AflaTest™ WB SR+

LC or LC-MS Detection

Instruction Manual

Waters™ | VICAM™

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1.0 Introduction

1.1 Intended User

The AflaTest™ WB SR+ immunoaffinity columns (IACs) can be used to cleanup and quantify total and individual aflatoxins (AF) for HPLC, UPLC, LC-MS or LC-MS/MS in many commodities. VICAM's advanced biotechnology permits the measurement of all the major aflatoxins (including AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFM_2) and sterigmatocystin. AflaTest WB SR+ and our complete family of AflaTest columns can be used in any laboratory with LC or LC-MS system - from food processing and Quality Control laboratories to food and feed company laboratories to commercial and government testing laboratories.

1.2 Principle

Aflatoxins are a group of naturally occurring toxins produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin B_1 is a Group 1 carcinogen proven to cause cancer in humans. Aflatoxin can also cause economic losses in livestock due to disease or reduced efficiency of production. AflaTest immunoaffinity columns are a fast, simple, safe, and highly accurate method for quantitatively measuring aflatoxin in many commodities.

Samples are prepared by mixing with an extraction solution, blending, and filtering. The extract is then applied to the AflaTest column containing specific antibodies to aflatoxin. At this stage, the aflatoxin binds to the antibody on the column. The column is then washed with water or buffer to rid the immunoaffinity column of impurities. By passing a mixture of methanol and acetonitrile through the column, the aflatoxin is removed from the antibody. This solution can then be injected into an LC or LC-MS system. These steps are outlined in section 1.7, AflaTest WB SR+ Overview.

1.3 Applicability and Approvals

The AflaTest WB SR+ columns have been optimized for quantitative measurement of aflatoxins in many commodities. The column can tolerate high concentrations of organic solvent, especially acetonitrile (ACN) which is often needed for better extraction. The column capacity is as high as 1,000ng. The Table of Contents lists the testing protocols developed for specific commodities as of the publication date of this manual. Assistance in measuring aflatoxin in commodities not listed in this manual may be obtained by contacting our Technical Services Department (techservice@vicam.com).

	Cartridge Size	Capacity	Storage Temp	Shelf Life*	QC Recovery Criteria
AflaTest WB SR+	3 mL	1,000 ng for total aflatoxins	2 - 30°C	18 months	B_1, B_2, G_1, G_2 ≥ 90% @2ng and @ 500ng

*At room temperature from the date of production.

AflaTest is cited in the AOAC® Official Methods Program, as official method 991.31 applicable for the determination of aflatoxin B_1, B_2, G_1 and G_2 both by fluorometry and HPLC analysis in corn, peanuts and peanut butter. AOAC Official Method 991.31 has final

action status. AflaTest immunoaffinity columns can also be used in AOAC Official Methods 999.07 for aflatoxin determination in peanut butter, pistachio paste, fig paste and paprika powder, in AOAC Official method 2003.02 for the determination of aflatoxin in cattle feed, as well as in AOAC Official Method 2013.05 for Olive Oil, Peanut Oil, and Sesame Oil. The AflaTest WB SR+ column has a very high affinity for aflatoxin which gives its exceptional recoveries and solvent tolerance. **When using the AflaTest WB SR+ column with the AOAC methods, elute the column with 0.75 ml of ACN:MeOH (1:2). Wait 3 to 10 minutes, then add an additional 0.75 mL ACN:MeOH (1:2) for the best recoveries.**

1.4 Limitations

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

Attention for intended user of the AflaTest WB SR+ column, the elution method provided in this report must be used to ensure the highest recovery of each aflatoxin. Any other elution method must be evaluated when using this column to ensure maximal aflatoxin recovery.

1.5 Sampling

Mycotoxins do not occur in every kernel in a lot and may only occur in a small percentage of the kernels in a lot. Because of the wide range in mycotoxin concentrations among individual kernels in a contaminated lot, variation from sample to sample can be large. It is important to obtain a representative sample from a lot. Product should be collected from different locations in a static lot based on a probing pattern. The probe should draw from the top to the bottom of the lot. The samples obtained from the probes should be ground and mixed well and a subsample taken for testing. For further information on grain sampling, refer to the following United States Federal Grain Inspection Service (FGIS) and European Community publications:

