# AflaOchra™

## LC or LC-MS Detection

## Instruction Manual







VICAM™

A Waters Business

34 Maple Street, Milford, MA 01757 USA
Tel: 800.338.4381, 508.482.4935
Email: vicam@vicam.com

Effective Date: September, 2020

Table of Contents

## 1.0 Introduction

### 1.1 Intended User

VICAM's AflaOchra™ LC immunoaffinity (IA) columns enable laboratories to simultaneously isolate aflatoxins $B_1$, $B_2$, $G_1$, $G_2$, $M_1$, $M_2$ and ochratoxin A (OTA) from complex organic sample matrices in a single test run. This combination immunoaffinity column significantly reduces the time, labor, and material costs of assessing the safety and quality of products that are subject to both aflatoxin and OTA contamination, such as grains, feed, nuts, dried fruit, spices, cocoa, coffee, beer, and wine. Based on the same monoclonal antibody technology used in VICAM's AOAC- and FGIS-certified AflaTest®, the AflaOchra™ combination column optimizes sample cleanup, streamlining procedures, and yielding pure, highly concentrated sample extracts for analysis by HPLC or UPLC with fluorescence detection, or LC-MS. Used in conjunction with these highly sensitive instrumental techniques, AflaOchra™ column cleanup ensures accurate, precise, reliable detection and measurement of these commonly co-occurring mycotoxins at parts-per-billion levels.

### 1.2 Principle

Aflatoxins are a group of naturally occurring toxins produced by *Aspergillus flavus* and *Aspergillus parasiticus*, two common mold species that can infect susceptible crops such as corn, wheat, and other grains; tree nuts; peanuts; and fruits in the field and during storage and transportation. Like all mycotoxins, aflatoxins can persist in agricultural commodities even after mold removal, drying, and heating. Of the four main forms of aflatoxin ($B_1$, $B_2$, $G_1$, and $G_2$), the most potent, $B_1$, is of particular concern. A Group 1 carcinogen, known to cause cancer in humans, aflatoxin $B_1$ is also linked to immunosuppression and liver and kidney damage. Aflatoxin $M_1$, a hydroxylated metabolite of aflatoxin $B_1$, is less potent than its precursor, but its occurrence in milk and dairy products raises concerns primarily because of heavy consumption of these products by children and the increased sensitivity of this population to the toxic effects of aflatoxins.

Another mycotoxin with carcinogenic, immunosuppressant, and nephrotoxic properties, ochratoxin A (OTA) derives from several *Aspergillus* and *Penicillium* species that frequently co-occur with aflatoxigenic molds, compounding the risk of toxicologically significant levels of mycotoxin contamination in commodities exposed to mold-friendly environmental conditions. OTA is strongly linked to the chronic kidney disease Balkan endemic nephropathy.

In addition to posing these public health risks, aflatoxins and OTA can cause reduced productivity as well as disease and organ damage in animals that consume contaminated feed.

The Federal Food and Drug Administration (FDA) maintains an action level of 20 ppb for total aflatoxins (the sum of $B_1$, $B_2$, $G_1$, $G_2$,) in food and feed but, to date, has set no regulatory guidelines for OTA contamination. The EU stipulates maximum levels (MLs) for OTA ranging from 0.5 to 10 ppb for unprocessed cereals, dried vine fruits, coffee, wine, and grape juice. The EU's aflatoxin regulations are also far more stringent than those in the U.S., with MLs of 2 ppb for

aflatoxin $B_1$ and 4 ppb total aflatoxins in ready-to-eat almonds, pistachios, hazelnuts, peanuts, and cereals. The ML for aflatoxin $M_1$ in milk is 0.5 ppb in the U.S. and 0.05 ppb in the EU.

Aflatoxins and OTA are naturally fluorescent compounds. Their innate fluorescence facilitates their determination by HPLC and UPLC systems with fluorescence detectors.

Sample cleanup with the AflaOchra™ column is a fast, simple, and effective method of extracting aflatoxins and OTA that minimizes the use of organic solvents and removes potentially interfering substances in one easy step. No specialized training is required to perform the procedure. A small amount of ground sample is mixed with an extraction solution. The sample and extraction solution are blended, filtered, diluted, and filtered again before the extract is run through the IA column. The column contains stationery beads that are treated with antibodies that are highly specific and selective for aflatoxins and OTA. As the sample extract flows over the beads, the target analytes strongly bind with their respective antibodies. With the mycotoxins of interest immobilized on the beads, the user can then flush out matrix impurities by running PBS and/or water through the column. To complete the separation process, the user passes a small amount of methanol through the column to detach the aflatoxins and OTA from the antibody-coated beads and collects the eluate in a cuvette. The eluate is injected into an HPLC, UPLC, or LC-MS system to measure the concentrations of the target analytes. These steps are outlined in section 1.7, AflaOchra™ Procedure Overview.

## 1.3 Applicability and Approvals

AflaOchra™ has been optimized for quantitative measurement of aflatoxins and ochratoxin A in corn, wheat, raisins, ginseng, ginger, nuts, spices, cocoa, feed, baby food, and milk. Assistance in measuring aflatoxins and ochratoxin A in commodities not listed in this manual can be obtained by contacting our Technical Assistance Department.

AflaOchra™ immunoaffinity columns can be used with AOAC Official Method 2008.02 for the measurement of total aflatoxins and ochratoxin A in ginseng and ginger. References for other methods are listed in section 4.5.

## 1.4 Limitations

This test kit has been designed for use with the procedures and reagents described on the following pages. Do not use materials beyond the expiration date. Deviation from these instructions may not yield optimal results.

## 1.5 Sampling

Improper sampling techniques can lead to test results that misrepresent the true mycotoxin concentration of a product lot. These faulty results reflect the tendency of mycotoxins to occur in isolated “hot spots.” In shipments of grains or nuts, for example, the total amount of mycotoxin contamination in a lot may be confined to just few kernels. Consequently, obtaining a representative picture of the lot requires collecting a

