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Trusted Answers

Complex Matrices: Minimizing Lipids, Maximizing Recovery

Food Testing Application Compendium: Volume 3

Complex Samples Don't Have to Complicate Your Analysis

Reducing matrix interference is a must for maintaining sensitivity standards—particularly for multiresidue, multiclass analysis of food samples.

Minimize interferences in complex samples by applying the latest SPE, filtration, and QuEChERS sample preparation techniques

Effective sample preparation allows you to selectively remove interferences while leaving analytes of interest intact. That means your lab will benefit from:

- More reproducible results and greater data accuracy
- Reduced sample variability and error
- Time and resource savings on sample-related instrument maintenance

What's more, your lab can improve productivity by standardizing its sample preparation processes.

What's Inside?

In this compendium, you'll find current and emerging applications covering challenging food matrices, such as high-fat produce, fish, meat, and milk products. And as always, our product and application experts are available to help you maximize productivity and analyte recovery.

Sample Preparation Techniques for Food Testing

Sample preparation for food testing applications has long been recognized as time-consuming and error-prone, due to the number of steps involved. Traditionally, analytes of interest are selectively retained by the SPE sorbent, while other matrix components are washed away, and analytes of interests are eluted.

To help you reduce the need for repeated runs, and minimize interferences that can compromise your results, we developed the Agilent Food Testing Application Compendium series.

Volume 1 covers SPE techniques used in food analysis

By striking a balance between your sample preparation budget, required method ruggedness, and mandated selectivity and sensitivity, you can achieve the right level of cleanup.

Volume 2 focuses on QuEChERS techniques

For multiclass, multiresidue analysis of food samples, QuEChERS methodology significantly reduces your burden by switching the focus from analyte enrichment to matrix removal. Together with modern Tandem Quadrupole technology, QuEChERS techniques have been widely adopted throughout the industry.

Volume 3 highlights Agilent Captiva EMR–Lipid

For complex samples that include large amounts of fats/lipids, existing QuEChERS dispersive SPE (dSPE) can be insufficient for cleanup, leading to inaccurate analyses. EMR—Lipid is an innovative material that efficiently removes major lipid classes from sample matrices without analyte loss. It works through a unique combination of size exclusion and hydrophobic interaction, and includes these formats:

- Bond Elut EMR—Lipid dSPE streamlines the QuEChERS workflow for fatty samples.
- Captiva EMR—Lipid allows a "pass-through" cleanup workflow, further simplifying sample preparation.

On the following pages, you'll find application notes that describe the use of EMR—Lipid in complex food sample preparation and analysis.

Maximize cleanup in complex matrices.
Visit **www.agilent.com/chem/captiva-emr-lipid**

Promoting The Quality of Our Food

The Agilent food analysis workflow helps your lab ensure consistent quality throughout the food production chain—including incoming inspection, new product development, and packaging.

Preparation

SPE products

Agilent Bond Elut SPE products selectively remove interferences from complex matrices, and let you choose from over 40 phase functionalities in more than 30 formats.

QuEChERS

Easily clean up a variety of food samples with Bond Elut QuEChERS extraction and dispersive SPE kits.

Captiva EMR—Lipid

Save time and improve precision on cleanup of dirty complex samples with Captiva EMR—Lipid pass-through cleanup cartridges.

Analysis

Identify contaminants, authenticate ingredients, and ensure product quality with these Agilent solutions:

Multi-target screening: Agilent 6400 Series Triple Quadrupole LC/MS and 7000C QQQ GC/MS

Non-targeted analysis: Agilent accurate-mass 6500 Series LC/Q-TOF and 7250 GC/Q-TOF

Columns and supplies: Agilent J&W Ultra Inert GC columns and InfinityLab Poroshell 120 LC columns help you achieve lower LODs and more accurate data

Results

MassHunter Workstation

Produce high-quality MS data—and use that data to identify and quantify targets and unknowns.

OpenLab

Maximize the business value of scientific data across its entire life cycle.

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Pesticide Analysis

Multiresidue Analysis of Pesticides in Avocado with Bond Elut EMR—Lipid by LC/MS/MS

Multiresidue Analysis of Pesticides in Avocado with Bond Elut EMR—Lipid by GC/MS/MS

Benefits of EMR—Lipid Cleanup with Enhanced Post Treatment on Pesticides Analysis by GC/MS/MS

Multiresidue Analysis of Pesticides in Avocado with Agilent Bond Elut EMR—Lipid by LC/MS/MS

Application Note

Food Testing and Agriculture

Authors

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Abstract

Agilent Bond Elut QuEChERS Enhanced Matrix Removal-Lipid (EMR—Lipid) is the next generation of sample preparation products, and is used in convenient, dispersive solid phase extraction (dSPE) for highly selective matrix removal without impacting analyte recovery, especially for high-fat samples. This study demonstrates the application of this novel product for the analysis of 44 multiclass pesticides in avocado by LC/MS/MS. The procedure involves a QuEChERS AOAC extraction followed by the use of EMR—Lipid dSPE and EMR—Lipid polish salts, providing fast and effective sample cleanup. The matrix cleanup was evaluated by determining the amount of nonvolatile coextractives from an avocado extract after different dSPE cleanup, and by evaluating chromatographic matrix effects for target analytes. Compared to other matrix cleaning products, EMR—Lipid dSPE provides much more efficient matrix cleanup without impacting analyte recoveries. The optimized method delivers excellent accuracy and precision for all 44 LC-amenable pesticides in avocado by LC/MS/MS. The EMR—Lipid dSPE conveniently fits into a QuEChERS protocol, providing fast, robust, and effective sample preparation for pesticide residue analysis in high-fat avocado samples.

Agilent Technologies

Introduction

Pesticide residue analysis in food commodities is routine for many laboratories using the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method [1,2]. This allows analysis of hundreds of pesticides at low concentrations with a single extraction. While the method has worked well for various fruits and vegetables, foods high in fat such as avocado, nuts, and foods of animal origin present new challenges [3,4]. Overcoming these challenges is a high priority for laboratories tasked with reaching the stringent validation criteria required by government agencies to ensure that food is safe for consumption.

Analysis can use a combination of LC and GC to accommodate volatile, semivolatile and nonvolatile pesticides associated with many multiclass, multiresidue methods [4]. While many pesticides are amenable to both LC and GC, many are not. Each chromatographic technique has its inherent advantages and disadvantages in terms of analyte quantitation and adverse effects from coextracted matrix. Removal of these coextractives is essential to accurate quantitation in complex food matrices, requiring treatment with matrix removal sorbents such as C18, PSA, and GCB [5]. Other materials containing zirconia are commercially available, and generally improve lipid removal when compared to typical matrix removal sorbents. However, it does not target all lipid classes and can retain analytes of interest [6,7]. Samples high in lipid content may also require cleanup using solid phase extraction cartridges (SPE) [7,8,9] or gel permeation chromatography (GPC) [10], adding time and cost to an otherwise routine analysis.

Agilent Bond Elut EMR—Lipid is a novel sorbent material that selectively removes major lipid classes from sample matrix without unwanted analyte loss. Removal of lipid interferences from complicated matrices is especially important for techniques such as QuEChERS and protein precipitation, as these methods coextract large amounts of matrix with the target analytes. This study investigates sample preparation for the analysis of 44 LC-amenable representative pesticides in avocado using a QuEChERS AOAC extraction followed by EMR—Lipid dSPE cleanup. The pesticides represent 12 different chemical classes to establish proof of concept for analytes that were not included in this application note. Table 1 lists the LC-amenable pesticides and their classes. This application note demonstrates the exceptional cleanliness that EMR—Lipid provides for complex, fatty samples such as avocado, and the high recovery and precision for 44 multiclass pesticide residues at three levels.

Table 1. LC-amenable pesticides used in this study and their associated chemical classes.

Representative pesticide	Chemical class	Pesticide group
Methamidophos	Organophosphate	Insecticide
Acephate	Organophosphate	Insecticide
Omethoate	Organophosphate	Insecticide
Dimethoate	Organophosphate	Insecticide
Malathion	Organophosphate	Insecticide
EPN	Organophosphate	Insecticide
Tepp-A	Organophosphate	Insecticide
Monocrotophos	Organophosphate	Insecticide
Mexacarbate	Carbamate	Insecticide
Carbaryl	Carbamate	Insecticide
Propoxur	Carbamate	Insecticide
Carbofuran	Carbamate	Insecticide
Methiocarb	Carbamate	Insecticide
Chlorpropham	Carbamate	Insecticide
Propham	Carbamate	Insecticide
Aminocarb	Carbamate	Insecticide
Oxamyl	Carbamate	Insecticide
Methomyl	Carbamate	Insecticide
Aldicarb	Carbamate	Insecticide
Terbuthylazine	Triazine	Algaecide
Simazine	Triazine	Herbicide
Sebuthylazine	Triazine	Herbicide
Monuron	Urea	Herbicide
Chlorotoluron	Urea	Herbicide
Diuron	Urea	Herbicide
Fluometuron	Urea	Herbicide
Isoproturon	Urea	Herbicide
Metobromuron	Urea	Herbicide
Siduron	Urea	Herbicide
Linuron	Urea	Herbicide
Neburon	Urea	Herbicide
Fenuron	Urea	Herbicide
Metoxuron	Urea	Herbicide
Carbendazim	Benzimidazole	Fungicide
Thiabendazole	Benzimidazole	Fungicide
Thiophanate methyl	Benzimidazole	Fungicide
Cyprodinil	Anilinopyrimidine	Fungicide
Imazalil	Imidazole	Fungicide
Penconazole	Triazole	Fungicide
Imidacloprid	Neonicotinoid	Insecticide
Metazachlor	Chloracetanilide	Herbicide
2,4-D Acid	Chlorophenoxy acid	Herbicide
Dichlorprop	Chlorophenoxy acid	Herbicide
Bentazon	Unclassified	Herbicide

Experimental

All reagents and solvents were HPLC or analytical grade. Acetonitrile (ACN) and methanol were from Honeywell (Muskegon, MI, USA). Reagent grade acetic acid (AA) was from Sigma-Aldrich, Corp. (St Louis, MO, USA). Pesticide standards and internal standard were from Sigma-Aldrich, Corp. and AccuStandard (New Haven, CT, USA).

Solution and standards

Acetonitrile containing 1% AA was prepared by adding 10 mL acetic acid to 990 mL ACN. Standard and internal standard (IS) stock solutions were made for some of the pesticides in either ACN or methanol at 2.0 mg/mL. The rest of the pesticide standards were from commercial mixed standard stock solutions, which were used directly to prepare the standard working solution. A combined working solution was prepared in ACN at 25 µg/mL. A 25 µg/mL aliquot of TPP IS working solution was prepared in ACN.

Equipment

Equipment and material used for sample preparation included:

- Geno/Grinder (SPEX, Metuchen, NJ, USA)
- Centra CL3R centrifuge (Thermo IEC, MA, USA)
- Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)
- Vortexer and multitube vortexers (VWR, Radnor, PA, USA)
- Bottle top dispenser (VWR, So. Plainfield, NJ, USA)
- Eppendorf pipettes and repeater
- Agilent Bond Elut EMR—Lipid tubes (p/n 5982-1010) and Agilent Bond Elut Final Polish for Enhanced Matrix Removal—Lipid tubes(p/n 5982-0101)

Instrumentation

Analysis was performed on an Agilent 1290 Infinity LC consisting:

- Agilent 1290 Infinity Quaternary Pump (G4204A)
- Agilent 1290 Infinity High Performance Autosampler (G4226A) equipped with an Agilent 1290 Infinity Thermostat (G1330B), and an Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)

The UHPLC system was coupled to an Agilent 6490 Triple Quadrupole LC/MS system equipped with an Agilent Jet Stream electrospray ionization source and iFunnel technology. Agilent MassHunter workstation software was used for data acquisition and analysis.

Instrument conditions

HPLC conditions

Column:	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm (p/n 959759-902), Agilent ZORBAX RRHD Eclipse Plus C18 UHPLC Guard, 5 × 2.1 mm, 1.8 µm (p/n 821725-902)	
Mobile phase:	A) 0.1% FA in water B) 0.1% FA in acetonitrile	
Flow rate:	0.3 mL/min	
Column temp:	35 °C	
Autosampler temp:	4 °C	
Inj vol:	3 µL	
Needle wash:	1:1:1:1 ACN:MeOH:IPA:H_2O with 0.2% FA	
Gradient:	Time (min) 0 15 15.01	%B 10 95 100
Stop time:	16 min	
Posttime:	3 min	

MS conditions

Positive/negative mode

Gas temp:	120 °C	
Gas flow:	14 L/min	
Nebulizer:	40 psi	
Sheath gas heater:	400 °C	
Sheath gas flow:	12 L/min	
Capillary:	3,000 V	
iFunnel parameters:	Positive	Negative
High-pressure RF:	100 V	90 V
Low-pressure RF:	70 V	60 V

MS MRM conditions relating to the analytes are listed in Table 2, and a typical chromatogram is shown in Figure 1.

Table 2. LC triple quadrupole MRM parameters and retention times for the pesticides used in this study.

Analyte	RT (min)	Delta RT (min)	Polarity	Precursor ion (*m/z*)	Product ion (*m/z*)	CE (v)
Methamidophos	1.83	2	Positive	142	94.1	9
Aminocarb	2.03	2	Positive	209.1	137.2	24
Acephate	2.13	2	Positive	184	143	9
Omethoate	2.54	2	Positive	214	124.9	17
Carbendazim	3.40	2	Positive	192.1	132	33
Thiabendazole	3.89	2	Positive	202	131.1	41
Mexacarbate	3.99	2	Positive	223.1	151.1	20
Oxamyl	4.24	2	Positive	237.1	72	12
Monocrotophos	4.46	2	Positive	224.1	127	10
Methomyl	4.64	2	Positive	163.1	106	4
Fenuron	6.17	2	Positive	165.1	72	20
Imidacloprid	6.43	2	Positive	256.1	209.1	13
Dimethoate	6.63	2	Positive	230	199	5
TEPP-A	7.69	2	Positive	291.1	179	20
Aldicarb	7.87	2	Positive	213.1	89.1	15
Metoxuron	7.89	2	Positive	229	46.1	12
Imazalil	7.99	2	Positive	297.1	158.9	25
Simazine	8.31	2	Positive	202.1	132	22
Monuron	8.37	2	Positive	199.1	46.1	16
Thiophanate methyl	8.95	2	Positive	343.1	151.2	4
Propoxur	9.15	2	Positive	210.1	111.1	9
Carbofuran	9.30	2	Positive	222.1	123.1	30
Chlorotoluron	9.54	2	Positive	213.1	72	20
Diuron	9.65	2	Positive	233	72.1	20
Carbaryl	9.73	2	Positive	202.1	145.1	9
Bentazone	9.73	2	Negative	239	132	15
Isoproturon	9.96	2	Positive	207.1	46.1	20
2,3-D acid	10.06	2	Negative	219	161	15
Fluometuron	10.10	2	Positive	233.1	72	16
Metobromuron	10.48	2	Positive	259	148	10
Cyprodinil	10.53	2	Positive	226.1	93.1	41
Metazachlor	10.71	2	Positive	278.1	134.2	15
Propham	10.80	2	Positive	180.1	138.1	4
Terbuthylazine	10.98	2	Positive	230.1	174.1	15
Dichlorprop	10.99	2	Negative	233	161	10
Siduron	11.26	2	Positive	233.2	137.1	12
Sebuthylazine	11.47	2	Positive	230.1	174.1	16
Methiocarb	11.47	2	Positive	226.1	169	4
Linuron	11.69	2	Positive	249	160.1	20
Chlorpropham	12.53	2	Positive	214.1	172	5
Penconazole	12.76	2	Positive	284.1	70	17
Malathion	12.85	2	Positive	331	126.9	5
Neburon	13.29	2	Positive	275.1	57.1	20
TPP (IS)	13.99	2	Positive	327.1	51.1	80
EPN	14.96	2	Positive	324.1	296.1	8

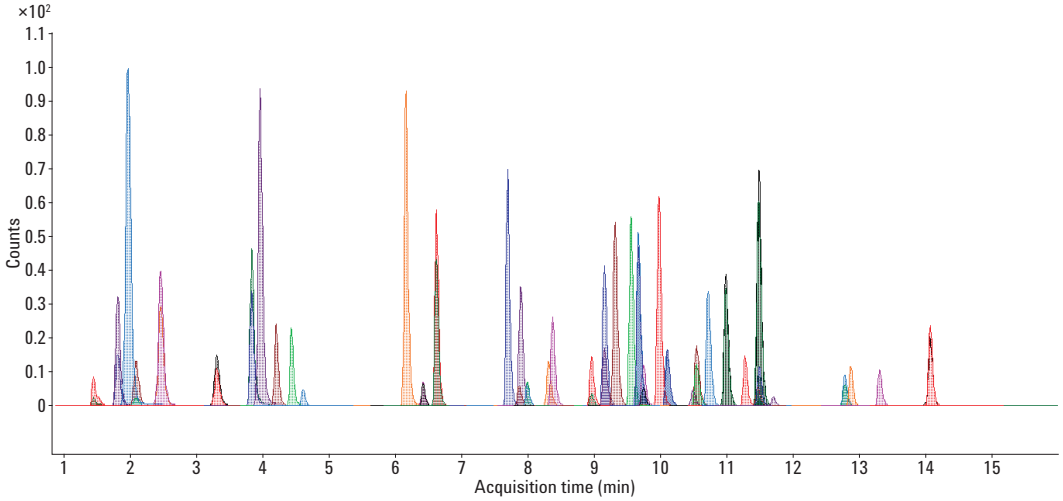

Figure 1. A typical LC/MS/MS chromatogram (MRM) of avocado sample fortified with 50 ng/g of pesticides and extracted by QuEChERS followed by cleanup with Agilent Bond Elut EMR—Lipid.

Sample preparation

The final sample preparation procedure was optimized using a QuEChERS workflow with the following steps:

1. Weigh 15 g (±0.1 g) homogenized avocado into 50 mL centrifuge tubes.
2. Add 15 mL acetonitrile (1% AA), and vortex for 10 s.
3. Add a packet of AOAC extraction salt.
4. Mix on a mechanical shaker for 2 min.
5. Centrifuge at 5,000 rpm for 5 min.
6. Add 5 mL water to a 15 mL EMR—Lipid dSPE tube.
7. Transfer 5 mL of supernatant to EMR—Lipid dSPE tube.
8. Vortex immediately to disperse sample, then for an extra 60 s on a multitube vortexer.
9. Centrifuge at 5,000 rpm for 3 min.
10. Transfer 5 mL of supernatant to a 15 mL EMR—Lipid polish tube containing 2 g salts (1:4, NaCl:$MgSO_4$), and vortex for 1 min.
11. Centrifuge at 5,000 rpm for 3 min.
12. Combine 200 µL of upper ACN layer and 800 µL water in a 2 mL sample vial and vortex.

The sample is now ready for LC/MS/MS analysis. The entire sample preparation flow path is shown in Figure 2.

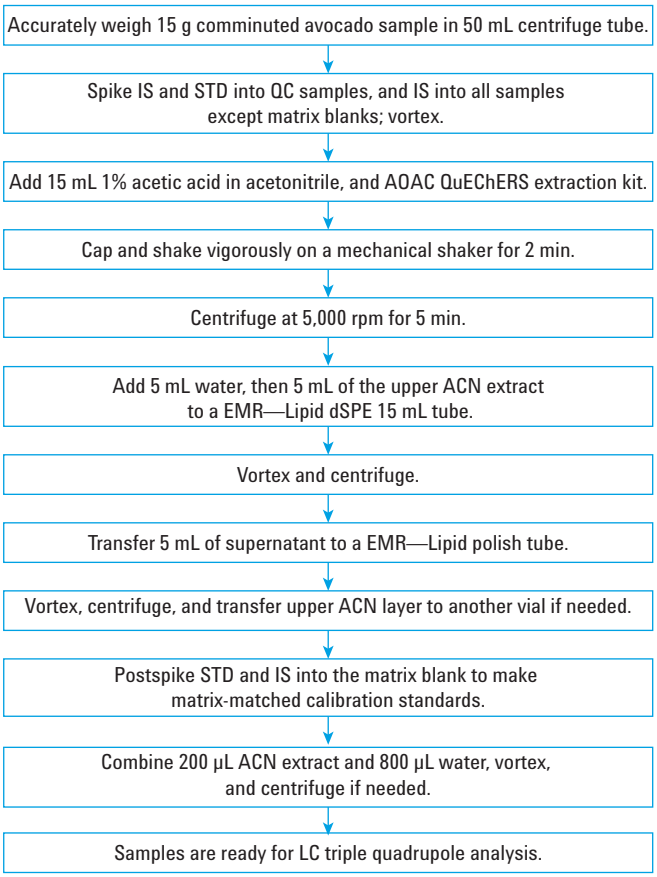

Figure 2. Sample preparation procedure using Agilent Bond Elut EMR—Lipid for the analysis of pesticides in avocado.

Calibration standards and quality control samples

Prespiked QC samples were fortified with combined standard working solution appropriately, after step 1, for six replicates. The QC samples correspond to 5, 50, and 200 ng/g in avocado. IS solution was also spiked into all the samples except the matrix blank, corresponding to 100 ng/g of TPP in avocado.

Matrix-matched calibration standards were prepared with standard and IS working solutions. Appropriate concentrations in the matrix blank samples after step 10 corresponded to 1, 5, 10, 50, 100, 150, and 200 ng/g and 100 ng/g IS (TPP). We diluted the final sample extract with water to make the sample amenable to the LC/MS/MS gradient and maintain peak shape integrity for early eluting analytes. The LC/MS/MS system provided excellent sensitivity using the final dilution as described and met the required limits of detection. If instrument sensitivity cannot meet the desired needs by sample dilution, a sample concentration step (evaporation and reconstitution), though less than ideal, should be considered

Determining amount of coextractives

The amount coextractive was determined by gravimetric measurements [2] for three different cleanup techniques: C18/PSA, zirconia sorbent, and EMR—Lipid. Samples were prepared as follows to collect data in duplicate.

1. Heat glass tubes for ~ 1 h at 110 °C to remove moisture.
2. Cool tubes to room temperature.
3. Preweigh test tubes.
4. Accurately transfer 1 mL of initial matrix blank extract (no cleanup) and the matrix blanks with various cleanups, each in duplicate.
5. Dry all samples on a CentriVap at 50 °C for 1 h, or until dry.
6. Heat the tubes for ~ 1 h at 110 °C to remove moisture.
7. Cool tubes to room temperature.
8. Reweigh the tubes.

The weight difference between after step 8 and after 3 is the amount of sample coextractive. The amount of coextractive removed by cleanup was the average weight difference of the matrix coextractives before and after cleanup.

Matrix effect assessment

Additionally, the analyte response (peak area) was compared between postspiked avocado extracts and the equivalent neat solutions. Postspiked avocado extracts were made by postspiking standard pesticide solution into the blank avocado matrix extract. The difference in response (peak area) is directly correlated to matrix effects.

Method comparison and validation

Currently, the QuEChERS method recommends fatty dSPE, which contain PSA, EC-C18, and $MgSO_4$, for the cleanup in high-fat samples such as avocado. Also, the zirconia sorbent claims to be a more efficient at lipid removal than C18/PSA dSPE. Our method comparison focused on EMR—Lipid cleanup and the other cleanup techniques. Recovery data compared pre- and postspiked samples corresponding to 50 ng/g in avocado. Extraction was carried out with the AOAC QuEChERS procedure, followed by dSPE with each cleanup protocol; EMR—Lipid, C18/PSA dSPE, and zirconia sorbent. For EMR—Lipid cleanup, the protocol shown in Figure 2 was followed. The EMR—Lipid dSPE, unlike traditional dSPE sorbents, requires extra water to activate the material, dramatically improving matrix removal performance. The supernatant from EMR—Lipid is transferred to the EMR—Lipid polish salts to phase separate the ACN/water, and remove dissolved solids. For QuEChERS with C18/PSA and zirconia cleanup, 1 mL of crude ACN extract was transferred into a 2 mL fatty dSPE tube (p/n 5982-5122), or into a 2 mL vial containing 100 mg zirconia sorbent. Samples were then vortexed for one minute and centrifuged at 13,000 rpm for three minutes on a microcentrifuge. An aliquot of 200 µL of supernatant was then transferred into a sample vial containing 800 µL water. A precipitate was generated with both the C18/PSA dSPE and zirconia sorbent cleanup protocols at this step, and samples must be filtered with a regenerated cellulose 0.45 µm filter vial before LC/MS/MS analysis. The precipitants are believed to be caused by unremoved lipids from the fatty dSPE and zirconia cleanups. This was not the case for the crude extract cleanup by EMR—Lipid, which, upon dilution, gave a clear solution with no precipitants. Filtration was, therefore, not required. It is important to make the postspiked calibrants in the corresponding matrix blanks, to prepare matrix-matched calibration standards. Recovery was calculated by the ratio of analyte peak areas from pre- and postspiked samples.

The EMR—Lipid method was validated in avocado at 5, 50, and 200 ng/g levels in six replicates using a 7-point matrix-matched calibration curve. An internal standard was used for quantitation, and data were reported as accuracy and precision.

Results and Discussion

Amount of coextractives

The results of sample coextractives weight determination are shown in Table 3, clearly demonstrating that EMR—Lipid dSPE provides the best matrix cleanup efficiency by weight.

Table 3. Avocado coextractive weights from QuEChERS extraction and various cleanup materials (n = 2).

Cleanup technique	Coextractives per 1 mL ACN final extract (mg)	Matrix coextractive removal efficiency by cleanup (%)
No further cleanup	14.7	–
EMR—Lipid cleanup	4.2	71.4
Zirconia cleanup	7.0	52.4
C18/PSA cleanup	9.5	35.4

$$\text{Matrix coextractive removal efficiency (\%)} = \frac{(\textit{Amount of coextractives without cleanup} - \textit{Amount of coextractives with cleanup})}{\textit{Amount of coextractives without cleanup}} \times 100$$

Matrix effect assessment

Analyte response between postspiked matrix blanks and neat standards was compared to evaluate matrix effects. Since the majority of coextracted lipids elute late in an LC gradient (reversed phase, low to high % organic), the hydrophobic analytes are impacted to a greater extent by the sample matrix. This effect is usually known as ion suppression, which correlates to low analyte response. Because of inefficient matrix lipid removal by C18/PSA and zirconia sorbent, significantly more matrix ion suppression was observed for the late eluting compounds. Figure 3 shows three compounds as examples of the reduced ion suppression resulting from EMR—Lipid cleanup. The three pesticides are compounds with relatively high log P values; chlorpropham (log P 3.6), penconazole (log P 3.7), and EPN (log P 4.5). The higher the log P value, the more hydrophobic the compound. These pesticides show up to 80% ion suppression caused by matrix interferences, especially by lipids, which were not effectively removed using C18/PSA dSPE and zirconia sorbent. For these compounds, EMR—Lipid produced no significant matrix effects, as seen in Figure 3.

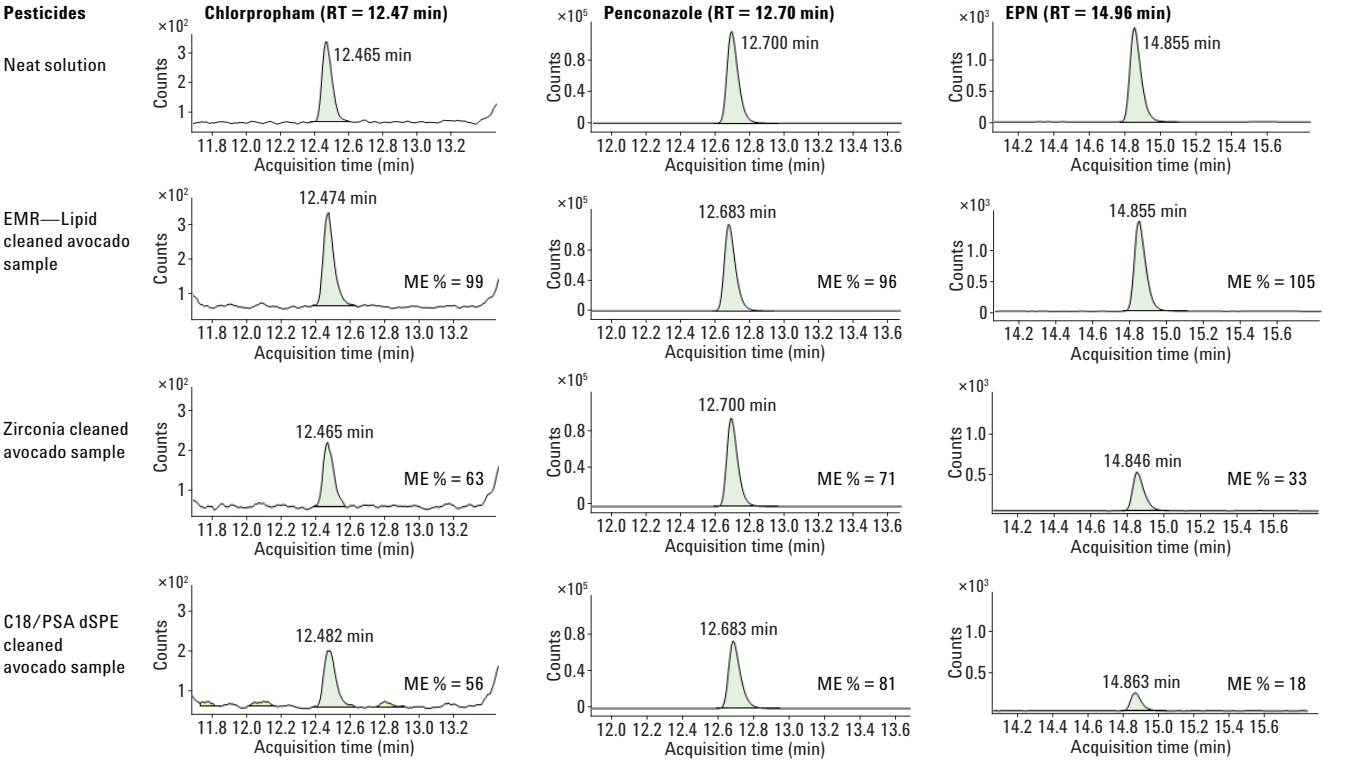

Figure 3. Matrix effect comparison for hydrophobic analytes. Matrix samples were postspiked at 50 ng/g with pesticide standard in a matrix blank.

Method comparison for analyte recovery

The optimized QuEChERS method with EMR—Lipid dSPE was then compared with C18/PSA and zirconia sorbent dSPE cleanup. Figure 4 shows the statistical recovery comparison results, and Figure 5 the selected problematic analyte comparison results.

The EMR—Lipid protocol provided overall excellent recovery and precision for most pesticides. Only two pesticides fell below the 70 to120% recovery window, namely cyprodinil (64%) and 2,4-D acid (65%), with RSD less than 10%. Therefore, they are considered as acceptable based on SANCO guidelines [11], as they meet acceptable reproducibility criteria. The recovery results for C18/PSA dSPE cleanup were good, except two acidic compounds. 2,4-D acid and dichlorprop gave very low recovery (<10%) caused by PSA. The recovery results from zirconia sorbent showed more analyte retention resulting in nine pesticide recoveries below 70%.

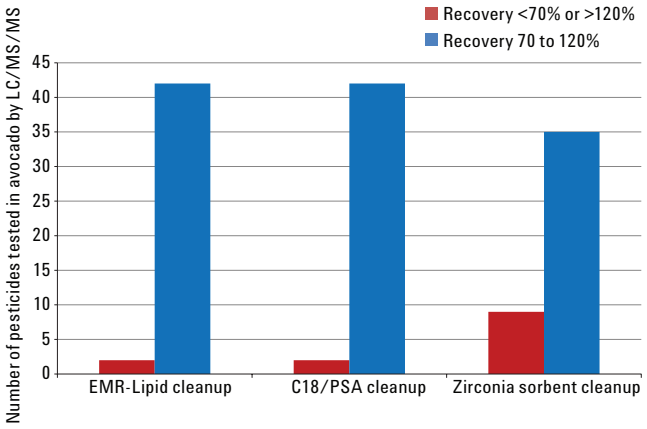

Figure 4, Statistical recovery results for the comparison of Agilent Bond Elut EMR—Lipid, C18/PSA dSPE, and zirconia sorbent.

Method validation

The EMR—Lipid protocol was validated by running a full quantitation batch. The methodology was described in the sample preparation section. An internal standard (TPP) was used for quantitation, and, therefore, the quantitation results are defined as accuracy and precision. However, the absolute recovery of IS (TPP) was above 90%, so the accuracy results correspond to absolute recovery.

Detailed validation results are listed in Table 4, and as a summarized figure (Figure 6) generated by average accuracy and precision calculated based on 18 total replicates of QC prespikes at three different levels. Accuracy results showed 95% of the 44 pesticides fell within the 70 to 120% window, except for 2,4-D acid and cyprodinil, which gave recoveries just below 70% with good RSD. The method reproducibility was exceptional with less than 10% RSD (n = 6) for 91% of the pesticides at 5 ng/g, 100% at 50 ng/g, and 98% at 200 ng/g. All other RSD values were well under 20% using the EMR—Lipid protocol. The instrumental detection limit is a likely contributor to the higher variation for these compounds above 10% RSD at the lowest spike level. The unbuffered EMR—Lipid polish step (NaCl, $MgSO_4$) is also a potential cause of variation and so buffered polish salts will be investigated in future work.

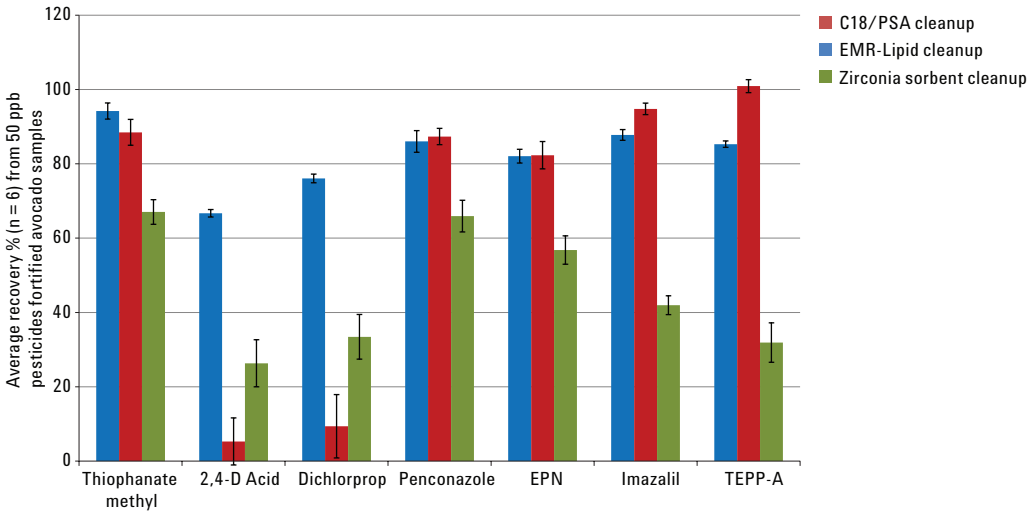

Figure 5, Recovery comparison results for Agilent Bond Elut EMR—Lipid (blue), C18/PSA (red), and zirconia sorbent (green) dSPE cleanup.

