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AOZ HPLC™

Instruction Manual

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1.1 INTENDED USER

Foods for human consumption, as well as animal feeds, are increasingly subject to more and more regulation. At the same time, mycotoxins are increasingly included in testing as an important toxicological parameter. Frequently, the levels of several mycotoxins in a sample have to be determined. Among the large number of mycotoxins, aflatoxins, ochratoxin A (OTA) and zearalenone (ZEA) are especially important.

AOZ HPLC™ is a quantitative method for the simultaneous detection of aflatoxin, ochratoxin A and zearalenone in several commodities.

1.2 PRINCIPLE

Determination of aflatoxins, ochratoxin A and zearalenone previously required several analyses, demanding considerable time and material. In earlier studies it was found that simultaneous determination of the mycotoxins aflatoxins B_1, G_1, B_2, G_2, zearalenone (ZEA) and ochratoxin A (OTA) is possible by high-pressure liquid chromatography (HPLC). Based on work by Dunne et al. (1993), HPLC conditions were correspondingly modified for this purpose. VICAM immunoaffinity columns were used for the preparation of samples. By combining the VICAM *AOZ HPLC™* columns with the aflatoxin, ochratoxin and zearalenone HPLC conditions, clean-up of an extract of grain, feedstuffs and the like was performed in a single operation (Göbel, 2000).

Samples are prepared by mixing them with an extraction solution, blending and filtering. The filtered extract is then applied to the *AOZ HPLC™* column bound with specific antibodies to aflatoxin, ochratoxin and zearalenone. At this stage, the toxins bind to the antibodies on the column. The column is then washed with water to rid the immunoaffinity column of impurities. By passing methanol through the column, the toxins are removed from the antibodies. This methanol solution can then be injected into an HPLC system. These steps are outlined in section 1.7 and 1.8, *AOZ HPLC™* Overviews.

1.3 APPLICABILITY AND APPROVALS

AOZ HPLC™ has been validated for quantitative measurement of aflatoxins, ochratoxin A and zearalenone in rice, barley and rye fodder.

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 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 Federal Grain Inspection Service (FGIS) publications:

FGIS Aflatoxin Handbook
FGIS Grain Inspection Handbook, Book 1, Grain Sampling
FGIS Mechanical Sampling Systems Handbook

These can be viewed online at:

http://www.usda.gov/gipsa/reference-library/handbooks/handbooks.htm
http://www.usda.gov/gipsa/reference-library/brochures/sampling.pdf

