Ind. J. Pharm. Edu. Res., 2026; 60(4s):s1782-s1794.
https://www.ijper.org

Original Article

Method Development and Validation for the Simultaneous Estimation of Aflatoxins in Botanical Origin Samples Using High-Performance Thin-Layer Chromatography

Vaishnavi Chaudhari, Shraddha Bhor, Swati Lade, Milind Umekar, Sushil Burle*, Radheshyam Lohiya, Diksha Undirwade

Smt. Kishoritai Bhoyar College of Pharmacy, Kamptee, Nagpur, Maharashtra, INDIA.

ABSTRACT

Background and Objectives: Aflatoxins are toxic secondary metabolites produced by certain *Aspergillus species* and are frequently found in contaminated food and herbal products. Their presence poses serious health risks, necessitating effective analytical methods for detection and control. In this study, a simple, sensitive, and cost-effective High-Performance Thin-Layer Chromatography (HPTLC) method was developed and validated for the simultaneous estimation of aflatoxins B_1, B_2, G_1, and G_2 in a polyherbal formulation, Haridrakhand. **Materials and Methods:** The method employed silica gel 60 F_{254} plates and a mobile phase of chloroform: acetone: water (140:20:0.3, v/v/v), with detection at 366 nm under UV light. The method was validated as per ICH Q2(R^2) guidelines for linearity, accuracy, precision, specificity, robustness, Limit of Detection (LOD), and Limit of Quantification (LOQ). **Results:** Calibration curves showed excellent linearity with Correlation Coefficients (R^2) greater than 0.996. Recovery studies at 80%, 100%, and 120% levels yielded recovery rates within 98-102%. The LOD and LOQ values confirmed the method's sensitivity, and all validation parameters met the required acceptance criteria. **Conclusion:** This HPTLC method is suitable for routine screening and quality control of aflatoxins in herbal formulations.

Keywords: Botanical Sample, Liquid Chromatography, Method Development, Validation, Toxins.

Correspondence:
Dr. Sushil Burle
Associate Professor, Smt. Kishoritai Bhoyar College of Pharmacy, Kamptee, Nagpur-441002, Maharashtra, INDIA.
Email: drsushilburle@gmail.com

Received: 14-05-2026;
Revised: 07-06-2026;
Accepted: 22-08-2026.

INTRODUCTION

Background on Natural Toxins and Mycotoxins

Natural toxins are hazardous chemical compounds produced by living organisms, including plants, fungi, algae, and bacteria. While these toxins serve defensive roles in the natural world, their unintended presence in food, medicinal plants, and dietary supplements poses a serious health risk to humans and animals. One hazardous group of natural toxins is mycotoxins (Figure 1), secondary metabolites formed by specific species of fungi, especially under humid or improperly stored conditions.[1,2]

Mycotoxins such as aflatoxins, ochratoxins, and zearalenone can contaminate a wide range of agricultural and herbal products. Their chemical diversity, combined with their toxicological potency, makes them a major concern for food and drug safety authorities worldwide.[3,4] These compounds are known to be carcinogenic, mutagenic, immunosuppressive, and hepatotoxic, even at trace levels.[5-8]

Out of the 400 known mycotoxins, only around 30 are considered to have significant health impacts, and aflatoxins are among the most dangerous. Produced predominantly by *Aspergillus flavus, A. parasiticus, and A. nomius,* aflatoxins regularly contaminate food items such as maize, nuts, spices, milk, and increasingly, herbal and botanical formulations. Due to their strong link to liver cancer and other chronic illnesses, aflatoxins (especially B_1) are classified as Group 1 carcinogens by the International Agency for Research on Cancer (IARC).[9]

Aflatoxins: Structure, Types, and Toxicity

The major aflatoxins of concern include Aflatoxin B_1, B_2, G_1, and G_2, designated based on their fluorescence (blue or green) under UV light and structural differences. B_1 is the most potent, exhibiting strong carcinogenic, hepatotoxic, teratogenic, and genotoxic properties (Table 1). Chronic exposure to even small quantities can result in severe long-term health effects such as liver cancer, immune dysfunction, and growth retardation.[10-12]

These compounds are highly stable and can persist through food processing, making their detection and quantification critical for consumer safety. Regulatory bodies have set Maximum Residue

DOI: 10.5530/ijper.20265177

Copyright Information :
Copyright Author (s) 2026 Distributed under Creative Commons CC-BY 4.0

Publishing Partner : Manuscript Technomedia. [www.mstechnomedia.com]

Limits (MRLs) for total aflatoxins in food products, typically 10-20 ppb depending on the country and product type.

Mycotoxins in Herbal and Botanical Products

The use of herbal medicine has grown significantly in the last few decades due to its perceived natural origin and fewer side effects compared to synthetic drugs. Botanicals are used in various forms powders, extracts, tablets, and decoctions, and are often consumed without stringent quality checks, especially in developing countries. Products such as Haridrakhand, a traditional Ayurvedic formulation, are commonly used in Indian households for skin and immune-related disorders.

However, botanicals are not immune to fungal contamination. Improper storage, high humidity, and lack of GMP/GLP standards during manufacturing or post-harvest processing allow for the proliferation of aflatoxin-producing fungi. Contamination in such products is frequently under-reported due to insufficient regulatory surveillance and a lack of validated analytical methodologies.

Although the WHO has issued guidelines to ensure the safe use of herbal medicines, significant gaps still exist in their standardization, especially regarding microbial and mycotoxin contamination. With increasing global trade and reliance on herbal formulations, ensuring the mycotoxin-free status of these products is imperative.[13-21]

Analytical Techniques for Aflatoxin Detection

Various analytical methods have been established to detect and quantify aflatoxins across different sample matrices.[22-24] These include High-Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), Enzyme-Linked Immunosorbent Assays (ELISA), Liquid Chromatography-Mass Spectrometry (LC-MS/MS), and High-Performance Thin-Layer Chromatography (HPTLC). Each of these techniques offers specific advantages in terms of sensitivity, selectivity, and suitability for particular sample types. Among them, HPTLC stands out for its unique advantages, especially in the analysis of complex herbal matrices. It enables the simultaneous analysis of multiple samples, which is highly beneficial in high-throughput testing environments. The method is also cost-effective, requiring relatively inexpensive equipment and consumables compared to LC-MS/MS. HPTLC requires a low volume of sample, which is particularly useful when only a limited sample quantity is available. Furthermore, it supports both visual and spectrometric detection, offering flexibility in identification and quantification. Most importantly, HPTLC is especially well-suited for complex herbal matrices, where multiple phytochemicals and excipients might interfere with analysis in other methods. These features make HPTLC an ideal tool for the routine screening of aflatoxins in herbal and nutraceutical products.[25-30]

HPTLC uses pre-coated silica gel plates and supports flexible detection methods such as fluorescence. The use of advanced instruments such as the CAMAG Linomat 5 applicator, TLC Scanner, and winCATS software enables semi-automated (Figure 2), reproducible analysis while ensuring strong adherence to ICH and other regulatory standards.

HPTLC offers several advantages over other analytical techniques, such as HPLC and spectrometric titrations.[31] Key benefits include:

The separation process is easy to follow, especially with coloured compounds.

