APPLICATION NOTE

Liquid Chromatography / Mass Spectrometry

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Analysis of Mycotoxins in Multi-Grain and Corn Cereals without Derivatization by LC-MS/MS Using Time-Managed MRMs

Introduction

Mycotoxins in feed, crops and various foods are known to pose a serious health hazard to both livestock and humans. They are produced by fungi as toxic secondary metabolites, with grains, maize and cereals being particularly vulnerable. With this in mind, and considering that an estimated 25% of all crops show some signs of mycotoxin contamination, many countries have established regulatory guidelines for maximum mycotoxin limits in not only feed and grain, but also in processed food products, notably cereal and baby food.

The current global regulatory limits for mycotoxins in processed cereals are shown in Table 1. The most demanding of these are for mycotoxin B1 (2 µg/kg maximum in EU) and ochratoxin A (3 µg/kg maximum in EU and Singapore).

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Table 1. Global maximum regulatory limits (µg/kg; ppb) for mycotoxins in processed cereal products intended for human consumption.[1]

Mycotoxin	EU	USA	China	Singapore	Brazil
Sum of B1, B2, G1 and G2	4	20	NA	5	5
B1 Only	2	NA	5* 20**	5	NA
Sum of T-2 and HT-2	75***	NA	NA	NA	NA
Sum of F-B1 and F-B2	800	NA	NA	NA	400
Ochratoxin A	3	NA	5*	3	10
Ergot Alkaloids	NA				

*Grain products **Corn/peanut products NA = none available at this time
***Indicative level (regulatory level under discussion)

Thereupon, the presented work details an effective, reliable and robust LC-MS/MS method, using time-managed MRM (Multiple Reaction Monitoring) transitions, for the monitoring of mycotoxins in multi-grain and corn cereals at low µg/kg levels, without the need for derivatization.

The analyzed mycotoxins included aflatoxins B1, B2, G1, G2, ochratoxin A, HT-2 and T-2 toxins, ergocristine and fumonisins F-B1 and F-B2.

Experimental

Hardware/Software

For the chromatographic separations, a PerkinElmer UHPLC System was used with a PerkinElmer QSight® 210 MS/MS detector. All instrument control, analysis and data processing was performed using the Simplicity 3Q™ software platform.

Method Parameters

The LC and MS/MS method parameters are shown in Tables 2 and 3, respectively.

Table 2. LC Method Parameters.

Column	PerkinElmer Analytical C18, 3 µm, 4.6 x 100 mm (Part# N9303863)
Mobile Phase	Solvent A: Water; 5 mM NH_4-formate and 0.1% formic acid Solvent B: 90:10 methanol/water; 5 mM NH_4-formate and 0.1% formic acid

	Time (min)	Flow Rate (mL/min)	%A	%B	Curve
1	Initial	1.00	45.00	55.00	
2	3.50	1.00	45.00	55.00	6
3	3.75	1.00	10.00	90.00	6
4	7.00	1.00	10.00	90.00	6
5	7.10	1.00	45.00	55.00	6

Analysis Time	7 min; re-equilibration time: 4 min
Pressure	6200 psi/413 bar (maximum)
Oven Temp.	35 °C
Injection Volume	50 µL

Table 3. MS/MS Method Parameters.

Ionization Mode	ESI (positive)
Drying gas (nitrogen): 120 (arbitrary units); HSID™ Temp: 320 °C; Electrospray V1: 4500 V; EV(V): 30	

