Analysis of Organochlorine Pesticide Residues in Whole Milk Using QuEChERS Followed by Enhanced Matrix Removal—Lipid Cleanup by GC-MS/MS

Application Note

Food Safety

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Abstract

Efficient, high-recovery extraction and cleanup of fatty sample matrices is important for obtaining reliable quantitative results and reducing GC-MS/MS system contamination. Though commonly used, the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) sample preparation method provides limited sample cleanup when lipid components are greater than 3% in sample matrix. This application note describes a simple, rapid, and effective extraction and cleanup method using the QuEChERS extraction protocol followed by EMR—Lipid cleanup to prepare samples for GC-MS/MS analysis. The method improved recovery of less polar organochlorine pesticides (OCPs) to SANCO/11945/2015 guideline-acceptable levels of 70 to 120 % [1]. Traditionally difficult to recover, *cis*-chlordane, 4,4'-DDE, 4,4'-DDT, Endrin, and HCB showed acceptable recoveries of 88, 76, 88, 86, and 74%, respectively, at 10 ng/mL, which were 25 to 40% higher than recoveries obtained using other extraction protocols. Reduced matrix effect and fewer matrix interferences provided better signal-to-noise and more accurate integration, and thus enhanced method quantitation performance. LOQs of 5 ng/mL or lower with RSDs of less than 20% were reached for all of the OCPs tested.

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Introduction

Due to their harmful effects, bioaccumulation of persistent organic pollutants (POPs) such as organochlorine pesticide (OCP) residues is a serious food safety concern. However, because of the hydrophobic characteristic of OCPs, their analysis is challenging in complex lipid matrices such as meat, egg, milk, edible oil, and fatty vegetable matrices.

The Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method is a common sample preparation technique for multiresidue analysis in various matrices. However, QuEChERS provides limited cleanup of fatty matrices containing more than 3% lipids. Extensive, labor-intensive sample extraction and cleanup protocols such as liquid-liquid partitioning, solid-phase extraction (SPE), gel-permeation chromatography (GPC), and matrix solid-phase dispersion (MSPD), have been used to remove unwanted lipid matrix while extracting OCPs of interest [2]. These methods usually require tradeoffs between incomplete lipid removal and acceptable analyte recovery. SANCO 11945/2015 guidelines require 70 to 120% recovery and RSDs of less than 20% for multiresidue methods [1].

To increase confidence in results, GC/MS or GC-MS/MS detection is often used for the analysis of OCPs. GC-MS/MS has the advantage of improved selectivity and lower detection limits. Lipids tend to be retained on the surface of the injection port, column, ion source, and mass analyzer of GC/MS systems, resulting in poor separation and MS performance, and increased maintenance over time. In addition, enhancement of analyte response caused by coeluted matrix interferences is of concern for achieving accurate and reproducible quantitative results. Removing interfering lipid matrix prior to instrumental analysis minimizes issues, and is essential for reliable testing of OCP contamination in food and feeds.

For these reasons, development of improved extraction and cleanup methods for fatty matrices has received substantial attention. Traditionally, QuEChERS extraction followed by dispersive SPE (dSPE) using a primary secondary amine (PSA) and end-capped-C18 (EC-C18) protocol has been used to clean up lipid-containing matrices, however, this protocol does not adequately remove unwanted lipids. This application note describes a sample preparation approach using QuEChERS extraction followed by an enhanced matrix removal (EMR—Lipid) dSPE cleanup to prepare whole milk samples for GC-MS/MS analysis. Whole milk samples spiked with 21 target OCPs at four levels (5, 10, 25, and 50 ppb) were analyzed (Table 1). The OCPs tested included *cis*-chlordane, 4, 4-DDE, 4,4-DDT-p,p', Endrin, and most notably HCB, where

recovery can be low because of compound hydrophobicity. After the extraction step, (for example, QuEChERS salting out) the extract was cleaned up using novel dispersive EMR—Lipid sorbent. A polish step was then used to remove water residue prior to GC-MS/MS analysis.

Table 1. Spiked OCPs analyzed using QuEChERS, followed by EMR—Lipid cleanup and GC-MS/MS.

