1.0 INTRODUCTION

An independent method validation study was conducted at PTRL West, Inc. (625-B Alfred Nobel Dr, Hercules, California) to determine the validity of a procedure to analyze malathion and malaoxon in well and pond water. See Appendix A for the study Protocol. The study was initiated on August 24, 2011. The independent laboratory validation was conducted from August 24, 2011 through February 9, 2012.

2.0 MATERIALS AND METHODS

2.1 Method

The analytical method for the analysis of malathion and malaoxon in water was conducted relative to a method validated at Morse Laboratories (Reference 1, See Also Appendix A- Protocol Appendix 1).

The determination of malathion and malaoxon was validated by spiking known concentrations of each analyte into control well and pond water samples. Extraction of the analytes from the matrix was achieved by performing a liquid-liquid partition with dichloromethane. This was followed by a centrifugation step to ensure phase separation which was a minor modification relative to method validated at Morse Laboratories (Reference 1, See Also Appendix A- Protocol Appendix 1). The dichoromethane phase was separated and evaporated to dryness. This extract was then reconstituted in 50:50 methanol: 0.088% formic acid, sonicated, diluted in 50:50 Methanol: 0.088% formic acid and submitted for HPLC/MS/MS analyses in the positive mode. If the samples are found to require further cleanup, a portion of the reconstituted extract is diluted 10 fold in 0.088% formic acid and applied to a pre-conditioned OASIS HLB cartridge. The cartridge is washed successively with ammonium hydroxide/5% methanol and acetic acid/5% methanol. The analytes are then eluted with methanol. The eluant is diluted with water/0.088% formic acid and submitted for HPLC/MS/MS analyses in the positive mode. The percent recovery was determined relative to an external calibration curve.

2.2 Test System

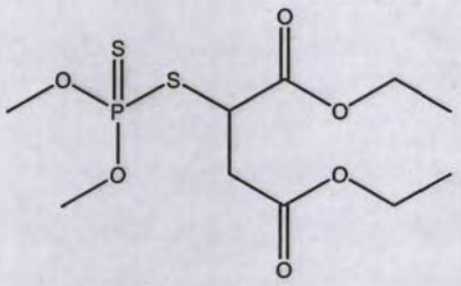
The test systems were pond and well water. The control pond water sample used for this study had an identification number of 2221W-004A and was collected from Refugio Park Pond, Hercules, CA on December 14, 2012. The control well water sample used for this

study had an identification number of 2221W-005A and was collected at North Gate road, Walnut Creek, CA on January 15, 2012.

2.3 Reference Substances

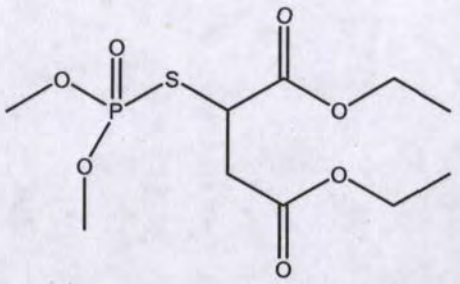
The malathion (1999W-003) and malaoxon (2144W-001) reference substances were obtained from Cheminova, Inc. Stock solutions of the reference substances were prepared at 1.0 mg/mL in acetonitrile and were assumed to be stable over the period of the study. Calibrant solutions appeared stable when stored refrigerated for at least 4 months, based on the comparison of LC-MS/MS chromatograms during this study. The certificates of analysis for the reference substances are provided in Appendix B.

Compound: **Malathion**
Chemical Name: Diethyl (dimethoxyphosphinothioylthio) succinate
Chemical Structure:



Purity: 99.6%
Lot Number: 650-OSJ-36E
PTRL West Inventory Number: 1999W-003
Date Received: July 16, 2010
Expiration Date: March 1, 2018
Storage Conditions: Freezer

Compound: **Malaoxon**
Chemical Name: 2-(dimethoxyphosphorylthio) butanedioic acid diethyl ester
Chemical Structure:



Purity: 96.2%
Lot Number: 849-Bse-39B
PTRL West Inventory Number: 2144W-001
Date Received: May 11, 2011
Expiration Date: October 6, 2014
Storage Conditions: Freezer

2.4 Equipment

Balance (various types)
Bottle, amber glass, 120 mL
Separatory funnel, 500 ml
Centrifuge bottle, HDPE, 1000 mL
Centrifuge (Sorvall RT7 plus)
Graduated cylinder, various sizes
Glass tubes, 50 mL Pyrex
Glass disposable centrifuge tubes 15 mL
Pasteur pipettes
Sonicator, Branson 2210
SPE cartridges Oasis HLB (60 mg, 3 cc)
Syringes, microliter, various sizes
Vacuum evaporator, (Zymark TurboVap LV evaporator)
Vacuum manifold with stopcocks for SPE cartridges
Volumetric flask, various sizes
Vacuum pump
Wrist-action shaker

2.5 Solvents and Reagents

All solvents and reagents (reagent grade or better) were obtained from Fisher Scientific or VWR. Ammonium hydroxide was obtained from Sigma Aldrich. All water used was HPLC grade.

Acetic acid
Methanol
Acetonitrile
Ethyl Acetate
Water
Formic Acid
Ammonium hydroxide

2.6 Reference Substance Stock Solution Preparation

Stock standard solutions of malathion and malaoxon (corrected for purity) were prepared at 1.0 mg/mL in acetonitrile. The stock standards were stored refrigerated (at 4°C) when not in use. These solutions were shown to be stable for the period of the study based on comparison of chromatograms during the study duration.

2.7 Preparation of Intermediate/Fortification Solutions

Individual solutions were prepared as follows:

100.0 µg/mL: 2.5 ml of each 1000 µg/mL stock solution was transferred into individual 25 ml volumetric flasks. Diluted to the mark with acetonitrile and mixed well.

10.0 µg/mL: 2.5 ml of each 100 µg/mL solution was transferred into individual 25 ml volumetric flasks. Diluted to the mark with acetonitrile and mixed well.

1.0 µg/mL: 2.5 ml of each 10 µg/mL stock solution was transferred into individual 25 ml volumetric flasks. Diluted to the mark with acetonitrile and mixed well.