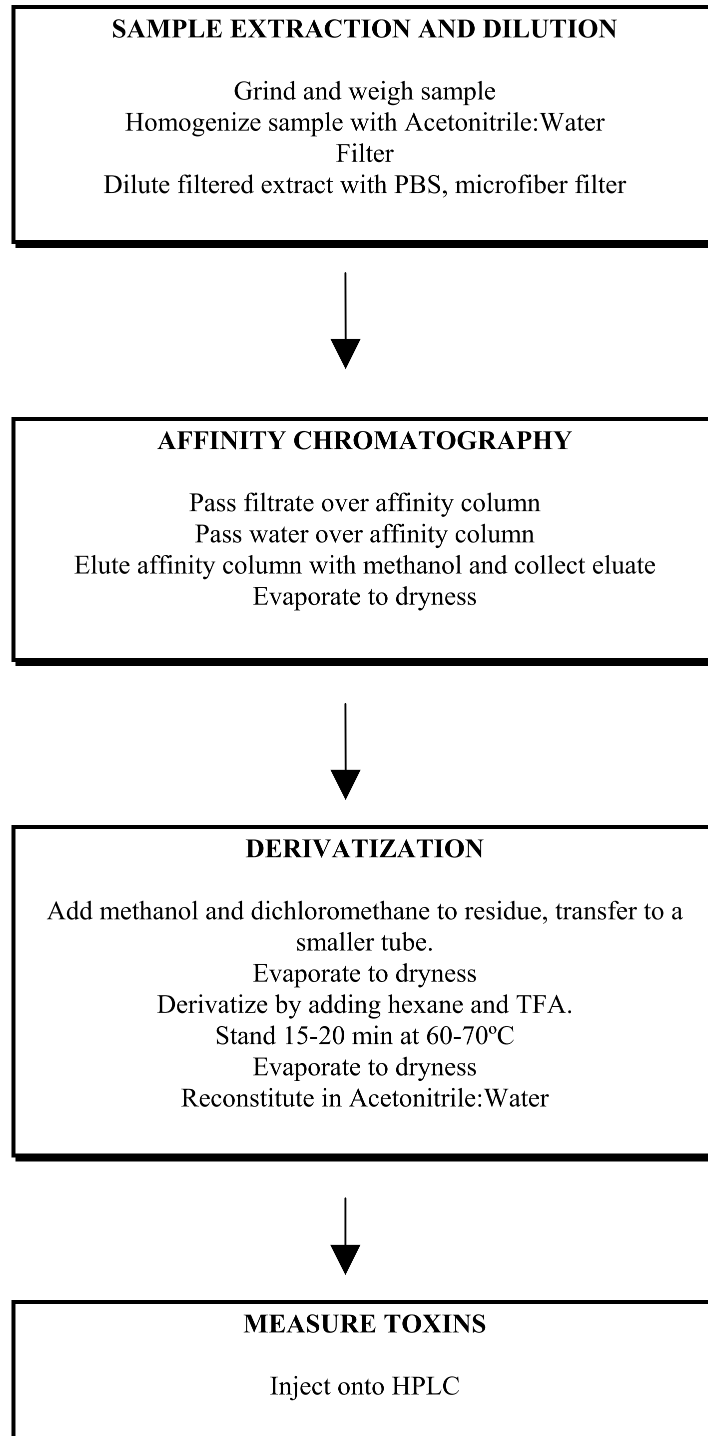
These can be viewed online at http://www.usda.gov/gipsa/reference-library/handbooks/hb.htm. Limited quantities of these publications are available free, upon request. Send requests to:

