INSTRUCTIONS FOR USE

aokin mycontrol AFLA IAC

Order No.: MY-IC-M-03

Sample preparation with aokinImmunoClean**M** columns (IAC)

aokin mycontrol AFLA **IAC**

Analytical-kit for rapid and quantitative determination of Aflatoxin B1 (AFLA).

Materials

aokinmycontrolAFLA IAC (Order No.: MY-IC-M-03-100)

Package content
Additional material may be needed (Methanol HPLC grade)

A) IC Consumables:
Filter paper
aokinExtractionSalt AFLA + spoon
aokinICElute AFLA
Reaction tubes 2 mL
IC Reservoirs (loading reservoirs) 25 mL

B) *IC Liquids:*
aokin*ExtractionSolvent* AFLA / or aokin*Extraction-Solvent Concentrate* AFLA **(in this case please add Methanol HPLC grade)**
aokin*ICDilute 10x*
aokin*ICWash*

C) *Immunoaffinity columns:*
aokin*ImmunoClean* M AFLA (Order No.: IC-M-03-100)

D) *Materials for analytical measurement:*
aokinReactionBuffer, Reaction buffer
aokinmycontrolAFLA, Reagent 1 (white cap), F-AFLA, (for 5 analyses each)
aokinmycontrolAFLA, Reagent 2 (green cap), A-AFLA , (for 5 analyses each)

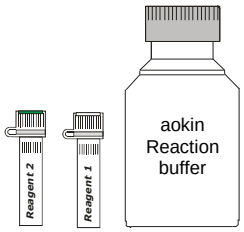


Figure 1: Reagent 1, F-AFLA (white cap), Reagent 2, A-AFLA (green cap) and Reaction buffer (1 L bottle)

E) *Materials for internal quality control:*
aokin*mycontrol* AFLA, negative control AFLA (transparent), for zero value measurements
aokinmycontrolAFLA, Reagent 1 (white cap), F-AFLA, (for 5 analyses each)
aokinmycontrolAFLA, Reagent 2 (green cap), A-AFLA , (for 5 analyses each)

Note: All substances provided are precisely weighed and calibrated. Control of the volume and concentration of the individual solutions are essential for the precision of the analysis.
Caution: The extraction solvent may contain methanol. Work with professional care.
Storage Conditions: Reagents 1, 2 and aokin *ImmunoClean* M AFLA columns must be stored at temperature of + 2 - 10°C. All other components may be stored at room temperature.
Quality Control: All materials and reagents are prepared according to strict quality control protocols. Exchanging reagents between kits having different Lot-numbers will lead to erroneous results and is not permitted.

Order Information:
aokinmycontrolAFLA (Order No.: MY-IC-M-03-100)

Introduction

aokinmycontrol AFLA is a rapid and precise quantitative method for analyzing total Aflatoxin B1 (AFLA). It has been specifically designed and calibrated for using aokinImmunoClean M columns. The sample preparation can be done in 25 minutes.
aokinmycontrolAFLA is available with a calibration, which has been validated for food products. Please use professional care and check the accuracy by regularly analyzing reference materials (***aokin****ReferenceMatrixMaterials*) and/or standards. Participation in proficiency tests is recommended.
aokin will gladly assist you to cust
omise this test for your specific sample type and application. Please do not hesitate to contact us.

Sample		cereals, peanuts
Time required for sample preparation		25 minutes
Time required for measurement		3 minutes
Analysis		
	Measurement range [µg/kg]	
Range 1	0,3 – 4,0	
Range 2	0,6 – 8,0	
Range 3	1,2 – 16,0	

Aflatoxin

Aflatoxins (AFLA) are mycotoxins produced by molds such as *Aspergillus flavus* and *Aspergillus parasiticus.* Aflatoxin B1 is acutely toxic and among the most carcinogenic substances known. As a consequence, it is necessary to monitor AFLA content in food and feed products.

