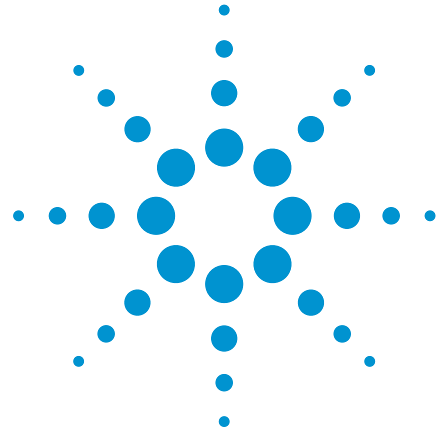

Aflatoxin Analysis in Infant Formula with Enhanced Matrix Removal—Lipid by LC/MS/MS

Application Note

Food Testing

Author

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Abstract

Aflatoxin M1 is the primary aflatoxin found in milk. It has European Commission (EC) maximum recommended levels of as low as 0.025 µg/kg in infant formula in Europe. In the United States, Food and Drug Administration (FDA) action levels for the aflatoxin are as low as 0.5 µg/kg in milk. This application note describes the determination of aflatoxins M1, G2, G1, B2, and B1 in infant formula by LC/MS/MS using Agilent Bond Elut Enhanced Matrix Removal—Lipid (EMR—Lipid). This study employed a QuEChERS extraction, followed by cleanup with EMR—Lipid dispersive SPE (dSPE). This method delivers excellent recoveries (88–113%), and precision (RSDs = 1.3–13.6%) for all aflatoxins at all levels. Due to the extensive matrix removal, limits of quantitation (LOQs) for this method were extended to below regulatory limits for both the U.S. and Europe. This simple and robust method requires minimal equipment and expertise, allowing for easy implementation in food laboratories.

Introduction

Mycotoxins are secondary metabolites of fungi, and are considered to be one of the most prevalent contaminants in food and feed supplies globally. The Food and Agriculture Organization (FAO) estimates that up to 25% of the world's agricultural production is contaminated with mycotoxins. This contamination results in devastating economic losses, particularly for the grain industry [1]. Aflatoxins (Table 1) are a class of mycotoxins produced by various species of fungi, particularly *Aspergillus flavus* and *Aspergillus parasiticus* [2]. Aflatoxin M1 is the most commonly found mycotoxin in milk, and is produced when cows ingest and metabolize feed contaminated with aflatoxin B1 [3]. Both the Food and Drug Administration (FDA) and European Commission (EC) have established limits for aflatoxin levels in various food commodities [4,5]. Table 2 summarizes FDA and EC aflatoxin limits for both the United States and Europe.

The regulatory limits for aflatoxins are very low, specifically in dairy matrices and formulations for infant consumption. Sample preparation is necessary to remove matrix interferences to improve analyte signals at low concentrations. Immunoaffinity columns are commonly used for the analysis of mycotoxins, including aflatoxins, in various matrices [6-9]. However, these columns can be expensive, and require a distinct and differentiated workflow that is not always convenient for food laboratories. Quick, Easy, Cheap, Effective, Rugged, Safe (QuEChERS) uses a simple three-step procedure (extract, cleanup, and analyze) for samples. Thus, it is an attractive method for the preparation of various analytes and matrices, including aflatoxins in grains and dairy products [3,10,11]. QuEChERS cleanup with C18 or PSA, however, has some limitations when analyzing high lipid-containing samples, such as meats and milk. These limitations result from nonselective interactions with target analytes and minimal removal of major lipid classes. Any remaining lipids can accumulate in the analytical flowpath resulting in increased maintenance, chromatographic anomalies, and poor data accuracy and precision.