Mixed solutions were prepared as follows:

100.0 µg/mL: 1.0 ml of each 1000 µg/mL stock solution was transferred into a single 10 ml volumetric flasks. Diluted to the mark with acetonitrile and mixed well.

10.0 µg/mL: 1.0 ml of each 100 µg/mL solution was transferred into a single 10 ml volumetric flasks. Diluted to the mark with acetonitrile and mixed well.

1.0 µg/mL: 1.0 ml of each 10 µg/mL stock solution was transferred into a single 10 ml volumetric flasks. Diluted to the mark with acetonitrile and mixed well.

0.1 µg/mL: 1.0 ml of each 1.0 µg/mL stock solution was transferred into a single 10 ml volumetric flasks. Diluted to the mark with acetonitrile and mixed well.

Intermediate/Fortification solutions were transferred to the amber glass bottles and stored frozen when not in use.

2.8 Preparation of Calibrants

Calibration standard solutions were prepared as follows:

- 2.5 ng/mL: 250 µL of the 0.1 µg/mL intermediate mixed solution was transferred into a 10 ml volumetric flask. Diluted to the mark with Acetonitrile and mixed well.
- 0.5 ng/mL: 50 µL of the 0.1 µg/mL intermediate mixed solution was transferred into a 10 ml volumetric flask. Diluted to the mark with Acetonitrile and mixed well.
- 0.25 ng/mL: 25 µL of the 0.1 µg/mL intermediate mixed solution was transferred into a 10 ml volumetric flask. Diluted to the mark with Acetonitrile and mixed well.
- 0.1 ng/mL: 400 µL of the 2.5 ng/mL mixed standard solution was transferred into a 10 ml volumetric flask. Diluted to the mark with Acetonitrile and mixed well.
- 0.05 ng/mL: 200 µL of the 2.5 ng/mL mixed standard solution was transferred into a 10 ml volumetric flask. Diluted to the mark with Acetonitrile and mixed well.

Standards were mixed well in volumetric flasks, then transferred to amber glass bottles. All calibration solutions were stored in refrigerator when not in use.

2.9 Sample Preparation

Aliquots of the water (250 ml) were measured into 500 mL glass separatory funnel prior to fortification (if necessary) and extraction.

2.10 Sample Fortification

Fortification of control pond and well water was performed to analyze method percent recoveries for ILV. A 250 ml (0.25 kg) portion of water was fortified with 45 µL of the listed fortification solution as follows:

Fortification Designation	Fortification Level (ng/kg)	Concentration of Fortification solution used
F1A	18 (malathion and malaoxon each)	0.1 µg/mL
F1B	18 (malathion and malaoxon each)	0.1 µg/mL
F1C	18 (malathion and malaoxon each)	0.1 µg/mL
F1D	18 (malathion and malaoxon each)	0.1 µg/mL
F1E	18 (malathion and malaoxon each)	0.1 µg/mL
F2A	180 (malathion and malaoxon each)	1.0 µg/mL
F2B	180 (malathion and malaoxon each)	1.0 µg/mL
F2C	180 (malathion and malaoxon each)	1.0 µg/mL
F2D	180 (malathion and malaoxon each)	1.0 µg/mL
F2E	180 (malathion and malaoxon each)	1.0 µg/mL

2.11 Extraction Method

1. Transfer 250 ml of a well- mixed sample into a 500 ml separatory funnel. Fortify as necessary.

2. Add 88 g of sodium chloride to the sample in the separatory funnel.

3. Add 50 ml of dichloromethane (DCM) to the sample and shake vigorously for 2 minutes (frequently vent the separatory funnel)

4. Drain complete sample into a 500 ml Centrifuge bottle and centrifuge at 2000 rpm for 5 minutes to effect complete phase separation.

5. Pass the resultant lower layer through a glass funnel which contains a glass wool plug and ~ 20 g of sodium sulfate into a 200 ml Zymark tube.

6. Repeat Steps 3 through 5 two additional times combining the DCM extracts.

7. Discard the aqueous layer

8. Rinse the sodium sulfate with 10 ml of DCM into the 200 ml Zymark tube.

9. Concentrate the sample to ~ 0.2 ml using a Turbo-Vap evaporator set to ≤ 40°C. Manually evaporate to dryness with Nitrogen. Add 10 ml of methanol:0.088% formic acid (50:50, v/v). Mix well and sonicate.

10. Transfer the solution from Step 9 to a 15 ml graduated polypropylene centrifuge tube and cap. Remove aliquots for analyses as specified in Step 11 a) and store the remainder at 1-8 °C as a retain original of the sample if needed for re-analysis.

11. a) For samples requiring no further cleanup, transfer 1.0 ml of the extract from Step 10 to a 15 ml graduated polypropylene centrifuge containing 4.0 ml of HPLC methanol:0.088% formic acid (50:50, v/v). Cap the centrifuge tube, mark the meniscus and vortex mix. Submit for analysis.

    b) For samples requiring additional cleanup transfer 1.0 ml of the extract from Step 9 into a 15 ml graduated polypropylene centrifuge containing 9.0 ml of 0.088% formic acid in DI water. Cap and Vortex mix. Proceed with Oasis HLB SPE cleanup (Step 12, i-x)

12. OASIS HLB SPE Cartridge Cleanup

    i. Set up an SPE cleanup system with Oasis HLB 60 mg, 3cc cartridge on a vacuum manifold connected to a vacuum pump. In general set vacuum to produce a flow rate of approximately 2 ml/minute (not continuous flow) for all elutions.

    ii. Condition an Oasis HLB cartridge (size 3 cc, 60mg) by passing 2 mL of methanol followed by 2 ml of deionized water, through the cartridge. Do not let the cartridge become dry in between any of the conditioning steps or between the conditioning and sample introduction. (Stop elution when each conditioning solvent reaches the top of the frit.) Discard all eluates.

    iii. Pass the sample extract (10 ml) from Step 2.11.7 through the SPE cartridge. Stop elution when the solvent reaches the top of the frit. Discard the eluate. Residues of malathion and malaoxon are retained on the cartridge.

