Instruction of the Clean-up Process Using *B-TeZ IAC Afla-Ochra 3ml*

Clean-up of Commodity Extracts of Food and Feed Samples containing Aflatoxins and Ochratoxins by Simultaneous Work-Up via Immunoaffinity Chromatography (IAC) with Bitox Functionality

Principle:
This instruction of simultaneous Aflatoxin and Ochratoxin (Afla-Ochra) determination in food and feed focuses on the enrichment step of extract using immunoaffinity column (IAC) and quantification with HPLC.

Accepted laboratory extraction methods could be maintained. Full performance of the IAC column is given if pronounced criteria regarding organic solvent tolerance, elution process of analyte and working range of column is followed.

Many pretreatment methods of Aflatoxin and Ochratoxin determination in food and feed show low sensitivity because of interfering substances if problematic matrices are applied.

This method of content determination of Aflatoxins and Ochratoxins combines the high selectivity of an immunoaffinity column (IAC) with its potential to concentrate eluate and additional step of purification by HPLC column.

Please notice that this instruction focuses on the handling with the IAC column. For the commodity extraction step a literature method is given. The given apparatus (e.g. HPLC system) might serve as example among other possibilities.

Extraction (Literature method given):
Samples which content of Aflatoxin and Ochratoxin are to be analyzed, e.g. wheat, maize etc., are extracted by the method of Krska et al.[1] using the solvent mixture of acetonitrile/water/acetic acid (79/20/1 v/v/v). E.g. to 25g of ground wheat kernels are added a volume of 100ml of the extraction solvent and processed as cited.

Enrichment Step IAC:
4ml extract (contains the quantity of toxins of 1g sample if above mentioned example extraction is followed) is diluted with extra 28ml 50mM PBS and then applied in a reservoir on top of the ***B-TeZ IAC Afla-Ochra 3ml*** column.

If the example is followed the resulting organic solvent concentration is 10% of acetonitrile which is tolerated by the column. The flow rate lies between 1 to 3ml/min. According to application and contents expected the applied extract volumes could vary.

Caution! The proportion of organic solvent of PBS diluted extract, which is applied on the column, should not exceed 20% methanol and 10% acetonitrile.

In case of very low contents even extract volumes of 20ml may be applied without significant loss of analyte as long as resulting pH is fairly neutral and methanol or acetonitrile content lies under stated limits. If latter is not the case the extract must be diluted with PBS until maximum allowed organic solvent content during enrichment step using ***B-TeZ IAC Afla-Ochra 3ml*** column is reached.

If samples are to be prepared in parallel, manifold of J.T. Baker for 12 samples has proven of value. Rate of flow through the affinity gel is 1 to 3 ml/min. In case of problematic matrices rate of flow should lie below 2ml/min.

Caution! Be aware that no big air bubbles are neither in the gel nor between gel and luer lock outlet of column which prevent a permanent flow or necessary exchange of matter.

Depending on application and on expected contents, larger or smaller extract aliquots can be applied. In such cases the sample calculation (see below) must be adapted.

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Wash:
After whole sample has passed through the gel the latter is washed with 5ml of 50mM PBS. Remaining liquids in the gel are removed by applying either pressure from top of the column or underpressure from bottom.

Elution:
Sample reservoir on top of the ***B-TeZ IAC Afla-Ochra 3ml*** column is removed and a appropriate vial is placed below the affinity column. The bound toxins are eluted by using a total of 2ml of methanol-acetic acid (98/2 v/v)[(1)] as elution solvent. The elution process is performed in two steps to ensure complete release of analytes. First, a volume of 1ml elution solvent is applied. After that volume has passed through column the second portion of 1ml of elution solvent is applied and half a minute is waited before eluting the liquid through the column. Remaining solvent solutions should be eluted by application of slight under- or overpressure. All methanolic-acetic acid fractions are unified to give the column eluate.

The column eluate may be injected into the HPLC directly or in case concentrations are low it may be concentrated by evaporation, e.g. using VLM evaporater at 50°C under a permanent stream of nitrogen[(2)]. The residue is redissolved in HPLC solvent, e.g. in 0.4ml, and a aliquot is finally injected into the system.

