FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA

Inspiring Trust, Assuring Safe & Nutritious Food

भाकृअनुप ICAR

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TRAINING MANUAL

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Program on
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Food Safety and Standards Authority of India
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Training Manual

For

Analysis of Mycotoxins

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Chapter 1

Mycotoxins and Significance of Analysis

Introduction

Contamination of foodstuffs with mycotoxins is one of the most concerning problems in food and feed safety. In most developing countries, agriculture is the backbone of the economy and export crops are greatly depended upon as a source of foreign exchange to finance productive activities and other essential services. Most of these crops are cereals and oil seeds that are highly susceptible to fungal growth and mycotoxin production. The mycotoxins are not only hazardous to consumer health but also affect food quality resulting in huge economic losses for these countries.

This Training Manual on Mycotoxins describes the origin of Mycotoxins, their classification and main emphasis is given on the analytical methods by reviewing methods given in AOAC and FSSAI Manual.

Definition of Mycotoxins

Mycotoxins are metabolic products produced by moulds that can grow under certain environmental conditions before harvest, during transport and storage and through processing procedures. Aflatoxins are capable of producing acute or chronic toxic effects (e.g. carcinogenic, mutagenic and teratogenic) on human beings and on animals at the level of exposure.

All Mycotoxins are low molecular weight natural products (i.e. small molecules) produced as secondary metabolites by filamentous fungi. These metabolites constitute a toxigenically and chemically heterogeneous assemblage that are grouped together only because the members can cause disease and death in human beings and other vertebrates. Many mycotoxins display overlapping toxicities to invertebrates, plants and microorganisms.

While all mycotoxins are of fungal origin, not all toxic compounds produced by fungi are called mycotoxins.

Toxic syndromes, resulting from the intake of mycotoxins by man and animals, are known as mycotoxicosis. Although mycotoxicosis caused by mould*Clavicepspurpurea*has been known for a long time. Mycotoxins remained neglected until the discovery of Aflatoxins in 1960. Mould growth in food is very common, especially in warm and humid climates. It can occur in fields or in storage after harvest. Mould infection of foods such as grains, seeds and nuts is often localized in pockets, especially in bulk storage and warehouses. Currently a few hundred mycotoxins are known, often produced by genera *Aspergillus, Penicillium* and *Fusarium.*

Table 1 :Classification of Mycotoxins

Mycotoxin(s)	Fungi	Favorable Conditions	Food Commodities
Aflatoxins	*Aspergillusflavus* *A.parasiticus* *A.parasiticus* Some*Penicillium*	Temperature: 25-30°C Grain Moisture	Cereal & Cereal Products (barley,corn, millet,oats, rice, wheat,spaghetti,sorghum) Pulses (soybeans, cowpeas, peas, soybean meal) Nuts & Products (peanuts,peanut butter, peanut meal) Dried figs Oil seeds (cotton seed, sesame) Spices
Ochratoxin A	*Aspergillusostianus* *A.petrakii* *A.alliaceus* *A.sclerotiorum* *A.sulphureus* *A.melleus*	Temperature: 24-25 °C	Wheat, barley and rye
Deoxynivalenol (Vomitoxin) Zearalenone	*Fusariumgraminearum* *Fusariumculmorum*	High moisture: 22-25% Alternating high and low temp (7-21°C)	Wheat,Corn, Oats, Rye, Barley, Durum

Mycotoxin(s)	Fungi	Favorable Conditions	Food Commodities
Patulin	*Penicilliumexpansum, P.clavifome, P.patulum, P.melinii, P.leucopus, P.urticae, P.equinum, P.cyclopium, Aspergillusclavatus,A. giganteus, A.terreus, Byssochlamys*	Temperature: 24–25 °C	Apple juice, Apple sap, Apple cider
Fumonisins	*Fusariumverticillioides Fusariumproliferatum*	Temperature: 30°C Max High humidity	Corn

Regulatory Limits of Mycotoxins

These compounds are toxic and can be carcinogenic to humans and animals. Due to this toxicity, government regulatory agencies impose strict limits on their content in foodstuffs. Regulatory limits as per Food Safety and Standards (Contaminants, Toxins and Residues) (Amendment) Regulations, 2015 are reflected in Table 2.

Table 2: Regulatory Limits of Mycotoxins

S. No.	Name of the Contaminants	Article of the food	Limit µg/kg
1.	Aflatoxin	Cereal and Cereal Products	15
		Pulses	15
		Nuts Nuts for further processing Ready to eat	 15 10
		Dried figs	10
		Oilseeds or oil Oil seeds for further processing Ready to eat	 15 10
		Spices	30
2.	Aflatoxin M_1	Milk	0.5
3.	Ochratoxin	Wheat, barley and rye	20
4.	Patulin	Apple juice and Apple juice ingredients in other beverages	50
5.	Deoxynivalenol	Wheat	1000

The US FDA has established maximum allowable levels of total aflatoxin in food commodities at 20 parts per billion. In the European legislation the maximum level of aflatoxin B1 allowed in cereals is 2.0 µg/kg, with a maximum level of the sum of B1, B2, G1 and G2 set at 4.0 µg/kg. For nuts, the levels vary between 2 and 12 µg/kg for B1 and 4 and 15 µg/kg for the sum of B1, B2, G1 and G2. Specifically in almonds, the levels are 8.0 µg/kg and 10.0 µg/kg, respectively. For M1 in raw milk the maximum level is 0.05 µg/kg. For infant formula and follow-on formula, the maximum permitted level of M1 is 0.025 µg/kg.

Aflatoxins

Aflatoxin is probably the most common and widely known mycotoxin contaminant. Aflatoxins, produced by the genus *Aspergillus*, are one of the most widely occurring mycotoxins. It is produced by moulds, *Aspergillus flavus* and *Aspergillus parasiticus*. In fact the name is a composite word derived from '*A.flavus* toxin'. Foods that are commonly affected include all nuts, especially groundnuts, tree nuts such as pistachio and Brazil nuts, cottonseed, copra, rice, maize, wheat, sorghum, pulses, figs and oilseed cakes. Unrefined vegetable oils made from contaminated seeds or nuts usually contain aflatoxins. However aflatoxin is destroyed in the refining process so that refined oils are safe.

There are six aflatoxins of analytical interest (figure 1). The main aflatoxins are B1, B2, G1 and G2 which occur in foods and two (M1 and M2) as metabolites that appear when dairy animals eat grain contaminated with B1 and B2 aflatoxins. B1 and G1 are more potent than B2 and G2 and this difference is reflected in the legislated levels.

Chemical Structure of Aflatoxins:

Aflatoxins B1, B2, G1 and G2 refer to toxins which fluoresce blue (B) or green (G) under ultraviolet light and are separable by thin layer chromatography (TLC). The only structural difference between B and G toxins is the inclusion of oxygen in the cyclopentanone ring. Aflatoxin M1 and M2 represent the toxin B1 and B2, which have been metabolized within the body of a lactating animal. Their finding in milk led to their designation as 'M'. The obvious structural difference between B and M is the addition of the hydroxyl group.

Aflatoxins are subject to light degradation. Therefore, all analytical materials must be adequately protected from light and standard Aflatoxin solutions should be stored using amber colored vials or aluminium foil.

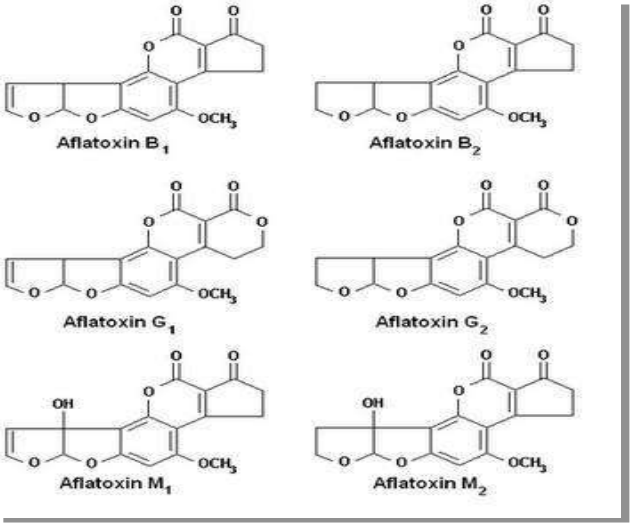


Figure 1: Chemical Structure of the six Aflatoxins

Chapter 2

Laboratory Safety

The methods and analytical procedures described in this Manual are designed to be carried out by properly trained personnel in a suitably equipped laboratory. In common with many laboratory procedures, the methods quoted frequently involve hazardous materials. For the correct and safe execution of these methods, it is essential that laboratory personnel follow standard safety procedures for the handling of hazardous materials.

Laboratory Safety:

1. Use laboratory coat/apron while working in the laboratory.
2. Wear safety glasses and gloves when working with aggressive or toxic chemicals.
3. Ensure that all chemicals are identified by suitable labels. Keep the quantities of the chemical to the bare minimum.
4. Read the characteristics of any unknown compound in the 'Material Safety Data Sheets' (MSDS).
5. Do not handle volatile solvents near an open flame or hot plate or heating mantle.
6. Do not store low flash point solvents like Hexane, Benzeneand Heptane etc. in domestic refrigerators.
7. Use fume hood for all operations with toxic or flammable materials.
8. Examine all glassware before use. Reject damaged ones.
9. Everyone should know the exact functioning and possible risks of apparatus before it is used.
10. Do not leave a heating operation unattended.
11. Do not use solvent for cleaning hands or body parts.
12. Avoid interrupting staff engaged in potentially dangerous operations. If you are interrupted, either stop operation or wait until you have finished before answering.
13. Do not use laboratory glass-ware for drinking purposes.
14. Do not keep eatables in refrigerator containing chemicals.
15. Use water bath for heating inflammable products.

Handling of food samples suspected of being contaminated with mycotoxins:

1. Use disposable gloves and protective masks if grinding the food as it creates dust. Aflatoxins are potent carcinogenic substances.
2. While handling pure aflatoxins reference material, extreme precautions must be taken as they are electrostatic.
3. All work must preferably be carried out in a hood.
4. Swab any accidental spill of toxin with 1% sodium hypochlorite bleach (NaOCl), leave 10 minutes and then add 5 % aqueous acetone.
5. Rinse all glassware exposed to aflatoxins with methanol, add 1% sodium hypochlorite solution and after 2 hours add acetone to 5 % of total volume. Let it react for 30 minutes and then wash thoroughly.
6. Use a laboratory coat or apron soaked in 5% sodium hypochlorite solution overnight and washed in water.
7. Reactive vapors i.e. O_2, SO_2, HCl can affect adsorbents used in TLC as well as the stability of adsorbed spots. TLC must, therefore, be performed only in a laboratory free of volatile reagents.
8. Always dry TLC plates thoroughly before exposure to UV light. UV light from sunlight or fluorescent lamps can catalyse changes to compounds being examined when exposed on adsorbent surface, particularly in the presence of solvent.
9. Avoid exposure to UV light of underdeveloped spots and expose developed plates to UV light for the minimum time needed for visualization.
10. Protect analytical material adequately from light and keep aflatoxin standard solutions protected from light by using amber vials or aluminium foil. Put a warning note on the label.
11. Use of non-acid washed glassware for aflatoxin aqueous solutions may cause loss of aflatoxin.
12. Before use, soak new glassware in dilute acid (carefully add 105 ml concentrated Sulphuric Acid to water and make upto 1 litre) for several hours, then rinse extensively with distilled water to remove all traces of acid (check with pH paper). (FAO Manual of Food Quality Control 14/7, 1986, page 185 / AOAC, Chapter 49, Subchapter 1 Mycotoxins /Subchapter 2 Aflatoxins).

Chapter 3

Mycotoxin Analysis Methods

Determination of aflatoxins concentration in food stuff and feeds is very important. However, due to their low concentration in foods and feedstuff, analytical methods for detection and quantification of aflatoxins have to be specific, sensitive and simple to carry out.

