Development and Validation of a High-Performance Thin-Layer Chromatographic–Densitometric Method for the Quantification of Apigenin in *Ocimum basilicum* L. Seed (Takhmaria)

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Key Words:

Apigenin
***Ocimum basilicum* seed (Takhmaria)**
High-performance thin-layer chromatography
Densitometric determination
Methanolic extract

Summary

A simple, selective, precise, and accurate high-performance thin-layer chromatographic (HPTLC) method has been developed for the quantification of *Ocimum basilicum* seeds for their apegenin content. Densitometric evaluation was carried out on precoated silica gel G60 F_{254} HPTLC plates using toluene–acetone–formic acid (5:4:1, *v*/*v*) as the mobile phase. Scanning and densitometric evaluations were done at 340 nm. The calibration curve was plotted in the concentration range of 100–600 ng band^{-1}, and the method was found linear with r^2 = 0.995. The developed method was validated according to the International Conference on Harmonization (ICH) guidelines for accuracy, precision, range, linearity, limit of detection (LOD), and limit of quantification (LOQ). The validated HPTLC method was used for the estimation of apigenin in *O. basilicum* seeds. The developed method can be applicable for the identification and quantification of apigenin in complex mixtures of phytochemical and marker-based standardization of plant samples as well as extracts containing *O. basilicum* seeds.

1 Introduction

Apigenin (4,5,7-trihydroxyflavone) is a natural product belonging to the flavone subclass of flavonoids and is the aglycone of several naturally occurring glycosides [1]. It is a common constituent present in fruits, plant-derived beverages, vegetables, wheat, sprouts, and some seasonings. Some of the popular and abundant sources of apigenin include chamomile tea [2] grapefruits, onions, oranges, and some spices such as parsley [3]. It is also found in higher ratios (relative to other foods) in yarrow, celery, basil, tarragon, cilantro, licorice, foxglove, coneflower, flax, passion flower, horehound, spearmint, [4, 5] red wine [6], and beer [7] and is an active ingredient in the memory herb *Gingko biloba [6].*

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Apigenin has anticancer activity in numerous human cancer cells including prostate cancer, colon carcinoma, and breast cancer with low cytotoxicity and without any mutagenic activity [8]. There are numerous data available on anti-inflammatory, antiviral, and purgative properties of apigenin in the literature [9].

The physicochemical properties of apigenin are as follows: molecular formula, $C_{15}H_{10}O_5$; molecular weight, 270.24 g mol^{-1}; melting point, 345–350°C; yellow crystalline powder; and it is practically insoluble in water, moderately soluble in hot alcohol, and soluble in dilute KOH and DMSO [10]. The structure of apigenin is shown in **Figure 1.**

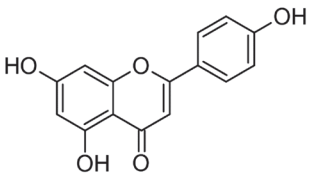

Figure 1

The structure of apigenin.

Ocimum basilicum (Lamiaceae), commonly known as holy basil, is an important medicinal plant found throughout India. Various medicinal properties are attributed to the plant, particularly to the *O. basilicum* leaves which possess antimicrobial [11], anti-inflammatory [12], antioxidant [13], antiulcerogenic [14], analgesic [15], cardiac stimulant [16], chemomodulatory [17], CNS depressant [18], hepatoprotective [19], hypoglycemic [20], larvicidal [21], and hypolipidemic [22] activities.

O. basilicum seeds, also known as Takhmaria, possess in-vitro antioxidant, antimicrobial, aphrodisiac, diuretic, and antidysenteric actions [23, 24].

Various phytoconstituents like ursolic acid, oleanolic acid, eugenol, methyl eugenol, mono, squiterpenes [25], isopropylpalmitate [26], linalool [27], methyl chavicol [28], bergamotene, germacrene D, δ-cadinene, γ-cadinene, β-selinene, and spathu-

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lenol [29] were isolated from *O. basilicum* leaves. D-xylose, L-arabinose, r-rhamnose, D-galacturonic acid, galactose, and glucose were isolated from *O. basilicum* seed [30]. The presence of other compounds was reported for the ethanolic extract of seeds which include alkaloids, flavonoids, amino acid, protein, saponins, fat, and oil [31].

