www.aokin.com

aokin ImmunoClean AFLA M1

High performance immunoaffinity columns (IAC)
for the quantification of aflatoxin M1

MANUAL

High performance immunoaffinity columns (IAC)
for the quantification of aflatoxin M1

Instructions for use

1.1 General information

aokin *ImmunoClean* ***AFLA M1*** columns are used for quantification of aflatoxin M1 in milk and milk products. The methods listed in this manual are intended for customers with HPLC systems.

To measure aflatoxin M1 levels, samples are prepared by defatting and extracting. 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.

AFLA M1 testing is used in a wide variety of locations from milk processing quality control laboratories to government testing laboratories – anyplace where quick, easy to perform and highly accurate aflatoxin M1 analysis can prevent contamination and improve the quality of the milk supply.

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. Aflatoxin M1 is a metabolite of aflatoxin B1 which is present in the milk of animals that ingest feed contaminated with aflatoxin B1.

aokin *ImmunoClean* ***AFLA M1*** columns are a fast, simple, safe and highly accurate method for quantitatively measuring aflatoxin M1 in powdered and liquid milk.

Samples are prepared by centrifuging and separating out the fat layer. The skim portion is then applied to the ***aokin*** *ImmunoClean* ***AFLA M1*** column, which is bound with specific antibodies to aflatoxin M1. At this stage, the aflatoxin M1 binds to the antibody on the column. The column is then washed with water to rid the immunoaffinity column of impurities. By passing an Acetonitrile/Methanol solution through the column, aflatoxin M1 is eluted from the antibody. Aflatoxin M1 can then be measured by analyzing a portion of the Acetonitrile/Methanol eluate by high pressure liquid chromatography (HPLC).

1.3 Application

aokin *ImmunoClean* ***AFLA M1*** columns have been tested and optimized for quantitative measurement of aflatoxin M1 in milk.

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).

aokin AG · Robert-Rössle-Str. 10 · 13125 Berlin · Germany
Tel. +49 30 94 89 21 60 · info@aokin.com · www.aokin.com

aokin is ISO 9001 certified and works according to GLP.
aokin laboratory is accredited ISO 17025.

FB-538-031 R9
Subject to change without notice.

aokin AG 05/2022

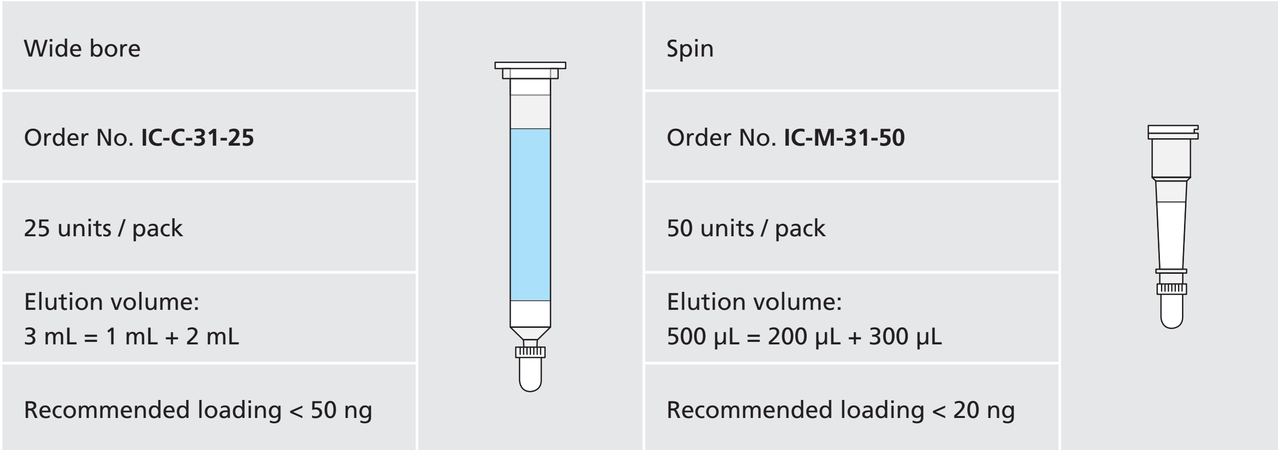
1.4 Limitations, shelf life and storage

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 M1*** 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



Wide bore	Spin
Order No. **IC-C-31-25**	Order No. **IC-M-31-50**
25 units / pack	50 units / pack
Elution volume: 3 mL = 1 mL + 2 mL	Elution volume: 500 µL = 200 µL + 300 µL
Recommended loading < 50 ng	Recommended loading < 20 ng

Use of adapters (adapter luer to column; Order no: LB-08-15-05) recommended for attaching a reservoir (luer syringe barrel) to the column.

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.

aokin AG · Robert-Rössle-Str. 10 · 13125 Berlin · Germany
Tel. +49 30 94 89 21 60 · info@aokin.com · www.aokin.com

FB-538-031 R9
Subject to change without notice.

aokin is ISO 9001 certified and works according to GLP.
aokin laboratory is accredited ISO 17025.

aokin AG 05/2022

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.

