2.0 INTRODUCTION

Vydate® insecticide / nematicide is used to control insects, mites, and nematodes during the production of various fruits and vegetables. The active ingredient of Vydate® is oxamyl. In water, oxamyl undergoes hydrolysis to produce oxime. An analytical method was developed and validated for the detection, quantitative analysis, and confirmation of the presence of oxamyl and its oxime metabolite in water. Henceforth in this report, the oxime metabolite will be referred to as "oxime". The method developed and validated is DuPont-2455 "**Analytical Method for the Determination of Oxamyl and its Oxime Metabolite in Water Using LC/MS/MS Analysis**". This method was used to monitor for oxamyl and oxime in soil-pore water and ground water following the application of Vydate® (References 1-2). The purpose of this study is to independently validate DuPont-2455. The ILV of analytical methods is required by the EPA (draft Ecological Effects Test Guidelines, OPPTS 850.7100, Data Reporting for Environmental Chemistry Methods).

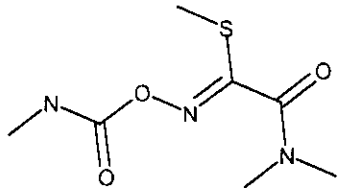
Ground water, three surface waters and drinking water were selected as the matrices for this ILV because they are representative of water analyzed using this method (References 1-2). The Limit of Quantitation (LOQ) and lowest fortification level tested for oxamyl and oxime in water using DuPont-2455 was 1.0 ng/g (ppb). During method ILV, the analytical method was tested at 1X and 10X the LOQ for oxamyl and oxime.

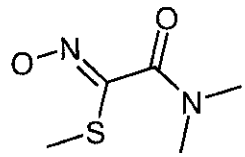
The analytical method involved the dilution of a 10 mL volume of ground water to 20 mL using an 0.1% aqueous formic acid solution. The samples were then syringe filtered and analyzed using LC/MS/MS detection. The instrument used for sample analysis was a triple quadrupole mass spectrometer. This analytical method passed ILV on the first attempt without any major modifications.

3.0 MATERIALS AND METHODS

3.1 Test Substances

Summaries of the test substances are provided in the tables below.

Common Name	Oxamyl
Structure	
DPX Number	DPX-D1410
CAS Chemical Name	(Methyl 2-(dimethylamino)-N-[[(methylamino)carbonyl]oxy]-2-oxoethanimidothioate)
CAS Number	23135-22-0
Lot Number	376
Purity	100.0%
Storage Conditions	Room Temperature

Common Name	Oxime
Structure	
DPX Number	IN-A2213
CAS Chemical Name	Methyl 2-(dimethylamino)-N-hydroxy-2-oxoethanimidothioate
CAS Number	66344-33-0
Lot Number	10
Purity	99.9%
Storage Conditions	Room Temperature

3.2 Test System

The analytical method was validated using ground water provided by the Sponsor Representative. This water was chosen because it is representative of water validated and analyzed using this method. The water was received in sealed plastic containers shipped frozen in dry ice. The samples used for method ILV were given the following identification numbers at the test site:

Identification Number	Location	Well
0006W09LCWR	Dorchester County, MD	Monitoring Well 09
0013W09LCWR	Dorchester County, MD	Monitoring Well 09

Upon receiving the containers, inspection conducted did not detect any damage resulting from the shipment of the controls. The control samples were allowed to

thaw at room temperature. The controls were then stored in a refrigerator set to 0-4°C.

The surface water analyzed was collected from local water supplies. A three gallon sample from each supply was collected on March 15, 2000. The water was intended to represent three different types of surface water: fresh (Brandywine River), pond (Lums Pond), and brackish (Delaware River). The samples were then stored in a refrigerator set to 0-4°C.

The drinking water used for validation was purchased from a local grocery store. A one gallon container of Great Bear Natural Spring Water from the Great Bear Spring Water Company, Breimgsville, PA 19464 was purchased. The sample was stored at room temperature and used on the same day it was opened.

All water samples were used without any preprocessing.

