1.0 INTRODUCTION

This report describes the independent laboratory validation (ILV) of Analytical Method No. REM 202.02 as performed by Enviro-Test Laboratories for the determination of the NOA-446510 and its metabolite CGA-380778 in soil using High Performance Liquid Chromatography with Triple Quadrapole Mass Spectrometric Detection (LC/MS/MS).

This study was conducted to satisfy guideline requirements described in the US EPA FIFRA Pesticide Assessment Guidelines for Subdivisions N, E, and K, and addenda for Data Reporting Guideline for Environmental Methods [2]. It also satisfies the requirements outlined in the harmonized guidelines from the OPPTS, "Public Draft" - Data Reporting for Environmental Chemistry Methods, OPPTS 850.7100 [3].

2.0 STUDY PERSONNEL

The following personnel from Enviro-Test Laboratories participated in the conduct of this study.

Gary Bruns	Study Director
Narinder Bains	Residue Analyst
Susan Nelson	Project Manager
Danuta Raszek	Log-In and Sample Control

3.0 MATERIALS

3.1 Test and Reference Substances

Reference substances were shipped from Syngenta US to Enviro-Test Laboratories and were received on September 4 and 8, 2003 (see the Standard disposition sheets in the Raw Data package). The following two substances were used:

Compound	Lot Number	Purity (%)	Expiration Date
NOA-446510	S03-2794	99.0	June 2005
CGA-380778	DAH-XXX-62	94.6	Jan. 31/05

The reference substances were logged in and then kept stored in a freezer after arrival at ETL. Syngenta Crop Protection, Inc. maintains the characterization and stability data for the reference substances.

On October 6, 2004 stock standards were prepared from the neat reference substances for use in preparing instrument calibration and fortification solutions. All stock standards were prepared as per the method. Fortification and working solutions were prepared from the stock standards on October 7, 2004. The stock standards and working solutions were kept stored in a refrigerator when not in use.

3.2 Control Soil

Control soil obtained from Syngenta Study T00053-03 0-6" was used to validate the method. The control soil sample was characterized by Agvise Laboratory. The trial I.D. number was 242.68, the date received was Aug. 15/03 and the date reported was Aug. 26/03.

The composite control soil was characterized for selected inorganic parameters as specified in the protocol. A certified copy of this soil characterization report can be found in Appendix 2.

The soil site was located in New York, USA.

Soil Characterization **Sample I.D.: PA.NY.T.CH48 0-6**	
Bulk Density	1.28
CEC	6.9
1/3 bar	15.6
15 bar	3.8
% OM	2.0
pH	6.7
Ca	554 ppm
Mg	149 ppm
K	79 ppm
Na	22 ppm
B	26

1. Agvise GLP soil characterization report. Lab Sample No. 03-1141

3.3 Equipment and Reagents

The equipment and reagents used for the method validation were as outlined in Method REM 202.02 (Section 2.0 Materials and Apparatus). Identical or equivalent apparatus and materials were used.

4.0 METHOD AND METHOD MODIFICATIONS

4.1 Modifications

No modifications were made to the extraction or cleanup sections of the method. They were performed exactly as written. As a result of the difference in LC/MS/MS systems the following specific modifications to the method are noted:

1. A PE Sciex API 4000 MS/MS system was used in place of the PE Sciex API-III+ Mass Spectrometer. The instrument specifications are listed in Table 1 and Table 2.
2. Samples were collected in15 mL culture tubes from the ENVI carb SPE cartridges in place of a round bottom.
3. The mobile phase flow rate was reduced from 800 µL/min to 700 µL/min.

4.2 Sample Preparation, Fortification, and Extraction

The validation trial consisted of one analytical set. This set consisted of 13 samples: one reagent blank, two matrix blanks, five matrix blanks fortified at the LOQ (0.5 ppb) and five matrix blanks fortified at 10X LOQ.

Twelve (10) g portions of soil were used as samples. Samples designated as spikes were fortified with either 50 µL of a 100 µg/L a mixed standard fortification solution (for LOQ fortifications) or 50 µL of 1000 µg/L (for 10X LOQ fortifications). Spikes were mixed and let stand 10 minutes. See detailed method below:

Extraction:

1. Weigh representative amounts of soil (10 ± 0.05 g) into separate 50 mL disposable plastic centrifuge tubes. At least one untreated control and two control samples fortified with known amounts of NOA-446510 and CGA-380778 in acetonitrile should be analyzed with each sample set, using the sample procedure, to verify method performance. No more than 0.5 mL of fortification solution should be added. Allow fortified control samples to equilibrate to at least 5 minutes before proceeding with the extraction.

