Application Note

Food

Multiclass Mycotoxin Analysis in Cheese Using Agilent Captiva EMR—Lipid Cleanup and LC/MS/MS

Authors

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Abstract

Agilent Captiva EMR—Lipid, a lipid removal product, was used for the cleanup of blue and parmesan cheese extract containing multiclass mycotoxins. The high fat content of cheese can present challenges for the accurate quantitation of mycotoxins at low levels. Captiva EMR—Lipid combines size exclusion and hydrophobic interaction to selectively capture lipid hydrocarbon chains and not target analytes. Available in 3 and 6 mL, Captiva EMR—Lipid cartridges allow pass-through cleanup of fatty sample extract. Cleaned extract can be directly injected onto the LC/MS or post treated as necessary to meet method requirements. This work describes the validation of 13 multiclass mycotoxins in blue and parmesan cheese using a QuEChERS workflow followed with Captiva EMR—Lipid cartridge cleanup. The method allows detection of mycotoxins down to 0.5 ng/g in cheese with recoveries of 70.7–111.8 % and RSD <20 %. Matrix removal efficiency was assessed using residue gravimetric analysis, GC/MS full scan, LC/MS detection of phospholipids, and lipid freeze-out.

Introduction

Mycotoxins are produced as secondary metabolites from fungi species on various crops, and are associated with mutagenic, carcinogenic, teratogenic, and immunogenic effects[1]. Contamination in cheese can originate from ingredients, or through the natural synthesis by incurred fungal strains. Cheeses are especially susceptible to mold growth, and influenced by storage conditions and chemical preservative content[2,3]. Detection and measurement of low concentrations of harmful classes of mycotoxins in complex samples can be accomplished using various immunoassays or LC/MS methods combined with a sample preparation technique such as immunoaffinity, SPE, QuEChERS[4], or stable isotope dilution[5]. High fat samples can be especially problematic, and many cleanup products such as immuno-based columns are expensive and specific to the analyte, class, and sample type. Other cleanup materials struggle to effectively and selectively remove matrix co-extractives, especially lipids, causing poor reproducibility, matrix effects, and accumulation on the instrument.

Agilent Captiva EMR—Lipid 3 and 6 mL tubes provide a simple pass-through cleanup, delivering selective lipid removal from fatty sample extract for multiclass mycotoxins analysis. A Quick Easy Cheap Effective Rugged Safe (QuEChERS) extraction was used for the extraction of 13 mycotoxins from cheese. QuEChERS is known for high extraction efficiency for a wide range of analyte classes, however QuEChRS can also extract a large amount of matrix. Captiva EMR—Lipid cartridges provide high lipid removal and allow accurate quantitation of the target mycotoxins. Removal of lipids was evaluated using gravimetric determination of matrix co-extractive residue, GC/MS full scan, phospholipid analysis by LC/MS/MS, and lipid freeze-out comparison. The method was validated for blue and parmesan cheese at three spike levels for aflatoxins (AF-B1, B2, G1, G2, M1), ochratoxins (OTA, OTB), fumonisins (FB1, FB2, FB3), zearalenone (ZON), mycophenolic acid (MPA), and sterigmatocystin (STC). The method delivered excellent recovery, precision, and sensitivity for trace level of mycotoxins in these high-fat sample matrices.

Experimental

Sample Preparation

- Agilent Captiva EMR—Lipid 3 mL tubes (p/n 5190-1003)
- Agilent QuEChERS original extraction salts (p/n 5982-5555)
- Agilent VacElut SPS-24 vacuum manifold (p/n 12234022)

LC configuration and Parameters

Configuration	
Components	Agilent 1290 Infinity II high speed pump (G7120A) Agilent 1290 Infinity II multisampler (G7167B) Agilent 1290 Infinity II multicolumn thermostat (G7116B)
Analytical column	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 ACN:Methanol + 0.1 % formic acid
Flow rate	0.5 mL/min
Gradient	Time(min) %B 0 5 1 50 4 60 7 98
Post time	2 minutes
Needle wash	1:1:1, H_2O, ACN, IPA 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 and Parameters

Configuration	
	Agilent 6460 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	3,500 V
EMV	500 V (+) 0 V (–)
Nozzle voltage	1,500 V (+) 0 V (–)