3.3 Equipment

Instrumentation

Mass Spectrometer System: Quattro II with ESI Interface (Micromass Inc., Beverly, MA)

LC system: HP1100 (Hewlett-Packard, Wilmington, DE)

Chromatographic Supplies

HPLC Column: 4.6 mm i.d. × 15 cm, Phenomenex® Phenyl-Hexyl analytical column with 3 µm diameter packing, PN 00F-4256-E0, (Phenomenex, Torrance, CA)

HPLC Vials, Target DP Amber Kit, T/S/T Septa, 100 PK, Part # 5182-0556 (Hewlett-Packard, Wilmington, DE)

6 Port Electrically Actuated Valve, Valco Instruments Co. Inc., PN 1384 (Alltech, Deerfield, IL)

Labware

VWR Brand Vortex Geni 2 Mixer, 115V, 60 Hz, Cat. No. 58815-178 (VWR Scientific, Boston, MA)

Biohit Proline Electronic Pipettors, Variable Volume with Tip Ejector, Vanguard, 5-100 µL Cat. No. 53495-200, 50-1000 µL Cat. No. 53495-205 and 0.10-5.0 mL Cat. No. 53495-290 (VWR Scientific, Boston, MA)

Balances - Mettler AE160 analytical and PE600 top-loading balances (Mettler Instrument Corp., Hightstown, N.J.)

VWR Brand Disposable Pasteur Pipettes, Borosilicate Glass, 9 in, Cat. No. 14673-043 equipped with 2 mL, 13 X 32 mm rubber bulbs, Cat. No. 56310-240 (VWR Scientific, Boston, MA)

Centrifuge Tubes - PYREX Brand Conical Centrifuge Tubes with Standard Taper Stopper, 50-mL capacity, Cat. No. 21048-050 (VWR Scientific, Boston, MA)

Miscellaneous

Filter - Non-sterile, Millex HV_{13}, 0.45 µm 13 mm Filter Unit, Cat. No. SJHV 013 NS (Millipore, Inc., Milford, MA)

3.4 Reagents

The reagents used were from a different vendor then the reagents used in the original method. A list of the reagents used has been provided below:

Water - EM Omni Solv®, HPLC-grade water, #WX0004-1, (EM Science, Gibbstown, NJ)

Acetonitrile - EM Omni Solv®, HPLC-grade acetonitrile, #AX0142-1, (EM Science, Gibbstown, NJ)

Methanol - EM Omni Solv®, HPLC-grade methanol, #MX0488-1 (EM Science, Gibbstown, NJ)

Acetic Acid - Baker Analyzed® glacial acetic acid, #9524-00 (J. T. Baker, Inc.)

Oxamyl Standard: Prepared by DuPont Agricultural Products, Global Technology Division, E. I. du Pont de Nemours and Company (DPX-D1410, Lot # 376, 100.0% Purity)

Oxime Standard: Prepared by DuPont Agricultural Products, Global Technology Division, E. I. du Pont de Nemours and Company (IN-A2213, Lot # 10, 99.9% Purity)

3.5 Principles of the Analytical Method

A dilute and analyze approach was taken for the analysis of oxamyl and oxime in ground water samples using DuPont-2455. The extraction and sample preparation was completed as follows:

1) A 10.0±0.1-mL aliquot of a ground water sample was transferred into a 50-mL centrifuge tube. The sample was fortified if necessary.
2) The sample was diluted to volume using a 10.0 ± 0.1-mL aliquot of an aqueous 0.1% formic acid solution. If the sample had been fortified in Step 1 the aliquot of formic acid solution was adjusted so that the final volume was 20.0-mL.
3) The sample was syringe filtered through an acrodisc filter into an HPLC autosampler vial.
4) The sample was then analyzed by LC/MS/MS.

3.6 Modifications, Interpretations, and Critical Steps

Quantitative analysis using LC/MS/MS with external standards faces several obstacles. The most troublesome obstacle involves matrix effects, which are attributed to the presence of co-eluting compounds during sample analysis that are not present during the analysis of standards. Matrix effects were initially observed during the instrument optimization process. Three parameters had a substantial effect on the

ionization of oxamyl and oxime in fortified ground water samples. The parameters were injection volume, source temperature and capillary voltage. As the injection volume decreased the matrix effects also decreased. For the analysis of ground water samples, the injection volume was set to 35 µL. As the source temperature increased the matrix effects observed decreased. We attributed this effect to enhanced volatilization of the eluate. The source temperature was set to 150°C, the highest setting for the Micromass Quattro II ion source. As the capillary (Needle) voltage increased, the observed matrix effect observed decreased. The capillary voltage was set to 4.5 kV, which is 90% of the maximum output for the system.

