Application Note

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Mycotoxin Analysis in Infant Formula Using Captiva EMR—Lipid Cleanup and LC/MS/MS

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Abstract

Several countries regulate the levels of mycotoxins in foods. However, the complexity of certain foodstuffs in terms of protein and lipid content can prove challenging in the accurate quantitation of low-level mycotoxins in these matrices. This Application Note describes the determination of 13 multiclass mycotoxins in solid and liquid infant formulations using a Quick Easy Cheap Effective Rugged Safe (QuEChERS) workflow followed by Agilent Captiva EMR—Lipid cartridge cleanup. Due to the high selectivity of the Captiva EMR—Lipid sorbent, excellent recoveries (70.4 to 106.8 %) and precision (<18 %) were achieved for all mycotoxins. This simple and robust methodology requires minimal equipment and expertise, which promotes easy implementation in food laboratories.

Introduction

Mycotoxins are produced as secondary metabolites by fungal species that grow on various crops such as grain, corn, and nuts. When cows ingest contaminated feed, mycotoxins and their metabolites can be excreted into the animal's milk[1]. Aflatoxin M1 is the most commonly found mycotoxin in milk, and is monitored and regulated in many countries, including the United States and European countries[2,3]. Despite a lack of regulations for other mycotoxins in milk, there is a growing interest to monitor additional mycotoxins such as fumonisins and ochratoxins.

Since the regulatory limits are very low, specifically in infant formulations, sample preparation is necessary to remove matrix interferences to improve analyte signals at low concentrations. Immunoassays or LC/MS methods, together with sample preparation techniques such as immunoaffinity, solid phase extraction (SPE), or stable isotope dilution[4], can be used to analyze mycotoxins. However, fatty, complex samples can be especially problematic due to the high concentrations of matrix components such as proteins and lipids. Immunoaffinity cartridges are expensive and often specific to the analyte, class, or sample type. Other cleanup products can lack selectivity towards target analytes, and be ineffective in removing lipids, causing poor reproducibility, matrix effects, and accumulation on the instrument.

Agilent Captiva EMR—Lipid, a lipid removal product, combines size exclusion and hydrophobic interaction to selectively capture lipid hydrocarbon chains without the loss of target analytes. Available in 3-mL and 6-mL volumes, Captiva EMR—Lipid tubes provide a simple pass-through cleanup, delivering selective lipid removal from fatty sample extracts for multiclass, multiresidue analysis. A QuEChERS extraction was used for the extraction of 13 mycotoxins from infant formula liquid and powder. QuEChERS is known for high extraction efficiency for a wide range of analyte classes, but it can also extract a large amount of matrix. The Captiva EMR—Lipid cartridges provide high lipid removal and allow accurate quantitation of the target mycotoxins. The method was validated for infant formula at three spike levels for aflatoxins (AF-B1, B2, G1, G2, and M1), ochratoxins (OTA and OTB), fumonisins (FB1, FB2, and FB3), zearalenone (ZON), mycophenolic acid (MPA), and sterigmatocystin (STC). The method delivered excellent recovery, precision, and sensitivity for trace mycotoxins in this complex, fatty matrix.

Experimental

Sample preparation

- Captiva EMR—Lipid 3-mL tubes (p/n 5190-1003)
- Captiva EMR—Lipid 6-mL tubes (p/n 5190-1004)
- QuEChERS original extraction salts (p/n 5982-5550)
- VacElut SPS 24 vacuum manifold (p/n 12234022)

