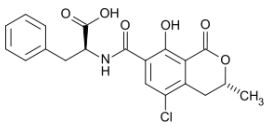
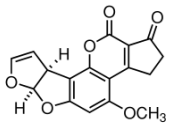
*aokin*ImmunoClean *AFLA / OTA*



Ochratoxin A



Aflatoxin B1

Manual

aokinImmunoClean Immunoaffinity columns for the quantification of Aflatoxin total and Ochratoxin

1.1. General information

aokinImmunoClean ***AFLA / OTA*** columns are used for quantification of Aflatoxin total (B1, B2, G1 and G2) and Ochratoxin (A and B) in one step for various sample types.

The methods listed in this manual are intended for customers with HPLC systems.

To measure Ochratoxin levels, samples are prepared by mixing with an extraction solution, followed by blending and filtering. The extract is then applied to the ***aokin***ImmunoClean column. The columns contain a mix of specific antibodies. The mycotoxin binds to the antibody on the column. The column is then washed to remove impurities of the sample. By passing methanol through the column, the antibody gets denatured and mycotoxin is released. Methanol can then be injected into an HPLC system.

1.2. Aflatoxin and Ochratoxin

Aflatoxin (AFLA) is a mycotoxin which is produced by several *Aspergillus* species. Aflatoxin exposure produces an acute hepatic necrosis, resulting later in chronic liver problems. Acute hepatic failure is made manifest by hemorrhage, edema, alteration in digestion, and absorption and/or metabolism of nutrients and mental changes and/or coma.

Ochratoxins are mycotoxins which are produced by several *Aspergillus* and *Penicillium species*. Ochratoxins are considered a human carcinogen. Exposure to Ochratoxins through diet can lead to acute toxicity to mammalian kidneys, and may be carcinogenic. In addition Ochratoxins affect the immune system.

Mycotoxins are found in many different sample types, especially when samples are stored under wet and humid conditions.
In line with various regulatory laws, it is required to control the contamination in food and feed.

1.3. Application

aokinImmunoClean ***AFLA / OTA*** columns have been tested and optimized for quantitative measurement of Aflatoxin total and Ochratoxin A in wheat and corn.

aokinImmunoClean columns can be used with AOAC Official Methods for the measurement in baby food, barley, beer, wine, green coffee and roasted coffee .

They may also be used for testing in soy, silage, hay, peanuts, tree nuts, spices or for a QC of infant food.

For all questions relating to the optimal use of our columns, please contact our experienced technical staff who will be glad to assist you (*info@aokin.com*).

1.4. Limitations, shelf life and storage

This product has been designed for use with the protocol and reagents described on the following pages. Do not use materials beyond the expiration date. Deviation from these instructions may not yield optimum results. Do not freeze columns or reagents. Do not keep them in the heat. Store at 2-8°C. It is recommended that reagents should be at ambient temperature for usage, best at 18-22°C.

1.5. General recommendation

- Perform test from beginning to end without interruptions.
- Load sample on column immediately after centrifugation.
- Mix the eluate in the cuvette very well before injecting eluate into HPLC.
- Avoid contact of any test reagents or solutions (such as acetonitrile, methanol or column eluate) with rubber or soft flexible plastic. These materials may leach fluorescence into the sample.
- Maintain a slow and steady flow rate through the ***aokin****ImmunoClean* column (1-2 drops/second) during sample loading.
- Elute the column at a rate of 1 drop for every 2-3 seconds.

1.6. Types of columns

		
Column type	wide bore	spin
Order No.:	**IC-C-0304-25**	**IC-M-0304-50**
Package size:	25 units / pack	50 units / pack
Elution volume	3 mL = 1 mL + 2 mL	500 µL = 200 µL + 300 µL
Recommended loading:	< 200 ng Aflatoxin total < 200 ng Ochratoxin	< 40 ng Aflatoxin total < 40 ng Ochratoxin

Use of adapters and reservoirs for loading recommended (Order no.: LB-08-13)

1.7. Preparation

1.7.1. Cleaning

All equipment has to be clean and not contaminated with materials that might cause interference with the analysis. All equipment should be washed with a mild detergent solution and then rinsed thoroughly with purified water. This includes glass ware, adapters and syringe barrels used for sample reservoirs. In between assays it is sufficient to rinse with methanol and water. This helps to prevent cross-contamination of samples.