2. Add 20 mL of 80:20 (v/v) acetonitrile:purified water, cap and shake on a mechanical shaker at a speed that visibly agitates the samples for a minimum of 30 minutes. Tubes should be placed in a flat or horizontal orientation.

3. Centrifuge samples at 6000 rpm (or at a speed that visibly separates the solid sample from the supernatant) for about 5 minutes. Decant the supernatant liquid into a separate 50 mL centrifuge tube. NOTE: With some soils, particularly those with a high clay content, the solution may still be visibly cloudy even after centrifugation. This is normal and will not affect results.

4. Repeat extraction with an additional 20 mL of 80:20 acetonitrile:purified water. Add extraction solvent to the solid soil remaining in the centrifuge tube from the first extraction (step 3.). Cap and shake by hand or use a vortex mixer. If shaking cannot break up the compacted soil, use a **clean** suitable implement (e.g. a spatula) to facilitate this process. Shake on a mechanical shaker at a speed that visibly agitates the samples for a minimum of 30 minutes. Once again, tubes should be placed in a flat or horizontal orientation.

5. Centrifuge samples at 6000 rpm (or at a speed that visibly separates the solid sample from the supernatant) for 5 minutes. Decant the supernatant liquid into the plastic centrifuge tube containing the first extract. Cap the tubes and mix thoroughly by shaking.

6. Transfer a 10 mL aliquot of this sample into a 125 mL round bottom flask. Place on a rotary evaporator with a bath temperature of ca. 35-40°C and reduce to aqueous. NOTE: If you experience problems with frit blockage on the SPE, you may centrifuge the combined extracts at 6000 rpm (or at a speed that visibly separates the solid sample from the supernatant) for 3-5 minutes before aliquotting.

7. After removing the sample from the rotary evaporator add ca. 5 mL ultra-pure water and swirl to mix.

ENV Solid Phase Extraction Procedure:

- To minimize the possibility of contamination, the vacuum manifold, inlets and needles should be thoroughly cleaned or replaced prior to the analysis of each batch of samples.
- Once the SPE procedure has been started, do not stop and store the samples before the analytes have been eluted using methanol and collected (step 6).

1. Take one Varian ENV sold phase extraction cartridge (size 500 mg, 6 mL) for each sample to be analyzed and place on a suitable vacuum manifold. Add methanol (ca. 5 mL) and draw through under vacuum to the level of the top frit at steady drip rate. Do not allow the cartridges to become dry. Add ultra-pure water (ca. 5 mL) to the top of each cartridge and draw through under vacuum to the level of the top frit at the same rate. Discard the column eluates. Do not allow the cartridges to become dry.

2. Transfer the sample aliquots from Extraction step 7 to the top of the cartridges and draw through under vacuum at a steady drip rate, discarding the column eluates. NOA-446510 and CGA-380778 are retained on the column.

3. Add 3 mL of 30:70 (v/v) acetonitrile:ultra-pure water to the sample round bottom flask and swirl to rinse. Transfer to the SPE and draw through under vacuum at a steady drip rate discarding this wash solvent.

4. Dry under high vacuum for approximately 15-20 minutes. Remove any remaining droplets of water adhering to the inside of the cartridges with absorbent tissue.

5. Where achievable vacuums are less that specified or apparatus does not allow sufficient airflow through the cartridges, longer drying times may be required.

6. Place suitable collection tubes (e.g., 50 mL glass concentration/centrifuge tubes) under each port, as required, in the manifold rack. Elute NOA-446510 and CGA-380778 using 10 mL methanol at a steady drip rate. NOTE: The above ENV SPE procedure has been developed using columns from a specific manufacturer; however, it may be possible to carry out the procedure using similar columns from other manufacturers. In all cases, it

is strongly recommended that the elution profile of the chosen batch of columns be checked prior to commencing analysis to rule out any variation between manufacturers products and between batches.

7. Evaporate the samples to dryness by rotary evaporation using a bath temperature of 35-40°C. A trace (i.e., one drop or less) of water may be left in the tube. This small contribution to the sample final volume will not significantly affect the analytical results. NOTE: If you experience sample "bumping" it may be necessary to vent the vacuum several times in the beginning and/or gradually increase the vacuum to minimize this effect.

8. Add 2.5 mL of 30:70 (v/v) acetonitrile:ultra-pure water and mix on a vortex mixer and/or in an ultrasonic bath. Generally, 10-20 seconds of mixing will suffice.

9. Transfer an aliquot of sample to an amber glass autosampler vial for analysis by LC with triple quadrupole mass spectrometric detection (LC/MS/MS).