LC configuration and parameters

Configuration	
Agilent 1290 Infinity II high-speed pump (G7120A)	
Agilent 1290 Infinity II multisampler (G7167B)	
Agilent 1290 Infinity II multicolumn thermostat (G7116B)	
Analytical columns	Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 100 mm, 2.7 µm, LC column (p/n 695775-902) Agilent InfinityLab Poroshell 120, EC-C18, 2.1 × 5 mm, 2.7 µm, guard column (p/n 821725-911)
Column temperature	40 °C
Injection volume	5 µL
Mobile phase A	5 mM Ammonium formate in H_2O + 0.1 % formic acid
Mobile phase B	1:1 Acetonitrile(ACN):methanol + 0.1 % formic acid
Flow rate	0.5 mL/min
Gradient	Start at 5 %B, Hold 1 minute, Then ramp from 50 to 60 %B at 4 minutes, Then to 98 %B at 7 minutes, Hold 1 minute
Post time	2 minutes
Needle wash	1:1:1 H_2O:ACN:isopropanol for 10 seconds
Vials	2 mL vial (p/n 5190-4044) PTFE cap (p/n 5182-0725) Insert (p/n 5183-2086)

MS/MS configuration

Configuration	
Agilent 6490 triple quadrupole LC/MS with Agilent Jet Stream	
MS/MS mode	Dynamic MRM
Ion mode	Positive/negative
Drying gas temperature	250 °C
Drying gas flow	8 L/min
Nebulizer pressure	40 psi
Sheath gas temperature	350 °C
Sheath gas flow	11 L/min
Capillary voltage	5,000 V
EMV	500 V(+) 0 V(–)
Nozzle voltage	1,500 V(+) 0 V(–)

MS/MS parameters

Compound	Precursor ion	Quantifier ion (CE)	Qualifier ion (CE)	Fragment (V)	Retention time (min)
Aflatoxin M1	329.1	313.0 (24)	115.1 (88)	135	1.842
Aflatoxin G2	331.1	313.0 (24)	115.1 (88)	165	1.916
Aflatoxin G1	329.1	243.2 (24)	200.0 (44)	175	2.018
Aflatoxin B2	315.1	287.0 (28)	259.0 (32)	175	2.104
Aflatoxin B1	313.1	285.2 (24)	128.1 (84)	170	2.223
Fumonisin B1	722.4	352.3 (36)	334.4 (44)	200	2.810
Ochratoxin B	370.0	205.0 (16)	120.1 (96)	120	3.200
Mycophenolic acid	321.1	302.9 (4)	206.9 (20)	90	3.235
Fumonisin B3	706.4	336.3 (36)	318.5 (40)	200	3.676
Zearalenone	317.1	175 (24)	131 (28)	175	4.217
Fumonisin B2	706.4	336.3 (36)	318.5 (40)	200	4.398
Ochratoxin A	404.1	239.0 (24)	120.1 (96)	120	4.398
Sterigmatocystin	325.0	310.0 (24)	281 (40)	150	4.525

Table 1. Sample QC concentrations.

Analyte	LQ (ng/g)	MQ (ng/g)	HQ (ng/g)
Aflatoxin B1 (AF-B1)	2	10	20
Aflatoxin B2 (AF-B2)	2	10	20
Aflatoxin G1 (AF-G1)	2	10	20
Aflatoxin G2 (AF-G2)	2	10	20
Aflatoxin M1 (AF-M1)	1	5	10
Fumonisin B1 (FB1)	10	50	100
Fumonsin B2 (FB2)	10	50	100
Fumonisin B3 (FB3)	10	50	100
Mycophenolic acid (MPA)	2	10	20
Ochratoxin A (OTA)	2	10	20
Ochratoxin B (OTB)	1	5	10
Sterigmatocystin (STC)	2	10	20
Zearalenone (ZON)	2	10	20

Chemicals and reagents

Food samples bought from a local grocery store were used for method quantitation and matrix-removal studies. Standards and internal standards were purchased as premixed solutions from Sigma-Aldrich (St Louis, MO, USA) or Romer Labs (Getzersdorf, Austria). LC solvents were bought from Honeywell (Muskegon, MI, USA).

