2.0 INTRODUCTION

Described in this report is the independent laboratory validation (ILV) of Syngenta Analytical Method GRM020.08B entitled "Trinexapac-ethyl - Residue Method (GRM020.08B) for the Determination of CGA300405 and CGA313458 Metabolites of Trinexapac-ethyl in Surface Water" (1) as performed by ABC Laboratories, Inc.

This study was designed to satisfy harmonized guideline requirements described in OCSPP 850.6100 (Data Reporting for Environmental Chemistry Methods). This study was conducted in compliance with EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160 (3) and EC Guidance Document SANCO/825/00 rev.7 (4).

The residue analytical method is suitable for the determination of CGA300405 and CGA313458 metabolites of trinexapac-ethyl in surface water.

Water samples were analyzed directly by LC-MS/MS after dilution with 30/70 acetonitrile/ultrapure water. Matrix matched standards may be required for certain water types.

The LOQ of the method is 10.0 µg/L (10.0 ppb) for water.

Untreated control and reagent blank samples analyzed with each set contained ≤20% of the LOQ in the matrices tested and are considered negligible.

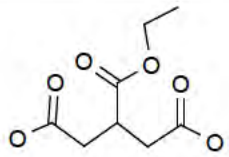
Recovery efficiency is generally considered acceptable when the mean values are between 70% and 120% and with a relative standard deviation of ≤ 20%.

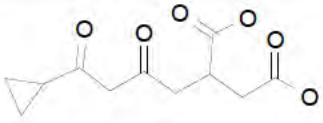
The method was used as written. Communication with the Sponsor Monitor occurred to discuss items such as 1) questions regarding instrumentation conditions, 2) clarification of some technical aspects of the method prior to validation trial 1, and 3) discussion regarding results from method validation trial 1. A complete list of communications is provided in Appendix 6.

3.0 MATERIALS AND METHODS

3.1 Test/Reference Substance

The test/reference substances were obtained from Syngenta Crop Protection, LLC. The following test/reference substances were used:

Compound Structure	 $C_8H_{12}O_6$
Syngenta Code:	CGA300405
Common Name:	Not available
IUPC Name:	3-Ethoxycarbonyl-pentanedioic acid
CAS Name:	1,2,3-Propanetricarboxylic acid, 2-ethyl ester
CAS Number:	Not Assigned
Molecular Weight:	204.17
Standard Reference:	NV-XXVII-89-1
Storage Conditions:	Refrigerated
Purity:	92.6%
Expiration Date:	30 June 2013

Compound Structure	 $C_{11}H_{14}O_6$
Syngenta Code:	CGA313458
Common Name:	Not available
IUPC Name:	2-(4-Cyclopropyl-2,4-dioxo-butyl)-succinic acid
CAS Name:	Butanedioic acid, (4-cyclopropyl-2,4-dioxobutyl)-
CAS Number:	Not Assigned
Molecular Weight:	242.00
Standard Reference:	DAH-XXXV-15
Storage Conditions:	Refrigerated
Purity:	97.1%
Expiration Date:	31 March 2014

Characterization data for the test/reference standard are maintained by the Sponsor, Syngenta Crop Protection, LLC. The Certificates of Analysis is included in Appendix 3.

The test/reference substance (analytical standard) used in this study was procured from the Sponsor and stored as directed on the Certificate of Analysis. All solutions made from the reference substances (analytical standards) were stored according to the method.

3.2 Test System

The test system evaluated in this study was surface water. This matrix was chosen because it is representative of the matrices the method was designed for. The control sample used in this study was provided by the Sponsor. The control water samples were characterized by

Syngenta Crop Protection, LLC under Syngenta Study Number TK0044976. GLP characterization results are presented in Table 1 and summarized below:

Sample ID	pH	Calcium (ppm)	Magnesium (ppm)	Hardness CaCO3 (mg/L)	TDS (ppm)	TSS (ppm)	DOC (ppm)
Surface (WC06)	6.4	3.5	1.6	15	126	NA	8.9

The surface water sample was were shipped on dry ice from Morse Laboratories, LLC on 02 July 2012 and received in good condition at ABC Laboratories on 03 July 2012. The control sample was held frozen until used in the study.

These control samples were checked for contamination prior to use in this ILV study by employing the same extraction and detection method as described in Syngenta Method GRM020.08B.

