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HPTLC quantitation of eugenol from leaf and berry powder of *Pimenta dioica*(L.) merr.

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ABSTRACT

A sensitive and accurate high performance thin layer chromatographic method has been developed and validated for the determination of eugenol from the leaf and berry powder of *Pimenta dioica*(L.) Merr..The leaf and berry powders were extracted with methanol and the extracts were separated on TLC Silica gel 60 F_{254} using toluene as the mobile phase.Detection and quantification were carried out densitometrically using deuterium lamp at λ=280 nm.The proposed method was validated for linearity, precision and accuracy.Linear response to eugenol was found to be in the concentration range of 200-600 ng per band.The developed method can be used for routine quantitative monitoring of eugenol from the dried leaf and berry powder of *Pimenta dioica*(L.) Merr.

KEYWORDS

Eugenol;
Pimenta dioica(L.) merr;
HPTLC;
Quantitation.

INTRODUCTION

Pimenta dioica(L.) Merr. is a bushy evergreen tree belonging to the Myrtaceae family[1]. It is native to West Indies and Tropical America[2] and is grown in India in the states of Bangalore,Bihar ,Karnataka,Kerala,Orissa, Tamilnadu and West Bengal[3]. The dried unripe fruits of *Pimenta dioica*(L.) Merr.form the Allspice, Jamaican Pepper or Pimento of Commerce[1]. The berries are the source of important spice in the food industry[4]. The berry oil has been reported to have anti-microbial, antifungal and insect-repellant properties[5]. *Pimenta dioica*(L.)Merr. leaf oil has been used as an adulterant of the more expensive berry oil[1]. It is also used for the isolation of eugenol[1].

Most of the recent work for the determination of the constituents of leaf and berry oils of *Pimenta dioica*(L.) Merr. has been carried out by GC methods[6-9].

Eugenol(4-hydoxy-3-methoxyallylbenzene) is identified as one of the major constituents of *Pimenta dioica* (L.) Merr. leaf and berry oils[6-9]. Eugenol is used as a fragrance and flavouring agent[10]. It acts as an antioxidant,carminative and anti-spasmodic agent[11].

A literature survey reveals that a HPLC method is reported for the quantification of eugenol from the berries *Pimento dioica* (L.) Merr.[4]. Also, GC methods are reported for the identification of the constituents of the leaf and berry oil of *Pimenta dioica*(L.) Merr[6-9]. A HPTLC method is reported for the quantification of eugenol from leaf powder and capsule formation of *Ocimum sanctum*[12]. A HPTLC method for simultaneous determination of eugenol, luteolin, ursolic acid and oleanolic acid in black and green varieties of *Ocimum sanctum* Linn.is reported[13]. Also, there are HPTLC methods reported for quantification of eugenol

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from *Syzygium aromaticum*(L.) Merr. and Perry[14] and from leaf powder of *Cinnamomum tamala* nees & eberm[15]. Another, HPTLC densitometric method for simultaneous determination of cinnamaldehyde, eugenol and piperine in pepper contaminated cinnamon is reported[16].Also, a HPTLC method for simultaneous analysis of umbelliferone, psoralen and eugenol in the fruit pulp of *Aegle marmelos* and in fruits of *Trachyspermum ammi*(Linn.) Sprague and *Foeniculam vulgare* Mill. is reported[17]. A HPTLC and GC-MS method for separation and identification of eugenol in clove, nutmeg cinnamon, herb bennet, calamus and valerian is reported[18].

However, no HPTLC method for the quantitative determination of eugenol from crude leaf and berry powder of *Pimenta dioica*(L.) Merr. is reported. As eugenol is a significant constituent of the leaves and berries of *Pimenta dioica*(L.) Merr., a simple HPTLC method is developed for its quantitation.

2. EXPERIMENTAL

2.1. Reagents and standards

Methanol and toluene were of analytical grade, each with a purity of 99.8 % and 99.5% respectively. Eugenol was given as a gift from S.H.Kelkar and Co.Pvt.Ltd., Mumbai, India and had a purity of 98.76%.Aluminium backed TLC Silica gel 60 F_{254} plates having a thickness of 0.2mm were purchased from Merck(Mumbai,India).