Compound	Log P
Aldrin	5.32
α-1,2,3,4,5,6-Hexachlorocyclohexane (α-BHC)	3.99
β-BHC	3.99
δ-BHC	3.99
α-Chlordane	5.57
γ-Chlordane	5.57
1,1-Dichloro-2,2-*bis*(4-chlorophenyl)ethane (4,4'-DDD)	5.39
4,4'-DDE	6.37
4,4'-DDT	5.92
Dieldrin	4.88
α-Endosulfan	3.83
β-Endosulfan	3.62
Endosulfan sulfate	4.30
Endrin	4.88
Endrin aldehyde	2.76
Endrin ketone	2.68
HCB	4.89
Heptachlor	5.46
Heptachlor exo-epoxide	5.47
Lindane	3.99
Methoxychlor	4.56

Experimental

Reagents and chemicals

All reagents and solvents were HPLC or analytical grade. Acetonitrile (ACN), toluene, hexane, and ethyl acetate were from Honeywell (Muskegon, MI, USA), Pesticide standards were purchased from Sigma-Aldrich, Corp. (St. Louis, MO, USA).

Solutions

A standard mix of 17 pesticides from Sigma-Aldrich (Organochlorine Pesticides Mix) at 2,000 µg/mL (toluene/hexane, 1:1) and four individual pesticides (α-chlordane, *trans*-chlordane, Endrin ketone, and HCB) at 2,000 µg/mL (toluene/hexane, 1:1) were used to make a combined working solution in ACN at 100 µg/mL.

Calibration standards and quality control samples

Prespiked quality control (QC) samples were fortified with standard working solution to the appropriate concentrations in replicates of six. The QC samples corresponded to 5, 10, 25, and 50 ng/mL in whole milk.

Matrix-matched calibration standards (STD) prepared with standard working solutions were post-spiked, corresponding to 0.5, 1.0, 10, 25, 50, and 100 ng/mL in whole milk extract.

Equipment and instrumentation

Table 2 provides the list of the equipment and instrumentation used to perform the analysis.

Table 2. Equipment and instrumentation used for sample preparation and analysis.

	Part number
Sample preparation	
Agilent Bond Elut QuEChERS EN Extraction Salts	5982-5650
Agilent Bond Elut EMR-Lipid tubes	5982-1010
Agilent Bond Elut Polish Tubes	5982-0101
Agilent QuEChERS Ceramic Homogenizers	5982-9313
GenoGrinder (SPEX, NJ, USA)	
Centra CL3R centrifuge (Thermo IEC, MA, USA)	
Heidolph Reax (Heidolph NA, IL, USA)	
Bottle top dispenser (VWR, NJ, USA	
Eppendorf pipettes and repeater (VWR, NJ, USA)	
Gas chromatograph system	
Agilent 7890B GC	
Multimode Inlet (MMI)	
Purged Ultimate Union	
Agilent DB-5ms Ultra Inert Column (×2)	19091S-431UI
Agilent dimpled Ultra Inert liner, 2 mm id	5190-2297
Mass spectrometer system	
Agilent 7000C Triple Quadrupole GC/MS system	
Agilent MassHunter Software	

GC-MS/MS analysis

An Agilent 7890 GC coupled to an Agilent 7000C Triple Quadrupole GC/MS system was used for the GC-MS/MS analysis. GC and MS conditions are provided in Tables 3 and 4, respectively.

Table 3. GC conditions.

Parameter	Value
Column 1:	Agilent DB-5ms Ultra Inert, 15 m × 0.25 mm, 0.25 µm
Flow rate, column 1:	1.1 mL/min
Column 2:	Agilent DB-5ms Ultra Inert, 15 m × 0.25 mm, 0.25 µm
Flow rate, column 2:	1.2 mL/min
Injection mode:	PTV solvent vent
Injection volume:	2 µL (syringe size 10 µL)
Solvent washes:	Pre-injection, 1; Post-injection, 5
Sample wash:	2 µL × 2
Sample pumps:	5
MMI inlet temperature program:	60 °C for 0.35 minutes, then 900 °C/min to 280 °C (hold 15 minutes), then 900 °C/min to 300 °C (to the end of the analysis)
Purge flow to split vent:	50 mL/min at 1.5 minutes
Vent flow:	25 mL/min
Vent pressure:	5 psi to 0.3 minutes
Gas saver:	On, 20 mL/min at 5 minutes
Septum purge flow:	3 mL/min
Cryo air cooling:	On at 200 °C
Oven temperature program:	60 °C for 1.0 minute, then 40 °C/min to 170 °C, then 10 °C/min to 310 °C, hold 3 minutes
Total run time:	20.75 minutes

Table 4. MS conditions.