Compound	Precursor ion	Quantifier ion (CE)	Qualifier ion (CE)	Fragment (V)	Retention time (min)
Aflatoxin M1 (AF-M1)	329.1	313.0 (24)	115.1 (88)	135	1.842
Aflatoxin G2 (AF-G2)	331.1	313.0 (24)	115.1 (88)	165	1.916
Aflatoxin G1 (AF-G1)	329.1	243.2 (24)	200.0 (44)	175	2.018
Aflatoxin B2 (AF-B2)	315.1	287.0 (28)	259.0 (32)	175	2.104
Aflatoxin B1 (AF-B1)	313.1	285.2 (24)	128.1 (84)	170	2.223
^{13}C Aflatoxin B1 (IS)	330.1	301.1 (24)	–	170	2.223
Fumonisin B1 (FB1)	722.4	352.3 (36)	334.4 (44)	200	2.810
Ochratoxin B (OTB)	370.0	205.0 (16)	120.1 (96)	120	3.282
Mycophenolic acid (MPA)	321.1	302.9 (4)	206.9 (20)	90	3.304
Fumonisin B3 (FB3)	706.4	336.3 (36)	318.5 (40)	200	3.780
Zearalenone (ZON)	317.1	175 (24)	131 (28)	175	4.155
Fumonisin B2 (FB2)	706.4	336.3 (36)	318.5 (40)	200	4.511
Ochratoxin A (OTA)	404.1	239.0 (24)	120.1 (96)	120	4.604
Sterigmatocystin (STC)	325.0	310.0 (24)	102.1 (96)	120	4.685

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 from Sigma-Aldrich (St Louis, MO, USA) or Romer Labs (Getzersdorf, Austria) as premixed solutions. LC solvents were bought from Honeywell (Muskegon, MI, USA).

Validation Study

The validation of mycotoxins in cheeses 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 five (n = 5) and injected in between two sets of calibration curves. Calibration curves were generated using six levels: 0.25, 1, 5, 10, 20, and 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, and 20 ng/mL for AF-M1 and OTB; and 1.25, 5, 25, 50, 100, and 200 ng/mL for FB1, FB2, and FB3. Isotopically labeled internal standard ^{13}C-AF-B1 was spiked at 5 ng/mL.

Table 1. Sample QC concentrations.

Analyte	LQ (ng/g)	MQ (ng/g)	HQ (ng/g)
AF-B1	1	5	10
AF-B2	1	5	10
AF-G1	1	5	10
AF-G2	1	5	10
AF-M1	0.5	2.5	5
FB1	5	25	50
FB2	5	25	50
FB3	5	25	50
MPA	1	5	10
OTA	1	5	10
OTB	0.5	2.5	5
STC	1	5	10
ZON	1	5	10

Sample Preparation Detailed Procedure

Two grams of cheese were weighed into a 50-mL centrifuge tube. Calibrators and QCs were prespiked at appropriate levels, and thoroughly soaked into the cheese matrix for >1 hour before extraction. Next, 10 mL of water was added and allowed to soak into the sample for 5 minutes. The sample was extracted with 10 mL of acetonitrile with 2 % formic acid using vertical shaking on a Geno/Grinder for 20 minutes. QuEChERS original salts (4 g $MgSO_4$, 1.5 g NaCl) was added to the sample, and vertically shaken again for 2 minutes. Samples were centrifuged at 5,000 rpm for 5 minutes. The upper acetonitrile layer was transferred to a clean tube (8 mL) and diluted with 2 mL of water (20 % water by volume) and vortexed. The extract was loaded (2.5 mL) onto a 3 mL Captiva EMR—Lipid tube, and allowed to flow under gravity. Once the extract was 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. Then, 1.25 mL of eluent was transferred to a clean test tube, dried at 40 °C under nitrogen flow, and reconstituted with 200 µL of 85:15, 5 mM ammonium formate:acetonitrile using vortex and sonication. The sample was transferred to autosampler vials for LC/MS/MS analysis.

Results and Discussion

Linearity

The data were processed with Agilent MassHunter quantification software. Calibration curves gave R^2 values >0.990 for 13 mycotoxins using linear regression fit and $1/x^2$ weighting. The accuracy of all calibrators was within ±10 % of expected values.

Recovery and Precision Results

The summary in Table 2 shows that the study produced outstanding results. Recovery for all QCs were 70–120 %, and %RSD <20 at all levels, most of which had a %RSD <10. Parmesan cheese gave better overall reproducibility, likely due to lower matrix complexity relative to blue cheese. Early studies avoided the use of a final concentration step, however, it was necessary to concentrate considering the small sample size and low detection limits. Due to limited extractability using acetonitrile, fumonisins were the only challenging class of mycotoxin in this study. Optimization revealed that the addition of 2 % formic acid greatly enhanced analyte solubility without adversely affecting other classes.

Table 2. Recovery and precision results for 13 mycotoxins in blue and parmesan cheeses (n = 5).

