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Determination of Organochlorine Pesticides in Wildlife Liver and Serum Using Gas Chromatography Tandem Quadrupole Mass Spectrometry

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Abstract

A single laboratory validation of a quantitative capillary gas chromatography tandem quadrupole mass spectrometer (GC-MS/MS) method utilizing a Quick, Easy, Cheap, Effective, Rugged, Safe (QuEChERS) approach for the extraction of 24 organochlorine analytes in liver and blood serum is presented here. The QuEChERS approach utilizes an acetonitrile extraction, partitioning facilitated by the addition of salts and a dispersive solid phase extraction cleanup. This method simultaneously monitored 24 organochlorine pesticide residues representing four different classes, including chlordecone, cyclodienes, dichlorodiphenylethanes, and hexachlorocyclohexanes. Calculated limits of detection (LOD) varied from 0.002 to 2.4 ppb and limits of quantification (LOQ) varied from 0.01 to 7.4 ppb. This multi-residue method proved to be a sensitive approach to the measurement of persistent organic pollutants in biological matrices.

Keywords: QuEChERS; Organochlorine pesticides; Veterinary toxicology; GC-MS/MS

Introduction

Organochlorine pesticides (OCPs) were widely used from the 1940's through 1970's and some are still used in developing countries today [1]. OCPs were highly effective in agricultural applications, improved crop production and enhanced public health through the reduction of diseases spread by insects [2]. However, OCPs are chemically stable, have high lipid solubility, low volatility and low rates of degradation leading to their classification as persistent organic pollutants (POPs). Organisms at higher trophic levels exhibit greater body burdens because OCPs bioconcentrate and biomagnify in the food chain [2]. When present in high concentrations, OCPs can produce a variety of toxic effects dependent on pesticide class [2]. Accordingly, nations observing the Stockholm Convention have eliminated or restricted the use and production of 12 POPs of high priority to protect the environment and human health [2]. Thus, it is imperative that veterinary diagnostic laboratories have the capability to identify and quantify low level traces of these compounds in animals that may be consumed by people, as well as in wildlife, particularly those animals with diets that lead to extensive accumulation such as certain avian species.

Traditional extraction methods for pesticide analysis in biological samples often use large quantities of solvent, are time consuming and may use expensive equipment to aid in the extraction process. Common pesticide extraction methods for a variety of matrices have been the subject of several reviews [3-7]. The QuEChERS extraction method was developed in 2003 by Anastassiades et al. in an effort to minimize time and analytical cost associated with multiresidue pesticide analysis in produce [8]. This approach to analyte extraction was a significant advancement over traditional multi-residue methods in that it utilized significantly less solvent and sample preparation time, making it a more economical approach for diagnostic labs to adopt. In addition to the economic advantages, the QuEChERS approach does not use harsh extraction conditions therefore making it is less likely to cause thermal breakdown of compounds in contrast to pressurized liquid extraction (PLE), supercritical fluid extraction (SFE) or microwave-assisted extraction (MAE) [7]. Additionally, the cleanup of samples prior to analysis by gas chromatography (GC) or liquid chromatography (LC) systems is performed by a dispersive solid phase extraction (dSPE) step. The dSPE step uses sorbents such as PSA and C18 to remove interfering matrix components and this step is faster and has less chance of carryover compared to gel permeation chromatography (GPC), silica gel or solid phase extraction (SPE) cleanup methods [8].

Since the sentinel publication of this method in 2003, QuEChERS has been further developed, modified and adapted to many laboratory panels. The method was developed and utilized extensively by agricultural laboratories for pesticide residue testing of fruits and vegetables and environmental samples. The QuEChERS method has been the subject of recent review of pesticide analysis in food [9] and in environmental samples [10]. The extensive use of the method has led to commercial QuEChERS extraction kits from several vendors and an official AOAC method for analysis of pesticide residues in food [11]. Although the method has been widely used for pesticide analysis in a variety of food types, there have been few extensions to analysis of chemical residues in animal tissues [12,13]. The QuEChERS sample preparation method has been reported for the detection of pharmaceuticals in animal liver [14,15] and human whole blood [16]. To the best of the authors' knowledge, the method has not been applied to pesticide quantification in wildlife samples. With slight modifications to the extraction procedure, the method was readily adapted to pesticide analysis in biological samples of interest for veterinary diagnostic laboratories.

Analysis by gas chromatography tandem mass quadrupole spectrometry greatly enhances selectivity and sensitivity in comparison to single quadrupole techniques. Monitoring multiple precursor and

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product ions per analyte along with retention time provides greater selectivity nearly eliminating false positive results. With organic pesticides, the use of multiple reaction monitoring (MRM) with the tandem mass spectrometer provides sensitivity to at least the same order of magnitude as electron capture detection [17]. Previous reports have shown the validation of GC-MS/MS analysis of OCPs in biological samples [18,19].

The present work describes the development and single-laboratory validation of a multi-residue method for the detection of organochlorine pesticides in biological matrices using a QuEChERS based extraction approach and GC-MS/MS targeted analysis by MRM. This method allowed for the simultaneous monitoring of 24 organochlorine pesticide residues in liver and blood serum.

Materials and Methods

Pesticide standards

CLP organochlorine pesticide mix (alpha-chlordane, methoxychlor, gamma-chlordane, aldrin, alpha-BHC [benzene hexachloride or hexachlorocyclohexane], beta-BHC, gamma-BHC, delta-BHC, 4,4'-DDD [Dichlorodiphenyldichloroethane], 4,4'-DDE [Dichlorodiphenyldichloroethylene], 4,4'-DDT [dichlorodiphenyltrichloroethane], dieldrin, endosulfan I [alpha], endosulfan II [beta], endosulfan sulfate, endrin, endrin aldehyde, endrin ketone, heptachlor, heptachlor exo-epoxide isomer B) 2000 µg/mL in 50:50 toluene/hexane and trans-Nonachlor 100 µg/mL in hexane were obtained from Supelco Analytical (Sigma-Aldrich Corp. St. Louis, MO, USA); oxychlordane, chromatography purity, and decachlorobiphenyl (internal standard), 99.5% purity were purchased from Cerilliant Corporation (Round Rock, TX) and ChemService (West Chester, PA), respectively.

Solvents and reagents

Acetonitrile UV (chromatography grade) and Isooctane (chromatography grade) were purchased from Honeywell, Burdick and Jackson brand (Muskegon, MI). Glacial acetic acid was purchased from EMD Millipore USA (Billerica, MA). Anhydrous magnesium sulfate (Baker analyzed reagent) and sodium chloride (ACS reagent grade) were purchased from JT Baker (Phillipsburg, NJ). Bondesil-PSA, 40 µm was purchased from Varian (Division of Agilent, Palo Alto, CA). C18, octadecyl-functionalized silica gel, 200-400 mesh was purchased from Aldrich (Sigma-Aldrich Corp. St. Louis, MO, USA). Sodium acetate trihydrate (HPLC grade) was purchased from Fisher Scientific (Waltham, MA). 200-proof ethyl alcohol was purchased from Decon Labs (King of Prussia, PA). Solutions of 1% (v/v) acetic acid in acetonitrile UV and 80:20 isooctane:ethanol were prepared prior to extraction procedures.