iv. Add 1 ml of 5% methanol in DI water to the empty centrifuge tube that contained the sample extract. Shake to rinse the tube and pass the rinse through the cartridge. Stop elution when the solvent reaches top of frit. Discard eluate.

v. Wash the sample laden cartridge with 1 ml of ammonium hydroxide:5% methanol in DI water (2:98, v/v). Stop elution when the solvent reaches top of frit. Discard eluate.

vi. Wash the sample laden cartridge with 1 ml of acetic acid: 5% methanol in DI water (2:98, v/v). Stop elution when the solvent reaches top of frit. Discard eluate.

vii. Place a 15 ml polypropylene centrifuge tube calibrated at 1.0 and 2.0 ml under the SPE cartridge

viii. Elute the analytes with 2 mL of HPLC methanol into the calibrated 15 mL polypropylene tube. Allow the cartridge dry under vacuum.

ix. To each sample eluate add 0.5 ml HPLC Methanol. Bring to a final volume of 5.0 ml with 0.088% formic acid in HPLC water. Mix well. Submit to LC-MS/MS analysis.

2.12.1 LC-MS/MS Analysis of malathion and malaoxon

An Applied Biosystems MDS/SCIEX API 4000 LC/MS/MS system with electrospray ionization and an Agilent 1100 series LC system were used.

2.12.1 LC System Components

SCIEX 4000 (HPLC/Turbo Ion Spray Mode)

Pump: Agilent 1100 series, model G1312A

Autosampler: Agilent 1100 series, model G1329A

Micro-Degasser: Agilent 1100 series, model G1379A

Column Compartment: Agilent 1100 series, model G1316A

2.12.2 LC Parameters

Column: Phenomenex Synergi Fusion RP, 100A (100 mm x 2.0 mm I.D.), plus a 4 x 2 mm Phenomenex Fusion security guard pre column cartridge.
Flow Rate: 200 µL/minute

Injection Volume: 10 µL

Temperature: 40°C

Solvent System: Solvent A = Water (0.1% formic acid)

Solvent B = Acetonitrile

Solvent Program:

Minutes	Solvent A	Solvent B
0	75	25
0.5	75	25
5.0	5	95
9.0	5	95
9.5	75	25
13.0	75	25

Divert Valve: Divert LC flow from column to waste (bypassing MS) from 0 to 6.0 minutes and again from 9.0 to 13.0 minutes.

Approximate Retention Times: malaoxon: 7.1 minutes, malathion: 8.6 minutes,

2.12.3 Mass Spectrometer Parameters

An Applied Biosystems MDS/SCIEX API 4000 LC/MS/MS system was used with electrospray ionization in positive polarity mode to acquire data by Multiple Reaction Monitoring (MRM):

Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)	Declustering Potential	Collision Energy	Collision Activation Dissociation Gas	Collision Cell Exit Potential
315.2	126.9	150	40	18	6	11.0
315.2	99.1	150	40	35	6	7.0
315.2	143.1	150	40	16	6	12.0
331.1	127.0	150	40	19	6	11.0
331.1	99.1	150	40	33	6	8.0
331.1	285.3	150	40	11.5	6	7.0

Source Dependent Settings:

Nebulizer Temperature (TEM):	350°C
Nebulizer Gas (GS1):	40
Turbo Gas (GS2):	80
Ion Spray Voltage (IS):	5500
Curtain Gas (CUR):	10
Interface Heater (ihe):	on
Entrance potential:	10

Separation of the analytes was achieved by high performance liquid chromatography. The analyte was identified by the coincidence of it retention time with that of the respective reference standards as well as by monitoring specific ion transitions.

3.0 METHODS OF CALCULATION

3.1 Preparation of Stock Standards

$$\text{Final Concentration (mg/mL)} = \frac{(W) \times (P)}{(VS)}$$

where W = Milligrams of neat standard
P = Chemical purity of neat standard
VS = Volume of Solvent (mL)

3.2 Residue in Matrices

Each analyte was quantified by peak area relative to an external calibration curve. A calibrant peak area (y) from the quantitation ion transition relative to the concentration of the calibrant in ng/mL (x) yielded a linearity curve, where $y = mx + b$ was plotted using a least squares fit with 1/x weighting. Curves are determined by Applied Biosystems/MDS SCIEX Instruments Analyst Software version 1.4.2 or the equivalent

The residue of each analyte (malathion and malaoxon) in water was calculated as follows:

$$\text{ng/kg (ppt)} = \frac{\text{ng/mL analyte} \times \text{Final vol.(mL)} \times 1000\ \text{pg/ng} \times \text{Dil factor}}{\text{Extraction volume (ml)}}$$

Where:

ng/mL analyte = ng/mL of analyte found from standard curve

Final vol. (mL)	=	Volume of final HPLC ready extract (10 mL)
Extraction volume	=	Volume of extraction solvent (250 mL)
Dil. Factor	=	5.0
1000 pg/ng	=	Unit conversion factor

And:

ng/mL analyte	=	[(PA – b) ÷ m]
Where:	=	
PA	=	Peak area analyte
B	=	y-intercept of calibration curve
m	=	slope of calibration curve

$$\% \text{ Recovery} = \frac{\text{Analyte Residue Detected (mg/kg) - Avg. Control Residue}}{\text{Analyte Fortification Level (mg/kg)}} \times 100$$

An example calculation for the malathion residue in well water (Fort 1A at 0.01 mg/kg) is shown below:

Linear regression analysis (with 1/x weighting) of the malathion standards gave a curve as calculated by the Analyst Software version for the quantitation ion transition 331.1/285.3 with the equation

$$y = 207470.18762\ x + 6227.0322583\ (r^2 = 0.998365303339)$$

The ng/mL malathion injected determined by this curve was:

ng/mL malathion = [((22863.70021 - 6227.0322583) ÷ 207470.18762= 0.0802 ng/mL

Where:

22863.70021 = peak area malathion

6227.0322583= y-intercept of calibration curve

207470.18762 = slope

The malathion residue (ng/kg)

= (0.0802 ng/mL x 10.0 mL x 5.0 x 1000 pg/ng) ÷ (250 ml)

= 16.04 pg/ml or ng/kg

$$\text{Percent Recovery} = \frac{16.04\,\text{ng/kg} - 0.00000\ \text{mg/kg}}{18\,\text{ng/kg}} \times 100 = 89\%$$

4.0 LIMIT OF QUANTITATION AND DETECTION

The limit of detection (LOD) is defined as the concentration of the lowest linearity calibrant injected – 0.05 ng/ml malathion and malaoxon. Using the current methodology this is equivalent to an LOD of 10 ppt for both analytes. The limit of quantitation (LOQ) is defined as the lowest concentration validated which is 18 ppt for malathion and malaoxon.

