File No. 11014/04/2021-QA (E file 1349)

Food Safety and Standards Authority of India

(A statutory Authority established under the Food Safety and Standards Act, 2006)

(Quality Assurance Division)

FDA Bhawan, Kotla Road, New Delhi – 110002

Dated, the 20 July, 2021

ORDER

Subject: **Revised FSSAI Manual of Methods of Analysis of Foods – Mycotoxins – reg.**

Revised FSSAI Manual of Methods of Analysis of Foods – Mycotoxins was uploaded on the website of FSSAI vide Order No. 11014/08/2020-QA dated 04.12.2020.

2. The 'Method for Determination of Patulin in Apple and Apple Juice' which has been approved by the Food Authority in its 35th meeting held on 24.06.2021 has been included in the 'Revised FSSAI Manual of Methods of Analysis of Foods – Mycotoxins'.

3. Accordingly, the enclosed 'Revised FSSAI Manual of Methods of Analysis of Foods – Mycotoxins' shall be treated as a substitution of the manual uploaded on 04.12.2020 and shall be used by the laboratories with immediate effect.

4. Since the process of updation of test methods is dynamic, any changes happening from time to time will be notified separately. Queries/concerns, if any, may be forwarded to *email: sp-sampling@fssai.gov.in, dinesh.k@fssai.gov.in*

Encl: as above

(Harinder Singh Oberoi)
Advisor (QA)

To:

1. All FSSAI Notified Laboratories
2. All State Food Testing Laboratories

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA

Inspiring Trust, Assuring Safe & Nutritious Food

Ministry of Health and Family Welfare, Government of India

MANUAL OF METHODS OF ANALYSIS OF FOODS

MYCOTOXINS

PREFACE

Food safety requires an assurance that food will not cause any harm to the consumer, when it is prepared and/or consumed according to its intended use. There is a significant challenge in ensuring food safety to protect public health. Safeguarding food safety in today's complex world is a formidable task and is possible only with an intensive effort of all the stakeholders including regulatory authorities, industry and consumers.

The FSSAI Manual of Methods for Analysis of Mycotoxins is principally intended to provide unified, up-to-date testing methods for regulatory compliance. The manual brings together testing methodologies approved by FSSAI for use in surveillance and implementing the regulatory program. The objective here is to adopt "One Parameter - One Method" approach. These methods are dynamic and will be constantly updated, commensurate with the latest technological advancements in food analysis. The FSSAI notified laboratories shall use these testing methods only for analyzing samples under the Food Safety and Standards Act, 2006 and Food Safety and Standards Regulations, 2011.

Any suggestions/feedback from the stakeholders, which will contribute towards updating the manuals from time to time are welcome.

Shri ArunSinghal
Chief Executive Officer,
Food Safety and Standards Authority of India,
FDA Bhawan, Kotla Road,
New Delhi – 110002

ACKNOWLEDGEMENT

My deepest sense of gratitude and indebtedness to all the Members of the Panel on "Methods of Sampling and Analysis" especially Dr. Lalitha R Gowda whose help, knowledge and insight has led to the successful revision of this manual.

Sincere thanks to the Panel, Chairman for their valuable guidance and encouragement and the Secretariat of this panel who have extended their support during this revision process.

Deepest appreciation to the Chairperson, FSSAI and CEO, FSSAI for their cooperation, support and constant encouragement without which the work would not have seen the light of day.

July 2021

Dr. Harinder Singh Oberoi
Advisor (QA),
Food Safety and Standards Authority of India,
FDA Bhawan, Kotla Road,
New Delhi – 110002

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA

Inspiring Trust, Assuring Safe & Nutritious Food

Ministry of Health and Family Welfare, Government of India

List of Contributors

Name	Organization
Dr. Rajan Sharma	ICAR-National Dairy Research Institute, Karnal
Dr. Ajit Dua	Punjab Biotechnology Incubator, Mohali
Dr. AK Dikshit	Retd. from Division of Agricultural Chemicals, Indian Agricultural Research Institute (IARI), Pusa, New Delhi
Dr. Alok Kumar Srivastava	CSIR-Central Food Technological Research Institute (CFTRI), Mysuru
Dr. Jagan Mohan Rao	Retd. from CSIR-Central Food Technological Research Institute (CFTRI), Mysuru
Dr. Kiran N Bhilegaonkar	ICAR-Indian Veterinary Research Institute Regional Station, Pune
Dr. Lalitha Ramakrishna Gowda	Retd. from CSIR-Central Food Technological Research Institute (CFTRI), Mysuru
Dr. Mohana Krishna Reddy Mudiam	CSIR- Indian Institute of Chemical Technology, Hyderabad
Dr. Rajesh R Nair	CALF, National Dairy Development Board, Anand
Dr. Raju Khan	CSIR-Advanced Materials & Processes Research Institute, Bhopal
Dr. Reena Das	Postgraduate Institute of Medical Education and Research, Chandigarh
Dr. Kaushik Banerjee	ICAR-National Research Centre for Grapes, Pune
Dr. Sanu Jacob	Food Safety and Standards Authority of India
Dr. Dinesh Kumar	Food Safety and Standards Authority of India
Shri Shailender Kumar	Food Safety and Standards Authority of India
Ms. Kanika Aggarwal	Food Safety and Standards Authority of India
Shri Ratish Ramanan	Food Safety and Standards Authority of India

TABLE OF CONTENTS

		Performance Liquid Chromatography with fluorescence detection	
16.	FSSAI 07.013:2020	Determination of Aflatoxins M1 and M2 in Fluid Milk Liquid Chromatographic Method	60-63
17.	FSSAI 07.014:2020	Method for Determination of Aflatoxin M1 In Liquid Milk: Immunoaffinity Column Chromatography followed by Liquid Chromatography	64-67
18.	FSSAI 07.015:2020	Determination of Aflatoxins B1, B2, G1, And G2 in Foodstuffs other than described above	68-70
19.	FSSAI 07.016:2020	Determination of Deoxynivalenol (DON) in Wheat (Thin Layer Chromatographic Method)	71-73
20.	FSSAI 07.017:2020	Determination of Ochratoxin (OTA) in Barley Thin Layer Chromatographic Method	74-78
21.	FSSAI 07.018:2020	Ochratoxin A in Barley: Immunoaffinity by Column HPLC and fluorescence detection	79-83
22.	FSSAI 07.019:2020	Direct analysis of Aflatoxins (AF) and Ochratoxin A (OTA) in cereals and their processed products by Ultra-High-Performance Liquid Chromatography with fluorescence detection	84-86
23.	FSSAI 07.020:2021	Determination of Patulin in Apple and Apple Juice	87-89

Note: The test methods given in the manual are standardised/ validated/ taken from national or international methods or recognised specifications, however it would be the responsibility of the respective testing laboratory to verify the performance of these methods onsite and ensure that it gives proper results before putting these methods in to use.

List of Abbreviations

AF	Aflatoxin
DON	Deoxynivalenol
ELISA	Enzyme Linked Immunosorbent Assays
FLD	Fluorescence detector
HPLC	High Performance Liquid Chromatography
HP-TLC	High Performance Thin Layer Chromatography
IAC	Immuno-Affinity Chromatography
LC	Liquid Chromatography
LC-MS/MS	Liquid chromatography tandem mass
OTA	Ochratoxin A
PAT	Patulin
PBS	Phosphate Buffered Saline
PHRED	Photochemical Reactor Enhanced Detection
TLC	Thin Layer Chromatography
UPLC	Ultra Performance Liquid Chromatography

1.0 Introduction

Mycotoxins—toxic secondary metabolites of filamentous fungi—are biological in origin. Only a few of the thousands of mycotoxins present significant food safety challenges to the farm-to-fork food continuum. The natural fungal flora associated with food safety are dominated by three genre: *Aspergillus, Fusarium,* and *Penicillium.*

These fungal metabolites when present in sufficiently high levels in food, can have toxic effects that range from acute (for example, liver or kidney deterioration), to chronic (for example, liver cancer), mutagenic, and teratogenic; and resulting symptoms range from skin irritation to immunosuppression, neurotoxicity, and death (ICMSF 1996). Aflatoxin B1, fumonisins, and patulin are suspected human carcinogens.

The chemical structures of some important mycotoxins are shown in Figure 1.

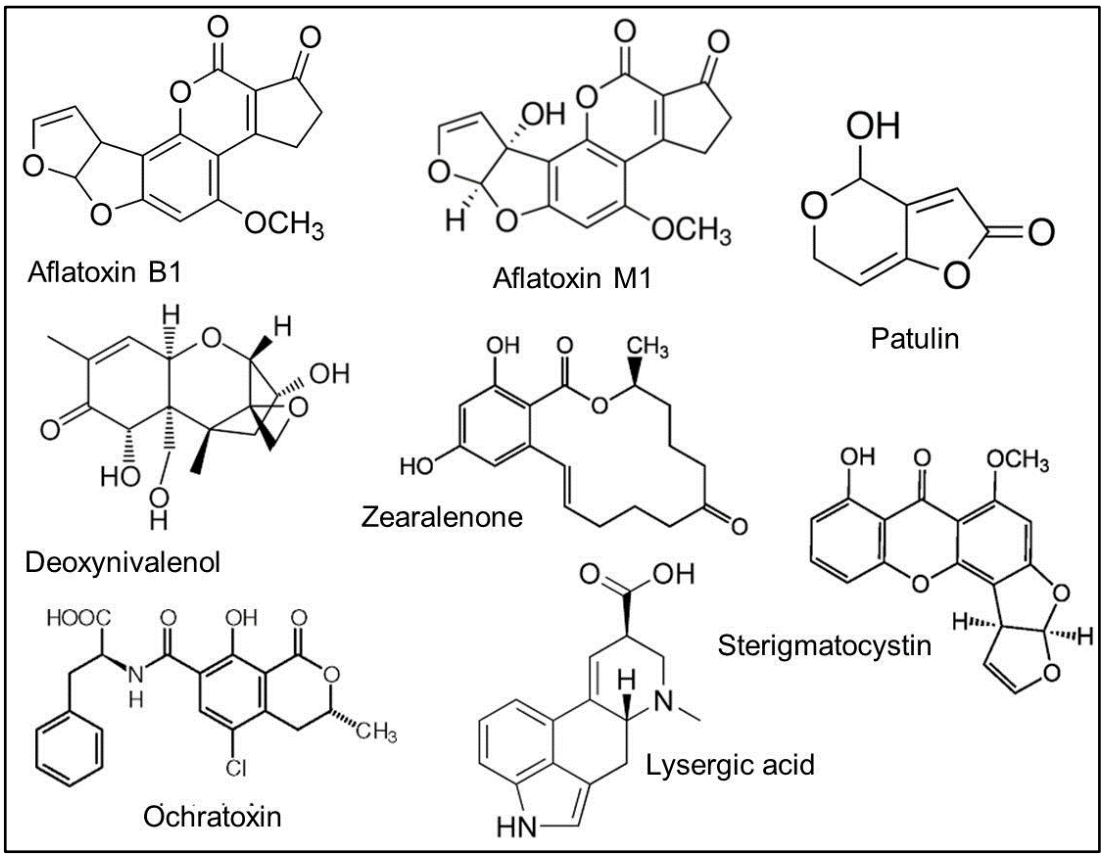


Figure 1: Chemical structures of a few mycotoxins that are of food safety concern.

Aflatoxins

Aflatoxin, ahighly toxic secondary metabolite derived from polyketides produced by fungal species *Aspergillus flavus, A. parasiticus,* and *A. nomius*,is probably the most common and widely known mycotoxin contaminant. Aflatoxin-producing fungi can contaminate crops in the field, at harvest, and during storage.Some of the more common crops susceptible to contamination with aflatoxins are cereals (e.g. maize, rice and wheat), tree nuts (e.g. pistachios, walnuts and Brazil nuts), cottonseed and groundnuts and can lead to serious threats to human and animal health. Unrefined vegetable oils made

from contaminated seeds or nuts usually contain aflatoxin. However, during the refining process aflatoxin is destroyed therefore, refined oils are safe. The most ambient climates for aflatoxin-production are high temperature and humidity typically found in tropical and subtropical regions of the world including sub-Saharan Africa and Southern Asia.

There are more than 20 known aflatoxins, but the four main ones are aflatoxin B1, aflatoxin B2, aflatoxin G1 and aflatoxin G2. Aflatoxin M1 and M2 are the mono-hydroxylated derivatives of B1 and B2, respectively, and occur in the milk of lactating mammals including humans, after ingestion of food or feed contaminated with the toxins. The chemical structures of the aflatoxins are show in Figure 2. The level of toxicity associated with aflatoxin varies with the types present, with the order of toxicity beingB1> G1> B2>G2

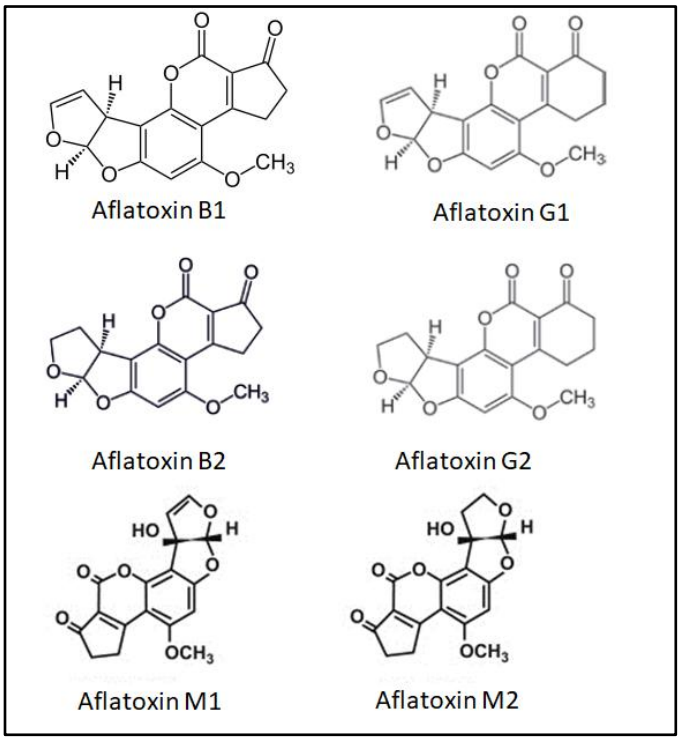


Figure 2:Chemical structures of the six aflatoxins

Aflatoxin 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 an oxygen in the cyclopentanone ring.

The stringent regulations worldwide place more emphasis on estimating the aflatoxin content in food and feed. The current methods for quantitative aflatoxin suitable for use in regulatory laboratories include 1) thin layer chromatography (TLC), 2) high performance thin layer chromatography (HP-TLC)3) high performance liquid chromatography (HPLC), and4) the more recent liquid chromatography tandem mass spectrometry (LC-MS/MS). Several semiquantitative and qualitative methods including Enzyme Linked Immunosorbent Assays (ELISA) and immunoaffinity column followed by fluorescence spectrometry are also used.Rapid in-field and laboratory involve the lateral flow dip-stick kits, hyperspectral imaging and electronic nose.

Deoxynivalenol (DON)

Deoxynivalenol (DON) also known as vomitoxin is a trichothecene mycotoxin mainly produced by Fusarium fungi (Fusarium molds). Major producing fungi include Fusarium species *F. graminearum* and *F. culmorum*, one of plant pathogens that cause scab mainly in wheat and barley etc., and damages cereals the most widely by contamination in the field. The main commodities affected are cereals such as wheat, rice, barley, oats and maize etc.

Trichothecene mycotoxins are classified into three groups by structural characteristics, and deoxynivalenol is classified into Group B.

Generic name: Deoxynivalenol
IUPAC Name: (3α,7α)-3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one
Molecular weight: 296.13
Molecular formula: C15H20O6

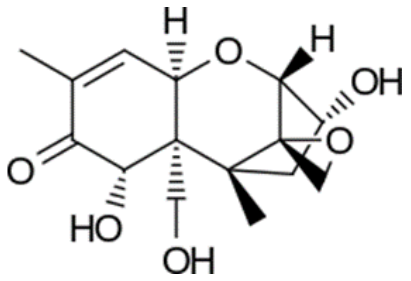


Figure 3 Chemical structure of DON

Trichothecene mycotoxins act on serotonin-mediated neurons and induce anorexia and vomiting**.** FSSA(I) has established a level of restriction.

The current methods suitable for use in regulatory laboratories for DON estimation include 1) thin layer chromatography (TLC), 2) high performance liquid chromatography (HPLC), and 3) the more recent liquid chromatography tandem mass spectrometry (LC-MS/MS).

Patulin

Patulin(Figure 4) is a mycotoxin that is produced by certain species of *Penicillium, Apergillus*, and *Byssochylamys* molds that may grow on variety of foods including fruit, grains, and cheese.

Generic name: Patulin
IUPAC Name: 4-hydroxy-4*H*-furo[3,2-*c*]pyran-2(6*H*)-one
Molecular weight: 154.12
Molecular formula: $C_7H_6O_4$

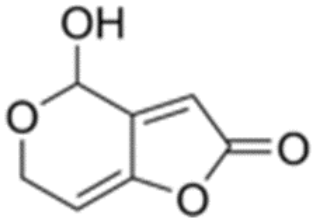


Figure 4. Structure of Patulin

Patulin is a furopyran (Figure 4)Patulin has been found to occur in a number of foods including apple juice, apples, and pears. Patulin contamination is primarily associated with damaged and rotting fruits and fruit juicesmade from poor quality fruits.The amount of patulin in apple products is generally viewed as a measure of the quality of the apples used in production. It is not a particularly potent toxin, but a number

of studies have shown that it is genotoxic, which has led to some theories that it may be a carcinogen, though animal studies have remained inconclusive.

Ochratoxin A

Ochratoxin A (OTA) is a naturally occurring foodborne mycotoxin found in a wide variety of agricultural commodities worldwide, ranging from cereal grains to dried fruits to wine and coffee. Ochratoxins A, B, and C contain a phenylalanine moiety attached to a dihydroisocoumarin group via an amide bond (Figure 5). OTA is the most prevalent, most important from an animal and human health standpoint, while ochratoxins B and C are of lesser importance. It is produced by several fungal species including *Aspergillus ochraceus*, *A. carbonarius*, *A. niger* and *Penicillium verrucosum*. Contamination generally occurs as a result of poor storage of commodities and suboptimal agricultural practices during the drying of foods. Ingestion is the main source of exposure to OTA. OTA is a chemically stable compound; hence, ordinary food processing measures fail to substantially reduce its presence in foods and beverages. OTA has been shown to be toxic and carcinogenic in animals. It is nephrotoxic to multiple species, and is a potent renal carcinogen in rodents. The kidney is the main target organ.

Generic name: Ochratoxin
IUPAC Name: *N*-{[(3*R*)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1*H*-isochromen-7-yl]carbonyl}-L-phenylalanine
Molecular weight: 403.81
Molecular formula: $C_{20}H_{18}ClNO_6$

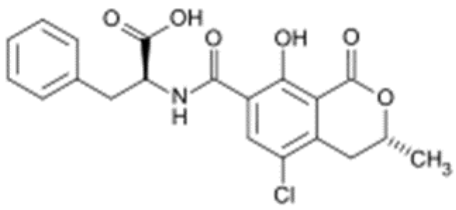


Figure 5 Structure of Ochratoxin A

2.0 The **regulatory limits** for the presence of these contaminant is listed in Table 1 below:

CURRENT FSSA(I) REGULATORY LIMITS FOR MYCOTOXINS IN FOODS		
MYCOTOXIN	Food product	FSSA(I) Regulatory limit (µg /Kg)
AFLATOXIN	Cereal and Cereal Products	15
	Pulses	15
	Nuts	
	Nuts for further processing	15
	Ready to eat	10
	Dried figs	10
	Oilseeds or oil	
	Oilseeds for further processing	15
	Ready to eat	10
	Spices	30
	Betelnut/Arecanut	15
AFLATOXIN M1	Milk	0.5
OCHRATOXIN A	Wheat, barley and rye	20
PATULIN	Apple juice and Apple juice ingredients in other beverages	50
DEOXYNIVALENOL	Wheat	1000 (1ppm)

3.0Safety requirements while handling mycotoxins

All food samples suspected of being contaminated with mycotoxins must be handled with extreme care. Aflatoxins are potent carcinogenic substances. Refer to MSDS for specific information.

I.Personal Safety precautions

a) Use disposable gloves and protective face masks while grinding the food creates dust.
b) Prepare samples in area separate from analytical laboratory.
c) Wear a full sleeved lab coat, safety goggles, closed shoes and gloves when carrying out analyses.
d) The laboratory coat or apron must be soaked in 5% sodium hypochlorite solution over-night and washed in water
e) All work must preferably be carried out in a hood
f) While handling pure aflatoxin reference material, extreme precautions must be taken as they are electrostatic.
g) Weighing and transferring mycotoxins in dry form should be avoided; they should be dissolved in a solvent. The electrostatic nature of a number of the mycotoxins in dry form results in a tendency for them to be easily dispersed in the working area, and to be attracted to exposed skin and clothes. Their concentrations should be determined spectrophotometrically.
h) Protect eyes with UV-absorbing filter when using UV-viewing chamber.
i) Swab any accidental spill of toxin with 1% sodium hypochlorite bleach (NaOCl), leave 10 min and then add 5% aqueous acetone.

II. Precautions during analysis

a) 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.
b) Always dry TLC plates thoroughly before exposure to UV light.
c) 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.
d) Avoid exposing to UV light underdeveloped spots and expose developed plates to UV light for the minimum time needed for visualization.
e) Protect analytical material adequately from light and keep aflatoxin standard solutions protected from light by using amber vials or cover with aluminium foil. Put a warning note on the label.

III. Handling glassware for aflatoxin analysis

a) Use of non-acid washed glassware for aflatoxin aqueous solutions may cause loss of aflatoxin.
b) Before use soak new glassware in dilute acid (carefully add 105 mL concentrated Sulphuricacid to water and make upto 1 L) for several h, then rinse extensively with distilled water to remove all traces of acid. (Check with pH paper).
c) Rinse all glassware exposed to aflatoxin with methanol, add 1% sodium hypochlorite (NaOCl) solution and after 2 h add acetone to 5% of total volume. Let it react for 30 min and then wash thoroughly.

Reference:FAO Manuals of Food Quality Control 14 /7, 1986, page 185 / AOAC 17th edn, 2000, Chapter 49, subchapter 1 Mycotoxins /Sub chapter 2 Aflatoxins).