Livers and serum

Control livers were obtained from bovine necropsy submissions to the veterinary diagnostic center and pooled control blood in ethylenediaminetetraacetic acid (EDTA) was obtained from canine clinical chemistry panel (CBC) submissions from the veterinary teaching hospital. Livers from bald eagles were obtained through submissions of birds found dead or moribund by the Michigan Department of Natural Resources (Lansing, MI).

Equipment

An Agilent Technologies 7890A GC system coupled with an Agilent 7000 GC/MS Triple Quad detector (Agilent, Santa Clara, CA) was used to perform the GC-MS/MS analysis. The GC separation was conducted with an Agilent 19091S-433UI fused silica capillary column (30 m × 250 µm × 0.25 µm) and was performed with a 4 µL injection into an inlet operated in splitless mode. The liner was an Agilent 5062-3587 split/splitless glass cylinder with single taper from which the glass wool was removed. The inlet was at 13.4 psi with an initial temperature of 210°C held 1 min, ramped 100°C/min to 280°C, held 10 min: then ramped 100°C/min to 210°C. Helium was used as the GC carrier gas at 1.5 mL/min. The GC oven initial temperature was 60°C, held 5 min, then ramped 8°C/min to 300°C, held 0.5 min for a total run time of 35.5 min. The MSD transfer line was held constant at 300°C.

The triple quadrupole mass analyzer was operated in negative EI mode at 70 eV with a source temperature of 230°C. Nitrogen gas was used as the collision gas at 1.5 mL/min and helium was used as the quench gas at 2.25 mL/min. MRM scanning was initiated after 3.75 min solvent delay. Acquisition and data processing were performed with MassHunter software (Agilent, Santa Clara, CA).

Backflushing

Installation of the T-union involved combination of two Agilent 19091S-431 fused silica capillary columns (each 15 m × 250 µm × 0.25 µm) with the first column in sequence operated at 1.2 mL/min constant He flow and the second at 1.4 mL/min constant flow. Backflush involved He flow introduced at the T-union at 17.3 mL/min with a backflush pressure of 100 psi for a total time of 0.52 min while the oven was held at 320°C.

GC/MS/MS Multi-mode Inlet

The Agilent Multi-mode Inlet (MMI) was temperature programmed as described above, but temperatures lower than the 210°C initial temperature were studied, specifically the range 50-75°C at 5-degree increments. Each temperature was held 0.3-min, and then ramped to 325°C at 600°C/min.

QuEChERS extraction procedure

Control bovine liver and serum was found to be uncontaminated with organochlorine pesticides. The liver and serum were stored at -17°C and were thawed before use. The following extraction was performed on all samples and in the preparation of matrix matched calibration standards. 2.0 g of liver was placed in a 50 mL glass test tube and spiked with 20 µL of 20 µg/mL decachlorobiphenyl (internal standard) and appropriate amount of stock or working solution of OC pesticide solution for calibration curve and QC samples. 5 mL of 1% acetic acid in acetonitrile was added to the test tube and the mixture was homogenized using a Brinkmann PTMR 3000 (Kinematica, Bohemia, NY) homogenizer. 0.5 g sodium chloride, 0.5 g sodium acetate, and 2.0 g of magnesium sulfate were added to the sample. The sample was vortexed for 30 seconds and centrifuged 5 min at 3000 rpm. 4 mL of the supernatant layer was transferred to a 20 mL glass test tube containing 0.60 g anhydrous magnesium sulfate, 0.10 g primary secondary amine, and 0.10 g C18. The extract was vortexed 30 seconds and centrifuged 5 min at 3000 rpm. A 2.5 mL aliquot of the supernatant was transferred to a clean test tube and dried on an N-EVAP (Organomation, Berlin, MA) just to dryness. The analytes were reconstituted in 0.5 mL 80:20 isooctane: ethanol and transferred to GC autosampler vials. The same procedure was followed for the serum samples with 1 mL being the initial sample volume.

Use of commercial QuEChERS tubes

The extraction method was simplified with the use of commercially available extraction and clean-up tubes available from Waters

Corporation (Milford, MA). The Waters extraction tube (PN 186004571) contained 1.5 g sodium acetate and 6.0 g magnesium sulfate. The Waters clean up tube (PN 186004834) contained 150 mg PSA, 150 mg C18 and 900 mg magnesium sulfate. The use of the commercially available tubes reduced possible contamination of glassware and streamlined the extraction process. No change in extraction efficiencies were observed in the transition to the commercial tubes.

Construction of calibration curve

Matrix matched standards were prepared from blank matrix samples that underwent the QuEChERS extraction and reconstituted with the addition of appropriate pesticide spikes. Neat standards in pure solvent were prepared from appropriate dilution of standard solution to make working solution of pesticide solution in same concentration as the matrix matched standards. Standard levels were 1, 10, 100, 200, 500 and 1000 ppb in 80:20 isooctane ethanol.

Results and Discussion

Analytical performance

The method's linearity, recovery, repeatability, selectivity, limit of detection and limit of quantitation were evaluated as measures of analytical performance [20]. Neat standards in pure solvent were prepared to examine matrix effects. Matrix may enhance the signal of a compound due to the binding of active sites in the inlet and chromatography column leading to higher amounts of analyte reaching the detector and producing significant increases in recovery [5,21,22]. The effects were apparent in serum samples with an average increase in response of 20.6%. Matrix effects were negligible in liver samples, but matrix matched standards were nevertheless utilized. For the purposes of this work, the general assumption was made that healthy organs such as liver or fluids such as serum provided roughly equivalent matrices on interspecific comparisons. By this reasoning, a commonly available or plentiful tissue such as bovine liver would be an appropriate matrix for determining avian liver pesticides. The authors are aware that there is a toxicology specialty dealing with extrapolation between matrices, usually between environmental categories such as air and water, and between organisms, such as from laboratory animals to humans [23]. Nevertheless, it seemed an appropriate assumption to make, particularly in light of the paucity of unadulterated eagle livers available for the study of subtle matrix effects.

Linearity

Matrix matched calibration curves were constructed in the range of 1 to 1,000 ppb. Calibration curves were constructed by a plot of peak area versus analyte concentration. Linearity was evaluated by the calculation of a six-point linear plot with three replicates, based on linear regression and coefficient of determination, r^2, which should be >0.990. 45 of the 48 calibration curves met this criterion. Experimental linear regression r^2 varied from 0.997 to 0.999 for liver and 0.985 to 0.996 for serum. These correlations improved slightly when calibration curves were fitted to quadratic regressions.

LOD and LOQ

The lower limits of detection (LOD) and of quantitation (LOQ) were calculated based on FDA guidance for bioanalytical method validation [20]. LOD was defined as 3.3 times the standard deviation of the blank divided by the slope of the calibration curve. The standard deviation was calculated from the responses of each analyte in 5 blank matrix samples. LOQ was defined as 10 times the standard deviation of the blank divided by the slope of calibration curve. The working upper and lower LOQ were taken as the maximum and minimum of linear calibration curve, 1000 ppb and 10 ppb, respectively. The calculated LOD is likely to be far below that of the actual amount detectable. This is due to the way that the data analysis software calculates abundances for analytes in the blanks. A peak is identified in the RT window defined in the program for a specific analyte. In the blank sample, the algorithm for peak integration and start and end points are likely to be variable leading to very low peak areas on a very flat chromatogram. Because of this, a conservative LOD of the lowest consistently detected concentration in the calibration curve was used, 10 ppb (ng/mL). The r^2, LOD, and LOQ calculated values for the OCPs analyzed are summarized in Table 1; Figure 1 illustrates chromatographic separation with the mixed OCP standards.