There are different techniques of mycotoxin analysis since their discovery in 1960. Many methods have been developed for the analysis of aflatoxins, including Thin layer chromatography (TLC), Immunoaffinity chromatography, High performance liquid chromatography (HPLC), Enzyme-linked immune sorbent assay (ELISA) and Liquid Chromatography Mass Spectrometry (LC/MS/MS). Each of these methods has advantages and limitations in aflatoxins analysis. LC/MS/MS provides the ultimate in selectivity and sensitivity for quantitative analysis, but requires significant investment for laboratories that do not already possess the required instrumentation or skill sets. The combination of selective immunoaffinity separations with highly sensitive fluorescence detection is an alternative technology for this application. However, since reverse phase eluents quench the fluorescence of aflatoxins B1 and G1, derivatization is common to enhance the response of these analytes.

I. Sample Preparation:

1.Preparation of Lot Sample:

Mould contamination is by nature non homogeneous and hence the amount of mycotoxin is not uniformly distributed throughout the food stuff. Mycotoxin contamination, particularly in grains and nuts is likely to occur in pockets of high concentration, which may not be randomly distributed. Hence sampling and sample preparation are to be performed with this factor in mind. Include total laboratory sample in sample preparation. Aim at maximum particle size reduction and the thoroughness of mixing to achieve effective distribution of contaminated portions. One contaminated peanut (ca 0.5 g) can contain enough aflatoxin to result in significant level when mixed with 10,000 peanuts (ca 5 kg). To obtain 1 piece of

contaminated nut in each 50 g portion, the single nut must be reduced to 100 pieces and these 100 pieces must be uniformly blended through entire mass.

To achieve this degree of size reduction, nuts must be ground to pass through a No. 20 sieve and thorough mixing of sample is needed before taking sample for analysis. When handling large samples, coarse grind and mix entire sample, remove about 1/20 and regrind this portion to a finer size. In case of liquids, mix thoroughly to obtain a homogeneous sample.

2.Preparation of Laboratory Sample:

Draw with the same precaution as with a lot sample. Wherever practical, divide by riffling or similar random dividing procedure until sub-division is close to the desired analytical sample (AOAC 977.16, Sampling of Aflatoxinsand Preparation of Sample).

Caution:

Grinding of dry samples may result in air borne dust. Even if no toxin is present there is potential harm from inhalations of mould spores or from allergic response to inhaled dust. Use protective mask and/or dust collector. Prepare samples in area separated from analytical laboratory.

II. Methods for Detection and Quantification of Aflatoxins

Chromatographic Methods

Chromatographic techniques are based on the physical interaction between a mobile phase and a stationary phase. The components to be separated are distributed between the two phases (stationary phase and mobile phase).

1. ThinLayer Chromatography (TLC)

Thinlayer chromatography has been regarded by the Association of Official Analytical Chemists (AOAC) as the method of choice since 1990. Thin-layer chromatography is one of the most widely used separation techniques in aflatoxins analysis. It consists of a stationary phase made of either silica or alumina or cellulose immobilized on an inert material such as glass or plastic, called the matrix. The mobile phase is comprised of methanol: acetonitrile: water mixture, which carries the sample along as it moves through the

solid stationary phase. In TLC, the distribution of aflatoxins between the mobile and stationary phases is based primarily on differences in solubility of the analytes in the two phases. Different analytes, depending on their molecular structures and interaction with the stationary and mobile phases, either adhere to the stationary phase more or remain in the mobile phase, thereby allowing for quick and effective separation. Thin-layer chromatography has been widely used in the determination of aflatoxins in different foods and as low as 1–20 ppb of aflatoxins has been reported.

Advantages:

- It can detect several types of mycotoxins in single test sample.
- It has excellent sensitivities.

Disadvantages:

- It requires pretreatment of sample and expensive equipment.
- In addition, it lacks precision due to accumulated errors during sample application, plate development and plate interpretation.

Developments in TLC

Attempts to improve TLC have led to the development of automated form of TLC, called the Highperformance thinlayer chromatography (HPTLC). The HPTLC has since overcome the problems associated with the conventional TLC techniques through automation of sample application, development and plate interpretation. Currently HPTLC is one of the most efficient and precise methods in aflatoxins analysis. Nevertheless, the requirement for skilled operators, the costs of the equipment coupled with its bulkiness and the extensive sample pretreatment, limit the HPTLC to the laboratory and thus it is inapplicable in field situations.

2. Highperformance liquid chromatography (HPLC)

- Highperformance liquid chromatography (HPLC) is the most popular chromatographic technique for separation and determination of organic compounds. The HPLC technique makes use of a stationary phase confined to either a glass or a plastic tube and a mobile phase comprising aqueous/organic solvents, which flow through the solid adsorbent.

- In practice, the HPLC technique employs a stationary phase such as C-18 chromatography column, a pump that moves the mobile phase(s) through the column, a detector that displays the retention times of each molecule and mobile phases.
- The sample to be analyzed is usually injected into the stationary phase and the analytes are carried along through the stationary phase by the mobile phase using high pressure delivered by a pump.
- The analytes are distributed differently within the stationary phase through chemical as well as physical interactions with the stationary and mobile phases.
- The time at which a specific analyte elutes is recorded by a detector as its retention time. The retention time depends on the nature of the analyte and composition of both stationary and mobile phases. Programmable detectors such as either the Fluorescentdetector (FLD) or the Ultraviolet (UV) detector or the Diode array detector (DAD) may be used in the detection and identification of aflatoxins.
- HPLC methods used for the determination of aflatoxins in foods include the normal-phase and reversed-phase high pressure liquid chromatography techniques.
- The reversed phase HPLC method is the most widely used for separation and determination of aflatoxins.
- Occasionally, chemical derivatization of aflatoxins B1 and G1 may be required to enhance sensitivity of HPLC during analysis since the natural fluorescence of aflatoxins B1 and G1 may not be high enough to reach the required detection limit. The derivatization reactions of aflatoxin B1 with both the acid and halogens are presented. While in the first reaction step, the second furan ring of aflatoxin B1 is hydrolyzed by trifluoroacetic acid (TFA) into a highly fluorescent aflatoxin B2a, in the second and the third derivatization reaction steps, bromine and iodine are used as reagent, respectively. They react with aflatoxin B1 to form highly fluorescent aflatoxin B1 derivatives of these halogens, respectively.

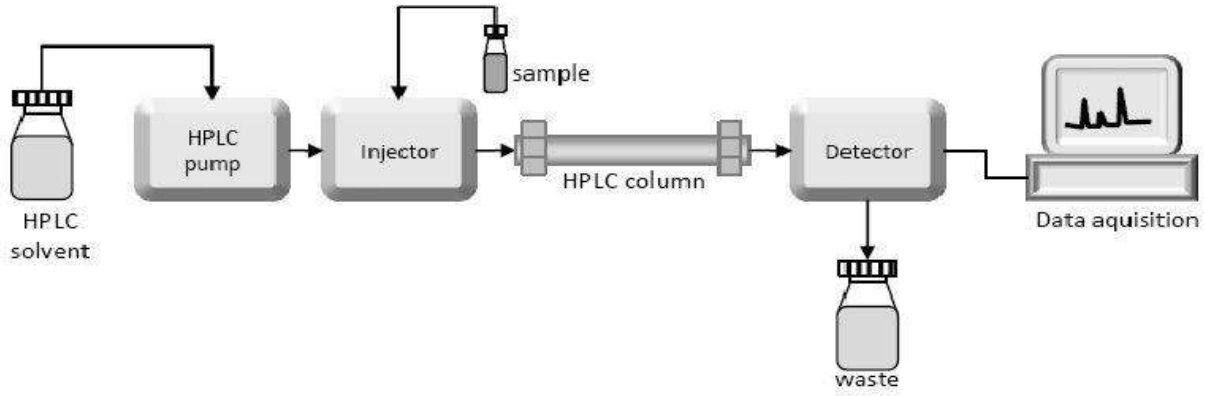


Figure2: Schematic diagram of HPLC

Two widely used post-derivatisation methods are described below:

a. HPLC-FLD with KOBRA®CELL:

Aflatoxins fluorescenaturally under UV light (i.e. B's fluoresce blue and G's fluoresce green) with the subscripts relating to their relative chromatographic mobility.

Aflatoxin B_1 and G_1 do not fluoresce naturally at high degree and must be derivatised using iodine or bromine.During derivatisation the chemical structures of Aflatoxins B_1 and G_1 are changed to a more fluorescent form, increasing the fluorescent signal in each case for detection by HPLC.

Derivatisation with KOBRA®CELL

Kobra cell**= KO**k**BR**omine **A**pparatus

The KOBRA CELL is a unique system which offers a popular alternative derivatisation method with testing for aflatoxins in conjunction with HPLC.

The KOBRA®CELL is an electrochemical cell consisting of a platinum working electrode and a stainless steel auxiliary electrode separated from another by a membrane. These layers are sandwiched between a rigid plastic housing.

The CELL is fitted between the HPLC column and the detector that generates the derivatisationagent, bromine, on-line from potassium bromide and nitric acid present in the

mobile phase.The derivatisation results in the significant results of the fluorescent signals of the modified forms of Aflatoxin B1 and G1.

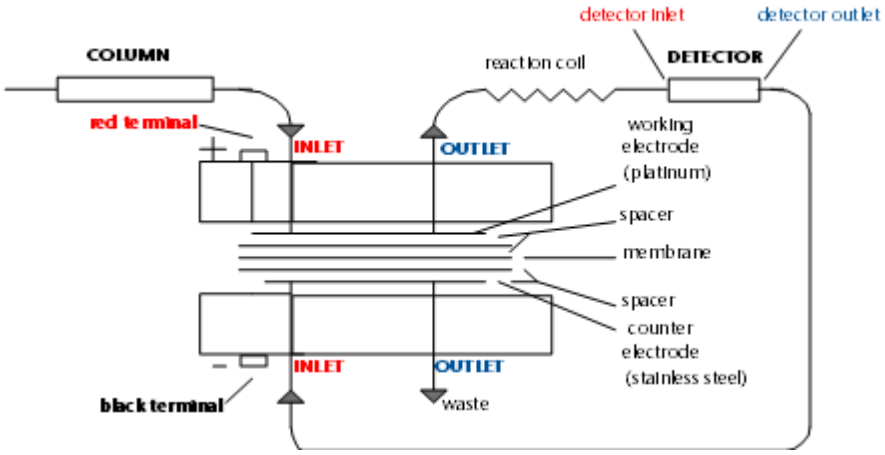


Figure 3: Schematic diagram of KOBRA CELL

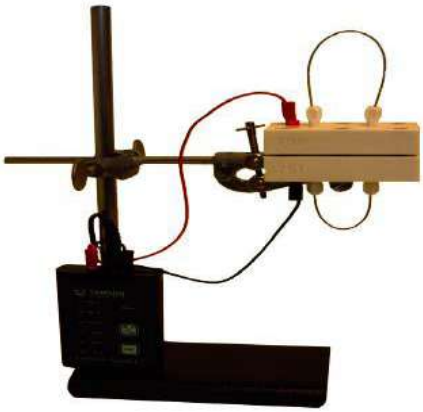


Figure 4: KOBRA CELL

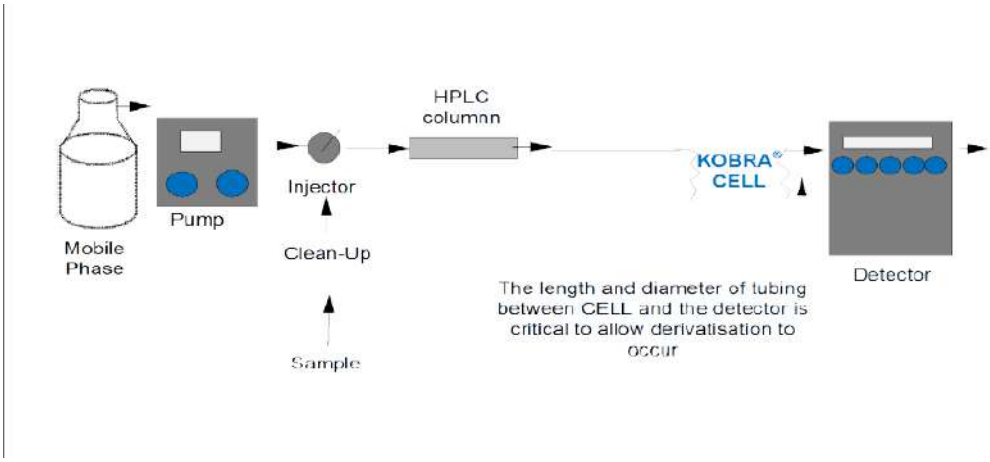


Figure 5:Schematic diagram of HPLC with KOBRA CELL

Advantages of KOBRA Cell compared to traditional methods:

a. No daily preparation of derivatising reagents.
b. No second pump required.
c. No water bath or column heater required.
d. Comparable detection limit to other derivatisation methods.
e. Low maintenance Electro Chemical cell.
f. Easy to install.
g. Sharp peaks, no risk of peak broadening normally associated with the introduction of derivatisation agent.
h. No odour, non-hazardous derivatising agent.

b. HPLC-FLD with Photochemical Reactor:

Detection enhancement of aflatoxins

Photochemical derivatization is a simple, inexpensive and flexible technique that improves sensitivity and selectivity of broad range of analytes. It consists of 254 nm UV low pressure lamp with cooled reflector tube.

