RESEARCH ARTICLE

Simultaneous Estimation of Monoterpenes (Eugenol, Eucalyptol And R-Limonene) by HPTLC Densitometric Method and Its Validation

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ABSTRACT

Plant-derived monoterpenes have various pharmacological applications, but the problem lies with their detection and estimation. Hence, it is crucial to develop a robust technique to overcome this problem. This work was aimed at developing a swift, accurate and reproducible technique for the simultaneous detection of three monoterpenes, namely, eugenol, eucalyptol (1,8-cineole), and R-limonene, using the high-performance thin-layer chromatography technique, as per ICH guidelines. The best results were obtained in the eluent mixture prepared by using hexane: toluene: ethyl acetate (6:3:1, v/v/v), and silica gel-coated aluminum TLC plates $60F_{254}$ as a stationary phase that produced very sharp and well-resolved symmetrical peaks for eugenol, eucalyptol, and R-limonene at Rf values of 0.47 ± 0.03, 0.56 ± 0.03, and 0.71 ± 0.03, respectively. The linear range for eugenol, eucalyptol, and R-limonene was 1 to 15 ng/spot (r^2 = 0.9906), 100 to 600 ng/spot (r^2 = 0.99625), 50 to 300 ng/spot (r^2 = 0.9873), respectively. Furthermore, the LoD and LoQ values for eugenol were obtained at 0.0129 and 0.0391 ng/spot, followed by eucalyptol (0.82 and 2.48 ng/spot) and R-limonene (0.594 and 1.8 ng/spot), respectively. To the best knowledge, we are the first to report a quick and reproducible HPTLC technique for the simultaneous determination of eugenol, eucalyptol (1,8-cineole), and R-limonene. This method can further be used for the quantification of these secondary metabolites in plant extracts, enriched fractions, and pharmaceutical formulations.

Keywords: Monoterpenes, R-Limonene, Eugenol, Eucalyptol, Validation, HPTLC.

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INTRODUCTION

Clove oil is a natural source of monoterpenoid eugenol (90%), primarily extracted from *Syzygium aromaticum*. It is a clear or pale-yellow volatile oil with a spicy odor. Eugenol is highly susceptible to oxidation and other chemical processes.[1] Eugenol is known for its fragrant qualities and considerable antioxidant, antibacterial, anti-inflammatory, and anticancer actions against several types of bacteria, parasites, and fungi.[2] The antimicrobial (antibacterial, antifungal and anti-trichomonal) properties of eugenol are because of the presence of free hydroxyl group, which can induce morphological changes in microbial cells along with disruption of the cell membrane.[3] Eucalyptol or 1,8-cineole, is the primary monoterpene found in eucalyptus oil (70–80%) derived from *Eucalyptus globulus*. It is a clear, transparent, colorless liquid with a spicy aroma.[4] Eucalyptol has several therapeutic properties such as antibacterial, antioxidant, antifungal, hepato-protective effects, anticancer and anti-inflammatory.[5] The antimicrobial activity of eucalyptol was due to its interference in the signaling pathways of microbes that cause apoptosis and nuclear condensation of microbial cells.[6] R-limonene is a naturally occurring monoterpene obtained from citrus oil (95%), extracted from citrus plants of the family Rutaceae. It is a clear, transparent, volatile, oily liquid with a lemon-like odor. R-limonene converts into its epoxides after oxidation.[7,8] It has numerous medicinal properties, such as anti-carcinogenic, antimicrobial (against bacteria and fungi), neuroprotective, antioxidant, and anti-diabetic actions.[9] Fenton-mediated hydroxyl radical production in bacterial cells leads to DNA oxidation and ultimately changes the permeability of the bacterial cell membrane. ROS accumulation in fungal cells takes place after exposure to R-limonene, which ultimately leads to cell disruption.[10,11]

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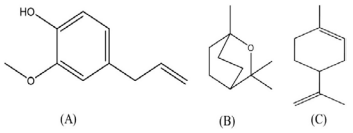