Recovery, repeatability and stability

Recovery of analytes was assessed at 3 spike levels with 3 replicates: 10, 20 and 1000 ppb. Acceptable levels of recovery for method validation are 70 to 120% [20]. Experimental recovery averages ranged from 46.8 to 117%, with 94.4% of samples within the accepted range. Repeatability is expressed as the relative standard deviation (RSD) of the QC sample recoveries. The accepted RSD values are <15% or <20% at the LOQ [20]. Overall, 131 of 144 of the analyte determinations in the validation met this criterion. Hexachlorobenzene was the only analyte that had lower than acceptable recoveries in more than one spike concentration level. A summary of average recovery and RSD for each analyte is given in Table 2. Stability was measured for each analyte at spike concentrations of 10 and 100 ppb in serum and liver matrices. Extracts were taken from spiked serum and livers at 72 hours and 1 hour prior to analysis. The percent recovery was evaluated for each analyte tested (Table 3). The percent recovery ranged from 96.3% to 111.4% in the 10 ppb serum spike and 96.2% to 122% in the 100 ppb serum spike demonstrating good sample stability over 3 days in this matrix. Alternatively, the percent recovery ranged from 32.2% to 117% in the 10 ppb liver spike and 73.7% to 161.3% in the 100 ppb liver spike. The complexity of the liver matrix was most likely responsible for the increased variability observed between analytes.

Selectivity

The pesticides evaluated in this study were identified by comparing retention time (RT) and precursor ion to product ion transitions. Two additional qualifier ion transitions were chosen for each analyte. The qualifier response to quantifier response ratio should not deviate more than 20%. Ions used for pesticide detection in identification are presented in Table 4. Retention time stability was expressed as the RSD of the time. RT should be ± 1% or six seconds for GC, whichever is greater [20]. Experimental data shows excellent RT reproducibility. The RT was also compared to the average RT of several solvent standard runs to examine the matrix effects on RT. No significant change in RT was detected. Retention times for the 24 analytes are provided in Table 4.

Precision

Precision of the organochlorine pesticide assay was assessed by determination of average refit values of standards to their respective standard curves over the course of three separate determinations, and calculation of the relative standard deviation (RSD) as %RSD. Precision was reasonable, with the average %RSD <30% across all analytes for the 100, 200, 500 and 1000 ppb standards. Table 5 lists the %RSD for each compound and all six standards.

Application of the method to real-life samples

Bald eagles have become victims of the farming industry and its

Compound Name	Liver			Serum		
	r^2	LOD (ppb)	LOQ (ppb)	r^2	LOD (ppb)	LOQ (ppb)
Alpha- BHC	0.998	0.1	0.32	0.995	0.04	0.13
Hexachlorobenzene	0.998	0.09	0.27	0.996	0.02	0.05
Gamma- BHC	0.998	0.07	0.22	0.994	0.02	0.06
Beta-BHC	0.998	0.08	0.24	0.993	0.03	0.1
Delta- BHC	0.998	0.14	0.43	0.995	0.06	0.17
Heptachlor	0.999	0.04	0.13	0.993	0.01	0.04
Aldrin	0.998	1.16	3.53	0.995	0.14	0.42
Oxychlordane	0.999	0.7	2.13	0.995	0.01	0.02
Heptachlor epoxide	0.999	0.1	0.32	0.993	0.01	0.03
Endosulfan I	0.999	1.83	5.56	0.993	0.01	0.03
Gamma-chlordane	0.999	0.07	0.21	0.993	0.01	0.03
Alpha-chlordane	0.999	0.03	0.09	0.993	0.01	0.03
Trans-nonachlor	0.999	0.08	0.25	0.993	0.01	0.02
4,4'-DDE	0.999	0.32	0.95	0.994	0.03	0.09
Dieldrin	0.999	2.44	7.4	0.991	0.14	0.42
Endrin	0.998	0.87	2.63	0.996	0.22	0.65
Endosulfane II	0.998	0.22	0.66	0.994	0.03	0.09
4,4'-DDD	0.998	0.14	0.43	0.993	0.02	0.04
Endosulfane Sulfate	0.998	0.05	0.15	0.989	0.02	0.07
4,4'-DDT	0.998	0.36	1.08	0.985	0.06	0.19
Endrin ketone	0.997	0.3	0.91	0.994	0.04	0.13
Endrin Aldehyde	0.999	0.32	0.97	0.994	0.1	0.29
Methoxychlor	0.998	0.31	0.93	0.985	0.05	0.16
Mirex	0.998	0.04	0.11	0.993	0.01	0.02

Table 1: Determined limits of detection (LOD) and quantitation (LOQ) in liver and serum matrices, including coefficient of determination (r^2) of corresponding standard curves.