USDA-APHIS, MSD-HSB PDMS
Room 1A28
4700 River Road
Riverdale, MD 20737

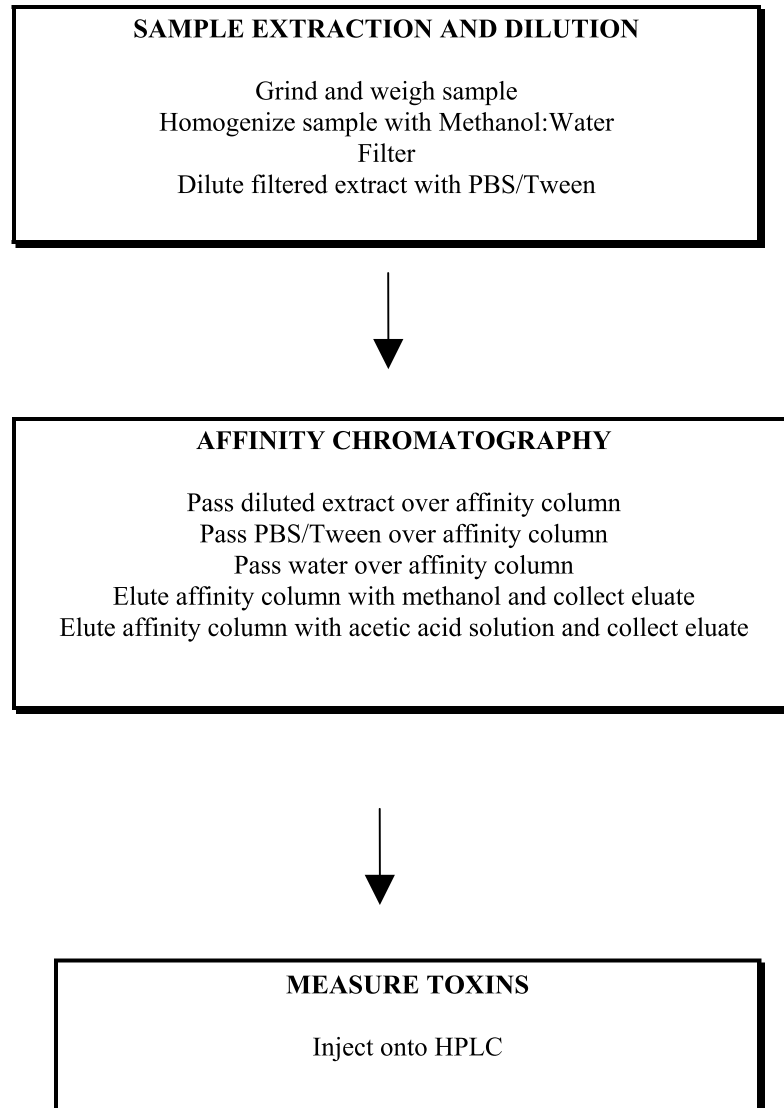
1.6 SHELF LIFE AND STORAGE CONDITIONS

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, columns may be stored at 4°C. It is recommended that columns should be at room temperature (18 - 22°C) for usage.

1.7 *AOZ HPLC™* OVERVIEW FOR RICE, BARLEY, RYE AND FEED USING TFA DERIVATIZATION



1.8 *AOZ HPLC™* OVERVIEW FOR CORN AND WHEAT USING POST COLUMN IODINE DERIVATIZATION



2.1 PREPARATION OF FILTRATION STEPS

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 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.

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 affinity column. Microfiber filtration is performed just after dilution with PBS prior to affinity chromatography.

2.2 PUMP STAND SETUP

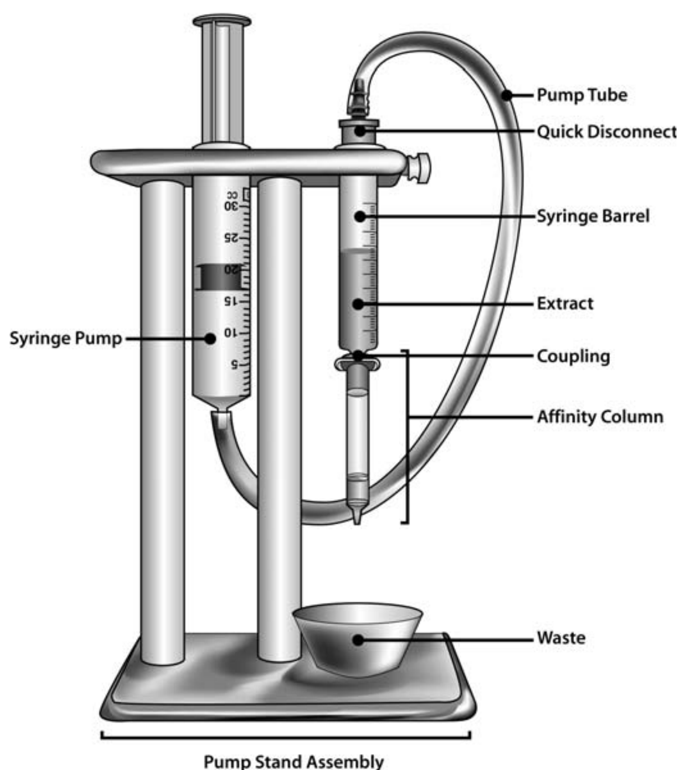
AOZ HPLC™ affinity chromatography is easily performed with the *AOZ HPLC™* affinity column attached to a pump stand (part # 21020). The stand has a 10 mL glass syringe barrel that serves as a reservoir for the column. A large plastic syringe with tubing and coupling provides air pressure to manually push liquids through the column. An adjustable air pump (part #20650) can be attached to the pump tube instead of the large pump syringe barrel to operate without using hand pressure. Double position pump stand (part # 21030), four-position pump stand with aquarium pumps (part #21045) and 12 position pump stand with pumps (part #G1104) are available for running multiple samples at one time. Alternatively, a vacuum manifold can be used to pull liquid through the *AOZ HPLC™* column.