Parameter	Value
MS source:	EI
Tune file:	Atune.u
Source temperature:	300 °C
Quadrupole temperature:	150 °C
Transfer line temperature:	280 °C
Solvent delay:	3.0 minutes
Helium quench gas:	2.25 mL/min
Nitrogen collision gas:	1.5 mL/min
Acquisition mode:	Multiple reaction monitoring (MRM)
MS1/MS2 resolution:	Wide
Dwell time:	10 ms

Triple quadrupole multiple reaction monitoring (MRM) acquisition parameters for each target OCP per time segment monitored are provided in Table 5.

The GC system was equipped with a multimode inlet (MMI) with air-cooling, and a backflushing system based on a purged ultimate union controlled by an AUX EPC module.

Agilent MassHunter Software was used for instrument control, and for qualitative and quantitative data analysis.

Table 5. Triple quadrupole MRM acquisition parameters per time segment.

Compound	Retention time (min)	Precursor ion	Product ion	CE	Compound	Retention time (min)	Precursor ion	Product ion	CE
Time segment 1 (0.00 minutes)					4,4'-DDE	11.94	318	246	25
α-1,2,3,4,5,6-Hexachlorocyclohexane (α-BHC)	7.93	219	183	7			246	176	35
							246	150	55
		217	181	7	Dieldrin	12.06	276.8	205.9	22
		181	145	15			262.9	192.9	40
HCB	8.07	283.9	178.9	53			262.9	190.1	38
		283.8	248.8	22	**Time segment 3 (12.20 minutes)**				
		283.8	213.9	40	4,4'-DDE	11.94	318	246	25
β-BHC	8.32	219	183	8			246	176	35
		217	181	7			246	150	55
		183	147	16	Dieldrin	12.06	276.8	205.9	22
Lindane	8.44	219	183	8			262.9	192.9	40
		217	181	7			262.9	190.1	38
		183	147	16	Endrin	12.45	316.9	172.9	52
δ-BHC	8.81	219	183	8			262.9	192.9	40
		217	181	7			262.9	190.9	38
		183	147	16	β-Endosulfan	12.61	240.9	205.9	15
Time segment 2 (9.25 minutes)							195	159	8
Heptachlor	9.65	274	239	15			195	125	27
		272.1	236.9	15	4,4'-DDT	12.70	237	165	25
		272.1	143	50			235	199	18
Aldrin	10.27	292.8	186	35			235	165	30
		262.8	193.1	30	Endrin aldehyde	12.95	278.9	209	25
		261	191	30			249.9	214.9	30
Heptachlor exo-epoxide	10.94	352.8	281.9	18	Endosulfan sulfate	13.36	386.9	288.9	5
		352.8	262.9	15			271.9	236.9	15
		352.8	317	42			271.9	116.9	48
γ-Chlordane	11.35	372.8	300.8	10	1,1-Dichloro-2,2-*bis*(4-chlorophenyl)ethane(4,4'-DDD)	13.37	235	199.1	20
		372.8	265.9	24					
		372.8	263.9	26			235	165.1	25
α-Endosulfan	11.59	240.9	205.9	15	Endrin ketone	14.22	316.8	280.7	5
		195	159	8			316.8	100.8	10
		195	125	27	Methoxychlor	14.36	227	169	28
α-Chlordane	11.63	372.8	300.8	10			227	141	40
		372.8	265.9	24			227	115	54
		372.8	263.9	26					

Optimized sample preparation procedure

1. Add 10 mL of whole milk and two ceramic homogenizers to a 50 mL centrifuge tube.

2. Spike STD into QC samples, except matrix blanks, and vortex.

3. Add 1 mL of ethyl acetate; vortex at 2,000 rpm for 10 minutes.

4. Add 9 mL of ACN; vortex at 2,000 rpm for 30 minutes.

5. Add QuEChERS EN extraction salt packet.

6. Shake on a mechanical vertical shaker, 2 minutes.

7. Centrifuge at 5,000 rpm for 5 minutes.

8. Add 2 mL of water to a 15 mL EMR—Lipid dSPE tube, and vortex immediately for 30 seconds.

9. Add 5 mL of supernatant to an EMR—Lipid tube.

10. Vortex immediately to disperse the sample, then extract the entire batch for 60 seconds on the multitube vortexer.

11. Centrifuge at 5,000 rpm for 5 minutes.

12. Decant the supernatant into a 50 mL centrifuge tube, add the entire contents from the Bond Elut Polish Tube, vortex, then place on a vertical shaker for 5 minutes.

