# Instruction of the Clean-up Process Using *B-TeZ IAC DonZon*

## Clean-up of Commodity Extracts of Food and Feed Samples containing DON and ZON by Simultaneous Work-Up via Immunoaffinity Chromatography with Bitox Functionality and Determination by HPLC-UV

Principle:
This instruction of Deoxynivalenol (**DON**) and Zearalenon (**ZON**) 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 DON and ZON determination in food and feed show low sensitivity because of interfering substances if problematic matrices are applied.

This method of content determination of DON and ZON combines the high selectivity of an immunoaffinity column (IAC) with its potential to concentrate elute 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 ground corn sample are extracted by a total of 100ml acetonitrile/water (75/25, v/v), as reported by Krska et al.[1] 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.

Enrichment Step IAC:
An aliquot of 1ml extract (see above, contains the quantity of toxins of 0.25g commodity) are diluted with 9ml 50mM PBS (pH=7.4) and then applied in a reservoir on top of the ***B-TeZ IAC DonZon*** 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 to small. A proportion of 7.5% acetonitrile, resulting in this example enrichment, does not affect column performance.

**The proportion of organic solvent of PBS diluted extract, which is applied on the column, should not exceed 15% methanol or 15% 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 20ml of deionized water. 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 DonZon*** 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 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 eluate 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:** As long as evaporation process is performed moderately, it is not necessary to add a keeper. If that is not the case, it is recommended to use a keeper, e.g. 100µl deionized water or PBS.

The residue then is redissolved in HPLC solvent (e.g. 0.5ml) and an aliquot is finally injected into the system.

**IAC Column Characteristics:**

**A) Working Range and Recovery Rates of *B-TeZ IAC DonZon* Column:**

| | |
|---|---|
| Working Range of Column: | |
| DON: | 5– 500ng per IAC |
| ZON: | 5– 500ng per IAC |
| Zero Contamination per Column: | |
| DON: | <5ng (LOD of HPLC method) |
| ZON: | <5ng (LOD of HPLC method) |
| Guaranteed Recovery Rates within the Working Range(*): | |
| DON: | >85% |
| ZON: | >85% |

(*) Recovery rates are confined to solvent content of diluted extract, which is applied on top of the IAC column, below 15% methanol or 15% acetonitrile (see details under Enrichment Step).

**B) Cross Reactivities(**) of *B-TeZ IAC DonZon* Column:**

| | |
|---|---|
| DON: | 100% |
| Nivalenol (NIV): | 31% |
| 15-Acetyl-DON: | 33% |
| 3-Acetyl-DON: | <1% |
| ZON: | 100% |
| α-Zearalanol: | 99% |
| β-Zearalanol: | 94% |
| α-Zearalenol: | 97% |
| β-Zearalenol: | 86% |

(**) Recovery rates if a total quantity of 500ng of DON, NIV, 3-Acetyl-DON and 15-Acetyl-DON (molar ratio of 1:1:1:1) and a total quantity of 500ng of Zearalenon, α-Zearalanol, β-Zearalanol, α-Zearalenol and β-Zearalenol (molar ratio of 1:1:1:1:1) is analyzed.

**C) Capacity(***) of *B-TeZ IAC DonZon* Column:**

| | |
|---|---|
| Maximum Column Capacity: | 1.8µg DON and 2.7µg ZON |

(***) An excess of DON and ZON, namely 5µg 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 nonbonded fraction is analyzed. The difference of added analyt and nonbonded analyt equals maximum column capacity.

**High commodity mycotoxin contents and IAC working range:**

If dilution factors of this instruction are followed, commodity contents upto 2000ng/g DON and 2000ng/g ZON are within working range of the product.

Only if commodity contents exceed these values, the IAC procedure and subsequent HPLC analysis should be repeated with a smaller extract volume. E.g. use instead of 1ml extract a volume of 0.5ml extract, or even less where applicable, and dilute with 10ml PBS and apply to the IAC column.

**Analytical Method:**

The HPLC multitoxin method (lc-ms/ms) of Chan et al.[2] is adapted for the use of UV absorbance 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

UV-Absorbance-Detection: $\lambda_{ABS}$ [nm]: 250nm (DON); only from 30 to 36min: 305nm (ZON).

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

**Example Sample Calculation of Zearalenon (ZON) content:**
(Calculation of DON content is analogous)

A) Calculation of Sample Gramm Equivalents per HPLC injection:

$$\frac{\text{25g Sample}}{\text{100ml Extraction Solvent}} \times \frac{\text{1ml Extract}}{\text{0.5ml}} \times \text{0.1ml injector volume} = \text{0.05g Sample Equivalents}$$

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

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

**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

**Consumables:**

- ***B-TeZ IAC DonZon***

**Elution Solvent**:

Methanol

**Evaporation:**

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

Apparatus:
HPLC; Shimadzu; pump: LC-6A (2 pieces); auto sampler: SIL 6B; UV-absorbence 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] "Performance of modern sample preparation techniques in the analysis of Fusarium mycotoxins in cereals", Rudolf Krska, *Journal of Chromatography A* **1998**, 815, 49–57

[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