For the analysis of surface water, a matrix effect was initially observed. The injection volume was decreased from 35 µL to 10 µL. Reanalysis of the extracts resulted in acceptable recoveries.

The detection was highly dependent on the instrument parameters. The parameters provided in the method report and this ILV report will need to be carefully examined and interpreted if an instrument from an alternative vendor is used.

*3.7 **Instrumentation***

3.7.1 Chromatography

Analysis was conducted using a gradient-elution on a reversed phase Phenomenex® Phenyl-Hexyl analytical column with 3 µm diameter packing. Conditions used for the generation of the validation data presented in this report are summarized in the following table:

System:	Hewlett-Packard HP1100 HPLC			
Column:	4.6 mm i.d. × 15 cm, Phenomenex® Phenyl-Hexyl, 3 µm diameter packing, PN 00F-4256-E0			
Column Temperature:	35 °C			
Injection Volume:	0.035 - 0.010 mL			
Flow Rate:	1.00 mL/min			
Conditions:	A: Water B: Methanol			
	Time	%A	%B	Flow (mL/Min.)
	0.0	80	20	1.00
	6.0	50	50	1.00
	7.0	30	70	1.00
	7.5	0	100	1.00
	8.5	0	100	1.00
	9.0	80	20	1.00
	11.0	80	20	1.00
Oxime Retention Time:	~ 5.6 min			
Oxamyl Retention Time:	~ 7.0 min			
Total Run Time:	11 min			

A six port electronically activated switching valve was used to direct the flow to waste prior to and following the elution of the compounds of interest. The use of this valve reduces source contamination and enables additional samples to be analyzed prior to source cleaning. The valve switching times are given in the following table.

Time (Minutes)	Column Eluate Flow
0.00-4.00	Waste
4.00-8.50	MS source
8.50-11.00	Waste

Since electrospray LC/MS systems perform optimally at low flow rates the eluate was split following the switching valve. Approximately 200 µL/min of eluate (5:1 split) flowed into the ion source with the remaining eluate flowing into a waste container.

3.7.2 LC/MS/MS Analysis

The quantitative analysis of oxamyl and oxime was performed using a Micromass Quattro II LC/MS/MS system. Quantitative analysis was based on the integration of a single ion transition. The system parameters were adjusted while a solution of oxamyl and oxime was infused directly into the electrospray ion source. The solution composition was 50% methanol/50% water, so that it would approximate the

composition of the mobile phase at the retention time of the analytes. A summary of the experimental conditions is provided in the following table:

Micromass Quattro LC ESI-LC/MS/MS Mass Spectrometer Conditions

Analytes	Ions Monitored	Cone Voltage	Collision Energy	Mode
Oxime	163.0→ 71.8 ± 0.1 AMU	17V	10V	MRM
Oxamyl	220.0→ 71.8 ± 0.1 AMU	11V	10V	MRM
Dwell Time:	0.50 seconds			
Electrospray Voltage:	4.5 kV			
Detector Voltage:	750 V			
Source Temperatures:	150 °C			
Collision Gas Pressure:	3.3 e-3 mBar			
Nebulizing Gas Flow:	15 L/h			
Drying Gas Flow:	300 L/h			

A complete list of the experimental parameters is given in Appendix 1.