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Preparation of a Homogenous Laboratory Sample for Analysis of Aflatoxin**		
Method No.	FSSAI 07.001:2020	**Revision No. & Date**	0.0
Caution	Follow all personal safety procedures while handling and disposing solution described earlier. Grinding of dry samples may result in airborne dust. Even if no toxin is present there is potential harm from inhalations. Use protective mask and or dust collector. Prepare samples in area separated from analytical laboratory.		
Preparation of Lot sample	Mold 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. Therefore, sampling and sample preparation is very important. Use the entire 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 one 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, grind entire sample to pass through a No 20 sieve. 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, and homogenize thoroughly to obtain a homogeneous sample.		
Preparation of Laboratory Sample	Draw with the same precaution as with a lot sample. Wherever practical, divide using riffling splitter or similar random dividing procedure until sub-division is close to the mass of desired analytical sample		
Reference	AOAC 17th edn, 2000, Official Method 977.16 Sampling of Aflatoxins, Preparation of Sample		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

	Preparation of Aflatoxin Standards for Thin Layer Chromatography Method		
Method No.	FSSAI 07.002:2020	**Revision No. & Date**	0.0
Caution	Follow all personal safety procedures while handling and disposing solution described earlier. Weighing and transferring mycotoxins in dry form should be avoided; they should be dissolved in a solvent. The electrostatic nature of a number of the mycotoxins in dry form results in a tendency for them to be easily dispersed in the working area, and to be attracted to exposed skin and clothes.		
Principle	Determining the concentrations of aflatoxin standards solutions spectrophotometrically.		
Materials and Reagents	1. Acetonitrile- HPLC grade 2. Benzene HPLC grade 3. Methanol- HPLC grade 4. Toluene -HPLC grade 5. Aflatoxin Standards		
Preparation of Reagents	1. Benzene-acetonitrile: Mix 98 mL benzene and 2 mL acetonitrile 2. Toluene-acetonitrile: Mix 90 mL toluene and 10 mL acetonitrile		
Preparation of standard	*Aflatoxin standards received as dry films or crystals***:** i. To containers of dry aflatoxins B1, B2, G1, G2 using the label statement of aflatoxin t as guide add the required volume of either one of the following solvents 1) acetonitrile, 2) benzene–acetonitrile (98+2), 3) methanol or 4) toluene–acetonitrile (9+1), calculated to give a concentration of 8-10 µg/mL. ii. For Aflatoxin M1 use benzene-acetonitrile (9+1). Use label statement of Aflatoxin weight as guide. iii. Vigorously agitate solution for one min on a vortex shaker and transfer without rinsing to a convenient sized glass flask. iv. Do not transfer dry Aflatoxins for weighing or other purposes unless facilities are available to prevent dissemination to the surroundings because of electrostatic charge on particles. v. For Aflatoxins received as solutions transfer solution to convenient sized glass stoppered flask. Dilute if necessary, to adjust the concentration to 8-10 µg/mL.		
Method of analysis	*Determination of aflatoxin concentration:* Record the UV-Vis spectrum of the aflatoxin solution from 200-500 nm. Determine the concentration of individual aflatoxin by measuring the absorbance (A) at wavelength of maximum absorption close to 350 nm and substitute in the following equation		

$$\text{Concentration of aflatoxin (mg/L)} = \frac{A_{350} \times Mw \times 1000}{\varepsilon}$$

Where A_{350} = the absorbance of the aflatoxin at 350 nm,
M_w = molecular weight of the aflatoxin (Table below),
ε = the molar absorptivity of the aflatoxin in benzene-acetonitrile solution. The Mw and molar absorptivity values are provided in the Table below

Aflatoxin	Molecular weight	Solvent	E
B1	312	Benzene-acetonitrile (98+2)	19800
		Toluene-acetonitrile (9+1)	19300
		Methanol	21500
		Acetonitrile	20,700
B2	314	Benzene-acetonitrile (98+2)	20900
		Toluene-acetonitrile (9+1)	21000
		Methanol	21400
		Acetonitrile	20,700
G1	328	Benzene-acetonitrile (98+2)	17100
		Toluene-acetonitrile (9+1)	16400
		Methanol	17700
		Acetonitrile	17600
G2	330	Benzene-acetonitrile (98+2)	18200
		Toluene-acetonitrile (9+1)	18300
		Methanol	19200
		Acetonitrile	18900
M1	328	Benzene-acetonitrile (9+1)	18000
		Acetonitrile	19000
M2	330	Acetonitrile	21000

Preparation and storage of working standards

1. Dilute portions of stock solution to a spotting concentration (0.5 µg/mL) with the same solvent used to prepare aflatoxin standards.
2. Use benzene–acetonitrile (9+1) to dilute Aflatoxin M1 solution.
3. Before storage, weigh flasks to nearest mg and record mass for future reference.
4. Wrap flasks tightly with aluminum foil and store at 0 °C. When the solution is to be used after storage, reweigh flask and record any change.
5. To avoid incorporation of water by condensation, bring all standards to room temperature (25 ±2 °C) before use.

	6. Do not remove aluminum foil until contents have reached room temperature. Standard solutions of aflatoxins B1, B2, G1, G2 are stable for more than one year. 7. The criteria of purity of the standards can be checked by determining chromatographic purity and molar absorption. 8. The absorbance close to 350 nm is determined and concentration calculated.
Preparation of Resolution Reference Standards	Prepare resolution reference standards by mixing B1, B2, G1 and G2 to give a final spotting concentration of 0.5 μg/mL for each aflatoxin.
Reference	AOAC 17th Edn 2000, Official Method 971.22 Standards of aflatoxin, sub Para E, Preparation and storage of TLC Standards)
Approved by	Scientific Panel on Methods of Sampling and Analysis

	TLC methodfor Determination of AflatoxinsBF Method (Applicable for groundnuts and groundnut products, oilseeds and food grains)		
Method No.	FSSAI 07.003:2020	**Revision No. & Date**	0.0
Caution	Follow all safety precautions described earlier. Inhalation of chloroform vapors can cause headaches, drowsiness, dizziness, and nausea. Disorientation, anesthetic effects, and loss of consciousness can occur at high concentrations. Wear laboratory safety goggles and mask. Perform work in a fume hood when using solvents. Protect eyes with UV-absorbing filter when using UV-viewing chamber. Refer to MSDS for specific information.		
Principle	Aflatoxins are extracted with aqueous methanol, concentrated and subjected to Thin Layer Chromatography. The resolved toxins are visualized using long wavelength UV lamp.		
Apparatus/Instruments	1. Stoppered Conical Flask 2. Measuring Cylinders – 25, 50, 250 mL 3. Chromatography column – 25 mm (i. d.) × 300 mm length 4. High speed blender 5. Funnel – 7.5 cm diameter or Buchner Funnel with Whatman No1 filter paper or equivalent 6. Wrist action shaker 7. Rotary evaporator 8. UV light Chamber equipped with Longwave UV lamp with an intensity of 430 mwatt/cm^2 at 15 cm at 365 nm 9. Adjustable Micropipette– 5-100 µL, 10. Vials, Borosilicate – screw cap lined with foil or Teflon 11. Microsyringe 12. TLC chamber		
Materials and Reagents	*Note:* Refer to Material Safety Data Sheets and ensure that safety guidelines are applied before using chemicals 1. Acetone 2. Aflatoxin Standard 3. Sodium chloride 4. Methanol 5. Chloroform ($CHCl_{3)}$ 6. Diatomaceous earth (Celite) 7. Glass Wool 8. Hexane 9. Methanol 10. Nitrogen Gas for Drying 11. Silica Gel (60 Mesh) or Precoated silica gel 60 (0.25 mm		

	thickness) plates 12. Screw capped borosilicate vial
Preparation of Reagents	1. *Methanol: Water (55: 45):* Add 55 mL of methanol to 45 mL of water in a glass conical flask and mix by inversion. 2. *Acetone: Chloroform (1: 9):* Add 20 mL of acetone to 180 mL of chloroform in a glass conical flask and mix by inversion *Note:* Prepare reagent fresh daily and in the fume hood. 3. *Aflatoxin standard solution: As described earlier under 'Preparation of Standards'* 4. *Silica gel for column chromatography*: Silica Gel 60 (0.063–0.2 mm) for 50 g test portions. Activate by drying 1 h at 105 °C. Add H_2O,1 mL/100 g, seal, shake until thoroughly mixed, and store ≥15 h in air-tight container
Sample Preparation	Peanut butter and peanut meal need no preparation unless they contain large particles, in which case reduce extraction by milling. Use hammer mill, rotary cutter, or disk (burr) type mill for meals. Grind raw materials and roasted peanuts and peanut butter with pieces of pea nuts to paste with disk (burr) type mill before extraction. Alternatively, prepare peanut samples by H_2O slurry method: Blend 1100 g peanuts comminuted in subsampling mill with 1.5L H_2O and 22 g NaCl 3 min at medium speed in 1 gal. blender cup. *Extraction* 1. Weigh 100 g of peanut meal or powder or 50 g peanut butter into a blender jar. 2. Add: 1) 250 mL methanol–water (55+45) and 100 mL hexane to peanut butter 2) 500 mL methanol–water (55+45), 200 mL hexane and 4 g NaCl to peanut powder. 3. Blend for one min at high speed. 4. Transfer to 250 mL centrifuge bottles and centrifuge for 5 min at 2000 rpm. Alternatively let mixture stand undisturbed in blender jar wherein separation will occur within 30 mins. 5. Pipette 25 mL of lower aqueous methanol phase into a separating funnel, add 25 mL chloroform, stopper and shake for 30–60 s. 6. Let layers separate and drain bottom chloroform layer through anhydrous Na_2SO_4 into a 250 mL beaker. 7. Repeat extraction with two 25 mL portions of chloroform. 8. Evaporate all combined chloroform extracts on a steam bath with a stream of N_2 to between 2 mL and just dryness or as soon as condensing vapor is no longer visible on beaker lip. 9. Do not leave beaker on hot plate after solvent has evaporated. 10. Transfer extract with careful washing to a screw capped borosilicate vial and evaporate to dryness under gentle stream of nitrogen Seal vial with hollow polyethylene stopper and cap.

	Save for TLC. 11. Re-dissolve the residue just prior to TLC.
Method of analysis	***Thin Layer Chromatography*** *Preparation of TLC plates* 1. Weigh 30 g silica gel, into 300 mL glass-stoppered Erlenmeyer, add H_2O as recommended by manufacturer, shake vigorously for 1 min, and pour into applicator. Adjust amount of H_2O to obtain best consistency of slurry for spreading. 2. Immediately coat five 20×20 cm glass plates with 0.25 mm thickness of silica gel slurry. 3. Rest the plates undisturbed until gelled (ca 10 min). Adjusting thick ness of spread to 0.5 mm, provides good resolution of aflatoxins and tightness of spots. 4. Dry coated plates ≥2 h at 80 °C or ≥1 h at 110 °C, and store in desiccating cabinet with active silica gel until further use. 5. Alternatively, Precoated silica gel 60 0.25 mm thickness, TLC plates of appropriate size may be used. *Preliminary TLC*: 1. Uncap vial containing the extract, add 200 µL benzene–acetonitrile and reseal with a polythene stopper. 2. Shake vigorously to dissolve. 3. Puncture polythene stopper to accommodate the needle of a 10 µL syringe. 4. Under subdued incandescent light and as rapidly as possible spot 2, 5 and 10 µL on an imaginary line 4-5 cm from bottom of the TLC plate. Keep vial for quantitative analysis. 5. On the same plate spot 2.5 and 10 µL of aflatoxin standards. Spot at least one 5 µL resolution reference standard, to show whether adequate resolution is attained. 6. Add 50 mL acetone–chloroform (10:90) to trough of unlined developing tank. Allow the chamber to be saturated with solvent before use. 7. Use only one plate per tank, placing trough to one side to permit maximum exposure of the coated surface to tank volume. Immediately insert spotted plate into the tank and seal tank. 8. Develop plate for 40 min 23°–25 °C or until aflatoxins reach a Rf 0.4-0.7. 9. Remove plate from the TLC chamber, evaporate solvent at room temperature. 10. View the plate using long wavelength UV lamp in a viewing chamber. 11. Observe pattern of the four fluorescent spots. Protect eyes with UV-absorbing filter *Note:* Composition of acetone–$CHCl_3$ can be varied from (5 + 95) to

	(15 + 85) to compensate for variations in Silica gel and developing conditions. *Quantitative TLC:* If preliminary TLC shows the need for further dilution/concentration of test solution, evaporate to dryness on a steam bath and re-dissolve in a calculated volume of benzene–acetonitrile. Spot successively 3.5, 5.0, and 6.5 µL of test solution. All spots should be approximately of the same size and ~ 0.5 cm in diameter. On the same plate spot 3.5, 5.0, 6.5 µL aflatoxin standard. Spot 5.0 µL of each standard used on top of one of the two 6.5 mL test solution origin spots as internal standard. To see whether adequate resolution is achieved. Spot at least one 5.0 µL resolution reference standard. After developing the plate, dry in subdued light. Compare fluorescent intensities of the sample spot with those of the standard aflatoxins and determine which of the sample spot matches the standards. If the spots of the smallest quantity of sample are too intense to match standards, the sample should be further diluted and re-chromatographed.
Inference (Qualitative Analysis)	Four clearly identifiable spots should be visible in resolution reference standard. Examine pattern from test solution spot containing internal standard for aflatoxin spots. Rf values of aflatoxins used as internal standards should be same as or only slightly different from those of respective standard aflatoxin spots. (Since spots from test solution are compared directly with standard aflatoxins on same plate, magnitude of Rf is not important. These may vary from plate to plate.) Compare test solution patterns with pattern containing internal standard. Fluorescent spots in test solution thought to be aflatoxins must have Rf values identical to and color similar to aflatoxin standard spots when un known spot and internal standard spot are super imposed. Spot from test solution and internal standard combined should be more intense than either test solution or standard alone
Calculation with units of expression	Calculate the concentration of Aflatoxin B1 from the formula $\mu g/kg\,(ppb) = \frac{S \times Y \times V}{X \times W}$ Where, S = µL Aflatoxin standar d, which matches the test solution Y = Concentration of Aflatoxin B1 standard (µg/mL) V = µL of final dilution of test extract applied X = µL of sample extract spotted giving a fluorescent intensity equivalent to S (B1 standard)

	W = mass of the sample (in g) contained in final extract (10 g if 50 mL Chloroform extract is used) Calculate Aflatoxin B2, G1, and G2 similarly
Reference	Official Method 968.22 'Aflatoxins in Peanuts and Peanut Products CB Method', AOAC 17th edn, 2000
Approved by	Scientific Panel on Methods of Sampling and Analysis

	TLC Method for Determination of Aflatoxins in Food and Feeds: Romer Mini Column Method		
Method No.	FSSAI 07.004:2020	**Revision No. & Date**	0.0
Scope	Applicable to detection of ≥5 ng/g total aflatoxins [B1 + B2 + G1 + G2] in almonds; ≥10 ng/g total aflatoxins in white and yellow corn, peanut and cot ton seed meals, peanuts, peanut butter, and pistachio nuts; and ≥15 ng/g total aflatoxins in mixed feeds.		
Caution	Follow all personal safety procedures while handling and disposing solution described earlier. Inhalation of Chloroform vapors can cause headaches, drowsiness, dizziness, and nausea. Disorientation, anesthetic effects, and loss of consciousness can occur at high concentrations. Wear laboratory safety goggles and mask. Perform work in a fume hood when using solvents. Protect eyes with UV-absorbing filter when using UV-viewing chamber. Refer to MSDS for specific information. Concentrated Sulphuric acid is corrosive and can cause severe burns.		
Principle	Aflatoxins are extracted with organic solvents and separated using small chromatographic columns (mini-columns) developed with solvent. The columns are examined under longwave ultraviolet (UV) light for the characteristic blue or bluish - green color that the aflatoxins emit when exited by light at 365 nm.		
Apparatus/Instruments	1. High Speed Blender 2. Ultraviolet light – Long wave UV Lamp with intensity of 430 μ watt/ cm^2 at 15 cm at 365 nm 3. Mini-column – Borosilicate standard wall tubing 6 mm (i.d.) x 150 mm, tapered at 1 end to 2 cm 4. Mini-column Support rack- Test tube rack may be used 5. Rubber bulb – with 7 mm bulb at one end		
Materials and Reagents	1. Chloroform 2. Acetone 3. Potassium hydroxide pellets 4. Sodium hydroxide pellets 5. Potassium Chloride 6. Concentrated Sulphuric acid 7. Copper carbonate 8. Ferric Chloride 9. Diatomaceous Earth 10. Florisil		
Preparation of Reagents	11. Potassium Hydroxide wash solution – 0.02 M KOH with 1% KCl. Dissolve 1.12 g KOH pellets and 19 g KCl in 1000 mL water		

	12. Sodium Hydroxide Solution – 0.02 M – 8.0 g NaOH/L 13. 0.03% Sulphuric acid Solution– Add 0.3 mL of concentrated Sulphuric acid in 1000 mL 14. Precipitating reagents – (1) Copper carbonate – Basic (2) Ferric Chloride Slurry – Mix 20 g of $FeCl_3$ with 300 mL water 15. Column packing (a) Florisil (100– 200 mesh) (b) Silica gel 60 (70-230 mesh) for column chromatography (c) Alumina Neutral, (80–200 mesh)- activate for two h at 110 °C (d) Calcium Sulfate anhydrous (20–40 mesh). Dry all packing material for 1-2 h at 110 °C. Store all packing materials and packed columns in vapour-tight containers. Aflatoxin solution for spiking - Dilute solutions of B1 and G1 to final concentration of 2 µg/mL
Preparation of mini column	Trap a small plug of glass wool into the tapered end of a column. To the column add to the height indicated in the following order: 1) 5-7 mm, Calcium Sulfate, 2) 5-7 mm, Florisil, 3) 18-20 mm, Silica gel, 4) 8-10mm, neutral alumina, and 5) 5-7 mm, Calcium Sulfate. Finally trap the column top with a small plug of glass wool. Tap column after each addition to settle packing and maintain uniform interfaces levels as possible. After packing apply pressure to top glass wool plug with a 5 mm dia. glass rod. Packed mini-columns are available commercially.
Sample Preparation	*Extraction:* 1. Weigh 50 g test sample into a blender jar, add 250 mL acetone–water (85+15) and blend for three min. Alternatively use a 500 mL glass stoppered Erlenmeyer flask and shake for 45 min on a mechanical shaker. 2. Filter through Whatman filter paper No 4 or equivalent into a 250 mL graduated cylinder. 3. Collect 150 mL filtrate and transfer to 400 mL beaker.
	Purification: 1. Quantitatively add 170 mL of 0.02 N Sodium hydroxide and 30 mL Ferric chloride slurry to a 600 mL beaker and mix well. 2. To the filtrate in the 400 mL beaker add about three grams basic Copper carbonate, mix well and add to the mixture in the 600 mL beaker. 3. To this add 150 mL diatomaceous earth and mix well. 4. Filter using a 160 mm funnel or Buchner funnel using Whatman No 4-filter paper or equivalent. 5. Quantitatively transfer 150 mL filtrate to a 500 mL separator, add 150 mL 0.03% Sulphuric acid and 10 mL Chloroform. 6. Shake vigorously for about two mins and let separate. 7. Transfer lower Chloroform layer (13-14 mL) to 125 mL separator.

	8. Add 100 mL Potassium hydroxide wash solution swirl gently for 30 s and let separate. 9. If emulsion occurs drain emulsion into 10 mL glass stoppered flask, add about one g anhydrous Sodium Sulfate, stopper shake 30 s and let separate (Chloroform phase need not be completely clear). 10. If emulsion is not broken, transfer emulsion to 125 mL separator and wash with 50 mL 0.03% Sulphuric acid. 11. Collect 3 mL of Chloroform layer in a 10 mL glass stoppered cylinder for chromatography
Method of analysis	1. Transfer two mL of Chloroform solution to a mini-column using a 5 mL syringe with 5-inch, 15- gauge needle. 2. Allow to drain by gravity (15–30 min). 3. When solvent reaches top of adsorbent, add 3mL elution solvent, Chloroform – acetone (9+1). 4. Allow to drain by gravity until solvent reaches the top of adsorbent. 5. Do not let columns run dry during determination. 6. Examine columns in darkened chamber using a UV lamp. Look for a blue fluorescent band at the top of the Florisil layer (ca 2.5 cm from bottom of column), which is indicative of aflatoxin. 7. Perform analysis with "clean" test portion and with test portion spiked with known amounts of aflatoxin to obtain comparison standards. 8. Some uncontaminated products show white, yellow or brown fluorescence at top of Florisil in sample column. If band has no definite bluish tint test portion is negative.
Reference	AOAC 17th edn, 2000 Official Method 975.36. Aflatoxins in Food and Feed – AACC- AOAC Method
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Thin Layer Chromatographic Method for Determination Aflatoxins in Corn and Peanuts (Groundnuts)		
Method No.	FSSAI 07.005:2020	**Revision No. & Date**	0.0
Scope	Applicable to determination of 5-50 ng B1/g corn, 3-15 ng B2/g corn, 10-50 ng G1/g corn, 3-15 ng G2/g corn, 5-25 ng B1/g raw peanuts and 1.5-7.5 ng B2/g raw peanuts by densitometry; 10-50 ng B1/g corn, 10-25 ng B1/g peanuts, 7.5 ng B2/g raw peanuts, and 10-25 ng G1/g raw peanuts by visual comparison.		
Caution	Follow all personal safety procedures while handling and disposing solution described earlier. Grinding of dry samples may result in airborne dust. Even if no toxin is present, there is potential harm from inhalation of mold spores or from allergic response to inhaled dust. Use protective mask and/or dust collector. Prepare samples in area separate from analytical laboratory. Inhalation of Chloroform vapors can cause headaches, drowsiness, dizziness, and nausea. Disorientation, anesthetic effects, and loss of consciousness can occur at high concentrations. Wear laboratory safety goggles and mask. Perform work in a fume hood when using solvents. Protect eyes with UV-absorbing filter when using UV-viewing chamber. Concentrated Sulphuric acid is corrosive and can cause severe burns. Refer to MSDS for specific information.		
Principle	Aflatoxins are extracted from samples with methanol-water. Filtrate is diluted with Sodium chloride solution and defatted with hexane. Aflatoxins are partitioned into chloroform which is then removed by evaporation. Aflatoxins are purified by chromatography on 0.5 g silica gel column, and quantitated by TLC/HPTLC on Silica gel 60 plate with densitometry or visual estimation.		
Apparatus/Instruments	1. Wrist-action shaker: Capable of holding four to eight 250 mL flasks. 2. Silica gel column: Disposable column (6 mL), packed with 40 µm (60Å) silica gel. 3. Vacuum apparatus: Equipped with vacuum gauge/flow controller and manifold fitted with 10 female Luer connectors. 4. Vials: Two dram (8mL), with foil or Teflon-lined screw caps. 5. TLC/HPTLC plate: 20×20 cm glass plate coated with 0.25 mm thick gel without fluorescent indicator (precoated Silica gel 60 plates can be used).		