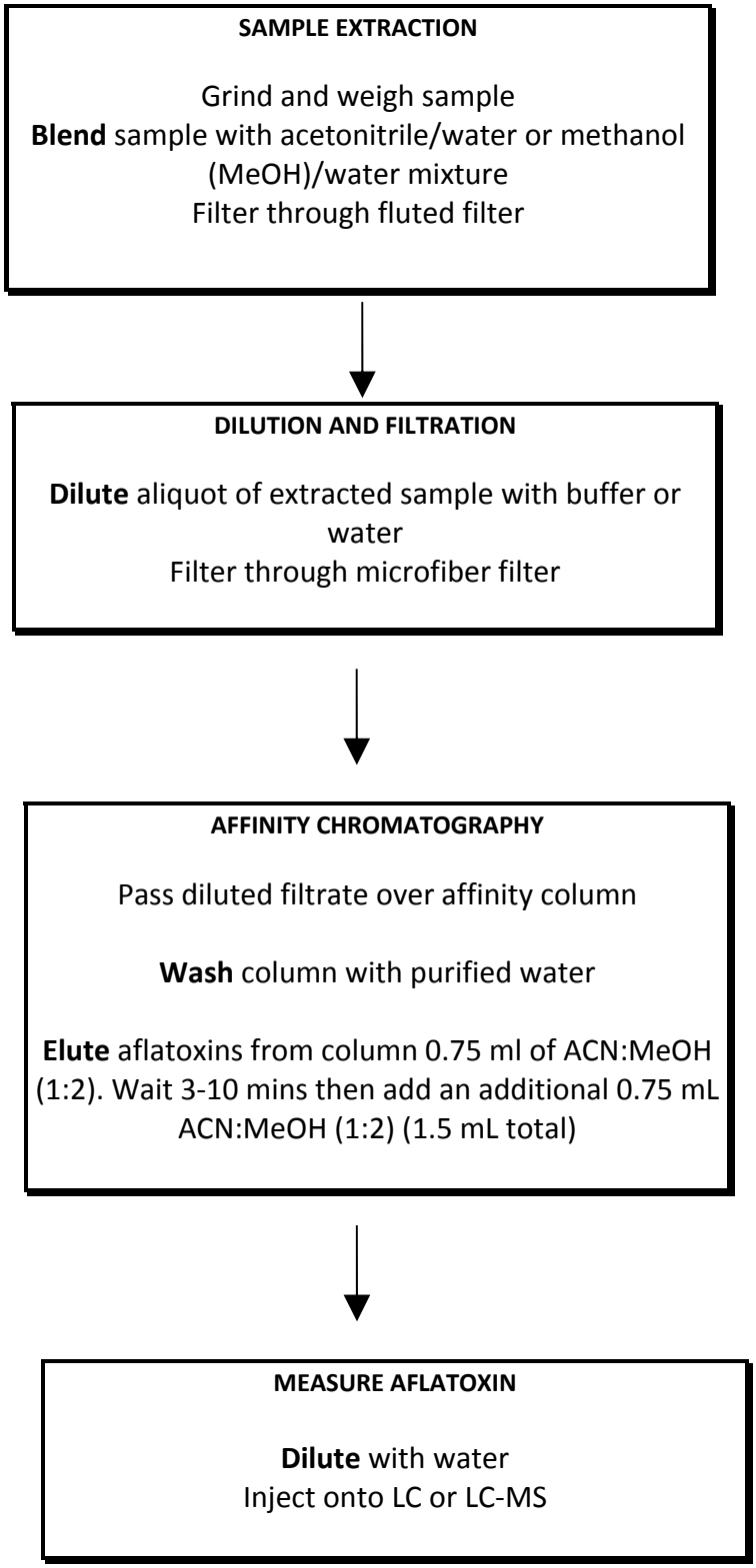
- FGIS Mycotoxin Handbook https://www.ams.usda.gov/sites/default/files/media/MycotoxinHB.pdf
- FGIS Grain Inspection Handbook, Book 1, Grain Sampling https://www.ams.usda.gov/sites/default/files/media/Book1.pdf
- European community sampling procedures can be found in Commission Regulation EC No 401/2006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

1.6 Storage Conditions

Store AflaTest WB SR+ 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 AflaTest WB SR+ Immunoaffinity Column Cleanup Overview



2.0 Equipment Preparation

2.1 Materials and Equipment Required

Materials Required

Description	Part #
AflaTest WB SR+ Columns (25/box)	176004746
AflaTest WB SR+ Kit (100 Columns and 1 set stds)	176004754
Disposable Plastic Pipets, 1 mL (50)	20652
VICAM Fluted Filter Paper, 24 cm (100)	31240
Microfiber Filters, 1.5 mm, 11 cm (100)	31955
5X Concentrate of 2% Tween PBS 300 mL	G1105
10X Concentrate PBS Wash Buffer 150mL	G1113
Disposable Cuvettes (250)	34000
Methanol, HPLC Grade (4 x 4 L)	35016
Disposable Plastic Beakers (25)	36010
Noniodized sodium chloride (salt, NaCl)	G1124
Distilled, reverse osmosis or deionized water	
Acetonitrile, HPLC	

Equipment Required

Description	Part #
Graduated Cylinder, 50 mL	20050
Digital Scale with AC Adapter	20100
Commercial Blender with Stainless Steel Container	20200
Eberbach Glass Blender Jar, 500 mL (for Chinese herbs and spices)	20300
Graduated Cylinder, 250 mL	20250
500 mL Bottle Dispenser for Methanol (0-3 mL range)	20501
Wash Bottle, 500 mL	20700
Cuvette Rack	21010
Filter Funnel, 65 mm (10 per pack)	36020
Filter Funnel, 105 mm (4 per pack)	36022
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
WB column coupling	G1118
PhCR Photochemical Reactor (for HPLC only)	600001222
HPLC, UPLC or LC-MS system as specified in procedure	

Suggested but not required

12-Position Pump Stand w/ air pump 20 mL syringe barrels for AOAC method	600001707
Micro-pipette Tips for 1 mL Micro-pipettor (100)	20656
Micro-pipettor, 1.0 mL	G4033

AflaTest WB SR+ Kit, P/N 176004754

PhCR Photochemical Reactor P/N 600001222

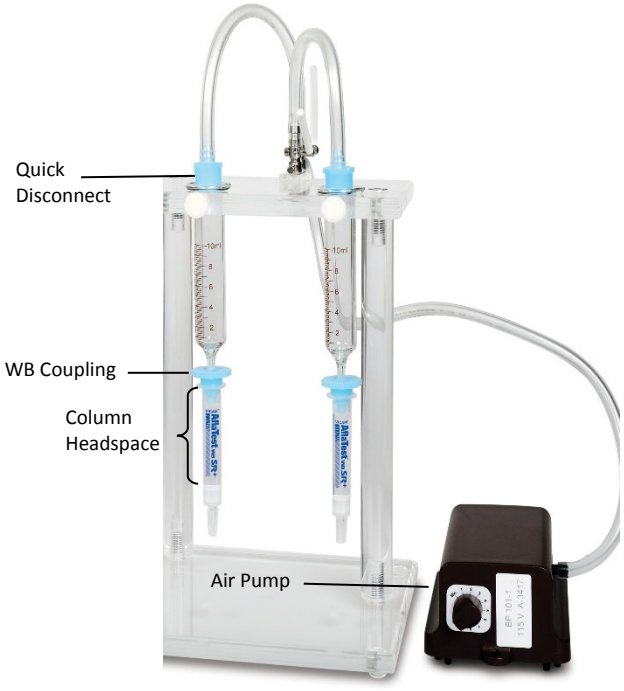
12-Position Pump Stand w/ 6 Pumps 10mL P/N G1104

2.2 Pump Stand Setup

The immunoaffinity chromatography is easily performed with the AflaTest immunoaffinity column attached to a pump stand. The pump stand has a 10 mL glass syringe barrel that serves as a reservoir for the column. An adjustable air pump (P/N 20650) is attached to the pump tube to push liquid through the column using positive pressure. Double position pump stands (P/N 21040), four-position pump stands with aquarium pumps (P/N 21045), and twelve-position pump stands with aquarium pumps (P/N G1104) are available for running multiple samples at one time.