4.3 LC/MS/MS Instrumentation

All samples were analyzed using an Applied Biosystems API-4000 Triple Quadrupole Mass Spectrometer with Turbo Ion Spray Interface. The following components completed the system:

HPLC: Two Perkin Elmer Series 200 Micropumps
Autoinjector: CTC HTS PAL
Column Heater: Waters Temp. Control module Millipore
Data System: Dell Precision 360, Intel(R) Pentium Computer, 4 CPU 3.00 GHz running Microsoft Windows 2000 Version 5 and Analyst Version 1.4

The HPLC operating parameters are shown in Table 1. The API 4000 MS/MS operating parameters are shown in Table 2.

4.4 Data Acquisition and Reporting

Peak integration and quantitation were performed by using Analyst, Version 1.4 (Applied Biosystems). Analytes were quantitated by external calibration. The MS detector response (peak area) versus the standard concentration was used to generate calibration curves for the analytes. Best-fit weighted 1/x linear regression equation for the curves were derived and these equations were used to calculate the concentration of analytes in the samples. Recovery results were computed for each sample. The equations used for quantitation are presented in Table 5.

Statistical treatment of the data includes calculation of averages, standard deviations, relative standard deviations and confidence limits. The calculations were performed using Excel 97 SR-2. Results were rounded off for reporting purposes but not during calculations.

8.0 TABLES

Table 1. HPLC System

Analytical Column:	Phenomenex-Kromasil, ODS, (C_{18}), 3.20 x 150 mm, 5 µm, Part No. #223195-1
Guard Column:	Phenomenex Security Guard C 18 (3.0 x 4 mm), Part No.: AJO-4287
Mobile Phase Flow Rate:	700 µL/min
Mobile Phase A:	4 mM Ammonium Formate/0.1% formic acid in water
Mobile Phase B:	4 mM Ammonium Formate/0.1% formic acid in methanol
Run time:	5 minutes
Injection Volume:	20 µL

Mobile Phase Isocratic Program:

Duration (min.)	%A	%B
0 - 1.0 (equil)	30	70
5	30	70

Analyte Retention times:

Analyte	Min.
NOA-466510	3:55
CGA-380778	2:50

Table 2. LC/MS/MS Operating Parameters

The samples were analyzed using positive ion detection. The MRM (multiple reaction monitoring) scan mode was used for the signal acquisition.

Interface:	Turbo Ion-Spray
Polarity:	Positive
Nebuliser Gas (GS1):	60
Turbo Gas (GS2):	60
Curtain Gas (CUR):	14 (arbitrary units)
Temperature (TEM):	550
Ion-Spray voltage:	5500
Collision gas (CAD):	Nitrogen 6 (arbitrary units)
Scan type:	MRM

	NOA-446510	CGA-380778
Q1 Mass	412.10	374.10
Q3 Mass	328.30	328.20
Dwell time (msec)	200	200
Resolution Q1	UNIT	UNIT
Resolution Q3	UNIT	UNIT
Declustering potential (DP)	46	61
Entrance potential (EP)	10	10
Collision energy (CE)	21	19
Collision cell exit potential (CXP)	10	10
Electron multiplier setting (CEM)	2200	2200

Table 4. Clarifications, Communication, and Recommendations to perform Analytical Method No. REM 202.02

Clarifications, Communication and Recommendations:

No communication was required for this ILV. The method was clearly written and easy to follow.

Table 5. Calculations

Peak areas and external calibrations were used for data analysis. The Analyst Version 1.4 quantitation software package was used to calculate a best fit, 1/x weighted line of the standards. Extract concentration found was determined from the analyte peak area versus the calibration.

a) Calculated Concentration in Samples:

$$\text{Calc.Conc.(ppb)} = \frac{(x - b)}{m} \times \text{D.F.}$$

Where:
x = Peak Area of the analyte
b = Intercept from weighted 1/x regression analysis (Peak Area)
m= Slope from weighted 1/x regression analysis (response per concentration)
D.F. = Dilution Factor

$$\text{D.F.} = \frac{\text{Extraction Volume(mL)}}{\text{Aliquot Volume(mL)}} \times \frac{\text{Final Volume(mL)}}{\text{Sample Weight (g)}} = \frac{40\,\text{mL}}{10\,\text{mL}} \times \frac{2.5\,\text{mL}}{10.0\,\text{g}} = 1.0$$

The Analyst data processing software generates both the slope and intercept.

The calculation of averages, standard deviations, relative standard deviations and 95% confidence limits were performed in Excel.

The report percent recoveries shown on Table 3 may not exactly match the corresponding recoveries on the Analyst Result tables shown in Appendix 3. This is because Analyst uses a large string of un-rounded numbers to calculate the percent recoveries.