

Analysis Of Aflatoxins: A Novel Approach To Enhance The Sensitivity And Detection Limits Of HPLC-FLD Technique Using Cyclodextrins.

Rajendra Bojanala*¹, Sundaram Ramachandran¹, Sheela Rani T.², Sujatha K.²

1 Analytical Development Laboratory, Himalaya Wellness Company,

Makali, Bengaluru-562162, India

2 Sri Ramachandra Institute of Higher Education and Research,

Chennai-600116, India

Abstract:

Several published reviews have proven the existence of mycotoxin contamination in herbal materials and related products. These reports indicated that mycotoxin contamination in herbal medicines is considered a global issue, particularly in the case of developing countries[16]. An High Performance liquid chromatography coupled with Fluorescence Detector (HPLC-FLD) method was developed and validated for simultaneous analysis of multi-class mycotoxins including aflatoxins (AFB1, AFB2, AFG1 and AFG2) in raw materials and Finished products of *Hygrophila Auriculata* seed samples collected from different regions and different seasons. The four Aflatoxins were extracted and cleaned up by using QuEChERS DSPE -based technique and were quantified under the influence of β -Cyclodextrin for increasing the sensitivity of the HPLC-FLD method and focusing on optimizing the extraction and clean-up conditions, as well as HPLC separation of targeted analytes, the developed method expressed good linearity for the four Aflatoxins within their respective linear ranges with correlation coefficients all higher than 0.99. The limits of detection (LODs) and quantification (LOQs) ranged from 0.02 to 0.05 $\mu\text{g}/\text{kg}$ and from 0.14 to 0.67 $\mu\text{g}/\text{kg}$, respectively. Recoveries for spiked *H. Auriculata* seed samples at three different levels were all above 85% with relative standard deviations (RSDs) below 6.69% for all analytes.

The proposed quantitative method with significant advantages including simple pre-treatment, rapid determination and high sensitivity would be the preferred candidate for the determination and quantification of Aflatoxin contaminants in complex matrixes, which well fulfilled the maximum residue limits (MRLs) from various countries.

Keywords: Aflatoxin, HPLC-FLD, QuEChERS, Cyclodextrin, Ph-Red Cell, phytopharmaceuticals, *Hygrophila Auriculata*.

Introduction:

For thousands of years, herbal medicines, also known as phytomedicines or botanical medicines, have been essential to maintaining global health. Herbal medications comprise herbs, herbal materials, herbal preparations, and completed herbal products that contain plant parts, other plant materials, or mixtures as active components, according to the World Health Organization (WHO)[1].

The usage of herbal medicines has grown significantly in popularity during the last ten years and all around the world.[2,3,4]. In affluent nations with relatively well-established health care system structures, the use of herbal medicines as supplemental therapy has increased recently [5,6].

The plant *Hygrophila auriculata* (K. Schum) Heine, of the Acanthaceae family, has long been used as a diuretic and to treat gout, oedema, urinary infections, and inflammation. It is described in ayurvedic literature as *Ikshura*, *Ikshugandha*, and *Kokilasha* and are characterised as having eyes like the *Kokila* or Indian Cuckoo. In India, Sri Lanka, Burma, Malaysia Nepal., and other countries, the plant is widely dispersed. Researchers have worked to confirm the effectiveness of the plant through scientific biological screening in response to numerous folk remedies

for a variety of ailments. The primary phytoconstituents of the plant are saponins, alkaloids, steroids, tannins, flavonoids, and triterpenoids. A review of the literature uncovered some noteworthy pharmacological actions, such as anti-nociceptive, anticancer, antioxidant, hepatoprotective, hypoglycaemic, haematinic, diuretic, free radical scavenging, anthelmintic, anti-inflammatory, antipyretic, anabolic and androgenic activities.⁷

Herbs, 40-100 cm tall with unbranched, sub quadrangular stems with numerous fasciculate, swollen node, hispid with long hairs. Leaves sub-sessile, lanceolate, 6-15×1.5-3 cm, acute, hairy, in whorls of 6 at each node, the two outer ones much larger than the four inner ones. Thorns from the axils of leaves sharp, 2-3 cm long, yellowish-brown. Flowers in axillary clusters of eight at each node in 4 pairs. Bracts lanceolate, hairy and ciliate, like the leaves; bracteoles linear-lanceolate, 1.5-2 cm long, with hyaline margins in the lower part, hairy and ciliate with long white hairs. Calyx 4 partite; upper sepals broader unequal, longer than the other three, all linear lanceolate, 1.2-2 cm long, with hairy on the back and hyaline ciliate margin. Corolla purple-blue, 2-3 cm long, bilipped; tube 11-13 mm long, swollen at top; stamens didynamous 4; filaments glabrous. Ovary 2 celled with 4 ovule, capsules linear-oblong, 4 seeded 5-7 mm long, pointed. Seeds, ovate, compressed, hairy, hygroscopic, black. Soil type: Wet soil of Marshy places. Locality: In all districts. Flowers and fruits: June to February.⁸