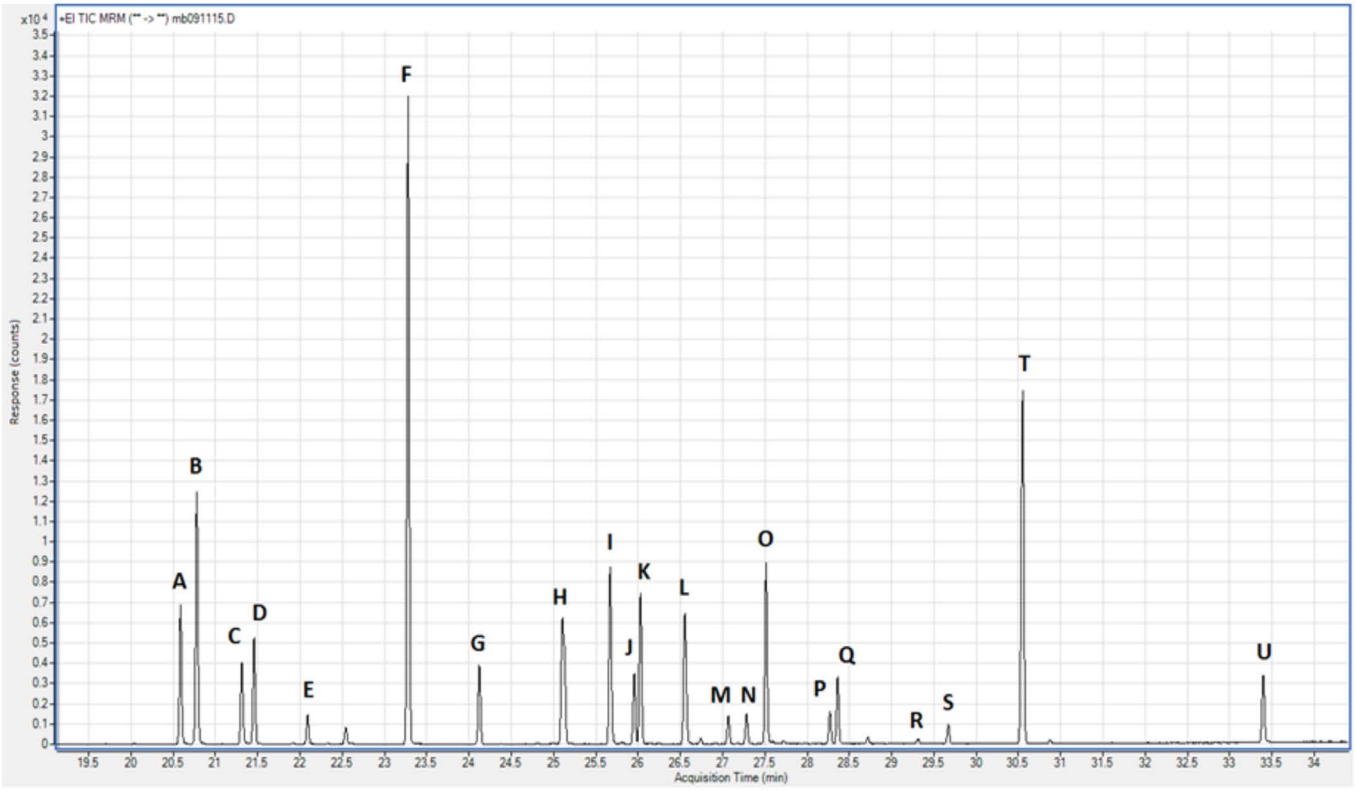

Figure 1: TIC MRM of 24 organochlorine analytes of study. Chromatograph peaks were assigned as follows: A) Alpha-BHC; B) Hexachlorobenzene; C) Gamma-BHC; D) Beta-BHC; E) Delta-BHC; F) Heptachlor; G) Aldrin; H) Oxychlordane, Heptachlor Epoxide; I) Endosulfan I; J) Alpha-Chlordane, Gamma-Chlordane; K) Trans-Nonachlor; L) 4,4'-DDE, Dieldrin; M) Endrin; N) Endosulfan II; O) 4,4'-DDD; P) Endosulfan Sulfate; Q) 4,4'-DDT; R) Endrin Ketone, Endrin Aldehyde; S) Methoxychlor; T) Mirex; U) Decachlorobiphenyl (internal standard).

Compound Name	Liver			Serum		
	10 ppb	200 ppb	1000 ppb	10 ppb	200 ppb	1000 ppb
Alpha-BHC	93.5 (2.4)	87.4 (9.0)	92.4 (7.4)	73.5 (9.9)	73.0 (15.9)	62.4 (20.5)
Hexachlorobenzene	69.6 (2.6)	75.2 (5.7)	77.4 (5.7)	56.9 (2.1)	60.8 (14.2)	46.8 (9.4)
Gamma-BHC	88.2 (1.9)	92.2 (6.4)	96.3(8.9)	88.3 (10.8)	87.7 (12.1)	83.2 (11.8)
Beta-BHC	94.8 (0.9)	88.0 (0.9)	94.2 (7.7)	83.2 (7.4)	80.4 (14.6)	70.7 (18.4)
Delta-BHC	87.8 (1.9)	94.7 (7.0)	94.6 (9.0)	91.3 (12.8)	91.7 (16.3)	77.3 (10.5)
Heptachlor	96.9 (3.6)	87.9 (9.3)	91.2 (9.6)	78.8 (8.8)	73.9 (12.7)	67.0 (15.9)
Aldrin	84.3 (3.9)	85.2 (7.6)	89.1 (8.0)	87.2 (5.6)	77.9 (10.1)	68.7 (12.9)
Oxychlordane	84.1 (0.8)	91.8 (9.0)	93.5 (9.9)	85.1 (6.9)	82.4 (9.7)	80.4 (11.5)
Heptachlor epoxide	89.3 (6.2)	92.7 (9.2)	95.0 (9.4)	87.9 (14.0)	83.5 (9.3)	81.4 (11.5)
Endosulfan I	80.1 (16.7)	89.1 (5.3)	94.4 (9.0)	73.7 (11.5)	81.0 (12.9)	81.8 (12.2)
Gamma-Chlordane	86.5 (2.0)	90.3 (9.7)	94.2 (9.1)	79.6 (3.7)	83.2 (10.0)	85.4 (9.1)
Alpha-Chlordane	80.6 (5.0)	92.1 (8.6)	94.5 (10.0)	88.8 (6.9)	85.3 (8.0)	85.9 (8.1)
Trans-Nonachlor	86.3 (2.3)	92.5 (8.3)	91.9 (11.3)	89.6 (11.4)	85.0 (8.3)	85.6 (8.9)
4,4'-DDE	81.0 (2.0)	90.0 (10.0)	92.7 (9.9)	83.9 (12.5)	82.6 (6.7)	86.3 (6.1)
Dieldrin	83.8 (3.2)	93.6 (8.0)	94.6 (9.5)	77.9 (4.1)	85.3 (8.0)	92.7 (1.7)
Endrin	94.7 (9.9)	93.0 (10.6)	95.8 (10.1)	83.6 (3.1)	84.6 (10.3)	78.3 (4.6)
Endosulfan II	98.0 (8.7)	93.6 (8.3)	97.5 (8.7)	91.9 (13.4)	85.9 (9.3)	89.8 (8.0)
4,4'-DDD	117.3 (1.8)	93.0 (6.2)	96.7 (8.1)	86.0 (4.4)	94.1 (8.0)	83.0 (5.6)
Endosulfan Sulfate	86.2 (0.6)	92.5 (6.2)	90.4 (6.4)	92.3 (18.6)	92.2 (23.8)	91.4 (10.0)
4,4'-DDT	85.2 (3.7)	90.1 (12.1)	93.6 (10.8)	82.9 (16.0)	79.3 (10.6)	87.5 (10.7)
Endrin Ketone	109.4 (15.4)	94.5 (8.1)	96.7 (9.6)	80.2 (15.1)	87.8 (16.7)	82.6 (6.8)
Endrin Aldehyde	56.7 (35.4)	94.6 (2.2)	96.3 (8.0)	84.3 (8.8)	86.3 (14.3)	82.3 (6.7)
Methoxychlor	84.6 (3.9)	92.5 (5.7)	96.4 (8.7)	76.9 (10.2)	88.5 (11.7)	81.5 (5.0)
Mirex	69.3 (3.0)	78.3 (7.2)	80.8 (6.5)	75.4 (8.0)	80.5 (4.5)	75.1 (4.0)

Table 2: Percent recovery and percent RSD (in parentheses) summary of spiked samples in two matrices at three spiked levels.

heavy reliance on chlorinated pesticides. For example, organochlorine chemicals appear as significant stressors on Great Lakes bald eagle populations when compared with stresses on successful populations of bald eagles continent-wide [24], despite the compounds' discontinuance in the US since the 1970-80s. Livers from three bald eagles submitted to the Diagnostic Center for Population and Animal Health at Michigan State University (DCPAH) were randomly chosen for assessment of organochlorine burden by the described GC-MS/MS method, and quantitative results are shown in Table 6. In addition, serum samples from three bald eagles, a common raven and three turkey vultures were also assessed (Table 7). Figure 2 illustrates the quantitative standard curves and confirmatory quantifier and qualifier ion chromatograms for two representative compounds in one of the eagles. There were significant amounts of the DDT metabolites 4,4'-DDE and 4,4'-DDD, as well as hexachlorobenzene, trans-nonachlor and mirex. DDT was banned by the EPA in 1976; hexachlorobenzene was used as a pesticide until 1965 and has been banned globally under the Stockholm Convention on persistent organic pollutants, 2001; trans-nonachlor is a bioaccumulating component of the insecticide chlordane, banned in 1983; and mirex has been banned since 1976 [25]. This finding is an unfortunate testament to the classification of the chlorinated pesticides as persistent organic pollutants.