Exp er. Group 1 (1.00 - 2.10 min)	MRM Transitions (amu)				
	Quantifier Ion	Qualifier Ion	CCL2(V)	CE(V)	Dwell Time (msec)
Aflatoxin G2	331.1/245.2	331.1/285.1	-100	-35	100
Exp er. Group 2 (1.90 - 3.50 min)	**MRM Transitions (amu)**				
	Quantifier Ion	**Qualifier Ion**	**CCL2(V)**	**CE(V)**	**Dwell Time (msec)**
Aflatoxin G1	329.0/243.2	329.0/283.2	-120	-30	100
Aflatoxin B2	315.1/259.2	315.1/287.2	-120	-32	100
Aflatoxin B1	313.3/285.2	313.3/241.3	-110	-30	100
Exp er. Group 3 (4.90 - 5.62 min)	**MRM Transitions (amu)**				
	Quantifier Ion	**Qualifier Ion**	**CCL2(V)**	**CE(V)**	**Dwell Time (msec)**
HT-2 Toxin	447.3/285.3	447.3/345.4	-120	-23	100
Fumonisin F-B1	722.8/352.4	722.8/334.4	-120	-47	100
Exp er. Group 4 (5.22 - 5.85 min)	**MRM Transitions (amu)**				
	Quantifier Ion	**Qualifier Ion**	**CCL2(V)**	**CE(V)**	**Dwell Time (msec)**
Ergocristine	610.6/223.3	610.6/592.6	-120	-23	50
T-2 Toxin	489.2/245.1	489.2/387.1	-115	-30	50
Ochratoxin A	404.2/239.1	404.2/358.2	-85	-25	50
Exp er. Group 5 (5.55 - 6.10 min)	**MRM Transitions (amu)**				
	Quantifier Ion	**Qualifier Ion**	**CCL2(V)**	**CE(V)**	**Dwell Time (msec)**
Fumonisin F-B2	706.8/336.5	706.8/354.3	-120	-30	50

HSID™ = Hot-surface induced desolvation

EV(V) = Entrance voltage; CCL2(V) = Collision cell lens 2; CE(V) = Collision cell energy

Standards and Sample Preparation

Standards were obtained from Sigma-Aldrich. A working standard (WS) was prepared in 80/20 ACN/water, used for both the initial calibration and as the spiked extraction solvent.

For the initial calibration (in straight solvent), a 5-level calibration set was prepared by diluting the WS 1:1 with water and then serially diluting with 40/60 ACN/water. All calibrants were run in triplicate.

Samples of multi-grain cereal (MGC) and corn cereal (CC) obtained from a local food market were analyzed for mycotoxins. 5 grams of each sample was homogenized for 45 seconds, each sample being prepared in duplicate. The homogenized samples were extracted with either 20 mL of 80/20 ACN/water (as a control) or with 20 mL of WS (serving as the spiked extraction solvent).

For extraction, all mixtures were first vortexed for 25 minutes, with intermittent hand shaking, followed by centrifugation at 3500 rpm for 10 minutes. For the calibration plots of the spiked matrix extracts, each supernatant was diluted 1:1 with water and then serially diluted with 40/60 ACN/water. For recovery testing, 5 grams of homogenized samples were spiked with 5 mL of 40/60 ACN/water containing 5 ppb aflatoxin B1 and ochratoxin A, both having the lowest regulatory limits (2 and 3 µg/kg, respectively). 5 mL of each supernatant was diluted 1:1 with water. For all extractions, the overall sample dilution was 8-fold.

All prepared calibrants and samples were filtered using 0.22-µm filters and then injected (50 µL) in triplicate on column.

Results and Discussion

Figure 1 shows the chromatography and the resulting chromatographic repeatability for 10 replicate injections.

For the calibration plots, developed in either straight solvent (40/60 ACN/water) or as matrix-matched extracts, the R^2 values for all analytes were > 0.995. A representative plot for aflatoxin B1 prepared in solvent is shown in Figure 2, while Figure 3 shows the corresponding plots for aflatoxin B1 prepared in matrix.

