SHIMADZU

High Speed Analysis of Mycotoxins

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Introduction

Mycotoxins are low-molecular-weight natural products produced by fungi and are capable of causing disease and death. Mycotoxins are typically found in grain products and their occurrence is increased under certain heat and humidity conditions. Regulatory levels have been set for mycotoxin levels, and are dependent on the end use of the food products. Mycotoxin levels can vary widely within the same crop, creating a potential need for increased testing to provide more accurate mycotoxin levels. A high-speed UHPLC method was developed that could provide separation of eight common mycotoxins in less than two minutes to allow for increased testing. Details of the method will be presented.

Mycotoxins Tested

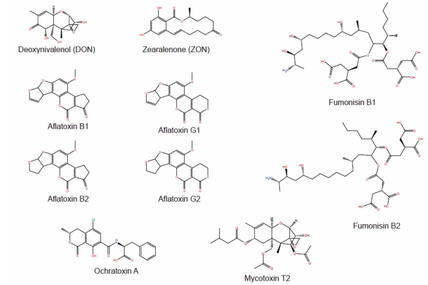
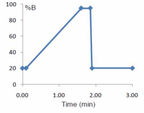


Figure 1: Structures of Selected Mycotoxins

Method Conditions

Method: Reference standards were obtained for each mycotoxin. Each was diluted in an 80/20 mixture of 5 mM ammonium formate/methanol at the concentrations listed in Table 1. A Shimadzu Shim-pack XR-ODS III UHPLC column (2 x 50 mm, 1.6 µm) was used at a flow rate of 0.4mL/min and a temperature of 50 °C.

A binary gradient of 5 mM ammonium formate and methanol was used and is shown in Figure 3. Electrospray ionization was used with polarity switching. The MRM transitions and MS parameters for each compound were optimzed and are listed in Table 2.



Time	%B
0.00	20
0.10	20
1.60	95
1.85	95
1.90	20
3.00	20

Figure 3: Binary Gradient Program

Mycotoxin Std Concentrations

	L2	L3	L4	L5	L6
Aflatoxin B1	67.2	22.4	7.5	2.5	0.8
Aflatoxin B2	20.3	6.8	2.3	0.8	0.3
Aflatoxin G1	67.2	22.4	7.5	2.5	0.8
Aflatoxin G2	20.3	6.8	2.3	0.8	0.3
Fumonisin B1	833.3	277.8	92.6	30.9	10.3
Fumonisin B2	833.3	277.8	92.6	30.9	10.3
Mycotoxin T2	1666.7	555.6	185.2	61.7	20.6
DON	8100.0	2700.0	900.0	300.0	100.0
Ochratoxin	81.0	27.0	9.0	3.0	1.0
ZON	2025.0	675.0	225.0	75.0	25.0

Table 1: Concentrations of Standards (ppb)

MRM Parameters

Type	Event#	+/-	Compound Name (m/z)	Dwell Time (msec)	Q1 Pre Bias(V)	CE	Q3 Pre Bias(V)	Measurement Time		
MRM	1	+	Aflatoxin B1 313>241	25	-25	-39	-25	1.25–1.75		
MRM	2	+	Aflatoxin B2 315>259	25	-25	-33	-25	1.25–1.75		
MRM	3	+	Aflatoxin G1 329>243	25	-25	-31	-25	1.25–1.75		
MRM	4	+	Aflatoxin G2 331>245	25	-25	-33	-25	1.25–1.75		
MRM	5	+	Fumonisin B1 722.4>334.4	25	-20	-40	-15		1.6–3.0	
MRM	6	+	Fumonisin B2 706.4>336.2	25	-20	-40	-24		1.75–3.0	
MRM	7	+	Mycotoxin T2 484.3>305.1	25	-24	-16	-21		1.75–3.0	
MRM	8	-	DON 295>138	25	15	19	30	0–1.0		
MRM	9	-	Ochratoxin 402.1>167	25	15	40	30		1.75–3.0	
MRM	10	-	ZON 317>131	25	15	30	25		1.75–3.0	