1.7.2. Preparation of reagents

Prepare solutions every week or as needed.

CAUTION: Methanol, Acetonitrile and the solutions made thereof are flammable. Keep containers in a safe place and tightly capped when not in use.

Extraction solvent: Methanol/PBS
Use Methanol HPLC grade only. Use 700 mL methanol and 300 mL PBS buffer, mix.

Diluting Buffer: PBS
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.

Wash Buffer: PBS/Methanol
Use 100 mL methanol and 900 mL PBS, mix.

Methanol for elution
Use HPLC Grade methanol only.

Tween-20 solutions
10% Tween-20
Tween 20: 100 mL
Purified Water: 900 mL
Total Volume: 1000 mL

15 % Tween-20
Tween 20: 150 mL
Purified Water: 850 mL
Total Volume: 1000 mL

HPLC Mobile Phases
Methanol/water (45:55)
HPLC Grade Methanol: 450 mL
Purified Water: 550 mL
Total Volume: 1000 mL

Water:Acetonitrile:Methanol (3:1:1)
Purified Water: 600 mL
Acetonitrile: 200 mL
HPLC Grade Methanol: 200 mL
Total Volume: 1000mL
Solutions should be filtered and degassed before use.

Iodine solution (0.05%)
Iodine: 0.5 g
Methanol: 100 mL
Purified water: 900 mL
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 filter.

Kobra Cell Mobile Phase
Methanol: 450 mL
Purified water: 550 mL
Potassium Bromide: 119 mg
Nitric Acid 16M (70%): 7.5 µL

Preparation of spiking solutions
Prepare a 0.25 ng/µL Aflatoxin standard by adding 100µL of a 2.5 ng/µL Aflatoxin standard stock solution to 900µL methanol.
Prepare a 0.025 ng/µL Aflatoxin standard by adding 100µL of the 0.25ng/µL Aflatoxin standard to 900µL methanol.

Preparing spiking corn with Aflatoxin at 25 ppb level
25 ppb (ng/g) X 50 g corn = 1250 ng
1250 ng ÷ 2.5 ng/µL = 500 µL
Add 500µL of the 2.5 ng/µL Aflatoxin standard to 50g of Aflatoxin-free corn. Allow the spiked sample to dry in a hood for at least 30 minutes before assaying. Alternatively spike directly into the mixture of 50 g corn and 100 mL extraction solvent.

Prepare HPLC standard diluent
Mixing equal volumes of eluting solution and HPLC quality water.

Prepare HPLC standards for 1g equivalent procedures
1.25 ppb x 1 g = 1.25 ng
1.25 ng ÷ 0.025 ng/µL standard = 50 µL
50 µL 0.025 ng/µL standard added to 950 µl methanol

2.5 ppb x 1g = 2.5 ng
2.5 ng ÷ 0.025 ng/µL standard = 100 µL
100 µL 0.025 ng/µL standard added to 900 µl methanol

25 ppb x 1g = 25 ng
25 ng ÷ 0.25 ng/µL standard = 100 µL
100 µL 0.25 ng/µL standard added to 900 µl methanol

50 ppb x 1g = 50 ng
50 ng ÷ 0.25 ng/µL standard = 200 µL
200 µL 0.25 ng/µL standard added to 800 µl methanol

