Analysis of Regulated Mycotoxins in Infant Formula Using Liquid Chromatography-Tandem Mass Spectrometry

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Introduction

The analysis of mycotoxins in infant formula is challenging due to the very low limits of quantitation required to comply with world-wide regulations. For example, the EU regulated limits (EC Regulation 1881/2006) for aflatoxin M1, aflatoxin B1, and ochratoxin A are 0.025, 0.1, and 0.5 µg/kg, respectively. Most current and traditional methods require labor intensive and time consuming sample purification and concentration steps to achieve these levels using liquid chromatography with fluorescence detection or liquid chromatography-mass spectrometry (LC-MS). In this study, an Agilent 1290 UHPLC coupled to an Agilent 6490 triple quadrupole mass spectrometer was utilized to analyze the EU regulated mycotoxins (aflatoxins B_1, B_2, G_1, G_2, M_1 and non-regulated M_2; ochratoxin A; deoxynivalenol; zearalenone; fumonisins B_1 and B_2; and T-2 and HT-2 toxins) in infant formula using a simple extraction without any concentration step. The triggered multiple reaction monitoring (tMRM) function of the system was employed for increased confidence in positive analyte identification even at the very low, sub-ppb concentration levels in the samples.

Experimental

Mycotoxin standards were obtained from Biopure and Enzo Life Sciences. Reagents were ACS, HPLC or MS grade.

Liquid infant formula samples were processed on a platform shaker using a two step mixed solvent extraction, followed by centrifugation. Stable isotope internal standards (obtained from Biopure) were added to the sample extracts before LC-MS/MS analysis.

The LC-MS/MS analysis was performed with an Agilent 1290 UHPLC coupled to an Agilent 6490 tandem mass spectrometer. Chromatographic separation (Figure 1) was achieved with a Zorbax Eclipse Plus C18 column (2.1 x 150 mm, 1.8 µm) using a binary gradient of methanol and ultra pure water containing 5 mM ammonium formate and 0.1% formic acid.

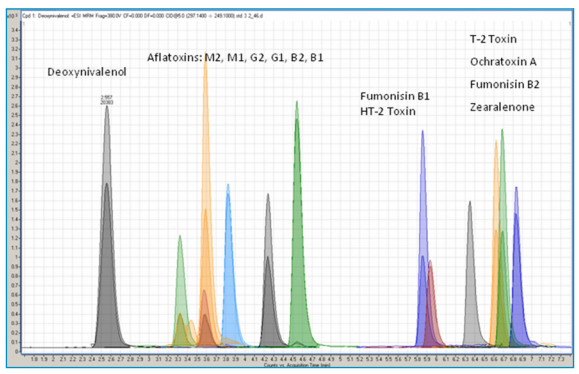

Figure 1. Extracted ion chromatogram (XIC) of a mycotoxin standard.

The MS/MS ionization was performed with Jet Stream electrospray ionization (ESI), using +/- polarity switching. Zearalenone was analyzed in ESI- with $[M-H]^-$ as the precursor ion. All other tested mycotoxins were analyzed in ESI+, mostly giving $[M+H]^+$ ions, except for T-2 and HT-2, which gave more intense $[M+NH_4]^+$ precursor ions. Two MS/MS transitions were collected in dynamic MRM (dMRM) mode for each analyte for quantification and identification purposes (Quant and Qual MRM, respectively, shown in Table 1). In addition, aflatoxin B1, aflatoxin M1, deoxynivalenol, fumonisin B1, ochratoxin A, and zearalenone were analyzed in tMRM mode, in which case collection of additional MRMs was triggered based on the Quant ion detection.