When using a pump stand:

1) Remove large top cap from column. **Do NOT discard the buffer in the column.**
2) Attach column to WB coupling (P/N G1118) and place waste collection cup under column outlet. Keep bottom cap on column.
3) Add desired amount of extract to glass syringe barrel.
4) Remove the bottom cap from columns. Inset quick disconnect on end of tube into syringe barrel. Frequently columns will flow by gravity without need of the pump.
5) 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 or by gravity flow. Repeat for wash and elution.
6) The methanol elution requires less pressure to maintain the 1 drop/2 seconds, or gravity flow. The quick disconnect can be loosened or pulsed to reduce the pressure for the methanol elution.



2.3 Cleaning Equipment

Before Starting AflaTest WB SR+ 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 brand 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. Other pieces of equipment that need to be cleaned with detergent before using are graduated cylinders, funnels and blender jars. Bottle top dispenser need only to be rinsed with methanol before use.

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 top dispenser with soap. Methanol bottle top dispenser needs only to be refilled with methanol.

In between each assay, the syringe barrel reservoir can be rinsed with methanol followed by a rinse with purified water. This will be enough to prevent cross-contamination of samples. After several samples have been tested, the glass syringe barrel should be washed with a brush and detergent and 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.

Other Important Precautions

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

High levels of aflatoxin can be decontaminated by NaOCl (bleach). Swab accidental spills of toxin with 1% NaOCl bleach, leave 10 min, and then add 5% aqueous acetone.

More details on decontamination can be found AOAC OFFICIAL METHODS OF ANALYSIS (2005) Laboratory Safety Appendix B, page 6 http://www.eoma.aoac.org/app_b.pdf

3.0 Solution Preparation

3.1 Preparation of Extraction Solutions

The procedures use acetonitrile/water or methanol/water solution to extract aflatoxin out of the sample.

To prepare extraction solution use reagent grade (or better - i.e. HPLC grade) acetonitrile (ACN) and methanol (MeOH).

ACN: Water	ACN	Purified Water	Total Volume
80:20	800 mL	200 mL	1000 mL
90:10	900 mL	100 mL	1000 mL

MeOH: Water	MeOH	Purified Water	Total Volume
70:30	700 mL	300 mL	1000 mL

CAUTION: Extraction solvent is flammable. Keep container tightly capped when not in use. Prepare extraction solution every week or as needed. The formulas above will prepare 1 liter of solution. Solution volume may be increased or decreased as needed provided the proportion of reagents is kept consistent.

3.2 Preparation of Elution Solution

The formulas below will prepare 150 mL of solution. Solution volume may be increased or decreased as needed provided the proportion of reagents is kept consistent.

Elution Solution should be prepared every week or as needed.

ACN: MeOH	HPLC Grade ACN	HPLC Grade MeOH	Total Volume
(1:2)	50 mL	100 mL	150 mL

3.3 Preparation of HPLC or UPLC Mobile Phases

MeOH: Water	HPLC Grade MeOH	Purified Water	Total Volume
45:55	450 mL	550 mL	1000 mL

ACN: Water	HPLC Grade ACN	Purified Water	Total Volume
60:40	600 mL	400 mL	1000 mL

Solutions should be filtered and degassed before use.

4.0 Immunoaffinity Column Clean Up Procedures for LC and LC-MS

4.1 AflaTest WB SR+ Procedure for Aflatoxins and Sterigmatocystin in Corn, Wheat, Oats, and other Grains (0 - 1600 PPB)

4.1.1 LC Set up:

See HPLC and UPLC conditions or alternative instrumental conditions listed in section 5.0

4.1.2 Sample Extraction*:

4.1.2.1 Place 25g finely ground sample and 100 mL 80% acetonitrile/water (80/20, V/V) in a blender jar.

4.1.2.2 Cover blender jar and blend at high speed for 2 minutes.

4.1.2.3 Remove cover from jar and pour extract into fluted filter paper (P/N 31240). Collect filtrate in a clean vessel.

*Alternatively, 5g of salt can be added to the extraction at step 4.1.2.1 and the extract diluted with water at step 4.1.3.2

4.1.3 Extract Dilution

4.1.3.1 Pipet or pour 10 mL filtered extract into a clean vessel.

4.1.3.2 Dilute extract with 40 mL of 1 X PBS (P/N G1113). Mix well.

4.1.3.3 Filter dilute extract through microfiber filter (P/N 31955) into a clean vessel.

4.1.4 Column Chromatography

4.1.4.1 Pass 10 mL filtered diluted extract (10 mL = 0.5g sample equivalent) through AflaTest WB SR+ column at a rate of about 1 drop/second or by gravity until entire extract enters the resin bed. Do not dry column at this step.

4.1.4.2 Pass 10 mL of purified water through the column at a rate of about 1-2 drops/second. Pump air through the column for a few seconds at the end of this step.

4.1.4.3 Place glass cuvette (P/N 34000) under column and add 0.75 mL eluting solution (section 3.2) directly into column headspace.

4.1.4.4 Elute column by gravity (do not pump air through column). Let it stand for at least 3 minutes, but no longer than 10 minutes.

4.1.4.5 Add another 0.75 mL of the eluting solution to column headspace, eluting by gravity.

4.1.4.6 At the end of second 0.75mL elution, connect air pump, turn to maximum for a few seconds, and collect all the eluting solution residues from column (1.5mL). Gently vortex to mix eluate.

4.1.4.7 Add 1.5 mL of purified water to eluate and vortex briefly to mix. For aflatoxin detection: inject 50 µL onto **HPLC**. Alternatively, pipette 200 µL of eluate, mix well with 200 µL purified water, and inject 6 µL onto Waters Acquity **UPLC** system. For sterigmatocystin detection: inject 20 µL of diluted sample eluates onto UPLC system. The UPLC conditions are outlined at step 5.4.

