SOLUTIONS BY LC Tech

CrossTOX®



# Multi-Mycotoxin Analysis CrossTOX® Sample Clean-up and Matrix Removal

# Content

## 1. Introduction

In the last years increasing numbers of mycotoxin contamination in food and feed were reported. Furthermore not only single mycotoxins but up to 90 % of all sample material contains more than one mycotoxin. With this the multi-mycotoxin analysis becomes more relevant. To overcome the workload issue with increasing sample numbers and to remain in compliance with regulated levels of mycotoxins.

The matrices which are investigated for mycotoxins are not only cereals or cereal based anymore, but more food and feed stuff that contains nuts or peanut with high fat components or contain oils are investigated. These disturb the analytical devices and lead to implementation of more and more complicated extraction procedures. Dried fruits as another matrix group is described in literature to be affected by toxic mycotoxins as well.

The CrossTOX® column allows the analysis of at least 16 mycotoxins extracted from one sample with only one extraction protocol for most matrices and an impressive matrix removal. CrossTOX® is the next step to common protocols for mycotoxin sample clean-up for multi mycotoxin analysis in the LC-MS/MS environment. The column clean-up is not restricted to only one type of matrix, but allows the mycotoxin clean-up and matrix removal from cereals, dried fruits or nut matrices as well as some spices could be processed with the CrossTOX® column. This leads to cost reduction and decreases the downtime of the analytical devices due to higher clearance of the sample with lower matrix interferences.

# 2. Method Development

## 2.1 Reagents and Materials

- CrossTOX® column (P/N 17900)
- UPLC-MS/MS H-ESI,
- Accucore Biphenyl 100 mm x 2.1mm; 2.6 µm with defender Guard column
- Labmill or equivalent
- Blender jar
- Syringe (10 mL), single use
- Beaker
- Laboratory balance (+/- 0.1 gram)
- Pipettes (0.2 – 1.0 mL)
- Optional plaited filter or centrifuge (3000xg)
- GC –vials with cap
    - Acetonitril p.a.
    - Water hplc grade
    - Acetic acid p.a.
    - Extraction solvent: Acetonitrile /water/acetic acid (84/15/1 (v/v/v))
    - n-hexane

## 2.2 Material for Automated Clean-up with Direct Injection

### 2.2.1 Configuration FREESTYLE

| | | |
|---|---|---|
| 1. | FREESTYLE BASIC | P/N 12663-12 |
| 2. | FREESTYLE SPE module | P/N 12668 |
| 3. | QuEChERS Set (Hardware and Software) | P/N 16269 |
| 4. | HPLC Direct-Injection module | P/N 16278 |
| 5. | Sample loop, stainless steel, 10 µL | P/N 14486 |
| 6. | Relay cable assembly for AGILENT-system | P/N 13679 |
| 7. | Rack for solvent delivery | P/N 13156 |
| 8. | Overflow Sensor for Waste Level Control | P/N 12709 |
| 9. | Frame for trays | P/N 11915 |
| 10. | Rack for 60 SMART columns | P/N 13497 |
| 11. | Adapter/Outlet for SPE-columns | P/N 13544 |
| 12. | Frame for trays | P/N 11915 |
| 13. | Tray for 60 pcs - 1 mL GC vials | P/N 11920 |
| 14. | Tray for 60 pcs - 4 mL vials | P/N 11926 |
| 15. | Tray for 30 pcs - 16 mL vials | P/N 11933 |
| 16. | Retention plate for 1mL and 4 mL rack | P/N 13429 |

## 2.3 Sample Preparation for Cereal, Nut or Dried Fruit Matrices

20 g of sample material for cereals, nuts or dried fruits are homogenized and weight in. 100 mL of extraction solvent are added. The sample is extracted in a blender jar to ensure highest extraction efficiency. Check extraction time in your laboratory (according to experience the extraction time is 5 - 10 min). Let the sample settle for a few seconds. The supernatant could be used immediately. To avoid backpressure in the CrossTOX® columnm, an optional filtration through a plaited filter or centrifugation could be used for clearance of flour samples. The CrossTOX® column will trap smaller particles efficently and remove those from the sample. A reduction of sample volume could help to overcome the backpressure in the column.

## 2.4 Sample Preparation for Spices

10 g of sample material for spices are homogenized and weight in. 100 mL of extraction solvent are added. Add 50 mL of n-hexane to remove fats and oils from the raw material during the extraction procedure. The sample is extracted in a blender jar to ensure highest extraction efficiency. Check extraction time in your laboratory (according to experience the extraction time is 5 - 10 minutes). Let the sample settle approx. one minute. The n-hexane free (lower) phase could be used immediately. A centrifugation could help to improve phase separation. Use the lower solvent phase (n-hexane free) for the further sample clean-up.