Figure 1: Structure of eugenol (A), eucalyptol (B), and R-limonene (C)

These three monoterpenes have been identified individually or in various combinations in different disorders because of their broad therapeutic effects. These biomarkers can be present in plant-derived raw materials, natural products, and pharmaceutical complex mixtures and they can also be used in the preparation of pharmaceutical formulations. A rapid, accurate, and consistent approach is needed to measure and quantify these biomarkers simultaneously. Eugenol, eucalyptol, and R-limonene (Figure 1) have been simultaneously estimated through GC chromatography.[12] HPTLC technique has been used to measure and confirm the secondary metabolites of plants since the beginning because it is very selective, efficient, accurate, precise, and reliable.[13] In the present study, to overcome the simultaneous quantification problem faced with these three monoterpenes, we developed a robust and reproducible HPTLC method for the simultaneous estimation of these three biomarkers along with its validation. After preparing various mobile phase combinations, the optimal separation was achieved by repeatedly developing TLC plates and validating the results using the HPTLC method. The proposed approach was validated using ICH recommendations.[14]

MATERIALS AND METHODS

Chemicals

Pure extracts of eugenol (99% pure), eucalyptol (99% pure) and R-limonene (99.2% pure) were procured from Sigma-Aldrich (St. Louis, USA). Anisaldehyde-H_2SO_4 reagent was prepared by adding 0.5 mL of anisaldehyde (Central Drug House, New Delhi) in 10 mL of GAA or glacial acetic acid (Molychem, Mumbai), followed by the addition of 85 mL of methyl alcohol or methanol (Molychem, Mumbai) and 5 mL of conc. sulphuric acid (Merck, India). The other solvents that were used in this method were of HPLC grade and all the chemicals used were of pharmaceutical grade.

Method of Sample and Standard Solution's Preparation

A volume of 0.5 mL each of eugenol (purity 99%), eucalyptol (purity 99%), and R-limonene (purity 99%) were conveyed into three individual volumetric flasks previously containing 5 mL of methanol. By adding methanol, the volume was made up to 10 mL to get 500 µL/mL solutions as primary stock solution. Then, 50 µL of eugenol, 100 µL of eucalyptol, and 200 µL of R-limonene were taken from the primary stock solution and put into a 5 mL graduated volumetric flask. Sonicate the mixture for 5-10 minutes to obtain a physical mixture that can be utilized for HPTLC analysis. To make standard solutions for each essential oil, 0.1 mL of eucalyptol (99% pure), eugenol (99% pure), and R-limonene (99% pure) were taken into three individual 10 mL volumetric flasks that each had 5 mL of methyl alcohol in them. The final volume was marked up to 10 mL by adding methanol.

Mobile Phase Selection

A unique mobile phase combination of hexane, toluene, and ethyl acetate in the ratio (6:3:1, v/v/v) was chosen following multiple attempts with various solvent systems. This combination was then utilized for the simultaneous separation of standards and sample mixtures. To our best knowledge, this specific mobile phase combination is being employed for the first time to concurrently estimate these phytoconstituents.

Instrumentation and Chromatographic Conditions

HPTLC technique was performed by using TLC aluminum (E-Merck, Germany) plates (previously coated by silica-gel $60F_{254}$; thickness of 0.20 mm; width× length-20×10 cm). A Linomat V (semiautomatic TLC sampler, Camag, Switzerland) was used to make separate spots on the plates for the standard solution (which was a pure extract of eugenol, eucalyptol, and R-limonene) and the sample solution, which was a physical mixture of essential oils. After being positioned 8 mm from the bottom, the application rate was 150 nL per second on the plate. The band-width was 8 mm, and the interval in between two consecutive bands was 21.20 mL. The spotted TLC plates were kept and further developed (linear ascending) in a glass chamber (twin-trough, 20×10 cm, Camag, Switzerland). The chamber was previously saturated with eluent phase in a volume of 20mL (hexane: toluene: ethyl acetate, 6:3:1, v/v/v) for 15 minutes at the temperature of 25 ± 4˚C and a relative humidity of 60 ± 4%. The HPTLC chromatogram was recorded up to 70 mm for the sample application. A continuous air current was supplied with a hair dryer in standard mode and the plates were dried. The slit-dimension of plates was 4.0 × 0.30 mm and the scanning speed was 20 mm/s for quantification of plates.[15,16]

