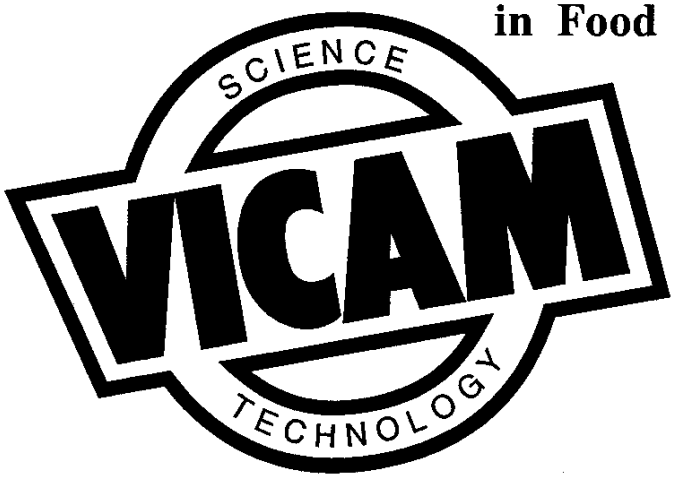
Your Silent Partner in Food Safety



AflaOchra HPLC™

Instruction Manual

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Effective date: August 3, 1999

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AflaOchra HPLC™ from Vicam is the only test that employs a single column to produce precise numerical results for both Aflatoxin and Ochratoxin. Using monoclonal affinity chromatography, the AflaOchra HPLC™ can isolate Aflatoxins B_1, B_2, G_1 and G_2 from grain, food, feed and nuts and Ochratoxin A and its stereoisomer from coffee, beer, grain and feed. AflaOchra HPLC™ is based on the same technology that has already been approved by the AOAC and FGIS for the widely used AflaTest™. You have the best of all the worlds: sensitivity, simplicity and speed – quick test for parts per billion levels. AflaOchra HPLC™ is the ideal cleanup step for any HPLC analysis. In fact, no other test comes close for speed, quantification and economy.

1.1 INTENDED USER

AflaOchra HPLC™ from Vicam is the only test that employs a single column to produce precise numerical results for both Ochratoxin A and the Aflatoxins B_1, B_2, G_1 and G_2 in a variety of commodities. AflaOchra HPLC™ is safe and simple. It can be performed in less than 30 minutes (excluding sample preparations and extraction) and requires only basic HPLC skills. Unaffected by heat or humidity, AflaOchra HPLC™ can be performed virtually anywhere.

1.2 PRINCIPLE

Ochratoxin A is a mycotoxin produced by the fungus *Aspergillus ochraceous* and also by several species of *Penicillium* fungi. Ochratoxin has been known to cause kidney damage and decreased egg production in animals. It is an immunosuppressant and is considered a potential carcinogen. Aflatoxin, a toxin from a naturally occurring mold, is a Group I carcinogen proven to cause cancer in humans. Aflatoxin can also cause economic losses in livestock due to disease or reduced efficiency of production.

Samples are prepared by mixing with an extraction solution, blending and filtering. The extract is then applied to the AflaOchra™ column bound with specific antibodies for both aflatoxins and ochratoxin A. Both aflatoxin and ochratoxin A bind to the appropriate antibody. The column is then washed with water to rid the immunoaffinity column of impurities. By passing methanol through the column, the aflatoxins as well as ochratoxin A are removed from the antibody. This methanol solution can then be injected into an HPLC system to determine aflatoxin or ochratoxin. These steps are outlined in section 1.6, AflaOchra HPLC™ Overview.

1.3 APPLICABILITY AND APPROVALS

AflaOchra HPLC™ has been optimized for quantitative measurement of aflatoxins and ochratoxin A in corn and wheat. Assistance in measuring aflatoxins and ochratoxin in commodities not listed in this manual can be obtained by contacting our Technical Assistance Department.

AflaOchra HPLC™ uses the same technology that has been approved by the AOAC as an Official Method for aflatoxin testing in corn, peanuts and peanut butter [1]. The AflaOchra HPLC™ column also uses the same technology to detect ochratoxin as the one used in Vicam's widely used OchraTest™.

