2.0 INTRODUCTION

Described in this report is the independent laboratory validation (ILV) of Syngenta Analytical Method AG-498 entitled "Analytical Method for the Determination of CGA136872 in Soil by High Performance Liquid Chromatography," as performed by ADPEN Laboratories, Inc. (Reference 1).

This study was designed to satisfy harmonized guideline requirements described in OCSPP 850.6100, Environmental Chemistry Methods and Associated ILV (Reference 2). This study was conducted in compliance with EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160 (Reference 3).

3.0 MATERIALS AND METHODS

3.1 Reference Substances

The reference substance was obtained from Syngenta Crop Protection and stored as directed. All fortification and calibration solutions made from the reference substance (analytical standards) were stored according to the method.

The following reference substance was used:

Common Name:	**Primisulfuron-Methyl**
IUPAC Name:	2-{3-[4,6-bis(difluoromethoxy)-pyrimidin-2-yl]-ureidosulfonyl} benzoic acid methyl ester
CAS Number:	86209-51-0
Lot Number:	685483
Molecular Formula:	$C_{15}H_{12}F_4N_4O_7S$
Molecular Weight:	468.3
Storage Conditions:	Room temperature in Standard cabinet C-1
Batch Identification:	AMS 256/2
Purity:	99.5%

Characterization data for the reference standard is maintained by the Sponsor, Syngenta Crop Protection. The Certificate of Analysis is presented in Appendix 3.

3.2 Test System

The following are the representative soil samples used for this study:

- Clay loam soil - Sample ID: RIMV00213-0001 (Underwood Farm, OH, 0-6" under protocol number TK0002309)
- Sandy loam soil - Sample ID: RIMV00213-0002 (San Luis Obispo Farm, CA, 0-6" collected on 01/07/2011)

Soil samples were sent from Syngenta to ADPEN on 08/19/2013 by FedEx priority overnight and received at room temperature on 08/20/2013. Upon receipt, samples were logged into LIMS and assigned unique laboratory codes, which are cross-referenced to the Syngenta sample ID numbers on raw data and detailed residue reports. Samples were stored in freezer E-23, which had a temperature range during the course of this study of -29 to -6 °C. Sample extracts were stored in refrigerator E-54 while awaiting HPLC analysis. The temperature range during the course of this study for refrigerator E-54 was 6–7 °C.

3.3 Preparation of Standard Solutions

All stock and concentrated standard solutions were prepared as specified in the analytical method and were stored in freezer E-51. Calibration standard solutions for HPLC-UV analysis were prepared in acetonitrile (ACN) as specified in the analytical method and were stored in freezer E-51. Calibration standard solutions for LC-MS/MS analysis were prepared in 90:10 (v/v) 0.2 M ammonium acetate in water/ACN to be amenable to instrument conditions and were stored in refrigerator E-51.

3.4 Analytical Procedure

Analytical Method AG-498 was independently validated for sandy loam soil and clay loam soils. See the

3.4.1 Reagents and Apparatus

The reagents and apparatus used for the method trial were as outlined in the analytical method with equivalent substitutions as necessary.

3.4.2 Fortifications

Untreated control soil samples were fortified using microliter amounts of the appropriate fortification standard at LOQ (0.01 ppm) and 10× LOQ (0.1 ppm) concentrations as per the method. Fortifications used in this method validation are as follows:

Matrix	Fortification Volume (mL)	Fortification Concentration (µg/mL)	Sample Weight (g)	Final Concentration (ppm)	Replicates
Soil	0.25	1	25.0 ± 0.02	0.01	5
	0.25	10	25.0 ± 0.02	0.1	5

3.4.3 Extraction Procedure

Extraction

1. Accurately weigh and transfer 25 g of each soil sample into a separate 175-mL plastic disposable centrifuge bottles.
2. Fortify samples, if necessary.
3. Accurately add 125 mL acetonitrile-water-NH_3 (90:8:2 v/v) into the sub-sample.

4. Properly cap the bottle and shake well at room temperature for one hour.
5. Centrifuge samples at 4160 rpm for 15 minutes.
6. Decant supernatant through a Whatman 2V filter inside the hood.