Table 2: MRM Parameters

Mycotoxin Standards

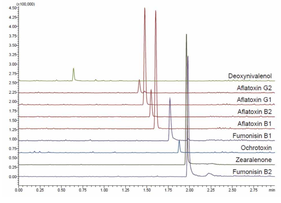
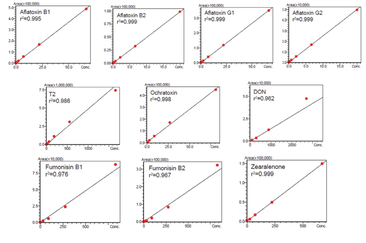


Figure 2: Chromatogram of Mycotoxins Standards

Calibration Curves



Accuracy

	L2	L3	L4	L5	L6
Aflatoxin B1	99.48	100.52	99.72	100.40	99.90
Aflatoxin B2	100.86	101.20	102.90	93.68	102.72
Aflatoxin G1	97.94	100.54	101.70	100.26	99.72
Aflatoxin G2	101.58	100.88	103.90	91.36	104.60
DON	137.40	114.40	93.42	81.68	107.58
Ochratoxin	102.58	96.40	94.30	113.40	97.10
ZON	84.30	101.86	123.68	132.42	82.62

Table 3: Percent Accuracy of Mycotoxin Standards in Solution (n=5)

Reference Samples

Reference samples of feed corn and wheat with independently determined levels of various mycotoxins were prepared by solid phase extraction and analyzed by LC-MS-MS. Alternatively, oatmeal spiked with mycotoxins was prepared by reconstitution and dilution before analysis.

Mass spectra were collected at 15,000 u/sec in positive and negative mode continuously throughout the run. Data-dependent MS-MS was triggered based on these survey scans at a product ion scan speed which was also set at 15,000 u/sec.

Sample Agreement

Aflatoxin B1	Measured (ppb)	Expected (ppb)
Corn	145.0	135.0
	5.7	4.2
	24.4	13.8
	27.7	18.8
Oatmeal	26.7	26.0
	11.9	15.0

Aflatoxin B2	Measured (ppb)	Expected (ppb)
Corn	14.4	5.2
	1.8	0.8
	1.3	0.3
Oatmeal	7.6	7.8
	3.4	4.5

Aflatoxin G1	Measured (ppb)	Expected (ppb)
Corn	4.6	2.5
Oatmeal	25.4	26.0
	11.1	15.0

Aflatoxin G2	Measured (ppb)	Expected (ppb)
Corn	ND	0.0
Oatmeal	7.3	7.8
	3.9	4.5

Zearalenone (ZON)	Measured (ppb)	Expected (ppb)
Corn	203.4	352.0
	112.5	105.5
	125.3	105.5
	1053.8	1195.0
Oatmeal	31.2	40.0
	118.3	75.0

Ochratoxin	Measured (ppb)	Expected (ppb)
Corn	86.3	94.5
	45.5	61.9
	76.5	64.3

Deoxynivalenol (DON)	Measured (ppb)	Expected (ppb)
Wheat	903.9	500.0
	3271.8	5100.0
	1798.9	2500.0
	1267.2	500.0
	5707.4	6200.0
	6245.9	5100.0

Mycotoxin T-2	Measured (ppb)	Expected (ppb)
Corn	265.6	263.7
	284.7	253.5

Fumonisin B1	Measured (ppb)	Expected (ppb)
Corn	616.2	900.0
	894.8	900.0
	5268.4	28000.0
	7969.4	6760.0
	35394.4	28000.0
	3185.0	2750.0

Fumonisin B2	Measured (ppb)	Expected (ppb)
Corn	209.0	300.0
	292.4	300.0
	1930.9	7100.0
	2107.6	1850.0
	9436.9	7100.0
	622.6	700.0

Table 4: Measured and Expected Amounts of Mycotoxins in Each Grain Sample. Expected Values Based on Measurement by an Independent Method

Untargeted Screening

Additionally, a feed corn sample was spiked with the pesticide Bromacil to demonstrate untargeted screening with data-dependent MS-MS and spectral pattern matching.