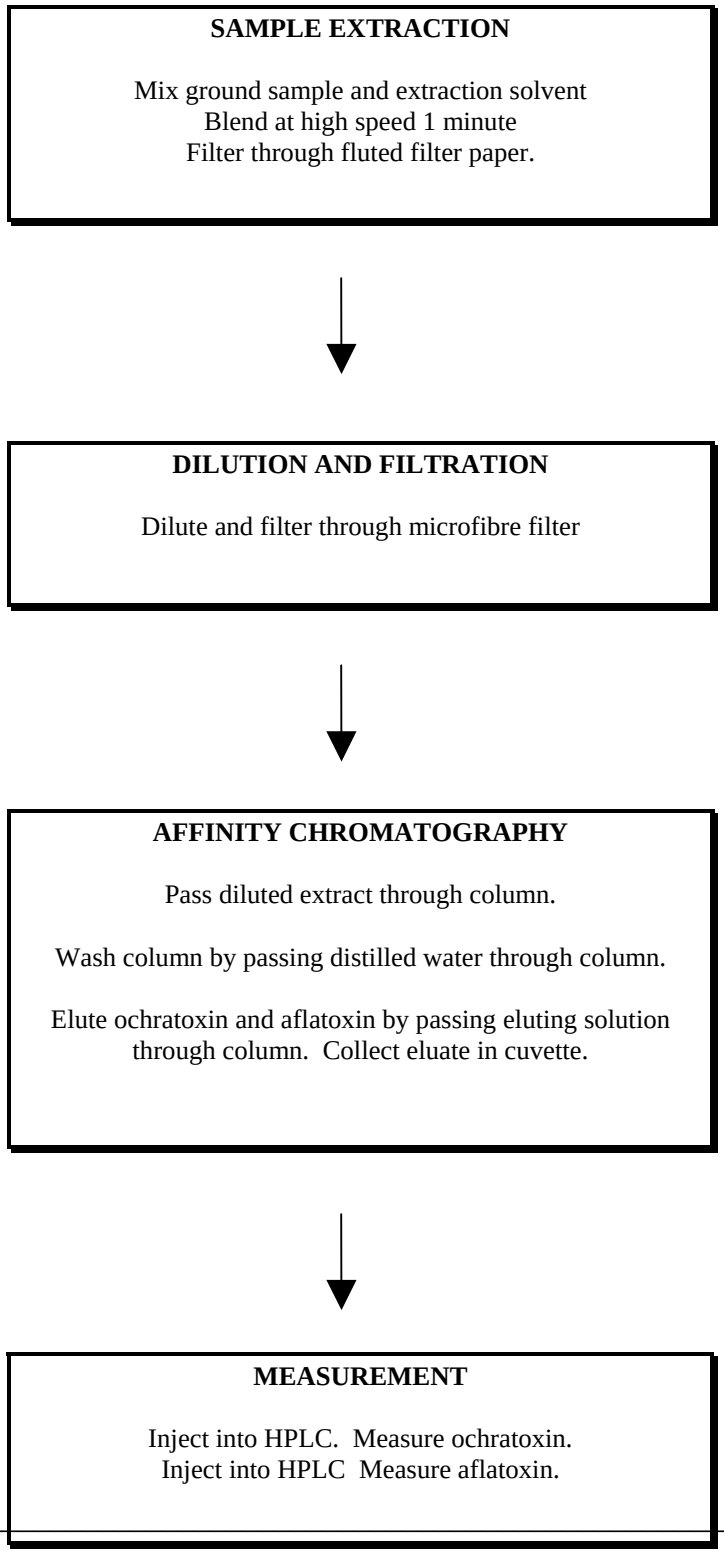
1.4 LIMITATIONS

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.