The sample is passed thorugh the CrossTOX® column. Depending on the sample, impurities and dyes (0.5 - 3 mL) could be passed through the column. The filtrate is collected in the GC-vial and analysed in the LC-MS/MS for multi-mycotoxin status. Reduced sample volume increase matrix clearance, volumes down to 0.25 mL could be applied on the CrossTOX® column.

## 2.5 Instrumentation

UPLC column Accucore Biphenyl 100 mm x 2.1 mm; 2.6 µm with defender Guard was used. The sample is passed through the CrossTOX® column. Depending on the sample, impurities and dyes (0.5 - 3 mL) could be passed through the column. The filtrate is collected in the GC-vial and analysed in the LC-MS/MS for multi-mycotoxin status.

Column temperature was set to 38 °C. Eluent a): HPLC water/methanol (98 / 2 (v/v))+ 1 % acetic acid + 5 mM ammonium acetate. Eluent b): HPLC water/methanol (2 / 98 (v/v)) + 1 % acetic acid + 5 mM ammoinum acetate. Flow rate 0.4 mL / min.

*Table 1: UPLC gradient*

| Time [min] | Eluent A (%) | Eluent B (%) | Curve |
|---|---|---|---|
| 0 - 2 | 95 | 5 | 5 |
| 2 - 5 | 15 | 85 | 5 |
| 5 - 11 | 5 | 95 | 5 |
| 11 - 13 | 95 | 5 | 5 |
| 13 - 16 | 95 | 5 | 5 |

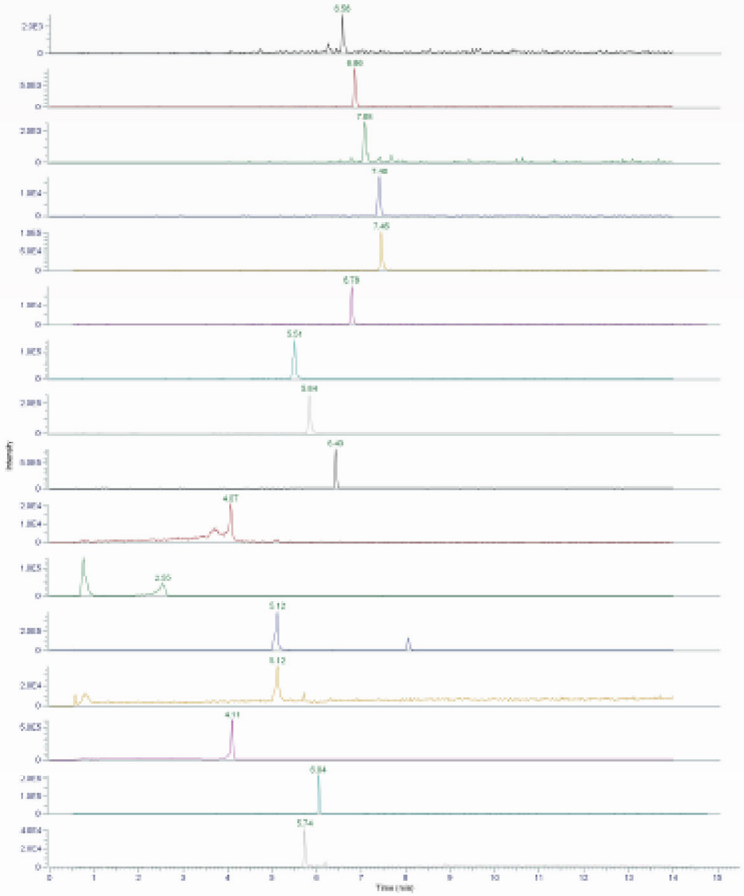
MS settings: heated ESI, 3500 V positive / 1500 V negative ion voltage. Ion transfer tube temperature 325 °C, vaporizer temperature 350 °C.

For all analytes at least one quantifier and two qualifier product ions were chosen.

The matrices were spiked prior and after extraction to investigate extraction efficency and matrix interferences. The spiking was performed using a broad range of toxin contamination levels ranging from 2 - 20 ppb for aflatoxins and 10 - 50 ppb for ochratoxin A and sterigmatocystin 15 - 50 ppb for T2 /H-T2 toxin and 50 - 500 ppb for zearalenone. Deoxynivalenol, derivatives thereof and nivalenol were investigated from 200 - 1000 ppb.