	6. UV-Viewing cabinet: 270×270 mm base minimum, equipped with 15 W long wave ultraviolet (UV) lamp. 7. Fluoro-densitometer (TLC/HPTLC scanner): Capable of scanning in reflectance mode by fluorescence, equipped with high-pressure Hg lamp, monochromator for adjustment to excitation 366 nm, and emission cutoff filter 420 nm.
Preparation of Reagents	1. *Solvents*: Methanol, hexane, chloroform, anhydrous ethyl ether (100%), dichloromethane, acetone and isopropanol. 2. *Aflatoxin standard solution*: Prepared in benzene-acetonitrile (98+2) to contain 0.5 μg/mL each B1 and G1 and 0.15 μg/mL each B2 and G2.
Sample Preparation	*Extraction:* 1. Weight 50 g (ground to pass No. 20 sieve) corn or peanuts into 500 mL glass-stoppered Erlenmeyer flask. 2. Add 200 mL methanol-H_2O (85+15) and secure stopper with masking tape. 3. Shake vigorously by hand until samples show no clumps. 4. Shake 30 min on wrist-action shaker and filter mixture through medium fluted paper. Collect 40 mL filtrate in 50 mL graduated cylinder. 5. Transfer filtrate to 125 mL separatory funnel. 6. Add 40 mL 10% Sodium chloride solutions, mix, and add 25 mL hexane. 7. Shake one min. Let the phases separate, drain lower (aqueous) phase into second 125 mL separatory funnel, and discard upper phase. 8. Extracts aflatoxins from aqueous phase with two 25 mL portions chloroform 9. Shake one min each time. 10. Combine chloroform fractions in 125 mL Erlenmeyer flask and evaporate to dryness on steam bath
Method of analysis	*Silica Gel Column Chromatography:* 1. Attach silica gel column, to extraction system, (or clamp to stand if using gravity flow only). 2. Condition the column by washing with three mL hexane, followed by three mL dichloromethane using vacuum (flow rate 6 mL/min), or let drip freely unassisted by suction. 3. Check column suitability by adding aflatoxin B1 standard (three mL dichloromethane containing 100 ng aflatoxin B1) to 0.5 g silica gel column. Recovery must be >90% by this method. 4. Dissolve residue of extracted sample, in 3mL dichloromethane and add to column. Let drip freely (flow rate ca 3 mL/min, apply vacuum if needed). 5. Rinse residue container with two × one mL portions of

	dichloromethane and add rinses to column. 6. Wash column with 3 mL hexane, 3 mL anhydrous ethyl ether, and then 3 mL dichloromethane. (Use vacuum, flow rate 6 mL/min, or use syringe and adapter to apply pressure to increase solvent flow if necessary. Do not pull up syringe plunger while it is still attached to column.) 7. Turn off vacuum, remove extraction system cover, and place vial, under each column (test tube rack can be used to hold vials). 8. Elute aflatoxins (without vacuum) with two to four 3 mL portions (according to results of column suitability test) of chloroform-acetone (9+1). 9. Evaporate eluate to dryness on steam bath under stream of nitrogen. *Thin-Layer Chromatography: Fluro-densitometry:* 1. Dissolve residue from above in 250 µL chloroform. 2. Spot plate, with 5 µL chloroform test solution in duplicate and 2, 5, 10, and 20 µL aflatoxin standard solution. 3. Randomize standard and test solution spots across plate so duplicate test solution spots are not next to each other and standard spots are dispersed evenly. 4. To avoid errors, prepare spotting plan, either on plate or in notebook, prior to spotting. 5. Develop plate for one h with chloroform-acetone (9 + 1). 6. Evaporate solvent for five min in fume hood followed by 2 min at 50 °C forced draft oven. 7. Examine plate under long wave UV light to determine presence or absence of aflatoxins. 8. Quantitate by fluoro-densitometric measurement. Scan test and aflatoxin reference spots (transmission or reflectance mode, excitation 365 nm and emission cutoff 430 nm). 9. At end of plate scan, rescan 1st or 2nd lane. Scans of test spots should be within +5%; if not, rescan entire plate.
Calculation with units of expression	Calculate concentration of aflatoxin B1 in test portion, using following formula: $\text{Concentration of aflatoxin B1 (ng/g)} = \frac{250 \times R_u}{5 \times R_s \times 10}$ Where, 250 = µL test solution volume Ru = average densitometer response for B1 spots of test solution duplicates 5 = µL test solution spotted; Rs = calculated average densitometer response/ng for 4 B1

	standard spots; 10 = g corn or peanut represented by extract. Calculate concentrations of aflatoxins B2, G1, and G2 similarly.
Reference	AOAC Official Methods of Analysis (2000), Ch.49.2.15 Method, 993.17
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Aflatoxin in Corn and PeanutPowder/ButterLiquid Chromatographic Method		
Method No.	FSSAI 07.006:2020	**Revision No. & Date**	0.0
Caution	Follow all personal safety procedures while handling and disposing solution described earlier. Inhalation of Chloroform vapors can cause headaches, drowsiness, dizziness, and nausea. Disorientation, anesthetic effects, and loss of consciousness can occur at high concentrations. Wear laboratory safety goggles and mask. Perform work in a fume hood when using solvents. Protect eyes with UV-absorbing filter when using UV-viewing chamber. Refer to MSDS for specific information. Concentrated Sulphuric acid is corrosive and can cause severe burns. Trifluoroacetic acid is corrosive chemical and contact can severely irritate and burn the skin and eyes with possible eye damage. Use face shield or eye protection (safety goggles) in combination with breathing protection. Concentrated HCl is corrosive and can cause severe burns. Use gloves, protective clothing, safety goggles or eye protection in combination with breathing protection.		
Principle	Aflatoxins are extracted, purified and derivatized with trifluoroacetic acid (aflatoxins B1 and G1 to B2a and G2a, respectively), separated by reverse phase liquid chromatography and detected by fluorescence. Method can measure 0.1 ng of aflatoxin B1, B2, G1, and G2. Detection limit is about 0.3 ng/g.		
Apparatus/Instruments	I. High performance liquid chromatograph (HPLC) equipped with 1. A binary pump, 2. Rheodyne septum-less injector (or autosampler), 3. Fluorescence detector (Excitation 360 nm and Emission 440nm) fitted with flow cell, 4. Integrator /recorder and appropriate software for peak identification and area under the curve. II. Chromatography conditions 1. Flow rate 1.0 mL/min. 2. Set up detector give minimum half scale deflection with 1.25 ng aflatoxin B1 or G1. For optimum performance detector should be left on continuously. 3. Column – 15 cm×4.6 mm i. d. C-18 (Octadecyl), Particle size 5µ or equivalent. Note: - New LC columns or those that have been stored in methanol for extended periods require conditioning with concentrated standards in order to achieve optimum resolution and sensitivity to aflatoxin B1		

<table>
<tr><td></td><td>and G1.
III. Clean Up Column – 20 cm × 1cm i. d. with Teflon stopcock and coarse frit bed support, detachable glass solvent reservoir with 24/40 fitting
IV. Adjustable autopipettes – 10-100 and100–200 µL with disposable tips
V. Filter tube – glass 15 × 2.5 cm i. d. with coarse frit bed support (glass wool not recommended)</td></tr>
<tr><td>Materials and Reagents</td><td>1. Solvents: HPLC grade: methanol, hexane, methylene chloride, benzene, acetone, acetonitrile. Anhydrous ethyl ether stored in metallic container (Glass bottled ether forms peroxides soon after opening which degrades aflatoxins)
2. Hydrochloric acid (0.1 M): Prepare in a fume hood. Dilute 5.0 mL of concentrated HCl (11.6M) to 580 mL with distilled water. Caution: Add acid to water.
3. LC elution solvents – Water: acetonitrile: methanol (700:170:170). Adjust ratio of water to obtain baseline resolution of aflatoxin B2 and G2. Note: Mix the solvents do not makeup volume.
4. Silica gel for Column chromatography – Silica gel 60, (0.063-0.2 mm). activated by drying at 110 °C. Cool to room temperature. Weigh desired quantity (100 g) into glass stoppered container. Add one ml water in small increments, agitate silica gel between additions. Shake or tumble mechanically 4-6 h. Let stand 16 h
5. Trifluoroacetic acid (TFA) – ≥98.5% pure. Transfer 1-2 mL TFA to a one-dram vial with a Teflon lined cap. Keep in freezer when not in use. Discard if discoloration appears.
Anhydrous Sodium sulfate: Sift out fines to obtain 20–40 mesh. Heat for 2-3 h at 600 °C to remove organic impurities</td></tr>
<tr><td>Preparation of Reagents</td><td>Aflatoxin standard solutions:
Aflatoxin stock solution – 10 µg/mL. Prepare individual stock solution in benzene-acetonitrile (98+2) and determine concentration of each by measuring UV absorption if desired.
Working standard solutions - Use an autopipette (Pipetman) to transfer an appropriate quantity stock solution to each 4-dram vial (15 mL) to obtain the final concentrations of aflatoxins in each vial as indicated in Table below
<table>
<tr><td>Table</td><td colspan="2">Working Aflatoxin Standards</td><td colspan="2">Final concentration of Aflatoxins</td></tr>
<tr><td>Vial Number</td><td>B1& G1 (ng)</td><td>B2 & G2 (ng)</td><td>B1& G1 (µg/10.05 mL)</td><td>B2 & G2 (µg/10.05 mL)</td></tr>
<tr><td>1</td><td>250</td><td>125</td><td>0.25</td><td>0.125</td></tr>
<tr><td>2</td><td>500</td><td>250</td><td>0.50</td><td>0.25</td></tr>
<tr><td>3</td><td>1000</td><td>500</td><td>1.0</td><td>0.50</td></tr>
<tr><td>4</td><td>2000</td><td>1000</td><td>2.0</td><td>1.</td></tr>
</table></td></tr>
</table>

	Evaporate solutions to dryness under a gentle stream of nitrogen (drying may be facilitated by warming to 40 °C). Using Eppendorf pipette add 200 µL hexane and 50 µL of TFA to each vial, cap and vortex for 30 s. Let solutions stand 5 min, then add 10 mL water: acetonitrile (9+1) and vortex for 30 s. Let layers separate for 5 -10 min or centrifuge at 1000 rpm for 30 s. Final concentration of aflatoxins shall be as shown in the table above.
Sample Preparation	*Extraction and partition:* 1. Transfer 50 g prepared corn, or peanut powder or peanut butter to a jar (Capacity 1L) 2. Add 200 mL of methanol followed by 50 mL of 0.1 M HCl and blend for three min at high speed. 3. Filter through 24 cm Whatman No 1 filter paper or equivalent. Filtrate may not be completely clear. 4. Collect 50 mL filtrate. 5. Transfer to 250 mL separatory funnel. 6. Add 50 mL 10% Sodium chloride solution, swirl. 7. Add 50 mL hexane and shake gently for about 30 s. 8. Let phases separate then drain lower aqueous layer into another 250 mL separator funnel. Discard hexane layer. 9. Add 25 mL methylene chloride and shake moderately for 30 s. If emulsion occurs break up with clean pipette. 10. Let phases separate then drain lower methylene chloride layer through coarse granular anhydrous sodium sulfate in glass filter tube. 11. Collect elute in a 250 mL beaker. 12. Evaporate elute, on steam bath under a gentle stream of nitrogen to 2-3 mL.
Method of analysis	*Column Chromatography:* 1. Make a slurry of two g silica gel with about 10 mL ether–hexane (3+1) in a 30 mL beaker. 2. Pour slurry into a clean-up column and wash beaker with additional 5 mL ether–hexane solvent to effect complete transfer. 3. Keep stop cock closed and let silica gel settle without tamping. 4. Wash sides of column with 2-3 mL ether–hexane using squeeze bottle. 5. After gel settles, open stop cock and while column drains, add about 1 cm anhydrous sodium sulfate. 6. Transfer eluate collected after extraction to column. 7. Wash beaker with about 2 mL of methylene chloride and add wash to column. Do not use more than 5-6 mL methylene chloride to transfer eluate to column. 8. With stop cock fully open, add 25 mL benzene–acetic acid (9+1) and the 30 mL ether–hexane (3+1) to column, draining each wash

	to top of sodium sulfate. 9. Discard washes. 10. Elute aflatoxin with 100 mL methylene chloride–acetone (90+10) 11. Collect elute in 250 mL beaker. 12. Evaporate elute on steam bath under a gentle stream of nitrogen to about 6 mL. Quantitatively transfer to 3-dram vial. 13. Evaporate elute to dryness using a steam bath or an aluminum block under a gentle stream of nitrogen. 14. Evaporate remaining 200 µL just to dryness under a gentle stream of nitrogen by holding vial in palm of hand and slowly rotating vial *Derivatization:* 1. Add 200 µL hexane to the residue obtained above. 2. Then add 50 µL of TFA using Eppendorf pipette, cap the vial and vortex vigorously for 30 s (exactly). This procedure must be followed closely to ensure consistent reaction yields. 3. Let mixture stand 5 min. 4. Using transfer pipette add 1.950 mL water-acetonitrile (9+1). 5. Vortex vigorously for exactly 30 s and let layers separate 10 min. Concentration is 10 g/2 mL aqueous acetonitrile. [Note: Post column derivatization with Kobra Cell may also be used] *HPLC:* 1. Using a HPLC equipped with a fluoresce detector and C-18 column set at a flow rate of 1 mL/min equilibrate the column with solvent (Water: acetonitrile: methanol (700:170:170). 2. Inject 25 µL of derivatized standard solutions. 3. Prepare standard curve to check linearity of responses. 4. Inject 25 µL of derivatized test solution (lower aqueous phase). If test peaks are outside the dynamic linear range, dilute aliquot of derivatized test solution to suitable volume with water –acetonitrile, remix on vortex mixer and inject another 25 µL portion.
Calculation with units of expression	Calculate individual aflatoxin concentration as follows: Use responses of standard containing 500 ng B1 and G1, and 250 ng B2 and G2 for calculations. Aflatoxins, ng/g = $(P/P`) \times C \times (2/10) \times 1000 \times D$ where $P$ and $P`$ = peak areas (integrator counts) or height for test solution and standard, respectively, per 25 µL injection; C =concentration of individual aflatoxins in standard solution (0.5 or 0.25 mg/10.05 mL); D = dilution factor if 2 mL test solution for injection is diluted.
Reference	AOAC 17th edn, 2005 Official Method 990.33 Aflatoxins in Corn and Peanut Butter, Liquid Chromatographic Method)
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Aflatoxins B1, B2, and G1 in Corn, Cottonseed, Peanuts, and Peanut Butter Enzyme-Linked Immunosorbent (Immuno-dot Screen Cup) Screening Assay		
Method No.	FSSAI 07.007:2020	**Revision No. & Date**	0.0
Scope	Applicable to screening aflatoxin B1, B2, and G1 contamination in whole cotton seed and peanut butter at ≥20 ng/g and in corn and raw peanuts at ≥30 ng/g		
Caution	Follow all personal safety procedures while handling and disposing solution described earlier. Grinding of dry samples may result in airborne dust. Prepare samples in area separate from analytical laboratory. Inhalation of solvent vapors can cause headaches, drowsiness, dizziness, and nausea. Perform work in a fume hood when using solvents. Refer to MSDS for specific information.		
Principle	Antibodies specific to aflatoxins B1, B2, and G1 are immobilized on a filter, and toxin (aflatoxin B1) is labeled with an enzyme (horseradish peroxidase). Binding of toxin-enzyme conjugate by immobilized antibodies is inhibited by addition of free toxin present in test sample. Since fixed number of antibody reaction sites are available, enzyme activity is proportional to amount of bound toxin-enzyme conjugate. Antibody-toxin-enzyme complex concentration is inversely proportional to concentration of free toxin added. Bound enzyme catalyzes oxidation of substrate to form blue complex. Development of color indicates that test sample contains aflatoxins at <20 ng/g; no color development indicates that test sample contains aflatoxins at ≥ 20 ng/g		
Determining the specificity of antibodies	Antibodies have specific ability to bind structurally related compounds, namely, aflatoxins B1, B2, and G1. Determine specificity of purified rabbit anti-aflatoxin B1 polyclonal antibodies by direct competitive ELISA method. 1. Coat serially diluted antibodies on microtiter plates. 2. Prepare standard solutions of aflatoxins B1, B2, G1, G2, and M1; zearalenone; T-2 toxin; and deoxynivalenol, and add to individual microtiter well 3. Add solution of aflatoxin B1 conjugated to horseradish peroxidase to each well. 4. Add substrate solution of tetramethylbenzidine and hydrogen peroxide, and measure development of color with scanner. 5. Least color development indicates highest reactivity of toxin-antibody reaction.		

	6. Cross-reactivity to aflatoxin B1 for antibody should be 100, 70, 75, and <10% for aflatoxins B1, B2 and G1 and G2, respectively. All other toxins tested should show no cross-reactivity.
Sensitivity of *ELISA* Reagent	(**a**) *Negative control test solution:* Use 100 µLofbuffer solution in the cup. Follow procedure in enzyme immunoassay steps 7-9 (**b**) *Thresh old-level standard solution:* Used to define lower limit of determination. Dispense 100 mL working standard into test tube. Add 350 mL methanol–buffer, (30 + 70), and mix. Follow procedure in enzyme immunoassay steps 7-9 (**c**) *Positive control test solution:* Use working standard solution; follow procedure for enzyme immunoassay steps 7-9 Negative control test solution should develop blue color; positive control test solution should have no color development. Threshold standard solution should show no color development
Preparation of Reagents	Reagents from commercial suppliers can be used provided requirements listed below are met. 1. Antibody-coated solid support: Antibody-coated filter material attached to analytical cup made of porous polyethylene (3.2 cm diameter, 2.5 cm high, capacity 4 mL). Coated cup is specified by manufacturer to be stable for 6 months stored at4-8°C. Coated 8/12/96 well strips or plates can be used. 2. Aflatoxin-enzyme conjugate- Aflatoxin B1-horseradish peroxidase conjugate at toxin-enzyme molar ratio of 10-15:1. Conjugate is specified by manufacturer to be stable for 6 months at 4-8°C. 3. Wash solution (Phosphate-buffer saline (PBS) solution). Dissolve 0.23 g $NaH_2PO_4.H_2O$, 1.95 g $K_2HPO_4.3H_2O$, 8.70 g Sodium chloride, 0.125 mL Tween 20 (polyoxyethylenesorbitan monolaurate), and 10 mg thimerosal (Ethylmercurithiosalicylic acid, sodium salt), in 900 mL H_2O adjust pH to 7.2, and dilute to 1 L. 4. Buffer –Bovine serum albumin (0.1% w/v) in PBS containing 0.05% thimerosal. 5. Substrate solution A – Tetramethylbenzidine (TMB) (0.4 g/L H_2O), pH 8.3. 6. Substrate solution B – Hydrogen peroxide (0.02% H_2O_2 in 0.13% aqueous citric acid solution), pH 3.0. 7. Methanol, hexane, and chloroform – Reagent grade. 8. Standard aflatoxin B1 – Approximately 28 µg as dry film.
Apparatus/Instruments	Equipment specified is not restrictive; other suitable and compatible equipment may be used**.** 1. High- speed blender – With 500 mL jar 2. Micropipette and tips- recommended range 100-1000 µL; with disposable polypropylene tips.

	3. Glass culture (test) tubes- 10×75 mm; 3 mL. 4. Microplates (96-well)/ 8/16 well strips 5. Filters- Whatman No. 4 or equivalent. 6. Timer- Graduated in 1 s intervals. 7. Carborundum boiling chips.
General Instructions	1. Store all kit components at 4-8 °C. Do not freeze. 2. Before use, allow one h for antibody coated cups/ plates/strips and reagents to reach room temperature (23-29 °C). 3. Use separate disposable pipet tips for each solution to avoid cross contamination. 4. Include one negative control with each group (20 cups/wells) of test samples. Negative control must be functioning properly (must develop blue color in center of cup/wells) for test to be valid. 5. Positive controls must be used with each group of test portions and must show no color in the center of the cup/well. 6. Threshold level standard should also be used and must show no color development. If color develops, repeat the test. Color development in more than 2 tests indicates a defective kit. 7. Reagents are stable for 6 h at room temperature. To ensure shelf life of kit components promptly return reagents to refrigerator after use. 8. Addition of reagents to cups/wells must be successively spaced at convenient time intervals e.g. 60 s or higher for making observations.
Sample Preparation	(a*) Corn, raw peanuts, and whole cottonseed*: Weigh 50 g test portion into blender jar. Add 100 mL methanol-water (8+2). Blend for three min at high speed. Filter mixture and recover filtrate. Alternatively, let mixture stand 10-15 mins and recover supernatant liquid. Dilute extract in ratio 1:1 with extraction solvent. *(b) Peanut butter-*: Weigh 50 g test portion into blender jar. Add 100 mL hexane and 250 mL methanol-water (55+45). Blend for three min at high speed. Filter mixture and transfer filtrate to separator funnel. Let layers separate for 10 mins. Place 20 mL lower layer in 150 mL beaker. Add minimum of 15 boiling chips and heat in steam bath or on hot plate. Boil for 3 mins and let cool. *Preparation of Aflatoxin B1 Standard Solutions:* (a) Stock solution- Add 3 mL chloroform to vial containing 28 µL aflatoxin B1 standard (ca 9 ng/µL). Cap vial, mix contents, and store vial in refrigerator. (b) Working solution- Prepare fresh daily. Dispense 300 µL stock solution into vial. Add 2400 µL methanol (1 ng/µL), mix and store solution in refrigerator. Dispense 10 µL diluted standard (1 ng/µL) into test tube. Add 300 µL methanol and 700 µL buffer, Prepare ≤2h

	before use.
Method of analysis	*Enzyme Immunoassay[#] for Corn, raw peanuts and whole cottonseed:* 1. Allow 1 h for all reagents to reach room temperature (23-29 °C). 2. Prepare fresh substrate in a small culture (test) tube by mixing 500 µL substrate solution A with 500 µL substrate solution B for each cup/well being used. Do not combine substrate solution A with solution B more than 15 min before use. 3. Run 1 negative control and 1 positive standard control each day to ensure that all reagents are functional. Threshold-level standard should be run with each set of new reagents. Negative control should be run by using 100 µL buffer. For positive standard control, using working standard. 4. Add 200 µL test extract to 400 µL PBS (600 µL total). 5. Thoroughly mix diluted test extract and apply one 150 µL aliquot to cup/well. 6. Using timer, after exactly 60 s add second 150 µL aliquot of diluted test extract to same well cup/well. Using timer, wait additional 1 min before proceeding to next step. 7. Apply 100 µL enzyme solution to center of cup/well. Using timer, wait one min. 8. Wash with 1.5 mL wash solution added drop wise. If more than 1 cup is being used, wash successively with 500 µL per cup 3 times. 9. Add entire contents of substrate solution 1.0 mL from each test tube to each cup. (Start time as soon as substrate mixture is added to cup.). Wait one min and immediately observe the disk (center of cup) for blue color development (negative) or no color development (positive). *Enzyme Immunoassay[#] for Peanut butter:* 1. Allow 1 h for all reagents to reach room temperature (23-29 °C). 2. Prepare fresh substrate solution in small culture (test) tube by mixing 500 µL (10 drops) substrate solution A with 500 µL (10 drops) substrate solution B for each cup being used. Do not combine substrate solution A with substrate solution B more than 15 min before use. 3. Add 500 µL test extract to 500 µL PBS (1000 µL total). 4. Thoroughly mix diluted test extract and apply one 200 µL aliquot to center of cup. Using timer, after exactly 60 s add second 200 µL aliquot of diluted test extract. After exactly additional 60 s third 200 µL aliquot of diluted test extract and after 60 s add fourth 200 µL aliquot of diluted test extract before proceeding to next step. 5. Proceed as for corn steps 7-9.

Inference (Qualitative Analysis)	Observe well/cup for blue color or no color development at exactly after 60 s of adding substrate A and B mixture. *Negative*- If it turns light blue or darker, test sample total aflatoxin B1, B2 and G1 is < 20 ng/g (cottonseed, butter). *Positive*- If no color is observed in disk (center of cup/plate) and disk remains completely colorless (no color change) for at least 60 s, test sample contains total aflatoxin B1, B2 and G1 at >20 ng/g. *Negative control*- Negative control cup must develop blue color in center of cup. *Positive control*-Positive standard cup must remain completely white (no color change) for at least 60 s. *Threshold-level standard*- Cup must remain completely white (no color change) for 60 s.
[#] ***Note***	The ELISA kits are meant for primary screening purposes and results obtained must be confirmed with other analytical methods. Various manufacturers have different protocols for using their kits. It would be the responsibility of the lab to validate these kits prior to use.
Reference	AOAC Official Methods of Analysis (2000), Method, 990.34. Ch.49.2.07
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Method for Determination of Aflatoxins B1, B2, and G1 in Corn: Enzyme-Linked Immunosorbent Assay method (Afla-20 cup Test)		
Method No.	FSSAI 07.008:2020	**Revision No. & Date**	0.0
Scope	Applicable to the detection of ≥20 ng total aflatoxins /g of corn (maize).		
Caution	Follow all personal safety procedures while handling and disposing solution described earlier. Grinding of dry samples may result in airborne dust. Even if no toxin is present, there is potential harm from inhalation of mold spores or from allergic response to inhaled dust. Use protective mask and/or dust collector. Prepare samples in area separate from analytical laboratory. Inhalation of solvent vapors can cause headaches, drowsiness, dizziness, and nausea. Disorientation, anesthetic effects, and loss of consciousness can occur at high concentrations. Wear laboratory coat, gloves, safety goggles and mask. Perform work in a fume hood when using solvents. Refer to MSDS for specific information.		
Principle	Antibodies specific to aflatoxins B1, B2, and G1 are immobilized on a filter, and toxin (aflatoxin B1) is labeled with an enzyme (horseradish peroxidase). Binding of toxin-enzyme conjugate by immobilized antibodies is inhibited by addition of free toxin present in test sample. Since fixed number of antibody reaction sites are available, enzyme activity is proportional to amount of bound toxin-enzyme conjugate. Antibody-toxin-enzyme complex concentration is inversely proportional to concentration of free toxin added. Bound enzyme catalyzes oxidation of substrate to form blue complex. Development of color indicates that test sample contains aflatoxins at <20 ng/g; no color development indicates that test sample contains aflatoxins at ≥ 20 ng/g.		
Determining the specificity of antibodies	Antibodies have specific ability to bind structurally related compounds, namely, aflatoxins B1, B2, and G1. Determine specificity of purified rabbit anti-aflatoxin B1 polyclonal antibodies by direct competitive ELISA method. 1. Coat serially diluted antibodies on microtiter plates. 2. Prepare standard solutions of aflatoxins B1, B2, G1, G2, and M1; zearalenone; T-2 toxin; and deoxynivalenol, and add to individual microtiter well. 3. Add solution of aflatoxin B1 conjugated to horseradish peroxidase to each well. 4. Add substrate solution of tetramethylbenzidine and hydrogen peroxide, and measure development of color with scanner.		