Table 4. Validation results from EMR—Lipid protocol for 44 pesticides in avocado at 5, 50, and 200 ng/g levels (n = 6).

	Calibration curve			Method accuracy and precision					
				5 ng/g QCs		50 ng/g QCs		200 ng/g QCs	
Analyte	Regression fit/weight	R^2	Cal. range (ng/g)	Rec. %	RSD	Rec. %	RSD	Rec. %	RSD
Methamidophos	Quadratic, 1/x	0.9993	1-200	69.1	9.5	93.8	8.4	109.8	6.0
Aminocarb	Linear, 1/x	0.9990	1-200	74.6	8.4	88.0	2.7	87.0	2.0
Acephate	Linear, 1/x	0.9948	1-200	55.8	12.4	88.8	2.3	86.6	4.0
Omethoate	Linear, 1/x	0.9996	1-200	84.5	6.0	85.3	1.4	84.4	2.6
Carbendazim	Linear, 1/x	0.9995	1-200	87.1	6.3	86.2	2.2	85.4	1.2
Thiabendazole	Linear, 1/x	0.9995	1-200	49.4	24.3	76.7	1.7	79.0	2.0
Mexacarbate	Linear, 1/x	0.9993	1-200	83.6	7.8	90.4	3.3	89.0	2.1
Oxamyl	Linear, 1/x	0.9991	1-200	81.1	7.6	96.7	2.6	94.4	3.5
Monocrotophos	Linear, 1/x	0.9979	1-200	85.2	6.1	85.1	1.9	101.5	4.6
Methomyl	Linear, 1/x	0.9993	1-200	77.8	8.2	88.6	3.3	92.8	4.5
Fenuron	Linear, 1/x	0.9969	1-200	86.5	9.9	103.4	2.5	91.7	1.7
Imidacloprid	Linear, 1/x	0.9996	1-200	81.7	5.9	94.1	2.6	87.9	2.5
Dimethoate	Linear, 1/x	0.9993	1-200	83.3	8.0	99.2	3.1	94.8	2.5
TEPP-A	Linear, 1/x	0.9989	1-200	50.2	6.5	88.3	1.6	78.4	3.1
Aldicarb	Linear, 1/x	0.9989	1-200	88.6	5.6	101.2	3.5	76.2	1.9
Metoxuron	Linear, 1/x	0.9987	1-200	102.0	5.4	105.8	2.5	89.9	2.6
Imazalil	Linear, 1/x	0.9988	1-200	81.4	6.9	86.2	2.0	82.5	2.7
Simazine	Linear, 1/x	0.9984	1-200	91.8	5.4	93.8	1.9	85.4	1.6
Monuron	Linear, 1/x	0.9990	1-200	82.5	9.9	96.0	3.7	88.4	1.8
Thiophanate methyl	Linear, 1/x	0.9977	1-200	89.4	10.8	104.6	5.5	86.0	7.1
Propoxur	Linear, 1/x	0.9993	1-200	84.7	8.1	97.6	1.4	94.5	2.2
Carbofuran	Linear, 1/x	0.9993	1-200	88.3	8.5	98.9	5.1	97.2	2.4
Chlorotoluron	Linear, 1/x	0.9990	1-200	96.3	5.0	97.9	3.1	89.9	2.0
Diuron	Linear, 1/x	0.9995	1-200	86.6	6.7	98.7	2.8	97.5	3.5
Carbaryl	Linear, 1/x	0.9991	1-200	80.7	7.4	101.1	3.2	90.5	2.1
Bentazone	Quadratic, 1/x	0.9993	1-200	111.2	5.5	102.3	4.7	97.4	7.9
Isoproturon	Linear, 1/x	0.9993	1-200	98.7	4.1	98.9	2.3	92.1	2.6
2,3-D acid	Linear, 1/x	0.9985	1-200	64.3	7.6	65.4	5.1	65.6	2.6
Fluometuron	Linear, 1/x	0.9975	1-200	86.2	5.7	87.8	3.9	88.0	3.0
Metobromuron	Linear, 1/x	0.9977	1-200	96.0	6.6	100.3	4.6	92.4	4.5
Cyprodinil	Linear, 1/x	0.9986	1-200	60.3	8.3	67.0	2.6	65.5	3.6
Metazachlor	Linear, 1/x	0.9992	1-200	99.8	5.7	99.4	3.4	94.3	2.8
Propham	Linear, 1/x	0.9985	1-200	85.8	9.7	89.3	3.8	87.0	3.8
Terbuthylazine	Linear, 1/x	0.9993	1-200	90.7	6.5	91.1	2.6	85.8	2.0
Dichlorprop	Linear, 1/x	0.9992	1-200	75.6	9.7	73.3	4.6	76.9	2.3
Siduron	Linear, 1/x	0.9990	1-200	90.2	8.6	92.4	3.5	91.5	2.2
Sebuthylazine	Linear, 1/x	0.9992	1-200	95.3	4.8	89.5	2.5	83.7	2.1
Methiocarb	Linear, 1/x	0.9984	1-200	77.6	8.8	94.7	3.2	86.3	1.9
Linuron	Linear, 1/x	0.9984	1-200	84.7	7.4	85.2	3.6	84.6	3.6
Chlorpropham	Linear, 1/x	0.9994	5-200	91.6	10.0	84.3	9.3	81.1	3.8
Penconazole	Linear, 1/x	0.9992	1-200	83.0	6.3	81.1	2.4	80.7	1.5
Malathion	Linear, 1/x	0.9991	1-200	76.2	7.1	100.5	2.2	100.0	1.0
Neburon	Linear, 1/x	0.9994	1-200	66.9	6.8	83.0	1.6	84.8	1.3
EPN	Linear, 1/x	0.9995	1-200	76.4	4.7	73.8	3.9	62.9	13.2

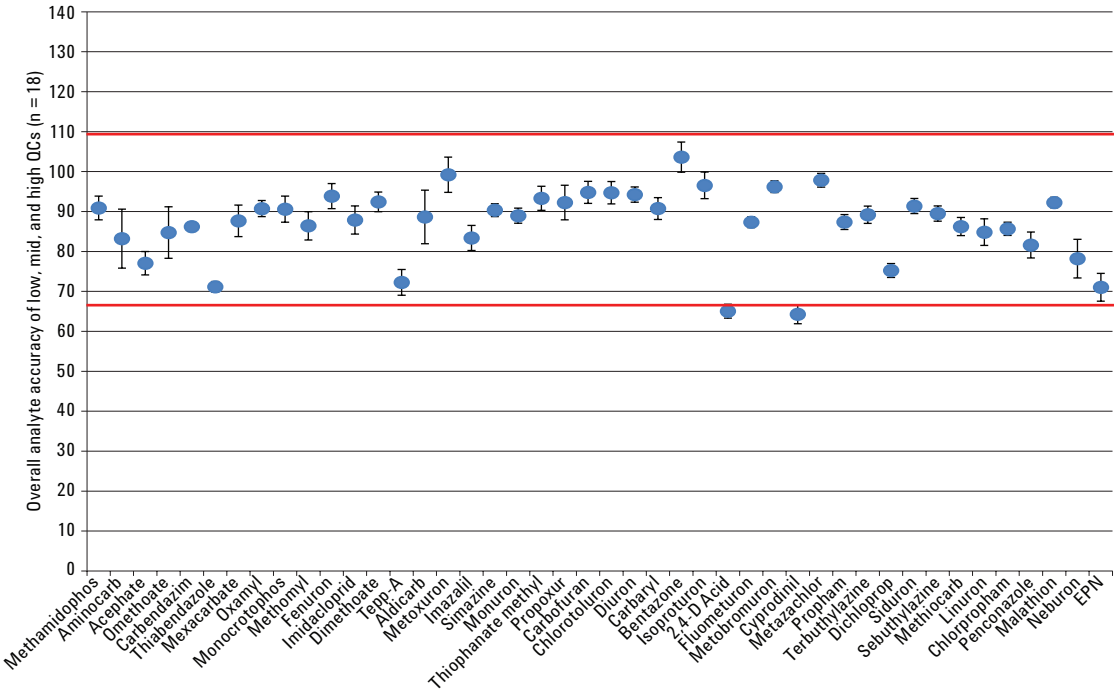

Figure 6, Quantitation results for 44 representative pesticides in avocado using the Agilent Bond Elut EMR—Lipid workflow. The accuracy and precision data were calculated based on 18 total replicates at three different concentrations.

Conclusions

A rapid, reliable, and robust method using a QuEChERS AOAC extraction followed by Agilent Bond Elut EMR—Lipid dSPE cleanup was developed and validated for the analysis of 44 LC-amenable pesticides in avocado. Matrix effect was carefully assessed and compared with traditional C18/PSA dSPE and zirconia sorbent cleanup. Results demonstrate that EMR—Lipid provides superior matrix cleanup than C18/PSA dSPE and zirconia sorbent by weight and matrix effect. Analyte recoveries and method precision were extensively compared between the three different cleanup techniques. EMR—Lipid cleanup provides comparable analyte recoveries relative to C18/PSA dSPE with dramatically fewer coextractives. Both EMR—Lipid and fatty dSPE cleanup delivered much better recovery than zirconia sorbent, due to nonselective analyte interactions with the zirconia. The data suggest that EMR—Lipid removes most matrix, especially lipids, without significantly affecting analyte recovery.

This work demonstrates the superior cleanliness that can be achieved using EMR—Lipid as a dSPE sorbent in a QuEChERS workflow. The sorbent's high selectivity for coextracted lipids makes it ideal for the analysis of fatty samples regardless of the fat content and target analyte list. EMR—Lipid gives high recovery, precision, superior matrix removal, and ease-of-use for the quantitation of pesticides in avocado. Future work will continue to focus on multiresidue analysis in complex, high-fat samples.

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Multiresidue Analysis of Pesticides in Avocado with Agilent Bond Elut EMR—Lipid by GC/MS/MS

Application Note

Food Testing and Agriculture

Authors

Limian Zhao and Derick Lucas
Agilent Technologies, Inc.

Abstract

Agilent Bond Elut Enhanced Matrix Removal—Lipid (EMR—Lipid) is the next generation of sample preparation products, and is used in convenient, dispersive solid phase extraction (dSPE) for highly selective matrix removal without impacting analyte recovery, especially for high-fat samples. This study demonstrates the application of this novel product for the analysis of 23 GC-amenable pesticides in avocado by GC/MS/MS. The procedure involves a QuEChERS AOAC extraction followed by EMR—Lipid dSPE and polish salts. EMR—Lipid provides far superior matrix removal by weight, GC/MS full scan, and matrix effect determination when compared to C18/PSA and zirconia-based sorbents. Furthermore, less matrix is introduced into the analytical flow path. The data also demonstrate dramatically improved reproducibility for the analytes over 100 injections relative to C18/PSA and especially zirconia, which experience significant response deviations. EMR—Lipid is highly selective for lipids and does not negatively affect analyte recovery. Analyte recoveries are high and precision is outstanding. This work demonstrates that EMR—Lipid dSPE fits into a QuEChERS workflow and delivers fast, robust, and effective sample preparation with the most complete matrix removal available for multiresidue analysis of pesticides in avocado.

Agilent Technologies

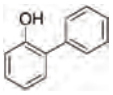
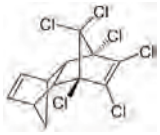
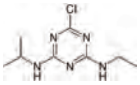
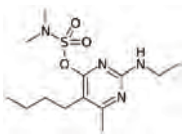
Introduction

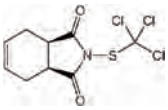
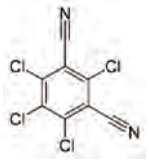
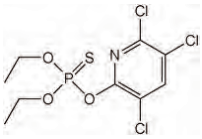
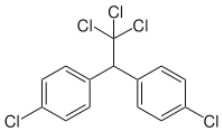
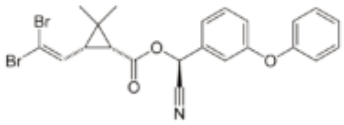
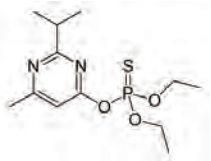
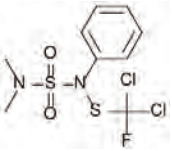
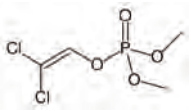
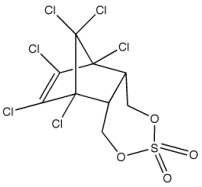
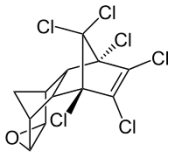
Pesticide residue analysis in food commodities is routine for many laboratories that use the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method [1,2]. This allows analysis of hundreds of pesticides at low concentrations with a single extraction. While the method has worked well for various fruits and vegetables, foods high in fat such as avocado, nuts, and foods of animal origin present new challenges [3,4]. Overcoming these challenges is a high priority for laboratories tasked with reaching the stringent validation criteria required by government agencies to ensure that food is safe for consumption.

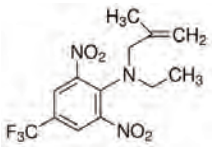
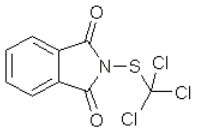
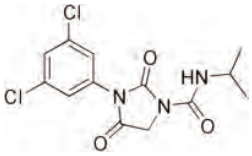
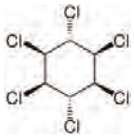
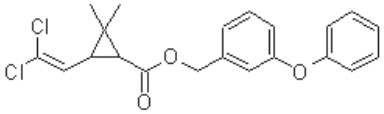
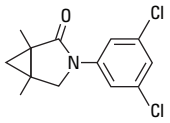
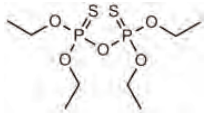
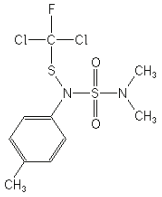
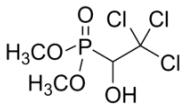
Analysis can use a combination of LC and GC to accommodate volatile, semivolatile and nonvolatile pesticides associated with many multiclass, multiresidue methods [4]. While many pesticides are amenable to both LC and GC, many are not. Each chromatographic technique has its inherent advantages and disadvantages in terms of analyte quantitation and adverse effects from coextracted matrix. Removal of these coextractives is essential to accurate quantitation in complex food matrices, requiring treatment with matrix removal sorbents such as C18, PSA, and GCB [5]. Other materials containing zirconia are commercially available, and generally improve lipid removal when compared to typical matrix removal sorbents. However, it does not target all lipid classes and can retain analytes of interest [6,7]. Samples high in lipid content may also require cleanup using solid phase extraction cartridges (SPE) [7,8,9] or gel permeation chromatography (GPC) [10], adding time and cost to an otherwise routine analysis.

Agilent Bond Elut EMR—Lipid is a novel sorbent material that selectively removes major lipid classes from the sample extract without unwanted analyte loss. Removal of lipid interferences from complicated matrices is especially important for QuEChERS, where large amounts of matrix are extracted with the target analytes. Avocado is known as a difficult matrix due to its high lipid content (15 to 20%), and was, therefore, selected as a representative sample for the evaluation of EMR—Lipid. This study investigates the sample preparation for the analysis of 23 GC-amenable pesticides in avocado using a QuEChERS AOAC extraction followed by EMR—Lipid dSPE and polishing salts. The pesticides are from 10 different classes to broaden the scope of the application (Table 1). This application note demonstrates the exceptional cleanliness that EMR—Lipid provides for complex, fatty sample such as avocado, and the high recovery and precision for 23 multiclass pesticide residues at three levels.

Table 1. Target analytes, class, log P, water solubility, and chemical structure [11].

Name	Category	Log P	Solubility in water (mg/L)	Molecular formula	Structure
2-Phenylphenol	Phenol	3.18	560	$C_{12}H_{10}O$	
Aldrin	Organochlorine	6.5	0.003	$C_{12}H_8Cl_6$	
Atrazine	Triazine	2.7	33	$C_8H_{14}ClN_5$	
Bupirimate	Pyrimidinol	2.2	22	$C_{13}H_{24}N_4O_3S$	

Name	Category	Log P	Solubility in water (mg/L)	Molecular formula	Structure
Captan	Phthalimide	2.5	5.1	$C_9H_8Cl_3NO_2S$	
Chlorothalonil	Chloronitrile	2.94	1.0	$C_8Cl_4N_2$	
Chlorpyrifos methyl	Organophosphate	4.0	2.74	$C_7H_7Cl_3NO_3PS$	
DDT	Organochlorine	6.91	0.006	$C_{14}H_9Cl_5$	
Deltamethrin	Pyrethroid	4.6	0.0002	$C_{22}H_{19}Br_2NO_3$	
Diazinon	Organophosphate	3.69	60	$C_{12}H_{21}N_2O_3PS$	
Dichlofluanid	Sulphamide	3.7	1.3	$C_9H_{11}Cl_2FN_2O_2S_2$	
Dichlorvos	Organophosphate	1.9	18,000	$C_4H_7Cl_2O_4P$	
Endosulfan sulfate	Organochlorine	3.13	0.48	$C_9H_6Cl_6O_3S$	
Endrin	Organochlorine	3.2	0.24	$C_{12}H_8Cl_6O$	

Name	Category	Log P	Solubility in water (mg/L)	Molecular formula	Structure
Ethalfluralin	Dinitroaniline	5.11	0.01	$C_{13}H_{14}F_3N_3O_4$	
Folpet	Phthalimide	3.02	0.8	$C_9H_4Cl_3NO_2S$	
Iprodione	Dicarboxamide	3.1	12.0	$C_{13}H_{13}Cl_2N_3O_3$	
Lindane	Organochlorine	3.5	8.52	$C_6H_6Cl_6$	
Permethrin	Pyrethroid	6.1	0.006	$C_{21}H_{20}Cl_2O_3$	
Procymidone	Dicarboxamide	3.3	2.46	$C_{13}H_{11}Cl_2NO_2$	
Sulfotep	Organophosphate	3.99	10	$C_8H_{20}O_5P_2S_2$	
Tolylfluanid	Sulphamide	3.9	0.9	$C_{10}H_{13}C_{l2}FN_2O_2S_2$	
Trichlorfon	Organophosphate	0.43	120,000	$C_4H_8Cl_3O_4P$	

Experimental

All regents and solvents were HPLC or analytical grade. Acetonitrile (ACN) and methanol were from Honeywell (Muskegon, MI, USA). Reagent grade acetic acid (AA), pesticide standards, and internal standard were purchased from Sigma-Aldrich, Corp. (St Louis, MO, USA).

Solution and standards

Acetic acid 1% in ACN was prepared by adding 10 mL acetic acid to 990 mL ACN. Standard and internal standard (IS) stock solutions were made in either ACN or methanol at 2.0 mg/mL. A combined working solution was prepared in ACN at 25 µg/mL, except for captan, folpet, trichlorfon, and bupirimate. Due to relatively low responses on the instrument, the concentration was made five times higher for those four compounds in the combined working solution, which was 125 µg/mL. A 25 µg/mL aliquot of combined IS working solution was prepared in ACN, including TPP, parathion ethyl d_{10}, and ^{13}C-DDT.

Equipment

Equipment and material used for sample preparation included:

- Geno/Grinder (SPEX, Metuchen, NJ, USA)
- Centra CL3R centrifuge (Thermo IEC, MA, USA)
- Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)
- Vortexer and multitube vortexers (VWR, Radnor, PA, USA)
- Bottle top dispenser (VWR, So. Plainfield, NJ, USA)
- Eppendorf pipettes and repeater
- Agilent Bond Elut EMR—Lipid tubes (p/n 5982-1010) and Agilent Bond Elut Final Polish for Enhanced Matrix Removal—Lipid tubes (p/n 5982-0101)

Instrumentation

Analysis was completed on an Agilent 7890A GC equipped with an Agilent 7693B Autosampler and an Agilent 7000C Triple Quadrupole GC/MS system. Column backflushing was used, which is highly recommended for complex sample matrices [12]. The total run time for a sample spiked with standard was 23 minutes, with two minutes for column backflushing.

Instrument conditions

GC conditions

Autosampler:	Agilent 7693 Autosampler and sample tray 10 µL syringe (p/n G4513-80220), 1 µL injection volume Three post injection solvent A (acetonitrile) washes Three sample pumps Three post injection solvent B (isopropanol) washes
Column:	Agilent J&W DB-5ms Ultra Inert, 0.25 mm × 15 m, 0.25 µm (p/n 122-5512UI)
Carrier:	Helium, constant pressure
Gas filter:	Gas Clean carrier gas filter kit, 1/8 inch (p/n CP17974)
Inlet liner:	Agilent Ultra Inert single taper splitless liner with wool (p/n 5190-2293)
Inlet:	MMI inlet at pulsed cold splitless mode, 75 °C initially, hold for 0.02 min, then ramp to 350 °C at 750 °C/min
Injection pulse pressure:	36 psi until 0.75 min
Purge flow to split vent:	60 mL/min at 0.75 min
Inlet pressure:	17 psi during run, and 1.0 psi during backflushing
Oven:	60 °C for 2.57 min, then to 150 °C at 50 °C/min, to 200 °C at 6 °C/min, to 300 °C at 16 °C/min, hold for 3 min
Post run:	2 min at 300 °C
Capillary Flow Technology:	UltiMetal Plus Purged Ultimate Union (p/n G3182-61581) for backflushing the analytical column and inlet
Aux EPC gas:	Helium plumbed to Purged Ultimate Union
Bleed line:	0.0625 inch od × 0.010 inch id × 100 cm, 316SS tubing, on top of the oven
Aux pressure:	4 psi during run, 75 psi during backflushing
Connections:	Between inlet and Purged Ultimate Union
Restrictor:	Inert fused silica tubing, 0.65 m × 0.15 mm (p/n 160-7625-5)
Connections:	Between Purged Ultimate Union and the MSD

MSD conditions

MSD:	Agilent 7000C Triple Quadrupole GC/MS, inert, with performance electronics
Vacuum pump:	Performance turbo
Mode:	MRM
Tune file:	Atune.u
Transfer line temp:	280 °C
Source temp:	300 °C
Quad temp:	150 °C for Q1 and Q2
Solvent delay:	2.57 min
Collision gas flow:	He quench gas at 2.35 mL/min, N_2 collision gas at 1.5 mL/min
MS resolution:	MS1 and MS2 = 1.2u

The MRM parameters were easily optimized for each analyte using the Agilent Pesticides and Environmental Pollutants MRM Database (G9250AA), which contains MS/MS conditions and retention time information for over 1,070 compounds [13]. Table 2 lists the MRM transitions for the target analytes used in this study. An example of a typical GC/MS/MS chromatogram is shown in Figure 1 for the 23 pesticides under investigation.

Table 2. GC/MS/MS MRM conditions and retention time for pesticide analysis.

		MRMs			
Analyte	**RT (min)**	**Quant channel**	**CE (v)**	**Qual channel**	**CE (v)**
Dichlorvos	4.70	184.9 → 93	10	109 → 79	5
Trichlorfon	5.94	110.8 → 47	30	81.8 → 47	50
2-Phenylphenol	6.39	169 → 115.1	25	170 → 141.1	25
Ethalfluralin	7.58	275.9 → 202.1	15	315.9 → 275.9	10
Sulfotep	7.83	237.8 → 145.9	10	201.8 → 145.9	10
Atrazine	8.69	214.9 → 58.1	10	214.9 → 200.2	5
Lindane	8.83	181 → 145	15	216.9 → 181	5
Chlorothalonil	9.20	263.8 → 168	25	265.8 → 231	20
Diazinon	9.22	137.1 → 54	20	199.1 → 93	20
Chlorpyriphos methyl	10.30	285.9 → 92.9	20	124.9 → 47	15
Dichlorfluanid	11.31	223.9 → 123.1	20	123 → 77	20
Aldrin	11.55	262.9 → 192.9	35	254.9 → 220	35
Parathion ethyl D_{10} (IS)	11.96	98.7 → 67	10	114.9 → 82.9	20
Tolylfluanid	12.80	136.9 → 91	20	136.9 → 65	30
Captan	12.96	151 → 79.1	15	149 → 79.1	10
Forpet	13.13	259.8 → 130.1	15	261.8 → 130.1	15
Procymidone	13.13	282.8 → 96	10	96 → 67.1	10
Bupirimate	15.44	272.9 → 193.1	15	272.9 → 108	5
Endrin	15.68	316.7 → 280.8	5	244.8 → 173	30
Endosulfan sulfate	17.44	273.9 → 238.9	15	271.9 → 237	15
^{13}C-DDT (IS)	17.69	246.5 → 177.1	15	248.5 → 177.1	15
DDT	17.69	235 → 165.2	20	237 → 165.2	20
TPP (IS)	18.20	325.9 → 169	30	325.9 → 233	27
Iprodione	18.82	313.8 → 55.9	20	187 → 124	25
Permethrin	20.68	183.1 → 153.1	15	183.1 → 153.1	15
Deltamethrin	22.51	252.9 → 93	15	181 → 152.1	25

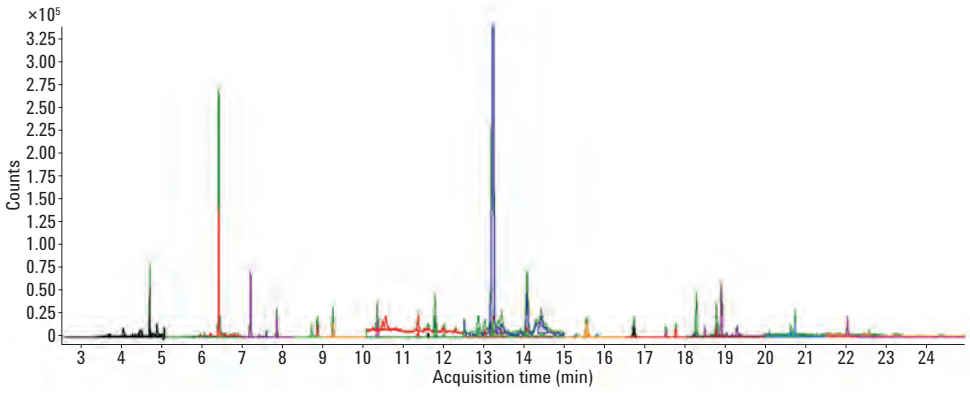


Figure 1. A typical GC triple quadrupole chromatogram (MRM) of an avocado sample fortified with a 50 ng/g pesticide standard. The sample preparation used QuEChERS followed by cleanup with Agilent Bond Elut EMR—Lipid.

Sample preparation

The final sample preparation procedure was optimized as follows:

1. Weigh 15 g (±0.1 g) homogenized avocado into 50 mL centrifuge tubes.
2. Add 15 mL of acetonitrile (1% AA) and vortex for 10 s.
3. Add AOAC extraction salt packet.
4. Mix on a mechanical shaker for 2 min.
5. Centrifuge at 5,000 rpm for 5 min.
6. Add 5 mL water to a 15 mL EMR—Lipid dSPE tube, and transfer 5 mL of supernatant to EMR—Lipid tube.
7. Vortex immediately to disperse sample, then for an extra 60 s with the entire batch on a multitube vortexer.
8. Centrifuge at 5,000 rpm for 3 min.
9. Transfer 5 mL supernatant to a 15 mL EMR—Lipid polish tube containing 2 g salts (1:4, NaCl:$MgSO_4$), and vortex for 1 min.
10. Centrifuge at 5,000 rpm for 3 min.
11. Transfer the upper ACN layer to a sample vial for GC/MS/MS injection.

The entire sample preparation workflow is shown in Figure 2.

Calibration standards and quality control samples

Prespiked QC samples were fortified with combined standard working solution at appropriate concentrations, after step 1, in replicates of six. The QC samples correspond to 5, 50, and 300 ng/g in avocado. The QC samples were 25, 250, and 1,500 ng/g for captan, folpet, trichlorfon, and bupirimate. An IS solution was also spiked into all samples except the matrix blank, corresponding to 250 ng/g in avocado.

Matrix-matched calibration standards prepared with standard and IS working solutions were added appropriately into the matrix blank samples after step 10, corresponding to 1, 5, 10, 50, 100, 200, 300, and 400 ng/g in avocado, and 250 ng/g IS. The four compounds used calibration standards at 5, 25, 50, 250, 500, 1,000, 1,500, and 2,000 ng/g.

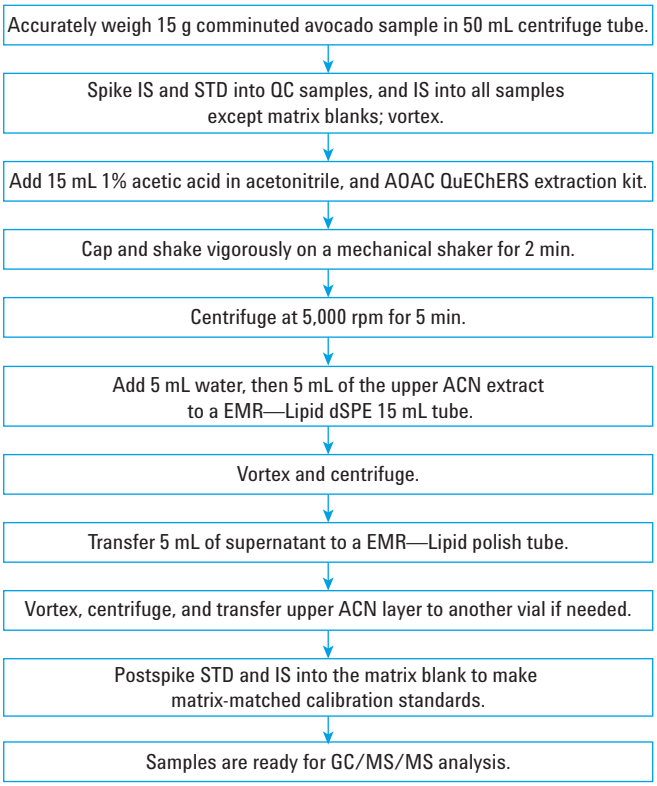


Figure 2. Sample preparation workflow showing a QuEChERS extraction with Agilent Bond Elut EMR—Lipid cleanup for the analysis of pesticides in avocado by GC/MS/MS.

Matrix cleanup assessment

The avocado extracts were applied to three different cleanup materials, fatty dSPE (C18/PSA), zirconia sorbent, and EMR—Lipid. An experiment compared the GC/MS full-scan profile of the final extract, before and after cleanup. Chromatograms were overlaid to compare the amount of matrix cleanup by chromatographic background. To quantitatively evaluate matrix cleanup efficiency, the GC/MS full-scan chromatogram was manually integrated across the entire window, and the matrix removal efficiency was then calculated according to Equation 1.

$$\% \text{ Matrix removal} = \frac{\textit{Total peak area}_{\textit{Sample without cleanup}} - \textit{Total peak area}_{\textit{Sample with cleanup}}}{\textit{Total peak area}_{\textit{Sample without cleanup}}} \times 100$$

Equation. 1

A gravimetric experiment comparing the weight of avocado coextracts after treatment with EMR—Lipid, C18/PSA, and zirconia sorbent has been published [14].

Method comparison and validation

An analyte recovery experiment compared prespiked and postspiked samples at 50 ng/g in avocado. Samples were treated using the QuEChERS AOAC extraction procedure followed by EMR—Lipid, C18/PSA, or zirconia cleanup. For EMR—Lipid cleanup, the protocol shown in Figure 2 was followed. The other materials applied the same QuEChERS extraction with a C18/PSA and zirconia sorbent cleanup. An aliquot of 1 mL crude ACN extract was then transferred to a 2 mL C18/PSA dSPE tube (p/n 5982-5122) or a 2 mL vial containing 100 mg zirconia sorbent. All samples were vortexed for one minute and centrifuged at 13,000 rpm for three minutes on a microcentrifuge. The ACN layer was then transferred into a sample vial for GC/MS/MS analysis. Matrix-matched calibration standards were prepared by postspiking the blank avocado extract with standards and internal standards. Recovery was calculated by the ratio of analyte peak areas from pre- and postspiked samples.

The EMR—Lipid method was validated in avocado at three levels in six replicates using an 8-point matrix-matched calibration curve. An internal standard (IS) was used for quantitation and data were reported as accuracy and precision.

Matrix impact on GC/MS/MS system performance

The matrix impact on GC/MS/MS system performance was investigated by evaluating the consistency for analyte response over multiple injections of avocado samples. The experiment compared the analyte response on GC/MS/MS over time by making multiple injections of avocado extracts treated with EMR—Lipid, C18/PSA, or zirconia sorbent. Each testing batch included matrix blanks and postspiked 50 ppb QC samples. The sequence injected four blanks with a QC sample on the fifth injection, and was carried out for 100 total injections. This was to determine the effect of unremoved matrix accumulation on GC/MS flow path surfaces on analyte instrument response using the different cleanup options. For each cleanup, the analyte response (peak area) was used to calculate the %RSD over the 100-injection run. To exclude the contribution of the GC flow path, Agilent Inert Flow Path consumables were used, with a new Agilent Ultra Inert liner and column for each cleanup method.

Results and Discussion

Matrix cleanup assessment

Complex matrices significantly impact GC/MS performance as matrix forms active sites on the GC flow path surface, induces matrix effects in the mass spectrometer, and introduces interferences in the final chromatogram. While GC/MS (SIM) and GC/MS/MS (MRM) show enhanced selectivity for the target ions, unremoved matrix can still cause interference and decrease performance over time. To remedy these negative effects from high-fat matrices such as avocado, more complete sample preparation cleanup methods must be applied to make samples more amenable to GC/MS analysis.

Figure 3A shows the overlaid GC/MS full-scan chromatograms for an avocado matrix blank and the chromatographic profiles obtained from EMR—Lipid, C18/PSA, and zirconia cleanup methods. The chromatogram from the sample without further cleanup (black trace) shows a high abundance of matrix interferences, which will hinder the analysis of target analytes. The chromatograms from extracts treated with C18/PSA (blue) and zirconia sorbent (green) cleanup show 36% and 55% matrix removal, respectively, as determined by Equation 1. However, the EMR—Lipid dSPE trace (red) shows near baseline removal of these interferences on the GC/MS full-scan chromatogram, corresponding to 95% matrix removal. The large amount of cleanup achieved with EMR—Lipid has obvious implications for the analysis of pesticides in avocado as there is dramatically less matrix in the sample to affect instrument performance. Furthermore, this is achieved using a simple dSPE with EMR—Lipid in a conventional QuEChERS workflow.

Figure 3B shows the overlapped GC/MS/MS MRM chromatograms for avocado samples fortified with 50 ppb of pesticide standard. Due to the improved selectivity of the MS/MS system, the matrix background is less significant than a GC/MS SIM or full-scan chromatogram. Despite the superior selectivity for analytes of interest, interference peaks are still present between 11 and 20 minutes on the chromatogram for C18/PSA (blue) and zirconia (green). These interferences affect the accurate integration for some analyte signals. The EMR—Lipid extracts show a substantially cleaner background as evident in the red trace in Figure 3B, dramatically improving the accuracy of integration.