The quality control procedures around herbal medicines have emerged as the key issue for both health authorities and the general population as the use of herbal medicines increases globally. Contamination is crucial to keep an eye on in the case of herbal remedies since it is possible for toxicities to be caused by external factors, which are often linked to unwanted poisonous compounds, rather than by the herbs themselves. In particular, microbial and fungal contamination has long been of concern on a global scale. Previous studies have shown that toxigenic fungus species that are produced from the soil or plants themselves can contaminate herbal medications. Species from the genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* are among these toxic fungus.^{9,10,11,12}

These fungi produce mycotoxins under unfavorable environmental conditions, which are secondary metabolites that could contaminate different plants in the field or at any time during the collection, handling, transportation, or storage of the plants (for example, mycotoxin contamination produced by *Fusarium* species can occur in the field and build up during the harvesting and drying stage, while additional toxins primarily produced by *Penicillium* and *Aspergillus* species can coexist with those produced by *Fusarium* and The most often contaminated mycotoxin species, according to reports on mycotoxin contamination screening of medicinal herbs and related products, are aflatoxins (AFs), ochratoxins, Fumonishins (FBs), trichothecenes, and zearalenones (ZENs).^{13,14,15}

Chemical characteristics of aflatoxins are they exhibit intense blue or green fluorescence under UV: aflatoxins B1, B2, G1 and G2 They encompass a considerable variety of low molecular weight compounds with diverse chemical structures and biological activities.

Solubility: soluble in moderately polar organic solvents (e.g., chloroform, methanol, dimethylsulfoxide), scarcely soluble in water (10–30 mg/mL) and insoluble in non-polar organic solvents

The B-type aflatoxins are characterized by a cyclopentane E-ring. These compounds have a blue fluorescence under long-wavelength ultraviolet light.

(B) The G-type aflatoxins, with a green fluorescence, have a xanthone ring in place of the cyclopentane.

(C) Aflatoxins of the B2 and G2 type have a saturated bis-furanyl ring.

Analytical techniques have been published in great numbers. Thin-layer chromatography (TLC)^{2,3}, enzyme linked immunoassay (ELISA)⁴⁻⁷, gas chromatography^{8,10} paired with tandem mass spectrometry¹², or thermal spray liquid chromatography–mass spectrometry¹³ are the techniques that are used the most commonly.

Solvation also affects the emission maxima, causing a shift towards shorter wavelengths (blue shift) when the environment becomes less polar. This effect was seen for AFB1 in multiple solvents (Dirr & Schabert 1987, Va´zquez et al. 1991) The inherent fluorescence of the aflatoxins can be activated by UV light (360–365 nm). AFG1 and AFG2 have a blue/green fluorescence, while AFB1 and AFB2 have a blue fluorescence (emission around 440 nm). The nomenclature of the "B" or "G" aflatoxins was obtained from the colour of the fluorescence (emission circa 460 nm). The content of the solvent, the temperature, and interactions with solid phases like silica gel can all

have an impact on the emission of aflatoxin (Robertson & Pons 1968, Van Duuren et al. 1968, Dirr & Schabert 1987, Va'zquez et al. 1991).

In their original study, Holland and Sepaniak (1993) separated ten mycotoxin standards using a combination of α , β and γ CDs, sodium dodecyl sulphate (SDS), and acetonitrile as buffer additives.

Potential impacts on fluorescence were unfortunately not disclosed because the study used absorbance rather than fluorescence for detection. Aflatoxin B1, B2, G1, and G2 retention was barely impacted by α , β and γ CDs (Holland & Sepaniak 1993). Wei et al's work was the first to include the fluorescence enhancement effects of CDs with capillary electrophoresis of aflatoxins (2000). Aflatoxin B1, B2, G1, and G2 were excited using a titanium: sapphire laser (730–770 nm) with either carboxymethyl-CD or sulfated-CD present at 2–10 mM in the electrophoretic buffer. The carboxymethyl β CD served as a helpful tool¹⁴.

The sample can be purified using the immunoaffinity column, which can pick out and adsorb aflatoxins (B1, B2, G1, G2) from the sample solution. The HPLC analysis can then be done right away using the purified sample solution^{15,16}.

To speed up testing, boost signal-to-noise ratio, and increase detection method accuracy, affinity columns can be employed in conjunction with HPLC.