A literature survey described that high-performance thin-layer chromatography (HPTLC) [32, 33] and reversed-phase high-performance liquid chromatography (RP-HPLC) [34, 35] methods were developed for the determination of apigenin in various plant samples. Till date, no HPTLC method has been reported for the quantification of apigenin in *O. basilicum* seed. This paper describes a novel HPTLC–densitometry method for the quantification of apigenin in plant samples of *O. basilicum* seeds.

2 Experimental

2.1 Materials

The *O. basilicum* seeds were collected from the local market, Ahmedabad, Gujarat, and were authenticated by Dr. Bhasker L. Pungani (Head of P.G. Center in Botany, Smt. S.M. Panchal Science College, Talod, Gujarat). The reference standard of apigenin (HPLC purity >98.0%) was obtained from Sigma-Aldrich, Bangalore, Karnataka, India. All solvents used in this study were of analytical grade.

2.2 Instruments

A CAMAG HPTLC system (Muttenz, Switzerland) comprising of a 100-µL Hamilton syringe, a Linomat 5 automatic sample applicator, a CAMAG twin-trough chamber (20 × 20 cm), a CAMAG TLC Scanner III, CAMAG CATS IV integration software, and UV cabinet with dual wavelength UV lamp was used for the HPTLC analysis. HPTLC analyses were performed on 20 cm × 10 cm HPTLC plates coated with silica gel G60 F_{254}.

2.3 Methods

2.3.1 Preparation of Standard Solution of Apigenin

The stock solution of standard apigenin (1 mg mL^{-1}) was prepared in methanol. One milliliter of stock solution was diluted with 9 mL of methanol to make the concentration 100 ng μL^{-1}. For calibration curve, 1, 2, 3, 4, 5, and 6 µL standard solutions were applied to the HPTLC plate to obtain a concentration range of 100–600 ng $band^{-1}$. Each concentration was spotted three times on the HPTLC plate. The plate was developed under optimized chromatographic conditions. Peak areas were plotted against corresponding concentrations to obtain the calibration graph.

2.3.2 Extraction and Preparation of Test Samples

Air-dried powder (100 g) of *O. basilicum* seeds (Takhmaria) was defatted by refluxing with 250 mL petroleum ether (60–80°C) for 4 h. The residue was dried and then subjected to extraction by soxhlet method using methanol as the solvent. The flask was refluxed for 4 h and filtered through Whatman filter paper. This procedure was repeated three times to get complete extraction from the powder. All powder extracts were combined and evaporated to dryness on water bath to get the residue. The residues of test samples were dissolved in 10 mL of methanol. These test solutions were spotted against standard apigenin for assay on a HPTLC plate.

2.3.3 Chromatographic Conditions

Chromatographic analyses were performed on (20 cm × 10 cm) HPTLC silica gel G60 F_{254} plates. The plates were cleaned by predevelopment to the top with methanol and dried in an oven at 105°C for 5 min. Sample and standard zones were applied to the layer as bands by means of a CAMAG Linomat 5 automatic sample applicator equipped with a 100-µL syringe and operated with the following settings: band length, 6 mm; application rate, 150 µL s^{-1}; and distance from the bottom of the plate, 2 cm. The plates were developed to a distance of 80 mm with toluene–acetone–formic acid (5:4:1, *v*/*v*) as the mobile phase in a CAMAG twin-trough glass chamber, previously saturated with mobile phase vapor at room temperature for 30 min. After removal of the plates from the chamber, they were completely dried in air at room temperature; the peak areas for samples and standard were recorded by densitometry at λ_{max} 340 nm, by means of a CAMAG TLC Scanner III with winCATS software. The calibration curves were constructed by plotting peak area *versus* concentration (100–600 ng $band^{-1}$) corresponding to each band.

2.4 Method Validation

The method was validated according to the International Conference on Harmonization guidelines for validation of analytical procedures [36].