Note: To increase detection sensitivity of STC, drying eluate is required. Take 1mL of eluate to dry at 45°C under nitrogen for 10-15min (check during drying). Once dried, add 200µl of 100% ACN, briefly vortex (3 times at 10 second each), then add 200µl of water, and mix well by vortex. Inject 20µl onto UPLC.

4.1.5 Limit of Detection and Limit of Quantitation: (Using UPLC)

Aflatoxins	LOD (ng)	LOQ (ng)
G_2	0.008	0.015
G_1	0.034	0.083
B_2	0.007	0.012
B_1	0.037	0.057

Sterigmatocystin LOD and LOD in Corn

Eluate	LOD (ppb)	LOQ (ppb)
With Drying	0.96	1.42
Without Drying	2.31	3.23

4.2 AflaTest WB SR+ Procedure for Raw Peanuts (0 - 800 PPB)

(This procedure can be used for Cashews, Apricot Nuts, Almonds, Pistachios, Walnuts, and Pecans.)

4.2.1 HPLC Set up:

See HPLC and UPLC conditions or alternative instrumental conditions listed in section 5.0.

4.2.2 Sample Extraction:

4.2.2.1 Weigh 25g ground sample with 5g salt (NaCl) and place in blender jar.
4.2.2.2 Add to jar 125 mL methanol: water (70:30).
4.2.2.3 Cover blender jar and blend at high speed for 2 minutes.
4.2.2.4 Remove cover from jar and pour extract into fluted filter paper (P/N 31240). Collect filtrate in a clean vessel.

4.2.3 Extract Dilution

4.2.3.1 Pipet or pour 15 mL filtered extract into a clean vessel.
4.2.3.2 Dilute extract with 30 mL of purified water. Mix well.
4.2.3.3 Filter dilute extract through microfiber filter (P/N 31955) into a clean vessel.

4.2.4 Column Chromatography

4.2.4.1 Pass 15 mL filtered diluted extract (15 mL = 1g sample equivalent) through AflaTest column at a rate of about 1 drop/second until entire extract enters the resin bed. Do not dry column at this step.
4.2.4.2 Pass 10 mL of purified water through the column at a rate of about 1-2 drops/second.
4.2.4.3 Repeat step 4.2.4.2 once more. Pump air through the column for a few seconds at the end of this step.
4.2.4.4 Place glass cuvette (P/N 34000) under column and add 0.75 mL of the eluting solution (section 3.2) directly into column headspace.
4.2.4.5 Elute column by gravity (do not pump air through column). Let it stand for at least 3 minutes, but no longer than 10 minutes.
4.2.4.6 Add another 0.75 mL of the eluting solution to column headspace, eluting by gravity.
4.2.4.7 At the end of second 0.75mL elution, connect air pump, turn pump to maximum for a few seconds, and collect all the eluting solution residues from column (1.5 mL). Gently vortex to mix eluate.
4.2.4.8 Add 1.5 mL of purified water to eluate and vortex briefly to mix. Inject 50 µL onto **HPLC**. Alternatively, pipette 200 µL of eluate, mix well with 200 µL purified water, and inject 6 µL onto Waters Acquity **UPLC** system.

4.3 AflaTest WB SR+ Procedure for Chinese Herbs, Cocoa, Roasted Coffee, Dog Food, and Spices (0 - 3200 PPB)

4.3.1 HPLC Set up:
See HPLC or UPLC conditions listed in section 5.1 and 5.2.

4.3.2 Sample Extraction:

4.3.2.1 Place 10 g finely ground sample and 100 mL 90% acetonitrile/water (90/10, V/V) in a blender jar (use a blender jar size of 500 mL or less).

4.3.2.2 Cover blender jar and blend at low speed for 2 minutes, then at high speed for 1 minute.

4.3.2.3 Remove cover from jar and pour extract into fluted filter paper (P/N 31240). Collect filtrate in a clean vessel.

4.3.3 Extract Dilution

4.3.3.1 Pipet or pour 10 mL filtered extract into a clean vessel.

4.3.3.2 Dilute extract with 30 mL of 1 X 2%Tween/PBS (P/N G1105). Mix well.

4.3.3.3 Filter dilute extract through microfiber filter (P/N 31955) into a clean vessel. **Note: if the diluted extract remains clear without precipitation, no filtration is needed.**

4.3.4 Column Chromatography

4.3.4.1 Pass 10 mL filtered diluted extract (10 mL = 0.25g sample equivalent) through AflaTest WB SR+ column at a rate of about 1 drop/second or by gravity until entire extract enters the resin bed. Do not dry column at this step.

4.3.4.2 Pass 10 mL of purified water twice through the column at a rate of about 1-2 drops/second. Pump air through the column for a few seconds at the end of second wash step.

4.3.4.3 Place glass cuvette (P/N 34000) under column and add 0.75 mL eluting solution directly into column headspace.

4.3.4.4 Elute column by gravity (do not pump air through column). Let it stand for at least 3 minutes, but no longer than 10 minutes.

4.3.4.5 Add another 0.75 mL of the eluting solution to column headspace, eluting by gravity.

4.3.4.6 At the end of second 0.75mL elution, connect air pump, turn to maximum for a few seconds, and collect all the eluting solution residues from column (1.5mL). Gently vortex to mix eluate.

4.3.4.7 Add 1.5 mL of purified water to eluate and vortex briefly to mix. Inject 50 µL onto **HPLC**. Alternatively, pipette 200 µL of eluate, mix well with 200 µL purified water, and inject 6 µL of samples and standards onto Waters Acquity **UPLC** system.

4.3.5 Recovery: For spiked *Fallopia multiflora*:

Spiked Levels (total aflatoxins)	Recovery %			
	G_2	G_1	B_2	B_1
5 ppb	98.7	104.7	96.0	102.7
20ppb	96.0	99.0	93.3	98.0

4.4 AflaTest WB SR+ Procedure for Peanut Oil (0 - 3200 PPB)

4.4.1 HPLC Set up:

See HPLC or UPLC conditions listed in section 5.1 and 5.2.

4.4.2 Sample Extraction:

4.4.2.1 Weigh 3 g of peanut oil in a 50 mL conical tube and then add 30 mL of 90% acetonitrile/water (90/10,V/V). Vortex vigorously for 2 min using Vortex Genie II or equivalent at high speed.