When using a pump stand:

1. Remove large top cap from column.
2. Vicam part G1118 HPLC Column Coupling provides a reusable coupling for attaching the column to the syringe barrel reservoir.
3. Pour extract after microfiber filtration into glass syringe barrel reservoir.
4. Pull up on the plastic syringe piston.
5. Insert coupling on end of tube into syringe barrel. Remove column bottom cap.
6. 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.

Affinity Column Syringe Barrel Connection



2.3 CLEANING EQUIPMENT

Wash blender jar, blender blade assembly, funnel and gasket with soap and hot water. Rinse thoroughly with cold tap water and then dry completely.

3.0 REAGENT PREPARATION

1. Extraction Solution

The *AOZ HPLC™* procedure uses either an acetonitrile:water or a methanol:water solution to extract aflatoxin, ochratoxin A and zearalenone out of the sample. Use reagent grade (or better - i.e. HPLC grade) acetonitrile or methanol when preparing extraction solutions.

Solution desired (acetonitrile:water)	Acetonitrile (mL)	Purified Water (mL)	Total Volume (mL)
60:40	600	400	1000 (1 liter)

Solution desired (methanol:water)	Methanol (mL)	Purified Water (mL)	Total Volume (mL)
80:20	800	200	1000 (1 liter)

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.

2. Phosphate Buffered Saline (pH 7.4) for use with procedure 4.2

0.20 g KCl
0.20 g KH_2PO_4
2.92 g $Na_2HPO_4 \bullet 12H_2O$
8.00 g NaCl

Dissolve in 900 mL purified water. Adjust to pH 7.4 with 0.1M HCl or 0.1M NaOH and dilute to 1000 mL. Commercial buffered saline tablets may also be used.

A 10X concentrate of PBS may also be purchased from Vicam (part # G1113). 10X PBS Concentrate should be diluted to 1X with purified water as needed - i.e. dilute 100 mL of 10X concentrate with 900 mL purified water.

3. Phosphate Buffered Saline (pH 7.0) with 0.01% Tween for use with procedure 4.3

Make **PBS first**
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

Add 0.1 mL Tween-20 to
1000 mL Phospahte Buffered saline

A 10X concentrate of 0.01% Tween-20/PBS may also be purchases from Vicam (part # G1114). 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.

Saturated 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.

4.1 MATERIALS AND EQUIPMENT REQUIRED FOR HPLC PROCEDURES

Materials Required

Description	Part #
AOZ HPLC™ Columns (25/box)	G1031
VICAM Fluted Filter Paper, 24 cm (100)	31240
Pointed Flask, 25 mL	
Methanol, HPLC Grade (4 x 4 L)	35016
Acetonitrile, HPLC Grade	
Phosphoric Acid	
Dichloromethane	
Trifluoroacetic acid (TFA)	
Disposable Plastic Beakers (25 per pack)	36010
Distilled, reverse osmosis or deionized water	
Hexane	
10 X Concentrate Phosphate Buffered Saline	G1113
10 X Concentrate 0.01% Tween-20/PBS (for Post Column Iodine method)	G1114
Aflatoxin, Ochratoxin, Zearalenone Standards	

Equipment Required

Description	Part #
Graduated cylinder, 50 mL	20050
Digital Scale with AC Adapter	20100
Ultra Thurax or mechanical shaker	
Graduated cylinder, 250 mL	20250
Wash Bottle, 500 mL	20700
Single Position Pump Stand (or vacuum manifold)	21020
or 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
HPLC System as specified in procedure	
Vacuum Rotary Evaporator	
Heating Block (50 °C) and nitrogen tank	
Adjustable micropipettors and micropipette tips	