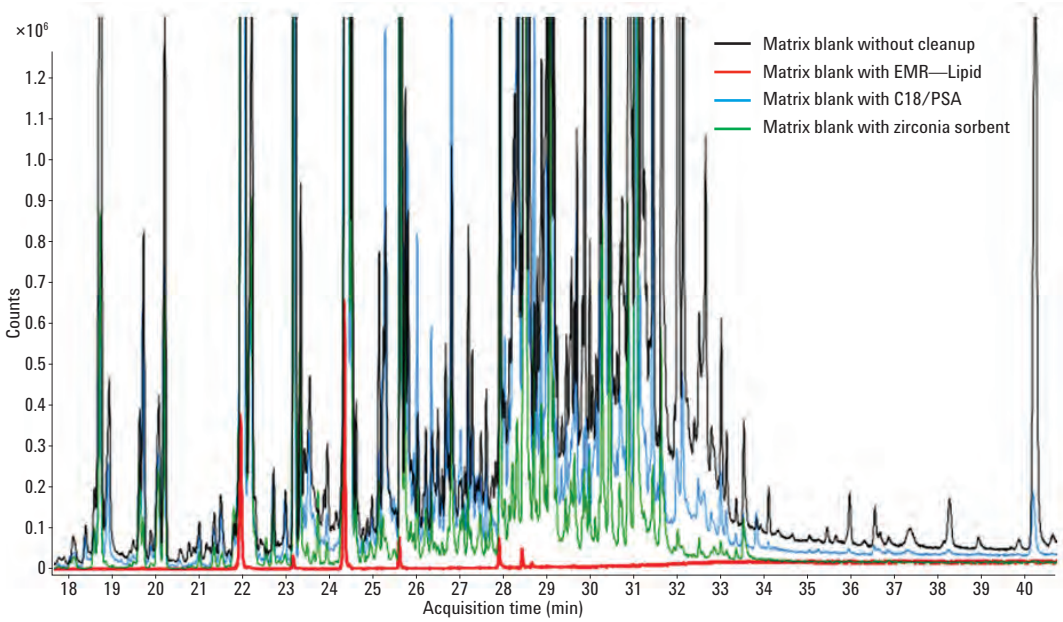


Figure 3A. GC/MS full-scan chromatogram overlay of avocado matrix blanks prepared by a QuEChERS AOAC extraction followed by dSPE using Agilent Bond Elut EMR—Lipid (red), zirconia (green), PSA/C18 (blue), or no cleanup (black).

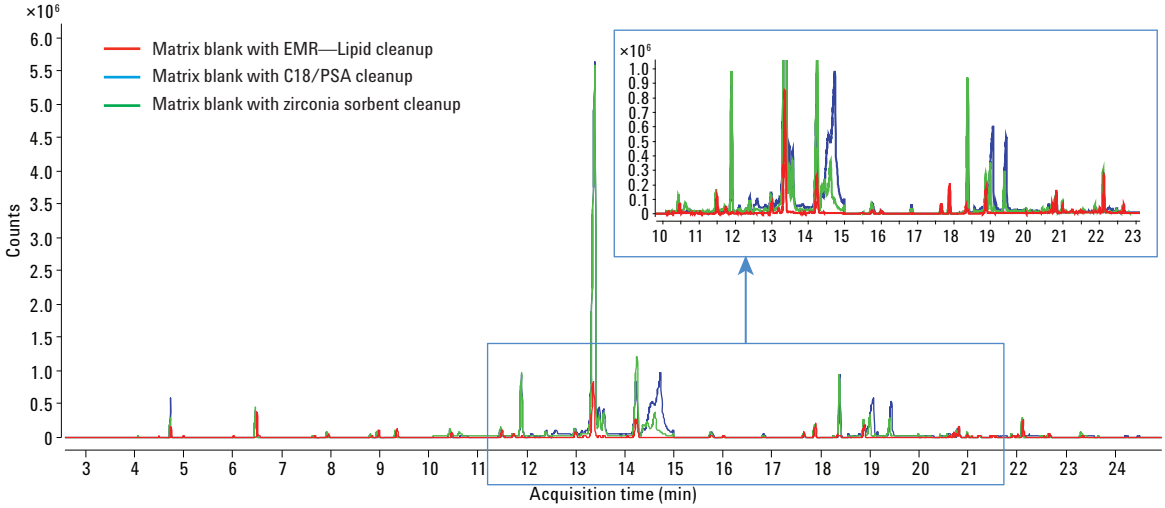


Figure 3B. GC/MS/MS MRM chromatogram overlay of an avocado sample prepared using a QuEChERS AOAC extraction followed with Agilent Bond Elut EMR—Lipid (red), C18/PSA (blue), and zirconia sorbent (green). All samples were fortified with a 50 ppb pesticide standard.

The improved matrix cleanup of EMR—Lipid and the positive effect of superior matrix removal for three example analytes are demonstrated in Figure 4. In all cases, chromatograms using EMR—Lipid cleanup show fewer interference peaks, better signal/noise, and consistent baseline integration. These improvements make data processing and review faster, and easier, and build a high degree of confidence in the analytical method.

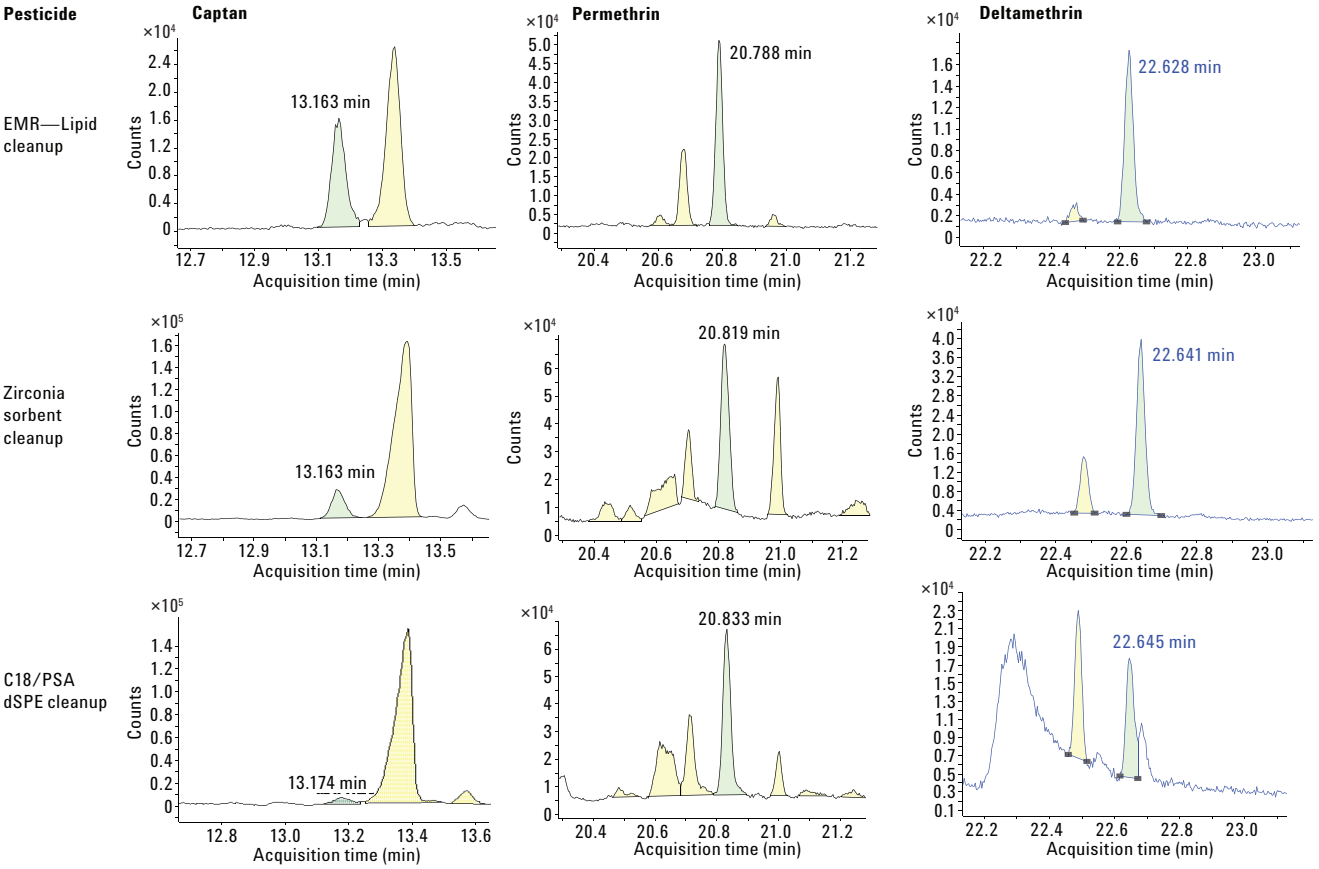


Figure 4. Chromatogram comparison for analytes of interest and the affect of matrix on peak response, peak quality, and interferences in the MRM window. Blank samples were treated with either Agilent Bond Elut EMR—Lipid, zirconia, or C18/PSA and the final sample postspiked with a 50 ppb pesticide standard.

Method comparison for analyte recovery

The optimized EMR—Lipid method was then compared with a traditional QuEChERS method using C18/PSA or zirconia sorbent. Figure 5 shows the recovery comparison for all 23 pesticides using these different cleanup materials. The results demonstrate that EMR—Lipid cleanup does not cause significant analyte retention, and thus provides comparable recovery results to C18/PSA cleanup. However, we have shown that C18/PSA and zirconia sorbents do not provide efficient matrix removal.

There are some analytes with lower absolute recoveries regardless of the cleanup method. Aldrin, endrin, and DDT had less than 60% recovery, and permethrin and deltamethrin were 63% and 75%, respectively. C18/PSA cleanup provided a slightly higher recovery than EMR—Lipid and zirconia sorbent cleanup. These pesticides are highly lipophilic (high log P) with very poor solubility in water, and are readily incorporated into high-lipid sample matrices such as avocado, making them challenging to extract with polar solvents such as acetonitrile. The use of stronger solvents may increase the extraction efficiency of these lipophilic analytes from the fatty matrix, increasing extraction efficiency and improving absolute recovery. Future work will investigate the extraction efficiency of lipophilic compounds from high-fat matrices followed by enhanced matrix removal.

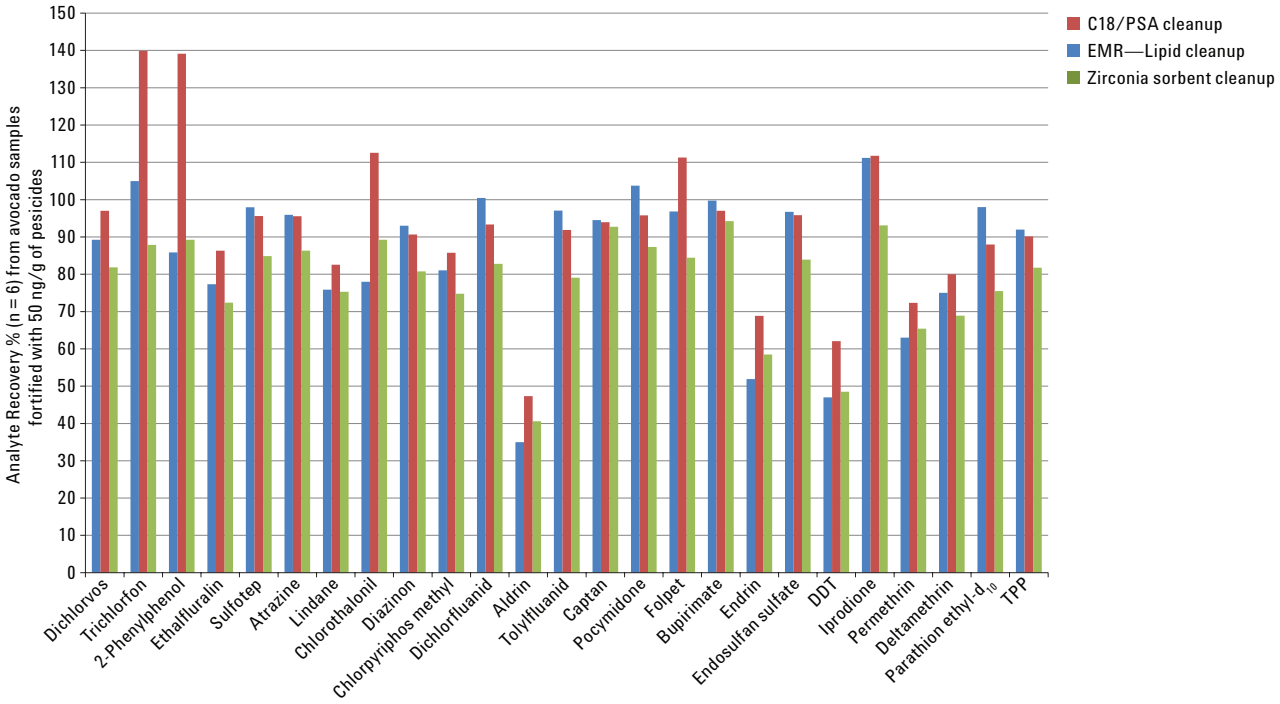


Figure 5. Recovery comparison between Agilent Bond Elut EMR—Lipid, C18/PSA, and zirconia cleanup at 50 ppb in avocado.

To correct for these compounds low in absolute recovery, a stable labeled internal standard, ^{13}C-DDT, was used to improve the accuracy of DDT, aldrin, and endrin in the final quantitation results. The use of TPP as internal standard for permethrin and deltamethrin was suitable for quantitation.

Method validation

The EMR—Lipid method was validated by running a full quantitation batch. Internal standards were used for quantitation, and results were reported as accuracy and precision. Three internal standards were used for the quantitation, namely parathion ethyl-D_{10}, ^{13}C-DDT, and TPP. The analytes with retention times before 12 minutes used parathion ethyl-D_{10} as IS, and those after 12 minutes used TPP as IS. As previously mentioned, ^{13}C-DDT was used as an IS for aldrin, endrin, and DDT to correct analyte loss due to poor extraction efficiency.

Detailed validation results are listed in Table 3. Figure 6 is a summary generated using the average accuracy and precision calculated for 18 total replicates of QCs (three levels, n = 6). Pesticide accuracy was between 70% and 120% for all but one analyte (67%), and precision was less than 20% RSD for all analytes, with 80% less than 10% RSD. Aldrin accuracy was still slightly lower than 70%, but with good precision (RSD < 6%), and is acceptable based on SANCO guidelines [15].

Table 3. Quantitation results for pesticides in avocado spiked at 5, 50, and 300 ng/g levels for six replicates.

	Calibration curve			Method accuracy and precision (ng/g QCs[1])					
				5 (25)		50 (250)		300 (1,500)	
Analyte	Regression fit/weight	R^2	Cal. range (ng/g)	Rec%	RSD	Rec%	RSD	Rec%	RSD
Dichlorvos	Linear, 1/x	0.9967	1-400	97	8.2	108	4.9	111	12.7
Trichlorfon	Linear, 1/x	0.9964	5-2000[1]	98	7.8	95	7.3	84	4.7
2-Phenylphenol	Linear, 1/x	0.9996	10-400[2]	97	14.0	104	1.7	105	5.1
Ethalfluralin	Linear, 1/x	0.9969	1-400	109	3.2	98	7.6	110	6.5
Sulfotep	Linear, 1/x	0.9958	1-400	96	5.8	76	3.9	85	9.8
Atrazine	Linear, 1/x	0.9967	1-400	91	5.0	80	2.1	76	3.9
Lindane	Linear, 1/x	0.9991	1-400	92	6.7	104	4.0	98	12.5
Chlorothalonil	Linear, 1/x	0.9944	1-400	89	13.5	103	8.6	92	19.4
Diazinon	Linear, 1/x	0.9993	1-400	102	6.8	116	5.1	108	8.9
Chlorpyrifos methyl	Linear, 1/x	0.9984	1-400	101	6.2	123	4.5	113	15.0
Dichlofluanid	Linear, 1/x	0.9989	1-400	96	10.2	85	5.1	91	4.3
Aldrin	Linear, 1/x	0.9982	1-400	76	4.8	59	2.3	65	5.1
Tolylfluanid	Linear, 1/x	0.9990	10-400	108	10.0	93	6.2	93	5.4
Captan	Linear, 1/x	0.9959	25-2000[1,2]	89	8.2	109	11.0	87	18.1
Folpet	Linear, 1/x	0.9897	5-2000[1]	76	9.5	79	9.9	87	13.2
Procymidone	Linear, 1/x	0.9977	1-400	87	5.0	76	1.9	79	7.2
Bupirimate	Linear, 1/x	0.9957	5-2000[1]	101	6.5	100	5.6	85	10.3
Endrin	Linear, 1/x	0.9967	1-400	75	10.8	88	6.7	80	13.6
Endosulfan sulfate	Linear, 1/x	0.9996	1-400	96	9.9	97	6.4	95	4.9
DDT	Linear, 1/x	0.9995	1-400	103	4.5	105	2.6	107	4.6
Iprodione	Linear, 1/x	0.9995	1-400	97	6.7	105	2.7	97	4.2
Permethrin	Linear, 1/x	0.9992	1-400	87	6.6	97	4.3	84	14.0
Deltamethrin	Linear, 1/x	0.9963	1-400	89	13.8	92	8.3	98	11.5

[1] Compounds were prepared at five times higher concentration in the combined standard working solution due to a low response. Therefore, the QC spiking and calibration standard spiking levels were five times higher than those of the other compounds.

[2] Raised LOQ due to either poor sensitivity or matrix interference peak interfered the detection of analyte at original LOQ.

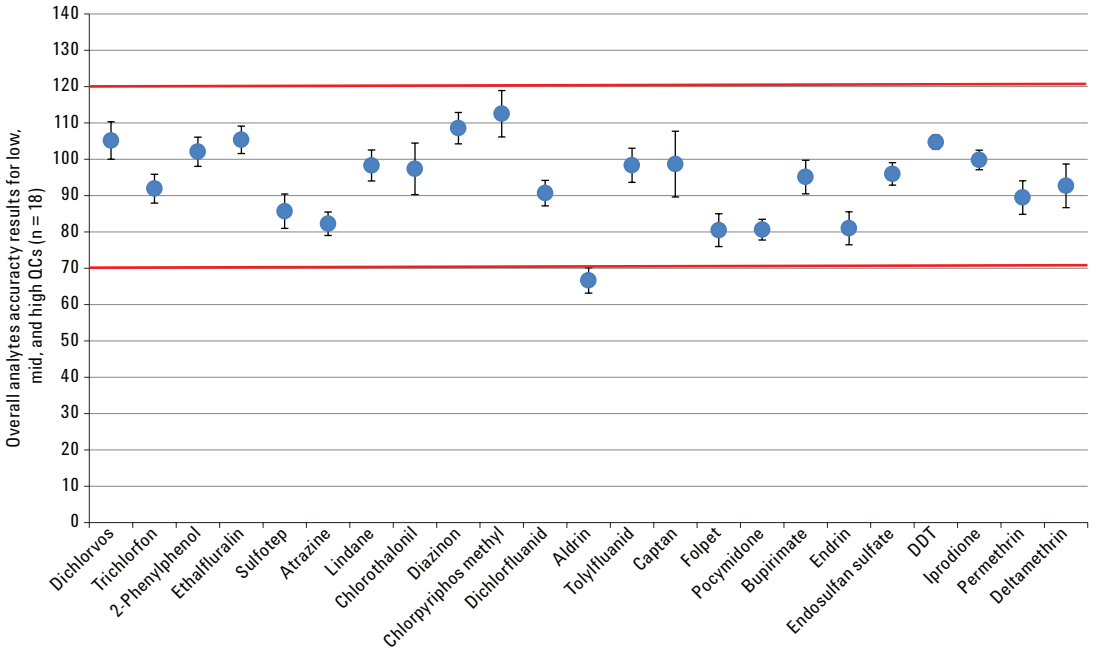


Figure 6. Quantitation results for 23 pesticides in avocado using a QuEChERS extraction with Agilent Bond Elut EMR—Lipid, dSPE. The data points represent accuracy and precision and were calculated at three levels in six replicates. Error bar = 95% CI.

Matrix impact on GC/MS/MS system performance

Matrix interferences will affect GC/MS/MS system performance over time as more samples are injected into the system. GC flow path active sites can negatively impact instrument performance. Agilent Inert Flow Path components provide the best deactivation for the entire GC flow path and significantly reduce negative interactions between analytes and active sites that result in analyte loss and chromatographic anomalies. However, if the matrix is laden with high-boiling compounds (high fat) it will accumulate on the flow path surface and generate new active sites. Over time, this can lead to analyte response variations, greatly impacting method reliability and reducing the number of injections per batch. To fix this, laboratories must perform more instrument maintenance such as liner change or column trim/change, leading to decreased laboratory productivity.

As demonstrated in the matrix cleanup assessment and gravimetric determination [14], samples that are treated with EMR—Lipid provided significantly cleaner background, showing that dramatically less matrix is being introduced into the GC/MS/MS system. The number of active sites that accumulate in the GC/MS flow path are decreased, preserving the analytical integrity of the instrument. This is demonstrated with better analyte precision (RSDs) for over 100 injections of avocado samples on the GC/MS/MS (Table 4). Samples treated with EMR—Lipid achieved RSDs <15% for 91% of the analytes, most in single digits. Two compounds, captan (RSD 29.9%) and DDT (RSD 21.6%) gave higher RSDs over the 100 injection experiment, but gave 11.1% and 6.4% RSD for the first 50 injections, respectively.

Table 4. Comparison of analyte reproducibility (RSDs) over 50 and 100 injections of avocado samples treated with Agilent Bond Elut EMR—Lipid, C18/PSA, or zirconia sorbent by GC/MS/MS. The samples were fortified at 50 ng/g. Analyte peak areas were used to calculate RSD results.

	Analyte RSD over 100 injections (n = 20)			RSD over 50 injections (n = 10)		
Pesticides	**EMR—Lipid cleanup**	**C18/PSA cleanup**	**Zirconia sorbent cleanup**	**EMR—Lipid cleanup**	**C18/PSA cleanup**	**Zirconia sorbent cleanup**
Dichlorvos	6.2	10.5	16.8	2.2	9.4	6.3
2-Phenylphenol	7.0	13.6	19.5	5.0	12.4	8.4
Ethalfluralin	12.4	18.8	32.0	5.8	10.3	7.9
Sulfotep	7.1	11.8	17.2	3.1	6.4	10.8
Atrazine	6.8	12.2	19.1	3.2	12.2	5.2
Lindane	8.5	10.8	20.0	4.6	10.9	5.1
Chlorothalonil	12.5	11.7	37.4	8.0	12.9	11.0
Diazinon	6.6	11.7	16.9	4.4	10.5	5.6
Chlorpyriphos methyl	8.4	8.9	14.9	3.8	8.6	6.6
Dichlorfluanid	11.7	9.0	25.9	5.4	9.9	5.5
Aldrin	9.8	19.3	25.7	8.6	19.3	7.1
Tolylfluanid	10.5	6.6	17.8	4.2	6.9	6.6
Captan	29.9	51.9	47.1	11.1	24.9	21.7
Procymidone	6.8	14.3	22.5	5.6	13.8	4.8
Bupirimate	6.8	10.4	20.7	7.6	11.0	6.2
Endrin	8.3	12.6	24.1	5.9	13.8	5.4
Endosulfan sulfate	8.5	12.1	22.4	5.3	12.7	6.4
DDT	21.6	22.4	42.6	6.4	12.0	11.8
Iprodione	11.0	10.7	40.0	8.2	10.9	16.3
Permethrin	6.8	11.8	18.8	5.2	11.2	8.6
Parathion ethyl-d_{10} (IS)	11.8	7.2	13.0	4.7	6.8	7.0
TPP (IS)	9.1	19.9	28.3	9.0	22.5	12.8

In comparison, C18/PSA produced RSDs <15% for 74% of analytes, and zirconia dramatically fewer, at only 9%. The zirconia-treated extract was especially problematic with 100% of the analytes above 10% RSD, 57% of which were well above 20% RSD over 100 injections. This indicates that the higher level of matrix remaining in the C18/PSA and zirconia cleanup extract is negatively affecting instrument performance, resulting in significant variability of analyte response. These results attest to the excellent matrix removal provided by EMR—Lipid, which results in less activity in the GC flow path, higher precision over multiple injections, and more samples being run before instrument maintenance.

Conclusions

A rapid, reliable, and robust method using QuEChERS AOAC extraction followed by Agilent Bond Elut EMR—Lipid cleanup was developed and validated for the analysis of 23 GC-amenable pesticides in avocado. Matrix effects were assessed and compared with traditional C18/PSA and zirconia sorbent cleanup. Results demonstrate that the EMR—Lipid provides superior chromatographic cleanliness with both GC/MS and GC/MS/MS versus C18/PSA and zirconia sorbent. Implementing EMR—Lipid cleanup facilitates the use of GC/MS for sample analysis in high-fat matrices. The recovery comparison demonstrates that EMR—Lipid cleanup produced comparable analyte recoveries relative to C18/PSA, and better recovery than zirconia sorbent. The greatest advantage of EMR—Lipid in this application was attributed to the high degree of matrix removal, providing outstanding reproducibility over 100 injections on the GC/MS/MS. The analyte responses of C18/PSA and especially zirconia-treated samples were highly variable over this 100-injection experiment. The use of EMR—Lipid as a dSPE cleanup material in a QuEChERS workflow, therefore, improves overall laboratory productivity, increases sample throughput, decreases data process and review, reduces batch reruns, and reduces instrument maintenance. Future work will examine the advantages of enhanced matrix removal for other complex, high-fat samples and target analytes.

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Benefits of EMR—Lipid Cleanup with Enhanced Post Treatment on Pesticides Analysis by GC/MS/MS

Application Note

Food Testing

Author

Limian Zhao
Agilent Technologies, Inc.

Abstract

Agilent Bond Elut Enhanced Matrix Removal—Lipid (EMR—Lipid) is a next-generation sample preparation product designed for the selective cleanup of lipids in fatty samples. The product is implemented in a convenient dispersive solid phase extraction (dSPE) format for the treatment of extracts from widely accepted workflows such as QuEChERS and protein precipitation. The EMR protocol is modified after the EMR—Lipid cleanup, with the use of anhydrous $MgSO_4$ in a pouch format. Anhydrous $MgSO_4$ is used for the separation of the aqueous and acetonitrile solvent phases, and the subsequent drying step to completely remove residual water and any water-soluble residues. The enhanced post-sample treatment has significant impact on GC-type applications by improving instrumental analysis reproducibility, especially for labile analytes. This study investigates the modified EMR protocol for the analysis of GC amenable pesticides in avocado by GC/MS/MS. The modified EMR protocol improves instrumental analytical reproducibility, reliability, and long-term usability, especially for labile pesticides, while maintaining high matrix removal efficiency and acceptable analyte recovery.

Agilent Technologies

Introduction

The analysis of pesticide residues in food commodities is routine for many laboratories. The adoption of the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method [1,2], allows the analysis of hundreds of pesticides at low concentrations. The methodology has worked well for various fruits and vegetables. However, foods high in fat such as avocado, nuts, and foods of animal origin present new challenges [3,4]. Overcoming these challenges is a high priority for laboratories tasked with reaching the stringent validation criteria required by government agencies to ensure that food is safe for consumption.

Agilent Bond Elut Enhanced Matrix Removal—Lipid (EMR—Lipid) is a novel sorbent material that selectively removes major lipid classes from the sample extract without unwanted analyte loss. A previous application note demonstrated the exceptional cleanliness that EMR—Lipid provides for complex, fatty samples such as avocado. EMR—Lipid also meets the recovery and precision requirements for multiclass pesticide residues [5]. Advancements in post-sample treatment have determined that removal of NaCl from the polish step is advantageous. The presence of NaCl could allow a small percentage of water and, therefore, nonmatrix water-dissolved residues to be present in the final extract. Complete removal of water residue is important for reliable GC and GC/MS analysis.

The enhanced post sample treatment incorporates anhydrous $MgSO_4$ for phase separation and sample drying. This significantly improves the removal of water and water-dissolved residue without sacrificing the matrix removal of EMR—Lipid cleanup. This study demonstrates the benefits of using enhanced post-sample treatment after EMR—Lipid cleanup for pesticide analysis in avocado by GC/MS/MS. The four difficult labile pesticides: captafol, phosmet, coumaphos, and pyraclostrobin, were added to evaluate the impact of water residue on labile pesticide analysis.

Experimental

Reagent and chemicals

All regents and solvents were HPLC or analytical grade. Acetonitrile (ACN) and methanol were from Honeywell (Muskegon, MI, USA). Reagent grade acetic acid (AA) was from Sigma-Aldrich. The pesticide standards and internal standard were purchased from Sigma-Aldrich (St Louis, MO, USA).

Solution and standards

A solution of 1% AA in ACN was prepared by adding 10 mL of acetic acid to 990 mL of ACN. Standard and internal standard (IS) stock solutions were made in either ACN or methanol at 2.0 mg/mL. A combined working solution was prepared in ACN at 25 µg/mL. A 25 µg/mL solution of combined IS working solution was prepared in ACN, including TPP, Parathion ethyl d10, and ^{13}C-DDT.

Equipment and materials

Equipment and material used for sample preparation included:

- Geno Grinder (Metuchen, NJ, USA)
- CentraCL3R centrifuge (Thermo IEC, MA, USA)
- Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)
- Vortexer and Multi-Tube Vortexer (VWR, Radnor, PA, USA)
- Bottle top dispenser (VWR, So. Plainfield, NJ, USA)
- Eppendorf pipettes and repeater
- Agilent Bond Elut AOAC extraction kit (p/n 5982–5755)
- Agilent Bond Elut EMR—Lipid dSPE (p/n 5982–1010) and EMR—$MgSO_4$ polish pouches (p/n 5982–0102)

Instrument conditions

The GC and MS conditions were used in previous application notes [5]. Analysis was completed on an Agilent 7890A GC equipped with an Agilent 7693B Autosampler and an Agilent 7000C Triple Quadrupole GC/MS system. Column backflushing was used, which is highly recommended for complex sample matrices.

GC conditions

Parameter	Value
GC:	Agilent 7890A GC
Column:	Agilent J&W DB-5ms Ultra Inert, 0.25 mm × 15 m, 0.25 µm (p/n 122–5512UI)
Carrier:	Helium, constant pressure
Gas filter:	Gas Clean carrier gas filter kit, 1/8 inch (p/n CP17974)
Inlet liner:	Agilent Ultra Inert single taper splitless liner with wool (p/n 5190–2293)
Inlet:	MMI inlet at pulsed cold splitless mode, 75 °C initially, hold for 0.02 min, then ramp to 350 °C at 750 °C/min
Pulsed splitless injection:	36 psi until 0.75 min
Purge flow to split vent:	60 mL/min at 0.75 min
Inlet pressure:	17 psi during run, and 1.0 psi during backflushing
Oven:	60 °C for 2.57 min, then to 150 °C at 50 °C/min, to 200 °C at 6 °C/min, to 300 °C at 16 °C/min, hold for 3 min
Postrun:	2 min at 300 °C
Capillary Flow Technology:	Agilent UltiMetal Plus Purged Ultimate Union (p/n G3182-61581) for backflushing the analytical column and inlet
Autosampler:	Agilent 7693 Autosampler and sample tray 10 µL syringe (p/n G4513-80220), 1 µL injection volume

MSD conditions

Parameter	Value
MSD:	Agilent 7000C Triple Quadrupole GC/MS, inert, with performance electronics
Vacuum pump:	Performance turbo
Mode:	MRM
Transfer line temp:	280 °C
Source temp:	300 °C
Quad temp:	150 °C for Q1 and Q2
Solvent delay:	2.57 min
MS resolution:	MS1 and MS2 = 1.2u

Table 1 lists the MRM transitions for the four additional labile pesticides used in this study. The MRM transitions for other pesticides were listed in reference [5].

Table 1. GC/MS/MS MRM parameters and retention times for the additional labile pesticides used in this study.

		MRMs			
Labile analyte	RT (min)	Quant channel	CE (v)	Qual channel	CE (v)
Captafol	18.20	183 → 79	10	150 → 79	5
Phosmet	18.77	160 → 77.1	20	160 → 133.1	20
Coumaphos	20.67	361.9 → 109	10	210 → 182	10
Pyraclostrobin	22.03	132 → 77.1	20	164 → 132	15

Sample preparation

The modifications only apply to the polishing step after EMR—Lipid cleanup. There are no changes to the QuEChERS extraction step and the EMR—Lipid cleanup step. After the EMR—Lipid cleanup, the ACN layer was phase separated from the aqueous phase, and further dried with anhydrous $MgSO_4$. Figure 1 shows the protocol diagram. There are two points to be emphasized for the modified procedure after EMR—Lipid cleanup:

- First, adding $MgSO_4$ to the sample minimizes the exothermic effect of $MgSO_4$ and water, and reduces salt clumping.
- Second, drying tubes were preweighed into 2 mL tubes using 300 mg of anhydrous $MgSO_4$ salt (from an EMR—Polish pouch) for 1 mL of ACN extract after EMR—Lipid cleanup.

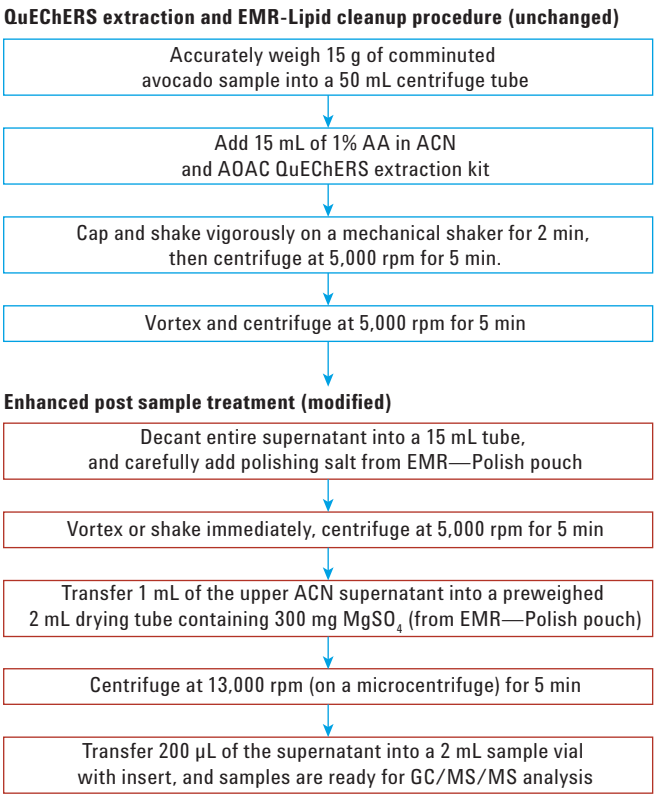

Figure 1. Sample preparation workflow showing the recommended protocol for the analysis of pesticides in avocado by GC/MS/MS, using unchanged QuEChERS extraction and EMR—Lipid cleanup steps followed with the enhanced post sample treatment procedure.