4.4.2.2 Let it sit for 10 min on a table, then take an adequate amount of top layer and filter it with microfiber filter (P/N 31955). Optional: centrifuge at 5000g for 10 min.

4.4.3 Extract Dilution

4.4.3.1 Pipet 10mL of filtrate from step 4.4.2.2 (or 10 mL from top layer of centrifuged sample) into a clean vessel and add 30 mL of 1 X 2%Tween/PBS (P/N G1105). Mix well.

4.4.3.2 Filter diluted extract through microfiber filter (P/N 31955) into a clean vessel. **Note: if the diluted extract remains clear without precipitation, no filtration is needed.**

4.4.4 Column Chromatography

4.4.4.1 Pass 10 mL filtered diluted extract (10 mL = 0.25g sample equivalent) completely through AflaTest WB SR+ column at a rate of about 1 drop/second or by gravity until entire extract enters the resin bed. Do not dry column at this step.

4.4.4.2 Pass 10 mL of purified water through the column at a rate of about 1-2 drops/second. Pump air through the column for a few seconds at the end of this step.

4.4.4.3 Place glass cuvette (P/N 34000) under column and add 0.75 mL eluting solution directly into column headspace.

4.4.4.4 Elute column by gravity (do not pump air through column). Let it stand for at least 3 minutes, but no longer than 10 minutes.

4.4.4.5 Add another 0.75 mL of the eluting solution to column headspace, eluting by gravity.

4.4.4.6 At the end of second 0.75mL elution, connect air pump, turn to maximum for a few seconds, and collect all the eluting solution residues from column (1.5mL). Gently vortex to mix eluate.

4.4.4.7 Add 1.5 mL of purified water to eluate and vortex briefly to mix. Inject 50 µL onto **HPLC**. Alternatively, pipette 200 µL of eluate, mix well with 200 µL purified water, and inject 6 µL of samples and standards onto Waters Acquity **UPLC** system.

4.5 AflaTest WB SR+ Procedure for Aflatoxins and Sterigmatocystin in Infant Formula (0 - 3200 PPB)

4.5.1 HPLC Set up:

See HPLC or UPLC conditions listed in section 5.3 and 5.4.

4.5.2 Sample Extraction:

4.5.2.1 Weigh 2 g of infant formula in a 50 mL conical tube, add 4 mL of distilled water, then vortex briefly to completely dissolve infant formula. Add 6 mL 100% acetonitrile to the conical tube, then vortex vigorously for 2 min by using Vortex Genie II or equivalent at high speed.

4.5.2.2 Let it sit for 2-5 min on a table.

4.5.3 Extract Dilution

4.5.3.1 Pipet 5mL of supernatant to another 50 mL tube containing 20 mL 1x PBS (P/N G1113) and mix well by vortexing.

4.5.4 Column Chromatography

4.5.4.1 Pass 10 mL diluted extract (10 mL = 0.4 g sample equivalent) completely through AflaTest WB SR+ column at a rate of about 1 drop/second or by gravity until entire extract enters the resin bed. Do not dry column at this step.

4.5.4.2 Pass 10 mL of purified water through the column at a rate of about 1-2 drops/second. Pump air through the column for a few seconds at the end of this step.

4.5.4.3 Place glass cuvette (P/N 34000) under column and add 0.75 mL eluting solution directly into column headspace.

4.5.4.4 Elute column by gravity (do not pump air through column). Let it stand for at least 3 minutes, but no longer than 10 minutes.

4.5.4.5 Add another 0.75 mL of the eluting solution to column headspace, eluting by gravity.

4.5.4.6 At the end of second 0.75mL elution, connect air pump, turn to maximum for a few seconds, and collect all the eluting solution residues from column (1.5mL). Gently vortex to mix eluate.

4.5.4.7 Add 1.5 mL of purified water to eluate and vortex briefly to mix. For aflatoxin detection: inject 50 ul onto **HPLC**. Alternatively, pipette 200 µL of eluate, mix well with 200 µL purified water, and inject 10 µL of samples and standards onto Waters Acquity **UPLC** system. For STC detection: inject 20 µL of diluted sample eluates and standards onto UPLC system.

Note: To increase detection sensitivity of STC, drying eluate is required. Take 1mL of eluate to dry at 45°C under nitrogen gas for 10-15min (check during drying). Once dried, add 200µl of 100% ACN, briefly vortex (3 times at 10 second each), then add 200µl of water, and mix well by vortex. Inject 20µl onto UPLC.

4.6 Other Previously Established Procedures

When using the AflaTest WB SR+ column with previously established methods, elute the column with 0.75 ml of Acetonitrile:Methanol (1:2). Wait 3-10 minutes then elute with an additional 0.75 mL Acetonitrile:Methanol (1:2) for the best recoveries. Please refer to section 4.0 for detailed elution protocols.

5.0 LC and LC-MS/MS Setup

5.1 HPLC Condition for Simultaneous Detection of Four Aflatoxins (B_1, B_2, G_1, and G_2)

HPLC System	Waters Alliance e2695 with Multi-Fluorescence Detector 2475
Column	Waters Nova-Pak C18, 4 mm, 3.9 mm X 150 mm (P/N WAT086344)
Post column derivatization	Photochemical reactor: PhCR (P/N 600001222)
Mobile phase	Water:methanol (55:45 v/v) Isocratic
Flow rate	0.8 mL/min
Injection volume	50 µL
Fluorescence condition	Excitation 360 nm : Emission 440 nm
Run time	12 minutes
Software	Waters Empower™

5.2 UPLC Conditions for Simultaneous Detection of Four Aflatoxins (B_1, B_2, G_1, and G_2)

UPLC System	Waters Acquity H-Class with FLR Detector
Column	Waters Acquity UPLC HSS T3, 1.8 µm, 2.1 x 100 mm (Waters P/N 186009468)
Mobile phase	Water:methanol (55:45 v/v) Isocratic
Flow rate	0.3 mL/min
Injection volume	6 µL
Fluorescence condition	Excitation 360 nm : Emission 440 nm
Flow cell	FLR Large Volume (Waters P/N 205000609)
Run time	8 minutes
Software	Waters Empower™