To help identify any untargeted compounds of potential interest, data-dependent product ion scans were compared to a database containing authentic tandem mass spectra of hundreds of substances including pesticides.

Untargeted Screening

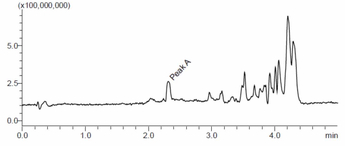


Figure 5: Total Ion Chromatogram From a Feed-Corn Sample. The Peak at 2.25 Minutes Was Automatically Selected for Data-Dependent MS-MS

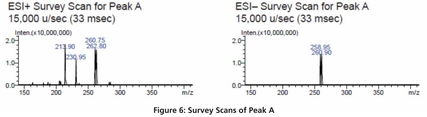
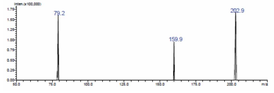


Figure 6: Survey Scans of Peak A

Library Match

ESI– Triggered Product Ion Scan of m/z 258.95 at 5,000 u/sec (38 msec)



ESI– Product Ion Scan of Bromacil Matched From Tandem MS Library

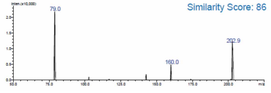


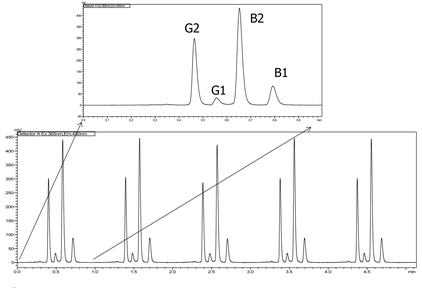
Figure 7: Data Dependend Product Ion Scan From Peak A Matched to Library Spectrum of Bromacil

UHPLC Analysis of Aflatoxins

Method conditions:

XR-ODSIII 2 x 50 mm, 1.6 um
Isocratic 70/30 H2O/50-50 ACN-MeOH
1 mL/min 80°C
Aflatoxin mix: 0.3 to 1 ug/mL
Inj: 1 uL
Ex/Em: 365/450 nm
Runtime: 1 minute

High Throughput UHPLC



Discussion

ESI was found to give the best overall signal intensity for the mycotoxins. The protonated molecules and sodium adducts of each of the mycotoxins were observed by ESI, APCI, and DUIS (a combination of ESI and APCI sources). A formic acid buffered mobile phase was found to depress ionization of the protonated molecules, whereas ammonium formate increased their signal dramatically. Sodium adducts of the mycotoxins had the same or lower signal intensity when ionized in the presence of ammonium formate. The four aflatoxins ionized well in positive mode, while some of the other mycotoxins ionized more efficiently in negative mode.

Injection solvent was found to be critical to achieving proper peak shape. Therefore samples were diluted in mobile-phase matched solvent (80/20 5 mM ammonium acetate/methanol). Calibration curves were prepared for each mycotoxin with a weighting factor of 1/A^2. The curves were linear (r^2 >0.95) in the tested range. Five replicates of each standard were analyzed for the accuracy results. The lower limit of quantitation (LLOQ) for each mycotoxin standard in solution corresponds approximately to the most dilute level reported in Table 1. The LLOQ for Fumonisin B1 and B2 was lower than 10 ppb and significantly lower than the lowest tested concentration of 20.6 ppb for Mycotoxin T2.

Measured values for each mycotoxin are reported in Tables 3 and 4. The rapid chromatographic method enables fast analysis of many samples regardless of sample prep procedure. Survey scanning at extremely fast scan speeds of 15,000 u/sec enables quantitative data to be collected without compromising quantitative performance. Automatically triggered fast tandem mass spectra were collected and used to search a library of known contaminants for untargeted screening. The mass and tandem MS spectrum of Peak A matched the pesticide Bromacil as shown in Figure 7. The presence of Bromacil could be confirmed even though the transitions specifically corresponding to Bromacil were not a part of the MRM method.

Conclusion

A rapid, selective, and sensitive LC-MS-MS method for the determination of mycotoxins and untargeted screening was developed. A high throughput UHPLC method for aflatoxins B and G is also presented.