Improvements to the method

The method as described has been validated; however, improvements were sought to provide increased robustness for daily operation and the best sensitivity possible. The approach took the tact of 1) introducing a T-union into the chromatographic column in order to reduce inlet contaminant buildup by enabling backflushing, 2) making use of an installed multi-mode inlet for temperature programming and large volume injection and 3) use of commercial extraction tubes. Chromatography with backflushing post-run had relatively minor effects, in general with some increase in retention; for example, alpha-BHC increased from 20.5 to 22.8 min RT, and methoxychlor increased from 29.6 to 31.8 min RT (not shown). This was principally an effect of the lower flow rate. The principal advantages of introducing backflush were improvements in chromatographic column and inlet cleanliness, as judged by decreased need for inlet cleaning or column maintenance. Its mention here was crucial in affirming that no deleterious effects were seen on the organochlorine pesticide chromatography or sensitivity.

The Agilent multi-mode inlet was studied with the same GC oven conditions but with decreased initial inlet temperatures substantially lower than 210°C. This offered advantages in being able to invoke larger injection volumes if desired as well as some chromatographic advantages from solvent focusing in cases where the initial oven temperature could also be lowered [26]. Initial inlet temperatures of 50, 55, 60, 65, 70 and 75°C were studied, and the lower temperatures did offer improvement on overall TIC area relative to the original starting conditions of 210°C. As shown in Table 8 for 55°C initial temperature, there was substantial increase in areas for compounds G-S, with little change in relative retention time, but some relatively minor decreases for the initial group of compounds, particularly the BHC family. Compound chromatograms are compared in Figure 3. Although it was difficult to determine differences within the relatively narrow range of inlet temperatures from 50 to 75°C, it was found that matching the inlet temperature to a reduced oven starting temperature was more important in improving sensitivity. Figure 4 compares the MMI 55°C inlet matched to a 45 to 300°C oven program in comparison to the original 210°C splitless inlet matched to the 60 to 300°C program. Note the shift in RTs accompanied by substantial increases in TIC peak areas.

The Agilent vapor volume software program [27] enables the chromatographer to judge whether a given injection volume will overload the injection liner volume and thereby shunt injected

Compound Name	Liver		Serum	
	10 ppb	100 ppb	10 ppb	100 ppb
Alpha- BHC	94.1%	75.6%	100.4%	117.5%
Hexachlorobenzene	ND	ND	ND	ND
Gamma- BHC	96.9%	91.3%	100.7%	122.7%
Beta-BHC	102.3%	89.9%	99.5%	108.4%
Delta- BHC	112.3%	83.8%	98.8%	111.5%
Heptachlor	78.8%	108.1%	101.4%	113.4%
Aldrin	94.1%	96.6%	105.2%	108.0%
Oxychlordane	74.7%	112.5%	97.9%	107.5%
Heptachlor epoxide	37.5%	118.7%	102.6%	111.2%
Endosulfan I	90.1%	106.2%	100.9%	104.1%
Gamma-chlordane	32.2%	101.6%	99.5%	106.8%
Alpha-chlordane	74.4%	97.0%	101.4%	104.4%
Trans-nonachlor	74.4%	95.2%	100.5%	107.6%
4,4'-DDE	104.2%	104.4%	99.9%	99.4%
Dieldrin	72.3%	161.3%	101.3%	102.9%
Endrin	78.2%	80.0%	101.9%	102.8%
Endosulfane II	99.3%	73.7%	100.9%	112.8%
4,4'-DDD	110.7%	86.2%	100.0%	101.0%
Endosulfane Sulfate	48.7%	115.8%	97.1%	98.4%
4,4'-DDT	117.0%	99.6%	96.3%	97.0%
Endrin ketone	78.3%	99.9%	101.0%	109.7%
Endrin Aldehyde	ND	ND	ND	ND
Methoxychlor	85.0%	81.4%	99.2%	96.2%
Mirex	ND	ND	ND	ND

Table 3: Stability of OCP analytes in liver and serum matrices over three days represented by percent recovery. ND: Not determined.

Compound Name	RT (min)	Quantitation MRM (CE)	Qualifier MRM 1 (CE)	Qualifier MRM 2 (CE)
Alpha- BHC	20.55	180.9→145 (15)	217 -> 181 (5)	219→183 (5)
Hexachlorobenzene	20.74	283.8→213.9 (30)	283.8→248.8 (15)	281.8→211.9 (30)
Gamma- BHC	21.28	180.9→145 (15)	217 -> 181 (5)	219→183 (5)
Beta-BHC	21.42	180.9→145 (15)	217 -> 181 (5)	219→183 (5)
Delta- BHC	22.06	180.9→145 (15)	217 -> 181 (5)	219→183 (5)
Heptachlor	23.33	272→236.9 (15)	236.8→118.9 (25)	273.9→238.9 (15)
Aldrin	24.09	263→193 (35)	263→191 (35)	263→263 (0)
Heptachlor epoxide	25.07	272→236.9 (15)	262.9→192.9 (35)	273.9→238.9 (15)
Oxychlordane	25.09	114.9→51.1 (25)	184.9→121 (15)	236.9→142.9 (25)
Gamma-chlordane	25.63	272→236.9 (15)	273.9→238.9 (15)	277→241 (5)
Endosulfan I	25.91	195.1→159 (5)	272→236.9 (15)	273.9→238.9 (15)
Alpha-chlordane	26.00	272→236.9 (15)	273.9→238.9 (15)	277→241 (5)
Trans-nonachlor	26.12	409→409 (0)	407→407 (0)	411→411 (0)
4,4'-DDE	26.53	246.1→176.2 (30)	315.8→246 (15)	317.8→317.8 (0)
Dieldrin	26.54	263→191 (35)	277→241 (5)	263→193 (35)
Endrin	27.03	263→193 (35)	263→191 (35)	263→263 (0)
Endosulfan II	27.25	195.1→159 (5)	206.9→172 (15)	180.9→145 (15)
4,4'-DDD	27.48	235.1→165.2 (20)	237→165.1 (27)	237→237 (0)
Endrin Aldehyde	27.70	184.9→121 (15)	206.9→172 (15)	195.1→159 (5)
Endosulfan Sulfate	28.23	272→236.9 (15)	273.9→238.9 (15)	277→241 (5)
4,4'-DDT	28.33	235.1→165.2 (20)	237→237 (0)	237→165.1 (27)
Endrin ketone	29.27	180.9→145 (15)	114.9→51.1 (25)	195.1→159 (5)
Methoxychlor	29.64	227→141.1 (40)	227→169.1 (30)	227→212 (17)
Mirex	30.51	272→236.9 (15)	273.9→236.9 (15)	273.9→238.9 (15)

Table 4: Mass spectrometer settings for the OCP GC-MS/MS method, including precursor ion→product ion m/z values and collision energies, kV, (CE) in parentheses, with compounds arranged in retention time (RT) order.

