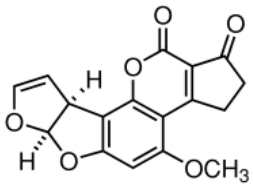
Manual

aokin*ImmunoClean* Immunoaffinity columns for the quantification of Aflatoxin total



Aflatoxin B1

1.1. General information

aokin*ImmunoClean* ***AFLA*** columns are used for quantification of Aflatoxin in various sample types.

These products are safe and simple. The methods listed in this manual are intended for customers with HPLC systems.

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

1.2. Aflatoxin

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. In line with various regulatory laws, it is required to control the AFLA content in food and feed.

Aflatoxins 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

aokin*ImmunoClean* ***AFLA*** columns have been tested and optimized for quantitative measurement of Aflatoxin B1 in corn and other grain, nuts and feed.

aokin*ImmunoClean* ***AFLA*** columns can be used with AOAC Official Methods for the measurement of Aflatoxin B1 in baby food.

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* ***AFLA*** 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-03-25**	**IC-M-03-50**
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	< 40 ng

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 and 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 100 µL of a 2.5 ng/µL Aflatoxin standard stock solution to 900 µL methanol.
Prepare a 0.025 ng/µL Aflatoxin standard by adding 100 µL 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

aokinImmunoClean **C** ***AFLA***	(IC-C-03-25, 25 units/pack)
aokinImmunoClean **M** ***AFLA***	(IC-M-03-50, 50 units/pack)
aokin Filter Paper	(LB-05-10-100, 100 units/pack)
Glass fiber filters GF/F	(LB-04-13-GF/F-100, 100 units/pack)
Reaction tubes (2 ml, with lid)	(LB-05-05, 500 units/pack)
Test tubes (15 ml, with lid)	(LB-05-01-100, 100 units/pack)
Test tubes (50 ml, with lid)	(LB-05-02-250, 250 units/pack)
Methanol, HPLC Grade	(LB-03-02-1000, 1 L)
Sodium Chloride, pure	
Acetonitrile, HPLC Grade	
Distilled, reverse osmosis or deionized water	
Graduated cylinder stand (50 mL)	(LB-08-16, 1 unit)
Graduated cylinder stand (250 mL)	(LB-08-17, 1 unit)
Cuvette Rack	(LB-05-04)
Digital Scale	(LB-07-04, 1 unit)
Commercial blender, with metal or glass beaker for use with Acetonitrile mixtures	(EX-08, 1 unit)
Commercial blender, with plastic beaker (200 mL) for use with Methanol mixtures	(EX-07-06, 1 unit)
Vacuum-pump (diaphragm pump)	(LB-04-10, 1 unit)
Trap for Vacuum-pump (vacuum bottle), 500 mL	(LB-04-12, 1 unit)
Vacuum manifold	(LB-04-09, 1 unit)
Filter funnel (for retainig paper filters)	(LB-06-01, 1 unit)
Adjustable Micropipette, 1000 µL	(LB-04-05-1000, 1 unit)
Micropipette tips for adjustable Micropipette, 1000 µL	(LB-04-08-1000L, 500 units/pack)
aokinReferenceMatrixMaterial ***AFLA***	(RMM-03)

1.9. Set up and equilibration of columns

Allow column to be at ambient temperature. Remove bottom cap first 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.

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 % max 15 % solvent.
- Adjust to neutral pH.
- Remove all precipitation by glassfiber-filtration using a 1.7 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 1 mL Methanol. Incubate for 3 minutes by stopping flow. Apply 2 mL Methanol (Alternatively use back flushing technique).
- Elute by vacuum or air pressure at 1 mL/minute or by back flushing with a syringe.
- Dilute with purified water.
- Quantify the Aflatoxin concentration by comparing the sample peak height or area to the standard.

aokin*ImmunoClean* ***AFLA*** columns have been optimized for quantitative measurement of Aflatoxins 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 50 g 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 5 mL filtered extract into another clean vessel.
- Dilute extract 1:7 by adding 30 mL of PBS. Precipitation takes place.
- Check pH to be neutral, if required neutralize by adding small amounts of HCl or NaOH.
- Filter diluted extract through a glass microfibre filter into a clean vessel.