Derivatization and Quantitative Evaluation

The derivatization of plates was done by immersing them in anisaldehyde-H_2SO_4 reagent for 1 to 2 seconds, followed by heating at 100 to 105˚C for 10 minutes on a hot plate. Within 10 min of derivatization, plates were further scanned by using a scanner (densitometric, WinCATS, Camag, Absorption mode) equipped with deuterium and a white light source (D2 and W) at 450 nm to quantify eugenol, eucalyptol, and R-limonene. The spots and their corresponding peaks were observed at their R_f values.

Calibration Curve by HPTLC Densitometric Method

The calibration process involved applying different concentrations of standard solutions of eugenol, eucalyptol, and R-limonene on HPTLC plates using a semiautomatic TLC sampler. The bandwidth measured 8 mm, with a spacing of 21.2 mm between each pair of bands in a six-point spotting. A six-point calibration curve was constructed for eugenol, eucalyptol, and R-limonene within the linear range of 1-15 nanogram/spot, 100-600 nanogram/spot, and 50 to 300 nanogram/spot, respectively, at 450 nm. The area of peak and sample concentration statistics were analyzed using the least

square regression method. The calibration curves were plotted as peak area against the amount per spot. Throughout the analysis, all of the experimental parameters were kept constant.

Validation of Developed Method

Linearity and specificity

Linearity was assessed using six concentration levels: 1 to 15 µL for the eugenol standard and 1 to 6 µL for both the eucalyptol and R-limonene standards. Linear least squares regression analysis was used to assess linearity and to plot the calibration curve using the corresponding peak area versus concentration. By comparing R_f values with the standards and spectral analysis, the specificity of this suggested approach was calculated. The peaks were evaluated for their different positions, such as the start point of the peak, peak height, and end point of the peak per spot.[17]

Precision and accuracy

The accuracy of this identified approach was analysed by using the standard-addition method and further determined by the sample's spiking technique with known additional amounts of standard eugenol, eucalyptol, and R-limonene. The experiment was performed in triplicate, and then the samples were subsequently reanalysed by the HPTLC method. Recovery studies were conducted, and the %RSD (relative standard deviation) values were calculated[17]. Instrumental precision was assessed by using 9 ng/spot of eugenol, 370 ng/spot of eucalyptol, and 170 ng/spot of R-limonene, each applied six times. The precision of their correlated R_f values was determined and evaluated. The precision for this developed method was evaluated by three time repetition within the same day and over three consecutive days at three different concentration levels for eugenol, eucalyptol, and R-limonene.

Robustness

The method robustness was determined by investigating a small deliberate change in chromatographic conditions such as chromatogram run or distance front (± 5 mm), changes in time between spotting and chromatography, alteration in volume of mobile or eluent phase, and changes in polarity and combinations of mobile phase.[18]

LoD and LoQ

Plotting calibration curves, the limit of quantification, LoQ and limit of detection, LoD for eucalyptol, R-limonene, and eugenol were assessed that was based on the lower concentration detection and quantification. The values were driven using the SD value (standard deviation) and the S value (slope of calibration).[18]

RESULTS AND DISCUSSIONS

Fingerprinting and Chromatography of Standards and Sample

Chromatographic fingerprinting is a reliable and reproducible analytical technique used for the simultaneous quantification and identification of phytoconstituents in pharmaceutical preparations. A HPTLC method was identified, developed and further validated to simultaneously estimate eugenol, eucalyptol, and R-limonene in a physical combination intended for formulation development. The standards and sample mixture were quantified by using TLC aluminum pre-coated silica gel $60F_{254}$ plates with mobile phase using hexane, toluene, ethyl acetate in a ratio (6:3:1,v/v/v). This resulted in precise and well-defined symmetrical peaks for eugenol, eucalyptol, and R-limonene at R_f values of 0.47 ± 0.03, 0.56 ± 0.03, and 0.71 ± 0.03, respectively. Figure 2 shows the densitometric