1.5 SHELF LIFE AND STORAGE CONDITIONS

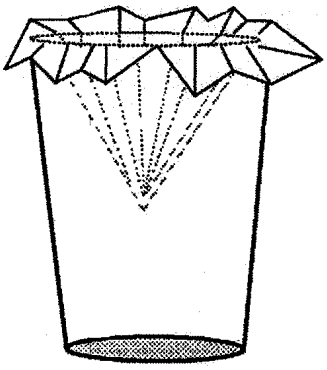
Store at room temperature. Storage at temperatures above 30°C for prolonged periods of time may reduce shelf life. If storage temperatures above 30°C are anticipated, all components may be stored at 4°C. It is recommended that reagents should be at room temperature (18 - 22°C) for usage.

1.6 AFLAOCHRA HPLC™ PROCEDURE OVERVIEW



2.1 PREPARATION OF FILTRATION STEPS

Fluted Filter



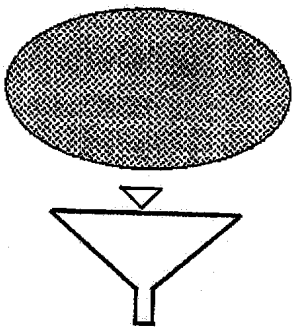
Fluted Filter Assembly

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 funnel may be used to hold the filter).

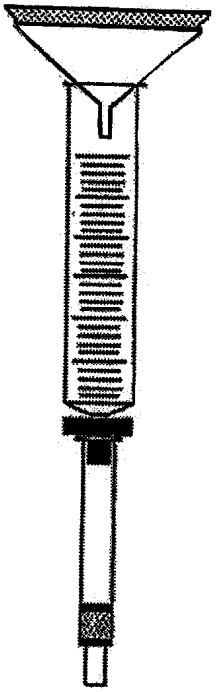
2. Fold edges of filter over rim of cup to hold in place. Maintain the fluted folds of the filter paper to maximize surface area. This will increase speed of filtration.

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



Microfibre Filter

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



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

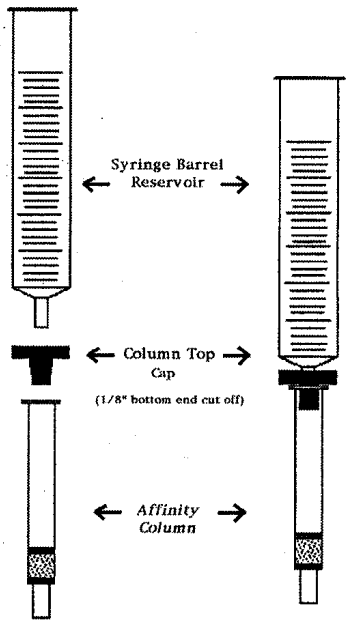
2. Place one microfibre filter gently into small funnel by pressing filter into funnel with index finger. Be careful not to rip or puncture the filter.

2.2 PUMP STAND SETUP

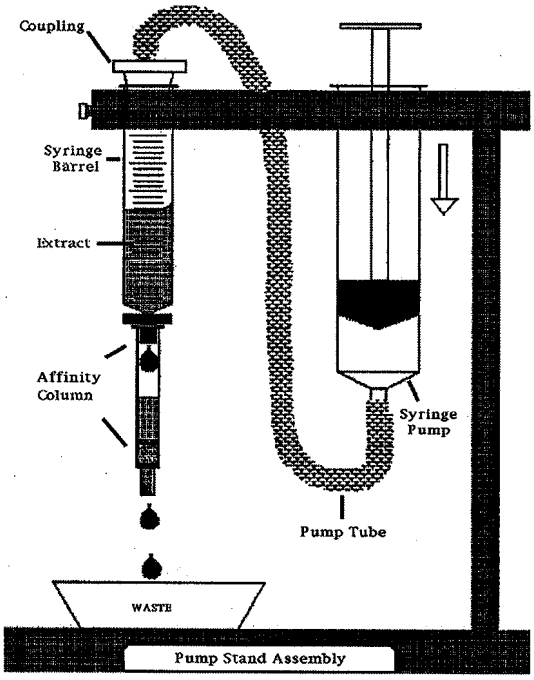
AflaOchra HPLC™ affinity chromatography is easily performed with the AflaOchra HPLC™ affinity column attached to a pump stand (part # 21020). The stand has a 10 mL glass syringe barrel that serves as a reservoir for the column. A large plastic syringe with tubing and coupling provides air pressure to manually push liquids through the column. An adjustable aquarium pump (part #20650) can be attached to the pump tube instead of the large pump syringe barrel to operate without using hand pressure. Double pump stand (part # 21030), four-position pump stand with aquarium pumps (part #21045), and twelve-position pump stand with aquarium pumps (part # G1104) are available for running multiple samples at one time.

