mins
  - Aluminium plates kept between two glass plates
  - Higher temperature: Very active & risk of decomposition of sample applied
- No activation is required for freshly opened box

Plate size
- Pre-coated TLC/HPTLC plate size: 20 x 20 cm
- Cutting can be made with scissors at per requirement

Precaution:
- Scissors must have sharp blades and inclined slightly to right for cutting of plates
- The loosened layer should be removed by lightly drawing the spatula over the cut edge to obtained constant RF

Note:
- It is important to note direction and the application of sorbant
Sample preparation

Sample is prepared for complete recovery of intact components of interest and minimum matrix along with suitable concentration and analysis.

For normal phase chromatography:
- S absorb- Non-polar and volatile to avoid circular chromatography.

For Reverse phase Chromatography:
- S absorb- Polar (aromatic-water-like) layer uniformity.

Storage: Refrigerator.

Reference substance and sample- In same solvent.

Application of sample

It is most critical step for good resolution for quantification HPTLC:

1. Ensure complete fill and empty of capillary.
2. Apply through upper end of capillary hence reverse capillary after filling.

- Band length, 2-4 mm TLC, 0.5-1 mm HPTLC.
- Concentration, 0.1-1 µg/ml TLC/HPTLC.

Mobile phase

Analytical grade, AR solvents are used.

Selection: Trial and error experience.

Savings:
- Composition can total volume is 100 parts.
- Measure the solvent separately then place in mixing vessel then introduced to developing chamber.
- Solvent volume at room temperature should not be used for long time.
- Polar solvent portion of mobile phase may get adsorbed during development.
- Mobile phase should be ample.

Preconditioning (chamber saturation)

Chamber should be saturated (by lining with filter paper) prior to development.

Saturation affects the Rf value.

Low polarity solvent e.g. Hexanic hydrocarbons, toluene and their mixture, pre-saturation is not recommended.

High polarity solvent e.g. Methanol pre-equilibrium is recommended.

Phase separation mixture:
- Partial saturation (n-butanol, water and glacial acetic acid).
- RP-TLC: Saturation with methanol is recommended.
- For humidity control: Suitable liquids is placed in one of the chamber trough.

Drying of plates

After development of plate, mobile phase is removed completely & quickly as possible preferably be performed in fume cup board.

HAIR DRYERS NEED PRECAUTIONS:
- Essential oil components may evaporate.
- Compounds sensitive to oxygen may get destroyed due to rise in temperature while using hot hair dryer.

Detection and visualization

Visualizing reagent help in further confirmation of findings.

Non-destructive:
1. UV detection.
2. Iodine: Universal detection reagent.
3. Fluorescent chemicals (Rhodamine B, phosphomolybdic acid and Antimony trichloride).

Destructive:
1. Charring on heating after spraying with corrosive reagent.
2. Dipping technique.
3. Embedding 10-20% Na₂SO₄.

Quantization (evaluation)

In-situ Determination:

Instrumental measurement of visible, UV absorbance, fluorescence or fluorescence quenching directly on the layer without resorting to scraping or eluting sample.

Quantitative evaluation is based on visual comparison of size & intensity of fluorescent or UV absorbing compound.

Scanner converts the spot, splash band into chromatogram consisting of peaks similar in appearance to HPLC.

The position of scanned peak is correlated to Rf value of the spot/band and height/area is related to the concentration of the substance on the spot.

Figure drawn separation of six components, with the solvent DCM/MeOH, 9:1, 100 µl each spotted in relation to the concentration of the substance on the spot.

Quantization (evaluation)

1. Single level calibration:
   - Suitable for known target as stability testing, dissolution profile.
2. Multiple level calibration:
   - Three level of calibrator, curve serve the purpose of for quantitative analysis & scanning is done at different wavelengths.

If adsorption measure of individual component of the formation is quite apart then the chromatogram must be scan at individual adsorption maxima for obtaining meaningful result.
Advantages of HPTLC

- TLC and especially HPTLC provides fingerprinting analysis for the complex components and quantitative determination of the marker compounds.
- No limitation to the composition of mobile phase to maximize the selectivity of the separation.
- Chamber requires very little time for equilibration.
- Choice of detection: 200 to 700 nm, with or without derivitization of plates.

Cost and time efficiency:
- Multiple samples can be analyzed on one plate – affects the cost.
- Automation sample application takes 0.5 to 2.0 min per sample.
- Plate development requires 8 minutes.
- Equilibration time 10-15 min is sufficient.
- Drying normally does not exceed 10 minutes.
- Densitometric evaluation of a plate can be accomplished within 10 minutes.