Column chromatography:
- Pass 15 mL filtered diluted extract completely through column at a rate of about 1 drops/second until air comes through column.
- Pass 15 mL of wash buffer 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. Corn, raw peanuts, peanut butter

Sample Extraction:
- Weigh 25 g ground sample with 5 g salt (NaCl) and place in blender jar.
- Add to jar 125 ml Extraction solvent.
- Cover blender jar and blend at high speed for 2 minutes.
- Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

Dilution:
- Pipet or pour 10 ml filtered extract into a clean vessel.
- Dilute extract with 30 ml of purified water. Mix well.
- Filter diluted extract through glass microfibre filter into a clean vessel.

Column chromatography:
- Pass 20 ml filtered diluted extract completely through column at a rate of about 1-2 drops/second until air comes through column.
- Pass 10 ml of wash buffer or purified water through the column at a rate of about 2 drops/second.
- Repeat washing step once more until air comes through the column.
- 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.
- Add 1 mL destilled water to eluate 1:1.
- Inject 20 to 100 µl into HPLC.

A3. Fluid milk samples

Sample Extraction:
- Add 1g NaCl to 40 ml fluid milk sample and mix well.
- Centrifuge milk at greater than 15.000g for 25 minutes.
- Carefully remove the skim portion (bottom layer) of the milk for analysis without disturbing the top, fat layer (a syringe needle can be used to poke a hole into the bottom of a plastic centrifuge tube).
- Immediately before affinity chromatography analysis, filter the skim sample through glass microfiber filter paper.

Column chromatography:
- Pass 25 ml of filtered milk sample through the ***aokin***ImmunoClean column at a steady slow flow rate of about 1-2 drops/second.
- After milk sample has completely passed through column, transfer column to a clean syringe barrel and pass 10 ml 10% Methanol/ 90% water solution through column twice at about 2 drops/second flow rate.
- Make sure all the liquid has passed through the ***aokin***ImmunoClean column. A few seconds of air can come through the column.
- Place collection vial under the column and add 1 mL Methanol/water (80:20) into syringe barrel.

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.
- Add 1 mL destilled water to eluate 1:1.
- Inject 20 to 100 µl into HPLC.

A4. Nutmeg

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 1 minute.
- Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

Extract dilution:
- Pipet or pour 5.0 ml filtered extract into a clean vessel.
- Dilute extract with 20 ml of 15% Tween-20 solution. Mix well.
- Filter diluted extract through glass microfibre filter into a clean vessel.

Column chromatography:
- Pass 4 ml of filtered diluted extract (4 mL = 0.2 g sample equivalent) completely through the ***aokin***ImmunoClean column at a rate of about 1-2 drops/second until air comes through column.
- Pass 10 ml of purified water 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.
- Add 1 mL destilled water to eluate 1:1.
- Inject 20 to 100 µl into HPLC.

A5. Dried onions

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 1 minute.
- Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

Extract dilution:
- Pipet or pour 10 ml filtered extract into a clean vessel.
- Dilute extract with 40 ml of purified water. Mix well.
- Filter diluted extract through glass microfibre filter into a clean vessel.

Column chromatography:
- Pass 4 ml of filtered diluted extract completely through the ***aokin***ImmunoClean column at a rate of about 1-2 drops/second until air comes through column.
- Pass 10 ml of purified water through the column at a rate of 1-2 drops/second.
- Repeat washing 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.
- Inject 20 to 100 µl into HPLC.

A6. Oregano

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 1 minute.
- Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

Extract dilution:
- Pipet or pour 5.0 ml filtered extract into a clean vessel.
- Dilute extract with 20 ml of 10% Tween-20 solution. Mix well.
- Filter diluted extract through glass microfibre filter into a clean vessel.

Column chromatography:
- Pass 4 ml of filtered diluted extract (4 ml = 0.2 g sample equivalent) completely through the ***aokin***ImmunoClean column at a rate of about 1-2 drops/second until air comes through column.
- Pass 10 ml of purified water through the column at a rate of about 2 drops/second.
- Repeat the washing once more until air comes through column.
- Place collection vial under column and add 1 ml HPLC grade Methanol into syringe barrel.