The antigen-antibody response serves as the measurement's foundation. The aflatoxin in the sample is removed, filtered, and diluted before being gently fed through the aflatoxin immunoaffinity column, where antibodies are bound. The immunoaffinity column is then washed to get rid of other unrelated chemicals that haven't been bound after the toxins bind to the antibodies in the column. Aflatoxin is then administered after being eluted with methanol^{17,18}.

2. Materials and Methods

Aflatest-p Immunoaffinity column (Make-R-Biopharm)

rOQ QuEChERS salts of catalogue number (KS0-8910)

Aflatoxin standards G₂, G₁, B₂ & B₁ (Manufacturer: Supelco, Purity-99.0%)

3. Sampling, Extraction and Cleanup

3.1 Sampling

Sampling plays a critical role in how precise the determination of mycotoxin levels are due to the fact that the molds that generate mycotoxins do not grow uniformly on the substrate and existing contamination in natural samples is not homogeneous. A study carried out in 2003 demonstrated that the actual mycotoxin concentration of a bulk lot cannot be determined with 100% certainty due to the variability associated with each step in the mycotoxin test procedure. Thus, the sampling procedure could dramatically impact the final results regarding the determination of mycotoxins¹⁹.

3.2 Extraction Procedure

The purpose of extraction is to remove mycotoxin from the herbal medicine matrix as much as possible into a solvent that is suitable for subsequent clean up or direct analysis. The extraction solvent and method used are the two most important considerations for the extraction procedure.

3.3 Extraction Solution

Currently, the most common solvents used for the extraction of mycotoxins from herbal medicines are methanol-water and acetonitrile-water. Acetonitrile–water (84:16, v/v) methanol: water (80:20, v/v) are the most commonly

used solvents. However, in order to enable higher extraction efficiencies and lower matrix effects (MEs), the extraction solvents still need to be compared across many studies.

3.4 Extraction Method

In addition to the type of extraction solvent used, the extraction method is another critical determinant of the extraction efficiency. The conventional solid-liquid extraction technology used for mycotoxin extraction involves the use of ultrasonic extraction, homogenization, and shaking. Vortexing and blending are also used sometimes for the detection of mycotoxins in herbal medicines. When selecting the extraction method, the matrix constitution should be considered. A recent report demonstrated that samples with different matrix types required their own specific extraction method. For example, in the case of matrices with high fatty oil and polysaccharide contents that are more viscous, an ultrasonography extraction method was found to be prone to aggregating the extracts and thereby prevented the dissolution of AFs from the matrices²⁰.

3.5 Clean-up

Considering the low residue level of mycotoxins (generally at $\mu\text{g kg}^{-1}$ level) and the complex chemical composition of herbal medicine samples, a clean-up step was required prior to instrumental analysis in most cases. This clean-up step may function to further concentrate mycotoxins in addition to removing sample impurities. A variety of clean-up methods have been implemented and shown to contribute to the accurate measurement of mycotoxins in herbal medicine, including solid phase extraction (SPE) and immunoaffinity column (IAC).

4. Chromatographic Techniques for Detecting/Quantifying Mycotoxins

In addition to Thin-layer chromatography (TLC) methods, chromatographic methods, such as Liquid Chromatography (LC) and Gas Chromatography (GC) coupled to a specific detector, are the most commonly used techniques to date for obtaining highly accurate results. In the case of single mycotoxin analysis, e.g., AF, ochratoxin, the traditional LC with a FLD detector is the most widely used method for herbal medicine matrices. Currently, the co-occurrence of multiple mycotoxins has gained increasing attention. Therefore, liquid chromatography–tandem mass spectrometry (LC-MS-MS) is the technique choice for the simultaneous determination of various mycotoxins that belong to different chemical families.

Standards used from R-biopharma in concentration of:

Name of toxin	Concentration in ppm in Methanol
B ₁	1.5
B ₂	1.5
G ₁	0.3
G ₂	0.3

4.1. Chemicals/Reagents used:

Methanol (HPLC Grade) used as diluent Sodium phosphate (AR Grade), Acetonitrile (HPLC Grade), Formic acid (AR Grade), Beta Cyclodextrin- (Molecular weight ≥ 1000) (β -Cyclodextrin/BCD) & Purified water

4.2. Mobile phase preparation:

Measure 630 ml of 20 Mmol sodium phosphate buffer (pH 7.2), transfer to 1000 ml volumetric flask. Similarly measure 220 ml of Methanol and 150 ml of acetonitrile, transfer to same volumetric flask. Add 3G of Beta Cyclodextrin and mix gently, filter the mobile phase through 0.45 μ filter.

Note: The usage of 119mg Potassium Bromide is necessary if Ph-Red Cell is used and not required when Kobra cell is used.