Sample matrix impact on GC/MS/MS system performance

To directly measure the impact of the sample matrix on GC/MS/MS instrument performance, avocado matrix blank sample was prepared following the original polish step and the enhanced post sample treatment after EMR—Lipid cleanup. The matrix blank was then post-spiked with pesticide standards at 50 ppb to determine matrix effects on GC/MS/MS system performance.

Labile compounds were investigated for analyte responses (peak area), peak shape, and reproducibility over multiple injections.

The injection sequence consisted of injecting four matrix blank samples followed by a post-spiked sample. This injection pattern was repeated until 100 injections, therefore, 80 matrix blank sample injections and 20 post-spiked sample injections were run in the testing sequence. The liner was replaced, and the column head was trimmed between sequences using original polish or enhanced post sample treatment. Since both UI single taper splitless liner with wool and UI dimple liner have been usually used for the analysis of complicated matrix samples, they were evaluated for their appearance after 100 injections of avocado samples prepared using enhanced post sample treatment.

Matrix removal efficiency and analyte recovery

Matrix removal efficiency was confirmed by running the avocado matrix blank by GC/MS under full scan mode, and comparing the entire chromatographic profile using the efficiency calculation, as previously described [5]. Analyte recovery was evaluated by comparing the pre-spike and post-spike peak area of each analyte at 50 ppb.

Results and Discussion

Higher analyte responses and better peak shape

The enhanced post treatment after EMR—Lipid cleanup removes the residual water and water-dissolved residues. Figure 2 shows the chromatographic comparison for labile compound response and peak shape on GC/MS/MS using enhanced post treatment versus the original polishing step. Analyte responses were increased more than threefold, especially for pyraclostrobin and trichlorfon, where a 10 fold increase was observed. Chromatography was also improved, with more symmetrical peak shape and less tailing, providing easier data processing. These improvements indicated that these labile compounds passed through the GC flow path without significant interactions on the flow path surface.

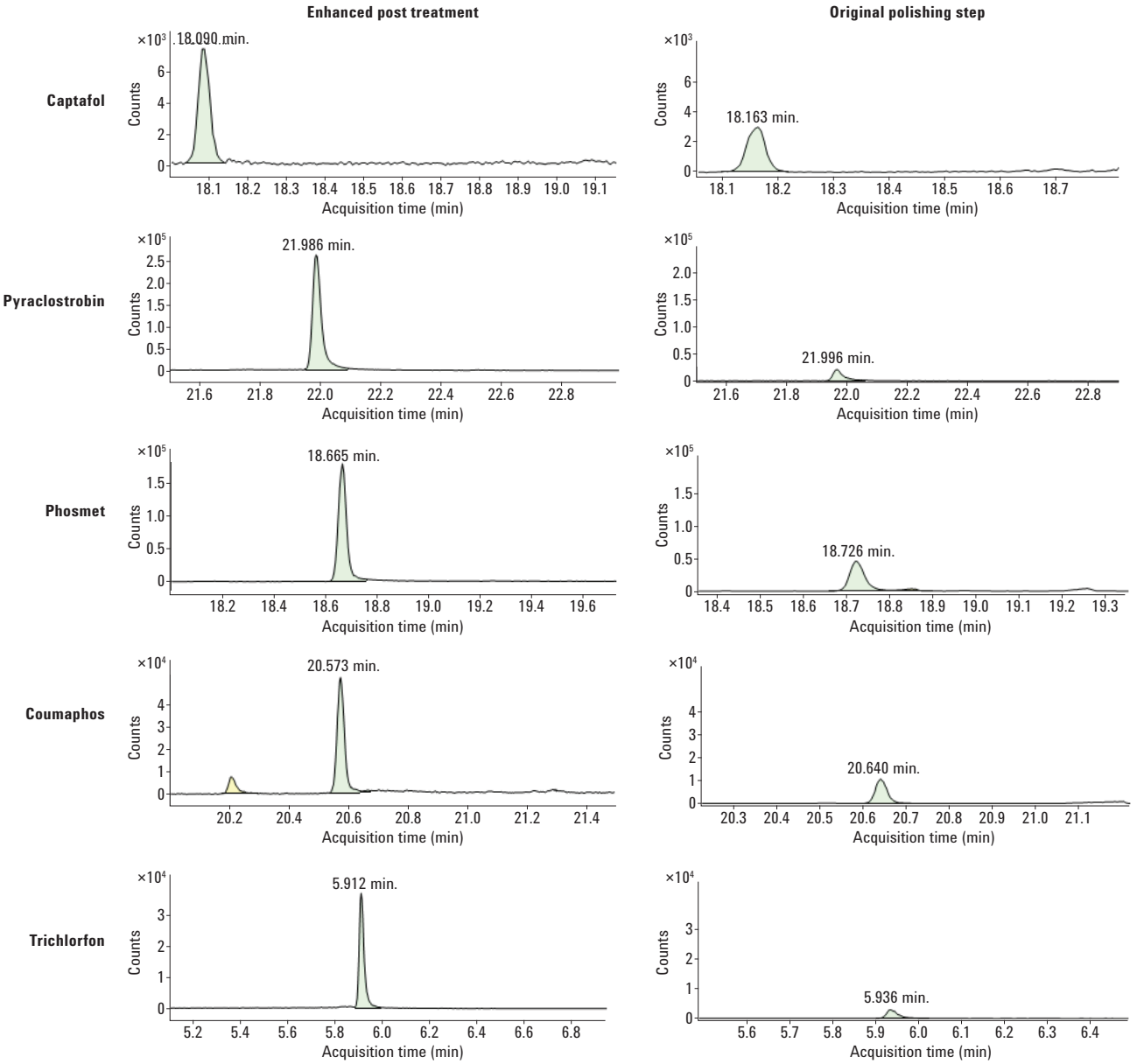

Figure 2. Chromatographic comparison for labile compounds responses and peak shape on GC/MS/MS using enhanced post treatment and original polishing step after EMR—Lipid cleanup.

Improved system reproducibility

Method reproducibility is arguably the most important aspect of analysis as it directly impacts the reliability of quantitation results. As matrix accumulates in the flow path over multiple injections, analyte responses can vary over multiple injections, especially for labile compounds. These inconsistent responses make the quantitation difficult and unreliable. Our previous results demonstrated significant improvements in GC/MS/MS system reproducibility over multiple injections of complex samples prepared using EMR—Lipid cleanup [5]. Despite these improvements, some labile compounds still showed variability over multiple injections. This variability is mostly caused by trace amounts of water residues remaining in the final sample extract. The enhanced post treatment after EMR—Lipid cleanup, $MgSO_4$ salt partition, and drying steps were implemented to eliminate water residue and water dissolved solid residue from the final sample extract, thus improving the GC/MS/MS system reproducibility.

In Figure 3, pyraclostrobin was used as an example to show the improved reproducibility when injecting avocado samples prepared by the enhanced post treatment after EMR—Lipid cleanup. The comparison includes results from samples prepared using the enhanced post treatment and original polishing step after EMR—Lipid cleanup, as well as using traditional PSA/C18 cleanup. The data clearly demonstrate the dramatically improved reproducibility of pyraclostrobin response in samples prepared using the enhanced post treatment after EMR—Lipid cleanup. When using traditional PSA/C18 cleanup or EMR original protocol to prepare samples, the pyraclostrobin signal drops to 30–40% of the initial response after 100 injections. This inconsistency will cause quantitative analysis to fail for this compound. However, when using enhanced post treatment after EMR—Lipid cleanup, excellent signal reproducibility for pyraclostrobin (±10% deviations) was obtained. The improved reproducibility gained using the enhanced post treatment after EMR—Lipid cleanup makes quantitative analysis of labile analytes reliable and robust.

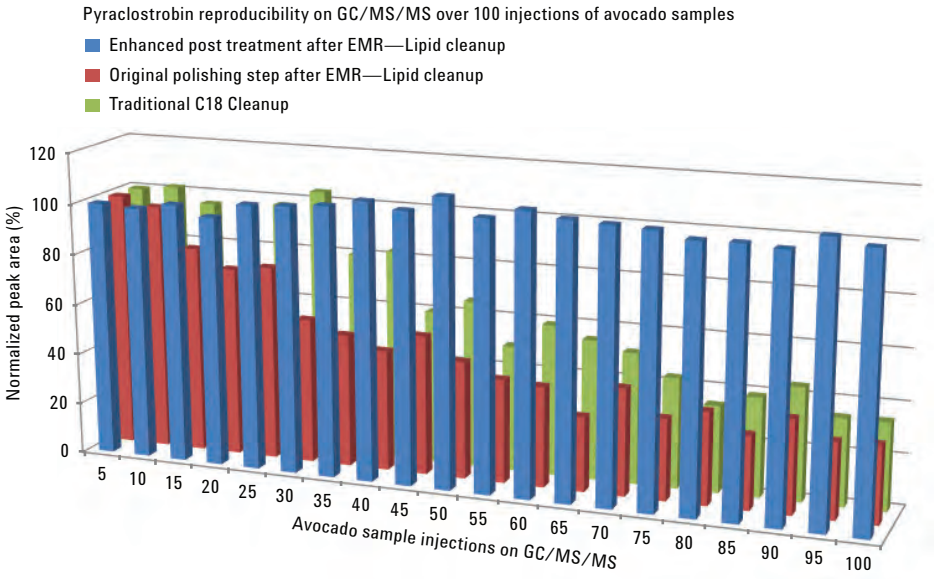

Figure 3. Labile compound pyraclostrobin response reproducibility on GC/MS/MS over 100 avocado sample injections prepared using enhanced post treatment and original polishing step after EMR—Lipid cleanup, and traditional C18 cleanup.

Table 2 lists all the pesticides tested in this study and their respective RSD over 100 injections of avocado using the method described. The EMR—Lipid cleanup followed with enhanced post treatment gives less than 10% RSDs for 24 of the 29 compounds. Captan, Folpet, Captafol, and DDT are problematic compounds on GC/MS/MS, and the high RSDs, especially from PSA/C18 cleanup, reflect a significant signal variation within 100 injections. However, the signal reduction for samples prepared by EMR—Lipid cleanup and enhanced post treatment was reduced, and the reproducibility of these four labile pesticides within 50 injections met the acceptance criteria, with less than 10% RSD.

Table 2. Analytes GC/MS/MS reproducibility (peak area RSD %) over 100 injections of avocado samples.

	Analyte RSD over 100 injections (n = 20)		
Pesticide	**EMR-Lipid cleanup with enhanced post treatment**	**EMR-Lipid cleanup with original polishing step**	**C18/PSA cleanup**
Dichlorvos	8.5	6.2	10.5
Trichlorfon	9.2	35.0	73.0
2-Phenylphenol	2.5	7.0	13.6
Ethalfluralin	4.6	12.4	18.8
Sulfotep	3.1	7.1	11.8
Atrazin	2.1	6.8	12.2
Lindane	3.1	8.5	10.8
Chlorothanil	2.2	12.5	11.7
Diazinon	2.6	6.6	11.7
Chlorpyrifos-Me	2.6	8.4	8.9
Dichlorfluanid	5.4	11.7	9.0
Aldrin	2.1	9.8	19.3
Tolyfluanid	6.6	10.5	6.6
Captan	29.8	29.9	51.9
Folpet	22.0	53.8	52.2
Procymidone	2.1	6.8	14.3
Bupirimate	3.1	6.8	10.4
Endrin	4.0	8.3	12.6
Endosulfan sulfate	3.6	8.5	12.1
DDT	16.1	21.6	22.4
Captafol	38.5	53.8	63.7
Iprodione	3.7	11.0	10.7
Phosmet	6.2	24.0	12.5
Coumaphos	4.3	19.8	9.7
Permethrin	3.0	6.8	11.8
Pyraclostrobin	3.7	43.7	38.8
Deltamethrin	8.7	22.5	9.8
Parathion ethyl –d10 (IS)	4.9	11.8	7.2
TPP (IS)	2.1	9.1	19.1

Longer GC inlet liner and column lifetime

Another advantage of using enhanced post treatment after EMR—Lipid cleanup is the reduction of nonvolatile salt residue, which can remain dissolved in trace water residues. We tested two types of UI liners for 100 injections of avocado samples, Agilent Ultra Inert single taper splitless liner with wool (p/n 5190-2293) and Agilent UI dimple liner (p/n 5190–2297). After the test, the appearance of the liner was visually inspected for residue deposition. Figure 4 shows that both liners are virtually clean after 100 injections. These results attest to the superior cleanliness achieved using EMR—Lipid cleanup following enhanced post treatment. It results in longer liner and column lifetime and less system maintenance.

Figure 4. Typical GC inlet liners appearance after 100 injections of avocado samples prepared by EMR-Lipid cleanup followed with enhanced post treatment. A) Agilent Ultra Inert single taper splitless liner with wool, B) Agilent UI dimple liner.

Equivalent matrix removal efficiency and analyte recovery

Matrix removal efficiency was evaluated using the GC/MS full scan profile comparison before and after cleanup [5]. Results showed that equivalent matrix removal efficiency can be achieved using the enhanced post treatment and the original polishing step after EMR—Lipid cleanup (Figure 5).

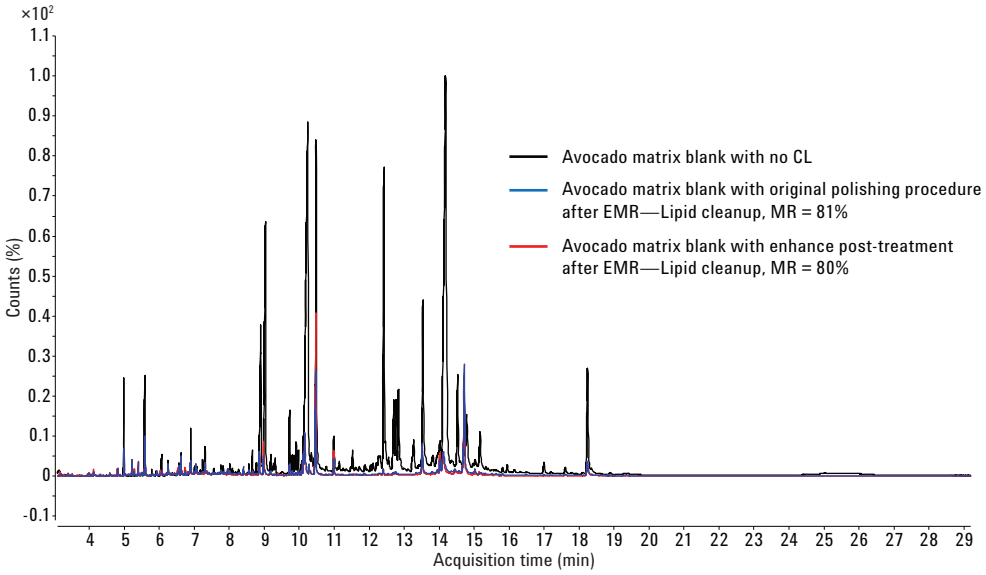

Figure 5. GC/MS full scan chromatograph comparison demonstrate the equivalent matrix removal efficiency provided by enhanced post treatment and original polishing step after EMR—Lipid cleanup.

Figure 6 shows the pesticides recovery comparison for 50 ppb fortified avocado samples (n = 6) prepared by EMR—Lipid cleanup followed with the enhanced post treatment and original polishing step, respectively. Some analytes show slightly lower recoveries using enhanced post treatment. However, the drastic improvements in reproducibility with less than 5% RSD for all compounds is significant.

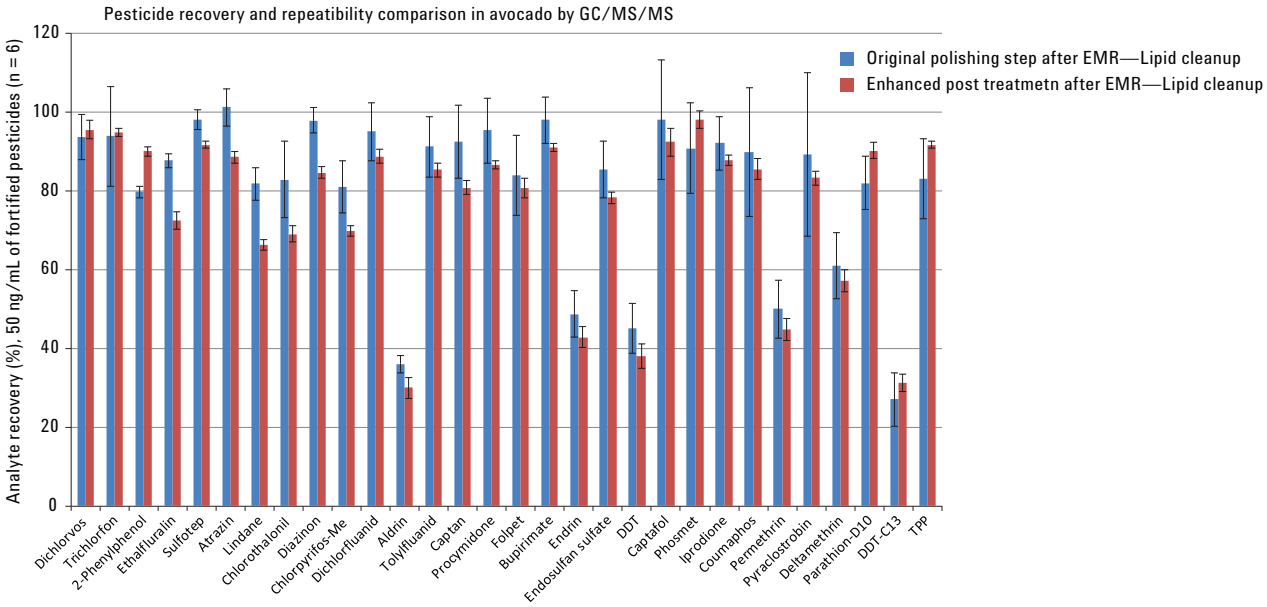

Figure 6. Pesticides recovery of avocado sample fortified at 50 ng/mL prepared by enhanced post treatment and original polishing step after EMR—Lipid cleanup.

Conclusions

The enhanced post sample treatment after EMR—Lipid cleanup implements a polish step and a drying step with anhydrous $MgSO_4$ to remove residual water and water-dissolved residue before sample injection on GC/MS/MS. It improves the GC/MS/MS analysis by providing higher analyte response, better peak shape, excellent instrument reproducibility, and longer inlet liner and column life. This approach is ideal for analysts seeking to improve their sample preparation for complex, fatty samples, especially when labile analytes are of interest. The enhanced post sample treatment after EMR—Lipid cleanup also maintains high matrix removal efficiency for complicated samples, and delivers acceptable analyte recovery for multiresidue pesticides analysis. The polish salt (anhydrous $MgSO_4$) is available in a pourable pouch for easy dispensing into samples, and better storage.

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Veterinary Drug Analysis

Multiresidue Analysis of Veterinary Drugs in Bovine Liver with Bond Elut EMR—Lipid by LC/MS/MS

Multiclass Multiresidue Veterinary Drug Analysis in Beef with Agilent Captiva EMR—Lipid by LC/MS/MS

Macrolide Analysis in Pork Using Bond Elut EMR—Lipid and InfinityLab Poroshell 120

Multiresidue Analysis of Veterinary Drugs in Bovine Liver by LC/MS/MS

Agilent Bond Elut Enhanced Matrix Removal—Lipid

Application Note

Food Testing and Agriculture

Authors

Limian Zhao, Derick Lucas
Agilent Technologies, Inc.

Abstract

Agilent Bond Elut Enhanced Matrix Removal—Lipid (EMR—Lipid) is the next generation of sample preparation product, and is available for convenient dispersive solid phase extraction (dSPE). The material is highly selective towards coextracted matrix, especially from fatty samples (fat content > 5%) without negatively impacting analyte recovery. This study demonstrates the application of this novel product for the analysis of 30 representative veterinary drugs in bovine liver. The procedure involves a rapid and efficient protein precipitation extraction by acidified acetonitrile, followed by the use of EMR—Lipid dSPE and a polish kit for further cleanup. The amount of matrix removed by the EMR-Lipid protocol was determined by the weight of coextractives and postcolumn infusion experiments. Compared to other matrix cleanup products, EMR—Lipid dSPE provides more effective matrix removal and better analyte recoveries. The optimized EMR—Lipid method delivers superior cleanliness, and excellent accuracy and precision for all 30 veterinary drug compounds at all levels, providing fast, robust, and effective analysis of high-fat samples.

Agilent Technologies

Introduction

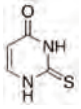
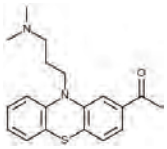
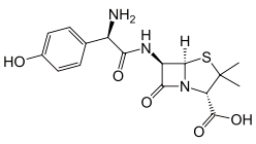
Veterinary drugs are widely used for animals in the food production industry to prevent diseases, or as growth promoters. These drugs accumulate in animal tissue, and improper use can lead to drug residue build-up in edible tissues, which are a known risk to human health. With increased attention on food safety, regulations have been put in place in nearly every country to limit the drugs used in food animal production [1-4].

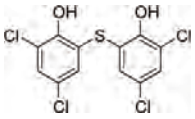
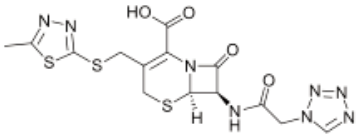
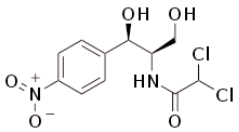
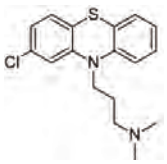
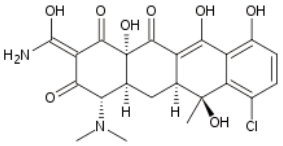
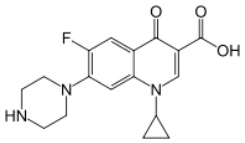
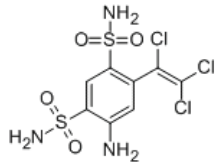
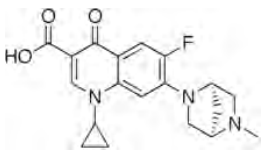
Foods from animal origin such as muscle, liver, and eggs are usually chemically complex and, therefore, it is critical to apply an efficient sample preparation method that includes general extraction and efficient cleanup. The established sample preparation methods include traditional solvent extraction, solid phase extraction (SPE), or a combination of multiple techniques. These methods are usually labor-intensive, time consuming, only suitable for limited classes of compounds, and require additional method development.

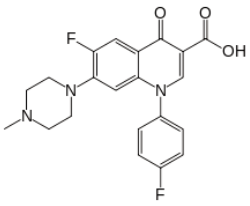
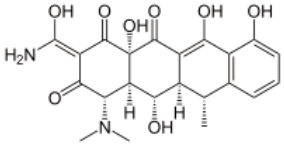
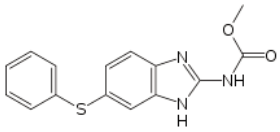
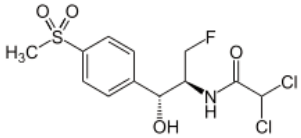
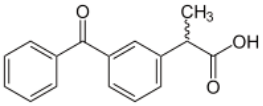
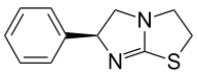
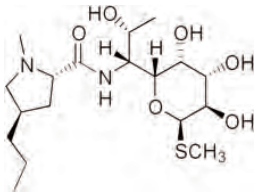
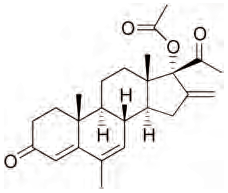
Multiclass, multiresidue methods are becoming increasingly popular in regulatory monitoring programs due to their increased analytical scope and laboratory efficiency. The number of veterinary drugs being monitored has increased in the past few years, and now there are more than 100 reported [5-8]. Sample pretreatment usually involves extraction with a mixture of acetonitrile:water, followed by C18 cleanup, other cleanup techniques, or both. Sorbents such as C18 only provide limited removal of coextracted lipids, which can result in precipitation in the final sample on dilution or reconstitution. The generation of precipitate requires sample filtration before LC/MS/MS injection, and may cause analyte loss. Hexane can be added during the dispersive solid phase extraction (dSPE) to remove coextracted lipids but is nonselective, time-consuming, and removes hydrophobic analytes. The use of zirconia sorbent materials for cleanup provides improved matrix cleanup compared to C18, but also results in more analyte loss, especially for carboxylic acid- and hydroxyl-containing compounds such as fluoroquinolones, tetracyclines, and macrolides [7,8].

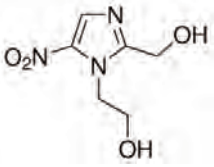
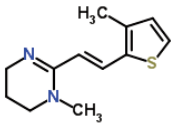
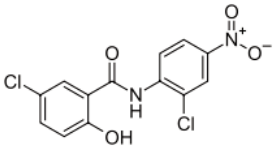
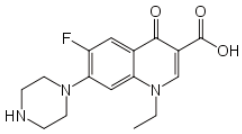
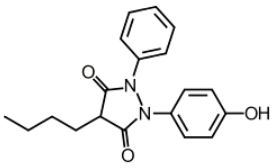
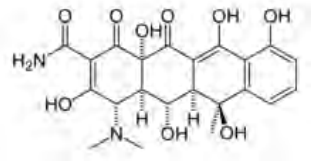
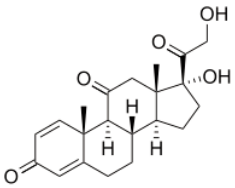
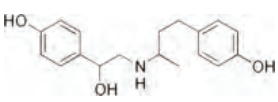
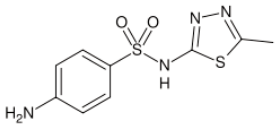
Agilent Bond Elut Enhanced Matrix Removal—Lipid (EMR—Lipid) is a novel sorbent material that selectively removes major lipid classes from the sample without unwanted analyte retention. Removal of lipid interferences from complex matrices is especially important for techniques such as QuEChERS and protein precipitation. Since these simple sample preparation methods cannot remove a large percentage of lipids, the coextractives will remain in the final sample extract with the target analytes. This causes chromatographic anomalies, poor data precision and accuracy, and increased maintenance issues. In this study, we investigate a novel sample preparation approach for the analysis of 30 representative and challenging veterinary drugs in bovine liver using a simple protein precipitation extraction followed by EMR—Lipid cleanup. The selected veterinary drugs represent 17 different classes, including hydrophilic to hydrophobic, acidic, neutral, and basic drugs. Table 1 shows the chemical and regulatory information for these veterinary drugs.

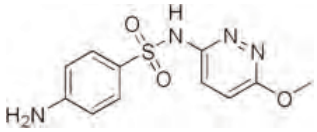
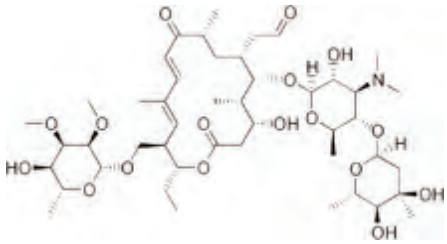
Table 1. Chemical and physical properties of veterinary drugs.

Name	Drug class	Log P	pKa	Molecular formula	Structure	US tolerance (µg/g)
2-Thiouracil	Thyreostat	–0.28	7.75	$C_4H_4N_2OS$		N.A
Acetopromazine	Tranquilizer	3.49	9.3	$C_{19}H_{22}N_2OS$		N.A
Amoxicillin	β-Lactam	0.86	2.4	$C_{16}H_{19}N_3O_5S$		0.01

Name	Drug class	Log P	pKa	Molecular formula	Structure	US tolerance (µg/g)
Bithionol	Flukicide	5.51	4.82	$C_{12}H_6Cl_4O_2S$		N.A
Cefazolin	Cephalosporin	–1.5	2.3	$C_{14}H_{14}N_8O_4S_3$		N.A.
Chloramphenicol	Phenicol	1.14	5.5	$C_{11}H_{12}C_{12}N_2O_5$		(c)
Chlorpromazine	Tranquilizer	5.20	9.3	$C_{17}H_{19}ClN_2S$		N.A
Chlortetracycline	Tetracycline	0.24	3.3, 7.4, 9.3	$C_{22}H_{23}ClN_2O_8$		2 (d)
Ciprofloxacin	Fluoroquinolone	0.28	6.09, 8.74	$C_{17}H_{18}FN_3O_3$		(c)
Clorsulon	Flukicide	1.25	–	$C_8H_8Cl_3N_3O_4S_2$		0.1 (e)
Danofloxacin	Fluoroquinolone	1.2	6.04	$C_{19}H_{20}FN_3O_3$		0.02 (a, e)

Name	Drug class	Log P	pKa	Molecular formula	Structure	US tolerance (µg/g)
Difloxacin	β-Lactam	2.78	5.85	$C_{21}H_{19}F_2N_3O_3$		N.A.
Doxycycline	Tetracycline	–0.54	3.4	$C_{22}H_{24}N_2O_8$		N.A
Fenbendazole	Anthelmintic	3.75	10.27	$C_{15}H_{13}N_3O_2S$		0.4 (e, h), 2 (b)
Florfenicol	Phenicol	–0.12	10.73	$C_{12}H_{14}C_{12}FNO_4S$		0.2 (b)
Ketoprofen	Tranquilizer	2.81	3.88	$C_{16}H_{14}O_3$		N.A
Levamisole	Anthelmintic	1.85	8.0	$C_{11}H_{12}N_2S$		0.1 (e)
Lincomycin	Lincosamide	0.91	7.8	$C_{18}H_{34}N_2O_6S$		0.1(b)
Melengesterol acetate (MGA)	Other	4.21	11.42	$C_{25}H_{32}O_4$		0.025 (g)

Name	Drug class	Log P	pKa	Molecular formula	Structure	US tolerance (µg/g)
Methonidazole-OH	Nitroimidazole	–0.81	3.09	$C_6H_9N_3O_4$		(c)
Morantel	Anthelmintic	1.97	>12	$C_{12}H_{16}N_2S$		N.A
Niclosamide	Flukicide	5.41	5.6	$C_{13}H_8C_{12}N_2O_4$		N.A
Norfloxacin	Fluoroquinolone	0.82	6.32	$C_{16}H_{18}FN_3O_3$		(c)
Oxyphenylbutazone	NSAID	2.72	4.87	$C_{19}H_{20}N_2O_3$		N.A
Oxytertracycline	Tetracycline	–1.5	3.27	$C_{22}H_{24}N_2O_9$		2 (d)
Prednisone	Corticosteroid	1.57	12.58	$C_{21}H_{26}O_5$		N.A
Ractopamine	β-Agonist	1.65	9.4	$C_{18}H_{23}NO_3$		0.03 (e)
Sulfamethizole	Sulfonamide	0.51	5.45	$C_9H_{10}N_4O_2S_2$		N.A

Name	Drug class	Log P	pKa	Molecular formula	Structure	US tolerance (µg/g)
Sulfamethoxypyridazine	Sulfonamide	0.32	6.7	$C_{11}H_{12}N_4O_3S$		N.A
Tylosin	Macrolide	3.27	7.7	$C_{46}H_{77}NO_{17}$		0.2 (f)

a Tolerance in cattle liver (for tulathromycin, a marker residue has been established)

b Tolerance in swine muscle

c Banned for extralabel use

d Tolerance is the sum of residues of tetracycline including chlortetracycline, oxytetracycline, and tetracycline in muscle

e Tolerance in cattle muscle

f Tolerance in uncooked cattle fat, muscle, liver, and kidney

g Tolerance in cattle fat

h Tolerance in goat muscle

Experimental

All regents and solvents were HPLC or analytical grade. Acetonitrile (ACN) was from Honeywell (Muskegon, MI, USA). Dimethyl sulfoxide (DMSO), vet drug standards, and internal standard were from Sigma-Aldrich, Corp. (St Louis, MO, USA). Reagent-grade formic acid (FA) was from Agilent (p/n G2453-86060). Ammonium acetate (NH_4OAc) was from Fisher Chemicals (Fair Lawn, NJ, USA).

Solution and standards

Formic acid (5%) in ACN was freshly prepared by adding 5 mL formic acid to 95 mL ACN. Ammonium acetate stock solution (1 M) was made by dissolving 19.27 g NH_4OAc in 250 mL Milli-Q water. The solution was stored at 4 °C. A 5 mM ammonium acetate in water solution was made by adding 5 mL of 1 M ammonium acetate stock solution to 1 L of Milli-Q water.

Standard and internal standard (IS) stock solutions were made in DMSO at 2.0 mg/mL except for danofloxacin stock solution in DMSO at 1.0 mg/mL and ciprofloxacin stock solution in DMSO at 0.25 mg/mL. Amoxicillin and cefazolin stock solutions were made in water at 2.0 mg/mL. All stock solutions were prepared in amber glass vials, except plastic vials for amoxicillin and cefazolin stock solutions. All stock solutions were stored at –20 °C. The 30 compounds were allocated to two groups, G1 and G2, based on instrument response. A combined 25/5 µg/mL (G1/G2) standard working solution was prepared in 1/1 ACN/water. Flunixin-d_3 IS working solution (25 µg/mL) was prepared in 1/1 ACN/water.

Equipment

Equipment and material used for sample preparation included:

- Geno/Grinder (SPEX, Metuchen, NJ, USA)
- Centra CL3R centrifuge (Thermo IEC, MA, USA)
- Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)
- Vortexer and multitube vortexers (VWR, Radnor, PA, USA)
- Bottle top dispenser (VWR, So. Plainfield, NJ, USA)
- Eppendorf pipettes and repeater
- Agilent Bond Elut EMR—Lipid tubes (p/n 5982-1010) Agilent Bond Elut Final Polish for Enhanced Matrix Removal—Lipid (p/n 5982-0101)

Instrumentation

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

- Agilent 1290 Infinity Quaternary Pump (G4204A)
- Agilent1290 Infinity High Performance Autosampler (G4226A) equipped with an Agilent 1290 Infinity Thermostat (G1330B), and an Agilent1290 Infinity Thermostatted Column Compartment (G1316C)

The UHPLC system was coupled to an Agilent 6490 Triple Quadrupole LC/MS system equipped with an Agilent Jet Stream electrospray ionization source and iFunnel technology. Agilent MassHunter workstation software was used for data acquisition and analysis.

Instrument conditions

HPLC conditions

Column:	Agilent Poroshell 120 EC-C18, 2.1 × 150 mm, 2.7 µm (p/n 693775-902), Agilent Poroshell 120 EC-C18 UHPLC Guard, 2.1 × 5 mm, 2.7 µm (p/n 821725-911)
Mobile phase:	A) 0.1% FA in water B) 0.1% FA in acetonitrile
Flow rate:	0.3 mL/min
Column temp:	40 °C
Autosampler temp:	4 °C
Inj vol:	3 µL
Needle wash:	1:1:1:1 ACN:MeOH:IPA:H2O with 0.2% FA
Gradient:	Time (min) %B 0 10 0.5 10 8.0 100
Stop time:	12 min
Posttime:	3 min

Conditions, MS

Positive/negative mode

Gas temp:	120 °C		
Gas flow:	14 L/min		
Nebulizer:	40 psi		
Sheath gas heater:	400 °C		
Sheath gas flow:	12 L/min		
Capillary:	3,000 V		
iFunnel parameters:		Positive	Negative
	High-pressure RF	90 V	90 V
	Low-pressure RF	70 V	60 V

MS DMRM conditions relating to the analytes are listed in Table 2, and a typical chromatogram is shown in Figure 1.

