Taking the Fat Out: Improved Sample Preparation Techniques for Toxicologic Testing of Postmortem Liver Tissue by GC-MS in Veterinary Diagnostics

By

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Abstract

An efficient and effective method for the detection of a wide variety of toxicants in liver tissue was developed and validated using gas chromatography-mass spectrometry (GC-MS). The aim of this research was to address the need for non-targeted analysis in postmortem liver samples. We wanted to find a faster, less labor-intensive method for fat removal from liver. A variety of clean-up sorbents and cartridges were assessed for their efficacy in lipid removal from liver tissue as well as analyte recovery. The study focused on the evaluation of QuEChERS solvent extraction with Enhanced Matrix Removal-Lipid (EMR-Lipid™) clean-up to remove lipids from liver tissue samples. Qualitative analysis was performed on 15 representative pesticides and 2 drugs commonly analyzed for in veterinary diagnostics. Increased sensitivity was achieved by using 5 μL injections into the GC-MS running on solvent vent mode. Probability of detection (POD) was used as the statistical model for method evaluation and validation. Three sources of liver (bovine, chicken, and caprine), with varying amounts of fat present, were tested. The QuEChERS extraction with EMR-Lipid clean-up was found to be as effective as gel permeation chromatography (GPC) in removing fats from liver tissue with minimal analyte loss. GPC, the typical clean-up method for fatty tissue samples, was the reference method used for comparison. It was determined by the POD model that the candidate method had better detection than GPC at 5 μg/g for all the analytes investigated and that the difference was statistically significant at 5 μg/g but not below that. In addition, EMR-Lipid uses substantially less solvent and is twice as fast compared to GPC.

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Table of Contents

List of Abbreviations

The following table includes the various abbreviations and acronyms used throughout the thesis.

Abbreviation	Meaning
AOAC	Association of Official Analytical Chemists
AMDIS	automated mass spectral deconvolution and identification system
C_{18}	octadecyl silica (18 carbon atoms)
CI	confidence interval
CIPS	cold-induced aqueous acetonitrile phase separation
cSPE	cartridge solid phase extraction
dPOD	difference in POD values
dSPE	dispersive solid phase extraction
EMR-Lipid	Enhanced Matrix Removal-Lipid
EI	electron ionization
EPA	environmental protection agency
FDA	Food and Drug Administration
FVM	Foods and Veterinary Medicine
GC-IT/MS	gas chromatography- ion trap mass spectrometry
GC-MS	gas chromatography-mass spectrometry
GC-MS/MS	gas chromatography-mass spectrometry/ mass spectrometry
GPC	gel permeation chromatography
HPLC	high performance liquid chromatography
LC/MS/MS	liquid chromatography/ mass spectrometry/ mass spectrometry
NIST	National Institute of Standards and Technology
OCP	organochlorine pesticide
OP	organophosphate
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
PFTBA	perfluorotributylamine
POD	probability of detection
PSA	primary secondary amine
PTV	programmable temperature vaporization
QuEChERS	quick, easy, cheap, effective, rugged, and safe
MMI	multimode inlet
RT	retention time
SIM	selected ion monitoring
SPE	solid phase extraction
TIC	total ion chromatogram

1. INTRODUCTION

Animals and people can be exposed to a large number of chemically diverse pesticides used for agricultural and home use. Due to their toxicity, illness and death can occur. When postmortem samples such as blood, brain, liver, or stomach contents are submitted to a laboratory for toxicologic analysis, information is not always available with regard to what toxicant(s) an individual might have been exposed to and, as a result, determining what the best sample for analysis is challenging. Liver tissue samples are commonly used for non-targeted analysis of toxicants in postmortem samples using gas chromatography-mass spectrometry (GC-MS). This is because the liver is the organ that is exposed to large amounts of xenobiotics following absorption from the gastrointestinal tract. It is also an organ of substantial metabolism. In many cases a history of a specific pesticide exposure is unavailable, so untargeted analytical screening methods that are sensitive and rapid are crucial to help with identifying exposure in postmortem samples such as liver.

One limitation of analyzing liver tissue is that it contains lipids that make it difficult to detect toxicants by GC-MS. Lipids are a class of organic compounds commonly known as fats and oils; these include free fatty acids, cholesterol, and triglycerides (Stevens, 2015). Lipid interferences adversely affect the extraction efficiency and must be removed in order to reduce matrix effects and baseline disturbances (Chamkasem *et al.*, 2013). This improves detection of analytes that might be present in the sample. Without sufficient lipid clean-up, liver samples can clog GC liners quickly and result in analyte carryover. When high amounts of fatty acids remain in sample extracts, it can also affect instrument performance by degrading the column lifetime so their removal prior to analysis helps protect the instrument and reduce potential cross contamination (Parrilla Vázquez *et al.*, 2016).

A method often used to prepare liver samples for GC-MS analysis involves extraction of the tissue using ethyl acetate and clean-up of the extract by gel permeation chromatography (GPC), a type of size exclusion chromatography that separates molecules based on their size to remove lipids. GPC has been used for the removal of fats for pesticide residue determination in food since 1972 (Jain & Gupta, 2006). It has been the usual method for the clean-up of liver extracts due to its removal of lipids without loss of analytes of interest. However, GPC clean-up is both time and solvent consuming. This method depends on labor intensive processes and the cost of analysis can be quite expensive. It takes roughly 1 hour for the extraction procedure to prep a sample for GPC clean-up. It takes almost an hour for each individual sample to run on the GPC instrument, so the process for a batch of samples usually takes an overnight run on the GPC instrument resulting in a 2-day turnaround time. GPC requires its own specially designed equipment that utilizes columns filled with a porous gel. After the clean-up procedure, additional evaporation and concentration steps are needed which takes about another hour. A high-volume nitrogen evaporator, such as TurboVap®, is an additional instrument necessary to complete the final steps. In total, it takes approximately 3 hours to prepare a liver sample for GC-MS analysis when using GPC clean-up. Methylene chloride, a carcinogen, is also used in the process. The goal of this study was to find an efficient, inexpensive clean-up method for liver samples that is at least as effective as GPC in removing fats, retains analytes of interest, but decreases the use of potentially hazardous solvents.

Possible solutions are a QuEChERS extraction with dispersive solid phase extraction (dSPE) or solid phase extraction (SPE) clean-up prior to GC analysis. Anastassiades *et al.* (2003) developed a quick, easy, cheap, effective, rugged, and safe (QuEChERS) method that has been widely used to extract pesticides from food and agricultural products. This simple multiresidue

method utilizes loose extraction salts and sorbent material in combination with shaking and centrifugation. The procedure involves initially combining of the sample with water and acetonitrile, then the addition of anhydrous $MgSO_4$ and NaCl to the centrifuge tube to induce phase separation. The acetonitrile is purified by dSPE clean-up in a secondary tube containing clean-up sorbents and anhydrous $MgSO_4$ (Anastassiades *et al.*, 2003). Modifications to the original QuEChERS method have been done for applications on fatty complex mixtures such as avocado (Chamkasem *et al.*, 2013), olive and soybean oil (Parrilla Vázquez *et al.*, 2016), and salmon (Shao *et al.*, 2017).

