AOZ™ LC

LC or LC-MS Detection

Instruction Manual

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1.0 Introduction

1.1 Intended User

Foods for human consumption, as well as animal feeds, are increasingly subject to strict global regulation and preventive management requirements. At the same time, mycotoxins have increased in importance as a serious risk to raw materials, as well as finished food and agricultural products – resulting in the need for testing methods that enable confirmatory testing for single and multiple mycotoxins in a single commodity or sample type. Among several mycotoxins of importance, aflatoxins, ochratoxin A (OTA) and zearalenone (ZEA) have been identified as prevalent and likely to co-occur in a wide range of products. t.

AOZ™ LC 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, reagents and consumables. 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 using HPLC, UPLC or LC-MS. 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. Combining the VICAM AOZ LC columns with the aflatoxin, ochratoxin and zearalenone LC conditions, clean-up of a sample extract of grain, feedstuffs and the like was performed in a single operation (Göbel, 2004).

Samples are prepared by mixing with an extraction solution, blending and filtering. The filtered extract is then applied to the AOZ LC column containing immobilized antibodies selected for their ability to isolate aflatoxin, ochratoxin and zearalenone. At this stage, the toxins bind to the antibodies on the column. The column is then washed with water to remove f impurities. Finally, methanol is used to release the toxins from the antibodies, which remain suspended in the methanol eluate. This methanol solution is then be injected into an LC system. These steps are outlined in section 1.7 and 1.8, AOZ LC overviews.

1.3 Applicability and Approvals

AOZ LC column has been validated for quantitative measurement of aflatoxins, ochratoxin A and zearalenone in corn, wheat, rice, barley, rye, and feed.

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 the instructions provided in this manual may not yield optimum results.

1.5 Sampling

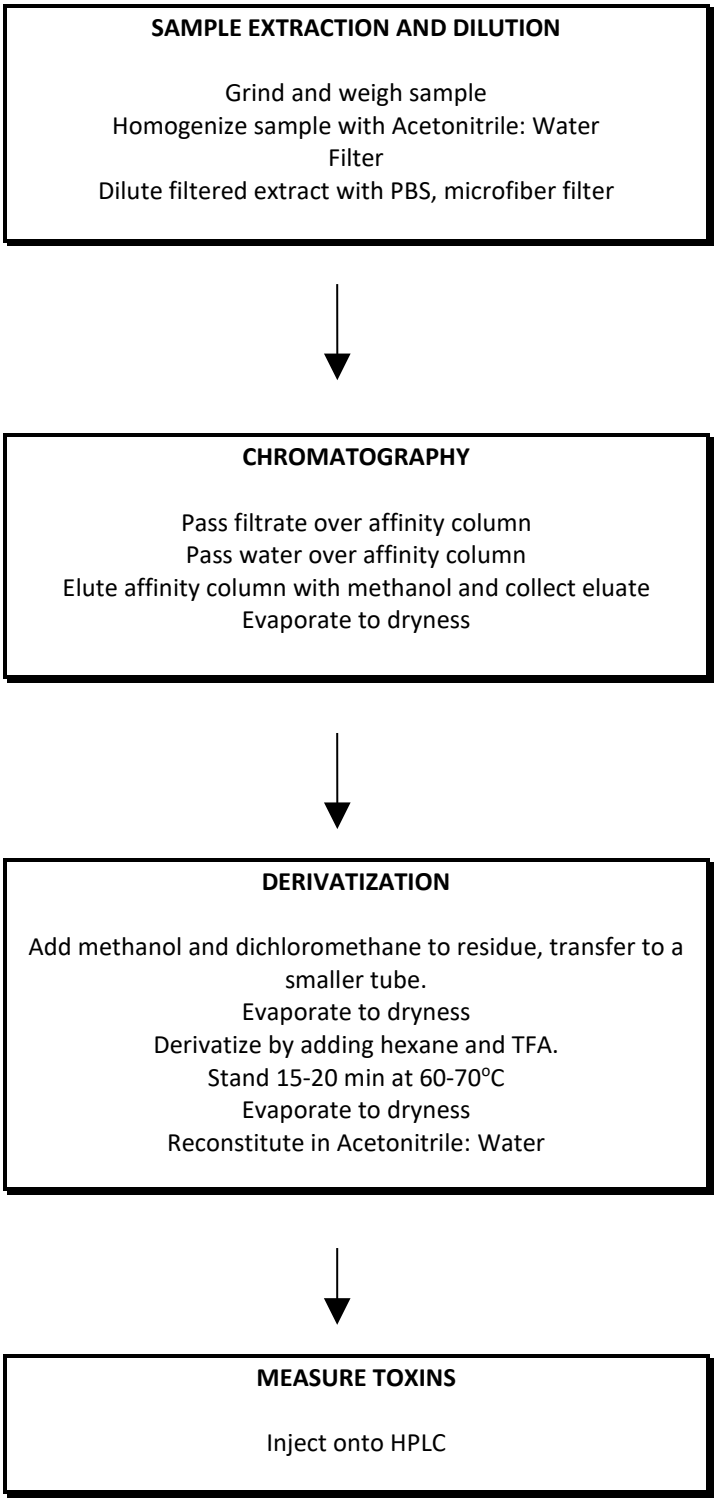
Mycotoxins do not occur in every kernel in a lot and may only occur in a small percentage of the kernels in a lot. Because of the wide range in mycotoxin concentrations among individual kernels in a contaminated lot, variation from sample to sample can be significant. It is important to obtain a representative 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