2.2. Plant material

The leaves and berries of *Pimenta dioica*(L.) Merr. were collected from the trees from Nagercoil, Tamilnadu,India.The samples were authenticated by National Botanical Research Institute, Council of Scientific and Industrial Research, Lucknow,India(voucher specimen no: 95394). The leaves and berries were dried and powdered. Each powder was passed through a BSS no.85 mesh sieve.Each sample powder was stored in a separate air-tight container at room temperature(28± 2^0C).

2.3. Preparation of stock and working standard solutions of eugenol

A stock solution of eugenol (0.5 mg mL^{-1}) was prepared by transferring accurately weighed about 5 mg of eugenol into a 10 mL standard volumetric flask, followed by dissolving in 5 mL methanol.The flask was sonicated for 10 minutes and then diluted to volume with methanol. 1 mL of this stock solution was transferred to a separate 10 mL standard volumetric flask.Volume of this flask was made upto the mark with methanol(0.05mg mL^{-1}).

2.4. Preparation of sample solutions

Accurately weighed about 100 mg of leaf and berry powder of *Pimenta dioica* (L.) Merr. was transferred to a 10 mL standard volumetric flask. 5 mL of methanol was added to each flask and the flasks were sonicated for 45 minutes, with intermediate shaking.After cooling to room temperature(28 ± 2^0C), the contents of each flask were diluted to volume with methanol. Each of the sample solution was then filtered separately through Whatman no.41 filter paper(Merck,Mumbai,India) and filtrate of each sample solution was collected. For the determination of eugenol from the berry powder, 1 mL of the filtrate obtained was diluted to 2 mL with methanol.

2.5. Chromatographic conditions

Chromatography was performed on 20 cm × 10 cm aluminium backed TLC Silica gel 60 F_{254} plates. Before application, the plates were pre-washed with methanol by ascending chromatography and then activated in an oven at 110^{0}C for 10 minutes. The standard and sample solutions were applied as sharp bands of 8mm length, by means of Camag (Muttenz,Switzerland) Automatic TLC Sampler 4 (ATS 4), equipped with 25µL Hamilton syringe. They were applied at a distance of 8mm from bottom, the distance from the sides was 15mm.The delivery speed of application was 150nL/sec.

The plates were developed to a distance of 60mm using toluene as the mobile phase. Before application, the Camag glass twin trough chamber lined with filter paper was saturated with mobile phase vapours for 20 minutes. After development, the plates were dried for 4 minutes using hot air. The plates were scanned by means of TLC Scanner 3 (Camag), with win CATS software, version.1.4.4, in absorbance-reflectance mode using deuterium lamp at λ=280nm.The photodocumentation

of the plates was done with TLC Visualiser(Camag).

2.6. Validation of the proposed HPTLC method

2.6.1. Linearity

Different volumes of the eugenol standard solution of concentration 0.05 mg mL^{-1} were applied to a 20 x10cm pre-washed TLC plate.Volumes of 4, 6, 8, 10, 12μL equivalent to 200, 300, 400, 500 and 600 ng per band were applied. The plates were developed and scanned as described above.The calibration plot for eugenol was obtained by plotting obtained peak areas against corresponding concentrations. The linearity experiment was repeated three times on three different plates and the mean was used for the calculations. The linear response to eugenol was found in the concentration range of 200-600 ng per band and correlation coefficient was 0.998.

2.6.2. Limit of detection(LOD) and limit of quantification(LOQ)

The limit of detection and limit of quantification were calculated by the use of the equations LOD = 3 × N/B and LOQ = 10 × N/B, where N is the standard deviation of peak areas of eugenol(n=5) taken as a measure of noise, and B is the slope of the corresponding calibration plot. The LOD and LOQ values for eugenol were found to be 40 ng per band and 120 ng per band respectively.