Advantages:

- Long term stability of lamp and coil.
- High light transmission.
- Robust steel housing to meet laboratory requirements.
- Special designed fluorocarbon coil.
- Photochemical post-column derivatization of Aflatoxins in a special reactor loop with UV light.
- Result: clear peaks
- Comparable to electrochemical derivatization with Kobra cell.

Figure 6: PHRED

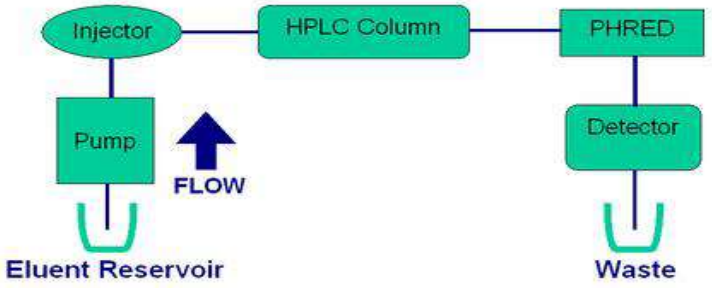


Figure 7: Schematic diagram of HPLC with PHRED

- By comparison of two post-column derivatization methods for the determination of aflatoxins B1, B2, G1 and G2 by fluorescence detection after liquid chromatographic separation, the results showed that both bromination and irradiation by UV light were suitable for the determination of aflatoxins in various foods and animal feed matrices and both generated comparable results for fluorescence amplification and repeatability.
- The fluorescence of aflatoxins B1 and G1 was significantly enhanced after derivatization reaction either by bromination or by irradiation by UV light.

Advantages:

- Highperformance liquid chromatography provides fast and accurate aflatoxin detection results within a short time.
- A sensitivity of detection as low as 0.1 ng/kg using FLD has been reported.

Disadvantages:

- HPLC for aflatoxin analysis requires rigorous sample purification using immunoaffinity columns.
- In addition, HPLC requires tedious pre and post-column derivatization processes to improve the detection limits of aflatoxin B1.

In this training program, the Ultra High Performance LC (UPLC/UHPLC) system coupled with Fluorescence (FLR) Detector has been used.

The UPLC system with the large volume flow cell removes the need for time-consuming and laborious derivatization steps.

The response of B1 and G1 are less than B2 and G2 due to the quenching effect of the solvents on the fluorescenceof these two compounds. However, the UPLC FLR Detector with the large volume flow cell is able to detect B1 and G1 at the required levels without derivatization.

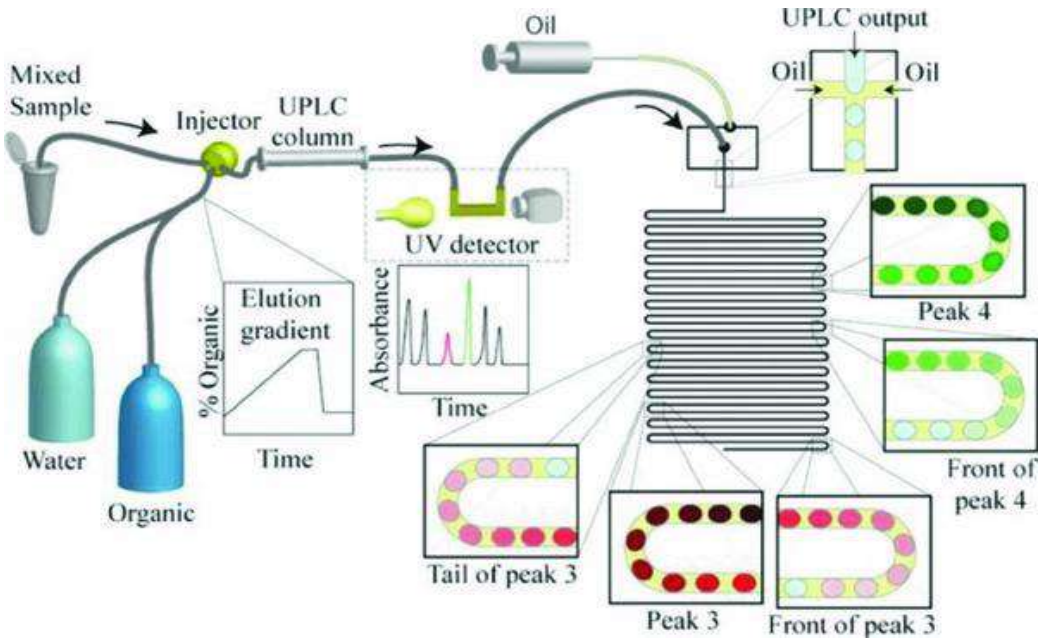


Figure 8: Schematic Diagram of UPLC

3. Liquid Chromatography Mass Spectrometry (LC-MS/MS)

To overcome the challenges associated with derivatization processes in aflatoxins analysis, a modification of the HPLC method, whereby the HPLC is coupled to mass spectroscopy, has been made and is currently employed in the determination of aflatoxins. Since the mass spectrometer requires neither use of UV fluorescence nor the absorbance of an analyte, the need for chemical derivatization of compounds is eliminated. The HPLC-MS/MS uses small amounts of sample to generate structural information and exhibits low detection limits. LC-MS/MS is one of the most advanced techniques for mycotoxin analysis and many labs are moving towards this technique which is particularly suitable for multi-toxin analysis.

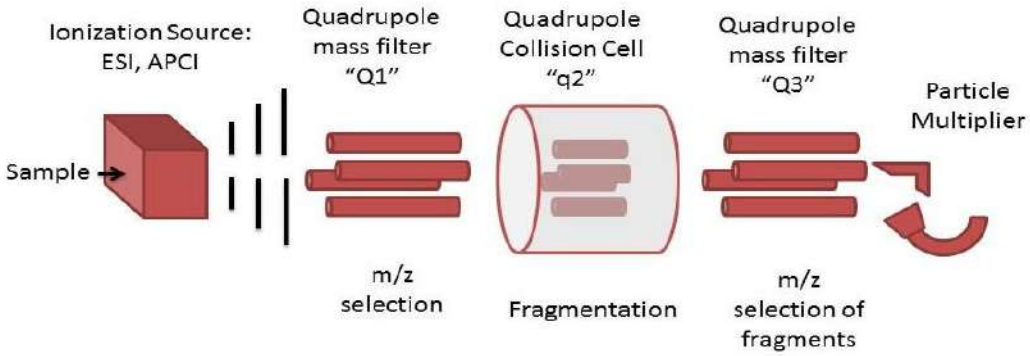


Figure 9: Schematic diagram of LC-MS/MS

Advantages:

- LC-MS/MS is one of the most advanced techniques for mycotoxin analysis and many labs are moving towards this technique which is particularly suitable for multi-toxin analysis.
- No requirement for derivatisation of samples when used in conjunction with LC-MS/MS.
- Multi toxin analysis leads to more efficient use of time.

Disadvantages:

- LC-MS/MS is bulky and very expensive equipment which can only be operated by trained and skilled personnel. This also limits its use to only laboratory environment and not under field conditions.
- Matrix effect is a common problem encountered in LC-MS/MS analysis which might suppress the signal intensity significantly in comparison to the solvent standard. To

eliminate matrix effects and minimize errors in quantification, it might be necessary to use isotopically labelled internal standards of aflatoxins (and other mycotoxins) which are expensive and may not be easily available. Alternatively, matrix-matched standards may be tried to correct any such errors.

4. Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme immunoassay (EIA) and typically the ELISA have become the methods of choice for medical diagnostic laboratories, research institutions and regulatory bodies for quality assessment and proficiency-testing, among others. The principle of enzyme immunoassays is essentially the same as other immunochemical methods; that is, it relies on the specificity of antibodies for antigens and the sensitivity of the assay is increased by labeling either the antibodies or the antigens with an enzyme that can be easily assayed by use of specific substrates. Hence, an antibody immobilized onto a solid support may capture an unlabeled antigen in the analyte, which is subsequently detected by a labeled antibody.

The ELISA technique is currently used in the detection of aflatoxins in agricultural products and a number of commercially available ELISA kits based on a competitive immunoassay format are widely used. Most of the kits use horseradish peroxidase (HRP) and alkaline phosphatase (AP) enzymes as labels in analysis of aflatoxins.

Advantages:

- It is possible to perform the test on a 96-well assay platform, which means that large number of samples can be analysed simultaneously.
- ELISA kits are cheap and easy to use and do not require extensive sample cleanup.
- There are no inherent health hazards associated with enzyme labels as there are for isotopes.

Disadvantages:

- The ELISA technique requires multiple washing steps, which may at times prove not only laborious but also time consuming.

Table 3:AOAC Official methods of analysis of Mycotoxins are summarised below:

Sr. No.	Title	AOAC Reference No.	Technique/ Method	Chapter 49, Page No.
1.	Preparation of standards for aflatoxins	970.44	TLC	3
2.	Standards for aflatoxins	971.22	TLC	4
3.	Identification of aflatoxins	975.35	TLC	5
4.	Identification of aflatoxinB1	975.37	TLC	5
5.	Aflatoxin in coconut, copra and copra meal	971.24	TLC	32
6.	Aflatoxins in corn	972.26	TLC	15
7.	Aflatoxins in corn and peanuts	993.17	TLC	15
8.	Aflatoxins in cotton seed products	980.20	TLC	25
9.	Aflatoxin B_1 in eggs	978.15	TLC	28
10.	Aflatoxins in green coffee	970.46	TLC	30
11.	Toxicity of AflatoxinB1	970.48	Chicken embryo Bioassay	34
12.	Identification of Aflatoxin B_1	970.47	TLC	31
13.	Aflatoxin M1 in dairy products	974.17	TLC	47
14.	Aflatoxin B1 and M1 in Liver	982.24	TLC	48
15.	Aflatoxin M1 in milk and cheese	980.21	TLC	47
16.	Ochratoxin A in barley	973.37	TLC	63
17.	Ochratoxin A in green coffee	975.38	TLC	65
18.	Ochratoxin in roasted coffee	2000.09	TLC	65

Sr. No.	Title	AOAC Reference No.	Technique/ Method	Chapter 49, Page No.
19.	Ochratoxin A in corn and barley	991.44	Liquid Chromatography Method	69
20.	Patulin in apple juice	974.18	TLC	75
21.	Aflatoxin in food and feeds-Romer Minicolumn method	975.36	RomerMinicolumn Method	5
22.	Aflatoxin in corn	979.18	Holaday-Velasco Minicolumn	6
23.	Aflatoxin B_1,B_2, and G_1 in corn cotton seed, peanuts and peanut butter	990.34	ELISA (Screening)	7
24.	Aflatoxin in peanuts and peanut products	968.22	C. B. Method IUPAC-AOAC	9
25.	Aflatoxin in Cocoa beans	971.23	Modified C. B. Method IUPAC-AOAC	9
26.	Aflatoxin in peanut and peanut products BF method	970.45	BF Method AOCS.AOAC	11
27.	Total Aflatoxins (B_1,B_2 and G_1) in corn	993.16	ELISA	14
28.	Aflatoxin B_1 in corn and roasted peanuts	990.32	ELISA	17
29.	Aflatoxins in corn, raw peanuts and peanut butter	991.31	Immuno Affinity Column	21
30.	Aflatoxin in corn, raw peanuts and peanut butter	2005.08	Liquid chromatography with post column photochemical derivatization	23
31.	Aflatoxin in corn, almonds, Brazil nuts, Peanuts and pistachio nuts	994.08	Multifunctional column (Mycosep) method	26