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.

Methanol for elution
Use HPLC Grade methanol or Acetonitrile/Methanol in a mixture with 30 mL acetonitrile and 20 mL methanol resulting in 50 mL elution solvent.

Preparation of HPLC mobile phase
Use HPLC grade solutions only. Mix 680 mL water, 240 mL acetonitrile and 80 mL methanol resulting in 1000 mL HPLC mobile phase solution.

Preparation of spiking solutions
Prepare a 1.0 ng/µL aflatoxin standard by adding 100 µL of a 10 ng/µL aflatoxin M1 standard to 900µL acetonitrile.
Prepare a 0.1 ng/µL aflatoxin standard by adding 100 µL of a 1.0 ng/µL aflatoxin M1 solution to 900µL acetonitrile.

Preparing spiked milk
Spiking milk with aflatoxin M1 at 0.1 and 0.05 ppb level
0.1 ppb (ng/g) × 50 g (mL) milk = 5 ng
5 ng ÷ 0.1 ng/µL = 50 µL
Add 50 µL of the 0.1 ng/µL aflatoxin M1 solution to 50 mL defatted milk
0.05 ppb (ng/g) × 50 g (mL) milk = 2.5 ng
2.5 ng ÷ 0.1ng/µL = 25 µL
Add 25 µL of the 0.1 ng/µL aflatoxin M1 solution to 50 mL defatted milk

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

Prepare HPLC standards
1.0 ppb (ng/g) × 50 g(mL) milk = 50 ng
50 ng ÷ 1.0 ng/µL (aflatoxin M1 spiking solution) = 50 µL
50 µL 1.0 ng/µL aflatoxin M1 solution added to 2.45 mL standard diluent

0.1 ppb (ng/g) × 50 g(mL) milk = 5 ng
5 ng ÷ 0.1 ng/µL (aflatoxin M1 spiking solution) = 50 µL
50 µL 0.1 ng/µL aflatoxin M1 solution added to 2.45 mL standard diluent

0.05 ppb (ng/g) × 50 g(mL) milk = 2.5 ng
2.5 ng ÷ 0.1 ng/µL (aflatoxin M1 spiking solution) = 25 µL
25 µL 0.1 ng/µL aflatoxin M1 solution added to 2.475 mL standard diluent

0 ppb (ng/g) use 2.5 mL HPLC standard diluent.

aokin AG · Robert-Rössle-Str. 10 · 13125 Berlin · Germany
Tel. +49 30 94 89 21 60 · info@aokin.com · www.aokin.com

aokin is ISO 9001 certified and works according to GLP.
aokin laboratory is accredited ISO 17025.

FB-538-031 R9
Subject to change without notice.

aokin AG 05/2022

1.8 Materials required for the sample preparation and the HPLC

aokin *ImmunoClean C* ***AFLA M1***	(IC-C-31-25, 25 units/pack)
aokin *ImmunoClean M* ***AFLA M1***	(IC-M-31-50, 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, 250 units/pack)
aokin *reference matrix material* ***AFLA M1***	(RMM-031)

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 buffer. 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.

aokin AG · Robert-Rössle-Str. 10 · 13125 Berlin · Germany
Tel. +49 30 94 89 21 60 · info@aokin.com · www.aokin.com

aokin is ISO 9001 certified and works according to GLP.
aokin laboratory is accredited ISO 17025.

FB-538-031 R9
Subject to change without notice.

aokin AG 05/2022

2 Points of critical importance for reproducibility and recovery

2.1 Sample preparation

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

- Skim milk.
- Equilibrate column to room temperature, best by equilibrating 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 elution solution. Incubate for 3 minutes by stopping flow. Apply 2 mL elution solution (Alternatively use back flushing technique to allow full release of analyte).
- Quantify the aflatoxin concentration by comparing the sample peak height or area to the standard.

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 Milk Powder

- Weigh 10g milk powder into a 250 mL beaker.
- Heat 100 mL purified water to 30 – 40 °C.
- Add 80 mL preheated water in small amounts to the milk powder.
- Mix continually until a homogeneous mixture is obtained.
- Transfer milk mixture to a 250 mL measuring cylinder and bring the volume to 100 mL with the remaining preheated water.
- Centrifuge 50 mL samples at greater than 15.000 × g for 15 minutes.
- Separate fat (top) layer from defatted (skim) layer. Use defatted (skim) milk for further analysis.

A2 Fluid Milk

- Measure 50 mL of fluid milk into a 50 mL cylinder.
- Centrifuge sample at greater than 15.000 × g for 15 minutes.
- Separate fat (top) layer from defatted (skim) layer. Use defatted (skim) milk for further analysis.