1.8. Materials required for the sample preparation and the HPLC

aokin Filter Paper	(LB-05-07-100, 100 units/pack)
Glass fiber filters GF/F	(LB-04-13-GF/F-100, 100 units/pack)
Collection tubes 2 mL	(LB-05-05, 500 units/pack)
Collection tubes 15 mL	(LB-05-01-100, 100 units/pack)
Collection tubes 50 mL	(LB-03-02-1000, 1L)
Methanol, HPLC Grade	
Sodium Chloride, pure	
Acetonitrile, HPLC Grade	
Sodium bicarbonate	
Glacial acetic acid, 99% purity	
Distilled, reverse osmosis or deionized water	
Graduated Cylinder, 50 mL	(LB-08-16, 1 unit)
Graduated Cylinder, 250 mL	(LB-08-17, 1 unit)
Digital Scale	(LB-07-04, 1 unit)
Commercial Blender, with metal beaker for use with acetonitrile mixtures	
Commercial Blender, with plastic beaker (200 mL) for use with methanol mixtures	(EX-07-06, 1 unit)
Wash Bottle, 500 mL	
Cuvette Rack	
Pump Stand with Air Pump	
Vacuum pump	(LB-04-10, 1 unit)
Vacuum manifold	(LB-04-09, 1 unit)
Filter Funnel, 65 mm	(LB-06-01, 1 unit)
Adjustable Micro-pipettor, 1000 µL	(LB-04-05-1000, 1 unit)
Micro-pipette Tips for adjustable Micro-pipettor, 1000 µL	(LB-04-08-1000L, 100 units/pack)

1.9. Set up and equilibration of columns

Allow column to be at ambient temperature. Remove bottom cap and place the column onto a vacuum manifold, or in a pump stand or collection tube. Open top cap and fill column with PBS/Methanol (90/10). Connect adapter and a reservoir to the column. Use a flow rate of 1 mL/min and have 1-2 ml pass through the column. This step ensures an equilibration of the column. Close the valve again to stop the flow.

2. Points of critical importance for reproducibility and recovery

2.1. Representative sampling

A representative sample is essential for accurate and reliable results. Samples should be collected and ground before taking a subsample. Contamination of mycotoxin may differ significantly within a single batch and from kernel to kernel.

2.2. Sample preparation

Different procedures require different reagents. Please make sure that your protocol consists of the following points:

- Extract with high solvent ratio.
- Dilute to 10 % solvent.
- Adjust to neutral pH.
- Remove all precipitation by glassfiber filtration using a 0.7 µm mesh size.
- Equilibrate column to room temperature, best by rinsing with wash buffer.
- Load column with flow rate of 1 mL/min.
- Wash column with wash buffer.
- Dry column by vacuum or air pressure.
- Apply 2 mL Methanol/1%Acetic Acid. Incubate for 3 minutes by stopping flow. Apply 2 mL Methanol/1%Acetic Acid. (Alternatively 100 % Methanol can be used)
- Elute by vacuum or air pressure at 1 mL/minute or by back flushing with a syringe.
- Dilute with purified water.
- Quantify the concentration by comparing the sample peak height or area to the standard.

aokinImmunoClean columns have been optimized for quantitative measurement of Ochratoxins in many commodities. Test methods vary in the amount of sample passed through the affinity column resulting in different limits of detection.

General recommendation:

- Perform test from beginning to end without interruptions.
- Load sample on column immediately after centrifugation.
- Mix the eluate in the cuvette very well before injecting eluate into HPLC.
- Avoid contact of any test reagents or solutions (such as acetonitrile, methanol or column eluate) with rubber or soft flexible plastic. These materials may leach chemicals into the sample.
- Maintain a slow and steady flow rate through the column during sample loading.
- Elute the column slowly, do an incubation step.

Example Procedures:

A1. Corn, grains and feed

Sample extraction:

- Place 25g ground sample with 5 g salt (NaCl) into blender jar.
- Add to jar 100 mL Methanol/PBS buffer (80:20) or alternatively Methanol/water.
- Cover jar and blend at high speed for at least 3.5 minutes.
- Remove cover and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

Dilution:

- Transfer 2 mL filtered extract into another clean vessel.
- Dilute extract by adding 18 mL of PBS. Precipitation may take place.
- Check pH to be neutral, if required neutralize by adding small amounts of 2 M HCl or 2 M NaOH.
- Filter diluted extract through a glass microfibre filter into a clean vessel.

Column chromatography:

- Pass 10 mL filtered diluted extract completely through column at a rate of about 1 drops/second until air comes through column.
- Pass 20 mL of PBS through the column at a rate of about 2 drops/second.
- Dry column with air flow.
- Place new collection tube under the ***aokin***ImmunoClean column.