5.0 STATISTICAL METHODS

The residue data included the following statistical calculations: averages, standard deviations, relative standard deviation and linear regression analysis with 1/x weighting.

6.0 TIME REQUIRED FOR ANALYSIS

A sample set consisting of 12 samples and a reagent blank sample can be completed by one analyst in the following amount of time:

Extraction/Cleanup: 14 hrs.

Analysis: 13 hrs for analysis (includes 4 hours for instrument tuning).

Total person-hours = 16 (does not include 7 hrs of unattended LC-MS/MS analysis or 4 hrs for tuning)

Days Required for the Sample Set = 2 Days

7.0 ARCHIVING STATEMENT

The original project specific data files will be stored at PTRL-West (Hercules CA). The project specific data files will be transferred to a location specified by the Sponsor upon their authorization. No data will be discarded without the Sponsor's written consent. Facility records and a copy of the final report are to be maintained at PTRL West. The test system will be retained at PTRL West. Following Sponsor's authorization, the test system may be sent to the Sponsor or disposed of, in accordance with PTRL West standard operating procedures.

DETERMINATION OF MALATHION AND MALAOXON IN WATER BY LC-MS/MS

Reason for Revision: To correct the title of the original version, dated May 20, 2011, to read as initially intended (adding "by LC-MS/MS").

1 PRINCIPLE

The method described herein is capable of determining malathion and malaoxon in water. To summarize, residues of malathion and malaoxon are extracted from water by partitioning three times with dichloromethane. The lower dichloromethane layer is passed through sodium sulfate, evaporated to dryness and re-dissolved in HPLC methanol:0.088% formic acid (50:50, v/v). The resulting solution is analyzed for malathion and malaoxon by LC/MS/MS in the positive ionization mode. The limit of quantitation (LOQ) is 18 ppt for both analytes.

The method provides for an optional Oasis® HLB cartridge solid phase extraction (SPE) cleanup if further cleanup of the extract is deemed necessary (as determined by unacceptable chromatography resulting from co-elution of interfering compounds or analyte response enhancement/suppression). If this cleanup is employed the LOQ remains 18 ppt.

2 EQUIVALENCE STATEMENT

During the conduct of this analysis, comparable apparatus, solvents, glassware, and techniques (such as sample extract evaporation) may be substituted for those described in this method, except where specifically specified. In the event a substituted piece of equipment or technique is used, its use will be documented in the study records.

3 APPARATUS AND EQUIPMENT

Assorted laboratory glassware

Balances:	Analytical balance capable of weighing to ±0.1 mg
	Top-loading balance capable of weighing to ±0.01 g
Evaporation tubes:	Zymark, glass; 200 mL (Zymark Corp., Hopkinton, MA)
Evaporators:	N-Evap Laboratory Sample Evaporator Model 115 attached to a N_2 source (Organomation Assoc., South Berlin, MA)
	Zymark Turbo-vap Concentrator 200 (Zymark Corporation, Hopkinton, MA)
Graduated cylinders:	Glass; various sizes

Graduated mixing cylinders:	Glass; various sizes
LC/MS/MS system:	Applied Biosystems/Sciex API 4000 LC/MS/MS System with ACQUITY UPLC System including Sample Organizer with Applied Biosystems/MDS Sciex Analyst Software for data collection and system control (version 1.5)
Microliter syringes:	Various sizes (Hamilton Co., Reno, NV)
Pasteur pipets:	Glass, disposable; 5½-inch and 9-inch
Pipets:	Glass, graduated, serological; various sizes
Pipets, adjustable:	Finnpipette digital pipettors: 40-200 µL: VWR Scientific Catalog #53515-052 100-1000 µL: Fisher Catalog #14-386-74 Finnpipette pipet tips: 1-200 µL: VWR Scientific Catalog #53508-810 100-1000 µL: VWR Scientific Catalog #53516-164 Eppendorf Research Series 2100: 1-10 mL: Eppendorf Catalog # 022472208
Sample solution storage vessels:	Polypropylene (PP), graduated; 15-mL
Separatory funnel:	Glass; 500-mL
Solid Phase Extraction Apparatus:	Visiprep 12 or 24-port SPE vacuum manifold with disposable flow control liners (Supelco, Bellefonte, PA)
Standard bottles:	Glass, amber; 50 and 25-mL
Syringes:	Glass, Hamilton Teflon® Luer-Lok; 2.5 mL (Hamilton Co., Reno, NV)
Ultrasonic bath:	Branson Model 2210 ultrasonic bath (VWR Scientific, Bridgeport, NJ)
Volumetric flasks:	Glass; 250, 50, 25, and 10-mL

	Vortex Mixer:	VWR Mini Vortexer (VWR Scientific, Bridgeport, NJ)

4 REAGENTS AND MATERIALS

Acetic acid:	Glacial, 100%, HPLC grade, (Fisher Scientific, Fairlawn, NJ)
Acetonitrile:	Optima® (Fisher Scientific, Fairlawn, NJ) HPLC grade (Fisher Scientific, Fairlawn, NJ)
Ammonium hydroxide:	28-30% GR ACS (EMD Chemicals, Gibbstown, NJ)
Dichloromethane:	Optima® (Fisher Scientific, Fairlawn,
Formic acid:	88%, certified, A.C.S. (Fisher Scientific, Fairlawn, NJ) 98% GR ACS (EMD Chemicals, Gibbstown, NJ)
HPLC column:	100 mm × 2.0 mm i.d. Phenomenex Luna C18(2)-HST, 2.5µ particle size
Methanol:	Optima® (Fisher Scientific, Fairlawn, NJ) HPLC grade (Fisher Scientific, Fairlawn, NJ)
Reference standards:	Malathion: Analytical grade Malaoxon: Analytical grade
Sodium chloride:	99% GR ACS (EMD Chemicals, Gibbstown, NJ)
Sodium sulfate:	Anhydrous granular (10-60 mesh), AR® (ACS), (Mallinkcrodt Chemicals, Philipsburg, NJ)
Solid phase extraction cartridges:	Oasis® HLB extraction cartridges; 3cc, 60 mg (Waters Corporation, Milford, MA; Catalog #WAT094226)
Water:	Deionized (DI) water (Polymetrics System, Morse Laboratories) HPLC Grade water (Fisher Scientific, Fair Lawn, NJ)