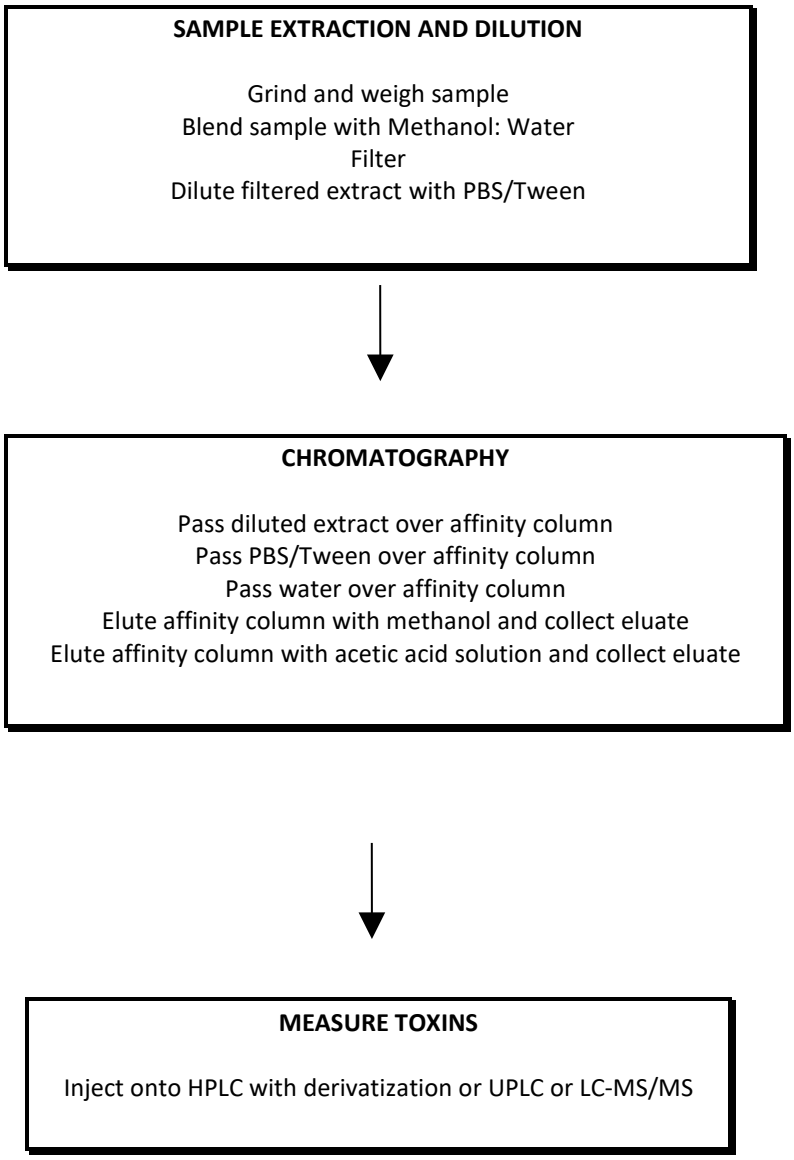
1.6 Storage Conditions

Store AOZ LC 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 AOZ LC Clean up Overview for Rice, Barley, Rye and Feed Using TFA Derivatization