Table 1. MS/MS Conditions for the Tested Mycotoxins and Their ^{13}C-Isotope-Labeled Internal Standards (IS)

	Quant MRM			Qual MRM			Triggered MRM		
Analyte	Precursor (m/z)	Product (m/z)	CE (V)	Precursor (m/z)	Product (m/z)	CE (V)	Precursor (m/z)	Product (m/z)	CE (V)
Aflatoxin B1	313.07	128	80	313.07	241.1	44	313.07	285	24
							313.07	269	36
							313.07	212.9	52
							313.07	185	60
							313.07	157	68
							313.07	126.8	80
							313.07	115	76
Aflatoxin B1 IS	330.1	301.1	21						
Aflatoxin B2	315.09	287.1	28	315.09	115.1	80			
Aflatoxin B2 IS	332.2	303	21						
Aflatoxin G1	329.07	243	24	329.07	200	48			
Aflatoxin G1 IS	346.1	212.2	41						
Aflatoxin G2	331.08	313.1	24	331.08	189	48			
Aflatoxin G2 IS	348.1	259.1	25						
Aflatoxin M1	329.07	273.1	20	329.07	229	44	329.07	301	20
							329.07	258.9	24
							329.07	128	72
							329.07	115.1	80
Aflatoxin M1 IS	346.01	288.2	24						
Aflatoxin M2	331.08	273.1	24	331.08	229.1	48			
Deoxynivalenol	297.14	249.1	5	297.14	203	13	297.14	115	76
							297.14	90.9	52
							297.14	79	48
							297.17	77	80
							297.14	65	76
							297.14	55	36
							297.14	43.1	60
Deoxynivalenol IS	312.2	263.1	4						
Fumonisin B1	722.4	334.3	40	722.4	352.3	36	722.4	704.2	32
							722.4	95.1	80
							722.4	80.9	72
							722.4	74	44
							722.4	70	68
Fumonisin B1 IS	756.5	374.4	37						
Fumonisin B2	706.4	336.4	40	706.4	318.2	48			
Fumonisin B2 IS	740.5	358.3	41						
HT-2 Toxin	442.24	214.9	8	442.24	105	64			
HT-2 Toxin IS	464.3	278.1	9						
Ochratoxin A	404.09	239	24	404.09	102.1	80	404.09	220.9	40
							404.09	192.9	48
							404.09	136.9	64
Ochratoxin A IS	424.2	250.1	25						
T-2 Toxin	484.25	215.1	16	484.25	185.1	20			
T-2 Toxin IS	508.3	322.1	8						
Zearalenone	317.1	175	24	317.1	273.1	16	317.1	131	28
							317.1	40.9	76
Zearalenone IS	335.2	290	17						

Results and Discussion

Fast MS/MS Method Development with Automated Tools

The Agilent MassHunter software contains two useful components for automated MS/MS method development; Optimizer, and Source and iFunnel optimizer. The optimizer program allows for the automated development of compound dependent parameters on the MS/MS. After the user defines settings such as a generic LC method, generic source settings, compound molecular formula and potential precursor ions, compound tuning solutions are applied sequentially to the MS/MS with the autoinjector. Prominent precursor and fragment ions are selected and optimized via ramping collision energy over several injections. After suitable chromatography is created using the optimized compound data, the Source and iFunnel optimizer script is used to create optimal source settings. Source settings are varied incrementally over multiple injections, and corresponding compound responses can be evaluated in MassHunter quantification.

Triggered MRM (tMRM)

Triggered MRM is a variation of dMRM where a predetermined response on a selected MRM transition will 'trigger' the collection of additional MRM transitions. This allows for higher confidence in analyte identification while maximizing the dwell of the quant and qual MRMs. Up to ten total MRM transitions can be collected, each with optimized collision energies to increase sensitivity. The collected ions are formulated into a 'spectra', which is compared to a library 'spectra' for confirmation.

The following figures show chromatograms, calibration curves, and tMRM library match for selected mycotoxins spiked in infant formula matrix at very low concentration levels.

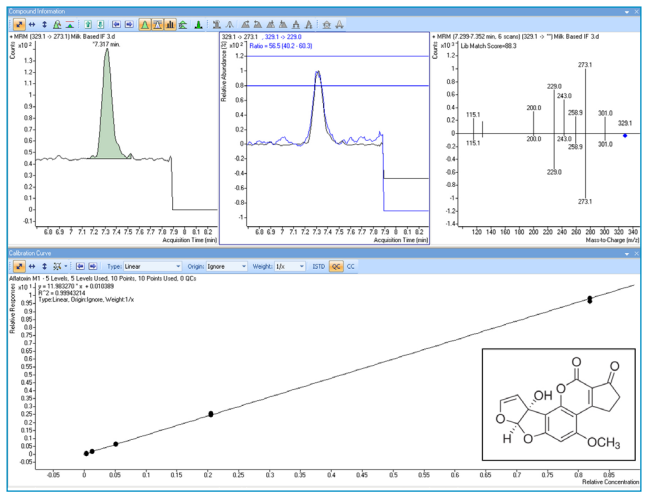

Figure 2. Aflatoxin M1 in Infant Formula, fortified at 0.02 µg/kg.