Several samples can be separated in parallel on the same plate, resulting in a high throughput and a rapid, low-cost analysis.

Two-dimensional separations are easy to perform. Specific and sensitive colour reagents can be used to detect separated spots.

HPTLC can combine and consequently use different modes of evaluation, allowing the identification of compounds having different light-absorption characteristics or different colors.

Contact detection allows radiolabelled compounds to be monitored and microbial activity in spots to be assessed.

The TLC plates are disposable; therefore, neither regeneration nor essential clean-up is required.

Chromatographic development and detection of the separated spots on a plate are generally separate processes in time; therefore, after separation, the plates can be stored for a long time, and detection performed at a later stage to obtain the analytical information.

Need for Method Development and Validation

There is an urgent need to develop simple, rapid, economical, and validated methods to quantify aflatoxins in botanical origin formulations. The majority of existing literature emphasizes food matrices such as cereals and nuts, whereas research on herbal and Ayurvedic products is relatively limited. The complexity of herbal matrices poses additional analytical challenges, making robust method validation essential.

This study focuses on developing an HPTLC method for Aflatoxins B_1, B_2, G_1, and G_2, with comprehensive validation as per ICH Q2 (R^2) guidelines. The goal is to make this method suitable for routine quality control testing of herbal formulations, particularly those marketed in bulk or sold as traditional remedies.[32-36]

MATERIALS AND METHODS

Chemicals and Reagents

The chemicals and reagents used in the study included HPLC-grade acetonitrile, methanol, dichloromethane, chloroform, and acetone. Analytical Reagent (AR/GR) grade

petroleum ether was used, along with sodium chloride solution (10%), sodium sulphate, and distilled water.

Aflatoxins B_1, B_2, G_1, and G_2 in acetonitrile were obtained as a mixed standard from Pribolab Pvt. Ltd. for analysis. The herbal sample, Haridrakhand, was procured from the local market.

Instruments

High-Performance Thin Layer Chromatography (HPTLC) was used to analyse Toxin. Data processing was done using the winCATS Planar Chromatography Manager. Silica Gel 60 F254 served as the stationary phase on the Thin Layer Chromatography (TLC) plates, and the CAMAG Linomat 5 (Linomat 5_70644) was utilized for the semi-automated band-wise application of samples. The development chamber was a Twin Trough Chamber. The CAMAG TLC Scanner (Scanner_171005) was used for detection and scanning, while the CAMAG Visualizer (Visualizer_171113) was used for visualization.

Experimental

Preparation of Mobile Phase

A mixture of Chloroform, Acetone, and Water in a ratio of 140:20:0.3 v/v/v was prepared in a conical flask.

Aflatoxin Standard Solution

An aflatoxin standard solution comprising B_1, B_2, G_1, and G_2 in acetonitrile was utilized for HPTLC analysis.

The concentrations of the individual aflatoxins in the standard solution were as follows:

Aflatoxin B_1: 1.00 µg/mL.

Aflatoxin G_1: 1.00 µg/mL.

Aflatoxin B_2: 0.300 µg/mL.

Aflatoxin G_2: 0.300 µg/mL.

Selection of Stationary Phase, Chromatographic Conditions, and Mobile Phase

The selection of an analytical technique is greatly influenced by the nature and composition of the sample. In this study, the chosen aflatoxins are polar. HPTLC was employed for preliminary separation due to its simplicity, broad applicability, and overall effectiveness. To establish optimal chromatographic conditions and an appropriate mobile phase for the separation, elution, and quantification of Aflatoxins B_1, B_2, G_1, and G_2 method parameters were systematically varied across trials, and

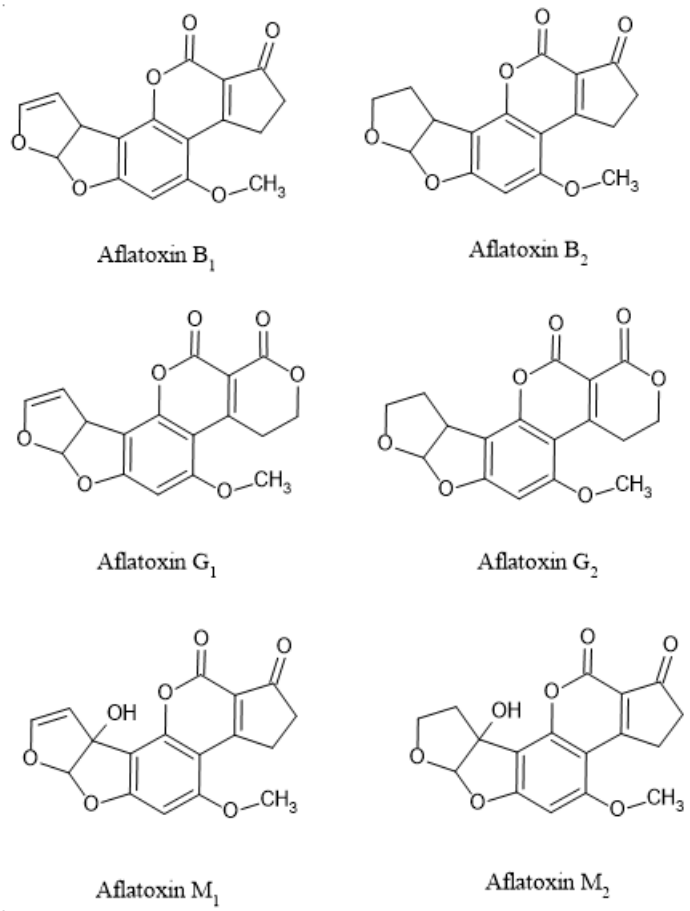

Figure 1: Chemical Structure of Aflatoxins.

corresponding densitograms were analysed. The choice of mobile phase plays a critical role in chromatographic performance. Standard drug solutions were prepared in mobile phases with varying compositions, and multiple trials were conducted to identify the most suitable mobile phase, with densitograms recorded for each attempt.

Optimized Chromatographic Parameter

Ultimately, the selected mobile phase and optimized chromatographic conditions proved effective for the clear resolution of intact Aflatoxins B_1, B_2, G_1, and G_2, without any interference from excipients. The optimized chromatographic parameters are shown in Table 2.

Procedure

The chromatographic conditions were optimized, and estimations were performed on a stationary phase, pre-coated silica gel 60 F_{254} aluminium sheets (20 × 20 cm), which were pre-washed with methanol and dried in air. With a mobile phase of Chloroform: Acetone: Water (140:20:0.3 v/v/v), the twin-trough chamber and plate were allowed to saturate for about 30 min. The wavelength scanning was performed at 366 nm, which is a universal wavelength for the detection of aflatoxins, keeping the slit dimension at 4.00 × 0.30 mm. The source of radiation was a mercury vapor lamp emitting a continuous fluorescent spectrum in the UV region, and scanning was carried out at a speed of 20 mm/s. The standard solutions of aflatoxins (B_1, B_2, G_1, and G_2) were spotted by leaving 15 mm from the bottom edge and 8 mm from the side edge using a Linomat 5 semi-automatic applicator, and then developed at a constant temperature in a twin-trough chamber. After development, the plates were dried in air and visualized under long-wave UV light at 366 nm. Densitometric scanning was conducted in fluorescence mode with the detection of emission in the range of 440-450 nm. The densitogram was recorded, and peak areas were measured for quantitative analysis (Figure 3).