3.3 Equipment and Reagents

The equipment and reagents/supplies used for the method validation were as outlined in the method. Identical or equivalent equipment and materials were used, as permitted by the method. The equivalent equipment and reagents used were as follows:

3.3.1 Equipment

Balance:	Analytical balance capable of weighing to ±0.01 mg Model BB2440 (Mettler Toledo)
	Analytical balance capable of weighing to ±0.01 mg Model XP205DR (Mettler Toledo)
HPLC vial:	National Scientific Target Snap-Its vial
Centrifuge Tubes:	Polypropylene (PP), 15-mL, BD Falcon
Pipettes:	Various sizes Gilson Brand

3.3.2 Reagents

HPLC water:	Fisher Scientific
Acetonitrile (ACN, MeCN):	Fisher Scientific
Ammonium acetate:	Fisher Scientific
Methanol:	Fisher Scientific

3.3.3 Preparation of Reagents

30:70 Acetonitrile:H_2O (Extraction Solvent)
Combine 300 mL of acetonitrile and 700 mL of HPLC grade water.

50/50 Methanol/water (v/v) in 10 mM Ammonium acetate (Mobile Phase A)
Add 0.77 g of ammonium acetate to 500 mL of HPLC grade water in a 1 L carboy bottle and add 500 mL of methanol to bring final volume to 1 L and mix well.

90/10 Acetonitrile/water (v/v) in 10 mM Ammonium acetate (Mobile Phase B)
Add 1.54 g of ammonium acetate to 200 mL of HPLC grade water in a 2 L carboy bottle and add 1800 mL of acetonitrile to bring final volume to 2 L and mix well.

Needle Wash 1:1:1 MeOH/ACN/Water (Needle Wash)
Combined 4-L each of acetonitrile, methanol, and high purity water. Swirled until mixed well.

10 mM Ammonium acetate in (90:10) Acetonitrile/water
Add 20 mL of 1 M ammonium acetate to a mixture of 1800 mL of acetonitrile and 200 mL of water in to a carboy bottle. Swirl until mixed well.

Needle Wash 1:1:2 MeOH/ACN/Water (Needle Wash, control screen and matrix test)
Combined 1000 mL of acetonitrile, 1000 mL of methanol, and 2000 mL of high purity water into an appropriate container. Swirled until mixed well.

50:50 Acetonitrile:H_2O
Separately measure equal amounts of acetonitrile and HPLC grade water. Add to an appropriate container. Cap and invert several times to mix well.

3.4 Preparation of Standard Solutions

The preparation of CGA300405 and CGA313458 metabolites of trinexapac-ethyl standard solutions used for this study is described below. The solutions were stored as recommended in the method when not in use (refrigerated).

3.4.1 Stock Standard Solution

Approximately 10 mg (adjusted for purity) of CGA300405 analytical standard were accurately weighed and quantitatively transferred into a volumetric flask via methanol. The flask was brought to a volume of one hundred milliliters of methanol before calculating concentration. The CGA300405 concentration was determined to be 100 µg/mL.

Approximately 10 mg (adjusted for purity) of CGA313458 analytical standard were accurately weighed and quantitatively transferred into a volumetric flask via methanol. The flask was brought to a volume of one hundred milliliters of methanol before calculating concentration. The CGA313458 concentration was determined to be 100 µg/mL.

3.4.2 Fortification Standards and Intermediate Solutions

1.00 µg/mL	1.0 mL of a 100-µg/mL standard solution were transferred to a 100-mL volumetric flask. The contents were brought to volume with methanol and mixed well.
0.10 µg/mL	10.0 mL of a 1.00-µg/mL standard solution were transferred to a 100-mL volumetric flask. The contents were brought to volume with methanol and mixed well.
0.01 µg/mL	1.0 mL of a 1.00-µg/mL standard solution were transferred to a 100-mL volumetric flask. The contents were brought to volume with methanol and mixed well.

3.4.3 Calibration Standard

Calibration standards were prepared by adding a known volume of standard to a known volume of 30:70 acetonitrile:water.

Initial Standard Concentration (µg/mL)	Initial Standard Volume (mL)	Amount 30:70 ACN:Water added (mL)	Final Standard Concentration (ng/mL)
1.00	0.100	9.90	10.0
1.00	0.050	9.95	5.00
1.00	0.020	9.98	2.00
1.00	0.010	9.99	1.00
0.10	0.050	9.95	0.50
0.10	0.020	9.98	0.20
0.10	0.010	9.99	0.10

For matrix match calibration standards, control matrix was added to a known volume of each concentration level of the standards.