Sample preparation

The final sample preparation procedure was optimized with the following steps.

1. Weigh 2 g (±0.1 g) homogenized bovine liver into 50 mL centrifuge tube.
2. Add 10 mL acidified acetonitrile (containing 5% FA).
3. Mix samples on a mechanical shaker for 2 min.
4. Centrifuge at 5,000 rpm for 5 min.
5. Add 5 mL ammonium acetate buffer (5 mM) to a 15 mL EMR—Lipid dSPE tube.
6. Transfer 5 mL of supernatant to EMR—Lipid tube.
7. Vortex immediately to disperse sample, followed by 60 s mixing on a multiposition vortexer table.
8. Centrifuge at 5,000 rpm for 3 min.
9. Transfer 5 mL of supernatant into a 15 mL EMR—Lipid polish tube containing 2 g salts (1:4 NaCl:$MgSO_4$) and vortex for 1 min.
10. Centrifuge at 5,000 rpm for 3 min.
11. Combine 200 µL of upper ACN layer and 800 µL water in a 2 mL sample vial, and vortex.

Table 2. LC/MS/MS DMRM parameters and retention times for target analytes.

Analyte	RT (min)	Delta RT (min)	Polarity	Precursor ion (*m/z*)	Product ion			
					Quant ion	CE (V)	Qual ion	CE (V)
2-Thiouracil	1.41	2	Negative	127	57.9	17	–	–
Amoxicillin	1.84	2	Positive	366.1	349.2	5	114	25
Methonidazole-OH	2.07	2	Positive	188.1	123.1	9	126.1	13
Levamisole	3.4	2	Positive	205.1	178.1	21	91.1	41
Lincomycin	3.35	2	Positive	407.2	126.1	37	70.1	80
Norfloxacin	4.22	2	Positive	320.1	302.2	21	276.1	17
Oxytetracycline	4.24	2	Positive	461.2	426.1	17	443.2	9
Ciprofloxacin	4.31	2	Positive	332.1	231	45	314.3	21
Danofloxacin	4.42	2	Positive	358.2	340.2	21	81.9	53
Ractopamine	4.4	2	Positive	302.2	107	33	77	77
Morantel	4.9	2	Positive	221.1	123.1	37	76.9	80
Cefazolin	4.65	2	Positive	455	323.1	9	156	13
Sulfamethizole	4.65	2	Positive	271	156.1	13	92	29
Sulfamethoxypyridazine	4.69	2	Positive	281.1	92	33	65.1	57
Difloxacin	4.83	2	Positive	400.2	382	25	356.3	17
Chlortetracycine	5.11	2	Positive	479.1	444.2	21	462.1	17
Doxycycline	5.24	2	Positive	445.2	428.1	17	410.2	25
Florfenicol	5.47	2	Negative	300.1	268.1	25	159.1	41
Chloramphenicol	5.68	2	Negative	321	152	17	257.1	9
Tylosin	5.85	2	Positive	916.5	173.9	45	772.5	33
Closulon	5.86	2	Negative	377.9	341.9	9	–	–
Prednisone	5.88	2	Positive	359.2	147.2	33	341.2	9
Acetopromizine	5.93	2	Positive	327.2	86	21	58	45
Chlorpromazine	6.49	2	Positive	319.1	86	21	58.1	45
Fenbendazole	6.77	2	Positive	300.1	268.1	25	159.1	41
Ketoprofen	6.8	2	Positive	255.1	208.9	13	77	57
Oxyphenbutazone	7.27	2	Negative	323.1	133.9	25	295	17
Flunixin-d_3 (NEG)	7.53	2	Negative	298.1	254.2	17	192	37
Flunixin-d_3 (POS)	7.53	2	Positive	300.1	282	25	264	41
Melengestrol acetate	8.78	2	Positive	397.2	337.4	13	279.2	21
Niclosamide	8.82	2	Negative	325	170.9	25	289.1	13
Bithionol	9.49	2	Negative	352.9	161	21	191.8	25

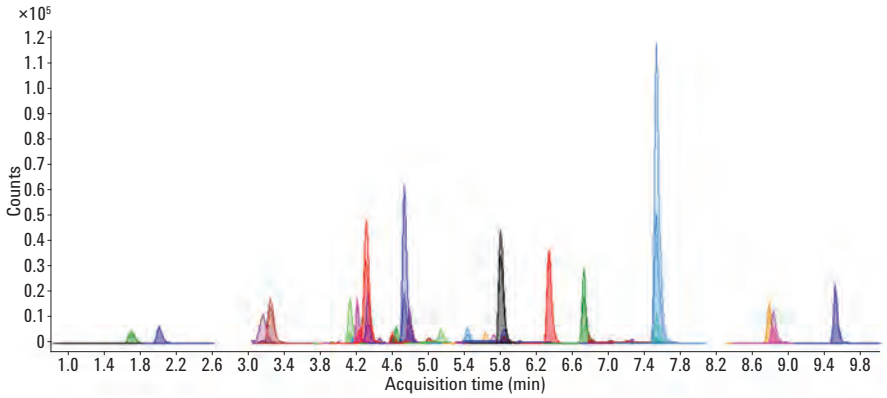


Figure 1. A typical LC/MS/MS chromatogram (DMRM) of a bovine liver sample fortified with a 50 ng/g veterinary drug standard and extracted by protein precipitation followed by cleanup with Agilent Bond Elut EMR—Lipid.

The sample is now ready for LC/MS/MS analysis. The entire sample preparation flow path is shown in Figure 2.

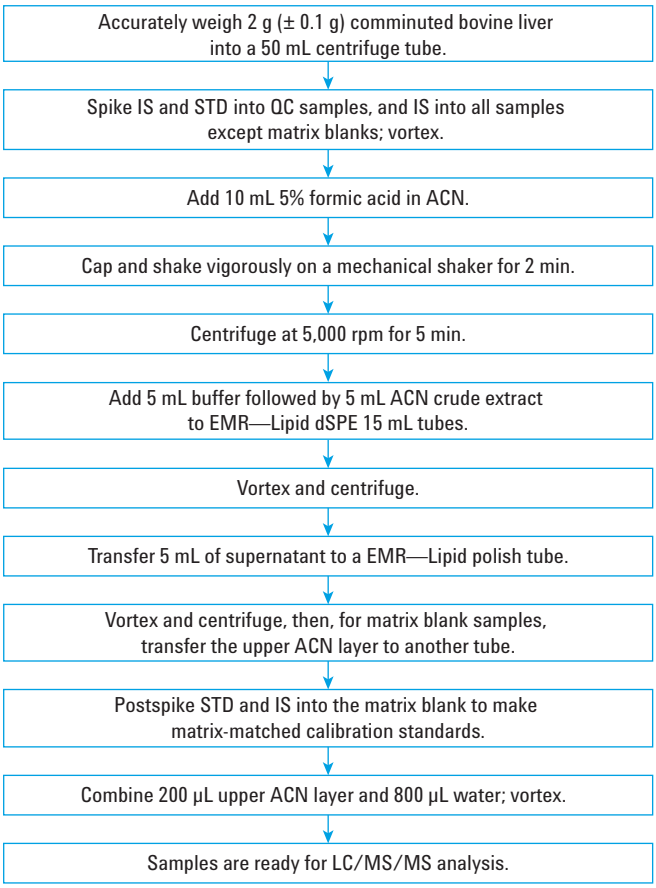


Figure 2, Sample preparation procedure using Agilent Bond Elut EMR—Lipid for the analysis of vet drugs in bovine liver.

Calibration standards and quality control samples

Prespiked QC samples were fortified with combined standard working solution appropriately, after step 1, for six replicates. For G1 analytes, the QC samples corresponded to 10, 50, 250, and 750 ng/g in liver. For G2 analytes, QC samples corresponded to 2, 10, 50, and 150 ng/g in liver. IS solution was also spiked into all samples except the matrix blank, corresponding to 200 ng/g of flunixin-d_3 in liver.

Matrix-matched calibration standards were prepared with standard and IS working solutions. Appropriate concentrations into the matrix blank samples after step 8 corresponded to 5, 25, 50, 250, 750, and 1,000 ng/g in liver (G1), or 1, 5, 10, 50, 150, and 200 ng/g in liver (G2), and 200 ng/g IS in liver.

Determining amount of coextractives

The amount of coextractives was determined by gravimetric measurement [7] for three different cleanup techniques; C18, zirconia sorbent, and EMR—Lipid. Samples were prepared as follows.

1. Heat glass tubes for ~1 h at 110 °C to remove moisture.
2. Cool tubes to room temperature.
3. Preweigh test tubes.
4. Accurately transfer 1 mL of initial matrix blank extract (no cleanup) and the matrix blanks with various cleanups, each in duplicate.
5. Dry all samples on a CentriVap at 50 °C for 1 h or until dry.
6. Heat the tubes for ~1 h at 110 °C to remove moisture.
7. Cool tubes to room temperature.
8. Reweigh the tubes.

The weight difference between after step 8 and after 3 is the amount of sample coextractive. The amount of coextractives removed by cleanup was the average weight difference of the matrix coextractives before and after cleanup.

Matrix effect assessment

Chromatographic matrix effect was assessed by a postcolumn infusion experiment. The matrix blank samples were injected with simultaneous postcolumn infusion of 10 ppb neat standard vet drug solution at 60 µL/min. All compound transitions were monitored through the entire LC cycle.

Additionally, the analyte response (peak area) was compared between postspiked liver extracts and the equivalent neat solutions. Postspiked liver extracts were made by postspiking standard solution into the blank liver matrix extract. The difference in response (peak area) is directly correlated to matrix effects.

Method optimization, validation, and comparison

Different optimization tests were assessed; 5% FA in ACN versus 1% FA in ACN for protein precipitation, ammonium acetate buffer versus water for EMR—Lipid cleanup, and with and without polish salts after EMR—Lipid cleanup. Results were evaluated based on analyte recovery, precision, and other quantitation parameters. The final optimized method was then validated by running a full quantitation batch with duplicated calibration curve standards bracketing all QC samples.

Recovery comparison data were gathered by pre- and postspiking liver samples at 50 ng/g. The extracts were then processed with acceptable cleanup protocols. For the EMR—Lipid protocol with protein precipitation, the cleanup procedure described in Figure 2 was employed. For QuEChERS, a C18 or zirconia sorbent dSPE cleanup was used, as follows.

1. Weigh 2 g of liver in a 50 mL tube.
2. Add 8 mL phosphate buffer (pH 7.0) and 10 mL acidified ACN (5% FA).
3. Vortex sample for 30 s.
4. Add EN salts for partitioning/extraction, and shake vigorously on a mechanical shaker.
5. Centrifuge at 5,000 rpm for 5 min.

The crude ACN liver extract used for further cleanup was prepared as follows.

1. Add 1 mL crude ACN liver extract to a 2 mL vial containing 25 mg C18 and 150 mg $MgSO_4$ (p/n 5982-4921), or into a 2 mL vial containing 100 mg zirconia sorbent.
2. Cap and vortex for 1 min.
3. Centrifuge at 13,000 rpm for 3 min (microcentrifuge).
4. Transfer 200 µL of supernatant into another vial containing 800 µL water.
5. Vortex and filter with a regenerated cellulose 0.45 µm filter.

Samples are then ready for LC/MS/MS analysis. The recovery was calculated by the ratio of analyte peak areas from pre- and postspiked samples.

Results and Discussion

Amount of coextractives

Table 3 shows the results from the sample coextractive gravimetric test, clearly demonstrating that EMR—Lipid dSPE provides the best matrix cleanup efficiency by weight than dSPE with C18 or zirconia sorbent.

Table 3. Bovine liver matrix coextractive gravimetric results for Agilent Bond Elut Enhanced Matrix Removal—Lipid, zirconia, and C18 cleanup.

Cleanup technique	Coextractives per 1 mL of ACN final extract (mg) (n = 2)	Matrix coextractive removal efficiency by cleanup (%)
No further cleanup	12.1	–
EMR—Lipid dSPE	5.3	56.2
Zirconia separation with dSPE	6.0	50.4
C18 dSPE	7.8	35.5

Matrix effect assessment

Postcolumn infusion (PCI) of vet drug standards was used for evaluation of matrix effects in the crude ACN extracts and three final ACN extracts cleaned up by EMR—Lipid, C18, and zirconia sorbent. All analytes were monitored through the entire LC cycle. The PCI chromatograms reflect the matrix impact for analytes monitored under positive and negative mode. The final ACN extract was not diluted before injection, and the ACN was injected directly. The PCI profiles are shown in Figure 3.

As shown in the red trace, significant matrix suppression (lower baseline) and matrix enhancement (large peaks) were observed with the injection of crude liver matrix blank without any cleanup. These matrix effects will have dramatic negative impacts on method reliability and data quality. In comparison, as shown in the blue trace, the use of EMR—Lipid cleanup gave significant improvements, as observed by reduced matrix suppression and enhancement.

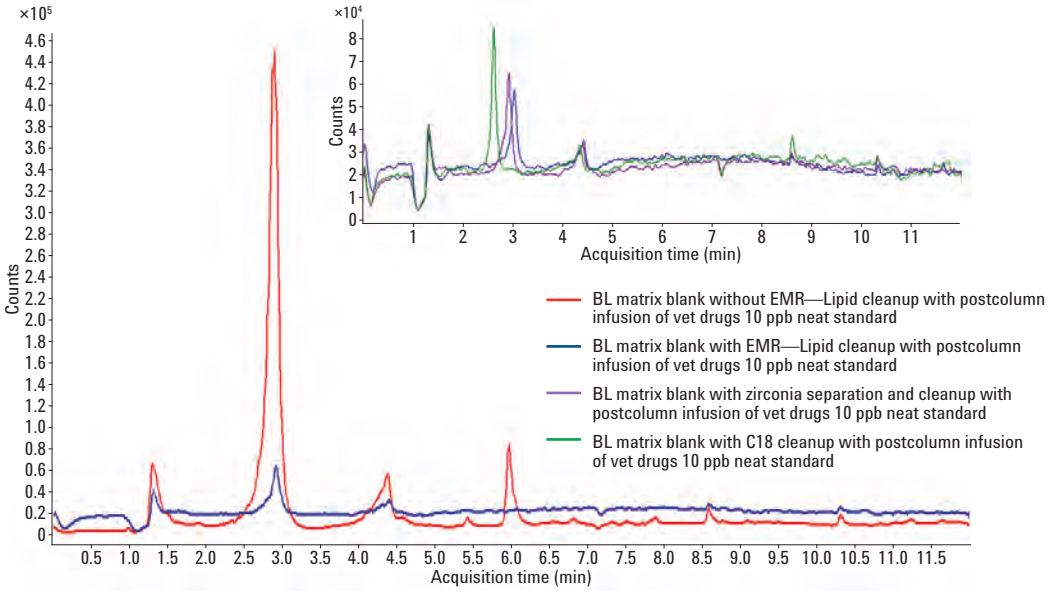


Figure 3. Postcolumn infusion profiles were generated by injecting a bovine liver (BL) matrix blank sample with simultaneous postcolumn infusion of 10 ppb of veterinary drug standard solution. All analytes were monitored for the chromatographic run, and the profile was a combined TIC of all monitored analyte transitions.

The chromatogram insert in Figure 3 shows the PCI profiles with the injection of matrix blank samples using different cleanup. The profiles of EMR—Lipid (blue) and zirconia sorbent (purple) are similar. The C18 cleanup (green) profile also shows similarity with the other two, but with more regions of matrix enhancement and suppression. This comparison corresponds to that of the gravimetric coextractive evaluation, in which EMR—Lipid cleanup gives slightly better cleanup efficiency than zirconia sorbent, and both give better cleanup than C18.

Analyte response comparisons between the postspiked matrix samples and neat standards were also used to evaluate matrix effect. For most analytes, there were no significant differences in analyte responses. However, for the later eluting compounds, which are more hydrophobic, more matrix ion suppression was seen in samples cleaned by C18 and zirconia sorbent. Since most lipid interferences elute late, the reduced matrix ion suppression on hydrophobic analytes confirms that EMR—Lipid efficiently removes coextracted lipids compared to dSPE C18 and zirconia sorbent. Figure 4 shows two examples of how EMR—Lipid cleanup reduced matrix ion suppression effects.

Method optimization

The solvent used in the protein precipitation step was also investigated. It is known that acetonitrile precipitates proteins efficiently at the sample:organic ratio of 1:3 to 1:5, and acidified acetonitrile offers more protein precipitation. BL is a complex matrix, and the removal of proteins is critical not only for matrix removal, but also to facilitate efficient EMR—Lipid cleanup. In this study, 1% and 5% FA in ACN were evaluated in the protein precipitation step followed by EMR—Lipid cleanup and analysis by LC/MS/MS. Using 5% FA in ACN provided better precision as shown by the calibration curves (Figure 5). The analyte recoveries were also compared, and again 5% FA in ACN provided better precision.

It is important that extra water or buffer is added to activate the EMR-Lipid material. This improves its interaction with unwanted sample matrix, especially lipids, leading to efficient matrix removal. The effect of using an ammonium acetate buffer (5 mM) during EMR—Lipid cleanup was investigated, and results were compared with those using water. Generally, the use of ammonium acetate buffer improved many analyte recoveries by 5 to 10%, except for tetracyclines.

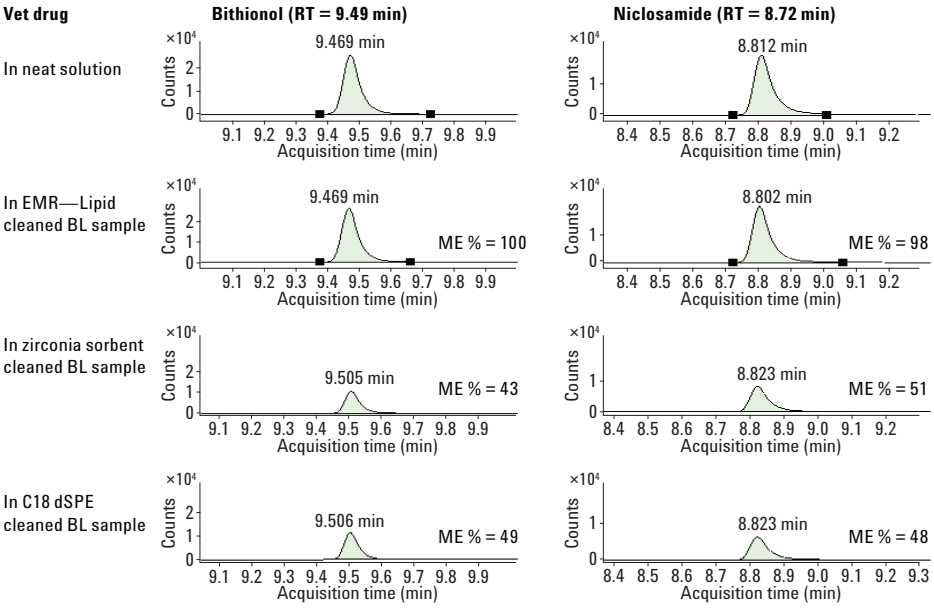


Figure 4. Matrix ion suppression effect comparison for hydrophobic analytes. Matrix effect (ME) was calculated from the ratio of peak area in postspike liver extract and corresponding neat standard.

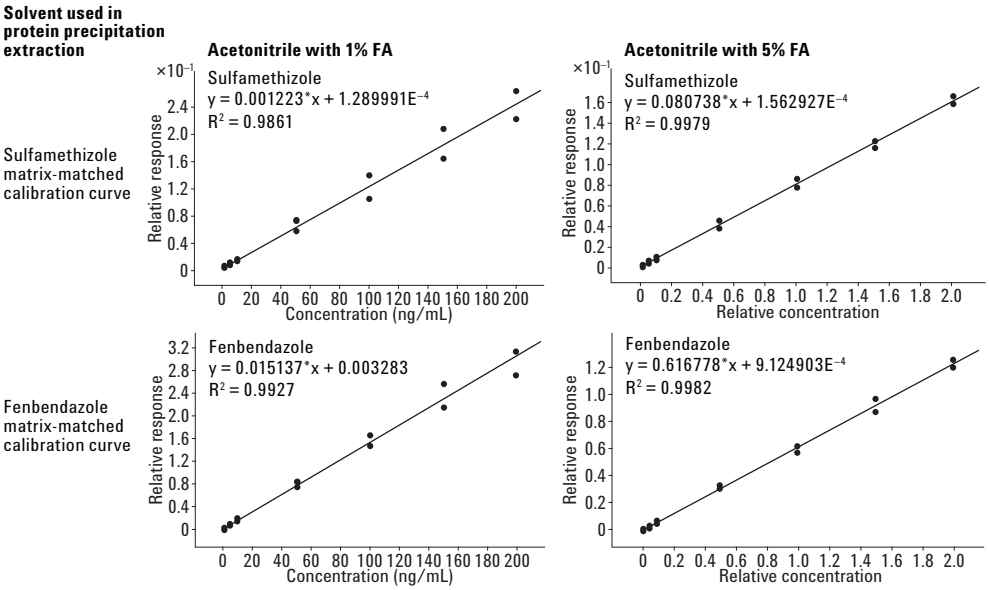


Figure 5. Calibration curve linearity comparison when using 1% FA in ACN versus 5% FA in ACN for protein precipitation.

For these compounds, the use of buffer results in approximately 5% lower recoveries when comparing to the use of unbuffered water. Analytes with obvious recovery differences when buffer versus water was employed were selected for comparison in Figure 6. Since more analyte recoveries improved with the use of buffer, and the tetracycline loss caused by using buffer was minimal, the 5 mM ammonium acetate buffer solution was used for the rest of the study.

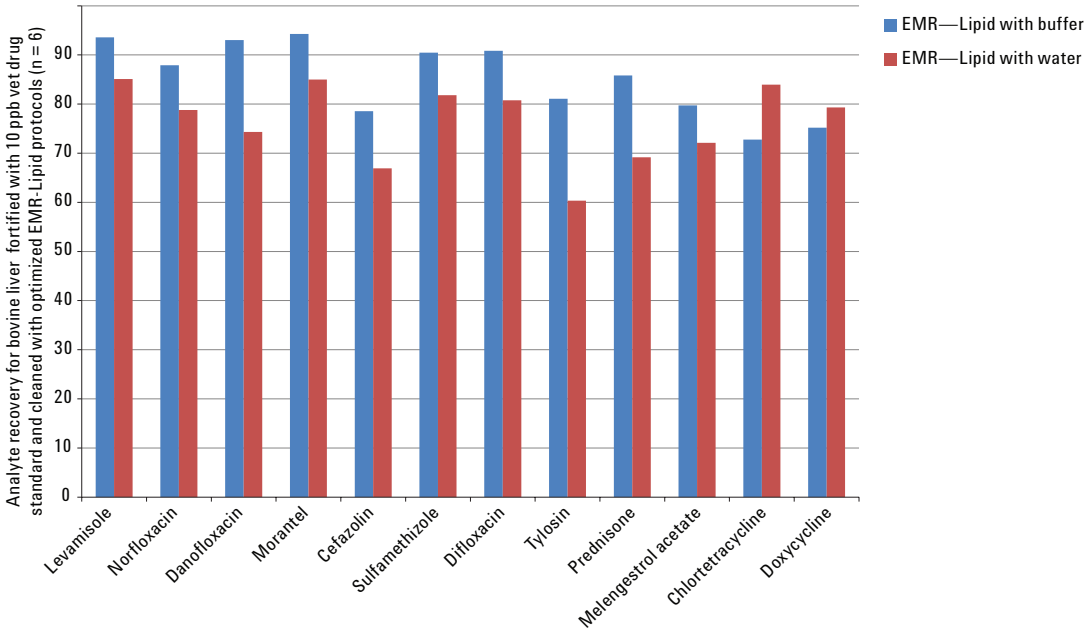


Figure 6. Analyte recovery comparison when using 5 mM ammonium acetate buffer versus water in the Enhanced Matrix Removal dSPE cleanup step.

After EMR—Lipid cleanup, the supernatant is approximately a 1:1 ACN:aqueous mixture. An aliquot of 5 mL supernatant is then transferred into EMR—Lipid polish tube, containing 2 g of salt mix (4:1 $MgSO_4$:NaCl) to separate organic and aqueous phases by salt partition. This step not only partitions but also facilitates further matrix cleanup and removes dissolved extra sorbents and salts. Therefore, this step is highly recommended for both GC and LC analysis. During our method development, good analyte recoveries and precision were achieved for all analytes except tetracyclines. With further investigation, we noticed that the low recoveries of tetracyclines (45 to 68%) were related to the salt partition step. To reduce tetracycline loss, an alternative protocol (Figure 7) was investigated that omits the EMR—Lipid polish salts. The procedure is similar, except that 400 µL of extract (from EMR—Lipid dSPE) and 600 µL of water were mixed in a microcentrifuge vial and vortexed for one minute without using the polish step. Samples were centrifuged on a microcentrifuge at 13,000 rpm for three minutes, and then the supernatant was transferred to a 2 mL sample vial for LC/MS/MS analysis. The standards and IS postspiking in matrix blank was conducted before the mixing/dilution step. This protocol can be considered as nonpartitioning, since the partition salts were not used.

The results in Figure 8 clearly demonstrate that the recoveries for tetracyclines can be substantially improved with this alternative protocol. However, the results from using the polish protocol (as shown in Figure 2) for tetracyclines could be acceptable, since the precision is for quantitation. The low recoveries can be corrected by using an appropriate stable labeled internal standard. In this study, we split extracted samples for the alternative nonpolish protocol after EMR—Lipid cleanup, and reported three results for tetracyclines (oxytetracycline, chlortetracycline, and doxycycline) from the alternative protocol.

Method comparison

The optimized EMR—Lipid method was then compared with a traditional QuEChERS method with C18 dSPE cleanup and zirconia sorbent cleanup. QuEChERS is often used for analyte or residue extraction, and employs a dSPE cleanup step. Figure 9 shows the statistical recovery comparison results, and Figure 10 shows the comparison for selected, problematic analytes. The optimized EMR—Lipid protocol provides significant improvements for recovery and precision of the problematic analytes, especially with respect to zirconia sorbent, which gives low recoveries for fluoroquinolone and tetracycline classes. Only oxytetracycline and niclosamide gave absolute recoveries of 67% and 68%, respectively. However, the precision for these two compounds for six

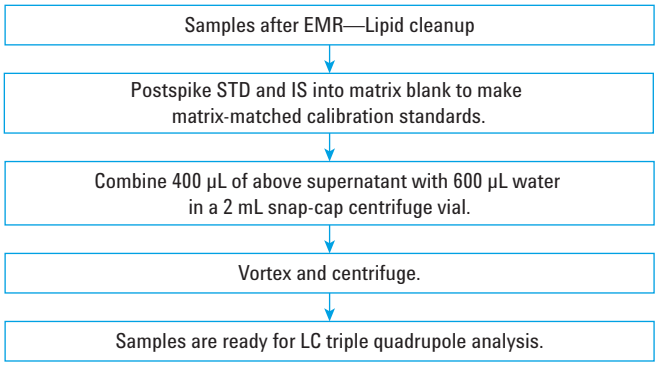


Figure 7. Optional procedure after Agilent Bond Elut EMR—Lipid. cleanup to improve tetracycline recoveries.

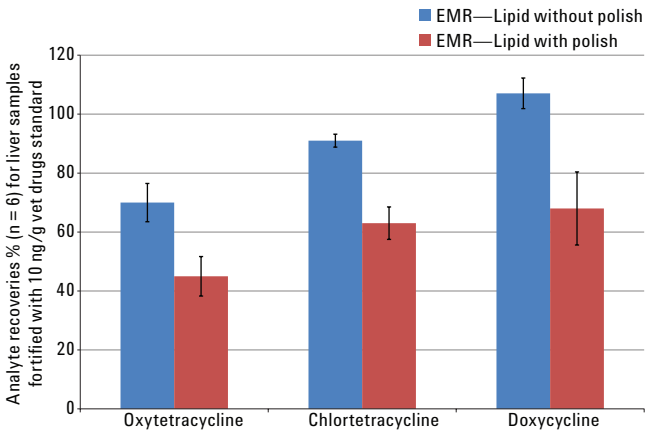


Figure 8. Tetracycline recovery and precision comparison for liver samples prepared with and without a polish step following cleanup with Agilent Bond Elut EMR—Lipid.

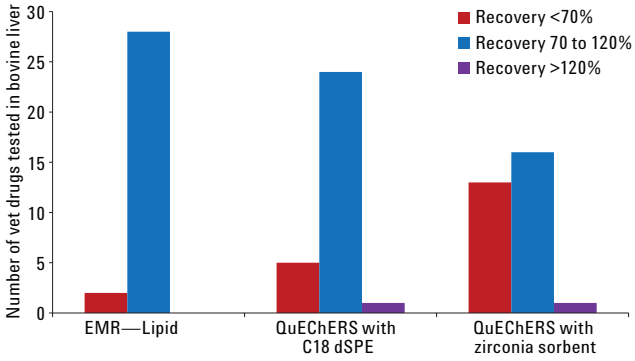


Figure 9. Statistical recovery results for comparison of Agilent Bond Elut EMR—Lipid protocol with traditional QuEChERS protocols.

replicates was acceptable with RSD of 12.8% and 2.0%, respectively, considered as acceptable based on SANCO guidelines [9]. These results are superior to results from the other two protocols employing dSPE with C18 and zirconia sorbents.

Method validation

The optimized EMR—Lipid method was validated by running a full quantitation batch, using the method described in the sample preparation section. Internal standard (flunixin-d_3 for positive and negative mode) was used for quantitation of accuracy and precision. The absolute recovery of flunixin-d_3 was from 90 to 100%. Therefore, accuracy closely corresponds to absolute recoveries. Table 4 shows the quantitation results. Summarized accuracy (Figure 11) was generated by determining accuracy and precision for 24 QCs at four different levels (G1 = 10, 50, 250, and 750 ppb and G2 = 2, 10, 50, and 150 ppb; see calibration standard section), with six replicates at each level. Acceptable accuracies (70 to 120%) were achieved for 93% of analytes, except for two outliers, which are slightly below 70% recovery, with good RSDs. The RSD values for six replicates at each level were exceptional, at below 10% for most compounds. It should be noted that ractopamine and ketoprofen were detected at low levels in the BL blank, resulting in the modified calibration range.

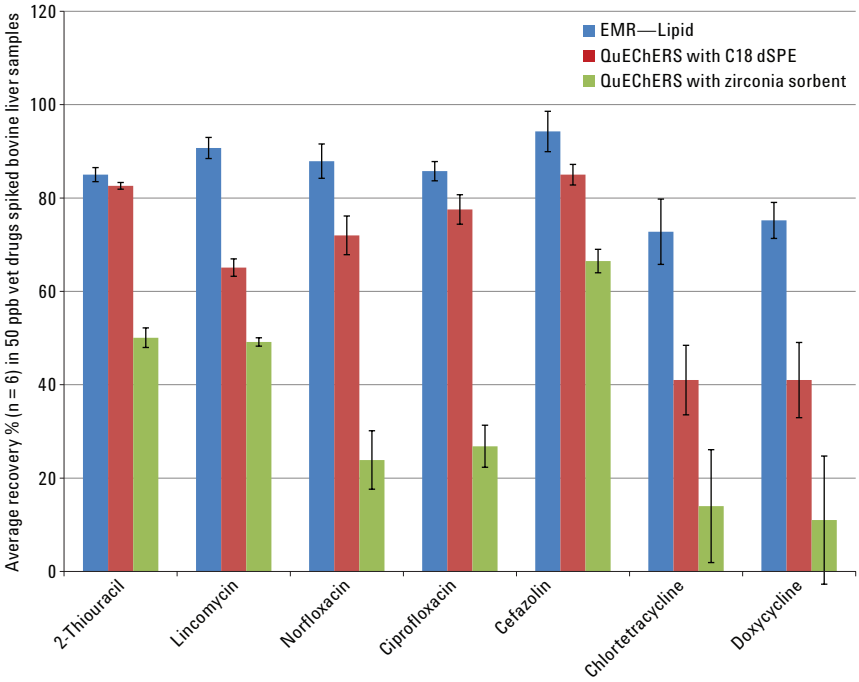


Figure 10. Selected analyte recovery results comparing the Agilent Bond Elut Enhanced Matrix Removal-Lipid protocol with traditional protocols. Chlortetracycline and doxycycline results were generated from an alternative protocol shown in Figure 6. The rest of the compounds used the protocol in Figure 1.

Table 4. Quantitation results for target analytes using Agilent Bond Elut Enhanced Matrix Removal—Lipid. Each analyte was assessed at four concentration levels for six replicates at each level.