Optional Materials

Description	Part #
Microfiber Filters, 1.5µm, 11 cm (100)	31955

4.2 *AOZ HPLC™* PROCEDURE FOR RICE, BARLEY, RYE AND FEED USING TFA DERIVATIZATION

1.0 HPLC Set Up*:

1.1 Column: 150 x 4.6 mm, 5µm, Prodigy ODS-2 from Phenomenex
1.2 Mobile phases:
Solution A = water:methanol:acetonitrile (55:30:15)
Solution B = acetonitrile:0.1% phosphoric acid (50:50)
1.3 Gradient: 0 - 8 min = 100% A
8.01 -13 min gradient = 30% A, 70% B
13.01 - 25 min isocratic = 30% A, 70% B
25.01 - 30 min = 100% A
1.4 Flow rate: 0.7 mL/min.
1.5 Column temperature: 30°C
1.6 Wavelength settings on the fluorescence detector:

Time (min)	Mycotoxin	Excitation wavelength	Emission wavelength
0 – 18	Aflatoxins	360 nm	440 nm
18 – 25	Zearalenone	276 nm	460 nm
25 - 30	Ochratoxin A	330 nm	460 nm

1.7 Injection volume: 70 µL

1.8 Retention times:

Mycotoxin	Retention Time (min)
Aflatoxin G_1	4.0
Aflatoxin B_1	4.9
Aflatoxin G_2	6.3
Aflatoxin B_2	8.3
Zearalenone	24.5
Ochratoxin A	27.2

*Although specific equipment and HPLC columns are listed in this document, there are a number of equally suitable components that can also be used.

2.0 Sample Extraction and Dilution:

2.1 Measure 25g of sample and homogenize with 100 mL Acetonitrile: Water (60:40). Homogenize for 2-3 minutes if using an Ultra-Turrax T25-model, with the color yellow to green (speed) or shake for 30 minutes on a mechanical shaker.
2.2 Pour extract into fluted filter paper. Collect filtrate in a clean vessel.
2.3 Pipet 10 mL filtered extract into a clean vessel.
2.4 Dilute extract with 40 mL of PBS. Mix well.
2.5 If the diluted solution is cloudy, filter the diluted extract through a glass microfiber filter into a clean vessel.

3.0 Column Chromatography

3.1 Remove two end caps from *AOZ HPLC™* affinity column.

3.2 Attach column to outlet of reservoir on pump stand or put in automated system.

3.3 Siphon off any column supernatant. Wash column by passing 3 mL of purified water through the column at a rate of about 1-2 drops per second. Condition column by passing 10 mL PBS buffer through column at a rate of 1-2 drops per second.

3.4 Pass 20 mL (for feed samples only pass 10 mL) filtered diluted extract completely through *AOZ HPLC™* affinity column at a rate of about 1-2 drops/second. Pass sample completely through the column until there is no liquid on the top of the column bed but do not dry out the column by passing air through.

3.5 Pass 20 mL of purified water through the column at a rate of about 1-2 drops/second. Shortly blow dry column.

3.6 Elute affinity column by passing 6.0 mL HPLC grade methanol through column at a rate of 1-2 drops/second and collecting all of the sample eluate in a 25 mL pointed flask. The solvent is evaporated to dryness using a vacuum rotary evaporator.

4.0 Sample Derivatization

4.1 Add 0.5 mL methanol to the 25 mL pointed flask and swirl to redissolve the evaporated eluate. Transfer the methanol to a 1.5 mL LC sample bottle. Then add 0.5 mL dichloromethane to the same 25 mL pointed flask, again swirling to redissolve the evaporated eluate. Add the dicholormethane to the same 1.5 mL LC sample vial.

4.2 The solvent is evaporated with nitrogen at approximately 50°C.

4.3 Derivatize the sample by adding 200 µL hexane and 50 µL TFA (trifluoroacetic acid) and vortexing. Allow the sample to stand for 15 – 20 minutes at 60 - 70°C.