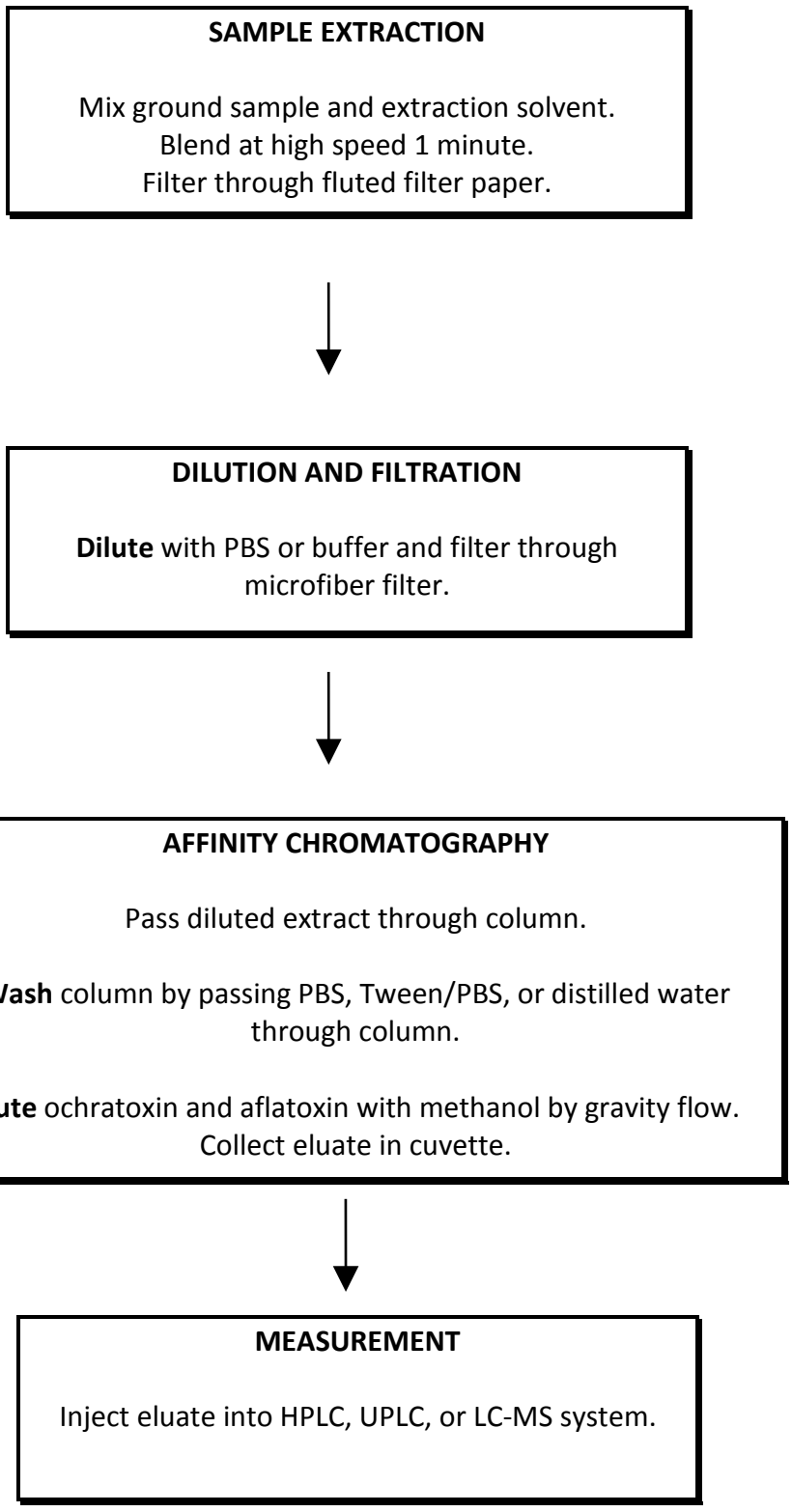
large aggregate sample that consists of multiple incremental samples from different areas of the lot. To draw a representative cross-section of kernels from a static lot, a manual or mechanical probe that extends from the top to bottom of the lot should be inserted into a series of locations that conforms to an officially recommended probe pattern. A diverter-type sampler should be used to gather incremental samples at regular intervals from a moving stream. To evenly distribute any contaminated material that may be present, the aggregate sample should be finely ground and thoroughly mixed before a subsample is taken for testing. For more information on grain sampling, refer to the following United States Federal Grain Inspection Service (FGIS) and European Community publications:

- FGIS Mycotoxin Handbook https://www.ams.usda.gov/sites/default/files/media/MycotoxinHB.pdf
- FGIS Grain Inspection Handbook, Book 1, Grain Sampling https://www.ams.usda.gov/sites/default/files/media/Book1.pdf
- European community sampling procedures can be found in Commission Regulation EC No 401/2006 of 23 February 2006. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02006R04010140701&from=EN

### 1.6 Shelf Life and Storage Conditions

Store AflaOchra columns at 2 - 30°C (36 - 86°F). Storage at temperatures above 30°C for prolonged periods of time may reduce shelf life. If storage temperatures above 30°C are anticipated, all components may be stored in the refrigerator (2 - 8°C). Columns and reagents must be brought to room temperature (18° - 25°C) before using. Do not freeze columns or reagents.

## 1.7 AflaOchra Immunoaffinity Column Cleanup Overview



## 2.0 Equipment Preparation

### 2.1 Materials and Equipment Required

**Materials Required**

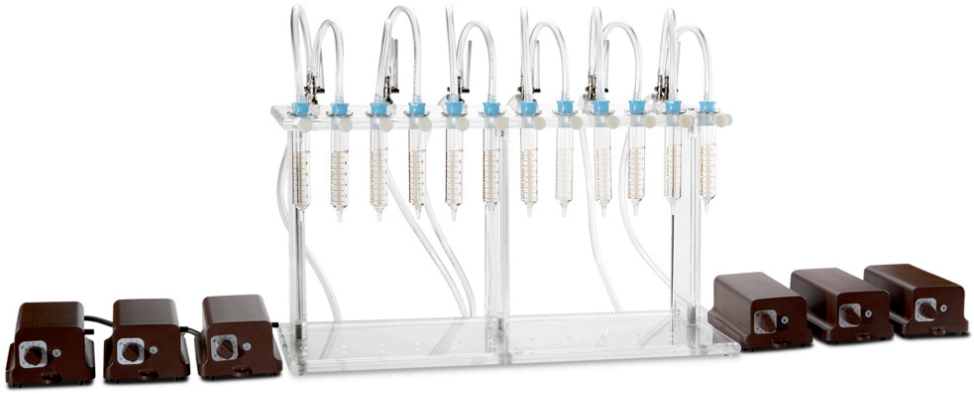
| Description | Part Number |
|---|---|
| AflaOchra™ Columns | G1017 |
| AflaOchra HPLC Kit (100 Columns and 1 set each std) | G1056 |
| Fluted Filter Paper, 24 cm (100 filters per pack) | 31240 |
| Microfiber Filters, 11 cm (100 filters per pack) | 31955 |
| Disposable Cuvettes (250 per pack) | 34000 |
| 10X Concentrate PBS Wash Buffer | G1113 |
| Methanol, HPLC Grade (4 x 4 L) | 35016 |
| Disposable Plastic Beakers | 36010 |
| Distilled, reverse osmosis or deionized water | |
| Noniodized sodium chloride (salt, NaCl) | |
| Acetonitrile, HPLC Grade | |

**Equipment Required**

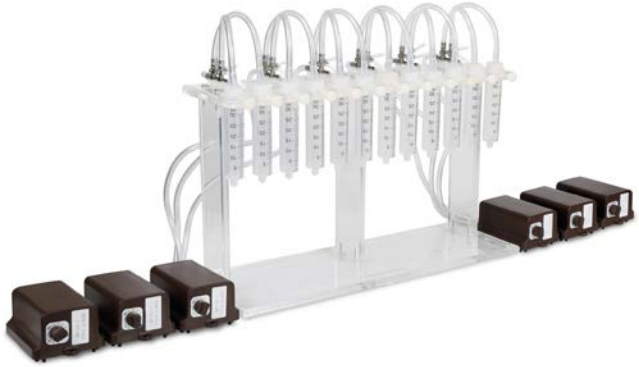
| Description | Part Number |
|---|---|
| Graduated Cylinder, 50 mL | 20050 |
| Graduated Cylinder, 250 mL | 20250 |
| Digital Scale with AC Adapter | 20100 |
| Commercial Blender with Stainless Steel Container | 20200 |
| Wash Bottle, 500 mL | 20700 |
| Cuvette Rack | 21010 |
| Single Position Pump Stand | 21020 |
| 2-Position Pump Stand w/ Air Pump (10 mL) | 21040 |
| or 4-Position Pump Stand w/2 Air Pumps (10 mL) | 21045 |
| or 12-Position Pump Stand w/6 Air Pumps (10 mL) | G1104 |
| or 12-Position Pump Stand with 30 mL Syringe Barrels and 6 Pumps | 60001708 |
| Vortex Mixer | 23040 |
| PhCR Photochemical Reactor | 600001222 |
| 500 mL Bottle Dispenser for Methanol (0-3 mL range) | 20501 |
| Disposable Plastic Beakers | 36010 |
| Filter Funnel, 65 mm (10 per pack) | 36020 |
| Filter Funnels, 105 mm (4 per pack) | 36022 |
| WB Column Coupling (6) | G1118 |
| Adjustable Micro-pipettor, 1.0 mL | G4033 |
| Micro-pipette Tips for 1 mL Micro-pipettor (100) | 20656 |


AflaOchra HPLC Kit 100 Columns and 1 set each std P/N G1056


PhCR Photochemical Reactor P/N 600001222



12 position with 10 mL syringe barrel, P/N G1104



12 position with 30 mL syringe barrel, P/N 60001708

## 2.2 Preparation of Filtration Steps

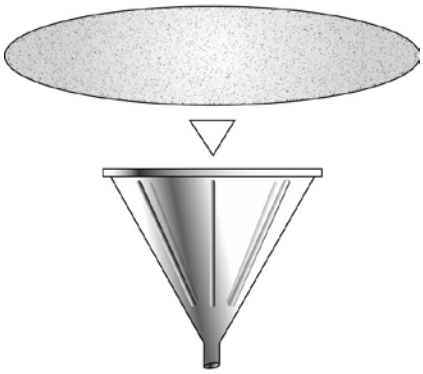
### 2.2.1 Fluted Filter

The first filtration step is a simple gravity filtration through fluted filter paper to separate the sample extract solution from the coarse particulate sample solids. The filtrate is collected in a clean container or graduated cylinder.

