Development and validation of a high performance thin layer chromatographic method for determination of 1, 8-Cineole in *Callistemon Citrinus*

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ABSTRACT

A new, simple, precise, rapid, and selective high performance thin layer chromatographic (HPTLC) method has been developed and validated for the estimation of 1, 8-cineole in volatile oil of leaves of *Callistemon citrinus* obtained by hydro distillation. The method was validated as per ICH guidelines and can be utilized for routine analysis. The retention factor for 1, 8-cineole was found to be 0.52. The linearity was found to be in the range of 3 μg-12 μg. The recovery obtained for 1, 8-cineole was 98%, which is satisfactory. The result obtained in validation indicate the accuracy, reproducibility, and reliability of the developed HPTLC method for determination of 1, 8-cineole.

Key words: 1, 8-cineole, *Callistemon citrinus*, essential oil, high performance thin layer chromatographic

INTRODUCTION

Callistemon is a genus of 34 species of shrubs in the family Myrtaceae, all of which are endemic to Australia. The genus *Callistemon* is known in folk medicine for its anti-cough, anti-bronchitis, and insecticidal effects and its volatile oils have been used as anti-microbial and anti-fungal agents.[1] *Callistemon lanceolatus* D.C. (Myrtaceae) is a slow-growing ornamental shrub that grows to a height of around 10 meters. It is commonly known as crimson bottle brush tree because of its spiky inflorescence that resembles a bottle brush. The plant has been used by tribal communities of India for the treatment of gastrointestinal disorders, pain, and infectious diseases.[2]

Essential oils (EO) are plant secondary metabolites that are known for their fragrance and food flavor properties. They consist of a complex mixture of mono- and sesquiterpenes, phenyl propanoids, and oxygenated compounds. EOs can be present in different plant organs and materials, and their storage is related to specialized secretory structures.[3]

Therapeutically, the essential oils exert wide spectrum of activities like antiseptic, stimulant, carminative, diuretic, anthelmintic, analgesic, anti-rheumatic, aromatic, counter irritant and many other activities. Apart from food and pharmaceutical uses, they are also used as insect repellants, insecticides, pesticides, and deodorants.[4]

Callistemon citrinus (Curtis) Skeels (syn: *Metrosideros citrina* Curtis; commonly known as crimson or lemon bottlebrush) is a handy medium shrub to large tree (ca. 5-7 m tall). *C. citrinus* is the most widely cultivated member of the genus *Callistemon*. The main component in the essential oil of *Callistemon citrinus* is 1, 8-cineole (61.4%) and alpha pinene (13.4%).[5] *Callistemon citrinus* syn. *Callistemon lanceolatus* has shown to possess activities like anti-caries, antioxidant, spasmolytic, anti-inflammatory, hypoglycemic, hepatoprotective, cytotoxic, cardio-protective, anti-*Helicobacter pylori*, and anti-bacterial activity.[6-15]

The literature revealed that no HPTLC method has yet been reported for estimation of 1, 8-cineole in the essential oil of *Callistemon citrinus (Curtis) Skeels*. The method validation has been carried out as per ICH guidelines.[16] Thus, this method is simple, accurate, precise, and cost-effective method for estimation of 1, 8-cineole in the volatile oil of *Callistemon citrinus*.

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MATERIALS AND METHODS

Reagents and materials

All the chemicals and solvents used were of analytical grade and obtained from E. Merck (Darmstadt, Germany). The standard 1, 8-cineole was procured from Carol Aromas, Mumbai, India (purity > 99%). The aluminum-backed pre-coated TLC plates (silica gel $60F_{254}$, 20×20 cm, 0.2 mm thick) were obtained from E. Merck.

Plant material

The fresh leaves of *Callistemon citrinus* were collected from Karad, Maharashtra in July 2012. A voucher specimen (APS 01) for the plant material was identified at Yashwantrao Chavan College of Science (Karad). The leaves were washed and shade-dried for 24 hours. The leaves were then grounded to form coarse powder.

Extraction of essential oil for HPTLC analysis

The coarsely powdered leaves (100 g) of *Callistemon citrinus* were hydro-distilled for 3 hours using Clevenger type apparatus. The resulting oil was collected, dried over anhydrous sodium sulfate, preserved in an amber-colored glass bottle, and stored in refrigerator till further analysis.

Instrumentation

The method was developed on CAMAG HPTLC system consisting of Linomat V applicator (Camag, Muttenz, Switzerland), CAMAG twin trough chamber (10×10 cm and 20×20 cm), CAMAG TLC Scanner equipped with WinCATS software (version 1.4.6), CAMAG syringe of 100 µl capacity, CAMAG derivatization chamber, and CAMAG TLC plate heater. Separation, derivatization, and identification of 1, 8-cineole were done on aluminum-backed pre-coated TLC plates.

Preparation of working standard solution of 1, 8- cineole

The standard stock solution of standard 1, 8-cineole was prepared by dissolving 15 mg of 1, 8-cineole up to 10 ml with methanol. The concentration of resulting stock solution was 1500 µg/ml of 1, 8-cineole.

