1 INTRODUCTION

Morse Laboratories, LLC Analytical Method# Meth-207, Revision #1, entitled "Determination of Malathion and Malaoxon in Soil by LC-MS/MS" (Appendix I) was the procedure validated in this study. No changes were made to the method during the course of this study. [**Note:** The title of the method used during the validation, "Determination of Malathion and Malaoxon in Soil," was not as intended; therefore Revision #1 to the method was generated to make the change which now correctly reads "Determination of Malathion and Malaoxon in Soil by LC-MS/MS." It is the method of record. The original method (dated May 25, 2011) and Revision #1 (dated June 16, 2011) are identical in every way except for the title.]

The method was validated on two different textural classes of soil (sandy loam and sandy clay loam) at two concentrations, the limit of quantitation (LOQ) of 0.01 ppm and at 1.0 ppm (100 × LOQ). The validation results are reported herein.

This study was conducted by Morse Laboratories, LLC (Morse Labs) of Sacramento, California, U.S.A., according to Study Protocol No. 66797, entitled "Validation of the Residue Analytical Method: 'Determination of Malathion and Malaoxon in Soil by LC-MS/MS '" (Appendix II).

This report contains the following: reference material information, experimental details, method summary, method comments, calculations, results and discussion, example chromatography, and the data generated from the analyses performed by Morse Laboratories, LLC personnel.

2 PRINCIPLE

The method described herein is capable of determining residues of malathion and malaoxon in soil.

To summarize, residues of malathion and malaoxon were extracted from the sample with acetonitrile using multiple extractions (3 extractions). Following each extraction, the mixture was centrifuged and the crude extract (supernatant) was decanted and combined into a graduated cylinder. The combined extracts were brought to a final known volume, and then mixed thoroughly. An aliquot of the combined extract was purified by means of an Oasis® HLB solid phase extraction (SPE) cleanup. The methanolic SPE eluate was concentrated to 1.0 mL, brought to a final volume of 2.0 mL with HPLC grade methanol:0.088% formic acid (50:50, v/v), then submitted to high performance liquid chromatographic (HPLC) analysis. Determination and quantitation for malathion and malaoxon were conducted using HPLC employing tandem mass

spectrometric (MS/MS) detection (LC/MS/MS) in the positive ionization mode. The limit of quantitation (LOQ) was 0.01 ppm for both analytes.

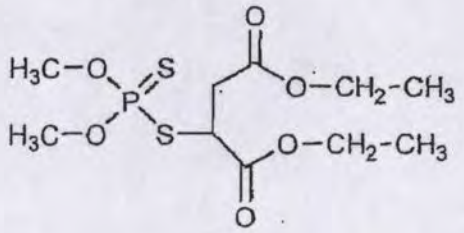
During the conduct of this analysis, comparable apparatus, solvents, glassware, and techniques (such as sample extract evaporation) were allowed to be substituted for those described in this method, except where specifically noted otherwise. In the event a substituted piece of equipment or technique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

3 REFERENCE MATERIALS

The analytical standards used for this study were:

Malathion:

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

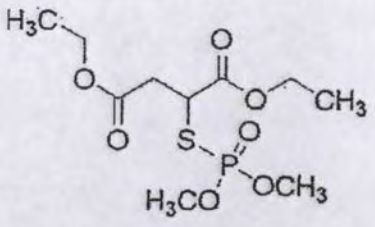


CAS No.: 121-75-5
Molecular weight: 330.4 g/mol
Source: Cheminova A/S
Purity: 99.6% w/w
Lot no.: 650-OSJ-36E
Date received: July 1, 2009
Expiration date: March 1, 2018
Storage: -8 °C to -22 °C

Malaoxon:

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

Structural Formula:



CAS No.:	1634-78-2
Molecular weight:	314.3 g/mol
Source:	Cheminova A/S
Purity:	96.2% w/w
Lot no.:	849-BSe-39B
Date received:	October 15, 2007
Expiration date:	October 6, 2014
Storage:	-8 °C to -22 °C

4 TEST SYSTEMS

Two textural classes of soil were evaluated in this study: sandy loam and sandy clay loam. Both soils were provided by the Sponsor. The soils were received homogenized on April 27, 2011. No further processing was required.