The proposed study involved assessing the efficacy of different dSPE sorbents/ combination of sorbents and SPE cartridges by analyzing multiple replicates of spiked liver tissue. The clean-up sorbents chosen for comparison were primary secondary amine (PSA) exchange material, C_{18}, Florisil®, Supel™ QuE Z-Sep™, and Enhanced Matrix Removal-Lipid (EMR-Lipid). PSA sorbent is commonly used as a base sorbent to remove fatty acids (Misselwitz & Cochran, 2015). C_{18} can remove nonpolar interferences such as lipids, fats, and free fatty acids (Kowalski *et al.*, n.d.). Florisil sorbent is used for the separation of polar lipids/ compounds (Raina-Fulton, 2015). Z-Sep sorbent, zirconia bonded to silica, can remove lipids (Raina-Fulton, 2015). A cartridge solid phase extraction (cSPE) cleanup, as recommended by Misselwitz & Cochran (2015) for high fat matrices that may require a more thorough cleanup than dSPE, was also tested. A PSA cartridge allows more sorbent capacity than dSPE without requiring elution solvents or extract concentration steps that are needed for traditional SPE. In one study, a "pass through" procedure on a 500 mg PSA cartridge removed more fatty acids than dSPE in tuna fish (Misselwitz & Cochran, 2015). Agilent's Bond Elut EMR-Lipid is a proprietary sorbent mixture, containing C_{18} and proprietary polymers, that removes components

with long alkyl chains (Vuković *et al.*, 2016). The EMR-Lipid sorbent also uses water "activation" which enables the sorbent to interact with straight chain, "lipid-like" functional groups. This mechanism removes lipids by size exclusion and increased hydrophobic interaction (Stevens, 2015). After the EMR-Lipid dSPE step, a final polishing step is highly recommended. The EMR-Lipid polish tube contains NaCl and $MgSO_4$ salts that help improve extraction efficiency by removing residual water and any dissolved solids from the extracted samples. EMR-Lipid has been used to detect pesticides in avocado using a QuEChERS extraction, veterinary drugs in bovine liver, and polycyclic aromatic hydrocarbons (PAHs) in salmon using a modified protein precipitation extraction (Stevens, 2015). Avocado and salmon have higher approximate total lipid contents than liver, with 21% and 27% respectively (Stevens, 2015). Stevens (2015) lists beef and pork liver to have approximate percent lipid values of 4% for both.

Cold-induced aqueous acetonitrile phase separation (CIPS) and freezing out steps were also investigated since improved detection has been noted when used for pesticide analysis. A study on CIPS-QuEChERS presented a modified procedure that led to higher analyte recovery due to lower background signals when applied to salmon by GC-MS (Shao *et al.*, 2017). Other research found that a QuEChERS protocol, including a freeze-out step with dry ice prior to dSPE EMR-Lipid clean-up, had better removal of co-extracted matrix compounds and recoveries than PSA and QuE Z-Sep in edible vegetable oils by GC-MS/MS (Parrilla Vázquez *et al.*, 2016).

There is little information in the literature about pesticide residue analysis in animal liver samples using this approach. EMR-Lipid has been previously applied to the multi-residue analysis of veterinary drugs in bovine liver by liquid chromatography with tandem mass spectrometry (LC-MS/MS) using a modified liquid extraction that selectively removed major lipid classes without impacting recoveries (Zhao *et al.*, 2015). Molina-Ruiz *et al.* (2014)

completed a study using a modified QuEChERS method, involving an overnight freezing out step to reduce the amount of matrix co-extractives, in chicken liver samples by gas chromatography-ion trap mass spectrometry (GC-IT/MS).

The objective of this study was to develop a quick, sensitive, and reliable pesticide screening method for liver samples based on QuEChERS methodology using GC-MS analysis. Fifteen representative pesticides were chosen for this study from the following chemical groups: organophosphates (OPs) [methamidophos, dichlorvos, phorate, diazinon, malathion, chlorpyrifos, and coumaphos], organochlorines (OCs) [trans-chlordane, p,p'-DDE, and endrin], pyrethroids (bifenthrin, allethrin, permethrin, and deltamethrin), and a carbamate pesticide (carbofuran). Two drugs, tiletamine and zolazepam, were also included in this study. Three types of liver sources were used in this study with increasing percent lipid content, respectively: bovine (beef), chicken, and caprine (goat). Different injector inlets and modes of operation were also tested on the GC system to determine which inlet and mode provided the best chromatographic separation for analyte detection.

Probability of detection (POD) is a statistical model that was approved by the Association of Official Analytical Chemists (AOAC) Official Methods Board for the use in validation of qualitative methods (Wehling *et al.*, 2011). This concept considers POD as a conditional probability with concentration as a continuous variable for method response. In other words, this probability is viewed as dependent upon the concentration of the analyte in the sample. The POD model offers an approach for graphical representation of response curves for qualitative methods as a POD curve. The POD model was used to study binary qualitative methods of analysis in which the output would be 1 for a positive response and 0 for a negative response. POD is defined as the probability of a method giving a positive result at a given concentration (Wehling

et al., 2011). The graph for the POD curve uses concentration on the x-axis and POD value on the y-axis. POD values range from 0 to 1.00 which is equivalent to 0 to 100%. The estimation method for the POD curve implies that the probability of a positive response is near 1 when the analyte is present and near 0 when the analyte is absent. It is also expected that the probability of a positive response should increase, or approach 1, as the analyte concentration increases.

2. MATERIALS AND METHODS

2.1 Chemicals and Materials

Certified pesticide standards of high purity (>95%) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), U.S. EPA National Pesticide Standard Repository (Fort Meade, MD, USA), and Chem Service (West Chester, PA, USA). The certified pesticide standards were used to prepare stock standard solutions. These stock standard solutions were then diluted to prepare standard mixes of 250 µg/mL in either ethyl acetate or methanol. Tiletamine and zolazepam already had stock standard solutions prepared by another analyst in the laboratory. Tiletamine was diluted to a standard solution of 250 µg/mL in methanol and zolazepam was diluted to 100 µg/mL in methanol. These standard solutions were used as spike mixes (total of 17 compounds) and stored in a refrigerator at 9°C in amber screw-capped glass vials. The compounds were combined into different spike mixes which were grouped by relative detector response. The analytes that were grouped in each spiking mix are specified in Table 1A in appendix A.

Acetonitrile, ethyl acetate, methanol, and formic acid were all of HPLC grade or equivalent and supplied by Fisher Scientific (Fair Lawn, NJ, USA). Eighteen Mega-Ohm water from an Aqua Solutions, Inc. purification system (Georgia, USA) was used throughout the study.

Fifty mL QuEChERS centrifuge tubes prepackaged with 6 g of anhydrous magnesium sulfate ($MgSO_4$) and 1.5 g of sodium acetate (NaOAc) were purchased from United Chemical Technologies (Bristol, PA, USA). Prepackaged 15 mL centrifuge tubes containing 1.0 g of Bond Elut QuEChERS dSPE EMR-Lipid sorbent and prepackaged 15 mL QuEChERS EMR-Lipid polish tubes containing 0.4 g of sodium chloride (NaCl) and 1.6 g of $MgSO_4$ were purchased from Agilent Technologies (Folsom, CA, USA).

2.2 Studied Analytes

Fifteen representative pesticides were chosen for method validation based on factors such as elution time, polarity, volatility, and chemical class. The pesticides involved in this study are a group of OPs, OCs, pyrethroids, and a carbamate insecticide (Table 1). The insecticides from the pyrethroid family as well as the OC pesticides (OCPs) are relatively non-polar, highly lipophilic compounds that tend to remain in fat (Parrilla Vázquez *et al.*, 2016). This presented the challenge in being able to remove the interfering lipids without losing those analytes of interest. Some analytically challenging pesticides were also included, such as methamidophos and dichlorvos. Methamidophos is a polar compound with poor chromatographic characteristics such as early elution and peak tailing. Dichlorvos is poorly retained and has low sensitivity by GC-MS. Carbamates are thermally labile and are typically analyzed by liquid chromatography, so carbofuran was chosen to determine if thermal degradation would be an issue. Deltamethrin, a pyrethroid insecticide, is strongly retained which helped determine analysis time; it is also non-volatile and has lower sensitivity by GC-MS. Tiletamine and zolazepam were included in the residue analysis as anesthetic drugs in addition to pesticides (Table 1).