1.8 AOZ LC Clean up Overview for Corn and Wheat Using Post Column Derivatization



2.0 Equipment Preparation

2.1 Materials and Equipment Required

Materials Required

Description	P/N
AOZ LC Columns (25/box)	G1031
AOZ LC Kit (100 Columns and 1 set standards)	G1057
VICAM Fluted Filter Paper, 24 cm (100)	31240
Microfiber Filters, 1.5 mm, 11 cm (100)	31955
10 X Concentrate 0.01% Tween-20/PBS150 mL	G1114
10X Concentrate PBS Wash Buffer 150 mL	G1113
Disposable Cuvettes (250)	34000
Methanol, HPLC Grade (4 x 4 L)	35016
Disposable Plastic Beakers (25)	36010
Non-iodized sodium chloride (salt, NaCl)	G1124
Distilled, reverse osmosis or deionized water	
Acetonitrile, HPLC	
Pointed Flask, 25 mL	
Phosphoric Acid	
Dichloromethane	
Trifluoroacetic acid (TFA)	
Hexane	

Equipment Required

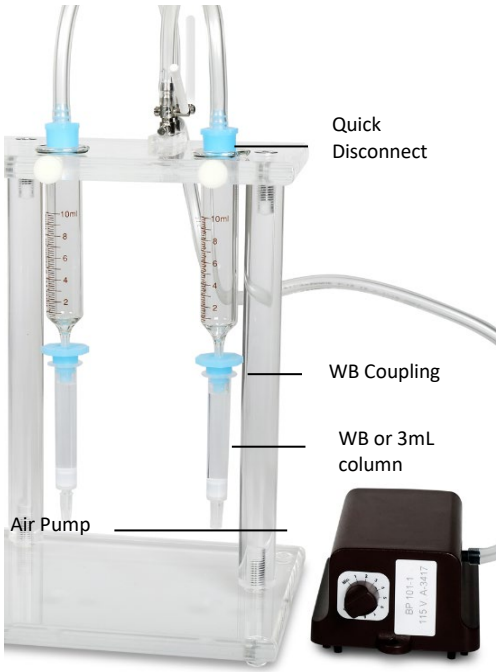
Description	P/N
Graduated Cylinder, 50 mL	20050
Digital Scale with AC Adapter	20100
Commercial Blender with Stainless Steel Container	20200
Eberbach Glass Blender Jar, 500 mL	20300
Graduated Cylinder, 250 mL	20250
500 mL Bottle Dispenser for Methanol (0-3 mL range)	20501
Wash Bottle, 500 mL	20700
Cuvette 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	
Ultra Thurax or mechanical shaker	
Vacuum Rotary Evaporator	
Heating Block (50 °C) and nitrogen tank	

2.2 Pump Stand Setup

The immunoaffinity chromatography is easily performed with the AOZ LC 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. Alternatively, a vacuum manifold can be used to pull liquid through the AOZ LC column.

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

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 Solution Preparation

3.1 Preparation of Extraction Solutions

The AOZ LC procedure uses either an acetonitrile: water or a methanol: water solution to extract aflatoxin, ochratoxin A and zearalenone from 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.

3.2 Preparation of Phosphate Buffered Saline

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 (P/N 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.

3.3 Preparation of *0.01% Tween/PBS*

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 and bring to 1 liter with 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 Phosphate Buffered Saline
1000 mL Phosphate Buffered Saline

A 10X concentrate of 0.01% Tween-20/PBS may also be purchases from VICAM (P/N 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.

3.4 Preparation of saturated Iodine solution

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.

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

4.1 AOZ LC Procedure for Rice, Barley, Rye and Feed Using TFA Derivatization*

4.1.1 HPLC Set Up: See 5.1

4.1.2 Sample Extraction and Dilution:

4.1.2.1 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 yellow to green (speed) color or shake for 30 minutes on a mechanical shaker.

4.1.2.2 Pour extract into fluted filter paper. Collect filtrate in a clean vessel.

4.1.2.3 Pipet 10 mL filtered extract into a clean vessel.

4.1.2.4 Dilute extract with 40 mL of PBS. Mix well.

4.1.2.5 If the diluted solution is cloudy, filter the diluted extract through a glass microfiber filter into a clean vessel.

4.1.3 Column Chromatography

4.1.3.1 Remove two end caps from AOZ LC column.

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

4.1.3.3 Pass 20 mL (for feed samples only pass 10 mL) filtered diluted extract completely through AOZ LC 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.

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

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

4.1.4 Sample Derivatization

4.1.4.1 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.

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

4.1.4.3 Derivatize the sample by adding 200 µL hexane and 50 µL TFA (trifluoroacetic acid) and vortexing. Then incubate at 60 - 70°C in heat block for 15-20 minutes.