Compound Name	Calibrator levels					
	1000 ppb	500 ppb	200 ppb	100 ppb	10 ppb	1 ppb
Alpha-BHC	1%	4%	13%	21%	11%	104%
Hexachlorobenzene	2%	4%	32%	23%	42%	124%
Gamma-BHC	1%	4%	10%	27%	33%	116%
Beta-BHC	7%	10%	15%	11%	64%	97%
Delta-BHC	8%	16%	10%	25%	3%	65%
Heptachlor	0%	2%	10%	19%	18%	87%
Aldrin	1%	0%	7%	13%	20%	106%
Heptachlor epoxide	1%	8%	5%	10%	10%	19%
Oxychlordane	1%	6%	6%	10%	10%	56%
Trans-Chlordane	2%	9%	5%	9%	11%	12%
Endosulfan I	1%	7%	3%	10%	61%	49%
Cis-Chlordane	2%	9%	4%	5%	12%	26%
4,4'-DDE	1%	6%	5%	8%	6%	23%
Dieldrin	2%	7%	6%	6%	6%	19%
Trans-Nonachlor	1%	5%	5%	13%	16%	46%
Endrin	1%	6%	7%	9%	14%	40%
Endosulfan II	2%	9%	7%	14%	54%	88%
4,4'-DDD	3%	6%	10%	13%	25%	64%
Endrin aldehyde	14%	22%	28%	18%	65%	142%
Endosulfan sulfate	5%	10%	9%	10%	38%	88%
4,4'-DDT	5%	7%	14%	22%	33%	23%
Endrin ketone	2%	6%	7%	13%	39%	141%
Methoxychlor	5%	5%	15%	18%	29%	47%
Mirex	2%	6%	7%	8%	10%	21%
Average across all compounds	3%	7%	10%	14%	26%	67%

Table 5: Average percent RSD for all OCPs measured as a refit of calibrators to their respective standard curves across three separate calibrations performed on different days.

Compound (RT order)	Bald Eagle 1	Bald Eagle 2	Bald Eagle 3
Alpha-BHC	<10	<10	<10
Hexachlorobenzene	311	355	407
Gamma-BHC	<10	<10	<10
Beta-BHC	<10	<10	<10
Delta-BHC	<10	<10	<10
Heptachlor	<10	<10	<10
Aldrin	<10	<10	<10
Heptachlor epoxide	<10	<10	<10
Oxychlordane	<10	<10	<10
Trans-Chlordane	<10	<10	<10
Endosulfan I	<10	<10	<10
Cis-Chlordane	<10	<10	<10
4,4'-DDE	1950	977	2600
Dieldrin	<10	<10	<10
Trans-Nonachlor	812	132	803
Endrin	<10	<10	<10
Endosulfan II	<10	<10	<10
4,4'-DDD	<10	<10	62
Endrin aldehyde	<10	<10	<10
Endosulfan sulfate	<10	<10	<10
4,4'-DDT	<10	<10	<10
Endrin ketone	<10	<10	<10
Methoxychlor	<10	<10	<10
Mirex	2111	570	464

Table 6: OCP contaminants found in three separate bald eagle liver samples. All values expressed in ppb.

Compound (RT order)	Bald Eagle 1	Bald Eagle 2	Bald Eagle 3	Common Raven	Turkey Vulture 1	Turkey Vulture 2	Turkey Vulture 3
Alpha-BHC	<10	<10	<10	<10	<10	<10	<10
Hexachlorobenzene	ND	ND	ND	ND	ND	ND	ND
Gamma-BHC	<10	<10	<10	<10	<10	<10	<10
Beta-BHC	<10	<10	<10	<10	<10	<10	<10
Delta-BHC	<10	<10	<10	<10	<10	<10	<10
Heptachlor	<10	<10	<10	<10	<10	<10	<10
Aldrin	<10	<10	<10	<10	<10	<10	<10
Heptachlor epoxide	<10	<10	<10	<10	<10	<10	<10
Oxychlordane	<10	<10	<10	<10	<10	<10	<10
Trans-Chlordane	<10	<10	<10	<10	<10	<10	<10
Endosulfan I	<10	<10	<10	<10	<10	<10	<10
Cis-Chlordane	<10	<10	<10	<10	<10	<10	<10
4,4'-DDE	62	288	133	121	15	10	22
Dieldrin	11	10	<10	<10	<10	<10	<10
Trans-Nonachlor	<10	41	12	<10	<10	<10	<10
Endrin	<10	<10	<10	<10	<10	<10	<10
Endosulfan II	<10	<10	<10	<10	<10	<10	<10
4,4'-DDD	<10	<10	<10	<10	<10	<10	<10
Endrin aldehyde	<10	<10	<10	<10	<10	<10	<10
Endosulfan sulfate	<10	<10	<10	<10	<10	<10	<10
4,4'-DDT	<10	<10	<10	<10	<10	<10	<10
Endrin ketone	<10	<10	<10	<10	<10	<10	<10
Methoxychlor	<10	<10	<10	<10	<10	<10	<10
Mirex	ND	ND	ND	ND	ND	ND	ND

Table 7: OCP contaminants identified in the serum of three avian species: bald eagle, common raven, and turkey vulture. All values expressed in ppb. ND = not determined

Compound	Inlet 55°C RT, min	Inlet 210°C RT, min	TIC area ratio, t55/t210	RT ratio, t55/t210
A	22.84	22.87	0.1986	0.9985
C	23.52	23.62	1.5822	0.9958
D	23.69	23.73	0.4915	0.9982
E	24.28	24.38	0.4663	0.9962
F	25.53	25.56	0.4214	0.9990
G	26.39	26.42	4.2794	0.9991
H	27.34	27.39	2.1271	0.9982
J	27.89	27.95	10.2979	0.9979
I	28.20	28.25	3.2446	0.9982
K	28.26	28.30	7.1228	0.9986
L	28.70	28.76	8.8798	0.9980
O	29.65	29.73	5.9753	0.9974
Q	30.50	30.57	3.4425	0.9978
S	31.76	31.82	3.2488	0.9982

Table 8: Study of effects of inlet temperature in the MMI. Shown are retention times (RT) at 55 and 210°C for 14 of the compounds under study, and the relative changes in total ion chromatogram (TIC) area and RT on comparing temperature 55 divided by temperature 210 (t55/t210).