Partition

7. Aliquot 25 mL of this solution (5 g ESW) into a 125 mL round bottom flask.
8. Concentrate under vacuum until acetonitrile is evaporated, leaving a few drops of water.
9. Add 50 mL of 0.1M Na_2CO_3 to the round bottom flask then transfer to a 250 mL separatory funnel.
10. Add 50 mL of toluene. Shake vigorously for 30 seconds. Allow to separate. Transfer the lower aqueous layer to another 250 mL separatory funnel and discard the toluene layer.
11. Slowly add 10 mL of 1.2M H_3PO_4 and swirl gently. Allow the CO_2 to escape.
12. Check the pH (should be between 2 and 3) and add 25 mL of dichloromethane. Shake vigorously for 30 seconds and allow separation.
13. Collect the lower dichloromethane layer into a 125 mL round bottom flask.
14. Repeat steps 12 and 13.
15. Evaporate the dichloromethane under vacuum to dryness at no more than 45°C. Usually there will be a few droplets of water.
16. Immediately add 5 mL of acetonitrile to the flask, swirl and evaporate to dryness again. Do not allow the flask to stay under vacuum a prolonged time before the ACN is added. The acetonitrile evaporation removes the residual water.
17. Dissolve the residue in the round bottom flask in 5 mL of acetonitrile as soon as evaporation is finished.

Cleanup

18. Prepare a set of SPE Sep-Pak Alumina A (Waters). Condition the SPE with 5 mL of methanol:acetonitrile (15:85 v/v) followed by 5 mL of acetonitrile. Help the flow of solvents with vacuum or rubber bulb.
19. Transfer the acetonitrile solution from the 125 mL round bottom flask on the SPE columns and allow draining under gravity. Discard the acetonitrile.
20. Elute the SPE columns with 30 mL of methanol:acetonitrile (15:85, v/v) and collect the eluate into a 50 mL round bottom flask. Evaporate to dryness under vacuum.
21. Add 0.500 mL of acetonitrile. Swirl to dissolve. Prepare dilutions if necessary and vial sample for analysis by HPLC.

3.4.4 Modifications for Sandy Loam Soils

The following was a necessary modification for the successful validation of the procedure with sandy loam soils:

1. Step 5.3.3 of the method's cleanup procedure was modified to use 30 mL of methanol:ACN (15:85, v/v). Column profiling during the method control stage

demonstrated incomplete elution of CGA136872 in the first 15-mL portion. Potentially due to lot variation, significant amounts of CGA136872 were found in the second 15-mL portion. Subsequent portions of methanol:ACN were unnecessary.
2. The mobile phase composition used for HPLC-UV analysis was modified in order to improve the resolution of the analyte peak from matrix interferences (see section 3.5).

3.4.5 Modifications for Clay Loam Soils

The following were necessary modifications for the successful validation of the procedure with clay loam soils:

1. Step 5.1.3 in the extraction procedure (5.1) was modified. Only 5 mL of the extract was filtered through a 0.45 µm syringe filter. A 0.5-mL aliquot was then diluted to 10 mL with 10:90 ACN: 0.2 M ammonium acetate and vialed for analysis by LC-MS/MS.
2. The procedures in 5.2 (Partition) and 5.3 (Cleanup) were bypassed due to recovery losses.
3. The detector and instrument conditions were modified to allow analyses by LC-MS/MS (see section 3.6).

These modifications can be incorporated into the original method as a suitable alternative for analyzing difficult soil matrices or can be written as a new method.

