1 INTRODUCTION

Morse Laboratories, LLC Analytical Method #Meth-206, Revision #1, entitled "Determination of Malathion and Malaoxon in Water 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 Water," 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 Water by LC-MS/MS." It is the method of record. The original method (dated May 20, 2011) and Revision #1 (dated June 16, 2011) are identical in every way except for the title and some typographical error corrections.]

The method was validated on two types of water (surface and ground) at two concentrations, the limit of quantitation (LOQ) of 0.018 ppb (18 ppt) and at 18,000 ppb (1,000,000 × 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. 66799, entitled "Validation of the Residue Analytical Method: 'Determination of Malathion and Malaoxon in Water by LC-MS/MS'" and Protocol Amendment 1 (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 water.

To summarize, residues of malathion and malaoxon were extracted from water by partitioning three times with dichloromethane. The lower dichloromethane layer was passed through sodium sulfate, evaporated to dryness, and re-dissolved in HPLC grade methanol:0.088% formic acid (50:50, v/v). The resulting solution was submitted to high performance liquid chromatography (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 18 ppt (0.018 ppb) 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). It was included in the analyses conducted in this study.

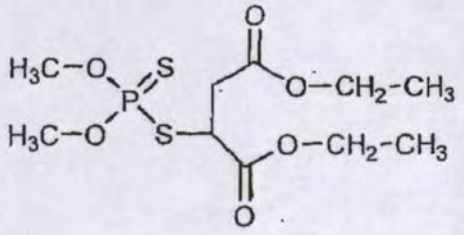
During the conduct of this analysis, comparable apparatus, solvents, glassware, and techniques (such as sample extract evaporation) were allowed to be substituted for those described in this method, except where specifically noted otherwise. In the event a substituted piece of equipment or technique 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:	June 1, 2009
Expiration date:	March 1, 2018
Storage:	-8 °C to -22 °C

Malaoxon:

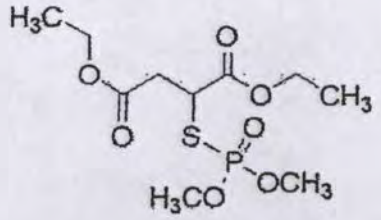
Common Name: Malaoxon

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 types of water were evaluated in this study: surface water and ground water. All waters were obtained by Morse Labs personnel in one-gallon plastic containers from local sources. Surface (river) water was collected April 15, 2011 from the American River near Sunrise Boulevard in Sacramento, CA. Ground (well) water was obtained April 14, 2011 from a residence in Sacramento, CA.

Upon receipt of the samples at the laboratory (within 2 hours of collection) they were immediately placed in refrigerated storage (typically 1-8°C), where they remained pending analysis for suitability. Once determined by analysis to be suitable for the study, an aliquot of each bulk sample was removed for subsequent characterization analysis.

The waters were characterized under GLP for pH, calcium, magnesium, sodium, hardness, conductivity, sodium absorption ratio (SAR), total dissolved solids, and turbidity by Agvise Laboratories, Inc. of Northwood, ND. Five hundred (500)-mL aliquots of each control water sample (2) were sent to Agvise Labs for analysis. The results are summarized below:

	Ground Water	Surface Water
Location	Sacramento (Well)	Sacramento (American River)
State	CA	CA
Sample ID	66799A	66799B
pH	7.7	7.7
Calcium (ppm)	48	6.2
Magnesium (ppm)	33	2.9
Sodium (ppm)	20	2.5
Hardness (mg equiv. $CaCO_3$/L)	259	28
Conductivity (mmhos/cm)	0.55	0.07
Sodium Absorption Ratio (SAR)	0.53	0.21
Total Dissolved Solids (ppm)	330	14
Turbidity (NTU)	0.93	0.82

The Agvise analysis report is found in Appendix IV.

5 ANALYTICAL METHOD

Morse Laboratories, LLC Analytical Method #Meth-206, Revision #1, entitled "Determination of Malathion and Malaoxon in Water 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 "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: ~ 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 ppb) in the sample is calculated according to the following equation:

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

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 10.0 mL)
aliq. 2 (mL)	=	aliquot of sample extract taken for dilution or SPE (typically 1.0 mL)
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:

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

7.2 Example Calculations

Malathion and malaoxon were calculated in exactly the same manner. Only examples of malathion calculations in surface (river) water will be provided and thus serve to illustrate the calculations of both analytes in both water types.