Agilent Bond Elut Enhanced Matrix Removal—Lipid (EMR—Lipid) is an innovative sorbent that uses a unique combination of size exclusion and hydrophobic interactions to selectively remove major lipid classes from samples without unwanted analyte retention. This sorbent can be used as a dSPE cleanup with QuEChERS and protein precipitation workflows, facilitating a simple and effective cleanup [12,13]. In this study, the analysis of five aflatoxins in infant formula was investigated. Infant formula was chosen, due to its lipid content and aflatoxin regulatory levels, for this matrix. A QuEChERS extraction followed by EMR—Lipid dSPE cleanup and enhanced post sample treatment using anhydrous $MgSO_4$ provided excellent matrix removal. This application note demonstrates the effectiveness of EMR—Lipid for aflatoxin analysis at three different concentration levels.

Table 1. Chemical and physical information for five aflatoxins.

Aflatoxin	Molecular formula	Structure	pKa	logP
M1	$C_{17}H_{12}O_7$		11.4	0.93
B1	$C_{17}H_{12}O_6$		–	1.58
B2	$C_{17}H_{14}O_6$		–	1.57
G1	$C_{17}H_{12}O_7$		–	1.37
G2	$C_{17}H_{14}O_7$		–	1.36

Table 2. Aflatoxin limits in associated matrices according to the EC and the FDA.

Aflatoxin	Limit (µg/kg)	Matrix	Regulatory agency
M1	0.025	Infant formula	EC [5]
	0.05	Raw milk	EC [5]
	0.50	Raw milk	FDA [14]
B1	0.10	Baby food	EC [5]
	2–12*	General food	EC [5]
B1 + B2 + G1 + G2 (total concentration)	4–15*	Nuts, figs, dried fruits, cereals, maize, spices	EC [5]
	20	General food	FDA [15]**
	20–300	Animal feeds	FDA [16]**

* See EC Commission Regulation No 1881/2006 for details regarding specific matrices [5].

** Identity of aflatoxin B1 must be confirmed by chemical derivative formation [15,16].

Experimental

Reagents and chemicals

All reagents were HPLC grade or higher. Acetonitrile (ACN) and methanol were purchased from Honeywell (Muskegon, MI, USA). Water was purified using an EMD Millipore Milli-Q Integral System (Darmstadt, Germany). Reagent-grade formic acid (FA, p/n G2453-85060) was from Agilent Technologies. European Reference Material ERM-BD283 (Whole milk powder low-level aflatoxin M1) was purchased from LGC Standards (Teddington, Middlesex, UK). Aflatoxin M1 (10 µg/mL in ACN), an aflatoxin mix (B1, G1, B2, G2: 20 µg/mL each in ACN), and ammonium formate were purchased from Sigma-Aldrich, Corp. (St. Louis, MO, USA). Aflatoxin stock standards were stored at 2–8 °C (M1) and –20 °C (B1, B2, G1, G2 mix) per manufacturer's recommendations. Liquid, ready-to-use, infant formula was purchased from a local grocery store.

Equipment

Equipment and materials used:

- Eppendorf pipettes and repeater
- Vortexer and multitube vortexers (VWR, Radnor, PA, USA)
- Geno/Grinder (SPEX, Metuchen, NJ, USA)
- Centra CL3R centrifuge (Thermo IEC, MA, USA)
- Turbovap LV (Biotage, Charlotte, NC, USA)
- Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)
- Agilent Bond Elut Original QuEChERS method (nonbuffered) extraction kits (10 g sample) with ceramic homogenizers (p/n 5982-5550CH)
- Agilent Bond Elut EMR—Lipid dSPE (p/n 5982–1010)
- Agilent Bond Elut EMR—Lipid $MgSO_4$ Polish Pouch (p/n 5982–0102)

Instrumentation

Analysis was performed on an Agilent 1290 Infinity LC system consisting of:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity High Performance Autosampler (G4226A) equipped with an Agilent 1290 FC/ALS Thermostat (G1330B),
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)

The LC system was coupled to an Agilent 6460A Triple Quadrupole LC/MS/MS system equipped with Agilent Jet Stream electrospray ionization technology. Agilent MassHunter workstation software was used for all data acquisition and analysis.