Standard preparation:

Aflatoxin mix standard should be allowed to attain the room temperature, place 5 ml of methanol into a 10 ml volumetric flask. Add 100 μ l of undiluted standard mix to the volumetric flask and mix well. Make-up the volume with methanol.

[Aflatoxin Standards are procured from R-Bio Pharma consisting of B1 & G1 (1.5 ppm/mL), B2 & G2 (0.3 ppm/mL)]

HPLC Condition:	
Column	: C18 Luna ODS Phenomenex (250 X 4.6) mm ,5 μ particle size
Mobile Phase	: Purified Water: Methanol: Acetonitrile (63:22:15) + 3g β -Cyclodextrin
Flow rate	: 1.0 ml/min
Injection Volume	: 20 μ L
Column temperature	: 25°C
Detector (Fluorescent detector)	: Excitation -355nm Emission -460
Run time	: 40 Mins
External Derivatization	: Ph-red cell/Kobra Cell
Elution Order	: G ₂ , G ₁ , B ₂ & B ₁
Retention times	: 13.0, 15.5, 18.0 & 21.7 respectively

5. Plant material and preparation of extract:

To obtain a representative sample from the purchased lots, the collected Seeds are sampled using the $\sqrt{n} + 1$ formula or any appropriate sampling approach. They are first ground for 5–10 minutes at a low speed in a mixer grinder or other suitable milling device, then moved to a 25# mesh and sieved, transferred to a suitable tray, and any residue transferred back into the milling device and ground once more. The above-mentioned steps of sieving and grinding

are repeated until 90–95 percent of the material has been processed. The powder collected in the tray is then thoroughly mixed, ensuring the homogeneity of the sample that was taken.

5.1 Extraction from Sample:

1. Weigh and transfer 25 g of sample in to a 50 mL extraction tube
2. Add 10 mL of water and 10 mL of methanol with 1.0 % formic acid
3. Shake the contents vigorously for 5 mins
4. Dispense contents of the included RoQ QuEChERS extraction packet containing 4.0 g MgSO₄, 1.0 g NaCl, (KS0-8910) into the 50 mL tube containing homogenized sample.
5. Shake vigorously by hand for 1 minute
6. Centrifuge for 5 minutes @ 4000 rpm, making sure that the solid material is at the bottom of the tube and a liquid layer forms on top of the solid material.

5.2 Clean up using dispersive Solid Phase Extraction (dSPE)

1. Transfer the supernatant from Step 6 of the extraction process into a 15 mL centrifuge tube containing 900 mg MgSO₄ and 150 mg PSA (KS0-8924)
2. Shake vigorously by hand for 30 seconds
3. Centrifuge for 5 minutes at 4000 rpm to separate solid material from the liquid layer

5.3 Immuno-Affinity column clean-up:

1. Attach an Aflatest-P column to the pump stand (precondition using methanol and water as per the Immunoaffinity column instructions)
2. Pipette 10 ml of supernatant on the column and allow to absorb on the column.
3. Adjust the air-pressure to have a flow rate of 1–2 drops/second.
4. After all the liquid has flowed through, add 10 mL of water to wash the column at a flow rate of 2–3 drops per second. Repeat this wash step one more time.
5. Once the sample is passed through the column, rinse the column with 10 ml of purified water. Repeat purified water rinse.
6. Place an HPLC vial under the tip of the column and add 1 ml of methanol to the column. Collect methanol added at a flow rate of 15 drops per minute and collect the eluate and bring to volume of 1 mL with methanol.
7. Filter using 0.22 µm nylon filter.

The sample is now ready for injection into the HPLC

5.4 Procedure:

After the stabilization of the instrument with the mobile phase, inject 20 µl of blank (methanol), standard five injections followed by sample solution into the column of the HPLC instrument and record the chromatogram for 35 minutes.

The method should pass the system suitability parameters. i.e; Tailing factor not more than 2, Theoretical plates not less than 5000 & resolution is not less than 2.0 between the peaks.

6. Method validation parameters:

Method validation is an integral and important part of the method development; it is the process by which a method is put to test by the developer for accuracy, reliability, and preciseness of the method [101]. different criteria's must be explored to verify the methodological procedure meets per the existing regulatory requirements.