Sr. No.	Title	AOAC Reference No.	Technique/ Method	Chapter 49, Page No.
32.	Aflatoxin in Peanut butter, Pistachio Paste, Fig Paste, and paprika powder almonds, Brazil nuts, Peanuts and pistachio nuts	999.07	Immunoaffinity column Liquid chromatography with post column	34
33.	Aflatoxin B_1 in baby food	2000.16	Immunoaffinity column HPLC method	37
34.	Aflatoxin in corn, peanut butter	990.33	Liquid chromatography	19
35.	Aflatoxin B_1 in cattle feed	2003.02	Immunoaffinity column liquid chromatography	40
36.	Aflatoxin B1, B2, G1, G2 and Ochratoxin A in Ginseng and Ginger	2008.02	Multitoxinimmunoaffinity column cleanup and liquid chromatographic quantification	
37.	Aflatoxin M_1 in liquid milk	2008.08	Immunoaffinity column by liquid chromatography	52
38.	Aflatoxin M_1 in fluid milk	986.16	Liquid Chromatography	51
39.	Deoxynivalenol in wheat	986.17	TLC	54
40.	Deoxynivalenol in wheat	986.18	Gas chromatographic method	55
41.	Ochratoxin A in green coffee	2004.10	Immunoaffinity column cleanup and LC method	67
42.	Ochratoxin A in barley	2000.03	immunoaffinity column HPLC	71
43.	Patulin in apple juice	995.10	Liquid Chromatography	76

Sr. No.	Title	AOAC Reference No.	Technique/ Method	Chapter 49, Page No.
44.	Patulin in clear and cloudy apple juices and apple puree	2000.02	Liquid Chromatography	78

Table 4 :FSSAI Manual of Methods of Analysis of Mycotoxinsis summarised below:

Sr. No.	Title	Reference No.	Technique/ Method	Page No.
1.	Determination of Aflatoxins (For Groundnuts and groundnut products, Oilseeds and Food grains)	S.No. 2	CB Method by TLC	7
2.	Determination of Aflatoxins (For Peanut and Peanut products, Cereals and pulses)	S.No. 3	BF Method	10
3.	Determination of Aflatoxins	S.No. 4	RomerMinicolumn Method	10
4.	Determination of Aflatoxins in Corn and Peanut Powder / Butter	S.No. 5	Liquid Chromatographic method	13
5.	Determination of Aflatoxin M_1 in Milk, Cheese and Koya	S.No. 6	TLC	19
6.	Determination of Aflatoxin B1, B2, G1 & G2, in Spices, Tea, Coffee, Nutmeg and Rice	S.No.7	HPLC-MS/MS	20
7.	Determination of Aflatoxin B1, B2, and G1 in Corn, Cottonseed, Peanuts and Peanut Butter	S.No. 8	ELISA	25
8.	Determination of Total Aflatoxins in Corn	S.No. 9	ELISA	32
9.	Determination of Aflatoxins in Corn and Peanuts	S.No. 10	TLC	34
10.	Determination of Aflatoxin M1 and M2 in Fluid Milk	S.No. 11	Liquid Chromatographic	38
11.	Determination of Aflatoxin M1 in Liquid Milk	S.No. 12	Immunoaffinity Column by Liquid Chromatography	42
12.	Deoxynivalenol (DON)	S.No. 13	TLC	47

Sr. No.	Title	Reference No.	Technique/ Method	Page No.
13.	Determination of Patulin	S.No. 14	TLC	52
14.	Determination of Ochratoxin	S.No. 15	TLC	51
15.	Determination of Aflatoxins B_1, B_2, G_1 and G_2 in Food stuffs	S.No. 16	TLC	56

Patulin in Apple juice by Liquid Chromatography Method (AOAC 995.10)

Principle:

Patulin is extracted with ethyl acetate and isolated by extraction with Na_2CO_3 Solution. Extract is dried with anhydrous Na_2SO_4. After evaporation of ethyl acetate, patulin is determined by reverse phase LC column with UV detection.

Ochratoxin A in Barley by Immunoaffinity Column HPLC (AOAC 2000.03)

Principle:

Test portion is extracted by blending with acetonitrile- water. The extract is cleaned up by passing through an immunoaffinity column. OchratoxinA (OTA) is eluted with methanol, further purified and identified by LC and quantified by fluorescence.

Chapter 4

Procedure of preparation of Aflatoxin standards and Stability Check

(Ref.: NRCG-NRL Standard Operating Procedure)

1. Purpose:

To specify the procedure for preparation of intermediate standard stock solution and determining its concentration by UV –Spectroscopy.

2. Scope:

This procedure is applicable for intermediates of individual aflatoxins standard solution viz. B1, B2, G1, G2, prepared in organic solvents**.**

3. Requirements:

i.Reference standards:

The reference standard used for the test must possess a certificate of analysis mentioning their quantity, purity, storage conditions, production date and expiry date. Wherever possible the reference standards must be traceable to SI systems of Measurement to a Certified Reference Material (CRM) or to the national or international standards like NIST.

Acceptance criteria:

The expiry date of reference material must be at least 6 months from the date of receipt.

The organic solvents are checked for purity for every Lot or Batch. The toxins prepared should be soluble and stable in it and it should be inert to the same.

ii.Solvents:

The organic solvents are checked for purity for every Lot or Batch. The toxins prepared should be soluble and stable in it and it should be inert to the same.

iii.Apparatus:

a. Beaker
b. Micro pipettes
c. Quartz cuvettes

All these apparatus must be calibrated for their volume capacity and uncertainty associated with it.

iv.Equipment:

a. UV –Visible Spectrophotometer
b. Precision weighing balance

4. i. Preparation of individual standard stock solution:

Aflatoxin standards are received in minimal quantity (with exact amount mentioned) in amber coloured containers. Hence, for preparing a required concentration, appropriate volume of solvent is directly added to the bottle and stock solution is prepared. Calculate the Concentration of the standard in µg/mL.

a. Weight of standards (corrected, a) = [weight of standards × Purity]/100
b. Volume of solvent (mL, b) = [Weight of solvent / specific gravity]
c. Calculated Conc. (µg/mL) = [Corrected weight of standard (a) / volume of solvent (b)] x 1000
d. Label the container with lab code, name of chemical, conc.in µg/mL, name of solvent, date of presentation, date of expiry.
e. Enter the data in the respective log book and registers.

ii.Preparation of intermediate standard stock solutions:

a. Before use, the stock solution is sonicated and vortexed thoroughly.
b. From the above standard stock, using dilution process and the formula C1V1=C2V2, a intermediate standards solution concentration 4µg/mL of individual aflatoxins prepared in required volume.

iii.Analysis by UV-Spectrophotometer:

a. Since aflatoxins are photosensitive and get degraded upon exposure to light during storage, their accurate concentration is determined by UV-Spectroscopy.

b. Since these individual aflatoxins contain chromophore groups, which can absorb light of a particular wavelength, they can be detected and qualified using Beer Lambert's law.

c. After switching on the spectrophotometer, wait for initialization of instrument.

d. Initially, baseline corrections are performed specifying the range for measurementviz.200 to 800 nm absorbance as 0 to 1.

e. Using UV-spectrum mode, lambda max (λ max) was identified respectively for each aflatoxin for the range described above.

f. Further, the absorbance value was measured at λ max in equation mode.

g. The absorbance value was used to calculate the actual concentration from a formula derived from Beer Lambert's law, as follows:

$$A = \varepsilon l c$$

Absorbance = molar absorptivity x path length of the cuvette (1 cm) x concentration

Concentration (mg/ml)=(A x Mol.wt x 1000) /(molar absorptivity x path length)

h. This is considered as correct concentration which is applied for preparation of working standard solution mixture and calibration standards.

iv.Preparation of working standard solution mixture:

a. Similarly, a working standard solutions mixture (100ppb) is prepared from individual intermediate standard stock solution (4ppm) by dilution and use of $C_1V_1=C_2V_2$ formula.

b. Applicationof correction factor to the concentration of individual intermediate standard stock solution will assure correct preparation of further mixture and dilutions.

v. Storage of Working Standards:

Before storage, weigh flasks to nearest mg and record weight for future reference. Wrap flasks tightly with Aluminium foil and store at 0°C. When the solution is to be used after storage, reweigh flask and record any change.

vi. Stability check:

a. The correct concentration of individual intermediate standard stock solution is determined since actual value may differ from theoretical value due to the degradation of standard over a period of time.

b. The value of concentration is calculated from the formula as described in SOP for preparation of aflatoxin standards and estimation of its concentration by UV-Spectroscopy for the intermediate stock solutions.

c. This result is recorded after a regular interval of two months and analysed for % degradation for the intermediate stock solution from day '0'. The same stock solution of intermediate solution is used throughout for this procedure.

d. The same individual intermediate standard stock solutions as well as the working standard mixture or calibration standards (Prepared from intermediate standard stock) can be used until the intermediate standard stock shows ±10% rate of degradation.

e. Beyond this duration, a new working standard mixture or calibration standard are prepared from the available individual intermediate standard stock only upon application of correct concentration factor (considering the actual rate of degradation at that point).

Hence, a stability check is maintained for the individual intermediate standard stock as well as working standard mixture or further dilution.

This will avoid the error in preparation of working standard mixture and its dilutions.

Chapter 5

Procedure for the Estimation of Concentration of Aflatoxins in Peanut and Peanut products using HPLC-FLD

(Ref.: NRCG-NRL Standard Operating Procedure)

1. Purpose:

This procedure describes the process for the Estimation of Aflatoxins contamination in peanut and peanut products**.**

2. Scope:

These procedures apply to estimation of aflatoxins contamination in peanuts and peanut products.

3. References :

i. AOAC Official Method 2005.08 (49.2.18A). Aflatoxins in Corn, Raw peanuts and Peanut Butter.

ii. A.E. Waltking, D.Wilson, J. AOAC. Int. 89, no.3. 2006, 678-692.

4. Procedure:

4.1 Chemicals and Reagents:

i. Gradient grade Methanol
ii. Acetic acid (Glacial)
iii. Sodium chloride
iv. Ammonium formate
v. Potassium chloride
vi. Potassium dihydrogen phosphate
vii. Anhydrous disodium hydrogen phosphate

viii. Hydrochloric acid

ix. Sodium hydroxide

x. Distilled, reverse osmosis or deionized water

4.2 Phosphate buffer saline (PBS):

Dissolve 0.2 g Potassium chloride, 0.2 g Potassium dihydrogen phosphate, 0.93g and anhydrous disodium hydrogen phosphate and 8 g Sodium chloride in 900 ml HPLC grade water.

Then adjust the pH to 7.4(±0.1) with 0.1M HCl or 0.1 M NaOH.

4.3 Reference standards:

Use Aflatoxin mix standard solution with known concentration (as an example: Sigma Aldrich Aflatoxin Mix Solution 33415).