## 3. Results

Chromatographic separation



**Aflatoxin G2**
**Aflatoxin G1**
**Aflatoxin B2**
**Aflatoxin B1**
Sterigmatocystin (STC)
**Ochratoxin A (OTA)**
**Fumonisin B1**
**Fumonisin B2**
**Zearalenone**
**Deoxynivalenol (DON)**
Nivalenol
3-Acetyl-DON
15-Acetyl-DON
DON-3Glc
**T2 Toxin**
**H-T2 Toxin**

*Figure 1: Chromatographic appearance for individual toxins using CrossTOX® clean-up procedure (regulated mycotoxins are marked in bold)*

## 3.1 Cereal Matrices

*Table 2: Results for cereal based matrices (the abbreviations used in the table correspond in the order in which the diagrams are labelled)*

| | Afla B1 | Afla G1 | Afla B2 | Afla G2 | OTA | ZEA | DON | F-B1 | F-B2 | T2-Toxin | HT2-Toxin | NIV | 3-AcDON | 15-AcDON | DON 3-Glc | STC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Spelt** | 95 | 79 | 95 | 113 | 84 | 81 | 94 | 116 | 116 | 74 | 79 | 94 | 88 | 73 | 94 | 83 |
| **Rice** | 102 | 100 | 127 | 76 | 107 | 98 | 72 | 113 | 120 | 99 | 93 | 105 | 104 | 113 | 82 | 119 |
| **Wheat** | 100 | 98 | 114 | 73 | 100 | 87 | 104 | 103 | 111 | 81 | 77 | 103 | 102 | 90 | 78 | 112 |
| **Oats** | 110 | 91 | 84 | 88 | 108 | 85 | 93 | 109 | 105 | 100 | 78 | 91 | 87 | 77 | 77 | 116 |
| **Corn** | 108 | 109 | 84 | 93 | 104 | 84 | 104 | 115 | 115 | 110 | 84 | 86 | 97 | 80 | 78 | 112 |