Table 1. Retention Times (RT) and Target Ions of Analytes of Interest in the Study by Class

RT (min)	Analyte	Pesticide/Drug Class	Ions (m/z)
21.6	permethrin	pyrethroid	163, 183
19.2	bifenthrin	pyrethroid	166, 181
25.0	deltamethrin	pyrethroid	181, 253
14.7	allethrin	pyrethroid	123, 136

16.6	endrin	organochlorine	81, 263
15.1	trans-chlordane	organochlorine	272, 373
16.0	p,p'- DDE	organochlorine	176, 246
11.3	carbofuran	carbamate	149, 164
7.8	methamidophos	organophosphate	47, 94
12.4	malathion	organophosphate	125, 173
11.8	diazinon	organophosphate	137, 179
7.3	dichlorvos	organophosphate	109, 185
13.8	chlorpyrifos	organophosphate	197, 314
21.9	coumaphos	organophosphate	109, 226
10.8	phorate	organophosphate	75, 121
11.0	tiletamine	dissociative anesthetic	166, 195
18.3	zolazepam	benzodiazepine	257, 286

2.3 Equipment

Homogenization of the liver was performed with a Waring commercial blender (model 32BL80). A Mettler Toledo Model PM4600 laboratory balance was used to weigh the liver samples. A Mettler Toledo Model XS204 laboratory balance was used to weigh the solid reagents. SPEX SamplePrep GenoGrinder (model 2010) and Beckman Coulter Avanti J-E centrifuge were used for sample preparation. Fisher Vortex Genie 2 vortex mixer and Heidolph Reax 2 rotary mixer were used to mix the samples. Organomation Associates Inc. N-EVAP Model 112 was used as a temperature-controlled nitrogen gas evaporator for the final extracts.

2.4 GC-MS Analysis

An Agilent 7890B/5977A Series Gas Chromatograph/Mass Selective Detector (Agilent Technologies, CA, USA) equipped with an Agilent 7693 autosampler was used for analysis. Chromatographic separations were carried out using an Agilent J&W GC HP-5MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness). Five μL samples were injected using a multimode injector inlet in solvent vent mode using Agilent's ultra-inert inlet liner (2mm dimpled, splitless). The inlet temperature was programmed as follows: initial temperature, 50°C for 1.2 min, then raised at 400°C/min to 350°C for 32 min. The GC oven temperature program was initially set at 55°C (3 min hold), ramped at 25°C/min to 190°C (1 min hold), and then ramped at 8°C/min to 340°C (6 min hold). The total run time was 34.15 minutes.

The septum purge flow was set at 3 mL/min. The purge flow to split vent was set to 40 mL/min at 2.5 min. Vent was programmed to 45 mL/min at 5 psi until 1 min. Solvent delay of 5.50 min was used to prevent filament damage. Helium (99.999%) was used as the carrier gas at a constant flow rate of 1.0 mL/min. The ion source temperature was 230°C and the electron ionization (EI) energy was 70 eV. The mass spectrometer was tuned and calibrated daily using PFTBA (perfluorotributylamine). All analytes were monitored with acquisition over the mass range of *m/z* 45-650 (scan mode). Data acquisition and processing were performed using the Enhanced Data Analysis Software Chemstation™ (Agilent Technologies) and an automated mass spectral deconvolution and identification system (AMDIS™). Selected ion monitoring (SIM) mode was also helpful in finding compounds that were not easily identifiable in scan mode. Identifications were verified based on retention times as well as the presence and ratios of two fragment ions. The National Institute of Standards and Technology (NIST) mass spectral

database (NIST 11) was used concurrently with a Wiley/NIST combined mass spectral library (W10N11) for reference data for analyte identification.

2.5 Spiking Procedure

All liver samples were obtained from a local supermarket (Sacramento, CA) and homogenized in a blender. The samples were stored in a freezer at -12°C until use. A prior GC-MS and LC-MS analysis of the store-bought samples was performed using existing lab techniques to ensure that they did not contain any analytes of interest. These samples were used as matrix blanks and for fortification. For validation studies, the samples were fortified with the spiking solutions (Table 1A) at the desired concentration of the compound in the homogenate. The spiking concentrations levels were 1, 2.5, and 5 µg/g.

2.6 Clean-up Sorbents

Each clean-up sorbent was tested individually as well as in different combinations by GC-MS analysis: (a) dSPE with PSA, (b) PSA "pass through" SPE cartridge, (c) dSPE with C_{18}, (d) dSPE with PSA + C_{18}, (e) Florisil SPE cartridge, (f) Z-Sep SPE cartridge, (g) dSPE with EMR-Lipid, (h) dSPE with PSA followed by EMR-Lipid, and (i) dSPE with PSA + C_{18} followed by EMR-Lipid. Cold-induced phase separation was also tested on PSA "pass through", EMR-Lipid, and PSA followed by EMR-Lipid.

The extraction and clean-up methods were evaluated for lipid removal and target analyte recoveries. Lipid removal was measured by comparing the chromatographic peak areas and heights of 2 fatty acids that were chosen as markers using target ions in the SIM mode: hexadecanoic acid (256 m/z) and octadecadienoic acid (280 m/z). Analyte loss was evaluated by

comparing the number of analytes that were detected on the GC-MS system at the highest fortification level (5 µg/g).

2.7 Sample Preparation Procedure

A 1.0 ± 0.02 g aliquot of liver was weighed and placed in a 50 mL polypropylene centrifuge tube (Corning Science Mexico S.A. de C.V.). Five mL of deionized water and 1-2 grinding balls were added to the tube. The tube was shaken in the GenoGrinder (750 rpm) for 5 min. After the sample was taken off the GenoGrinder, a magnet was used to remove the grinding balls from the tube. Fifteen mL of 0.1% formic acid in acetonitrile was added and the sample was shaken in the GenoGrinder again (750 rpm) for 5 min. The sample was then centrifuged (2500 rpm and 10°C) for 5 min. The supernatant was decanted into a 50mL QuEChERS extraction tube and shaken well by hand before placing it in the centrifuge (2500 rpm and 10°C) for 5 min. Four mL of deionized water was added to the 15 mL centrifuge tube containing the EMR-Lipid sorbent to "activate" the sorbent. Four mL of the extract was transferred into the EMR-Lipid tube and vortexed immediately. The tube was placed on a tube rotator (~35% speed) for 10 min and then centrifuged (2500 rpm and 10°C) for 5 min. The sample was then decanted into the 15 mL EMR-Lipid polish tube and vortexed immediately. The tube was rotated for 10 min and then centrifuged (2500 rpm and 10°C) for 5 min. Only the upper, acetonitrile phase was transferred into a 15 mL glass tube using a Pasteur pipet. The extracts were evaporated to dryness under a stream of N_2 (45°C) and reconstituted by adding 200 µL of ethyl acetate to the tube. The sample was vortexed then pipetted into an autosampler vial equipped with a glass insert. After the vial was capped, the sample was ready for analysis on the GC-MS system.

2.8 Method Validation

The method validation was performed in accordance with the guidelines for the validation of chemical methods for the Food and Drug Administration (FDA) Foods and Veterinary Medicine (FVM) program, 3rd edition (2019). The guidelines for the program describes four method validation levels that can be followed depending on the method's intended purposes. Level one has the lowest level of validation requirements with methods designed for emergency/limited use. Level three is multi-laboratory validation level in which at least two laboratories must be employed, and level four is a full collaborative study. Level two is a single laboratory validation level with methods intended for routine regulatory testing, minor method modifications, as well as analyte and matrix extensions of screening methods. Level two was the standard level of performance chosen as the most appropriate procedure to follow for the validation study.