2.6.3. Precision

Th method was validated for instrumental precision, repeatability and intermediate precision. Instrumental precision was studied by repeated analysis (n=10) of standard eugenol containing 400ng per band.The results were expressed as percent RSD of peak area.The repeatability of the method was tested by preparing three different concentrations of leaf powder of *Pimenta dioica* (L).Merr. (6, 12 and 18 mg mL^{-1}) and three different concentrations of berry powder(3, 7 and 11 mg mL^{-1}). Each sample was extracted with methanol as described earlier. Each concentration of the leaf and berry powder extracts was applied in triplicate, on the same plate, on the same day and analysed by the above procedure.

The intermediate precision of the method was determined in the same way as repeatability, but on three successive days. The values of precision for each of three parameters for the leaf and berry powder solutions were found to be below 2, indicating that the method is precise to carry out this analysis.

Spectra comparison

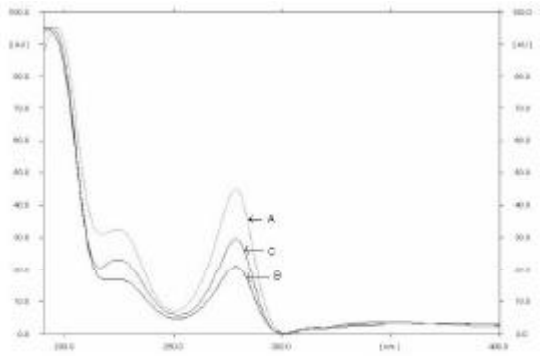

Figure 1: Overlay of UV spectra of ; (A) Standard eugenol and; (B) Eugenol present in the leaf powder of *Pimenta dioica*(L.) Merrill; (C) Eugenol present in berry powder of *Pimenta dioica*(L.) Merrill

2.6.4 Specificity

The specificity of the proposed HPTLC method was ascertained by overlapping UV spectra of standard eugenol with UV spectra of each of the sample solutions(Figure 1). The eugenol band in both the samples was compared at three positions, the peak start, peak middle and peak end.There was a good correlation between all spectra obtained at each of the three positions of bands. The peak of eugenol was thus not masked by the peak of any other component in the sample which was indicative of peak purity.

2.6.5 System suitability

The system suitability tests were carried out to confirm that system used is adequate to carry out this analysis and gives reproducible results.Parameters that were studied to evaluate the suitability of the system were peak areas and retardation factors(R_F) of eugenol. To ascertain the effectiveness of the method developed in this study, system suitability tests were carried out by applying standard eugenol solutions containing 600 ng to the plate, six times. The plates were developed under optimized chromatographic conditions. The results were again expressed as percent RSD of peak area. The values of percent RSD were found to be below 2, indicating that the method is reproducible and hence

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TABLE 1: Results from recovery analysis of eugenol from leaf and berry powder of *Pimenta dioica* (L.)Merr

Part used	Level	Amount of sample weighed[mg]	Amount of eugenol added[mg]	Mean amount found[mg][a]	Percent recovery
Leaves	0	100.3	0	0.90±0.072	98.5
	1	100.5	0.22	1.10±0.053	
	2	100.7	0.45	1.33±0.060	
	3	100.4	0.67	1.55±0.038	
Berries	0	100.7	0	2.30±0.046	99.0
	1	100.4	1.15	3.42±0.032	
	2	100.3	1.73	3.98±0.045	
	3	100.5	2.3	4.56±0.051	

[a]Mean ± SD(n=7)

TABLE 2: Results obtained from assay of eugenol from the leaf and berry powder of *Pimenta dioica*(L.)Merr

	Leaf	Berry
Mean weight of sample[mg]	100.6	100.4
Mean amount of eugenol found in sample[mg][a]	0.90±0.037	2.30±0.042
% RSD of peak area of eugenol	0.77	0.64
Mean amount of eugenol found[%]	0.90	2.30

[a]Mean ± SD(n=7)

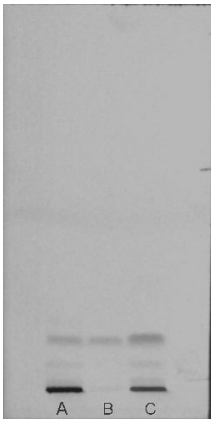

Figure 2 : TLC plate showing separation of eugenol in; (A) Methanolic extract of leaf powder of *Pimenta dioica* (L.) Merrill; (B) Standard eugenol and; (C) Methanolic extract of berry powder of *Pimenta dioica*(L.) Merrill

suitable for routine chromatographic analysis.