2.4.1 Linearity

The linear response of apigenin was determined by analyzing six independent levels of the calibration curve in the range of 100–600 ng $band^{-1}$. The result was expressed in terms of correlation coefficient.

2.4.2 Precision

Intra-day and inter-day precisions were performed to check the repeatability and intermediate precision of the method, respectively. It was achieved by using three different concentration levels of 100, 300, and 600 ng $band^{-1}$ three times on the same day for intra-day and on three different days for inter-day precision. Method precision was evaluated by analyzing sample solutions (n = 6). Results were statistically evaluated in terms of percent relative standard deviation (% RSD).

2.4.3 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The calibration curve was repeated five times, and the standard deviation (SD) of the intercepts (response) was calculated. Then, LOD and LOQ were measured by using the mathematical expressions: LOD = 3.3 δ/S and LOQ = 10 δ/S, where S is the slope of the calibration curve and δ is the standard deviation of the Y-intercept of the regression line.

2.4.4 Accuracy (% Recovery)

The accuracy is expressed as % recovery by the assay of known and added amount of analyte. It is the measure of the exactness of the analytical method. The recovery experiments were car-

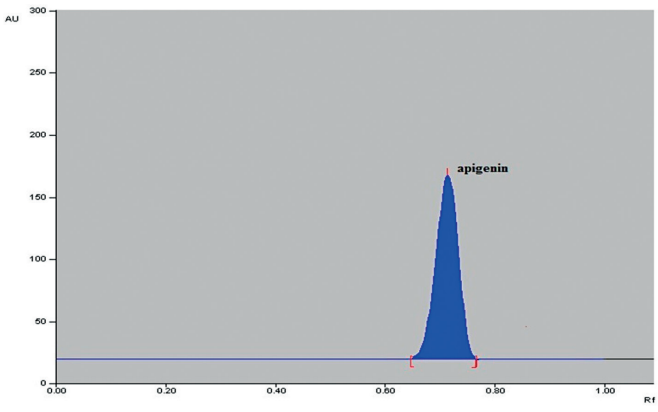

Figure 2

Densitometric HPTLC chromatogram of standard apigenin (200 ng band^{-1}) at R_F = 0.71.

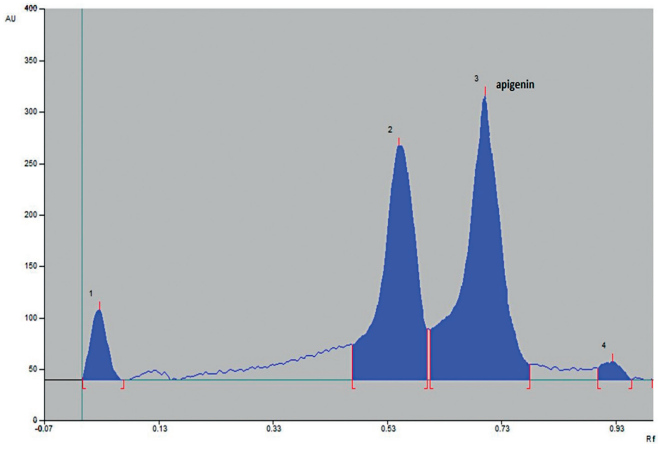

Figure 3

Densitometric HPTLC chromatogram of methanolic extract of apigenin *Ocimum basilicum* seed at R_F = 0.71.