These key validation parameter requirements for chemical methods were followed: 3 sources of liver matrix, 3 analyte spike levels, and at least 3 replicates per matrix source at each level tested. Three sources of liver were used for replicate analyses: bovine (beef), chicken, and caprine (goat). Each liver source was analyzed in duplicate at ~1/2X, X, and 2X fortification levels (1, 2.5, and 5 μg/g) as suggested by the FDA FVM guidelines. Possible analyte carryover and contamination were evaluated using blank matrix samples. Reagent blanks were used to help confirm that the reagents were analyte free. The performance of the method was evaluated in terms of sensitivity, specificity, false positive rate, false negative rate, minimum detectable concentration, ruggedness, and confirmation of identity.

2.9 Percent Lipid Determination

All three sources of liver underwent a percent lipid determination procedure to compare the amount of fat present. Percent lipid is calculated based on sample weight. Two replicates from each source of liver were tested and the average percent was taken. Since fresh liver is a wet matrix, the samples had to initially be dried in an oven overnight to determine percent moisture content from its wet and dry weight. A solvent mixture of petroleum ether: isopropanol (3:2) was added to the centrifuge tubes containing the dried liver samples, shaken by hand, and then the liquid portion was transferred to a separate tube that was used for evaporation using the N-EVAP; these steps were repeated three times before the final drying process in the oven to obtain the samples' final dry weight.

Bovine liver had the lowest concentration of fat present with an average of 9.11%. Chicken liver had an average percent lipid of 13.59%. Caprine liver had the highest concentration of fat present with an average of 15.04%. The percent lipid values can be found in Table 3A in appendix A. Chocolate, the reference material with sample ID listed as NIST 2384, was used as quality control to determine percent recovery. Percent recovery was 105% indicating that the average percent lipid values calculated could be slightly higher than the true values.

2.10 Inlets and Modes of Operation

Various injector inlets and modes of operation were assessed on the gas chromatography system: (a) splitless inlet/ splitless mode, (b) splitless inlet/ pulsed splitless mode, (c) programmable temperature vaporization (PTV) inlet/ splitless mode, (d) PTV inlet/ solvent vent mode, and (e) multimode inlet (MMI)/ solvent vent mode. One µL injections were tested on splitless mode and 5 µL injections were tested on solvent vent mode to compare data. Higher volume injections can help with analyte detection due to more sample being introduced into the

GC system, but issues such as column overloading and detector saturation can occur. The mass spectral data was used to determine which inlet and mode had the greatest analyte recovery with the least matrix effects at the lowest fortification level (1 μg/g).

2.11 Probability of Detection Statistical Model

In this study, GPC was the reference method for comparison with the candidate method of QuEChERS extraction with EMR-Lipid clean-up. Chicken liver was used as the representative source of the matrix to compare the candidate method with the reference method because it had an average percent lipid in between the other two sources of liver. The PODs for these methods were compared by the difference in POD values (dPOD) at a given concentration. If the confidence interval (CI) on dPOD includes zero, then the difference between the methods is not statistically significant (Wehling *et al.*, 2011). The CIs were calculated for a binomial probability using the Clopper-Pearson "exact" method at 95% confidence.

3. RESULTS

3.1 Method Selection

The QuEChERS methodology with various clean-up procedures was studied on post-mortem liver samples to find a method that was as effective as GPC in removing fats while retaining toxicants of interest. Sixteen out of the 17 analytes were detected after GPC clean-up at 5 µg/g. The EMR-Lipid dSPE clean-up sorbent and polishing step provided the greatest lipid removal and analyte recovery in comparison to the other clean-up methods. The Florisil SPE clean-up had the highest amount of fat still present in the final extract. The SPE with Z-Sep and dSPE with PSA, C_{18}, PSA + C_{18}, PSA "pass through", and PSA followed by EMR-Lipid clean-up displayed insufficient lipid removal with significant matrix peaks that masked analytes of interest. The total ion chromatogram (TIC) of the liver samples that underwent clean-up using EMR-Lipid dSPE, PSA "pass through" cSPE, and Z-Sep dSPE are overlayed in Figure 1. The Florisil, PSA, C_{18}, and PSA + C_{18} clean-up sorbents had similar chromatograms to Z-Sep. The CIPS [PSA "pass through"], CIPS [EMR-Lipid], and CIPS [PSA followed by EMR-Lipid] clean-up did a better job at removing fat than those previously listed, but each technique resulted in the loss of 2 analytes. PSA + C_{18} followed by EMR-Lipid clean-up was able to detect all analytes except 1.

The dSPE with EMR-Lipid clean-up sorbent demonstrated the most complete lipid removal, with the smallest peak areas and heights of the 2 fatty acid markers, while retaining all 17 target analytes at 5 µg/g. This indicates even better detection than GPC clean-up at 5 µg/g. Agilent suggests a 1:1 ratio of water/extract for the sorbent's water "activation strength" (Stevens, 2015). Various amounts, between 3 to 6 mL, were tested and 4 mL of water/extract exhibited optimal matrix removal and analyte recovery. As a result, 4 mL was the amount of

water used to “activate” the sorbent and 4 mL of the extract was transferred into the tube containing the EMR-Lipid.

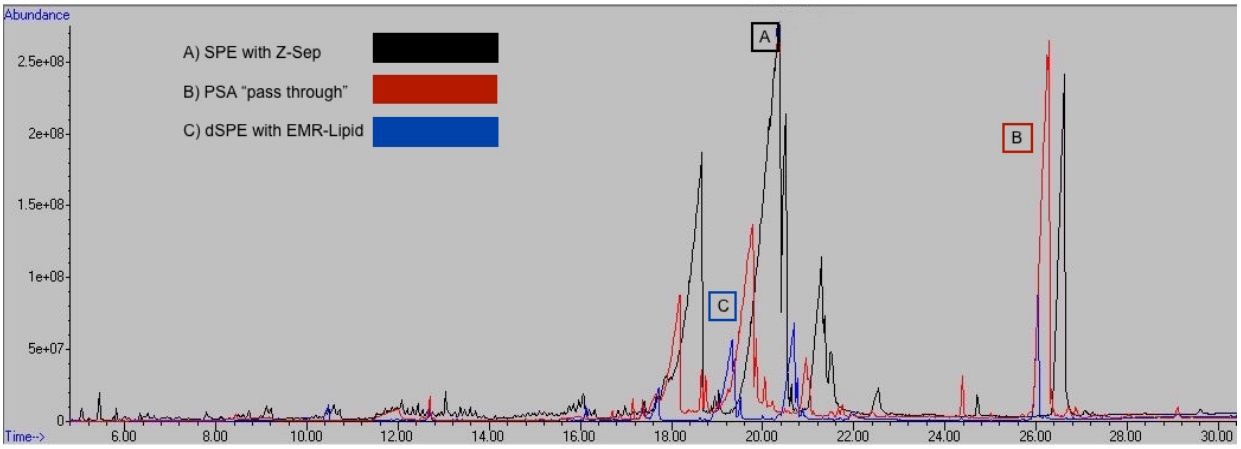

Figure 1. GC-MS full-scan chromatogram of liver extracts using QuEChERS methodology followed by [A] SPE with Z-Sep, [B] PSA “pass through”, [C] dSPE with EMR-Lipid
*Refer to figure 1B in appendix B for this chromatogram with a lighter color background

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3.2 Inlet and Mode of Operation Selection

For the samples that underwent EMR-Lipid clean-up, we only detected 14 out of the 17 analytes using splitless and pulsed splitless mode at 1 µg/g, whereas 15-16 out of the 17 analytes were detected after GPC clean-up using 1 µL injections. Better analyte detection for EMR-Lipid was achieved on solvent vent mode with larger volume injections (5 µL). Sixteen out of the 17 compounds, were detected at 1 µg/g using the PTV and MMI inlet on solvent vent mode. Both inlets worked well for this research due to the similarity in results, but validation experiments were carried out using 5 µL injections with the MMI inlet and solvent vent mode.