		Calibration curve			Method recovery and precision (ng/g QCs)											
Group		Regression		Cal. range	2		10		50		150		250		750	
no.	Analyte	fit/weight	R^2	(ng/g)	Rec%	RSD	Rec%	RSD	Rec%	RSD	Rec%	RSD	Rec%	RSD	Rec%	RSD
1	2-Thiouracil	Linear, 1/x	0.9976	5-1,000	--	--	106.6	4.9	93.6	5.4	--	--	87.0	3.0	85.4	8.3
1	Amoxicillin	Linear, 1/x	0.9978	5-1,000	--	--	65.9	12.1	74.3	8.8	--	--	79.5	4.1	79.7	4.8
1	Methonidazole-OH	Linear, 1/x	0.9981	5-1,000	--	--	92.6	15.1	89.4	6.6	--	--	89.4	3.5	89.7	3.3
1	Oxytetracycline	Linear, 1/x	0.9963	5-1,000	--	--	73.9	19.1	71.2	14.0	--	--	67.0	12.8	63.4	9.0
1	Cefazolin	Linear, 1/x	0.9966	5-1,000	--	--	72.1	9.4	81.2	2.7	--	--	86.7	3.8	82.9	3.0
1	Difloxacin	Linear, 1/x	0.9978	5-1,000	--	--	79.0	8.3	86.5	5.9	--	--	104.4	5.1	97.7	6.3
1	Chlortetracycine	Linear, 1/x	0.9928	5-1,000	--	--	100.6	14.1	96.9	9.5	--	--	93.8	14.0	85.0	11.6
1	Doxycycline	Linear, 1/x	0.9972	5-1,000	--	--	118.9	12.0	102.7	10.0	--	--	110.0	7.7	104.5	8.6
1	Florfenicol	Linear, 1/x	0.9942	5-1,000	--	--	103.4	6.2	107.9	6.0	--	--	115.2	13.5	107.0	4.0
1	Chloramphenicol	Linear, 1/x	0.9962	5-1,000	--	--	103.6	6.6	107.1	7.9	--	--	113.7	9.8	100.9	5.2
1	Closulon	Linear, 1/x	0.9954	5-1,000	--	--	77.9	10.3	104.4	6.0	--	--	102.2	7.1	94.3	3.3
1	Prednisone	Linear, 1/x	0.9984	5-1,000	--	--	105.9	9.1	92.1	11.1	--	--	103.9	10.5	94.5	2.3
1	Oxyphenbutazone	Linear, 1/x	0.9903	5-1,000	--	--	93.6	3.4	91.9	5.2	--	--	93.7	5.6	97.0	4.6
1	Melengestrol acetate	Linear, 1/x	0.9994	5-1,000	--	--	70.6	1.4	77.3	3.0	--	--	82.8	2.1	77.1	2.6
1	Bithionol	Quadratic, 1/x	0.9981	5-1,000	--	--	69.4	6.2	90.4	2.9	--	--	91.3	4.3	83.1	3.7
2	Levamisole	Linear, 1/x	0.9967	1-200	84.5	11.3	95.5	5.1	103.8	5.2	89.4	9.7	--	--	--	--
2	Lincomycin	Linear, 1/x	0.9950	1-200	89.5	16.4	79.6	10.6	74.1	4.5	74.8	11.2	--	--	--	--
2	Norfloxacin	Linear, 1/x	0.9960	1-200	89.5	9.7	89.2	4.7	95.8	7.4	93.5	7.2	--	--	--	--
2	Ciprofloxacin	Linear, 1/x	0.9980	1-200	81.0	5.6	83.6	6.9	96.9	4.1	99.5	5.9	--	--	--	--
2	Danofloxacin	Linear, 1/x	0.9985	1-200	78.2	7.8	86.2	5.8	99.5	7.8	96.9	4.8	--	--	--	--
2	Ractopamine	Linear, 1/x	0.9961	10-200[b]	--	--	98.1	15.5	105.0	10.4	102.5	6.8	--	--	--	--
2	Morantel	Linear, 1/x	0.9960	1-200	89.5	4.9	95.1	4.5	101.0	8.6	94.1	7.4	--	--	--	--
2	Sulfamethizole	Linear, 1/x	0.9928	1-200	85.7	14.6	89.2	8.1	93.9	4.9	88.0	10.4	--	--	--	--
2	Sulfamethoxypyridazine	Linear, 1/x	0.9973	1-200	84.7	8.1	84.4	2.9	89.9	5.7	84.9	6.2	--	--	--	--
2	Tylosin	Linear, 1/x	0.9967	1-200	80.6	11.2	75.6	2.7	71.0	4.5	65.3	2.3	--	--	--	--
2	Acetopromizine	Linear, 1/x	0.9973	1-200	74.1	6.3	73.5	3.0	77.2	4.9	75.2	5.3	--	--	--	--
2	Chlorpromazine	Linear, 1/x	0.9967	1-200	66.1	6.1	67.8	3.8	73.4	4.3	72.7	6.5	--	--	--	--
2	Fenbendazole	Linear, 1/x	0.9988	1-200	74.6	7.7	82.3	4.6	97.9	9.9	84.9	3.5	--	--	--	--
2	Ketoprofen	Linear, 1/x	0.9978	5-200[c]	--	--	88.3	7.2	98.1	6.9	94.5	3.8	--	--	--	--
2	Niclosamide	Linear, 1/x	0.9996	1-200	60.0	15.3	66.6	4.7	71.7	2.0	67.6	3.1	--	--	--	--

[a] Group 1 analytes have a calibration range of 5 to 1,000 ng/g, and QC spiking levels of 10, 50, 250, and 750 ng/g. Group 2 analytes have a calibration range of 1 to 200 ng/g, and QC spiking levels of 2, 10, 50, and 150 ng/g.

[b] Modified calibration range due to ractopamine detected in the BL control blank.

[c] Modified calibration range due to ketoprofen detected in the BL control blank.

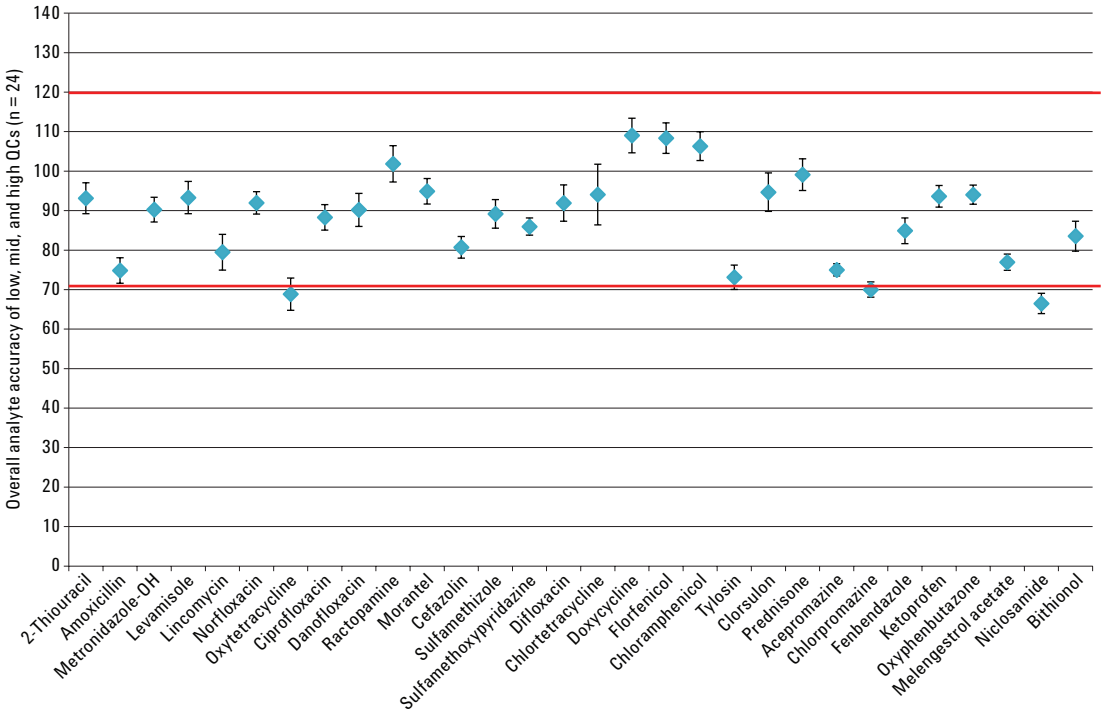


Figure 11. Quantitation of 30 representative vet drugs analyzed in BL using the optimized Agilent Bond Elut Enhanced Matrix Removal—Lipid protocol. The accuracy and precision data were calculated using 24 total replicates at four different spike levels (n = 6 at each level). Error bar = 95% CI. Three tetracycline compound results were generated from an alternative protocol shown in Figure 6. The rest of the compounds used the protocol in Figure 1.

Conclusions

A rapid, reliable, and robust method using protein precipitation extraction followed by Agilent Bond Elut EMR—Lipid and EMR—Lipid polish cleanup was optimized and validated for the analysis of veterinary drug multiresidues in BL. Matrix effects were carefully assessed and compared with traditional C18 dSPE and zirconia sorbent cleanup. Results demonstrate that the optimized EMR—Lipid method provided superior matrix cleanup, and excellent recovery and precision for this type of application.

It is important to note that direct dilution with water was used before injection to make samples amenable to LC/MS/MS and maintain peak integrity of the early eluting analytes. The LC/MS/MS system we used in this study provided adequate sensitivity for using direct sample dilution while still meeting the detection limit requirement. Compared to common dry-and-reconstitution, this workflow saved significant time and effort, and also prevented potential deviation and analyte loss. If instrument sensitivity cannot meet the desired needs by direct sample dilution, a sample concentration step at the end should still be considered. This is usually achieved by sample evaporation and reconstitution. This application demonstrates that selective matrix removal using EMR—Lipid provides significant advantages for complex samples such as BL, in the form of cleaner samples and higher recoveries and precision for multiresidue veterinary drug analysis.

Future work will investigate other complex, high-lipid matrices and target analytes to demonstrate the advantages of clean samples when using enhanced matrix removal.

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Application Note

Food Testing

Multiclass Multiresidue Veterinary Drug Analysis in Beef Using Agilent Captiva EMR—Lipid Cartridge Cleanup and LC/MS/MS

Authors

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Agilent Technologies, Inc.

Abstract

Agilent Captiva Enhanced Matrix Removal—Lipid (EMR—Lipid) cartridges are the second generation of Agilent EMR—Lipid products, and are implemented in a solid phase extraction (SPE) format for highly selective lipids removal without impacting analyte recovery. The SPE cartridges provide a simple pass-through cleanup workflow that requires minimal method development. The tube is optimized to facilitate hands-free gravity elution for large volume sample cleanup without the hassle of control on vacuum or pressure. To facilitate lipid capture and increase hydrophobic compound recoveries, the Captiva EMR—Lipid sorbent requires the addition of 20 % water to activate the EMR sorbent for cleanup. This study demonstrates the application of Captiva EMR—Lipid in the analysis of 39 representative multiclass, veterinary drugs in beef. A two-step sample extraction was used to achieve satisfactory recoveries for both hydrophilic and hydrophobic compounds. The extracts were then combined and applied to a Captiva EMR—Lipid cartridge for cleanup. The method was assessed for matrix effect, analyte recovery, and method reproducibility. When compared to other cartridge pass-through cleanup products, the Captiva EMR—Lipid cartridge provided more efficient matrix cleanup and better recovery of hydrophobic analytes.

Introduction

Veterinary drugs are widely used in animal food to prevent animal diseases, or as a growth promoter. These drugs can accumulate in animal tissues, and improper use can result in drug residues in edible tissues, resulting in risks to human health. With increased public attention on food safety, regulation of veterinary drugs used in animal food production is imposed in most countries[1,2]. Animal foods, such as muscle, liver, and eggs, are complex matrices; thus it is critical to use an efficient preparation method for sample extraction, cleanup, and concentration (when needed) before instrumental analysis. The established sample pretreatment methods include traditional solvent extraction, solid phase extraction (SPE), or a combination of techniques. These methods are usually labor-intensive, time-consuming, and only suitable for limited classes of compounds, requiring method development.

Multiclass, multiresidue methods are increasingly becoming popular in regulatory monitoring programs due to their increased analytical scope and laboratory efficiency. Analysis of more than 100 veterinary drugs has been reported in literature in the past few years[3-5]. The sample pretreatment usually involves pre-extraction with a mixture of acetonitrile (ACN)/water, followed by C18 cleanup or a combination of other cleanup techniques. However, current cleanup techniques have limitations such as inefficient lipid removal and unwanted analyte loss. The ACN/water mixture direct extraction can compromise the protein removal efficiency and hydrophobic analyte extractabilities during the extraction step.

Agilent Enhanced Matrix Removal—Lipid (EMR—Lipid) dSPE cleanup has gained a lot of attention since it was introduced in 2015. The EMR—Lipid sorbent specifically interacts with the unbranched hydrocarbon chains of lipid compounds using a combined mechanism of size exclusion and hydrophobic interaction. This combined mechanism provides highly selective lipid removal without unwanted impact on target analytes. This technology has been used for multiclass, multiresidue pesticides analysis in complex matrices, providing superior matrix cleanup and optimum results[6,7]. The second-generation product, Agilent Captiva EMR—Lipid cartridges, reduces the water percentage needed for sorbent activation, and eliminates the need for a polishing step subsequently. This simplifies the workflow and improves the solubility of hydrophobic compounds during cleanup.

This study investigates the use of Captiva EMR—Lipid cartridge cleanup during sample preparation for the analysis of 39 representative and challenging veterinary drugs in beef. The selected representative veterinary drugs were from 17 different classes including hydrophilic and hydrophobic drugs, acidic, neutral, and basic drugs, and some of the most difficult classes, such as tetracycline and β-lactam. Table 1 shows the drug class, regulatory information, retention time, and MS/MS conditions for analysis of these veterinary drugs.

Experimental

Reagent and Chemicals

All reagents and solvents were HPLC or analytical grade. Acetonitrile (ACN) was from Honeywell (Muskegon, MI, USA). Dimethyl sulfoxide (DMSO) and ethylenediaminetetraacetic acid, disodium salt, dehydrate (NaEDTA) were from Sigma-Aldrich (St Louis, MO, USA). Reagent grade formic acid (FA) was from Agilent (p/n G2453-86060). The veterinary drugs standards and internal standard were purchased from Sigma-Aldrich (St Louis, MO, USA).

Solution and Standards

Standard and internal standard (IS) stock solutions were made in DMSO at 2.0 mg/mL, except for the following:

- Danofloxacin stock solution was made in DMSO at 1.0 mg/mL.
- Ciprofloxacin stock solution was made in DMSO at 0.25 mg/mL.

All the β-lactam drugs and cefazolin stock solutions were made in water at 2.0 mg/mL. All stock solutions were prepared in amber glass vials, except for β-lactam drugs, Cefazolin, and tetracycline drugs stock solutions, which were prepared in polypropylene plastic tubes. All solutions were stored at –20 °C. The 39 compounds were separated into two groups: group 1 (G1) and group 2 (G2), based on their instrument response. Two combined standard working solutions, 25/5 µg/mL and 5/1 µg/mL (G1/G2), were prepared in 1:1 ACN/water. A 25 µg/mL working solution of Flunixin-d3 IS was prepared in 1:1 ACN/water.

A cold extraction solvent was prepared daily by adding 2 mL of formic acid and 2 mL of DMSO into 100 mL of precooled ACN. A 0.1 M Na EDTA solution was made by dissolving 1.8612 g of NaEDTA powder in 50 mL Milli-Q water. The solution was stored at room temperature. A 80:20 ACN/water was made by combining 80 mL of ACN with 20 mL of Milli-Q water.

Table 1. List of selected veterinary drugs for analysis; drug class, US tolerance, retention time, and MRM conditions.

Analyte	Drug class	US tol. (µg/g)	Retention time (min)	Polarity	Precursor ion (*m/z*)	Product ion			
						Quant ion	CE (v)	Qual ion	CE (v)
2-Thiouracil	Thyreostat	–	1.41	NEG	127	57.9	17	–	–
Amoxicillin	β-Lactam	0.01	1.94	POS	366.1	349.2	5	114	25
Metronidazole-OH	Nitroimidazole	[d]	2.21	POS	188.1	123.1	9	126.1	13
Lincomycin	Lincosamide	0.1[c]	3.80	POS	407.2	126.1	37	70.1	80
Levamisole	Anthelmintic	0.1[f]	3.90	POS	205.1	178.1	21	91.1	41
Minocycline	Tetracycline		4.14	POS	458.2	440.9	17	282.9	49
Ampicillin	β-Lactam	0.01	4.15	POS	350.1	106	33	79.1	61
Norfloxacin	Fluoroquinolone	[d]	4.36	POS	320.1	276.1	17	302.2	21
Oxytetracycline	Tetracycline	2[e]	4.42	POS	461.2	426.1	17	443.2	9
Ciprofloxacin	Fluoroquinolone	[d]	4.43	POS	332.1	231	45	314.3	21
Tetracycline	Tetracycline	2e	5.37	POS	445.2	409.9	17	153.9	33
Danofloxacin	Fluoroquinolone	0.2[b,f]	4.53	POS	358.2	340.2	21	81.9	53
Ractopamine	β-Agonist	0.03[f]	4.55	POS	302.2	107	33	77	77
Cefazolin	Cephalosporin	–	4.78	POS	455	323.1	9	156	13
Sulfamethizole	Sulfonamide	–	4.88	POS	271	156.1	13	92	29
Sulfamethoxypyridazine	Sulfonamide	–	4.91	POS	281.1	92	33	65.1	57
Demeclocycline	Tetracycline	–	4.94	POS	465.1	429.9	21	448.0	13
Difloxacin	β-Lactam	–	4.97	POS	400.2	356.3	17	382.0	25
Morantel	Anthelmintic	–	5.08	POS	221.1	123.1	37	76.9	80
Gamithromycin	Macrolide	0.15	5.22	POS	777.6	157.9	41	83.1	65
Chlortetracycline	Tetracycline	2[e]	5.24	POS	479.1	444.2	21	462.1	17
Doxycycline	Tetracycline	–	5.36	POS	445.2	428.1	17	410.2	25
Florfenicol	Phenicol	0.2[c]	5.69	NEG	356.0	336.0	5	185.1	13
Chloramphenicol	Phenicol	[d]	5.86	NEG	321	152	17	257.1	9
Tylosin	Macrolide	0.2[g]	5.94	POS	916.5	173.9	45	772.5	33
Prednisone	Corticosteriod	–	6.02	POS	359.2	147.2	33	341.2	9
Clorsulon	Flukicide	0.1[f]	6.09	NEG	377.9	341.9	9	–	–
Acetopromazine	Tranquilizer	–	6.09	POS	327.2	86	21	58	45
Chlorpromazine	Tranquilizer	–	6.69	POS	319.1	86	21	58.1	45
Penicillin V	β-Lactam	0.05[a]	6.70	POS	351.6	160.1	9	113.9	45
Oxacillin	β-Lactam	–	6.93	POS	402.1	160.0	17	242.9	9
Fenbendazole	Anthelmintic	–	6.98	POS	300.1	268.1	25	159.1	41
Cloxacillin	β-Lactam	0.01[a]	7.20	POS	436.1	159.9	9	276.8	13
Nafcillin	β-Lactam	–	7.35	POS	415.1	199.0	13	171.0	41
Ketoprofen	Tranquilizer	–	7.44	POS	255.1	208.9	13	77	57
Oxyphenbutazone	NSAID	–	7.47	NEG	323.1	295	17	133.9	25
Flunixin-d3 (NEG)	–	–	7.81	NEG	298.1	254.2	17	192	37
Flunixin-d3 (POS)			7.81	POS	300.1	282	25	264	41
Melengestrol acetate	Other	0.025[h]	9.05	POS	397.2	279.2	21	337.4	13
Niclosamide	Flukicide	–	9.07	NEG	325	170.9	25	289.1	13
Bithionol	Flukicide	–	9.07	NEG	352.9	161	21	191.8	25

[a] Tolerance in uncooked edible tissue of cattle
[b] Tolerance in cattle liver
[c] Tolerance in swine muscle
[d] Banned for extralabel use
[e] Tolerance is for the sum of residue of tetracycline including chlortetracycline, oxtetracycline, tetracycline in muscle
[f] Tolerance in cattle muscle
[g] Tolerance in uncooked cattle fat, muscle, liver, and kidney
[h] Tolerance in cattle fat

Equipment and Material

Separation was carried out using an Agilent 1290 Infinity UHPLC system consisting of an:

- Agilent 1290 Infinity binary pump (G4220A)
- Agilent 1290 Infinity high performance autosampler (G4226A)
- Agilent 1290 Infinity thermostatted column compartment (G1316C)

The UHPLC system was coupled to an Agilent G6490 Triple Quadrupole LC/MS system equipped with an Agilent Jet Stream electrospray ionization source. Agilent MassHunter workstation software was used for data acquisition and analysis.

Other equipment used for sample preparation:

- 2010 Geno/Grinder (Metuchen, NJ, USA)
- Centra CL3R centrifuge (Thermo IEC, MA, USA)
- Multi Reax Test Tube Shaker (Heidolph, Schwabach, Germany)
- Eppendorf pipettes and repeater
- Agilent Captiva EMR—Lipid cartridge, 6 mL, 600 mg (p/n 5190-1004) and, 3 mL, 300 mg (p/n 5190-1003)
- Agilent Vac Elut SPS 24 Manifold with collection rack for 16 × 100 mm test tubes (p/n 12234004)

Instrument Conditions

Figure 1 shows the typical chromatograms for A) beef extract matrix blank, and B) beef extract fortified with 5/1 ng/g (G1/G2) veterinary drug standards (limit of quantitation level).

Sample Preparation

Figure 2 shows the final sample preparation procedure to prepare beef samples. The following points need to be emphasized for the optimized extraction and cleanup method for the beef samples:

- Beef purchased from a local grocery store was used for method development and the validation study. Samples were homogenized and stored at –20 °C.
- After prespiking the standard and IS into the homogenized beef sample, the samples stood at room temperature for 20 minutes. This allowed the spiked standards to infiltrate the sample matrix and equilibrate prior to sample extraction.
- The use of water for sample extraction is necessary to achieve homogeneous mixing with the beef, and ensure the recovery and stability of the polar drug compounds.

HPLC conditions

Parameter	Value
Column	Agilent InfinityLab Poroshell 120 EC-C18, 150 × 2.1 mm, 2.7 µm (p/n 693775-902) Agilent InfinityLab Poroshell 120 EC-C18 UHPLC guard, 5 × 2.1 mm, 2.7 µm (p/n 821725-911)
Flow rate	0.3 mL/min
Column temperature	40 °C
Autosampler temperature	4 °C
Injection volume	3 µL
Mobile phase	A) 0.1 % FA in water B) 0.1 % FA in acetonitrile
Needle wash	1:1:1:1 ACN/MeOH/IPA/H_2O w/ 0.2 % FA
Gradient	Time (min) %B Flow rate (mL/min) 0 10 0.3 0.5 10 0.3 8.0 100 0.3
Stop time	12 minutes
Post time	3 minutes

MS conditions

Parameter	Value	
Positive/negative mode		
Gas temperature	120 °C	
Gas flow	14 L/min	
Nebulizer	40 psi	
Sheath gas heater	400 °C	
Sheath gas flow	12 L/min	
Capillary	3,000 v	
iFunnel parameters	Positive	Negative
	High-pressure RF 90 V	90 V
	Low-pressure RF 70 V	60 V

A one-step extraction with a 20:80 water/ACN mixture greatly reduces the solvent extractability of hydrophobic compounds and protein removal efficiency. Therefore, a two-step extraction protocol was used: a 2 mL aqueous extraction followed by an 8 mL solvent extraction.

- To prevent loss of tetracycline compounds due to chelation, a 0.1 M EDTA buffer solution was used for aqueous extraction.
- To improve solvent extractability for difficult drug compounds, such as tetracycline, β-lactam, and fluoroquinolones, 2 % formic acid and 2 % DMSO was added into the extraction solvent, ACN.
- To improve the phase separation with solid residues, especially in the first aqueous extraction step, a cooled centrifugation (4 °C) was used.
- To ensure the complete elution of analytes from the cartridge, a secondary elution after EMR—Lipid cartridge cleanup was performed.

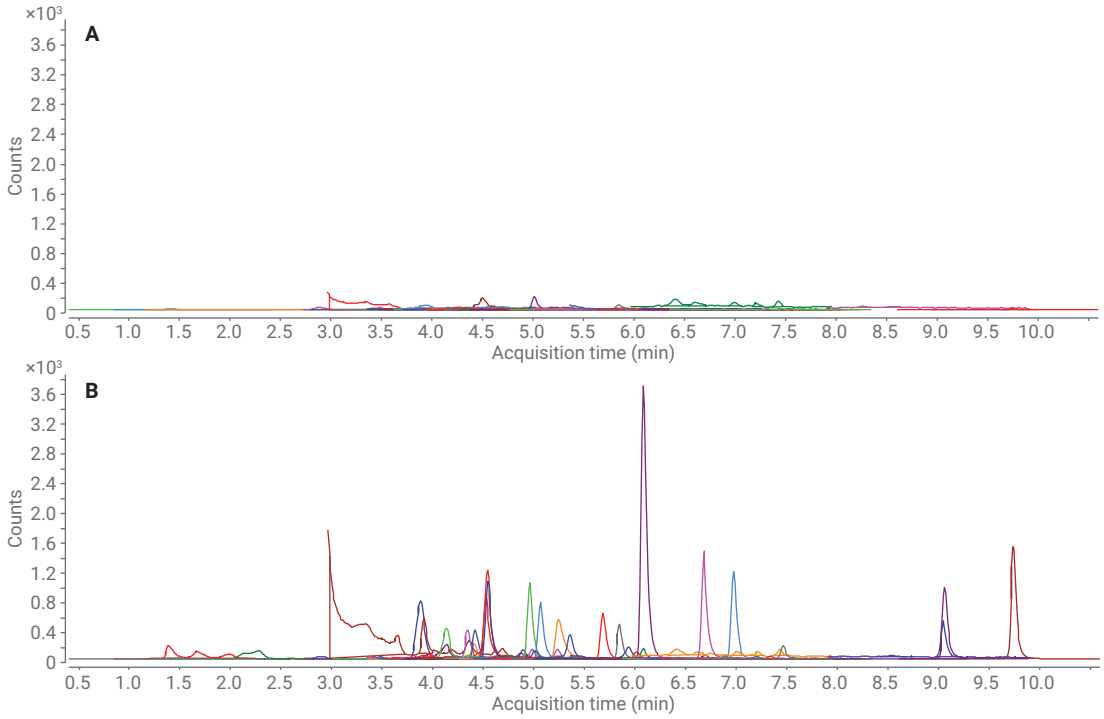

Figure 1. LC/MS/MS chromatograms for A) beef extract matrix blank, and B) beef extract fortified with 5/1 ng/g (G1/G2) vet drug standards. Group 1 (G1) analytes correspond to a 5 ng/g fortification level, while group 2 (G2) compounds correspond to a 1 ng/g fortification level. Refer to Table 1 for analyte identification with elution order, and Table 2 for compound group identification.

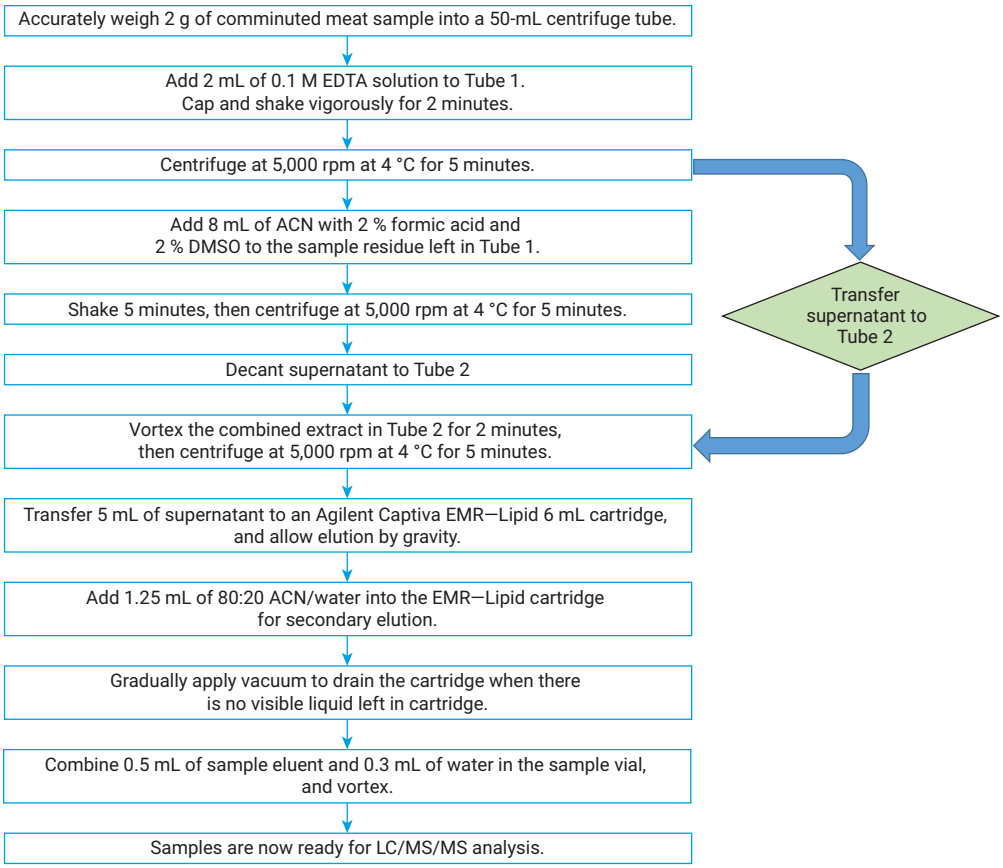

Figure 2. Beef sample extraction and following cleanup procedure using an Agilent Captiva EMR—Lipid 6 mL cartridge.

Calibration Standards and Quality Control (QC) Samples

Prespiked QC samples were fortified by spiking appropriate standard working solution into the homogenized beef samples with six replicates of low, mid, and high levels.

- For G1 analytes, the spiking levels were 10, 50, and 750 ng/g.
- For G2 analytes, the spiking levels were 2, 10, and 150 ng/g.

A standard 25/5 µg/mL (G1/G2) working solution was used to spike high-level QC samples; while a 5/1 µg/mL (G1/G2) standard solution of was used to spike low and mid QC samples. The IS solution was also spiked into all samples except matrix blank, corresponding to 200 ng/g of Flunixin-d3.

Matrix-matched calibration standards and postspiked QC samples were prepared by spiking appropriate standard and IS working solutions into the matrix blank eluent after cartridge cleanup. The spiking concentrations for calibration standards were 5, 25, 50, 250, 750, and 1,000 ng/g (G1) or 1, 5, 10, 50, 150, and 200 ng/g in beef (G2), and 200 ng/g IS; spiking concentrations for postspiked QC samples were 10, 50, and 750 ng/g (G1) or 2, 10, and 150 ng/g (G2).

Determine the Amount of Co-extractives

The amount of co-extractive residue was determined by gravimetric measurements[5] for EMR—Lipid cartridge and other manufacturer's cartridge cleanup. The co-extractive residue weight was collected based on 1 mL of ACN final extract, while the matrix co-extractives removal efficiency by cleanup was calculated by comparing the ratio of the difference of co-extractive residue weight with and without cartridge cleanup.

Matrix Effect Assessment

Chromatographic matrix effect was assessed by a post column infusion test. The matrix blank samples were injected with simultaneous post column infusion of a 10 ng/mL neat standard veterinary drug solution at 90 µL/min. All compound transitions were monitored through the chromatographic window.

Analyte Recovery Assessment by Cartridge Cleanup

Cartridge cleanup impact on analyte recovery was evaluated by prespiking standards into beef extract blank before cartridge cleanup, and postspiking standards into beef extract blank eluent after cartridge cleanup. The collected recovery results only reflect the impact of cartridge cleanup on analyte recovery, and exclude other contributions from the extraction procedure. It is a more direct comparison of cartridge cleanup impact on analyte recovery. The EMR—Lipid cartridges, 3 mL and 6 mL, were compared to corresponding other manufacturer's cartridges. For 3 mL cartridges, the sample loading volume was 2.5 mL, and the secondary elution volume was 0.625 mL.

Method Validation

To ensure calibration reproducibility, the developed method was validated by running a full quantitation batch with two separate calibration curves run before and after the QC samples.

Results and Discussion

Ease of Cartridge Cleanup

An important feature of using Captiva EMR—Lipid cartridges for complex sample matrix cleanup is ease-of-use. The EMR—Lipid sorbent targets unwanted lipid interferences instead of analytes, and implements a pass-through approach. The sample mixture is loaded onto the cartridge and is allowed to pass through the packed Captiva EMR—Lipid sorbent in the cartridge. Lipids are trapped in the sorbent, while target analytes pass through the cartridge, foregoing the need for traditional SPE steps such as conditioning, washing, and elution. Therefore, the use of Captiva EMR—Lipid cartridges is greatly simplified, saving a significant amount of time and solvent. The pass-through cleanup does not need traditional SPE method development for washing and elution steps. A possible method modification for Captiva EMR—Lipid is the use of a secondary elution step to achieve complete elution. It is recommended to use a 20:80 water/ACN mixture at approximately 20–25 % of the sample loading volume for secondary elution (for example, a 5 mL load followed by a 1–1.25 mL second elution). Lastly, the product design for gravity elution allows hands-free operation once the sample is loaded onto EMR—Lipid cartridges. Control of the elution flow rate by manipulating vacuum or positive pressure is not required. These features provide increased lab productivity when using Captiva EMR—Lipid cartridge cleanup to prepare complex food samples.

Amount of Co-extractives

Table 2 shows the sample co-extractives gravimetric test results. The co-extractive residue weight study is an important method to evaluate how efficient sample extraction and cleanup method can control the residue of matrix co-extractives, including proteins, lipids, salts, and other matrix components in the final sample being injected onto the instrument. Matrix co-extractives residue weight shows the entire co-extractives amount, whether they are detectable on an instrument or not. Co-extractive residues, whether being detectable on an instrument or not, can introduce matrix effects, impact method reliability and data quality, accumulate on the instrument flow path, such as the column and MS source, and deteriorate the detection system's long-term performance.

The less matrix co-extractive residue, the better method reliability and instrument performance. The results clearly demonstrated that Captiva EMR—Lipid cartridge cleanup provided better matrix cleanup efficiency with less co-extractive residue weight than the other manufacturer's cartridge cleanup.

Matrix Effect Assessment

Postcolumn infusion (PCI) of veterinary drug standards was used to evaluate matrix effects in beef extract with and without Captiva EMR—Lipid cartridge cleanup. All analytes were monitored through the entire acquiring window. The PCI profiles reflect the matrix impact for analytes monitored under both positive and negative mode. Figure 3 shows the PCI profiles.

Table 2. Beef matrix co-extractives residue amount and matrix removal by cartridge cleanup.

Cleanup technique	Co-extractives per 1 mL of ACN final extract (mg)	Matrix co-extractives removal efficiency by cleanup (%)
No further cleanup	7.68	–
Agilent Captiva EMR—Lipid 3 mL cartridge	4.38	43
Agilent Captiva EMR—Lipid 6 mL cartridge	4.03	48
Other manufacturer's 3 mL cartridge	5.91	23
Other manufacturer's 6 mL cartridge	6.30	18

$$\text{Matrix co-extractives removal efficiency (\%)} = \frac{(\textit{Amount of co-extractives without cleanup} - \textit{Amount of co-extractives with cleanup})}{\textit{Amount of co-extractives without cleanup}} \times 100$$

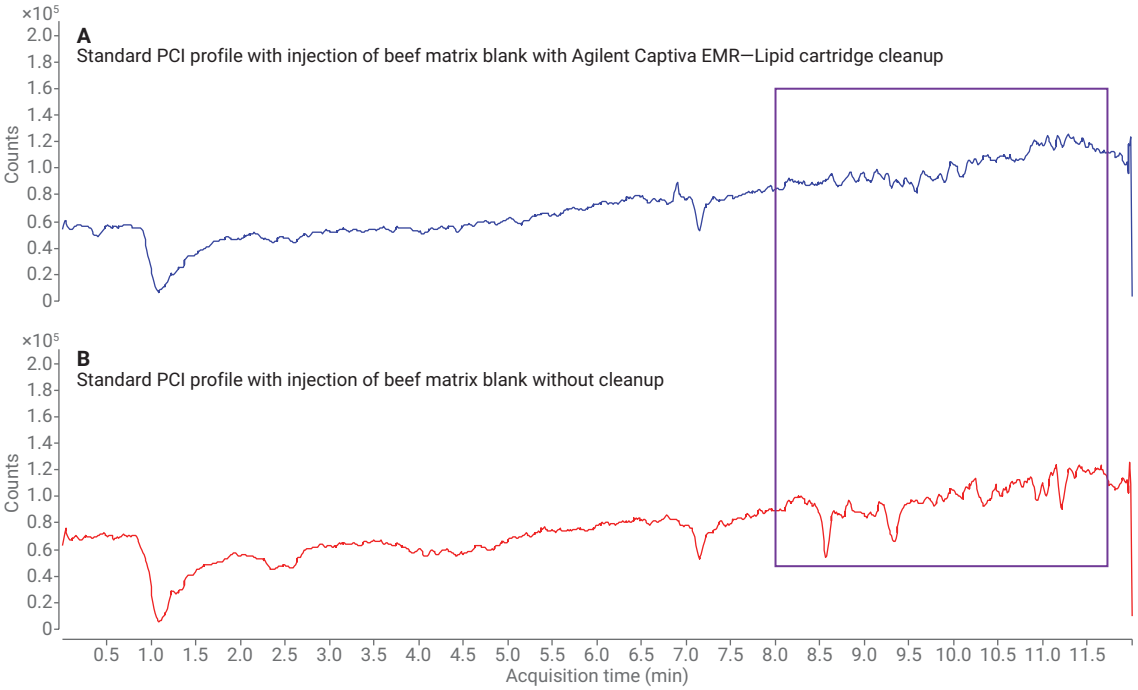

Figure 3. Matrix effect study by standard PCI with the injection of beef matrix blank with Agilent Captiva EMR—Lipid cartridge cleanup (A) and without any cleanup (B).

