APPLICATION NOTE AN0044

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CrossTOX®

Comparison of Analytical Results of Reference / QC Material Cleaned-up by CrossTOX® Column

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Content

1. Introduction

Mycotoxins are widly found in various matrices and various combination. Mycotoxins are hazardous for human health and impact economically all agricultural areas, either by direct mycotoxin presence and loss of quality or by influencing animal health with massive impact on productivity.

Due to the prevalence of mycotoxin in various materials, more than one mycotoxin could be found in samples. This requires multi-toxin clean-up to cover most or all mycotoxin in one analytical approach. The different properties of matrices bring more challenges in the field of sample clean-up, as fat or oil, starch or etheric oils or dyes impact sample analysis and needed to be compensated by internal standards for analysis . A single clean-up column, which allow matrix removal without rendering mycotoxin concentration in a sample should overcome this challenges. The removal of matrix leads to a higher analytical precision, reduction of costs for internal standards and by the clean-up of the sample the drift or maintenance downtime of analytical devices become dramatically reduced. The CrossTOX® column allows extraction and sample clean-up from cereals, nut and fruit matrices. Even spices and animal feed could be investigated by this non dispersive clean-up technology.

The sample clean-up is dedicated for LC-MS/MS analysis, which allows the parallel analysis for multiple mycotoxin components in one sample. The clean-up approach removes matrix interferences to allow higher throughput and a significant reduction in downtime of analytical devices.

2. Method Development

For direct comparison of analytical performance reference materials, tested in interlaboratory studies were used and analyzed by immunoaffinity clean-up or SPE or by the CrossTOX® clean-up approach.

2.1 Reagents and Materials

- Extraction solvents
  - Methanol/ Water (80/20 (v/v)) (for extraction of samples for immunoaffinity clean-up)
  - n-hexane for defatting of matrices
  - Acetonitril/water (84/16) for extraction and clean-up of Deoxynivalenol and derivatives thereof by DONeX column.
  - Acetonitrile/water/acetic acid (84/15/1) for CrossTOX® clean-up.

- Sample dilution buffer
  - 5 x PBS buffer
    1) Disodiumhydrogenphosphate (12 H2O) 100,279 g
    solved in 1400 mL deionised water

    2) Sodiumdihydrogenphsophate monohydrate 19,319 g
    solved in 700 mL deionised water.

    Sodiumchloride 85 g
    Solution 1 and parts of solution 2 were mixed to adjust pH to 7.2; finally the sodium chloride was added.

  - PBS buffer
    200 mL of the 5 x PBS was added to 800 mL deionized water and mixed thoroughly.

  - PBS buffer containing 8 % Tween20
    8 mL Tween20 was added to 92 mL PBS buffer and mixed thoroughly.

- Test materials

Material	Name	Origin	Toxins
FCMM4 SPI14QC	T04364QC	Bell Pepper	Aflatoxins B/G, Aflatoxins ∑, OTA
FCMM5 AFE2QC	T04375QC	Animal feed	AFB1, ZEA; DON
FCMM14-CCP32QC	TCL0405QC	Maize flour	AFB1, Aflatoxins ∑, DON, OTA, Total T2/H-T2, total FB1+2, ZEA
FCMM8-CCP28QC	T17200QC	Flour	CIT, OTA
FCMM3-CCP30QC	T04384QC	Maize flour	AFB1, DON, ZEA, OTA, FB1; FB2, T2, H-T2

- Sample clean-up column
  - AflaCLEAN 3mL immunoaffnity column 25 pc PN # 10514
  - OtaCLEAN 3mL immunoaffinity column 25 pc PN # 10515
  - Afla-OtaCLEAN immunoaffinity column 25 pc PN # 11022
  - ZeaCLEAN SMART column 100 pc PN # 14741
  - DONeX column 25 pc PN # 12792
  - CrossTOX® column 100 pc PN # 17900
  - CrossTOX® column for automated processing 100 pc PN # 17901
- Glassware, accessories and devices
  - Beaker 100 mL and 200 mL
  - Measuring cylinder 2 mL
  - Pipettes 10 mL and 50 mL (single use)
  - Microliter pipettes 10-100 and 100-1000 µL
  - Plaited filter
  - Glass fibre filter whatman GF/A
  - Syringe 1 mL, 10 mL and 20 mL
- UHPLC-MS/MS, Thermo Fisher Scientific
- HPLC-FLD (-UV)

2.2 Sample Preparation

The samples were extracted according to the extraction and clean-up procedure of the individual clean-up column. The crude extract was filtered prior to further sample dilution. In case of turbidity the diluted sample was filtered through a whatman GF/A filter.

