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

Clean-up of Commodity Extracts of Food and Feed Samples containing Aflatoxins Via Immunoaffinity Chromatography and Subsequent HPLC Analysis with Postcolumn Derivatization using Pyridinium Perbromide

Principle:
This instruction of Aflatoxin total determination in food and feed focuses on the enrichment step of extract using immunoaffinity column (Immunoaffinity Chromotography (IAC)) in combination with quantification with HPLC (High Performance Liquid Chromotography).

Accepted laboratory extraction methods should be followed throughout the procedure. If the criteria of organic solvent tolerance, elution process of analyte, and working range of column are followed you will see full performance with the IAC column.

If problematic matrices are applied, many pretreatment methods of aflatoxin (AFG_2, AFG_1, AFB_2 and AFB_1) determination in food and feed will show low sensitivity because of interfering substances. This method of content determination of aflatoxins combines the high selectivity of an immunoaffinity column (IAC) with its potential to concentrate eluate with the 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 please note that a literature method is provided. The given apparatus (e.g. HPLC system) might serve as example among other possibilities.

Extraction (Literature method given):
Assuming that 25g sample is extracted by a total of 100ml methanol/water (60/40 v/v) and if organic solvent proportion is varied, the dilution of extract with PBS should be adapted accordingly in the enrichment step. On the other hand, if proportion of sample quantity and volume of extraction solvent is altered, the calculation of gram equivalents must be corrected.

For groundnut meal as an example of a problematic matrix, literature method of *Roch et al.*[1] *could be applied.*

Enrichment Step IAC:
4ml extract (see above, contains the quantity of aflatoxins of 1g sample) is diluted with **16ml 50mM PBS (pH=7.4**) and then applied in a reservoir on top of the ***B-TeZ IAC Aflatoxin 3ml*** column.

To maintain full performance of the column, please make sure that proportion of dilution buffer in the solution on top of the column is not too small. A proportion of 12% methanol, resulting in this example enrichment, does not affect column performance.

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

If organic solvent proportion lies above these limits, recovery rates are diminished. Increase of diluted extract volume by diluting extract with additional PBS, on the other hand, has almost no consequences to column performance.

If samples are to be prepared simultaneously, 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 cautious that there are no big air bubbles in the gel or between gel and the lock outlet of column. This can prevent a permanent flow or the 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 10mM PBS/Methanol (90/10 v/v).** Remaining liquids in the gel are removed by applying either pressure from top of the column or pressure from bottom.

Elution:
Sample reservoir on top of the ***B-TeZ IAC Aflatoxin 3ml*** column is removed and an appropriate vial is placed below the affinity column. The bound toxins are eluted by using a total **of 2ml of methanol** 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, half a minute is waited before the **second portion of 1ml of elutions solvent** is eluted through the column. The flow rate of the elution process should not exceed **1ml/min** very much. Remaining solvent solutions should be eluted by application of slight under or overpressure. All methanolic fractions are unified to give the column eluate.

The column eluate may be injected into the HPLC directly or if the contents are low (<2ng/g, related to commodity content), it may be concentrated by evaporation (e.g. using VLM evaporator at 50°C under a permanent stream of nitrogen).

Caution: If you follow concentration by evaporation, add 100µl of acetic acid/water (50/50 v/v) as keeper. To avoid loss of analytes, stop concentration at a small volume of residue, e.g. 50 to 100µl.

The residue then is redissolved in HPLC solvent (e.g. 0.4ml) and an aliquot is finally injected into the system.

IAC Column Characteristics:

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

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

(*) Recovery rates are confined to the complete IAC separation procedure (not including recovery rates of commodity extraction process), that means for the IAC Enrichment Step, organic solvent content of diluted extract below 20% methanol or 10% acetonitrile, for the Wash and Elution Step in the manner as described in this instruction

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

Aflatoxin B_1 (AFB_1):	100%
Aflatoxin B_2 (AFB_2):	86%
Aflatoxin G_1 (AFG_1):	101%
Aflatoxin G_2 (AFG_2):	82%

(**) Recovery rate of AFB_2, AFG_1, AFG_2, divided by recovery rate of AFB_1 if a total of 1.8µg Aflatoxin total (with molar ratio of B_1, B_2, G_1, G_2, = 4:1:4:1) is analyzed per column. Please notice that this quantity is near the capacity limit of column where binding sites of column are limited. For this reason, cross reactivities of AFG_1 and AFG_2 are much higher at aflatoxin concentrations within the given working range where antibody binding sites are in excess (see table A above).

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

Maximum Column Capacity:	1.8µg Aflatoxin total

(***) An excess of aflatoxins, e.g. 4µg, 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:

HPLC: Shimadzu
Column: Trentec Reprosil-Pur RP C18 120 ODS3 5µm; 125x3,0mm with guard column
Mobile Phase A: methanol / deionized water (85/15 v/v)
Mobile Phase B: methanol / acetonitrile / deionized water (18/18/64 v/v/v)
Gradient: 0.01 min B 100 %; 16 min B 100 %; 17 min B 0 %; 19 min B 0 %; 20 min B 100 %
Flow Rate: 0.5ml/min
Time of Analysis: 30min
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.
Temperature: Machine and eluents are at room temperature.
Eluents are degassed with helium gas.

HPLC Method Characteristics

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).

Balance of commodity contamination and HPLC measuring range:

If stated dilution steps (enrichment step, eluate concentration step) are followed, aflatoxin B_1 contents in commodites of 0.04 to 2ng/g lie within measuring range of HPLC method. If contents are higher, from 2 to 200ng/g, IAC column eluate should be diluted accordingly with HPLC eluent or, alternatively, injector volume should be adjusted.

Only if commodity contents lie above working range of IAC column of 200ng/g total aflatoxin, the 1g equivalent per column of this instruction must be lowered. Thus, the IAC enrichment step 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.

Left Aflatoxin G_1, Aflatoxin B_2; Aflatoxin G_2:

Sensitivities [AREA Peak / pg] of aflatoxins AFG_1, AFB_2 und AFG_2 related to that of AFB_1 are 37%; 136% and 67%, respectively.

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).

Because left aflatoxins (AFG_1, AFB_2 and AFG_2) mostly occur in common and are thus analyzed simultaneously with key aflatoxin B_1, different levels of commodity contents and established HPLC measuring ranges are balanced by the same manner as stated for aflatoxin B_1.

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Example Sample Calculation of AFB_1 content:
(Calculation of AFB_2, AFG_1 and AFG_2 content is analogous)
A) Calculation of Sample Gramm Equivalents per HPLC injection:

25g Sample / 100ml Extraction Solvent	x	4ml Extract / 0.4ml	x	0.1ml injector volume	=	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
- methanol, HPLC grade
- acetic acid, 100% ultrapure
- deionized water
- dipotassium hydrogenphosphate, >98%
- potassium dihydrogenphosphate, >98%
- sodium chloride

Consumerables:

- ***B-TeZ IAC Aflatoxin 3ml*** [BTAF310005]

Elution Solvent:

methanol

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; 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]

[1] "Determination of aflatoxins in groundnut meal by high-performance liquid chromatography: a comparison of two methods of derivatisation of aflatoxin B_1" G. Roch, G. Blunden, J. Haig, D.Coker and C. Gay *B. J. Biomed Sci* **1995**, 52, 312-316.

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