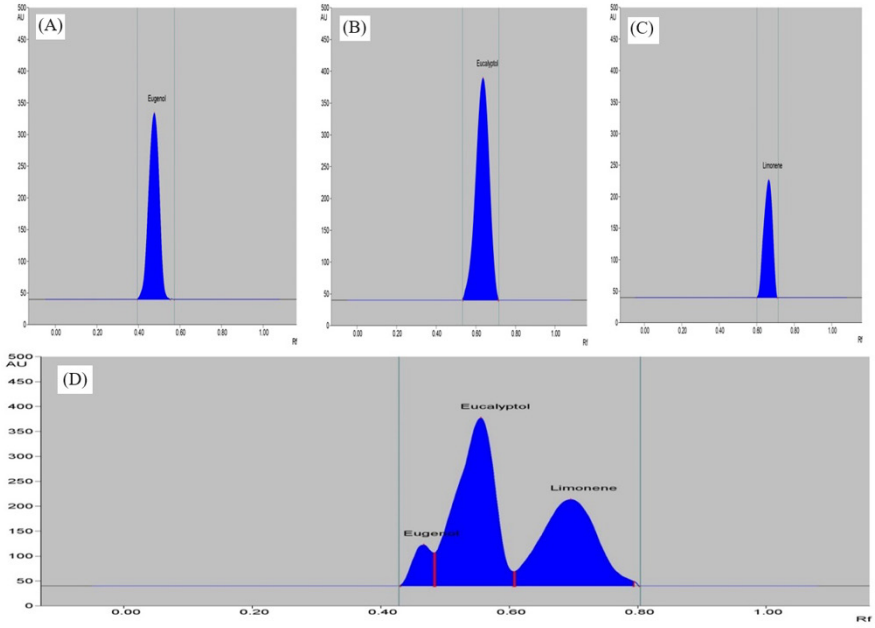

Figure 2: Densitometric chromatogram of eugenol (standard) (A), eucalyptol (standard) (B), R-limonene (standard) (C), and sample mixture (D) at 450 nm.

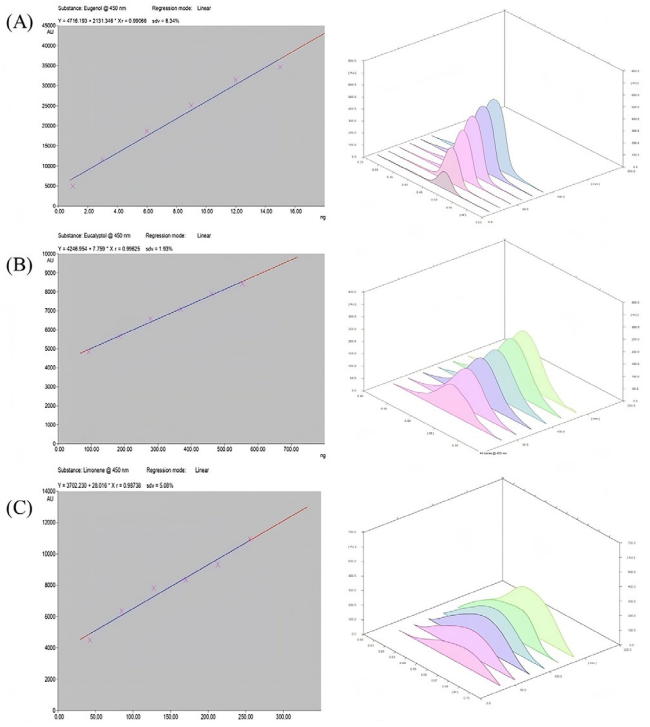

Figure 3: Calibration curve and spectra of eugenol (standard) (A), eucalyptol (standard) (B), and R-limonene (standard) (C) at 450 nm

chromatograms of both the standards and the sample mixture. The peaks, bands, and R_f values obtained were compared between the sample mixture and the standard references.

