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"EXTRACTION OF LIMONENE FROM ORANGE PEEL; ANALYTICAL METHOD DEVELOPMENT AND VALIDATION BY HPTLC METHOD"

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ABSTRACT

The orange peel is used as essential oil which has a variety of uses ranging from food flavouring to cosmetics. Orange oil is extracted using a variety of processes including simple distillation and solvent extraction and also Novel techniques such as supercritical CO2 extraction and turbo distillation have also been used. Where focuses on simple distillation, which involves preheating the orange peels before distillation. The preheating of the orange peels improves the oil output. This method of extraction can also be used to extract aromatic oils.

HPTLC is a versatile technique that is known for its precision and accuracy of results as well as its uniformity, purity profile, and assay values. It can handle a large number of samples of various types and compositions. HPTLC is an advanced analytical separation technology with numerous applications and saves time and money. The goal of this work is to develop a new, accurate, robust, and validated HPTLC method for limonene. The analysis was performed on a silica gel 60F254 plate with mobile phase Chloroform: Methanol (8:2 v/v). The detection wavelength is 254 nm. The Rf value for limonene is 0.24 ± 0.05. the goal of this study was to develop such a method and validation in accordance with International Council of Harmonisation (ICH) guidelines.

INTRODUCTION

- HPTLC is a most versatile technique and is known for uniformity, purity profile, assay values, precision, and accuracy of results.
- It can handle several samples of even divergent nature and composition.
- It is accepted as a time-saving and most economical machine practically with minimum trouble shootings.
- It speeds up analysis work which is usually not possible with other parallel chromatographic techniques available.
- HPTLC is a most versatile technique and is known for uniformity, purity profile, assay values, precision, and accuracy of results.
- It can handle several samples of even divergent nature and composition.

- ❖ It is accepted as a time-saving and most economical machine practically with minimum trouble shootings.
- ❖ It speeds up analysis work which is usually not possible with other parallel chromatographic techniques available.
- ❖ Limonene is an excellent cleaning surfactant and effective emulsifier.

Boiling point of limonene is 174 -176ºC

Properties of Limonene

a) Anti-inflammatory

b) Antioxidant

c) Anti-stress.

RATIONALE

- During the literature review, it was found that HPTLC methods were not developed for the limonene.
- HPTLC method is not harmful to the environment because in the HPTLC method less amount of mobile phase uses.
- HPTLC method is cost-effective and multi-component separate at a time.

AIM AND OBJECTIVES

AIM: ‘’Extraction of Limonene from Orange Peel; Analytical Development and Validation by HPTLC Method.

Objective:

a) To optimize the mobile phase for better resolution of the peak from essential oil.

b) To separate and sharp peaks of essential oil without interference.

c) To reduce the time for saturation.

d) To validate the method developed as per ICH guidelines for parameters (Q_2R_1As per ICH guidelines)

- Specificity
- Accuracy
- Precision
- Limit of detection
- Limit of quantitation
- Linearity
- Robustness

PLAN OF WORK

Stage -1

1 . Literature review.

2 . Extraction of essential oil using the Simple Distillation Method.

3 . Preliminary characterization of essential oil.

a. Color

b. Odor

c. Appearance

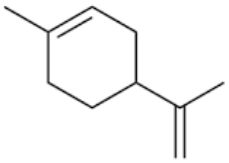
4. Identification test.

5. Spectral identification- UV spectroscopy .

Stage - 2

1. Selection of mobile phase.
2. Selection of scanning wavelength.
3. Confirmation of optimized run.
4. Validation of the method developed as per ICH (Q_2R_1) guidelines,
   - Linearity
   - Specificity
   - Accuracy
   - Precision
   - Limit of detection
   - Limit of quantitation
   - Robustness

ESSENTIAL OIL PROFILE

Structure	 Limonene
Obtained from	Citrus sinensis
Molecular formula	$C_{10}H_{16}$
IUPAC Name	1-methyl-4-(1-methyl ethyenyl)-cyclohexene
Boiling Point	176-178^{o}C
Category	Anti-inflammatory, Antioxidant
Solubility	Insoluble in water, soluble inorganic solvent
Density	0.844g/ml at 25^{o}C