13. Centrifuge at 5,000 rpm for 5 minutes.

14. Transfer the supernatant into a 15 mL centrifuge tube, add 200 mg MgSO$_4$, vortex for 30 seconds, then centrifuge at 5,000 rpm for 5 minutes.

15. Transfer 1 mL to a sampler vial with 10 µL of 1% formic acid in ACN for GC-MS/MS analysis.

The EMR—Lipid protocol was optimized to improve traditionally low recoveries of hydrophobic OCPs from the high-fat matrix (steps 3, 4, and 8 in blue). Most notably, ethyl acetate was added, and the amount of extraction solvent was adjusted to 10 mL of 9:1 ACN/ethyl acetate for QuEChERS EN extraction. In addition, less water, 2 mL versus the 5 mL commonly used, was added to activate the EMR—Lipid sorbent. Increasing the speed and time that the extraction solvents were vortexed or vertically shaken with the sample also improved recovery of the nonpolar OCPs. The EMR—Lipid sorbent was immediately mixed after adding the extract to suspend its particles to ensure rapid and consistent interaction with the matrix and to avoid clumping.

Formic acid (1%) was added to the autosampler vials just prior to GC-MS/MS analysis, which improved the stability of the base-sensitive endosulfan sulfate.

Results and Discussion

Unwanted lipid matrix removal

The EMR—Lipid approach is simple and universally applicable to reducing matrix effects and improving analyte recoveries for the analysis of polar, mid-polar, and nonpolar target analytes. EMR—Lipid ingeniously replaces the PSA C18-EC used following QuEChERS extraction with a unique sorbent chemistry. When activated by water, the EMR—Lipid sorbent selectively traps lipids by size exclusion and hydrophobic interaction (Figure 1). Unbranched hydrocarbon chains (lipids) enter the sorbent, but bulky analytes do not. Lipid chains that enter the sorbent are then trapped by hydrophobic interactions.

EMR—Lipid retains aliphatic compounds with a long carbon chain. EMR—Lipid does not interact with functional groups such as carboxylic acids of fatty acids, phospho-amines on phospholipids, amides, carbonyls, or hydroxyls of triglycerides and sphingolipids. EMR—Lipid also does not interact with bulkier compounds such as OCPs.

Size Exclusion: Unbranched hydrocarbon chains (lipids) enter the sorbent; bulky analytes do not.

Analyte

Lipid

EMR Sorbent

Sorbent Chemistry: Lipid chains that enter the sorbent are trapped by hydrophobic interactions
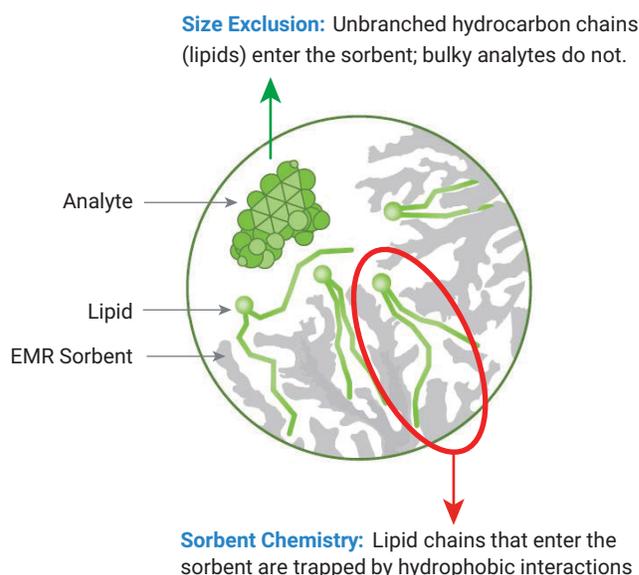
Figure 1. EMR—Lipid mechanism of action: size exclusion and sorbent chemistry.