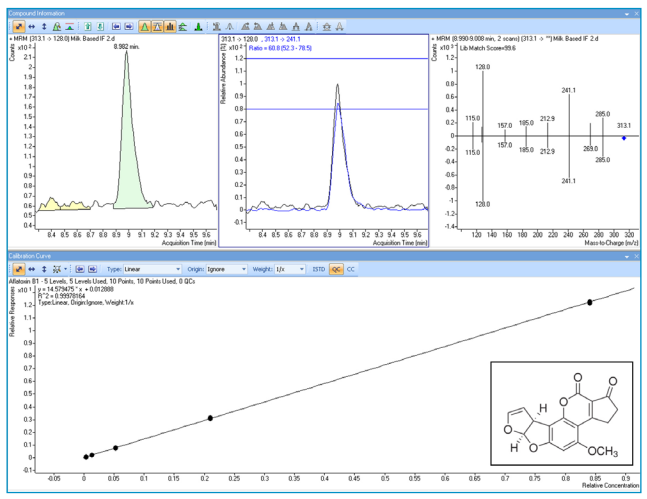

Figure 3. Aflatoxin B1 in Infant Formula, fortified at 0.02 µg/kg.

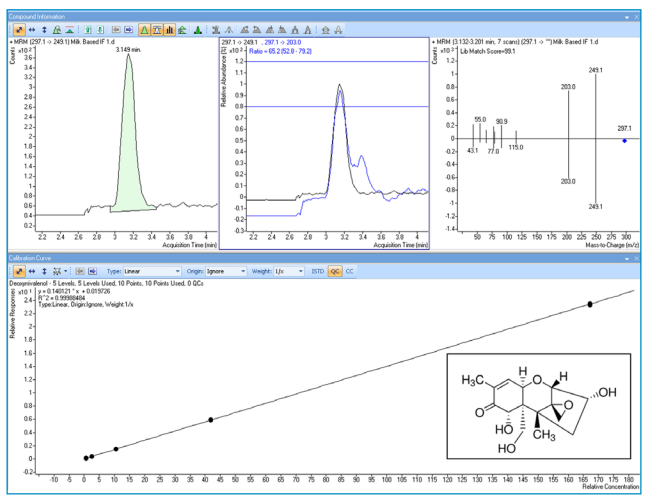

Figure 4. Deoxynivalenol in Infant Formula, fortified at 4.0 µg/kg.

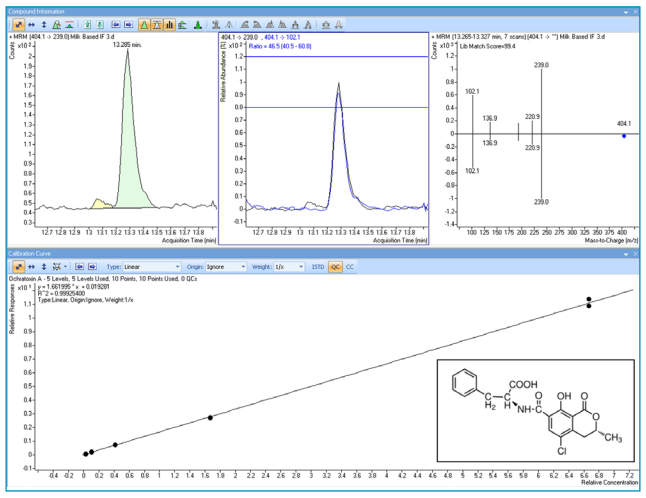

Figure 5. Ochratoxin A in Infant Formula, fortified at 0.16 µg/kg.