EQUIPMENT

Equipment	Model Name
Linomat 5 Applicator	Camag
Camag TLC Scanner 3	Camag
UV Cabinet	Lab Link Instrument
UV Spectrometer	Shimadzu 1900
Twin Trough Chamber	Camag
Oven	Star Scientific
Glass ware	Borosil

EXPERIMENTAL WORK

Extraction of limonene by simple distillation method:

Procedure;

- ✓ Setup the simple distillation assembly.
- ✓ Cutting the orange peels by using scissors.
- ✓ After cutting the peels small pieces are obtained.
- ✓ To take 250 ml volumetric flask and add 100 g of small cut pieces in a volumetric flask.
- ✓ Then add 100 ml distilled water to a 250ml volumetric flask.
- ✓ Then 250ml volumetric flask attaches to the condenser and heating the volumetric flask by heating mental.
- ✓ The temperature does not exceed 100^{o}C. The temperature is maintained by the heating mantel.

- ✓ After half an hour condensed vapours are collected in another 250 ml volumetric flask. In a 250 ml volumetric flask, two layers are obtained one is oil and another is water.
- ✓ Two layers are separated by a separating funnel above one layer is oil and another is water.

Fig : Assembly of simple distillation

Fig : Extracted limonene

- **Preliminary testing of the standards and the essentials:**

The essential oils possess a characteristic odor and which very much specific to each of the essential oils as well as the standard so in this test the colour, appearance, and odor.

UV Spectroscopy Method:

- The UV spectroscopy is carried out on the standards of essential oils that are limonene accurately measured at 0.1 ml and then transferred into the 100 ml volumetric flask and make up the volume by using methanol(1000 ug/ml).
- 1 ml solution pipette out from the standard stock solution and diluted up to 10 ml to produce a 100 ug/ml solution.
- The order to obtain the λ_{max} scanning the wavelength range between 200- 400.

Optimization of the mobile phase on thin-layer chromatography:

- In order to achieve separation of the standards on the percolated HPTLC plates, different solvents are used such as ethyl acetate, n-butanol, toluene, and methanol in the different ratios were used, but the separation was not achieved. The separation occurs using chloroform and hexane in the ratio of (8:2 v/v).

Selection of the wavelength for densitometric scanning :

- The various trials on HPTLC were taken to check the wavelength which gives an optimum peak at 254 and 366 nm were wavelengths reported for scanning of the essential oil in HPTLC method development. The favourable wavelength for scanning of standards limonene is 254 nm.

SAMPLE PREPARATION IN HPTLC:

a). Preparation of standard solution.

- 0.1ml of standard limonene is measured accurately and transferred to a 100 ml volumetric flask and make up the volume by chloroform to make a 1000ug/ml solution of standard limonene.

b). Preparation of sample solution of oil.

- 0.1 ml of extracted limonene is transferred into the 100 ml volumetric flask and volume makeup by chloroform to make a solution.

CHROMATOGRAPHIC CONDITION

Parameters	Limits
Mobile Phase	10ml
Stationary Phase	Loba chem TLC Plate Silica Gel 60 F_{254} 10*10 cm
Saturation time	30 min
Derivatizing agent for visulization	Anisaldhyde is used as a derivatizing agent and then the plate is heated for visualization of bands
Qualitative detection	Plates after derivatization are observed at 360nm and 254nm
Saturation temperature	25°C
Migration	85% plate
Quantification	The densitometric scanning was done at 254 nm and hence quantification done
Syringe volume	2 μl

Validation of the HPTLC method according to ICH (Q_2R_1) guidelines.

Specificity

- The specificity of the developed method is established by analyzing the sample solutions in relation to interferences from another component.
- The spot for the sample is confirmed by comparing retardation factor (Rf) values of the spot with that of the standard.

