Instruction of the Clean-up Process Using SENSI*Column* IAC Aflatoxin M_1 *3ml*

Product Code: EFAFM3115

Clean-up of Milk of Milk Products containing Aflatoxin M_1 (AFM_1) Via Immunoaffinity Chromatography and Subsequent HPLC Analysis with Postcolumn Derivatization with Perbromide

(for joint determination of Aflatoxin B_1)

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 **SENSI*Column* IAC Aflatoxin M_1 *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 **SENSI*Column* IAC Aflatoxin M_1 *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 elution 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 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. in 400µl in example calculation see below, and a aliquot is finally injected into the system.

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IAC Column Characteristics:

A) Working Range and Recovery Rates of SENSI*Column* IAC Aflatoxin M_1 *3ml*:

Working Range of Column:	0.04 – 50ng AFM1 per IAC
Zero Contamination of Column:	<0.04ng (LOD of HPLC-FLD method)
Guaranteed Recovery Rates for AFM_1 within the Working Range(*):	>85%

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

B) Cross Reactivities(**) of SENSI*Column* IAC Aflatoxin M_1 *3ml* Column:

Aflatoxin M_1 (AFM_1):	100%
Aflatoxin B_1 (AFB_1):	98%
Aflatoxin B_2 (AFB_2):	99%
Aflatoxin G_1 (AFG_1):	50%
Aflatoxin G_2 (AFG_2):	98%

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

C) Capacity(***) of SENSI*Column* IAC Aflatoxin M_1 *3ml* Column:

Maximum Column Capacity:	0.9µg AFM_1

(***) 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 B_1 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. Eluents are degassed with helium gas.

Example Sample Calculation of AFM_1 content:
(Calculation of AFB_1 is analogous)

A) Calculation of Sample Gramm Equivalents per HPLC injection:

$$\frac{\text{50g Sample}}{\text{55ml Extraction Mixture}} \times \frac{\text{55ml Extract}}{\text{0.4ml}} \times \text{0.1ml injector volume} = \text{12.5g Sample Equivalents}$$

B) Calculation of Aflatoxin M_1 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_2PO_4
72.7g K_2HPO_4
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:

- **SENSI*Column* IAC Aflatoxin *M_1 3ml***

Elution Solvent:

Methanol

Mycotoxin Standard:

- Aflatoxin M1 [e.g. Biopure]

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. Mix thoroughly.

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]

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[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