Chromatographic performance

The MRM chromatogram of spiked whole milk at 10 ng/mL (Figure 2) shows the chromatographic performance that can be obtained using the EMR—Lipid protocol. Ideal peak shape due to reduced matrix effect and interferences resulted in better signal-to-noise (S/N) and more accurate integration.

The full scan MS chromatogram of whole milk blank matrix (Figure 3) shows the effectiveness of the QuEChERS extraction followed by the EMR—Lipid cleanup protocol compared to QuEChERS extraction with dSPE PSA C18-EC cleanup. EMR—Lipid provides substantially better cleanup and improved analytical results, while preventing fatty matrix buildup in the GC-MS/MS instrument.
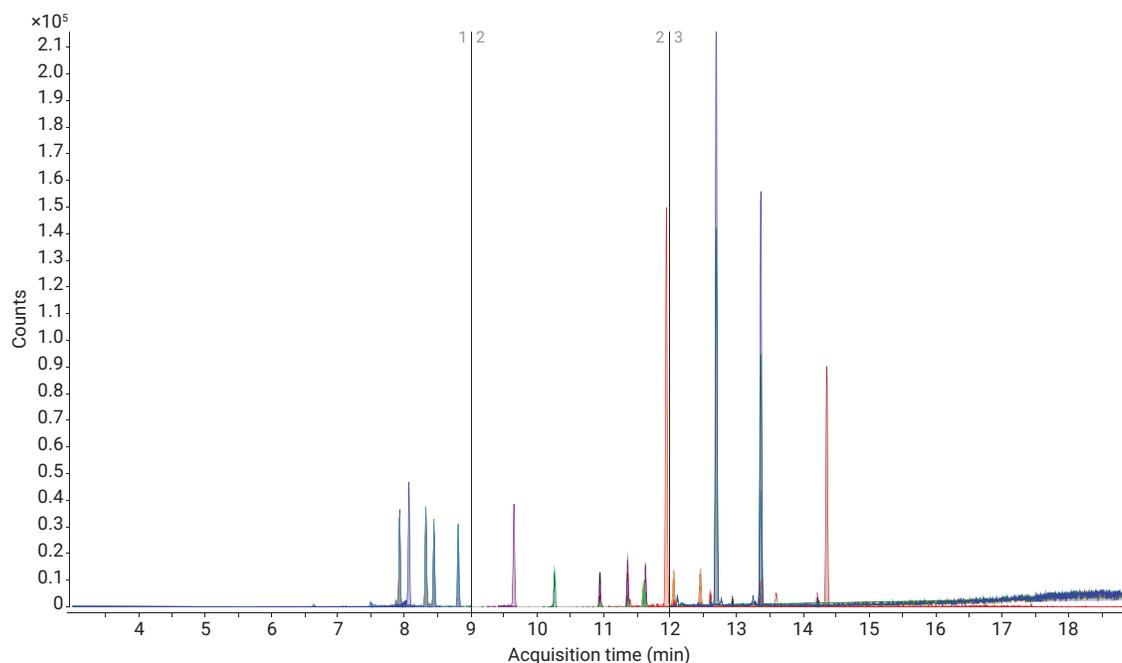
Figure 2. MRM chromatogram of whole milk spiked at 10 ng/mL.
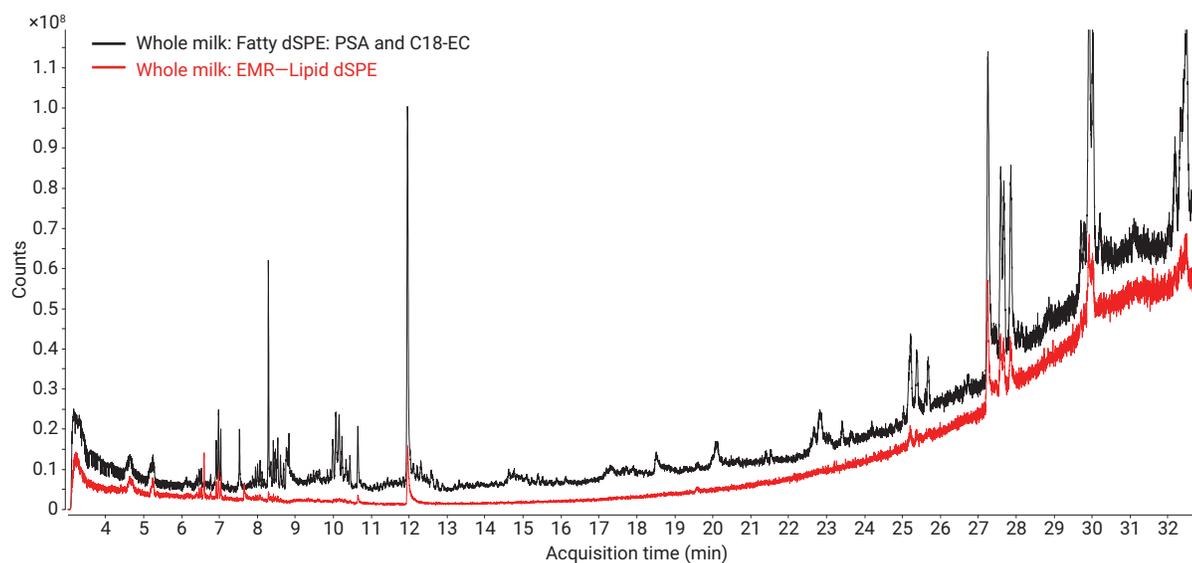
Figure 3. GC/MS full scan chromatograms of whole milk with fatty dSPE PSA C18-EC cleanup (black) and whole milk with dSPE EMR—Lipid cleanup (red).

