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Chlorothalonil

Analytical Method (GRM005.07A) for the Determination of Chlorothalonil Degradates R182281, R611966, R611968, R611965, R613636, R417888, SYN510573 and SYN546669 in Soil

Analytical Method

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VOLUME 1 OF 1 OF STUDY

1.0 INTRODUCTION

1.1 Scope of the Method

Analytical method GRM005.07A is suitable for the determination of chlorothalonil soil degradates R182281, R611966, R611968, R611965, R613636, R417888, SYN510573 and SYN546669 (Figures 1 - 8) in soil. The limit of quantification (LOQ) of the method has been established at 5 ppb (µg/kg) for R182281, R611966, R611968, R611965, R613636, R417888, SYN510573 and SYN546669 in soil.

This method satisfies US EPA guidelines EPA OCSPP 850.6100 and EC Guidance Documents SANCO/3029/99 rev 4 and SANCO/825/00 rev 8.1.

1.2 Method Summary

Typically, 20 gram sub-samples of soil are initially extracted with 50/50 (v/v) MeOH/ultrapure water by sonication and then shaking followed by second extraction with 50/50 (v/v) acetonitrile/ultrapure water at room temperature. The extracts are combined after centrifuging and adjusted to a final volume. An aliquot of the combined soil extract is diluted and injected onto LC-MS/MS with negative electrospray ionization (ESI) for analysis for R182281, R611968, R611965, R417888, SYN510573 and SYN546669. A separate aliquot of the combined soil extract is carried through QuEChERS treatment followed by a solid phase extraction (SPE) clean-up to facilitate proper sample treatments for R611966 and R613636 prior to final residue determination using GC-MSD.

The limit of quantification (LOQ) of the method has been established at 5 ppb (µg/kg) for R182281, R611966, R611968, R611965, R613636, R417888, SYN510573 and SYN546669 in soil.

2.0 MATERIALS AND APPARATUS

2.1 Apparatus

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

2.2 Reagents

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. A list of reagents used in this method along with details of preparation of solutions is included in Appendix 2.

2.3 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 gloves and laboratory coat.
3. Prevent inhalation and contact with mouth.
4. Wash any contaminated area immediately.

2.3.1 Stock Solutions

Prepare individual 100 µg/mL stock solutions for R182281, R611966, R611968, R611965, R613636, R417888, SYN510573 and SYN546669 by one of the following methods.

Weigh out accurately, using a five figure balance, sufficient individual analytical standards (purity corrected) into an amber "Class A" volumetric flask (100 mL). Dilute to the mark with MeOH to give a 100 µg/mL stock solution for R182281, R611966, R611968, R611965, R613636, R417888, SYN510573 and SYN546669, individually.

Alternatively, the appropriate volume of MeOH to add to a known amount of standard material may be determined using the equation below. The standard concentration is corrected for its chemical purity.

$$V = \frac{W \times P}{C} \times 1000$$

P	=	Standard purity in decimal form (P(%)/100)
V	=	Volume of methanol required
W	=	Weight, in mg, of the solid analytical standard
C	=	Desired concentration of the final solution, (µg/mL)
1000	=	Unit conversion factor

In this case, the standard material is weighed directly into an appropriate storage vessel.

2.3.2 Fortification Solutions

Sample fortification solutions containing R182281, R611966, R611968, R611965, R613636, R417888, SYN510573 and SYN546669 should be prepared by serial dilution with MeOH. It is recommended that the following mixed fortification solutions are prepared for fortification purposes: 1 µg/mL and 0.1 µg/mL.

2.3.3 Preparation of Calibration Standards for LC-MS/MS

Calibration standards should be prepared by diluting the 1 µg/mL mixed fortification standard with 10/90 (v/v) MeOH/ultrapure water. It is recommended that the following calibration standard solutions are prepared: 5, 2, 1, 0.5, 0.2, 0.1 and 0.05 ng/mL.

Any significant matrix effects observed may be compensated by use of matrix-matched standards at the discretion of the study director, or by further dilution of the sample fraction with 10/90 (v/v) MeOH/ultrapure water should instrument sensitivity permits. To prepare matrix-matched standard solutions, intermediate calibration standards must be prepared with 10/90 (v/v) MeOH/ultrapure water to yield concentrations at 50, 20, 10, 5, 2, 1 and 0.5 ng/mL. Mix 100 µL of the intermediate standards with 900 µL of control final fractions to yield matrix-matched standards at 5, 2, 1, 0.5, 0.2, 0.1 and 0.05 ng/mL.

Calibration curves should be generated to quantify residues of R182281, R611968, R611965, R417888, SYN510573 and SYN546669 in a sample set using 1/x weighing factor. Standards over an appropriate concentration range should be prepared in a manner similar to description above. The linear regression coefficient should be >0.99.

2.3.4 Preparation of Calibration Standards for GC-MSD

Calibration standards should be prepared by diluting the 1.0 µg/mL mixed calibration standard with HPLC grade toluene (Note: Solvent exchange is required to prepare the working mixed calibration standard from individual stock standards as described in Section 2.3.1). It is recommended that the following calibration standard solutions are prepared: 100, 50, 20, 10, 5 and 2 ng/mL in HPLC grade toluene.