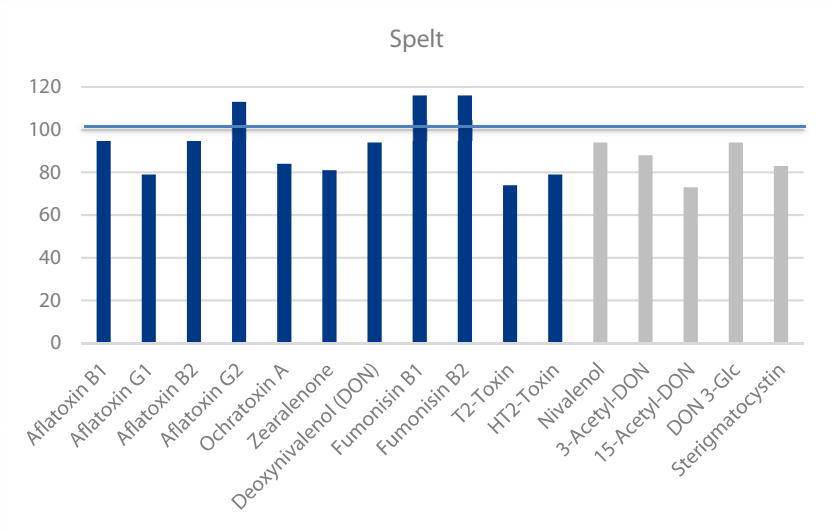



*Figure 2: Recovery rates for Spelt*

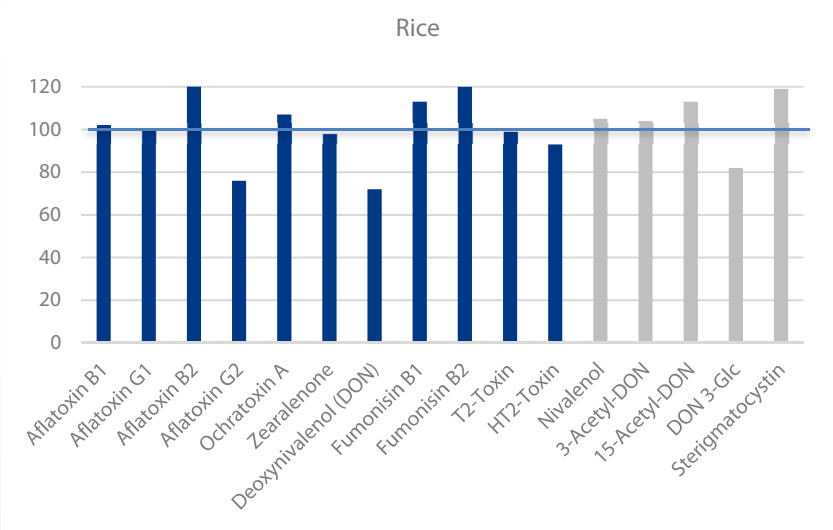



*Figure 3: Recovery rates for Rice*

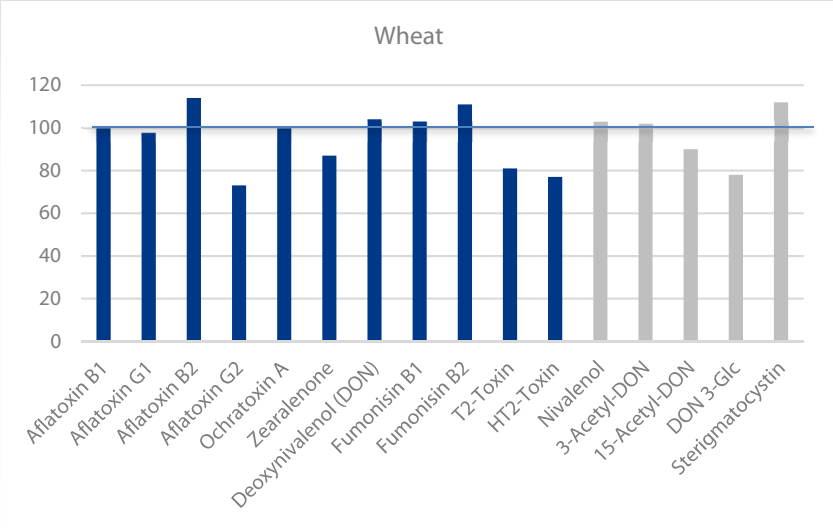



*Figure 4: Recovery rates for Wheat*

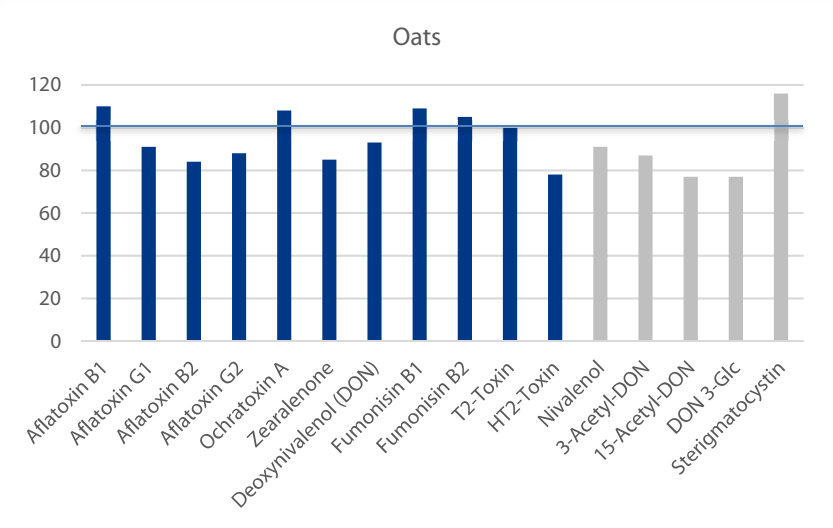



*Figure 5: Recovery rates for Oats*

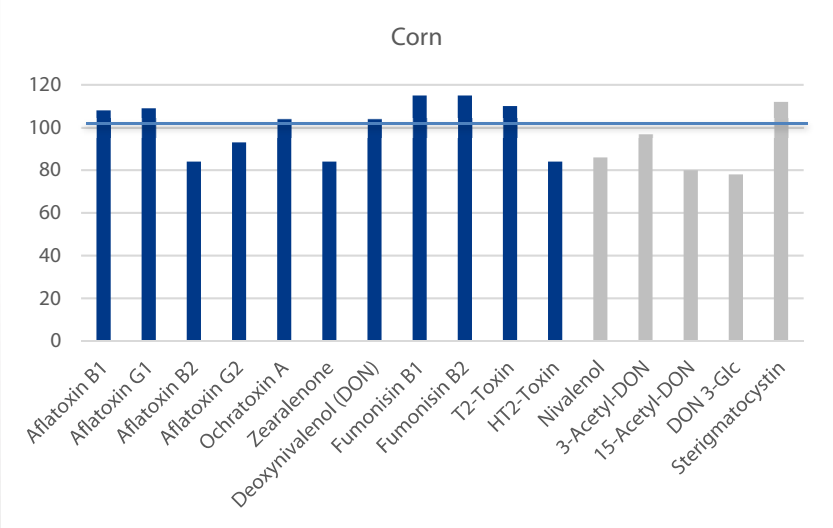



*Figure 6: Recovery rates for Corn*

## 3.2 Nuts or Nut-like Matrices

*Table 3: Results for nuts / nut-like matrices (the abbreviations used in the table correspond in the order in which the diagrams are labelled)*