1) Open one fluted filter carefully and insert into clean container. (Optional: a large funnel may be used to hold the filter).

2) Fold edges of filter over rim of cup to hold in place. Maintain the fluted folds of the filter paper to maximize surface area. This will increase speed of filtration.

3) It is not necessary to wait for all the extract to pass through the filter before continuing.



### 2.2.2 Microfiber Filter

The second filtration step is the gravity filtration of the extract through a microfiber filter. This removes any precipitates in the extract and assures that the extract will easily pass through the immunoaffinity column. Microfiber filtration is performed just prior to chromatography.

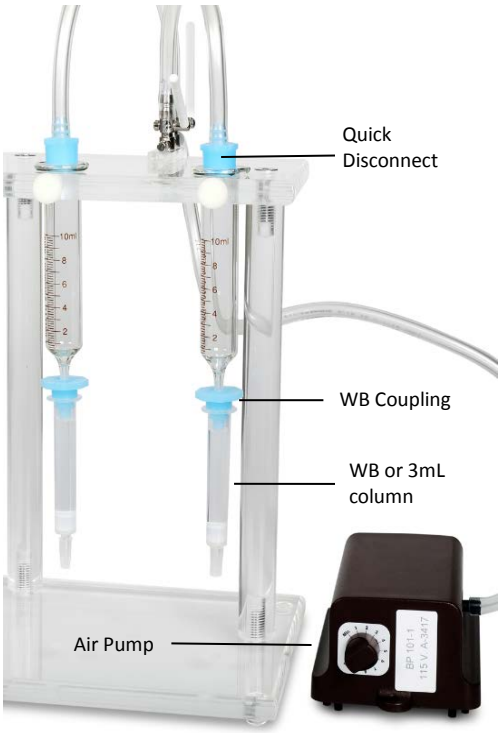
1) Place a small funnel in top outlet of syringe barrel or clean collecting cup.

2) Place one microfiber filter gently into small funnel by pressing filter into funnel with index finger. Be careful not to rip or puncture the filter.

## 2.3 Pump Stand Setup

AflaOchra™ immunoaffinity chromatography is easily performed with the column attached to a pump stand (P/N 21020). The stand has a 10 mL glass syringe barrel that serves as a reservoir for the column. A large plastic syringe with tubing and coupling provides air pressure to manually push liquids through the column. An adjustable aquarium pump (P/N20650) can be attached to the pump tube instead of the large pump syringe barrel to operate without using hand pressure. Double pump stand (P/N 21030), four-position pump stand with aquarium pumps (P/N21045), and twelve-position pump stand with aquarium pumps (P/N G1104) are available for running multiple samples at one time. Alternatively, a vacuum manifold can be used to draw the extract through the column.

1) Remove large top cap from column.
2) Attach column to coupling (P/N G1118) and place waste collection cup under column outlet. Keep bottom cap on column.
3) Attach column to couplings and place waste collection cup under column outlet. Keep bottom cap on column.
4) Pour extract into microfiber filter (see previous section) and collect desired amount of extract in glass syringe barrel using markings on the syringe barrel to measure extract. For more accuracy, measure extract with a pipet.
5) Inset quick disconnect on end of tube into syringe barrel. Remove the bottom cap from columns.
6) Use the dial on the air pump to set the air pressure applied to the contents of the syringe barrel. Maintain enough air pressure to push all the liquid in the syringe barrel through the column at a flow rate of 1 drop/second. Repeat for wash and elution.
7) The methanol elution requires less pressure to maintain the 1 drop/2 seconds, or gravity flow rate. The quick disconnect can be loosened or pulsed to reduce the pressure for the methanol elution.



## 2.4 Cleaning Equipment

### 2.4.1 Before Starting AflaOchra™ Testing

To eliminate background fluorescence, make sure the equipment is clean and not contaminated with materials that might cause background fluorescence. This is particularly important when using new equipment or equipment that has not been used for a long period of time.

Before using the equipment, it should be washed with a mild detergent solution and then rinsed thoroughly with purified water. This includes the glass syringe barrels used for sample reservoirs. The syringe barrels may be treated with a lubricant for use with a piston plunger. The lubricant needs to be washed off with a mild detergent and the syringe barrel rinsed thoroughly with purified water before using for AflaOchra™. Other pieces of equipment that need to be cleaned with a mild detergent and rinsed thoroughly with purified water before using are graduated cylinders, funnels and blender jars. Bottle dispensers need only to be rinsed with methanol before use.

### 2.4.2 Between Assays

After each assay, the blender jar assembly needs to be washed with a mild detergent solution and rinsed thoroughly with purified water. The same cleaning procedure must be performed for any equipment that will be reused to hold, collect or transfer sample extracts.

Do not wash bottle dispensers with soap. Methanol bottle dispensers needs only to be refilled with HPLC grade (or higher) methanol.

Between each assay, the syringe barrel reservoir can be rinsed with purified water. This will be enough to prevent cross-contamination of samples. After a large number of samples have been tested, the glass syringe barrel should be washed with a brush and mild detergent then rinsed well with water.

It is not recommended to wash and reuse the cuvettes. These cuvettes are designed for one-time use and should be discarded.

### 2.4.3 Other Important Precautions

Use only equipment specified by VICAM. Avoid contact of any test reagents or solutions (such as methanol, water, extract, column eluate or developer) with rubber or soft flexible plastic. These materials may leach contaminating fluorescent materials into the sample and thereby affect results.

**Note**: Some blender jar lids are lined with waxed cardboard. These liners are not resistant to methanol and water solutions and will breakdown when used for sample extraction. The extract will then become contaminated with materials which may cause background fluorescence. Lids with a cardboard liner should not be used.

## 3.0 Reagent Preparation

### 3.1 Preparation Extraction Solutions

Prepare extraction solutions every week or as needed. All formulas below will prepare 1,000 mL (1 liter) of solution. Solution volume may be increased or decreased as needed provided the proportion of reagents is kept consistent.

The procedures use a methanol:water or methanol:2% Tween/PBS buffer solution to extract aflatoxins and/or ochratoxin A from the sample.

To prepare extraction solution: Use reagent-grade (or better—i.e., HPLC-grade) methanol when preparing extraction solutions.

| **Methanol:Water** | **Methanol** | **Purified Water** | **Total Volume** |
|---|---|---|---|
| 80:20 | 800 mL | 200 mL | 1000 mL |
| 70:30 | 700 mL | 300 mL | 1000 mL |
| **Acetonitrile:Water** | **Acetonitrile (mL)** | **Purified Water** | **Total Volume** |
| 60:40 | 600 mL | 400 mL | 1000 mL |
| **Methanol:0.5% NaHCO3*** | **Methanol (mL)** | **0.5% $NaHCO_3$** | **Total Volume** |
| 70:30 | 700 mL | 300 mL | 1000 mL |

CAUTION: Extraction solvent is flammable. Keep container tightly capped when not in use.
*** Prepare fresh daily.** Others can prepare solution every week or as needed.

### 3.2 Preparation of Dilution and Wash Solutions

#### 3.2.1 2.5% sodium chloride, 0.5% sodium bicarbonate

2.5 g NaCl
0.5 g $NaHCO_3$
bring to 100 mL with purified water

#### 3.2.2 1X PBS

A 10X PBS concentrate can be purchased from VICAM (P/N G1113). The 10X PBS concentrate should be diluted to 1X with purified water as needed - i.e. dilute 100 mL of 10X PBS concentrate with 900 mL purified water.