3.8 Calculations

3.8.1 Methods

Average Response Factor (RF_{ave}) was calculated as follows:

$$RF_{ave} = \frac{(\text{Area A} \div \text{Conc. A}) + (\text{Area B} \div \text{Conc. B}) + (\text{Area C} \div \text{Conc. C})}{3}$$

where Conc. X and Area X are the concentration (µg/mL) and corresponding peak area (counts) for standards run with the analysis set. The analyte concentration in fortified samples (ppm found) was calculated as follows:

$$\text{ppm Found} = \frac{(\text{Peak area, counts}) \times (\text{Final Volume, mL})}{(RF_{ave}, \text{counts}/\mu\text{g/mL}) \times (\text{Sample Weight, g})}$$

ppm Found = µg/g

The sample weight was calculated using grams. This was done since 10 mL of water is equivalent to 10 grams of water.

ppb Found = μg/g×1000ng/μg = ng/g

The percent recovery found was calculated as follows:

$$\%\ \text{Recovery} = \frac{(\text{ppm Found})}{(\text{Fortification level, ppm})} \text{ x } 100$$

3.8.2 Example

For a 1.0-ppb fortified oxime sample (Data Sheet Number 080999, sample 0809rs-3 in Appendix 2), the concentration found was calculated as follows:

Average Response Factor was calculated as follows:

$$RF_{ave} = \frac{(1220\,AC \div 0.0005\mu g/mL) + (2420\,AC \div 0.001\mu g/mL) + (5150\,AC \div 0.002\mu g/mL)}{3}$$

(AC ≡ Area Counts)

$$RF_{ave} = 2478333\,AC/\mu g/mL$$

ppm found was calculated as follows:

$$\text{ppm Found} = \frac{(1410\,AC)\times(20\,mL)}{(2478333\,AC/\mu g/mL)\times(10\,g)}$$

ppm Found = 0.00113786 µg/g

(ppm values are rounded to two significant figures)

ppb Found = 0.00113786 µg/g x 1000 ng/µg = 1.13786 ng/g

(ppb values are rounded to two significant figures)

The percent recovery found was calculated as follows:

$$\%\ \text{Recovery} = \frac{(1.13786\,ng/g)}{(1.00\,ng/g)}\ x\ 100$$

% Recovery = 114%

(percent recoveries are rounded to the nearest whole number)

Unit Analysis :

$$RF_{ave} = \frac{(AC \div \mu g/mL) + (AC \div \mu g/mL) + (AC \div \mu g/mL)}{3}$$

RF_{ave} = AC/ µg/mL

$$\text{ppm Found} = \frac{(AC)\times(mL)}{(AC/\mu g/mL)\times(g)}$$

ppm Found = µg/g

ppb Found = µg/g × ng/µg

ppb Found = ng/g

APPENDIX 3
SYNOPSIS OF COMMUNICATIONS BETWEEN JAMES J. STRY (STUDY DIRECTOR) AND JANET C. RÜHL (SPONSOR REPRESENTATIVE)

1) Protocol Step 2 – Areas Requiring Clarification (All other areas did not require any additional information)

Method Section

4.2.6 Analyte Extraction Procedure

The 10 mL sample aliquot will be placed in a 20 mL scintillation vial. Fortified if necessary. The volume is adjusted to 20 mL.

Clarification Required

The method is not clear on how the volume should be adjusted to 20 mL. This becomes a problem for fortified samples because the volume is no longer 10 mL. In addition, the scintillation vials are not graduated. The 0.1% formic acid solution must be added by pipette. Since the vials are not graduated a measurement can not be taken. Also, there is no information indicating if the sample should be mixed prior to analysis.

Interpretation

A pipette will be used to add all 0.1% formic acid solutions. For fortified samples the amount of 0.1% acid added will be adjusted to 9.9 mL for LOQ fortifications and 9.0 mL for 10XLOQ fortifications. The samples must be mixed prior to filtering 2 mL into an autosamplier vial.

Effect on Result

Provided standard analytical practices are followed, there will be no effect on the results.

4.3 Instrumentation

A description of the interface and equipment used for the detection of oxamyl and oxime was provided.

Clarification Required

The method did not provide any information pertaining to splitting the eluate flow before entering the ion source. Many API systems will not perform optimally when the flow into the API source is 1.0 mL/minute.

Interpretation

The eluate flow will be split for optimal performance for the LC/MS/MS system performing the analysis.

Effect on Result

If a split is not added to some systems the sensitivity will be below the required detection limits for this method.

