The Quantitation of Mycotoxins in Cereals Using a Simple Sample Extraction and LC-MS/MS with Fast Polarity Switching and the *Scheduled* MRM™ Algorithm

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Overview

A rapid, robust, sensitive and specific LC-MS/MS assay has been developed for the detection of several major classes of known toxic mycotoxins. The method uses a simple solvent extraction followed by a dilution and injection of extracts to achieve detection of mycotoxins below the regulatory requirements. Fast polarity switching and the *Scheduled* MRM™ algorithm were used with the AB SCIEX Triple Quad™ 5500 system to cover all mycotoxins of interest and to detect them with the best sensitivity, accuracy, and reproducibility.

Introduction

Mycotoxins are produced by several strains of fungi both in the field, during storage, mixing and delivery of grain, human and animal food. Mycotoxins are known to be toxic and harm humans and animals as they are carcinogenic or otherwise cytotoxic and impair the immune system. Mycotoxins fall into several major classes and those which can affect the health of humans or animals include the aflatoxins, ochratoxins, Fusarium toxins, including fumonisins, zearalenone (ZON), trichothecenes, and ergot alkaloids.[1]

Regulations for mycotoxin contamination for some of the major classes have been set in different countries. In the European Union the mycotoxin limits were harmonized in the regulation for contaminants in foodstuffs[2,3] and amended by regulations in September 2007.[4] Traditionally mycotoxin analyses have been carried out using multiple methods, each method just suitable for one single mycotoxin or a group of chemically similar compounds e.g. aflatoxins.[5] This has been due to the wide range of polarities and physical properties of these compounds. These single mycotoxin methods include two new analytical methods for measuring aflatoxin B1 (AFB1) and ZON in baby food which were adopted as European benchmark methods in July 2010.[6] Both methods are based on an immuno-affinity column cleanup of the sample followed by HPLC with fluorescence detection. However, it is possible that many different classes of mycotoxins could be present in the same sample of food or feed[7,8] and not just AFB1 and ZON.

In this work we show the ability to analyze AFB1 and ZON at comparable detection levels, to the benchmark methods, as well as implementing these two mycotoxins into an LC-MS/MS screening method. For these measurements the AB SCIEX Triple Quad™ 5500 system was used (Figure 1). In one single LC-MS/MS run of 13 minutes 17 compounds were detected; 12 of them in the positive ionization mode and 5 of them in the negative ionization mode. The crude extracts of different foods were diluted and injected without any extensive sample clean up or concentration steps. Detection limits of AFB1 and ZON were found to be comparable to the required values set by EN standards[6] and reproducibility was found to be better than 20% without the use of any internal standards. The method itself incorporates fast polarity switching using the *Scheduled* MRM™ algorithm, unlike previous work[9], and expands on the previous number of toxins detected.

Experimental

Sample Preparation

A very simplified sample preparation was used[10] similar to one that has been developed by SGS GmbH (Hamburg, Germany).[9] Homogenized sample (2 g) was mixed with acetonitrile/water (8 mL, 80/20) and roller mixed for 20 minutes. The sample was centrifuged for 10 minutes at 3500 rpm and filtered using a Phenomenex PHENEX filter (15 mm RC Membrane 0.45 µm). The filtrate was then diluted 1:4 with water containing 5 mM ammonium acetate prior to injection.

LC

Samples were injected onto a Shimadzu Nexera UHPLC system containing LC-30AD pumps, SIL-30AC autosampler and a CTO-20A column oven. The column used for the separation was a Phenomenex Kinetex 2.6 µm XB-C18 (50x2.1 mm) column and was run at a flow rate of 450 µL/min and at a temperature of 40°C using a gradient of water to methanol with both phases containing 5 mM ammonium acetate and 0.5% acetic acid (see Table 1 for the gradient profile). An injection volume of 30 µL was used.

Table 1. Gradient profile for mycotoxin analysis

Time (min)	Flow (µL/min)	A (%)	B (%)
0	450	98	2
2	450	98	2
5	450	20	80
5.2	450	2	98
8	450	2	98

MS/MS

An AB SCIEX Triple Quad™ 5500 LC/MS/MS System equipped with the Turbo V™ source and Electrospray Ionization (ESI) probe was used for MS/MS detection using the selective and sensitive Multiple Reaction Monitoring (MRM) mode.