Preparation of Sample

Grind the sample and homogenize. Weigh accurately about 20 g of the sample and add 80 mL of methanol, mix for 3 min. Add

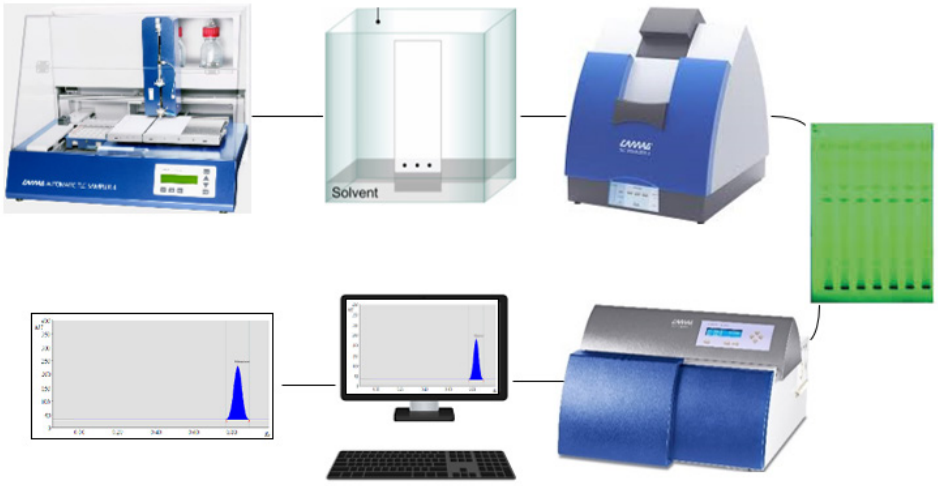

Figure 2: Instrumentation of HPTLC.

Table 1: Aflatoxins: Physicochemical and Toxicological Profile.

Aflatoxins	MW (g/mol)	Formula	Melting Point (°C)	LD_{50} (mg/kg)	Adverse Health Effect
Aflatoxin B_1	312.27	$C_{17}H_{12}O_6$	268-269°C	0.5-10	Hepatotoxicity, Carcinogenicity, Teratogenicity, Genotoxicity, Immunotoxicity
Aflatoxin B_2	314.29	$C_{17}H_{14}O_6$	286-289°C	1.7	Hepatotoxicity, weak Mutagenic, Carcinogenicity
Aflatoxin G_1	328.27	$C_{17}H_{12}O_7$	244-246°C	0.78-15	Hepatotoxicity, Carcinogenicity, Mutagenicity,
Aflatoxin G_2	330.29	$C_{17}H_{14}O_7$	237-239°C	2.4	Low toxicity
Aflatoxin M_1	328.27	$C_{17}H_{12}O_7$	299-300°C	0.3-16	Carcinogenicity, Hepatotoxicity, Nephrotoxicity
Aflatoxin M_2	330.29	$C_{17}H_{14}O_7$	285-287°C	0.28	Mild Hepatotoxicity, Carcinogenicity,

Table 2: **Optimized Chromatographic Parameters.**

Parameter	Condition
Stationary Phase	Pre-coated silica gel 60 F_{254} aluminium sheets (20 × 20 cm)
Mobile Phase	Chloroform: Acetone: Water (140:20:0.3 v/v/v)
Detection wavelength	366 nm

Table 3: **Observations of system suitability parameters.**

Sl. No.	Volume of Standard taken (µL)	Peak Area (AU)			
		Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2
1.	~ 10	16280.23	4550.64	13703.5	3790.44
2.		16450.9	4535.4	13758.7	3825.69
3.		16410.7	4490.6	13732.3	3770.32
4.		16295.3	4515.98	13620.4	3840.7
5.		16305.69	4475.82	13632.1	3795.03
6.		16439.3	4500.66	13541	3785.42
Mean		16363.7	4511.52	13664.7	3801.3
± SD		71.3433	28.1091	74.4261	26.5195
%RSD		0.4359	0.6231	0.5446	0.6977
R_f		0.46	0.41	0.38	0.33

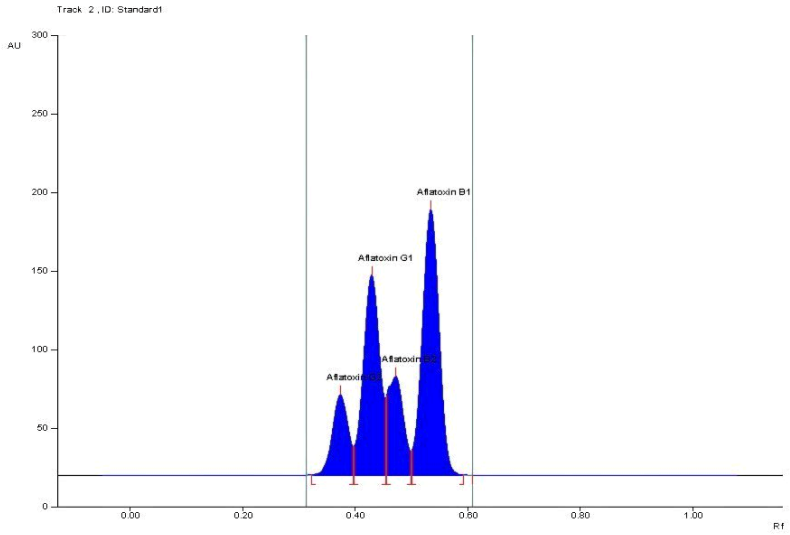

Figure 3: Densitogram of the mixed standard solution showing simultaneous detection of aflatoxins B_1, B_2, G_1, and G_2

20 mL distilled water, sonicate for 4 min, allow to stand for 10 min, and then filter. Collect the filtrate to 100 mL in a separating funnel. Add 50 mL NaCl solution and 20 mL petroleum ether, shake for 2 min, allow to separate for 10 min, and discard the ether layer. Shake the aqueous phase with 50 mL dichloromethane for 1 min and allow to separate (extraction of aflatoxin in dichloromethane). Filter the Dichloromethane layer through sodium sulphate anhydrous and evaporate to dryness. Dissolve the residue in 0.1 mL Acetonitrile.

Development of Plate

TLC plates were first developed in diethyl ether up to 8 cm. After drying, the plates were observed under UV light at 366 nm. The chamber was then dried and refilled with a solvent mixture of chloroform: acetone: water (140:20:0.3 v/v/v). The plate was developed in this mixture, 80 mm. After development, the plate was dried and visualized under UV light at 366 nm to detect aflatoxins.