Sample preparation

Liquid infant formula (10 mL) was added to a 50 mL centrifuge tube, and spiked as appropriate with standards for quality control (QC) samples. Two ceramic homogenizers and 10 mL of ACN were added, and the sample was vortexed for two minutes. An Original (nonbuffered, 10 g sample) QuEChERS extraction salt packet was added to the sample. The sample was mixed on a mechanical shaker for two minutes, followed by centrifugation at 5,000 rpm for five minutes. Water (5 mL) was added to the EMR—Lipid dSPE tube, vortexed, and followed by the addition of 5 mL of crude sample extract. The sample was vortexed immediately, then vortexed for an extra 60 seconds on a multitube vortexer. The sample was then centrifuged at 5,000 rpm for five minutes, and decanted into an empty 15 mL centrifuge tube. Anhydrous magnesium sulfate ($MgSO_4$) from a final polish pouch was added to the extract. The sample was vortexed immediately to disperse the salt followed by an extra 60 seconds, and centrifuged at 5,000 rpm for three minutes. The supernatant was transferred to a separate 15 mL centrifuge tube containing 1.5 g of $MgSO_4$ (from a new polish pouch). The sample was vortexed immediately and then again for 60 seconds. After centrifugation at 5,000 rpm for three minutes, the final sample (1 mL) was transferred to a 16 × 100 mm glass test tube and evaporated under nitrogen at 50 °C until dry. As necessary, blank matrix was spiked with calibration standards before dry down. The sample was reconstituted with 100 µL of H_2O with 0.1% FA/ACN (80/20), and vortexed for a minimum of two minutes. The sample was then sonicated and centrifuged if necessary. The final sample was then transferred to a vial with insert for LC/MS/MS analysis. Figure 1 shows the entire sample preparation procedure.

Instrument conditions

HPLC

Column:	Agilent Poroshell 120 SB-C18 2.1 × 100 mm, 2.7 µm (p/n 685775-902)
Guard:	Agilent Poroshell 120 SB-C18 UHPLC Guard, 2.1 mm × 5 mm, 2.7 µm (p/n 821725-912)
Mobile phase:	A) 5 mM Ammonium formate in water with 0.1% FA B) 5 mM Ammonium formate in 50/50 ACN/methanol with 0.1% FA
Flow Rate:	0.3 mL/min
Column temp:	40 °C
Autosampler temp:	4 °C
Injection volume:	5 µL
Needle wash:	1:1:1:1 ACN/methanol/isopropanol/water with 0.2% FA
Gradient:	Time (min) %B 0 20 7 70 7.25 95
Stop time:	11 min
Post time:	2 min

MS

Electrospray Ionization (ESI), Positive Mode	
Gas temp:	325 °C
Gas flow:	10 L/min
Nebulizer:	50 psi
Sheath gas heater:	350 °C
Sheath gas flow:	11 L/min
Capillary:	4,000 V
Delta EMV (+):	300 V
Time segments:	No. Time (min) Diverter valve 1 0 To Waste 2 2 To MS