4.1 Reagents and Materials to be Prepared (including typical preparation instructions)

4.1.1 *0.088% formic acid in HPLC grade water:* Add 0.25 mL formic acid (88%) to a 250-mL mixing cylinder containing ~100 mL HPLC grade water. Bring to 250-mL final volume with HPLC grade water. Invert several times to mix. *Prepare weekly.*

4.1.2 *0.088% formic acid in deionized water:* Add 0.25 mL formic acid (88%) to a 250-mL mixing cylinder containing ~100 mL deionized water. Bring to 250-mL final volume with deionized water. Invert several times to mix. *Prepare weekly.*

4.1.3 *Methanol:0.088% formic acid (50:50, v/v):* Add equal amounts of HPLC methanol and 0.088% formic acid in HPLC grade water in an appropriate container. Mix well. *Prepare weekly.*

4.1.4 *5% methanol in DI water:* Add 15 mL methanol to a 500-mL mixing cylinder. Bring to 300-mL final volume with DI water. Invert several times to mix. *Prepare weekly.*

4.1.5 *Ammonium hydroxide:5% methanol in DI water (2:98, v/v):* Add 2.0 mL ammonium hydroxide (~30%) to a 100-mL mixing cylinder. Bring to 100-mL final volume with 5% methanol in DI water. Invert several times to mix. *Prepare weekly.*

4.1.6 *Acetic acid:5% methanol in DI water (2:98, v/v):* Add 2.0 mL acetic acid (glacial) to a 100-mL mixing cylinder. Bring to 100-mL final volume with 5% methanol in DI water. Invert several times to mix. *Prepare weekly.*

4.1.7 *HPLC mobile phase:*

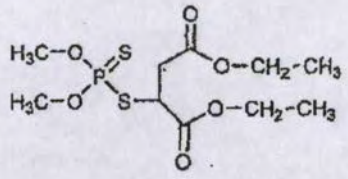
0.1% formic acid in HPLC water: To a 1-liter graduated cylinder, add 1.0 mL of 98% formic acid using a 1.0-mL graduated pipet. Bring to 1-liter final volume with HPLC grade water. Transfer entire solution to the HPLC solvent reservoir and once transferred, mix thoroughly.

5 REFERENCE STANDARDS

5.1 Malathion

Common Name: Malathion
Chemical Names:
CAS: O,O-dimethyl phosphorodithioate of diethyl mercaptosuccinate
IUPAC: diethyl (dimethoxythiophosphorylthio)succinate

Structural Formula:



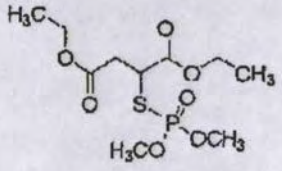
Malathion

CAS No.:	121-75-5
Molecular weight:	330.4
Source:	Cheminova A/S
Storage:	Freezer (typically -8 to -22 °C)

5.2 Malaoxon

Common Name:	Malaoxon
Chemical Names:	
CAS:	Butanedioic acid, [(dimethoxyphosphinyl)-thio]-, diethylester]
IUPAC:	2-(dimethoxyphosphorylthio) butanedioic acid diethyl ester

Structural Formula:



Malaoxon

CAS No.:	1634-78-2
Molecular weight:	314.3
Source:	Cheminova A/S
Storage:	Freezer (typically -8 to -22 °C)

6 STANDARD PREPARATION

All standard solutions prepared in this section are stored refrigerated (1 to 8 °C) in amber bottles, when not in use. Typically the following standard concentrations are prepared:

6.1 Stock Standard Solutions

Twenty-five (25.0) mg (corrected for purity) of malathion and malaoxon are accurately weighed and quantitatively transferred to separate 25-mL volumetric flasks and brought to volume with HPLC acetonitrile. The resulting stock solution concentrations are 1000 µg/mL.

Because it is difficult to weigh small amounts (generally ≤25 mg) of both liquid and solid analytical standards to specific predetermined values, they may be weighed to ±10% of the target value. When calculated, the actual concentration of the stock solution produced is expressed to three significant figures. Appropriate adjustments in the preparation of subsequent dilutions can be made in order to produce concentrations that are more manageable to work with.

6.2 Intermediate/Fortification Standard Solutions

Prepared as individual solutions:

100 µg/mL: Transfer 2.5 mL of the targeted 1000-µg/mL stock standard solution to a 25-mL volumetric flask. Bring to volume in HPLC acetonitrile. Mix well. *Prepare every 12 months.*

10 µg/mL: Transfer 2.5 mL of the targeted 100-µg/mL standard solution to a 25-mL volumetric flask. Bring to volume in HPLC acetonitrile. Mix well. *Prepare every 6 months.*

1.0 µg/mL: Transfer 2.5 mL of the targeted 10-µg/mL standard solution to a 25-mL volumetric flask. Bring to volume in HPLC acetonitrile. Mix well. *Prepare every 3 months.*

Prepared as mixtures:

100 µg/mL: Transfer 2.5 mL of each targeted 1000-µg/mL stock standard solution to a 25-mL volumetric flask. Bring to volume in HPLC acetonitrile. Mix well. *Prepare every 12 months.*

10 µg/mL: Transfer 2.5 mL of a 100-µg/mL mixed standard solution to a 25-mL volumetric flask. Bring to volume in HPLC acetonitrile. Mix well. *Prepare every 6 months.*

1.0 µg/mL: Transfer 2.5 mL of a 10-µg/mL mixed standard solution to a 25-mL volumetric flask. Bring to volume in HPLC acetonitrile. Mix well. *Prepare every 3 months.*

0.10 µg/mL: Transfer 2.5 mL of a 1.0-µg/mL mixed standard solution to a 25-mL volumetric flask. Bring to volume in HPLC acetonitrile. Mix well. *Prepare monthly.*

6.3 HPLC Calibration Standard Solutions

Prepare these mixtures monthly.