Under the optimized HPTLC conditions, standard aflatoxins B_1, B_2, G_1, and G_2 showed clear and well-resolved peaks at their

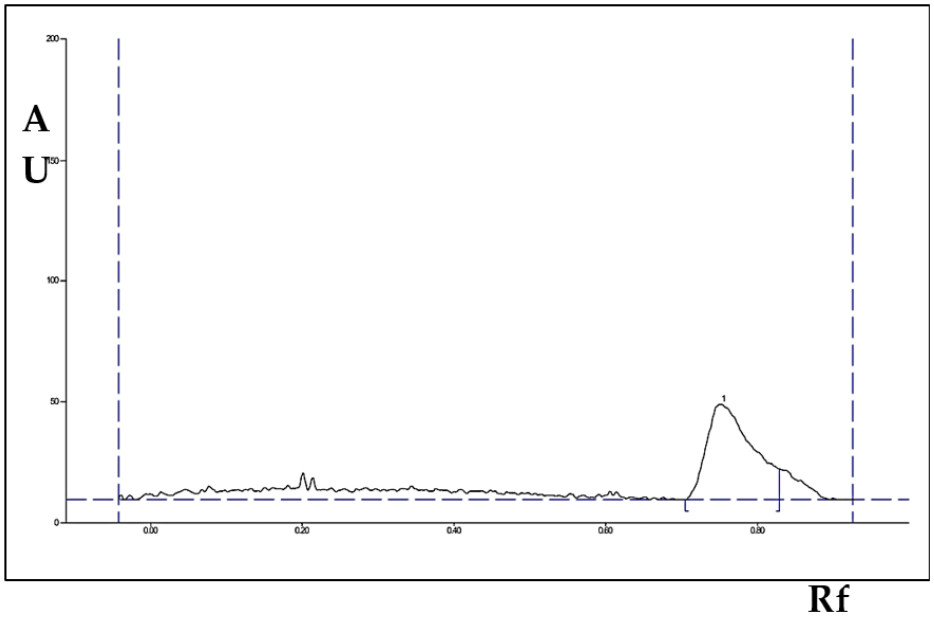

Figure 4: Densitogram of Sample.

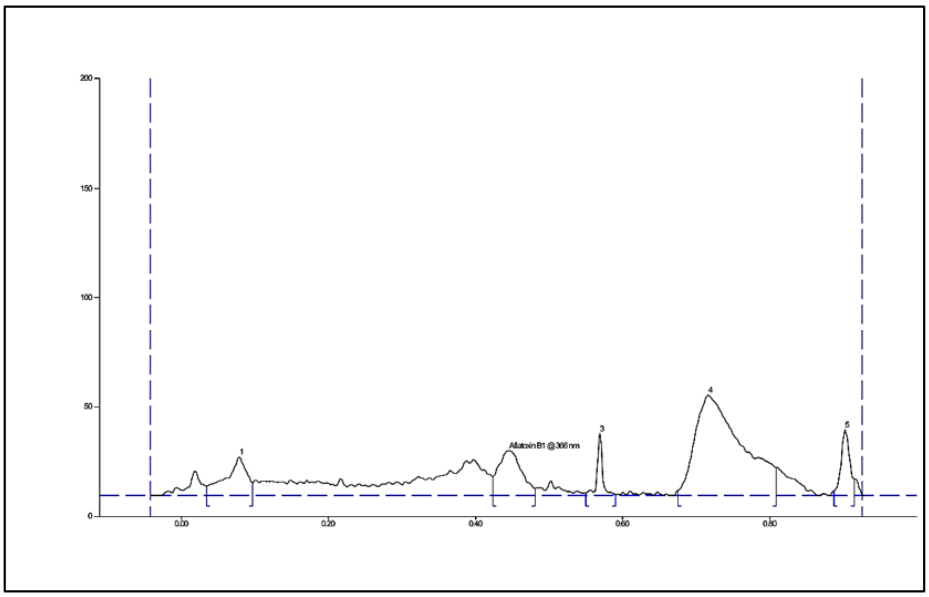

Figure 5: Densitogram of Standard during trial-1.

respective R_f values. However, analysis of the Haridrakhand sample revealed no detectable peaks at these Rf positions, indicating the absence of aflatoxins in the sample (Figure 4). These findings indicate that the investigated aflatoxins were not detected in the tested commercial Haridrakhand sample under the established analytical conditions.

RESULTS AND DISCUSSION

Mobile Phase Selection

The selection of an appropriate mobile phase is critical in the method development and validation of aflatoxins in botanical origin samples using HPTLC. Multiple solvent systems were evaluated to achieve optimal peak resolution and sharpness. Initially, a mixture of Acetonitrile: Dichloromethane (3:17 v/v) was tested, but it resulted in low resolution of aflatoxin peaks (Figure 5). A second trial using Water: Methanol: Acetonitrile (60:30:15 v/v/v) (Figure 6) led to irregular and poorly defined peaks, indicating instability in migration and poor compatibility with the stationary phase. In the third trial, Chloroform: Acetone (9:1 v/v) (Figure 7) showed some improvement but still resulted in broad peak shapes.

The fourth and final trial used a ternary mixture of Chloroform: Acetone: Water (140:20:0.3 v/v/v), which provided well-resolved, sharp (Figure 8), and symmetrical peaks for aflatoxins B_1, B_2, G_1, and G_2. The addition of a small amount of water likely improved the chromatographic behavior by reducing tailing and enhancing

Table 4: Observations of the Calibration Curve for Aflatoxin B_1 and G_1.

Sl. No.	Concentration (ng/band)	Peak Area (AU)	
		Aflatoxin B_1	Aflatoxin G_1
1.	2 ng	3572.7	3432.94
2.	4 ng	6405.5	5465.88
3.	6 ng	9808.22	8188.82
4.	8 ng	13391	10731.76
5.	10 ng	16320.7	13664.7

Table 5: Observations of the Calibration Curve for Aflatoxin B_2 and G_2.

Sl. No.	Concentration (ng/band)	Peak Area (AU)	
		Aflatoxin B_2	Aflatoxin G_2
1.	0.6 ng	1087.9	926.24
2.	1.2 ng	1941.64	1520.52
3.	1.8 ng	2719.91	2280.78
4.	2.4 ng	3610.22	3021.04
5.	3 ng	4566.52	3831.3

Table 6: Recovery Study for Aflatoxin B_1 and G_1.

Sl. No.	% level	Amount of Standard added (ng)	Peak Area of standard (AU)	Peak Area (AU)		Amt. Estimated (ng)		% Recovery	
				Aflatoxin B_1	Aflatoxin G_1	Aflatoxin B_1	Aflatoxin G_1	Aflatoxin B_1	Aflatoxin G_1
1.	80%	8.0 ng	16363.7 (Aflatoxin B_1) 13664.7 (Aflatoxin G_1)	12843.54	10782.5	7.85	7.89	98.10	98.63
2.	100%	10.0 ng		16086.09	13367	9.83	9.78	98.30	97.8
3.	120%	12.0 ng		19454.66	16180.23	11.89	11.84	99.08	98.67
Mean								98.49	98.37
±SD								0.5178	0.4911
%RSD								0.5257	0.4993

Table 7: Recovery Study for Aflatoxin B_2 and G_2.