3.3 POD- Comparing Method Responses

Figure 2 suggests that the candidate method had better detection than the reference method at 2.5 and 5 µg/g with higher POD values. Although, the 95% confidence interval (CI)

on dPOD includes zero at 2.5 µg/g (-0.06, 0.06), so the difference is not statistically significant. The 95% CI on dPOD was (0.02, 0.06) at 5 µg/g which does not include zero, thus it was determined that the difference between the methods being compared is significant at 5 µg/g but not at lower concentrations. The data that was used to calculate the PODs and the CIs for the two methods can be found in Table 2A.

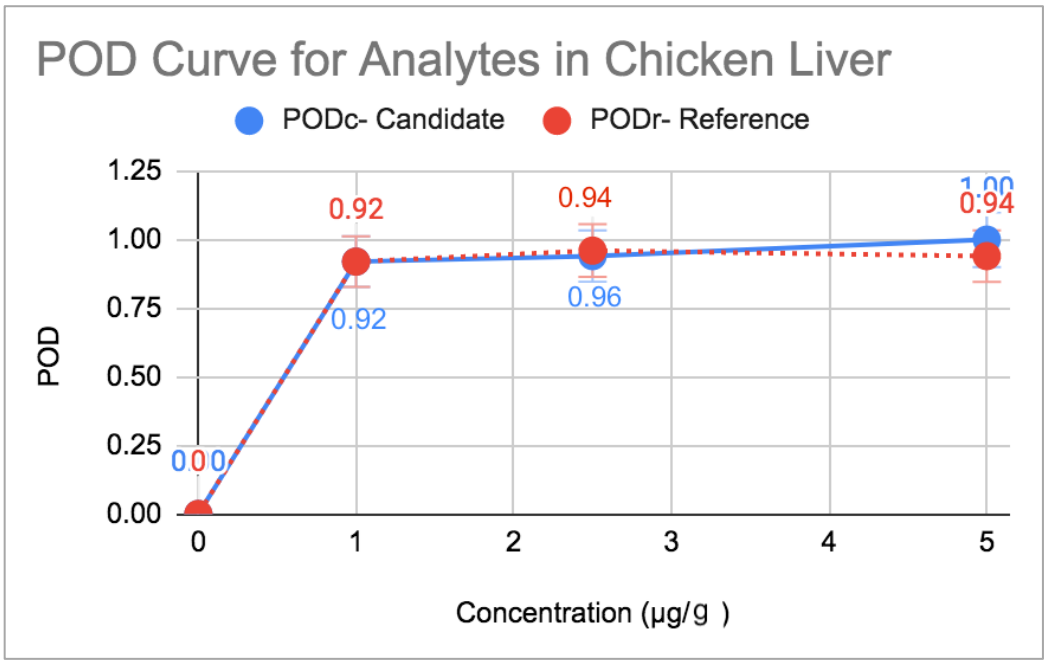


Figure 2. POD response curve for the candidate method (QuEChERS extraction with EMR-Lipid clean-up) and the reference method (GPC clean-up).

3.4 Method Validation

The POD model incorporates sensitivity, specificity, false positive, and false negative rates into a single parameter, POD (Wehling *et al.*, 2011). Its graphical representation can be seen in Figure 3. Matrix blanks were analyzed after standards and samples; analyte carryover was not observed. Method validation was also assessed on the following performance characteristics: minimum detectable concentration, ruggedness, and confirmation of identity.

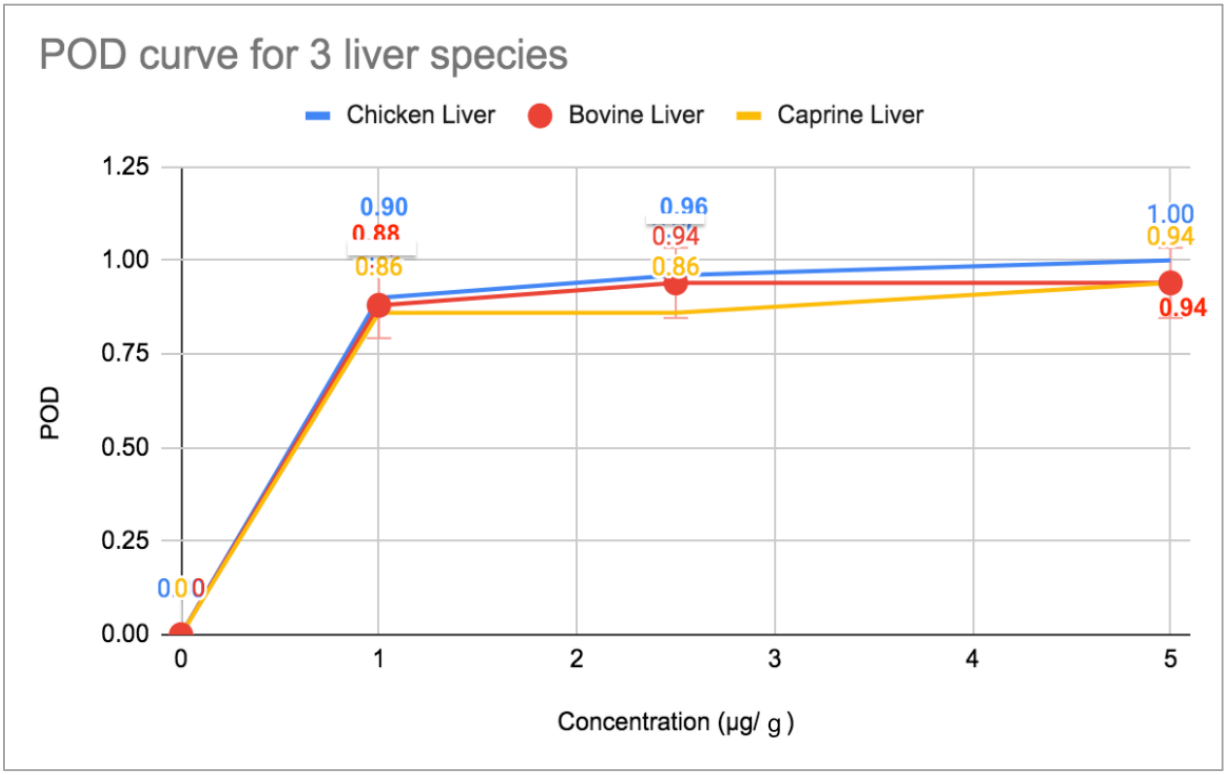


Figure 3. POD curve for comparison of detection of analytes spiked in replicate liver samples for three sources of liver.

Sensitivity

Sensitivity, the probability of a positive response at a given concentration, is equivalent to POD(c) which is the value of the POD curve at any given concentration (Table 2). The POD curve showed good sensitivity in all liver sources with POD values > 0.85 at every concentration. Chicken liver exhibited the greatest sensitivity with having the highest POD at every concentration in comparison to bovine and caprine liver. Sensitivity generally increased with higher concentration.

Table 2. Single-laboratory data for POD for liver samples fortified at 3 different spike mixture concentrations.