Table 2 shows the MRM transitions corresponding to the analyzed compounds in a negative-positive switching method using the *Scheduled* MRM™ algorithm.

The following MS/MS parameters were kept constant during the whole acquisition: TEM: 550ºC; CUR: 25 psi; Gas 1: 60 psi; Gas 2: 70 psi; CAD: medium; IS (negative polarity): -4000V; IS (positive polarity): +5000V.

Figure 1. The AB SCIEX Triple Quad™ 5500 LC/MS/MS System

Data was acquired and processed using Analyst® software version 1.6 and MultiQuant™ software version 2.1.

Results and Discussion

With this study we wanted to investigate the possibility to analyze AFB1 and ZON at the defined baby food levels without sample concentration and implemented this into an LC-MS/MS screening method.

The studied mycotoxins are listed in Table 2. An example chromatogram with all mycotoxins is shown in Figure 2.

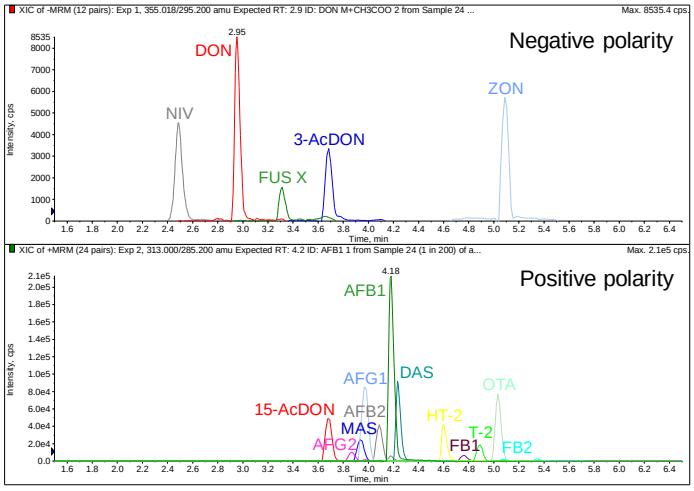

Figure 2. TIC of all mycotoxins analyzed in a single method with negative and positive polarity switching and the *Scheduled* MRM™ algorithm

Table 2. MRM transitions with their retention times (RT) used to detect target mycotoxins using a single method with negative and positive polarity switching

Mycotoxin	RT (min)	Polarity	Ion	MRM (quantifier)	MRM (qualifier)
15-Acetyldeoxynivalenol (15-AcDON)	3.7	positive	$[M+H]^+$	339/321	339/137
3-Acetyldeoxynivalenol (3-AcDON)	3.7	negative	$[M+CH3COO]^-$	397/307	397/59
		negative	$[M-H]^-$		337/307
Aflatoxin B1 (AFB1)	4.2	positive	$[M+H]^+$	313/285	313/128
Aflatoxin B2 (AFB2)	4.1	positive	$[M+H]^+$	315/287	315/259
Aflatoxin G1 (AFG1)	4	positive	$[M+H]^+$	329/243	329/200
Aflatoxin G2 (AFG2)	3.9	positive	$[M+H]^+$	331/313	331/245
Deoxynivalenol (DON)	3	negative	$[M+CH_3COO]^-$	355/295	355/59
Diacetoxyscirpenol (DAS)	4.2	positive	$[M+H]^+$	384/307	384/247
Fumonisin B1 (FB1)	4.8	positive	$[M+H]^+$	722/334	722/352
Fumonisin B2 (FB2)	5.1	positive	$[M+H]^+$	706/336	706/318
Fusarenon X (FUS X)	3.3	negative	$[M+CH_3COO]^-$	413/353	413/59
HT-2 toxin	4.6	positive	$[M+NH_4]^+$	442/263	442/105
Monoacetoxyscirpenol (MAS)	3.9	positive	$[M+H]^+$	342/265	342/307
Nivalenol (NIV)	2.5	negative	$[M+CH_3COO]^-$	371/281	371/59
Ochratoxin A (OTA)	5	positive	$[M+H]^+$	404/239	404/102
T-2 toxin	4.9	positive	$[M+NH_4]^+$	484/215	484/185
Zearalenon (ZON)	5.1	negative	$[M-H]^-$	317/131	317/175

To test the new method several products including baby cereals, adult cereals and a beer were prepared according to the documented sample preparation and standards were spiked into them. Internal standards have not yet been used.