Upon receipt of the samples at the laboratory the soils were immediately placed in frozen storage (typically −20 ± 5 °C), where they remained pending analysis for suitability.

Characterization data for the soils, provided by the Sponsor, is summarized below.

	Sandy Loam	Sandy Clay Loam
Location	Fresno	Northwood
State	CA	ND
Sample ID	1810W-029	1810W-033
Characterization:		
% sand	75	63
% silt	14	16
% clay	11	21
USDA textural class	sandy loam	sandy clay loam
FAO Textural Class	coarse	medium
Bulk density (gm/cc)	1.36	1.02
Cation exchange capacity (meq/100g)	8.8	17.0
Moisture at 1/3 bar	9.4	21.9
% organic matter	0.6	3.4
pH (1:1 soil:water)	7.5	6.8

The Agvise soil characterization reports, as provided by the Sponsor, are found in Appendix IV.

5 ANALYTICAL METHOD

Morse Laboratories, LLC Analytical Method #Meth-207, Revision #1, entitled "Determination of Malathion and Malaoxon in Soil by LC-MS/MS" (Appendix I) was the procedure validated in this study. No changes were made to the method during the course of this study. A summary of the method was provided in the "Principle" section of this report.

6 INSTRUMENTATION

All samples were analyzed by HPLC employing tandem mass spectrometric (MS/MS) detection. Typical conditions were as follows:

6.1 Operating Conditions

Instrument: Applied Biosystems/Sciex API 4000 LC/MS/MS System with Shimadzu LC-20AD Liquid Chromatographs and Shimadzu SIL-20AC Autosampler, Shimadzu DGU-20A5 degasser, and Shimadzu CBM-20A Communications Bus Module (System Controller) 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

Column Divert Program: Programmed to divert LC flow from column to waste (bypassing detector) from 0 to 5.1 minutes and again from 9.0 to 10.0 minutes. LC flow is directed to detector during the 5.1 to 9.0 minute window. Diversion time settings can be adjusted as necessary depending on the retention time of the analytes.

Flow rate: 0.2 mL/min.

Interface probe: TIS

Ionization mode: Positive (+)

Acquisition mode: MRM

Source temperature: 350 °C

Curtain gas: Nitrogen @ setting of "20"

Collision gas: Nitrogen @ setting of "6"

Injection volume: 10 µL

Column temperature: 40 °C

Autosampler tray temperature: ambient

Transitions monitored:

	Ion, *m/z*		Time, *ms*	CE, *v*	
	Q1	Q3			
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: ~ 7.6 minutes
Malaoxon: ~ 6.2 minutes

6.2 Calibration/Sample Analysis

For the analysis of samples, a five-point standard curve was prepared by injecting constant volumes of standard solutions containing both malathion and malaoxon. Constant volume injections were used for sample extracts as well. Sample responses greater than those produced by the highest concentration of applicable standard in the standard curve required dilution and reinjection. A curve check standard was typically injected every 2-3 sample injections.

7 CALCULATIONS

7.1 Equations

Calculations for malathion and malaoxon were conducted using a validated software application to create a standard curve based on linear regression. The regression functions were 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) was 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

Note: A standard curve is generated by plotting the standard concentration (in µg/mL) on the x-axis and the respective peak response on the y-axis.

The standard (calibration) curve generated for each analytical set was used for the quantitation of both malathion and malaoxon in the samples. For this study, the correlation coefficient (r) for each calibration curve was equal to or greater than 0.990 (r^2 equal to or greater than 0.98).