Sl. No.	% level	Amount of Standard added (ng)	Peak Area of Standard (AU)	Peak Area (AU)		Amt. Estimated (ng)		% Recovery	
				Aflatoxin B_2	Aflatoxin G_2	Aflatoxin B_2	Aflatoxin G_2	Aflatoxin B_2	Aflatoxin G_2
1.	80%	2.4 ng	4511.52 (Aflatoxin B_2) 3801.3 (Aflatoxin G_2)	3597.5	3017.4	2.37	2.38	98.75	99.17
2.	100%	3.0 ng		4456.2	3776.7	2.96	2.98	98.66	99.33
3.	120%	3.6 ng		5248.6	4482.56	3.51	3.54	97.5	98.33
Mean								98.30	98.94
±SD								0.5692	0.5371
%RSD								0.5790	0.5429

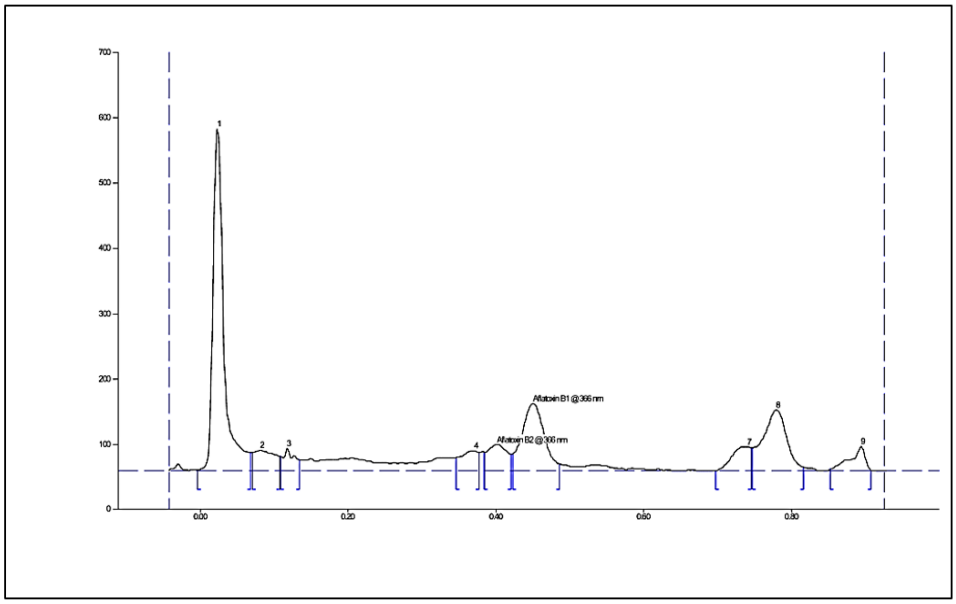

Figure 6: Densitogram of Standard during trial-2.

separation. This mobile phase was therefore selected as the optimized system for the validated HPTLC method.

System Suitability Study

The system suitability characteristics of the suggested method were represented as the SD and %RSD of a measurement series. Through repeated examination of a homogeneous reference solution, the system suitability study of the suggested approach was determined. The calculated %RSD values for each aflatoxin, B1 (0.43%), B2 (0.62%), G1 (0.54%), and G2 (0.69%), were all well below 2%, confirming the repeatability of the method. The R_f values remained consistent for each aflatoxin: B1 (0.46), B2 (0.41), G1 (0.38), and G2 (0.33), further validating system stability and suitability for quantitative analysis (Table 3).

Study of Calibration Curve

In the present study, aflatoxins B_1, B_2, G_1, and G_2 were detected and quantified using High-Performance Thin Layer Chromatography (HPTLC). The concentration range selected for Aflatoxin B_1 and G_1 was 2 to 10 ng per band (Table 4), while for Aflatoxin B_2 and G_2, it was 0.6 to 3 ng per band (Table 5). Standard solutions of these aflatoxins were applied to pre-coated silica gel 60 F_{254} plates and developed using a mobile phase consisting of chloroform, acetone, and water in the ratio 140:20:0.3 (v/v/v). The plates were then scanned under UV light at 366 nm, and the resulting fluorescent bands were recorded. The peak area corresponding to each concentration was measured and used to plot calibration curves. A consistent and linear increase in peak area with increasing concentration was observed for all aflatoxins (Figures 9-12), which confirms that the method is suitable for quantifying even low levels of these toxins.

Validation Study

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value, and the value found. This is sometimes termed trueness.

For accuracy assessment, an aflatoxin-free extract was spiked with known amounts of aflatoxin standard at 80%, 100%, and 120% concentration levels. A 10 µL volume of each spiked solution was applied to silica gel plates and developed using a mobile phase of chloroform: acetone: water (140:20:0.3). After development, the plates were scanned at 366 nm under UV light, and the peak areas were recorded to calculate the percentage recovery, which confirmed the accuracy of the method (Tables 6 and 7).

$$\text{Amt. of estimated drug} = \frac{\text{Area of Spiked Sample}}{\text{Area of standard}} \times \text{Standard Concentration}$$

$$\%\text{Recovery} = \frac{\text{Amount of drug recoverd}}{\text{weight of pure drug}} \times 100$$

Precision

The precision of the proposed method was assessed by calculating the Standard Deviation (SD) and Relative Standard Deviation (%RSD) from a series of replicate measurements. Six replicates of a homogeneous standard solution were analysed under optimized conditions to determine the precision of the method for aflatoxins B_1, B_2, G_1, and G_2. The %RSD values for each aflatoxin were found to be within acceptable limits, demonstrating the method's repeatability and confirming its reliability for routine quantitative analysis of these mycotoxins (Table 3).

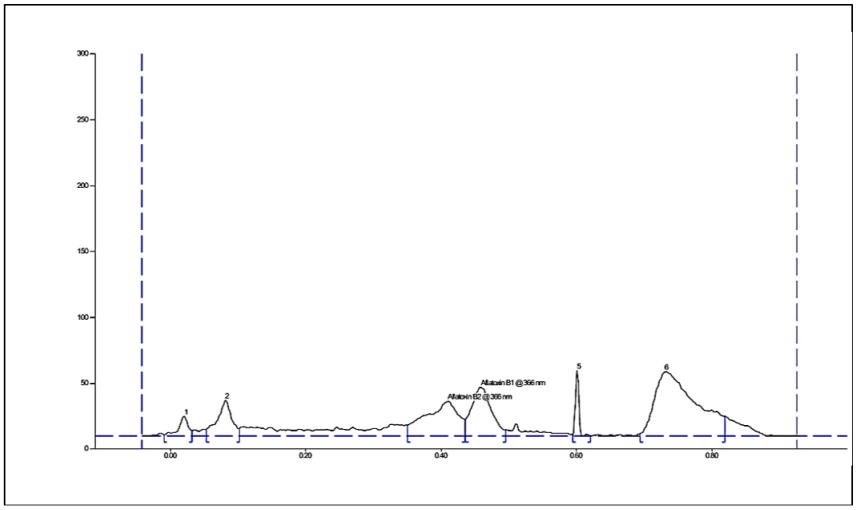

Figure 7: Densitogram of Standard during trial-3.