Quantitative performance

Calibration curve linearity for each pesticide was evaluated. Good linearity of response was observed for all pesticides at the concentration levels tested. The average coefficient of determination (R^2) for each OCP analyzed was greater than 0.990. Figure 4 shows the calibration curves generated for the more-difficult-to-analyze compounds *cis*-chlordane (A), 4,4'-DDE (B), and HCB (C).

A
cis-Chlordane *alpha*
y = 5484.201110x − 201.731931
$R^2 = 0.9928$

B
4,4'-DDE
y = 25273.021721x − 125.544266
$R^2 = 0.9932$

C
HCB
y = −2.960282x² + 6907.955807x − 1416.939876
$R^2 = 0.9940$
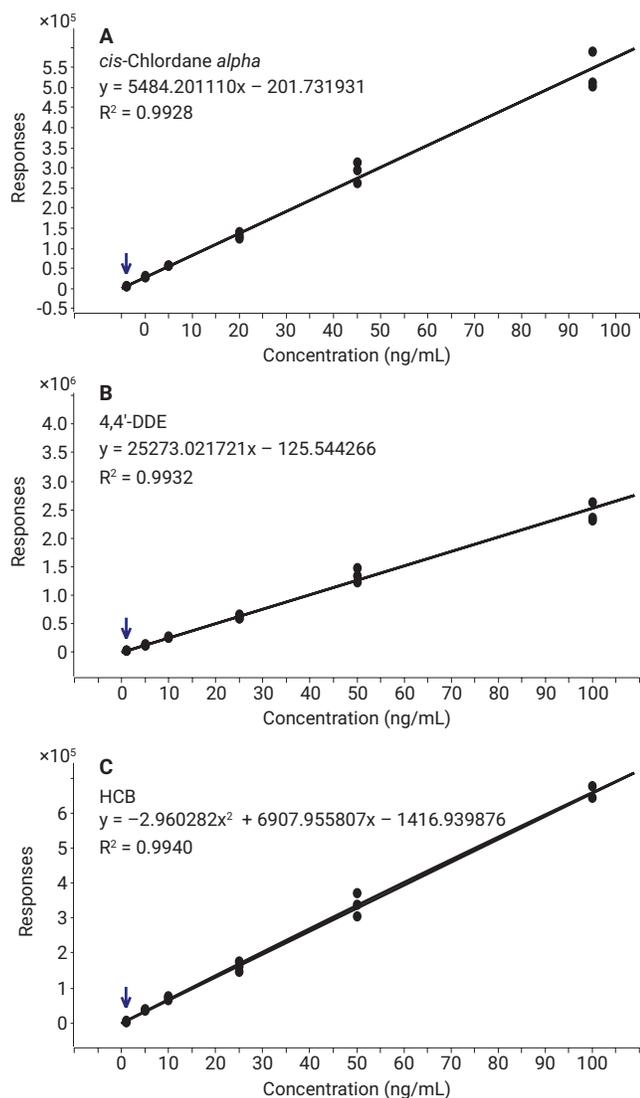
Figure 4. Selected calibration curves. A) *cis*-chlordane; B) 4,4'-DDE; C) HCB; calibration range 0.5-100 ng/mL.