The calculations for ppm found and percent recovery (for fortified samples) were:

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

$$ppm = ng/mL\ found \times \frac{final\ vol.(mL)}{sample\ wt.(g)} \times \frac{ext.solv.(mL)}{aliq.(mL)} \times \frac{1\ \mu g}{1000\ ng} \times HPLC\ dil.\ factor$$

where:

ng/mL found	=	ng/mL of analyte found from standard curve
final vol. (mL)	=	volume of final extract submitted to instrumentation (typically 2.0 mL)
sample wt. (g)	=	gram weight of sample extracted (typically 10.0 g)
ext. solv. (mL)	=	volume of extraction solvent (typically 500 mL)

aliq. (mL)	=	volume of extract taken through Oasis® HLB SPE cleanup (typically 1.0 mL)
1 µg/1000 ng	=	conversion factor
HPLC dil. factor	=	dilution of sample extract required to produce analyte responses bracketed by standards

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

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

*Average of two control samples analyzed

7.2 Example Calculations

Malathion and malaoxon were calculated in exactly the same manner. Only examples of malathion calculations in sandy loam soil will be provided and thus serve to illustrate the calculations of both analytes in both soil types.

1. ML ticket #87621, Malathion, Sandy loam soil, Set #1, 1810W-029, **Control** 1:

$$2890\ peak\ response\ (area) \rightarrow 0.0244\ ng/mL$$

$$ppm = 0.0244\ ng/mL \times \frac{2.0\ mL}{10.0\ g} \times \frac{500\ mL}{1.0\ mL} \times \frac{1\ \mu g}{1000\ ng} \times 1$$

$$ppm = 0.00244$$

$$Reported\ ppm = < 0.01$$

- $Average\ of\ two\ control\ samples\ analyzed = \frac{0.00244\ ppm + 0.00240\ ppm}{2} = 0.00242\ ppm$

2. ML ticket #87621, Malathion, Sandy loam soil, Set #1, 1810W-029, **Fortified Control** 2 @ 0.01 ppm:

$$12500 \text{ peak response (area)} \rightarrow 0.119 \text{ ng/mL}$$

$$ppm = 0.119 \text{ ng/mL} \times \frac{2.0 \text{ mL}}{10.0 \text{ g}} \times \frac{500 \text{ mL}}{1.0 \text{ mL}} \times \frac{1 \mu g}{1000 \text{ ng}} \times 1$$

$$ppm = 0.0119$$

$$\text{Reported ppm} = 0.0119$$

$$\% \text{ Recovery} = \frac{0.0119 \text{ ppm} - 0.00242 \text{ ppm}}{0.01 \text{ ppm}} \times 100$$

$$= 95\%$$

3. ML ticket #87621, Malathion, Sandy loam soil, Set #1, 1810W-029, **Fortified Control** 10 @ 1.0 ppm, 1→10 dilution:

$$92500 \text{ peak response (area)} \rightarrow 0.912 \text{ ng/mL}$$

$$ppm = 0.912 \text{ ng/mL} \times \frac{2.0 \text{ mL}}{10.0 \text{ g}} \times \frac{500 \text{ mL}}{1.0 \text{ mL}} \times \frac{1 \mu g}{1000 \text{ ng}} \times 10$$

$$ppm = 0.912$$

$$\text{Reported ppm} = 0.912$$

$$\% \text{ Recovery} = \frac{0.912 \text{ ppm} - 0.00242 \text{ ppm}}{1.0 \text{ ppm}} \times 100$$

$$= 91\%$$

8 EXPERIMENTAL PROCEDURES

8.1 Sample Processing

The control samples were received homogenized and required no further processing.

8.2 Control Suitability

The control samples used in this study were analyzed in a related non-GLP method development phase (Reference 1) prior to initiation of this study. No malathion or malaoxon residues ≥30% of the LOQ were found in the control samples and were therefore judged suitable for use in this study.

8.3 Fortification Procedures

Aliquots of bulk control sample were fortified with microliter amounts of appropriate analytical standard solution(s). Fortification was conducted with mixed standards (standards containing both analytes). During fortification, the standard solution was evenly distributed over the exposed sample as much as possible.