Validation study

The validation of mycotoxins in infant formula was carried out in batches consisting of two double blanks, two blanks, six calibrators, and three QC levels. QCs were prespiked as shown in Table 1 in replicates of six (n = 6), and injected between two sets of calibration curves. Calibration curves were generated using six levels, as follows:

- 0.25, 1, 5, 10, 20, 40 ng/mL for AF-B1, AF-B2, AF-G1, AF-G2, MPA, OTA, STC, and ZON
- 0.125, 0.5, 2.5, 5, 10, 20 ng/mL for AF-M1 and OTB
- 1.25, 5, 25, 50, 100, 200 ng/mL for FB1, FB2, and FB3

Isotopically labeled internal standard $^{13}C_{17}$-AF-B1 was spiked at 5 ng/mL.

Sample preparation detailed procedure

For infant formula powder (5 g), calibrators and QCs were prespiked at appropriate levels, and thoroughly soaked for at least one hour before extraction. Next, 10 mL of water were added and allowed to soak into the sample. For infant formula liquid, 10 g of sample was used for extraction, and calibrators and QCs were prespiked accordingly. No additional water was needed. For both infant formula powder and liquid, the sample was extracted with 10 mL of acetonitrile with 2 % formic acid and QuEChERS original salts (4 g $MgSO_4$, 1 g NaCl) using vertical shaking on a Geno/Grinder for 10 minutes. This was followed by centrifugation at 5,000 rpm for five minutes. The upper acetonitrile layer (8 mL) was transferred to a clean 15-mL tube, diluted with 2 mL of water (20 % water by volume), and vortexed. The extract (2.5 mL) was loaded onto a 3-mL Captiva EMR—Lipid tube, and allowed to flow under gravity. Once the extract had completely eluted through the Captiva EMR—Lipid tube (approximately 10 minutes), vacuum was applied and ramped from 1–10 in. Hg to drain the tube. For prespiked samples, 0.500 mL of eluent was transferred to autosampler tubes, and 0.300 mL of 5 mM ammonium formate with 0.1 % formic acid was added. Matrix-matched calibrants were prepared by transferring 0.500 mL of blank eluent to autosampler tubes, with 0.270 mL of 5 mM ammonium formate with 0.1 % formic acid and 0.030 mL of appropriate working standards.

Results and discussion

Linearity

The data were processed with Agilent MassHunter quantification software. Calibration curves gave R^2 values between 0.992 and 0.998 for 13 mycotoxins using linear regression fit and $1/x^2$ weighting. The accuracy of all calibrators was within ±10 % of expected values.

Accuracy and precision results

The study produced outstanding results, as shown by the summary in Table 2. Recovery for all QCs was 70 to 120 % and %RSD was <20 at all levels, with most %RSD <10. Fumonisins were the only challenging class of mycotoxin in this study due to poor extractability using acetonitrile. Optimization revealed that the addition of 2 % formic acid greatly enhanced analyte solubility without adversely affecting other classes.

EMR—Lipid mechanism

The EMR—Lipid selectivity is attributed to the combined mechanism of size exclusion and hydrophobic interaction. Lipids possess a linear, unbranched hydrocarbon chain, which is sufficiently small enough to enter the EMR—Lipid sorbent. Once inside the sorbent, the lipids are trapped in place by hydrophobic interaction. Most analytes do not contain a linear, unbranched hydrocarbon chain, and will not enter the sorbent, remaining in solution for analysis. Shorter hydrocarbon chains (<six carbons) are not as strongly bound by EMR—Lipid, and are not removed as efficiently as longer lipids. The unique EMR—Lipid mechanism is well suited to multiclass, multiresidue analysis where matrix interferences are targeted instead of diverse groups of analytes.

Table 2. Recovery and precision results for 13 mycotoxins in infant formula (n = 6).