	5. Least color development indicates highest reactivity of toxin-antibody reaction. 6. Cross-reactivity to aflatoxin B1 for antibody should be 100, 70, 75, and <10% for aflatoxins B1, B2 and G1 and G2, respectively. All other toxins tested should show no cross-reactivity.
Sensitivity of *ELISA* Reagent	(**a**) *Negative control test solution:* Use 100 µLofbuffer solution in the cup. Follow procedure in enzyme immunoassay steps 7-9 (**b**) *Thresh old-level standard solution:* Used to define lower limit of determination. Dispense 100 mL working standard into test tube. Add 350 mL methanol–buffer, (30 + 70), and mix. Follow procedure in enzyme immunoassay steps 7-9 (**c**)*Positive control test solution:* Use working standard solution; follow procedure for enzyme immunoassay steps 7-9 Negative control test solution should develop blue color; positive control test solution should have no color development. Threshold standard solution should show no color development.
Materials and Reagents	Reagents from commercial suppliers can be used provided requirements listed below are met. 1. Antibody-coated solid support: Antibody-coated filter material attached to analytical cup made of porous polyethylene (3.2 cm diameter, 2.5 cm high, capacity 4 mL). Coated cup is specified by manufacturer to be stable for 6 months stored at 4-8 °C. Coated 8/12/96 well strips or plates can be used. 2. Aflatoxin-enzyme conjugate- Aflatoxin B1-horseradish peroxidase conjugate at toxin-enzyme molar ratio of 10-15:1. Conjugate is specified by manufacturer to be stable for 6 months at 4-8 °C. 3. Wash solution (Phosphate-buffer saline (PBS) solution). Dissolve 0.23 g $NaH_2PO_4.H_2O$, 1.95g $K_2HPO_4.3H_2O$, 8.70 g Sodium chloride, 0.125 mL Tween 20 (polyoxyethylenesorbitan monolaurate), and 10 mg thimerosal (Ethylmercurithiosalicylic acid, sodium salt), in 900 mL H_2O adjust pH to 7.2, and dilute to 1 L. 4. Buffer –Bovine serum albumin (0.1% w/v) in PBS containing 0.05% thimerosal. 5. Substrate solution A – Tetramethylbenzidine (TMB) (0.4 g/L H_2O), pH 8.3. 6. Substrate solution B – Hydrogen peroxide (0.02% H_2O_2 in 0.13% aqueous citric acid solution), pH 3.0. 7. Methanol, hexane, and chloroform – Reagent grade. 8. Standard aflatoxin B1 – Approximately 25 µg as dry film.
Apparatus/Instruments	Equipment specified is not restrictive; other suitable and compatible equipment may be used**.** 1. High- speed blender – With 500 mL jar 2. Micropipette and tips- recommended range 100-1000 µL; with

	disposable polypropylene tips. 3. Glass culture (test) tubes- 10×75 mm; 3 mL. 4. Microplates (96-well)/ 8/16 well strips 5. Filters- Whatman No. 4 or equivalent. 6. Timer- Graduated in 1 s intervals. 7. Carborundum boiling chips
General Instructions	1. Store all kit components at 4-8 °C. Do not freeze. 2. Before use, allow one h for antibody coated cups/ plates/strips and reagents to reach room temperature (23-29 °C). 3. Use separate disposable pipet tips for each solution to avoid cross contamination. 4. Include one negative control with each group (20 cups/wells) of test samples. Negative control must be functioning properly (must develop blue color in center of cup/wells) for test to be valid. 5. Positive controls must be used with each group of test portions and must show no color in the center of the cup/well. 6. Threshold level standard should also be used and must show no color development. If color develops, repeat the test. Color development in more than 2 tests indicates a defective kit. 7. Reagents are stable for 6 h at room temperature. To ensure shelf life of kit components promptly return reagents to refrigerator after use. Addition of reagents to cups/wells must be successively spaced at convenient time intervals e.g. 60 s or higher for making observations.
Sample Preparation	1. Weigh 50 g test portion into blender jar. 2. Add 100 mL methanol-water (8+2). 3. Blend for three min at high speed. 4. Filter mixture and recover filtrate. 5. Alternatively, let mixture stand 10-15 mins and recover supernatant liquid. 6. Dilute extract in ratio 1:1 with extraction solvent. *Preparation of Aflatoxin B1 Standard Solutions:* 1. Stock solution: Add 2.5 mL methanol to vial containing 25 µg aflatoxin B1 standard (10 ng/µL). Cap vial, mix contents, and store vial below -20 °C. Stable for six months 2. Working solution: Dispense 250 µL stock solution into vial. Add 2250 µL methanol (5 ng/µL), mix and store solution at 5 °C. May be stored for one months (1ng/ µL) 3. Buffer solution of standard: Prepare fresh (<2 h before use). Dispense 5 µL of working solution into test tube. Add 300 µL of methanol and 700 µL of PBS, mix by agitation. 4. Proceed as below (Steps 5-8)
Method of Analysis	*Enzyme Immunoassay[#]:* 1. Allow 1 h for all reagents to reach room temperature (23-29 °C).

	2. Prepare fresh substrate in a small culture (test) tube by mixing 500 µL substrate solution A with 500 µL substrate solution B for each cup/well being used. Do not combine substrate solution A with solution B more than 15 min before use. 3. Run 1 negative control and 1 positive standard control each day to ensure that all reagents are functional. Threshold-level standard should be run with each set of new reagents. Negative control should be run by using 100 µL buffer. For positive standard control, using working standard 4. Add 100 µL test extract to 200 µL PBS (300 µL total). 5. Thoroughly mix diluted test extract and apply one 100 µL aliquot to center of cup/well. 6. Using timer, after exactly 60 s add 100 µL enzyme solution to center of cup/well. Using timer, wait one min. 7. Wash one time with 1.5 mL wash solution added drop wise. If more than 1 cup is being used, wash successively with 500 µL per cup 3 times. 8. Add entire contents of substrate solution 1.0 mL from each test tube to each cup. (Start time as soon as substrate mixture is added to cup.). Wait one min and immediately observe the disk (center of cup) for blue color development (negative) or no color development (positive)
Inference (Qualitative Analysis)	Observe well/cup for blue color or no color development at exactly after 60 s of adding substrate A and B mixture. 1. *Negative*- If it turns light blue or darker, test sample total aflatoxin B1, B2 and G1 is < 20 ng/g. 2. *Positive*- If no color is observed in disk (center of cup/plate) and disk remains completely colorless (no color change) for at least 60 s, test sample contains total aflatoxin B1, B2 and G1 at ≥20 ng/g. Positive samples must be confirmed by quantitative method. 3. *Negative control*- Negative control cup must develop blue color in center of cup. 4. *Positive control*-Positive standard cup must remain completely white (no color change) for at least 60 s. 5. *Threshold-level standard*- Cup must remain completely white (no color change) for 60 s.
[#] ***Note:***	The ELISA kits are meant for primary screening purposes and results obtained must be confirmed with other analytical methods. Various manufacturers have different protocols for using their kits. It would be the responsibility of the lab to validate these kits prior to use.
Reference	AOAC Official Methods of Analysis (2000), Method, 990.16. Ch.49.2.11
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Aflatoxin B1 and Total Aflatoxins using Immunoaffinity Column Cleanup, Post-column Derivatization, and LiquidChromatography/Fluorescence Detection**		
Method No.	FSSAI 07.009:2020	**Revision No. & Date**	0.0
Caution	Follow all personal safety procedures while handling and disposing solution described earlier. Read MSDS of all chemicals.		
Principle	Test portion is either extracted with Methanol–H2O (8 + 2) or Methanol–H_2O (8 + 2) plus hexane (or cyclohexane). Extract is filtered, diluted with water, and applied to an immune affinity column (IAC) containing antibodies specific to aflatoxins B1, B2, G1, and G2. Aflatoxins eluted from affinity column with Methanol and are quantified by reversed-phase liquid chromatography (RP-HPLC) with post-column derivatization involving bromination, achieved either electrochemically generated bromine (Kobra cell) or with pyridinium hydrobromide perbromide and determined by fluorescence detection.		
Apparatus/Instruments	1. Blender-Explosion proof (minimum 8000 rpm). 2. Vertical shaker:Adjustable (for maximum solid–liquid agitation); holding 500 mL Erlenmeyer flasks. 3. Filter paper—24 cm diameter, pre-folded, retention: 30 µm or better. 4. Erlenmeyer flask: 500 mL, screw top or glass stopper. 5. Glass microfiber filter paper:5 cm diameter, retention:1.6 µm (or better). 6. Reservoir: 75 mL with Luer tip connector for affinity column. 7. 20 mL syringe with Luer lock or rubber stopper. 8. Class A Volumetric glassware: 2, 3, 10, and 20 mL 9. High Performance Liquid Chromatograph equipped with a. Pump: Suitable for flow rate at 1.000 ± 0.005 mL/min. b. Injection system: Valve with 200 µL loop or equivalent. c. Column: C-18 (Octadecyl 25 cmx 4.6 mm i.d. ×, 5 µm. d. Fluorescence detector: Wavelength 360 nm excitation filter and 420 nm cut-off emission filter, or equivalent 10. Post column derivatization system a. For pyridinium hydrobromide perbromide reagent: Second LC pulseless pump, zero-dead volume T-piece, reaction tubing minimum dimensions 45 cm × 0.5 mm id PTFE. b. For electrochemically generated bromine: Kobra cell. 11. Disposable filter unit: Cellulose or cellulose nitrate, 0.45 µm. 12. Pipets: 10 mL. 13. Analytical balance: Weighing to 0.1 mg. 14. Laboratory balance: Weighing to 0.1 g. 15. Calibrated microliter syringes or micropipette(s): 25 and 500 µL. 16. Affinity Columns: Vicam (Watertown, MA) or Rhone-Diagnostics		

	have been found to meet the criteria.
Criteria for acceptance of immunoaffinity column	The aflatoxin IACs to contain monoclonal antibodies that are cross reactive with AFB1, B2, G1, and G2. Thecolumns should have capacity of not less than 100 ng total AFand should give a recovery of not less than 80% for AFB1, B2, G1,and G2 when 5 ng of each AF is applied in 10 mL methanol–PBS(10 + 90, v/v). The columns should have a shelf life of 18 monthsat 4°C or 12 months at room temperature.
Materials and Reagents	All chemicals should be of analytical grade 1. Water, except where specified, should be produced by single distillation, deionization, or reverse osmosis 2. Potassium chloride (KCl) 3. Dihygrogen potassium phosphate (KH_2PO_4) 4. Disodium mono hydrogen phosphate (Na_2HPO_4) 5. Sodium chloride (NaCl) 6. Hydrochloric acid 7. Pyridinium hydrobromide perbromide (PBPB)—CAS-39416-48-3. 8. Potassium bromide 9. Acetonitrile: HPLC grade 10. Methanol: HPLC grade 11. Methanol: Technical grade, pure, or distilled 12. Water:HPLC grade; complying with grade 1 of ISO 3696 13. Hexane or cyclohexane 14. Concentrated Nitric acid 15. Toluene *Reagents:* 1. Phosphate buffered saline solution (PBS): Dissolve0.20 gKCl, 0.20 g KH_2PO_4, 1.16 g anhydrous Na_2HPO_4 (or 2.92 g$Na_2HPO_4{\cdot}12H_2O$), and 8.00 g NaCl in 900 mL water. Adjust topH 7.4 with 0.1M HCl or NaOH and dilute to 1 L. (Commercial buffered saline tablets may be used.) 2. Extraction solvent: Methanol–water solution (8 + 2, v/v). 3. 4M Nitric acid: Dilute 28.1 mL concentratedHNO_3 (65%) or 26.1 mL 70% HNO_3) in water to final volume of100 mL. 4. Mobile phase A—Water–acetonitrile–methanolsolution (6 + 2 + 3, v/v/v). Mix 600 mL of HPLC grade water, 200 mL of acetonitrile and 300 mL of HPLC grade methanol. 5. Mobile phase B —For use with electrochemically generated Br:water: acetonitrile: methanol solution (6:2:3 v/v/v). To each liter of mobile phase, add 350 µL nitric acid [4M] and 120 mg potassium bromide, and mix to dissolve. 6. Post column reagent (B): Dissolve 25 mg Pyridinium hydrobromide perbromide in 500 mL H_2O. Solution can be used for up to 4 days if

	stored in dark at room temperature. 7. Toluene–acetonitrile: 9:1(v/v): Toluene-acetonitrile: Mix 90 mL toluene and 10 mL acetonitrile
Preparation of standards	*Stock Aflatoxin standards* 1. To containers of dry aflatoxins B1, B2, G1, G2 using the label statement of aflatoxin t as guide add the required volume of toluene–acetonitrile (9+1), calculated to give a final concentration of 1000 ng B1, 200 ng B2, 1000 ng G1, and 200 ng G2/mL. 2. Vigorously agitate solution for one min on a vortex shaker and transfer without rinsing to a convenient sized glass flask. 3. Do not transfer dry Aflatoxins for weighing or other purposes unless facilities are available to prevent dissemination to the surroundings because of electrostatic charge on particles. 4. For Aflatoxins received as solutions transfer solution to convenient sized glass stoppered flask. Dilute if necessary, to adjust the concentration as above 5. Record the UV-Vis spectrum of the aflatoxin solution from 200-500 nm. Determine the concentration of individual aflatoxin by measuring the absorbance (A) at wavelength of maximum absorption close to 350 nm and substitute in the following equation $$\text{Concentration of aflatoxin (mg/L)} = \frac{A_{350} \times Mw \times 1000}{\varepsilon}$$ Where A_{350} = the absorbance of the aflatoxin at 350 nm, M_w = molecular weight of the aflatoxin (Table below), ε = the molar absorptivity of the aflatoxin in Toluene–acetonitrile solution. 1. Wrap flasks tightly with aluminum foil and store at 0 °C.Do not remove aluminum foil until contents have reached room temperature. Standard solutions of aflatoxins B1, B2, G1, G2 are stable for more than one year. *Intermediate Working Standard solutions.* 1. Prepare solution by pipetting exactly 2.0 mL of stock standard solution into 20.0 mL volumetric flask (or 2.5 mL into 25 mL volumetric flask). Dilute to mark with toluene–acetonitrile solution andshake well. Concentration of 1) B1 and G1 will be 100 ng/mL and 2) B2and G2 will be 25 ng /mL. These intermediate working

Aflatoxin	Mol Wt	Solvent	E
B1	312	Toluene-acetonitrile (9+1)	19300
B2	314	Toluene-acetonitrile (9+1)	21000
G1	328	Toluene-acetonitrile (9+1)	16400
G2	330	Toluene-acetonitrile (9+1)	18300

standards can be stored in dark brown bottles covered with Aluminium foil

Working Standard Solution (To be prepared daily)

2. Pipet the volumes of the IntermediateWorking Standardas shown in the Table below into a set of 10.0 mL volumetric flasks. Evaporate toluene–acetonitrile solution just to dryness under stream of nitrogen at room temperature.

3. To each flask, add 4 mL methanol, mix, dilute to 10.0 mLwith water, and mix again

4. Prepare working solutions daily

Working Standard	Aliquot taken from Intermediate working standard (µL)	Final mass concentration of AFs in the working Standard (ng/mL)			
		B1	B2	G1	G2
1	40	0.400	0.080	0.400	0.080
2	120	1.200	0.024	1.200	0.024
3	200	2.000	0.400	2.000	0.400
4	280	2.800	0.560	2.800	0.560
5	360	3.600	0.720	3.600	0.720

Sample Preparation

Extraction:

Peanut butter and pistachio paste: Weigh, to nearest 0.1 g, 50 g test portion into 500 mL Erlenmeyer flask, add 5 g NaCl, 300 mL methanol–water extraction solvent, and 100 mL hexane or cyclohexane. Blend 3 min with high speed blender. Filter and pipette 10.0 mL clear filtrate into reservoir containing 60 mL PBS solution placed on conditioned immunoaffinity column. Mix with plastic spatula and rinse residues with 1–2 mL PBS from spatula into reservoir. Transfer solution to column as described below.

Chilli, paprika powder and other spice powders: Weigh, to the nearest 0.1 g, 50 g test portion into 500 mL Erlenmeyer flask with screw top or glass stopper. Add 5 g NaCl and 300 mL methanol–water solvent. Shake intensively by hand for 15–30 s and then for 30 min on a shaker. Filter extract using pre-folded paper. Pipette 10.0 mL clear filtrate into reservoir containing 60 mL PBS solution placed on conditioned immunoaffinity column. Mix with plastic spatula and rinse residues with 1–2 mL PBS into reservoir. Apply solution on immune affinity column as described below.

	Dried figs and other dried fruits: Weigh, to nearest 0.1 g, 50 g test portion into 500 mL Erlenmeyer flask, add 5 g NaCl, 300 mL methanol–water extraction solvent. Blend 3 min with high speed blender. Filter and pipette 10.0 mL clear filtrate into reservoir containing 60 mL PBS placed on conditioned immunoaffinity column. Mix with plastic spatula and rinse residues with 1–2 mL PBS from spatula into reservoir. Transfer solution on column as described below.
Method of Analysis	*Immunoaffinity Chromatography:* 1. Bring the immunoaffinity columns to room temperature prior to conditioning. 2. Apply 10 mL PBS solution on top of column and let flow at a speed of 2–3 mL/min through column by gravity. 3. Make sure that 0.5 mL of PBS remains on column until test solution is applied. [*Note: Methods for loading onto affinity columns, washing the column, and elution vary slightly between manufacturers. Follow manufacturer's instructions supplied with columns. In general, procedures involve extraction with methanol–water, filtration or centrifugation, possible dilution with PBS orwater, loading under pressure onto (possibly prewashed) column, washing of column with distilled water, and elution of aflatoxinswith methanol or acetonitrile*.] 4. Pass filtrate of the extractions through column at flow rate of ca 1 drop/s (ca 3 mL/minby gravity). Do not exceed 5 mL/min. 5. Wash column with 15 mL waterand dry by applying little vacuum for 5–10 s or passing air throughwith a syringe for 10 s. 6. Elute aflatoxins by adding0.5 mL methanol on column and letpass through by gravity. Collect eluate in 3.0- or 5.0-mLClass A volumetric flask. 7. Wait 1 min and apply second portion of 0.75 mL methanol. 8. Collect applied elution solvent by pressing air through. 9. Dilute to mark with water and mix. 10. If solution is clear, it can be useddirectly for LC analysis. 11. If solution is not clear, pass through disposal syringe filter unit (0.45 µm) before injection on the LC column. *HPLC with Fluorescence Detection and Post-Column Derivatization:* 1. When using PBPB, mount mixing T-piece and reaction tubing,then operate using the following parameters: flow rates, 2. mL/min (mobile phase A) and 0.30 mL/min (reagent). 3. When using electrochemically generated bromine (Kobra cell), 4. Follow instructions for installation of cell supplied by manufacturer and operate using the following parameters:

	5. Flow rate, 1.00 mL/min (mobile phase B); current, 100 µA. 6. Inject 200 µL working standard mixture (covering the range of 1–4 ng/g for aflatoxin B1) into injector, following manufacturer's instructions to ensure complete filling of the injection loop. 7. Prepare calibration curve using calibration solutions described and check curve for linearity. 8. Inject 200 µL extract into injector and identify each aflatoxin peak in chromatogram by comparing retention timeswith those of corresponding reference standards. Determine quantity of aflatoxin in eluate injected from standard curve.
Results	Aflatoxins elute in the order G2, G1, B2, and B1 with retention times of ca 6, 8, 9, and 11 min, respectively, and should be baseline resolved.
Calculation with units of expression	Calculate concentration of aflatoxin in test sample as follows: Plot data [concentration of aflatoxin (ng/mL; y-axis) from calibrant solution experiments against peak area (units; x-axis)] Carry out a linear regression analysis. Use resulting function (y = ax + b) to calculate concentration of aflatoxin in injected sample solution according to: C_i (ng/mL) = a × peak area (u of Aflatoxin B1)+ b Where C_i= concentration of B1 in injected sample Calculate B1 concentration in the sample using the equation $$\text{Aflatoxin B1 (ng/g)} = \frac{\text{Ci (ng/mL)} \times \text{Solvent volume (mL)} \times \text{Elution volume (mL)}}{\text{Sample weight (g)} \times \text{Aliquot taken (mL)}}$$ Where Ci (ng/mL) = concentration of aflatoxin B1 calculated from linear regression Sample Weight in (g) Solvent volume (mL) = Solventtaken for extraction Elution volume (mL) = final volume collected after elution from IAC; Aliquot (mL) = aliquot loaded onimmunoaffinity column for cleanup Add mass fractions of the 4 aflatoxins to obtain a total aflatoxin mass fraction. Construct individual calibration curves for each of the aflatoxins.

Reference	J. AOAC Int. 83, 320(2000). AOAC Official Method 999.07 Aflatoxin B1 and Total Aflatoxins in Peanut Butter,Pistachio Paste, Fig Paste, and Paprika Powder Immunoaffinity Column Liquid Chromatography with Post-Column Derivatization.
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Aflatoxin B1 in Baby Food using ImmunoaffinityColumn Cleanup, Post-column Derivatization, and Liquid Chromatography/ Fluorescence Detection**		
Method No.	FSSAI 07.010:2020	**Revision No. & Date**	0.0
Scope	Applicable to determination of ≥ 0.1 ng/g aflatoxin B1 in baby food.		
Caution	Follow all personal safety procedures while handling and disposing solution and washing glassware as described earlier. Soak new glassware before use in dilute acid (e.g., sulfuric acid, 2 mol/L) for several hours; then rinse extensivelywith distilled water to remove all traces of acid(check using pH paper). Read MSDS of all chemicals.		
Principle	Test portion is either extracted with Methanol–H_2O (8 + 2). Extract is filtered, diluted with water, and applied to an immune affinity column (IAC) containing antibodies specific to aflatoxins B1. Aflatoxins eluted from affinity column with Methanol and are quantified by reversed-phase liquid chromatography (RP-HPLC) with post-column derivatization involving bromination, achieved either electrochemically generated bromine (Kobra cell) or with pyridinium hydrobromide perbromide and determined by fluorescence detection.		
Apparatus/Instruments	1. Blender-Explosion proof (minimum 8000 rpm). 2. Vertical shaker: Adjustable (for maximum solid–liquid agitation); holding 500 mL Erlenmeyer flasks. 3. Filter paper—24 cm diameter, pre-folded, retention: 30 µm or better. 4. Erlenmeyer flask: 500 mL, screw top or glass stopper. 5. Glass microfiber filter paper:5 cm diameter, retention:1.6 µm (or better). 6. Reservoir:75 mL with Luer tip connector for affinity column. 7. 20 mL syringe with Luer lock or rubber stopper. 8. Class A Volumetric glassware: 2, 3, 10, and 20 mL 9. High Performance Liquid Chromatograph equipped with a. Pump: Suitable for flow rate at 0.2-1.000 ± 0.005 mL/min. b. Injection system: Total loop injection valve with loop between 100 and 1000 µL. For the volume (100–1000 µL) of the injection system, it must be guaranteed that the relative standard deviation (RSD) of the aflatoxin B1 peak for a multiple injection (n = 10) of a standard solution of aflatoxin B1 reflecting a contamination level of 0.1 ng/g results in a value of maximum 10%. c. Column: C-18 (Octadecyl 25 cmx 4.6 mm i.d. ×, 5 µm or ODS-2 column of 5 µm pore size.12% carbon loading; notend-capped is suitable. d. Fluorescence detector: Wavelength 360 nm excitation filter and 420 nm cut-off emission filter, or equivalent		

	10. Post column derivatization system a. For pyridinium hydrobromide perbromide reagent: Second LC pulseless pump, zero-dead volume T-piece, reaction tubing minimum dimensions 45 cm × 0.5 mm id PTFE. b. For electrochemically generated bromine: Kobra cell. 11. Disposable filter unit: Cellulose or cellulose nitrate, 0.45 µm. 12. Pipets: 10 mL. 13. Analytical balance: Weighing to 0.1 mg. 14. Laboratory balance: Weighing to 0.1 g. 15. Calibrated microliter syringes or micropipette(s):25 and 500 µL. 16. Calibrated UV spectrophotometer 17. Affinity Columns: Vicam (Watertown, MA) or Rhone-Diagnostics have been found to meet the criteria.
Criteria for acceptance of immunoaffinity column	The affinity column must contain antibodies raised againstaflatoxin B1 with a capacity of not less than 50 ng aflatoxin B1and should give recovery of not less than 80% when applied asa standard solution in methanol–H_2O containing 5 ng aflatoxin B1.
Materials and Reagents	1. All chemicals should be of analytical grade 2. Water, except where specified, should be produced by single distillation, deionization, or reverse osmosis 3. Potassium chloride (KCl) 4. Dihygrogen potassium phosphate (KH_2PO_4) 5. Disodium mono hydrogen phosphate (Na_2HPO_4) 6. Sodium chloride (NaCl) 7. Hydrochloric acid 8. Pyridinium hydrobromide perbromide (PBPB): CAS-39416-48-3 9. Potassium bromide 10. Acetonitrile: HPLC grade 11. Methanol: HPLC grade 12. Methanol: Technical grade, pure, or distilled 13. Water: HPLC grade; complying with grade 1 of ISO 3696 14. Hexane or cyclohexane 15. Concentrated Nitric acid 16. Toluene *Reagents:* 1. Phosphate buffered saline solution (PBS): Dissolve 0.20 g KCl, 0.20 g KH_2PO_4, 1.16 g anhydrous Na_2HPO_4 (or 2.92 g $Na_2HPO_4 \cdot 12H_2O$), and 8.00 g NaCl in 900 mL water. Adjust to pH 7.4 with 0.1M HCl or NaOH and dilute to 1 L. (Commercial buffered saline tablets may be used.) 2. Extraction solvent: Methanol–water solution (8 + 2, v/v). 3. 4M Nitric acid: Dilute 28.1 mL concentrated HNO_3 (65%) or 26.1 mL 70% HNO_3) in water to final volume of 100 mL.