	LQ		MQ		HQ	
Analyte	% Recovery	%RSD	% Recovery	%RSD	% Recovery	%RSD
Parmesan cheese						
AF-M1	111.8	1.5	95.6	5.9	96.3	1.7
AF-G2	101.8	2.2	98.5	3.8	104.6	3.2
AF-G1	102.2	2.8	89.1	2.2	93.9	6.6
AF-B2	108.5	1.5	101.5	4.2	103.5	2.4
AF-B1	103.2	5.1	84.9	2.7	90.3	2.9
FB1	79.4	6.7	71.3	3.2	74.2	2.2
OTB	109.6	1.7	98.5	7.2	106.0	1.8
MPA	111.3	8.6	103.6	2.1	107.5	4.6
FB3	98.2	7.1	90.6	8.1	92.0	5.0
ZON	98.0	7.8	85.8	4.0	88.2	2.8
FB2	101.9	5.5	92.4	7.8	95.6	3.8
OTA	104.7	10.4	89.4	5.7	92.6	2.5
STC	85.4	3.4	70.7	2.3	75.7	2.5
Blue cheese						
AF-M1	97.0	17.8	101.2	8.8	107.9	5.8
AF-G2	88.6	12.4	96.1	6.3	98.3	8.6
AF-G1	91.8	9.1	97.5	2.5	105.5	3.2
AF-B2	98.2	13.8	99.7	8.8	108.5	8.1
AF-B1	91.8	7.9	93.5	5.7	102.4	6.2
FB1	103.9	7.9	83.5	5.4	85.3	5.8
OTB	81.5	7.1	79.9	3.9	89.0	5.8
MPA	92.4	10.3	95.0	1.8	95.4	8.0
FB3	101.9	5.7	93.9	5.0	94.3	7.7
ZON	76.1	3.9	83.3	9.6	90.2	9.3
FB2	102.0	4.7	100.6	5.9	99.4	3.9
OTA	89.0	3.4	82.5	7.9	84.9	5.5
STC	100.0	3.0	74.3	13.4	70.9	6.8

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, thereby remaining in solution for analysis. Shorter hydrocarbon chains (<6 carbons) are not as strongly bound by EMR—Lipid, and are not completely 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.

Competitive Comparison – Recovery and Precision

Recovery and precision were evaluated for Captiva EMR—Lipid and a commercially available pass-through cleanup cartridge from another manufacturer 6 mL, 500 mg. In this evaluation, cheese extracts were spiked directly to negate any extraction contributions on recovery and precision. Table 3 summarizes the results, and indicates higher recovery provided by Captiva EMR—Lipid cartridge cleanup, especially for compounds 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.

Matrix Removal

Cheeses contain various different lipid classes including free fatty acids, triglycerides, and some low-level phospholipids. Proteins are effectively removed during the acetonitrile-based QuEChERS extraction. Lipid removal was evaluated using quantitative and qualitative methods including gravimetric determination of residue, GC/MS full scan, LC/MS/MS for phospholipids, and lipid freeze-out.

Table 3. Recovery and precision comparison of Agilent Captiva EMR—Lipid and another manufacturer's cartridge pass-through cleanups (parmesan cheese extract, 5 ng/mL, n = 4).

	Agilent Captiva EMR—Lipid cartridge		Other manufacturer's cartridge	
	% Recovery	%RSD	% Recovery	%RSD
AF-M1	96.1	3.6	93.5	4.4
AF-G2	100.9	0.5	89.5	4.4
AF-G1	102.4	1.6	86.1	4.8
AF-B2	100.8	3.2	84.2	4.7
AF-B1	98.4	4.0	85.3	5.5
FB1	96.6	3.4	77.3	3.8
OTB	104.9	6.4	76.7	7.5
MPA	90.8	7.2	79.3	7.0
FB3	103.1	11.6	76.8	11.5
ZON	96.1	3.1	46.7	7.5
FB2	85.0	6.9	85.1	9.6
OTA	95.1	10.9	66.4	11.7
STC	99.6	4.1	50.1	10.3

Monitoring Matrix Removal by GC/MS

Although the validation is accomplished using LC/MS/MS, the GC/MS full scan comparison of sample final extract can give valuable information regarding the removal of matrix and lipids. $MgSO_4$-based salting out was used to remove the water residue in the sample extract after Captiva EMR—lipid cleanup. Figure 1 shows the GC/MS full scan chromatograms of blue and parmesan cheeses before and after cleanup with Captiva EMR—Lipid. As shown, the black traces are the chromatograms generated from sample extract without cleanup, and represent lipids as well as other matrix co-extractives. Red traces are the chromatograms generated from sample extract with Captiva EMR—Lipid cartridge cleanup. Blue cheese after Captiva EMR—Lipid cleanup (red) shows 61 % matrix removal, and parmesan cheese after cleanup (purple) shows 68 % matrix removal, and was calculated using Equation 1. While later-eluting matrix is completely removed, early-eluting matrix is significantly reduced but not completely removed. It is also evident that although the chromatographic profiles of cheese are compositionally similar, blue cheese contains more free fatty acids than parmesan cheese.