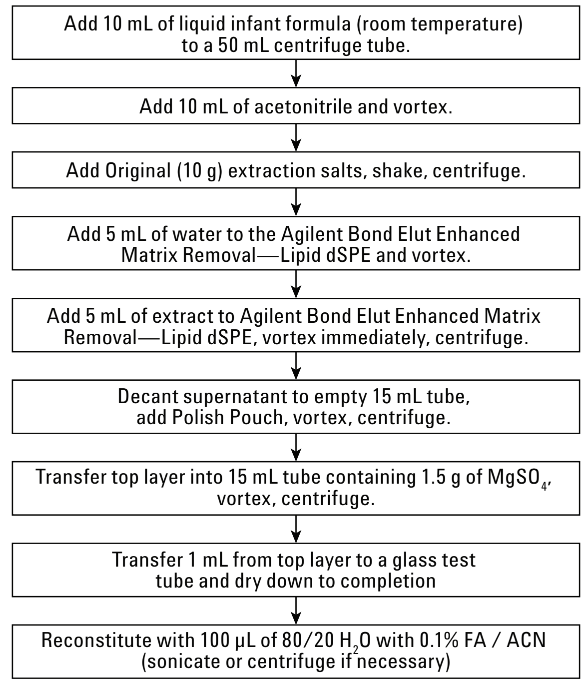

Figure 1. Sample preparation protocol for aflatoxins in liquid infant formula with Agilent Bond Elut Enhanced Matrix Removal—Lipid cleanup and enhanced post sample treatment using $MgSO_4$.

Table 3. LC/MS/MS dMRM parameters and retention times for target analytes.

Analyte	RT (min)	Precursor ion (*m/z*)	Frag (V)	Product ion			
				Quant ion (*m/z*)	**CE (V)**	**Qual ion (*m/z*)**	**CE (V)**
Aflatoxin M1	4.28	329.1	143	273.1	21	229	45
Aflatoxin G2	4.55	331.1	164	313.1	25	245	33
Aflatoxin G1	4.88	329.07	149	243.1	25	115.1	80
Aflatoxin B2	5.13	315.09	174	287.1	25	259.1	29
Aflatoxin B1	5.44	313.07	169	241	41	128	80

Calibration standards and quality controls

An aflatoxin working solution was prepared in ACN at 2 µg/mL (aflatoxin M1) and 10 µg/mL (aflatoxin G2, G1, B2, and B1) from the stock solutions. The concentration of aflatoxin M1 was made five times lower than the additional aflatoxins to meet the different regulatory limits. Calibrator and QC standards were made at 100x the final concentration by appropriately diluting the working solution. Table 4 shows the final concentration for calibration and QC samples in infant formula. The working solution and all calibration and QC standards were stored in amber vials at 2–8 °C. Matrix blank infant formula was spiked with 100 µL of the corresponding QC standard before extraction. For matrix matched calibration standards, 10 µL of the appropriate calibration standard was spiked into 990 µL of matrix blank ACN extract before dry down.

Table 4. Final concentrations for prespike QC and postspike calibration samples in infant formula.

Standard*	Aflatoxin M1 (ng/mL)	Aflatoxin B1, B2, G1, G2 (ng/mL)
Cal 1	0.01	0.05
Cal 2	0.02	0.10
Cal 3	0.10	0.50
Cal 4	0.50	2.50
Cal 5	1.00	5.00
Cal 6	2.50	12.5
Low QC	0.025	0.125
Mid QC	0.25	1.25
Hi QC	2.00	10.0

*Standards made at 100x final concentration for spiking.

Results and Discussion

Method Optimization

EMR—Lipid can be used in both protein precipitation and QuEChERS workflows. Initial testing showed that aflatoxin analysis in liquid infant formula was amenable to both the protein precipitation and QuEChERS procedures. However, due to the increased dilution factor associated with traditional protein precipitation, the QuEChERS methodology was chosen for this application. After preliminary experimentation, original QuECHERS extraction salts were chosen for this application, but both AOAC and EN extraction salts are also acceptable.

Based on signal-to-noise (S/N) criteria, it was determined that a concentration step was required to reach the desired limit of quantitation (LOQ) for aflatoxin M1. Concentrations of 5x, 10x, and 20x were evaluated, and with the given instrumental setup, a 10x concentration was suitable for LOQ and method needs. Figure 2 shows the LC/MS/MS dMRM chromatogram for aflatoxins in infant formula after QuEChERS extraction, EMR—Lipid cleanup, and enhanced post sample treatment with $MgSO_4$.