Alternatively, a 1X PBS solution can be prepared as follows:

8.0 g NaCl
1.2 g $Na_2HPO_4$
0.2 g $KH_2PO_4$
0.2 g KCl
dissolve in approximately 990 mL purified water
adjust pH to 7.0 with concentrated HCl
bring to 1 liter with purified water

### 3.2.3 0.1%Tween-20/PBS Wash Buffer

A 10X concentrate of 0.1% Tween-20/PBS may be purchased from VICAM (P/N G1112). The 10X concentrate should be diluted to 1X with purified water as needed - i.e. dilute 100 mL of 10X concentrate with 900 mL purified water.

Alternatively, a 1X 0.1%Tween-20/PBS Wash Buffer solution can be prepared as follows:

Measure 1 mL Tween-20
bring to 1 liter with 1X PBS.

### 3.2.4 1%Tween-20/PBS Wash Buffer

Measure 10 mL Tween-20
bring to 1 liter with 1X PBS.

## 3.3 Preparation of LC Solutions

### 3.3.1 Iodine solution (0.05%)

0.5 g Iodine
100 mL Methanol
900 mL purified water

Dissolve iodine in methanol, stirring until completely dissolved. While stirring, add purified water. Mix solution for at least 30 minutes. Filter solution through 0.45 micron nylon filter. This solution can be used for 2 weeks from preparation.

### 3.3.2 Aflatoxin HPLC mobile phase

| Methanol:Water | HPLC Grade Methanol | Purified Water | Total Volume |
|---|---|---|---|
| 45:55 | 450 mL | 550 mL | 1000 mL |

Solution should be filtered and degassed before use.

### 3.3.3 Ochratoxin HPLC mobile phase

| Acetonitrile:Water:Acetic Acid | Acetonitrile | Purified Water | Acetic Acid | Total Volume |
|---|---|---|---|---|
| 99:99:2 | 495 mL | 495 mL | 10 mL | 1000 mL |

Solution should be filtered and degassed before use.

## 4.0 Immunoaffinity Column Clean Up Procedures for LC and LC/MS

### 4.1 AflaOchra Procedure for Corn (0 - 100 PPB)

#### 4.1.1 LC Set up: See section 4.6

#### 4.1.2 Sample Extract

1) Weigh 50g ground sample with 5g salt (NaCl) and place in blender jar.
2) Add to jar 100 mL methanol:water (80:20 by volume).
3) Cover blender jar and blend at high speed for 2 minutes.
4) Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

#### 4.1.3 Extract Dilution

1) Pipet or pour 10 mL filtered extract into a clean vessel.
2) Dilute extract with 40 mL of purified PBS. Mix well.
3) Filter extract through Microfiber filter and collect filtrate in a clean vessel.

#### 4.1.4 Column Chromatography

1) Pass 10 mL (10 mL = 1.0 g sample equivalent) diluted extract completely through AflaOchra column at a rate of about 1 drop/second until air comes through column.
2) Fill column headspace with PBS, reattach the column to syringe barrel, fill the syringe barrel with 5 mL of PBS then pass PBS through AflaOchra column at a rate of 1-2 drops/second until air comes through the column.
3) Pass 5 mL water through AflaOchra column about 1-2 drops/second until 5 seconds of air come through the column.
4) Place a glass cuvette under the AflaOchra column. Elute column with 1.5 mL HPLC grade methanol by gravity (or 1 drop/ 2 seconds) until air comes through column. Turn air pump to maximum for 2 seconds to collect all the methanol. Backflush technique can be used to enhance elution (See section 4.8 for more information).
5) Add 1.5 mL purified water to reservoir and eluate column again. Collect all the sample eluate (1.5 mL MeOH + 1.5mL water) in the same glass cuvette. Vortex. Inject 50-200μL onto HPLC or 10 -20 μL onto UPLC.

#### 4.1.5 Limit of Detection: Less than 0.25 ppb.

#### 4.1.6 Recovery: QC spec for recovery. Aflatoxin B1 > 90%, Aflatoxin B2 > 80%, Aflatoxin G1 > 90%, Aflatoxin G2 > 70% and Ochratoxin A > 85% over the range of 0-100 ng for both aflatoxin and ochratoxin.

## 4.2 AflaOchra Procedure for Wheat (0 - 100 PPB)

### 4.2.1 LC Set up: See section 4.6

### 4.2.2 Sample Extraction:

1) Place 50g ground sample into a blender jar.
2) Add to jar 100 mL* acetonitrile:water (60:40).
3) Cover blender jar and blend at high speed for 2 minutes.
4) Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

### 4.2.3 Extract Dilution:

1) Pipet or pour 10 mL filtered extract into a clean vessel.
2) Dilute extract with 40 mL of purified PBS. Mix well.
3) Filter dilute extract through Microfiber filter into clean container or directly into glass syringe barrel.

### 4.2.4 Column Chromatography

1) Pass 10 mL filtered extract (10 mL = 1 g sample equivalent) completely through AflaOchra column at a rate of about 1 drop/second until air comes through column.
2) Fill column headspace with PBS, reattach the column to syringe barrel, fill the syringe barrel with 5 mL of PBS then pass PBS through AflaOchra column at a rate of 1-2 drops/second until air comes through the column.
3) Pass 5 mL water through AflaOchra column about 1-2 drops/second until 5 seconds of air come through the column.
4) Place a glass cuvette under the AflaOchra column. Elute column with 1.5 mL HPLC grade methanol by gravity (or 1 drop/ 2 seconds) until air comes through column. Turn air pump to maximum for 2 seconds to collect all the methanol. Backflush technique can be used to enhance elution (See section 4.8 for more information).
5) Add 1.5 mL purified water to reservoir and eluate column again. Collect all the sample eluate (1.5 mL MeOH + 1.5mL water) in the same glass cuvette. Vortex. Inject 50-200µL onto HPLC or 10 -20 µL onto UPLC.

### 4.2.5 Limit of Detection: 0.25 ppb

### 4.2.6 Recovery: Greater than 70% recovery over the 0.25 - 100 ppb range.

* If ground wheat sample does not blend properly in 100 mL acetonitrile:water (60:40) use 200 mL acetonitrile:water . Double the extract volume passed through the column to 20 mL to keep the same gram equivalency.

## 4.3 AflaOchra Procedure for Corn Ginseng and Ginger (0 - 100 PPB)

**4.3.1 LC Set up:** See section 4.6

**4.3.2 Sample Extraction:**

1) Weigh 5g test sample in a 50 mL centrifuge tube. Add 1g NaCl and 25 mL MeOH–0.5% $NaHCO_3$ (700 + 300, v/v). Mix on a Vortex mixer until sample particles and extract solvent are well mixed.
2) Shake at 400 rpm for 10 min.
3) Centrifuge for 10 min at 7000 rpm (g value = 5323 mm/$s^2$) or at speed that can create a firm pellet of residues.

**4.3.3 Extract Dilution:**

1) Immediately pipet 7 mL of supernatant into a 50 mL centrifuge.
2) Add 28 mL 1% Tween 20/PBS, mix well.
3) Filter through glass microfiber paper.
4) Collect 25 mL filtrate into a 25 mL graduate cylinder and proceed **immediately** with column chromatography

**4.3.4 Column Chromatography**

1) Pass 25mL filtered extract (25 mL = 1 g sample equivalent) completely through AflaOchra column* by gravity force until air comes through column.
2) Remove column from syringe barrel, add about 2 mL 10 mM PBS into column, and reattach column to syringe barrel. Add an additional 3 mL 10 mM PBS to syringe barrel (5 mL total) and pass through column at a rate of about 1 drop/second.
3) Pass 5 mL water through AflaOchra column at a rate of about 1 drop/second.
4) Force 3 mL air through column with a syringe or turn on pump to maximum.
5) Place a 3 mL volumetric flask under column. Elute and collect the aflatoxin and ochratoxin in the 3 mL volumetric flask with 1 mL HPLC grade methanol; let drip freely by gravity. Let column run dry.
6) Let stand for 1 min, then elute with additional 1 mL methanol and collect into the same volumetric flask. Let column run dry and force 10 mL air through column. Dilute eluate to volume with water. Vortex.
7) Perform LC analysis for aflatoxins immediately. Perform LC analysis for OTA next. Inject 50-200μL onto HPLC for Aflatoxin and 50-200μL for Ochratoxin. Alternatively, aflatoxins and ochratoxin can be injected together onto an HPLC or UPLC. For UPLC, inject 10 -20 μL.