In previous work we used chromatographic separation and a fixed switch between the negative and positive polarity experiments. Here we applied a single looped experiment containing both negative and positive polarity and combined it with the *Scheduled* MRM™ algorithm (Figure 2).

To test the effect of the *Scheduled* MRM™ algorithm two experiments were compared: one using polarity switching with traditional MRM mode and a second with polarity switching and *Scheduled* MRM™.

The use of *Scheduled* MRM™ not only increased the number of data points across the peak but also the signal-to-noise (S/N) observed (Figure 3). This increase in sensitivity was affected by retention time but was shown to be at least a factor of 3 fold for all mycotoxins. The scheduling of MRM transitions also allowed the addition of more mycotoxins to the method, so that a total of 17 mycotoxins were now detected in comparison to 10 in the previous method.[9]

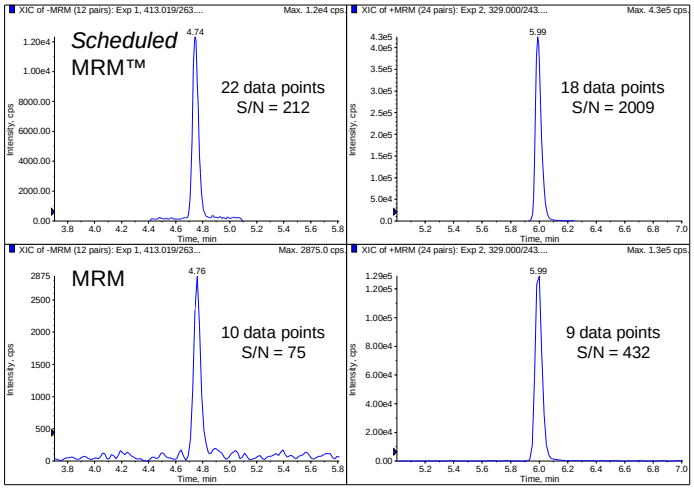

Figure 3. Comparison of a positive and negative switching experiment with and without *Scheduled* MRM™, FUS X in negative polarity (left) and AFG1 in positive polarity (right)

This method produced a linear regression coefficient greater than 0.99 for ZON (Figure 4). It was found to be reproducible with a %CV of 9.2 (Table 3), robust, and reliable for ZON with no major matrix effects like signal suppression or shift in retention times observed.

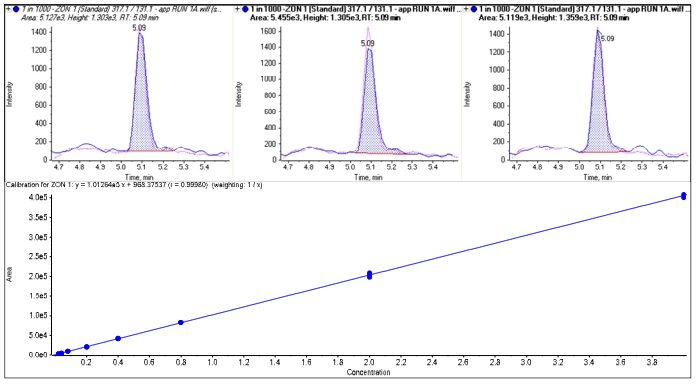

Figure 4. Triplicate injections of ZON at 0.04 ng/mL (top) and calibration line from 0.02 to 4 ng/mL with r = 0.9998 (bottom)

Diluted spiked matrix standards at several levels including 4 µg/kg (Figure 5) were injected three times and ZON was detected easily at these levels well below the EU legislation.

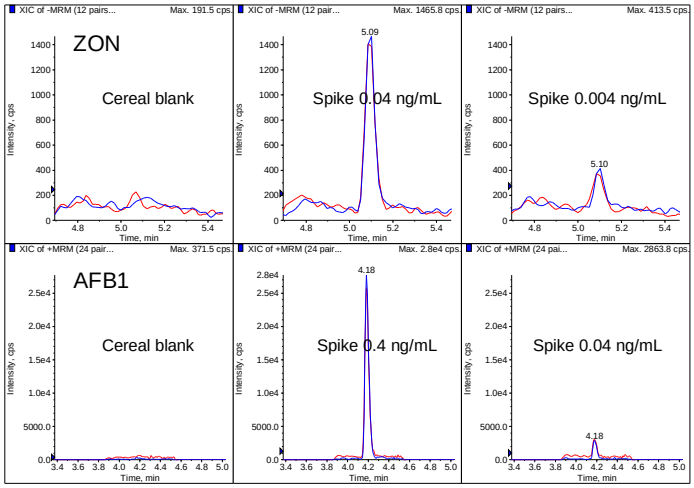

Figure 5. Chromatograms of ZON and AFB1 spiked into baby cereal compared with a matrix blank. The samples had been diluted 20 times.