For CrossTOX® clean-up a 84/15/1 Acetonitrile/water/acetic acid (v/v/v) as extraction solvent was used, the crude extract was filtered prior to use.

Animal feed samples were diluted by pbs tween buffer prior to application on immunoaffinity column.

Bell Pepper and spices were extracted by methanol/ water and n-hexane for immunoaffinity clean-up. For CrossTOX® clean-up matrix was extracted with acetonitrile/water (84/16) and 50 ml n-hexane was added to remove fat and oil. For further clean-up the n-hexane free phase was used.

2.3 Experimental procedure

2.3.1 **CrossTOX® clean-up:**
3 mL centrifuged crude extract (84/16) was applied under constant flow (1mL/min) on CrossTOX® column. The flowthrough was collected in a gc vial for direct LC-MS/MS analysis.

2.3.2 **ZeaCLEAN SMART clean-up:**
20 mL of the diluted sample was applied under constant flow on a ZeaCLEAN SMART column, the sample reservoir was washed with 20% acetonitrile. After drying the column by a flush of nitrogen, the toxins were eluted in 400µL acetonitrile and subsequently analysed by hplc- FLD or LC-MS/MS.

2.3.3 **DONeX clean-up:**
20 mL of the extract was applied under constant flow (2 mL/min) on a DONeX column, the flowthrough was collected in a beaker. The sample reservoir was washed by 10 mL acetonitrile/water (84/16) and applied onto the column. The flowthrough was collected and mixed with the first fraction. 7.5 mL representing 1gram matrix was evaporated under nitrogen and resolved in HLPC solvent for subsequent analysis by HPLC-UV or LC-MS/MS.

2.3.4 **AflaCLEAN clean-up**
7 mL of the crude extract were diluted with pbs buffer to a final volume of 50 ml. The sample was applied under constant flow (1-2 mL/min) onto a AflaCLEAN column. The sample reservoir was washed with 10 ml deionized water, which was then used for washing the column. After drying the column by a flush of nitrogen, the toxins were eluted by methanol into a 2 mL measuring cylinder.

2.3.5 Afla-OtaCLEAN clean-up

2 mL of the crude extract were diluted with pbs buffer containing Tween20 to a final volume of 14 ml. The sample was applied under constant flow (1-2 mL/min) onto a Afla-OtaCLEAN column. The sample reservoir was washed with 10 ml deionized water, which was then used for washing the column. After drying the column by a flush of nitrogen, the toxins were eluted by methanol into a 2 mL measuring cylinder.

2.3.6 OtaCLEAN clean-up

10 mL of the crude extract were diluted with pbs buffer to a final volume of 50 ml. The sample was applied under constant flow (1-2 mL/min) onto a OtaCLEAN column. The sample reservoir was washed with 10 ml deionized water, which was then used for washing the column. After drying the column by a flush of nitrogen, the toxins were eluted by methanol into a 2 mL measuring cylinder.

3. Results

3.1 Efficiency

The results of the individual clean-up procedures were analyzed by HPLC-FLD or HPLC-UV and LC-MS/MS to compare for matrix interferences and analytical conformity.