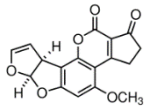


Figure 2: Chemical formula for Aflatoxin B1, $C_{17}H_{12}O_6$. Molecular weight: 312.3 g/mol

Recommended Accessories

All required materials are available from ***aokin***.
Tel.: +49 30 9489 2160

	Order No.
aokin*extractor* (food blender)	EX-07-06
aokin*watchbox* (timer for food blender)	EX-07-06-4
Weighing scale, d = 0,01 g	LB-03-04
Eppendorf centrifuge, variable g-force	LB-04-04
Variable pipettes (1000 µl)	LB-04-05-1000
Pipette tips (1000 µl)	LB-04-08-1000
Funnel	LB-05-04
Dispensette	LB-08-01
aokin*ReferenceMatrixMaterial*	RMM-03
Reaction tubes, 2mL	LB-05-02
aokin*ICadapter*	LB-08-10-05
aokin*VacuumAdapter*	LB-08-11
Vacuum-pump (diaphragm pump)	LB-04-10
Trap for vacuum-pump (vacuum-bottle)	LB-04-12
Vacuum manifold	LB-04-09
Glass fiber filters, GF/F	LB-04-13-100
Vacuum bottle and funnel for glass fiber filters (Büchner-Funnel)	LB-04-14
pH-indicator strips	LB-08-18
Magnetic stirrer plate (not heated)	LB-04-15
Magnetic stirrer (to degas)	LB-04-16-05

Sample preparation

The following protocol is an example. The quantification ranges are dependent on dilutions. Actual volume settings in the software may vary.

Note: It is of critical importance to use the correct sample preparation protocol for each quantification range. Use volumes displayed in the *aokin* software.

1. Quality control

There are free materials included in the kit, for your internal quality control: ***Reagent 1***, ***Reagent 2***, as well as ***negative control*** solutions for measurements of zero values (corresponding to samples free of mycotoxin).

Please regularly carry zero value measurements to ensure the accuracy of your measurements.

If you should measure increased zero values, please contact the ***aokin*** team.

2. Preparation (if needed)

Prepare aokinExtractionSolvent by adding of 800g Methanol HPLC grade to each delivered 1 L bottle of aokinExtractionSolvent Concentrate AFLA.

3. Sample collection, homogenisation, and grinding

The analysis sample is collected, ground, and homogenised according to an approved procedure. Small samples may be ground using the ***aokin****extractor.*

4. Weighing and extraction

Weigh 15 g of your sample, add one spoon (1,5 g) of *aokinExtractionSalt* AFLA and 30,4 g extraction solution (35 ml aokinExtractionSolvent AFLA at 20°C) directly into the extraction beaker (Figure 3). Preferentially the exact volume is applied using a dispensette.

Figure 3: Weighing

Close the extraction beaker with the lid (with the blending knifes). Blend for 3.5 minutes. The recommended protocol has blending times alternating with resting time to avoid heating of the sample and is as follows: mix for 30 seconds, pause for 1 minute, mix for 30 seconds and so on (until 3.5 minutes of blending time). Use the ***aokin***watchbox (a preprogrammed timer) to conveniently and automatically complete this extraction protocol.

Figure 4: Extracting with the aokinextractor *(blender)*

5. Filtration

Place the filter on a suitable funnel and the funnel onto a collection container. Open the extraction beaker, pour the extract onto the filter and collect the filtrate. Discard the filter paper and filter cake. Shake/stir the filtrate to ensure homogeneity.

Figure 5: Filtration

6. Dilution

Dilute 3 mL of your collected filtrate with 2 mL of aokinICDilute10x and 19 mL deionized water. Shake softly.

Check the pH value. If necessary, readjust the pH value by adding diluted HCl or NaOH to a neutral pH (pH 6.5 - 7.5).

Important: all precipitate has to be removed before loading the column by glass fiber filtration to ensure a good flow through the column (see step 6b).

Figure 6: Filtration through a glass fiber filter using a vacuum (shown as an example for a coffee sample)

6. Clean up with aokinImmunoClean **M**

a) Preparation

Attach the vacuum manifold to a pump. Attach the aokinImmunoClean M column to the vacuum manifold (or alternatively onto an aokin VacuumAdapter) and transfer aokinICWash solution onto the column. Attach the aokin*ICadapter* to the aokinImmunoClean M column and connect it to the loading reservoir. Pipette aokinICWash into the loading reservoir.

Important: No air bubbles should be visible in the column. Let the aokinICWash solution elute, leaving only a little liquid in the reservoir and so that the aokin*ICadapter* and aokinImmunoClean M column are still filled with liquid.

b) Loading

Load the column with 20 ml of the diluted filtrate. Adjust the vacuum so that a flow rate of approximately **1 ml/min** is produced (total running time approximately 20 minutes). The running speed can be reduced by closing the stopcock, or increased with applying vacuum. Alternatively, a positive pressure from above can be used.

