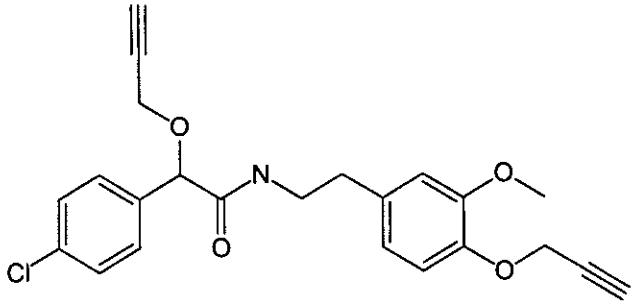
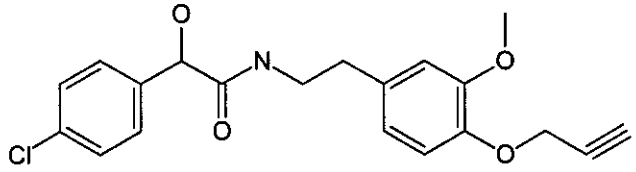
1.0 Introduction and Summary

1.1 Scope

Analytical method REM 202.02 has been shown to be suitable for the determination of residues of NOA-446510 and its metabolite CGA-380778 in soil samples. The method LOQ has been set at 0.5 ppb (μg kg^{-1}). Method REM 202.02 was validated under Syngenta Study Number T000056-03[1]. Chemical structures and CAS names for NOA-446510 and CGA-380778 are shown below.

Code:	NOA-446510
CAS Name:	Benzeneacetamide, 4-chloro-N-[2-[3-methoxy-4-(2-propynyloxy)phenyl]ethyl]-alpha-(2-propynyloxy)-
CAS Number:	Not assigned
	

Code:	CGA-380778
CAS Name:	Benzeneacetamide, 4-chloro-alpha-hydroxy-N-[2-[3-methoxy-4-(2-propynyloxy)phenyl]ethyl]-
CAS Number:	282720-26-7
	

1.2 Method Summary

Soil samples (10 g) are extracted twice with 80:20 (v/v) acetonitrile:water (20 mL). Extracts are combined, an aliquot taken and the organic solvent removed by rotary evaporation. The

samples are diluted with additional water and taken through a Varian ENV solid phase extraction (SPE) procedure. NOA-446510 and CGA-380778 are eluted from the SPE cartridge using methanol. Samples are evaporated to dryness by rotary evaporation then redissolved in 30:70 (v/v) acetonitrile:ultra-pure water. Final determination is by high performance liquid chromatography with tandem mass spectrometric detection (LC/MS/MS). External standards in 30:70 (v/v) acetonitrile:ultra-pure water are used for calibration. The method is also summarized in flow chart form in Figure 1.

2.0 Materials and Apparatus

The recommended equipment and apparatus are listed in Appendix 1. Equipment with equivalent performance specifications may be substituted.

2.1 Reagents and Analytical Standards

All solvents and other reagents must be of high purity, e.g. glass distilled/HPLC grade solvents and analytical grade reagents. Particular care must be taken to avoid contamination of the reagents used. Reagents of comparable purity may be substituted as long as acceptable performance is demonstrated. See Appendix 2 for a list of reagents and analytical standards used in this method.

2.2 Preparation of Analytical Standard Solutions

It is recommended that the following precautions should be taken when weighing the analytical materials.

1. Ensure good ventilation.
2. Wear approved eye protection, gloves and a laboratory coat.
3. Prevent inhalation and contact with mouth.
4. Wash any contaminated area immediately.

All stock solutions are stored in amber glass bottles in a freezer (ca. -20°C) when not in use. No analyte stability or solubility problems have been observed in the standard solutions used in this study. The expiration date for the primary (100 ng/µL) stock standard solutions is the reassay date of the analytical standard. Whenever a fresh primary stock solution is prepared, dilutions of the "new" versus the "old" solutions should be analyzed to verify solution stability.

Prepare individual 100 ng/µL stock solutions for NOA-446510 and CGA-380778 by one of two methods. The first method is to weigh exactly 10.0 mg of analyte (corrected for purity) into a weighing dish and quantitatively transfer (using acetonitrile) to a "Class A" 100-mL volumetric flask. Add additional acetonitrile to the 100 mL mark on the flask.