5.3 UPLC Conditions for Simultaneous Detection of Six Aflatoxins (B_1, B_2, G_1, G_2, M_1 and M_2)

UPLC System	Waters Acquity H-Class with FLR Detector
Column	Waters Acquity UPLC HSS T3, 1.8 µm, 2.1 x 100 mm (Waters P/N 186009468)
Injection volume	10 µL
Fluorescence condition	Excitation 365 nm : Emission 445 nm
Column Temperature	25°C
Flow cell	FLR Large Volume (Waters P/N 205000609)
Run time	7 minutes
Software	Waters Empower™

Mobile Phase: gradient seen below

Minutes	Flow rate	% water	% methanol	% acetonitrile	Curve
initial	0.4ml/min	64	18	18	
6	0.4ml/min	66	11	23	6
6.5	0.4ml/min	64	18	18	11

5.4 UPLC Conditions for Detection of Sterigmatocystin

UPLC System	Waters Acquity H-Class with UV Detector
Column	Waters Acquity UPLC HSS T3, 1.8 µm, 2.1 x 100 mm (Waters P/N 186009468)
Mobile phase	Water:acetonitrile (40:60 v/v) Isocratic
Flow rate	0.4 mL/min
Injection volume	20 µL
UV detector	Wavelength: 325nm
Column Temperature	30°C
Run time	3 minutes
Software	Waters Empower™

5.5 UPLC-MS/MS Conditions

UPLC Condition		MS Condition	
UPLC System	ACQUITY UPLC I-Class	MS System	Xevo TQS-XS
Column	CORTECS UPLC®, C_{18},, 2.1 x 50 mm, 1.65 µm Waters P/N 186007093	Ionization mode	ESI positive
Column Temp	25°C	Collision gas (N2)	3.00 x 10^{-3} mbar
Sample Temp	25°C	Capillary voltage	1.0 kV
Mobile Phase A	H_2O with 10 mM ammonium acetate	Cone voltage	Aflatoxin B_1, B_2, G_1 & G_2 – 35 V,
Mobile Phase B	Methanol	Source temp	150°C
Flow Rate	0.450 mL/minute	Desolvation temp	550°C
Injection Volume	5 µL	Desolvation gas	1000 L/hr
Retention Time	Varies per analyte; refer to MS Parameters section (below)	Cone gas	250 L/hr

Gradient:

35 to 85% B over 2 minutes,
85 to 100% B over 0.5 minutes,
100 to 35% B over 1 minutes.

MS/MS Parameters for the Analytes

Toxin	Pseudo-molecular	RT*(min)	Precursor Ion	Product Ion		CV*	CE*
				Quantification	qualifier		
AFG_2	$[M+H]^+$	1.15	331.1	245.1	313	45	28/24
AFG_1	$[M+H]^+$	1.39	329.1	243.1	311.1	45	26/22
AFB_2	$[M+H]^+$	1.58	315.1	259.1	287.1	40	26/26
AFB_1	$[M+H]^+$	1.74	313.1	241.1	285.1	40	40/22

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

5.6 HPLC Standard Preparation and Sample Spiking

A Hamilton Syringe is preferred for spiking samples and preparing standards, but an adjustable micropipettor with disposable plastic tips can also be used. Use only HPLC grade methanol and acetonitrile when preparing standard solutions.

5.6.1 Aflatoxin solutions

The Supelco aflatoxin standard product #CRM46304 comes in sealed ampules. The concentration of this aflatoxin standard stock solution is about 2.6ng/µL in methanol. This standard is prepared according to AOAC Official methods. The certificate of analysis will show the exact concentration of each of the 4 different aflatoxins. An opened ampule should be able to be used for up to two weeks when stored at 2 – 8 °C.

Prepare a working solution I: 0.26 ng/µL aflatoxin standard by adding 100µL of the 2.6ng/µL aflatoxin standard stock solution to 900µL methanol.

Prepare a working solution II: 0.026 ng/µL aflatoxin standard by adding 100µL of the 0.26ng/µL aflatoxin standard to 900µL methanol.

5.6.2 Spiking corn with aflatoxin at 26 ppb level

26 ppb (ng/g) X 25g corn = 650 ng
650 ng ÷ 2.6 ng/µL = 250 µL
Add 250 µL of the 2.6 ng/µL aflatoxin standard to 25 g of aflatoxin-free corn

Allow the spiked sample to dry in a hood for at least 30 minutes before assaying.

5.6.3 Preparing aflatoxin LC standards for 0.5-gram equivalent procedures

1.3 ppb (0.5 B_1:0.15 B_2:0.5 G_1:0.15 G_2 ng/g) X 0.5 g = 0.65 ng
0.65 ng ÷ 0.026ng/µL standard = 25 µL
25 µL 0.026ng/µL standard added to 1475 µl eluting solution (a mixture of acetonitrile and methanol with ratio 1/2 , V/V)

2.6 ppb (1.0 B_1:0.3 B_2:1.0 G_1:0.3 G_2 ng/g) X 0.5 g = 1.3 ng
1.3 ng ÷ 0.026ng/µL standard = 50 µL
50 µL 0.026ng/µL standard added to 1450 µl eluting solution

26 ppb (10 B_1:3.0 B_2:10 G_1:3.0 G_2 ng/g) X 0.5 g = 13 ng
13 ng ÷ 0.26ng/µL standard = 50 µL
50 µL 0.26ng/µL standard added to 1450 µl eluting solution

52 ppb (20 B_1:6.0 B_2:20 G_1:6.0 G_2 ng/g) X 0.5 g = 26 ng
26 ng ÷ 0.26ng/µL standard = 100 µL
100 µL 0.26ng/µL standard added to 1400 µl eluting solution

Add 1.5 mL water to the 1.5 mL of eluting solution for all standards and samples before injecting onto the LC. Adding water to the standards and samples makes them like the LC mobile phase.

Or for UPLC, pipette 200 µL of standard and mix well with 200 µL purified water.

5.6.4 Sterigmatocystin solutions

The Sigma sterigmatocystin(STC) standard product #S3255 is in powder form. To make 5 ng/µL stock solution, dissolve 5 mg STC in 1 mL acetonitrile completely, then store it at 4°C.