Linearity

- The Linearity of the method is evaluated by plotting calibration curves at different concentration levels.
- A calibration curve is plotted over a different concentration range of analytes. The calibration curve is developed by plotting peak area vs. concentrations.

Acceptance criteria

The correlation coefficient (r^2) ranges from -1 to +1.

- **The linearity range is selected over the range 50%-300%.**

Concentration %	Limonene (μg/ml)
50%	100
100%	200
150%	300
200%	400
250%	500
300%	600

Accuracy

The accuracy in analytical procedure means the closeness of the value that is accepted as a true value or a reference value to the value that is obtained. The accuracy is carried out over 50%, 100%, and 150 % of the STD solution. prepared these solutions in triplicate and percentage recovery is calculated.

Precision

The precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple Samplings of a homogenous sample. The precision of an analytical method is usually expressed as a standard deviation and relative standard deviation**.**

Acceptance Criteria:

% RSD is NMT 2% for test results.

Robustness

The effect results from a deliberate change in mobile phase composition, saturation time, and a slight change in solvent migration distance.

Limit of Detection

The lowest conc. of the analyte in the sample is detected. but not necessarily quantified under the stated experimental conditions simply indicates that the sample is below or above a certain level.

It may be calculated based on the response's standard deviation (SD) and the slope of the curve(S).

LOD= 3.3 *(SD)/S

Where, SD= Standard deviation, S= Slope

8.6.6. Limit of Quantitation

The limit of quantitation (LOQ) is the lowest amount of analyte in a sample the sample is determined with acceptable precision and accuracy under the stated experimental conditions. It may be calculated based on the standard deviation (SD) of the response and slope of the curve(S).

LOQ= 10 *(SD)/S

Where, SD= Standard deviation, S= slope

RESULT AND DISCUSSION

Organoleptic Test :

Parameter	Observation
Colour	Yellow
Odour	Lemon Like
Appearance	Liquid

Table: Organoleptic test for extract limonene

Parameter	Obsevation
Colour	Pale yellow
Odour	Lemon like
Appearance	Liquid

Table: Organoleptic test for standard limonene

Identification test for limonene

Salkowski test:

The extract is mixed with chloroform and concentrated sulphuric acid giving a color of pale yellow to reddish-brown.

Fig : Salkowski test for extracted limonene

UV Spectroscopy of the Standard Limonene

- Determination of wavelength maxima using UV Spectroscopy.
- The standard solution was scanned between 200 -400 nm. The maximum wavelength of limonene was found to be 237 nm.