**4.3.5 Recovery** Average recoveries of Aflatoxins from ginseng and ginger ranged from 70 to 87% (at spiking levels ranging from 2 to 16 μg/kg), and of OTA, from 86 to 113% (at spiking levels ranging from 1 to 8 μg/kg).

* (*Caution*: For immunoaffinity column cleanup, columns must be kept at room temperature for at least 15 min before use.)

## 4.4 AflaOchra Procedure for Red Pepper (0 - 100 PPB)

**4.4.1 LC Set up**: See section 4.6

**4.4.2 Sample Extraction:**

1) Place 25g ground sample into a jar and add 2.5 g NaCl.
2) Add to jar 100 mL methanol:water (80:20, V/V).
3) Shake sample on a wrist action shaker for 60 minutes. Alternatively, sample can be blended at high speed for 2 minutes.
4) Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

**4.4.3 Extract Dilution:**

1) Pipet or pour 15 mL filtered extract into a clean vessel.
2) Dilute extract with 60 mL of 0.1% Tween-20/PBS.
3) Filter dilute extract through Microfiber filter into clean container or directly into glass syringe barrel.

**4.4.4 Column Chromatography**

1) Pass 20 mL filtered extract (10 mL = 1 g sample equivalent) completely through AflaOchra column by gravity (not more than 1 drop/second) until air comes through column.
2) Fill column headspace with PBS, reattach the column to syringe barrel, fill the syringe barrel with 10 mL of PBS, then pass PBS through AflaOchra column at a rate of 1-2 drops/second until air comes through the column.
3) Fill syringe barrel with 10mL purified water and pass it through AflaOchra column until 5 seconds of air comes through the column.
4) Place a glass cuvette under the AflaOchra column. Elute column with 2.0 mL HPLC grade methanol by gravity (or 1 drop/ 2 seconds) until air comes through column. Turn air pump to maximum for 2 seconds to collect all the methanol.
5) Add 2.0 mL purified water to reservoir and eluate column again. Collecting all the sample eluate (2.0 mL MeOH + 2.0 mL water) in the same glass cuvette. Vortex. Inject 50-200µL onto HPLC or 10 -20µL onto UPLC or UPLC/MS/MS.
6) Alternatively, 2.0 mL methanol at step 4.4 can be evaporated to dryness under $N_2$ at 50°C. The dried eluate can be reconstituted with 1 mL 10% TFA (TFA-10% acetonitrile = 1:9), vortex mixed, and kept for 3 hrs. in a dark place for TFA derivatization of AFB1 and AFG1.

**4.4.5 Recovery** Greater than 90 % aflatoxins and ochratoxin A.

## 4.5 Other Published Procedures

**Cocoa**
Turcotte AM, Scott PM, Tague B, *Mycotoxin Research*, Analysis of cocoa products for ochratoxin A and aflatoxins, 2013; 29:193–201

**Baby Food and Milk**
Beltrán E, Ibáñez M, Sancho JV, Cortés MA Yusà V, Hernández F, *Food Chemistry,* UHPLC–MS/MS highly sensitive determination of aflatoxins, the aflatoxin metabolite M1 and ochratoxin A in baby food and milk 126 (2011) 737–744

**Feed**
Kim HJ, Lee MJ, Kim HJ, Cho SK, Park HJ, Jeong MH, *Cogent Food & Agriculture,* Analytical method development and monitoring of Aflatoxin B1, B2, G1, G2 and Ochratoxin A in animal feed using HPLC with Fluorescence detector and photochemical reaction device 2017; 3: 1419788

**Ginseng and Ginger (AOAC Method 2013.05)**
Trucksess MW, Weaver CM, Oles CJ, Fry FS, JR, Noonan GO., BETZ JM, Rader JL., *Journal of AOAC International,* Determination of Aflatoxins B1, B2, G1, andG2 and Ochratoxin A in Ginseng and Ginger by Multitoxin Immunoaffinity Column Cleanup and Liquid Chromatographic Quantitation: Collaborative Study 2008; 91(3): 511-523

**Nutmeg**
Kong WJ, Liu SY, Qiu F, Xiao XH, Yang HM, *The Royal Society of Chemistry Analyst*, Simultaneous multi-mycotoxin determination in nutmeg by ultrasound-assisted solid–liquid extraction and immunoaffinity column clean-up coupled with liquid chromatography and on-line post-column photochemical derivatization-fluorescence detection, 2013, 138, 2729–2739

**Spices**
Wan Ainiza W.M., Jinap S. & Sanny M., *Food Control*, Simultaneous determination of aflatoxins and ochratoxin A in single and mixed spices, April 2015, 50: 913-918

Iha MH, Rodrigues ML, Trucksess MW, *Journal of AOAC International,* Multitoxin immunoaffinity analysis of aflatoxins and ochratoxin A in spices, 2020

## 4.6 AflaOchra LC and LC/ MS/MS Set Up:

### 4.6.1 HPLC Set Up for Simultaneous Quantification of Aflatoxin and Ochratoxin

**Column:** reverse phase C18 (Waters NovaPak C18, 3.9 mm x 150 mm, 4.0 µm)
**Mobile Phase:** Gradient*

**Flow Rate:** 0.8 mL/minute
**Injection volume:** 50-200 µL
**Fluorescence Detector:** Waters 2475 Scanning Fluorescence detector,
excitation 365 nm/emission 455 nm for 0–16 minutes for aflatoxins
excitation 333 nm/emission 477 nm for 16–30 minutes for ochratoxin
**Post-column:** PhCR (Photochemical Reactor, P/N 600001222)
**Reaction Temperature:** Room temp ~22°–25°C

***Gradient for HPLC**

| | Time | Flow | %A | %B | %C | %D | Curve |
|---|---|---|---|---|---|---|---|
| 1 | | 0.800 | 0.0 | 25.0 | 15.0 | 60.0 | |
| 2 | 12.00 | 0.800 | 0.0 | 25.0 | 15.0 | 60.0 | 6 |
| 3 | 14.00 | 0.800 | 0.0 | 0.0 | 70.0 | 30.0 | 6 |
| 4 | 25.00 | 0.800 | 0.0 | 25.0 | 15.0 | 60.0 | 11 |
| 5 | 30.00 | 0.800 | 0.0 | 100.0 | 0.0 | 0.0 | 11 |
| 6 | 37.00 | 0.800 | 0.0 | 0.0 | 100.0 | 0.0 | 11 |

Line A = Milli-Q Water or (Reverse Osmosis/Deionized)
Line B = 100% HPLC-Grade Methanol
Line C = 100% Acetonitrile
Line D = 0.1% Acetic Acid in Water

**Retention times:**

| Peak | G2 | G1 | B2 | B1 | OTA |
|---|---|---|---|---|---|
| Time (min) | 6.75 | 7.96 | 9.08 | 11.0 | 18.15 |

### 4.6.2 HPLC Setup for Quantification Aflatoxin and Ochratoxin in Separate runs

**HPLC Setup for Aflatoxin:**

1) Column: reverse phase C18 (Waters Nova pak C18, 3.9 mm X 150mm, 4µm cartridge WAT086344,
2) Mobile phase: methanol:water (45:55) isocratic degassed.
3) Flow rate: 1.0 ml/min.
4) Fluorescence detector: excitation 360 nm, emission 440 nm.
5) Post column: PhCR Photochemical reactor, VICAM product #600001222

**HPLC Setup for Ochratoxin:**

1) Column: reverse phase C18 column (Waters Nova-Pak® C-18, 3.9 X 150mm, 4µm)
2) Mobile phase: water:acetonitrile:acetic acid (99:99:2, v/v/v), degassed
3) Flow rate: 0.9 mL/min.
4) Fluorescence detector: Waters 470 Scanning Fluorescence detector

5) Detection wavelength: 333 nm excitation and 477 nm emission

### 4.6.3 UPLC Set Up for Simultaneous Quantification of Aflatoxin and Ochratoxin

**LC Instrument:** ACQUITY UPLC with Fluorescent detector and **large volume flow cell**
**LC Column:** CORTECS UPLC C18 1.6um (2.1x150mm)
**Flow rate:** 0.3mL/min
**Injection volume:** 20 µL