Further explanations:

Acc. [(1)]: If 100% methanol is used instead of recommended methanol-acetic acid (98/2 v/v) mixture as elution solvent a loss of up to 20% in recovery of Ochratoxin A could be encountered compared to the recommended elution solvent because of incomplete release of this mycotoxin of the gel

Acc. [(2)]: No loss of analytes, neither Aflatoxins nor Ochratoxin , despite of the presence of acetic acid, is observed in the concentration process as long as the eluate is not evaporated to complete dryness. The concentration should be stopped at a small volume of residue, e.g. 50 to 100µl.

IAC Column Characteristics:

A) Working Range and Recovery Rates of *B-TeZ IAC Afla-Ochra 3ml* Column:

Working Range of Column:	
Aflatoxins:	0.04 – 200ng Aflatoxins per IAC
Ochratoxin:	0.04 – 200ng Ochratoxin per IAC
Zero Contamination of Column:	
Aflatoxin:	<0.04ng (LOD of HPLC method[(**)])
Ochratoxin:	<0.04ng (LOD of HPLC method)
Guaranteed Recovery Rates within the Working Range[(*)]:	
Aflatoxin total:	>85% ± 5%
Aflatoxin B_1 (AFB_1):	>95% ± 5%
Aflatoxin B_2 (AFB_2):	>85%% ± 5%
Aflatoxin G_1 (AFG_1):	>85%% ± 5%
Aflatoxin G_2 (AFG_2):	>85% ± 5%
Ochratoxin A (OTA):	>90% ± 5%

(*) Recovery rates are confined to solvent content of diluted extract below 20% methanol or 10% acetonitrile (see details under Enrichment Step).

(**) Limit of detection (LOD) of method depends on the sensitivity of HPLC-FLD system in use; the lowest quantitiy of Aflatoxins and Ochratoxin which is bonded by the IAC is even lower

B) Cross Reactivities(***) of *B-TeZ IAC Afla-Ochra 3ml* Column:

Aflatoxin B_1 (AFB_1):	100%
Aflatoxin B_2 (AFB_2):	99%
Aflatoxin G_1 (AFG_1):	48%
Aflatoxin G_2 (AFG_2):	34%
Ochratoxin A (OTA):	100%
Ochratoxin B (OTB):	103%

(***) Recovery rate of AFB_2, AFG_1, AFG_2, divided by recovery rate of AFB_1 and recovery rate of OTA and OTB divided by recovery rate of OTA if a total of 1.5µg Aflatoxin total (with molar ratio of B_1, B_2, G_1, G_2 = 4:1:4:1) and a total of 1.5µg Ochratoxins (with molar ratio of OTA, OTB = 1:1) are simultaneously analyzed per column. Please notice that toxin quantities applied in the cross reactivity test are close to the capacity limit of the column where binding sites of column are limited. Thus, when analyzing within the working range of the column, cross reactivities of AFG_1 and AFG_2 as well lie much higher (above 85%).

C) Capacity(****) of *B-TeZ IAC Afla-Ochra 3ml* Column:

Maximum Column Capacity:	1.8µg Aflatoxin total and 3µg Ochratoxin

(****) An excess of Aflatoxin, Ochratoxin, namely 4µg of each, in a small volume of 2ml PBS is incubated with the IAC for 5 minutes; then the IAC is washed with 2ml PBS and the non-bonded fraction is analyzed. The difference of added analyte and non-bonded analyte equals maximum column capacity.

Analytical Method:

The HPLC multitoxin method (lc-ms/ms) of Chan et al.[2] is adapted for the use of fluorescence detector. Following conditions might serve as possibility among others.

HPLC: Shimadzu; Column: Trentec Reprosil-Pur RP C18 120 ODS3 5µm; 125x3,0mm with guard column; Mobile Phase A: methanol / deionized water / phosphoric acid (90/10/0.1 v/v/v); Mobile Phase B: methanol / deionized water / phosphoric acid (20/80/0.1 v/v/v); Gradient: 0.01 min B 100 %; 1 min B 100 %; 2 min B 60 %; 18 min B 60 %; 19 min B 40 %; 34 min B 0 %; 35 min B 100 %; Flow Rate: 0.5ml/min; Time of Analysis: 50min; Injector Volume: 100µl

Post Column Derivatization: 32 ppm pyridinium hydrobromide perbromide in dioxan/ deionized water (0.1/99.9, v/v); Flow Rate: 0.5ml/min; reaction capillary (put between end of column and detector by T-device):): PEEK-capillary, 1/16´´ x 0.25 mm ID; length: 40 cm

Fluorescence-Detection: λ_{EX} [nm]: 362nm; λ_{EM} [nm]: 440nm (Aflatoxins); only from 22 to 45min: λ_{EX} [nm]: 333nm; λ_{EM} [nm]: 460nm (Ochratoxin A, B).