Limits of quantitation (LOQs) for the 21 OCPs were 5 ng/g or lower. Recoveries were within the acceptable range of 70 to 120% (Figure 5). Increasing the duration and speed when vortexing the sample with the extraction solvents improved recovery. The addition of ethyl acetate with acetonitrile during extraction improved the recovery of less polar compounds such as chlordane, 4,4'-DDE, 4,4'-DDT, aldrin, and heptachlor to acceptable levels of 70%. Using less water to activate the sorbent likewise improved recovery of traditionally difficult to recover, *cis*-chlordane, 4,4'-DDE, 4,4'-DDT, Endrin, and HCB to 88, 76, 88, 86, and 74%, respectively at 10 ng/g, which were 25-40% higher than recoveries commonly obtained using other extraction protocols.

Method reproducibility for all compounds was determined by spiking the standards in whole milk to 5, 10, 25, and 50 ppb in replicates of six. Figure 6 shows that RSDs were less than the required ≤20% RSDs specified by SANCO 11945/2015 [1].

Method LOQs were based on % RSD ≤20 (n = 6). LOQs of 5 ng/g or lower were achieved for all OCPs tested in whole milk, which is less than the maximum residue limit (MRL) of 10 ng/g specified by the United States Department of Agriculture (USDA) Pesticide Data Program (PDP) [3].
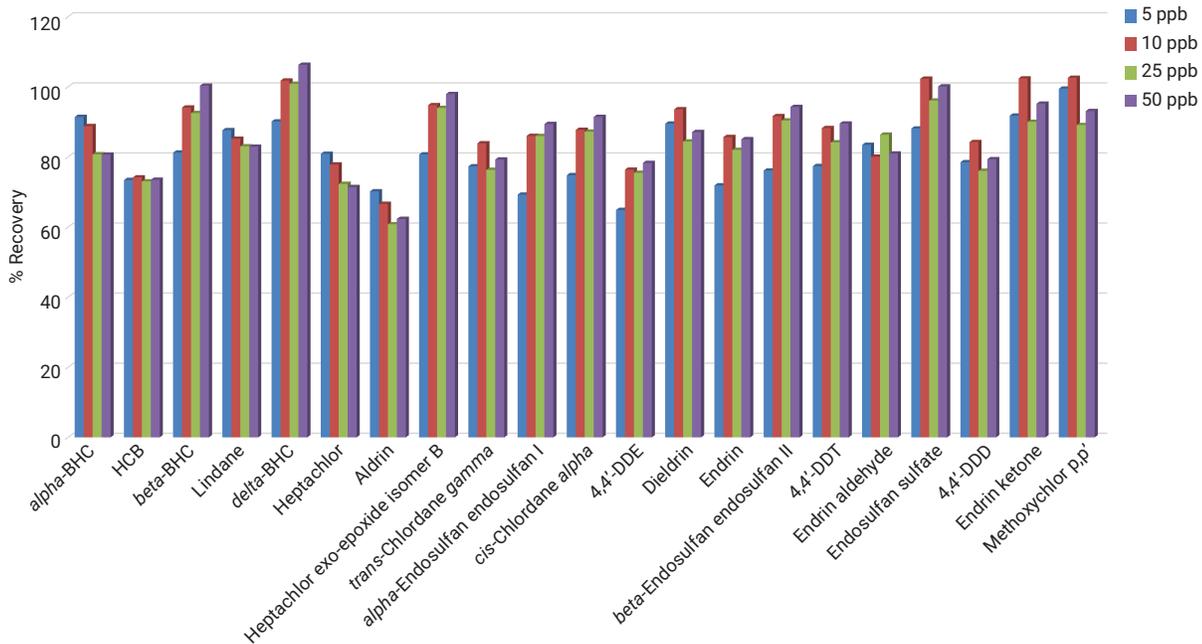
Figure 5. Recovery of OCPs in whole milk using QuEChERS with EMR—Lipid cleanup, n = 6.
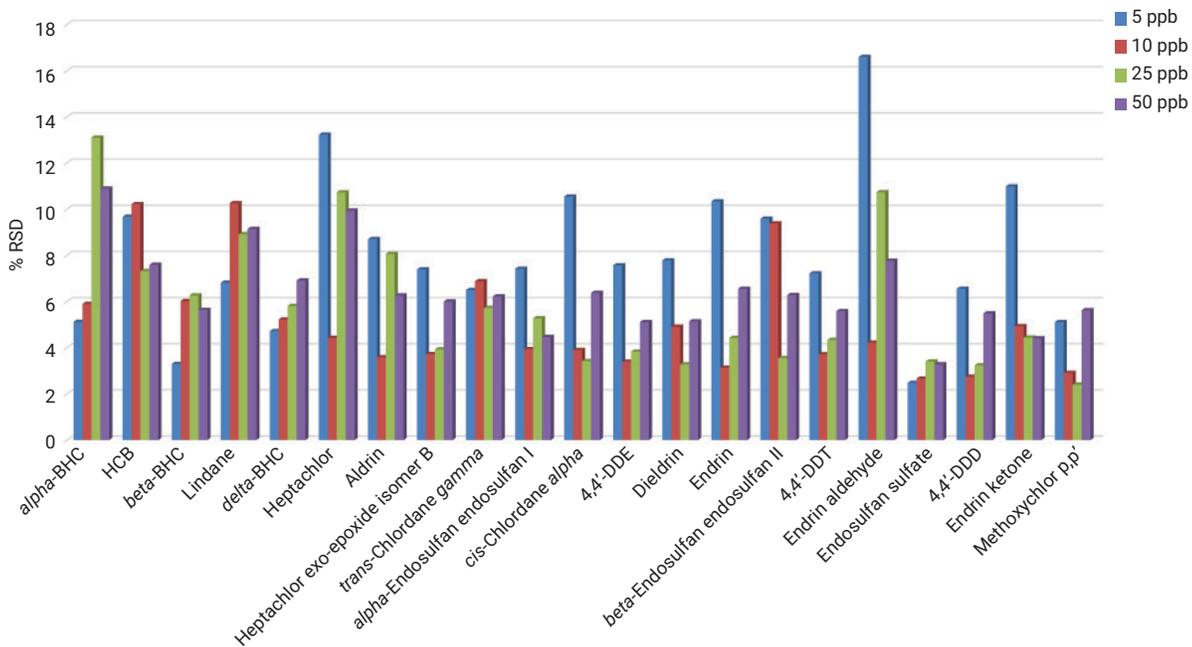
Figure 6. %RSDs for OCPs spiked in whole milk, n = 6.