Alternatively, you may determine the appropriate volume of acetonitrile to add to a known amount of standard material using the equation below. The concentration of the analytical standard is corrected for its chemical purity.

$$V\ (mL) = \frac{wt.\ (mg) \times P}{C\ (ng/uL)} \times 10^3$$

Where "V" is the volume of acetonitrile needed; "wt." is the weight, in mg, of the solid analytical standard; "P" is the purity, in decimal form, of the analytical standard; "C" is the desired concentration of the final solution, in ng/µL; and 10^3 is a conversion factor. In this second case, the standard material is weighed directly into the amber glass storage bottle.

Sample fortification solutions are prepared in acetonitrile from the two primary stock solutions. It is recommended that, as a minimum, the following concentrations be prepared: 5 ng/µL, 0.1 ng/µL and 0.01 ng/µL. The preparation of LC calibration standards is discussed in section 3.6.

Fortification standards should be stored in amber glass bottles refrigerated at ≤ 7°C. An expiration date of three months is recommended unless additional data are generated that show a longer expiration date is appropriate. In which case, the expiration date may be extended to a maximum of six months.

2.3 Safety Precautions and Hazards

Whereas most of the chemicals in this method have not been completely characterized, general laboratory safety precautions are advised (e.g., safety glasses, gloves, etc.). The user(s) should consult the relevant MSDS for commonly used reagents and materials.

3.0 Analytical Procedure

Note: Due to the low detection limit of the method it is important that precautions be taken to avoid cross contamination in the laboratory.
Specifically:

- Where possible disposable glassware/plastic-ware has been specified, new glassware/plastic-ware should be used for each batch of samples.
- Each solvent used in the method should be checked prior to use to verify that it is free from contamination.
- Existing glassware should be solvent (acetone or acetonitrile) rinsed, after washing and before use in the method.

3.1 Sample Preparation

It is important that a homogeneous soil sample be available for analysis. All samples should be prepared using an approved method of preparation for residue analysis prior to analysis.

3.2 Extraction

a) 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 same procedure, to verify method performance. No more than 0.5 mL of fortification solution should be added. Allow fortified control samples to equilibrate for at least 5 minutes before proceeding with the extraction.

Add 20 mL 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.

b) Centrifuge samples at 6000 rpm (or at a speed that visibly separates the solid sample from the supernatant) for about five minutes. Decant the supernatant liquid into a separate plastic 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.

c) Repeat extraction with an additional 20 mL 80:20 (v/v) acetonitrile:purified water. Add extraction solvent to the solid soil remaining in the centrifuge tube from the first extraction at 3.2 (b). 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.

d) 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.

e) 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.

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

3.3 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 3.3(e)).

a) Take one Varian ENV solid 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.

b) Transfer the sample aliquots from section 3.2 (f) 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.

c) Add 3 mL 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.

d) 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.

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

e) 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.

f) 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.

g) Add 2.5 mL 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.

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

3.4 Time Required for Analysis

The methodology may be performed with a batch of 12 samples. One person should be able to complete the analyses in 1 day (8 working hour period), LC/MS/MS instrument time excluded.

3.5 Work Flow

The analytical procedure can be stopped at various points for overnight and weekend storage unless otherwise specified in the analytical procedure. Acceptable external standard recoveries will validate any work interruptions. Samples should be stored in sealed vessels at a temperature of ≤7°C when the analyses cannot be completed in a single working day.

3.6 Preparation of Calibration Standards for LC/MS/MS

Standards for external calibration should be prepared in 30:70 (v/v) acetonitrile:ultra-pure water. It is recommended that the following concentration levels be prepared: 200 pg/µL (0.2 ng/µL), 10 pg/µL, 5 pg/µL, 2 pg/µL, 1 pg/µL, 0.5 pg/µL and 0.2 pg/µL. The 200 pg/µL is not used for calibration, but as a convenient stock to prepare subsequent dilutions.

A minimum of four standard levels is recommended for generation of the external calibration curve. Solutions of analytical standards are interspersed with the samples to form a sequence of analyses. The first and the last injections used in an analytical set must be standards. The smallest standard within a set will determine the limit of detection (LOD) for the set. The smallest standard generally corresponds to about 50% of the limit of quantitation (LOQ) of the analytical method.

The MS/MS response of the samples should fall within the limits of the standard curve. One exception would be for control samples containing residues less than the method LOQ. Any samples with residues less than the method LOQ are typically reported as <0.5 ppb or as nondetect (ND), whichever is appropriate. See section 5.0 of this report for details regarding calculation of results.