Certificate of Analysis

Product Name:	AFLATOXIN MIX 4 SOLUTION analytical standard
Product Number:	33415
Batch Number:	BCBS8757V
Brand:	Sigma-Aldrich
CAS Number:	
Formula:	
Formula Weight:	
Storage Temperature:	
Expiration Date:	SEP 2018
Quality Release Date:	19 SEP 2016

TEST	SPECIFICATION	RESULT
CONCENTRATION COMP1	– 20 UG/ML B1	21.02 UG/ML
CONCENTRATION COMP2	– 20 UG/ML G1	21.03 UG/ML
CONCENTRATION COMP3	– 20 UG/ML B2	20.96 UG/ML
CONCENTRATION COMP4	– 20 UG/ML G2	21.05 UG/ML
MEASURING TOLERANCE P±	0.0 - 2.0 UG/ML	+/- 0.42 UG/ML
SOLVENT	ACETONITRILE	ACETONITRILE
PURITY (HPLC) COMP1	≥ 98% B1	99%
PURITY (HPLC) COMP2	≥ 98% G1	99%
PURITY (HPLC) COMP3	≥ 98% B2	99%
PURITY (HPLC) COMP4	≥ 98% G2	99%
MEASURING TOLERANCE P± COMP1	≤ 2.0 %	+/- 0.6%

4.4 Apparatus:

i. Mixer and grinder (20 L capacity)

ii. Homogenizer/Blender

iii. Analytical and precision balance

iv. HPLC-Fluorescence

v. Pipettes
vi. Test tubes
vii. Vacuum manifold
viii. Immunoaffinity column
ix. Fluted filter paper (24 cm), funnel and Erlenmeyer flask
x. Glass microfiber filter GF/A (11 cm) and funnel
xi. Syringe (glass or other material compatible with methanol)
xii. Bottles of 250 ml

4.5 Calibration Standard Preparation:

Prepare an intermediate mixture of 1µg/mL by pipetting appropriate volume of all four aflatoxins. Prepare a set of calibration standards in range of 0.25, 0.5, 1, 2, 5, 10ng/mL for linearity as below.

i. Preparation of 1 µg/mL of Aflatoxin intermediate stock standard:

a. Prepare 1 µg/mL intermediate mix of std. from 20µg/mL purchased stock standard by taking 50 µL in 1 ml volumetric flask and making upto 1 ml by adding 950 µl of methanol.
b. Store stock standard solution at -18^{o}C. Equilibrate to room temperature before use.

ii. Preparation of 100 ng/mL of Aflatoxin intermediate standard:

Prepare 100 ng/mL intermediate std. by taking 100 µl from 1 µg/mL standard solution in 1 ml volumetric flask and making upto 1 ml by adding 900 µl of methanol.

iii. Working Aflatoxin Standard Solutions:

a. From 100 ng/mL, prepare working standard solutions of 10 ng/mL, then make serial dilutions for 5 ng/mL, 2 ng/mL, 1 ng/mL, 0.5 ng/mL, 0.25 ng/mL.
b. Prepare daily 6 calibration standards in separate 1mL volumetric flasks according to Table 4.

c. Dilute to volume with methanol. Store in refrigerator and equilibrate to room temperature before use.

Table 4: Preparation of Working Aflatoxins calibration solutions by serial dilution:

Working standard solutions	Standard (ng/mL)	Volume required (µl)	Final volume (to 1 ml) with methanol
1.	0.25	500 µl of 0.5 ng/mL	500 µl
2.	0.5	500 µl of 1 ng/mL	500 µl
3.	1	500 µl of 2 ng/mL	500 µl
4.	2	400 µl of 5 ng/mL	600 µl
5.	5	500 µl of 10 ng/mL	500 µl
6.	10	100 µl of 100 ng/mL	900 µl

4.6 Sample Extraction:

Individual samples of ground nutswere divided into two 25 g portions. One portion was kept as a blank. The other was spiked with aflatoxin standards at the regulatory levels. Both portions were then carried through the Immunoaffinity column cleanup procedure.

i. Homogenize the sample with water in equal amount (1:1) to have paste.

Figure 10: Grinder

ii. Weigh 25g sample in 250 ml capacity centrifuge bottle followed by addition of 100ml extraction solvent methanol : water (80:20) and 5g sodium chloride.

iii. Keep the centrifuge bottle on orbital shaker for 30min with speed of 200 rpm.

iv. Then centrifuge the sample at 5000 rpm for 5 min. Filter with fluted filter paper.

Figure11: Rotary Shaker

Figure12: Centrifuge

v. Take 3 ml supernatant in 50 ml centrifuge tube and dilute with water (for Aflatest, VICAM) or PBS Solution (Aflaprep, R-Biopharmimmunoaffinity column) to 15 ml. Filter through glass micro fiber filter paper.

4.7 Immunoaffinity column cleanup:

Objective: To isolate aflatoxins from a test sample extract using an immunoaffinity column work-up.

Applicability: LC-FLD.Can be used for LC-MS/MS if matrix effects are very large.

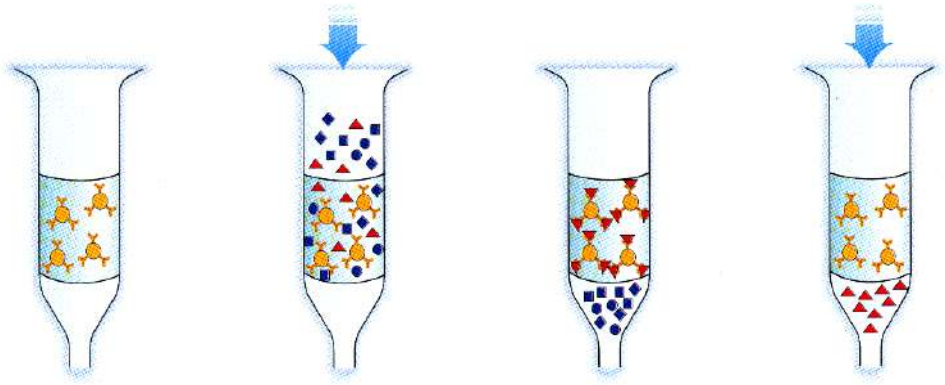
Advantage: Very clean sample

Figure 13:Immunoaffinity Column

Priniciple of Immunoaffinity column:

Affinity Chromatographyis a method of separating mixtures based on a highly specific interaction between an antigen and an antibody.

SAMPLE **WASHING** **ELUTION**



The sample extract is passed through the column.

The antibody isolates and concentrates the mycotoxin and retains it in the column.

Passage of solvent through the column denatures the antibody and releases the mycotoxin.

Figure14:Principle of Immunoaffinity column

Procedure:

i. Load the diluted sample to Immunoaffinitycolumn at flow rate 1.5 - 2 mL /min and then wash the column with 10 mL water.
ii. Elute with methanol (0.5ml×2).
iii. Dilute the final extract (0.5ml) with 0.5 ml of 1% acetic acid in water.

Precautions:

i. The immunoaffinity column should be kept refrigerated.
ii. The column needs to be brought to room temperature before use.
iii. Be careful not to let the column run dry in the steps where it is specified. But do make sure it runs dry right before you elute the mycotoxins to ensure you donot dilute your sample.

4.8. Determination of Aflatoxins:

4.8.1 HPLC –Fluorescence:

i. HPLC column: BEH 18 (100×2.1mm, 1.7µm)
ii. Column oven: 40°C
iii. Mobile phase: Methanol: Acetonitrile: Water (18:18:64)
iv. Flow rate: 0.4mL /min
v. Injection volume: 10 µl
vi. Detector: Emission 429 nm, 456 nm and excitation 360 nm.

Calculation:

Concentration of Aflatoxin (ng/g) = **[(X-Y)/m] × [(B/A) × (D/C)]**

Where,

X=Area of sample,

Y=Intercept value,

m= slope of calibration line,

B=Volume of extraction solvent,

A= Weight of sample (g),

C= Volume of extract taken for cleanup

D= Final volume of extract.

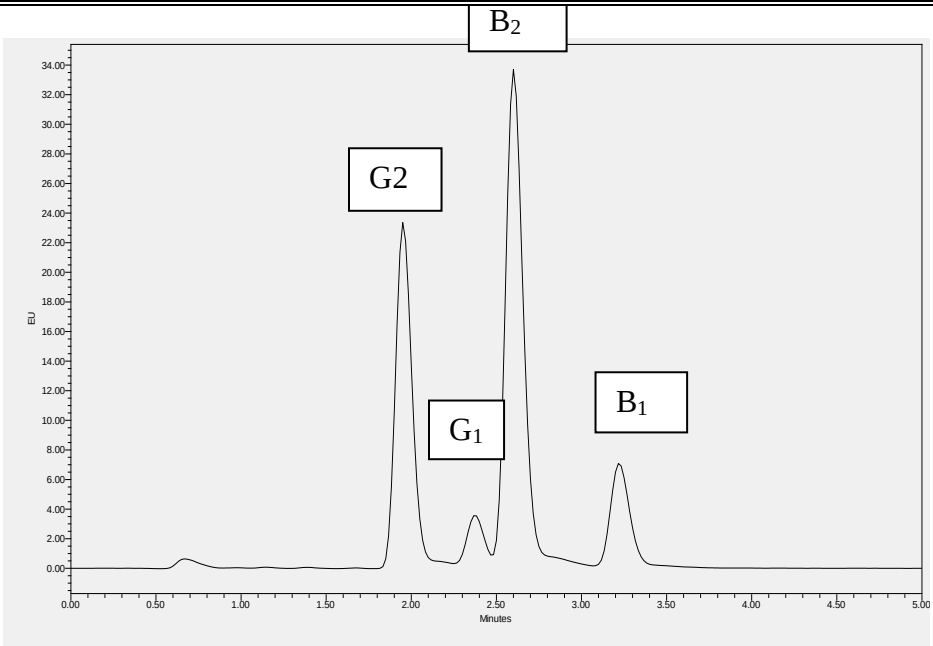


Figure15:Chromatogram of Standard 2 ppb Aflatoxin standard mix.

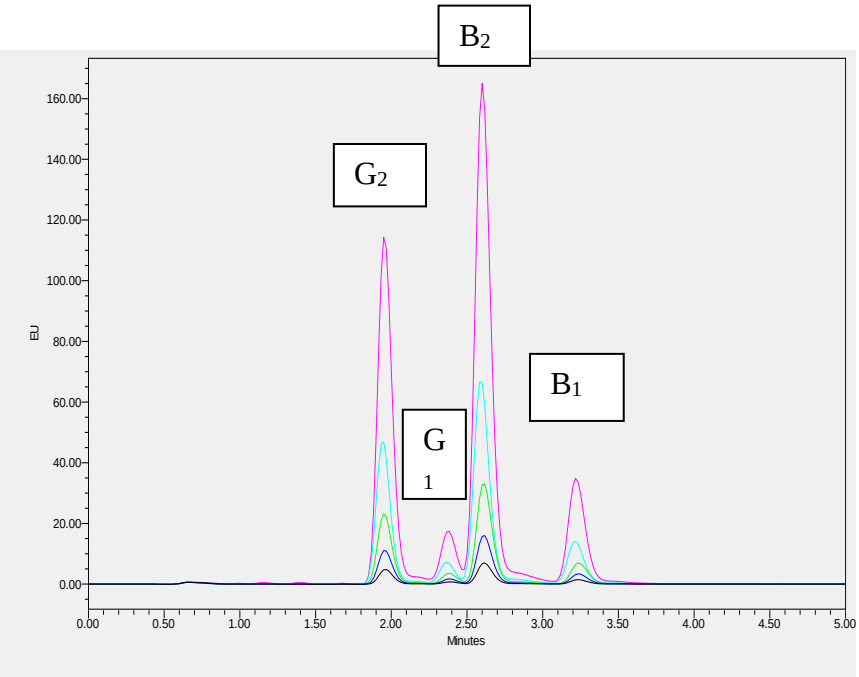
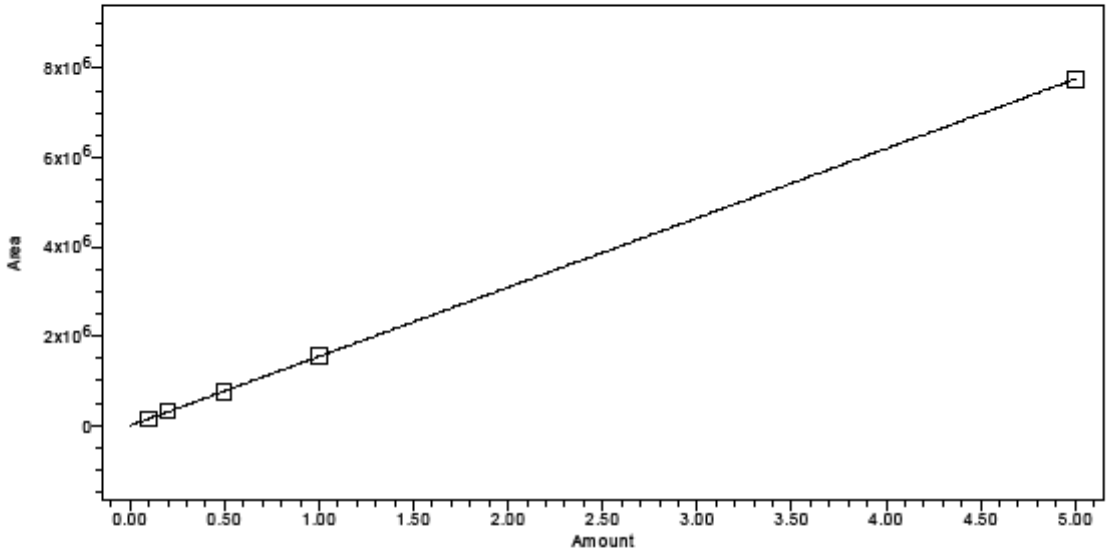