2.6.6 Accuracy

Accuracy of the method was checked by performing recovery experiment from the leaf and berry powder, by standard addition method, at three different levels. For the determination of recovery from the leaf powder, known amounts(0.22 mg, 0.45 mg and 0.67 mg) of standard eugenol were added to 100 mg of leaf powder of *Pimenta dioica*(L.)Merr. For determination of recovery from the berry powder, known amounts (1.15 mg, 1.73 mg and 2.3 mg) of standard eugenol were added to 100 mg of berry powder of *Pimenta dioica*(L.) Merr. Each sample was extracted as described earlier and each extract was analysed seven times by the developed HPTLC method. The amount of eugenol recovered from both the samples were determined at each level. The percent recovery was determined. The results of recovery analysis from the leaf and berry powder from *Pimenta dioica*(L.) Merr. are given in TABLE 1.

2.7. Estimation of eugenol from the dried leaves and berry powder of *Pimenta dioica* (L.)Merr.

Seven replicates of the leaf and berry powder solutions (prepared as described in section 2.4, 3μL for leaf powder solution and 2μL for berry powder solution) were analysed by the developed HPTLC method.Peak areas and mean peak areas of eugenol from both the sample solutions were recorded.The identity of eugenol from each sample solution was confirmed by comparison of R_F value of standard eugenol. Figure 2 shows a developed TLC plate indicating the separation of eugenol from the methanolic extract of leaf and berry powder of *Pimenta dioica*(L.) Merr. From the calibration plot of eugenol, the amounts of eugenol from each sample solution was determined. The results obtained from assay are given in TABLE 2.

3. RESULTS AND DISCUSSION

The HPTLC methods reported for the quantification of eugenol in different plants as mentioned earlier have used toluene:ethyl acetate:formic acid in varying ratios[12-15]. The HPTLC method reported for simultaneous determination of cinnamaldehyde, eugenol and piperine has used petroleum ether:dichloromethane: formic acid(2:4:0.1, v/v/v)as mobile phase[16]. The other HPTLC method reported for simultaneous determination of umbelliferone, psoralen and eugenol has used toluene:methanol(9.5:0.5, v/v) as mobile phase[17]. The HPTLC method reported for the separation and identification of eugenol in clove,nutmeg,cinnamon, herb bennet,calamus and valerian has used n-heptane:ethyl acetate(60:40, v/v)as mobile phase[18].

In this experiment, toluene as single component was used as mobile phase and was efficient to resolve eu-

genol from the other components present in the the leaf and berry powder of *Pimenta dioica*(L.) Merr. The R_F value obtained for eugenol was found to be 0.23. The quantification of eugenol was carried at λ =280 nm which is the wavelength of maximum absorption of eugenol.

The method is specific as eugenol in the methanolic extract of leaf and berry powder of *Pimenta dioica*(L.) Merr. was well resolved from the other components present in the extracts.

The values of percent RSD for instrumental precision, repeatability and intermediate precision were found to be below 2, which indicated that the method is precise to carry out analysis.

The percent recovery values for eugenol for leaf and berry powder were found to be 98.5 and 99.0 indicating high accuracy of the method. The average percent of eugenol in the leaf and berry powder of *Pimenta dioica*(L.) Merr. were found to be 0.9 and 2.3 respectively.

4. CONCLUSION

This high performance thin layer chromatographic method developed for the quantification of eugenol from dried leaf and berry powder of *Pimenta dioica* (L.) Merr. is simple, precise and accurate and can be used for routine quality control of the leaves and berries of *Pimenta dioica*(L.) Merr.

5. ACKNOWLEDGEMENTS

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