4.4 The solvent is evaporated to dryness with nitrogen. Reconstitute sample in 0.5 mL Acetonitrile:Water (50:50) and inject onto HPLC.

5.0 Recovery (from a rice extract):

Spiking levels: Aflatoxins = 5 µg/kg, zearalenone = 100 µg/kg, Ochratoxin = 10 µg/kg

Mycotoxin	Spiked level µg/kg	Recovery µg/kg	% Recovery
Aflatoxin G_1	5	4.79	95.8
Aflatoxin B_1	5	4.51	95.8
Aflatoxin G_2	5	4.67	93.4
Aflatoxin B_2	5	5.83	116.6
Zearalenone	100	105.80	105.8
Ochratoxin A	10	10.05	100.5

Spiking levels: Aflatoxins = 10 µg/kg, zearalenone = 200 µg/kg, Ochratoxin = 20 µg/kg

Mycotoxin	**Spiked level µg/kg**	**Recovery µg/kg**	**% Recovery**
Aflatoxin G_1	10	8.30	83.0
Aflatoxin B_1	10	7.95	79.5
Aflatoxin G_2	10	7.99	79.9
Aflatoxin B_2	10	10.94	109.4
Zearalenone	200	181.4	90.7
Ochratoxin A	20	17.3	86.3

4.3 *AOZ HPLC™* PROCEDURE FOR CORN AND WHEAT USING POST COLUMN IODINE DERIVATIZATION

1.0 HPLC Set Up*:

1.1 Column: Waters Symmetry C18 column, 4.6 x 150mm, 3.5 µm particles, WAT200632 with guard column symmetry C18, WAT054225

1.2 Mobile phases:
Solution B = Methanol
Solution C = Acetonitrile
Solution D = 0.1% Acetic Acid

1.3 Gradient: 0 – 12.0 min isocratic = 25%B, 15%C, 60% D
12.0 –14.0 min linear gradient to 10%B, 50%C, 40% D
14.0 - 24 min hold at 10% B, 50 %C, 40% D
24.0 min immediate return to 25% B, 15%C, 60% D
2 min delay of next injection for equilibration

1.4 Flow rate: 1 mL/min.

1.5 Column temperature: 30°C

1.6 Wavelength settings on the Waters 474 fluorescence detector:

Time (min)	Mycotoxin	Excitation wavelength	Emission wavelength
0.1 – 14.0	Aflatoxins	365 nm	455 nm
14.0 – 21	Zearalenone	276 nm	460 nm
21 - 25	Ochratoxin A	330 nm	460 nm

1.7 Injection volume: 100 µL

1.8 Post Column Waters reagent manager pump with Saturated Iodine at 0.2mL/min and 70C controlled by a switch to go on at 0 minute, off at 14 minutes, and then back on at 25 minutes for the next injection. The iodine is added to the HPLC flow through a "T" fitting and pass through a circular heated reaction coil for one minute before going through the fluorescence detector.

1.9 Retention times: G2 = 7.1min
G1 = 8.6 min
B2 = 9.6 min
B1 = 11.9 min
Zear = 19.3
Ochra = 22.0

* Although specific equipment and HPLC columns are listed in this document, there are a number of equally suitable components that can also be used.

2.0 Sample Extraction:

2.1 Place 25 g of ground sample into blender jar (no salt).
2.2 Add to jar 100 mL methanol:water (80:20).
2.3 Cover jar and blend at high speed for 2 minutes.
2.4 Remove cover and pour suspension onto a fluted filter paper.