Figure 16: Overlay of Chromatograms for standard mixes: 0.2 ppb,0.5ppb,1 ppb, 2 ppb, 5ppb

Empower 3

LC Calibration Report

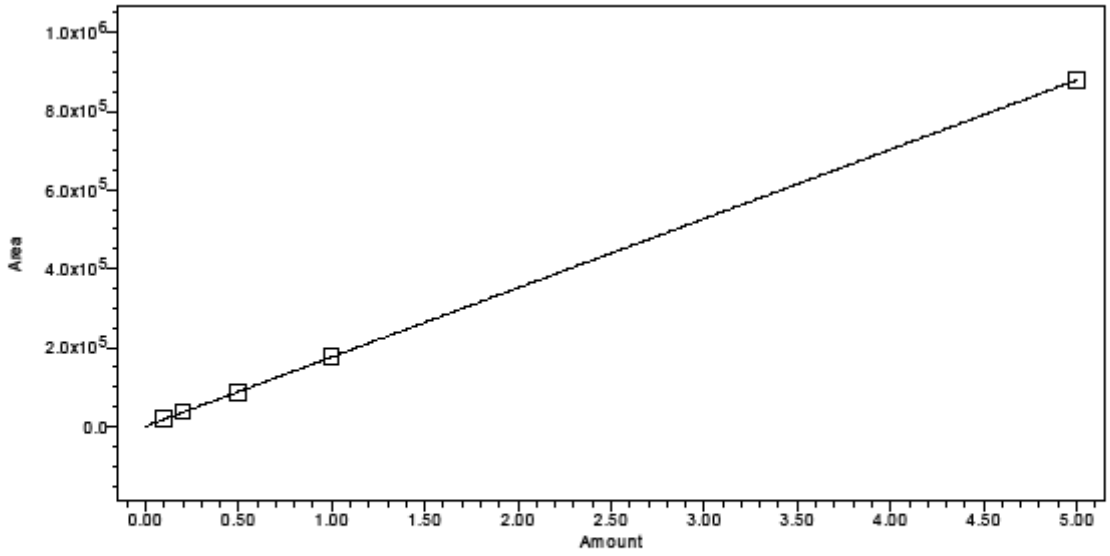
Processing Method:	Injection Calibration	System:	H Class_FLR
Processing Method ID:	7721	Channel:	ACQUITY FLR ChA
Calibration ID:	7938	Proc. Chnl. Descr.:	ACQUITY FLR ChA Ex365,Em456 nm
Date Calibrated:	18-Mar-17 12:10:11 PM IST		



Peak Name: AF G2; RT: 1.977; Fit Type: Linear (1st Order); Cal Curve Id: 7939; R: 0.999984; R^2: 0.999968; Weighting: None; Equation: Y = 1.55e+006 X - 7.73e+002; Normalized Intercept/Slope: -0.000196; RSD(E): 0.995817

Peak: AF G2

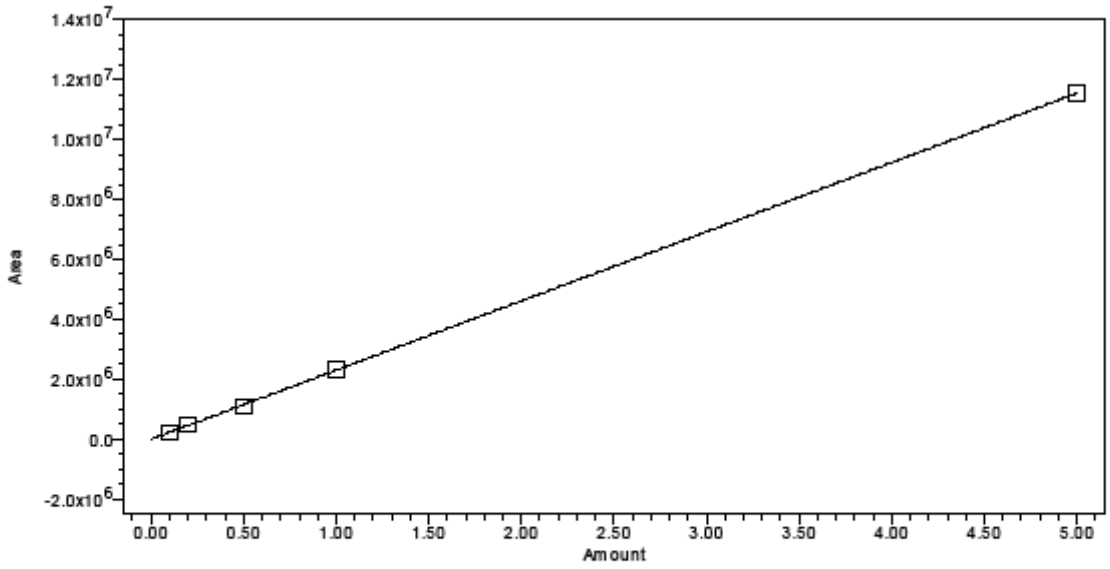
	Sample Name	Result Id	Peak Name	Level	X Value	Response	Calc. Value	% Deviation	Manual	Ignore
1	0.1 ppb	7937	AF G2		0.100	161406.254	0.105	4.61	No	No
2	0.2 ppb	7943	AF G2		0.200	320595.711	0.207	3.64	No	No
3	0.5 ppb	7944	AF G2		0.500	743134.784	0.480	-4.03	No	No
4	1 ppb	7945	AF G2		1.000	1562431.728	1.008	0.83	No	No
5	5 ppb	7947	AF G2		5.000	7750908.275	5.000	-0.00	No	No



Peak Name: AF G1; RT: 2.412; Fit Type: Linear (1st Order); Cal Curve Id: 7940; R: 0.999985; R^2: 0.999970; Weighting: None; Equation: Y = 1.76e+005 X + 6.06e+002; Normalized Intercept/Slope: 0.001353; RSD(E): 0.953937

Peak: AF G1

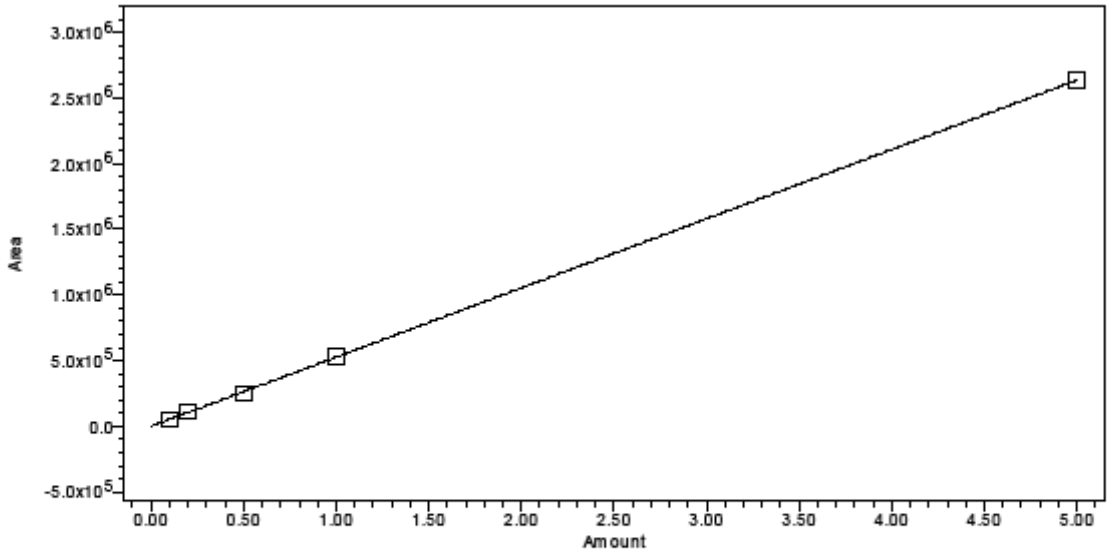
	Sample Name	Result Id	Peak Name	Level	X Value	Response	Calc. Value	% Deviation	Manual	Ignore
1	0.1 ppb	7937	AF G1		0.100	19326.172	0.107	6.55	No	No
2	0.2 ppb	7943	AF G1		0.200	37280.274	0.209	4.37	No	No
3	0.5 ppb	7944	AF G1		0.500	85043.947	0.481	-3.88	No	No
4	1 ppb	7945	AF G1		1.000	176884.770	1.003	0.33	No	No
5	5 ppb	7947	AF G1		5.000	879218.791	5.001	0.02	No	No



Peak Name: AF B2; RT: 2.640; Fit Type: Linear (1st Order); Cal Curve Id: 7941; R: 0.999980; R^2: 0.999961; Weighting: None; Equation: Y = 2.31e+006 X + 2.97e+003; Normalized Intercept/Slope: 0.000505; RSD(E): 1.093871

Peak: AF B2

	Sample Name	Result Id	Peak Name	Level	X Value	Response	Calc. Value	% Deviation	Manual	Ignore
1	0.1 ppb	7937	AF B2		0.100	238554.694	0.102	2.11	No	No
2	0.2 ppb	7943	AF B2		0.200	478691.418	0.206	3.09	No	No
3	0.5 ppb	7944	AF B2		0.500	1108310.575	0.479	-4.19	No	No
4	1 ppb	7945	AF B2		1.000	2341504.032	1.014	1.35	No	No
5	5 ppb	7947	AF B2		5.000	11537255.970	4.999	-0.02	No	No



Peak Name: AF B1; RT: 3.280; Fit Type: Linear (1st Order); Cal Curve Id: 7942; R: 0.999981; R^2: 0.999962; Weighting: None; Equation: Y = 5.27e+005 X + 1.99e+002; Normalized Intercept/Slope: 0.000148; RSD(E): 1.075799

Peak: AF B1

	Sample Name	Result Id	Peak Name	Level	X Value	Response	Calc. Value	% Deviation	Manual	Ignore
1	0.1 ppb	7937	AF B1		0.100	54599.611	0.103	3.18	No	No
2	0.2 ppb	7943	AF B1		0.200	109130.862	0.207	3.31	No	No
3	0.5 ppb	7944	AF B1		0.500	252675.788	0.479	-4.22	No	No
4	1 ppb	7945	AF B1		1.000	533710.951	1.012	1.19	No	No
5	5 ppb	7947	AF B1		5.000	2636006.002	4.999	-0.01	No	No

Chapter 6

Procedure for the Estimation of Concentration of Aflatoxins in Nut and Nut products & Cereal and Cereal products using LC-MS/MS

(Laboratory Validated Method)

1. Purpose:

This procedure describes the process for the Estimation of Aflatoxins contamination in Nut and Nut products& Cereal and Cereal products.

2. Scope:

These procedures apply to estimation of aflatoxins contamination in Nut and Nut products& Cereal and Cereal products.

3. Procedure:

3.1 Chemicals and Reagents:

i. Ammonium acetate
ii. Toluene
iii. Acetonitrile
iv. Methanol
v. Phosphate buffered saline
vi. Immunoaffinity Column (R-Biopharm)
vii. Distilled, reverse osmosis or deionized water

3.2 Phosphate buffered saline preparation method:

Potassium dihydrogen phosphate:	0.2 g/L
Disodium hydrogen phosphate Anhydrous:	1.16g/L
Potassium chloride:	0.2g/L
Sodium chloride:	8.0g/L

The final buffer should be pH 7.2 to 7.4 and is stable for 2 weeks at 2-4°C.

3.3 Reference standards:

Use Aflatoxin mix standard solution with known concentration (as an example: SupelcoAflatoxin Mix 46300-U).