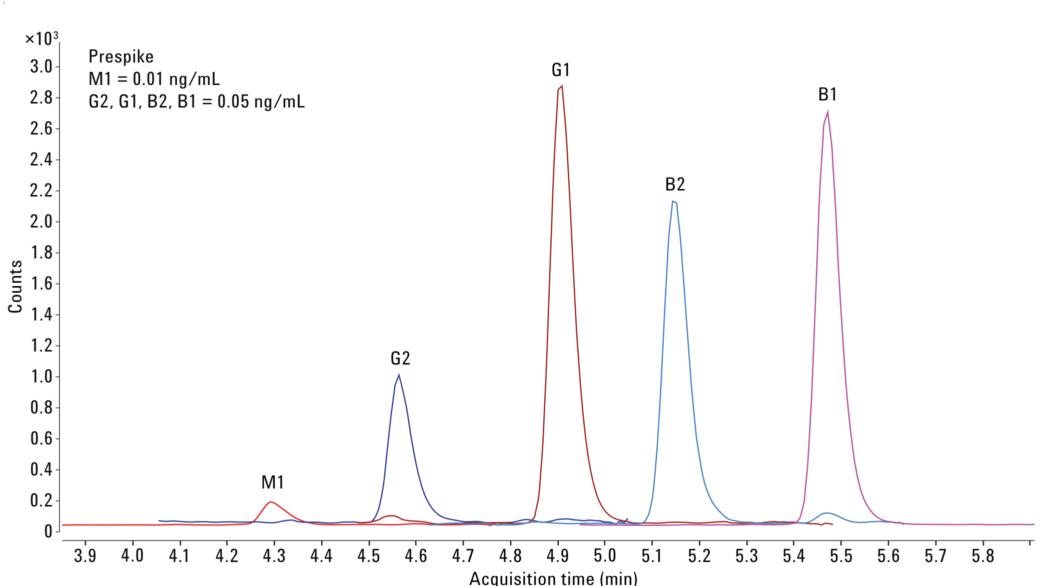

Figure 2. LC/MS/MS dMRM chromatogram of aflatoxins in liquid infant formula after QuEChERS extraction and Agilent Bond Elut Enhanced Matrix Removal—Lipid dSPE cleanup with enhanced post sample treatment with $MgSO_4$.

Matrix removal

Liquid infant formula has various matrix components such as fat, protein, carbohydrates, vitamins, and minerals. This complex matrix can make sample preparation more challenging especially since aflatoxin concentrations are generally very low. Figure 3 shows a GC/MS full scan overlay chromatogram for an infant formula matrix blank sample without cleanup, and with C18/PSA or EMR—Lipid dSPE cleanup with 10x sample concentration. When compared to the matrix blank without cleanup, C18/PSA removes very little matrix particularly in the later eluting region of the chromatogram. However, EMR—Lipid, shown in red, through its unique mechanisms of action, removes a significant amount of matrix even with a final 10x concentration during sample preparation.

Matrix effects were evaluated by comparing peak response (area) from postspiked infant formula and equivalent solvent standards (Table 5). Samples were spiked at a concentration of 0.025 ng/mL for aflatoxin M1 and 0.125 ng/mL for aflatoxin G2, G1, B2, and B1. This method demonstrates acceptable matrix removal, with no significant matrix effects present.

Table 5. Matrix effects (%) for the five aflatoxins. Matrix effects were evaluated by comparing peak response from postspiked infant formula and equivalent solvent standards (n = 5).

