# Analysis of Mycotoxins Regulated in Europe Using Stable Isotope Labeled Internal Standards and New Triggered MRM Acquisition in LC-MS/MS

Elisabeth Varga[1, 2]; Katharina Mayer[2]; Franz Berthiller[1, 2]; Rainer Schuhmacher[2]; Michael Sulyok[2]; Rudolf Krska[2]; Thomas Glauner[3]

[1]Christian Doppler Laboratory for Mycotoxin Metabolism, Tulln, AUSTRIA; [2]Center for Analytical Chemistry, IFA-Tulln, Tulln, AUSTRIA; [3]Agilent Technologies GmbH, Waldbronn, GERMANY

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## Introduction

Mycotoxins are low molecular weight metabolites of fungi which can cause immunosuppressive, hepatotoxic, mutagenic, carcinogenic, or estrogenic effects if ingested. Grain spoilage by molds occurs on the field or during storage. European Commission Regulation 1881/2006 and its amendments set maximum residue levels (MRLs) for 13 mycotoxins with very low MRLs in various food products. The determination of all regulated mycotoxins in a multi-target method can be achieved by applying liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with positive and negative electrospray ionization (ESI). Due to its selectivity LC-MS/MS offers the best sensitivity even in very complex matrices. However, ESI can be heavily effected by the sample matrix resulting in signal enhancement as well as signal suppression of the target compounds. There are several strategies to overcome matrix effects including sample dilution, matrix matched standards, standard addition, and internal calibration using isotopically labeled compounds.

The goal of this work was the development of a fast and robust analytical method for all mycotoxins currently regulated in Europe in solid food matrices. Dynamic multiple reaction monitoring enabled for fast polarity switching was chosen to allow the monitoring of the most abundant species for each toxin in a single analytical run. For unique identification of the toxins additional confirmatory ions have been triggered and resulting product ion spectra have been compared with a MS/MS library for mycotoxins. A stable isotope dilution assay (SIDA) was chosen since this approach offers the best possible accuracy and sensitivity for a wide variety of matrices. Since the use of $^{13}$C-labeled compounds is considered to be expensive, an estimation of the additional costs has been performed.

## Experimental

### Sample extraction

5 g ground and homogenized sample has been extracted with 20 mL acetonitrile/water/formic acid (80/19.9/0.1, v/v/v) for 60 min on a rotary shaker. After centrifugation (10 min @ 3800 g) the residue is extracted with 20 mL acetonitrile/water/formic acid (20/79.9/0.1, v/v/v) for another 30 min on a rotary shaker. The supernatants of both extraction steps are combined and a mixture of the internal standards is added to a 400 µL aliquot of the extract. After evaporation to dryness the residue is reconstituted in 100 µL acetonitrile/water/formic acid (30/69.9/0.1, v/v/v) and is transferred to a HPLC vial. 5 µL of the extract were injected into the UHPLC-MS/MS system without further clean-up.

## Experimental

ISTDs: [$^{13}C_{17}$]-aflatoxin $B_1$, [$^{13}C_{17}$]-aflatoxin $B_2$, [$^{13}C_{17}$]-aflatoxin $G_1$, [$^{13}C_{17}$]-aflatoxin $G_2$, [$^{13}C_{15}$]-deoxynivalenol, [$^{13}C_{34}$]-fumonisin $B_1$, [$^{13}C_{34}$]-fumonisin $B_2$, [$^{13}C_{22}$]-HT-2 toxin, [$^{13}C_{24}$]-T-2 toxin, [$^{13}C_{20}$]-ochratoxin A, [$^{13}C_{18}$]-zearalenone

Target compounds: aflatoxin $B_1$, aflatoxin $B_2$, aflatoxin $G_1$, aflatoxin $G_2$, deoxynivalenol, fumonisin $B_1$, fumonisin $B_2$, HT2-toxin, T2-toxin, ochratoxin A, zearalenone

Additional compounds: 3-acetyldeoxynivalenol, diacetoxy-scirpenol, fumonisin $B_3$, fusarenone X, nivalenol

All reference compounds and isotopically labeled mycotoxins have been provided by Biopure, Tulln, Austria.

### UHPLC-MS/MS parameters

An Agilent 1290 Infinity UHPLC system consisting of a G4220A binary pump, G4226A high performance sampler, and G1316C thermostated column compartment has been coupled to an Agilent G6460AA QQQ system. Separation has been done on an Agilent Poroshell 120 EC-C18 column (2.1 x 100 mm, 2.7 µm) @ 30°C with (A) Methanol/Water/Acetic acid (10/89/1, v/v/v) and 5 mM ammonium acetate and (B) Methanol/Water/Acetic acid (97/2/1, v/v/v) and 5 mM ammonium acetate as the mobile phase at a flow rate of 0.3 mL/min.

