Instruction of the Clean-up Process
Using
B-TeZ IAC Aflatoxin M₁, 3ml

Clean-up of Milk of Milk Products containing Aflatoxin M₁ (AFM₁)
Via Immunoaffinity Chromatography
and
Subsequent HPLC Analysis with Postcolumn Derivatization with Perbromide
(for joint determination of Aflatoxin B₁)

Principle:
Immunoaffinity chromatography stands for an efficient enrichment process of aflatoxins with concomitant effective separation from matrix components. The eluate is best suited for determination by HPLC. By varying washing process, that means exchange PBS buffer with 0.2M ammonium acetate solution, GC can also be applied.

Sample Preparation of Milk:
According to the method of Shundo et al.¹ the milk to be tested is first centrifuged by 1540g for 15 minutes. The cream is then separated using e.g. a separation funnel. If separation is incomplete, the milk sample should be filtered to avoid that remaining fatty particles block the column in next step below.

Enrichment Step IAC:
50ml skimmed milk are diluted with 5ml 10X PBS (= 10fold concentrated 50mM PBS buffer) and then applied in a reservoir on top of the B-TeZ IAC Aflatoxin M₁, 3ml column. Rate of flow through the affinity gel is 1 to 3 ml/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.

If samples are to be prepared simultaneously, manifold of J.T. Baker for 12 samples has proven of value.

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 Aflatoxin M₁, 3ml column is removed and a appropriate vial is placed below the affinity column. The bound toxins are eluted by using a total of 1.5ml of methanol as elution solvent. The elution process is performed in two steps to ensure complete release of analytes. First, a volume of 0.5ml 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. 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 elute 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).

Caution: If you follow concentration by evaporation, add 100µl of acetic acid/water (50/50 v/v) as keeper. To avoid loss of analytes, stopp concentration at a small volume of residue, e.g. 50 to 100µl.
Instruction of the Clean-up Process
Using
B-TeZ IAC Aflatoxin M₁, 3ml

The residue then is redissolved in HPLC solvent, e.g. in 400µl in example calculation see below, and a aliquot is finally injected into the system.

IAC Column Characteristics:

A) Working Range and Recovery Rates of B-TeZ IAC Aflatoxin M₁, 3ml Column:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Range of Column</td>
<td>0.04 – 50ng AFM₁ per IAC</td>
</tr>
<tr>
<td>Zero Contamination of Column</td>
<td>&lt;0.04ng (LOD of HPLC-FLD method)</td>
</tr>
<tr>
<td>Guaranteed Recovery Rates for AFM₁ within the</td>
<td></td>
</tr>
<tr>
<td>Working Range ($)</td>
<td>85%</td>
</tr>
</tbody>
</table>

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

B) Cross Reactivities (**) of B-TeZ IAC Aflatoxin M₁, 3ml Column:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Recovery Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFM₁</td>
<td>100%</td>
</tr>
<tr>
<td>AFB₁</td>
<td>98%</td>
</tr>
<tr>
<td>AFB₂</td>
<td>99%</td>
</tr>
<tr>
<td>AFG₁</td>
<td>50%</td>
</tr>
<tr>
<td>AFG₂</td>
<td>98%</td>
</tr>
</tbody>
</table>

(****) Ratio of IAC recovery rates if a quantity of 5ng Aflatoxin total per column is analyzed

C) Capacity (***) of B-TeZ IAC Aflatoxin M₁, 3ml Column:

<table>
<thead>
<tr>
<th>Capacity</th>
<th>0.9µg AFM₁</th>
</tr>
</thead>
</table>

(****) An excess of AFM₁, e.g. 3µg, 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 nonbonded fraction is analyzed. The difference of added analyt and nonbonded analyt 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 (only advisable in case of concomitant aflatoxin B₁ analysis): 32 ppm pyridinium hydrobromide perbromide in dioxan/ deionized water (0.1/99.9, v/v); Flow Rate: 0.2ml/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. Fluenst are degassed with helium gas.

Example Sample Calculation of AFM₁ content:

(Calculation of AFB₁ is analogous)

A) Calculation of Sample Gramm Equivalents per HPLC injection:

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>50g Sample</td>
<td>12.5g</td>
</tr>
<tr>
<td>0.1ml injector</td>
<td>55ml Extract x 0.4ml Mixture x 55ml</td>
</tr>
</tbody>
</table>

Instruction of the Clean-up Process
Using
B-TeZ IAC Aflatoxin M₁, 3ml

The residue then is redissolved in HPLC solvent, e.g. in 400µl in example calculation see below, and a aliquot is finally injected into the system.