Concentration (µg/g)	Bovine Liver (POD)	Chicken Liver (POD)	Caprine Liver (POD)
0	0	0	0
1	0.88	0.90	0.86
2.5	0.94	0.96	0.86
5	0.94	1.00	0.94

Specificity

Specificity, the probability of the method giving a negative response when the sample is truly without analyte, is equivalent to 1-POD(0) which is the distance along the POD y-axis from POD = 1 to the POD curve value. The POD(0) = 0 for all sources of liver so the specificity of the method is equal to 1.00 or 100% at all concentration levels tested (Table 2).

False positive

False positive, the probability of the method giving a positive response when the sample is truly without analyte, is equivalent to POD(0) which is the value of the POD curve when the concentration equals zero. Since the POD(0) = 0 for all sources of liver, the false positive rate is 0% at all concentration levels. At least 59 samples were tested over the course of the study, as suggested by FDA guidelines, to state that with 95% confidence that the false positive rate is < 5%.

False negative

False negative, the probability of a negative response when an analyte is present at a given concentration, is equivalent to 1-POD(c) which is the distance from the POD curve to POD = 1 on the y-axis. The false negative rates vary between 0 to 0.14 from the highest concentration and the lowest concentration fortified, respectively (Table 3). Chicken liver exhibited the lowest false negative rates with 0% at 5 µg/g. Caprine liver demonstrated the highest false negative rates at 0.14 or 14% at both 2.5 and 1 µg/g.

Table 3. Values for false negative rates using data from Table 2.

Concentration (µg/g)	**Bovine Liver (1-POD(c))**	**Chicken Liver (1-POD(c))**	**Caprine Liver (1-POD(c))**
1	0.12	0.10	0.14
2.5	0.06	0.04	0.14
5	0.06	0	0.06

Minimum detectable concentration

The lowest concentration evaluated was 1 µg/g. Refer to Table 1 for the full list of analytes and drugs that were spiked into the samples. For bovine liver, all analytes except dichlorvos and malathion could be detected at 1 µg/g. All analytes except dichlorvos, malathion, and zolazepam could be detected at 1 µg/g for chicken liver. For caprine liver, all analytes except methamidophos, dichlorvos, and malathion could be detected at 1 µg/g.

Ruggedness

Over the course of 16 weeks, more than 100 liver samples were analyzed with routine GC/MS maintenance. No significant loss in sensitivity or specificity was observed during this time. The method did not require any unusual instrument maintenance even though large volume injections (5 µL) were used. Replicate samples were analyzed using liver from 3 animal species. The method worked well on different days as well as with different analysts and instruments. Multiple lots of reagents, materials, and standards were used in this study. Reproducible and reliable results were observed during normal usage under a variety of conditions, thus indicating the method's ruggedness.

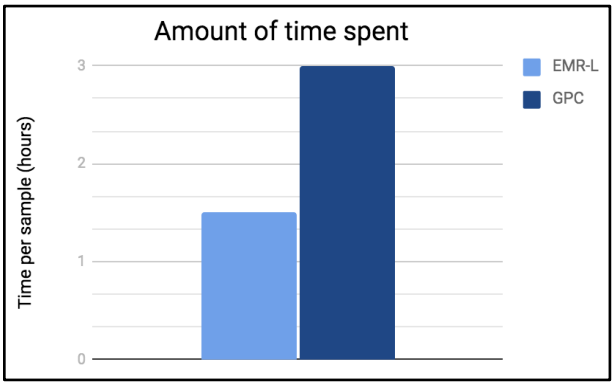
Confirmation of identity

The analytes were identified according to retention times and qualifier ions, along with comparing mass spectra from the reference libraries on Chemstation. The SIM acquisition method was used to find the target ions of the analytes. The target ions (m/z) that were monitored for each analyte can be found in Table 1. In addition, AMDIS was used for manual evaluation in which mass spectral fragmentation patterns from a library of approximately 1800 spectra were searched for in the sample. Analytes were fortified into the samples for the validation study, so the identity of the pesticides and drugs were also already known.

4. DISCUSSION AND CONCLUSION

4.1 Comparing Proposed Method with GPC

QuEChERS extraction followed by EMR-Lipid clean-up has several advantages over the reference method (GPC) such as saving time, using less solvent, avoiding hazardous chemicals, and decreasing the cost per sample. Sample preparation time was cut in half when using the EMR-Lipid method (Figure 4). This allows for better productivity as the number of samples that can be processed within a given amount of time is also significantly increased. The EMR-Lipid approach is a simple way to reduce solvent usage as it uses 93% less solvent per sample than the reference method (Figure 5). Furthermore, the EMR-Lipid procedure does not require specialized equipment or any carcinogens unlike GPC.



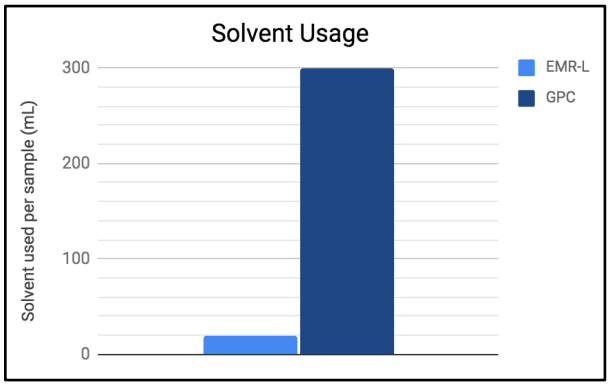


Figure 4 (left). Extraction/ clean-up time was decreased by 50% using the EMR-Lipid method
Figure 5 (right). The EMR-Lipid method uses 93% less solvent per sample

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The proposed method calls for a sample size of 1.0 g, whereas 2.5 g is recommended when preparing samples for GPC clean-up for optimal analyte detection. The need for smaller sample sizes is useful when small samples are submitted to the laboratory. Two µL injections were used on solvent vent mode after GPC clean-up since it required 2.5x more sample size than EMR-Lipid clean-up. Liver samples that underwent GPC clean-up had a large peak of glycerol, a

compound derived from animal fats, present during the early run which could mask the presence of other early-eluting chemicals. In addition, there was a very strong response for cholesterol, a type of lipid interference. Glycerol and cholesterol were both reduced along with other matrix interferences when the EMR-Lipid sorbent was used (Figure 6).

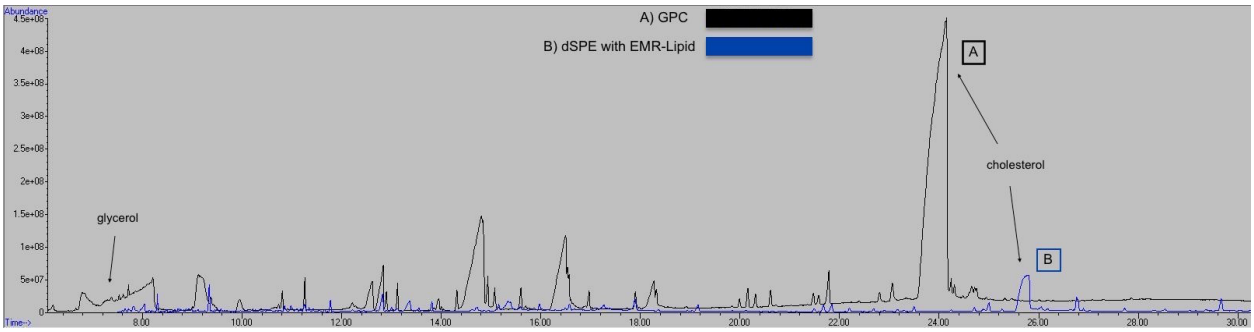

Figure 6. GC-MS full-scan chromatogram of liver extracts using [A] GPC clean-up and QuEChERS methodology followed by [B] dSPE with EMR-Lipid
*Refer to figure 2B in appendix B for this chromatogram with a lighter color background

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4.2 Comparison of Results Between Sources of Livers

The analytes that were not detected after EMR-Lipid clean-up in 3 replicate samples for each liver source at each fortification level are listed in Table 4. Caprine liver had the least number of detected analytes compared to the other two liver sources, which was expected due to its higher percent lipid content. The most analytes were detected in the chicken liver even though bovine liver had a lower percent lipid. The difference is that malathion was detected in the chicken liver samples. This could have been due to front-end maintenance, which included trimming the column, that was performed on the GC-MS instrument before the chicken liver sample extracts were analyzed. Malathion could not be found in any of the other liver samples because its targets ions were buried underneath matrix peaks. Dichlorvos was also problematic as a result of not having a strong enough signal for detection. Zolazepam was not detectable in the

chicken liver at 1 µg/g because it co-eluted with octadecanamide, a metabolite of octadecanoic acid.