4.1.4.4 The solvent is evaporated to dryness with nitrogen. Reconstitute sample in 0.5 mL acetonitrile: water (50:50) and inject onto HPLC.

Reference: Göbel et al, (2004).

4.2 AOZ LC Procedure for Corn and Wheat Using Post Iodine Column Derivatization

4.2.1 HPLC Set Up: See 5.2

4.2.2 Sample Extraction and Dilution:

4.2.2.1 Place 25 g of ground sample into blender jar (no salt).
4.2.2.2 Add to jar 100 mL methanol: water (80:20).
4.2.2.3 Cover jar and blend at high speed for 2 minutes.
4.2.2.4 Remove cover and pour suspension onto a fluted filter paper.
4.2.2.1 Pipet 10 mL filtered extract into a clean vessel.
4.2.2.2 Dilute extract with 40 mL PBS with 0.01% Tween-20. Mix well. Do not filter

4.2.3 Column Chromatograph

4.2.3.1 Remove two end caps from AOZ LC column.
4.2.3.2 Attach column to outlet of reservoir on pump stand.
4.2.3.3 Pass 20 mL of diluted extract (20 mL = 1g sample equivalent) through the AOZ LC column at a steady slow flow rate of about 1-2 drops per second. Do not let column completely dry out.
4.2.3.4 After extract has passed through column, pass 10 mL PBS with 0.01% Tween-20 solution through the AOZ LC column at about 1-2 drops per second flow rate. Do not let column completely dry out.
4.2.3.5 Pass 10mL water through the AOZ LC column. Gently pass a few seconds of air through the column.
4.2.3.6 Elute AOZ LC 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.
4.2.3.7 Pass 1.5mL 0.1% acetic acid solution through the AOZ LC 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.
4.2.3.8 Vortex and inject 100 µL onto HPLC.

4.2.4 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

4.2.5 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
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

The full performance report, VP-1035-0, may be obtained by emailing VICAM's technical services at techservice@vicam.com.

4.3 Other Published Procedures

Barley

Ibáñez-Vea, M., Corcuera, L., Remiro, R., Murillo-Arbizu, M., González-Peñas, E., Lizarraga, E., "Validation of a UHPLC-FLD Method for the Simultaneous Quantification of Aflatoxins, Ochratoxin A, and Zearalenone in Barley," (2011) *Food Chemistry* **127,** 351–358.

Breakfast Cereal

Ibáñez-Vea, M., Martínez, R., González-Peñas, E., Lizarraga, E., López de Cerain, A., "Co-occurrence of Aflatoxins, Ochratoxin A, and Zearalenone in Breakfast Cereals from Spanish Market, (2011) *Food Control* **22** (12), 1949–1955.

Cereals

Li, J., Yu, Y., Tian, M., Wang, H., Wei, F., Li, L., Wang, X., "Simultaneous Determination of Aflatoxins, Zearalenone and Ochratoxin A in Cereal Grains by Immunoaffinity Column and High-Performance Liquid Chromatography Coupled with Post-column Photochemical Derivatization," (2006) *Chinese Journal of Chromatography* **24** (6), 581–584.

Rahmani, A., Jinap, S., Soleimany, F., Khatib, A., Tan, C. P., "Sample Preparation Optimization for the Simultaneous Determination of Mycotoxins in Cereals," (2011) *European Food Research and Technology* **232** (4), 1438–2385.

Rahmani, A, Jinap, S., Soleimany, F., "Validation of the Procedure for the Simultaneous Determination of Aflatoxins, Ochratoxin A, and Zearalenone in Cereals Using HPLC-FLD," (2010) *Food Additives and Contaminants* **27** (12), 1683–1693.

Feeds

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.

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.

Poultry House Air

Wang, Y., Chai, T., Lu, G., Quan, C., Duan, H.,Yao, M., Zucker, B., Schlenker, G. "Simultaneous Detection of Airborne Aflatoxin, Ochratoxin, and Zearalenone in a Poultry House by Immunoaffinity Cleanup and High-Performance Liquid Chromatography," (2008) *Environmental Research* **107** (2), 139–290.