Advantages over TLC:

- Layer of Sorbent: 100 µm (AmD), 200 µm, 250 µm.
- Efficiency: High due to smaller particle size generated (5-6 µm) vs (10-12 µm).
- Separations: 3-5 cm vs 10-15 cm.
- Analysis Time: Shorter migration distance and the analysis time is greatly reduced.
- Solid support: Wide choice of stationary phases like silica gel for normal phase and C8, C18 for reversed phase.

Advantages over HPLC:

- Precision & Accuracy: Simultaneous processing of sample & standard leads to better Precision & Accuracy.
- Internal Standard: Less need for internal standard, Always needed.
- Flexibility: Extreme flexibility in stationary phase, mobile phase, developing technique, detection.
- Co-chromatography: Co-chromatography possible & often practiced.
- Sample preparation: Technically simple to learn & operate.
- Cost: Low cost per analysis & low cost pre-coated HPTLC plate available.

Benefits of HPTLC compared to HPLC:

- Enable concentration during application by up to a factor of 10,000.
- Is capable of high throughput (300 or 1000 runs per day at one workplace) with minimal cost.
- Enables multiple detection (UV/VIS, Fluorescence, MS, NMR etc).

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<thead>
<tr>
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<th>HPLC</th>
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Using TLC with electrospray ionization, the mobile phase used for separation can be chosen independently of MS consideration because it is evaporated after chromatographic separation.

- The methodology have been developed, which enables the detection of TLC spots on the basis of the relative molecular mass of the compounds under analysis.

### Adsorption Separation (Silica Gel)

- Adsorption is a surface phenomenon, sample interaction with silica group.-Dipole induced interaction occur depending on the nature of the solute.
- The weakly adsorbed analytes move with the solvent front (capillary action) leaving behind the more strongly adsorbed.
- Hydrocarbons < ethers < nitro compounds < tertiary amines < esters < primary amines.

### Partition separation

- Polar liquid (stationary phase) is held by a solid support (cellulose or a weak adsorbent).
- Separation of sample is effected by virtue of the difference in solubility of its components in the developing solvents and stationary phase (water, formamide, polyethylene glycol etc).
- Developing solvents
  - Butanol:acetic acid:water (40:10:50)
  - Butanol:acetonitrile:water (30:10:2:5)
  - 2-propanol:formic acid:water (42:10)

### Ion exchange separation

- Dependent on sorbent containing ions that are capable of exchanging with one of like charge in the sample or mobile phase.
  - The degree of separation is dependent on
  - pH value of the developing solvent
  - Ionic strength of the developing solvent
  - Adsorption property of ion exchange material.

### Mechanism of TLC/HPTLC separation

Three main types of separation mechanism:
- Adsorption
- Partition
- Ion-exchange

Separation that occur can't be attributed to just one mechanism may be result of two or more different types of interaction including those above:-
- ion pairing
- charge transfer
- π-π interactions

### Procedure of HPTLC

- TLC > 600 Th. plates
- HPTLC typically > 5000 Th. Plates
**HPTLC INSTRUMENTATION**

**SYSTEM MANAGER**

- An optimized TLC method is developed for the chromatograms to be run
- Method development depends upon solubility and chemical profile of the component (marker) to be detected in the mixture
- Method is developed in a view to standardize the drug mixture with respect to its marker component

**Step 2 - Sample application**

- BAND APPLICATION, NOT SPOTS
- ACCURATE POSITONING
- ACCURATE VOLUMES
- SHARP START ZONES

**HPTLC COMPLETE SYSTEM**

- SYSTEM MANAGER - DOCUMENTATION
- SAMPLE BAND APPLICATOR
- CHROM. DEVELOPMENT - CHAMBERS
- VISUALISATION - UV CABINET
- QUANT. EVALUATION - SCANNER
- PHOTODOCUMENTATION
HPTLC development chambers

- Twin Trough Chambers
- Automatic Development Chamber (ADC)
- Development (Gradient Chamber) (AMD)

Gradient

- Up to 25 developments
- In same direction
- Polar Non-polar
- Each time front moves further
- Computer-controlled
- All polarity covered
- Up to 40 components separated

POST CHROMATOGRAPHY

- VISUALISATION
- PHOTO DOCUMENTATION
- DERIVATISATION

POST - CHROMATOGRAM DERIVATIZATION

FOR

- SPECIFICITY
- ULTRA HIGH SENSITIVITY
- ADDITIONAL INFORMATION
- VISUAL CONFIRMATION
  (Rarely required if scanner available)

HPTLC QUANTITATIVE EVALUATION

SCANNED DATA
(Quantification)
Validation of Method

"Validation is establishing documented evidence that provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes.