1. Remove large top cap from column.
2. Attach the plastic coupling (part # G1109) to the column. This coupling can be reused for additional columns.
3. Place waste collection cup under column outlet. Keep bottom cap on column.
4. Pour extract into microfibre filter (see previous section) and collect desired amount of extract in glass syringe barrel using markings on the syringe barrel to measure extract.
5. Pull up on the plastic syringe piston.
6. Inset coupling on end of tube into syringe barrel. Remove column bottom cap.
7. Apply pressure to piston of plastic syringe to push liquid through the column. Maintain a flow rate of 1-2 drops per second. Push all liquid through the column. Repeat for wash and elution steps (see procedures).

Note: Avoid pulling up on plastic syringe piston while coupling is attached to glass syringe barrel. This may displace the antibody coated support beads and affect test results. The



Affinity Column Syringe Barrel Connection



2.3 CLEANING EQUIPMENT

Before Starting AflaOchra HPLC™ 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 are 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 HPLC™. 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.

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 methanol.

Between each assay, the syringe barrel reservoir can be rinsed with purified water. This will be sufficient 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.

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.

3.1 PREPARATION OF SOLUTIONS

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

1. acetonitrile:water (60:40)

   600 mL acetonitrile
   400 mL purified water

   Prepare solution every week or as needed.
   CAUTION: Extraction solvent is flammable. Keep container tightly capped when not in use.

2. methanol:water (80:20)

   800 mL methanol
   200 mL water

   Prepare solution every week or as needed.
   CAUTION: Extraction solvent is flammable. Keep container tightly capped when not in use.

3. 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.

4. Aflatoxin HPLC mobile phase

Solution desired (methanol:water)	HPLC Grade Methanol (mL)	Purified Water (mL)	Total Volume (mL)
45:55	450	550	1000 (1 liter)

   Solution should be filtered and degassed before use.

5. Ochratoxin HPLC mobile phase

Solution desired (acetonitrile:water :acetic acid)	Acetonitrile (mL)	Purified water (mL)	Acetic Acid (mL)	Total Volume (mL)
99:99:2	495	495	10	1000 (1 liter)

   Solution should be filtered and degassed before use.

4.1 MATERIALS AND EQUIPMENT REQUIRED FOR HPLC PROCEDURES

Materials Required

Description	Part #
AflaOchra HPLC™ Columns	G1017
Fluted Filter Paper, 24 cm (100 filters per pack)	31240
Microfibre Filters, 11 cm (100 filters per pack)	31955
Disposable Cuvettes (250 per pack)	34000
Methanol, HPLC Grade (4 x 4 L)	35016
Disposable Plastic Beakers	36010
Distilled , reverse osmosis or deionized water	
Noniodized sodium chloride (salt, NaCl)	
Acetonitrile	

Equipment Required

Description	Part #
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
Vortex Mixer	23040
500 mL Bottle Dispenser for Methanol (0-3 mL range)	20501
Filter Funnel, 65 mm (10 per pack)	36020
HPLC column (see description in specific procedure)	

Suggested But Not Required

Description	Part #
Filter Funnels, 105 mm (4 per pack)	36022
Adjustable micropipetter and tips	

4.2 AFLAOCHRA HPLC™ PROCEDURE FOR CORN (0 - 100 PPB)

1.0 HPLC Set up for Ochratoxin:

1.1 Column: reverse phase C18 (Waters NovaPak C18, 3.9mm X 150 mm, 4µm)
1.2 Mobile phase: water:acetonitrile:acetic acid (99:99:2, v/v/v), degassed
1.3 Flow rate: 0.9 mL/min.
1.4 Fluorescence detector: Waters 470 Scanning Fluorescence detector
1.5 Detection wavelength: 333 nm excitation and 477 nm emission