	Infant formula liquid					
	LQ		MQ		HQ	
Analyte	%Recov.	%RSD	% Recov.	%RSD	% Recov.	%RSD
Aflatoxin M1	92.6	5.5	96.1	3.7	94.5	3.8
Aflatoxin G2	82.6	2.6	91.4	5.5	87.5	5.1
Aflatoxin G1	86.9	2.4	97.7	3.2	93.7	3.4
Aflatoxin B2	86.9	2.6	97.5	2.7	91.1	4.2
Aflatoxin B1	88.3	1.8	99.6	4.3	92.0	3.2
Fumonisin B1	80.6	2.1	89.2	3.7	82.5	2.0
Ochratoxin B	89.2	6.7	96.8	2.7	93.2	3.1
Mycophenolic acid	83.0	2.7	92.1	4.6	93.3	3.7
Fumonisin B3	87.0	4.4	88.1	5.8	90.1	4.6
Zearalenone	91.0	2.2	92.5	7.2	88.2	3.4
Fumonisin B2	82.2	5.3	88.5	4.9	88.0	5.0
Ochratoxin A	84.4	2.3	92.8	5.7	90.5	2.0
Sterigmatocystin	85.8	1.9	88.2	7.0	91.4	3.6

	Infant formula powder					
	LQ		MQ		HQ	
Analyte	%Recov.	%RSD	% Recov.	%RSD	% Recov.	%RSD
Aflatoxin M1	97.5	5.1	96.9	4.9	100.4	4.6
Aflatoxin G2	100.6	5.5	98.4	1.5	101.9	3.7
Aflatoxin G1	101.5	1.4	98.2	3.3	101.7	5.1
Aflatoxin B2	101.0	3.4	99.3	3.5	102.1	4.2
Aflatoxin B1	99.7	3.6	97.5	3.6	100.5	4.7
Fumonisin B1	85.1	11.8	85.7	11.1	92.9	12.0
Ochratoxin B	102.5	6.5	98.4	2.8	106.8	4.6
Mycophenolic acid	106.2	6.3	98.8	1.4	101.8	4.6
Fumonisin B3	76.0	12.8	94.2	17.2	96.8	5.1
Zearalenone	99.9	5.6	100.6	2.4	103.2	4.0
Fumonisin B2	70.4	13.7	73.2	7.6	72.4	10.4
Ochratoxin A	98.1	5.9	97.1	4.3	102.3	3.7
Sterigmatocystin	87.5	4.7	87.3	2.7	93.1	3.3

Competitive comparison—recovery and precision

Recovery and precision were evaluated for Captiva EMR—Lipid (6-mL cartridge) and a commercially available pass-through cleanup product (competitor A, 6 mL, 500 mg). In this evaluation, infant formula extracts were spiked directly to negate any extraction contributions to recovery and precision. Table 3 summarizes the results, and indicates higher recovery for Captiva EMR—Lipid, especially for zearalenone, ochratoxin A, and sterigmatocystin. The unique sorbent chemistry of Captiva EMR—Lipid allows selective capture of lipids while currently available products often give unwanted analyte retention, especially for more hydrophobic analytes.

Monitoring matrix removal by GC/MS

Although validation is accomplished using LC/MS, the GC/MS full scan comparison of sample cleanups can give valuable information regarding the removal of matrix and lipids. Figure 1 shows the GC/MS full scan chromatogram of infant formula before and after cleanup with Captiva EMR—Lipid. The black trace is the chromatogram generated from no sample cleanup, and represents lipids as well as other matrix co-extractives. Liquid infant formula after Captiva EMR—Lipid cleanup (purple) shows 90 % removal, calculated using Equation 1. While later-eluting matrix is completely removed, early-eluting matrix is significantly reduced but not completely removed.

Table 3. Recovery and precision comparison of Captiva EMR—Lipid and competitor A pass-through cleanups (infant formula, 5 ng/g, n = 4).