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.
- Add 1 mL destilled water to eluate 1:1.
- Inject 20 to 100 µl into HPLC.

A7. Paprika, chili pepper and red pepper

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 1 minute.
- Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

Extract dilution:
- Pipet or pour 10 ml filtered extract into a clean vessel.
- Dilute extract with 40 ml of purified water. Mix well.
- Filter diluted extract through glass microfibre filter into a clean vessel.

Column chromatography:
- Pass 4 ml of filtered diluted extract (4 ml = 0.2 g sample equivalent) completely through the ***aokin***ImmunoClean column at a rate of about 1-2 drops/second until air comes through column.
- Pass 10 ml of Methanol/water (20:80) 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.
- Add 1 mL destilled water to eluate 1:1.
- Inject 20 to 100 µl into HPLC.

A8. Parsley

Sample Extraction:
- Weigh 25 g ground sample with 5 g salt (NaCl) and place in blender jar.
- Add to jar 200 ml Methanol/water (80:20).
- Cover blender jar and blend at high speed for 1 minute.
- Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

Extract dilution:
- Pipet or pour 10 ml filtered extract into a clean vessel.
- Dilute extract with 40 ml of purified water. Mix well.
- Filter diluted extract through glass microfibre filter into a clean vessel.

Column chromatography:
- Pass 8 ml of filtered diluted extract (8 ml = 0.2 g sample equivalent) completely through the ***aokin***ImmunoClean column at a rate of about 1-2 drops/second until air comes through column.
- Pass 10 ml of purified water through the column at a rate of about 2 drops/second.
- Repeat the washing once more until air comes through column.
- Place collection vial under column and add 1 mL HPLC grade Methanol into syringe barrel.

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.
- Add 1 mL destilled water to eluate 1:1.
- Inject 20 to 100 µl into HPLC.

A9. Peanuts, cashews, apricot nuts, almonds, pistachios, walnuts and pecans

Sample Extraction:
- Weigh 25 g ground sample with 5 g salt (NaCl) and place in blender jar.
- Add to jar 125 ml Methanol/water (60:40).
- Cover blender jar and blend at high speed for 1 minute.
- Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

Extract dilution:
- Pipet or pour 20 ml filtered extract into a clean vessel.
- Dilute extract with 20 ml of purified water. Mix well.
- Filter diluted extract through glass microfibre filter into a clean vessel.

Column chromatography:
- Pass 10 ml of filtered diluted extract (10 ml = 1 g sample equivalent) completely through the ***aokin***ImmunoClean column at a rate of about 1-2 drops/second until air comes through column.
- Pass 10 ml of purified water 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.
- Add 1 mL destilled water to eluate 1:1.
- Inject 20 to 100 µl into HPLC.

A10. Black pepper and turmeric

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 1 minute.
- Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

Extract dilution:
- Pipet or pour 5 ml filtered extract into a clean vessel.
- Dilute extract with 20 ml of 15% Tween-20 solution. Mix well.
- Filter diluted extract through glass microfibre filter into a clean vessel.

Column chromatography:
- Pass 4 ml of filtered diluted extract (4 ml = 0.2 g sample equivalent) completely through the ***aokin***ImmunoClean column at a rate of about 1-2 drops/second until air comes through column.
- Pass 10 ml of purified water through the column at a rate of about 2 drops/second.
- Repeat the washing once more until air comes through column.
- Place collection vial under column and add 1 mL HPLC grade Methanol into syringe barrel.

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.
- Add 1 mL destilled water to eluate 1:1.
- Inject 20 to 100 µl into HPLC.

A11. Mixing pellets, safflower seeds, safflower meal, canola seeds, canola meal, dried distillers grain and high fiber samples

Sample Extraction:
- Weigh 50 g ground sample with 10 g salt (NaCl) and place in blender jar.
- Add to jar 200 ml Methanol/water (80:20).
- Cover blender jar and blend at high speed for 1 minute.
- Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

Extract dilution:
- Pipet or pour 10 ml filtered extract into a clean vessel.
- Dilute extract with 40 ml of purified water. Mix well.
- Filter diluted extract through glass microfibre filter into a clean vessel.