Calibration Curve by Densitometric method

The specific volume was measured, i.e., 0.1 mL of eugenol (purity 99%) and put into a 10 mL graduated flask. The volume was marked up to 10 mL with methanol. One mL of the eugenol primary solution was further diluted up to 10 mL with methyl alcohol. Subsequently, the standard was spotted in various volumes (1, 3, 6, 9, 12, and 15 µL) on a TLC plate. Using peak area *versus* amount per spot, the six-point standard graph was plotted in the linear range of 1 to 15 ng/spot (Figure 3A). To achieve a concentration of 92.25ng/µL, 0.1 mL of eucalyptol (purity 99%) was precisely measured, put into a 10 mL graduated flask, and the volume was marked up to 10 mL with methyl alcohol. The plates were then spotted with the volumes, which were 1 to 6 µL. Using peak areas versus its correlated amount; the linear calibration curve was generated for the six points in the 100-600 ng/spot range (Figure 3B). For the calibration curve of R-limonene, accurately measured 0.5 mL (purity 99%) of R-limonene was taken into a 10 mL graduated flask. The final volume was marked up to 10 mL by methyl alcohol to get a concentration of 42.55 ng/µL. TLC plates were spotted with different six volumes (1–6 µL). A six-point calibration curve constructed within the linear range of 50 to 300 ng/spot by plotting peak area against their relative amount (Figure 3C). Figure 4 displays the HPTLC spectra of the sample mixture at a wavelength of 450 nm.

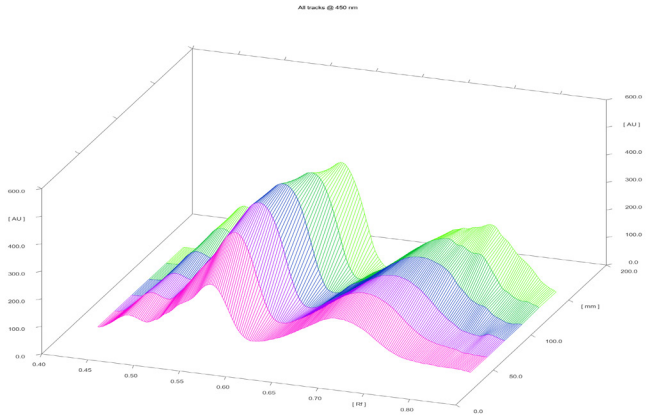

Figure 4: HPTLC spectra of sample mixture at 450 nm

Method Validation

The developed method was further validated for various parameters such as linearity, specificity, accuracy, precision and robustness. The linearity range for eugenol, eucalyptol, and R-limonene was 1 to 15 ng ($r^2 = 0.9906$), 100-600 ng/spot ($r^2 = 0.99625$), and 50 to 300 ng/spot ($r^2 = 0.9873$), respectively (Table 1). Standard calibration plots were plotted by taking peak area against concentration for eugenol, eucalyptol, and R-limonene standards separately and for a mixture of samples. Eugenol, eucalyptol, and R-limonene had regression equations of y = 4716.19 + 2131.34x, 4246.95 + 7.76x, and 3702.23 + 28.01x, respectively. The eugenol, eucalyptol, and R-limonene correlation coefficients were 0.9906, 0.99625, and 0.9873, respectively. The slopes were 2131.34x ± 0.054, 7.76x ± 0.0683 and 28.01x ± 0.076 and the intercepts were 4716.19 ± 21.33, 4246.95 ± 35.11 and 3702.23 ± 25.78. These values show that the proposed method is linear (Table 1).