Table 4. Analytes not detected after EMR-Lipid clean-up in replicate samples

Concentration (µg/g)	Bovine Liver	Chicken Liver	Caprine Liver
1	-dichlorvos ND* in 3/3 samples -malathion ND* in 3/3 samples	-dichlorvos ND* in 2/3 samples -malathion ND* in 2/3 samples -zolazepam ND* in 1/3 samples	-dichlorvos ND* in 3/3 samples -malathion ND* in 3/3 samples -methamidophos ND* in 1/3 samples
2.5	-malathion ND* in 3/3 samples	-dichlorvos ND* in 1/3 samples	-dichlorvos ND* in 3/3 samples -malathion ND* in 3/3 samples -methamidophos ND* in 1/3 samples
5	-malathion ND* in 3/3 samples	all analytes detected	-malathion ND* in 3/3 samples

*ND, not detected

4.3 Solvent Vent Mode

One µL injections were used on splitless mode and 5 µL injections were used on solvent vent mode and the difference in analyte response was examined on the MMI inlet; a 5x increased signal was observed with the higher injection volume without signs of overloading. Solvent vent mode is programmed to handle large volume injections which is useful in detecting target analytes that could not be found in splitless or pulsed splitless mode. Splitless mode was programmed to start with an initial temperature hot enough to quickly vaporize injected samples. Conversely, the samples were injected into a cold inlet when solvent vent mode was used and ramped quickly at a high temperature.

Solvent vent mode on the MMI inlet was able to boost sensitivity on the GC-MS system while minimizing matrix effects. Solvent vent mode helped reduce the lipid interference from the hexadecanoic acid (palmitic acid) and octadecanoic acid (stearic acid) in the liver samples as shown in Figure 7. Five (max volume) solvent washes of ethyl acetate were done pre-injection and post-injection to reduce potential sample carryover from large volume injections.

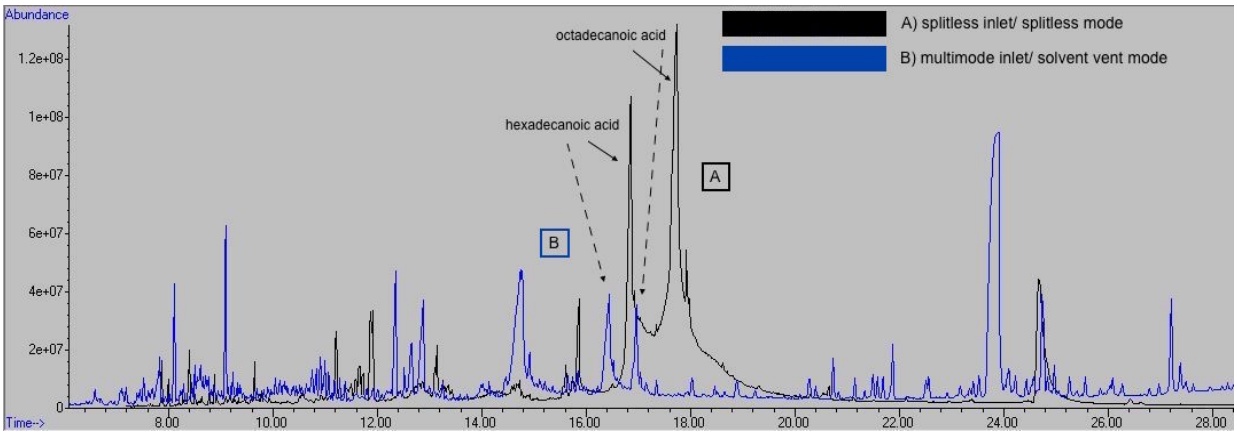

Figure 7. GC-MS full-scan chromatogram of liver extracts using QuEChERS methodology followed by dSPE with EMR-Lipid
*Refer to figure 3B in appendix B for this chromatogram with a lighter color background

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4.4 Application

The proposed extraction and clean-up method was tested on diagnostic liver samples from other species in addition to those used in the validation study. These included cougar, bear, canine, bison, and avian samples that contained toxicants not limited to those in the validation study (Table 1). These samples also underwent GPC clean-up to compare analyte detection. Dieldrin and heptachlor epoxide are two analytes that were not detected in avian liver after EMR-Lipid clean-up which could have been due to low analyte responses. Although, most of the analytes that were detected after GPC clean-up were also found in the extracts that underwent EMR-Lipid clean-up. The proposed method even detected a few analytes, such as strychnine and

acephate, that were not found when GPC clean-up was used. The detection of strychnine, a highly toxic substance, is significant because it is a compound not normally detectable by GC-MS analysis after GPC clean-up; this is most likely due to solubility issues. Strychnine is a natural substance primarily used as a pesticide, but there are some uncommon cases in which it has been found mixed with illicit drugs such as cocaine and heroin (Patocka, 2015). A list of the other compounds detected in these samples after the GC-MS analysis are listed in Table 4. These favorable results confirm that the method will work across species and for other analytes.

Table 4. Other analytes detected using EMR-Lipid clean-up in addition to the 17 spiked compounds listed in Table 1

Analyte
acephate
strychnine
chlordane
nonachlor
Cl(4-10) PCBs
phenytoin
pentobarbital
diazepam
midazolam
nordazepam
sulfamethazine
xylazine

4.5 Conclusion

A cheaper, faster, more efficient and environmentally-friendly extraction/clean-up method for liver samples was developed. The QuEChERS extraction followed by EMR-Lipid dSPE and polishing salts for the clean-up of liver samples led to the validation of a non-targeted GC-MS method using solvent vent injection. This method was optimized for high sensitivity with accurate, reliable, and consistent data. In addition to efficient clean-up and analyte recovery, all the validated parameters and diagnostic samples confirm that the method is suitable for the detection of a broad range of toxicants in the matrix of liver.

The proposed extraction and clean-up procedure would shorten the turnaround process by a day as well as significantly reduce waste from solvent usage. This would increase the number of samples that can be processed while minimizing labor and material costs along with the use of toxic solvents. These advantages make this an effective method of choice for non-targeted analysis in liver samples by GC-MS. This method could especially be beneficial in forensic laboratories by saving time and possibly reducing case backlogs. To our knowledge, this is the first study on the application of the QuEChERS methodology followed by EMR-Lipid in liver samples for GC-MS analyses. A possible next step is a multi-laboratory validation so that this method could be extended for widespread use. This method might be applicable to other high-fat samples, but further study and validation would be required.

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APPENDIX A

Table 1A. List of the analytes that were grouped into each spike mix and the concentrations of the spiking solutions used to fortify the samples.