Column chromatography:
- Pass 4 ml of filtered diluted extract (4 ml = 0.2 g sample equivalent) completely through the ***aokin***ImmunoClean column at a rate of about 1-2 drops/second until air comes through column.
- Pass 5 ml of purified water 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.
- Add 1 mL 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 wash buffer to ensure equilibration.

C-Mini: Alternative protocol for spin columns only (IC-M-03-50)

- Pass 50 mL of defatted skim milk over affinity column at a rate of about 1 drops/second (1 mL/min) until air comes through column. DO NOT EXCEED RECOMMENDED FLOW RATES. This can result in decreased recovery. If it flows too fast, adjust the stopcock to slow down the flow rate so the sample flows through the column at 1-2 drops/second by gravity. If the flow is too low apply vacuum or positive pressure by using the ***aokin****ICAdapter* and a syringe.
- Remove syringe barrel from column and fill the column head space with water. Pass 10 mL of purified water through the column at a rate of 1-2 drops/second. Pass again 10 mL water to the column and let run dry until air comes through column.
- Dry column with air flow. Disconnect column from adapter and place in 2 mL reaction tube for centrifugation, centrifuge 1 min at 1000 g for complete removal of excess liquid.
- Place new 2 mL reaction tube under column.
- Add 600 mL elution solvent. The elution solvent should be methanol or acetonitrile/methanol (3:2). Push about a third of the methanol through the gel bed by placing the lid onto the column. Incubate for 3 minutes.
- Centrifuge at 3000 x g for 2 minutes.
- Optional: add 100 µl distilled water to elute. Vortex sample.

D. Recovery

- Recovery of 80% Aflatoxin 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.

E. HPLC conditions

Absorbance detection is possible at 365 nm. For greater sensitivity, add purified water to eluate and concentrate the volume of the eluate to about 100 - 200 µL on a steam plate, under nitrogen or on an evaporator. Inject entire sample quantitatively. If drying is performed, use siliconized vials to avoid irreversible binding of Aflatoxins to the tube walls.

Aflatoxins B2 and G2 are naturally much more fluorescent than Aflatoxins B1 and G1. Aflatoxin B1 and G1 fluorescence can be increased for HPLC with fluorescence detection by derivatization using one of the four methods:

- Pre-column Trifluoroacetic acid (TFA)
- Post-column iodine derivatization
- Post-column electrochemically generated bromine (KOBRA or COBRA cell)
- Post-column in-line photochemical derivatization (PHRED)

E. HPLC setup

Example 1

- Column: reverse phase C18
- Mobile phase: Methanol/water (45:55) isocratic degassed.
- Flow rate: 0.8 mL/min.
- Fluorescence detector: excitation 360 nm, emission 440 nm.
- Post column iodine: 0.05% iodine solution.
- Flow rate: 0.2 mL/min.
- Reaction temperature: 70 °C.
- Reaction time: ~1 minute.

Example 2

- Column: reverse phase C18
- Mobile phase: Methanol/water (45:55) isocratic degassed.
- Flow rate: 1.0 ml/min.
- Fluorescence detector: excitation 360 nm, emission 440 nm.
- Post column: Photochemical reactor.

Example 3

- Column: 4.6 mm x 25 cm, 5 µm, C18
- Mobile phase: Water/acetonitrile/methanol (3:1:1) degassed.
- Flow rate: 1.0 ml/min.
- Fluorescence detector.
- Post column iodine: 0.05% iodine solution.
- Flow rate: 0.3 ml/min.
- Reaction temperature: 70 °C.
- Reaction time: ~1 minute.

Example 4

- Column: C18 4.6 X 150 mm
- Mobile phase: Methanol/water (45:55) isocratic degassed.
- Flow rate: 0.8 mL/minute.
- Fluorescence Detector: excitation 360 nm, emission 440 nm.
- Peak retention time: ~5 minutes.