	4. Mobile phase A—Water–acetonitrile–methanol solution (6 + 2 + 3, v/v/v). Mix 600 mL of HPLC grade water, 200 mL of acetonitrile and 300 mL of HPLC grade methanol. 5. Mobile phase B —For use with electrochemically generated Br: water: acetonitrile: methanol solution (6:2:3 v/v/v). To each liter of mobile phase, add 350 µL nitric acid [4M] and 120 mg potassium bromide, and mix to dissolve. 6. Post column reagent: Dissolve 25 mg Pyridinium hydrobromide perbromide in 500 mL H_2O. Solution can be used for up to 4 days if stored in dark at room temperature. 7. Toluene–acetonitrile: 9:1(v/v): Toluene-acetonitrile: Mix 90 mL toluene and 10 mL acetonitrile.
Preparation of standards	*Stock Aflatoxin standards:* *Option A For aflatoxin B1 standard received as a dry films or crystal:* 1. To container of dry aflatoxin B1 add volume of toluene– acetonitrile (9 + 1), calculated to give concentration of 8–10 µg/mL. 2. Use label statement of aflatoxin weight as guide. 3. Vigorously agitate solution 1 min on Vortex shaker and transfer without rinsing to convenient-sized glass-stoppered flask. Record UV spectrum and calculate exact concentration. 4. Return aflatoxin solution to original glass-stoppered flask and dilute with toluene–acetonitrile (9 + 1) to obtain a concentration of 5.00 ng/mL. (Note: Dry films on glass are not completely recoverable because ofadsorption. Continued contact with solvent may result in slowdissolution.) ***Caution: Do not transfer dry aflatoxin for weighing orother purposes unless facilities are available to prevent dispersion of aflatoxin to surroundings because of electrostatic charge on particles.*** *Option B For aflatoxin B1 standard received as solution:* 1. Transfer solution to convenient-sizedglass-stoppered flask. 2. Dilute, if necessary, to adjust concentrationto 8–10 µg/mL. 3. Record UV spectrum of aflatoxin B1solution from 200 to 400 nm against solvent used for dissolutionin reference cell. Determine concentration of aflatoxin B1 solutionby measuring absorbance (A) at wavelength of maximumabsorption close to 350 nmand substitute in the following equation $$\text{Concentration of aflatoxin (mg/L)} = \frac{A_{350} \times Mw \times 1000}{\varepsilon}$$

Where A_{350} = the absorbance of the aflatoxinB1 at 350 nm,

M_w = molecular weight of the aflatoxin B1 =312

ε = the molar absorptivity of the aflatoxinB1 in Toluene–acetonitrile solution= 19300 [from J. AOAC Int. 82, 252(1999)].

4. Return aflatoxin solution to original glass-stoppered flaskand dilute with toluene–acetonitrile (9 + 1) to obtain a concentration of 5.00 ng/mLWrap flasks tightly with aluminum foil and store at 0 °C. Do not remove aluminum foil until contents have reached room temperature. Standard solutions of aflatoxin B1 is stable for more than one year.

Working Standard Solution Option, A (To be prepared daily)

I. Use this solution (5ng/mL) for pipetting the volumes listed in Table below into a set of 10 mL calibrated volumetric flasks.

II. Evaporate toluene–acetonitrile solution just to dryness under stream of N_2 at room temperature (22–25 °C).

III. To each flask, add 3.5 mL methanol, and mix; dilute to volume (10 mL) with water and mix again

Preparation of Working Calibration Solutions Option A Prepared fresh daily		
Working standard	Aliquot taken from stock solution, µL	Final concentration of working calibrant, ng AfB1/mL
1	20	0.01
2	40	0.02
3	60	0.03
4	80	0.04
5	100	0.05
6	120	0.06
7	140	0.07

Working Standard Solution Option, B (To be prepared daily)

1. Pipet from aflatoxin standard solution (5 ng/mL) volumes as listed in Table below into a set of 10 mL calibrated volumetric flasks.
2. Evaporate the toluene–acetonitrile solution just to dryness under stream of N_2 at room temperature (22–25 °C).
3. Add 3.5 mL methanol, let aflatoxins dissolve, fill to the mark with methanol, and shake well.
4. Transfer exactly 1 mL of this working calibrant into an acid-washed vial and evaporate to dryness.
5. Re-dissolve in exactly the same amount of aqueous methanol that will be used for test solutions.
6. Calculate concentration of aflatoxin B1 in the re-dissolved working

<table>
<tr><td></td><td>calibrant solution in ng/mL.
7. Use these values for the calculation (the calibration range in ng/g will remain unchanged).

<table>
<tr><td colspan="3">Preparation of Working Calibration Solutions Option B
Prepared fresh daily</td></tr>
<tr><td>Working standard</td><td>Aliquot taken fromstock solution, µL</td><td>Final concentration of workingcalibrant, ng AfB1/mL</td></tr>
<tr><td>1</td><td>100</td><td>0.05</td></tr>
<tr><td>2</td><td>200</td><td>0.10</td></tr>
<tr><td>3</td><td>300</td><td>0.15</td></tr>
<tr><td>4</td><td>400</td><td>0.20</td></tr>
<tr><td>5</td><td>500</td><td>0.25</td></tr>
<tr><td>6</td><td>600</td><td>0.30</td></tr>
<tr><td>7</td><td>700</td><td>0.35</td></tr>
</table></td></tr>
<tr><td>Sample Preparation</td><td>Extraction:

1. Weigh to nearest 0.1 g ca 50 g test portion of baby food into 500 mL Erlenmeyer flask with screw top or glass stopper.
2. Add 5 g NaCl and 250 mL methanol–water solvent.
3. Shake intensively by hand for first 15–30 s and then for 30 min with ashaker.
4. Filter extract using pre-folded filter paper.
5. Pipet 15 mL clear filtrate into calibrated 150 mL volumetric flask and fill with PBS or water.
6. Refilter through glass fiber filter
7. Apply volume of 50-or 100 mL clear filtrate in a reservoir placed on a conditioned immunoaffinity column.
8. A volume of 50 mL will normally be adequate, although 100 µL can be used if fluorescence detection does not provide adequate sensitivity.</td></tr>
<tr><td>Performance Standard for Affinity Column</td><td>The affinity column must contain antibodies raised againstaflatoxin B1 with a capacity of not less than 50 ng aflatoxin Bland should give recovery of not less than 80% when applied asa standard solution in methanol–H_2O containing 5 ng aflatoxin B1.</td></tr>
<tr><td>Method of Analysis</td><td>Immunoaffinity Chromatography:

1. Bring the immunoaffinity columns to room temperature (22-25 °C) prior to conditioning.
2. Apply 10 mL PBS solution on top of column and let flow at a speed of 2–3 mL/min through column by gravity.
3. Make sure that 0.5 mL of PBS remains on column until test solution is applied.
4. [Note: Methods for loading onto affinity columns, washing the column,</td></tr>
</table>

	and elution vary slightly between manufacturers. Follow manufacturer's instructions supplied with columns. In general, procedures involve extraction with methanol–water, filtration or centrifugation, possible dilution with PBS or water, loading under pressure onto (possibly prewashed) column, washing of column with distilled water, and elution of aflatoxins with methanol or acetonitrile.) 5. Pass filtrate of the extractions through column at flow rate of ca 1 drop/s (ca 3 mL/min by gravity). Do not exceed 5 mL/min. 6. Wash column with 15 mL water in 5 mL portions, and dry by applying small vacuum for 5–10 s or passing air through by means of syringe for 10 s. 7. Elute aflatoxin B1 in two steps, First, apply 0.5 mL methanol on the column and let it pass through by gravity. Collect eluate in either 5 mL volumetric flask (option A below) or LC injection vial (option B below). *Option A (recommended):* This option requires appropriate fluorescence detector and injection system. Option B only applies if detector signal is insufficient for analysis by option A. 1. Collect elute in calibrated 5 mL volumetric flask. 2. Fill to mark with water and shake well. 3. If solution is clear, it can be used directly for LC analysis. 4. If solution is not clear, pass it through disposable 0.45 mm filter unit prior to LC injection. 5. Injection by total loop mode provides maximum accuracy. 6. Depending on injection system, e.g., syringe or autosampler, 7. Take volume of 3 times the injection loop size and inject at least 2/3 this volume into the valve to ensure that the middle fraction remains in the injection loop. 8. Thus, the loop is rinsed with the filtered eluate while enough liquid remains in the valve. *Option B (only if applicable*). If detector signal is not sufficient to provide the required RSD (10%), include an additional evaporation step to meet the required RSD. 1. Collect methanol eluate from affinity column in an acid-washed LC injector vial. 2. Evaporate methanol to dryness under gentle stream of N_2 at 40 °C. Re-dissolve residue in aqueous methanol solution (3.5 mL methanol diluted to 10 mL with water). Use exactly the same volume for the evaporated analyte residues as that used for evaporated calibrants. 3. The volume for re-dissolving will depend on the size of injection loop. 4. Use total loop mode for injection as in option A. *HPLC with Fluorescence Detection and Post-Column Derivatization:*

	When using PBPB, mount mixing T-piece and reaction tubing, then operate using the following parameters: flow rates, 1.0 mL/min (mobile phase A) and 0.30 mL/min (reagent). When using electrochemically generated bromine (Kobra cell),Follow instructions for installation of cell supplied by manufacturer and operate using the following parameters: Flow rate, 1.00 mL/min (mobile phase B); current, 100 µA. Inject working standard mixture (covering range of 0.05–0.35 ng/g for aflatoxin B1) into injector, following manufacturer'sinstructions to ensure complete filling of injectionloop. Prepare calibration curve using calibrationsolutions. Check curve for linearity. Inject same volumeof working standards and extract into injector and identifyeach aflatoxin peak in the chromatogram by comparing retentiontimes with those of corresponding reference standards. Determine quantity of aflatoxin B1 in injected eluate from thestandard curve.
Results	Aflatoxins elute in the order G2, G1, B2, and B1 with retentiontimes of approximately 6, 8, 9, and 11 min, respectively, and should be base-line resolved to measure aflatoxinB1 as a discrete peak.
Calculation with units of expression	Plot the data: concentration of aflatoxin (ng/mL) as they-axis against peak area (units) as the x-axis, from thecalibrant solutions. Calculate the resulting function, y = ax+b, from linear regression, where a is the slope and b is the y-valuewhere the line intercepts the y-axis (x = 0). Use resulting function (y = ax + b) to calculate concentration of aflatoxin in injected sample solution according to: C_i (ng/mL) = a × peak area (unknown of Aflatoxin B1)+ b Where C_i= concentration of B1 in injected sample Calculate B1 concentration in the sample using the equation $$\text{Aflatoxin B1 (ng/g)} = \frac{C\ (ng/g) \times V_{extraction}\ (mL) \times V_{dilution}\ (mL) \times V_{Elution}\ (mL)}{\text{Sample weight (g)} \times V_{Extract\ Aliquot}\ (mL) \times V_{AFC}\ mL}$$ Where C = concentration of aflatoxin B1 (ng/g) from linear regression;

	g = test portion (g; 50); $V_{extraction}$ = volume extraction solvent (250) $V_{extract\ aliquot}$ = volume aliquot extractionsolvent (15); V_{diln} = volume diluted with PBS or water (150); V_{AFC} = volume applied to column (50 or 100); V_{elutn}= volumeafter elution (5).
Reference	J. AOAC Int. 84, 1118–1121(2001) AOAC Official Method 2000.16, Aflatoxin B1 in Baby FoodImmunoaffinity Column HPLC Method, First Action 2000.
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Aflatoxins B1, B2, G1, and G2 in Olive Oil, Peanut Oil, and Sesame Oil using Immunoaffinity Column Cleanup, Post-column Derivatization, and LiquidChromatography/Fluorescence Detection		
Method No.	FSSAI 07.011:2020	**Revision No. & Date**	0.0
Scope	Applicable to the determination of total aflatoxins (AFs; sum of AFB1, AFB2, AFG1, and AFG2) in olive oil, peanut oil, andsesame oil at 2–20 µg/kg and AFB1 in the matrixes at 1–10 µg/kg.		
Caution	Follow all personal safety procedures while handling and disposing solution and washing glassware as described earlier. Theuse of non-acid-washed glassware (e.g., vials, tubes, flasks) for aflatoxin aqueous solutions maycause a loss of aflatoxin. Methanol and acetonitrile arehazardous and must be poured in a fume cupboard. Read MSDS of all chemicals.		
Principle	A test portion is extracted with methanol–water (55 + 45, v/v).After shaking and centrifuging, the extract is filtered, dilutedwith water, and applied to an IAC containing antibodies specificfor AFs. After washing with methanol–water (10 + 90, v/v), thetoxin is eluted from the column with methanol and determinedand quantified by Liquid Chromatography/Fluorescence Detection. For AF post-column derivatization, aphotochemical derivatization device or Kobra cell is used.		
Apparatus/Instruments	1. Blender-Explosion proof (minimum 8000 rpm). 2. Orbital shaker: or equivalent shaker capable of 400 rpm. 3. Centrifuge tubes: 50 mL, polypropylene, plug seal cap; 4. Centrifuge 5. Filter paper: Qualitative folded filter papers, Grade597½, 185 mm Whatman or equivalent. 6. Erlenmeyer flask: 500 mL, screw top or glass stopper. 7. Glass microfiber filter paper: 5 cm diameter, retention:1.6 µm (or better). 8. Reservoir: 75 mL with Luer tip connector for affinity column. 9. 20 mL syringe with Luer lock or rubber stopper. 10. Class A Volumetric glassware: 2, 3, 10, and 20 mL 11. High Performance Liquid Chromatograph equipped with a. Pump: Suitable for flow rate at 0.8 ± 0.005 mL/min. b. Injection system c. Column: C-18 (Octadecyl 15 cmx 4.6 mm i.d. ×, 3 µm or ODS-2column of 5 µm pore size. 12% carbon loading; not end-capped is suitable. d. Fluorescence detector: Wavelength 362 nm excitation filter and 440 nm cut-off emission filter, or equivalent 12. Post column derivatization a. Post column derivatization systemin a special reactor loop with UV light, standard reactor volume is 1.0 mL b. PHRED cell (post column photochemical derivatization cell (Caution: Avoid		

	visual exposure to the UV light). c. Kobra cell (electrochemical cell, post column bromination derivatization cell. (Caution: Set at 100 mA. Do not turn on current until LC pump is operating to avoid overheating the cell membrane.) 13. Disposable filter unit: Cellulose or cellulose nitrate, 0.45 µm. 14. Graduated measuring cylinders: 25 and 50 mL. 15. Analytical balance: Weighing to 0.1 mg. 16. Laboratory balance: Weighing to 0.1 g. 17. Calibrated microliter syringes or micropipette(s): 25 and 500 µL. 18. Calibrated UV spectrophotometer 19. Immunoaffinity columns—AflaTest WB columns (G1024; VICAM, meet the criteria below.
Criteria for acceptance of immunoaffinity column	The aflatoxin IACs to contain monoclonal antibodies that are cross reactive with AFB1, B2, G1, and G2. Thecolumns should have capacity of not less than 100 ng total AFand should give a recovery of not less than 80% for AFB1, B2, G1,and G2 when 5 ng of each AF is applied in 10 mL methanol–PBS(10 + 90, v/v). The columns should have a shelf life of 18 monthsat 4 °C or 12 months at room temperature.
Materials and Reagents	All chemicals should be of analytical grade 1. Water, except where specified, should be produced by single distillation, deionization, or reverse osmosis 2. Potassium chloride (KCl) 3. Dihygrogen potassium phosphate (KH_2PO_4) 4. Disodium mono hydrogen phosphate (Na_2HPO_4) 5. Sodium chloride (NaCl) 6. Hydrochloric acid 7. Pyridinium hydrobromide perbromide (PBPB): CAS-39416-48-3. 8. Potassium bromide 9. Acetonitrile: HPLC grade 10. Methanol: HPLC grade 11. Methanol: Technical grade, pure, or distilled 12. Water: HPLC grade; complying with grade 1 of ISO 3696 13. Hexane or cyclohexane 14. Concentrated Nitric acid 15. Toluene *Reagents:* 1. Phosphate buffered saline solution (PBS): Dissolve 0.20 g KCl, 0.20 g KH_2PO_4, 1.16 g anhydrous Na_2HPO_4 (or 2.92 g $Na_2HPO_4 \cdot 12H_2O$), and 8.00 g NaCl in 900 mL water. Adjust to pH 7.4 with 0.1M HCl or NaOH and dilute to 1 L. (Commercial buffered saline tablets may be used.) 2. Extraction solvent: Methanol–H_2O (55 + 45, v/v), mix, equilibrate to room temperature.

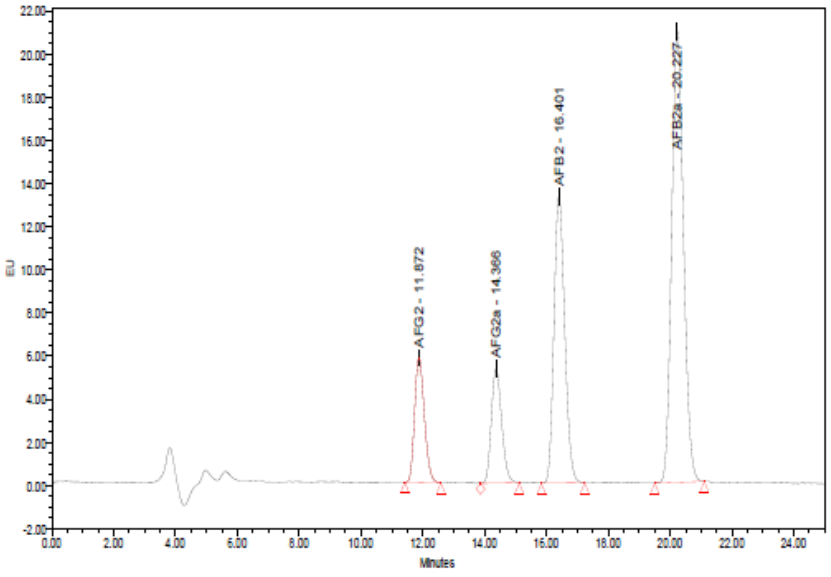
	3. Washing solution: Methanol–H_2O (10 + 90, v/v), mix, equilibrate to room temperature. 4. 4M Nitric acid: Dilute 28.1 mL concentrated HNO_3 (65%) or 26.1 mL 70% HNO_3) in water to final volume of 100 mL. 5. Mobile phase A: For AF post column derivatization with PHRED cell or UVE device. Methanol–acetonitrile–water (25 + 17 + 60, v/v/v). Mix 600 mL of HPLC grade water, 170 mL of acetonitrile and 250 mL of HPLC grade methanol. 6. Mobile phase B: For AF post column derivatization with Kobra cell. Methanol–acetonitrile–water (25 + 17 + 60, v/v/v) + 350 µL of 4 M nitric acid + 120 mg potassium bromide, and mix.
Preparation of standards	*Stock Aflatoxin standards* Prepare stock standard solutions of each of the fourAFs at 10 µg/mL in acetonitrile as described above for 'Thin Layer Chromatography Method'(RevisedAOACOfficial Method 971.22) *Preparation of 400 ng/mL AF second stock standard solution (mixture of AFB1, B2, G1, and G2 at 200, 50, 100, and 50 ng/mL).* 1. Add appropriate amount of each AF stock standard to the same volumetric flask and dilute to volume with acetonitrile. 2. Use the 400 ng/mL AF second stock standard as the spiking solution for the recovery study. 3. Store stock standard solution at –18 °C. 4. Equilibrate to room temperature before use. *Preparation of working AF calibrant solution.* Prepare daily 6 calibrates in separate 5 mL volumetric flasks according to Table below. Dilute to volume with methanol–water (1 + 1, v/v). Store in refrigerator and equilibrate to room temperature beforeuse. Prepare working calibration solutions daily. *Note: Silanized vials were used for storage of AF stock standard solutions*.

<table>
<tr><td></td><td>
<table>
<tr><th rowspan="2">Working Standard solution</th><th rowspan="2">Aliquot of 400 ng/mL AFs second stockstandard solution (µL)</th><th colspan="5">Final aflatoxin concentration of working standard solution, ng/mL</th></tr>
<tr><th>B1</th><th>B2</th><th>G1</th><th>G2</th><th>Sum</th></tr>
<tr><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0.0</td></tr>
<tr><td>2</td><td>10</td><td>0.40</td><td>0.10</td><td>0.20</td><td>0.10</td><td>0.80</td></tr>
<tr><td>3</td><td>25</td><td>1.00</td><td>0.25</td><td>0.50</td><td>0.25</td><td>2.00</td></tr>
<tr><td>4</td><td>50</td><td>2.00</td><td>0.50</td><td>1.00</td><td>0.50</td><td>4.00</td></tr>
<tr><td>5</td><td>100</td><td>4.00</td><td>1.00</td><td>2.00</td><td>1.00</td><td>8.00</td></tr>
<tr><td>6</td><td>250</td><td>10.0</td><td>2.50</td><td>5.00</td><td>2.50</td><td>20.0</td></tr>
</table>
</td></tr>
<tr><td>Sample Preparation</td><td>Extraction:
1. Weigh 5.0 g test portion in a 50 mL centrifuge tube.
2. Add 1.0 g NaCl and 25 mL extraction solvent.
3. Vortex until oil and extract solvent are well mixed.
4. Shake at 400 rpm for 10 min.
5. Centrifuge at 7000 rpm (g value = 5323 mm/s^2) for 10 min.
6. Aspirate and discard the upper oil layer.
7. Pass the lower aqueous methanol layer through folded filter paper. Measure 15 mL filtrate with a 25 mL graduated cylinder and place in a 50 mL centrifuge tube.
8. Add 30 mL water, mix, and filter through glass microfiber paper.
9. Collect 30 mL filtrate (equivalent to 2 g test portion) into a 50 mL graduate cylinder and proceed immediately with IAC chromatography.</td></tr>
<tr><td>Performance Standard for Affinity Column</td><td>The affinity column must contain antibodies raised againstaflatoxin B1 with a capacity of not less than 50 ng aflatoxin Bland should give recovery of not less than 80% when applied asa standard solution in methanol–H_2O containing 5 ng aflatoxinB1.</td></tr>
<tr><td>Method of Analysis</td><td>Immunoaffinity Chromatography:
1. After removing from storage at 4 °C, IACs are equilibrated at room temperature for at least 15 min before use.
2. Remove top from column and connect to reservoir of column manifold.
3. Remove bottom cap from column and let liquid in column pass through until liquid reaches 2 mm above the column packing.
4. Add 30 mL filtrate into column reservoir.
5. Let filtrate flow through IAC by gravity force until the liquid level reaches 2 mm above the column packing.
6. Add 10 mL washing solution to column reservoir.</td></tr>
</table>

7. Let column run dry and then force 10 mL air through column with a syringe.
8. Place a 2 mL volumetric flask under column.
9. Elute with 0.6 mL LC grade methanoland collect AFs in a 2 mL volumetric flask; let drip freely. Let column run dry.
10. Elute with additional 0.6 mL methanol and collect into the same volumetric flask.
11. Let column run dry and force 10 mL air through column.
12. Dilute eluate to volume with water and perform LC analysis.