3.4 Apparatus:

i. Mixer and grinder (20 L capacity)
ii. Homogenizer/Blender
iii. Analytical and precision balance
iv. LC-M/MS
v. Pipettes
vi. Test tubes
vii. Vacuum manifold
viii. Immunoaffinity column
ix. Fluted filter paper (24 cm), funnel and Erlenmeyer flask
x. Glass microfiber filter (11 cm) and funnel
xi. Syringe (glass or other material compatible with methanol)
xii. Bottles of 250 ml

3.5 Calibration Standard Preparation:

i. Standard Aflatoxin Mix Stock Solution:

B_1: 945 ng/ml, B_2: 286 ng/ml, G1: 924 ng/ml, G_2: 305 ng/ml. Purity: 99.5 %,99.5 %,99.7 %, 98 % respectively.

ii. IntermediateAflatoxin Mix Standard Solution:

Take 212 µl quantity of Standard Aflatoxin mix stock solution. Make volume upto 2 ml with mobile phase. The prepared standard contains:

B_1: 100.17 ng/ml, B_2: 30.3 ng/ml, G1: 97.9 ng/ml, G_2: 32.33 ng/ml.

iii. Working Aflatoxin Standard Solutions:

Table 6: Preparation ofWorkingaflatoxin calibration solutions:

Working standard solutions	Volume taken fromintermediate std.(µl)	Final aflatoxin concentration of working standard solution in ng/mL & volume makeup to 1mL			
		AF B_1	**AFB_2**	**AFG_1**	**AFG_2**
1	50	5	1.52	4.90	1.62
2	100	10	3.03	9.79	3.23
3	150	15	4.55	14.7	4.85
4	200	20	6.06	19.6	6.47
5	250	25	7.58	24.5	8.08
6	300	30	9.09	29.3	9.70

3.6 Instrumental Conditions:

I. LC Conditions:

i. Column: ZORBAX SB C 18, 150mm x 4.6 mm, 5 micron

ii. Column Temp: 40^0C

iii. Mobile phase: A = 10mM ammonium acetate in water 60%

B = Methanol 40%

iv. Flow rate: 0.3mL/min

v. Injection Volume: 5µL

II. MS/MS Conditions:

i. Source: Positive ESI

ii. Drying gas flow: 10L/min

iii. Nebulizer: 50psig

iv. Drying gas temp.: 350^0C

v. V_{cap}: 4000V

vi. Scan: M/Z 100-550

Table 7: Data Acquisition parameters of MRM Transitions for each Aflatoxin:

Aflatoxins	Precursor Ion	Product Ion	Dwell	Fragmentor Voltage	Collision Energy	Polarity
Aflatoxins B1	313.1	285.1	20	130	20	Positive
Aflatoxins B1	313.1	241.1	20	130	40	Positive
Aflatoxins B2	315.1	287	20	130	25	Positive
Aflatoxins B2	315.1	283.1	20	130	27	Positive
Aflatoxins G1	329.1	283.1	20	130	25	Positive
Aflatoxins G1	329.1	243.1	20	130	25	Positive
Aflatoxins G2	331.1	285.1	20	130	25	Positive
Aflatoxins G2	331.1	245	20	130	30	Positive

3.7 Sample Extraction and Clean-up:

i. Take 25g fine ground sample in a 250 ml bottle.

ii. Add 5 g NaCl followed by 100 ml 80% methanol.

iii. Cap and seal bottle. Shake at about 140 rpm on a horizontal shaker for 30 minutes.

iv. Centrifuge at 3000 rpm for 10 min. Filter through fluted filter paper.

v. Dilute 2 ml of filtrate with 18 ml of phosphate buffered saline.

vi. Adjust pH to 7.2 to 7.4 by using 2N NaOH, if required. Filter through glass micro fiber filter. Filtrate should be clear.

vii. Pass the dilute filtrate through the column at a flow rate of 2 ml per minute (or the sample can be allowed to pass through the column by gravity if preferred).A slow, steady flow rate is essential for the capture of the toxin by the antibody.

viii. Wash the column by passing 20 ml of PBS through at a flow rate of approximately 5 ml per minute. Pass air through the column to remove residual liquid.

ix. Elute the toxins from the column at a flow rate of 1 drop per second by passing 1 ml of 100% methanol and collect in a 5 ml amber glass vial. Back flushing is recommended.

x. Following elution, pass 1 ml of water through the column and collect in the same vial to give a 2 ml total volume.

xi. Inject 5 µL sample extract into the LC-MS/MS system.

3.8 Analysis on LC-MS/MS:

Injection sequence:

i. Inject calibration standards
ii. Inject recovery sample
iii. Inject the blank sample and verify the absence of analytes above 5 % of the recovery or sample concentration.
iv. Inject sample extracts
v. Re-inject the calibration standard at the appropriate level at least after every 20 injections and at the end of the run to verify instrument response.

3.9Calculations:

I. For Quantification of each compound

i. Review the chromatograms to verify that the analyte peaks are within the retention time windows and that the peaks are integrated correctly.
ii. Generate a linear curve fit to each analyte in standard curve using normalized response.
iii. Standard curve must have a correlation coefficient greater than or equal to 0.995.
iv. Blank must exhibit a response of less than 5% of the recovery.

II. For Confirmation:

i. Choose a standard or recovery containing the analyte of interest.
ii. Identify 2 product ion peaks in the sample and verify that their peaks are present with a signal to noise ratio ≥ 3. Auxiliary ions may be used if necessary.
iii. Identify the retention time of the two product ion peaks in the standard or recovery and in the sample of interest. The sample peak retention times must be within ± 5% of the standard or recovery retention times.
iv. Calculate the ratio of the response of product ion #2 to product ion #1 in the standard or recovery for the analyte of interest:

Ratio = Product ion#2/ Product Ion #1

Note: Ion ratio should be less than 1. If not, then invert the ratio.

Aflatoxins (B1, B2, G1, G2)=ng/ml (ppb) concentration from calibration curve X DF

Inng/g (µg/kg)Weight of sample taken in g

Analysis of Aflatoxin G2on LC-MS/MS

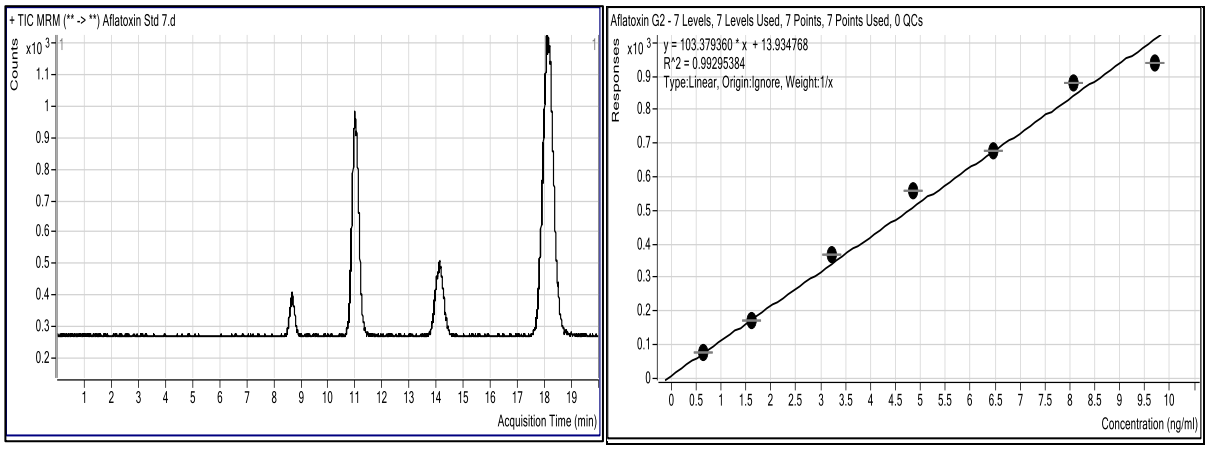


Figure17:Total Ion Chromatogram (TIC)Figure18:Calibration Curve

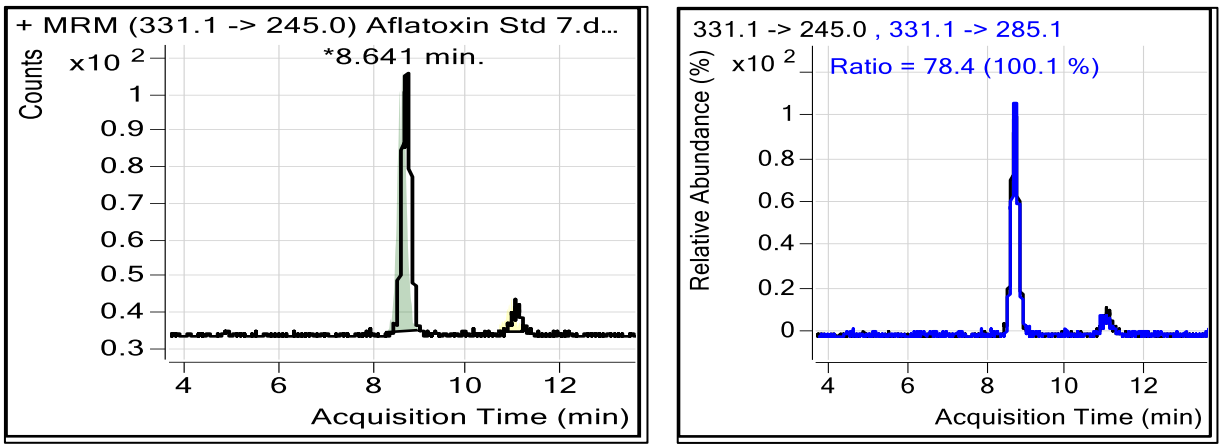


Figure 19: Retention Time of Aflatoxin G2Figure 20:Qualifier Quantifier Ratio

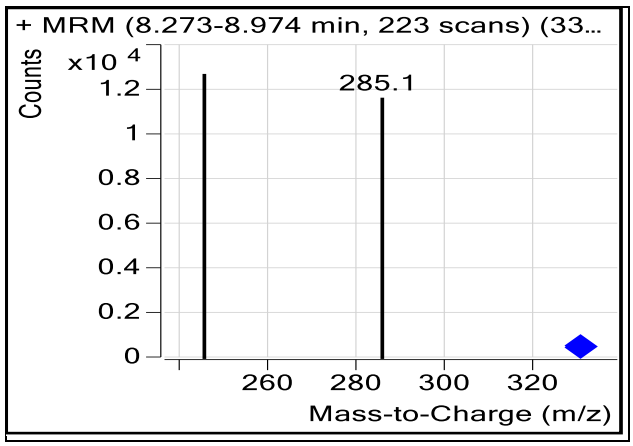


Figure21:Transitions of Aflatoxin G2

Analysis of Aflatoxin G1 on LC-MS/MS

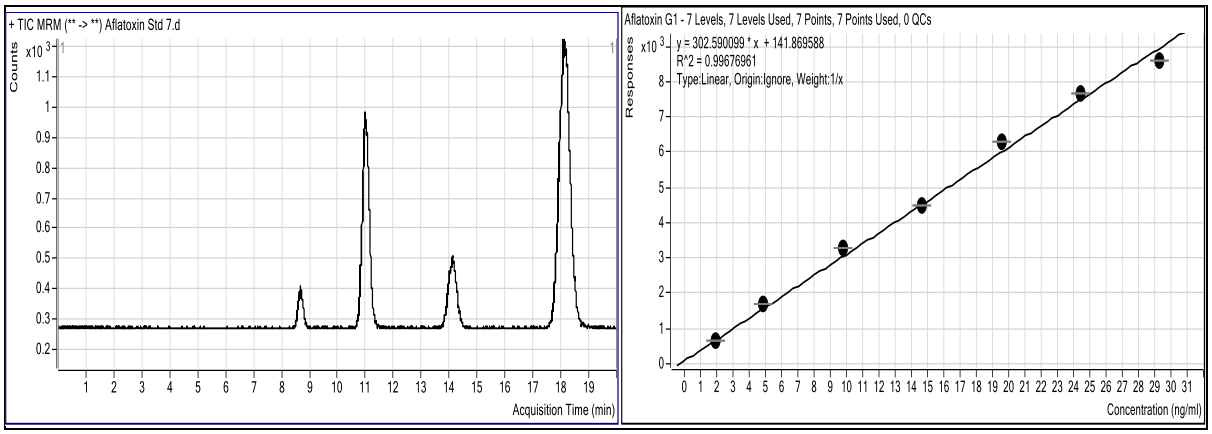
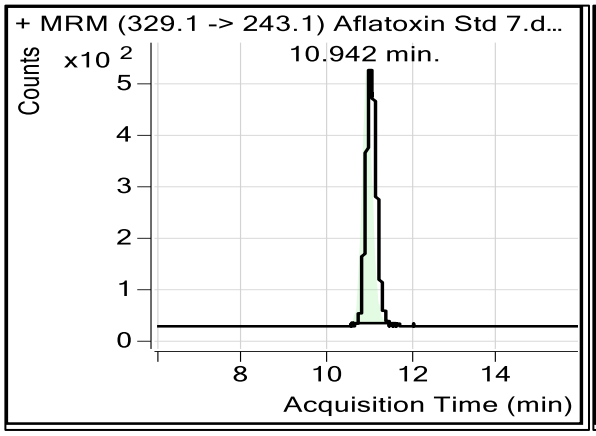