2.5 ng/mL: Transfer 625 µL of the appropriate 0.10-µg/mL mixed standard solution to a 25-mL volumetric flask. Bring to volume in HPLC methanol:0.088% formic acid (50:50, v/v). Mix well.

0.50 ng/mL: Transfer 125 µL of the 0.10-µg/mL mixed standard solution to a 25-mL volumetric flask. Bring to volume in HPLC methanol:0.088% formic acid (50:50, v/v). Mix well.

0.25 ng/mL: Transfer 125 µL of the 0.10-µg/mL mixed standard solution to a 50-mL volumetric flask. Bring to volume in HPLC methanol:0.088% formic acid (50:50, v/v). Mix well.

0.09 ng/mL: Transfer 900 µL of the 2.5-ng/mL mixed standard solution to a 25-mL volumetric flask. Bring to volume in HPLC methanol:0.088% formic acid (50:50, v/v). Mix well.

0.05 ng/mL: Transfer 500 µL of the 2.5-ng/mL mixed standard solution to a 25-mL volumetric flask. Bring to volume in HPLC methanol:0.088% formic acid (50:50, v/v). Mix well.

7 SAMPLE PREPARATION

1. If the samples are stored frozen, thaw just prior to analysis. (Completion of thawing should coincide with the start of analysis--optimally within 1-2 hours.)

2. Thawing of samples may be conducted in a refrigerator overnight up to a maximum of 3 days. If necessary, the thawing process may be completed in a 40 °C water bath (applies especially to samples thawed overnight).

3. Place sample bottles in a clean beaker when thawing to prevent sample loss.

4. Thawed samples must be well-mixed prior to handling. Shake manually.

8 SAMPLE FORTIFICATION

1. Transfer 250 mL of a well-mixed control sample to a 500-mL separatory funnel and fortify the sample with the appropriate amount (≤1.0 mL) of standard solution. Disperse solution over as much of the sample as possible.

2. Proceed to Section 9.2.

9 SAMPLE EXTRACTION

1. Transfer 250 mL of a well-mixed sample into a 500-mL separatory funnel. As applicable, fortify appropriate samples at this time.

2. Add 88 g of sodium chloride to the sample in the separatory funnel.

3. Add 50 mL dichloromethane (DCM) to the sample and shake vigorously for 2 minutes (frequently vent the separatory funnel).

4. Allow the solvents to completely separate (~5 minutes).

5. Drain the lower DCM layer through a glass funnel, which contains a glass wool plug and ~20 g of sodium sulfate, into a 200-mL Zymark tube.

6. Repeat steps 9.3 through 9.5 two additional times, combining the DCM extracts.

7. Discard the aqueous layer.

8. Rinse the sodium sulfate with 10 mL of DCM into the 200-mL Zymark tube.

9. Concentrate the sample to ~0.2 mL using a Turbo-Vap evaporator set to ≤40 °C. Manually evaporate to dryness with nitrogen. Add 10.0 mL methanol:0.088% formic acid (50:50, v/v). Mix well and sonicate.

10. Transfer the solution from Step 9.9 to a 15-mL graduated polypropylene centrifuge tube and cap. Remove aliquots for analysis as specified in Step 9.11, and store remainder at 1 to 8 °C as a retain extract of the sample if needed for reanalysis.

11. a) For samples **requiring no further cleanup**, transfer 1.0 mL of the extract from Step 9.9 to a 15-mL polypropylene centrifuge tube containing 4.0 mL HPLC methanol:0.088% formic acid (50:50, v/v). Cap the centrifuge tube, mark meniscus and vortex-mix. Submit to HPLC analysis. Final sample concentration is 1 mL = 5.0 mL sample.

b) For samples **requiring additional cleanup,** transfer 1.0 mL of the extract from Step 9.9 to a 15-mL polypropylene centrifuge tube containing 9.0 mL of 0.088% formic acid in DI water. Cap and vortex-mix. Proceed with Oasis® HLB SPE cleanup (Section 10.1).

10 OASIS® HLB SOLID PHASE EXTRACTION (SPE) CARTRIDGE CLEANUP

Note: Check or calibrate the SPE cartridges prior to use in order to ensure optimum method performance. In general, check one column per lot number. This assessment should be conducted well in advance of needing the cartridges for sample analysis. Recovery of >90% is desired to ensure that a box of columns is suitable for use. The analyses are conducted on an "analyte with no matrix present" basis. See Appendix II for detailed instructions on assessment of the SPE cartridges.

Procedure:

1. Set up the Visiprep system and support apparatus and proceed with Oasis® HLB SPE cleanup. In general, set vacuum to produce a flow rate of approximately 2 mL/minute (not continuous flow) for all elutions.

2. Condition an Oasis® HLB cartridge (size 3 cc, 60 mg) by passing 2 mL methanol followed by 2 mL deionized water, through the cartridge. Do not allow the cartridge to go dry in between any of the conditioning steps or between conditioning and sample introduction. (Stop elution when each conditioning solvent reaches top of frit.) Discard all eluates.

3. Pass the sample extract (10 mL) from Step 9.11 b) through the SPE cartridge. Stop elution when the solvent reaches the top of the frit. Discard the eluate. Residues of malathion and malaoxon are retained on the cartridge.

4. Add 1 mL of 5% methanol in DI water to the empty centrifuge tube that contained the sample extract. Shake to rinse the tube and pass the rinse through the cartridge. Stop elution when solvent reaches top of frit. Discard eluate.

5. Wash the sample laden cartridge with 1 mL of ammonium hydroxide:5% methanol in DI water (2:98, v/v). Stop elution when solvent reaches top of frit. Discard eluate.

6. Wash the sample laden cartridge with 1 mL of acetic acid:5% methanol in DI water (2:98, v/v). Stop elution when solvent reaches top of frit. Discard eluate.