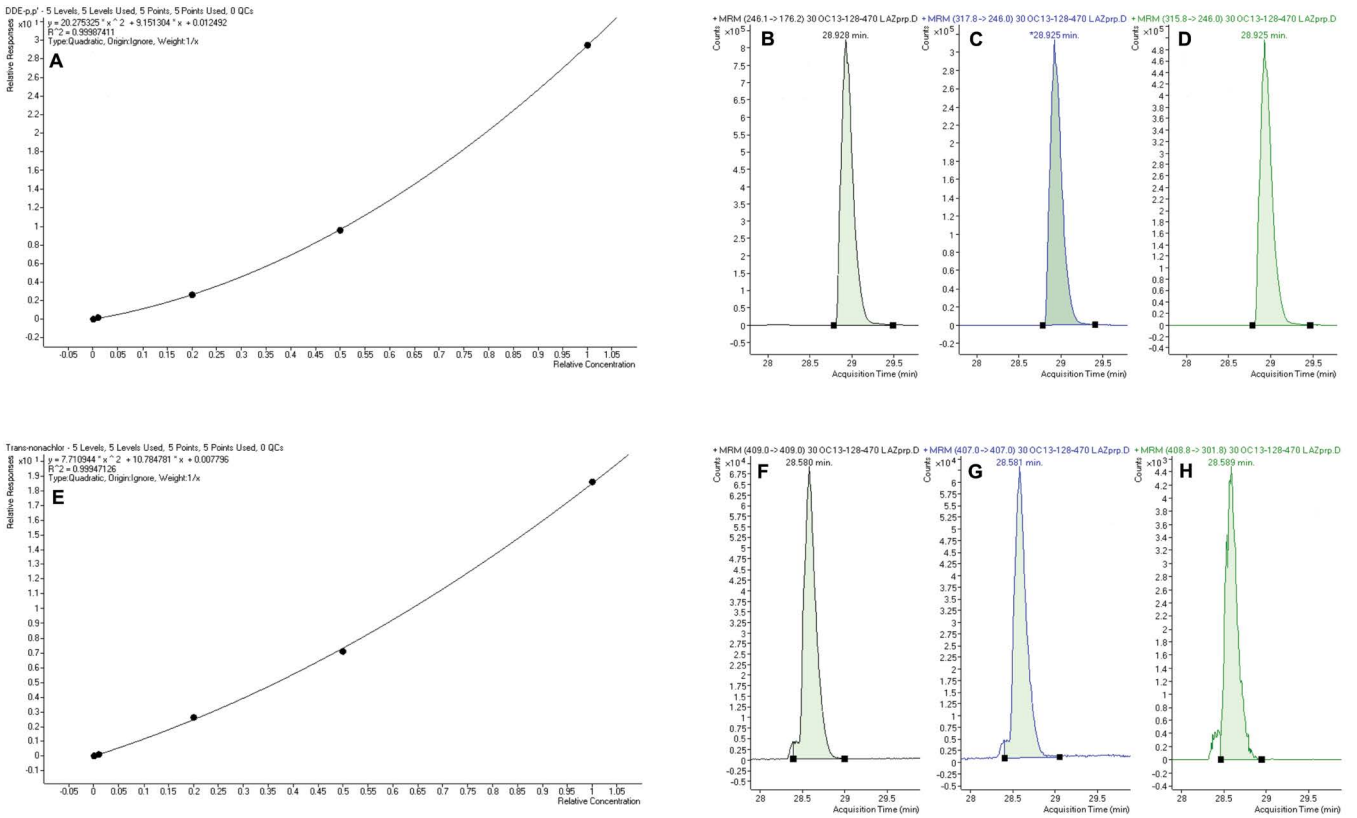

Figure 2: Example calibration curves and MRM qualifiers for real samples. **A)** Response relative to internal standard plotted as a function of concentration relative to internal standard for: 4,4'-DDE (quadratic calibration curve); **B-D**) ion chromatograms for 4,4'-DDE quantifier MRM (m/z 246→176) and two qualifier MRM transitions (m/z 318 → 246 and m/z 316→246), respectively, for bald eagle sample 3; **E**) Response relative to internal standard is plotted as a function of concentration relative to internal standard for: trans-nonachlor (quadratic calibration curve); **F-H**) ion chromatograms for trans-nonachlor quantifier MRM (m/z 409→409) and two qualifier MRM transitions (m/z 407→407 and m/z 409→302), respectively, for bald eagle sample 3. Note that this analysis made use of the described improvements of back flushing on a T-union joined chromatographic column as well as the commercial QuEChERS tubes.

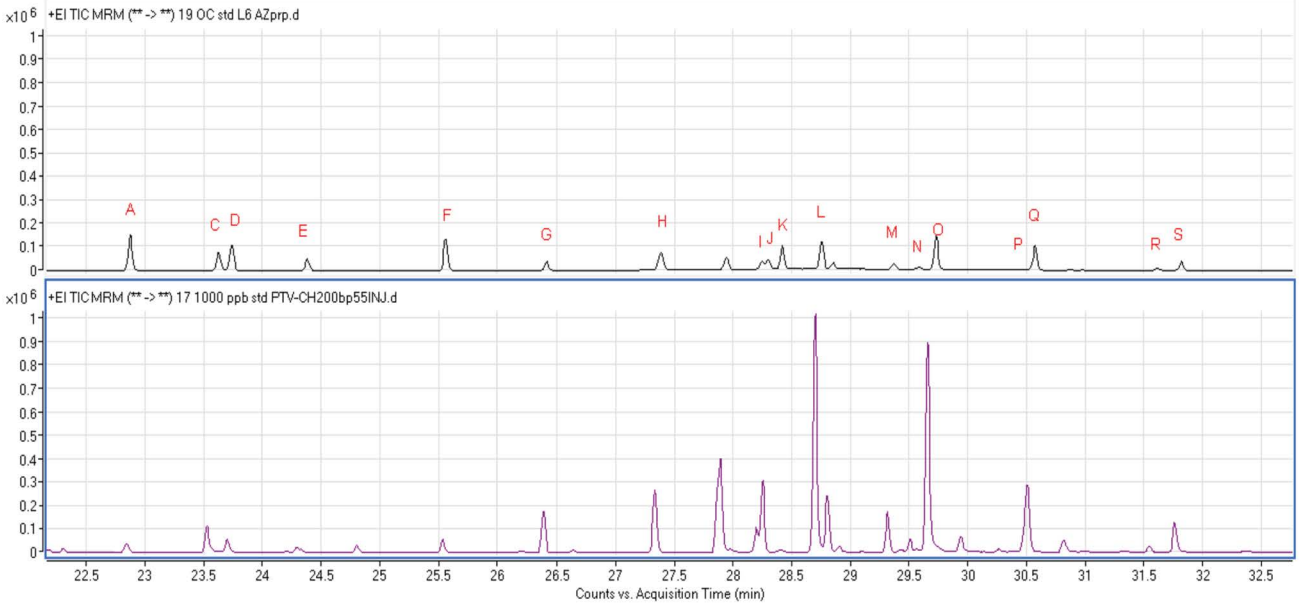

Figure 3: Comparison of 2 µL injections of the 1000 ppb organochlorine mixture at 210°C splitless (top) and 55°C (held 0.3 min, then ramped to 325°C at 600°C/min) (bottom) on the T-union chromatographic column. Same peak labels as in Figure 1.