HPLC with Fluorescence Detection and Post-Column Derivatization:

1. Inject 50 µL reagent blank (calibrate 1), AF working standards, or test sample into LC column.
2. Identify AF peaks in test sample by comparing retention time with those of standards.
3. AFs elute in the order of G2, G1, B2, and B1. After passing through theUVE device, PHRED cell, or Kobra cell, the AFG1 and AFB1 are derivatized to form G2a (derivative of G1) and B2a (derivative of B1). The retention times of AFG2, G2a, B2, and B2a are between about 11 and 21 min using the PHRED cell (see Figure); retention times are shorter using the Kobra cell.

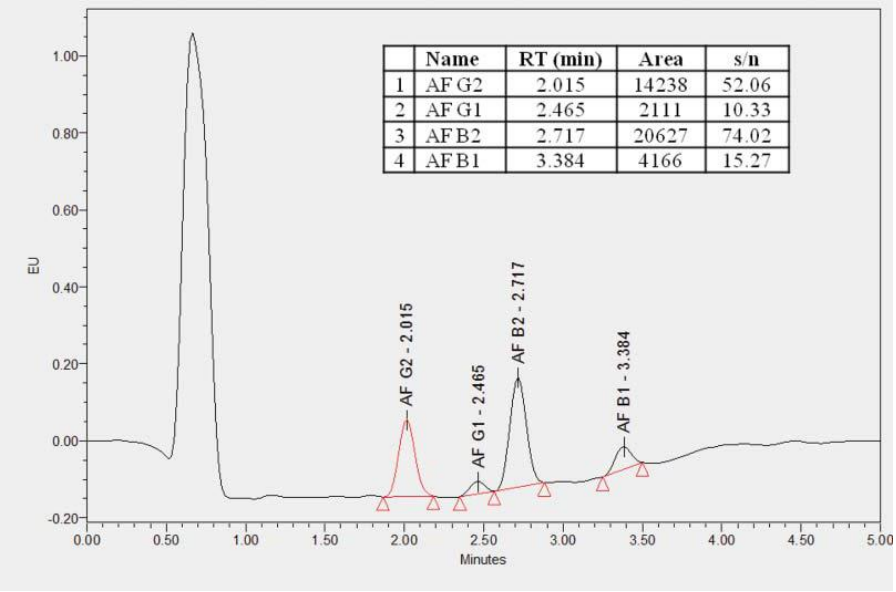


LC profile of AF standard solution (AFs 4.0 ng/mL) after post column derivatization with a PHRED.

4. The peaks should be baseline resolved (see Figure).
5. Construct standard curves of each AF.
6. Determine concentration of each AF in test solution from calibration curve.
7. Calibration curves should be prepared for each AF using the working calibration solutions containing the four AFs described in table.

	8. These solutions cover the range of 0.4–10.0 ng/mL for AFB1, 0.1–2.5 ng/mL for AFB2, 0.2–5.0 ng/mL for AFG1, and 0.1– 2.5 ng/mL for AFG2. 9. Check the curve for linearity. 10. If test portion area response is outside (higher) the calibration range, then the purified test extract should be diluted with methanol– water (50 + 50, v/v) and reinjected into the LC column.
Results	Aflatoxins elute in the order G2, G1, B2, and B1 and should be base-line resolved to measure each aflatoxinas a discrete peak. Quantitation of aflatoxins: Quantitation of AFs shouldbe performed by measuring peak areas at each AF retention timeand comparing them with the relevant calibration curve.
Calculation with units of expression	Plot peak area (response, Y-axis) of eachAF standard againstconcentration (ng/mL, X-axis) and determineslope (S) and Y-intercept Calculate level of toxin in testsample with the following equation, $$\text{Total Afs } (\mu g/Kg) = \left[\frac{R-a}{S}\right] \times \frac{V}{W} \times F$$ where R is the test solutionpeak area, V is the final volume (mL) of the injected test solution, F is the dilution factor (F is 1 when V is 2 mL), W is 2 g test sample passed through the IAC. S is the slope and a, the y intercept of the calibration curve. The total AFs is the sum of theAFB1, AFB2, AFG1, and AFG2
Reference	Journal of AOA C International 2012, 95, 1689-1700 AOAC Official Method Aflatoxins B1, B2, G1, and G2 in Olive Oil,Peanut Oil, and Sesame Oil, Immunoaffinity Column Cleanupand Liquid Chromatographic QuantitationFirst Action 2012.
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Direct analysis of Aflatoxins (AF) peanuts, peanut products and cereal matrices by Ultra-High-Performance Liquid Chromatography with fluorescence detection		
Method No.	FSSAI 07.012:2020	**Revision No. & Date**	0.0
Caution	Follow all personal safety procedures while handling and disposing solution and washing glassware as described earlier. Theuse of non-acid-washed glassware (e.g., vials,tubes, flasks) for aflatoxin aqueous solutions maycause a loss of aflatoxin. Methanol and acetonitrile arehazardous and must be poured in a fume cupboard. Read MSDS of all chemicals.		
Principle	A reverse phase based HPLC separation of the AFs and their detection by fluorescence. The AFs are extracted and then purified by using immunoaffinity columns specific for AFs.		
Apparatus/Instruments	Ultra-High-Performance Liquid Chromatography equipped with 1. Fluorescence detector [FLD with largevolume (13 µL) flow cell] 2. Column oven set at 40 °C 3. C18 column (2.1 × 50 mm, 1.7 µm)		
Materiasl and Reagents	1. Methanol (HPLC gradient grade), 2. Acetic acid, 3. Sodium chloride 4. Sodium hydroxide 5. HPLC grade water (18.2 MΩ cm) 6. Immunoaffinity (Monoclonal antibody) specific for AFs 7. Phosphate-buffered saline (PBS) 8. ***Reference standards*:** Individual AF standards (B1, B2, G1, and G2) with >95% purity		
Preparation of standards	*Stock standard*: Dissolve 5 mg each standard in 10 mL methanol in an amber-colored vial. The stock solutions containing 500 µg/mL of each AF is stored at –20 °C. Intermediate standard: Dilute the stock solutions in methanol. *Calibration standards*: Make serial dilutions of the intermediate solutions to obtain 0.02–10 ng/mL for each AF in 1:1 ratio of methanol: water (plus 0.2% acetic acid, v/v).		
Sample Preparation	*Grinding:* Raw peanuts, cereal grains and processed products (Chikki, peanut snacks, roasted, salted, spiced, peanut flips etc.) are thoroughly milled and allowed to pass through a No. 20 sieve. Defat 25 g of Peanut butter with 25 mL of hexane. Discard the hexane layer. Used defatted powder for extraction. *Extraction:* Add 12.5 g of finely ground dry matrix to 12.5 g distilled water to make a slurry. Mix the slurry with 100 mL of extraction solvent (methanol–water, 8+ 2, v/v)		

	and NaCl (5 g). Shake for 30 min,200 rpm), and then centrifuge (5000 rpm, 5 min). Take an aliquot (3 mL) and dilute with15 mL PBS and add 50 µL NaOH (2 M) solution. *IAC cleanup:* Load the diluted sample onto IAC connected to a vacuum manifold and allow to pass without any vacuum. Wash with 10 mL PBS. Elute with methanol (2 × 0.5 mL). Slowly evaporate the final extract (1 mL) to dryness. Reconstituted in 0.5 mL methanol–water (acidified with 0.1%acetic acid, 1:1), and finally inject 10 µL into the UHPLC-FLD instrument.
Method of Analysis	*Chromatography conditions:* 1. Column: C18 column (2.1 × 50 mm, 1.7 µm). 2. Column temperature: 40 °C, 3. Flow rate: 0.4 mL/min 4. Injection volume: 10 µL. 5. The mobile phase: methanol: acetonitrile: water (18:18:64) 6. Elution: Isocratic 7. Detector: a. Excitation wavelength 365 nm. b. Emission wavelength: 456 nm
Results	 The elution patter of AFs is as shown above
Calculation with units of expression	Prepare a calibration curve for 0.02–10 ng/g for each AF by injecting 10 µl each working standard. From the equation determine the concentration of the AF in the extracts prepared.
LOQ	LOQ is 0.008 µg/kg for the B1 and G1 and 0.003 µg/kg for the B2 and G2.

Reference	High-sensitivity direct analysis of aflatoxins in peanuts and cereal matrices by ultra-performance liquid chromatography with fluorescence detection involving a large volume flow cell' Oulkar, D., Goon, A., Dhanshetty, M., Khan, Z., Satav, S., & Banerjee, K (2018): Journal of Environmental Science and Health, Part B Pesticides, Food Contaminants, and Agricultural Wastes, 53, 255-260
Approved by	Scientific Panel on Methods of Sampling and Analysis

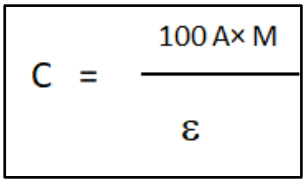
	Determination of Aflatoxins M1 and M2 in Fluid Milk Liquid Chromatographic Method		
Method No.	FSSAI 07.013:2020	**Revision No. & Date**	0.0
Caution	Follow all personal safety procedures while handling and disposing solution and washing glassware as described earlier. Methanol and acetonitrile arehazardous and must be poured in a fume cupboard. Trifluoroacetic acid is corrosive chemical and contact can severely irritate and burn the skin and eyes with possible eye damage. Use face shield or eye protection (safety goggles) in combination with breathing protection. Dichloro dimethyl silane is a lachrymator and is flammable. Read MSDS of all chemicals.		
Principle	Aflatoxins M1and M2 are extracted from milk using a C-18 cartridge, eluted with ether onto silica column and eluted with methylene chloride-alcohol. The eluted toxins are derivatized with trifluoracetic acid, resolved by liquid chromatography and detected fluorometrically compared with standard-TFA derivatives.		
Materials and Reagents	1. *Solvents:* HPLC grade Acetonitrile, methylene chloride, isopropyl alcohol, hexane, methanol; reagent grade alcohol, ether (0.01% ethyl alcohol preservative), and H_2O (deionized, filtered through 0.45 µm filter). 2. *Trifluoroacetic acid (TFA)* – ≥98.5% pure. Transfer 1-2 mL TFA to a one-dram vial with a Teflon lined cap. Keep in freezer when not in use. Discard if discoloration appears. 3. *Wash solution (*Water-acetonitrile *(*95+5)): Mix 950 mL of water and 50 mL acetonitrile 4. *Elution solution:* (Methylene chloride—alcohol (95+5)): Mix 950 mL of methylene chloride and 50 mL acetonitrile 5. *Mobile phase* (Water: isopropyl alcohol: acetonitrile (80:12: 8)). Measure and mix 800 mL water, 120 mL, isopropyl alcohol and 80 mL acetonitrile). Degas in ultrasonic bath, or equivalent for not more than 2 min. Alternative solvent proportions may be used to give optimum resolutions (84 + 11 + 5). 6. *Aflatoxin standard solution:* Aflatoxins M1 and M2 (high purity). a) *Stock Standard*: Prepare stock solutions (ca 200 µg M1/mL and 100 µg M2/mL) in acetonitrile. Determine concentrations spectrophotometrically using molar extinction coefficients of 19850 and 21400 for M1 and M2, respectively (Table 3.1). b) *Working standard solution*: Dilute stock standard with acetonitrile—benzene (1+9) to contain 0.50 and 0.10 µg /mL of M1 and M2 respectively for TFA derivative.		

	7. *Dichlorodimethylsilane (DDS):* 5% in toluene. Add 5 mL DDS (99%) to toluene and dilute to 100 mL. Store in glass-stoppered flask in cold. (Caution: DDS is a lachrymator and is flammable.)
Apparatus/Instruments	1. *Silica gel cleanup columns:* 0.8 ×4.0 cm polypropylene column with Luer tip, 35 µg porous polypropylene bed support disk, and 10 mL reservoir. 2. *Silica gel for cleanup columns packing and preparation:* Dry silica gel 60, particle size 0.40-0.063 mm for 1 h at 105 °C oven. Cool and add 1% H_2O by weight. Shake in sealed container and equilibrate overnight before use. Assemble polypropylene column and 25 mL vacuum flask fitted with 1-hole stopper**.** Fill column to ca 2 mL mark with silica gel (ca l g). Pull gentle vacuum to pack bed and add ca 1 g anhydrous Sodium sulfate to top of silica gel bed. 3. *Extraction cartridges:* C-18 Sep-Pak or equivalent cartridges 4. *Disposable pipet tips:* 50 and 200 µL Eppendorf or equivalent. 5. *Liquid chromatograph:* Any liquid chromatographic system which includes pump(s), injector/autosampler, and compatible computer and software for peak recognition and integration. 6. *Fluorescence detector:* A fluorescence detector capable of providing 365 nm excitation and ≥400 nm emission wavelength and sensitivity of 50-100% full-scale response for 1 ng M1-TFA derivative. 7. *LC analytical column:* Any 4.0 mm i.d.×25 cm column containing spherical 5 µm particle size C18 bonded silica gel 8. *Vacuum regulator:* Any commercial or custom device capable of creating and controlling partial and full vacuum with side arm vacuum flask. *Silylated vials for aflatoxin standard solutions:* Fill 1 or 1.5-dram (4or 6 mL) glass vials nearly full with 5% DDS and heat ca 40 min at 45-55 °C. Discard solution, and rinse vials three times with toluene and then three times with methanol. Heat vials in oven at 75°C for 20-30 min to evaporate methanol. Cap vials (with Teflon liners) and store for aflatoxin standard solutions.
Sample Preparation	1. Attach intel (longer) stem of C18 Sepak cartridge to Luer tip of 30-50 mL syringe. Assemble syringe, cartridge, and vacuum flask**.** Adjust vacuum to pull solvents through cartridge in fast drop wise manner (ca 5 mm Hg). Prime cartridge by adding 5 mL methanol, then 5 mL water in stem). Discontinue vacuum and move cartridge-syringe assembly from stopper to prevent loss of priming solution. 2. Warm milk (test sample) to room temperature. Gently invert test sample ≥ 10times to evenly distribute cream. 3. Transfer 20 mL milk to graduated test tube containing 20 mL hot (ca 80 °C) water. If necessary, more hot water may be used to thin milk solution. 4. Replace cartridge-syringe assembly in stopper. Pour entire 40 mL warm

	diluted milk into syringe and gently pull liquid through cartridge at flow rate ca 30 mL/min (very fast drops). (Caution: Too fast a flow will not allow sufficient time for aflatoxin to adsorb, resulting in low recoveries). 5. Add 10 mL water-acetonitrile wash solution to syringe and pull through. 6. Plug syringe barrel with stopper and pull hard vacuum on cartridge for ca 30 seconds to remove as much wash solution as possible from packing. 7. Remove cartridge and dry inside of both stems with cotton swab or tissue paper to eliminate any remaining wash solution. 8. Re-prime cartridge by adding 150 µL acetonitrile to inlet bed support disk and let solvent soak into packing for 30 seconds. Attach cartridge to dry glass or plastic 10 mL Leur tip syringe, retaining same stem as inlet. 9. Insert silica gel cleanup column into 250 mL vacuum flask fitted with one-hole rubber stopper. Wash column with five mL ether. 10. Add seven mL ether to syringe cartridge positioned above silica gel cleanup column. With plunger, slowly force through cartridge (in portions), collecting eluate in column reservoir. 11. Pull ether slowly through silica cleanup column, using vacuum to maintain flow rate ca 10 mL/min (fast drops). 12. Rinse silica column with 2 mL additional ether, continuing to use vacuum. Discard ether. 13. Remove column and stopper from flask and place 16 ×125 mm collection tube in flask to catch eluate from column. 14. Add 7 mL elution solution (Methylene chloride-alcohol) to column reservoir. Pull solvent through column with vacuum at ca 10 mL/min flow rate, collecting eluate in tube. 15. Discontinue vacuum and remove collection tube from assembly. Evaporate eluate just to dryness under nitrogen stream, using heat to keep collection tube near room temperature or under vacuum at ≤35 °C. 16. Transfer residue to one-dram vial with Methylene chloride and evaporate to dryness under nitrogen on steam bath or in heating block ≤50 °C (Do not overheat dry residue). 17. Save sample for derivative preparation. *Derivatization for LC:* 1. Prepare derivative of residue from above by adding 200 µL hexane and 200 µL trifluoroacetic acid to dry residue in vial. 2. Shake on vortex mixer ca 5-10 seconds. 3. Let mixture sit for 10 min at 40 °C, in heating block or bath; then evaporate to dryness under nitrogen on steam bath or heating block (<50 °C). 4. Add 2 mL water-acetonitrile (75 + 25) to vial to dissolve residue.

	5. Mix well using Vortex mixer for LC analysis. 6. Derivatization of standard containing M1 and M2: Add 200 µL hexane and 50 µL trifluoroacetic acid to silylated vial and mix. Add 50 µL M1-M2 working standard solution directly into hexane- trifluoroacetic acid mixture and mix using vortex mixer 5-10 seconds. Treat as described above (Steps 3-5).
Method of Analysis	*Liquid chromatography:* 1. Attach the C-18 analytical column to instrument. 2. Wash the column at flow rate of 1.0 mL/min with water-isopropanol-acetonitrile (80 + 12 + 8) for 30 min. 3. Allow the baseline to stabilize. 4. Adjust detector attenuator so that 50-100 µL injection of standard (0.625-1.25 ng M1, 0.125-0.25 ng M2) gives 50-70% full-scale recorder pen deflection for aflatoxin M1. 5. Inject LC standard 2-3 times until peak heights are constant. 6. Prepare standard curve from either peak heights or peak areas to ensure linear relationship. Inject test solutions (typically 50-100µL) with standard injections interspersed to ensure accurate quantitation. 7. Retention times M1 (as trifluoroacetic acid derivative) and M2 are ca 4-5 min and ca 7 min, respectively.
Calculation with units of expression	Calculate aflatoxin concentration using the following equation $\text{Concentration of aflatoxin M1 or M2 in µg/L (ppb)} = \frac{H \times C' \times V1' \times V}{H' \times V1 \times W}$ Where, H and H' = peak height or area of injected test solution and M1/M2 standard, respectively; C'=concentration of standard (ng/µL); V1' and V1 = volume injected of standard and test solution, respectively; V = final volume of test solution(µL); W= volume of milk represented by test solution (typically 20 mL). Separately calculate concentration for M1and M2
Reference	AOAC Official Methods of Analysis (2005), Ch.49.3.06 Method, 986.16
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Method for Determination of Aflatoxin M1 In Liquid Milk Immunoaffinity Column Chromatography followed by Liquid Chromatography		
Method No.	FSSAI 07.014:2020	**Revision No. & Date**	0.0
Scope	Applicable to determine aflatoxin M1 in raw liquid milk at >0.02 ng/mL.		
Caution	Follow all personal safety procedures while handling and disposing solution and washing glassware as described earlier. Theuse of non-acid-washed glassware (e.g., vials,tubes, flasks) for aflatoxin aqueous solutions maycause a loss of aflatoxin. Methanol and acetonitrile arehazardous and must be poured in a fume cupboard. Trifluoroacetic acid is a corrosive chemical and contact can severely irritate and burn the skin and eyes with possible eye damage. Use face shield or eye protection (safety goggles) in combination with breathing protection. Read MSDS of all chemicals.		
Principle	The test portion is extracted and cleaned up by passing through an immunoaffinity column containing M1 specific antibodies bound to a solid support. Antibodies selectively bind aflatoxin M1 (antigen) in the extract, to give an antibody-antigen complex. Other components of test sample do not bind and are washed off the column with water. AflatoxinM1 bound to the column is eluted with acetonitrile and concentrated. The amount of aflatoxin M1 is determined by LC and fluorometric detection.		
Performance Standards for Immunoaffinity Columns	The immune-affinity column shall contain antibodies against aflatoxin M1 with a capacity of binding not less than 100 ng aflatoxin M1 (which corresponds to 2 ng/mL when 50 mL test portion is applied). Recovery of not less than 80% must be obtained for aflatoxin M1 when a calibrant solution containing 4 ng toxin is applied (which corresponds to 80 ng/L for a load volume of 50 mL). Any immune-affinity column meeting the above specifications can be used. Check the performance of the column regularly, at least once for every batch of columns.		
Apparatus/Instruments	1. Disposable syringe barrels: To be used as reservoirs (10- and 50-mL capacity) 2. Vacuum system: For use with immunoassay columns 3. Centrifuge: To produce a radical acceleration of at least 2000 ×g 4. Volumetric pipets 5. Micro syringes: 100, 250, and 500 µL (Hamilton, or equivalent) 6. Glass beakers 7. Volumetric flasks: 50 mL 8. Water bath: 37±2 °C 9. Filter paper: Whatman No. 4, or equivalent 10. Conical glass tubes: 5 and 10 mL, stoppered 11. UV-Vis Spectrophotometer with quartz cells of optical length 1 cm		

	12. Liquid Chromatography System: a) With pump delivering a steady flow rate of 0.8 mL/min; loop injection system of 50-200 µL capacity; equipped with a fluorescent detector with 365 nm excitation and 435 nm emission; and recorder, integrator, or computer-based processing system. b) Reversed-phase LC analytical column: A suitable ODS (C18) column with particle size 5 µm may be used. Column dimensions can vary (mm): 100 ×2.3/4.6/5 i.d. or 125 ×4 i.d. or 200 ×2.1/3/4 i.d.; 250 ×4.6 i.d.; with orwithout guard columns. c) Mobile phases- Water –acetonitrile (75+25) or (67+33); water-acetonitrile-methanol (65+25+10);' or water-isopropanol-acetonitrile (80 + 12 + 8). Degas for 2 min before use.
Materials and Reagents	1. Chloroform-stabilized with 0.5-1.0% ethanol. 2. Nitrogen 3. Aflatoxin M1 standard solutions *a) Stock standard solution (1 µg/mL):* Suspend a lyophilized film of reference standard aflatoxin M1 in acetonitrile to obtain the required concentration. Determine the concentration of aflatoxin M1 by measuring its absorbance at the maximum (ca 365 nm) in a calibrated spectrophotometer against acetonitrile as a blank between 200-400 nm. Check purity by nothing an undistorted shape of the recorded peak. Calculate the mass concentration (C, µg/mL) from the equation:  Where, A is the measured absorbance at the maximum wavelength, M = molecular mass of aflatoxin M1 (328 g/mol), and ε is the Molar absorption coefficient of aflatoxin M1 in acetonitrile (198500/mol). Store this stock solution in a tightly stoppered amber vial below 4 °C. This is stable ca 1 year. *b) Working Standard Solution* (0.1 µg/mL): Transfer by means of a syringe 50 µL of the standard stock solution, into an amber vial and evaporate to dryness under a steady stream of Nitrogen. Dissolve the residue in 500 µL acetonitrile by vigorously vortexing in a Vortex mixer. Store this solution in a tightly stoppered amber vial below 4 °C. Solution is stable ca 1 months. *Calibrant standard solutions*: Prepare on the day of use. Bring working standard solution, to ambient temperature. Prepare a series of standard solutions in the mobile phase, of concentrations that depend upon the volume of the injection loop in order to inject, e.g. 0.05-1.0 ng aflatoxin M1.