Control samples were fortified according to the following scheme:

Matrix	Sample Type	Fortifying Compounds	Fortification Level	No. of Samples
Soil	Control	none	0.0 ppm	2
	Fortified control	Malathion and malaoxon	LOQ (0.01 ppm)	5
	Fortified control	Malathion and malaoxon	100×LOQ (1.0 ppm)	5

8.4 Analysis Scheme

Samples were analyzed in sample sets. For sandy loam soil, one sample set was analyzed and consisted of two replicates of unfortified control sample, five replicates of control sample fortified with malathion and malaoxon at 0.01 ppm (LOQ), and five replicates of control sample fortified with malathion and malaoxon at 1.0 ppm (100 × LOQ). For sandy clay loam soil, two sample sets were analyzed. One consisted of two replicates of unfortified control sample and five replicates of control sample fortified with malathion and malaoxon at 0.01 ppm (LOQ), and the other set consisted of two replicates of unfortified

control sample and five replicates of control sample fortified with malathion and malaoxon at 1.0 ppm (100 × LOQ).

9 CONFIRMATION

Ion-ratioing using data generated from 3 molecular ion transitions for both malathion and malaoxon was used to confirm the residues. Besides the monitoring of the primary quantitation transition, the MS/MS operating conditions included the monitoring of 2 additional transitions that made the determination of three confirmation ratios possible.

Analyte	Quadrupole-1 Ion (*m/z*)	MS/MS Fragment Ions (*m/z*)
Malathion	331	**285** 127 99
Malaoxon	315	**127** 143 99

*quantitation ion in bold type

Therefore for malathion, along with the quantitation transition of *m/z* 331→285 (Q_{ion}), additional confirmatory transitions of *m/z* 331→127 ($C\text{-}1_{ion}$) and *m/z* 331→99 ($C\text{-}2_{ion}$) were captured. For malaoxon, along with the quantitation transition of *m/z* 315→127 (Q_{ion}), additional confirmatory transitions of *m/z* 315→143 ($C\text{-}1_{ion}$) and *m/z* 315→99 ($C\text{-}2_{ion}$) were captured.

Three confirmation ratio pairs ($Q_{ion}/C\text{-}1_{ion}$, $Q_{ion}/C\text{-}2_{ion}$, $C\text{-}1_{ion}/C\text{-}2_{ion}$) were generated for each residue being confirmed and each applicable standard of the standard curve. An average ratio was determined for each ratio pair for the standards. The standard average was then compared to that calculated for each sample.

The acceptance criteria for the confirmation are: (1) the %RSD for the ion ratio of each ratio pair determined from the individual curve standards is ≤20%, (2) the ion ratio of each ratio pair for an analyte response in a sample is within ±25% (for fortifications above the LOQ) or ±30% (for fortifications at the LOQ of 0.01 ppm) of the average standard ion ratio for each pair.

10 INTERFERENCES

Detailed interference studies were not performed. No interferences due to solvents or labware were observed.

11 TIME REQUIRED FOR ANALYSIS

A set of 12 samples, from initial extraction to setting the samples up for HPLC analysis, required approximately 6 hours. Set up of the HPLC analysis required approximately 1.0 hour. The automated HPLC analysis took approximately 4.0 hours for the quantitation run. Data processing and review took approximately 1-2 hours.

12 COMMENTS/MODIFICATIONS/RECOMMENDATIONS

None.

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

Reason for Revision: To correct the title of the original version, dated May 25, 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 soil.

Residues of malathion and malaoxon are extracted from the sample with acetonitrile using multiple extractions (3 extractions). Following each centrifugation, the crude extract (supernatant) is decanted and combined into a graduated cylinder. The combined extracts are brought to a final known volume, and then mixed thoroughly. An aliquot of the combined extract is purified by means of an Oasis® HLB solid phase extraction (SPE) cleanup. The purified extract is submitted in HPLC methanol:0.088% formic acid (50:50, v/v) for HPLC analysis.

During routine analysis, determination and quantitation for malathion and malaoxon are conducted using HPLC employing mass spectrometric (MS/MS) detection (LC/MS/MS). The limit of quantitation (LOQ) is 0.01 ppm for malathion and malaoxon.