Red Pepper

Ok, H.E., Chung, S.H., Lee, N., Chun, Y.S., "Simple High-Performance Liquid Chromatography Method for the Simultaneous Analysis of Aflatoxins, Ochratoxin A, and Zearalenone in Dried and Ground Red Pepper," (2015) *Journal of Food Protection*, Vol. 78, No. 6, 1226–1231

5.0 LC with Fluorescent Detector and LC-MS/MS Setup

5.1 HPLC Conditions for TFA Derivatization

Column: 150 x 4.6 mm, 5μm, Prodigy ODS-2 from Phenomenex*****

Mobile phases:

Solution A = water:methanol:acetonitrile (55:30:15)

Solution B = acetonitrile:0.1% phosphoric acid (50:50)

Gradient: 0 - 8 min isocratic = 100% A

8.01 -13 min gradient to 30% A, 70% B

13.01 - 25 min isocratic = 30% A, 70% B

25.01 - 30 min isocratic = 100% A

Flow rate: 0.7 mL/min.

Column temperature: 30°C

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	329 nm	460 nm

Injection volume: 70 μL

Retention times:

Peak	AF G_1	AF B_1	AF G_2	AF B_2	Zearalenone	OTA
Time (min)	4.0	4.9	6.3	8.3	24.5	27.2

*Although specific HPLC column is listed here, there are several equivalent HPLC columns that can be used.

5.2 HPLC Conditions for Post Column Derivatization

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

Mobile phases:

Solution B = Methanol

Solution C = Acetonitrile

Solution D = 0.1% Acetic Acid

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 isocratic = 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

Flow rate: 1 mL/min.

Column temperature: 30°C

Wavelength settings on the Waters 2475 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	329 nm	460 nm

Injection volume: 100 µL

Post column derivatization EITHER:

5.2.1 Photochemical reactor: PhCR (P/N 600001222)

5.2.2 Post column iodine: 0.05% Iodine: 10% Methanol: 90% Water
Flow rate: 0.2mL/min.
Reaction temperature: 70°C (FIAtron FH-40 heater & FIAtron TC-50 controller)
Reaction time: Switch to go on at 0 min., off at 14 min., and then back on at 25 min. 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.

Retention times:

Peak	AF G_2	AF G_1	AF B_2	AF B_1	Zearalenone	OTA
Time (min)	7.1	8.6	9.6	11.9	19.3	22.0

5.3 UPLC Conditions

UPLC System	Waters Acquity H-Class with FLR Detector
Column	Waters Cortecs UPLC C18 1.6um, 2.1x150mm (P/N 186007096)
Injection volume	20 µL
Fluorescence condition	See wavelength settings chart
Column Temperature	25°C
Flow cell	FLR Large Volume (Waters P/N 205000609)
Run time	11.5 minutes
Software	Waters Empower™

Gradient:

Time (min)	Flow Rate (mL/min)	Percent Water	Percent Methanol	Percent Acetonitrile	Percent 0.1% Acetic Acid	Curve
initial	0.3	0	25	25	50	
5.0	0.3	0	25	25	50	6
5.50	0.3	0	30	30	40	6
10.0	0.3	0	30	30	40	6
10.5	0.3	0	25	25	50	6
11.0	0.3	0	25	25	50	11
11.5	0.3	0	25	25	50	6

Wavelength settings on the Waters Acquity FLR detector:

Time (min)	Mycotoxin	Excitation wavelength	Emission wavelength	PMT Gain
0.1 – 7.0	Aflatoxins	365 nm	455 nm	1
7.5 – 9.0	Zearalenone	276 nm	460 nm	10
9.0 – 11.5	Ochratoxin A	329 nm	460 nm	10

5.4 UPLC-MS/MS Conditions

System: Waters UPLC H-class with Xevo TQD
Column: Waters, CORTECS UPLC C_{18}, 2.1×30 mm, 1.6μm
Column temp: 25℃
Injection volume: 10μL
Gradient:

Time (min)	Flow Rate (mL/min)	Percent Water with 0.1% formic acid	Percent Acetonitrile with 0.1% formic acid
initial	0.4	90	10
3.0	0.4	90	10
10.0	0.4	30	70
10.1	0.4	10	90
12.0	0.4	10	90
12.1	0.4	90	10
15.0	0.4	90	10

MS/MS parameters for the analytes studied

Toxin	Pseudo-molecular	RT* (min)	Precursor Ion	Product Ion		CV*	CE*
				quantification	qualifier		
AFG_2	$[M+H]^+$	5.30	331.2	245.1	257.1	50	30/30
AFG_1	$[M+H]^+$	5.69	329.2	243.1	283.1	45	25/25
AFB_2	$[M+H]^+$	5.69	315.2	259.1	287.1	50	30/26
AFB_1	$[M+H]^+$	6.02	313.2	241.1	285.1	50	24/36
ZEN	$[M+H]^+$	7.75	319.2	187.0	185.0	20	19/23
OTA	$[M+H]^+$	7.84	404.2	239.1	358.2	30	19/14