Conclusion

This application note presents a simple and rapid extraction and cleanup method using QuEChERS with EMR—Lipid dSPE cleanup to prepare samples for GC-MS/MS analysis. EMR—Lipid efficiently removed unwanted lipids from the sample matrix, improving overall recovery of less polar OCPs to acceptable levels. Compounds that are traditionally difficult to recover, that is, *cis*-chlordane, 4,4'-DDE, 4,4'-DDT, Endrin, and HCB, showed recoveries of 88, 76, 88, 86, and 74%, respectively, at 10 ng/mL, which are 25 to 40% higher than recoveries achieved when using other extraction protocols. Ideal chromatographic peak shapes due to reduced matrix effect and interferences produced enhanced S/N, more accurate integration, and higher-confidence quantitative performance. LOQs of 5 ng/mL or lower, well below the desired screening level, with RSDs of less than 20%, were reached for all of the OCPs tested.

EMR—Lipid methodology can be readily incorporated into existing QuEChERS workflows, and does not require any additional sample preparation devices or glassware. EMR—Lipid can also significantly reduce the need for GC-MS/MS system maintenance, thereby reducing costly downtime.

References

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2. Gilbert-López, B.; García-Reyes, J. F.; Molina-Díaz, A. Sample treatment and determination of pesticide residues in fatty vegetable matrices: A review. *Talanta* **2009**, *79*, 109-128.

3. United Stated Department of Agriculture (USDA) Pesticide Data Program (PDP) https://www. ams.usda.gov/ datasets/pdp. Accessed July 21, **2017**.

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