Analyte	Captiva EMR—Lipid		Competitor A	
	% Recovery	%RSD	% Recovery	%RSD
Aflatoxin M1	96.1	3.6	93.5	4.4
Aflatoxin G2	100.9	0.5	89.5	4.4
Aflatoxin G1	102.4	1.6	86.1	4.8
Aflatoxin B2	100.8	3.2	84.2	4.7
Aflatoxin B1	98.4	4.0	85.3	5.5
Fumonisin B1	96.6	3.4	77.3	3.8
Ochratoxin B	104.9	6.4	76.7	7.5
Mycophenolic acid	90.8	7.2	79.3	7.0
Fumonisin B3	103.1	11.6	76.8	11.5
Zearalenone	96.1	3.1	46.7	7.5
Fumonisin B2	85.0	6.9	85.1	9.6
Ochratoxin A	95.1	10.9	66.4	11.7
Sterigmatocystin	99.6	4.1	50.1	10.3

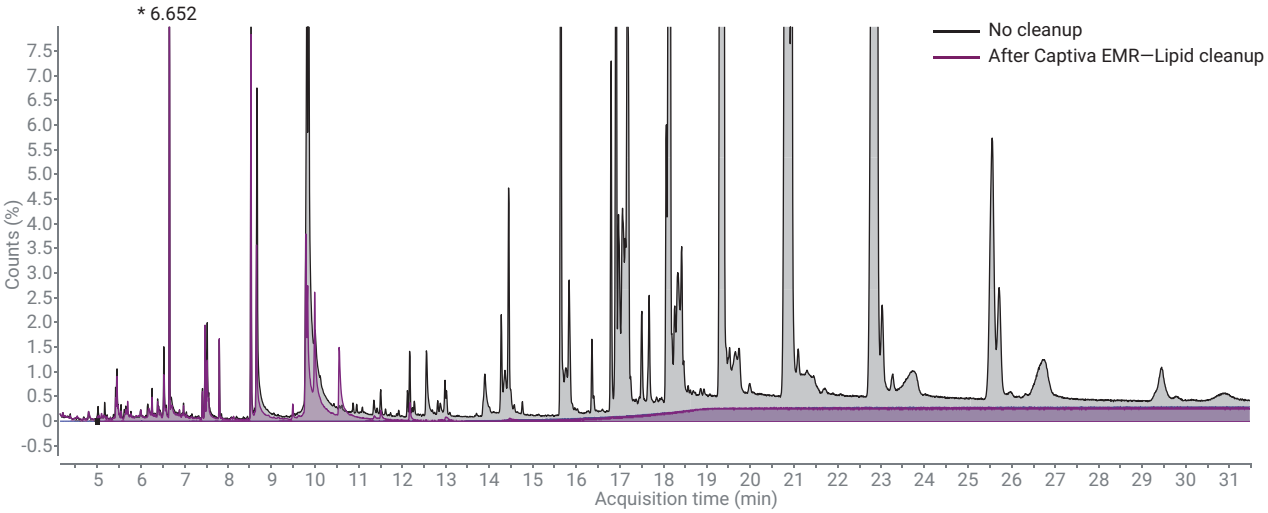

Figure 1. Matrix removal evaluation using GC/MS full scan chromatogram comparison of infant formula sample before and after Captiva EMR—Lipid cleanup.

$$\% \text{ Matrix Removal} = \frac{(\text{Peak Area}_{\text{Blank no cleanup}} - \text{Peak Area}_{\text{Blank Captiva cleanup}})}{(\text{Peak Area}_{\text{Blank no cleanup}} - \text{Peak Area}_{\text{Reagent blank}})} \times 100$$

Equation 1. Calculation for percent matrix removal using total peak area from chromatograms.

Conclusion

This work demonstrates that Captiva EMR—Lipid is an easy and effective cleanup option for multiclass mycotoxin analysis. Validation of infant formula gave excellent recovery (70.4 to 106.8 %), precision (<18 %), and sensitivity down to 1.0 ng/g. Efficient cleanup was demonstrated through GC/MS full scans. A product comparison shows significantly higher recovery of mycotoxins using Captiva EMR—Lipid than the other commercially available cleanup product. Matrix removal for lipids and analyte recovery was high for a wide variety of applications, some of which extend beyond the scope of this work[5,6]. Captiva EMR—Lipid represents a new generation in selective lipid cleanup for multiclass, multiresidue analysis, and is ideal for laboratories looking to simplify sample preparation while improving method performance.

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