3.5 HPLC Instrumentation Parameters

HPLC System:	Agilent 1100 HPLC with UV detector
Column:	Phenomenex Luna C18, 250 mm, 4.6 mm, 5 µm
Column temperature:	Ambient
Injection Volume:	20.0 µL
Flow Rate:	1.0 mL/min
Mobile Phase A:	39.5% buffer: 0.02 M KH_2PO_4 and 0.02 M H_3PO_4 (80/20 v/v)
Mobile Phase B:	60.5% acetonitrile
Gradient:	Isocratic
Run Time:	15 minutes
Detector:	Variable Wavelength UV Detector
Detector wavelength:	234 nm
Retention Times:	Primisulfuron-Methyl, 8.76 minutes

3.6 LC-MS/MS Instrumentation Parameters

Liquid Chromatography Conditions

LC System:	Agilent 1290 UPLC
Flow Rate:	0.3 mL/min
Column:	Zorbax Eclipse Plus C18, 2.1 x 50 mm, 1.8 µm
Column temperature:	40 °C
Injection Volume:	10.0 µL
Run Time:	9 minutes
Retention Times:	Primisulfuron-Methyl, 4.5 minutes
Mobile Phase A:	0.2 M Ammonium Acetate in HPLC water
Mobile Phase B:	Acetonitrile

Mass Spectrometer Conditions

Detector:	Agilent 6490 Triple Quad
Interface:	ElectroSpray
Curtain gas:	14 L/min
Temperature:	150 °C
Capillary (V):	3000
V Charging:	1500
Nebulizer (psi):	45
Sheath gas heater:	300
Sheath gas flow:	12
MRM Conditions	**Primisulfuron-Methyl**
MS1:	469.1
MS2:	253.9
Dwell time:	100
Frag (V):	380
Collision Energy (V):	0
Cell Acc (V):	7
Polarity:	Positive

3.7 Data Acquisition

Peak integration and peak area count quantitation were performed by Agilent's ChemStation for HPLC analyses and Agilent's MassHunter for LC-MS/MS analyses. Both software systems generate a best-fit, linear regression equation which was derived and used in conjunction with the analyte response in each sample to calculate the concentration of the analyte. The square of correlation coefficients (r) for the calibration curves for each analytical set was greater than 0.99.

TABLE 1 Flow Diagram of the Analytical Procedure Method AG-498 using HPLC/UV

Weigh and transfer sample (25 g) to a 175 mL centrifuge bottle
(Fortify recovery sample, as needed)

↓

Add 125 mL ACN:water:NH_3 (90:8:2, v/v; 125 mL)

↓

Shake sample at high speed for one hour then centrifuge sample at 4160 RPM for 15 minutes.

↓

Decant supernatant through a Whatman 2V filter and transfer to a clean bottle.

Aliquot 25 mL into a 125 mL round bottom flask.

Concentrate until ACN is evaporated.

Add 50 mL of 0.1M Na_2CO_3 to the round bottom flask and swirl.

↓

Transfer lower aqueous layer to 250 mL sep funnel. Discard toluene layer.
Add 10 mL of 1.2 M H_3PO_4 and swirl. Allow CO_2 to escape. Check pH.

↓

Add 25 mL of DCM. Shake and separate. Collect lower DCM layer into a 125 mL flask.

↓

Repeat last step.

↓

Evaporate the DCM to dryness. Add 5 mL of ACN, swirl and evaporate.

↓

Condition SPE column with 5 mL of MeOH:ACN (15:85 v/v) followed by 5 mL of ACN.
Transfer on the SPE columns and drain under gravity. Discard ACN.

↓

Elute column with 30 mL MeOH/ACN (15:85 v/v) and collect into 50 mL flask.

↓

Evaporate to dryness. Add 0.5 mL of ACN. Swirl to dissolve.
Prepare dilutions. Transfer samples into HPLC vials

↓

Analysis by HPLC

TABLE 2 Flow Diagram of the Analytical Procedure Method AG-498 Modified using LC-MS/MS

Weigh and transfer sample (25 g) to a 175 or 250-mL centrifuge bottle
(Fortify recovery sample, as needed)

↓

Add 125 mL ACN:water:NH_3 (90:8:2, v/v; 125 mL)

↓

Shake sample at high speed for one hour then centrifuge sample at 4160 RPM for 15 minutes.

↓

Decant supernatant through a Whatman 2V filter and transfer to a clean bottle.

↓

Filter 5 mL of the filtrate through a 0.45 µm syringe filter

↓

Aliquot 0.5 mL into a 15 mL test tube.