Gradient program: 0.1 min isocratic at 10% B, linear gradient to 60% B in 1.9 min, linear gradient to 100% B in 3 min, 1.4 min isocratic at 100% B, linear gradient to 10% B in 0.1 min. Total run-time 8 min.

MassHunter Workstation B.04.01. acquisition (FW A.00.06.25); acquisition in the triggered MRM mode enabled for fast polarity switching with two primary and up to 7 confirmatory transitions per target compound. One primary transition has been acquired for each ISTD.

## Results and Discussion

### UHPLC separation and triggered MRM

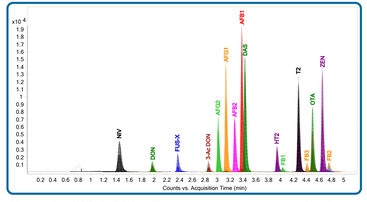


**Figure 1: UHPLC-MS/MS chromatogram of a spiked corn sample (100 ng/mL) acquired with Triggered MRM with fast polarity switching.**

## Results and Discussion

An UHPLC-MS/MS method has been developed for the analysis of 16 mycotoxins including 11 compounds currently regulated in Europe for food products. The resulting chromatogram is shown in figure 1.

The UHPLC separation allows the baseline separation of fumonisin $B_2$ and $B_3$ which is a key requirement since these isomeric compounds share the same MRM transitions and only fumonisin $B_2$ is currently regulated. Another requirement is the baseline separation of aflatoxin $G_1$ and $B_1$ as well as aflatoxin $G_2$ and $B_2$ since the native aflatoxins $G_1$ and $G_2$ produce a signal for the same MRMs as the ISTDs [$^{13}C_{17}$]-aflatoxin $B_1$ and [$^{13}C_{17}$]-aflatoxin $B_2$.

By applying Triggered MRM enabled for fast polarity switching each toxin could be monitored by its most abundant species, i.e. nivalenol, fusarenon X, and zearalenone have been measured in negative ion mode whereas all other toxins have been measured in positive mode.

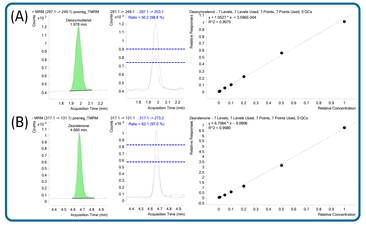


**Figure 2: Chromatograms of a spiked corn extract (5 ng/mL) and calibration curves for deoxynivalenol (A) acquired in positive tMRM mode and zearalenone (B) acquired in negative tMRM mode.**

### Sample preparation and matrix effects

Extraction efficiencies have been evaluated by spiking blank maize samples before extraction with native mycotoxins. The first extraction step with 80% acetonitrile resulted in recoveries of 80 to 110% for all regulated toxins except for $FB_1$ and $FB_2$. This could be improved by a second extraction step with a lower acetonitrile content of just 20% resulting in overall recoveries for $FB_1$ and $FB_2$ of 91 to 112%.

A preliminary validation of the method in maize, which is known for its matrix effects, showed signal suppression for DON, T-2, HT-2, OTA, ZEN, and the aflatoxins between 40 to 80% whereas signal enhancement has been observed for the fumonisins (10 to 30%).

## Results and Discussion

The addition of isotopically labeled standards after the extraction compensated for all matrix effects in the electrospray ionization and allowed the accurate quantitation of the mycotoxins even in complex matrices without extensive sample clean up.

### Preliminary validation for maize

Figure 2 shows chromatograms of a maize extract spiked to a concentration of 5 µg/kg and the calibration curves for deoxynivalenol and zearalenone acquired in triggered MRM mode enabled for fast polarity switching. For all target compounds linear calibration curves have been obtained with a linear range of 3 to 4 orders of magnitude.

**Table 1: Method performance characteristics for maize extracts and regulatory limits for different commodities based on European regulation (EC) 1881/2006.**

| Analytes | Linear range ng/mL | LOQs (maize) µg/kg | MRLs (EC Reg. No 1881/2006) | Commodities |
|---|---|---|---|---|
| Aflatoxin $B_1$ | 0.01-20 | 1.5 | 0.1 | processed cereal-based baby f. |
| | | | 2.0-12 Sum Aflatoxins: 4.0-15.0 | Nuts and cereals |
| Ochratoxin A | 0.5 - 206 | 2.0 | 0.5<br>2.0<br>3.0 / 5.0<br>10.0<br>15 / 20 / 80 | processed cereal-based baby f.<br>wine, grape juice<br>processed / unp. cereals<br>dried vine fruit<br>spices / liquorice root / extract |
| Fumonisin $B_1$ | 1.8-600 | 3.0 | 200<br>800<br>1000 / 4000 | processed maize-based baby f.<br>maize-based breakfast cereals<br>maize / unprocessed maize |
| Fumonisin $B_2$ | 2.0-600 | 4.0 | | |
| Deoxy-nivalenol | 5.0-500 | 30 | 200<br>500 - 1750 | processed cereal-based baby f.<br>processed / unp. cereals, bread, pasta, breakfast cereals |