| | Afla B1 | Afla G1 | Afla B2 | Afla G2 | OTA | ZEA | DON | F-B1 | F-B2 | T2-Toxin | HT2-Toxin | NIV | 3-AcDON | 15-AcDON | DON 3-Glc | STC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Peanut** | 106 | 106 | 103 | 98 | 108 | 103 | 107 | 108 | 104 | 65 | 75 | 83 | 108 | 100 | 71 | 102 |
| **Hazelnut** | 114 | 105 | 107 | 104 | 101 | 86 | 96 | 106 | 109 | 102 | 71 | 110 | 108 | 89 | 88 | 110 |
| **Walnut** | 108 | 109 | 108 | 82 | 103 | 100 | 111 | 101 | 105 | 87 | 85 | 83 | 87 | 76 | 82 | 102 |
| **Pistachio** | 103 | 108 | 109 | 96 | 100 | 107 | 107 | 102 | 96 | 107 | 103 | 81 | 106 | 101 | 81 | 106 |
| **Almonds** | 105 | 101 | 106 | 106 | 109 | 100 | 91 | 104 | 103 | 78 | 82 | 104 | 108 | 92 | 79 | 102 |

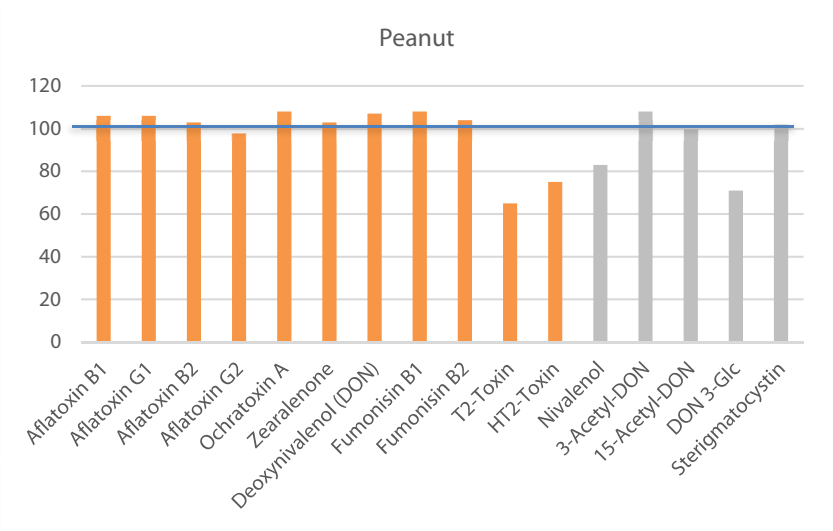



*Figure 7: Recovery rates for Peanut*