LC calibration standards should be stored in amber glass bottles refrigerated at ≤ 7°C. An expiration date of three months is recommended unless additional data are generated that show a longer expiration date is appropriate. In which case, the expiration date may be extended to a maximum of six months.

4.0 LC/MS/MS Parameters and Settings

The following instruments and conditions have been found to be suitable for this analysis in this laboratory. Other instruments may also be used, however optimization may be required to achieve the desired separation and sensitivity. Operating manuals for the instruments should always be consulted to ensure safe and optimum use.

4.1 LC System Description and Operating Conditions

LC Instrumentation:

Perkin-Elmer Series 200 LC Pump and Autosampler
Perkin-Elmer Series 200 Vacuum Degasser
Eppendorf Model CH-30 Column Heater
Eppendorf Model TC-50 Column Heater Controller
Perkin-Elmer Peltier Cooling Tray

LC Operating Conditions:

Column Temperature: 30°C
Injection Volume: 20 µL (may be adjusted, if necessary)
Autosampler Tray Temp.: cooled to ca. 5-10°C (if possible)
Mobile Phase Flow Rate: 0.8 mL/min (flow to MS-MS optimized using an in-line flow splitter)
Column: Phenomenex Kromacil® 100-5C18, 150 x 3.2 mm id (C18 guard column or cartridge recommended).
Mobile Phase A: HPLC grade water, 0.1% concentrated formic acid and 4 mM ammonium formate.
Mobile Phase B: HPLC grade Methanol, 0.1% concentrated formic acid and 4 mM ammonium formate.

Mobile Phase Program (Isocratic):

Step	Time, min.	%A	%B
0 (equil)	0.2	30	70
1	5	30	70

Periodically flush LC column with 95% methanol to remove highly retained matrix components.

Typical Analyte LC Retention Times:

Analyte	Approximate Retention time, min.
CGA-380778	2.2
NOA-446510	3.1

4.2 Mass Spectrometer System Description and Operating Conditions

Instrumentation:

Mass Spectrometer System:

PE Sciex API-III+ Mass Spectrometer
Instrument Control and Data Collection: Apple MacIntosh Quadra 950 Computer
Quantitation Software: Sciex MacQuan, version 1.6

All software programs written and provided by PE Sciex, except the system software by Apple.
Different versions of the system and applications software may be used provided they are able to collect and process the data properly.

Operating Conditions:

Interface Heater: 70°C
Curtain Gas Flow: 1.0-1.2 L/min
IonSpray™ Gas Pressure: generally 40-50 psi (optimized for maximum analyte response)
Sciex Turbo Ion Spray Interface (operated at ~450°C)

Typical State File Settings:

	State File Settings
Ionization Mode:	positive
ISV:	4500v
OR:	60.0v
CGT:	ca. 280
Dwell time (msec):	200
Scan rate (sec^{-1}):	1.67
Pause time (msec)	0.02
MS/MS Transition(s):	374.1 → 327.9 412.1 → 327.9

ISV = ion spray voltage
OR = orifice voltage
CGT = collision gas thickness
Note: These settings were specific for the Sciex API-III+ used for method development and validation. These settings will need to be optimized for the user's specific instrument and operating conditions.

5.0 Calculation of Results

Determination of Residues in Samples:

Inject the sample extract from 3.3(h) into the analysis system. The sample solution must be diluted if the analyte response exceeds the linear range of the calibration curve. Quantitation is achieved using a linear least squares curve fit to the external standards. The data may be linear, forced through zero, or weighted 1/x, as appropriate.

Determination of Residues in Fortified Samples:

Validate the method performance for each set of samples analyzed by including a control sample and two or more control samples fortified with known amounts of NOA-446510 and CGA-380778 prior to the extraction procedure. The fortification levels for external recoveries should approximate the expected residue levels in the study samples.

Recovery data are generally considered acceptable when the mean values are between 70% and 120% with a relative standard deviation of $\leq$20%.

Calculations:

Calculations may be performed by computer program (preferred) or manually as shown below.

Calculate the analyte concentration (in ppb) for field-incurred residues using the equation:

$$\text{RES(ppb)} = \frac{\text{Analyte found (ng)}}{\text{SWI (mg)}} \times 1000$$

where RES is the residue value in ppb, analyte found (ng) is calculated from the standard calibration curve, and SWI is the sample weight injected (mg). If the amount of analyte found is reported in picograms (pg) the multiplication factor of 1000 is dropped.