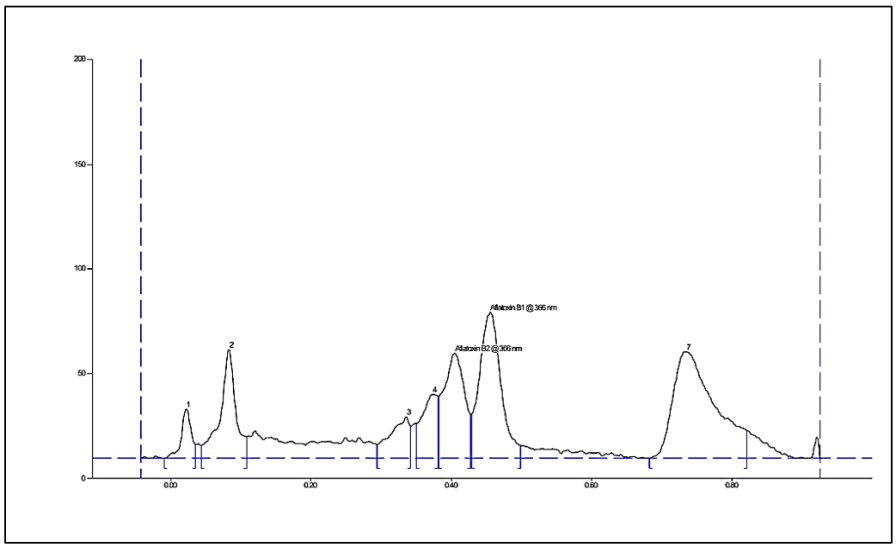

Figure 8: Densitogram of Standard during trial-4.

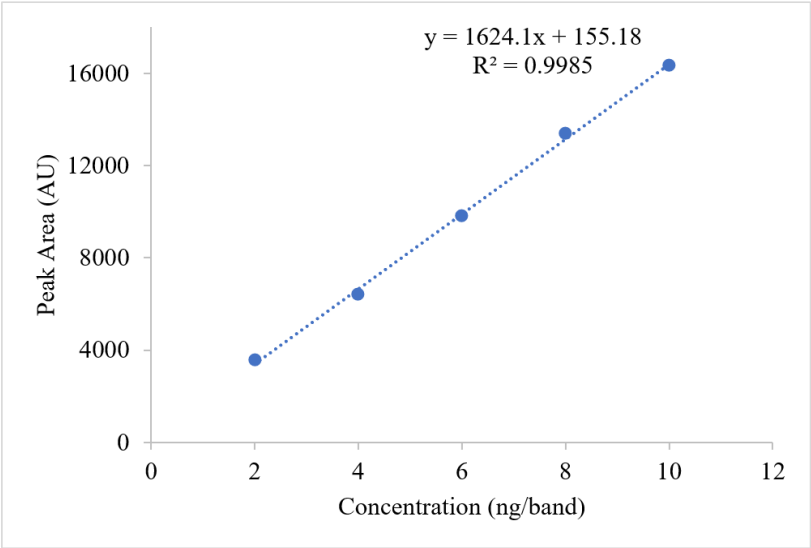

Figure 9: Calibration Curve of Aflatoxin B_1.

Table 8: Observations and Results of Intraday Study.

Time (hr)	The volume of Total Aflatoxin (µL)	Peak area (AU)			
		Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2
0 hr	~ 10	16353.61	4486.3	13646.4	3564.5
3 hr		16208.02	4561.07	13549.73	3523.64
6 hr		16189.6	4506.8	13500.96	3524.3
Mean		16250.41	4518.057	13565.7	3537.48
±SD		89.8471	38.63512	74.0229	23.4023
% RSD		0.5528	0.8551	0.5456	0.6615

Table 9: Observations and Results of Interday Study.

Days	The volume of std. Total Aflatoxin (µL)	Peak area (AU)			
		Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2
Day 1	~ 10	16250.41	4503.05	13465.7	3507.48
Day 2		16236.65	4486.3	13367.4	3459.99
Day 2		16184.3	4459.64	13346.8	3440.3
Mean		16223.79	4482.997	13393.3	3469.257
±SD		34.8816	21.8927	63.5406	34.5353
% RSD		0.2150	0.4883	0.4744	0.9954

Table 10: Results of LOD and LOQ of Total Aflatoxins.

Aflatoxins	LOD	LOQ
Aflatoxin B_1	0.1449	0.4392
Aflatoxin B_2	0.0645	0.1955
Aflatoxin G_1	0.1909	0.5785
Aflatoxin G_2	0.0718	0.2176

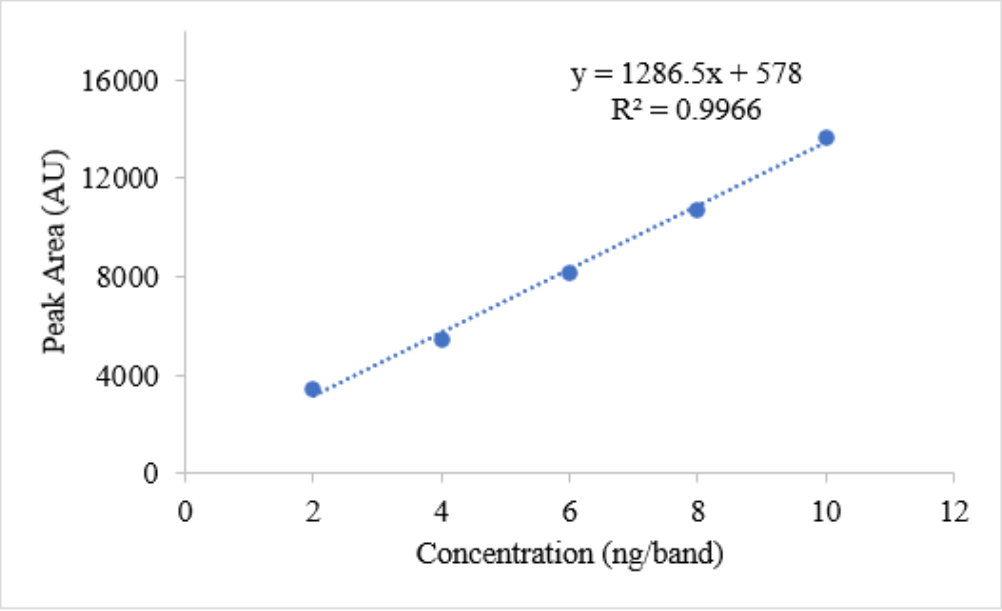

Figure 10: Calibration Curve of Aflatoxin G_1.

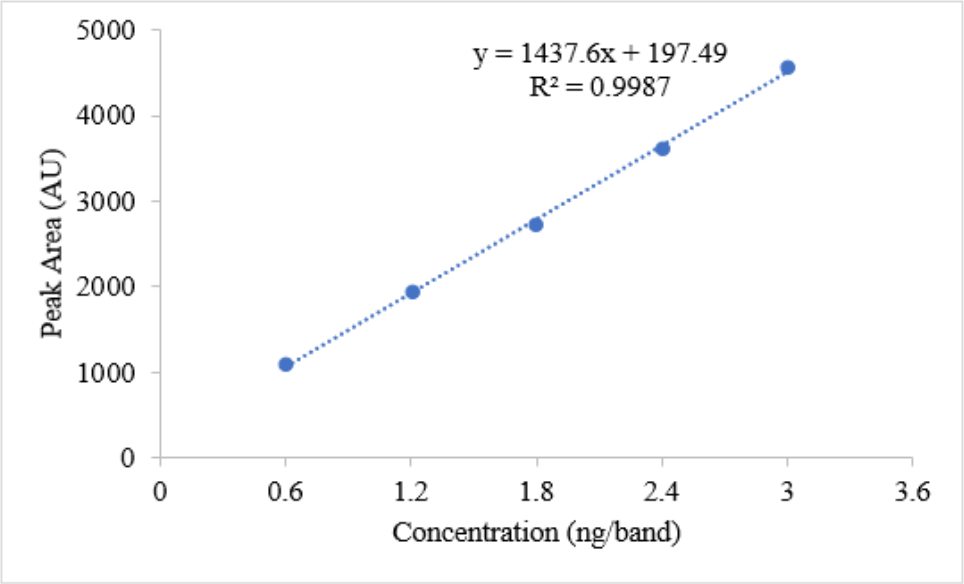

Figure 11: Calibration Curve of Aflatoxin B_2.