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

5.5 LC Standards 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.

The three standards for spiking, Supelco products (aflatoxin: CRM46304; zearalenone: CRM46916; ochratoxin A: CRM46912) come in sealed ampoules. The approximate concentration of the standard stock solution for each of these products is as follows: aflatoxins: 2.6 μg/mL (ng/μL) in methanol; zearalenone: 50 μg/mL (ng/μL) acetonitrile; ochratoxin A: 50 μg/mL (ng/μL) benzene: acetic acid (99:1). Use only HPLC-grade methanol and reagents when preparing standard solutions. The standards are prepared according to

AOAC Official methods. The certificate of analysis will show the exact concentration. An opened ampoule should be usable for as long as 2 weeks when stored at 2–8 °C.

5.5.1 Aflatoxin solutions

Prepare a 0.26 µg/mL (ng/µL) aflatoxin standard by adding 100µL of the 2.6 ng/µL aflatoxin standard stock solution to 900µL methanol.

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

5.5.2 Ochratoxin A solutions

Prepare a 5.0 ng/µL ochratoxin solution by adding 100 µL of the 50 ng/µL ochratoxin standard stock solution to 900 µL methanol.

Prepare a 0.5 ng/µL ochratoxin solution by adding 100 µL of 5.0 ng/µL ochratoxin solution to 900 µL methanol.

Prepare a 0.05 ng/µL ochratoxin solution by adding 100 µL of 0.5 ng/µL ochratoxin solution to 900 ng/µL methanol.

5.5.3 Zearalenone solutions

Prepare a 5 ng/µl zearalenone standard by adding 100 µL of the 50 ng/µL zearalenone standard stock solution to 900 µg/mL methanol.

Prepare a 0.5 ng/µl zearalenone standard by adding 100 µL of the 5 ng/µL zearalenone standard stock solution to 900 ng/µL methanol.

5.5.4 How to Spike a 25 gram of Sample

Aflatoxins:
26 ppb (ng/g) X 25 g sample = 650 ng
650 ng ÷ 2.6 ng/µL mixed aflatoxin standard = 250 µL

Ochratoxin A:
10 ppb (ng/g) X 25 g sample = 250 ng
250 ng ÷ 5 ng/µL OTA standard = 50 µL

Zearalenone:
200 ppb (ng/g) X 25 g sample = 5000 ng
5000 ng ÷ 50 ng/µL Zearalenone standard = 100 µL

Add above standards to 25 gram of sample and allow the spiked sample to dry in a hood for at least 30 minutes before assaying.

5.5.5 Preparing aflatoxins HPLC Standards for 1-gram equivalent procedures

2.6 ppb (B_1: 1.0; B_2: 0.3; G_1: 1.0: G_2: 0.3) X 1 g = 2.6 ng
2.6 ng ÷ 0.026 ng/µL standard = 100 µL

26 ppb (B_1:10; B_2: 3.0; G_1: 10; G_2 3.0; ng/g) X 1g = 26 ng
26 ng ÷ 0.26 ng/µL standard = 100 µL
100 µL 0.26 ng/µL standard added to 1,400 µL methanol

52 ppb (B_1: 20; B_2: 6.0; G_1: 20; G_2 6.0; ng/g) X 1 g = 52 ng
52 ng ÷ 0.26 ng/µL standard = 200 µL

5.5.6 Preparing ochratoxin A HPLC Standards for 1-gram equivalent procedures

2.0 ppb (ng/g) X 1.0 g sample equivalent = 2 ng
2 ng ÷ 0.05 ng/µL ochratoxin solution = 40 µL

10 ppb (ng/g) X 1.0 g sample equivalent = 10 ng
10 ng ÷ 0.05 ng/µL ochratoxin solution = 200 µL

50 ppb (ng/g) X 1.0 g sample equivalent = 50 ng
50 ng ÷ 0.5 ng/µL ochratoxin solution = 100 µL

5.5.7 Preparing zearalenone HPLC standards for 1-gram equivalent procedures

20 ppb (ng/g) X 1 g = 20 ng
20ng ÷ 0.5 ng/µL zearalenone standard = 40 µL

200 ppb (ng/g) X 1 g = 200ng
200 ng sample ÷ 5 ng/µL zearalenone standard = 40 µL