Figure 3B shows the PCI profile in red, matrix ion suppressions (overall low baseline) observed with the injection of beef extract without cleanup. Matrix ion suppression can dramatically impact the method sensitivity, reliability, and data quality for analytes within the coelution window. Conversely, Figure 3A shows that the PCI profile (in blue) becomes much smoother and more consistent with fewer troughs when injecting beef extract with EMR—Lipid cartridge cleanup. The highlighted RT window in Figure 3 shows the reduced matrix ion suppression effect comparison.

Cartridge Cleanup Recovery

Traditionally, the mechanism for lipid removal is based on hydrophobic interaction between lipids and sorbent. This mechanism can be efficient, especially when using strong hydrophobic interaction as the major sorbent function mechanism to trap and remove lipids. However, this interaction mechanism is not selective, and it does not differentiate unwanted lipids and wanted hydrophobic analytes from sample. Therefore, while the sorbent works on trapping lipids, it can also strongly interact with hydrophobic analytes, resulting in dramatic analyte loss during the cartridge cleanup. Furthermore, not all classes of lipids can be removed efficiently through hydrophobic interactions (for example, phospholipids).

Captiva EMR—Lipid sorbent uses a novel chemistry that combines size exclusion and hydrophobic interactions to significantly improve lipid removal selectivity. Only the lipid-like molecules containing straight unbranched hydrocarbon chains, preferably with more than six carbons, have access to EMR—Lipid sorbent pores. Once the lipids enter the EMR—Lipid sorbent, they are trapped inside with strong hydrophobic interaction. Other hydrophobic molecules that do not resemble lipids and are too bulky to enter the EMR—Lipid sorbent will stay in solution for subsequent analysis. Thus, EMR—Lipid sorbent can efficiently differentiate lipids from other hydrophobic molecules, dramatically improving selectivity, and decreasing hydrophobic compound loss during cleanup.

This mechanism has been well proven by the cartridge cleanup recovery study. In this study, the standards were prespiked into the beef blank extract before cartridge cleanup, and postspiked into the blank eluent after cartridge cleanup. The recovery data only indicate the cartridge cleanup impact on analytes. The comparison study included four types of cartridges: Captiva EMR—Lipid 3 mL (300 mg) and 6 mL (600 mg) cartridges, and other manufacturer's 3 mL (60 mg) and 6 mL (500 mg) cartridges. Figure 4 shows the study results. The analytes shown in the comparison are more hydrophobic compounds, eluting later on the C18 column. EMR—Lipid 3 mL and 6 mL cartridges provide consistent superior cartridge cleanup recoveries for compounds from mid to high hydrophobicity. However, for the other manufacturer's cartridge cleanup, which uses hydrophobic interaction mostly for lipids removal, the more hydrophobic (late eluting) analytes had lower recovery. When the comparable sorbent bed mass contained in 6 mL cartridges (500 mg) was used, the medium to high hydrophobic compounds were significantly retained. For example, as the last two most hydrophobic compounds, niclosamide and bithionol with log P >5, the other manufacturer's 6 mL cartridge cleanup recoveries were single digits, indicating substantial analyte loss on-cartridge. Their 3 mL cartridges use much less sorbent to balance hydrophobic analyte loss. In summary, the other cartridge cleanup tube with 60 mg sorbent sacrifices the cartridge matrix cleanup efficiency, and increasing to a higher bed mass lowers the recoveries for hydrophobic compounds to unacceptably low levels (<40 %). This study clearly demonstrates that EMR—Lipid sorbent provides a highly selective interaction mechanism for lipids, ensuring acceptable target analyte recoveries, especially for hydrophobic analytes.

Method Validation

The optimized extraction and cleanup method was validated by running a full quantitation batch. The methodology is described in the Experimental section. Internal standard (Flunixin-d3 for both positive and negative mode) was used for quantitation. However, as the absolute recoveries are the greatest concern when evaluating a new sample preparation method, the prespiked and postspiked QCs at three levels were included in the validation run. Table 3 lists the quantitation results in detail, and a summarized figure (Figure 5) was generated by average recovery and precision at each level. Acceptable recoveries (60–120 %) were achieved for most analytes at three levels (94 %), with the exception of two outliers, acepromazine and chlorpromazine. A confirmatory study reveals that these compounds can undergo analyte loss during the extraction step of the protocol. However, the RSD values for six replicates of these two compounds at each level were exceptional, with <10 % RSD for 91 % of analytes, and 10–20 % RSD for the remaining 9 % of analytes.

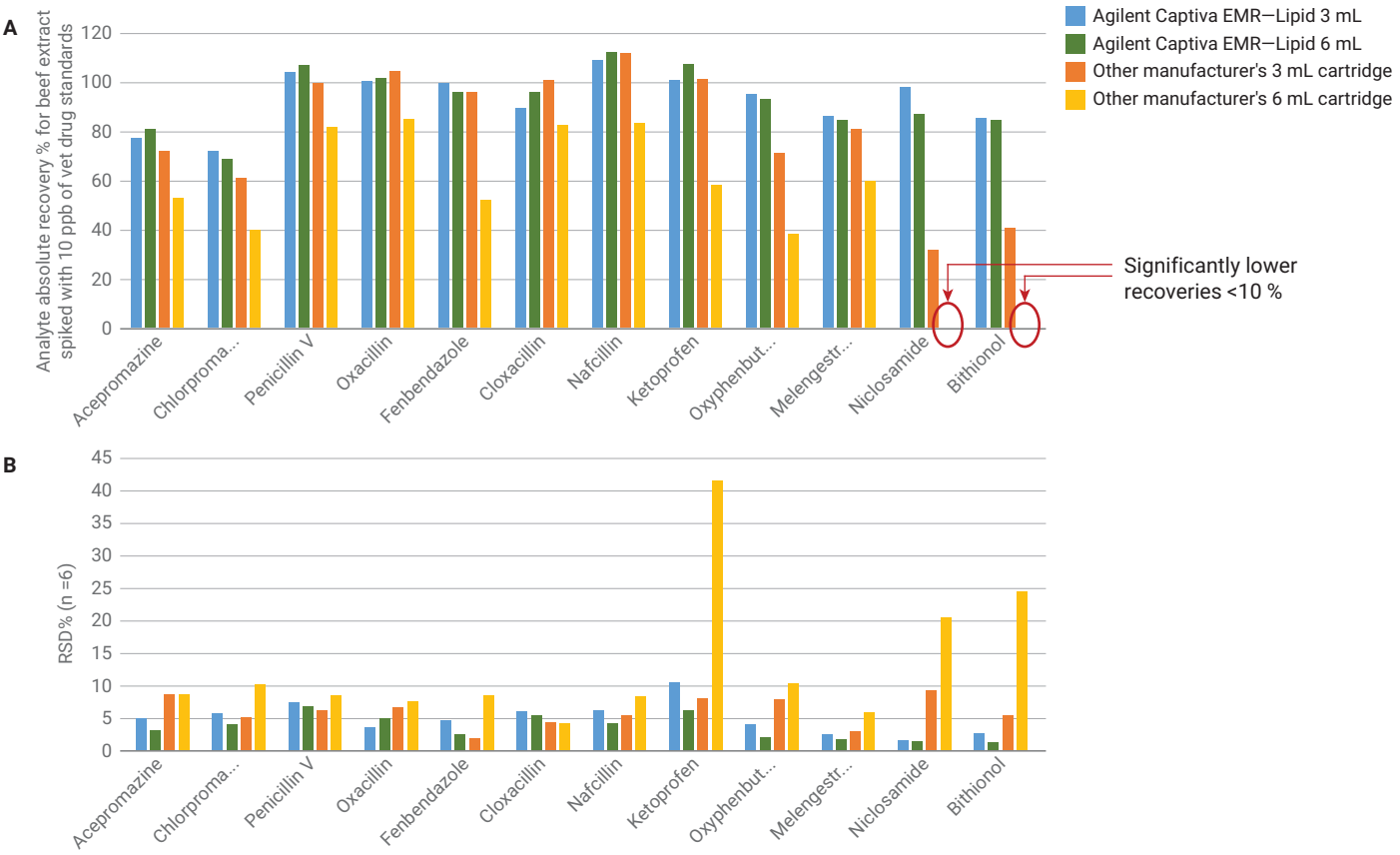

Figure 4. Cartridge cleanup comparison for hydrophobic analyte recovery (A) and reproducibility (B) from beef extract. The standard was spiked into beef extract before cartridge cleanup at 10 ng/mL. Analytes order from left to right with increasing hydrophobicity.

Table 3. Method quantitation results for veterinary drug analysis in beef.

Group no.[a]	Analyte	Calibration curve		Method absolute recovery and precision									
				2 ng/g QCs (n = 6)		10 ng/g QCs (n = 6)		50 ng/g QCs (n = 6)		150 ng/g QCs (n = 6)		750 ng/g QCs (n = 6)	
		R^2	Cal. range (ng/g)	Rec%	RSD	Rec%	RSD	Rec%	RSD	Rec%	RSD	Rec%	RSD
1	2–Thiouracil	0.9862	5–1,000	–	–	94	6.7	116	2.2	–	–	103	4.1
1	Amoxicillin	0.9964	5–1,000	–	–	88	8.6	77	3.9	–	–	69	2.7
1	Metronidazole–OH	0.9963	5–1,000	–	–	112	3.8	108	1.8	–	–	103	2.1
1	Ampicillin	0.9926	5–1,000	–	–	88	9.6	84	3.8	–	–	82	4.2
1	Minocycline	0.9943	5–1,000	–	–	72	13.3	69	11.2	–	–	62	2.9
1	Oxytetracycline	0.9941	5–1,000	–	–	84	6.2	87	11.0	–	–	72	5.1
1	Tetracycline	0.9919	5–1,000	–	–	87	9.0	86	5.2	–	–	90	4.1
1	Cefazolin	0.9933	5–1,000	–	–	109	5.8	94	2.8	–	–	87	5.4
1	Demeclocycline	0.9966	5–1,000	–	–	80	17.6	86	3.4	–	–	86	3.8
1	Difloxacin	0.9824	5–1,000	–	–	122	7.3	102	5.9	–	–	102	5.0
1	Gamithromycin	0.9901	5–1,000	–	–	100	8.9	92	5.3	–	–	89	6.1
1	Chlortetracycline	0.9976	5–1,000	–	–	80	7.8	86	8.5	–	–	81	4.1
1	Doxycycline	0.9936	5–1,000	–	–	77	11.2	70	5.4	–	–	73	4.7
1	Florfenicol	0.9920	5–1,000	–	–	116	4.4	110	3.8	–	–	99	7.1
1	Chloramphenicol	0.9928	5–1,000	–	–	113	7.6	104	1.9	–	–	103	3.4
1	Prednisone	0.9932	5–1,000	–	–	110	6.7	110	5.5	–	–	106	5.4
1	Clorsulon	0.9927	5–1,000	–	–	114	12.1	97	4.8	–	–	98	5.7
1	Penicillin V	0.9952	5–1,000	–	–	97	4.3	100	6.3	–	–	100	7.1
1	Oxacillin	0.9942	5–1,000	–	–	96	12.0	99	8.2	–	–	99	5.1
1	Cloxacillin	0.9932	5–1,000	–	–	103	8.1	101	6.0	–	–	97	5.7
1	Nafcillin	0.9926	5–1,000	–	–	107	8.9	110	6.5	–	–	95	5.6
1	Oxyphenbutazone	0.9910	5–1,000	–	–	106	8.1	98	3.0	–	–	86	2.8
1	Melengestrol acetate	0.9942	5–1,000	–	–	117	7.0	114	3.0	–	–	102	5.1
1	Bithionol	0.9807	5–1,000	–	–	63	8.2	81	5.7	–	–	92	1.4
2	Lincomycin	0.9961	1–200	94	8.5	99	3.0	–	–	88	6.4	–	–
2	Levamisole	0.9942	1–200	111	2.1	109	3.0	–	–	99	1.3	–	–
2	Norfloxacin	0.9974	1–200	111	5.5	91	4.9	–	–	100	8.0	–	–
2	Ciprofloxacin	0.9965	1–200	114	11.8	103	6.9	–	–	103	4.0	–	–
2	Danofloxacin	0.9969	1–200	101	8.3	94	5.8	–	–	99	5.6	–	–
2	Ractopamine	0.9858	1–200	120	6.5	110	5.5	–	–	109	3.2	–	–
2	Sulfamethizole	0.9950	1–200	102	11.0	105	2.5	–	–	97	5.0	–	–
2	Sulfamethoxypyridazine	0.9949	1–200	118	9.7	106	6.3	–	–	86	4.6	–	–
2	Morantel	0.9965	1–200	107	7.8	112	6.1	–	–	109	6.5	–	–
2	Tylosin	0.9946	1–200	125	5.3	105	4.8	–	–	98	7.5	–	–
2	Acetopromazine	0.9942	1–200	66	7.9	52	3.2	–	–	56	3.7	–	–
2	Chlorpromazine	0.9944	1–200	50	9.2	36	3.2	–	–	43	4.1	–	–
2	Fenbendazole	0.9910	1–200	76	5.6	99	1.6	–	–	90	4.9	–	–
2	Ketoprofen	0.9911	1–200	112	9.4	102	7.0	–	–	103	1.9	–	–
2	Niclosamide	0.9964	1–200	120	10.2	85	8.5	–	–	89	2.1	–	–

[a] Group 1 analytes have calibration range of 5–1,000 ng/g, QC spiking level of 10, 50, and 750 ng/g; while group 2 analytes have calibration range of 1–200 ng/g, QC spiking level of 2, 10, and 150 ng/g.

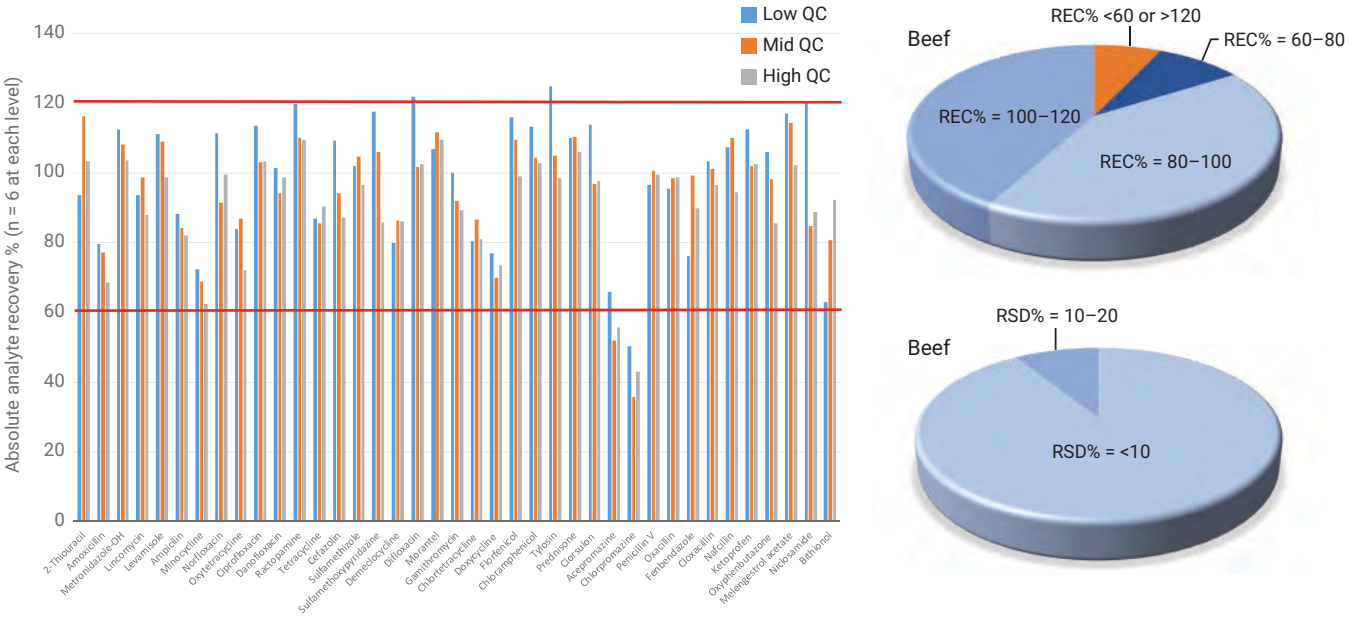

Figure 5. Absolute analyte recoveries and statistical summary of the analysis of veterinary drugs in beef method validation. Refer to Table 3 for more detailed information.

Conclusions

A rapid, reliable, and robust method using solid-liquid extraction followed by Agilent Captiva EMR—Lipid cartridge cleanup was developed and validated for the analysis of veterinary drug multiresidues in beef. A modified extraction procedure applied an aqueous extraction step, followed by an organic solvent extraction step to optimize analyte recovery. These extracts were combined and applied to the Captiva EMR—Lipid cartridge for cleanup. Matrix co-extractives and matrix effect was carefully assessed and compared with similar cartridges from another manufacturer. A study of the impact of cartridge cleanup on analyte recovery shows that the EMR—Lipid sorbent provides highly selective lipids removal, and does not cause unwanted target analytes loss. Results demonstrate that optimized solid-liquid extraction followed by the Captiva EMR—Lipid cartridge cleanup method provides superior matrix cleanup, excellent recovery, and precision results for this type of application.

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Macrolide Analysis in Pork Using Bond Elut QuEChERS dSPE EMR—Lipid and Poroshell 120

Application Note

Food Testing and Agiculture

Authors

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Agilent Technologies Shanghai Co. Ltd.

Abstract

This application note describes the use of a quick, easy, cheap, effective, rugged, and safe (QuEChERS) sample preparation approach for the extraction and cleanup of seven macrolide residues in pork. We analyzed residues of spiramycin, tilmicosin, oleandomycin, erythromycin, tylosin, roxithromycin, and josamycin. The analytes were extracted with Agilent Bond Elut QuEChERS dSPE Enhanced Matrix Removal—Lipid, and separated on an Agilent Poroshell 120 EC-C18 HPLC column. Quantification was achieved by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC/MS/MS) operated in positive ion multiple-reaction-monitoring mode. The method provided low limits of detection for all macrolides in pork. The dynamic calibration ranges for these compounds were obtained from 5 to 250 µg/kg. The overall recoveries ranged from 63.9 to 98.4%, with RSD values between 3.8 and 10.3%.

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Introduction

The use of antibiotics in food animal production has resulted in benefits throughout the food industry. However, their use has led to animal and human health safety concerns. Macrolides are a group of antibiotics that have been widely used to treat many respiratory and enteric bacterial infections in animals. Some of the more commonly used macrolides are spiramycin, tilmicosin, oleandomycin, erythromycin, tylosin, roxithromycin, and josamycin.

National agencies and international organizations have set regulatory limits on the concentrations of antibiotic residues in foods of animal origin [1,2]. The regulated residue limits vary from zero to 15 mg/kg. An application was developed previously for trace level macrolide residue analysis in honey [3]. The method used Agilent Bond Elut Plexa for sample preparation, and an Agilent Poroshell 120 EC-C18 column for separation. The recovery and reproducibility results based on matrix-spiked standards were acceptable for macrolide residue determination in honey under regulation.

The objective of this work was to develop a multiresidue method that would be simple and fast for routine regulatory analysis of macrolide residues in pork. A novel sorbent material, Agilent Bond Elut QuEChERS dSPE Enhanced Matrix Removal—Lipid, selectively removes major lipid components from high fat content matrices, such as pork, without unwanted analytes loss. Removal of lipid interferences from complicated matrices has many advantages, including reduced matrix effect to increased mass response, and helping extend the lifetime of LC columns. The superficially porous particle Poroshell 120 HPLC column provides high speed and efficiency with a low backpressure.

Table 1 shows details of the macrolides.

Table 1. Macrolide compounds used in this study (continued next page).

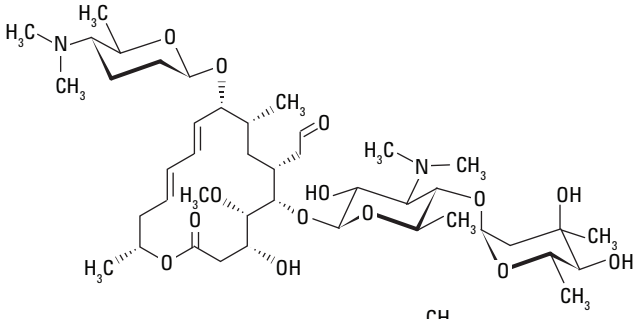
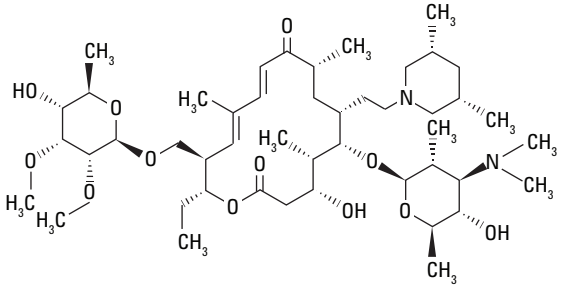
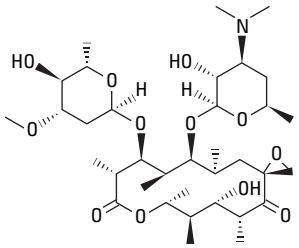
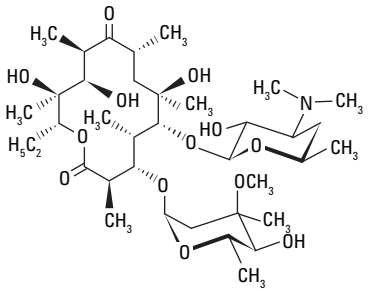
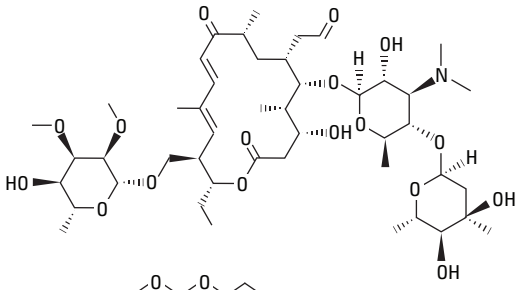
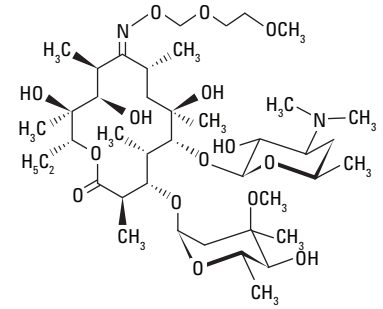
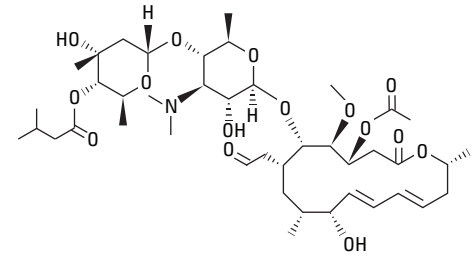
Compound	CAS No.	Structure
Spiramycin	8025-81-8	
Tilmicosin	108050-54-0	

Table 1. Macrolide compounds used in this study.

Compound	CAS No.	Structure
Oleandomycin	3922-90-5	
Erythromycin	114-07-8	
Tylosin	1401-69-0	
Roxithromycin	80214-83-1	
Josamycin	16846-24-5	

Materials and Methods

Reagents and chemicals

All reagents were MS, HPLC, or analytical grade. Acetonitrile and water were from Honeywell International, Inc. The standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Pork was purchased from a local supermarket. Standard solutions (1.0 mg/mL) were made in methanol individually, and stored at –20 °C. A combined working solution was made in acetonitrile:water (20:80) and also stored at –20 °C. The spiked solutions were then made daily by appropriately diluting the combined working solution with water.

Equipment and materials

- Agilent 1290 Infinity LC
- Agilent 6460 Triple Quadrupole LC/MS with electrospray ionization
- Agilent Bond Elut QuEChERS Extraction Kit EN (p/n 5982-5650)
- Agilent Bond Elut QuEChERS dSPE Enhanced Matrix Removal—Lipid (p/n 5982-1010)
- Agilent Bond Elut QuEChERS Final Polish for Enhanced Matrix Removal—Lipid (p/n 5982-0101)
- Agilent Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm (p/n 695975-302)
- Eppendorf 5810 R centrifuge (Brinkmann Instruments, Westbury, NY, USA)
- Digital vortex mixer (VWR International, LLC, Radnor, Pennsylvania, USA)

Sample preparation

The final sample preparation procedure was optimized with the following steps.

1. Weigh 2.5 g (±0.1 g) homogenized pork into 50 mL centrifuge tube.
2. Add 8 mL water, vortex for 1 min.
3. Add 10 mL acetonitrile.
4. Add salts in QuEChERS Extraction Kit EN method.
5. Mix sample by shaking for 1 min.
6. Centrifuge at 4,000 rpm for 5 min.
7. Add 5 mL water to a 15 mL EMR—Lipid dSPE tube.
8. Transfer 5 mL of supernatant to EMR—Lipid dSPE tube.
9. Vortex immediately to disperse sample, then vortex for 1 min.
10. Centrifuge at 4,000 rpm for 3 min.
11. Transfer 5 mL of supernatant to a 15 mL EMR—Lipid polish tube containing 2 g salts (1:4 NaCl:$MgSO_4$), and vortex for 1 min.
12. Centrifuge at 4,000 rpm for 3 min.
13. Combine 200 µL of upper ACN layer and 800 µL water in a 2 mL sample vial and vortex.

HPLC conditions

Column:	Agilent Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm (p/n 695975-302)	
Mobile phase:	A:10 mM ammonium acetate and 0.1% formic acid in water B: acetonitrile	
Inj vol:	2 µL	
Flow rate:	0.5 mL/min	
Gradient:	Time (min)	%B
	0	20
	5	65
	6	65
	8	20
Temp:	30 °C	

MS conditions

The macrolides were monitored in positive mode. Table 2 shows the multiple-reaction-monitoring details.

MS source parameters

Gas temp:	300 °C
Gas flow:	5 L/min
Nebulizer:	45 psi
Sheath gas temp:	400 °C
Sheath gas flow:	11 L/min
Nozzle voltage:	Positive, 0 V
Capillary:	Positive, 4,000 V

Table 2. Masses monitored by multiple-reaction monitoring.

Compound	Precursor ion	Product ion	Fragmentor (V)	Collision energy (V)	Retention time (min)
Spiramycin	843.4	540.0	270	35	2.178
Spiramycin	843.4	174.1	270	40	2.178
Tilmicosin	869.5	696.4	320	44	2.749
Tilmicosin	869.5	174.1	320	49	2.749
Oleandomycin	688.3	544.3	170	15	2.99
Oleandomycin	688.3	158.2	170	25	2.99
Erythromycin	734.4	576.3	180	14	3.204
Erythromycin	734.4	158.2	180	30	3.204
Tylosin	916.4	772.4	280	30	3.421
Tylosin	916.4	174.2	280	40	3.421
Roxithromycin	837.4	679.3	200	16	4.087
Roxithromycin	837.4	158.1	200	34	4.087
Josamycin	828.4	174.1	250	35	4.461
Josamycin	828.4	109.1	250	46	4.461

Results and Discussion

Linearity and limit of detection

Solutions used to create external calibration curves were prepared from a combined working solution to spike matrix blanks (5, 10, 20, 50, and 250 µg/kg). Matrix blanks were created by taking pork through the entire procedure, including pretreatment and QuEChERS procedures. The data of limits of detection (LODs) were calculated with a signal-to-noise ratio (S/N) of 3 by injecting the postspiked pork matrix at 0.1 µg/kg. All S/N were greater than 3:1. Therefore, all the LODs for these compounds were below 0.1 µg/kg, and conformed to the regulated method. Table 3 shows the results for the calibration curves.

Table 3. Linearity of macrolides in pork.

Compound	Regression equation	R^2
Spiramycin	Y = 386.144x + 19.317	0.9994
Tilmicosin	Y = 133.272x + 8.018	0.9999
Oleandomycin	Y = 317.284x + 43.963	0.9998
Erythromycin	Y = 848.506x + 119.918	0.9996
Tylosin	Y = 274.158x + 22.703	0.9997
Roxithromycin	Y = 477.739x + 53.019	0.9997
Josamycin	Y = 625.922x + 58.918	0.9998

Recovery and reproducibility

The recovery and repeatability for the method were determined at three levels of pork sample spiked at concentrations of 10, 20, and 100 µg/kg, with six replicates at each level. Table 4 shows the recovery and reproducibility data. Figure 1 shows the chromatograms of 20 µg/kg spiked pork extracts.

Table 4. Recoveries and reproducibility of macrolides in pork (n = 6).

Compound	Spiked level (µg/kg)	Recovery (%)	RSD (%)
Spiramycin	10	89.7	10.3
	20	94.0	8.3
	100	95.2	3.8
Tilmicosin	10	98.4	9.5
	20	90.0	9.7
	100	95.3	7.1
Oleandomycin	10	92.4	5.7
	20	96.4	7.1
	100	97.5	6.2
Erythromycin	10	64.5	8.8
	20	63.9	8.1
	100	68.7	5.1
Tylosin	10	84.1	10.2
	20	93.3	7.4
	100	94.6	5.5
Roxithromycin	10	89.9	9.8
	20	91.6	7.7
	100	92.6	5.1
Josamycin	10	87.9	7.4
	20	92.4	5.6
	100	93.2	4.9

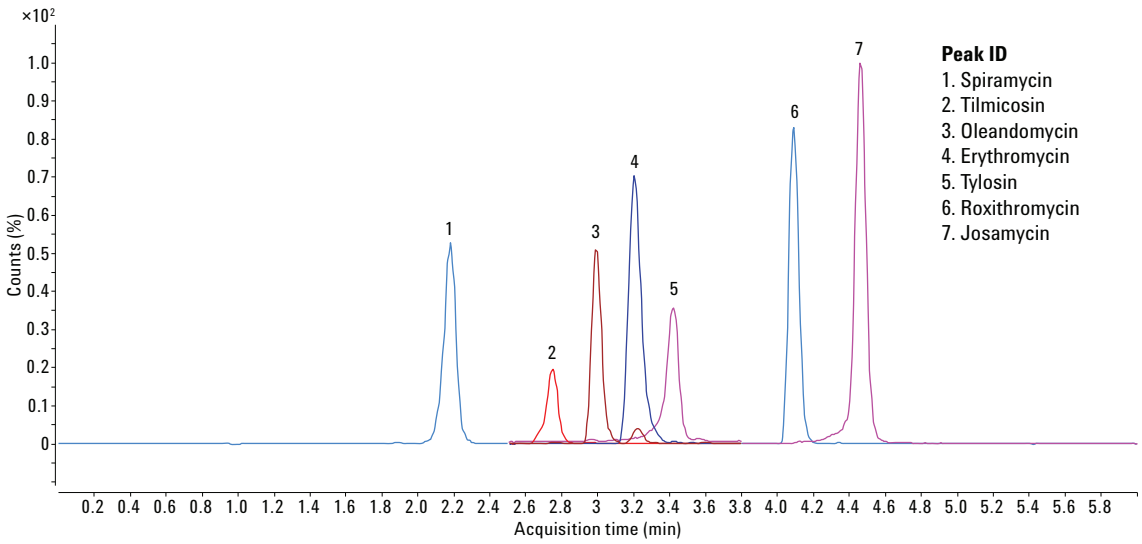


Figure 1. Chromatogram of 20 µg/kg spiked pork sample extract.

Conclusions

The method described in this application note is reliable, quick, and robust for the simultaneous quantification and confirmation of macrolides in pork. EMR—Lipid and Polish provide superior matrix cleanup and remove most matrix, especially lipids, without significantly affecting analyte recovery. The Agilent Poroshell 120 EC-C18 column provides quick separation for multiple macrolides with symmetrical peak shapes and high sensitivity.

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2. SN/T 1777.2-2007. *Determination of macrolide antibiotic residues in animal-derived food – part 2: LC-MS/MS method. China Standard*. General Administration of Quality Supervision, Inspection and Quarantine, Beijing, China.
3. Chen-Hao Zhai, Rong-jie Fu. *Macrolides in Honey Using Agilent Bond Elut Plexa SPE, Poroshell 120, and LC/MS/MS*; Application note, Agilent Technologies, Inc. Publication number 5991-3190EN, **2013**.

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Mycotoxin Analysis

Aflatoxin Analysis in Infant Formula with Bond Elut EMR—Lipid by LC/MS/MS

Multiclass Mycotoxin Analysis in Cheese with Captiva EMR—Lipid by LC/MS/MS

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.

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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.

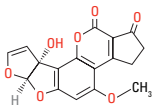
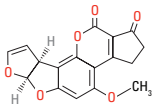
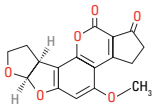
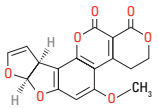
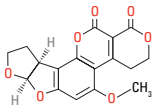
Aflatoxin	Molecular formula	Structure	pKa	logP
M1	$C_{17}H_{12}O_7$		11.4	0.93
B1	$C_{17}H_{12}O_6$		–	1.58
B2	$C_{17}H_{14}O_6$		–	1.57
G1	$C_{17}H_{12}O_7$		–	1.37
G2	$C_{17}H_{14}O_7$		–	1.36

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	4–15*	Nuts, figs, dried fruits, cereals, maize, spices	EC [5]
(total concentration)	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

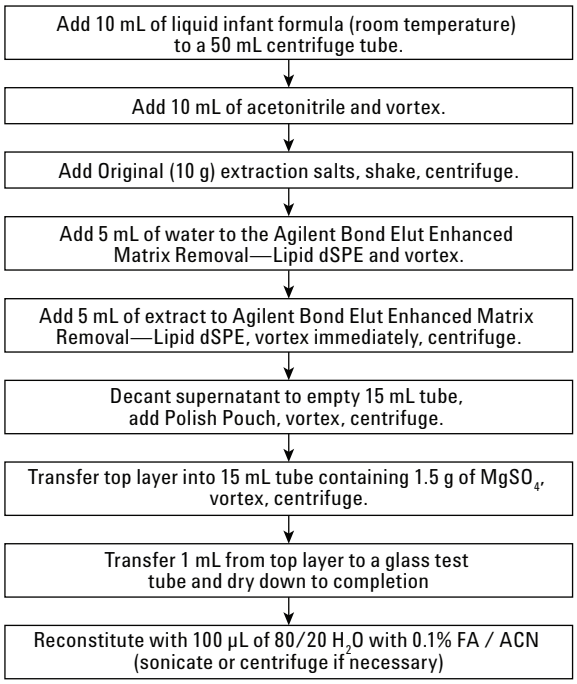


Figure 1. Sample preparation protocol for aflatoxins in liquid infant formula with Agilent Bond Elut Enhanced Matrix Removal—Lipid cleanup and enhanced post sample treatment using $MgSO_4$.