A properly designed system will provide a high degree of assurance that every step, process, and change has been properly evaluated before its implementation.

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METHOD CYCLE:

1. Validation
2. Development
3. Optimization

Quality topics - Method validation

Method is to be validated as per ICH guidelines using following parameters:

1. Linearity
2. Specificity
3. Precision
4. Limit of detection and quantification
5. Robustness
6. Accuracy
7. Ruggedness of Method

ICH Harmonised Tripartite Guideline, Validation of Analytical Procedures, 1994

LINEARITY

The linearity of the analytical method is its ability to elicit test results that are directly proportional to the concentration of the analyte in samples within a given range.

Acceptance criteria: Linear regression r^2 > 0.95 (min 5 concentration required)

Precision

Precision is a measure of "scatter or dispersion" about the mean and among a set of identical measurements. It is usually expressed in terms of standard deviation. Precision can be divided into:

a. Repeatability
b. Inter-day and inter-day precision

Method

Repeatability of the sample application and measurement of peak area can be carried out using six replicates of the same spot (e.g. 600 ng spot−1 of marker) and expressed in terms of percent relative standard deviation (%R.S.D.) and standard error (S.E.).

The intra- and inter-day variation for the determination of marker should be carried at three different concentration levels of e.g. 400, 600, and 800 ng spot−1 of marker.

The acceptance criteria is ≤ 10% (As per ICH guidelines)
**Accuracy**

The percent recovery study of an analytical procedure is the ability to estimate the amount of the substance that has traversed the chromatographic plate after development. It is thus a measure of the accuracy of the analytical method.

**Method**

The pre-analyzed samples are spiked with extra 50, 100, and 150% or with 80, 100, and 120% of the standard marker and the mixtures are reanalyzed by the developed method. The experiment can be conducted several times to get maximum accuracy. This is done to check for the recovery of the marker at different levels in the formulation.

**Accuracy**

- Consistency of the test results obtained by the method to the true value.
- It should be established across a specified range of analytical procedures.
- It should be assessed using a minimum of 3 concentration levels, each in triplicate (total of 9 determinations).
- The acceptance criteria is mean value ± 10% deviation from true value.

**LOD (Limit of detection)**

The limit of detection is a parameter of limit test. It is the "lowest concentration" of the analyte in a sample that can be detected, but not necessarily quantitatively (less than 400 AU).

**LOQ (Limit of quantification)**

It is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions.

**Robustness**

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

**Method**

By introducing small changes in the mobile phase composition, mobile phase volume, duration of mobile phase saturation and activation of pre-washed TLC plates with methanol, the effects on the results are examined. Robustness of the method should be done in triplicate at a single concentration level e.g. 600 ng spot⁻¹ and the %R.S.D and S.E. of peak areas are calculated.

**Ruggedness**

It is a degree of reproducibility of test results obtained by the analysis of the same sample under a variety of normal test conditions, such as different laboratories, different analyst, at different time intervals, etc.

**Specificity/Selectivity**

An analytical method is specific, if the analytical response arises from the analysis of interest only and does not arise from any other compound present in the sample.

**Method**

The specificity of the method can be ascertained by analyzing the standard drug and extract. The spot for marker in the sample is confirmed by comparing the Rf values and spectra of the spot with that of the standard. The peak purity of the marker can be assessed by comparing the spectra at three different levels, viz. peak start (S), peak apex (M) and peak end (E) positions of the spot.

**Experimental Methodology used in HPTLC**

**Analysis of a Marker in Extract and in Formulation**

- **Materials**
  - Standard (Marker), formulation and the drug used in the study were procured from authentic source.
  - All chemicals and reagents used were of Analytical grade and were purchased from Ranbaxy Fine Chemicals, New Delhi, India.

**Instrumentation and chromatographic conditions**

- **HPTLC software:** Wincats
- **Sample applicator:** Linomat 5
- **TLC chamber:** Twin-trough glass chamber
- **Detection scanner:** Camag scanner iii
- **Wavelength:** Desired
- **Lamp:** Deuterium
- **Slit dimension:** 0.2 x 0.2 mm
**Chromatographic Conditions**

**Stationary phase:** Pre-coated silica gel 60F<sub>254</sub> TLC

**Mobile phase:** Developed by hit and trial method

**Saturation time:** 20 min

**HPTLC System:** CAMAG, Switzerland.

**Sample application speed:** 80 nl sec<sup>-1</sup> (Depends upon type of solvent used)

**Application position:** 15 mm (Y axis)

**Position of Solvent front:** 75 mm

**Calibration curve**

A stock solution of marker (100 µg/mL<sup>-1</sup>) was prepared in methanol. Different volumes of stock solution 4, 6, 8, 10, 12, 14 µL were spotted on the TLC plate to obtain concentrations of 400, 600, 800, 1000, 1200, 1400 ng spot<sup>-1</sup> marker, respectively. The data of peak areas plotted against the corresponding concentrations were treated by least-square regression analysis.