7. Place a 15-mL polypropylene centrifuge tube, calibrated at 5.0 mL, under the SPE cartridge.

8. Elute both analytes with 2.0 mL of HPLC methanol into the calibrated test tube. Allow the cartridge to go dry under vacuum.

9. To each sample eluate, add 0.5 mL HPLC methanol. Bring to final volume of 5.0 mL with 0.088% formic acid in HPLC water. Mix well. Submit to LC/MS/MS analysis. Final sample concentration is 1 mL = 5.0 mL sample.

11 HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS

Note: The column and conditions stated in the method have been satisfactory for the matrices being analyzed. The specific column packing, mobile phase, column temperature and flow rate listed are typical conditions for this analysis. Alternate columns may be used depending on the need to resolve analyte and/or interfering responses. Specific conditions used will be noted on each chromatographic run and will not otherwise be documented.

11.1 Operating Conditions

Instrument: Applied Biosystems/Sciex API 4000 LC/MS/MS System with ACQUITY UPLC System including Sample Organizer with Applied Biosystems/MDS Sciex Analyst Software for data collection and system control (version 1.5)

HPLC column: 100 mm × 2.0 mm i.d. Phenomenex Luna C18(2)-HST, 2.5µ particle size

Mobile phase: *Fisher HPLC grade* water, *EMD GR ACS formic acid, Fisher HPLC grade acetonitrile*

Component A: 0.1% formic acid in HPLC water

Component B: 100% HPLC acetonitrile

Gradient:

Time (min.)	% A	% B
0.0-0.5	75	25
5.0-7.0	5	95
7.1-10	75	25

Flow rate: 0.2 mL/min.

Interface probe: TIS

Ionization mode: Positive (+)

Acquisition mode: MRM

Source temperature: 350 °C

Curtain gas: Nitrogen @ setting of "10"

Collision gas: Nitrogen @ setting of "6"

Injection volume: 10 µL

Column temperature: 40 °C

Autosampler tray temperature: ambient

Transitions monitored:

	Ion, *m/z* Q1	Ion, *m/z* Q3	Time, *ms*	CE, v	
Malathion:	331	285	150	11	(quantitation)
	331	127	150	19	(alternate)
	331	99	150	19	(alternate)
Malaoxon:	315	127	150	19	(quantitation)
	315	143	150	17	(alternate)
	315	99	150	17	(alternate)

Retention times: Malathion: ~ 5.75 minutes
Malaoxon: ~ 4.30 minutes

11.2 Sample Analysis

Prepare a five-point standard curve by injecting constant volumes of standard solutions. Use constant volume injections for sample extracts as well. For sample responses greater than those produced by the highest concentration of standard curve, dilution and reinjection are required. Inject a curve check standard every 3-4 sample injections.

12 CALCULATIONS

Calculations for instrumental analysis are conducted using a validated software application to create a standard curve based on linear regression. The regression functions are used to calculate a best-fit line (from a set of standard concentrations in ng/mL versus peak response) and to determine concentrations of the analyte found during sample analysis from the calculated best fit line. **Weighting (1/x) is used.**

The equation used for the least squares fit is:

$$y = mx + b$$

where:

y	=	peak response
m	=	slope
x	=	ng/mL found for peak of interest
b	=	y-intercept

The calculations for ppt found and percent recovery (for fortified samples) are:

1. The amount of analyte found (in ppt) in the sample is calculated according to the following equation:

$$ppt = ng/mL\ found \times \frac{final\ vol.(mL)}{samp.\ vol.(mL)} \times \frac{total\ ext.\ solv.(mL)}{aliq.\ 1\ (mL)} \times \frac{reconst.\ vol.(mL)}{aliq.\ 2\ (mL)} \times \frac{1000\ mL}{1\ L} \times HPLC\ dil.\ fact.$$

where:

ng/mL found	=	ng/mL of analyte found from standard curve
final vol. (mL)	=	volume of final extract submitted to instrumentation (typically 5.0 mL)
samp. vol. (mL)	=	volume of water sample (typically 250 mL)
total ext. solv. (mL)	=	total mL extraction solvent added (typically 150 mL)
aliq. 1 (mL)	=	volume of extract taken through the procedure (typically 150 mL)
reconst. vol. (mL)	=	volume of reconstitution solvent (typically 10 mL)

aliq. 2 (mL)	=	aliquot of sample extract taken for dilution or SPE (1.0 mL)
1000 mL/1 L	=	conversion factor for milliliter to liter
HPLC dil. factor	=	dilution of sample extract required to produce an analyte response bracketed by standards

2. The percent recovery for fortified control samples is calculated as follows:

$$\%\ Recovery = \frac{ppt\ found\ in\ fortified\ control - ppt\ found\ in\ control}{ppt\ added} \times 100$$