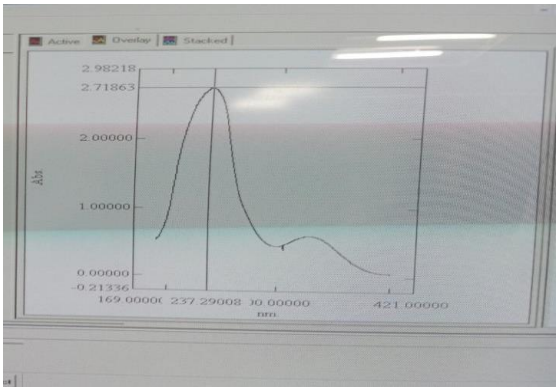
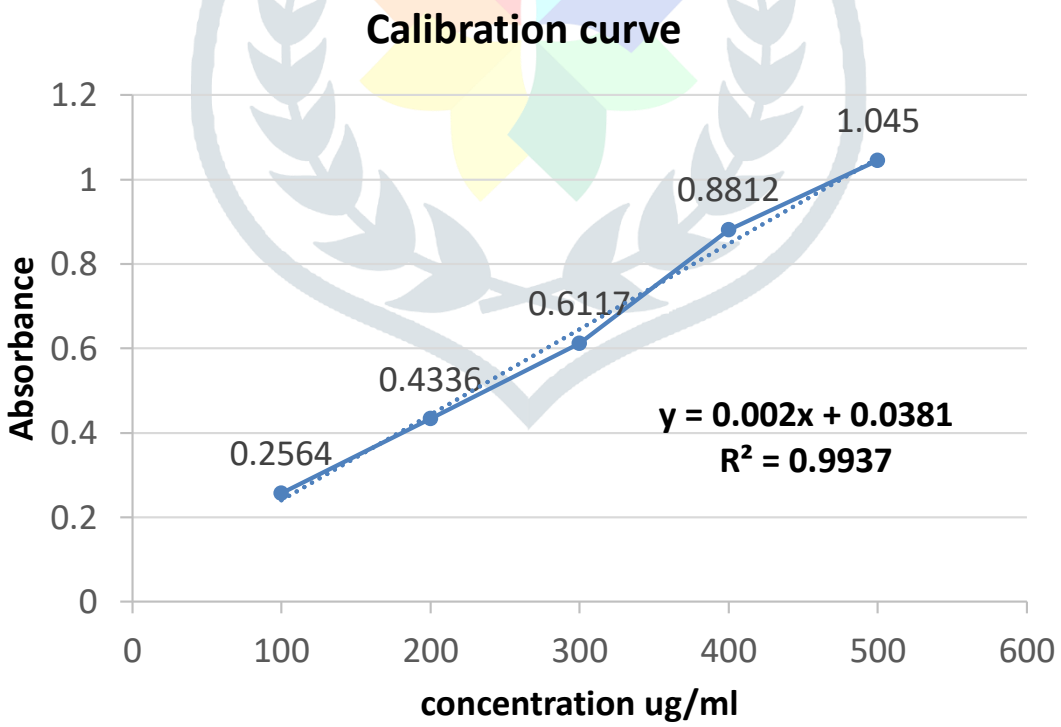


Fig: UV spectrum of standard limonene.

Concentration (µg/ml)	Absorbance at 237 nm
100	0.256
200	0.4434
300	0.6117
400	0.8812
500	1.048

Fig : UV Absorbance of standard limonene

Fig : Calibration curve of UV limonene



Method development:

For the method development, many solvent ratios are used. In this method development, chloroform and hexane (8:2) ratios are used.

Parameter	Discription
Stationary Phase	Silica gel F254
Detector	UV Detector
Injection volume	2 ug/ml per spot
Wavelength	254 nm
Mobile phase	Chloroform : hexane (8:2)
RF value	0.24± 0.05

Table : Chromatographic condition

Fig : Chromatogram of standard and extraction limonene

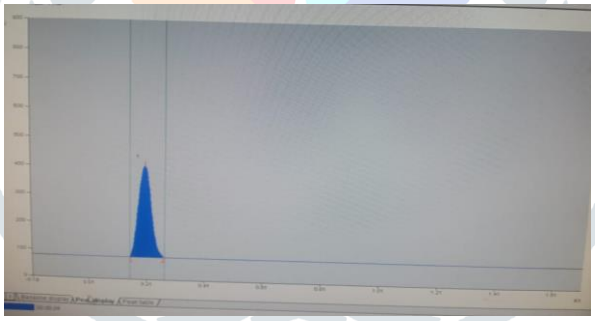


Fig : Chromatogram of standard limonene

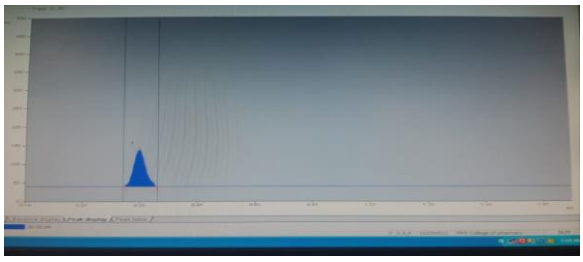


Fig : Chromatogram of extracted limonene

Validation of HPTLC Method:

Linearity: The linearity is carried out by preparing a solution of the standard substance limonene.