Sample Preparation	1. Warm milk before analysis to ca 37 °C in a water bath. 2. Gently stir with magnetic stirrer to disperse the fat layer. 3. Centrifuge liquid milk at 2000 × g to separate the fat and discard thin upper fat layer. 4. Filter through one or more paper filters, collecting at least 50 mL. 5. Let immuno- affinity column reach room temperature. 6. Attach syringe barrel to the top of immuno-affinity cartridge. 7. Transfer (Vs) of prepared test portion using a volumetric flask or volumetric pipet into syringe barrel and let it pass through immuno-affinity column at slow steady flow rate of ca 2-3 mL/min. Gravity or vacuum system can be used to control flow rate. 8. Remove syringe barrel and replace with a clean one. 9. Wash column with 20 mL water at steady flow rate. 10. After washing completely, blow dry column to dryness with nitrogen steam. 11. Put another dry clean barrel on the cartridge. 12. Slowly elute aflatoxin M1 from column with 4 mL pure acetonitrile. 13. Allow acetonitrile to be in contact with column at least 60 seconds. 14. Keep a steady slow flow rate. 15. Collect eluate in conical tube. 16. Evaporate eluate to dryness using gentle stream of nitrogen. 17. Dilute to volume V_f with mobile phase, i.e., 200 µL (for 50µL injections) or 1000 µL (for 250 µL injections).
Method of Analysis	*Liquid Chromatography using a fluorescent detector:* 1. Connect the C-18 LC column to the LC system. 2. Equilibrate the LC column with the mobile phase at a constant flow rate for at least 30 min. 3. Set the fluorescent detector at 365 nm excitation and 435 nm emission. 4. Depending on the kind of column, the acetonitrile-water ratio and flow rate of the mobile phase may be adjusted to ensure optimal separation of aflatoxin M1 from other extract components. As a guideline for conventional C-18 column (with a length of 250 ×4.6 i. d. mm), a flow rate of ca 0.8 mL/min gives optimal results. 5. Equilibrate column to obtain a stable baseline. 6. Check optimal conditions with aflatoxin M1 calibrant solution and spiked milk extract before analyzing test materials. 7. Check linearity of injection calibrant solutions and stability of chromatographic system. 8. Repeatedly inject a fixed amount of aflatoxin M1 calibrant solution until stable peak areas or heights are obtained. Peak areas or heights corresponding to consecutive injections must be within ±5%. 9. Retention times of aflatoxin M1 can vary as a function of temperature and must be monitored by injecting a fixed amount of aflatoxin M1 calibrant solution at regular intervals. 10. *Calibration curve of aflatoxin M1*: Inject in sequence suitable volumes (Vi)

	depending on the injection loop, aflatoxin M1 standard solutions containing from 0.05 to1 ng. Prepare a calibration graph by plotting the peak area or peak height versus the mass of injected aflatoxin M1. 11. *Analysis of purified extracts and injections scheme*: Inject suitable volume Vi (equivalent to at least 12.5 mL milk) of eluate into LC apparatus through injection loop. Using the same conditions as for calibrant solutions, inject calibrants and test extracts according to stipulated injection scheme. 12. Inject an aflatoxin M1 calibrant with every 10 injections. 13. Determine aflatoxin M1 peak area or height corresponding to the analyte, and calculate aflatoxin M1 amount $W_a$in test material from the calibration graph, in ng. 14. If aflatoxin M1 peak area or height corresponding to test material is greater than the highest calibrant solution, dilute the eluate quantitatively with mobile phase and re-inject the diluted extract. For best results this area must fall in the middle of the calibration curve.
Calculation with units of expression	Calculate aflatoxin M1 mass concentration of the test sample, using the following equation $W_m\ (\mu g/L \text{ or ppb}) = W_a \times (V_f/V_i) \times (1/V_s)$ Where W_m =the numerical value of aflatoxin M1 in the test sample in ng/mL (ppb or µg/L); W_a = the numerical value of the amount of aflatoxin M1 corresponding to the area or height of the aflatoxin M1 peak of the test extract (ng); V_f = the numerical value of the final volume of re-dissolved eluate (µL); V_i = the numerical value of the volume of injected eluate (µL) V_s = the numerical value of volume of prepared test portion passing through the column (mL). Express the results to 3 significant figures.
Reference	AOAC Official Methods of Analysis (2005), Ch.49.3.07 Method, 2000.08
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Aflatoxins B1, B2, G1, And G2 in Foodstuffs other than described above		
Method No.	FSSAI 07.015:2020	**Revision No. & Date**	0.0
Caution	Follow all personal safety procedures while handling and disposing solution and washing glassware as described earlier. Theuse of non-acid-washed glassware (e.g., vials, tubes, flasks) for aflatoxin aqueous solutions maycause a loss of aflatoxin. Methanol and acetonitrile arehazardous and must be poured in a fume cupboard. Trifluoroacetic acid is a corrosive chemical and contact can severely irritate and burn the skin and eyes with possible eye damage. Use face shield or eye protection (safety goggles) in combination with breathing protection. Read MSDS of all chemicals.		
Principle	Aflatoxins are extracted from samples with methanol-water. Filtrate is diluted with Sodium chloride solution. Aflatoxins are dissolved in dichloromethane. Aflatoxins are purified by chromatography on 0.5 g silica gel column followed by RP-column, and quantitated by TLC/HPTLC on Silica gel 60 plate with densitometry.		
Apparatus/Instruments	1. RP-18 cartridge (6 mL/1g sorbent), 2. Silica gel cartridge (3 mL/0.5g sorbent) 3. HPTLC plates or sheets silica gel, 20 × 10 cm or 20 × 20 cm. 4. Twin-trough TLC chambers 5. TLC/HPTLC scanner with CATS software		
Materials and Reagents	*Chemicals:* 1. Hexane 2. Diethylether (peroxide-free) 3. Dried petroleum ether 4. Toluene 5. Dichloromethane 6. Chloroform 7. Acetone, 8. Acetonitrile, 9. Methanol, 10. Water (HPLC grade) 11. Acetic acid 12. Trifluoroacetic acid 13. Sodium chloride 14. Sodium sulfate 15. Paraffin oil 16. Standard: aflatoxin B1, B2, G1, and G2, (Aflatoxin 17. standard Kit, 1 mg each).		

	Reagents: Sodium chloride solution (10%) Toluene–acetonitrile (98:2)
Sample Preparation	*Sample preparation for Spices* 1. Grind or homogenize sample and mix 5.6 g with 100 mL methanol for 3 min. 2. Add 40 mL water, mix for 4 min, leave to stand for 10 min and then filter. 3. Shake 20 mL of filtrate with 20 mL Sodium chloride solution (10%) and 20 mL petroleum ether for 2 min. 4. Leave to separate for 10 min (extraction of matrix in petroleum ether). 5. Shake aqueous phase with 50 mL dichloromethane for 1 min and leave to separate (extraction of aflatoxins in dichloromethane). 6. Dry, dichloromethane phase with 5 g sodium sulfate, filter and evaporate to dryness. 7. Dissolve residue in 0.5 mL toluene–acetonitrile (98:2). 8. Use extract (= 0.8 g sample) for application to the HPTLC layer. *Purification for critical matrices:* For some critical matrices such as paprika, it is advisable to dissolve the residue in 2 mL toluene–acetonitrile (98:2) and perform further purification as below 1. Purification of the extract on a silica gel cartridge a) Rinse the sorbent with 6 mL toluene–acetonitrile (98:2) (Do not let the sorbent run dry). b) Elute extract and rinse remaining matrix with 20 mL toluene-acetic acid (9:1) and 20 mL hexane–diethylether–acetonitrile (6:3:1) (dry the sorbent between and in the end). c) Elute the aflatoxins fraction with 7and 4mL dichloromethane–acetone (3:1) directly into a pear-shaped flask (dry sorbent between and in the end). 2. Evaporate eluate to dryness and take up the residue in 0.5 mL methanol. 3. Purification of the extract on an RP-18 cartridge Rinse cartridge with 2 mL methanol, dry and condition with 4 mL methanol–water (2:8) and 2mL water (Do not let the cartridge run dry). Load the extract and rinse remaining matrix with 5 mL methanol–water (2:8), dry for 1 min. Elute the aflatoxins with 4 × 2.5 mL methanol –water (5:5) direct in a pear shape flask (dry sorbent between and in the end). 4. Shake aqueous phase for 1 min with 20 mL Sodium chloride solution (10%) and 18 mL dichloromethane and leave to separate for 5 min (extraction of aflatoxins in dichloromethane). Separate dichloromethane phase. Repeat extraction of the aqueous phase with 2 mL dichloromethane. 5. Evaporate eluate to dryness and take up the residue in 0.5 mL toluene–

	acetonitrile (98:2). 6. Use extract (= 0.8 g sample) for application to the HPTLC layer. *Sample Preparation for Other Commodities:* Use a higher weighted amount (e.g. 80 g for nuts) if necessary and adjust the amounts of solvent, etc. accordingly
Preparation of standard solution	Make up a standard mixture of aflatoxins B1, B2, G1, and G2 in toluene–acetonitrile (98:2) containing 200 pg/L each of aflatoxins B1 and G1 and 100 pg/L each of G2 and B2
Sample Application	Apply band-wise, distance from lower edge of sheet 10 cm (for plates 6 cm), band length 8 mm, distance between tracks 4 mm, distance from left edge 15 mm. **Application Pattern** S1 U UUU S1 U UU S1 (S1 = standard mixture 5 µL each, U = sample of 100 µL each)
Method of Analysis	*Development of HPTLC plate* *2-Dimensional development (in opposing direction) in twin-trough chambers* *First dimension to removes the matrix from the start zone* 1. Fill the first chamber to a depth of 5 cm with peroxide-free, dried diethyl ether. 2. Place the sheet or plate (6 cm free side downwards) in the chamber: migration distance 50 mm (sheet) and 40 mm (plate), respectively. 3. View sheet or plate under UV 366 nm. 4. The fluorescent aflatoxins should have migrated little or not at all from the start zone. 5. Cut off the top 85-90 mm (sheet) and 25-30 mm (plate), respectively and turn the plate or sheet through 180°. *Second dimension, to separates the aflatoxins* Charge the second chamber normally (to a depth of about 8 mm) with chloroform: acetone: water (140:20:0.3) Insert plate or sheet; migration distance 80 mm (sheet) and 60 mm (plate), respectively. *Densitometric Evaluation:* TLC/HPTLC scanner with CATS software, fluorescence measurement at 366/>400 nm, single level calibration via peak height confirmed by a multilevel calibration. Aflatoxins are sensitive to light and oxidation. Store chromatographed HPTLC plates or sheets, standards, extracts, etc. in the dark at about 5 °C.
Reference	SOP A9024.01B, KantonalesLaboratorium Aargau, 13.03.1997
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Deoxynivalenol (DON) in Wheat (Thin Layer Chromatographic Method)		
Method No.	FSSAI 07.016:2020	**Revision No. & Date**	0.0
Scope	The main commodities affected are cereals such as wheat, rice, barley, oats and maize etc. Applicable at levels ≥300 ng /g of wheat.		
Caution	Methanol and acetonitrile arehazardous and must be poured in a fume cupboard. Inhalation of Chloroform vapors can cause headaches, drowsiness, dizziness, and nausea. Disorientation, anesthetic effects, and loss of consciousness can occur at high concentrations. Wear laboratory safety goggles and mask. Perform work in a fume hood when using solvents. Protect eyes with UV-absorbing filter when using UV-viewing chamber. Read MSDS of all chemicals.		
Principle	DON is extracted from the grain with acetonitrile-water and filtered through a column of mixed alumina-charcoal-Celite. The solvent is evaporated on a steam bath. Ethyl acetate is added to the residue and heated to dissolve DON. After cooling, the residue is transferred to a vial with additional ethyl acetate and is dissolved in $CHCl_3$-acetonitrile (4 + 1) for TLC on an $AlCl_3$-impregnated silica gel plate with $CHCl_3$-acetone-isopropanol (8 + 1 + 1). On heating the plate at 120 °C the presence of DON is indicated by a blue fluorescent spot under longwave ultraviolet light. DON is quantitated visually and/or by fluoro-densitometry.		
Apparatus/Instruments	1. Grinder 2. Chromatographic column – polypropylene (10 mm ×50 mm) 3. Filter flask – 125 mL fitted with a rubber stopper having a hole to hold chromatographic tube. 4. TLC/HPTLC Plates – Precoated 20 ×20 cm silica gel plates. Dip plates in 15% aluminium chloride solution prepared by dissolving 1.5 g $AlCl_3.6H_2O$ in 15 mL water and 85 mL alcohol. Let stand in vertical position for 5 min to drain. Remove residual aluminium chloride from back of plate with wet paper. Air dry for 2 h and activate for 1 h at 105 °C. Store in dust tight cabinet. 5. Viewing cabinet fitted with long wavelength UV lamp or Densitometer.		
Materials and Reagents	1. Activated Charcoal 2. Alumina, neutral- 80 – 200 mesh 3. Diatomaceous Earth – acid washed Celite 545 4. Aluminium chloride solution – spray reagent – 20 g $AlCl_3.6H_2O$ in 100 mL alcohol 5. Acetonitrile: Water (84 + 16) 6. DON Standard solution 7. Stock Solution – 0.5 mg/mL. Weigh 5.0 mg DON into a 10 mL glass		

	stoppered volumetric flask, dilute to volume with ethyl acetate – methanol (19 + 1) and shake to dissolve. 8. Working standard – 20 µg/mL – Pipette 1 mL of DON stock solution into a 25 mL volumetric flask and dilute to volume with ethyl acetate– methanol (19 +1) and shake to dissolve.
Sample Preparation	Grind large sample (2 – 4 Kg) to pass through a 20-mesh sieve. *Extraction*: 1. Weigh 50 g of ground sample into a 500 mL glass stoppered conical flask. 2. Add 200 mL acetonitrile- water (84 + 16) mixture, secure stopper with tape and shake vigorously for 30 min on shaker. 3. Filter and collect 20 mL filtrate in a 250 mL graduated cylinder.
Method of Analysis	*Column chromatography:* 1. Secure a chromatographic column to a 125 mL filter flask. 2. Plug the column with glass wool 3. Add about 0.1 g Celite. 4. Weigh 0.7 g charcoal, 0.5 g alumina, and 0.3 g Celite. Add to a 50 mL beaker and mix with a spatula. 5. Add mixture to chromatographic column. Tap lightly to settle packing. 6. Apply suction and place a ball of glass wool on top. 7. Add 20 mL of the extract (filtrate) to column and apply vacuum. 8. Flow rate should be 2-3 mL/min with 20 cm Hg vacuum. 9. As solution reaches top of packed bed rinse measuring cylinder with 10 mL acetonitrile – water (84 + 16) 10. Add rinse to column and continue aspiration till flow stops. 11. Do not let column go dry between addition of extract and rinse. 12. Cover vacuum nipple with Aluminium foil and evaporate solvent slowly to dryness on steam bath. Do not contaminate sample with water from condensing steam. 13. It is essential that no water droplets remain in flask on cooling. 14. Add 3 mL of ethyl acetate to residue and heat to boiling on steam bath and gently swirl to dissolve extracted DON. 15. Transfer solution to small vial, rinse with three 1.5 mL portions of ethyl acetate. 16. Evaporate to dryness and retain dry residue for TLC/HPTLC. 17. Final extract, represents 5 g of sample. *Thin Layer Chromatography:* 1. Dissolve above residue in vial in 100 µL chloroform – acetonitrile (4 + 1). 2. Apply 5 and 10 µL aliquots side by side 1, 2, 5. 10 and 20 µL working standard solution (20 µg DON/mL) on TLC plate. 3. Develop plate with chloroform – acetone – Isopropanol (8 + 1 + 1) in an unequilibrated tank (development time is about 1 h). 4. Remove plate, let solvent evaporate completely at room temperature.

	5. Residual solvent can result in fading of DON spots. 6. Heat plate for 7 min in upright position at 120 °C. 7. Place plate on cool surface in dark for 1 min. 8. DON appears as a blue florescent spot under long wave UV light at R_f about 0.6. 9. Quantify DON by comparing fluorescence intensity of test spots with those of standard DON using a densitometer
Calculation with units of expression	$$DON\ (ng/g) = S \times (C / X) \times (V / W)$$ Where, S = µL working standard equal to test spot C = concentration of standard solution (20 µg/mL) X = µL test solution that has florescence intensity equal to standard spot V = Final volume of test solution (µL) W = amount of test portion represented by final test solution
Reference	AOAC 17th edn, 2000 Official Method 986,17 Deoxynivalenol in Wheat–Thin Layer Chromatographic Method
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Ochratoxin (OTA) in Barley Thin Layer Chromatographic Method		
Method No.	FSSAI 07.017:2020	**Revision No. & Date**	0.0
Caution	Ochratoxin A (OTA) causes kidney and liver damage and is carcinogenic in some animals. Observe precautions while handling standards and material. Prepare BF_3 reagent in hood. Avoid contact with skin, eyes, and respiratory tract.		
Principle	OTA acids and its esters are extracted from barley by using chloroform and aqueous Phosphoric Acid. The acids are entrapped onan aqueous diatomaceous earth column. Esters and fat are removed with hexane and chloroform, and acids are eluted with formic acid–chloroform. Esters are isolated by entrapment on methanol–aqueous sodium bicarbonate-diatomaceous earth column, fats are removed with hexane-benzene, and esters are eluted with formic acid-hexane-benzene. Compounds are determined from fluorescence after TLC. All glassware must be free of alkaline soap or detergent residues to avoid loss of toxins.		
Apparatus/Instruments	1. Chromatographic columns with stop cocks: 700 × 17 mm and 350 × 25 mm 2. Wrist-action shaker 3. Büchner funnels: a) Glass, 9 cm diameter fitted with Whatman GF/B glass fiber paper, or equivalent and b) 24 cm diameter fitted with Whatman No. I paper, or equivalent. 4. Thin-layer chromatographic chamber 5. Densitometer		
Materials and Reagents	*Chemicals:* 1. Diatomaceous earth 2. Silica gel 3. Cotton 4. Chloroform 5. Hexane, 6. Acetic acid, 7. Methanol, 8. Formic acid (90%) 9. Phosphoric acid (85%). 10. Sodium bicarbonate 11. Boron trifluoride 12. Benzene *Reagents:* 1. Diatomaceous earth: Soak ~ 900 g acid-washed Celite 545 overnight in methanol. Filter through double thickness Whatman No. I paper or equivalent over Buchner and wash with 8 L water, and dry at 150 °C for 12 h.		

<table>
<tr><td></td><td>
2. Silica gel for thin-layer chromatography: Test adsorbent for resolution and fading of ochratoxins. Ochratoxins on occasion fade rapidly on some silica gel plates, especially when exposed to ≥60% humidity. Protect plate from humidity during spotting by placing in chamber under nitrogen or under stream of warm air from hair dryer, or by covering with clean glass plate. After development, dry plate at 50 °C for 15 min and immediately cover with clean glass plate, using tape on sides as spacers, for protection during scanning densitometry.
3. Methanolic sodium bicarbonate solution: Dissolve 0.3 g sodium bicarbonate in 30 mL water and add 70 mL methanol.
4. Alcoholic sodium bicarbonate solution: Dissolve 6.0 g sodium bicarbonate solution in 100 mL water and add 20mL alcohol.
5. Formic acid–benzene–hexane solution: Shake 100 mL benzene–hexane (20:80) with 10 mL of water–methanol (30 + 70), let layers separate. Discard lower layer. Shake upper layer with 5 mL formic acid, let separate, and discard lower layer.
6. Boron trifluoride 14% (w/v): Bubble gaseous BF_3 into chilled alcohol.
7. Developing solvents: (1) Benzene–methanol–acetic acid (18:1:1). Combine 2 volumes methanol–CH_3COOH (1 + I) with 18 volumes benzene. Adjust benzene (methanol–CH_3COOH) ratio, if necessary, to produce required resolution. Decrease benzene to increase Rf.
8. Hexane–acetone–acetic acid (18: 2: 1): Combine 3 volumes acetone–CH_3COOH (2 + 1) with 18 volumes hexane. Adjust hexane: (acetone–CH_3COOH) ratio, if necessary, to produce required resolution Decrease hexane to increase Rf.
Purified cotton: Wash 50 g absorbent cotton in beaker with 1 L of chloroform. Decant solution, evaporate residual solvent, and store cotton in closed container.
</td></tr>
<tr><td>Preparation of OTA standard</td><td>
Ochratoxin standard solutions
Prepare original solutions, each ca 40 µg/mL, in acetic acid-benzene (1:99) Determine concentration using the table of Molecular weights and molar absorptivity of ochratoxins given below. Dilute to required concentration (1-5 µg/mL) using benzene.
<table>
<tr><td>Ochratoxin</td><td>λ max (nm)</td><td>Molecular Weight</td><td>Molar absorption coefficient</td></tr>
<tr><td>A</td><td>333</td><td>403</td><td>5550</td></tr>
<tr><td>B</td><td>320</td><td>369</td><td>6000</td></tr>
<tr><td>A Ethyl ester</td><td>333</td><td>431</td><td>6200</td></tr>
<tr><td>B ethyl ester</td><td>320</td><td>397</td><td>6500</td></tr>
</table>
</td></tr>
<tr><td>Sample Preparation</td><td>
Extraction:
1. Weigh 50 g of sample into a 500 mL glass-stoppered Erlenmeyer flask
2. Add 25 mL 0.1M Phosphoric acid and 250 mL Chloroform, and secure stopper with masking tape.
3. Shake for 30 min using a wrist-action shaker
</td></tr>
</table>

	4. Filter through glass fiber paper, covered with ca 10 g diatomaceous earth, using a 9 cm Büchner funnel. *Separation of Ochratoxins* *Ia Removal of esters*: 1. Plug a 700 x 17 mm chromatographic tube with the purified cotton. 2. Mix 2.0 g diatomaceous earth with 1mL 1.25% sodium bicarbonate solution in 50 mL beaker. 3. Add to chromatographic tube and tap firmly. 4. Mix 50 mL filtrate with 40 mL hexane, and add to column. Reserve remainder of filtrate for confirmation of identity. 5. Elute at maximum flow rate; then elute with 75 mL chloroform. 6. Combine eluates, evaporate to dryness on steam bath, and reserve for ochratoxin ester separation. *Ib Removal of acids* 1. Elute Ochratoxins A and B with 75 mL freshly prepared formic acid-chloroform (1 + 99), and collect in 250 mL Erlenmeyer. 2. Immediately add 2 boiling chips and evaporate nearly to dryness on steam bath 3. Quantitatively transfer residue to 15 mL conical centrifuge tube with chloroform. 4. Evaporate to dryness under gentle stream of nitrogen on steam bath. (*Note*: Delay in evaporation of solvent may result in loss of ochratoxins.) Reserve residue for TLC. *Separation of Ochratoxin Esters:* 1. Prepare column as described above using 350 x 25 mm chromatographic tube with 2.5 mL methanolic sodium bicarbonate solution, and 4 g diatomaceous earth. 2. Dissolve residue of Ia (after removal of esters) in 50 mL hexane. Add to column. 3. Rinse extraction vessel with each subsequent eluting solvent in turn and add rinses to column. 4. Force eluting solvents through column at convenient rate with compressed gas at 1-2 psi (6.9-13.8 kPa). 5. Do not let liquid fall below top of column. 6. Eluting solvents a) Elute with 50 mL benzene-hexane (1 + 9) previously equilibrated with 2.5 mL methanolic sodium bi carbonate solution (discard). b) Then elute with 100 mL formic acid-benzene-hexane mixture. 7. Immediately evaporate eluate to dryness, quantitatively transfer to conical centrifuge tube with chloroform, evaporate to dryness under gentle stream of nitrogen on steam bath, and reserve for TLC
Method of Analysis	*Thin Layer Chromatography:* 1. Use appropriate silica gel and dissolve residue of Ib (from removal of acids) in 750 µL acetic acid-benzene (1 + 99).