3.0 Extract Dilution and Filtration:

3.1 Pipet 10 mL filtered extract into a clean vessel.
3.2 Dilute extract with 40 mL PBS with 0.01% Tween-20. Mix well. Do not filter

4.0 Column Chromatography:

4.1 Remove two end caps from *AOZ HPLC™* column.
4.2 Attach column to outlet of reservoir on pump stand.
4.3 Pass 20 mL of diluted extract (20 mL = 1g sample equivalent) through the *AOZ HPLC™* column at a steady slow flow rate of about 1-2 drops per second. Do not let column completely dry out.
4.4 After extract has passed through column, pass 10 mL PBS with 0.01% Tween-20 solution through the *AOZ HPLC™* column at about 1-2 drops per second flow rate. Do not let column completely dry out.
4.5 Pass 10mL water through the *AOZ HPLC™* column. Gently pass a few seconds of air through the column.
4.6 Elute *AOZ HPLC™* column at flow rate of about 1 drop per second or less with 1.5 mL HPLC grade methanol and collect in a clean glass cuvette. Gravity flow is good. Do not push air through the column to dry.
4.7 Pass 1.5mL 0.1% acetic acid solution through the *AOZ HPLC™* column at a rate of 1 drop per second or less and collect in the same cuvette as the methanol. Push air through the column to remove all the liquid.
4.8 Vortex and inject 100 µL onto HPLC.

5.0 Limit of detection: (using post iodine)

Commodity	Aflatoxin	Limit of Detection (ppb)
Corn	B_1	0.125
Corn	B_2	0.1
Corn	G_1	0.15
Corn	G_2	0.1
Corn	Ochratoxin	0.25
Corn	Zearalenone	5

6.0 Recovery (in corn): n = 4

Mycotoxin	Spiked level µg/kg	% Recovery
Aflatoxin G_2	0.2	90.8
Aflatoxin G_1	0.6	102
Aflatoxin B_2	0.2	92.6
Aflatoxin B_1	1.0	95.1
Zearalenone	20	93.3
Ochratoxin A	2	82.6

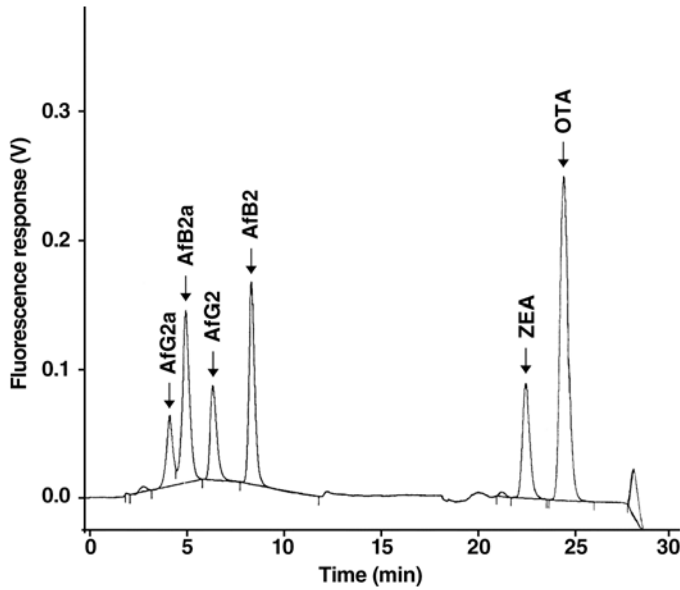
Recovery (in corn): n = 4

Mycotoxin	Spiked level µg/kg	% Recovery
Aflatoxin G_2	2	90.9
Aflatoxin G_1	6	96.1
Aflatoxin B_2	2	88.3
Aflatoxin B_1	10	87.9
Zearalenone	200	92.2
Ochratoxin A	20	83.2

See VP-1035-0 for full performance report of data using AOZ HPLC. VP-1035-0 can be obtained by calling VICAM technical service at 1-800-338-4381 or by emailing to techservice@vicam.com.

