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HPTLC method development and validation of stigmasterol from different extracts of *Tagetes erecta and Capsicum annuum*

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ABSTRACT

A new, simple, sensitive, selective, precise and robust high-performance thin-layer chromatographic (HPTLC) method for analysis of stigmasterol was developed and validated for the determination of stigmasterol in different extracts. A new, simple, sensitive, selective, precise and robust high-performance thin-layer chromatographic (HPTLC) method for analysis of stigmasterol was developed and validated for the determination of stigmasterol in different extracts. Analysis of stigmasterol was performed on TLC aluminium plates pre-coated with silica gel 60F-254 as the stationary phase. Linear ascending development was carried out in twin trough glass chamber saturated with mobile phase consisting of Hexane : Acetone (8:2 v/v) at room temperature. (25 °C ± 2 °C) Camag TLC scanner III was used for spectrodensitometric scanning and analysis in absorbance mode at 490nm. The system was found to give compact spots for stigmasterol. (R_f value of 0.44 ± 0.02) The linear regression analysis data for the calibration plots showed good linear relationship with r^2 =0.9997 ± 0.0002 in the concentration range 200-1200 ng spot^{-1} with respect to peak area. According to the International Conference on Harmonization (ICH) guidelines the method was validated for precision, recovery and robustness. Statistical analysis of the data showed that the method is reproducible and selective for the estimation of stigmasterol.

Keywords: *Tagetes erecta, Capsicum annuum*, stigmasterol, HPTLC, method validation

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INTRODUCTION:

Tagetes (Composite) is a genus of herbs, commonly known as marigold is however indiscriminately applied to several genera of Composite with golden or yellow capitula and there are about 33 species of the genus *Tagetes*, out of which, five species have been introduced into the Indian gardens viz. *Tagetes erectal* (Aztec or African Marigold), *Tagetes minutal* (Tagetes glandulifera Schrank), *Tagetes patula l.* (French Marigold), *Tagetes lucida Cav*. (Sweet-Scented Marigold), *Tagetes tenuifolia Cav.* (Striped Marigold) [1-3] whereas *Capsicum annum linn* is dicotyledonous flowering plant commonly grows worldwide, with many general names such as chilli, hot pepper, sweat pepper, bell pepper belonging to the family *Solanaceae*. It is used in various indigenous system due to its valuable medicinal property. [5-8] From the *Tagetes erecta* and *Capsicum annuum* extract, the stigmasterol is identified and quantified by the powerful chromatographic technique such as HPTLC.

Stigmasterol is a steroid derivative characterized by the hydroxyl group in position C-3 of the steroid skeleton, and unsaturated bonds in position 5-6 of the B ring, and position 22-23 in the alkyl substituent.

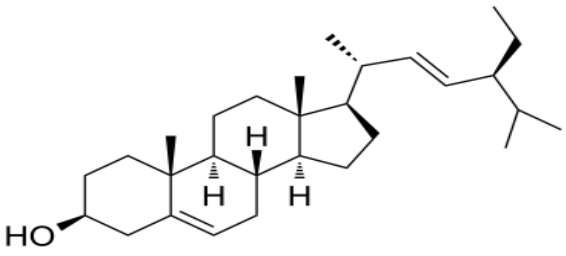

Fig. 2- Structure of stigmasterol [4]

Stigmasterol is an unsaturated plant sterol present in various medicinal plants. Stigmasterol is utilized in a number of chemical processes which are designed to yield numerous synthetic and semi-synthetic compounds for pharmaceutical industry. It acts as a precursor in the synthesis of progesterone and acts as an intermediate in the biosynthesis of androgens, estrogens and in the synthesis of vitamin D3. Stigmasterol is found in the fats and oils of soybean, calabar bean and rape seed, as well as several other vegetables,

legumes, nuts, seeds, and unpasteurized milk. Stigmasterol shows many biological activities like anti-osteoarthritic activity, anti-hypercholestrolemic activity, hypoglycemic activity, antioxidant. [9] The simple chromatographic method is developed for the quantitative and qualitative analysis of stigmasterol.

HPTLC is a powerful analytical technique due to its merits of reliability, simplicity, reproducibility and speed. This method is economical as it utilizes smaller amounts of solvents with minimum sample clean up. Also, in a short duration, a large number of samples are simultaneously analyzed. HPTLC has no limitation on the choice of the mobile phase and unlike HPLC, mobile phases having pH 8 and above can be employed. Direct application of suspensions, dirty or turbid samples are possible. Furthermore, it permits a simultaneous assay of several components in a multicomponent formulation or herbal extracts. [10-11] The aim of this work was to develop an accurate, specific, repeatable and robust method for the determination of stigmasterol. The proposed method was validated in compliance with ICH guidelines. [12-13]

EXPERIMENTAL:

2.1. Materials-

Standard stigmasterol was supplied by Tokyo Chemical Industry Co. Ltd., Japan. The different extracts obtained from *Tagetes erecta* and *Capsicum annuum* which are crude extract, aqueous extract, hexane extract and ethanol extract were procured as a fresh material from the local suppliers and was assessed biologically by the department of botany. All chemicals and reagents used were of analytical grade and were purchased from Rankem, Mumbai, India.