- The standard solution is prepared in the range of 100-500 µg/ml.
- The HPTLC plates are developed as per the above chromatographic conditions by scanning with 254 nm as detection and a 3D image of linearity overlain was obtained as shown in the figure. Standard solutions of limonene ranging from 100 to 500 µg/spot are applied on the HPTLC plate.
- **Fig : 3D Developed linearity plot**

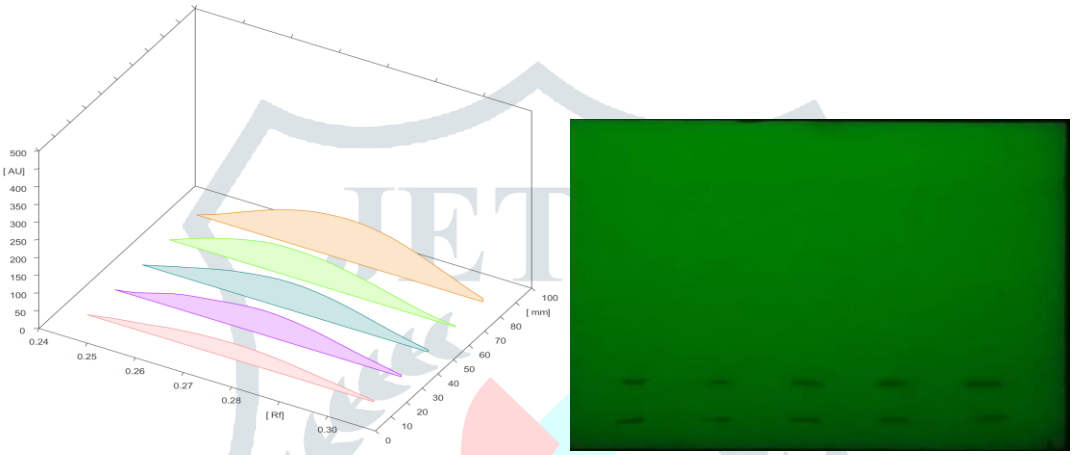


Fig : Chromatogram of linearity

Sr.no.	Concentration ug/ml	Peak area
1	100	1540
2	200	3112
3	300	4512
4	400	6130
5	500	7494

Table: Linearity of limonene

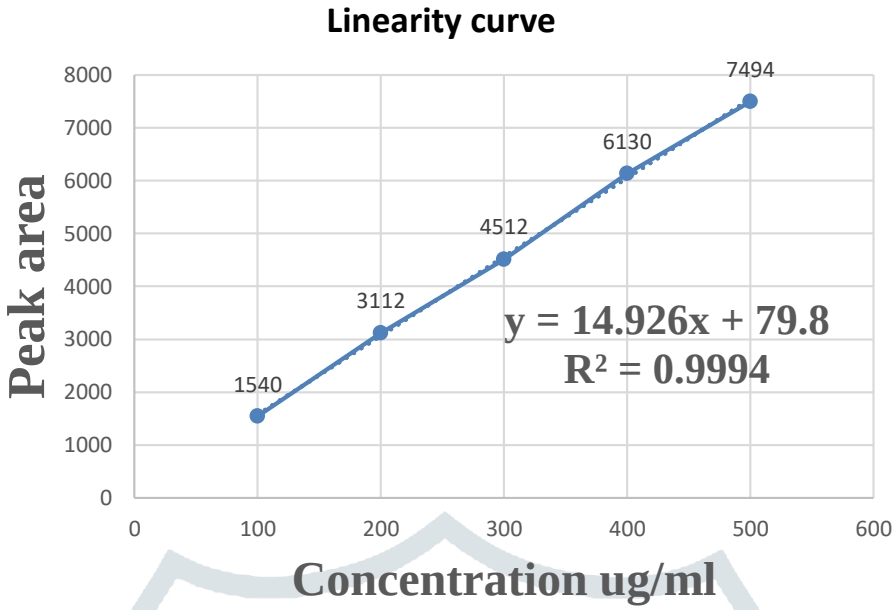


Fig: Calibration curve of linearity limonene

Accuracy: The accuracy is carried out over 50%, 100%, and 150 % of the STD solution. prepared these solutions in triplicate and percentage recovery is calculated.