Aflatoxin	Matrix Effects (%)
M1	+ 12
G2	– 13
G1	– 10
B2	– 11
B1	– 9

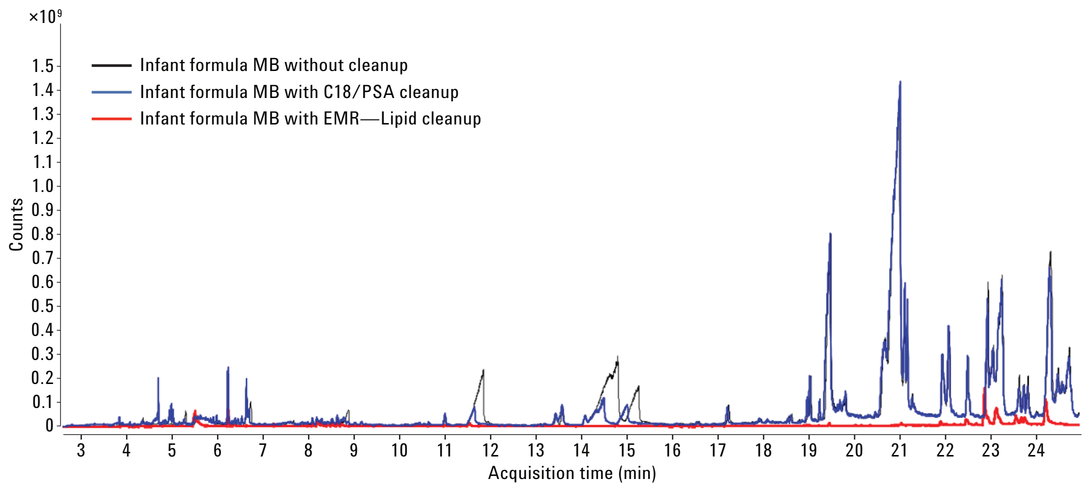

Figure 3. GC/MS full scan overlay chromatogram of infant formula matrix blank (MB) without cleanup, with C18/PSA dSPE cleanup, and with Agilent Bond Elut Enhanced Matrix Removal—Lipid cleanup.

Linearity and LOQ

The linear range was 0.01 to 2.50 ng/mL for aflatoxin M1, and 0.05 to 12.5 ng/mL for aflatoxins G2, G1, B2, and B1 in infant formula. Table 6 shows calibration range, regression/weight with R^2 values, and LOQ for each aflatoxin in this study. LOQs were determined experimentally based on method performance. The LOQ for aflatoxin M1 extends below U.S. action levels and European maximum levels.

Table 6. Calibration details (regression fit, weight, R^2 value, linear range, and LOQ) for each aflatoxin.

Aflatoxin	Regression fit, weight	R^2	Linear range (ng/mL)	LOQ (ng/mL)
M1	Linear, $1/x^2$	0.9931	0.01–2.50	0.01
G2	Linear, 1/x	0.9990	0.05–12.5	0.05
G1	Linear, 1/x	0.9994	0.05–12.5	0.05
B2	Linear, 1/x	0.9986	0.05–12.5	0.05
B1	Linear, 1/x	0.9987	0.05–12.5	0.05

Recovery and reproducibility

The recovery and reproducibility of this method were evaluated. QC standards (n = 6) were prepared by spiking blank liquid infant formula at concentrations of 0.025, 0.25, and 2.00 ng/mL for aflatoxin M1. In addition, QC standards were prepared at 0.125, 1.25, and 10.0 ng/mL for aflatoxins G2, G1, B1, and B2. The samples were then extracted using the procedure detailed in this application note. A series of calibrators were prepared as described previously and injected throughout the run, for a total of n = 6, to check for significant changes in response, of which there were none. Recoveries of the prespike QC samples were calculated based on their responses against the matrix-matched calibration curve. Figures 4 and 5 show the recovery and Relative Standard Deviation (RSD) data. The average recovery for all five aflatoxins at all three QC levels was approximately 101% with an average RSD <5.0%.