2.0 HPLC Set up for Aflatoxin:

2.1 Column: reverse phase C18 (Waters NovaPak C18, 3.9mm X 150 mm, 4µm)
2.2 Mobile phase: methanol:water (45:55) isocratic degassed.
2.3 Flow rate: 0.8 mL/min.
2.4 Fluorescence detector: Waters 470 Scanning Fluorescence detector, excitation 360 nm, emission 440 nm
2.5 Post column:
Post column iodine: 0.05% Iodine (see Section 3.1, Preparation of Solutions).
Flow rate: 0.2 mL/min.
Reaction temperature: 70°C (FIAtron FH-40 heater & FIAtron TC-50 controller)
Reaction time: ~1 minute.

3.0 Sample Extraction:

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

4.0 Extract Dilution

4.1 Pipet or pour 10 mL filtered extract into a clean vessel.
4.2 Dilute extract with 40 mL of purified water. Mix well.
4.3 Filter extract through microfibre filter and collect filtrate in a clean vessel.

5.0 Column Chromatography

5.1 Pass 10 mL (10 mL = 1.0 g sample equivalent) diluted extract completely through AflaOchra HPLC™ affinity column at a rate of about 1-2 drops/second until air comes through column.

5.2 Pass 10 mL of purified water through the column at a rate of 1-2 drops/second until air comes through column.

5.3 Elute affinity column by passing 1.5 mL HPLC grade methanol through column at a rate of ~1 drop/second and collecting all of the sample eluate (1.5 mL) in a glass cuvette.

5.4 Add 1.5 mL purified water to eluate. Vortex. Inject a 50-200µL portion of eluate into HPLC for Aflatoxin and another 50-200µL portion into HPLC for Ochratoxin.

6.0 Limit of Detection: Less than 0.25 ppb.

7.0 Recovery: Greater than 70% over the range of 0-100 ppb for both aflatoxin and ochratoxin.

4.3 AFLAOCHRA HPLC™ PROCEDURE FOR WHEAT (0 - 100 PPB)

1.0 HPLC Set up for Ochratoxin:

1.1 Column: reverse phase C18 column (Waters Nova-Pak® C-18, 3.9 X 150mm, 4μm)
1.2 Mobile phase: water:acetonitrile:acetic acid (99:99:2, v/v/v), degassed
1.3 Flow rate: 0.9 mL/min.
1.4 Fluorescence detector: Waters 470 Scanning Fluorescence detector
1.5 Detection wavelength: 333 nm excitation and 477 nm emission

2.0 HPLC Set up for Aflatoxin:

2.1 Column: reverse phase C18 column (Waters Nova-Pak® C-18, 3.9 X 150mm, 4μm).
2.2 Mobile phase: methanol:water (45:55) isocratic degassed.
2.3 Flow rate: 0.8 mL/min.
2.4 Fluorescence detector: Waters 470 fluorescence detector, excitation 360 nm, emission 440 nm
2.5 Post column:
Post column iodine: 0.05% Iodine (see Section 3.1, Preparation of Solutions).
Flow rate: 0.2 mL/min.
Reaction temperature: 70°C (FIAtron FH-40 heater & FIAtron TC-50 controller)
Reaction time: ~1 minute.

3.0 Sample Extraction:

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

4.0 Extract Dilution:

4.1 Pipet or pour 10 mL filtered extract into a clean vessel.
4.2 Dilute extract with 40 mL of purified water. Mix well.
4.3 Filter dilute extract through microfibre filter into clean container or directly into glass syringe barrel.