Temperature: Machine and eluents are at room temperature. Eluents are degassed with helium gas.

HPLC Method Characteristics:

Following characteristics are those of the machine used see above; they may differ from other machines.

Aflatoxin B_1:
Measuring range is linear from 10pg to 500pg Aflatoxin B_1 per injection (R^2=0.999) in given HPLC method. Lower limit of detection (LOD) is 2pg AFB_1 per injection (signal to noise (S/N) ratio = 3).

Remaining Aflatoxin G_1, Aflatoxin B_2; Aflatoxin G_2:
Sensitivities [AREA Peak / pg] of left aflatoxins AFG_1, AFB_2 und AFG_2 related to that of AFB_1 are 37%; 136% and 67%, respectively.

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AFG_1: Measuring range is linear from 25pg to 500pg Aflatoxin G_1 per injection (R^2=0.999). LOD of detection is 6pg AFG_1 per injection (signal to noise (S/N) ratio = 3).

AFB_2: Measuring range is linear from 2.5pg to 125pg Aflatoxin B_2 per injection (R^2=0.999). LOD is 1pg AFB_2 per injection (signal to noise (S/N) ratio = 3).

AFG_2: Measuring range is linear from 2.5pg to 125pg Aflatoxin G_2 per injection (R^2=0.999). LOD is 2pg AFG_2 per injection (signal to noise (S/N) ratio = 3).

Ochratoxin A:
Measuring range is linear from 20pg to 1000pg Ochratoxin A (OTA) per injection (R^2=0.999) in given HPLC method. Lower limit of detection (LOD) is 20pg OTA per injection (signal to noise (S/N) ratio = 3).

Balance of commodity contamination and HPLC measuring range:
If stated dilution steps (enrichment step, eluate concentration step etc.) are followed, Aflatoxin B_1 , Ochratoxin A contents in commodities of 0.04 to 2ng/g, 0.08 to 4ng/g respectively, lie within measuring range of HPLC method. If contents are higher, from 2 to 200ng/g in case of Aflatoxin or Ochratoxin, IAC column eluate should be diluted accordingly with HPLC eluent or, alternatively, injector volume should be adjusted.

Balance of commodity contamination and IAC working range:
Only if commodity contents lie above working range of IAC column of 200ng/g total Aflatoxin and of 200ng/g Ochratoxin A, the 1g commodity equivalent per column of this instruction should be lowered to guarantee stated recovery rates. Thus, the IAC procedure and subsequent HPLC analysis should be repeated with a smaller extract volume, e.g. instead of 4ml extract a volume of 2ml extract, or even less where applicable, are diluted with 10ml PBS and applied to the IAC column.

Example Sample Calculation of AFB_1 content:
(Calculation of AFB_2, AFG_1, AFG_2 , and OTA)

A) Calculation of Sample Gramm Equivalents per HPLC injection:

$$\frac{\text{25g Sample}}{\text{100ml Extraction Solvent}} \times \frac{\text{4ml Extract}}{\text{0.4ml}} \times \text{0.1ml injector volume} = \text{0.25g Sample Equivalents}$$

B) Calculation of Aflatoxin B_1 contamination of examined commodity in ng/g:

$$\frac{\text{\# ng injected } AFB_1}{\text{Sample Equivalents [g]}} = \text{ng/g } AFB_1 \text{ in e.g. ground nut meal}$$

Buffer, Chemicals, Apparatus and Literature:

Phosphate Buffered Saline pH 7.4 (= 50mM PBS)

1.24g KH_2PO_4
7.27g K_2HPO_4
8.76g NaCl

Dissolve in 1L deionized water. If necessary adjust pH to 7.4 (± 0.3) with 1N NaOH or 1N HCl

Chemicals:

- acetonitrile, HPLC grade

Consumables:

- ***B-TeZ IAC Afla-Ochra 3ml*** [BTCA321005]

•methanol, HPLC grade
•acetic acid, 100% ultrapure

•deionized water

•dipotassium hydrogenphosphate, >98%
•potassium dihydrogenphosphate, >98%
•sodium chloride