Prepare a working solution I: 0.5 ng/µL STC standard by adding 100 µL of the 5 ng/µL STC standard stock solution to 900µL eluting solution (a mixture of acetonitrile and methanol with ratio 1/2 , V/V).

Prepare a working solution II: 0.05 ng/µL STC standard by adding 100µL of the 0.5ng/µL STC standard stock solution to 900µL eluting solution.

5.6.5 Spiking corn with STC at 25 ppb level

25 ppb (ng/g) X 25g corn = 625 ng
625 ng ÷ 5 ng/µL (stock solution 5mg/ mL) = 125 µL
Add 125 µL of the 5 ng/µL STC standard to 25 g of aflatoxin and STC-free corn

Allow the spiked sample to dry in a hood for at least 30 minutes before assaying.

5.6.6 Preparing STC LC standards for 0.5-gram equivalent procedures

5 ppb (ng/g) X 0.5 g = 2.5 ng
2.5 ng ÷ 0.05ng/µL standard = 50 µL

50 µL 0.05g/µL standard added to 1450 µl eluting solution

10 ppb (ng/g) X 0.5 g = 5 ng
5 ng ÷ 0.05ng/µL standard = 100 µL
100 µL 0.05ng/µL standard added to 1400 µl eluting solution

25 ppb (ng/g) X 0.5 g = 12.5 ng
12.5 ng ÷ 0.5ng/µL standard = 25 µL
25 µL 0.5ng/µL standard added to 1475 µl eluting solution

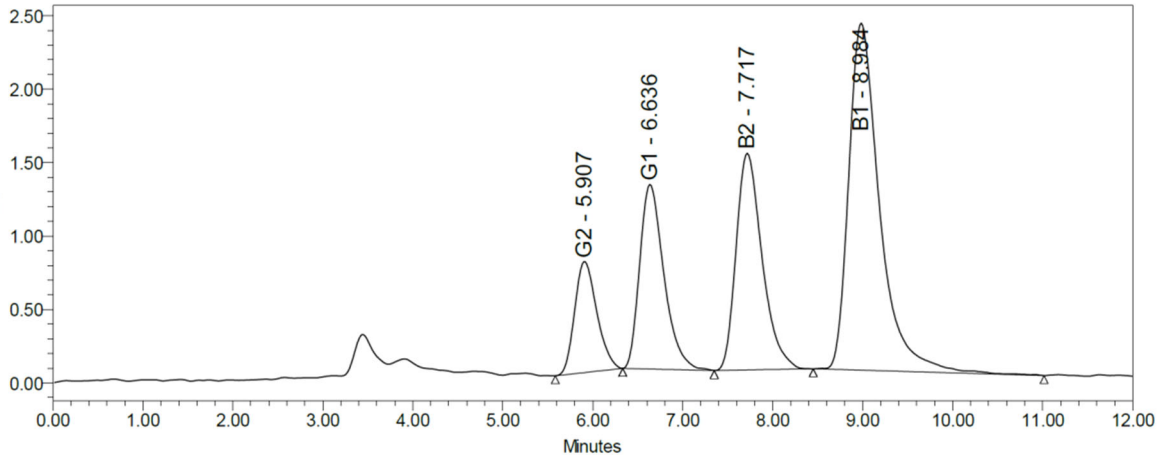
50 ppb (ng/g) X 0.5 g = 25 ng
25 ng ÷ 0.5ng/µL standard = 50 µL
50 µL 0.5ng/µL standard added to 1450 µl eluting solution

Pipette 200 µL of standard and mix well with 200 µL purified water for all standards and samples before injecting onto the LC. Adding water to the standards and samples makes them like the LC mobile phase.

Make a graph of ppb level of the standards vs peak area. The peak area of the unknown samples is then plugged into the standard curve to calculate the ppb value of the samples. This calculation can be done with the software provided by an HPLC manufacturer. In addition, this calculation can be done using Microsoft Excel software.