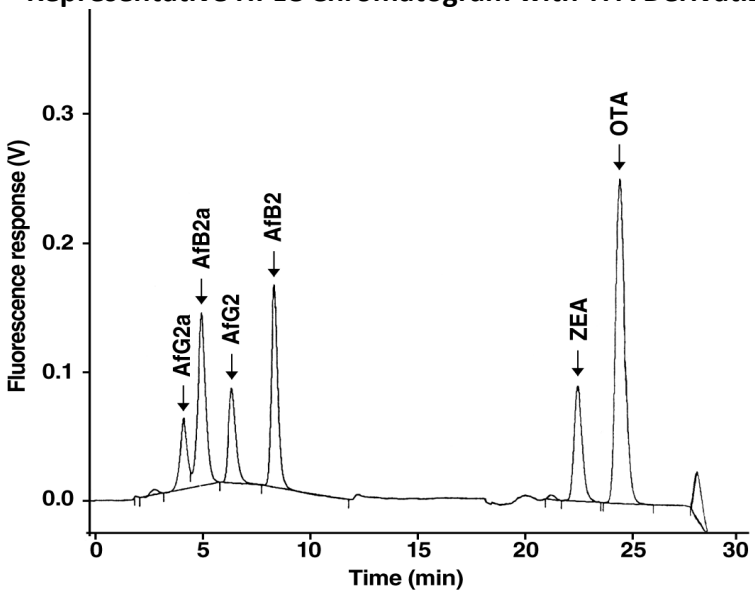
1000 ppb (ng/g) X 1 g = 1, 000 ng
1, 000 ng ÷ 5 ng/µL zearalenone standard = 200 µL

Measure all toxins calculated in 5.5.5-5.5.7 and mix with methanol to a total volume of 1.5 mL, and then add 1.5 mL 0.1% acetic acid. For example, add 100 µL of 0.026 ng/µL aflatoxin standard, 40 µL of 0.5 ng/µL zearalenone standard, and 40 µL of 0.05 ng/µL ochratoxin A standard to 1,320 µL methanol. Add 1.5 mL 0.1% acetic acid. This will make an AOZ HPLC standard at 1.0 ppb aflatoxin B_1, 0.3 ppb aflatoxin B_2; 1 ppb aflatoxin G_1, 0.3 ppb aflatoxin G_1, 20 ppb zearalenone, and 2 ppb ochratoxin A in the original sample.