1. ML ticket #87555, Malathion, River water, Set #5, 66799B, **Control** 9:

$$0\ peak\ response\ (area) \rightarrow 0.00\ ng/mL$$

$$ppb = 0.00\ ng/mL \times \frac{5.0\ mL}{250\ mL} \times \frac{150\ mL}{150\ mL} \times \frac{10.0\ mL}{1.0\ mL} \times 1$$

$$ppb = 0$$

$$Reported\ ppb = ND$$

2. ML ticket #87555, Malathion, River water, Set #5, 66799B, **Fortified Control** 33 @ 0.018 ppb:

$$10700\ peak\ response\ (area) \rightarrow 0.0822\ ng/mL$$

$$ppb = 0.0822\ ng/mL \times \frac{5.0\ mL}{250\ mL} \times \frac{150\ mL}{150\ mL} \times \frac{10.0\ mL}{1.0\ mL} \times 1$$

$$ppb = 0.01644$$

$$Reported\ ppb = 0.0164$$

$$\% \ Recovery = \frac{0.0164 \ ppb - 0.000 \ ppb}{0.018 \ ppb} \times 100$$

$$= 91\%$$

3. ML ticket #87555, Malathion, River water, Set #5, 66799B, **Fortified Control** 37 @ 18,000 ppb, 1→80,000 dilution:

$$104000 \ peak \ response \ (area) \rightarrow 0.818 \ ng/mL$$

$$ppb = 0.818 \ ng/mL \times \frac{5.0 \ mL}{250 \ mL} \times \frac{150 \ mL}{150 \ mL} \times \frac{10.0 \ mL}{1.0 \ mL} \times 80000$$

$$ppb = 13088$$

$$Reported \ ppb = 13088$$

$$\% \ Recovery = \frac{13088 \ ppb - 0.000 \ ppb}{18000 \ ppb} \times 100$$

$$= 73\%$$

8 EXPERIMENTAL PROCEDURES

8.1 Sample Processing

The control samples were stored refrigerated (REF-18-98), typically at 1 to 8°C, prior to subsampling for analysis (either characterization analysis or residue analysis). In either case, the samples were mixed well prior to removal of the aliquots for analysis.

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 both mixed standards (standards containing both analytes) and individual analyte containing standards, depending on the fortification level (mixed standards for LOQ fortifications and individual standards for high-level fortifications). Following fortification, the sample was hand-shaken for about 30 seconds so that the malathion and/or malaoxon was homogeneously distributed in the water sample.

Control samples were fortified according to the following scheme:

Matrix	Sample Type	Fortifying Compounds	Fortification Level[a]	No. of Samples
Water	Control	none	0.0 ppm	2
	Fortified control	Malathion and malaoxon	LOQ (0.018 ppb)	5
	Fortified control	Malathion and malaoxon	1,000,000×LOQ (18,000 ppb)	5

[a]0.018 ppb = 18 ppt (parts per trillion); 18,000 ppb = 18 ppm

8.4 Analysis Scheme

Samples were analyzed in sample sets. For surface (river) water, 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 0.018 ppb (LOQ), and five replicates of control sample fortified with malathion and malaoxon at 18,000 ppb (1,000,000 × LOQ). For ground (well) water, 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 0.018 ppb (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 18,000 ppb (1,000,000 × 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 0.018 ppb) 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.

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:*

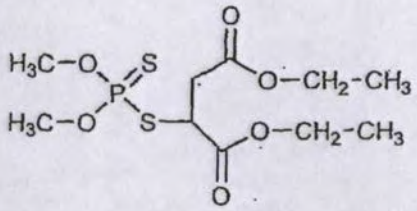
0.1% formic acid in HPLC water: To a 1-liter graduated cylinder, add 1.0 mL of 98% formic acid using a 1.0-mL graduated pipet. Bring to 1-liter final volume with HPLC grade water. Transfer entire solution to the HPLC solvent reservoir and once transferred, mix thoroughly.