6.1 Specificity:

The Specificity is performed by injecting diluent (blank), working standard and sample preparation, any interference at the retention time of the analytes were checked. Specificity is established based on system suitability, resolution factor and peak purity index.(Figure-1) (Table-1&2)

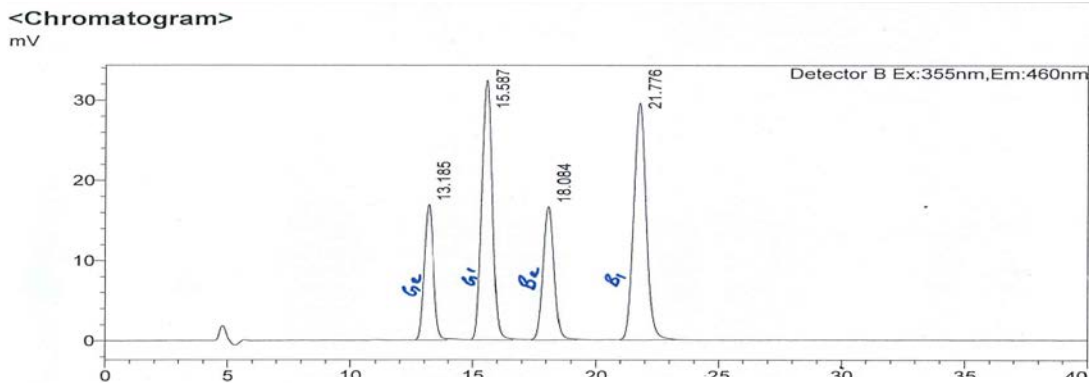


Figure-1: Chromatogram of Aflatoxins eluted

Table 1: System suitability observations

Name of the Analyte	Retention time (minutes)	Theoretical plates	Peak asymmetry	Resolution
G ₂	13.1	5821	1.04	-
G ₁	15.5	6339	1.03	3.25
B ₂	18.0	8221	1.07	3.14
B ₁	21.7	9216	1.06	4.33

Table 2: Peak purity index observations

Name of the Analyte	Standard		Sample	
	Peak purity index	Single point Threshold	Peak purity Index	Single point threshold
G ₂	1	0.999	1	0.999
G ₁	1	0.999	0.999	0.999
B ₂	1	0.999	0.999	0.999
B ₁	1	0.999	1	0.999

6.2 Repeatability:

System precision was established for working standard solution by injecting five injections from the single preparation. The average area under curve (AUC) and percentage of relative standard deviation (%RSD) was recorded. (Table-3)

Table 3: System precision observations

Injection No.	Aflatoxin G ₂ (AUC)	Aflatoxin G ₁ (AUC)	Aflatoxin B ₂ (AUC)	Aflatoxin B ₂ (AUC)
1	149039	283079	1653459	3600440
2	145000	281160	1653022	3609358
3	145846	282333	1659065	3603383
4	148229	284987	1665679	3604345
5	143316	284840	1658507	3604032
% RSD	1.60	0.58	0.31	0.09

6.3 Intermediate precision:

The intra-day precision of the method was established by injecting six injections from single preparation at the LOQ concentration respectively for B₁, G₁, B₂ & G₂ (established through 10 times the s/n ratio). The mean assay and % RSD values were calculated. (Table-4)

Table 4: Intra-day precision observations

Injection No.	Aflatoxin G ₂ (AUC)	Aflatoxin G ₁ (AUC)	Aflatoxin B ₂ (AUC)	Aflatoxin B ₂ (AUC)
1	146744	285237	1655511	3603278
2	146012	281967	1661381	3629243
3	140209	282333	1653212	3617230
4	145310	279050	1649994	3612517
5	143316	283595	1646839	3606148
6	143182	289562	1657928	3625252
% RSD	1.66	1.25	0.32	0.29

6.4 Inter-day precision:

The inter-day precision of the method was established by injecting six injections from single preparation at the LOQ concentration respectively for B₁, G₁, B₂ & G₂ (established through 10 times the s/n ratio). The mean assay and % RSD values were calculated. (Table-5)

The comparative %RSD's between intra and inter-day precision was recorded.

Table 5: Intra-day precision of method

Injection No.	Aflatoxin G ₂ (AUC)	Aflatoxin G ₁ (AUC)	Aflatoxin B ₂ (AUC)	Aflatoxin B ₂ (AUC)
1	145255	281846	1656111	3603821
2	143102	287835	1648788	3600345
3	142857	284764	1652616	3600201
4	146284	297387	1665577	3604747
5	145458	287574	1654577	3610769
6	145785	284177	1651429	3605120
% RSD	1.00	1.89	0.35	0.11

6.5 Linearity:

Linearity was performed by injecting eight different concentrations of the sample ranging from 5% to 150% of standard concentration (15ppb of B₁ & G₁, 3 ppb of B₂ & G₂). The regression coefficient (R²) was recorded and tabulated (Table-6).