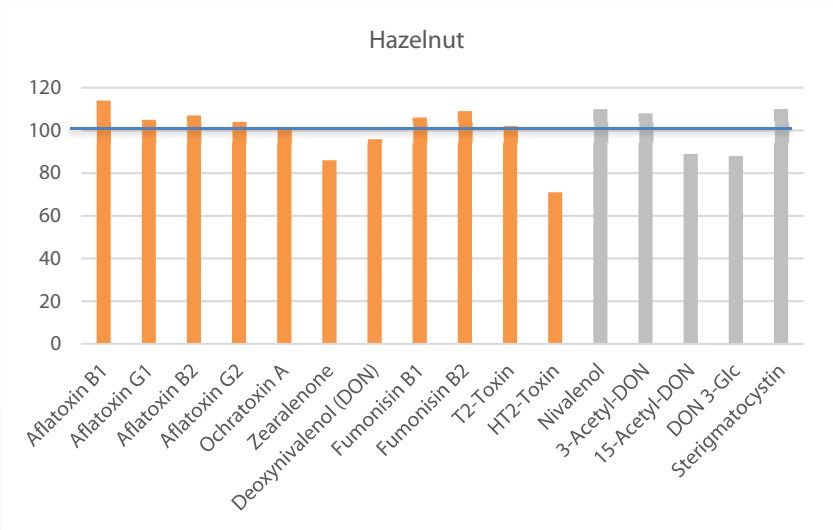



*Figure 8: Recovery rates for Hazelnut*

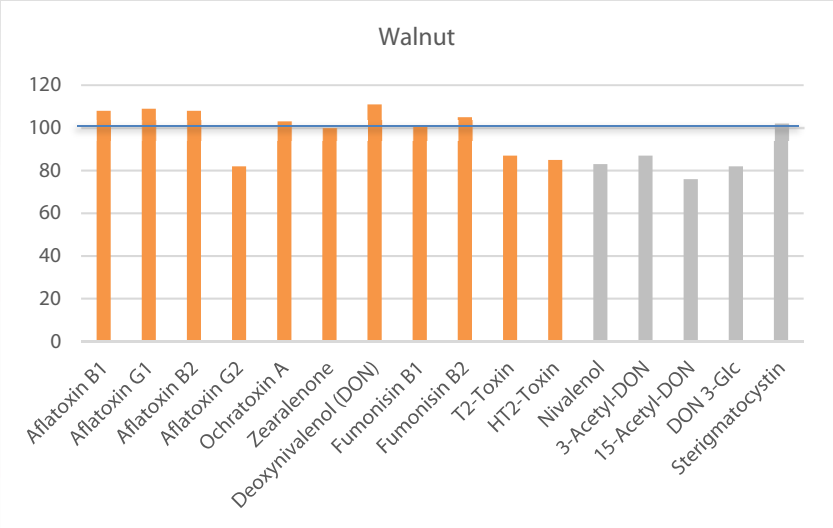



*Figure 9: Recovery rates for Walnut*

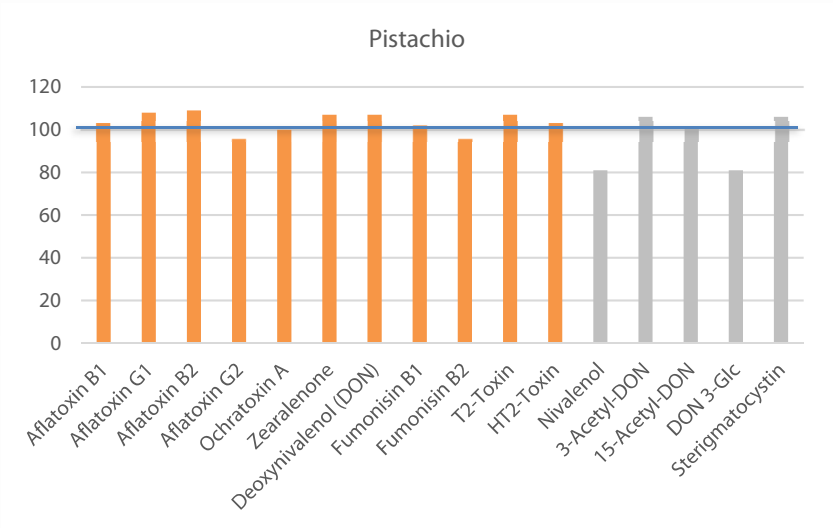



*Figure 10: Recovery rates for Pistachio*

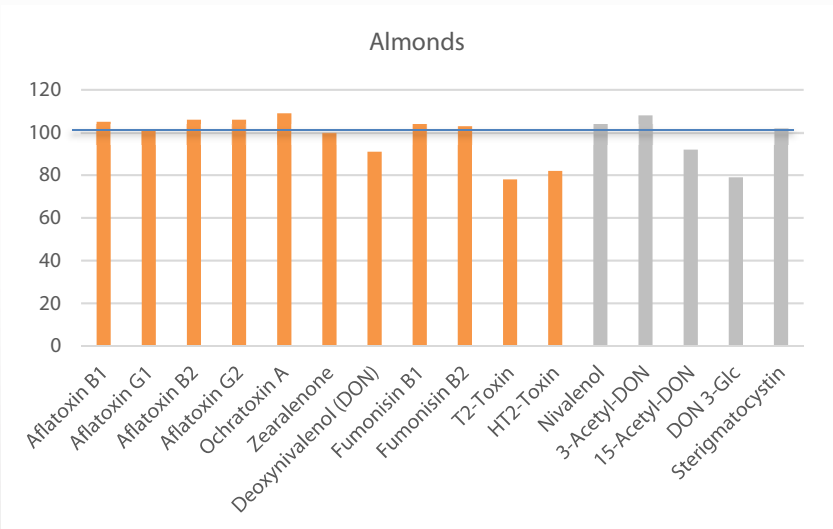



*Figure 11: Recovery rates for Almonds*

## 3.3 Dried Fruit Matrices

*Table 4: Results for dried fruit matrices (the abbreviations used in the table correspond in the order in which the diagrams are labelled)*

| | Afla B1 | Afla G1 | Afla B2 | Afla G2 | OTA | ZEA | DON | F-B1 | F-B2 | T2-Toxin | HT2-Toxin | NIV | 3-Ac-DON | 15-Ac-DON | DON 3-Glc | STC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Raisins** | 106 | 108 | 109 | 108 | 103 | 84 | 93 | 101 | 102 | 84 | 89 | 89 | 105 | 98 | 89 | 102 |