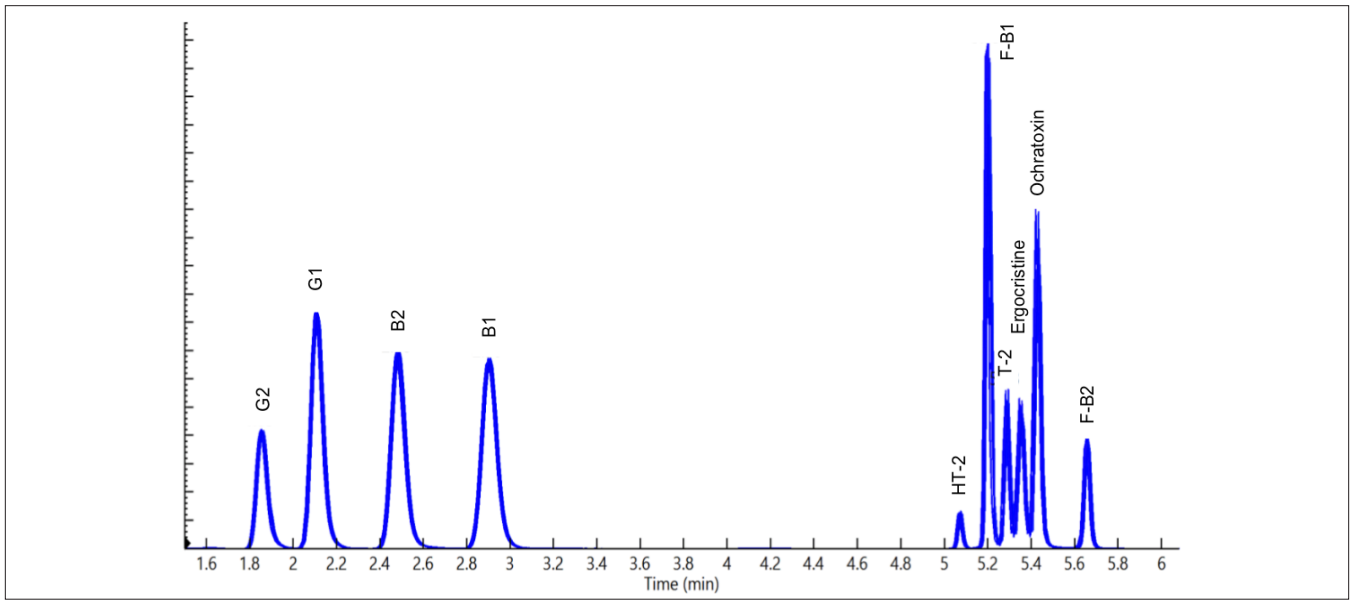

Figure 1. 10-replicate overlay of MRM quantifier transitions for all analytes.

To check for possible ion-suppression, calibration plots prepared in straight solvent were compared with the matrix-matched standards. Comparing the upper level (L5) values in Figures 2 and 3 shows an example of this for aflatoxin B1. Overall, ion suppression of 20-25% was observed from matrix effects.

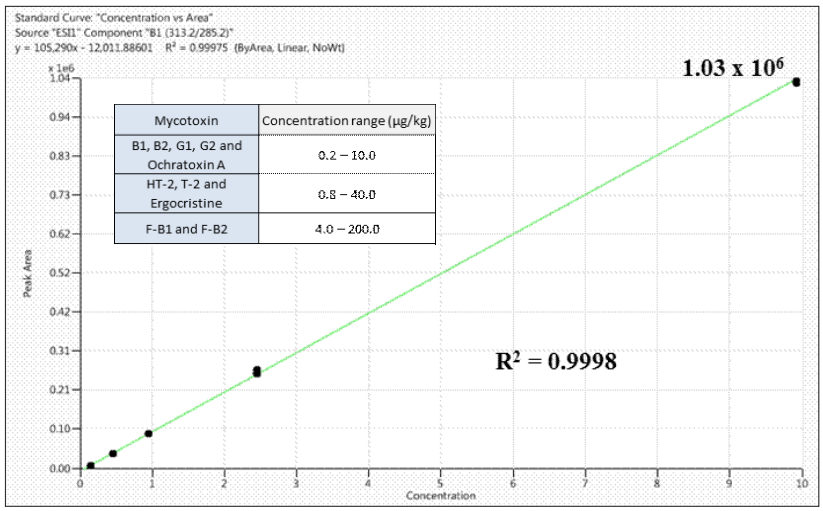

Figure 2. Calibration plot for B1 in 80/20 ACN/water. The B1 concentration range and that of all other analytes are provided in the inserted table.