Limits of quantitation (LOQ) for the UHPLC-MS/MS method have been below the MRLs specified in European regulation (EC) 1881/2006 for processed cereal-based baby food. However, the LOQs for the whole procedure including extraction are affected by the dilution and by the matrix suppression resulting in LOQs for aflatoxin $B_1$ and ochratoxin A in the maize matrix which are above the MRLs for processed cereal-based baby food. Whereas a further dilution of the extract could reduce the matrix effect for the aflatoxins resulting in a lower LOQ for aflatoxin $B_1$, a further dilution is not an option for ochratoxin A.

The additional analysis costs resulting from the addition of eleven $^{13}$C-labeled ISTDs after the extraction for the compensation of matrix effects in ESI account for $ 2.30 per sample.

### Compound confirmation by tMRM and library searching

Triggered MRM is a new acquisition mode which allows for the data dependant acquisition of additional confirmatory ions which can be searched against a user editable library.

## Results and Discussion

The triggered MRM spectra allow for product ion spectra with high sensitivity and very reproducible fragment patterns due to:

- Acquisition of each fragment with optimized collision energy
- Reasonably longer dwell times for each individual fragment

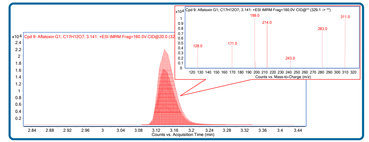


**Figure 3: Primary transitions (integrated), triggered MRM transitions (5 repeats), and resulting MRM spectrum for aflatoxin $G_1$.**

Figure 3 shows the chromatograms and the spectrum for aflatoxin $G_1$ acquired with tMRM. Whereas the two primary transitions have been acquired for the full retention time window, the confirmatory fragments are just triggered for a specified number of repeats. By keeping the cycle time of the MS constant, peak shapes of the primary transitions used for quantitation are not compromised by triggering of additional fragment ions.

Triggered MRM spectra of mycotoxins have been compared at different concentrations in corn extract ranging from 5 to 100 ng/mL. Figure 4 shows the comparison of the in-spectra fragment abundances for ZEN (neg. mode) and OTA (pos. mode) at 4 different concentrations.

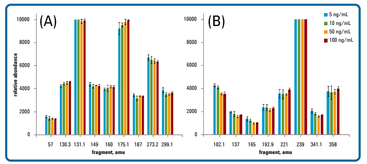


**Figure 4: Comparison of in-spectra fragment abundances for ZEN (A) and OTA(B) for 4 different concentrations spiked in maize extract (n = 4).**

Resulting in-spectra fragment ratios for all mycotoxins have been consistent over the whole concentration range with standard deviations between 0.3 to 12.3% and therefore have been in good agreement with the identification criteria specified in SANCO/10684/2009. Figure 6 shows the user interface of the MassHunter Quantitative software.

## Results and Discussion


**Figure 6: Screenshot of the MassHunter Quantitative software. Peak spectra are compared to library spectra (A) and library match scores are calculated (B).**

NIST-like library search algorithms are used for the calculation of library match scores of the acquired peak spectra. The library match scores are displayed in the spectral comparison as well as in the batch table and can be reported via specific reporting templates. At the 5 ng/mL spiking level the library match scores of all target compounds have been > 85.0 allowing for a fast and reliable unique identification of all mycotoxins.

## Conclusions

A sensitive, rapid and accurate method for the determination of mycotoxins currently regulated in solid food matrices in Europe has been established. The UHPLC separation improved the chromatographic resolution and allowed for short analytical runtimes. Acquisition in triggered MRM with fast polarity switching allowed both, quantitation and identification of all mycotoxins in a single analytical run. The addition of $^{13}$C-labeled ISTDs after extraction compensated for all matrix effects in ESI with reasonable additional costs. The in-spectrum ion ratios of the mycotoxins showed variation of less than 20% and are therefore in good agreement with the identification criteria specified in SANCO/10684/2009. The comparison of the acquired MRM spectra with library spectra via a library match score allowed the identification of mycotoxins in complex matrices with high confidence.

University of Natural Resources and Life Sciences, Vienna
Department for Agrobiotechnology (IFA-Tulln)

BOKU

IFA TULLN