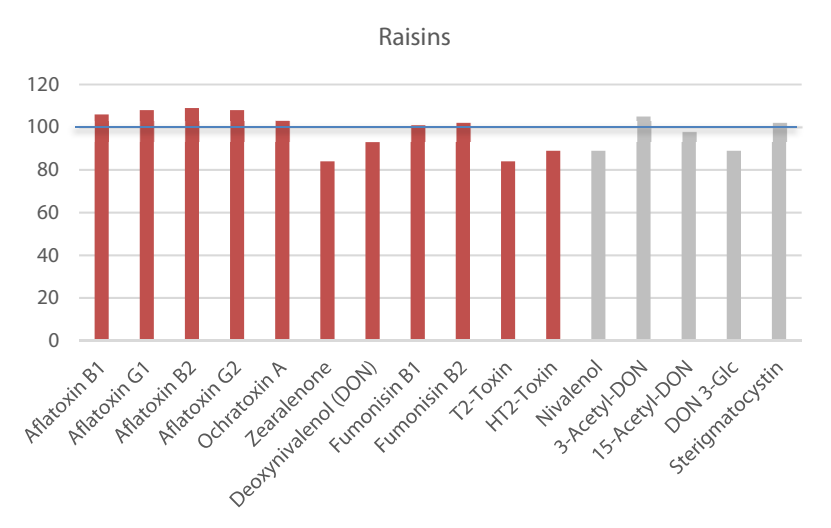



*Figure 12: Recovery rates for Raisins*

## 3.4 Spice Matrices

*Table 5: Results for spice matrices*

| | Aflatoxin B1 | Aflatoxin G1 | Aflatoxin B2 | Aflatoxin G2 | Ochratoxin A |
|---|---|---|---|---|---|
| **Bell Pepper / Chili** | 106 | 96 | 105 | 100 | 93 |
| **Ginger** | 74 | 93 | 106 | 146 | 76 |
| **Nutmeg** | 117 | 118 | 98 | 101 | 76 |
| **Turmeric** | 83 | 73 | 83 | 92 | 88 |