5.0 Column Chromatography

5.1 Pass 10 mL filtered extract (10 mL = 1 g sample equivalent) completely through OchraTest™ affinity column at a rate of about 1-2 drops/second until air comes through column.
5.2 Pass 10 mL of purified water through the column at a rate of 1-2 drops/second until air comes through the column.
5.3 Elute affinity column by passing 1.5 mL HPLC grade methanol through column at a rate of about 1 drop/second and collecting all of the sample eluate (1.5 mL) in a glass cuvette.
5.4 Add 1.5 mL purified water to eluate. Vortex. Inject 50-200μL into HPLC for Aflatoxin and 50-200μL for Ochratoxin.

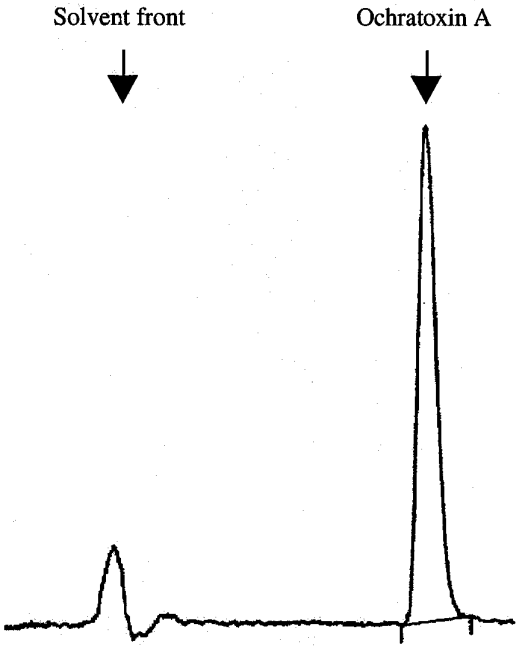
6.0 Limit of Detection: 0.25 ppb

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

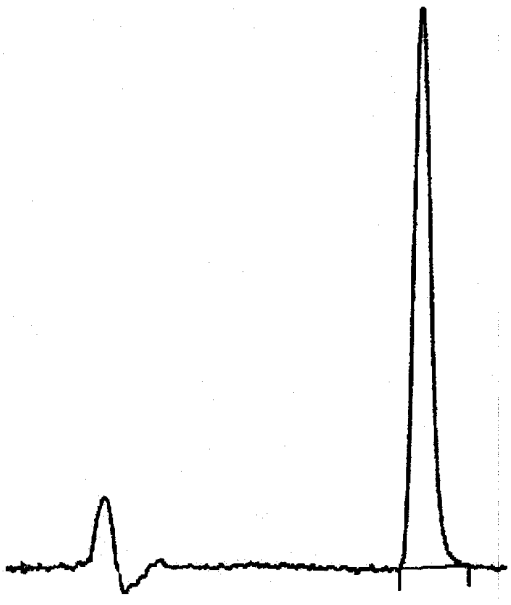
4.4 AFLAOCHRA HPLC™ REPRESENTATIVE CHROMATOGRAMS

(1 cm = 1 minute)