Table 6: Linearity of method

Name of the Aflatoxin	Regression equation	Regression coefficient (R ²)
G ₂	495400x-1631.5	0.9973
G ₁	288452x-2897.2	0.9991
B ₂	562665x-5060.1	0.9992
B ₁	30812x+1497.5	0.9998

6.6 Accuracy:

Accuracy can be defined as exactness of an analytical method or the closeness of obtained results. Accuracy was derived by spiking the 100% concentration of the standard to matrix matched blank and the recovery of the respective analyte is recorded. (Table-7)

Table 7: Recovery from the method

Injection No. (Spiked Concentration)	Aflatoxin G ₂ (% Recovery)	Aflatoxin G ₁ (% Recovery)	Aflatoxin B ₂ (% Recovery)	Aflatoxin B ₁ (% Recovery)
50%-1	102.11	92.12	106.4	97.08
50%-2	110.28	87.13	97.35	94.86
50%-3	104.61	87.77	99.24	94.54
100%-1	104.08	101.01	106.16	104.08
100%-2	105.99	95.13	97.52	104.1
100%-3	101.34	104.48	101.69	102.49
150%-1	101.11	99.15	103.8	98.35
150%-2	103.41	102.15	102.6	104.19
150%-3	102.46	101.78	101.32	107.54
Mean Recovery	103.93	96.75	101.79	100.80
%RSD	2.75	6.69	6.93	3.29

4.65

6.7 Robustness:

Reliability of the method was performed with respect to the deliberate changes in method parameters like variation in the flow rate of mobile phase (± 0.1 ml/min) and variation in the wavelengths Excitation -365nm (± 3 nm) which will impact the RT's and response of the analytes.

The sample solution, standard solution and diluent was analysed with deliberate changes in method parameters by injecting two replicates of single preparation and readings are recorded (Table-8 & 9).

Table 8: Robustness of the method

Robustness variables	Aflatoxin G ₂ (%RSD)	Aflatoxin G ₁ (%RSD)	Aflatoxin B ₂ (%RSD)	Aflatoxin B ₂ (%RSD)
Flow rate (0.9 ml/minute)	0.16	0.50	0.27	0.50
Flow rate (1.1 ml/minute)	0.05	0.01	0.01	0.01
Excitation -362nm &Emmission-435nm	1.68	0.83	0.54	0.25
Excitation -368nm &Emmission-435nm	0.98	1.14	0.14	0.12

Table 9: Comparison between assay of robustness and intraday precision

Robustness variables	%RSD	Comparison of % RSD between assay of robustness and intra-day precision
Flow rate (0.9 ml/minute)	0.37	0.05
Flow rate (1.1 ml/minute)	0.00	0.07
Excitation -352nm &Emmission-460nm	0.06	0.00
Excitation -358nm &Emmission-460nm	0.09	0.18

7. RESULTS and DISCUSSION

Detection and Screening of Mycotoxins

- Because of the diverse chemical structures of mycotoxins, the presence of trace amounts of toxins in very complicated matrices that interfere with analysis, and the uneven distribution of the toxins in the sample, analysis of mycotoxins is a difficult task.
- Because many steps are involved in the analysis, it is not uncommon that the analytical error can amount to 20–30%
- To obtain reliable analytical data, an adequate sampling program and an accurate analytical method are both important.
- To minimize the errors, studies have led to many improved and innovative analytical methods for mycotoxin analysis over the years.
- New, more sensitive TLC, HPLC, and GC techniques are now available.
- The MS methods have also been incorporated into HPLC systems.
- New chemical methods, including capillary electrophoresis and biosensors are emerging and have gained application for mycotoxin analysis.
- After a number of years of research, immunoassays have gained wide acceptance as analytical tools for mycotoxins in the last decade. Antibodies against almost all the mycotoxins are now available. Some quantitative and qualitative immunoassays have been approved. Many immunoscreening kits, which require less than 15 min. per test, are commercially available.
- Rather than analysis of toxin, PCR methods, based on the primers of key enzymes involved in the biosynthesis of mycotoxins, have been introduced for the determination of toxicogenic fungi present in foods.

- Detailed protocols for mycotoxin analysis can be seen in several of the most recent reviews and books and the most recent edition of AOAC .

Cyclodextrin forming inclusion complexes with different food matrices and toxins²¹. And according to simulations of AFB1 docking with α and β -CDs (Figure-2), it has been hypothesised that putting the fluorophore inside the CD cavity could lessen the solvent's quenching effect and boost fluorescence. Although the creation of a 1:1 inclusion complex has been proposed, the precise mechanism of the CDs' interaction with aflatoxins is unknown.

The combination of buffer and cyclodextrins were earlier studied and the suitable combinations of α and β -CDs with the sodium phosphate buffer was studied using HPLC technique for the enhancement of sensitivity and in turn the LOD & LOQ of the methods for better quantification of Aflatoxins.