Table 1: Spices / Bell Pepper - FCMM4-SPI14QC (T04364QC)

Toxin	µg/Kg	Accepted			Assigned	CrossTOX®	IAC HPLC-FLD	IAC LC-MS/MS
Aflatoxin B1	µg/Kg	1.98	-	5.08	3.53	4.94	3.71	4.42
Aflatoxin B2		0.83	-	2.14	1.49	1.65	1.44	1.49
Aflatoxin G1		1.36	-	3.49	2.43	3.26	2.68	1.82
Aflatoxin G2		0.81	-	2.08	1.44	<1.0*	1.17	0.92
Total Aflatoxin (B/G)		4.99	-	12.83	8.91	9.85	9.00	8.65
OTA		7.4	-	18.9	13.1	13.85	12.82	12.91

*Below LoQ

Table 2: Cookie / Biscuit Flour - FCMM8-CCP28QCT17200QC

Toxin	µg/Kg	Accepted			Assigned	CrossTOX®	IAC HPLC-FLD	IAC LC-MS/MS
Ochratoxin A	µg/Kg	3.31	-	8.51	5.91	7.52	5.57	5.51
Citrinin		67	-	173	120	74.52	n.d.	n.d.

Table 3: Animal Feed (Cereal-based) - FCMM5-AFE2QC (T04375QC)

Toxin	µg/Kg	Accepted			Assigned	CrossTOX® LC-MS/MS	DONeX HPLC-UV	DONeX LC-MS/MS	AflaCLEAN LC-MS/MS	AflaCLEAN HPLC-FLD	ZeaCLEAN SMART HPLC-FLD	ZeaCLEAN SMART LC-MS/MS
DON	µg/Kg	1958	-	3447	2702	3113	2349	2620				
AFB1		8.2	-	21.1	14.6	17.2			13.3	16.5		
ZEA		612	-	1200	906	733					869	912

Table 4: Maize / Corn - FCMM14-CCP32QC (TCL0405QC)

Toxin	µg/Kg	Accepted		Assigned	CrossTOX® LC-MS/MS	DONeX HPLC-UV	DONeX LC-MS/MS	AflaCLEAN LC-MS/MS	AflaCLEAN HPLC-FLD	ZeaCLEAN SMART HPLC-FLD	ZeaCLEAN SMART LC-MS/MS	OtaCLEAN HPLC-FLD	OtaCLEAN LC-MS/MS
DON	µg/Kg	835	1588	1211	1157	1169	1258						
AFB1		6.6	16.9	11.8	10.0			10.98	8.52				
Total AF		10.9	28.1	19.5	19.03			20.37	17.6				
ZEA		181	406	293	261					246	187		
OTA		2.82	7.25	5.04	5.88							5.26	5.03
Total FB		1034	1928	1481	1337								
T2 and HT-2		35.0	90.0	62.5	52.5								

Table 5: Maize / Corn: FCMM3-CCP30QC (T04384QC)

Toxin	µg/Kg	Accepted		Assigned	CrossTOX® LC-MS/MS	DONeX HPLC-UV	DONeX LC-MS/MS	AflaCLEAN LC-MS/MS	AflaCLEAN HPLC-FLD	ZeaCLEAN SMART HPLC-FLD	ZeaCLEAN SMART LC-MS/MS	OtaCLEAN HPLC-FLD	OtaCLEAN LC-MS/MS
DON	µg/Kg	578	1140	859	898	896	865						
AFB1		3.43	8.83	6.13	6.15			5.83	6.0				
ZEA		48.9	125.7	87.3	75.2					66.7	51		
OTA		2.70	6.94	4.82	4.86							4.58	4.43
FB1		293	622	457	379								
FB2		196	437	316	335								
Total FB		501	1003	752	714								
T-2		100	243	172	164								
HT-2		91	224	157	100								
T-2 and HT-2		205	455	330	264								

4. Conclusion

The results obtained by CrossTOX® and LC-MS/MS analysis are complying to the results of the alternative clean-up procedures and are within the accepted range of the interlaboratory testing specifications.

The CrossTOX® allows a fast, simple clean-up with results similar to immunoaffinity or SPE clean-up. The CrossTOX® column is suitable for different type of matrices , including animal feed, cereal, dried fruit, nuts and some spices or herbal material.
The CrossTOX® technology allows the removal of matrix interferences and reduces costs in terms of internal standards or downtime of the LC-MS/MS dramatically.

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