The percentage recovery or accuracy results for eugenol, eucalyptol, and R-limonene are mentioned in Table 2. The percentage recovery for these three phytoconstituents ranged from 96.71 to 99.24%, demonstrating the accuracy of the proposed approach. The instrumental precision and method

Table 1: HPTLC method's validation parameters for determination of eugenol, eucalyptol, and R-limonene

Validation parameters	*Eugenol*	*Eucalyptol*	*R-Limonene*
Regression equation	Y = 4716.19 + 2131.34X	Y = 4246.95 + 7.76X	Y = 3702.23 + 28.01X
Correlation coefficient, *n*=3	0.9906	0.99625	0.9873
Linearity range (ng/spot), *n*=3	1-15	100-600	50-300
R_f	0.47 ± 0.03	0.56 ± 0.03	0.71 ± 0.03
Limit of detection (ng/spot), *n*=3	0.0129	0.82	0.594
Limit of quantification (ng/spot), *n*=3	0.0391	2.48	1.8
Instrumental precision (RSD) *n*=3	0.61	0.85	0.87
Specificity	Specific	Specific	Specific

Table 2: Accuracy results for eugenol, eucalyptol, and R-limonene by proposed method

Additional standard put on to analyte (%)	*Theoretical content (ng)*	*Conc. Found (ng) ± SD*	*%Recovery*	*%RSD*
Eugenol				
0	90	87.7 ± 0.89	97.4	1.01
50	100	97.6 ± 1.82	97.6	1.86
100	110	106.8 ± 1.33	97.09	1.24
150	120	117.7 ± 0.96	98.08	0.82
Eucalyptol				
0	200	197.60 ± 2.42	98.8	1.22
50	300	293.05 ± 1.92	97.6	0.65
100	400	395.62 ± 1.04	98.9	0.26
150	500	496.22 ± 2.21	99.24	0.45
R-limonene				
0	100	97.78 ± 1.06	97.78	1.08
50	150	145.07 ± 2.05	96.71	1.41
100	200	194.23 ± 1.84	97.11	0.94
150	250	246.44 ± 1.53	98.57	0.62

Table 3: Precision results for eugenol, eucalyptol, and R-limonene by proposed HPTLC method

Concentration (ng/spot)	*Intra-day Precision*			*Inter-day Precision*		
	Average concentration ± SD	*Standard error*	*%RSD*	*Average concentration ± SD*	*Standard error*	*%RSD*
Eugenol						
60	18648.84 ± 68.73	28.05	0.37	18631.05 ± 53.12	21.68	0.28
90	25097.45 ± 52.43	21.40	0.21	25083.22 ± 45.77	18.68	0.18
120	31437.49 ± 55.67	22.72	0.17	31440.09 ± 67.92	27.72	0.22
Eucalyptol						
276.8	8766.87 ± 56.43	23.03	0.64	8789.34 ± 60.78	24.80	0.69
369.0	10049.53 ± 63.33	25.84	0.63	10050.22 ± 45.89	18.73	0.46
461.3	10826.95 ± 57.78	23.58	0.53	10820.60 ± 34.04	13.89	0.31
R-limonene						
316.12	2651.32 ± 25.78	10.52	0.97	2649.12 ± 22.20	9.06	0.83
421.50	2974.60 ± 23.43	9.56	0.78	2978.78 ± 25.05	10.22	0.84
526.87	3393.50 ± 27.81	11.35	0.82	3391.43 ± 21.43	8.74	0.63

Table 4: Robustness results for eugenol, eucalyptol, and R-limonene by proposed HPTLC method