The buffer was prepared at various concentrations and adjusted the pH to 7.2 using orthophosphoric acid where in best possible resolutions were achieved in combination of methanol and acetonitrile and the excitation response was noted, which is plotted as below graph (Figure-3):

The highest absorbance was noted for 20mmol concentration of buffer and the same was considered for the further experimentation.

The α and β cyclodextrins were studied at different concentrations like 1g/Lt, 2g/Lt, 3g/Lt, 5g/Lt of mixed buffer. And the highest response was obtained at 3g/Lt. concentration of β -cyclodextrin whereas α -cyclodextrin did not offer much resolution of the peaks as well as no increased absorbance was achieved.

Thus finalizing the concentration of the buffer as 20mmol (pH 7.2) and 3g/Lt of β -cyclodextrin the respective system suitability parameters were achieved as per the requirement and further validation procedure to finalize the method were formed as per the below details²².

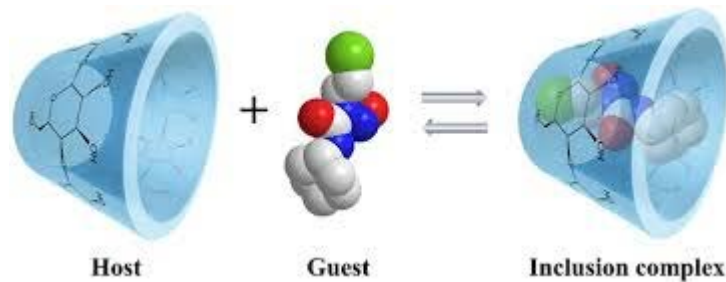


Figure-2: Molecular design image of the complex formed with Cyclodextrin

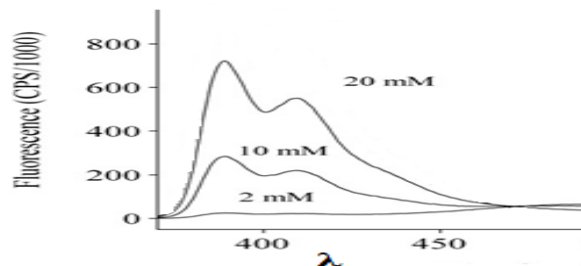


Figure-3 : Fluorescence Intensity changes observed after Complexing with Cyclodextrin

Selectivity: (No interference with the peaks of interest due to mobile phase or diluent)

Precession: (G_2, G_1, B_2 & $B_1 \leq 10\%$ for 6 injections at LOQ level)

Linearity: (G₂- 0.9973 G₁-0.9991, B₂-0.9992 & B₁-0.9998)

Accuracy: (G₂- 103.93% & 2.75% RSD G₁-96.75% & 6.69% RSD, B₂-101.79% & 3.29% RSD & B₁-100.80% & 4.65% RSD)

LOQ: (G₂- 0.02ppb G₁-0.05ppb, B₂-0.02ppb & B₁-0.05ppb)

Robustness performed with minor changes in

Flow rate (±10%): at 0.9 ml/min : (G₂- 0.16% RSD G₁-0.50% RSD, B₂-0.27% RSD & B₁-0.37% RSD), at 1.1 ml/min : (G₂- 0.05% RSD G₁-0.01% RSD, B₂-0.01% RSD & B₁-0.01% RSD)

Excitation Wavelength changes (±3nm): at 358nm : ((G₂- 1.68% RSD G₁-0.83% RSD, B₂-0.54% RSD & B₁-0.25% RSD), at 352nm : ((G₂- 0.98% RSD G₁-1.14% RSD, B₂-0.14% RSD & B₁-0.12% RSD))

The LOQ values obtained with respect to Buffer as such and buffer with beta cyclodextrin combination is provided below where in we can see significant increase of sensitivity in the analysis (Table-10).

Table 10: LOD & LOQ

	LOD values with only Buffer (ppb)	LOD values with Buffer+β-CD(ppb)
G2	0.24	0.02
G1	0.67	0.05
B2	0.14	0.02
B1	0.35	0.05

The results shows that the method is sensitive and robust enough to detect the aflatoxins in ppb level and the method was completely validated demonstrating its suitability, linearity, repeatability, are as per the ICH Q2 R1 guidelines proposed for validation of analytical methods. The results are complying as per the required acceptance criteria

8. CONCLUSIONS

In conclusion, the approach of adding Beta cyclodextrin into the usual method of analysis of aflatoxins increases the sensitivity of the method 3 folds, which provides support to trace level detection of toxins in the samples collected avoiding the false negative results because of lower sensitivity, this technique becomes more powerful in the monitoring of herbal products and phytopharmaceuticals for human and animal consumption. They assist in predicting shelf life or storage conditions in a much better way and enhancing the approval of herbal products & phytopharmaceuticals by the regulatory bodies.