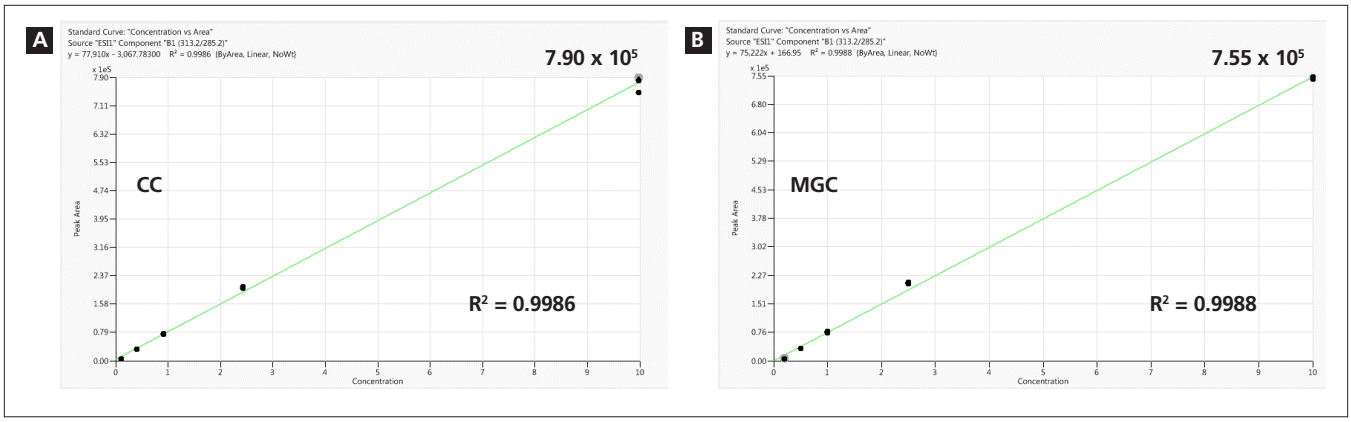

Figure 3. Calibration plots for B1 from supernatant of spiked extracts; A: CC; B: MGC.

However, the extent of ion suppression was considered inconsequential, since, per LOQ, the EU's 2-µg/kg regulatory limit for B1 was easily met. The calculated LOQ for B1, as well as those for all the other analyzed mycotoxins, are shown in Figure 4.

A 80/20 ACN/water blank injection showed no detectable mycotoxins. However, the control, obtained from injecting the supernatant from the unspiked extractions, showed quantitatable T-2 amounts for both MGC and CC (7.92 µg/kg and 13.8 µg/kg, respectively). This is chromatographically highlighted in Figure 4, showing quantitatable amounts of T-2 in the CC sample and confirmed via the qualifier MRM. However, the resulting T-2 amounts for both CC and MGC were well below the EU's regulated limit of 75 µg/kg for the sum of HT-2 and T-2.

For additional analyte ID confirmation, the qualifier/quantifier ion ratios for the lowest level CC supernatant calibration results are shown in Table 4. The asterisked analytes are the qualifiers. All analyte identities were positively confirmed.

The aflatoxin B1 and ochratoxin A recovery results for the 5-µg/kg spiked MGC homogenate are shown in Figure 5. The recoveries for both analytes were quite close to that expected. The bit higher ochratoxin A recovery was interesting but not further investigated.