Elution:

- Add 1 mL Methanol.
- Incubate for 3 minutes by stopping flow.
- Pass additional 2 mL Methanol through the ***aokin***ImmunoClean column at a rate of 1 drop/second. Apply air flow to collect all liquid out of the column.
- Transfer a definite amount into a new vial and add destilled water to eluate 1:1.
- Inject 20 to 100 µl into HPLC.

A2. Nuts

Sample Extraction:

- Weigh 25 g ground sample with 5 g salt (NaCl) and place in blender jar.
- Add to jar 100 ml Methanol/water (80:20).
- Cover blender jar and blend at high speed for 5 minute.
- Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

Extract dilution:

- Dilute 2 mL of extract with 18 mL of 10 % Tween 20 in PBS. Mix well.
- Adjust to around pH 7.4 using 2 M sodium hydroxide.
- Filter diluted extract through glass microfibre filter into a clean vessel.

Column chromatography:

- Pass 10 ml of filtered diluted extract (10 ml = 25 g sample equivalent) completely through the ***aokin****ImmunoClean* column at a rate of about 1-2 drops/second until air comes through column.
- Pass 20 ml of PBS through the column at a rate of about 2 drops/second.
- Repeat the washing once more until air comes through column.

Elution:

- Add 1 mL Methanol.
- Incubate for 3 minutes by stopping flow.
- Pass additional 2 mL Methanol through the ***aokin****ImmunoClean* column at a rate of 1 drop/second. Apply air flow to collect all liquid out of the column.
- Transfer a definite amount into a new vial and add destilled water to eluate 1:1.
- Inject 20 to 100 µl into HPLC.

B. Setup column

- Connect ***aokin****ICAdapter* and a 50 mL syringe barrel (best flow when bubble free).
- Place on vacuum manifold or pump stand.
- Flush with 2 mL PBS to ensure equilibration.

C. Recovery

- Recovery of 80% mycotoxin tested in PBS buffer.
- Exact results are found in the attached data sheet.
- Test the recovery of ***aokin****ImmunoClean* columns with your protocol and HPLC technique, and use a correction factor as determined.

D. Recommended HPLC conditions

Example 1

- Derivatization: KOBRA Cell at 100µA setting
- Guard Cartridge: Inertsil ODS-3
  5µm, 4 mm x 10 mm or equivalent
- Analytical Column: Inertsil ODS-3V
  5 µm, 4.6 mm x 150 mm or equivalent
- Mobile Phase: Solution A: Water/Methanol (55:45 v/v)
  Solution B: Water/Metanol (20/80 v/v)
  Add 119 mg potassium bromide and 350 µL 4 M Nitric Acid to 1 L of mobil phase A and B. Prepare fresh on day of analysis
- Flow rate: 0.8 mL/min
- Fluorescence Detector
- Column Heater 40 °C
- Injection Volume: 100 µL
- Elution Order: G2, G1, B2 B1 OTA

There are a number of equally suitable components that can be used for these examples.

Trouble shooting

3.1. Problem: Samples do not mix

- If samples are very absorbent double the amount of extraction liquid and double the extract volume passed through the column to 20 mL keep the same sensitivity of your analysis system.

3.2. Problem: Overestimation

- Check calculation for spiked sample and standard curve.

3.3. Problem: Underestimation

- Check the extraction procedure.
- Make sure you use the correct dilution rate. Check pH to be neutral before loading the column.
- Control the flow rates and the incubation step for elution.
- Check calculations for spiked samples.
- Make sure to use the correct HPLC procedure.
- Check calculation for spiked sample and standard curve.
- Control the procedure with analyzing a reference matrix material.

Liabilities

The customer is solely and fully responsible for educating oneself about the proper testing and sampling procedures using this product. ***aokin*** makes no warranty of any kind. ***aokin*** is not liable or responsible for any unsatisfactory or faulty results.

Ordering and technical support

To place an order please contact ***aokin*** at orders@aokin.com

For technical information please contact service@aokin.com

Please contact the application laboratory and service staff for all questions relating to the optimal use of our columns We will be glad to assist you.