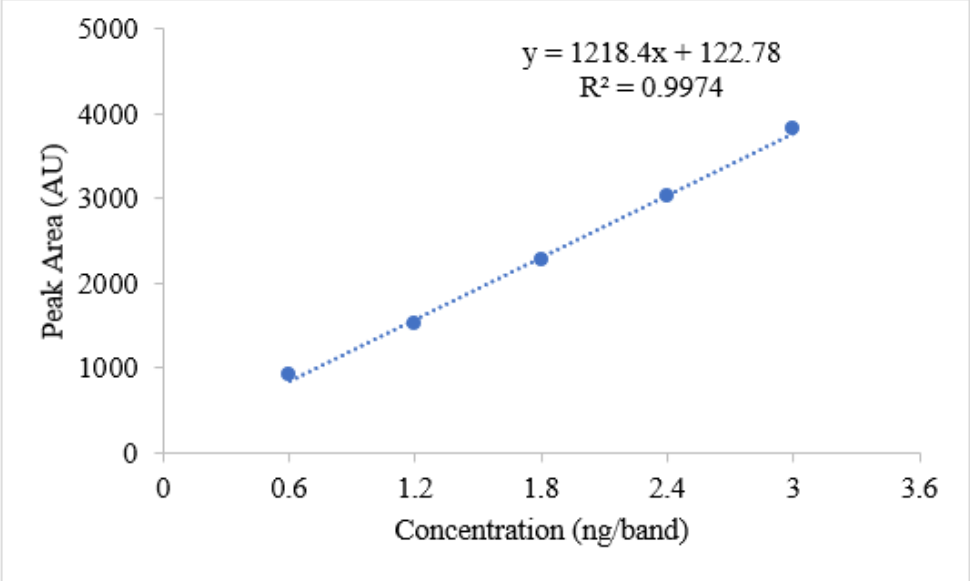

Figure 12: Calibration Curve of Aflatoxin G_2.

Intermediate Precision

A known concentration of aflatoxins B_1, B_2, G_1, and G_2 was spiked into the sample matrix to evaluate the method's intermediate precision, and 10 µL of each sample was applied onto the HPTLC plate for analysis. For the intraday precision study, samples were analysed at intervals of 3 hr up to 6 hr within the same day (Table 8). For the interday precision study, the analysis was carried out on three consecutive days (1st, 2nd, and 3rd days) (Table 9). The results obtained from both intraday and interday studies were used to assess the repeatability and reproducibility of the method. The precision was expressed in terms of Relative Standard Deviation (%RSD), demonstrating the reliability and consistency of the analytical procedure.

Limit of Detection and Limit of Quantification

The standard deviation of the Y-intercept and slope of calibration curves was used to calculate the LOD and LOQ for all the drugs using the following formulae (Table 10).

$$LOD = \frac{3.3\ (\sigma)}{S}$$

$$LOD = \frac{10\ (\sigma)}{S}$$

Where,

σ = Standard deviation.

S = Slope.

CONCLUSION

The HPTLC method established in this study proved to be accurate, precise, specific, and robust for the quantitative assessment of total aflatoxins. Its validation in line with ICH guidelines confirms its suitability for regulatory use and dependable performance. The method provides a reliable and economical approach for the routine analysis of herbal and botanical formulations. Additionally, it serves as a practical framework for analytical method development and validation aligned with international regulatory expectations. With further optimization, this technique can be extended to detect other mycotoxins and applied to more complex sample matrices, especially when combined with advanced technologies like HPTLC-MS. Its broad applicability across the food, pharmaceutical, and nutraceutical industries highlights its importance in enhancing product safety, quality control, and regulatory compliance.

ACKNOWLEDGEMENT

The authors are thankful to Qualichem Laboratories Nagpur for providing the research facility to carry out some of the research work.

ABBREVIATIONS

HPTLC: High-Performance Thin-Layer Chromatography; **UV:** Ultraviolet; **ICH:** International Council for Harmonisation; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **IARC:** International Agency for Research on Cancer; **MRLs:** Maximum Residue Limits; **ppb:** Parts per billion; **HPLC:** High-Performance Liquid Chromatography; **GC:** Gas Chromatography; **ELISA:** Enzyme-Linked Immunosorbent Assay; **LC-MS/MS:** Liquid Chromatography-Tandem Mass Spectrometry; **GMP:** Good Manufacturing Practice; **GLP:** Good Laboratory Practice; **WHO:** World Health Organization; **TLC:** Thin-Layer Chromatography; **SD:** Standard Deviation; **%RSD:** Percentage Relative Standard Deviation; **R_f:** Retention Factor; **MW:** Molecular Weight; **LD_{50}:** Lethal Dose, 50%; **v/v/v:** Volume-to-Volume-to-Volume Ratio.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

Vaishnavi S. Chaudhari: Data curation, Investigation, Visualization, Writing - original draft. **Shraddha D. Bhor:** Conceptualization, Investigation, Writing - original draft. **Swati N. Lade:** Methodology, Writing - original draft. **Diksha Undirwade:** Data Curation, Formal Analysis, Software. **Milind J. Umekar:** Formal Analysis, Investigation, Visualization. **Sushil S. Burle:** Conceptualization, Supervision, Writing - Review & Editing. **Radheshyam T. Lohiya:** Formal Analysis, Writing - Review & Editing