↓

Dilute to 10-mL mark with ACN – 0.2 M ammonium acetate 10-90

↓

Vial for analysis by LC-MS/MS

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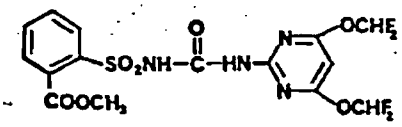
EDITION 2/24/86		DETERMINATION OF CGA-136872 IN SOIL BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
SUBMITTED BY: W. T. Beidler, K. P. Shoffner		
		APPROVED BY:

1.0 SCOPE

This method is used for the determination of residues of CGA-136872, structure shown below, in soil. The detection limit for the method is 0.01 ppm of CGA-136872.



CGA-136872

2.0 PRINCIPLE

Residues of CGA-136872 are extracted from soil by shaking for one hour at room temperature with acetonitrile:water: concentrated ammonium hydroxide, 90:8:2. An aliquot of the extract is evaporated to a small volume and diluted with 0.1M sodium carbonate. The alkaline aqueous solution is partitioned with toluene, then the aqueous portion is acidified with dilute phosphoric acid and partitioned again with dichloromethane. The dichloromethane is evaporated and the contents of the flask are dissolved in acetonitrile and the solvent evaporated again to remove any residual water. Final cleanup is performed with an Alumina-A Sep-Pak. Residues of CGA-136872 are determined by HPLC on a Zorbax-ODS column using a mobile phase comprised of 65% acetonitrile:28% 0.02M monobasic hydrogen phosphate:7% 0.02M phosphoric acid and UV detection at 234 nm. A flow diagram for the method is presented in Figure 1.

3.0 APPARATUS

3.1 Bottle, Boston round, 8-oz.

3.2 Bottle, Nalgene (polyethylene), 8-oz. wide-mouth.

3.3 Centrifuge (Sorvall RC2-B, equipped with a Type GSA rotor or equivalent).

3.4 Filter paper, Whatman 2V, 24-cm.

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		APPROVED BY:

3.5 Flask, round bottom, 100-ml and 50-ml.

3.6 Funnel, filter, 10-cm.

3.7 Funnel, separatory, 250-ml.

3.8 Graduated cylinder, 50-ml.

3.9 ISS-100 Microvial (Perkin-Elmer) or equivalent.

3.10 Mechanical shaker (Eberbach) or equivalent.

3.11 Rotary evaporator (Buchi) or equivalent.

3.12 Sep-Pak, Alumina-A (Waters Assoc.).

3.13 Syringe, Luer-Lok, 20-ml.

4.0 REAGENTS

4.1 Acetonitrile, HPLC grade.

4.2 Acetonitrile:water:conc. NH_4OH, 90:8:2.

4.3 Ammonium hydroxide, Conc., Reagent grade.

4.4 Dichloromethane, HPLC grade.

4.5 Methanol, HPLC grade.

4.6 Phosphoric acid, Reagent grade, 0.02M in deionized water.

4.7 Phosphoric acid, Reagent grade, 1.2M in distilled water.

4.8 Potassium dihydrogen phosphate, Reagent grade, 0.02M in deionized water.

4.9 Sodium carbonate, Reagent grade, 0.1M in distilled water.

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4.10 Standard CGA-136872 (available from CIBA-GEIGY Corp., P.O. Box 18300, Greensboro, NC 27419).

4.11 Toluene, HPLC grade.

4.12 Water, distilled.

4.13 Water, distilled, deionized.

5.0 PROCEDURE

5.1 Extraction

5.1.1 Weigh a 25-gram subsample from a well-homogenized, stone-free soil sample into an 8-oz. Nalgene bottle. Add 125 ml of the acetonitrile:water:NH_4OH (90:8:2) extraction mixture and shake for one hour at room temperature using a mechanical shaker.

5.1.2 Centrifuge for 10 minutes at 5,000 RPM using a Type GSA rotor.

5.1.3 Filter the sample through a Whatman 2V filter paper into an 8-oz. Boston round bottle.

5.2 Partition

5.2.1 Measure a 25-ml aliquot (5-g equivalent) of the extract from Step 5.1.3 into a graduated cylinder, pour the aliquot into a 100-ml round bottom flask and evaporate the solvent on a rotary evaporator until acetonitrile stops distilling. (There may be 1-2 ml of water remaining depending on the moisture content of the soil.)