The amount, in milligrams, of sample weight injected (SWI) can be calculated using the equation:

$$\text{SWI(mg)} = \frac{\text{FW(g)} \times \text{IV}(\mu\text{L})}{\text{FV(mL)}}$$

where FW = final sample weight (g), IV = LC injection volume (µL) and FV = final volume in which sample is dissolved (mL).

The final sample weight (FW) is calculated by the equation:

$$\text{FW(g)} = \left[\frac{\text{SWE(g)} \times \text{A1(mL)}}{\text{EV(mL)} + \{\text{SWE(g)} \times \text{M(\%)}/100\}}\right] \times \left[\frac{\text{A2(mL)}}{\text{INV(mL)}}\right]$$

where FW = final weight (g), SWE = sample weight extracted (g), A1 = aliquot 1 volume (mL), EV = total extraction solvent volume (mL), M = sample moisture in percent, A2 = aliquot 2 volume (mL), if needed, INV = interim volume (mL) is the total volume from which the 2nd aliquot is taken.

NOTE: If no sample dilutions are required, the second term in the equation (i.e., A2/INV) is equal to one.

Corrections may be made to the residue value (RES) calculated above. At the discretion of the study director, this value may be corrected to account for the average recovery and/or sample moisture.

The recovery factor, expressed as a percentage (R%), is calculated using the following equation.

$$R\% = \frac{\text{RES fortified (ppb)} - \text{RES control (ppb)}}{\text{ppb analyte added}} \times 100$$

To correct a residue value to its dry weight value, the following equation may be used:

$$\text{SDW(ppb)} = \left[\frac{\text{CR (ppb)}}{\frac{(100 - \text{M(\%)})}{100}} \right]$$

where SDW = soil dry weight residue (ppb), CR = corrected soil residue, and M = soil moisture (%).

The recovery corrected soil residue can be determined by the equation:

$$\text{CR (ppb)} = \left[\frac{\text{RES (ppb)}}{\text{AR(\%)}} \right] \text{x} 100$$

where CR = recovery corrected residue (ppb), RES = residue found (ppb) and AR = average recovery (%).

In general, if the average percentage recovery is greater than 100%, the sample residue values should not be corrected.

6.0 Interferences and Confirmation

Final determination by LC/MS/MS is considered to be highly specific; therefore no confirmatory conditions are included. Due to the high selectivity of the detection technique, interference arising from the sample matrix has not been observed. Final determination by LC/MS/MS is considered to be highly specific; therefore no confirmatory conditions are included.

This method uses disposable labware, wherever possible, and single use solid phase extraction cartridges. All reuseable laboratory items should be detergent washed then rinsed with HPLC grade solvent (acetone, acetonitrile or methanol) prior to use. If contamination is suspected, run reagent (method) blank samples using solvents only to help isolate the source of the problem.