2.2 Instrumentation and chromatographic conditions-

The samples were spotted in the form of bands of width 6 mm with a Camag microlitre syringe on precoated silica gel aluminium plate 60F-254 (20 cm × 10 cm) with 200 m thickness (E. Merck, Germany) using a Camag Linomat V (Switzerland) sample applicator. The syringe, 100 µL (from 2.5micron); the developing chamber was a CAMAG glass twin trough chamber (20 × 10 cm); the densitometer consisted of a CAMAG TLC Scanner 3 linked to win-CATS software; the experimental condition temperature 25 °C ± 2 °C, relative humidity 40 %. TLC plates were dried in a current of air with help of an air-dryer. Densitometric scanning was performed on Camag TLC scanner III in the absorbance mode at 490 nm. The source of radiation utilized was a deuterium lamp.

2.3 Calibration curve of stigmasterol-

A stock solution of stigmasterol (20 µg/ml) was prepared in dichloromethane. Different volumes of stock solution 10 µl, 20 µl, 30 µl, 40 µl, 50 µl and 60 µl were spotted on the TLC plate to obtain concentrations of 200 ng spot−1, 400 ng spot−1, 600 ng spot−1, 800 ng spot−1, 1000 ng spot−1, 1200 ng spot−1 of stigmasterol respectively. The data of peak areas plotted against the corresponding concentrations were treated by regression analysis.

2.4. Method validation:

2.4.1. Precision-

Repeatability of the sample application was carried out using six replicates of the same standard (800 ng spot−1 of stigmasterol) and was expressed in terms of percent relative standard deviation. (% RSD) The system precision and method precision were performed for the determination of stigmasterol carried by preparing standard and sample for six times.

2.4.2. Robustness of the method-

By introducing small changes in the scanning wavelength, the effects on the results were examined. Robustness of the method was done in at a concentration level of 800 ng spot−1 and the % RSD was calculated.

2.4.3. Recovery studies-

The pre-analyzed samples were spiked with extra 80 %, 100 % and 120 % of the standard stigmasterol and the mixtures were analyzed by the proposed method. The experiment was conducted for six times. This was done to check for the recovery of the stigmasterol at different levels in the formulations.

2.4.4. Specificity-

The specificity of the method was ascertained by analyzing the standard and extract. The spot for stigmasterol in the sample was confirmed by comparing the R_f values and spectra of the spot with that of the standard. The peak purity of the stigmasterol was assessed by comparing the spectra at three different levels, viz. peak start (S), peak apex (M) and peak end (E) positions of the spot.

2.5. Sample preparation:

Weigh 25 mg sample in 15 ml centrifuge tube. Add 10 ml dichloromethane and sonicate it for 2 min. Filter the solution through 2.5 micron filter paper. The filterate is then applied to the HPTLC system.

2.6 Standard preparation:

Weigh accurately 1 mg stigmasterol standard in 50 ml centrifuge tube. Make up volume by dichloromethane. Filter the solution through 2.5 micron filter paper. Use this stock standard solution for the analysis.

3. RESULTS AND DISCUSSION:

3.1. Development of the optimum mobile phase-

The TLC procedure was optimized with a view to quantify the herbal extract. Initially Hexane : Acetone, Hexane : Ethyl acetate in varying ratios was tried. The mobile phase Hexane: Acetone (8:2 v/v) gave good resolution with R_f = 0.44 for stigmasterol. (Fig. 1) Well-defined spots were obtained.

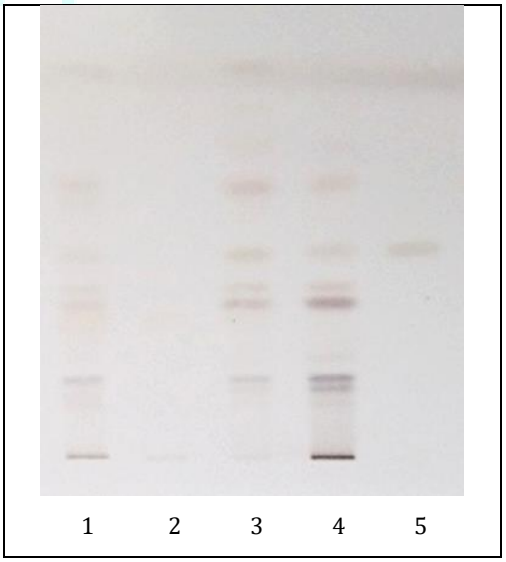

Fig. 1 TLC profile of different fractions from *Tagetes erecta* and *Capsicum annuum* after derivatization at 490 nm; Spot 1 indicates crude fraction, Spots 2 indicates aqueous fraction, Spot 3 indicates hexane fraction, Spot 4 indicates ethanol fraction and Spot 5 indicates stigmasterol standard.