Analyte(s)	Concentration
permethrin phorate chlorpyrifos endrin malathion diazinon	250 µg/mL in ethyl acetate
methamidophos deltamethrin	250 µg/mL in ethyl acetate
dichlorvos coumaphos	250 µg/mL in ethyl acetate
allethrin trans-chlordane tiletamine	250 µg/mL in methanol
bifenthrin carbofuran	250 µg/mL in methanol
p,p'- DDE	250 µg/mL in ethyl acetate
zolazepam	100 µg/mL in methanol

Table 2A. Combined single-laboratory data for detection of pesticides spiked in replicate chicken liver samples.

		Candidate Method			Reference Method				
Concentration(µg/ml)	N	x	POD	95% CI	x	POD	95% CI	dPOD	95% CI
0	85	0	0.00	(0.00, 0.04)	0	0	(0.00, 0.04)	0.00	(-0.04, 0.04)
1	85	78	0.92	(0.84, 0.97)	78	0.92	(0.84, 0.97)	0.00	(-0.09, 0.09)
2.5	85	83	0.94	(0.92, 1.00)	82	0.96	(0.90, 0.99)	-0.02	(-0.06, 0.06)
5	85	85	1.00	(0.96, 1.00)	80	0.94	(0.87, 0.98)	0.06	(0.02, 0.06)

N = (number of pesticides spiked) x (number of replicates) = 17 x 5
x = total number of positive responses
POD = x / N

Table 3A. The percent lipid values of 2 replicate liver samples from the 3 sources of liver used in the validation study.

Sample ID	Matrix	Tube (g)	Before (g)	After (g)	Evap. Tube (g)	Evap. Tube + lipid (g)	%Moisture	Sample Wet weight(g)	Sample Dry Weight (g)	%Lipid	% Recovery	Average of replicates
NIST 2384	Chocolate				10.345	10.5277			0.34	**53.74%**	105%	
Chicken (1)	Liver	10.49	12.54	10.96	10.34	10.4073	77.07%	2.05	0.47	**14.32%**		13.59%
Chicken (2)	Liver	10.49	12.52	10.97	10.5	10.5617	76.35%	2.03	0.48	**12.85%**		
Bovine (1)	Liver	10.43	12.53	11.04	10.51	10.5500	70.95%	2.10	0.61	**6.56%**		9.11%
Bovine (2)	Liver	10.48	12.53	11.08	10.51	10.58	70.73%	2.05	0.60	**11.67%**		
Goat (1)	Liver	10.42	12.66	11.15	10.5	10.6200	67.41%	2.24	0.73	**16.44%**		15.04%
Goat (2)	Liver	10.48	12.59	11.14	10.49	10.58	68.72%	2.11	0.66	**13.64%**		

APPENDIX B

*These chromatograms were added with a lighter background for easier viewing if printing in black and white.

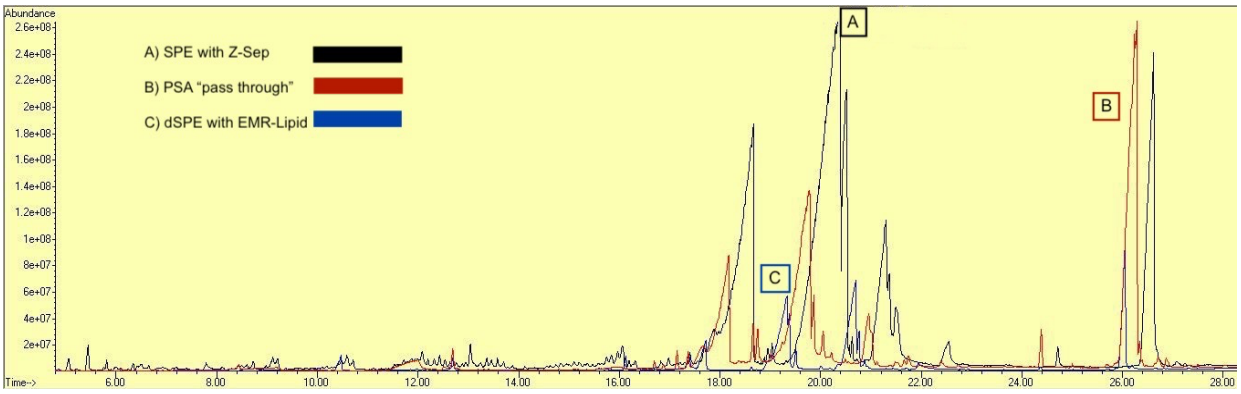

Figure 1B. GC-MS full-scan chromatogram of liver extracts using QuEChERS methodology followed by [A] SPE with Z-Sep, [B] PSA "pass through", [C] dSPE with EMR-Lipid

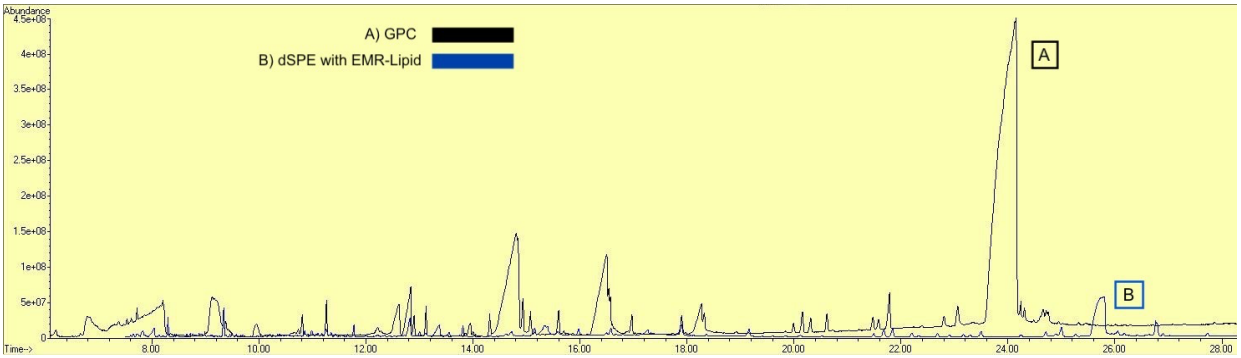

Figure 2B. GC-MS full-scan chromatogram of liver extracts using [A] GPC clean-up and QuEChERS methodology followed by [B] dSPE with EMR-Lipid

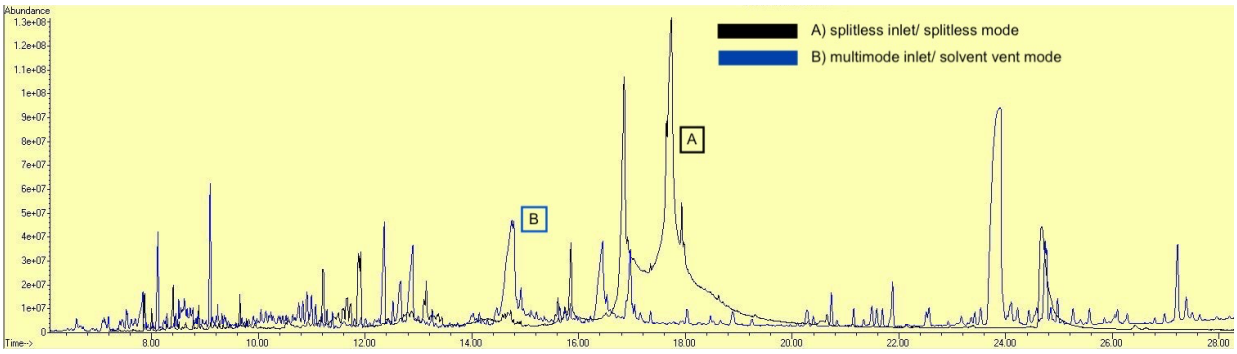

Figure 3B. GC-MS full-scan chromatogram of liver extracts using QuEChERS methodology followed by dSPE with EMR-Lipid