**Mobile Phase:**

| Time (min) | Methanol | Acetonitrile | 0.1% Acetic Acid |
|---|---|---|---|
| Initial | 25% | 25% | 50% |
| 5.0 | 25% | 25% | 50% |
| 5.5 | 30% | 30% | 40% |
| 10.0 | 30% | 30% | 40% |
| 10.5 | 30% | 30% | 40% |
| 11.0 | 25% | 25% | 50% |

**Fluorescent Detector:**

| Time (min) | Event | Parameter |
|---|---|---|
| 0.01 | Ex | 365 |
| 0.01 | Em | 455 |
| 0.01 | PMT Gain | 1 |
| 7.0 | PMT Gain | 30 |
| 8.0 | Ex | 329 |
| 8.0 | Em | 460 |

**Retention Time:**

| Peak | G2 | G1 | B2 | B1 | OTA |
|---|---|---|---|---|---|
| Time (min) | 1.5 | 1.6 | 1.8 | 2.0 | 9.0 |

### 4.6.4 UPLC-MS/MS Conditions

| UPLC Conditions | | MS Conditions | |
|---|---|---|---|
| UPLC System | ACQUITY UPLC I-Class | MS System | TQS-Micro |
| Column | X-Bridge® BEH, XP®, $C_{18,}$, 2.1 x 100 mm, 2.5 µm Waters P/N 186000031 and guard column Waters X-Bridge BEH XP $C_{18,}$ 2.5 mm x 2.1 mm, 2.5 µm Waters P/N 186003975 | Ionization mode | ESI positive |
| Column Temp | 30°C | Collision gas (N2) | $3.00 \times 10^{-3}$ mbar |
| Sample Temp | 20°C | Capillary voltage | 2.50 kV |

| | | | |
|---|---|---|---|
| Mobile Phase A | 5 mM ammonium formate with 0.02% formic acid in $H_2O$ | Cone voltage | Aflatoxin $B_1$, $B_2$, $G_1$ & $G_2$ – 35 V, Ochratoxin A – 45 V |
| Mobile Phase B | 5 mM ammonium formate with 0.02% formic acid in methanol | Source temp | 150°C |
| Flow Rate | 0.5 mL/minute | Desolvation temp | 500°C |
| Injection Volume | 10 µL | Desolvation gas | 1000 L/hr |
| Retention Time | Varies per analyte; refer to MS Parameters section (below) | Cone gas | 50 L/hr |

**Gradient:**

| Time (min) | Flow (mL/min) | A (%) | B (%) |
|---|---|---|---|
| 0 | 0.5 | 98 | 2 |
| 0.2 | 0.5 | 98 | 2 |
| 3.0 | 0.5 | 1 | 99 |
| 4.0 | 0.5 | 1 | 99 |
| 4.1 | 0.5 | 98 | 2 |
| 5 | 0.5 | 98 | 2 |

Mobile Phase A: Aqueous, 5 mM ammonium formate, 0.02% (v/v) formic acid
Mobile Phase B: Methanol, 5 mM ammonium formate, 0.02% (v/v) formic acid

**MS/MS Parameters for the Analytes Studied:**

| Toxin | Pseudo-molecular | RT*(min) | Precursor Ion | Product Ion | | CV* | CE* |
|---|---|---|---|---|---|---|---|
| | | | | quantification | qualifier | | |
| $AFG_2$ | $[M+H]^+$ | 2.46 | 331.1 | 245.1 | 189.1 | 35 | 40/28 |
| $AFG_1$ | $[M+H]^+$ | 2.53 | 329.1 | 243.1 | 311.1 | 35 | 25/21 |
| $AFB_2$ | $[M+H]^+$ | 2.60 | 315.1 | 259.1 | 287.1 | 35 | 28/25 |
| $AFB_1$ | $[M+H]^+$ | 2.67 | 313.1 | 241.1 | 285.1 | 35 | 35/21 |
| OTA | $[M+H]^+$ | 3.10 | 404.1 | 239.1 | 358.1 | 45 | 23/13 |

*RT: Retention Time, CV: Cone Voltage, CE: Collision Energy

**4.7 Procedure for Silanizing Glassware**

1) Make a 2% solution of Dimethyldichlorosilane in toluene.
2) Fill glassware with DMDCS/Toluene solution.
3) Heat at 40°C for about 30 minutes.
4) Rinse three times with toluene.
5) Rinse three times with methanol.
6) Bake in oven at 180°C for three hours.

Alternatively, you can use Pierce SurfaSil (product # TS-42800) from Fisher Scientific. Dilute Surfasil 1:10 in hexane, dip cuvettes into the solution, rinse 3 times with hexane, rinse 3 times

with methanol and let air dry without heating.

Sigmacote product # SL-2 from Sigma Aldrich can be used without dilution.

Silanized glass tubes and vials can also be purchased from Sigma Aldrich (sigmaaldrich.com)

**4.8 Backflush**

Backflushing will increase the time the elution solvent is in contact with the antibodies in the AflaOchra column, ensuring that all toxins are eluted. By gently pushing and pulling a syringe with plunger (P/N 600001145) with an attached coupling (VICAM part # G1118) placed on top of the AflaOchra column during elution, the elution solvent will move back and forth through the column to fully wet the resin. Repeat this process at least three times.

**4.9 How to Spike a Sample and Prepare LC Standard for AflaOchra**

Aflatoxin standard from Supelco, product #46304-U at 2.6µg/mL (ng/µL) in methanol (1.0:B1:0.3B2:1.0G1:0.3G2).

Ochratoxin A standard(std) from Supelco, product #4-6912 at 50µg/mL (ng/µL) in benzene:acetic acid (99:1). Dry this standard down and reconstitute it in methanol or dilute it directly in methanol.

Dilute all standards 1:10, then 1:100 in methanol. Dilute ochratoxin also 1:1000.

**For aflatoxin**:

**1:10** 100µL std at 2.6ng/µL + 900µL methanol = 0.26ng/µL std
**1:100** 100ul std at 0.26ng/µL + 900µL methanol = 0.026ng/µL std

**For ochratoxin:**

**1:10** 100µL std at 50ng/µL + 900µL methanol = 5ng/µL std
**1:100** 100ul std at 5 ng/µL + 900µL methanol = 0.5ng/µL std
**1:1000** 100ul std at 0.5 ng/µL + 900µL methanol = 0.05ng/µL std

**LC standards for 1g sample method over AflaOchra column**

**Aflatoxin**

Level 1: 2.6ppb (ng/g) X 1g = 2.6ng; 2.6ng ÷ 0.026ng/µL standard = 100µL (1B1:0.3B2:1G1:0.3G2)

Level 2: 26ppb (ng/g) X 1g = 26ng; 26ng ÷ 0.26ng/µL standard = 100µL
(10B1: 3B2:10G1: 3G2)

Level 3: 52ppb (ng/g) X 1g = 52ng; 52ng ÷ 0.26ng/µL standard =200µL
(20B1:6B2:20G1:6G2)

**Ochratoxin**

Level 1: 2 ppb (ng/g) X 1g = 2ng; 2ng ÷ 0.05 ng/µL standard = 40µL

Level 2: 10 ppb (ng/g) X 1g = 10ng; 10ng ÷ 0.05 ng/µL standard =200µL

Level 3: 50 ppb (ng/g) X 1g = 50ng; 50ng ÷ 0.5 ng/µL standard =100µL

Combine all toxins from the same level to a total of 1.5mL methanol, then add 1.5mL 0.1% acetic acid. For example, for level 1 standard, add 100µL of 0.026ng/µL aflatoxin standard, and 40µL of 0.05 ng/µL ochratoxin A standard to 1360µL methanol. Add 1.5mL 0.1% acetic acid. This will make an AO HPLC standard at 1.0ppb aflatoxin B1, 0.3ppb aflatoxin B2, 1ppb aflatoxin G1, 0.3ppb aflatoxin G2, and 2ppb ochratoxin A.