2) Protocol Step 2 – Questions Asked to the Sponsor Representative after reading the method

4.2.1 Preparation of Reagent Solutions – In our storeroom we do not have the brand reagents used in this method: Methanol (JT Baker), Water (Labconco), Acetonitrile (VWR Scientific)

Do we need to show equivalence for each substituted reagent or would a single experiment determining equivalence of all reagents be sufficient?

Sponsor Representative Response: Not an issue, A single experiment showing equivalence of all reagents will be sufficient. Record the purity of all reagents used.

- 4.2.2.1 Stock Standard Preparation and Stability – Is it necessary to correct for purity of standards?

Sponsor Representative Response: Not necessary for method validation.

- 4.2.2.3 Chromatographic Standard Preparation and Stability - Chromatographic standard section – 0.05 ng/mL standards used to show LOD. Since an LOQ sample is 0.5 ng/mL and we are assessing method validation can we eliminate all LOD work and use the 0.25 ng/mL as our lowest standard? Is it necessary to prepare the 0.05 ng/mL standard?

Sponsor Representative Response: The low standard will be 0.25 ng/mL. No work at the method LOD will be conducted.

- 4.2.4 Storage & Preprocessing of Samples – All samples are to be kept frozen until just prior to sample analysis? Does this include our control?

Sponsor Representative Response: Control samples may be thawed prior to use.

- 4.2.6 Analyte Extraction Procedure – 10 mL of sample should be adjusted to 20 mL with acidified water solution. The vials used are not graduated. Is this to be done using a pipette. Should I adjust the volume based on the spiking level for fortifications? Following dilution should the sample be vortex mixed?

Sponsor Representative Response: All 0.1% formic acid solutions will be added using a pipette. The amount of 0.1% acid added will be adjusted for fortified samples.

- Description -Was a split used? If so, what was the splitting ratio?

Sponsor Representative Response: Centre Analytical will be contacted to determine if a split was used. We will incorporate a split taking into account the operation of the system conducting the ILV.

- 4.4 Calculations In the event we are not linear over the calibration range or in the event our curve shows a bias can an average response factor be used to calculate recoveries?

Sponsor Representative Response: Yes, average response could be used. Add as a possible method upgrade.

3) Protocol Step 3 – Calibration Curve Generation, Interference Check and Test of Reagent Substitutions

I reported to the Sponsor Representative the recoveries for step 3 of DuPont 3214. The R^2 values for the calibration curves for oxime and oxamyl were 0.99998 and 0.99809, respectively. The values were within the acceptable range. The check for matrix effects gave recoveries of 115% for oxime and 108% for oxamyl. These values were also within the acceptable range. The test of equivalence for substituted reagent gave recoveries of 104, 103, 107, 108% for oxamyl and 122, 128, 122, and 124 for oxime. I stated that the recoveries were slightly high for oxime and we anticipated they could be improved through instrument tuning. However, if the Sponsor Representative did not have a problem with a 124% average recovery we would continue with the ILV and start step 4. The Sponsor Representative advised me that she would prefer to have us attempt to re-adjust the instrument and improve the recoveries for oxime. We discussed the matrix effects observed during the initial setup of the instrument and how the information collected then may help us optimize the system prior to starting step number 4. We agreed to do step 3 over again with improved instrument conditions.

4) Protocol Step 3 – Calibration Curve Generation, Interference Check and Test of Reagent Substitutions

I reported to the Sponsor Representative the recoveries for step 3 of DuPont 3214. The R^2 values for the calibration curves for oxime and oxamyl were 0.99847 and 0.99812, respectively. The values were within the acceptable range. The check for matrix effects gave

recoveries of 116% for oxime and 86% for oxamyl. These values were also within the acceptable range. The test of equivalence for substituted reagent gave recoveries of 84, 88, 99, 95% for Oxamyl and 110, 111, 117, and 117 for oxime. I stated that the recoveries were slightly high for oxime but in the acceptable range. Also, I stated that the recoveries were calculated using an average response factor bracketing the sample analyzed. When we used linear regression the curve had very good correlation however the intercept was effected by the more concentrated standards and did not accurately calculate the recoveries. All data was collected in a single sample analysis set.

The Sponsor Representative considered the recoveries to be acceptable and gave us the go-ahead to attempt the first validation set as described in step 4.