A3 BCR- Certified Reference Milk

- Weigh 5g milk powder into a beaker.
- Heat 50 mL purified water to 50 – 60 °C.
- Add 30 mL preheated water to the milk powder. Stir 10 minutes on a stir plate.
- Transfer to a measuring cylinder and bring the volume to 50 mL with the remaining preheated water.

aokin AG · Robert-Rössle-Str. 10 · 13125 Berlin · Germany
Tel. +49 30 94 89 21 60 · info@aokin.com · www.aokin.com

FB-538-031 R9
Subject to change without notice.

aokin is ISO 9001 certified and works according to GLP.
aokin laboratory is accredited ISO 17025.

aokin AG 05/2022

- Centrifuge sample at 5000 × g for 15 minutes.
- Freeze centrifuged sample for 15 minutes.
- Separate fat (top) layer from defatted (skim) layer. Use defatted (skim) milk for further analysis.

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 buffer to ensure equilibration.

C Using column

- 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.
- Place new collection tube (reaction tube) under column.
- Add 1 mL elution solvent. The elution solvent should be methanol or Acetonitrile/Methanol (3:2 v/v). Elute with drop/second.
- Incubate for 3 minutes by stopping flow.
- Pass additional 2 mL elution solvent through ***aokin****ImmunoClean* column at a rate of 1 drop/second. Apply air flow to collect all liquid out of the column. Vortex sample.
- Optional: add 100 µL destilled water to eluate. Vortex sample.

C-Mini: Alternative protocol for spin columns only (IC-M-31-50)

- 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 v/v). 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 × g for 2 minutes.
- Optional: add 100 µL distilled water to elute. Vortex sample.

aokin AG · Robert-Rössle-Str. 10 · 13125 Berlin · Germany
Tel. +49 30 94 89 21 60 · info@aokin.com · www.aokin.com

aokin is ISO 9001 certified and works according to GLP.
aokin laboratory is accredited ISO 17025.

D Recovery

- Recovery of 80 % aflatoxin M1 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 Set up

- Column: reverse phase ODS-2 (Spherisorb ODS-2, 4.6 mm × 250 mm, 5 µm) (Waters # PSS831915)
- Mobile phase: Water/Acetonitrile/Methanol (68:24:8 v/v)
- Flow rate: 1.0 mL/min.
- Fluorescence detector: Waters 474 scanning fluorescence detector, excitation 360 nm, emission 440nm
- Inject eluate into HPLC.

Trouble shooting

3.1 Problem: Sample eluate is cloudy

- Centrifuge at the specified g force for the length of time indicated in the procedure. The rpm value that corresponds to the specified g force will vary depending on the centrifuge rotor.
- Separate defatted portion from fat portion immediately after centrifuge has stopped, to avoid re-mixing. The bottom layer must be taken without disturbing the top layer of fat.
- Try removing bottom layer by piercing the bottom of a plastic centrifuge tube with an 18 gauge syringe needle.
- Filter eluate through 0.2 µm, 25 mm nylon membrane syringe filter before injecting onto HPLC.

3.2 Problem: Overestimation of aflatoxin M1

- Check calculation for spiked sample and standard curve.

3.3 Problem: Underestimation of aflatoxin M1

- 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.

Other published procedures

AOAC Method 2000.08 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, 84 (2) 437-443.

aokin AG · Robert-Rössle-Str. 10 · 13125 Berlin · Germany
Tel. +49 30 94 89 21 60 · info@aokin.com · www.aokin.com

aokin is ISO 9001 certified and works according to GLP.
aokin laboratory is accredited ISO 17025.

FB-538-031 R9
Subject to change without notice.

aokin AG 05/2022

CURD AND WHEY Battacone, G., Nudda, A., Palomba, M., Pascale, M., Nicolussi, P. And Pulina, G., Journal of Dairy Science, Transfer of Aflatoxin B1 from feed to Milk and from Milk to Curd and Whey in Dairy Sheep Fed Artificially Contaminated Concentrates, 88: 3063-3069.

CURD, WHEY, CHEESE AND PICKLED CHEESE Oruc, H., Cibik, R., Yilmaz, E. and Kalkanli, O., Food Additives and Contaminants, Distribution and stability of Aflatoxin M1 during processing and ripening of traditional white pickled cheese, 23 (2): 190-195.

MINAS CHEESE Prado, G., Oliveira, M., Pereira, M., Abrantes, F., Santos, L. and Veloso, T., Ciencia e Tecnologia de Alimentos, Aflatoxin M1 in samples of "minas" cheese commercialized in the city of Belo Horizonte – Minas Gerais/ Brazil, 20 (3): 398-400.

SOFT AND PARMESEAN CHEESE Prado, G., de Oliviera, M., de Carvalho, E., Veloso, T., de Sousa, L. And Cardoso, A., Revista do Instituto Adolfo Lutz, Aflatoxin M1 in soft and parmesan cheese by immunoaffinity column and liquid chromatography, 60 (2): 147-151.

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.

aokin AG · Robert-Rössle-Str. 10 · 13125 Berlin · Germany
Tel. +49 30 94 89 21 60 · info@aokin.com · www.aokin.com

aokin is ISO 9001 certified and works according to GLP.
aokin laboratory is accredited ISO 17025.

FB-538-031 R9
Subject to change without notice.

aokin AG 05/2022