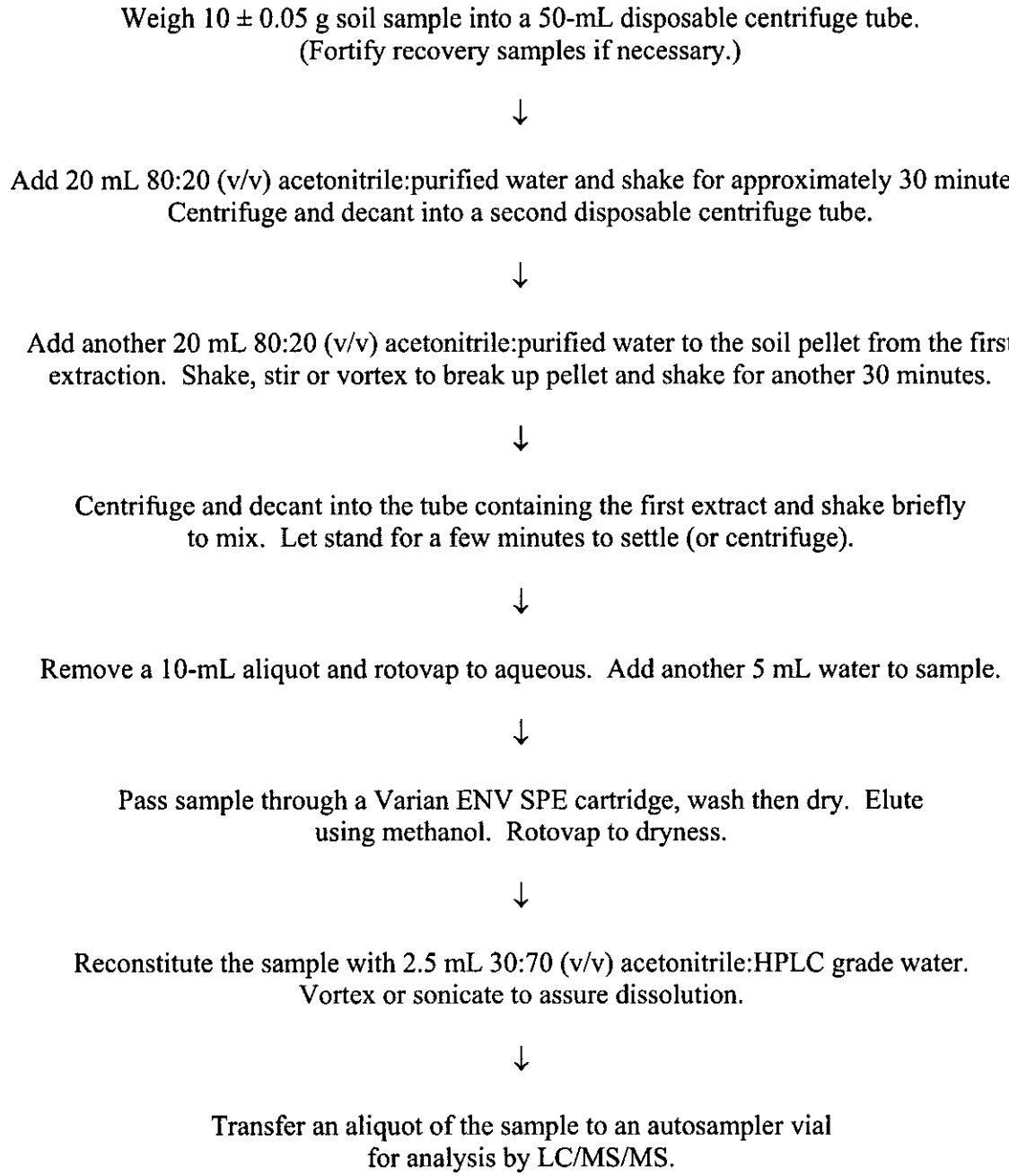
7.0 Modifications and Potential Problems

It is possible that contaminants from chemicals, solvents, glassware, etc. may interfere with the analysis and give a false positive result. It is recommended that reagent blank samples be included in a sample set if contamination is suspected.

Solvent foaming and "bumping" may occur during rotary evaporation. Periodic venting of the vacuum or a gradual increase in the applied vacuum can be used to minimize this effect. In addition, rotary evaporator solvent traps should be used when possible.

12.0 Figures

Figure 1. Method Flow Chart



13.0 Appendices

Appendix 1. Apparatus

General laboratory glassware (beakers, graduated cylinders, pipet bulbs, etc.) available from a general laboratory supply company.

Balance, analytical (Sartorius R160P), or equivalent. Electronic display of 0.01 mg, for preparation of the stock standard solutions.

Balance, laboratory (Mettler model BB1300), or equivalent. Electronic display of 0.01 g, for weighing soil samples.

Bottles, amber glass Boston round, 2 oz. and 4 oz., with Polyseal-lined cap (Fisher Scientific cat. nos. 03-320-4A and 03-320-4B) or equivalent.

Mixer, Vortex-Genie 2 (Fisher Scientific cat. no. 12-812) or equivalent.

Pipets, Pasteur, (Fisher Scientific cat. #13-678-7C) or equivalent.

Pipets, glass, Class A certified, assorted volumes. These pipets are used when an exact addition of liquid is required (i.e., sample fortification, standard solution preparation and dilutions).

Pipetter, Eppendorf Repeater, 100 – 1000 µL variable volume range (VWR cat. no. 53511-582) and 500-5000 µL variable volume range (VWR cat. no. 53513-412), or equivalent.

Mechanical reciprocating shaker, IKA Labortechnik Model HS501 digital, 300 rpm, or equivalent.

Centrifuge, Sorval Super T21, Kendro Laboratory Products, or equivalent.

Rotary evaporator, Buchi (VWR cat. no. 47728-920) or equivalent.