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.

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

				Product ion			
Analyte	RT (min)	Precursor ion (*m/z*)	Frag (V)	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

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 $MgSO_4$.

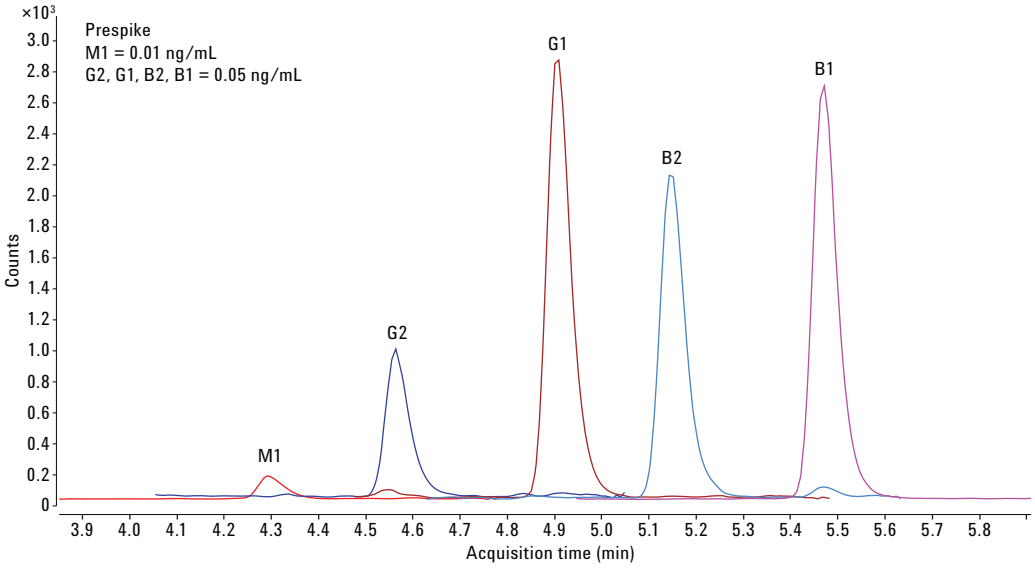


Figure 2. LC/MS/MS dMRM chromatogram of aflatoxins in liquid infant formula after QuEChERS extraction and Agilent Bond Elut Enhanced Matrix Removal—Lipid dSPE cleanup with enhanced post sample treatment with $MgSO_4$.

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

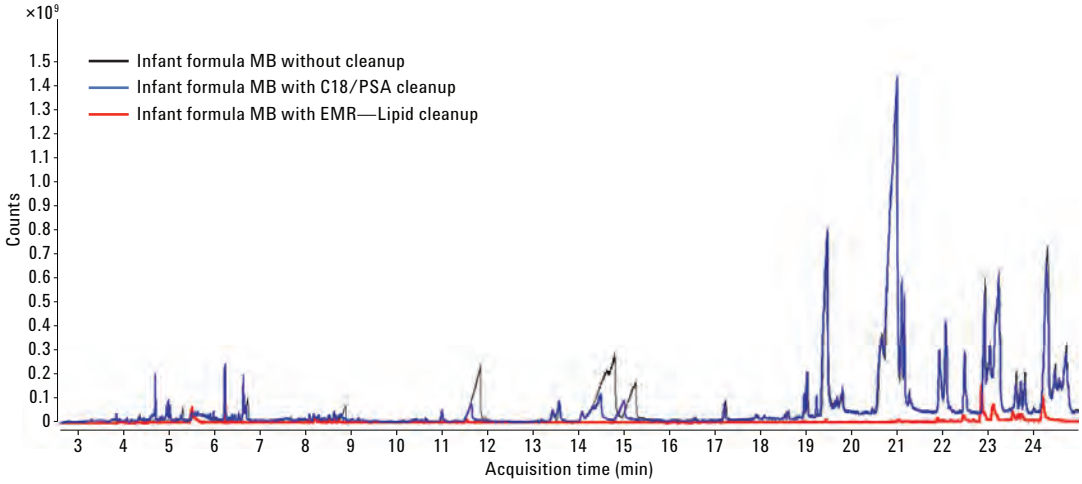


Figure 3. GC/MS full scan overlay chromatogram of infant formula matrix blank (MB) without cleanup, with C18/PSA dSPE cleanup, and with Agilent Bond Elut Enhanced Matrix Removal—Lipid cleanup.

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

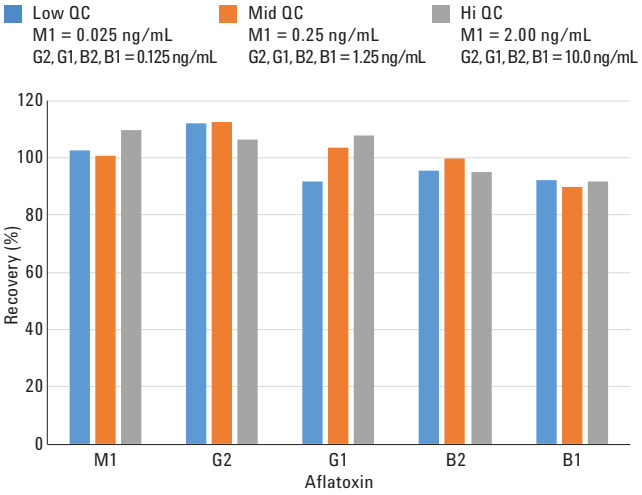


Figure 4. Recovery (%) for five aflatoxins in liquid infant formula using Agilent Bond Elut Enhanced Matrix Removal—Lipid cleanup. (n = 6)

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%.

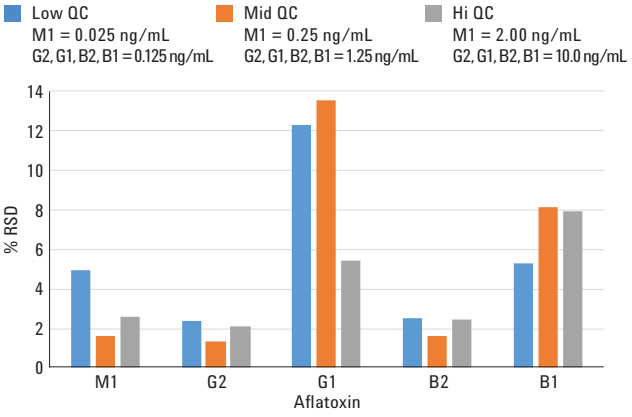


Figure 5. RSD (%) for five aflatoxins in liquid infant formula using Agilent Bond Elut Enhanced Matrix Removal—Lipid cleanup. (n = 6).

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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Agilent Technologies

Application Note

Food

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

Authors

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Agilent Technologies, Inc.

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.

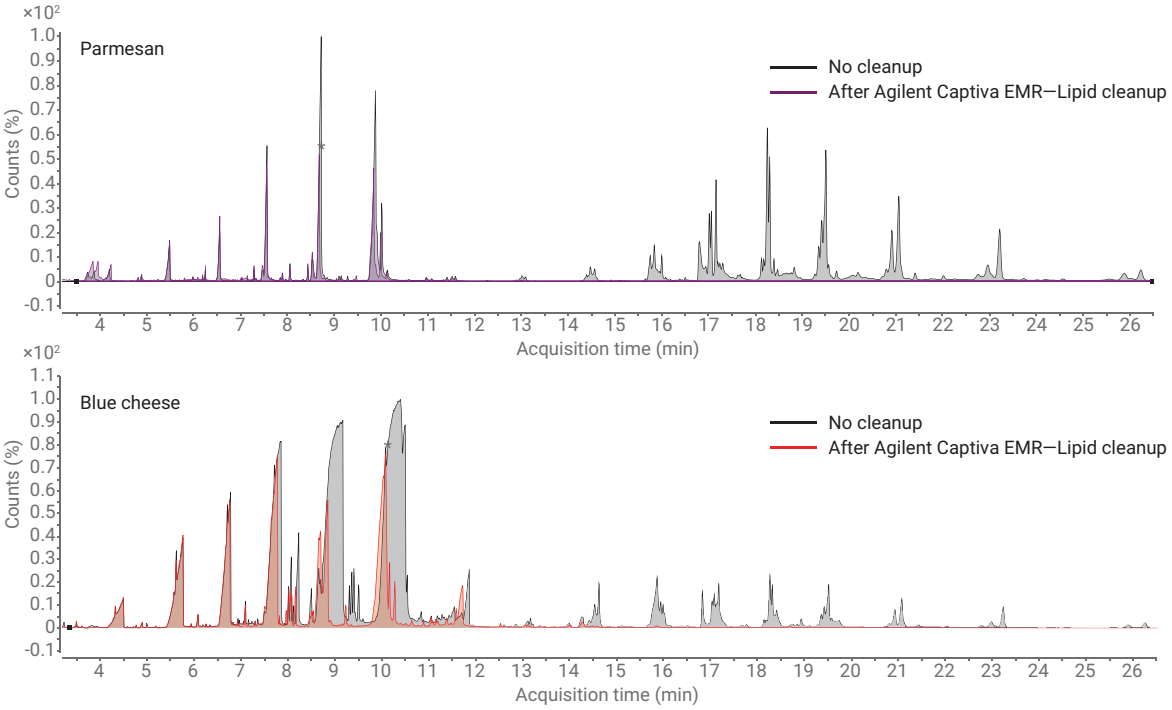

Figure 1. Matrix removal evaluation using GC/MS full scan chromatogram comparison of cheese samples before cleanup and after Agilent Captiva EMR—Lipid cleanup.

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

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.

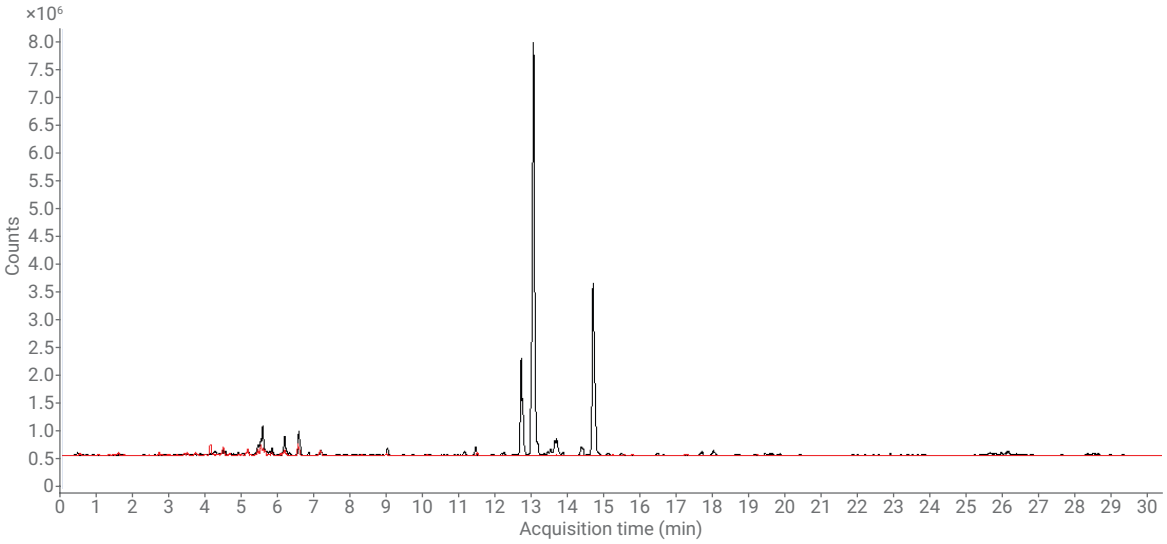

Figure 2. Phospholipid removal in blue cheese by LC/MS/MS precursor ion scan for *m/z* = 184.

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.

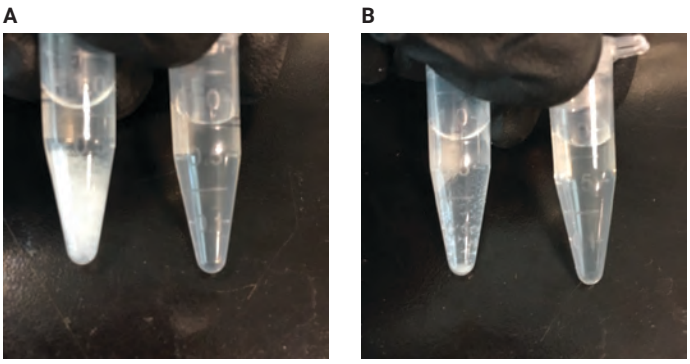

Figure 3. Lipid freeze-out experiment for Agilent Captiva EMR—Lipid cleanup of blue cheese (A) and parmesan cheese (B).

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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PAH Analysis

PAH Analysis in Salmon with Bond Elut EMR—Lipid

PAH Analysis in Salmon with Enhanced Matrix Removal

Application Note

Food Testing & Agriculture, Environmental

Authors

Derick Lucas and Limian Zhao
Agilent Technologies, Inc.

Abstract

Polycyclic aromatic hydrocarbons (PAHs) consist of fused benzene ring systems that resist degradation. They can be introduced to aquatic species by accumulation in the environment and cooking methods that use smoke. Analysis of PAHs in complex, high-fat food matrices can often present challenges as coextracted matrix hinders accurate quantitation in the form of interferences, matrix effects, and accumulation in the analytical flow path. Agilent Bond Elut Enhanced Matrix Removal—Lipid (EMR—Lipid) is the next generation of sample preparation products, and is used in convenient, dispersive solid phase extraction (dSPE) for highly selective matrix removal without impacting analyte recovery. This work demonstrates the effectiveness of this sample preparation methodology in the analysis of PAHs in salmon. The method delivers excellent accuracy (84 to 115%) and precision (RSD = 0.5 to 4.4%) for all 15 PAH compounds at all levels, providing a fast, robust, and effective analysis in high-fat samples.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants in the environment and may come from petrogenic or pyrogenic origins. They are composed of hydrogen and carbon arranged in two or more fused benzene rings, and can have substituted groups attached to one or more rings [1]. Concerns about PAHs arise from their persistence in the environment and known toxic, mutagenic, and carcinogenic effects on mammals for some of them [2]. Contamination of seafood can occur from accumulation of petroleum constituents in water sources and from cooking processes that introduce PAHs as combustion byproducts in smoke [3,4]. For these reasons, it is essential that analysts have robust and efficient methods for detecting contaminant PAHs at levels of concern.

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Detection of PAHs at low levels can be accomplished using GC/MS coupled with a robust and effective sample preparation method. Common preparation protocols include Soxhlet extraction [5], sonication assisted extraction [6], and pressurized solvent extraction [7]. Preparation can be coupled to cleanup procedures such as solid phase extraction [8] or gel permeation chromatography [9]. To overcome these labor-intensive and time-consuming techniques, protocols based on Quick, Easy, Effective, Rugged, and Safe (QuEChERS) [10,11] have also been implemented with good success [12,13,14]. Sample preparation is increasingly important for complex food samples, especially those high in lipids, as coextracted matrix has deleterious effects on analysis in the form of interferences, matrix effects, and accumulation in the analytical flow path.

Agilent Bond Elut Enhanced Matrix Removal—Lipid (EMR—Lipid) is a novel sorbent material that selectively removes major lipid classes from sample extracts without unwanted removal of analytes of interest. Removal of lipid species is especially important for techniques such as QuEChERS, which coextract large amounts of matrix with the target analytes. Traditionally, C18- and PSA-based sorbents were used for cleaning high-fat samples during a dispersive solid phase extraction (dSPE) step. However, these sorbents often fail to achieve adequate sample cleanup, and can exhibit nonselective interactions with analytes. This work investigates the sample preparation and analysis of 15 PAHs in salmon using a simple and effective workflow, delivering adequate cleanliness with EMR-Lipid as well as excellent accuracy and reproducibility on the GC/MS.

Experimental

Analysis was performed on an Agilent 7890 GC and an Agilent 5977 MSD equipped with multimode inlet (MMI), with an Agilent 7693 Automatic Liquid Sampler, and capillary flow technology for column backflushing. Table 1 shows the instrumental parameters, and Table 2 shows consumables and other equipment used in this work.

Table 1. Instrumental conditions for the Agilent GC/MS system used for PAH Analysis

GC:	Agilent 7890B
Autosampler:	Agilent 7693 Automatic Liquid Sampler, 10.0 µL syringe (G4513-80220)
Injection volume:	0.5 µL
Carrier gas:	Helium, constant flow
Gas filter:	Gas Clean filter GC/MS, 1/8 in (p/n CP17974)
Inlet:	MMI, hot splitless injection mode, 320 °C
Purge flow to split vent:	50 mL/min at 0.75 min
Flow rate:	2.0 mL/min
Oven program:	70 °C for 1 min, then 25 °C/min to 195 °C with a 1.5 min hold, then 7 °C/min to 315 °C
Column:	Agilent J&W DB-5ms UI, 20 m × 0.18 mm, 0.18 µm (p/n 121-5522UI)
Restrictor:	Deactivated silica tubing, 0.65 m × 0.15 mm (p/n 160-7625-5)
Postrun backflush:	5 min at 315 °C, 70 psi during backflush
Aux. pressure:	2 psi during run, 70 psi during backflush
MSD:	Agilent 5977 MSD
Mode:	SIM
Transfer line temperature:	340 °C
Source temperature:	325 °C
Quad temperature:	150 °C
Solvent delay:	3.5 min

Table 2. Other consumables and equipment.

Vials:	Amber, screw top, glass (p/n 5190-7041)
Vial caps:	PTFE, 9 mm, screw cap (p/n 5182-0717)
Vial inserts:	Glass, 150 µL, with polymer feet (p/n 5183-2088)
Septum:	Long-life, nonstick, 11 mm, 50/pk (p/n 5183-4761)
Ferrules:	Vespel:graphite, 85:15, 0.4 mm id (p/n 5181-3323), UltiMetal Plus Flexible Metal ferrules (p/n G3188-27501)
Inlet liner:	Single taper, splitless, Ultra Inert (p/n 5190-7041)
Capillary flow technology (CFT):	UltiMetal Plus Ultimate Union (p/n G3186-60580), CFT capillary fitting (p/n G2855-20530)
Bond Elut EMR—Lipid dSPE:	1 g in 15 mL tube (p/n 5982-1010)
Bond Elut Final Polish for Enhanced Matrix Removal—Lipid:	2 g in 15 mL tube (p/n 5982-0101)

Geno/Grinder, Metuchen, NJ, USA

Centra CL3R centrifuge, Thermo IEC, MA, USA

Eppendorf microcentrifuge, Brinkmann Instruments, Westbury, NY, USA

Vortexer and multitube vortexers, VWR, Radnor, PA, USA

Bottle top dispenser, VWR, So. Plainfield, NJ, USA

Eppendorf pipettes

Sample preparation

Salmon was homogenized and weighed (5 g) into 50 mL centrifuge tubes and spiked as necessary with standards and isotopically labeled internal standards. Acetonitrile (ACN) (10 mL) was added, and the sample was mixed on a mechanical shaker for two minutes. Tubes were centrifuged at 5,000 rpm for five minutes. The supernatant (8 mL) was transferred to a 15 mL centrifuge tube containing 1 g EMR—Lipid sorbent, vortexed immediately to disperse, and then for an extra 60 seconds on a vortex table. The slurry was then centrifuged at 5,000 rpm for three minutes. The entire supernatant was decanted into a second 15 mL polishing tube containing 2.0 g salts (1:4 NaCl:$MgSO_4$), and vortexed immediately to disperse, followed by centrifugation at 5,000 rpm for three minutes. The upper ACN layer was transferred to sample vials for GC/MS analysis (Figure 1).

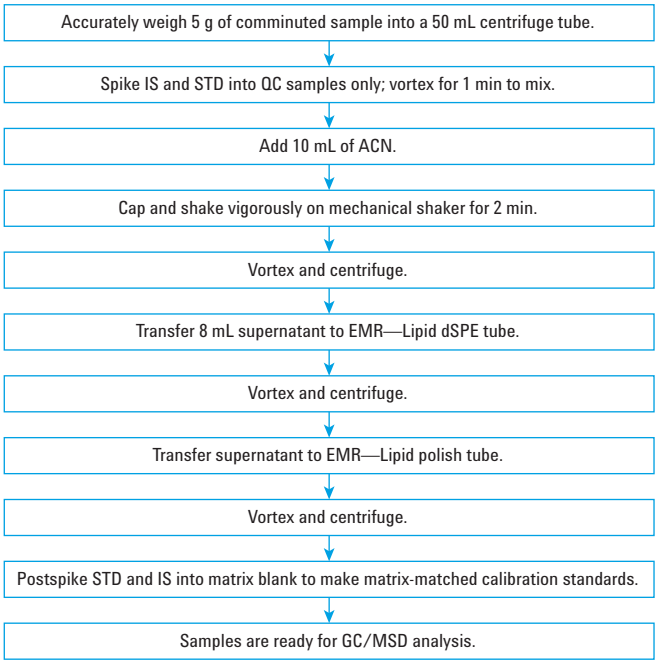

Figure 1. Sample preparation workflow for PAH in salmon using Agilent Bond Elut Enhanced Matrix Removal—Lipid before analysis by GC/MS.

Reagents and chemicals

All reagents and solvents were HPLC grade or higher. ACN was from Honeywell (Muskegon, MI, USA), and water was purified using an EMD Millipore Milli-Q Integral System (Darmstadt, Germany). PAH standards and internal standards were purchased from Ultra-Scientific as solutions (North Kingstown, RI, USA). Stock solutions were prepared at 100 µg/mL in acetone, and diluted in amber vials for working standards.

Calibration curves and quantitation

Matrix-matched calibration curves were generated over the calibration range, corresponding to 1, 10, 25, 50, 100, 250, 500, and 1,000 ng/g. Salmon blanks were carried through the entire sample preparation procedure and 950 µL of the blank extract, 25 µL standard working solution, and 25 µL stock internal standards. The internal standards were spiked into the salmon and postspiked into the matrix-matched calibration standards at 100 ng/g. All calibration curves gave exceptional linearity, with $R^2 > 0.999$ for all compounds. Salmon samples were prespiked at 25, 100, and 500 ng/g levels before extraction in six replicates. Agilent MassHunter Software was used to quantify the target analytes. Accuracy values were determined by calculating the spiked sample responses with respect to internal standards. Absolute recovery values were determined by measuring prespiked analyte response to the calibration curve without internal standard correction.

Results and Discussion

The 7890 GC and 5977 GC/MSD delivered excellent performance for the 15 PAHs and five internal standards, providing consistent results with high sensitivity. Figure 2 shows the separation achieved for the 15 PAHs on an Agilent DB-5ms UI column with a 25 ng/g prespike in salmon. The chromatogram shows baseline separation of all 15 PAHs, which is essential for accurate integration of PAH isomers phenanthrene, anthracene, benz[a]anthracene, chrysene, benzo[b]fluoranthene, and benzo[k]fluroanthene. Some minor interferences in the chromatogram are easily separated from the peaks of interest.

Excellent accuracy and precision was achieved at 25, 100, and 500 ng/g spike levels using the optimized procedure with EMR-Lipid. Figure 3 shows that accuracy was between 84 and 115% for all analytes at all levels using isotopically labeled internal standard correction, giving RSD from 0.5 to 4.4% (Figure 4). The accuracy data are grouped into recovery ranges in Figure 5, and show that most compounds fall between 90 and 120%, with two compounds falling slightly below 90% (indo[1,2,3-cd]pyrene and benzo[g,h,i]pyrene).

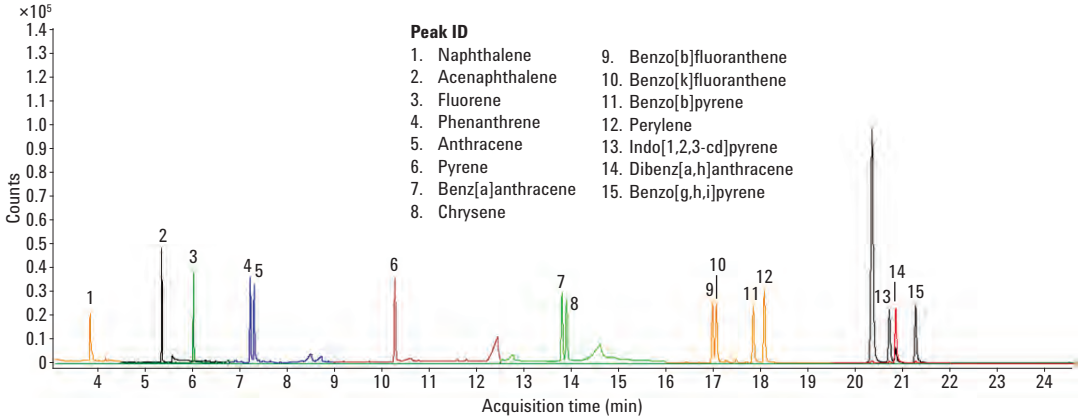

Figure 2. GC/MS SIM chromatogram of 15 PAHs from a 25 ng/g prespike in salmon.

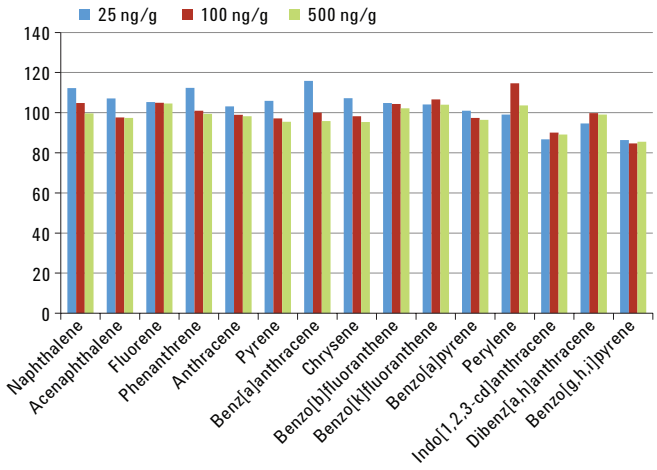

Figure 3. Accuracy results for 15 PAHs in salmon at 25 ng/g, 100 ng/g, and 500 ng/g levels.

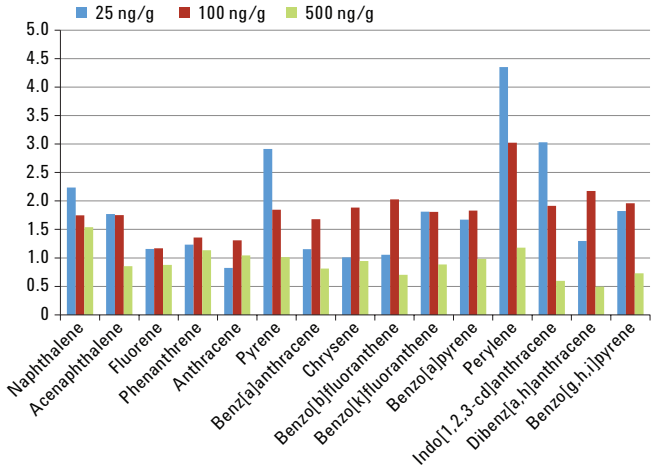

Figure 4. Precision results for 15 PAHs in salmon at 25 ng/g, 100 ng/g, and 500 ng/g levels.

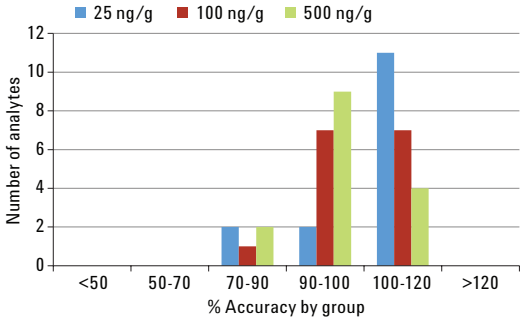

Figure 5. Grouped accuracy results for PAHs in salmon at 25 ng/g, 100 ng/g, and 500 ng/g levels.

Absolute recovery was from 62 to 98% without the use of internal standards (Table 3). Two compounds, indo[1,2,3-cd]pyrene and benzo[g,h,i]pyrene, gave recoveries slightly lower than 70%. The PAH absolute recoveries decrease with increasing molecular weight due to decreasing solubility in ACN. However, most recoveries are high and easily corrected using the internal standards. Internal standard absolute recoveries are also high as shown in Table 4. Despite the solubility limitation of ACN, this method gives good to excellent recoveries and highly reproducible results in the high-fat salmon sample.

EMR-Lipid dSPE

Salmon was chosen as a representative sample due to its high fat content relative to other seafood. The optimized procedure deviates from a typical QuEChERS protocol in several ways that streamline the workflow and take advantage of the EMR—Lipid dSPE cleanup step. First, the salmon is extracted directly with ACN without extra water or QuEChERS extraction salts. After centrifugation, the supernatant consists of ACN and a small amount of water from the sample. The supernatant is transferred to the EMR—Lipid tube for dSPE matrix removal. Finally, the dSPE supernatant is transferred to a polish tube containing 2.0 g NaCl/$MgSO_4$ (1:4) to induce phase separation. The upper ACN layer is then transferred to vials for analysis.

Table 4. Absolute recovery and precision (%RSD) for internal standards in salmon (n = 6).

	100 ng/g Spike	
Compound	**Rec.**	**%RSD**
Naphthalene-d8	87.8	1.0
Acenaphthalene-d10	93.3	0.8
Phenanthrene-d10	94.9	0.8
Chrysene-d12	87.1	1.0
Perylene-d12	86.4	3.1
Average	**89.9**	**1.3**

Table 3. List of PAHs used in this study and their accuracy, absolute recoveries, and relative standard deviations (RSDs) in salmon (n = 6).

	25 ng/g Spike			100 ng/g Spike			500 ng/g Spike		
Compound	**Acc.**	**Rec.**	**%RSD**	**Acc.**	**Rec.**	**%RSD**	**Acc.**	**Rec.**	**%RSD**
Naphthalene	112.2	86.7	2.2	104.8	89.7	1.7	99.7	85.8	1.5
Acenaphthalene	107.1	90.1	1.8	97.6	89.9	1.8	97.3	90.6	0.9
Fluorene	105.3	94.6	1.2	105.0	94.2	1.2	104.6	96.2	0.9
Phenanthrene	112.3	95.3	1.2	101.0	94.1	1.4	99.4	94.5	1.1
Anthracene	103.1	91.6	0.8	98.9	90.7	1.3	98.3	92.6	1.0
Pyrene	105.8	97.6	2.9	97.1	88.9	1.8	95.4	89.7	1.0
Benz[a]anthracene	115.8	91.2	1.2	100.1	84.7	1.7	95.8	85.7	0.8
Chrysene	107.2	83.6	1.0	98.2	83.2	1.9	95.4	85.4	0.9
Benzo[b]fluoranthene	104.8	78.3	1.1	104.3	76.1	2.0	102.2	79.2	0.7
Benzo[k]fluoranthene	104.1	78.8	1.8	106.6	77.5	1.8	104.0	80.3	0.9
Benzo[a]pyrene	101.0	74.2	1.7	97.4	71.8	1.8	96.4	74.8	1.0
Perylene	99.1	74.4	4.4	114.7	76.4	3.0	103.6	80.3	1.2
Indo[1,2,3-cd]pyrene	86.7	66.1	3.0	90.0	66.2	1.9	89.1	69.1	0.6
Dibenz[a,h]anthracene	94.7	73.9	1.3	99.7	72.2	2.2	99.0	76.2	0.5
Benzo[g,h,i]pyrene	86.4	64.7	1.8	84.7	62.3	2.0	85.6	66.3	0.7
Average	**103.0**	**82.7**	**1.8**	**100.0**	**81.2**	**1.8**	**97.7**	**83.1**	**0.9**

As is typical with protocols for enhanced matrix removal, this approach takes advantage of the enhanced cleanup by using a larger sample size, which in turn improves the overall sensitivity of the method. For conventional EMR—Lipid protocols, additional water is added to activate the sorbent material before dSPE. For this optimized protocol, it was found that extra water decreased the solubility of PAHs and negatively impacted some absolute recoveries. Therefore, the supernatant from extraction was transferred directly to the EMR—Lipid tube without additional water, providing adequate cleanup for GC/MS SIM analysis. Immediate mixing after the addition of supernatant to the EMR—Lipid and EMR—Lipid polish tubes suspends the solids to ensure maximum interaction with sorbent and avoids clumping. For optimal matrix removal, extra water can be added to the dSPE, and recoveries can be effectively corrected with the internal standards to give excellent accuracy and precision.

Conclusions

This work demonstrates a fast and easy method that effectively quantitates low to high-level concentrations of PAHs in high-fat salmon samples. The workflow is as easy as QuEChERS, but implements the new EMR—Lipid dSPE sorbent to minimize fat coextractives, maximize recovery, and give a high level of precision.

Although fat content in matrices such as salmon can vary greatly, Agilent Bond Elut Enhanced Matrix Removal—Lipid is a one-size-fits-all fat removal sorbent that does not interact with analytes of interest. Fat removal is maximized by using additional water with EMR—Lipid during the dSPE step. However, in this case, more water decreases solubility of PAHs and is not desirable for PAH sample preparation. Future work will continue to optimize EMR—Lipid for challenging sample types and applications to broaden its value on current and next generation chromatographic and detection systems.

Table 5. Target analytes, retention time, target ion, and internal standard designations for GC/MS SIM method.

	GC/MS (SIM)			
Compound	**RT**	**Target ion**	**Dwell (ms)**	**Internal standard**
Naphthalene	3.89	128.0	20	Naphthalene-d8
Acenaphthalene	5.37	152.0	20	Acenaphthalene-d10
Fluorene	6.05	166.0	20	Acenaphthalene-d10
Phenanthrene	7.25	178.0	20	Phenanthrene-d10
Anthracene	7.34	178.0	20	Phenanthrene-d10
Pyrene	10.31	202.0	20	Phenanthrene-d10
Benz[a]anthracene	13.83	228.0	20	Chrysene-d12
Chrysene	13.93	228.0	20	Chrysene-d12
Benzo[b]fluoranthene	16.99	252.0	20	Perylene-d12
Benzo[k]fluoranthene	17.08	252.0	20	Perylene-d12
Benzo[a]pyrene	17.85	252.0	20	Perylene-d12
Perylene	18.09	252.0	20	Perylene-d12
Indo[1,2,3-cd]pyrene	20.72	276.0	20	Perylene-d12
Dibenz[a,h]anthracene	20.87	278.0	20	Perylene-d12
Benzo[g,h,i]pyrene	21.29	276.0	20	Perylene-d12
Internal standards				
Naphthalene-d8	3.87	136.0	20	–
Acenaphthalene-d10	5.52	162.0	20	–
Phenanthrene-d10	7.22	188.0	20	–
Chrysene-d12	13.86	240.0	20	–
Perylene-d12	18.03	264.0	20	–

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