Table 4. Calculated S/N and LOQ values for the eight analyzed mycotoxins.

Mycotoxin	S/N of L1 calibrant*	LOQs (µg/kg) (per L1 calibrant)**	Sample LOQs (µg/kg)*** (Considering 8-fold dilution)
G2	73	0.03	0.24
G1	122	0.02	0.16
B2	91	0.02	0.16
B1	83	0.02	0.16
HT-2	27	0.30	2.40
F-B1	44	0.91	7.28
Ergocristine	40	0.20	1.60
Ochratoxin A	75	0.03	0.24
T-2	85	0.09	0.72
F-B2	19	2.11	16.84

* Lowest replicate-averaged S/N of CC and MGC L1 calibrants via spiked extraction
** As per lowest replicate-averaged S/N
*** Calculated based on LOQs of L1 calibrants

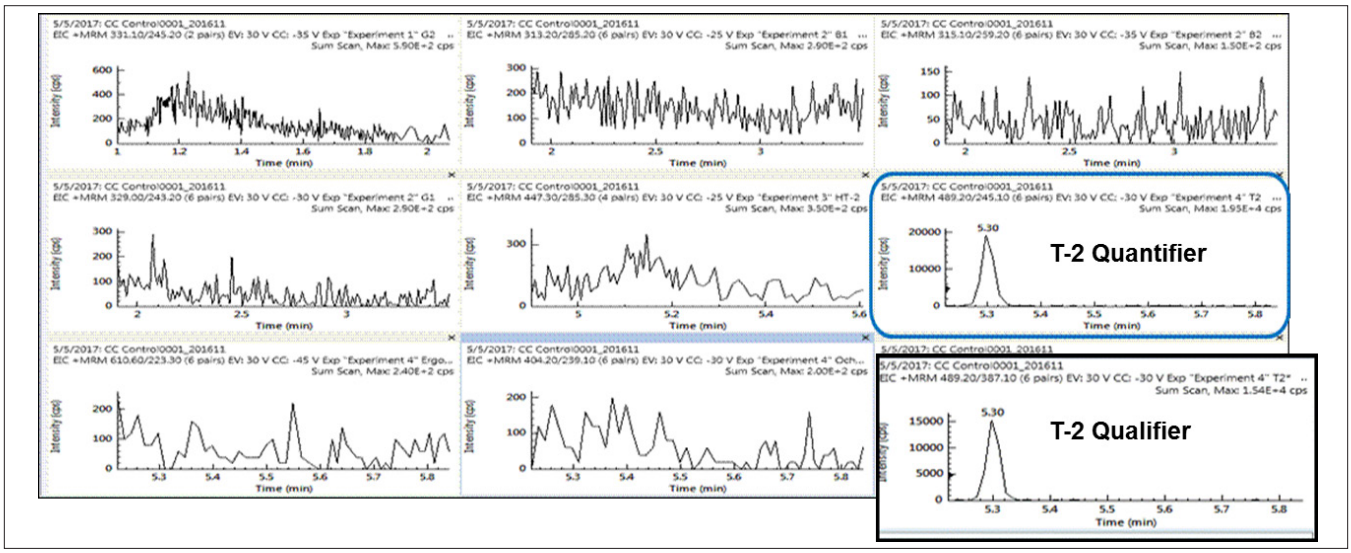

Figure 4. Quantifier MRM chromatograms of CC control, highlighting T-2 (blue); the insert at lower right shows the corresponding T-2 qualifier.

Table 5. Ion ratios for all ten analyzed mycotoxins. The green background indicates a positive match to within 20 % of ion ratio of the lowest calibration standard.