**Method validation**

**Linearity:**

- A representative calibration curve of marker is obtained by plotting peak area of marker against the concentration of marker over a range and the correlation coefficient is determined.

**Precision**

- The developed HPTLC method for extraction of marker showed a good precision with standard deviation (S.D.) = 1.08% and 0.012% respectively with standard error (S.E.) 0.4409.

- Low S.D. (3.11) and %R.S.D. (0.030) in the peak area with SE 0.89, proved the ruggedness of the method.

**Repeatability**

A spot of 800 ng was spotted in six replicates. The standard deviation(S.D.) and S.R.6.D. (Relative standard deviation) was found to be 1.08% and 0.012% respectively with standard error (SE) 0.4409.

**Precision**

The intra-day and inter-day variation for the determination of marker was carried out at three different concentration levels of 400, 600 and 800 ng spot<sup>-1</sup>.

**Ruggedness**

A solution of concentration 1000 ng spot<sup>-1</sup> was prepared and analyzed on day 0 and after 3, 6, 24, 48 and 72 h.

Data were treated for %R.S.D. to assess ruggedness of the method.
3. Specificity

The specificity of the method is ascertained by analyzing the standard drug and extract. The spot for marker was confirmed by comparing the Rf value of 0.31 and spectra of the spot with that of the standard. 

4. Detection of related impurities

The spots other than the principal spot for marker from formulation were examined. The spots other than the principal spot for marker from formulation show the presence of related impurities. The mixtures were reanalyzed and the results were examined. 

5. Robustness of the method

Robustness of the method was done in triplicate at a concentration level of 800 ng band and the % R.S.D and S.E of peak areas were calculated.

Robustness of Method

In order to determine the robustness of the method with respect to the mobile phase composition, a single spot at Rf = 0.31 was observed in the chromatogram of the extract of formulation along with other components. A single spot at Rf = 0.31 was observed in the chromatogram of the extract of formulation along with other components.

6. Recovery studies

The pre-analyzed samples were spiked with vicine 50, 100 and 150% of the standard vicine and the recoveries were quantified by diluting the known concentrations of vicine until the peak is detected and quantified. The recovery of the vicine at different levels in the formulation was studied. 

7. Limit of detection and quantification

The limit of detection and quantification was found to be 18.75 ng and 56.25 ng respectively, which indicates sensitivity of the method.

Recovery studies

The recovery studies were done in triplicate at a concentration level of 600 ng band and % R.S.D and S.E of peak areas were calculated. 

Estimation of Marker in formulation

A single spot at Rf = 0.31 was observed in the chromatogram of the extract of formulation along with other components. The total content of marker was found to be 0.140% (w/w).

Table 4. Recovery studies

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount (average)</th>
<th>CV%</th>
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<tbody>
<tr>
<td>Vicine</td>
<td>563.06 ng</td>
<td>0.253</td>
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</table>

Estimation of Marker in herbal extract

An accurately weighed 1 g of powdered formulation was transferred into 250 mL volumetric flask containing 10 mL methanol and sonicated for 30 min. The resultant solution was filtered and 40 μl of the filtrate was applied on the TLC plate followed by development and scanning as described. 

Table 5. Limit of detection and quantification

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<tr>
<td>Vicine</td>
<td>390 ng</td>
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HPTLC

- (I) HPTLC of isolated charantin was done in order to check its purity and absorbance pattern.
- (II) HPTLC of methanolic extract and standard vicine was done to do the qualitative and quantitative estimation of vicine in fruit pulp of Momordica balsamina.

Sample and Standard Preparation

- The isolated charantin was dissolved in methanol and a clear solution was prepared.
- For qualitative and quantitative estimation of vicine, 1g of powdered fruit pulp was ultrasonicated with 10ml of methanol at room temperature for 30 min. The extract was filtered using fine filter paper and two sample bands each of 40µl were applied.
- Stock solution of 100µg/ml of standard vicine was also prepared in pure methanol by sonication for 30 minutes at room temperature. For plotting the calibration curve of standard vicine different volumes of stock solution (100µg/ml) to obtain concentrations of 200, 400, 600, 800 and 1000ng per spot respectively.
- The presence of vicine in methanolic extract was confirmed by Rf value and spectral comparison of sample with standard vicine. Detection was done at 278nm. For quantitative estimation the calibration curve of standard vicine was plotted by applying different volumes of stock solution (100µg/ml) to obtain concentrations of 200, 400, 600, 800 and 1000ng per spot respectively. The sample (40µl) was applied along with standard vicine to determine the quantification of vicine in sample. The calibration curve of standard vicine was found to be linear showing standard deviation 2.67%.