Important: Do not let the aokinImmunoClean M column run dry before, or during loading.

Important: High flow rates lead to a reduced rate of recovery.

c) Washing

Fill 15 ml aokinICWash into the loading reservoir. Adjust the vacuum so that a high flow rate is achieved (running time approximately 2 minutes). The column bed should appear relatively colorless. If this is not observed, the wash step may be repeated. Let the column run dry.

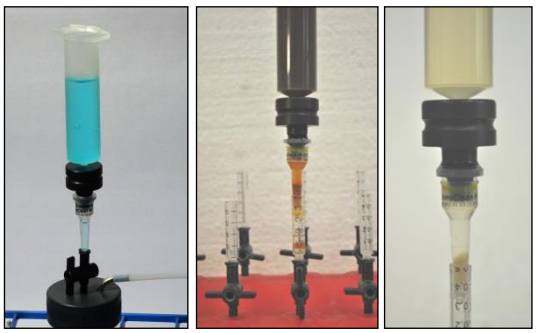


Figure 7: Attachment of reservoir, aokinICadapter and aokinImmunoClean M column to the aokin VacuumAdapter (left), to the vacuum manifold with a coffee sample (middle), and the coffee column after washing with aokinICWash (right).

d) Elution

Place the ***aokin****ImmunoClean* ***M*** column into a collection tube and centrifuge for 1 minute at 1000 x g to remove the residual liquid.

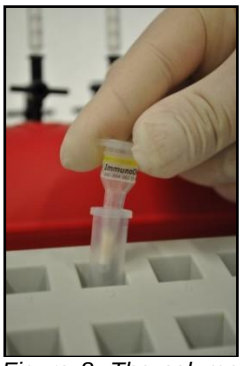


Figure 8: The column is placed into a collection tube before eluent is added, and then the column is centrifuged to remove residual liquid.

Set the column into a new clean collection tube and pipette 300 µl aokinICElute AFLA into the column, close (half closed) the column after that with the lid. When closing the column, about 100 µl aokinICElute AFLA will be **pushed through the gel**. After incubation for 3 minutes, centrifuge for 1 minute at 3000 x g. The lid must remain on the column during centrifugation. The liquid is present in the collection vessel after centrifugation.
Repeat the step, by placing the just used ***aokin****ImmunoClean* ***M*** column into a new collection tube and pipette again 300 µl aokinICElute AFLA into the column, following the procedure. Unite the eluates of both collection tubes and use it for the measurement (see step 7).

Important note: Eluates of some samples may show a precipitate. This leads to increased results. If you see or suspect a precipitation in the eluate than centrifuge eluate at high speed, than pipette supernatant into new collection tube and use for further analysis.

7. Analyzing

Use the eluate for analyzing in the aokinspectrometerFP470.

Important: Gas bubbles can lead to measurement inaccuracies. It is strongly recommended to degas the reaction buffer (aokinReactionBuffer) before each use. This is performed by applying vacuum and stirring for 30 min.

Figure 9: Setup for degassing the reaction buffer (aokinReactionBuffer). This consists of: vacuum pump (hose and vacuum trap, not shown) and the reaction buffer in a bottle with a magnetic stirrer rod on a magnetic stirrer plate.

Please follow detailed instructions for spectrometer use.

This includes:

1) Place *Reagents 1* and *2* into position A6 and B6 of the sample rack of your spectrometer.
2) Place a 25 ml *Clean1*-glass container, filled with Clean1 solution into the Clean1-position, on the left side, next to the palette.
3) Place a 25 ml *neg. control AFLA IAC*-glass container, filled with *negative control* AFLA IAC solution = aokinICElute AFLA = Methanol in the Clean2-position, on the left side of the palette.
4) Place an empty 2 mL vial in position A1 of the palette.
5) Place an empty waste bottle into the holder. Check presence of Reaction buffer and check if tubing is below the surface.
6) Place a new cuvette with a clean stirrer into the spectrometer.