		Solvent composition (hexane: toluene: ethyl acetate)				
Concentration (ng/spot)	*Actual*	*Used*	*Changed*	*Peak area ± SD (n = 3)*	*%RSD*	R_f
Eugenol						
150	6:3:1	6.1:2.9:1	+0.1, -0.1, 0.0	34612.65 ± 85.07	0.25	0.49
		6:3:1	0.0	34619.57 ± 94.21	0.27	0.47
		5.9:3:1.1	-0.1, 0.0, +0.1	34628.7 ± 73.54	0.21	0.45
Eucalyptol						
461.30	6:3:1	6.1:2.9:1	+0.1, -0.1, 0.0	12310.87 ± 67.09	0.55	0.52
		6:3:1	0.0	12319.65 ± 70.08	0.57	0.56
		5.9:3:1.1	-0.1, 0.0, +0.1	12326.67 ± 73.32	0.59	0.60
R-limonene						
210.75	6:3:1	6.1:2.9:1	+0.1, -0.1, 0.0	2540.98 ± 15.89	0.62	0.74
		6:3:1	0.0	2535.7 ± 23.65	0.93	0.71
		5.9:3:1.1	-0.1, 0.0, +0.1	2530.65 ± 20.09	0.79	0.67

precision were calculated, and the %RSD (relative standard deviation) is shown in Tables 1 and 2, respectively. The LoD and LoQ values for eugenol, eucalyptol, and R-limonene were found to be 0.0129 and 0.0391 ng/spot, 0.82 and 2.48 ng/spot, and 0.594 and 1.8 ng/spot, respectively. It shows that the proposed identified method is reliable and sensitive. The inter-day and intra-day findings are displayed in Table 3. For the developed method, the intra-day and inter-day (%RSD) were found out in a range of 0.17 to 0.37% and 0.18 to 0.28% for eugenol, 0.53-0.64% and 0.31-0.69% for eucalyptol, and 0.82 to 0.97% and 0.63 to 0.84% for R-limonene, respectively. The %RSD value was below 2% that, validating the precision and reproducibility of this approach. The %RSD robustness range was 0.21-0.27% for eugenol, 0.55 to 0.59% for eucalyptol, and 0.62 to 0.93% for R-limonene Table 4. The robustness determination shows that this method is reliable and can tolerate small changes in experimental settings.

CONCLUSION

An HPTLC approach has been identified, developed and further validated for the simultaneous measurement of eugenol, eucalyptol, and R-limonene. The method is accurate, reliable, exact, reproducible, sensitive, and rapid. The results obtained with this method can further be used for quality assurance and rapid estimation and quantification of monoterpenes in formulations, complex combinations derived from plants containing these monoterpenes, and essential oils. HPTLC chromatograms and calibration curves showed the precision of the instrument and the procedure, as well as the linearity and repeatability of this approach. When estimating these phytoconstituents on inter-days, this approach does not exhibit any significant influence. HPTLC chromatograms of standards and physical mixture showed that eugenol, eucalyptol, and R-limonene were separated without any tailing effect. Statistical analysis estimated the selectivity and repeatability of this method.