IAC Column Characteristics:

A) Working Range and Recovery Rates of B-TeZ IAC Aflatoxin M₁, 3ml Column:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Range of Column</td>
<td>0.04 – 50ng AFM₁ per IAC</td>
</tr>
<tr>
<td>Zero Contamination of Column</td>
<td>&lt;0.04ng (LOD of HPLC-FLD method)</td>
</tr>
<tr>
<td>Guaranteed Recovery Rates for AFM₁ within the</td>
<td></td>
</tr>
<tr>
<td>Working Range ($)</td>
<td>85%</td>
</tr>
</tbody>
</table>

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

B) Cross Reactivities (**) of B-TeZ IAC Aflatoxin M₁, 3ml Column:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Recovery Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFM₁</td>
<td>100%</td>
</tr>
<tr>
<td>AFB₁</td>
<td>98%</td>
</tr>
<tr>
<td>AFB₂</td>
<td>99%</td>
</tr>
<tr>
<td>AFG₁</td>
<td>50%</td>
</tr>
<tr>
<td>AFG₂</td>
<td>98%</td>
</tr>
</tbody>
</table>

(****) Ratio of IAC recovery rates if a quantity of 5ng Aflatoxin total per column is analyzed

C) Capacity (***) of B-TeZ IAC Aflatoxin M₁, 3ml Column:

<table>
<thead>
<tr>
<th>Capacity</th>
<th>0.9µg AFM₁</th>
</tr>
</thead>
</table>

(****) An excess of AFM₁, e.g. 3µg, 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 nonbonded fraction is analyzed. The difference of added analyt and nonbonded analyt 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 (only advisable in case of concomitant aflatoxin B₁ analysis): 32 ppm pyridinium hydrobromide perbromide in dioxan/ deionized water (0.1/99.9, v/v); Flow Rate: 0.2ml/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. Fluenst are degassed with helium gas.

Example Sample Calculation of AFM₁ content:

(Calculation of AFB₁ is analogous)

A) Calculation of Sample Gramm Equivalents per HPLC injection:

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>50g Sample</td>
<td>12.5g</td>
</tr>
<tr>
<td>0.1ml injector</td>
<td>55ml Extract x 0.4ml Mixture x 55ml</td>
</tr>
</tbody>
</table>
Instruction of the Clean-up Process
Using
B-TeZ IAC Aflatoxin M<sub>1</sub> 3ml

B) Calculation of Aflatoxin M<sub>1</sub> contamination of examined commodity in ng/g:

\[
\frac{\text{# ng injected AFM}_1}{\text{Sample Equivalents [g]}} = \text{ng/g AFM}_1 \text{ in commodity}
\]

Buffer, Chemicals, Apparatus and Literature:

10X PBS buffer:

12.4g KH<sub>2</sub>PO<sub>4</sub>  
72.7g K<sub>2</sub>HPO<sub>4</sub>  
87.6g NaCl

Dissolve in 1L deionised water. If necessary adjust pH to 7.2 (± 0.2) with 1N NaOH or 1N HCl

Phosphate Buffered Saline pH 7.4 (= 50mM PBS):

Dilute 100ml 10X PBS with deionized water to a final volume of 1L. Control pH to 7.4 (± 0.2)

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 M<sub>1</sub> 3ml  
  [BTAFM311005]

Elution Solvent:

Methanol

Mycotoxin Standard:

- Aflatoxin M<sub>1</sub> [e.g. Biopure]

Keeper:

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

Evaporation:

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

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. Mix thoroughly.
Instruction of the Clean-up Process

Using

*B-Tez IAC Aflatoxin M₁, 3ml*

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]

BioTez Berlin-Buch GmbH

[www.biotez.de](http://www.biotez.de)

---

1. "AFLATOXIN M₁ IN MILK BY IMMUNOAFFINITY COLUMN CLEANUP WITH TLC/HPLC DETERMINATION" Luzia Shundo; Myrna Sabino, *Brazilian Journal of Microbiology* **2006**, 37, 164-167
Warranty Information

Eagle Biosciences, Inc. warrants its Product(s) to operate or perform substantially in conformance with its specifications, as set forth in the accompanying package insert. This warranty is expressly limited to the refund of the price of any defective Product or the replacement of any defective Product with new Product. This warranty applies only when the Buyer gives written notice to the Eagle Biosciences within the expiration period of the Product(s) by the Buyer. In addition, Eagle Biosciences has no obligation to replace Product(s) as result of a) Buyer negligence, fault, or misuse, b) improper use, c) improper storage and handling, d) intentional damage, or e) event of force majeure, acts of God, or accident.

Eagle Biosciences makes no warranties, either expressed or implied, except as provided herein, including without limitation thereof, warranties as to marketability, merchantability, fitness for a particular purpose or use, or non-infringement of any intellectual property rights. In no event shall the company be liable for any indirect, incidental, or consequential damages of any nature, or losses or expenses resulting from any defective product or the use of any product. Product(s) may not be resold, modified, or altered for resale without prior written approval from Eagle Biosciences, Inc.

For further information about this kit, its application or the procedures in this kit insert, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.