Conversion factor: analyte concentration in cuvette (nM) to amount in sample (µg/kg)

aokin*mycontrol* ***AFLA*** ***IAC Standard***

Step 1: Extraction

- *Sample mass:* m $_{Sample}$ = 15 g
- *Volume extraction solvent:* V $_{Extraction\ solvent}$ = 35 ml
- *Molar mass Aflatoxin B1:* $MW_{Afla} = 312{,}3\left[\frac{g}{mol}\right]$

Mycotoxin concentration in the sample extract:

$$\boldsymbol{c}\left[\frac{\mu\boldsymbol{mol}}{\boldsymbol{l}}\right]_{\boldsymbol{Extract}} = \frac{m_{Sample}[kg]}{V_{Solvent}[l]*MW_{Mykotoxin}\left[\frac{g}{mol}\right]} * c\left[\frac{\mu g}{kg}\right]_{Sample} = \frac{0.015}{0.035*312{,}3} * c\left[\frac{\mu g}{kg}\right]_{Sample} = 0.001372 * c\left[\frac{\mu g}{kg}\right]_{Sample}$$

Step 2: Dilution

- *Volume Eluate:* V_{eluate} = 3 ml
- *Total volume:* V_{total} = 24 ml (2 ml $_{ICDilute\ 10x}$ + 19 ml $_{Water}$ + 3 ml $_{Eluate}$)

$$\boldsymbol{c}\left[\frac{\mu\boldsymbol{mol}}{\boldsymbol{l}}\right]_{\boldsymbol{Diluted}} = \frac{V_{eluate\,[ml]}}{V_{total\,[ml]}} * c\left[\frac{\mu mol}{l}\right]_{Extract} = \frac{3}{24} * c\left[\frac{\mu mol}{l}\right]_{Extract} = 0{,}125 * c\left[\frac{\mu mol}{l}\right]_{Extract}$$

Step 3: Purification

with *aokin* *IC M* ***AFLA***

- *Volume sample extract load to the* ***aokin*** *IC-M column:* $V_{loaded\ sample\ extract}$ = 20 ml
- *Volume "****aokin*** *Elute" used for the elution of the column:* V_{elute} = 0,6 ml

Mycotoxin concentration in the column eluate:

$$\boldsymbol{c}\left[\frac{\mu\boldsymbol{mol}}{\boldsymbol{l}}\right]_{\boldsymbol{Eluate}} = \frac{V_{load\,[ml]}}{V_{elute\,[ml]}} * c\left[\frac{\mu mol}{l}\right]_{Extract} = \frac{20}{0.6} * c\left[\frac{\mu mol}{l}\right]_{Extract} = 33.33 * c\left[\frac{\mu mol}{l}\right]_{Extract}$$

Step 4: Measurement

aokin FP 470 / *LHW 03*

- *Sample volume:* $V_{Column\ eluate} = V_{Sample} = 200\ \mu l$
- *Total volume in the cuvette:* $V_{Cuvette} = 2600\ \mu l$

Mycotoxin concentration in the cuvette:

$$\boldsymbol{c}\left[\frac{\mu\boldsymbol{mol}}{\boldsymbol{l}}\right]_{\boldsymbol{Cuvette}} = \frac{V_{Sample}\,[\mu l]}{V_{cuvette}\,[\mu l]} * c\left[\frac{\mu mol}{l}\right]_{Eluate} = \frac{200}{2600} * c\left[\frac{\mu mol}{l}\right]_{Eluate} = 0.0769 * \left[\frac{\mu mol}{l}\right]_{Eluate}$$

Conversion factor: Extraction, Purification and Measurement

It follows the conversion factor from 1 to 4 above:

$$\boldsymbol{c}\left[\frac{\mu\,\boldsymbol{mol}}{\boldsymbol{l}}\right]_{\boldsymbol{Cuvette}} = 0{,}001372 * 0.125 * 33.33 * 0.0769 * c\left[\frac{\mu g}{kg}\right]_{Sample} = 0.00043957 * c\left[\frac{\mu g}{kg}\right]_{Sample}$$ or

$$\boldsymbol{c}\left[\frac{\mu\,\boldsymbol{g}}{\boldsymbol{kg}}\right]_{\boldsymbol{Sample}} = \frac{1}{0.43957} * c\left[\frac{nmol}{l}\right]_{Cuvette} = \boldsymbol{2{,}274 * c}\left[\frac{\boldsymbol{nmol}}{\boldsymbol{l}}\right]_{\boldsymbol{Cuvette}}$$