REFERENCES

1. Fletcher MT, Netzel G. Food Safety and Natural Toxins. Toxins (Basel). 2020;12(4):236. doi: 10.3390/toxins12040236. PMID: 32276351; PMCID: PMC7232292
2. Visciano P, Schirone M, Berti M, Milandri A, Tofalo R, Suzzi G. Marine biotoxins: occurrence, toxicity, regulatory limits and reference methods. Frontiers in Microbiology. 2016;7:1051. Available from: https://doi.org/10.3389/fmicb.2016.01051
3. Iqbal SZ, Selamat J, Ariño A. Mycotoxins in food and food products: current status. In: Food Safety. 2016. p. 113–23. Available from: https://doi.org/10.1007/978-3-319-39253-0_6
4. Turner NW, Subrahmanyam S, Piletsky SA. Analytical methods for determination of mycotoxins: A review. Analytica Chimica Acta [Internet]. 2008;632(2):168–80. Available from: https://doi.org/10.1016/j.aca.2008.11.010
5. El-Sayed RA, Jebur AB, Kang W, El-Demerdash FM. An overview on the major mycotoxins in food products: characteristics, toxicity, and analysis. Journal of Future Foods. 2022;2(2):91–102. Available from: https://doi.org/10.1016/j.jfutfo.2022.03.002
6. Khan R, Anwar F, Ghazali FM. A comprehensive review of mycotoxins: Toxicology, detection, and effective mitigation approaches. Heliyon. 2024;10(8):e28361. Available from: https://doi.org/10.1016/j.heliyon.2024.e28361
7. Awuchi CG, Ondari EN, Ogbonna CU, Upadhyay AK, Baran K, Okpala COR, et al. Mycotoxins Affecting Animals, foods, humans, and Plants: Types, Occurrence, toxicities, Action Mechanisms, Prevention, and Detoxification Strategies—A Revisit. Foods. 2021;10(6):1279. Available from: https://doi.org/10.3390/foods10061279
8. Zhou H, George S, Hay C, Lee J, Qian H, Sun X. Individual and combined effects of Aflatoxin B1, Deoxynivalenol and Zearalenone on HepG2 and RAW 264.7 cell lines. Food and Chemical Toxicology. 2017;103:18–27. Available from: https://doi.org/10.1016/j.fct.2017.02.017
9. Umereweneza D, Kamizikunze T, Muhizi T. Assessment of mycotoxins types in some foodstuff consumed in Rwanda. Food Control. 2018;85:432-6.
10. Kumar P, Mahato DK, Kamle M, Mohanta TK, Kang SG. Aflatoxins: a global concern for food safety, human health and their management. Frontiers in Microbiology. 2017;07:2170. Available from: https://doi.org/10.3389/fmicb.2016.02170
11. Pickova D, Ostry V, Toman J, Malir F. Aflatoxins: history, significant milestones, recent data on their toxicity and ways to mitigation. Toxins. 2021;13(6):399. Available from: https://doi.org/10.3390/toxins13060399
12. Shaw D. Risks or remedies? Safety aspects of herbal remedies in the UK. Journal of the Royal Society of Medicine. 1998;91(6):294–6. Available from: https://doi.org/10.1177/014107689809100602
13. Miroddi M, Mannucci C, Mancari F, Navarra M, Calapai G. Research and development for botanical products in Medicinals and Food Supplements market. Evidence-based Complementary and Alternative Medicine. 2013;2013:1–6. Available from: https://doi.org/10.1155/2013/649720
14. Colombo F, Restani P, Biella S, Di Lorenzo C. Botanicals in Functional Foods and Food Supplements: tradition, Efficacy and regulatory aspects. Applied Sciences. 2020;10(7):2387. Available from: https://doi.org/10.3390/app10072387
15. Zhang L, Dou XW, Zhang C, Logrieco A, Yang MH. A review of current methods for analysis of mycotoxins in herbal medicines. Toxins. 2018;10(2):65. Available from: https://doi.org/10.3390/toxins10020065
16. Wei G, Guo X, Liang Y, Liu C, Zhang G, Liang C, *et al.* Occurrence of fungi and mycotoxins in herbal medicines and rapid detection of toxin-producing fungi. Environmental Pollution. 2023;333:122082. Available from: https://doi.org/10.1016/j.envpol.2023.122082
17. Zhu YP, Woerdenbag HJ. Traditional Chinese herbal medicine. Pharmacy World & Science. 1995;17(4):103–12. Available from: https://doi.org/10.1007/bf01872386
18. Baldé N, Youla A, Baldé M, Kaké A, Diallo M, Baldé M, et al. Herbal medicine and treatment of diabetes in Africa: an example from Guinea. Diabetes & Metabolism. 2006;32(2):171–5. Available from: https://doi.org/10.1016/s1262-3636(07)70265-3
19. Muyumba NW, Mutombo SC, Sheridan H, Nachtergael A, Duez P. Quality control of herbal drugs and preparations: The methods of analysis, their relevance and applications. Talanta Open. 2021;4:100070
20. Ekor M. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. Frontiers in Pharmacology [Internet]. 2014;4:177. Available from: https://doi.org/10.3389/fphar.2013.00177
21. Robards K, Ryan D. Principles and practice of modern chromatographic methods. Academic Press; 2021 Dec 3.
22. Shivhare H, Chincholikar P. Analytical chemistry: Overview (Techniques & Applications). Journal of Pharmaceutical Negative Results. 2022;13(4):1364-74.
23. Poole CF. The essence of chromatography. Elsevier; 2003.
24. Patil AshwiniR, Ghagare PrajktaM, Deshmane BhavnaJ, Kondawar ManishS. Review on Chromatography Principal Types and it's Application. Research Journal of Pharmaceutical Dosage Forms and Technology. 2020;12(1):27. Available from: https://doi.org/10.5958/0975-4377.2020.00005.1
25. Clegg DL. Paper chromatography. Analytical Chemistry. 1950;22(1):48-59.
26. Patil HD, Patil CB, Patil VV, Patil PS, Pawar AR. A brief review on gas chromatography. Asian Journal of Pharmaceutical Analysis. 2023;47–52. Available from: https://doi.org/10.52711/2231-5675.2023.00008

27. Rajmane AD, Shinde KP. A review of HPLC method development and validation as per ICH guidelines. Asian Journal of Pharmaceutical Analysis. 2023;13(2):143-.
28. Srivastava M. High-Performance Thin-Layer Chromatography (HPTLC).2010. Available from: https://doi.org/10.1007/978-3-642-14025-9
29. Renger B. Planar Chromatographic Procedures in Pharmaceutical Analysis. Journal of AOAC International.2001;84(4):1217–8. Available from: https://doi.org/10.1093/jaoac/84.4.1217
30. Agbaba D, Carapic M. Applications of thin-layer chromatography in the pharmaceutical industry. InInstrumental thin-layer chromatography 2023 Jan 1 (pp. 519-573).
31. Renger B, Végh Z, Ferenczi-Fodor K. Validation of thin layer and high performance thin layer chromatographic methods. Journal of Chromatography A. 2011;1218(19):2712-21.
32. Reich E, Schibli A, DeBatt A. Supplemental Information for: Validation of High-Performance Thin-Layer Chromatographic Methods for the Identification of Botanicals in a cGMP Environment. Journal of AOAC International. 2008;91(1):1Supp-4Supp.
33. Koll K, Reich E, Blatter A, Veit M. Validation of standardized high-performance thin-layer chromatographic methods for quality control and stability testing of herbals. Journal of AOAC International. 2003;86(5):909-15.
34. Sherma J. Review of HPTLC in drug analysis: 1996-2009. Journal of AOAC international. 2010;93(3):754-64.
35. Vyas A, Jain V, Sahu U, Kumar N, Joshi N. HPTLC method development of herbal drugs and its validation: An overview. Research Journal of Pharmacy and Technology. 2023;16(8):3964-76.

Cite this article: Chaudhari V, Bhor S, Lade S, Umekar M, Burle S, Lohiya R, *et al*. Method Development and Validation for the Simultaneous Estimation of Aflatoxins in Botanical Origin Samples Using High-Performance Thin-Layer Chromatography. Indian J of Pharmaceutical Education and Research. 2026;60(4s):s1782-s1794.