When integrating the toxin peaks, inhibit integration during the time periods of a gradient change or wavelength change.

**How to spike a 25 g sample**

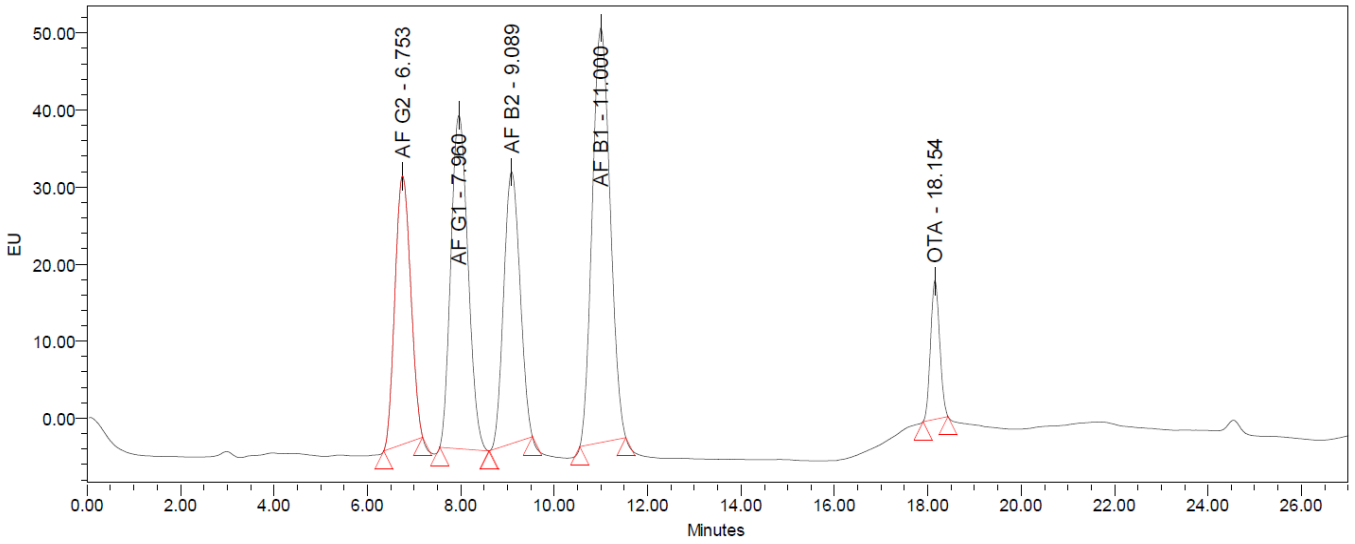
To spike a 25g ground grain sample at level 2:

**Aflatoxins**
26ppb (ng/g) X 25g sample = 650ng; 650ng ÷ 2.6ng/µL aflatoxin standard = 250µL spike

**Ochratoxin A**
10ppb (ng/g) X 25g sample = 250ng; 250ng ÷ 5ng/µL ochratoxin A standard = 50µL spike

## 4.10 Representation of LC and LC/MS Chromatograms

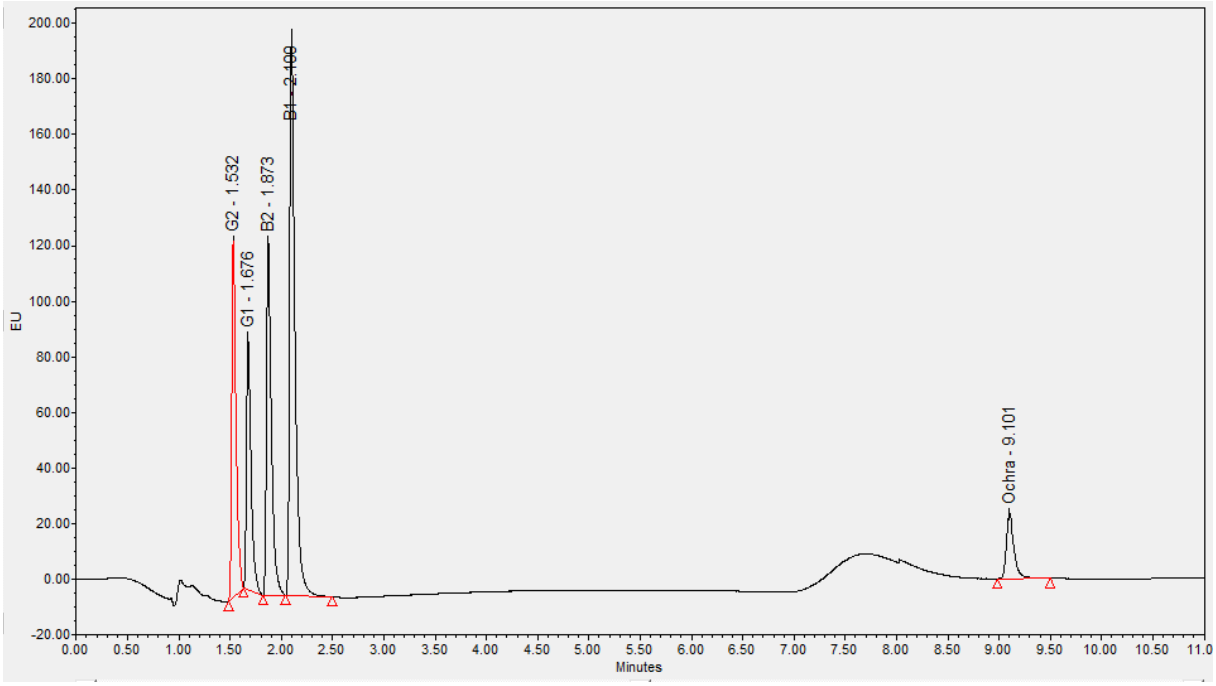
### 4.10.1 Single Injection HPLC with PhCR



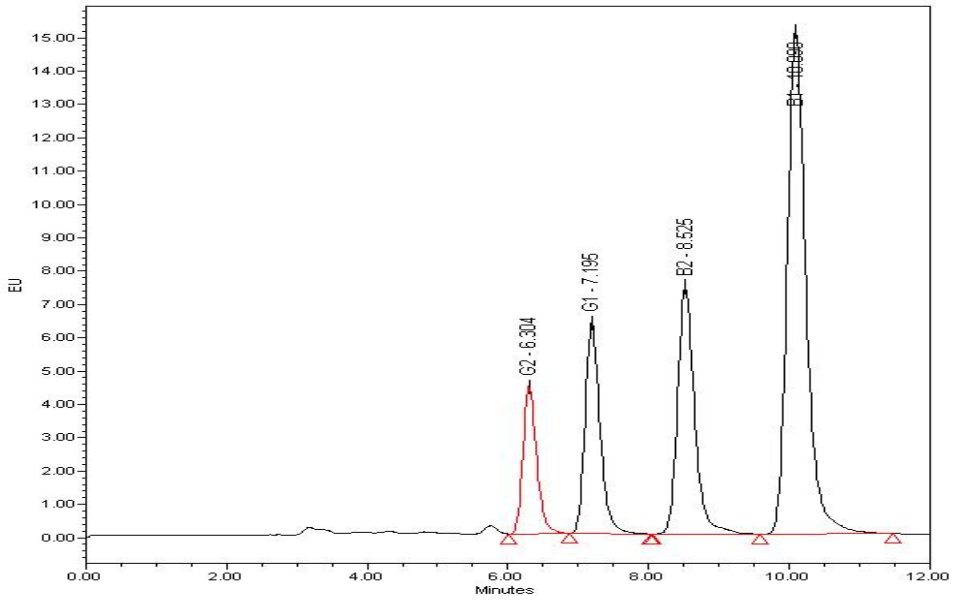

26 ppb total aflatoxins and 10 ppb ochratoxin A with PhCR photochemical derivatization