Dilution scheme of the standard solution sent for control purposes:
AFLA B1 [300 ng/mL] [960.61 nM] in Acetonitrile, 50 µl

aokin**mycontrol **AFLA** **IAC

Calibration:

Dilution scheme (example for standard experimental setup of 200 µl sample into 2600 µl of total cuvette volume):

AFLA B1 [300 ng/mL] [960.61 nM in vial]

↓ **1:20 diltution** in *MeOH*

15 ng/mL (48 nM in vial → **3.69 nM in cuvette**)

↓ **1:2 diltution** in *MeOH*

7.5 ng/mL (24 nM in vial → **1.85 nM in cuvette**)

↓ **1:2 diltution** in *MeOH*

3.75 ng/mL (12 nM in vial → **0.93 nM in cuvette**)

↓ **1:2 diltution** in *MeOH*

1.875 ng/mL (6 nM in vial → **0.46 nM in cuvette**)

MeOH
(→ **0.00 nM in cuvette**)

Positive control:

Dilution scheme (example for standard experimental setup of 200 µl sample into 2600 µl of total cuvette volume):

AFLA B1 [300 ng/mL] [960.61 nM in vial]

↓ **1:20 diltution** in *negative control AFLA SPE*

200 µl in RANGE 1
15 ng/mL (48 nM in vial ----------→ 3.69 nM in cuvette
in calculated volume for solid sample → 8.391 µg/kg)

100 µl in RANGE 2
15 ng/mL (48 nM in vial ----------→ 1.85 nM in cuvette
in calculated volume for solid sample → 4.196 µg/kg)

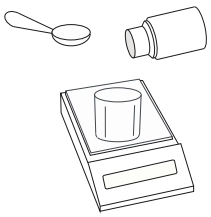
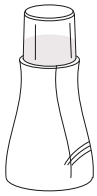
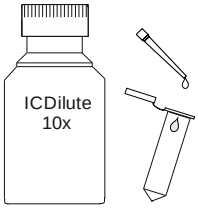
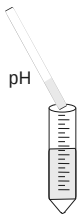
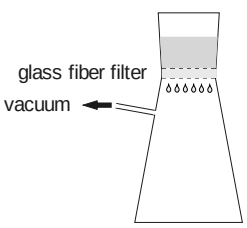
50 µl in RANGE 3
15 ng/mL (48 nM in vial ----------→ 0.93 nM in cuvette
in calculated volume for solid sample → 2.098 µg/kg)

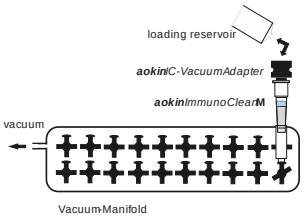
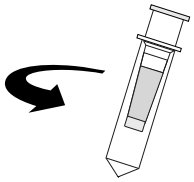
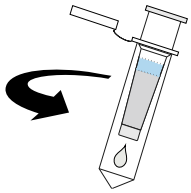
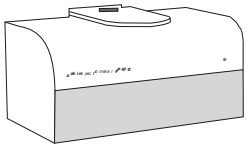
Aflatoxin / standard samples: *cereals, feeds, nuts*

- Recommended for standard types of samples:
  wheat, **corn**, **feeds**, **nuts**

Procedure:

***aokin**mycontrol AFLA IAC Standard*

Extraction		**Weighing:** 15 g sample 1,5 g ***aokin**ExtractionSalt **AFLA*** 35 mL ***aokin**ExtractionSolvent **AFLA***
		**Extraction:** 3,5 min mixing with ***aokin**watchbox*
		**Filtration:** collect filtrate (discard filter cake)
		3 mL filtrate 2 mL ***aokinIC**Dilute10x* 19 mL deionized water
		check and adjust to 6.5 - 7.5 pH, neutralize if necessary by adding NaOH or HCl
		filtrate through a glass fiber filter (optional)