13 REFERENCES

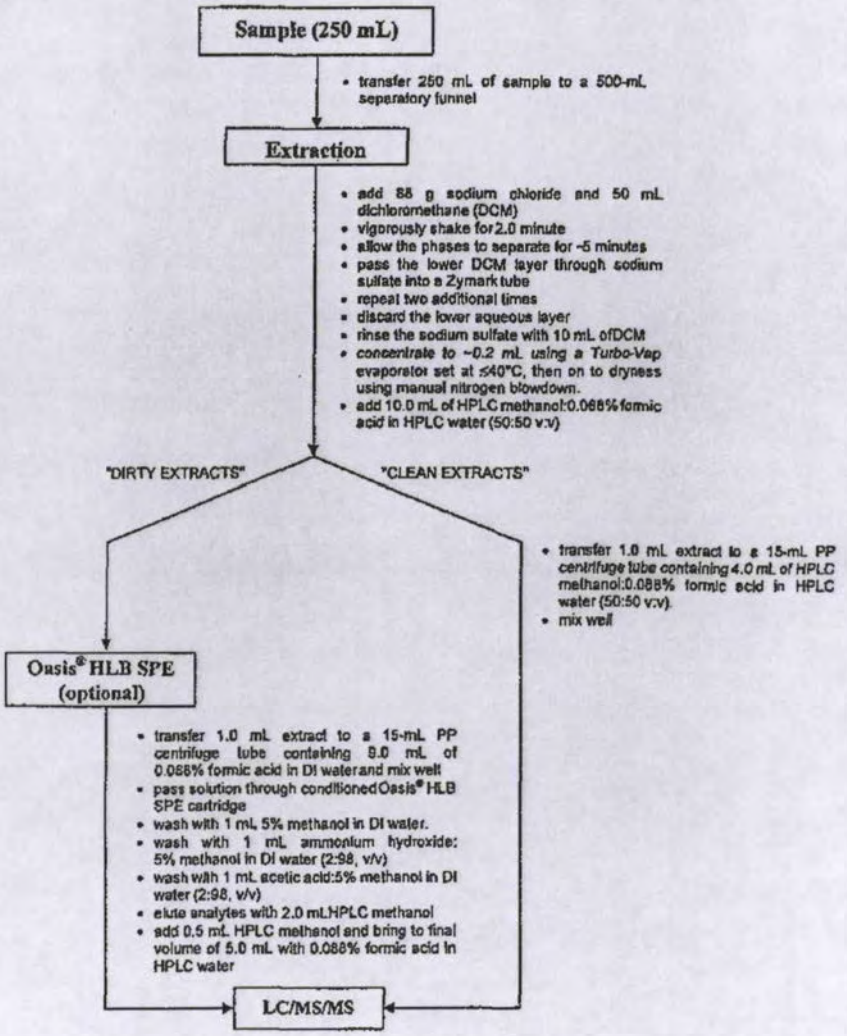
1. Institut Fresnius "Determination of Malathion in Water-Validation of the Method," Study Number IF-02/00004583, internal report dated May 14, 2002.

2. Morse Laboratories, "Determination of Malathion and its Metabolites Malaoxon, Desmethyl Malathion, Malathion Monocarboxylic Acid and Malathion Dicarboxylic Acid in Crops (Raw Agricultural and Processed Commodities)," Analytical Method #Meth-198, Original, dated December 16, 2009.

3. Morse Laboratories, "Determination of Selected Organophosphate Pesticides in Fruits and Vegetables," Analytical Method #Meth-117, Revision #3, dated January 14, 1999.

Method author: Kevin Clark

ANALYSIS FLOWCHART



Quality Control for Oasis® HLB SPE Cartridges:

1. Transfer 25 µL of a 0.10-µg/mL malathion and malaoxon mixed standard solution in acetonitrile to a 15-mL PP centrifuge tube containing 1.0 mL of methanol, 1.0 mL of DI water and 8.0 mL of 0.088% formic acid in DI water. Mix well.
2. Follow Steps 10.1 through 10.9 of the procedure.
3. Resulting analyte concentration is 1 mL = 0.50 ng.
4. Submit to LC/MS/MS analysis.

Appendix E Summary of communications regarding method between Sponsor, Study Director and Method developer.

Date: 10/11/2011

Communication between Study Director and Sponsor by e-mail

Summary of communication: Informing the Sponsor of the unacceptable – not within 70-120%- recovery of malathion from an initial trial of the pond water with the method. Also informing sponsor that SPE cleanup as specified by method did not lead to an improvement in the pond water recoveries.

Date: 10/13/2011

Communication between Sponsor and Study Director by e-mail

Summary of communication: Sponsor asks questions/comment about the reproducibility of the injections, difference in physical characteristics of pond water used in ILV from water used in development of method, further dilution of samples, and possible error in fortification.

Date: 10/18/2011

Communication between Sponsor and Study Director by e-mail

Summary of communication: Sponsor suggests that after the issues addressed in the 10/13/2011 communication have been ruled out as a source of the unacceptable recoveries in the pond water samples, Study director should have conversation with developer (Morse Laboratories) to compare instrumentation/equipment used in initial ILV with that used for method development

Date: 10/20/2011

Communication between Study Director and Sponsor by e-mail

Summary of communication: Study director details reproducibility of injection experiments which show that injection error is unlikely as a source of the poor recoveries in the pond water samples. Study Director details a reanalyses of the pondwater samples

which showed similar unacceptable recoveries as the initial analyses. Study director confirms that the method followed was identical to that developed by Morse Laboratories and provided to the Study Director by the sponsor at the commencement of the study. Study Director informs Sponsor of the pH of the water used.

Date: 10/26/2011

Communication between Sponsor and Study Director by e-mail

Summary of communication: Sponsor suggests that Study Director use the same HPLC column as used by method developer (Morse laboratories) and same mobile phase,. Sponsor requests Study Director to check all calculations as regards pond water recovery values.

Date: 10/27/2011

Communication between Study Director and Sponsor by e-mail

Summary of communication: Study Director confirms that the same HPLC column as used by method developer (Morse laboratories) and same mobile phase will be used for all work. Study Director confirms all calculations as reagrds pond water recovery values were checked.

Date: 10/31/2011

Communication between Study Director and Method developer (Morse Laboratories, Shawna Brown, browns@morselabs.com, Tel: 916-481-3141) by telephone

Summary of communication: Study Director confirms that method was performed in an identical manner by PTRL West and method developer for all samples. Method developer conforms that method was developed using river water and well water and not developed using pond water as a matrix. Developer confirms that SPE cleanup was performed for river water samples. Developer confirms that an emulsion upon Liquid Liquid partition was not observed in their development work with the river water and well water samples. Developer agrees that such an emulsion as observed by PTRL West in pond water may require additional experimental steps to disperse, additional to that described in the developed method.

Date: 11/08/2011

Teleconference between Study Director and PTRL West representatives (Janine Marin, Luis Ruzo) and Sponsor and Sponsor advisor (Mark Schocken)

Summary of communication: Means by which the emulsion which results from Step 2.11.3 – Dichloromethane Liquid Liquid partition of pond water samples could be dispersed were discussed. It was agreed that a simple centrifugation at that point of the method should be accepted a method modification and that a new trial should be attempted utilizing this method modification.

Date: 12/12/2011

Communication between Sponsor and Study Director by e-mail

Summary of communication: Sponsor requests that Study Director incorporate the minor modification of a centrifugation step into the method provided and perform a second ILV on Well and Pond water.