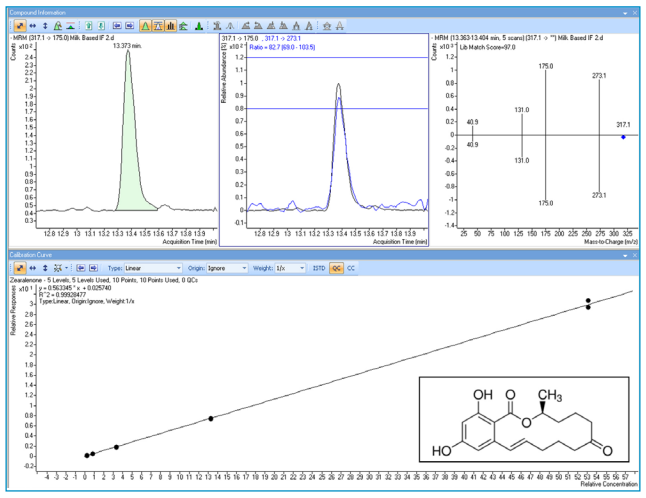

Figure 6. Zearalenone in Infant Formula, fortified at 2.6 µg/kg.

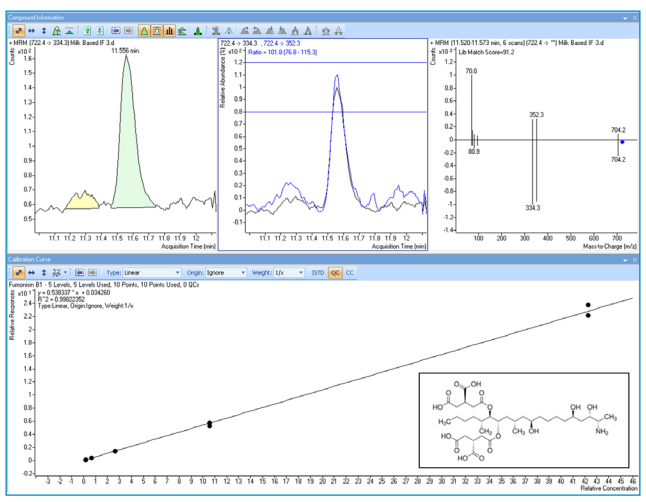

Figure 7. Fumonisin B1 in Infant Formula, fortified at 1.0 µg/kg.

Table 2 summarizes method performance results obtained for the tested mycotoxin in infant formula at a low fortification level (below the EU regulated levels).

Table 2. Mean Recoveries and Relative Standard Deviations (RSDs, *n* = 5)

Mycotoxin	Calibration Range (ng/mL)	EU Regulated Level (µg/kg)	Fortification Level (µg/kg)	Recovery (%)	RSD (%)
Aflatoxin M1	0.003 - 0.83	0.025	0.02	99.4	5.7
Aflatoxin B1	0.003 - 0.83	0.1	0.02	86.0	2.2
Aflatoxin B2	0.003 - 0.83		0.02	90.8	3.7
Aflatoxin G1	0.003 - 0.84		0.04	99.2	12.1
Aflatoxin G2	0.003 - 0.85		0.04	94.8	8.8
Aflatoxin M2	0.003 - 0.86		0.02	82.9	7.3
Fumonisin B1	0.17 - 43.0	200 FB1+FB2	1.0	93.1	11.2
Fumonisin B2	0.17- 43.0		1.0	86.2	7.9
Deoxynivalenol	0.65 - 167	200	4.0	93.9	4.5
Ochratoxin A	0.026 - 6.67	0.5	0.16	85.8	8.3
Zearalenone	0.21 - 53.1	20	2.6	90.5	10.6
HT-2	0.67 - 169		4.0	87.8	4.8
T2	0.067 - 16.9		0.4	93.4	3.4

Conclusions

This LC-MS/MS method has been proven to be effective for the analysis of regulated mycotoxins in infant formula samples. Acceptable results have been achieved for calibration curves, precision and accuracy. The use of dMRM with two MS/MS transitions satisfies requirements for positive compound identification, while the use of tMRM gives greater confidence in analyte identification without sacrificing sensitivity. Moving forward, this method will be adapted for the analysis of other infant and baby foods as required by regulations, such as processed cereal based foods and dietary foods for special medical purposes intended specifically for infants.

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