5.2.2 Add 50 ml of 0.1M Na_2CO_3 to the 100-ml round bottom flask, then transfer to a 250-ml separatory funnel.

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5.2.3 Partition the aqueous solution with 50 ml of toluene by shaking vigorously for 30 seconds, then, after the layers separate, drain the lower layer into another 250-ml separatory funnel. Discard the toluene.

5.2.4 Add 10 ml of 1.2M H_3PO_4 to the separatory funnel containing the lower layer from Step 5.2.3. and shake carefully with frequent venting until most of the CO_2 has dissipated.

5.2.5 Partition the acidified aqueous solution (pH should be 2-3) with two 25-ml portions of dichloromethane shaking vigorously each time for 30 seconds.

5.2.6 Collect both the dichloromethane portions in a 100-ml round bottom flask and evaporate on a rotary evaporator at a bath temperature of 40-45°C. When the dichloromethane no longer distills (there will usually be several water droplets left on the walls of the flask) stop the evaporation, immediately add 5 ml of fresh acetonitrile to the flask, swirl thoroughly and evaporate again to dryness. It is important not to leave the flask on the rotary evaporator for prolonged periods, especially before the acetonitrile evaporation (which removes any residual water) is performed.

5.3 Cleanup

5.3.1 Fit a 20-ml Luer-Lok syringe with an Alumina-A Sep-Pak and wash the Sep-Pak first with 5 ml of 15% methanol/acetonitrile, then with 5 ml of 100% acetonitrile. It may be necessary to start the solvent flow through the Sep-Pak by applying pressure with a pipette bulb or pressurized air.

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5.3.2 Dissolve the residue in the flask from Step 5.2.6 in 5 ml of acetonitrile and pipette into the syringe. Once the flow is started, allow the solvent to drain by gravity. When flow stops, discard the acetonitrile.

5.3.3 Elute the Sep-Pak with 15 ml of 15% methanol/acetonitrile, collecting the eluant in a 50-ml round bottom flask. Evaporate the solvent on a rotary evaporator.

5.3.4 Dissolve the contents of the 50-ml round bottom flask in 0.5 ml of acetonitrile or some multiple of 0.5 ml for higher residue levels.

5.3.5 Pipette the sample into a microvial for HPLC analysis.

6.0 HPLC ANALYSIS

6.1 Preparation of Standard CGA-136872

6.1.1 Weigh 100.0 mg of CGA-136872 analytical standard into a 100-ml volumetric flask and dilute the flask to the mark with acetonitrile.

6.1.2 Make serial dilutions of the 1 mg/ml standard solution with acetonitrile to give a series of injection standards in a range of 0.05 to 2.0 ng per µl.

6.2 Standardization

6.2.1 Standardize the HPLC under the conditions listed in Table I by making 20-µl injections in the range of 1 to 40 ng, depending on the concentrations being determined.

6.2.2 Measure the peak heights of the injected standards. Typical chromatograms for standards are shown in Figure 2 and

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standardization data generated from the chromatograms are listed in Table II.

6.2.3 Construct a standard curve by plotting, either manually or by computer, the detector response versus nanograms injected, or enter the data into an appropriate electronic calculator to obtain a least squares regression line.

6.3 Detection of Sample Residues

6.3.1 Inject a 20-µl aliquot of the sample from Step 5.3.5 into the HPLC under the same conditions employed for standards. Make dilutions of samples, as necessary, to maintain peak heights within the range of the standard curve. Compare the peak heights of the unknown samples with the standard curve or enter into the least squares program to determine the nanograms of CGA-136872 present in the injected aliquot.