Figure22:Total Ion Chromatogram (TIC)Figure23: Calibration Curve



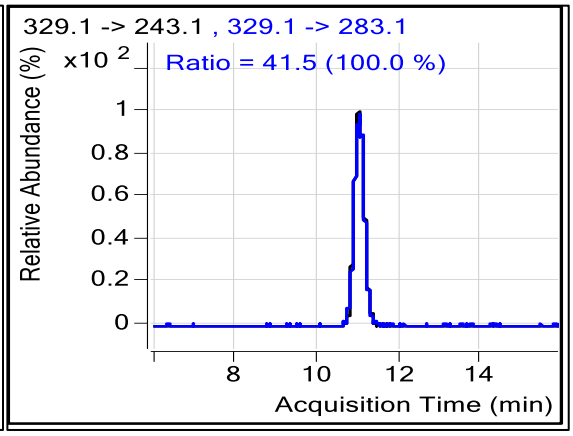


Figure 24:Retention Time of Aflatoxin G1 Figure 25:Qualifier Quantifier Ratio

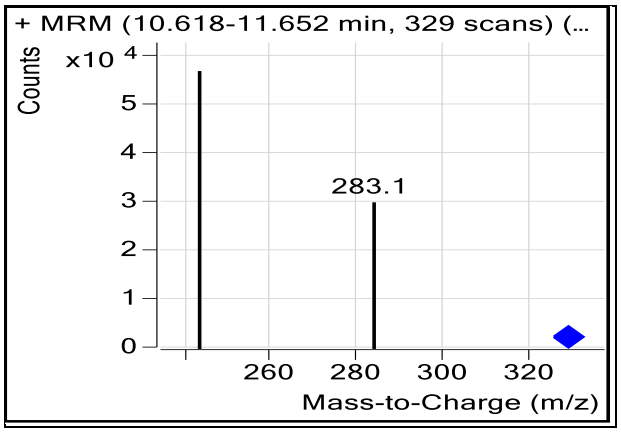
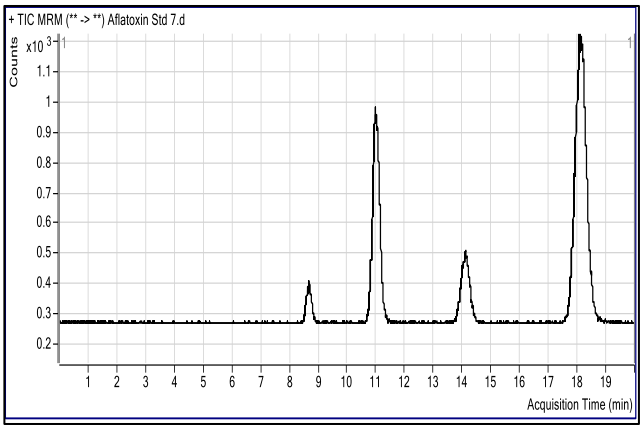


Figure 26:Transitions of Aflatoxin G1

Analysis of Aflatoxin B2 on LC-MS/MS



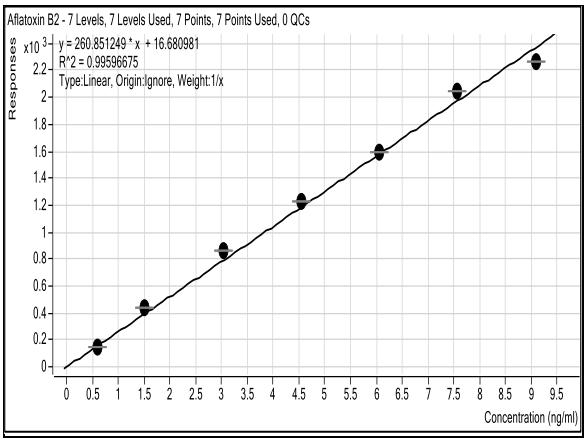
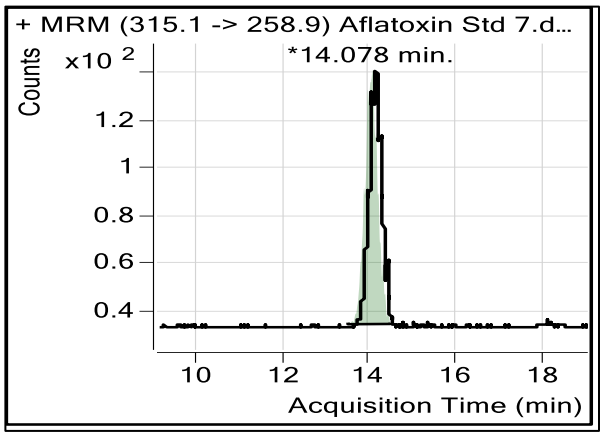


Figure 27:Total Ion Chromatogram (TIC)Figure 28:Calibration Curve



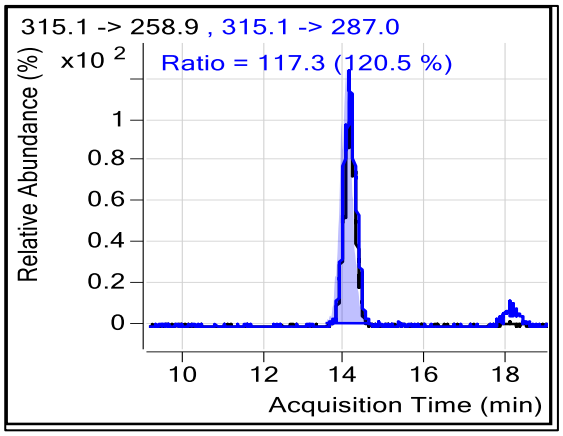


Figure29:Retention Time of Aflatoxin B2 Figure 30:Qualifier Quantifier Ratio

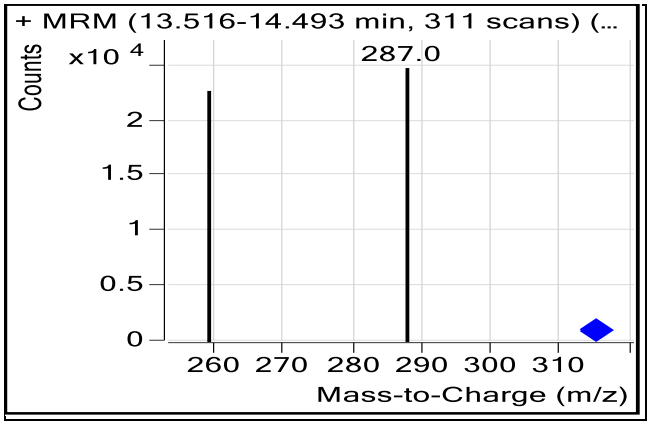
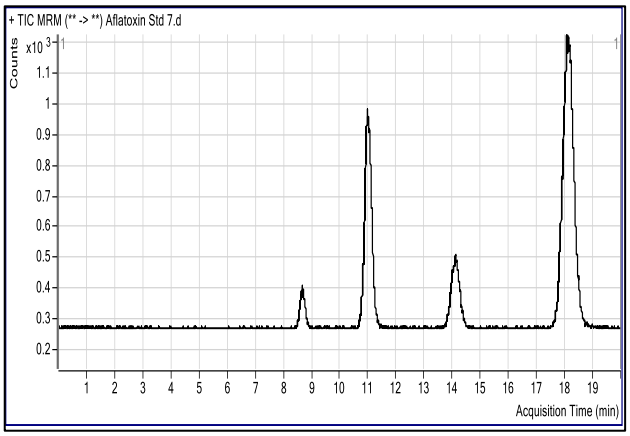


Figure 31:Transitions of Aflatoxin B2

Analysis of Aflatoxin B1 on LC-MS/MS



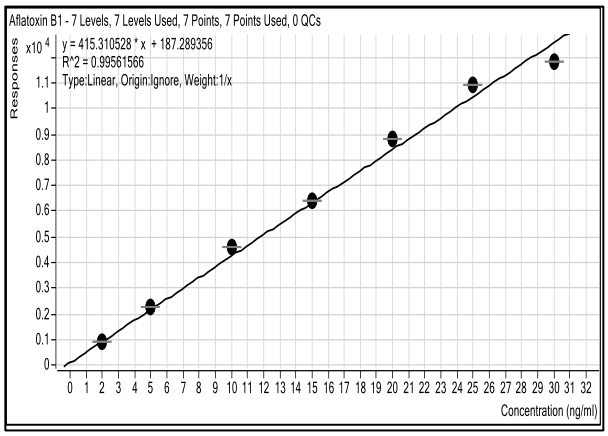
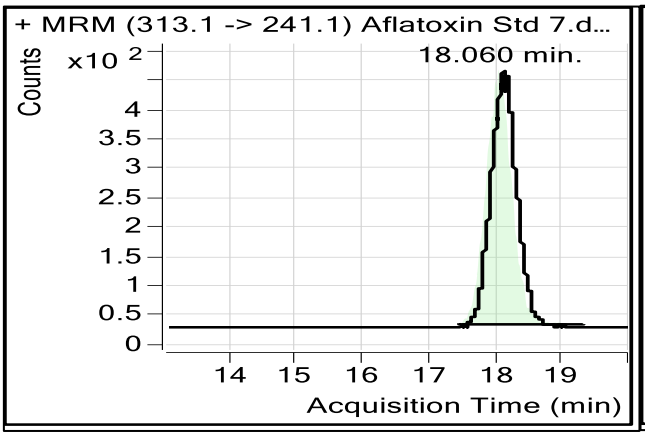


Figure 32:Total Ion Chromatogram (TIC)Figure 33:Calibration Curve



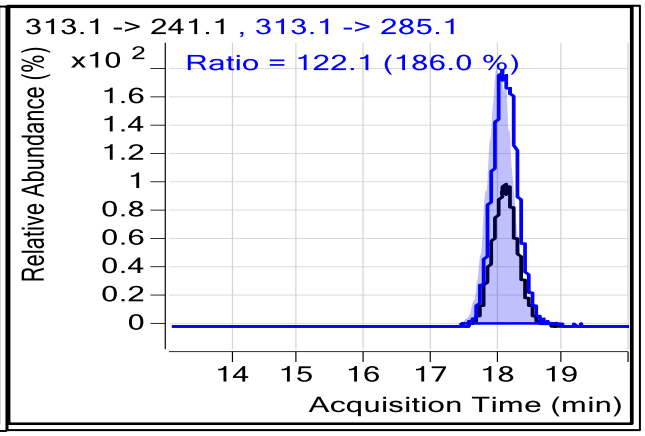


Figure34:Retention Time of Aflatoxin B1 Figure35:Qualifier Quantifier Ratio

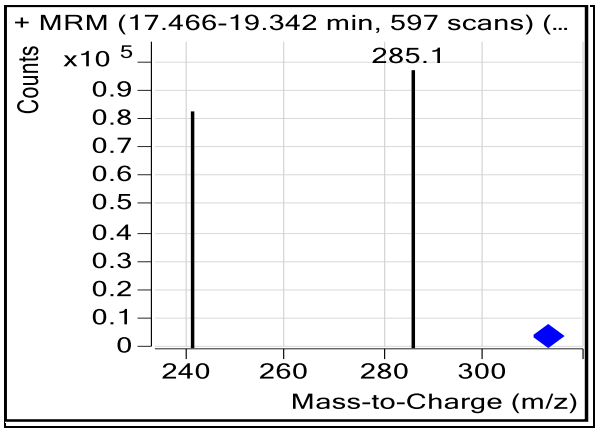


Figure36:Transitions of Aflatoxin B1

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2) AOAC Official Methods, Chapter 49, 20th Edition, 2016 AOAC International.
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WORK SHEETS

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