Analyte Component	Group	Mass Transition Q1/Q2	Ion Ratio Area
G2	10	331.1/245.2	-
G2*	10	331.1/285.1	0.67
B1	20	313.2/285.2	-
B1*	20	313.2/241.3	0.91
B2	30	315.1/259.2	-
B2**	30	315.1/287.2	0.92
G1	40	329/243.2	-
G1*	40	329/283.2	0.44
HT-2	50	447.3/285.3	-
HT-2*	50	447.3/345.4	0.92
F-B1	60	722.8/352.4	-
F-B1*	60	722.8/334.4	1.05
ERGOCRISTINE	70	610.6/223.3	-
ERGOCRISTINE*	70	610.6/592.6	0.43
OCHRATOXIN-A	80	404.2/239.1	-
OCHRATOXIN-A*	80	404.2/358.2	0.43
T2	90	489.2/245.1	-
T2*	90	489.2/387.1	0.80
F-B2	100	706.8/336.5	-
F-B2*	100	706.8/354.3	0.80

Mycotoxin B1 and ochratoxin A were chosen as examples herein, as they have the lowest regulatory limits of the analyzed mycotoxins.

Conclusions

- Repeatable chromatography was achieved in under seven minutes with all eight mycotoxins resolved.
- The LOQs were below the established regulatory limits in processed cereal for all analyzed mycotoxins, achieved by LC-MS/MS with time-managed MRMs, without the need for pre- or post-column derivatization.

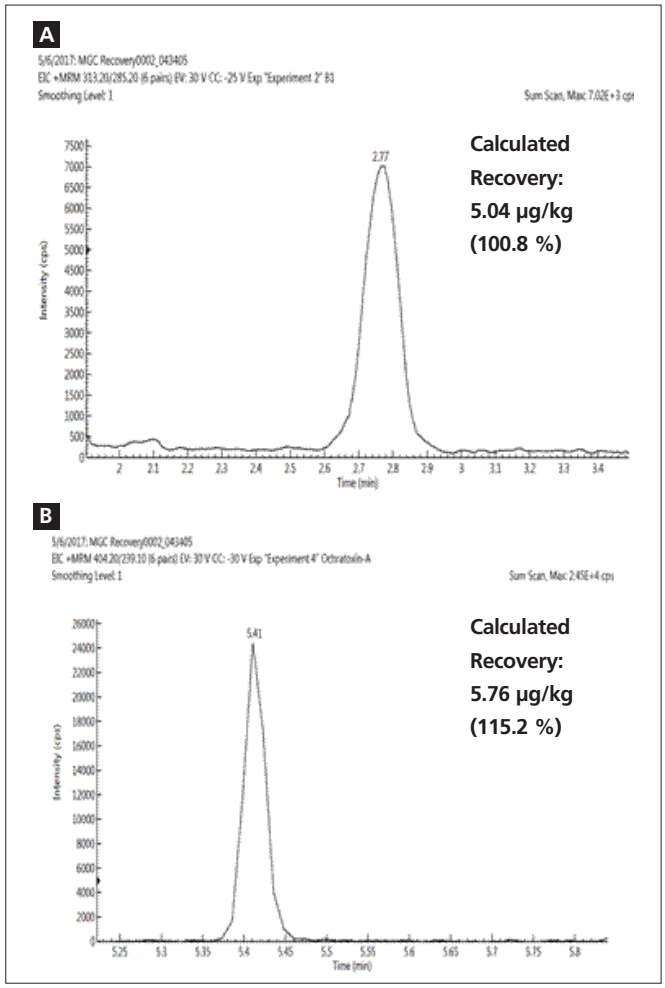

Figure 5. Chromatographic and quantitative recovery results for A) B1 and B) ochratoxin A, both spiked to 5 µg/kg in MGC.

- There was minimal ion suppression in the supernatant from the spiked extractions, allowing for simple, convenient sample preparation by liquid extraction.
- For analyte confirmation, product ion ratios could be used well below the regulatory concentration limits for all the analytes in this study.
- Though quantitatable levels of T-2 were found in both cereals, they were considerably below regulatory limits.

References

1. www.mycotoxins.info/myco_info/consum_regu.html.

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