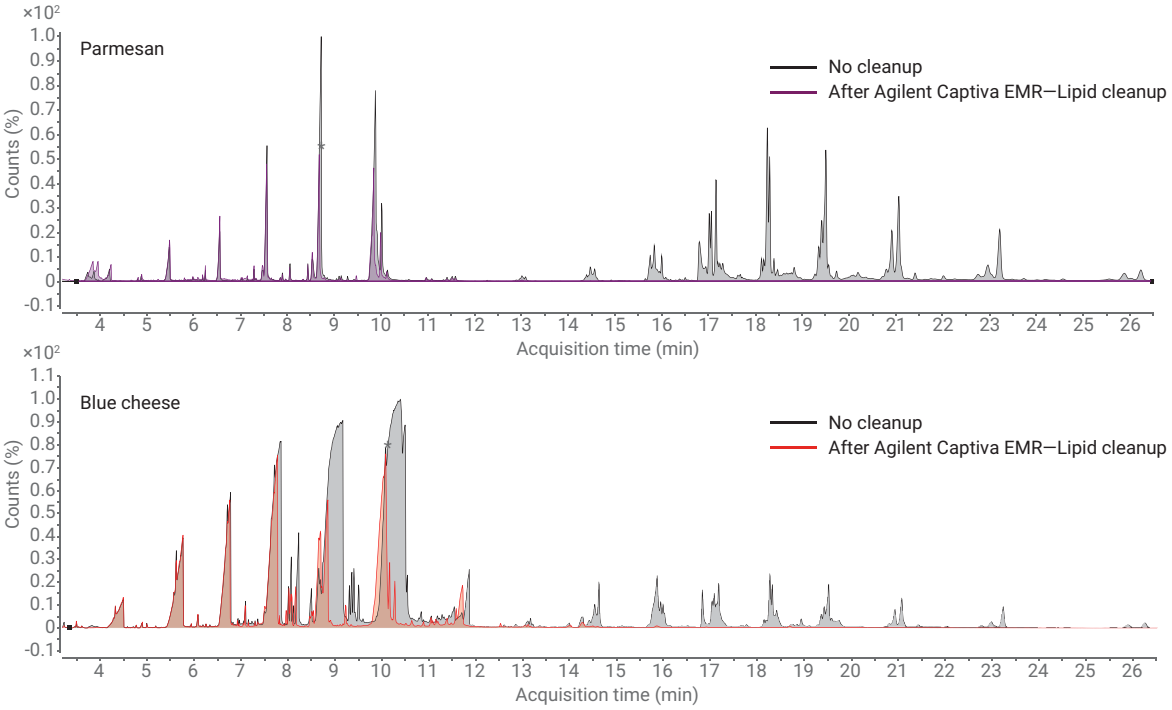

Figure 1. Matrix removal evaluation using GC/MS full scan chromatogram comparison of cheese samples before cleanup and after Agilent Captiva EMR—Lipid cleanup.

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

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

Phospholipid Removal Evaluation

The chromatogram comparison of phospholipid removal in Figure 2 was generated using LC/MS/MS precursor ion scan for *m/z* = 184 product ion. Overall, phospholipid concentration was low in blue cheese and insignificant in parmesan cheese. The black trace is unremoved phospholipids from the blue cheese extract, and the red trace is after Captiva EMR–Lipid cleanup. The matrix removal from Captiva EMR–Lipid was calculated at 92 % using Equation 1.

Gravimetric Determination of Co-extractive Residue

The co-extractive residue weight was collected by evaporating 1.25 mL of eluent, and removal efficiency by cleanup was calculated using co-extractive residue weight with cleanup and without clean up. Table 4 summarizes the results, demonstrating significant matrix removal.