Preparation of sample solution of oil of Callistemon citrinus

The stock solution of the sample was prepared by dissolving 15 mg of oil up to 10 ml with methanol, and the concentration of the sample solution was 1500 µg/ml of oil.

Chromatographic conditions

The experiment was performed on a silica gel 60F254 (0.2 mm thick) HPTLC plates (20×10 cm) and (10×10 cm) without pre-washing. Samples were applied to the plates as 8 mm bands with CAMAG Linomat V applicator. The plates were developed by ascending technique to a distance of 80 mm, at $25 \pm 5°$ C, relative humidity 50-60%, in a CAMAG twin trough chamber with a stainless steel lid. The mobile phase composed of toluene: Ethyl acetate: Methanol: Formic acid (9: 0.5: 0.5: 0.5) for 1, 8-cineole. The chamber saturation time was 10 mins. After development, the plates were dried and then viewed in a CAMAG visualizer at 254 nm, 366 nm, and white light. The plate was then derivatized using anisaldehyde sulfuric acid reagent in CAMAG derivatization chamber and then heated at 100ºC using CAMAG TLC plate heater. The plate was cooled and then scanned in CAMAG TLC scanner using WinCATS software (version 1.4.6) in absorption mode with slit dimensions 6.00×0.45 mm. The detection wavelength was 540 nm. The Rf value for 1, 8-cineole was found to be 0.52.

Calibration curve for standard 1, 8-cineole

The standard solutions of 1, 8-cineole of 3 µg-12 µg were applied on TLC plate, and further it was developed and scanned as per the chromatographic conditions. The peak areas were recorded. The calibration curve of 1, 8-cineole was obtained by plotting peak area against concentration of 1, 8-cineole applied.

VALIDATION OF PROPOSED METHOD

ICH guidelines were followed for the validation of the analytical method developed (precision, accuracy, ruggedness, robustness, linearity, LOD, LOQ, and specificity).

Linearity

Standard stock solution of 3-12 µg of 1, 8-cineole was prepared and diluted to appropriate concentrations for plotting the calibration curve. At least six concentrations of the analyte solutions were analyzed in triplicate, and then the calibration curve was constructed by plotting the mean peak areas versus the concentration of each analyte.

Precision

The inter-day precision (% RSD) was determined by analyzing standard solution of 1, 8-cineole over the entire calibration range for 3 different days. The intra-day precision (RSD) was determined by analyzing standard solution of 1, 8-cineole over the entire calibration range for 3 times on the same day.

Accuracy

Accuracy of the method was tested by carrying out recovery studies at different spiked level by standard addition method. Standard 1, 8-cineole solution was added

at 3 different levels (80%, 100%, and 120%). At each level, 3 determinations were performed, and the results were calculated by the difference between the spiked and un-spiked sample analyzed under same conditions. The percentage recovery of 1, 8-cineole was calculated at each level.

Limit of detection

The limit of detection (LOD) was determined using following formulae:

$$LOD = 3.3(S. D)/S,$$

Where S. D = Standard deviation of response, S = average of slope of the calibration curve. The LOD was calculated for 1, 8-cineole.

Limit of quantitation

The limit of quantitation (LOQ) was determined using following formulae:

$$LOQ = 10(S. D)/S$$

Where S. D = Standard deviation of response, S = average of slope of the calibration curve. The LOQ was calculated for 1, 8-cineole.

Specificity

It was observed that the other constituents present in the oil sample did not interfere with the peak of 1, 8-cineole. Therefore, the method was specific.

Robustness

By introducing small changes in the mobile phase composition and development distance, the effects on the results were examined. Robustness of method was done 3 times at a concentration level of 1.5 μg/spot, and the %RSD of peak area was calculated.

Ruggedness

Ruggedness of the method was assessed by spiking the standard 3 times with different analyst by using same equipment.

RESULT AND DISCUSSION

HPTLC procedure was optimized with a view to quantify the essential oil. Toluene: Ethyl acetate: Methanol: Formic acid (9: 0.5: 0.5: 0.5) gave good resolution with R_f value for 1, 8-cineole being 0.52. Well-defined band was obtained after chamber saturation for 10 min at room temperature. TLC plate was visualized after derivatization with anisaldehyde sulfuric acid reagent at 540 nm. The identity of 1, 8-cineole was confirmed by comparing chromatogram of standard 1, 8-cineole with that of oil extracted from *Callistemon citrinus* and by comparing the retention factor of the standard with that of sample.

Linearity

Calibration plot shown in Figure 1 indicates that the response was linear function of concentration in the range of 3-12 μg for 1, 8-cineole. The correlation coefficient, intercept, and the slope for 1, 8-cineole were 0.999, 491.577, and 862.495, respectively. The linearity study is shown in Table 1.

Precision

The measurement of peak area three times inter day and intra-day showed %RSD (<2%), which indicated precision of method [Table 2].