	2. Spot 3, 5, 7.5, and 10 µL on same plate. 3. Spot 10 µLextract superimposed with 10 ng each ochratoxin A and B standard solutions as internal standard. 4. Also spot 5, 7.5, and 10 µL, ochratoxin A and B standard solutions. 5. Develop plate to solvent stop line, but <90 min, with benzene-methanol-acetic acid (18 + 1 + 1) in an unlined, unequilibrated tank. 6. Remove plate, let solvent evaporate at room temperature, and view in dark under long- and shortwave UV lamp. 7. The Rf of Ochratoxins A and B should be in the range of 0.4-0.8. Ochratoxin A is above B, typically at 0.65 and 0.5, respectively. 8. Ochratoxin A fluoresces most intensely under long wave UV, while ochratoxin B is brightest under shortwave light. 9. Examine the pattern of test solution for fluorescent spots for spots with Rf close to those of standards and with similar appearance. 10. Compare fluorescence intensities of test solution spots with those of standard spots, and determine standard and test spot that match most closely, interpolating, if necessary. 11. If, concentration of test spots is outside range of standards, concentrate or dilute, solution and re-chromatograph. 12. Calculate concentration ochratoxin A in µg/kg. 13. Spray plate with alcoholic sodium bicarbonate and dry at room temperature. View spots in dark under long wavelength light. Fluorescence should have changed from greenish blue to blue and increased in intensity. 14. In case of disagreement, use estimate obtained before spraying. *Thin Layer Chromatography of OTA esters:* Perform TLC of ochratoxin A and B esters on TLC plate in same manner as above for acids, Develop plate in hexane-acetone-acetic acid (18 + 2 + 1). Rf value of Ochratoxin A ester is ca 0.5, above ochratoxin B ester *Densitometry:* Prepare and develop as described for visual analysis. In separate channels spot about 4-5 spots with increasing amounts standard ochratoxin A in range 3-10 ng/spot. Scan the plate in a densitometer or scanner following the manufacturer's instructions. Optimum spectral settings for ochratoxin A are excitation at 310-340 nm and emission, 440-475 nm. Plot standard curve from instrument response for linearity and system performance. Dissolve residue from I(b) in 0.5 mL acetic acid-benzene and spot replicates of at least two test extracts and standard of ≥ 3µL each. The test

	extract must have ochratoxin within the standard concentration range. *Confirmation of Identity of Ochratoxins A and B by formation of Ethyl Esters:* 1. Dissolve residue from I (b), containing equivalent of ≥ 10 g test sample, in 5 mL chloroform in a 25 mL Erlenmeyer. 2. Into separate 25 mL Erlenmeyer add 10 ng of ochratoxin A and B standard solution. (This step may be omitted when Ochratoxin A and B ester standards are available.) 3. Add 10 mL of 14% boron trifluoride. 4. Heat to boiling point and hold on steam bath 5 min. Transfer to a separator funnel containing 30 mL water. 5. Extract with three 10 mL portions of chloroform. 6. Combine extracts, wash with three 10 mL portions of water and evaporate to dryness. 7. Quantitatively transfer to a centrifuge tube with chloroform, and evaporate to dryness under a gentle stream of nitrogen. Dissolve residue in 250 μL acetic acid-chloroform, (1 + 99) and do TLC as described above, with the following modifications: 8. Spot 10 μL each of 1) underivatized test solution, 2) esterified (derivatized) test solution, 3) standard Ochratoxin esters, and 4) esterified test extract plus 10 μL standard esters. 9. Develop plate with benzene-methanol. 10. Examine plate under long- and short UV. 11. Ochratoxin A ester has a greater than Rf than that of Ochratoxin A ester, typically 0.8 and 0.7, respectively. The Rf of the Ethyl esters is greater than ochratoxins A and B, but have the same fluorescence intensity. For positive confirmation, the Ochratoxin A or B spots should be absent after esterification.
Reference	AOAC 17thedition, 2000 Official Method 973. 37 Ochratoxins in Barley. JAOAC, 56, 817, 822(1973).
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Ochratoxin A in Barley **Immunoaffinity by Column HPLC and fluorescence detection**		
Method No.	FSSAI 07.018:2020	**Revision No. & Date**	0.0
Scope	Applicable to the determination of ochratoxin A in barley at >1 ng/g		
Caution	OTA is toxic as well as carcinogenic in nature, use nitrile gloves while handling these substances. Prior to sample extract disposal, the solutions must be treated with 5–6% sodium hypochlorite. Allglassware exposed to the residues of these toxins must be rinsedwith methanol and 1% sodium hypochlorite solution and then washed.		
Principle	Sample is extracted by blending with acetonitrile–water.The extract is cleaned up by passing through animmunoaffinity column. Ochratoxin A (OTA) is eluted withmethanol, further purified and identified by HPLC, and quantifiedby fluorescence.		
Apparatus/Instruments	1. Silanized glass vials: Needed to ensure stability of OTA in aqueous solvents. Prepare vials by filling them with silanizing reagent and leaving this reagent in the vial for 1 min. Next, rinse vial with solvent of low polarity (e.g., toluene), and then with methanol. Finally, wash vials twice with distilled water, and dry before use. 2. Analytical balance: Accurate to 0.0001 g. 3. Blender: 1 L jar and cover, explosion-proof. 4. Displacement pipets: 5 mL, 1 mL, 200 µL with appropriatepipet tips. 5. Vacuum manifold: To accommodate immunoaffinitycolumns. 6. Reservoirs and attachments: To fit to immunoaffinitycolumns. 7. Vacuum pump: Producing a vacuum of 1.0 mPa andpumping 18 L/min. 8. Filter papers: Whatman No. 4 or equivalent. 9. Disposable syringe filters: 0.2 µm pore size, 25 mmdiameter polysulfone membrane. 10. HPLC /UPLC system equipped with 11. Pump: Mobile phase pump (isocratic) pumping 1 mL/min pulse-free. 12. Injection system: Valve injection system with 100 mL injection loop. 13. Column: C18 reversed-phase, 5 mm ODS (equivalent to ODS 1 or 2) with 11% carbon loading, fullyend-capped (pore size 10 nm), withcorresponding reversedreversed-phase guard column. 14. Detector: Fluorescencedetector with flow cell with emission wavelength 460 nm, excitationwavelength 333 nm, and data collection system. 15. Column oven: Controlling column at 45 ± 0.5°C. Maintainat a constant temperature, although the specified temperatureis not critical. 16. UV spectrophotometer: For checking concentrationof standard. 17. Immunoaffinity columns specific for OTA clean-up: The immunoaffinity column should contain antibodies raisedagainst OTA. The column should have maximum capacity of100 ng OTA and recover 85% OTA when applied as a standard solution in CH_3OH–PBS (phosphate buffered saline solution, (Columnsfrom Vicam LP, Rhône Diagnostics meet criteria criteria.)		

Materials and Reagents	1. Acetonitrile (CH_3CN): 99.9%, LC grade 2. Methanol 99.9%, LC grade, 3. Glacial Acetic acid, 4. HPLC grade water (18.2 MΩ cm) 5. Sodium chloride. 6. Disodium hydrogen orthophosphate (Na_2HPO_4,). 7. Potassium dihydrogen phosphate (KH_2PO_4) 8. Potassium chloride. 9. Sodium hydroxide. 10. Toluene. ***Reference standards*:** OTA with purity of >98%.
Preparation of Reagents	1. Extraction solvent (v/v): Mix 6 parts acetonitrile with 4 parts water 2. Injection solvent (v/v): Mix 30 parts methanol with 70 parts water and 1-part glacial acetic acid 3. Sodium hydroxide, 0.2M; Dissolve 8 g NaOH in 1 L water. 4. Phosphate buffered saline (PBS): Dissolve 8 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4 and 0.2 g KCl (j) in1 L water. Adjust pH to 7.4 with 0.2M NaOH. 5. HPLC mobile phase (Acetonitrile containing 1% acetic acid): Mix (v/v) 102 parts water with 96 parts CH_3CN and 2 parts glacial CH_3COOH; filter through0.22 µm filter Band degas. 6. Toluene–glacial acetic acid mixture (v/v): Mix9 parts toluene with 1-part CH_3COOH. 7. Silanizing reagent (v/v): Surface siliconizing fluid, 5% (v + v) solution (such as SurfaSilÔfrom Pierce ChemicalCo., PO Box 117, Rockford, IL 61105, USA, No. 42800). Mix1partSurfaSil with 19 parts toluene.

<table>
<tr>
<td>Preparation of standards</td>
<td>
Stock standard solution (10 µg/mL): Prepare OTA standard in tolueneacetic acid, (99 + 1,v/v).
Determine concentration of stock as follows:
Record UV spectrumof ochratoxin A solution against Toluene -acetic acid solution inreference cell.
Determine concentration of ochratoxin A solutionby measuring A at wavelength of maximum absorptionclose to 333 nm and using following equation:
$$\text{Ochratoxin (µg/mL)} = \frac{A \times MW \times 1000}{\varepsilon}$$

where A = absorbance,
MW = molecular weight ofochratoxin A (403),
ε = molar absorptivity (5440 in toluene–acetic acid, 99 + 1,v/v).
Preparation of working standards
Pipet 200 µL 10 µg/mLOTA stock standard into glass vial and dilute to 1 mLwith 800 µL toluene–acetic acid to give 2 µg/mL OTAsolution. Pipet 100 µL of 2 µg/mL OTA solution into silanizedglass vial Evaporate solvent under stream of nitrogen.

Re-dissolve in 10 mL injection solvent that has been filteredthrough 0.22 µm filter. This gives 20 ng/mL OTA solution.
HPLC calibrants
<table>
<tr><th>Calibrant No</th><th>Aliquot taken from working standard (20 ng/mL OTA) (µL)</th><th>Volume of Injection solvent to be added</th><th>Final concentration of OTA (ng/mL)</th></tr>
<tr><td>1</td><td>125</td><td>4875</td><td>0.5</td></tr>
<tr><td>2</td><td>250</td><td>4750</td><td>1.0</td></tr>
<tr><td>3</td><td>500</td><td>4500</td><td>2.0</td></tr>
<tr><td>4</td><td>1250</td><td>3750</td><td>5.0</td></tr>
<tr><td>5</td><td>2500</td><td>2500</td><td>10.0</td></tr>
</table>
From this solution, prepare 5 calibrants in separate5 mL volumetric flasks according to Table above. Diluteeach calibrant to volume (5 mL) with filtered injectionsolvent.
</td>
</tr>
<tr>
<td>Sample Preparation</td>
<td>
Extraction:
Weigh, to nearest 0.1 g, ca 25 g test portion of barley intoblender jar. Add 100 mL extraction solvent. Coverand seal blender; blend for 3 min. Filter extract through filterpaper.
</td>
</tr>
</table>

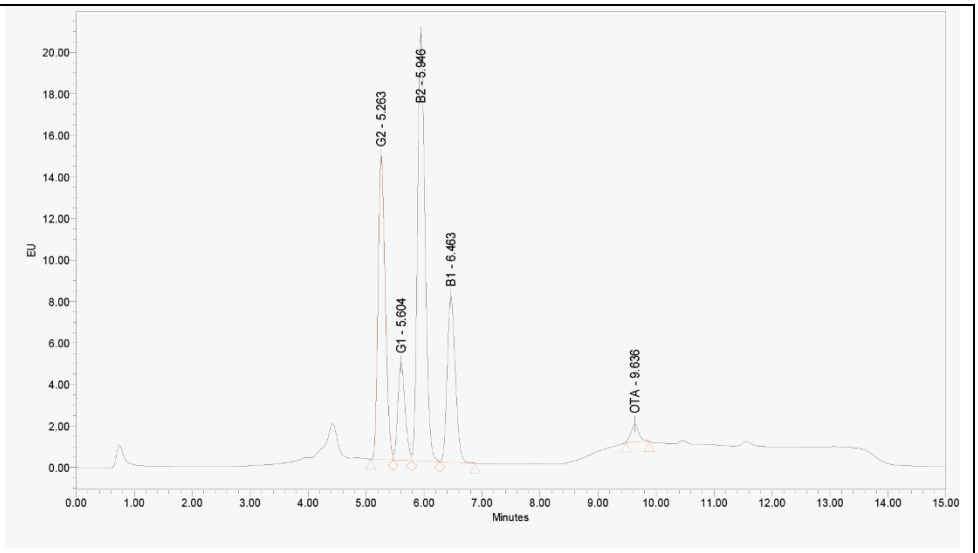
	Immuno-affinity cleanup: 1. Pipet 4 mL filtrate into 100 mL glass beaker (or similar) and dilute with 44 mL PBS. 2. Connect immunoaffinity column) to vacuum manifold and attach reservoir to immunoaffinity column. 3. Add diluted extract to reservoir and pass through immunoaffinity column at 0.5 mL/min flow rate. 4. The immunoaffinity column must not be allowed to run dry. 5. Wash beaker and column with 10 mL water, 6. Remove from vacuum manifold, and place over silanized vial. 7. Elute OTA into silanized vial with four 1 mL portions methanol. 8. Evaporate eluate to dryness over steam bath, under N. 9. Re-dissolve in 1 mL injection solvent which has been filtered through 0.2 mm filter. 10. Transfer to LC vial.
Method of Analysis	*Chromatography conditions:* 1. C18 reversed-phase, 5 mm ODS(equivalent to ODS 1 or 2) 2. Column temperature: 45 ± 1 °C, 3. Isocratic elution, Flow rate:1 mL/min 4. Injection volume: 100 µL. 5. Mobile phases: Acetonitrile containing 1% acetic acid 6. Detector: A. Excitation wavelength 333 nm. B. Emission wavelength: 460 nm Connect the HPLC column, set column temperature and detector wavelength Wash column thoroughly with mobile phase at a flow rate of 1 mL /min. Wash for 30 mins. Inject 100 µL of each calibrant solution to construct a calibration curve. Inject 100 µL sample in triplicate.
Calculation with units of expression	Determine, from calibration graph, masses in ng of OTA in aliquot of test solution injected ontothe LC column. The regression should be > 0.998 From the equations determine the concentration of the unknown (M_A). Calculate mass fraction,W_{OTA}, of OTA in mg/kg using theequation: $$\text{Ochratoxin A (mg/Kg)} = \frac{M_A \times V_1 \times V_3}{V_2 \times V_4 \times M_S}$$ Where M_A = mass of OTA in test solution extract, ng; V_1 = extractionsolvent, mL (100 mL); V_2 = acetonitrile–water filtratepassed through immunoaffinity column, mL(4 mL)

	V_3 = testsolution (1 mL); V_4 = test solution injected, mL M_S = Mass of test portion
LOD and LOQ	The LODs are 0.02 ng/g (S/N > 3) and 0.1 ng/g (S/N > 10) for AFs and OTA, respectively
Reference	J. AOAC Int. **83**, 1379–1383 (2000) AOAC Official Method 2000.03Ochratoxin A in BarleyImmunoaffinity by Column HPLCFirst Action 2000
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Direct analysis of Aflatoxins (AF) and Ochratoxin A (OTA) in cereals and their processed products by Ultra-High-Performance Liquid Chromatography with fluorescence detection**		
Method No.	FSSAI 07.019:2020	**Revision No. & Date**	0.0
Caution	As AFs and OTA are toxic as well as carcinogenic in nature, use nitrile gloves while handling these substances. Prior to sample extract disposal, the solutions must be treated with 5–6% sodium hypochlorite. Allglassware exposed to the residues of these toxins must be rinsedwith methanol and 1% sodium hypochlorite solution and then washed.		
Principle	A reverse phase based HPLC separation of the AFs and OTA and their detection by fluorescence. The AFs and OTA are extracted with methanol-water. The extract is cleaned by using immunoaffinity columns.		
Apparatus/Instruments	1. Ultra-High-Performance Liquid Chromatography equipped with a fluorescence detector[FLD with large volume (13 µL) flow cell] and column oven set at 40 °C and C18 column (2.1 × 50 mm, 1.7 µm). 2. Heavy-duty mixer/grinder 3. High-speed homogenizer 4. Centrifuge 5. Vacuum manifold		
Materials and Reagents	*Chemicals:* 1. Methanol (HPLC gradient grade), 2. Glacial Acetic acid, 3. Sodium chloride 4. Sodium hydroxide) 5. HPLC grade water (18.2 MΩ cm) 6. Monoclonal antibody-based immune-affinity columns (AFLAOCHRA PREP IAC (3 mL; R-Biopharm AG, Darmstadt, Germany) 7. Phosphate-buffered saline (PBS) 8. ***Reference standards*:** Individual AF standards (B1, B2, G1, and G2) with >95% purity OTA with purity of 98%. *Reagents:* 1. Sodium hydroxide, 0.2M; Dissolve 8 g NaOH in 1 L water. 2. Phosphate buffered saline (PBS): Dissolve 8 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4 and 0.2 g KCl (j) in 1 L water. Adjust pH to 7.4 with 0.2M NaOH. 3. HPLC mobile phase A (v/v): Mix 1 parts glacial acetic acid with 99 parts water and 2 parts CH_3COOH; filter through 0.22 µm filter B and degas. 4. HPLC mobile phase B(v/v): Mix 1 parts glacial acetic acid with 99 parts methanol; filter through 0.22 µm filter B and degas.		
Preparation of standards	*Stock standard*: Dissolve 5 mg each standard in 10 mL methanol in an amber-colored vial. The stock solutions containing 500 µg/mL of each AF and OTA is stored at –20 °C. Intermediate standard: Dilute the stock solutions in methanol.		

<table>
<tr><td></td><td>Calibration standards: Make serial dilutions of the intermediate solutions to obtain 0.02–10 ng/mL for each AF and 0.1–10 ng/mL for OTA in 1:1 ratio of methanol: water (plus 0.2% acetic acid, v/v).</td></tr>
<tr><td>Sample Preparation</td><td>Grinding:
Cereal grains and processed products (are thoroughly milled and allowed to pass through a No. 20 sieve.
Extraction:
Add 12.5 g of finely ground dry matrix to 12.5 g distilled water to make a slurry. Mix the slurry with 100 mL of extraction solvent (methanol–water,8+ 2, v/v) and NaCl (5 g). Shake for 30 min,200 rpm), and then centrifuge (5000 rpm, 5 min). Take an aliquot (3 mL) and dilute with 15 mL PBS and add 50 µL NaOH (2 M) solution.
IAC cleanup:
Load the diluted sample onto IAC connected to a vacuum manifold and allow to pass without any vacuum. Wash with 10 mL PBS. Elute with methanol (2 × 0.5 mL). Slowly evaporate the final extract (1 mL) to dryness. Reconstituted in 0.5 mL methanol–water (acidified with 0.2% acetic acid, 1:1), and finally inject 10 µL into the UHPLC-FLD instrument.</td></tr>
<tr><td>Method of Analysis</td><td>Chromatography conditions:
1. Column: C18 column (2.1 × 50 mm, 1.7 µm).
2. Column temperature: 40 °C,
3. Flow rate: 0.2 mL/min
4. Injection volume: 10 µL.
5. The mobile phases: (A) 1% acetic acid in water and (B) 1% acetic acid in methanol.
6. Detector:
7. Excitation wavelength 365 nm up to 8 min and subsequently switched to 333 nm and continued up to 15 min.
8. Emission wavelength: 456 nm t
9. Linear Gradient program

<table>
<tr><td>Time (mins)</td><td>% A</td><td>% B</td></tr>
<tr><td>Initial</td><td>90</td><td>10</td></tr>
<tr><td>0.25</td><td>90</td><td>10</td></tr>
<tr><td>2.50</td><td>58</td><td>42</td></tr>
<tr><td>6.00</td><td>58</td><td>42</td></tr>
<tr><td>7.00</td><td>20</td><td>80</td></tr>
<tr><td>11.50</td><td>20</td><td>80</td></tr>
<tr><td>12.00</td><td>90</td><td>10</td></tr>
<tr><td>15.00</td><td>90</td><td>10</td></tr>
</table>
</td></tr>
</table>

Results	 A representative chromatogram showing the elution profile of AFs and OTA
Calculation with units of expression	Prepare a calibration curve for 0.02–10 ng/g for each AF and 0.1–10 ng/g for OTA using the working standard. From the equations determine the concentration of the unknown.
LOD and LOQ	The LODs are 0.02 ng/g (S/N > 3) and 0.1 ng/g (S/N > 10) for AFs and OTA, respectively.
Reference	'Simultaneous Direct Analysis of Aflatoxins and Ochratoxin A in Cereals and Their Processed Products by Ultra-High-Performance Liquid Chromatography with Fluorescence Detection' Dhanshetty, M and Banerjee, K (2019) Journal of AOAC International, 102(6) 1666-1672.
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Patulin in Apple and Apple Juice**		
Method No.	FSSAI 07.020:2021	**Revision No. & Date**	0.0
Scope	This procedure is applicable for estimating patulin contamination in apple and apple juice.		
Caution	Wear protective gloves/protective clothing/eye protection/face protection while handling patulin solution, patulin-contaminated samples, and all solvents/reagents.		
Principle	Determination of patulin in apple and apple juice by liquid chromatography and tandem mass spectrometry		
Apparatus/Instruments	1. Mixer/grinder, 2. High-speed homogenizer, 3. Precision balance (0.0001 g), 4. Analytical balance, 5. Vortex shaker, 6. High-speed centrifuge, 7. Micro-centrifuge, 8. Ultrasonic bath, 9. Low-volume concentrator, 10. Ultra-High Performance Liquid Chromatograph with Tandem Mass Spectrometer		
Materials and Reagents	1. LC-MS grade acetonitrile, 2. Ethyl acetate, 3. Acetic acid, 4. Methanol, 5. Anhydrous Sodium sulfate, 6. Primary secondary amine (PSA) sorbent. It is a silica-based commercially available material, which usually has a bonded ethylenediamine-*N*-propyl phase containing both primary and secondary amine groups. 7. HPLC-grade water. 8. Reference standards: The reference standard of patulin, preserved at -20 °C. 9. Calibration standards: Prepare a set of calibration standards in the range of 0.002-0.05 µg/mL in methanol: water (2:8, v/v) for establishing the calibration linearity.		
Preparation of Reagents	Mobile phase A: One mL of acetic acid in 1000 mL of water Mobile phase B: One mL of acetic acid in 1000 mL of acetonitrile		

<table>
<tr><td>Sample Preparation</td><td>1. Take 10 g of homogenized sample of apple or 10 mL juice in a 50 mL polypropylene centrifuge tube.
2. Extract with 10 mL ethyl acetate in presence of 10 g anhydrous sodium sulfate.
3. Vortex for 2 min and centrifuge at 2795 ×g for 5 min.
4. Take 1 mL of the supernatant in a 2 mL Eppendorf tube and add 25 mg of PSA.
5. Centrifuge the Eppendorf tube at 5040 ×g for 5 min.
6. Evaporate the final extract under a gentle stream of nitrogen to dryness and reconstitute in 1 mL of methanol:water (2:8).
7. Inject 20 µL of the extract into LC-MS/MS.</td></tr>
<tr><td>Method of analysis</td><td>LC conditions (for reference only*):
a. Column: e.g., Bridged ethylene hybrid C_{18} column (2.1 × 100 mm,1.7 µm) or equivalent
b. Mobile Phase
A: Water with 0.1% acetic acid
B: Acetonitrile with 0.1% acetic acid
c. Column oven: 35 °C
d. Flow rate: 0.3 mL/min
e. Injection volume: 20 µL

Table 1. Gradient Programme
Time (min) | A% | B%
Initial | 90 | 10
1.0 | 90 | 10
1.5 | 01 | 99
2.0 | 01 | 99
2.5 | 90 | 10
5.0 | 90 | 10

Mass spectrometer parameters:
a. Ionization mode: ESI^- (negative mode)
b. Data acquisition: Multiple Reaction Monitoring;
Quantifier: 153>108.9 with collision energy ~7 eV and
Qualifier: 153>80.9 with collision energy ~11 eV
*The laboratory may use its own LC-MS/MS instrumentation method after appropriate optimization.
Prepare a calibration curve using a set of patulin standards in the range of 0.002-0.05 µg/mL in methanol: water (2:8, v/v).</td></tr>
<tr><td>Calculation with units of expression</td><td>1. Matrix effect (%) = [(Response in spiked matrix/ Response in spiked solvent) -1] × 100.
2. Recovery = (Observed concentration/Spiked concentration) × 100
3. Patulin level should be estimated based on matrix matched calibration, and expressed in the unit of mg/kg.</td></tr>
</table>

Inference (Qualitative Analysis)	The method provides sensitive analysis of patulin in apple and apple juice (LOQ-0.005 mg/kg). The detection is confirmed based on the detection of two selective reaction monitoring transitions and their ion ratio within a deviation of ±30%.
Reference	Shinde, R., Dhanshetty, M., Lakade, A., Elliott, C.T. and Banerjee, K., 2021. Development and validation of a liquid chromatographic tandem mass spectrometric method for the analysis of patulin in apple and apple juice. *Mycotoxin Research*, https://doi.org/10.1007/s12550-021-00422-2
Approved by	Scientific Panel on Methods of Sampling and Analysis

RAPID ANALYTICAL FOOD TESTING (RAFT) KIT/ EQUIPMENT

Alternate Rapid kits/equipment may be used to get quick results for screening and surveillance purposes, provided the kit/equipment is approved by FSSA(I). Details of the rapid food testing kit/ equipment approved by FSSA(I) are available at https://www.fssai.gov.in/cms/raft.php

Food Safety and Standards Authority of India
(Ministry of Health and Family Welfare)
FDA Bhawan, Kotla Road,
New Delhi-110002
www.fssai.gov.in
For any query contact: sp-sampling@fssai.gov.in