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
Centrifuges:	Centrific™ Centrifuge (Fisher Scientific, Fairlawn, NJ)
	IEC Clinical centrifuge (International Equipment Co., Needham Heights, MA)
Evaporator:	N-Evap Laboratory Sample Evaporator Model 115 attached to a N_2 source (Organomation Assoc., South Berlin, MA)
Extract storage containers:	Polypropylene (PP), graduated; 50-mL

Extraction vessels:	HDPE centrifuge bottles; 250-mL (VWR Scientific, Bridgeport, NJ)
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, 9-inch and 5½-inch, disposable
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
Platform shaker:	Eberbach Model 6000 (Eberbach Corp., Ann Arbor, MI)
Solid Phase Extraction Apparatus:	Visiprep 12 or 24-port SPE vacuum manifold with disposable flow control liners (Supelco, Bellefonte, PA)
Standard bottles:	Glass, amber; various sizes
Ultrasonic bath:	Branson Model 2210 ultrasonic bath (VWR Scientific, Bridgeport, NJ)
Volumetric flasks:	Glass; various sizes

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)
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
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 (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 of 88% formic acid 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 of 88% formic acid 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:*

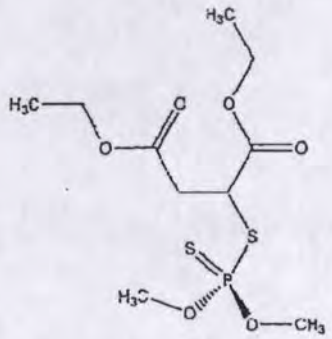
0.1% formic acid in HPLC water: To a 1-liter graduated cylinder, add 1.0 mL of 98% formic acid using a 1.0-mL graduated pipet. Bring to 1-liter final volume with HPLC grade water. Transfer entire solution to the HPLC solvent reservoir and once transferred, mix thoroughly.

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:



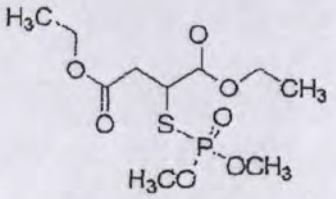
Malathion

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 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.

0.10 ng/mL: Transfer 1.0 mL 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 FORTIFICATION

1. Weigh 10.0 g of homogenized soil sample into a 250-mL HDPE centrifuge bottle.
2. Fortify the sample with the appropriate amount of standard solution. Disperse solution over as much of the sample as possible. Use a volume of ≤1.0 mL.
3. Proceed with Step 8.2.

8 SAMPLE EXTRACTION

1. Weigh 10.0 g of homogenized soil sample into a 250-mL HDPE centrifuge bottle. As applicable, fortify appropriate samples at this time.
2. Add 100 mL of acetonitrile. Cap and shake on a platform shaker at medium speed (~180 excursions per minute) for 30 minutes.
3. Centrifuge mixture at ~3000 rpm for ~10 minutes.
4. Decant supernatant (extract) into a 500-mL glass graduated mixing cylinder.
5. Repeat Steps 8.2 and 8.3 two additional times, decanting all extracts into the same cylinder from Step 8.4.
6. Bring the combined extracts to a final volume of 500 mL with acetonitrile. Mix well.

7. Transfer ~ 30 mL to a 50-mL graduated polypropylene centrifuge tube and cap. Mix well. Centrifuge, if necessary, at ~3000 rpm for ~10 minutes. Remove aliquots for analysis as specified in Step 8.8, and store remainder at 1 to 8 °C as a retain extract of the sample if needed for reanalysis.

8. Transfer 1.0 mL of the extract from Step 8.7 to a 15-mL polypropylene centrifuge tube containing 9.0 mL of 0.088% formic acid in DI water. Cap and vortex-mix. Proceed to Section 9.

9 OASIS® HLB SPE CARTRIDGE CLEANUP

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 8.8 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 1.0 mL and 2.0 mL, under the SPE cartridge.

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

9. Using an N-Evap with the water bath set @ 35 °C; concentrate the eluate to the 1.0 mL calibrated mark.

10. Bring to final volume of 2.0 mL with 0.088% formic acid in HPLC water. Sonicate and vortex to mix well. Final sample concentration is 1 mL = 0.01 g sample. Submit to LC/MS/MS analysis.

10 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 water, EMD formic acid, Fisher HPLC 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*		Time, *ms*	CE, *v*	
	Q1	Q3			
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

10.2 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.