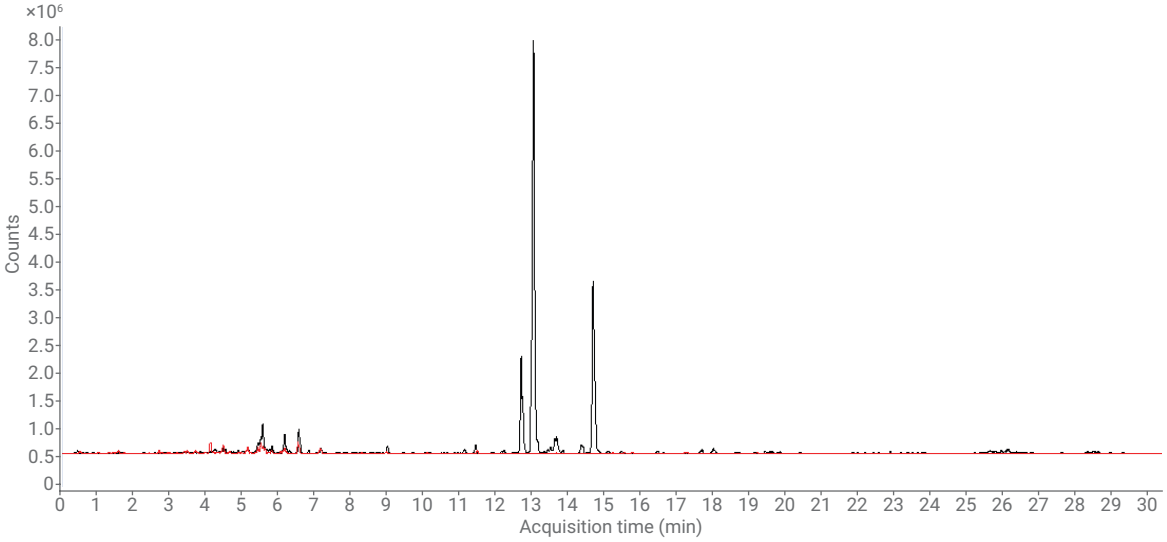

Figure 2. Phospholipid removal in blue cheese by LC/MS/MS precursor ion scan for *m/z* = 184.

Table 4. Co-extractive residue mass and matrix removal efficiency for blue cheese and parmesan cheese using Agilent Captiva EMR—Lipid cleanup.

	Co-extractive mass (mg)	Matrix co-extractive removal efficiency (%)
Blue cheese: no cleanup	12.76	–
Blue cheese: Agilent Captiva EMR—Lipid	6.22	51.3
Parmesan cheese: no cleanup	5.81	–
Parmesan cheese: Agilent Captiva EMR—Lipid	1.50	74.2

Lipid Freeze-Out

A qualitative comparison placed untreated cheese samples and Captiva EMR—Lipid treated samples in a freezer at 0 °C for 1 hour, and recorded precipitated lipid observations (Figure 3). As shown, untreated blue cheese contains a large amount of precipitated fats while parmesan shows a small amount clinging to the plastic vial. Captiva EMR—Lipid treated samples contained no observable fats after lipid freeze-out.

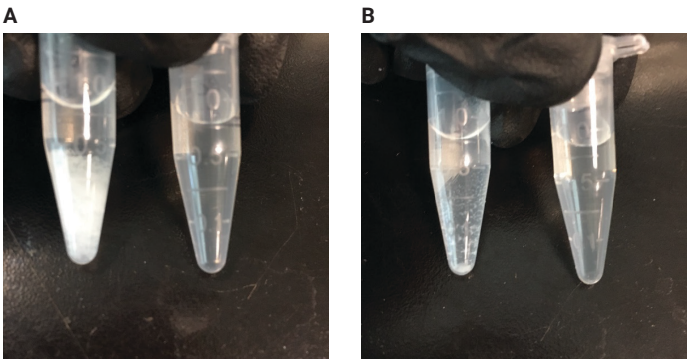

Figure 3. Lipid freeze-out experiment for Agilent Captiva EMR—Lipid cleanup of blue cheese (A) and parmesan cheese (B).

Conclusions

This work demonstrates that Agilent Captiva EMR—Lipid provides an easy and effective cleanup option for multiclass mycotoxins analysis. Method validation in blue and parmesan cheese gave excellent recovery (70.7–111.8 %), precision (<20 %), and sensitivity down to 0.5 ng/g in cheese. Efficient cleanup was demonstrated through gravimetric analysis, GC/MS full scan, phospholipid analysis, and lipid freeze-out comparisons. Blue cheese was more complex than parmesan, as demonstrated, and benefited from a 2 g sample size. Up to 5 g of parmesan cheese can be used by the validated protocol when there is a need for lower detection limit in cheese. A product comparison shows significantly higher recovery using Captiva EMR—Lipid than the other commercially available cleanup product. Matrix removal for lipids and analyte recovery is high for a wide variety of applications, some of which extends beyond the scope of this work[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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