Low QC
M1 = 0.025 ng/mL
G2, G1, B2, B1 = 0.125 ng/mL
Mid QC
M1 = 0.25 ng/mL
G2, G1, B2, B1 = 1.25 ng/mL
Hi QC
M1 = 2.00 ng/mL
G2, G1, B2, B1 = 10.0 ng/mL
Recovery (%)
0
20
40
60
80
100
120
M1
G2
G1
B2
B1
Aflatoxin

Figure 4. Recovery (%) for five aflatoxins in liquid infant formula using Agilent Bond Elut Enhanced Matrix Removal—Lipid cleanup. (n = 6)

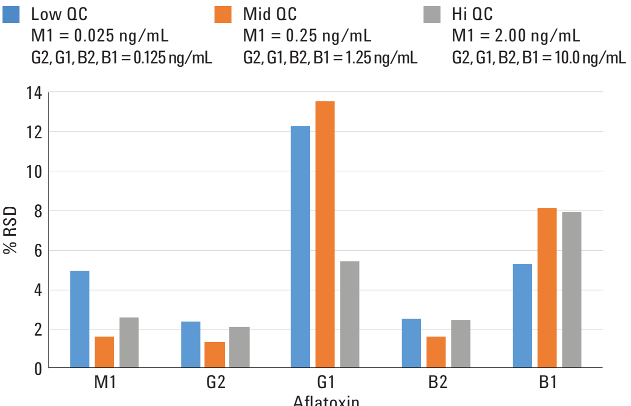

Figure 5. RSD (%) for five aflatoxins in liquid infant formula using Agilent Bond Elut Enhanced Matrix Removal—Lipid cleanup. (n = 6).

Incurred sample

An incurred infant formula reference standard containing aflatoxins was not available to buy. Therefore, European Reference Material (ERM) - BD283 (whole milk powder with low level aflatoxin M1) was obtained to further evaluate the method detailed in this application note. The reported certified value for this standard was 0.111 + 0.018 µg/kg [17]. The whole milk powder standard was reconstituted according to manufacturer's recommendations detailed in the ERM Certification Report. In brief, 100 mL of water was added to 10 g of whole milk powder, introducing a tenfold dilution factor. Therefore, the concentration of the reconstituted milk should be expected to be tenfold lower than the reported concentration, that is 0.0093–0.0129 ng/mL. This reconstituted sample was then extracted following the same procedure detailed previously. The incurred sample (n = 1) and was analyzed in a batch of samples that included a full set of calibrators and all three levels of QC (n = 3). Since the incurred sample was expected to quantitate close to the method's LOQ (0.01 ng/mL for aflatoxin M1), an extra LOQ QC (n = 3) at 0.01 ng/mL was included. Table 7 includes the results from this run. ERM-BD283 quantitated at 0.0101 ng/mL, which falls within the expected range for the reference material. This demonstrates the feasibility and versatility of this method for analyzing dry (with addition of water) and liquid infant formula samples.

Table 7. Results for aflatoxin M1 for incurred sample (n = 1) and QC samples (n = 3).

Sample	Expected conc. (ng/mL)	Calc. conc. (ng/mL)	Accuracy	RSD
Low QC	0.025	0.0219	88	1.9
Mid QC	0.25	0.2378	95	1.1
Hi QC	2.00	2.0285	101	0.6
LOQ QC	0.01	0.0107	107	4.7
ERM-BD283	0.0111	0.0101	91	n/a

Conclusions

A simple and effective method for the analysis of aflatoxins in infant formula by LC/MS/MS has been developed. Samples were extracted using a QuEChERS procedure followed by Agilent Bond Elut EMR—Lipid dSPE cleanup and enhanced post sample treatment using $MgSO_4$. This method provided excellent recoveries (average of 101%) and precision (average RSD < 5.0 %) across a broad linear range. LOQs were extended below regulations with a concentration step without unwanted matrix effects.

This methodology demonstrates the significant matrix removal achieved with EMR—Lipid without unwanted analyte loss. Superior instrument cleanliness provides improved chromatography and decreased maintenance and troubleshooting, saving both time and money. This solution requires little expertise and equipment, and can be easily implemented in food laboratories. While this application focuses on only one class of mycotoxins, EMR—Lipid is designed for multiresidue applications. Thus, unlike immunoaffinity columns, EMR—Lipid can easily be applied to multiclass mycotoxin analysis. Future work will continue to investigate EMR—Lipid cleanup with other complex, high-fat samples.

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