Example 5

- Column: 4.6mm X 25cm C18
- Mobile phase: Methanol/water (50:50) isocratic degassed.
- Flow rate: 0.7 ml/minute.
- Absorbance Detector.
- Peak retention time: ~8 minutes

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 of Aflatoxin

- Check calculation for spiked sample and standard curve.

3.3. Problem: Underestimation of Aflatoxin

- 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.
- Eluate is cloudy. Add water to eluate.
- Check the collection vials for their adsorbent properties. Aflatoxin may stick to surfaces. Use silanized glassware.

3.4. Sensitivity

- For greater sensitivity, more sample volume may be passed over column.
- Concentrate elute to 100 µl.
- Derivatization increases the sensitivity.

Other published procedures

Jaimez J, Fente CA, Vazquez BI, Franco CM, Cepeda A, Mahuzier G, Prognon P.
Journal of Chromatography A. Application of the assay of aflatoxins by liquid
chromatography with fluorescence detection in food analysis. 2000 Jun 16; 882(1-2): 1-10. Review.

Kok WT. *Journal of Chromatography B Biomed Application.* Derivatization reactions for
the determination of aflatoxins by liquid chromatography with fluorescence detection. 1994 Sep 23; 659 (1-2): 127-37.

Joshua, H., *Journal of Chromatography*, Determination of aflatoxins by reversed-phase high-performance liquid chromatography with post-column in-line photochemical derivitization and fluorescence detection, 654 (1993) 247-254.

Waltking, Arthur, *Journal of AOAC International*, Liquid Chromatographic Analysis of Aflatoxin Using Post-Column Photochemical Derivatization: Collaborative Study, 2006, 89 (3), 678-692.

PUBLISHED HPLC PROCEDURES:

Beer
Scott PM, Lawrence GA. *Journal of AOAC International*. Determination of aflatoxins in beer. 1997 Nov-Dec; 80(6): 1229-34.

Cattle Feed
Stroka, J.; von Holst, C.; Anklam, E.; Reutter, M. *Journal of AOAC Int'l.* Immunoaffinity Column Cleanup with Liquid Chromatography Using Post-Column Bromination for Determination of Aflatoxin B1 in Cattle Feed: Collaborative Study **2003**, *86(6)*, 1179-1186.

Corn, Peanuts and Peanut butter
Trucksess MW, Stack ME, Nesheim S, Page SW, Albert RH, Hansen TJ, Donahue KF. *Journal of Association Official Analytical Chemist.* Immunoaffinity column coupled with solution fluorometry or liquid chromatography postcolumn derivatization for determination of aflatoxins in corn, peanuts, and peanut butter: collaborative study. 1991 Jan-Feb; 74(1): 81-88.

Infant formula
Stroka J, Anklam E, Joerissen U, Gilbert J. *Journal of AOAC International* Determination of aflatoxin B1 in baby food (infant formula) by immunoaffinity column cleanup liquid chromatography with postcolumn bromination: collaborative study. 2001 Jul-Aug; 84(4): 1116-23.

Milk
Dragacci S, Grosso F, Gilbert J. *Journal of AOAC International*, Immunoaffinity column cleanup with liquid chromatography for determination of aflatoxin M1 in liquid milk: collaborative study. 2001 Mar-Apr; 84(2): 437-43

Milk
Hansen T.J., *Journal of Food Protection*, Affinity column cleanup and direct fluorescence measurement of Aflatoxin M1 in raw milk, **53** (1) (1990) 75-77.
Ioannou-Kakouri, E., Christodoulidou, M., Christou, E., Constantinidou, E., *Food and Agricultural Immunology,* Immunoaffinity column/HPLC determination of Aflatoxin M1 in milk, **7** (1995): 131-137.

Peanut butter, Pistachio paste, Fig paste, and Paprika powder (AOAC Method 999.07)
Stroka J, Anklam E, Jorissen U, Gilbert J. J. *Journal of AOAC International* Immunoaffinity column cleanup with liquid chromatography using post-column bromination for determination of aflatoxins in peanut butter, pistachio paste, fig paste, and paprika powder: collaborative study. 2000 Mar-Apr; 83(2): 320-40.

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.