Initial Standard Concentration (µg/mL)	Initial Standard Volume (mL)	Control Matrix added (mL)	Final Standard Concentration (ng/mL)
10.0	1.30	0.10	9.3
5.00	1.30	0.10	4.6
2.00	1.30	0.10	1.9
1.00	1.30	0.10	0.93
0.50	1.30	0.10	0.46
0.20	1.30	0.10	0.19
0.10	1.30	0.10	0.093

3.5 Analytical Procedures and Modifications

The analytical method independently validated in this study was a Syngenta method GRM020.08B entitled "Trinexapac-ethyl - Residue Method (GRM020.08B) for the Determination of CGA300405 and CGA313458 Metabolites of Trinexapac-ethyl in Surface Water." See Appendix 2 for the complete text of the method. The following is a summary of that method:

If water samples were received frozen they were allowed to defrost completely at room temperature. Defrosted samples were shaken thoroughly to ensure sample homogeneity prior to subsequent aliquoting for further treatment or analysis. 1.0 mL of water sample was accurately transferred into a 15 mL polypropylene falcon tube. Sample fortification was carried out at this time. Dilution was performed using the appropriate volume with 30/70 acetonitrile/water. The method LOQ (10 ppb) can be diluted 1 to 10 while maintaining acceptable signal/noise ratio at 1 ppb. The samples were vialed for analysis by LC-MS/MS in negative ion mode using turbo ion spray.

Control samples were analyzed with each set of samples to verify that the sample used to prepare recovery samples was free from contamination. A minimum of two control samples were analyzed with each batch of samples. Control samples were of the same matrix to monitor any instrumental matrix effects present.

The fortification levels were appropriate to the residue levels expected in the sample. Recovery efficiency is generally considered acceptable when the mean values are between 70% and 120% and with a relative standard deviation of ≤ 20%.

The method was used as written, with the minor modification shown below. Residue calculations were performed as specified in the analytical method and were conducted using a validated software application (Applied BioSystems/MDS Sciex Analyst Software, version 1.5) 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 concentration of the analyte found during sample analysis from the calculated best-fit line forced through zero. Equations used for calculation of residues and example calculations can be found in Appendix 4. The calculation

spreadsheets can be found in Appendix 5.

3.5.1 Modifications

Syngenta Method GRM020.08B was followed as written with exception:

A minimum of 50 equilibration injections were required to achieve constant instrumental response prior to acquiring data for the sample set. This was achieved by injecting the sample set (samples and matrix matched standards) three times and then acquiring data on the fourth injection.

3.5.2 Fortifications

Untreated control surface water samples were fortified using milliliter amounts of the appropriate fortification standard to LOQ and 10X LOQ concentrations as per method. Fortifications used in this method validation are as follows:

Matrix	Fortification Volume (mL)	Fortification Conc. (µg/mL)	Final Volume (mL)	Final Conc. (ppb)	Replicates
Surface Water	0.10	0.10	14.0	10 (LOQ)	5
	0.10	1.0	14.0	100 (10X LOQ)	5

3.5.3 Extraction Procedure

As indicated by method GRM020.08B the following extraction steps were performed:

1. Accurately measure 1.00 mL of the sample into a15-mL plastic centrifuge tube.
2. Fortified appropriate samples.
3. Dilute to 14 mL with 30:70 ACN:H_2O.
4. Vial for analysis.

3.6 Instrumentation

All samples were analyzed by HPLC (High Pressure Liquid Chromatography) tandem mass spectrometric (MS/MS) detection. The conditions listed below are those employed for the control suitability evaluation and the successful first validation trial.

Control Suitability and Method Validation Trial No. 1:

Operating Conditions

Instrument: Applied Biosystems/Sciex API 5000 LC/MS/MS System with Waters Acquity Column Manager, Waters Acquity

Sample Manager, Waters Acquity Binary Solvent Manager, and Waters Acquity Sample Organizer with Applied Biosystems/MDS Sciex Analyst Software for data collection and system control (version 1.5.1)

Analytical column: ACE 3 C18 3.0x50mm

Mobile phase A: Methanol/Water (50/50) in 10mM Amm. Acetate
Mobile phase B: MeCN/Water (90/10) in 10mM Amm. Acetate

Gradient:

Time (min.)	% A	% B	Flow Rate (mL/min.)
0.00	0	100	1.0
0.50	0	100	1.0
1.00	100	0	1.0
2.00	100	0	1.0
3.00	0	100	1.0

Divert valve: Not used

Injection volume: 20 µL(control suitability, matrix effect),
10 µL (Trial 1, storage stability)