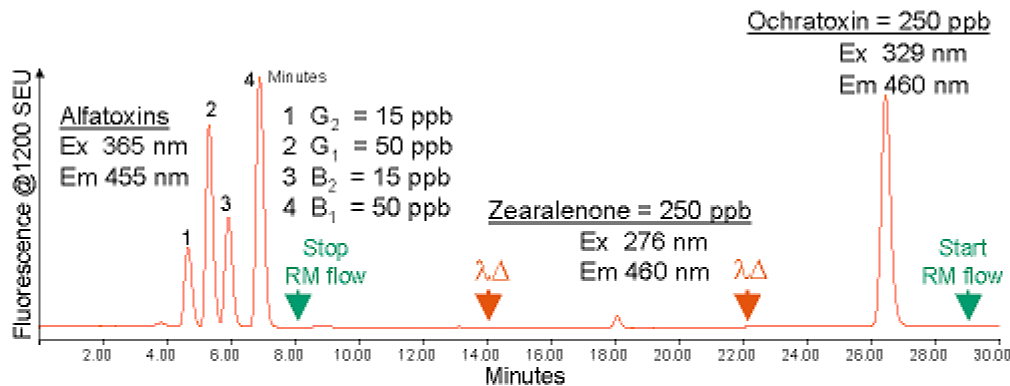
4.4 REPRESENTATIVE CHROMATOGRAMS

TFA DERIVATIZATION



Chromatogram of a rice mixture spiked with 5 µg/kg Afla, 75 µg/kg Zearalenone and 10 µg/kg Ochratoxin A and analyzed as described in the text with the VICAM AOZ column and TFA derivatization of eluates before HPLC chromatography

POST-COLUMN IODINE



A shift in the HPLC baseline will normally occur at each wavelength change. This becomes very noticeable when measuring low concentration samples.

5.0 GENERAL PRECAUTIONS AND TROUBLESHOOTING

5.1 We have found the best results when not drying down the sample eluate as aflatoxins often bind irreversibly to glassware when drying.

5.2 If passing the eluate through a disc filter before HPLC injection, be careful the toxins do not bind to the filter material. Test an unfiltered and filtered sample to confirm toxins are not binding to the disc filter.

5.3 Use high quality HPLC solvents. Some solvents can give background peaks, especially at the zearalenone retention time. We strongly recommend injecting a reagent blank before starting to make sure there are no background peaks in the chromatography.

6.0 TECHNICAL ASSISTANCE

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

Phone:	800-338-4381	Canada, Mexico and the United States
	617-926-7045	all International and United States customers
Fax:	617-923-8055	
e-mail:	techservice@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 *AOZ HPLC™* analytical procedures and products. Vicam makes no warranty of any kind, express or implied, other than that *AOZ 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 *AOZ HPLC™* 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 worldwide patents and trademarks.

8.0 REFERENCES

Some of the following work was done with a prototype AOZ HPLC column. The AOZ HPLC column currently sold by VICAM has been improved to give higher recovery of aflatoxin G2.

1. Dunne, C., Meaney, M., Smyth, M., *Journal of Chromatography*, "Multimycotoxin detection and clean-up method for aflatoxins, ochratoxin and zearalenone in animal feed ingredients using high-performance liquid chromatography and gel permeation chromatography.", **629** (1993) 229-235.

2. Göbel, R., Bohm, D., Grunow, M., "On the simultaneous determination of aflatoxins B_1, B_2, G_1, G_2, ochratoxin A and zearalenone." 22nd Mycotoxin Workshop, June 5-7, 2000, Bonn.

3. Göbel, R., Lusky, K., *Journal of AOAC International*, "Simultaneous Determination of Aflatoxins, Ochratoxin A, and Zearalenone in Grains by New Immunoaffinity Column/Liquid Chromatography.", **87(2)** (2004) 411-416.

9.0 ORDERING INFORMATION

To place an order contact your local Vicam distributor or Vicam at:

In the United States:

Phone:	877-228-4244	Canada and the United States
	800-338-4381	Mexico
	617-926-7045	all International and United States customers
Fax:	617-923-8055	
e-mail:	vicam@vicam.com	

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