Purification			set up column
		20 ml	filtrate, 1 drop / second (slow flow rate)
		15 ml	***aokin****ICWash*
		1 min	centrifuge at 1000 x g
		**Elution:** 1 x 300 µl 3 min 1 min 1 x 300 µl 3 min 1 min 2 x 300 µl	 ***aokin****ICElute* ***AFLA*** incubation with lid on centrifuge at 3000 x g ***aokin****ICElute* ***AFLA*** incubation with lid on centrifuge at 3000 x g transfer both eluates into clean 2 mL reaction tube
Measurement		**Automatic Analyse (FP470 / LHW03):** 2200 µl 200 µl 100 µl 100 µl	place the 2ml reaction tube in the sample holder of the *LHW03* ***aokin*** *Reaction buffer* sample *(diluted 1:1 - RANGE 1)* *(diluted 1:2 - RANGE 2)* *(diluted 1:4 - RANGE 3)* ***aokin****mycontrol* ***AFLA*** Reagent 1 ***aokin****mycontrol* ***AFLA*** Reagent 2
	AFLA = Aflatoxin, Conversion factor: 1 nmol AFLA/L in cuvette = 2.274 µg/kg in sample		

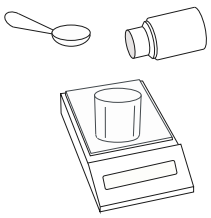
Aflatoxin / special samples: *spices, cocoa, roasted coffee*

- Recommended for colored sample types:
  spices, **cocoa**, **roasted coffee**, **some feeds, some nuts**

Procedure:

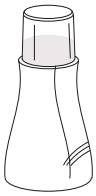
***aokin**mycontrol AFLA IAC*
Special Matrices

Extraction



Weighing:

15 g	sample
1,5 g	***aokin**ExtractionSalt **AFLA***
35 mL	***aokin**ExtractionSolvent **AFLA***

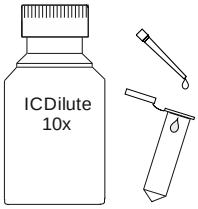


Extraction:

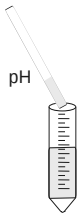
3,5 min mixing with ***aokin**watchbox*

Filtration:

collect filtrate
(discard filter cake)

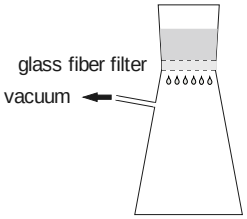


3 mL	filtrate
2 mL	***aokinIC**Dilute10x*
19 mL	deionized water

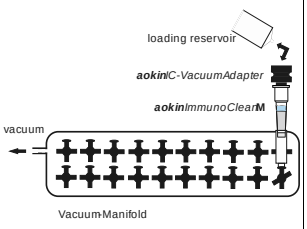
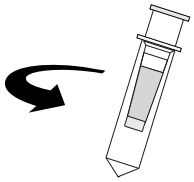
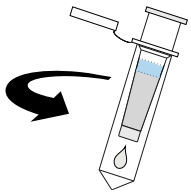
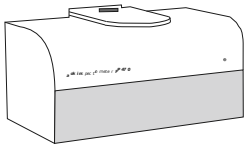


check and adjust to 6.5 - 7.5 pH,

neutralize if necessary by adding
NaOH or HCl



filtrate through a glass fiber filter (optional)

Purification			set up column
		20 ml	filtrate, 1 drop / second (slow flow rate)
		5 – 20 ml 2 x 5 ml	***aokin****ICWashTween* PBS
		1 min	centrifuge at 1000 x g
		**Elution:** 1 x 300 µl 3 min 1 min 1 x 300 µl 3 min 1 min 2 x 300 µl	***aokin****ICElute* ***AFLA*** incubation with lid on centrifuge at 3000 x g ***aokin****ICElute* ***AFLA*** incubation with lid on centrifuge at 3000 x g transfer both eluates into clean 2 mL reaction tube
Measurement		**Automatic Analyse (FP470 / LHW03):** 2200 µl 200 µl 100 µl 100 µl	place the 2ml reaction tube in the sample holder of the *LHW03* ***aokin*** *Reaction buffer* sample *(diluted 1:1 - RANGE 1)* *(diluted 1:2 - RANGE 2)* *(diluted 1:4 - RANGE 3)* ***aokin****mycontrol* ***AFLA*** Reagent 1 ***aokin****mycontrol* ***AFLA*** Reagent 2
			AFLA = Aflatoxin, Conversion factor: 1 nmol AFLA/l in cuvette = 2.274 µg/kg in sample