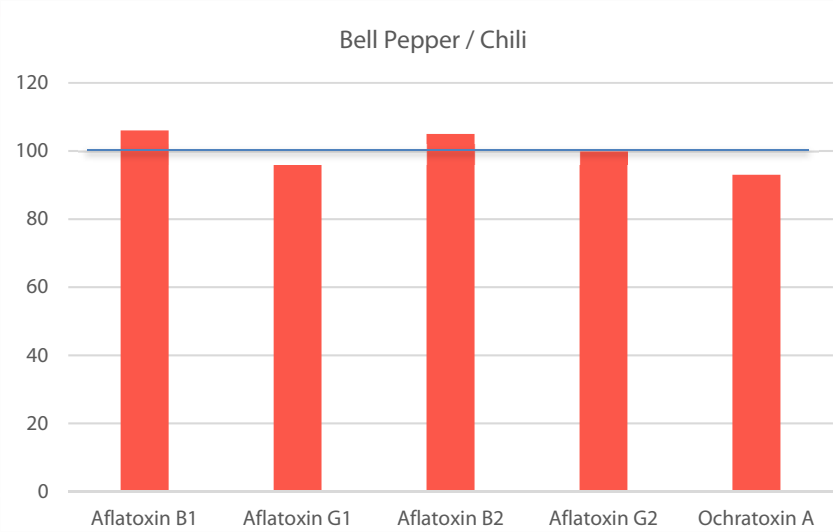



*Figure 13: Recovery rates for Bell Pepper / Chili*

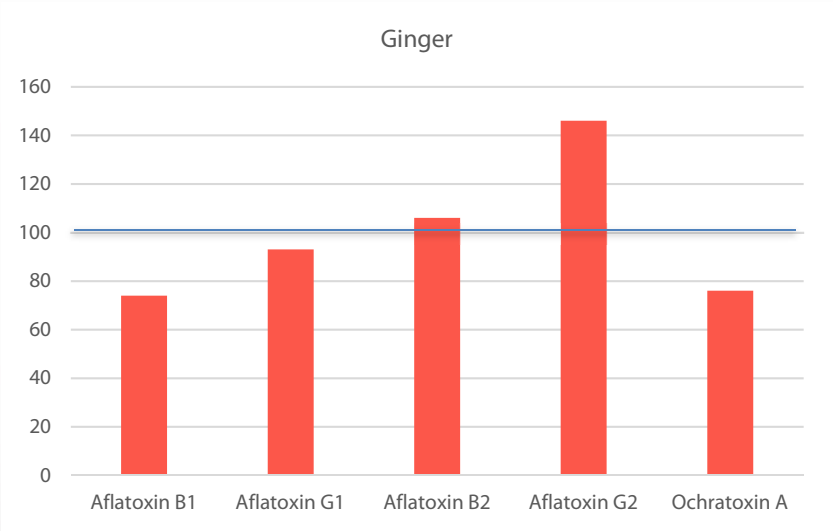



*Figure 14: Recovery rates for Ginger*

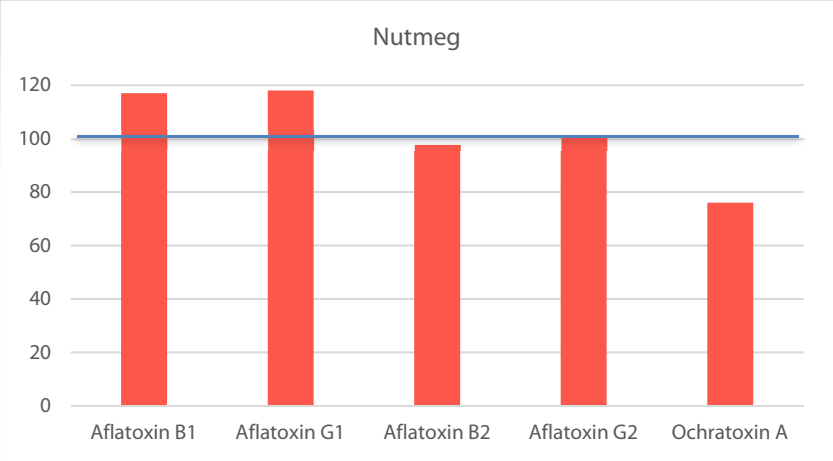



*Figure 15: Recovery rates for Nutmeg*

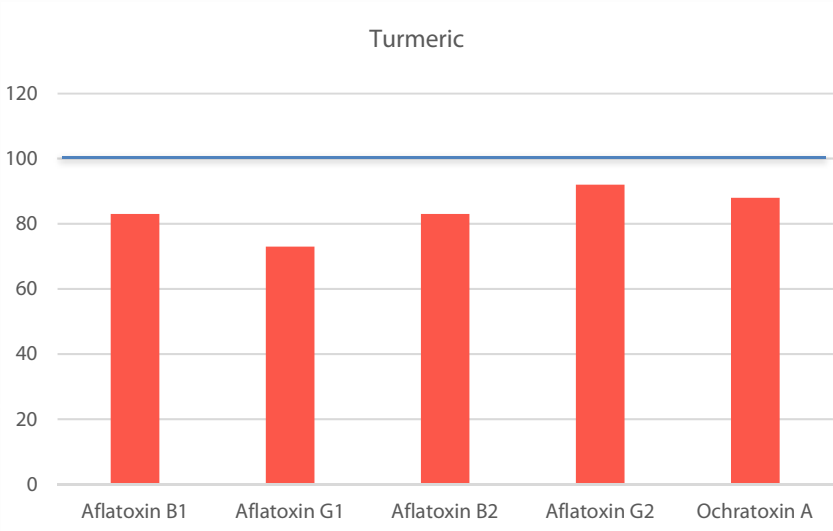



*Figure 16: Recovery rates for Turmeric*

## 3.5 Matrix Removal from Spice Samples – Reduction of Matrix Interferences

The matrix removal was observed for various matrices, matrix interferences and dye could be trapped in CrossTOX® column.

*Figure 17: Chili (highly coloured matrix) - left not cleaned crude extract, right purified with CrossTOX® column*

*Figure 18: Chili (highly coloured matrix) - left unused CrossTOX® column, right used CrossTOX® column to clean-up the extract*

*Figure 19: Pistachio (low coloured matrix) - left not cleaned crude extract, right purified with CrossTOX® column*

*Figure 20: Pistachio (low coloured matrix) - left unused CrossTOX® column, right used CrossTOX® column to clean-up the extract*

Matrix removal may lead to slight destaining of the sample, but the removal of matrix interferences, which inhibit LC-MS/MS analysis, is more dominant. Furthermore, the removal of matrix interferences lead to prolonged lifetime of the separation column and to higher analytical robustness of the LC-MS/MS and saving internal standards for analysis.
The analytes are not retained in the CrossTOX® column, this allows efficient clean-up even with small sample volume.

The analytical interference of dyes in the LC-MS/MS is no indicator for effects on ionisation, because retention time and impact on product ion formation are not correlated with colour in general.

After 150 samples of spices and peanut, the sweep cone was checked and shows almost no matrix remains. This documents in a visible way how the clean-up with CrossTOX® improves the removal of matrix interferences, which in common application physically reduces system consistency.

*Figure 21: Sweep cone after 150 spice and peanut samples, which were cleaned-up by CrossTOX®.*

The standards from the first (prior to matrix analysis) and after matrix analysis (150 samples) showed that in comparison with common application there is no drift, no changes in signal sensitivity, no interferences and no pressure increase in the LC , indicating the purity of the injected samples, and the compatibility of the clean-up with high throughput LC-MS/MS analysis.

## 4. Conclusion

The good recovery allows mycotoxin analysis not only from cereal but also from matrices containing higher fat or sugar content. The high matrix reduction and the robust sample clean-up allows standard reduction and increase in sample throughput for different food and feed mycotoxin analysis. By using the CrossTOX® column for sample preparation the sample number between maintenance intervals of your LC-MS/MS can be prolonged.

*Figure 22: CrossTOX® columns*

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