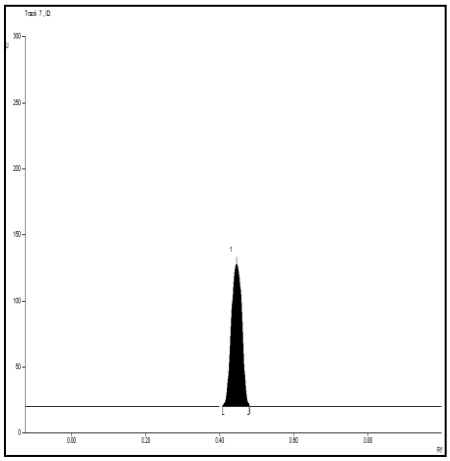

Fig. 2. Chromatogram of standard stigmasterol (800 ng spot−1), peak 1(R_f = 0.44 ± 0.02), mobile phase- Hexane : Acetone. (8:2 v/v)

3.2. Calibration curves-

The developed HPTLC method for estimation of stigmasterol showed a good correlation coefficient (r^2 = 0.9997 ± 0.0002) in concentration range of 200 ng spot−1 to 1200 ng spot−1 with respect to the peak area. The mean value (± SD) of slope and intercept were 0.0195 and -35.53 respectively. No significant difference was observed in the slopes of standard curves.

3.3. Method validation-

The % RSD for system precision of sample application (800 ng spot−1) was found to be 4.70. The % RSD shows value <10%. (Table 1) The method precision of sample application shows the % RSD 8.07. (Table 2) The low values of SD and % RSD obtained after introducing small deliberate changes in the developed HPTLC method indicated the robustness of the method. (Table 3) The proposed method when analysed for recovery study for estimation of stigmasterol affords recovery of 92.39 % to 96.71 % as listed in Table 4. Low % RSD values proved the ruggedness of the method indicating that stigmasterol is stable during the extraction procedure as well as during analysis.

Table 1- System precision of HPTLC method

Amount (ngspot−1)	Area
800ng	5356.00
800ng	5794.00
800ng	5741.00
800ng	5271.30
800ng	5931.40
800ng	5772.80
Average	5644.41
SD	365.68
% RSD	4.70

Table 2- Method precision of HPTLC method

Sample ID	% Content
Crude extract (1)	0.40
Crude extract (2)	0.41
Crude extract (3)	0.42
Crude extract (4)	0.40
Crude extract (5)	0.38
Crude extract (6)	0.33
Average	0.39
SD	0.031
% RSD	8.07

Table 3- Robustness of HPTLC method

Wavelength	Track 1	Track 2	Track 3
488nm	0.310	0.300	0.35
490nm	0.280	0.290	0.31
492nm	0.280	0.300	0.34
Average	0.290	0.290	0.33
SD	0.017	0.005	0.02
% RSD	5.970	1.940	6.24

Table 4- Percentage Recovery of HPTLC method

Accuracy level	Amount found	Amount added	% Recovery
80	4.16	4.50	92.39
100	5.13	5.58	91.85
120	5.92	6.12	96.71

3.4 Analysis of the prepared formulation-

A single spot at R_f = 0.44 was observed in the chromatogram of the stigmasterol. There was no interference from the other components present in the extracts of *Tagetes erecta* and *Capsicum annuum*. (Fig. 1) The total percent content of stigmasterol was found in different extracts is 0.78 %, 0.00 %, 2.31 %, 1.75% in crude extract, aqueous extract, hexane extract and ethanol extract respectively. For the method validation, hexane extract is considered as it contains stigmasterol in more amount as compared to other extracts. The percent recovery of the stigmasterol in sample was found to be in the range of 92.39 % to 96.71 %.

DISCUSSION:

Chromatographic fingerprint analysis has proven to be a rational and feasible approach for the assessment of quality and authentication of species of traditional medicine. [14-16] It effectively uses chromatographic techniques to form specific patterns of recognition for phytochemicals. The developed fingerprint pattern of components can thereafter be utilized to check the presence of markers of interest as well as the ratio of all detectable analytes. Though there are some shortcomings of high performance thin layer chromatography, such as the limited developing distance and lower plate efficiency as compared to high performance liquid chromatography and gas chromatography, still it is an effective tool for evaluation of herbal extracts due to its simplicity. Moreover, the formerly mentioned limitations can be curbed by separately developing fractions of different polarity on two or several thin layer plates. The unique feature of the picture like image of HPTLC coupled with the digital scanning profile is an attractive and useful tool for construction of herbal chromatographic fingerprint. The stigmasterol in sample of extract was identified and its presence was confirmed by comparing the R_f value of standard stigmasterol to that of the extract. This method is reproducible and has shown satisfactory results on precision, accuracy and recovery study data. [17] There is no report of detection and quantification of stigmasterol in *Tagetes erecta* and *Capsicum annuum* by HPTLC. Hence, we developed a simple and precise method for quantification of this marker.

CONCLUSION:

The developed HPTLC technique is a precise, specific, accurate and robust for the determination of stigmasterol. Statistical analysis proves that the method is reproducible and selective for the analysis of stigmasterol. Since the proposed mobile phase effectively resolves stigmasterol, the

method can be used for qualitative as well as quantitative analysis of stigmasterol in herbal extracts.

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