Chromatographic Conditions

- Stationary phase: Pre-coated silica gel 60F254 TLC plates
- Mobile phase:
  - Ethyl Acetate: Methanol: Water: Formic Acid (7:5 : 3 : 1 : 0.1 v/v/v/v)
- Saturation time: 20 min
- HPTLC System: CAMAG, Switzerland
- Application speed: 80slm/sec
- Application position: 15 mm
- Position of solvent front: 75 mm
Results

- One of the peak observed at Rf value 0.34 in chromatogram in test sample matched with the standard vicine and the peak purity was confirmed by spectral comparison at 278nm. This confirms the presence of vicine in fruit pulp.

- The amount of vicine in fruit pulp of Momordica balsamina is found to be 0.0975% w/w.

Validation of HPTLC Method

1. Linearity
   - The correlation coefficient for the method was found to be 0.99853 and the visible peak intensity between concentration and area.

2. Precision & repeatability
   - Reproducibility of the sample preparation and measurement of peak area was carried out using six replicates of the same level. Figure of gallic acid and was expressed in terms of percent relative standard deviation (RSD) and standard error (SE).

Intra-day & inter-day precision

The intra-day and inter-day precision for the determination of gallic acid was carried at three different concentration levels of 0.5 and 1 µg/ml.

Table: Intra-day & inter-day precision of gallic acid

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 µg/ml</td>
<td>0.03 µg/ml</td>
<td>0.04 µg/ml</td>
</tr>
<tr>
<td>1 µg/ml</td>
<td>0.05 µg/ml</td>
<td>0.06 µg/ml</td>
</tr>
</tbody>
</table>

3. Recovery studies

The pre-analyzed samples were spiked with extra 50, 100 and 150% of the standard gallic acid and the minimum was reanalyzed. The mean was evaluated six times. This was done to check the recovery of the gallic acid at different levels in the market formulations. The percentage recovery of gallic acid was found to be 99.89% ± 0.78% (mean ± standard deviation [SD]) with coefficient of determination (r²) = 0.9971 and standard error (SE) = 0.02.

Specificity

The spectrum of sample gallic acid was found to be similar and overlap with that of the reference standard spectrum and good correlation (r² = 0.9982) confirms of spectra were obtained between sample and standard.

Calibration curve of standard vicine

Different volumes of stock solution (50 µg/ml) were applied on the TLC plate to obtain concentrations of 0.5, 1, 2, 3 and 4 µg of gallic acid and Rf values were calculated by standard comparison method. Regression mode was found linear y=0.005x with the equation, Y=4.24% with this equation, Y=2000.060 + 2.754*X & S.D was 4.24%.

4. Specificity

The spectrum of sample gallic acid was found to be similar and overlap with that of the reference standard spectrum and good correlation (r² = 0.9982) confirms of spectra were obtained between sample and standard.

Table: Calibration curve of standard vicine

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Linear Area (µg)</th>
<th>Linear Area (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 µg/ml</td>
<td>11310.23</td>
<td>11088.20</td>
</tr>
<tr>
<td>1 µg/ml</td>
<td>7911.01</td>
<td>7409.32</td>
</tr>
<tr>
<td>2 µg/ml</td>
<td>5571.42</td>
<td>5000.060</td>
</tr>
<tr>
<td>3 µg/ml</td>
<td>3762.64</td>
<td>3220.77</td>
</tr>
<tr>
<td>4 µg/ml</td>
<td>2783.87</td>
<td>2240.90</td>
</tr>
</tbody>
</table>

Figure 5: Calibration curve of standard vicine

Figure 6: Spectral comparison of standard and sample levels.
The Rf value of gallic acid in these samples were found to be (0.33) which are similar and specific.

### 6. Limit of detection and limit of quantification (LOD & LOQ)

Limit of detection (LOD) was calculated by making the dilutions of stock solution and determining the minimum amount which could be detected densitometrically. Minimum detectable limit (LOD) = 60 ng. 

Limit of quantification (LOQ) = 180 ng.

### 7. Robustness

Robustness of the method was done in triplicate at a concentration level of 2 µg/band and the % R.S.D and S.E. of peak areas were calculated.