Rotary evaporator bump traps, 24/40 joint, (VWR cat. No. 80068-050), or equivalent.

Vacuum pump, Welch self-cleaning dry vacuum, model 2025 (VWR cat. no. 54994-126), or equivalent. The vacuum system should be capable of providing at least 28" Hg vacuum to each rotary evaporator.

Tube, glass concentration, 50-mL, with 19/38 ground glass joint (Fisher Scientific cat. no. 05-538-40B), or equivalent.

Appendix 1. Apparatus (continued)

Connecting adapter, 24/40 to 19/38 reducing (Fisher Scientific cat. no. 01-035D) or equivalent. (To connect the 50-mL glass concentration tube to the rotary evaporator.)

Solid phase extraction (SPE) column: Bond Elut ENV, 500 mg/6 mL capacity/volume (Varian cat. no. 1225-5011), or equivalent.

Tube, centrifuge, polypropylene, 50-mL graduated with plastic screw cap (VWR cat. # 21008-240), or equivalent.

Vacuum manifold, (J. T. Baker # SPE-12G column processor) or equivalent.

Utrasonic bath, Cole-Parmer Model 8893, or equivalent.

LC Column: Kromasil 5 µm, 150 x 3.2 mm (Phenomenex cat no. 00F-3033-R0), or equivalent.

LC Guard cartridge: Security guard kit (Phenomenex cat. no. AJ0-4282) with C18 guard cartridge, 4 x 3.0 mm (Phenomenex cat. no. AJ0-4287), or equivalent.

LC Vials, amber snap cap ID, 1.5 mL (National Scientific, Inc. cat no. C4001-6W) and pre-slit snap-it™ caps (National Scientific, Inc. cat no. C40011-55), or equivalent.

Appendix 2. Reagents and Analytical Standards

All solvents and other reagents must be of high purity, e.g. glass distilled/HPLC grade solvents and analytical grade reagents. Particular care must be taken to avoid contamination of the reagents used. All reagents and polypropylene glycols are stored at room temperature. Solid analytical standards are stored in a freezer (temperature < -10°C) unless specified otherwise on the sample shipment paperwork.

NOA-446510, obtained from the Technology Support/Chemistry group, Syngenta Crop Protection, Inc., Greensboro, North Carolina.

CGA-380778, obtained from the Technology Support/Chemistry group, Syngenta Crop Protection, Inc., Greensboro, North Carolina.

Acetone, HPLC grade (VWR cat. no. JT9002-33), or equivalent.

Acetonitrile, HPLC grade (VWR cat. no. EM-AX0145-1), or equivalent.

Methanol, HPLC grade (VWR cat. no. EM-MX0475P-1), or equivalent.

Formic acid, 90%, laboratory grade or better (Fisher cat. no. A119-1), or equivalent.

Water, ultra-pure or HPLC grade, purified in-house with a HYDRO® purification system or equivalent.

Polypropylene glycol, M.W. 425 (Aldrich cat. no. 20,230-4).

Polypropylene glycol, M.W. 1000 (Aldrich cat. no. 20,232-0).

Polypropylene glycol, M.W. 2000 (Aldrich cat. no. 20,233-9).

PPG tuning solution (for mass calibration of the LC/MS system). Dissolve 0.0014 grams of PPG 425, 0.0100 grams of PPG 1000, 0.0400 grams of PPG 2000, and 0.0126 grams of ammonium formate in 50 mL of methanol, 50 mL water, and 0.1 mL of acetonitrile. Mix well. Store refrigerated in an amber bottle. (Or use an equivalent mass tuning solution specified by the instrument manufacturer or SOP.)

Extraction Solvent: 80:20 (v/v) acetonitrile:purified water. To prepare one liter, combine 800 mL HPLC grade acetonitrile and 200 mL purified water. Mix well and store at room temperature.

Appendix 2. Reagents and Analytical Standards (continued)

SPE Wash and LC Sample Dilution Solvent: 30:70 (v/v) acetonitrile:purified water. To prepare one liter, combine 300 mL HPLC grade acetonitrile and 700 mL purified water. Mix well and store at room temperature.

LC Mobile Phase A: Add 1 mL of 90% formic acid and 250 mg ammonium formate to each liter of purified water. Mix well and store at room temperature.

LC Mobile Phase B: Add 1 mL of 90% formic acid and 250 mg ammonium formate to each liter of HPLC grade methanol. Mix well and store at room temperature.