REFERENCES

1. World Health Organization (WHO) *WHO Traditional Medicine Strategy 2002–2005*. WHO; Geneva, Switzerland: 2002.
2. Zhu Y.-P., Woerdenbag H.J. Traditional Chinese herbal medicine. *Pharm. World Sci.* 1995;17:103–112. doi: 10.1007/BF01872386.

3. Ramawat K.G., Goyal S. The Indian herbal drugs scenario in global perspectives. In: Ramawat K.G., Merillon J.M., editors. *Bioactive Molecules and Medicinal Plants*. Springer; Berlin/Heidelberg, Germany: 2008. pp. 325–347.
4. Baldé N.M., Youla A., Baldé M.D., Kaké A., Diallo M.M., Baldé M.A., Maugendre D. Herbal medicine and treatment of diabetes in Africa: An example from Guinea. *Diabetes Metab.* 2006;32:171–175.
5. Wu C.H., Wang C.C., Kennedy J. Changes in herb and dietary supplement use in the U.S. adult population: A comparison of the 2002 and 2007 National Health Interview Surveys. *Clin. Ther.* 2011;33:1749–1758.
6. Natural Health Products Directorate-Health Canada . *Natural Health Product Tracking Survey-2010 Final Report*. Ipsos-Reid; Toronto, ON, Canada: 2011
7. *Hygrophila auriculata* (K. Schum) Heine: Ethnobotany, phytochemistry and pharmacology / Asian Journal of Traditional Medicines, 2010, 5 (4)
8. Pharmacognosy and phytochemical evaluation of *Hygrophila auriculata* (Schumach.) Heine. root Salve SD, Bhuktar AS. *The Journal of Phytopharmacology* 2017; 6(4): 210-216
9. World Health Organization (WHO) *WHO Traditional Medicine Strategy: 2014–2023*. WHO; Geneva, Switzerland: 2013.
10. Abeywickrama K., Bean G.A. Toxigenic *Aspergillus flavus* and aflatoxins in Sri Lankan medicinal plant material. *Mycopathologia*. 1991;113:187–190
11. Halt M. Moulds and mycotoxins in herb tea and medicinal plants. *Eur. J. Epidemiol.* 1998;14:269–274
12. Logrieco A., Moretti A., Solfrizzo M. *Alternaria* toxins and plant diseases: An overview of origin, occurrence and risks. *World Mycotoxin J.* 2009;2:129–140.
13. Han Z., Ren Y.P., Zhu J.F., Cai Z.X., Chen Y., Luan L.J., Wu Y.J. Multianalysis of 35 mycotoxins in traditional Chinese medicines by ultra-high-performance liquid chromatography-tandem mass spectrometry coupled with accelerated solvent extraction. *J. Agric. Food Chem.* 2012;60:8233–8247.
14. C. M. Maragos , M. Appell , V. Lippolis , A. Visconti , L. Catucci & M. Pascale (2008) Use of cyclodextrins as modifiers of fluorescence in the detection of mycotoxins, *Food Additives & Contaminants: Part A*, 25:2, 164-171
15. Ashiq S., Hussain M., Ahmad B. Natural occurrence of mycotoxins in medicinal plants: A review. *Fungal Genet. Biol.* 2014;66:1–10.
16. Domijan A.M., Peraica M. 7.07—Carcinogenic Mycotoxins. In: McQueen C.A., editor. *Comprehensive Toxicology*. 3rd ed. Elsevier; Oxford, UK: 2018. pp. 154–167.
17. Brera C., De Santis B., Debegnach F., Miraglia M. Chapter 12 Mycotoxins. In: Picó Y., editor. *Comprehensive Analytical Chemistry*. Volume 51. Elsevier; Amsterdam, The Netherlands: 2008. pp. 363–427
18. Zhao S.P., Zhang D., Tan L.H., Yu B., Cao W.G. Analysis of aflatoxins in traditional Chinese medicines: Classification of analytical method on the basis of matrix variations. *Sci. Rep.* 2016;6:30822.
19. Sano, Y. Asabe, S. Takitani and Y. Ueno, *J. Chromatogr.*, 235 (1982) 257-265.
- 20 L. S. Lee and B. Skau, *J. Liq. Chromatogr.*, 4 (1981) 43-62.
- 21 <https://onlinelibrary.wiley.com/doi/abs/10.1002/pi.5992>
- 22 Ritesh Kumar Srivastava And S. Senthil Kumar “AN UPDATED REVIEW: ANALYTICAL METHOD VALIDATION” *European Journal Of Pharmaceutical And Medical Research* , 2017,4(09), 774-784