Percentage of concentration	Standard concentration amount add. (µg/ml)	Sample concentration (µg/ml)	Peak area	Mean of peak area	Recovered concentration	Percentrage recovery
50	200	100	4617			
50	200	100	4629	4612	299.77±0.7	99.9
50	200	100	4635			
100	200	200	6152			
100	200	200	6136	6154	399.16±0.84	99.79
100	200	20	6163			
150	200	300	7698			
150	200	300	7658	7687	499.65±0.44	99.93
150	200	300	7704			

Acceptance criteria:

% recovery should be between the range of 98%-102%.

Conclusion:

The % recovery of the essential oil was found to be within the range it is concluded that the method developed is accurate.

Precision

The precision of an analytical procedure is determined by evaluating a sufficient sequence of multiples sample used to generate an appropriate standard deviation and related standard deviation.(n=3).

Table : Precision results

Amount (μg/ml)	Intraday		Interday	
	Mean peak area ±SD	%RSD	Mean Peak area±SD	%RSD
300	4554±22.13	0.70	4578±22.45	0.713
400	6138±30.89	0.680	6242±33.56	0.738
500	7193±26.53	0.428	7155±28.68	0.466

Acceptance criteria:

% Relative standard deviation, not more than 2%.

Conclusion:

The % RSD of the limonene was found to be within the range it is concluded the method developed is precise.

Robustness:

The robustness performs on deliberate changes in the original mobile phase ratio (8:2). (n=6)

Conc.µg/ml	Mobile phase composition Chloroform:hexane		Results			
	Original	Used	Area mean	SD	%RSD	RF
300	8:2	8:5:1.5	4537	31.23	0.688	0.25
300	8:2	8:2	4542	33.56	0.738	0.24
300	8:2	7:5:2.5	4550	29.39	0.644	0.19

Table: Robustness (Change in MP)

Conc.µg/ml	Saturation time (min)		Results			
	Original	Used	Area mean	SD	%RSD	RF
300	30	28	4536	30.46	0.677	0.24
300	30	30	4542	33.56	0.738	0.24
300	30	32	4547	29.05	0.638	0.19

Table: Robutness (Change in saturation time)

Acceptance criteria:

%RSD is not more than 2%.

Conclusion:

The % RSD of the limonene was found to be within the range it is concluded the method developed is robust.

LIMIT OF DETECTION & LIMIT OF QUANTITATION

The limit of detection and limit of quantitation is calculated on the basis of the standard error of the response and slope of the curve (s)

SLOPE	14.92
STANDARD DEVIATION	136.8
LIMIT OF DETECTION	30.08 µg/ml
LIMIT OF QUANTITATION	91.15 µg/ml

SUMMARY AND CONCLUSION

- The limonene is an essential oil, in this research project the proposed HPTLC method was developed and validated for the essential oil was extracted from the orange peels.
- The method is simple and efficient for the extraction of limonene by simple distillation.

- The HPTLC method was developed by using mobile phase chloroform and hexane (8:2). with saturation time is 30 min.
- The UV detection is carried out at 254 nm. and retention factor value was found to be 0.24± 0.05.
- The HPTLC method was developed for estimation of limonene and parameter validated as per ICH guidelines (Q_2 R_1). Such as linearity, accuracy, precision, the limit of detection, the limit of quantification, and robustness.
- Linearity for the limonene was determined. The test results that are directly proportional to the concentration of analyte in the sample response and was found to be in the range concentration 100- 500 µg/ml and R^2=0.999 within the limit.
- LOD and LOQ were found to be 30.08 µg/ml and 91.15 ug/ml respectively.
- The %RSD was obtained at less than 2%.
- Thus, from the present work it was concluded that developed HPTLC method for extraction of limonene from orange peel.
- The HPTLC method is a cost-effective, accurate, precise, and specific method for essential oil.

FUTURE SCOPE

- The Quality by design approach can be applied for validation and estimation of limonene.
- This method can be applied for the quantitative estimation of essential oil in marketed preparation.
- This method can be developed for various essential oil commercialized to access purity.

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