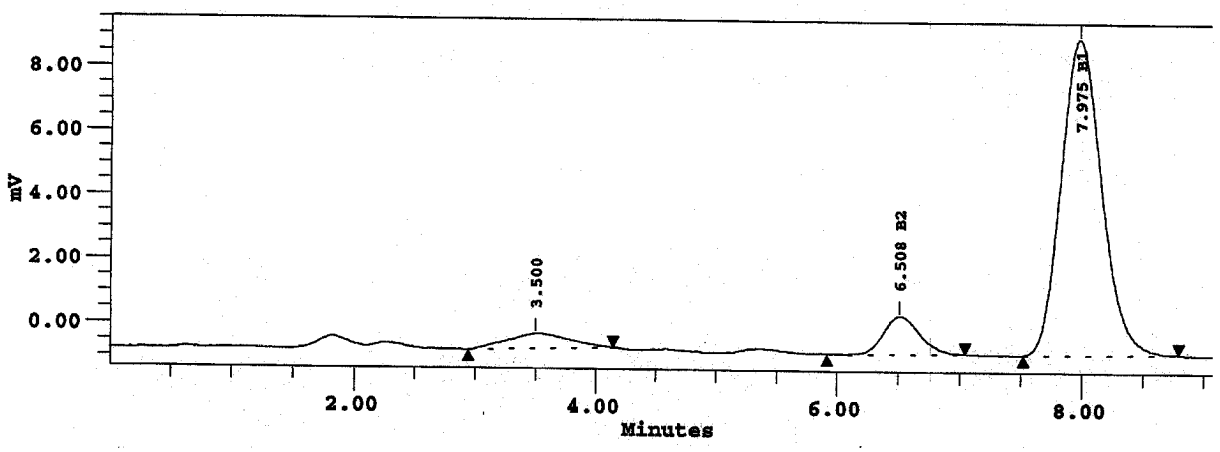
4.4.1 5 ppb ochratoxin standard



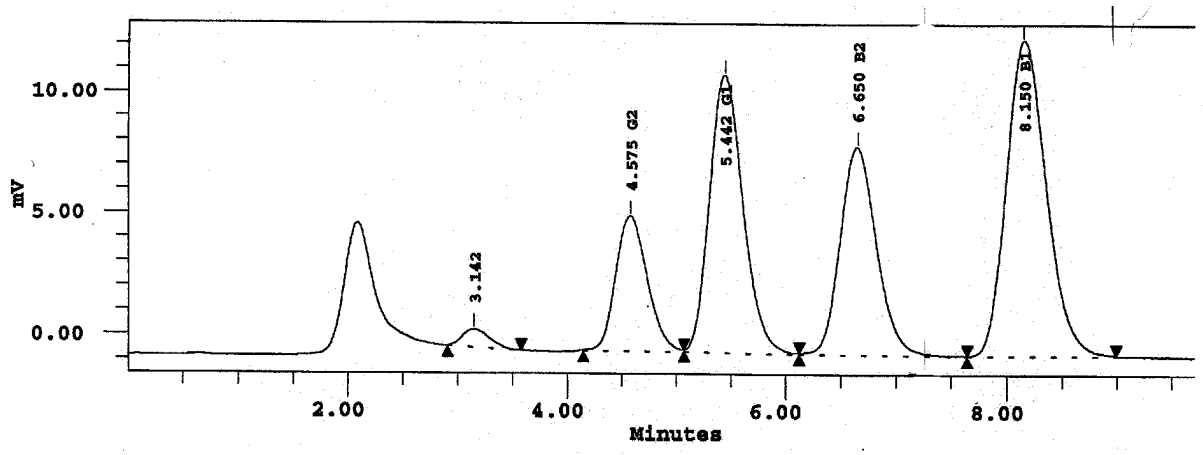
4.4.2 5 ppb ochratoxin sample



4.4.3 3.7 ppb aflatoxin contaminated corn (3.5 B1, 0.2 B2)



4.4.4 13 ppb total (5.0 B1, 1.5 B2, 5.0 G1, 1.5 G2) aflatoxin standard



5.0 GENERAL PRECAUTIONS FOR HPLC PROCEDURES

1. Ochratoxin may be lost if eluate is passed through nylon disc filter.

2. If wheat 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.

6.0 REFERENCES

Truckess, M. W., Stack, M. E., Nesheim, S., Page, S. W., Albert, R. H., Hansen, T. J., and Donahue, K. F., *Journal of the Association of the Official Analytical Chemistry*, Immunoaffinity column coupled with solution fluorometry or liquid chromatography post column derivitization for determination of alfatoxins in corn, peanuts and peanut butter: collaborative study, **74** (1) (1991) 81-88.

7.0 TECHNICAL ASSISTANCE

For assistance please contact your local distributor or Vicam Technical Services:

Phone:	800-338-4381	Canada, Mexico and the United States (outside Massachusetts)
	617-926-7045	International and United States customers
Fax:	617-923-8055	
e-mail:	techservice@vicam.com	

8.0 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 HPLC™ analytical procedures and products. Vicam makes no warranty of any kind, express or implied, other than that AflaOchra HPLC™ 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 HPLC™ product.

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	800-338-4381	Mexico
	617-926-7045	International and United States customers
Fax:	617-923-8055	
e-mail:	vicam@vicam.com	