Similarly AFB1 can be detected below the EU legislation. Figure 5 shows a spike into baby cereal of 0.040 ng/mL, which gave a S/N of 81. This clearly illustrated that AFB1 could be detected in baby food below the EU legislation limit of detection of 0.010 ng/mL (limit of detection of a method is normally defined as a signal to noise of 3 to 1 for the required analyte).

For AFB1 the method was found to be reproducible with a %CV of 4.2, robust and the linear regression coefficient was found to be greater than 0.99 (Figure 6).

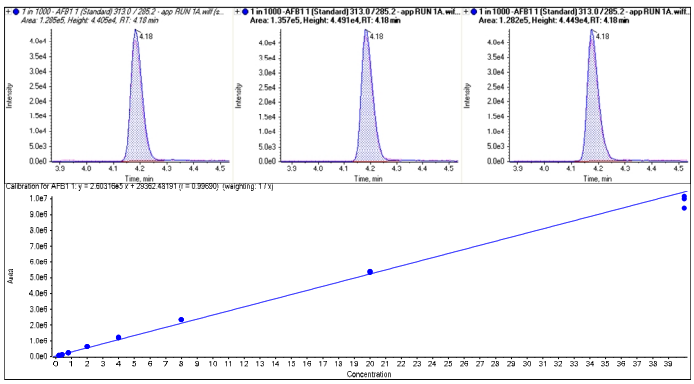

Figure 6 Triplicate injections of AFB1 at 0.4 ng/mL (top) and calibration line for ZON from 0.2 to 40 ng/mL with r = 0.9969 (bottom)

Table 3 shows the results for all the mycotoxins which all show good sensitivity, linearity and robustness.

Summary

The presented method has been tested on several cereal based samples and has been shown to be robust enough to detect these toxins below the required limits and met European Legislation.

The simple solvent extraction followed by dilution and the use of small particle size LC columns has meant that the method is fast and simple to apply. The use of polarity switching with *Scheduled* MRM™ acquisition has enabled shorter run times with an improvement in sensitivity, while extending the target list of compounds.

Table 3. Results from the multiple injections of standards for the 17 mycotoxins

Mycotoxin	Calibration range (ng/mL)	Linearity (regression coefficient)	Standard concentration (ng/mL)	S/N*	%CV
15-Acetyldeoxynivalenol (15-AcDON)	2 - 400	0.998	4	81	10.2
3-Acetyldeoxynivalenol (3-AcDON)	2 - 400	0.999	4	81	17
Aflatoxin B1 (AFB1)	0.2 - 40	0.997	0.4	621	4.2
Aflatoxin B2 (AFB2)	0.05 - 10	0.999	0.1	274.2	12.2
Aflatoxin G1 (AFG1)	0.2 - 40	0.998	0.4	573	8.4
Aflatoxin G2 (AFG2)	0.05 - 10	0.998	0.1	69	17
Deoxynivalenol (DON)	2 - 400	0.999	4	342	4.7
Diacetoxyscirpenol (DAS)	0.121 - 24.3	0.999	0.12	230	5.1
Fumonisin B1 (FB1)	0.2 - 40	0.993	0.4	36	15.1
Fumonisin B2 (FB2)	0.8 - 40	0.991	0.8	52	5.5
Fusarenon X (FUS X)	0.5 - 102	0.999	1.02	32	14.6
HT-2 toxin	0.4 - 80	0.999	0.8	148	5
Monoacetoxyscirpenol (MAS)	0.121 - 24.3	0.998	0.24	22	11.8
Nivalenol (NIV)	2 - 400	0.999	4	75	11.6
Ochratoxin A (OTA)	0.121 - 24.3	0.997	0.24	435	4.4
T-2 toxin	0.08 - 16	0.999	0.16	94	13.4
Zearalenon (ZON)	0.02 - 4	1.000	0.04	60	9.2

* Data calculated using signal / 1 x std dev noise

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