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REFERENCES

1. Khalil AA, Rahman U ur, Khan MR, Sahar A, Mehmood T, Khan M. Essential oil eugenol: sources, extraction techniques and nutraceutical perspectives. RSC Advances [Internet].2017;7(52):32669–81.Availablefrom: http://pubs.rsc.org/en/content/articlehtml/2017/ra/c7ra04803c
2. da Silva FFM, Monte FJQ, de Lemos TLG, do Nascimento PGG, de Medeiros Costa AK, de Paiva LMM. Eugenol derivatives: synthesis, characterization, and evaluation of antibacterial and antioxidant activities. Chemistry Central Journal [Internet]. 2018 Apr 3;12. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5880794/
3. Di Pasqua R, Betts G, Hoskins N, Edwards M, Ercolini D, Mauriello G. Membrane Toxicity of Antimicrobial Compounds from Essential Oils. Journal of Agricultural and Food Chemistry. 2007 Jun;55(12):4863–70.
4. Ferhat MA, Boukhatem MN, F. Chemat. Quality Assessment of the Essential Oil from Eucalyptus globulus Labill of Blida (Algeria) Origin. World Academy of Science, Engineering and Technology, International Journal of Biomedical and Biological Engineering. 2015 Dec 3;2(12).
5. Bhowal M, Gopal M. Eucalyptol: Safety and Pharmacological Profile. RGUHS J Pharm Sci. 2016 Jan 8;5(4):125–31.
6. Hendry ER, Worthington T, Conway BR, Lambert PA. Antimicrobial efficacy of eucalyptus oil and 1,8-cineole alone and in combination with chlorhexidine digluconate against microorganisms grown in planktonic and biofilm cultures. Journal of Antimicrobial Chemotherapy. 2009 Oct 16;64(6):1219–25.
7. Ravichandran C, Badgujar PC, Gundev P, Upadhyay A. Review of toxicological assessment of d-limonene, a food and cosmetics additive. Food and Chemical Toxicology [Internet]. 2018 Oct 1 [cited 2021 Oct 22];120:668–80. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0278691518304976
8. Eddin LB, Jha NK, Meeran MFN, Kesari KK, Beiram R, Ojha S. Neuroprotective Potential of Limonene and Limonene Containing Natural Products. Molecules [Internet]. 2021 Jan 1;26(15):4535. Available from: https://www.mdpi.com/1420-3049/26/15/4535
9. Chikhoune A, Hazzit M, Kerbouche L, Baaliouamer A, Aissat K. Tetraclinis articulata(Vahl) Masters essential oils: chemical composition and biological activities. Journal of Essential Oil Research. 2013 Aug;25(4):300–7.
10. Espina L, Somolinos M, Lorán S, Conchello P, García D, Pagán R. Chemical composition of commercial citrus fruit essential oils and evaluation of their antimicrobial activity acting alone or in combined processes. Food Control. 2011 Jun;22(6):896–902.
11. Brennan TJ, Krömer JO, Nielsen LK. Physiological and Transcriptional Responses of Saccharomyces cerevisiae to *d* -Limonene Show Changes to the Cell Wall but Not to the Plasma Membrane. Appl Environ Microbiol. 2013 Jun 15;79(12):3590–600.
12. Zhao C, Zeng Y, Wan MZ, Li R, Liang Y, Li C, et al. Comparative analysis of essential oils from eight herbal medicines with pungent flavor and cool nature by GC-MS and chemometric resolution methods. Journal of Separation Science. 2009 Feb 1;32(4):660–70.
13. Attimarad M, Mueen Ahmed KK, Aldhubaib BE, Harsha S. High-performance thin layer chromatography: A powerful analytical technique in pharmaceutical drug discovery. Pharmaceutical Methods [Internet]. 2011 Apr;2(2):71–5. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3658041/
14. International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Harmonised Triplicate Guideline on Validation of Analytical Procedures: Methodology, Recommended for Adoption at Step 4 of the ICH Process on November 1996 by the ICH Steering Committee. IFPMA, Switzerland.
15. Alam MN, Kaushik R, Singh L, Khan NA. Quantitative estimation of polyphenolic biomarker quercetin and chlorogenic acid in moringa oleifera leaves by hyphenated high-performance thin-layer chromatography (HPTLC) techniques. International Journal of Drug Delivery Technology. 2021;11:1123-9.
16. Kharate V, Kuchekar M, Harde M, Pimple B, Patole V, Salunkhe M, Wadgave P, Bhise M, Gaikwad A, Tare H. Development of Validated Stability Indicating HPTLC Method for Estimation

of Febuxostat in Bulk and Tablet Dosage Form by Using QBD Approach. International Journal of Drug Delivery Technology. 2023;13(2):542-50.
17. Ahmad S, Mujawar T, Batewal B, More P, Gaikwad A, Chumbhale D, Tare H. RP-UHPLC and HPTLC method development and validation for analysis of andrographolide from herbal hepatoprotective formulation. International Journal of Pharmaceutical Quality Assurance. 2023;14:96-104.
18. Krishti A, Baraka J, Dasvani B. Analytical Method Development and Validation of First Order Derivative Spectrophotometric Method for Simultaneous Estimation of Telmisartan and Metformin Hydrochloride in their Combined Pharmaceutical Dosage Form: Int. J. of Pharma. Quality Assurance (IJPQA). 2020;11(1):60-8.