11 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 ppm found and percent recovery (for fortified samples) are:

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

$$ppm = ng/mL\ found \times \frac{final\ vol.\ (mL)}{sample\ wt.\ (g)} \times \frac{ext.\ solv.\ (mL)}{aliq.\ (mL)} \times \frac{1\ \mu g}{1000\ ng} \times HPLC\ dil.\ factor$$

where:

ng/mL found	=	ng/mL of analyte found from standard curve
final vol. (mL)	=	volume of final extract submitted to instrumentation (typically 2.0 mL)
sample wt. (g)	=	gram weight of sample extracted (typically 10.0 g)
ext. solv. (mL)	=	volume of extraction solvent (typically 500 mL)
aliq. (mL)	=	volume of extract taken through Oasis® HLB SPE cleanup (typically 1.0 mL)
1 µg/1000 ng	=	conversion factor
HPLC dil. factor	=	dilution of sample extract required to produce analyte responses bracketed by standards

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

$$\%\ Rec. = \frac{ppm\ found\ in\ fortified\ control\ (spike) - ppm\ found\ in\ control}{fortification\ level\ (ppm)\ added} \times 100$$

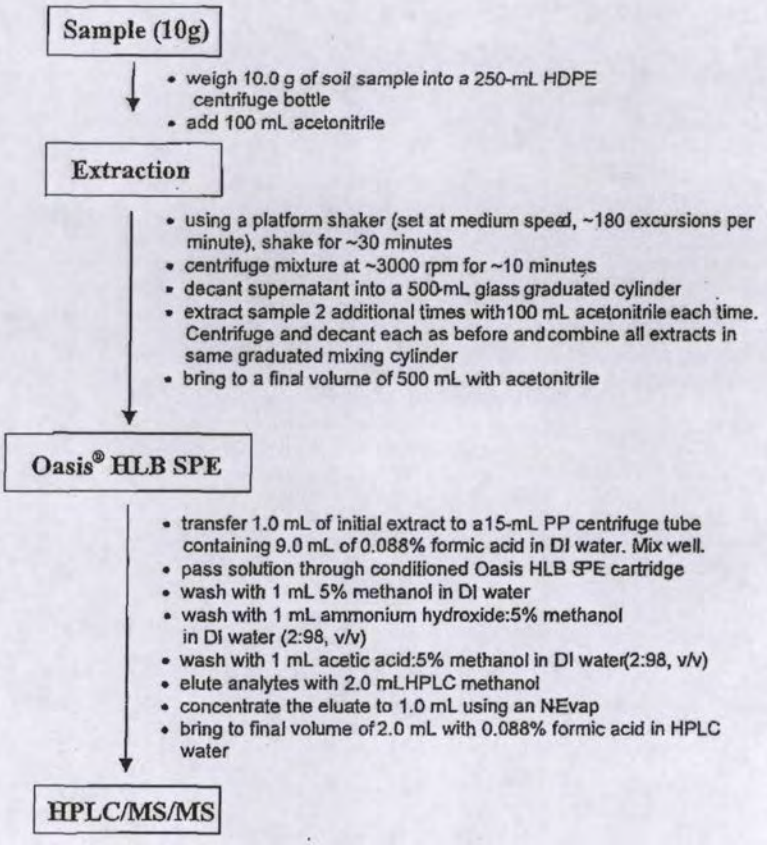
12 REFERENCES

1. Rice, F., Jacobson, B., Lochhaas, C., "Terrestrial Field Dissipation for Malathion in Cotton (California)," Analytical Bio-Chemistry Laboratories Inc., Report No. 38003, Unpublished Report, CHA Doc. No.: 43 FYF, MRID No. 41727701, dated December 3, 1990.

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.

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 acetonitrile, and 9.0 mL of 0.088% formic acid in DI water. Mix well.

2. Follow Steps 9.1 through 9.6 of the procedure.

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

4. Elute the analytes with 2.0 mL of HPLC methanol into the calibrated polypropylene centrifuge tube. Allow the cartridge to go dry under vacuum.

5. Add 0.5 mL HPLC methanol. Bring to final volume of 5.0 mL with 0.088% formic acid in HPLC water. Mix well.

6. Resulting analyte concentration is 1 mL = 0.50 ng.

7. Submit to LC/MS/MS analysis.