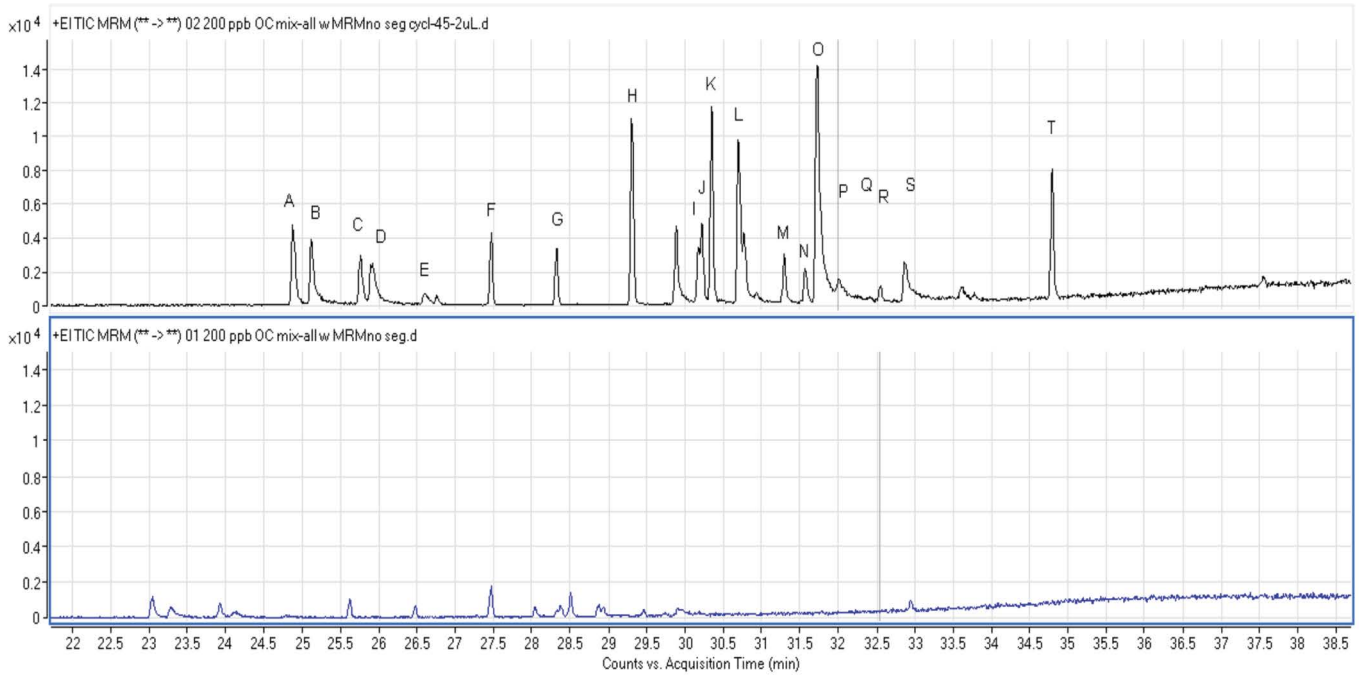

Figure 4: Comparison of 2 µL injections of the 200 ppb organochlorine mixture at 55°C (held 0.3 min, then ramped to 325°C at 600°C/min) with the oven temperature program starting at 45°C [top] with the original oven temperature program and splitless 210°C injector (bottom), both on the T-union chromatographic column. Same peak labels as in Figure 1. Note that peak retention times in the top TIC trace have increased by 2 min uniformly.

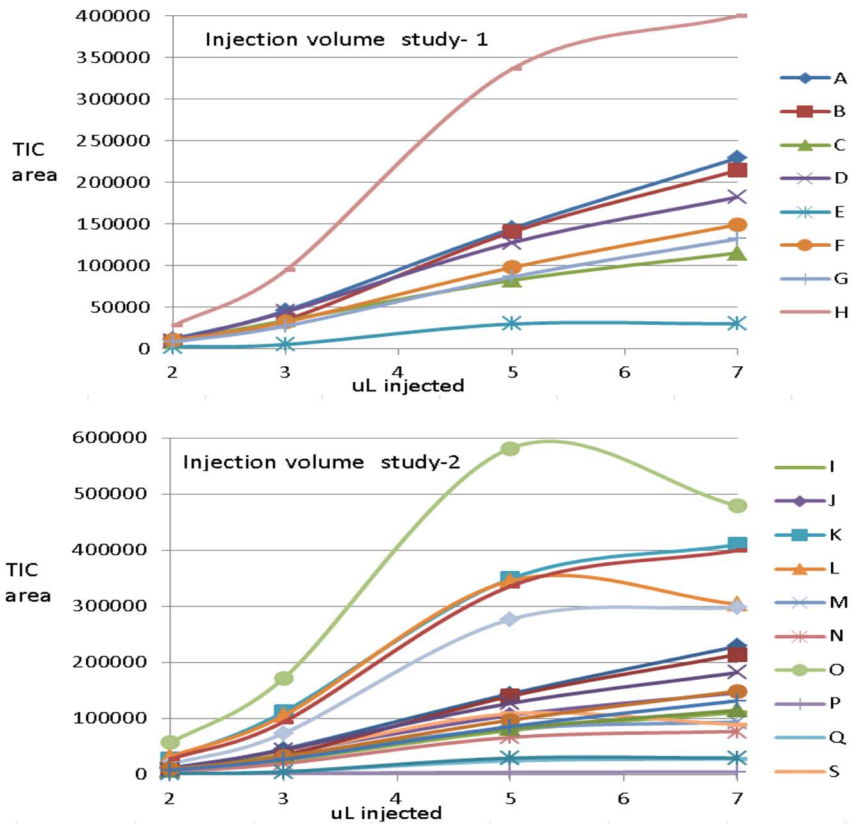

Figure 5: TIC area as a function of number µL injected for compounds A-H [top] and I-S [bottom]. The oven was temperature programmed from 45 (held 5 min) to 300°C at 8°C/min, and the MMI inlet was programmed from 55 to 320°C at 600°C/min.

material to the exhaust valve rather than onto the chromatographic column. By this criterion, 100% iso-octane theoretically appears suitable for a 4 µL injection at an estimated 94% of the 900 µL total liner volume at 210°C inlet temperature and 12 psi inlet pressure, whereas 100% ethanol would be unsuitable at 267% the inlet capacity. Reducing the inlet temperature to 55°C reduces these high percentages to 64% and 181% respectively, making it likely that large volume injection should not be a problem with the iso-octane:ethanol 80:20 solvent mixture. Injection volume capacity on the instrument was assessed with the optimized combination of 55°C programmed inlet with the 45 to 300°C oven temperature program, and the results are displayed in Figure 5. Most of the compounds demonstrated increase in response across the entire 2 to 7 µL range, whereas a few (E, O, L and S) were at maximum response around 5 µL.

Conclusion

The modified QuEChERS extraction approach coupled with GC-MS/MS analyte detection improved analytical turn-around time and increased sensitivity for analyte detection of organochlorine pesticides having a limit of detection of 10 ppb. The implementation of matrix matched calibration curves allowed for enhanced sensitivity demonstrated by the increased instrument response to the same concentration of analyte in matrix and solvent. This approach can clearly be extended to a variety of veterinary diagnostic panels that similarly use less efficient or lower specificity methodologies for assessing animal matrices for toxicant exposure. Finally, application of the optional temperature-programmable multi-mode inlet revealed techniques for improving sensitivity of the method as applied to analytes extracted from matrix, should greater sensitivity be required due to small sample size and/or a requirement for maximum detectability.

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