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

Product Code: EFOT3125

Clean-up of Commodity Extracts of Food and Feed Samples containing Ochratoxin A/B via Immunoaffinity Chromatography and Determination by HPLC-FLD

Principle:
This instruction of Ochratoxin 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 Ochratoxin determination in food and feed show low sensitivity because of interfering substances if problematic matrices are applied.

This method of content determination of 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):
Assuming that 25g sample are extracted by a total of 100ml methanol/water (80/20 v/v). 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, calculation of gram equivalents must be corrected.

Grains could be prepared by the literature method of Olsson et al.[1] For milk as an example of a problematic matrix, method of Zimmerli et al.[2] could be applied.

Enrichment Step IAC:
4ml extract (see above, contains the quantity of ochratoxins of 1g sample) are diluted with 16ml 50mM PBS (pH=7.4) and then applied in a reservoir on top of the **SENSI*Column* IAC Ochratoxin 3ml** column.

To maintain full performance of the column, please take care that proportion of dilution buffer in the solution on top of the column is not too small. A proportion of 16% 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 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.

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 underpressure from bottom.

Elution:
Sample reservoir on top of the **SENSI*Column* IAC Ochratoxin 3ml** column is removed and an appropriate vial is placed below the affinity column. The bound toxins are eluted by using a total of 1.5ml of methanol/acetic acid (98/2 v/v) 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 elution solvent is eluted 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. There is no danger of loss of analytes even though the eluate is evaporated to complete dryness.

The residue then is redissolved in HPLC solvent and an aliquot is finally injected into the system.

Caution!: If 100% methanol is used instead of recommended methanol-acetic acid (98/2 v/v) mixture as elution solvent of the column, 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.

IAC Column Characteristics:

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

Working Range of Column:	0.04 –200ng Ochratoxin per IAC
Zero Contamination of Column:	<0.04ng (det. lim. of method)
Guaranteed Recovery Rates within the Working Range(*):	>85% ± 5%

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

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

Ochratoxin A (OTA):	100%
Ochratoxin B (OTB):	103%

(**) Recovery rate of OTA and OTB divided by recovery rate of OTA if a total of 1.5µg Ochratoxins (with molar ratio of 1:1) is analysed per column. Please notice that this quantity is around half of the capacity limit of column where binding sites of column are limited. Thus, for analysis within the working range of the column, cross reactivities against OTA and OTB are practically the same in magnitude.

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

Maximum Column Capacity:	3µg Ochratoxin

(***) An excess of ochratoxin, 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 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: acetonitrile / deion. water (70/30, v/v); Mobile Phase B: 0.03 M sodium acetate (pH=4,0) / acetonitrile (65/35 v/v); Gradient: 0,01 min B 100 %; 10 min B 100 %; 11 min B 0 %; 13 min B 0 %; 15 min B 100 %; Flow Rate: 0.5ml/min; Time of Analysis: 25min; Injector Volume: 100µl; Fluorescence-Detection: λ_{EX} [nm]: 333nm; λ_{EM} [nm]: 460nm. Temperature: Machine and eluents are at room temperature. Eluents are degassed with helium gas.

HPLC Method Characteristics:
Measuring range is linear from 10pg to 1000pg Ochratoxin A (OTA) per injection (R^2=0.999) in given HPLC method. Lower limit of detection (LOD) is 10pg 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) are followed, OTA contents in commodities of 0.04 to 4ng/g lie within measuring range of HPLC method. If contents are higher, from 4 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 OTA, 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.

Example Sample Calculation of OTA content:
(Calculation of OTB content is analogous)

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 OTA contamination of examined commodity in ng/g:

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

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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 deionisized 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

Consumables:

- **SENSI*Column* IAC Ochratoxin 3ml**

Elution Solvent:

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

Evaporation:

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

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] "Detection and quantification of ochratoxin A and deoxynivalenol in barley grains by GC-MS and electronic nose" J. Olsson, T. Borjesson, T. Lundstedt , J. Schnurer, *International Journal of Food Microbiology* **2002,** 72, 203–214

[2] "Determination of ochratoxin A at the ppt level in human blood, serum, milk and some foodstuffs by high-performance liquid chromatography with enhanced fluorescence detection and immunoaffinity column cleanup methodology and Swiss data" B. Zimmerli, R. Dick *J. Chromatogr. B* **1995**, 666, 85-99