6.3.2 Calculate residue results in terms of ppm CGA-136872 by the following equation:

$$PPM = \frac{\text{CGA-136872 Found (ng)}}{\text{Mg Soil Injected}} \times \left[\frac{V+(W \times M/100)^*}{V}\right] \times \frac{100}{(100-M)} \times \frac{100}{R}$$

Where V = volume of extraction solvent (125 ml), W = weight of the soil sample (25 grams), M = percent moisture in soil and 100 = conversion factor. R = percent recovery based on fortified controls taken through the procedure.

This equation takes into account the volume increase in the extracting solvent due to water contained in the soil, the procedural recovery, and expresses the residue on the basis of soil dry weight.

*Generally, if the soil moisture is less than 10%, this portion of the equation may be omitted.

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6.4 Fortification Experiments

This method is validated for each set of samples analyzed by including an untreated control sample and one or more control samples fortified prior to extraction with 0.01 ppm or more of CGA-136872.

6.4.1 Add 0.25 ml of 1 µg/ml standard solution of CGA-136872 to 25 g of control soil prior to extraction (Step 5.1.1) for a 0.01 ppm fortification. Use correspondingly greater amounts of standards (volume not to exceed 2 ml) for higher fortifications. Analyze the control and fortified samples by the procedures of the method.

6.4.2 Calculate the final ppm value for the control and fortified samples according to the following equation:

$$PPM = \frac{\text{CGA-136872 Found (ng)}}{\text{Mg Soil Injected}} \times \frac{[V+(W \times M/100)]^*}{V}$$

*As in Section 6.3.2, this portion of the equation may be omitted if the soil moisture is less than 10%. The letters V, W and M have the same significance as in Section 6.3.2.

6.4.3 Correct the recovery value (ppm) by subtracting the ppm value, real or apparent, found in the control. Calculate the recovery factor in percent by the following equation:

$$R\ (\%) = \frac{\text{Ppm Found (corrected)}}{\text{ppm Added}} \times 100$$

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TABLE I: HPLC OPERATING CONDITIONS FOR DETERMINATION OF CGA-136872

Instrument:	Perkin-Elmer Series 4 Liquid Chromatograph with an LC85B Variable Wavelength UV Detector, an ISS-100 Sampling System, and a Chromatographics 3 Data Handling System or an equivalent HPLC pump and UV detector with or without automated data acquisition
Column:	Zorbax-ODS, 4.6 x 250 mm (DuPont Instruments)
Mobile Phase:	65% acetonitrile:28% 0.02M KH_2PO_4:7% 0.02M H_3PO_4
Flow Rate:	1.0 ml/min.
Temperature:	Ambient
Attenuation:	4
Detection:	Variable Wavelength UV Detector set at 234 nm
Minimum Detection Limit:	1.0 ng
Injection Volume:	20 µl
Chart Speed:	1.0 cm/min. from 4.5 to 5.5 minutes; 0.5 cm/min. all other times
Retention Time:	5.2 ± 0.15*

*The range of retention times is due to variations in ambient temperature.

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FIGURE 1: FLOW DIAGRAM FOR ANALYTICAL METHOD AG-498

25 g soil

↓

Shake for 1 hour with 125 ml of acetonitrile:H_2O:NH_4OH, 90:8:2.

↓

Centrifuge and filter.

↓

Evaporate the solvent from a 25-ml (5-g) aliquot.

↓

Add 50 ml of 0.1M Na_2CO_3 to the residue.

↓

Partition with 50-ml toluene.

↓

Toluene (discard) | Aqueous

↓

Acidify with 10 ml 1.2M H_3PO_4.

↓

Partition with dichloromethane (2x25 ml).

↓

Dichloromethane | Aqueous (discard)

↓

Evaporate solvent, add 5 ml acetonitrile and evaporate again. Dissolve residue in another 5 ml of acetonitrile.

↓

Prewash Alumina-A Sep-Pak first with 15% MeOH/acetonitrile then with 100% acetonitrile.

↓

Load the residue in acetonitrile onto the Sep-Pak.

↓

Discard the prewash and load solvents.

↓

Elute with 15 ml of 15% MeOH/acetonitrile.

↓

Collect the eluant and evaporate the solvent. Dissolve residue in 0.5 ml acetonitrile.

↓

HPLC