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:



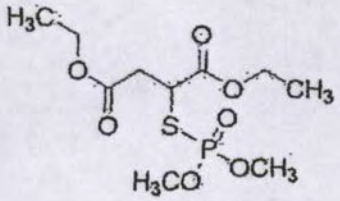
Malathion

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

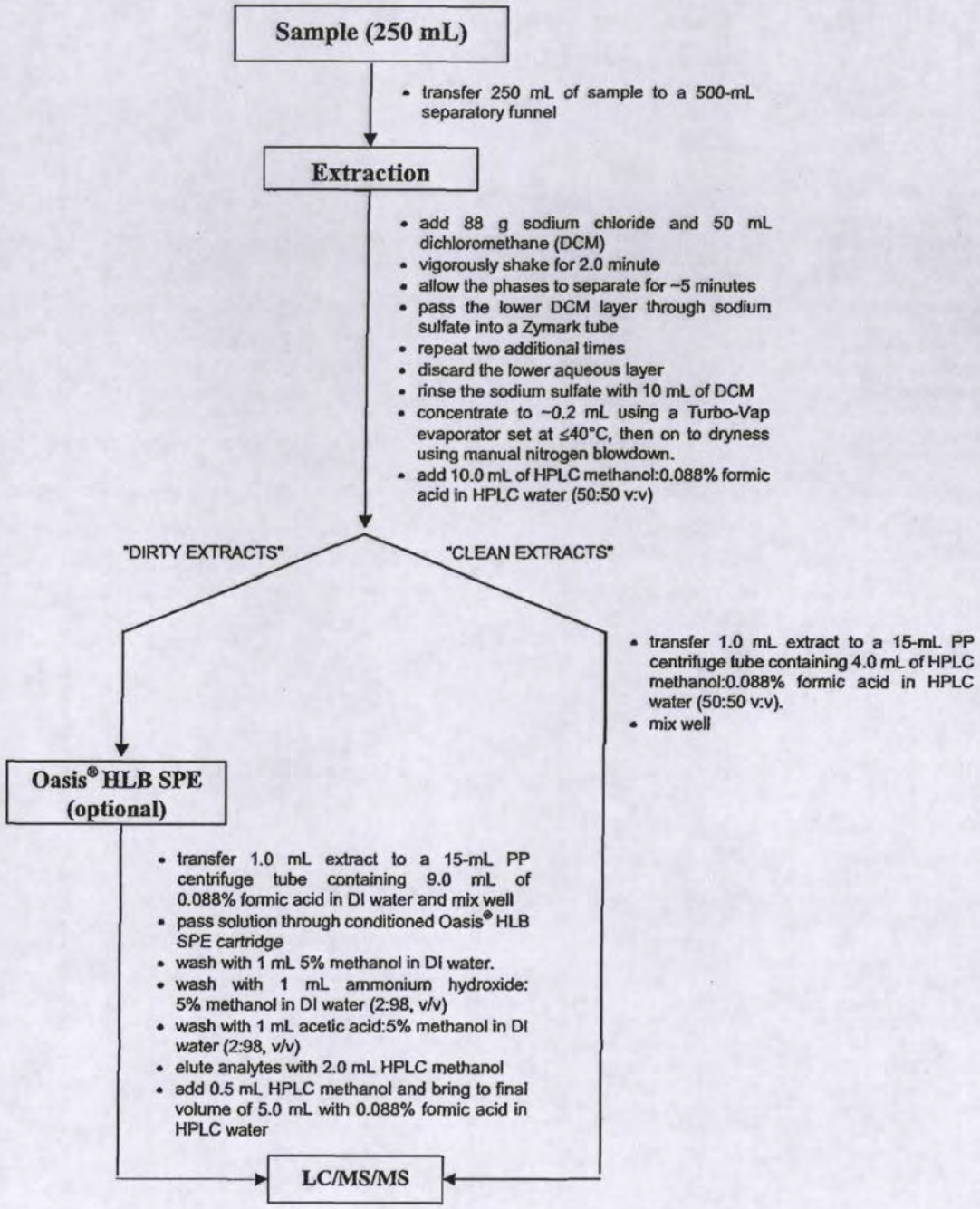
1. Institut Fresnius "Determination of Malathion in Water-Validation of the Method," Study Number IF-02/00004583, internal report dated May 14, 2002.

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.