Column temperature: 40°C

Retention time: CGA300405: 0.26 minutes
CGA313458: 0.26 minutes

Run Time: 3.00 minute

Mass Spectrometer Conditions

Interface	:	TIS (Turbo Ion Spray)
Polarity	:	Negative mode
Curtain gas (CUR)	:	25.00
Temperature (TEM)	:	600.00
Ionspray voltage	:	-2500.00
Collision gas setting (CAD)	:	4.00
Gas 1 (GS1)	:	50.00
Gas 2 (GS2)	:	50.00
Interface heater (ihe)	:	ON
Scan type	:	MRM

MRM Conditions		**CGA300405 Primary Transition**	**CGA300405 Confirmatory Transition**	**CGA313458 Primary Transition**	**CGA313458 Confirmatory Transition**
Q1 *m/z*	:	203.0	203.0	241.0	241.0
Q3 *m/z*	:	69.0	157.0	83.0	69.0
Dwell time	:	50.00	50.00	50.00	50.00
Resolution Q1	:	Unit	Unit	Unit	Unit
Resolution Q3	:	Unit	Unit	Unit	Unit
Declustering potential (DP)	:	-45.00	-45.00	-45.00	-45.00
Entrance potential (EP)	:	-10.00	-10.00	-10.00	-10.00
Collision energy (CE)	:	-28.00	-13.00	-33.00	-35.00
Collision cell exit potential (CXP)	:	-9.00	-9.00	-9.00	-9.00

3.7 Data Acquisition

Peak integration and peak area count quantitation were performed by Applied BioSystems/MDS Sciex Analyst Software, version 1.5. A best-fit, linear regression equation, with 1/x weighting, was derived and used in conjunction with the analyte response in each sample to calculate the concentration of analyte. The square of correlation coefficients (R^2)

for the calibration curves for each primary transition analytical set was greater than 0.99. Recovery results were computed for each sample.

A statistical treatment of the data includes the calculation of averages, standard deviations, relative standard deviations. Mean percent recoveries, standard deviations, and relative standard deviations were calculated using a current Microsoft Office Excel package.

APPENDIX 4 Example Calculations

Equations

Calculations for instrumental analysis 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 forced through zero.

The equation used for the least squares fit is:

$$y = mx + b \text{ forced through } 0$$

where:

y	=	peak response
m	=	slope
x	=	ng/mL found for peak of interest
b	=	y-intercept forced through 0

The standard (calibration) curves generated for each analytical set were used for the quantitation of Trinexapac-ethyl 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) for CGA300405 and CGA313458 are:

1. The amount of analyte (in ppb) found in the sample was calculated according to the following equations:

$$\text{ppb found } (\mu\text{g/kg}) = \frac{\text{ng/mL} \times \text{Aliquot Factor} \times \text{Final Vol (mL)} \times \text{Dilution Factor}}{\text{Sample Weight (g)}}$$

where:

ng/mL = (y –b)/m

Aliquot Factor = $\frac{\text{Extraction Volume (mL)}}{\text{Aliquot Volume (mL)}} = \frac{14 \text{ mL}}{14 \text{ mL}} = 1$

Final Volume (mL) = final volume of solution which is submitted for analysis (typically approximately 14.0 mL)

Dilution factor	=	dilution of sample extract required to produce an analyte response bracketed by standards (typically 1)
Sample Volume (g)	=	gram weight of sample extracted (typically 1 mL)

2. The percent recovery in fortified control samples is calculated as follows:

$$\% \text{ Recovery} = \frac{\text{ppm found in fortified control - ppm found in control}}{\text{fortification level (ppm) added}} \times 100$$

Example Calculations

1. ABC Laboratories, Inc. control water, 68826-027, Set 1, CGA300405, (Figure 9):

$$\text{ng/mL} = 0$$

$$\frac{0 \times 14 \text{ mL}/14 \text{ mL} \times 14 \times 1}{1} = 0.0 \text{ ppb (reported as ND)}$$

2. ABC Laboratories, Inc. fortified control water @ 10.0 ppb, 68826-029, Set 1, CGA300405, (Figure 11):

$$\text{ng/mL} = 0.76895$$

$$\frac{0.76895 \times 14 \text{ mL}/14 \text{ mL} \times 14 \times 1}{1} = 10.76527 \text{ ppb (reported as } 10.765 \text{ ppb)}$$

$$\frac{(10.765 - 0)}{10.0} \times 100 = 108\% \text{ Recovery}$$