Limit of detection and limit of quantitation

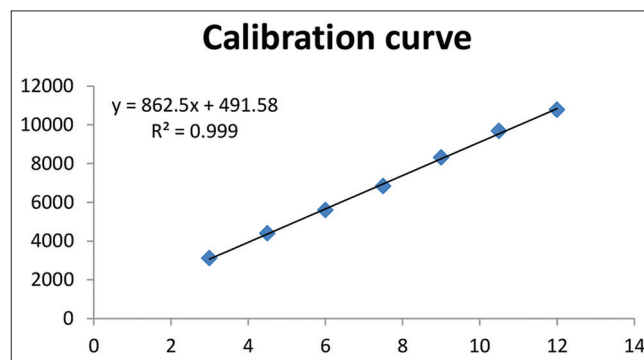The LOD and LOQ of 1, 8-cineole obtained indicate that the method is adequately sensitive [Table 3].

Figure 1: Calibration plot

Table 1: Linearity study							
Standard ⇨ Concentrations	**Peak area**						
Replicates ⇩	**3 µg/µl**	**4.5 µg/µl**	**6 µg/µl**	**7.5 µg/µl**	**9 µg/µl**	**10.5 µg/µl**	**12 µg/µl**
1	3122.54	4399.57	5598.34	6839.56	8308.15	9679.91	10774
2	3157.79	4268.47	5486.37	6670.58	8485.5	9736.47	10593.7
3	3157.33	4302.65	5527.57	6748.43	8247.46	9846.25	10468.3
Mean	3145.89	4323.56	5537.43	6752.86	8347.04	9754.21	10612
SD	20.2201	68.0061	56.632	84.5769	123.693	84.5771	153.659
% RSD	0.64275	1.57292	1.02271	1.25246	1.48188	0.86708	1.44797

SD=Standard deviation

Table 2: Precision of HPTLC method

Set	Intra-day Precision			Inter-days Precision		
	Time 1	Time 2	Time 3	Day 1	Day 2	Day 3
1	9272.16	9056.33	9145.89	9272.16	9049.39	8892.05
2	9161.9	9167.34	9267.26	9161.9	8845.84	9068.92
3	8972.87	9078.34	8956.2	8972.87	9193.69	9011.88
4	8664.62	9256.82	9029.56	8664.62	9038.85	9270.33
5	8940.38	9023.47	8878.33	8940.38	9250.9	9026.55
6	9236.37	8939.73	8911.69	9236.37	9140.99	8927.45
Mean	9041.38	9087.01	9031.49	9041.38	9086.61	9032.86
SD	229.193	111.385	136.722	229.193	131.054	121.868
% RSD	0.02535	0.01226	0.01514	0.02535	0.01442	0.01349

SD=Standard deviation

Table 3: LOD and LOQ for HPTLC quantification of 1, 8-cineole

	Value
Limit of detection of 1, 8-cineole	0.97949
Limit of quantitation of 1, 8-cineole	0.32323

LOD= Limit of detection; LOQ=Limit of quantitation

Table 4: Recovery study of 1, 8-cineole

Accuracy level (%)	% mean recovery	SD	% RSD
80	98.72	0.380395	0.385327
100	98.73666667	0.382797	0.387695
120	98.63	0.557494	0.565238

SD=Standard deviation

Table 5: Robustness of HPTLC method (n=3)

Replicate	Development distance (mm)		Mobile phase composition	
	15	15.1	(9/0.5/0.5/0.5)	(9.2/0.3/0.5/0.5)
1	3122.54	3202.46	3122.54	3368.47
2	3138.46	3256.36	3138.46	3398.57
3	3156.46	3288.46	3156.46	3402.57
Mean	3139.1533	3249.0933	3139.153333	3389.87
±SD	16.9706	43.4580	16.9706256	18.6405472
% RSD	0.5406	1.3375	0.540611554	0.549889736

SD=Standard deviation

Table 6: Ruggedness of HPTLC method (n=3)

Replicate	Analyst 1	Analyst 2
1	1704.77	1785.36
2	1758.44	1798.36
3	1705.88	1805.36
Mean	1723.03	1796.36
±SD	30.67098	10.14889
% RSD	1.780061	0.56497

SD=Standard deviation

Recovery

The results from recovery study were within acceptable limit indicating accuracy of method [Table 4].

Robustness

The low value of S. D and %RSD obtained after introducing small deliberate changes in the developed HPTLC method indicates the robustness of method [Table 5].

Ruggedness

Low %RSD values between the values of peak areas prove the ruggedness of method, which indicates that 1, 8-cineole gives reproducible results for the proposed method [Table 6].

Specificity

The peak purity of 1, 8-cineole was assessed by comparing the spectra at peak start, peak apex, and peak end position of the spot. Good correlation was obtained between standard and sample of 1, 8-cineole.

CONCLUSION

A rapid, simple, accurate, and specific HPTLC method for quantitative estimation of 1, 8-cineole present in oil of *Callistemon citrinus* has been developed and validated. The data could be used as a quality control standard. The method used resulted in good peak shape and enabled good resolution of 1, 8-cineole from other constituents of the essential oil since there was no interference with peak of 1, 8-cineole from other constituents of oil.

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