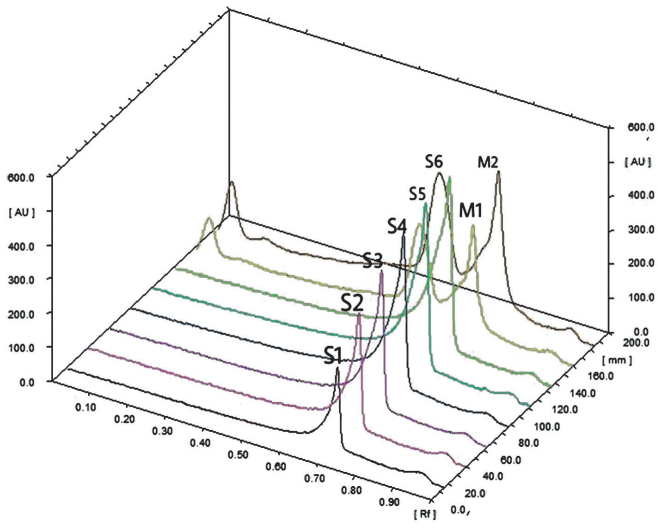

Figure 4

3D densitometric chromatograms of standard apigenin in 100–600 ng band^{-1} (S1–S6) and methanolic extract (M1–M2) at 340 nm.

ried out in triplicate by previously analyzed test samples with three different concentrations of standards at 50%, 100%, and 150%, respectively.

2.4.5 Robustness Study

Robustness was studied in triplicate at 300 ng band^{-1} by performing small changes in mobile-phase ratio, mobile-phase volume, and duration of saturation time. The results were examined in terms of % RSD and SD of peak areas. Mobile phase using toluene, acetone, and formic acid in different ratios (5:4:1, *v/v*; 5.5:3.5:1, *v/v*; 4.5:4.5:1, *v/v*; 4.75:3.75:1.5, *v/v*) were used for chromatography. Mobile-phase volume and duration of saturation investigated were 10 ± 2 mL (8, 10, and 12 mL) and 20 ± 10 min (10, 20, and 30 min), respectively.

2.4.6 Specificity

The identity of the spot of apigenin from test solutions was confirmed by comparing their R_F values in samples with those of the apigenin standard.

2.5 Quantification of Apigenin in Samples of the Plant

The extracts for the analysis were prepared as per section 2.3.2 and subjected to HPTLC analysis for apigenin content. Each analysis was performed in triplicate, and mean ± SD was recorded.

3 Results and Discussion

To obtain high resolution and reproducible peaks, mobile phases of different compositions were tried for HPTLC analysis. The desired profile was achieved in toluene–acetone–formic acid (5:4:1, *v/v*) at 340 nm. The R_F value of apigenin was found to be 0.71 ± 0.02. The densitometric chromatograms of standard apigenin and methanolic extract of *O. basilicum* seed are shown in **Figures 2 and 3,** respectively, and overlaid densitometric chromatograms of apigenin standard in the range of 100–600 ng band^{-1} are presented in **Figure 4.** The R_F values of apigenin standard and apigenin from seed extract were equal to 0.71 ± 0.02 for TLC–densitometric analysis, being identical with the densitogram of the apigenin standard (Figure 4). There was no interference in the analysis from other component present in the extract. The linearity data of standard apigenin are shown

Table 1

Linearity data of standard apigenin.

Conc. (ng band^{-1})	Area (mean ± SD, n = 3)	% RSD
100	8191 ± 22.33	0.27
200	11,296 ± 34.45	0.30
300	15,710 ± 43.22	0.27
400	18,682 ± 53.21	0.28
500	22,746.6 ± 68.28	0.30
600	25,147 ± 82.22	0.32
Mean % RSD 0.29		

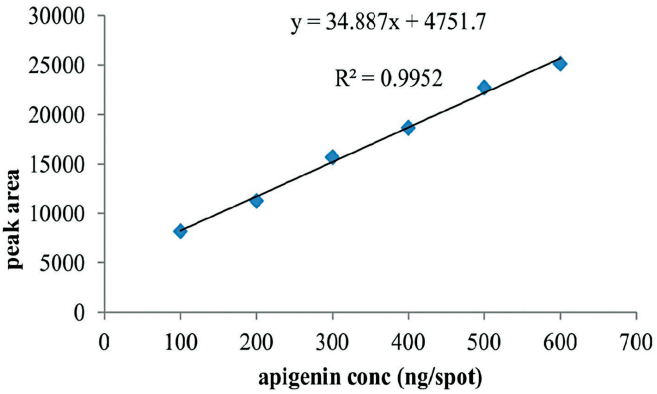

Figure 5

Linearity curve of standard apigenin (100–600 ng band^{-1}).