Elution Solvent:

methanol/acetic acid (98/2 v/v)
Pipetting 2ml acetic acid into 98ml methanol and mix

Evaporation:

•nitrogen gas 5.0 [Air Liquide M55763810] (to evaporate IAC-eluate)

Keeper:

acetic acid/water (50/50 v/v):
Mix 10ml acetic acid and 10ml deionized water

Postcolumn Derivatization:

•pyridine hydrobromide perbromide, >95%.
•dioxane, 99.5%

Reagent:

32 ppm pyridine hydrobromide perbromide in dioxan / deionized water (0.1/99.9, v/v):

Partially dissolve 32mg pyridine hydrobromide perbromide in 1ml dioxane and pipet the oily suspended liquid into 1L degassed deionized water

Apparatus:
HPLC; Shimadzu; pump: LC-6A (2 pieces); auto sampler: SIL 6B; UV-absorbance detector: SPD-10A; fluorescence detector: RF-10AXL; data handling: CLASS LC10

Evaporator (with tripod) [VLM EVA EC1-S]

Vacuum SPE Manifold (BAKER spe-24G Column Processor – process up to 24 samples) [J.T. Baker 7208]

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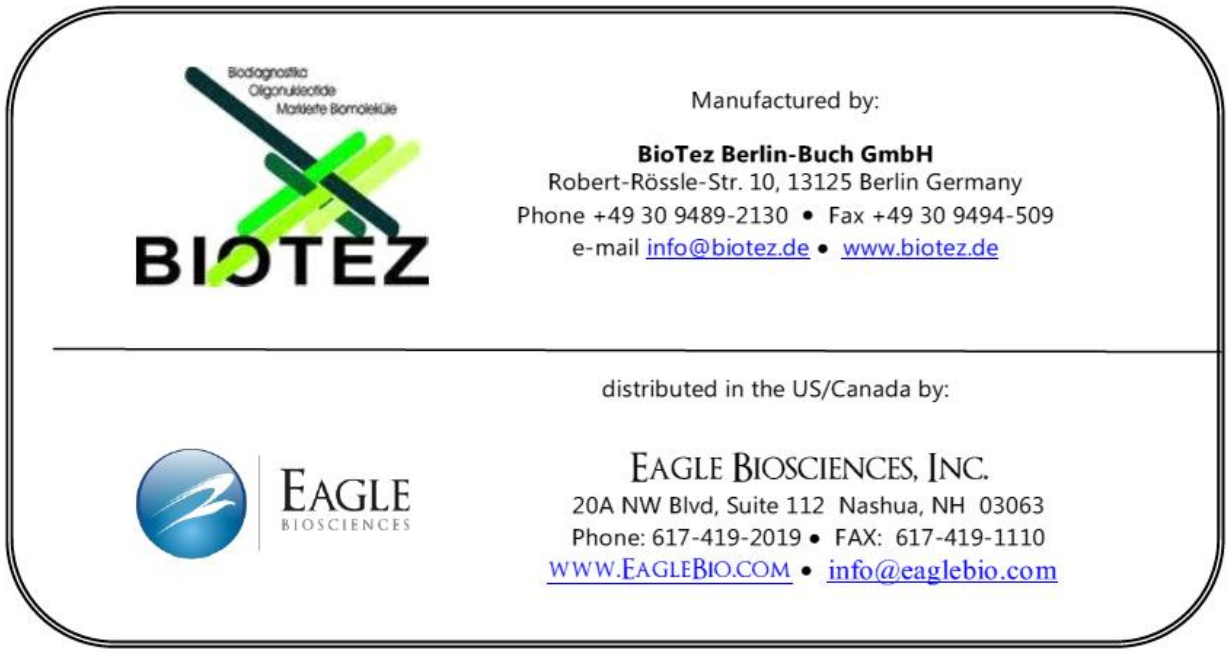
[1] "Development and validation of a liquid chromatography/ tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize" Michael Sulyok, Franz Berthiller, Rudolf Krska and Rainer Schuhmacher, Rapid Commun. Mass Spectrom. 2006; **20**: 2649–2659.

[2] "Simultaneous determination of aflatoxins and ochratoxin A in food using a fully automated immunoaffinity column clean-up and liquid chromatography–fluorescence detection", D. Chan, S.J. MacDonald, V. Boughtflower, P. Brereton *Journal of Chromatography A* **2004**, 1059: 13–16



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