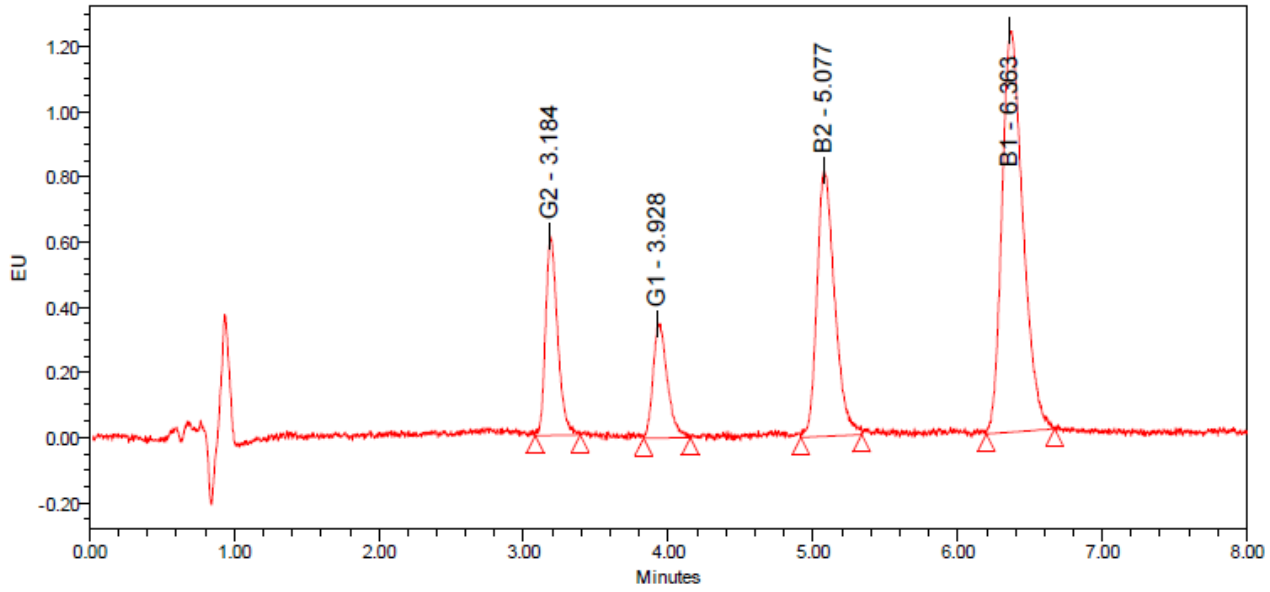
5.7 Representative Chromatograms

5.7.1 Representative Chromatogram for HPLC Condition

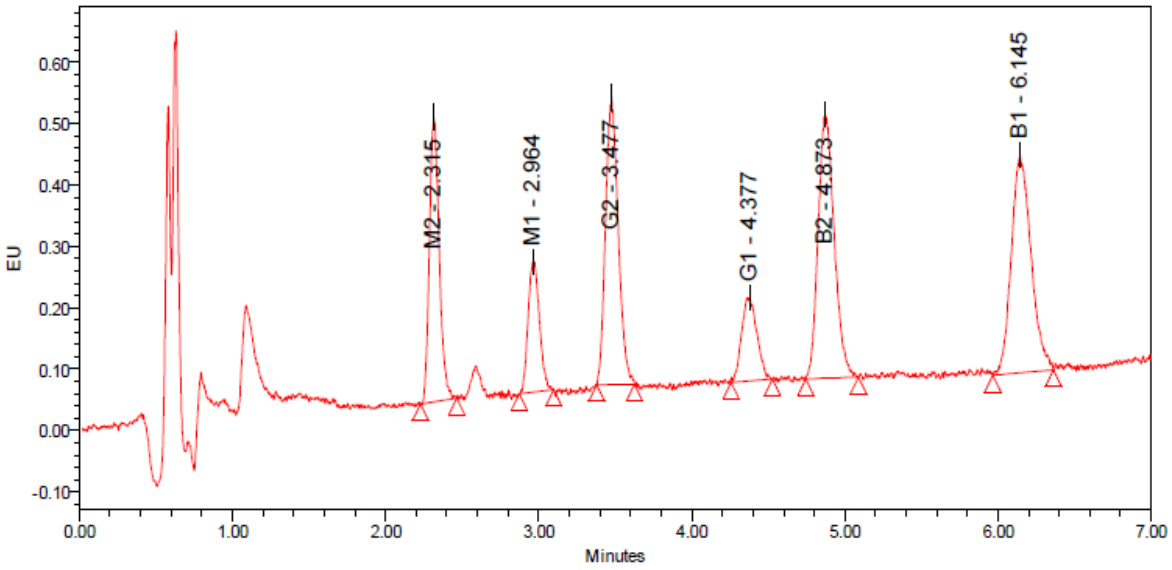


4 ppb total aflatoxin (B_1:B_2:G_1:G_2, 1: 0.3: 1: 0.3) spiked wheat sample

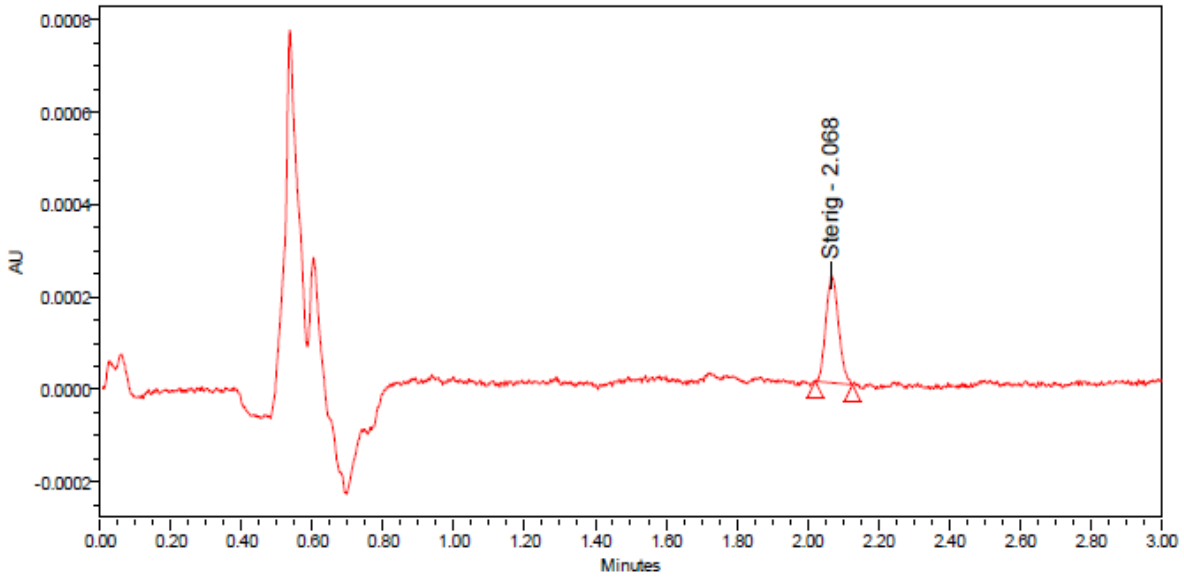
5.7.2 Representative Chromatogram for UPLC Condition



4 ppb total aflatoxin (B_1:B_2:G_1:G_2, 5:1:3:1) spiked corn sample



0.5 ppb aflatoxin M_2 , 0.5 ppb aflatoxin M_1 , and 2 ppb total aflatoxin mixture (B_1:B_2:G_1:G_2, 5:1:3:1) spiked infant formula



20 ppb sterigmatocystin spiked infant formula

6.0 General Precautions for LC

1. If sample eluate is dried, use silanized vials to avoid irreversible binding of aflatoxins to the tube walls.
2. Disc filters used to filter eluates before injection on to LC may bind aflatoxins. Eluates from immunoaffinity column chromatography are usually clean enough to dilute and inject without filtration.
3. For greater accuracy, 1.5 mL column eluate + 1.5 mL water and 1.5 mL standards + 1.5 mL water can be measured to exactly 3 mL total volume using a 3 mL volumetric flask.

7.0 Technical Assistance and Ordering Information

For technical assistance or ordering please contact VICAM or your local distributor

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

8.0 Liability

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