### 4.10.2 Single Injection UPLC

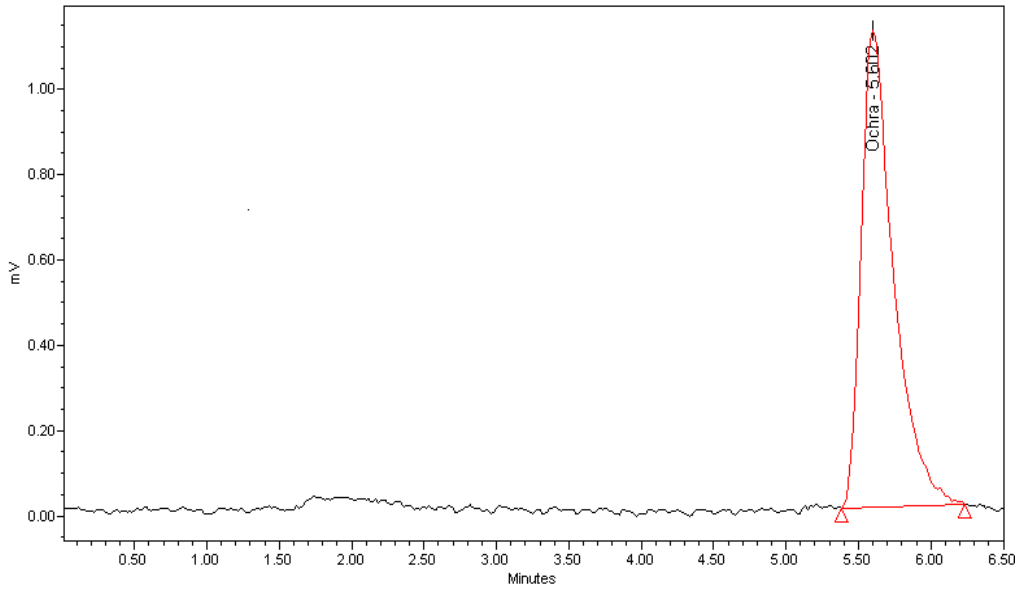
100 ppb total aflatoxins and 100 ppb ochratoxin A spiked corn sample



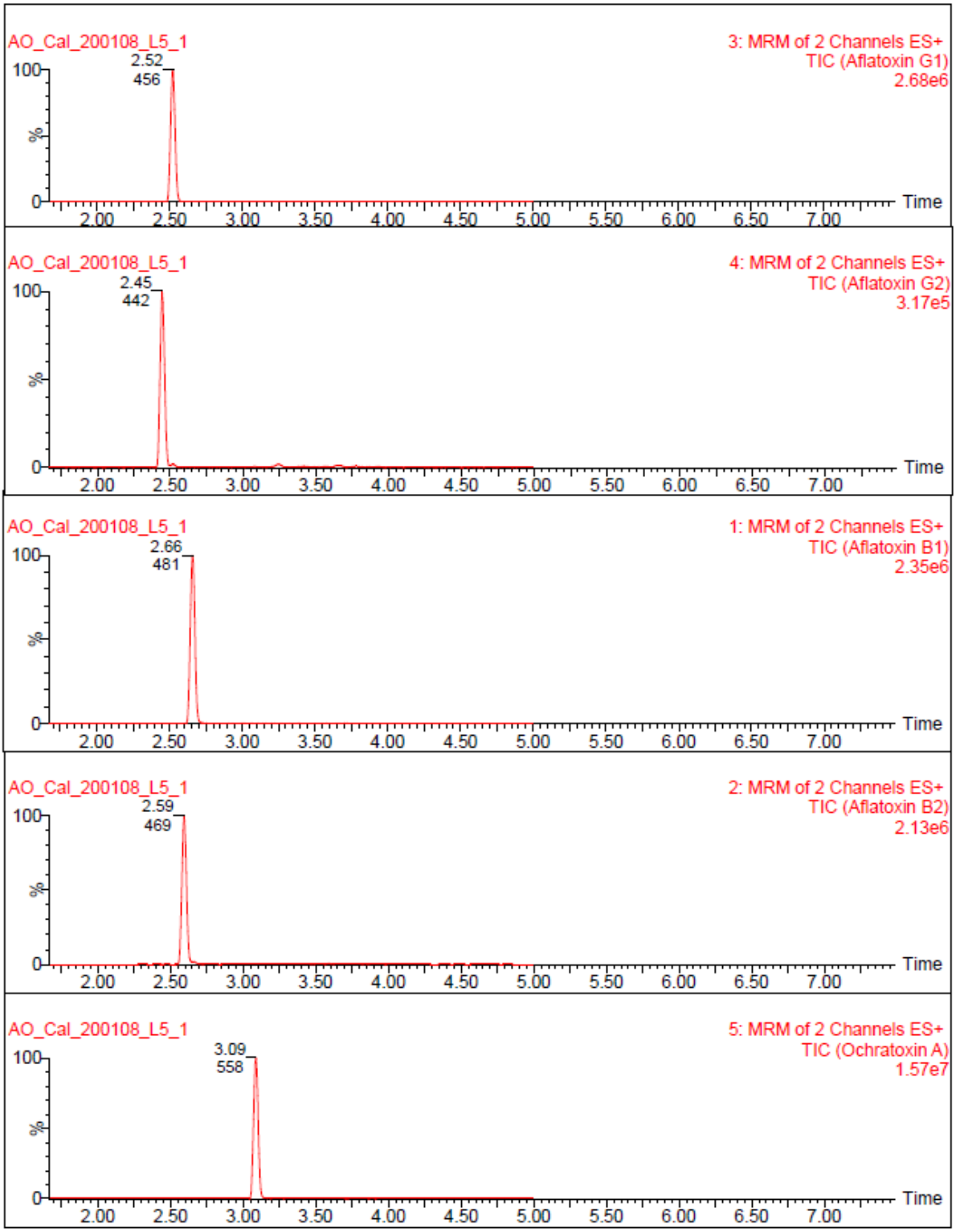
### 4.10.3 4.3 ppb aflatoxin contaminated corn




### 4.10.4 20ppb ochratoxin A spiked corn sample



### 4.10.5 Representative Total Ion Chromatogram:



## 5.0 General Precautions for LC and LC/MS Cleanup

5.1 Aflatoxin and Ochratoxin may be lost if eluate is passed through nylon disc filter.

5.2 If drying is performed, use silanized vials (See Section 4.7) to avoid irreversible binding of aflatoxins to the tube walls.

## 6.0 Technical Assistance and Ordering Information

For assistance please contact your local distributor or VICAM

Phone: 1-800-338-4381 or +1 508-482-4935
e-mail: techservice@VICAM.com

To place an order, contact your local distributor or VICAM

Phone: 1-877-228-4244 or +1 417-725-6588
Fax: +1 417-725-6102
e-mail: orders@VICAM.com

## 7.0 Liability

The analytical methods described above have been developed by VICAM to be used exclusively with the reagents in this test. The user assumes all risk in using AflaOchra™ analytical procedures and products. VICAM makes no warranty of any kind, express or implied, other than that AflaOchra™ products conform to VICAM's printed specifications and quality control standards. VICAM will at its option repair or replace any product or part thereof which proves to be defective in workmanship or material. VICAM's undertaking to repair or replace such products is exclusive and is in lieu of all warranties whether written, oral expressed, or implied, including any implied warranty of merchantability or fitness for a particular purpose. VICAM shall have no liability for anticipated or lost profits or any loss, inconvenience or damage whether direct, incidental, consequential or otherwise, to person or property, or for strict liability or negligence arising from or in connection with the use of these assay procedures or AflaOchra™ product.

The foregoing notwithstanding, protocols and other products developed by VICAM are periodically improved and revised in order to maximize reliability and optimize customer use and satisfaction. When an improved, new or substitute version of a protocol and product is available, VICAM shall not be held liable or responsible for any earlier protocol or product, even if use of earlier product or protocol be within the expiration date. Please inform yourself about any new protocols by either e-mailing, faxing or phoning VICAM or your local VICAM distributor.

VICAM shall not be liable or responsible for any unsatisfactory or faulty results or performance involving the use of VICAM protocols or products if the testing or sampling in question is not conducted properly. The customer is solely and fully responsible for educating oneself about the proper testing and sampling procedures using VICAM protocols and products.

All VICAM products are protected by world-wide patents and trademarks.

Effective Date: September 2020
Instruction Manual: GN-MC9521-2