Table 2

Results of intra-day precision.

Apigenin[a)]	Peak area	SD[b)]	% RSD
100	8123	25.32	0.31
300	15,695	45.32	0.28
600	25,120	83.30	0.33
Mean % RSD 0.30			

[a)]Concentration in ng band^{-1}
[b)]Mean of six determinations (n = 6)

Table 3

Results of inter-day precision.

Apigenin[a)]	Peak area	SD[b)]	% RSD
100	8200	38.52	0.46
300	15,795	59.72	0.37
600	25,110	95.42	0.38
Mean % RSD 0.40			

[a)]Concentration in ng band^{-1}
[b)]Mean of six determinations (n = 6)

Table 4

Results of repeatability studies.

Sr. No.	Apigenin conc. (ng band^{-1})	Area
1	300	15,710
2	300	15,665
3	300	15,760
4	300	15,775
5	300	15,814
6	300	15,610
SD	84.36	
% RSD	0.53	

Table 5

Results of recovery study.

% Amount estimated	Amount of apigenin present in preanalyzed sample (ng band^{-1})	Amount of apigenin standard added (ng band^{-1})	Amount recovered (ng band^{-1})	% Recovery ± SD	% RSD
50	200	100	98.4	98.4 ± 1.45	1.47
100	200	200	201.2	100.6 ± 2.02	1.00
150	200	300	296.5	98.83 ± 3.42	1.14

Table 6

Robustness of the method.

Conc. (ng band^{-1})	Original	Change	Peak area ± SD	% RSD
Mobile-phase composition (toluene–acetone–formic acid)				
300	(4: 5:1, *v*/*v*)	5.5:3.5:1	15,536 ± 82	0.52
		4.5:4.5:1	15,832 ± 93	0.58
		4.75:3.75:1.5	15,310 ± 75	0.48
Mobile-phase volume				
300	10 mL	8	14,982 ± 88	0.58
		10	15,145 ± 75	0.49
		12	15,422 ± 98	0.63
Duration of saturation				
300	20 min	10	14,567 ± 78	0.53
		30	14,898 ± 110	0.73

in **Table 1.** The calibration curve for apigenin was obtained by plotting the peak area of apigenin *versus* concentration over the range of 100–600 ng band^{-1}, and it was found to be linear with r^2 = 0.995 **(Figure 5).** LOD and LOQ were found to be 3.72 and 12.30, respectively. The % RSD values for intra-day, inter-day and repeatability studies were found to be 0.30, 0.40 and 0.53, respectively **(Tables 2–4).** The results of accuracy were 98.46–100.6% **(Table 5).** The % RSD for different parameters of robustness was found to be less than 2% **(Table 6).** The HPTLC

Table 7

Assay results of *Ocimum basilicum* seed (Takhmaria).

Sample	Apigenin content (% *w*/*w*)	SD	% RSD
Methanolic fraction	0.69	2.3	0.12

Table 8

Summary of the validation parameters of the developed HPTLC method.

Sr. No.	Parameters	Results
1	Linearity (ng band^{-1})	100–600
2	Linear regression equation	$y = 34.88x + 4751$
3	Correlation coefficient	0.995
3	Accuracy	98.46–100.6%
4	Precision (% RSD) Repeatability of sample application Inter-day Intra-day	 0.53 0.40 0.30
5	Limit of detection (ng band^{-1})	3.728
6	Limit of quantification (ng band^{-1})	12.30
7	Robustness	Robust

method was applied to the quantification of apigenin in plant samples of *O. basilicum* seed. The resolution was good, and the content of apigenin in seeds sample was found 0.69% *w/w* **(Table 7).** The summary of regression analysis and validation parameters is shown in **Table 8.**

4 Conclusion

A high-performance thin-layer chromatographic method has been developed and validated for the quantification of apigenin from methanolic extracts of dried seed powder of *O. basilicum*. From the above study, it can be concluded that the developed HPTLC technique is fast, simple, precise, specific, and accurate; hence, it can be employed for the quantification of apigenin in plant samples. The developed method could be used for the marker-based standardization of herbal formulations.

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