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

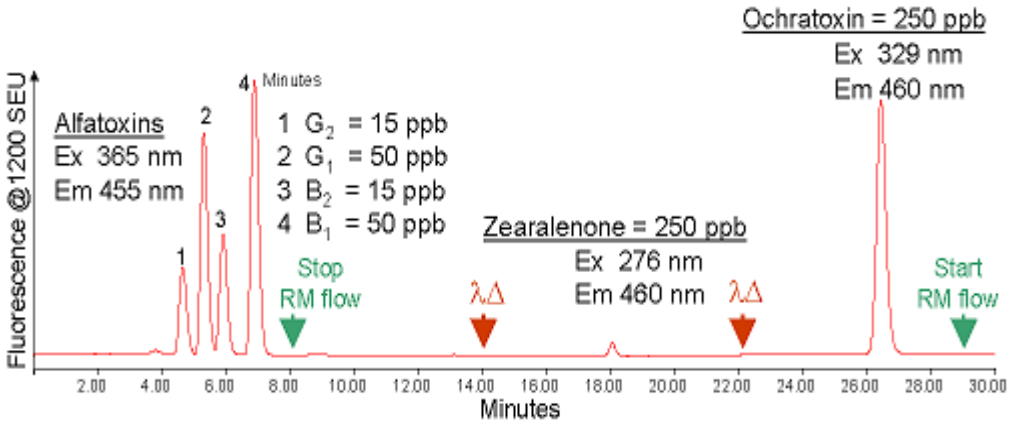
5.6 Representative Chromatograms

5.6.1 Representative HPLC Chromatogram with TFA Derivatization

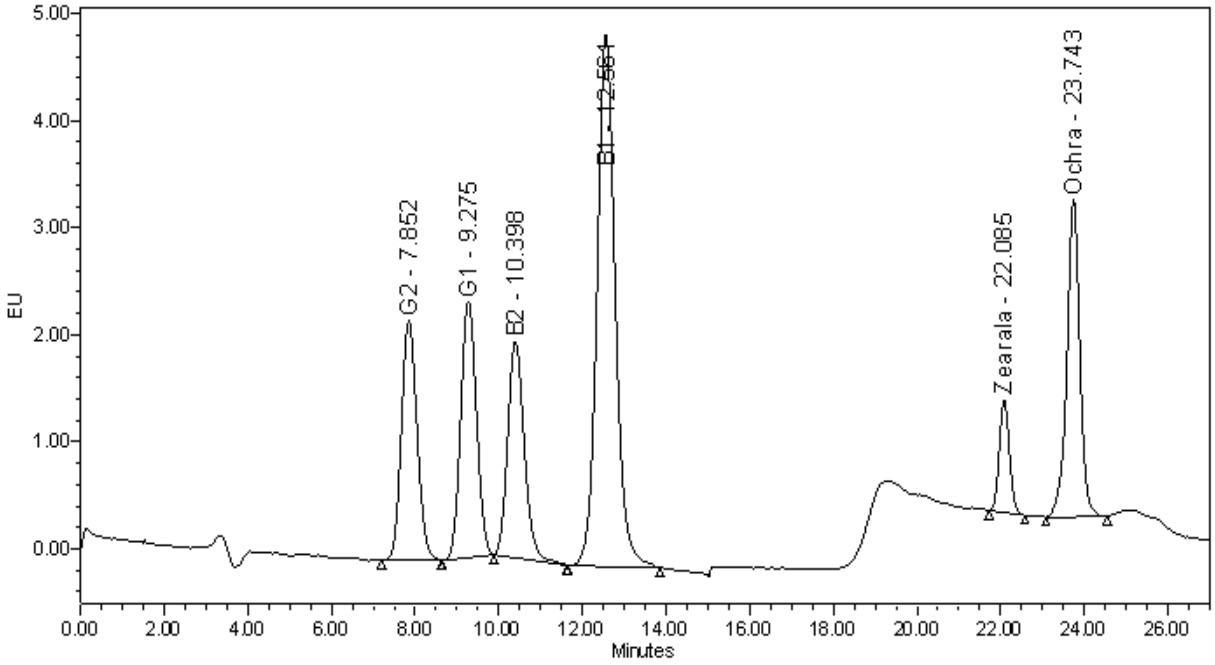


A rice mixture spiked with 5 µg/kg Aflatoxin, 75 µg/kg Zearalenone and 10 µg/kg Ochratoxin A.

5.6.2 Representative HPLC Chromatogram with Post-Column Iodine Derivatization



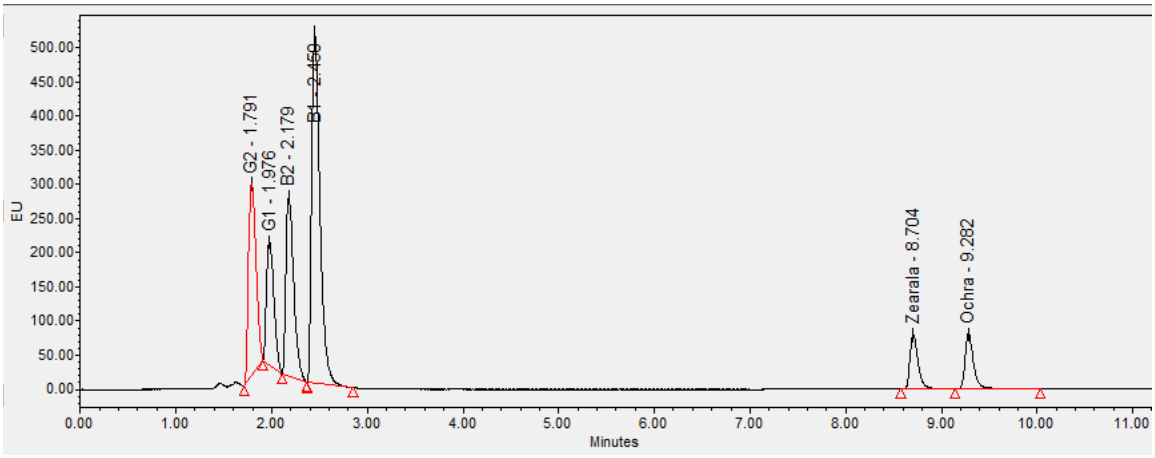
5.6.3 Representative HPLC Chromatogram with Post column PhCR derivatization



5ppb total Aflatoxins, 5ppb OTA, 50ppb Zearalenone.

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

5.6.4 Representative UPLC Chromatogram



100 ppb total Aflatoxins, 100 ppb OTA, 1000 ppb Zearalenone.

6.0 General Precautions for LC

5.1 We have found the best results when not drying down the sample eluate as aflatoxins often bind irreversibly to glassware when drying. If drying down, use silanized glassware to prevent aflatoxins from binding to the glassware.

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.

5.4 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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