Any significant matrix effects observed may be compensated by use of matrix-matched standards at the discretion of the study director, or by further dilution of the sample fraction with toluene should instrument sensitivity permits. To prepare matrix-matched standard solutions, intermediate calibration standards must be prepared with toluene to yield concentrations at 1000, 500, 200, 100, 50 and 20 ng/mL. Mix 100 µL of the intermediate standards with 900 µL of control final fractions to yield matrix-matched standards at 100, 50, 20, 10, 5 and 2 ng/mL.

Calibration curves should be generated to quantify residues of R611966 and R613636 in a sample set. Standards over an appropriate concentration range should be prepared in a manner similar to description above. The linear regression coefficient should be >0.99.

2.3.5 Standard Solution Storage and Expiration

All stock solutions should be stored in a refrigerator or freezer when not in use to prevent decomposition and/or concentration of the standard. Standard solutions should be allowed to equilibrate to room temperature prior to use.

An expiration date of six months for R182281, R611966, R611968, R611965, R613636, R417888, SYN510573 and SYN546669 in methanol is recommended unless additional data are generated to support a longer expiration date.

2.4 Safety Precautions and Hazards

The following information is included as an indication to the analyst of the nature and hazards of the reagents used in this procedure. If in any doubt, consult the appropriate MSDS or a monograph such as 'Hazards in the Chemical Laboratory', edited by S. G. Luxon, The Chemical Society, London (Reference 1).

Solvent and Reagent Hazards

	Acetonitrile	MeOH	Formic acid	Conc. NH_3	Toluene	Acetone
Harmful Vapor	✓	✓	✓	✓	✓	✓
Highly Flammable	✓	✓	✖	✖	✓	✓
Harmful by Skin Absorption	✓	✓	✓	✓	✓	✓
Irritant to respiratory system and eyes	✓	✓	✓	✓	✓	✓
Causes severe burns	✖	✖	✓	✓	✖	✖
Syngenta Hazard Category (SHC)	SHC-C, S	SHC-C, S	SHC-C, S	SHC-C, S	SHC-D, S	SHC-C, S
OES Short Term (mg/m^3)	105	310	N/A	24*	560	3,560
OES Long Term (mg/m^3)	70	260	9	17*	188	750

N/A not known; * Based on NH_3

At present there are insufficient data available to assign a Syngenta Hazard Classification for R182281, R611966, R611968, R611965, R613636, R417888, SYN510573 and SYN546669. They should be treated as a category SHC-D compounds until further information indicates otherwise. The Syngenta Hazard Category scale rates highly toxic chemicals as category SHC-E and non-toxic chemicals as category SHC-A. An additional hazard category of S indicates the compound is a severe skin and eye irritant.

In all cases avoid breathing vapor. Avoid contact with eyes and skin.

3.0 ANALYTICAL PROCEDURE

A summary of the method is included in flow-chart form in Appendix 4.

3.1 Sample Preparation

All samples should be prepared using an approved method of preparation to obtain a homogeneous sample prior to analysis.

3.2 Sample Fortification

In order to verify method performance and allow recovery corrections to be made (if appropriate), fortified control samples should be included with each sample set. To each pre-weighed control soil sample, add the appropriate amount of mixed fortification standard solution containing R182281, R611966, R611968, R611965, R613636, R417888, SYN510573 and SYN546669 in MeOH. Let each sample stand for at least five minutes after fortification to allow the spiking solution to soak into the matrix before proceeding with the extraction procedure. At least one untreated control and two fortified control samples should be analyzed with each sample set.

3.3 Extraction

A summary of the method is included in flow-chart form in Appendix 4.

a) Weigh a representative amount of soil (20 g) into a disposable polypropylene centrifuge tube (50 mL). Fortify samples with a mixed fortification standard solution in MeOH if necessary at this point. At least one untreated control and two control samples fortified with known amounts of analytes of interest should be analyzed with each sample set, using the same procedure, to verify method performance. No more than 1.0 mL of fortification standard solution should be added. Allow fortified control samples to equilibrate for at least 5 minutes before proceeding to the extraction;

b) Add 30 mL of 50/50 (v/v) MeOH/ultrapure water into the centrifuge tube (50 mL) and cap. Swirl the content followed by sonication in a water bath for 10 minutes;

c) Place the tube (horizontal orientation) on a mechanical shaker and shake at a speed (typically ~ 300 cps) that visibly agitates the samples vigorously for 30 minutes;

d) Centrifuge samples at approximately 6000 rpm with refrigeration at 10°C (or at a speed that visibly separates the solid sample from the supernatant) for about 10 minutes;

e) Carefully decant supernatant to a second clean polypropylene centrifuge tube (50 mL);

   Note: With some soils, particularly those with high clay contents, the solution may still be visibly cloudy even after centrifugation. This is normal and will not affect results.

f) Repeat extraction using second 20 mL of 50/50 (v/v) acetonitrile/ultrapure water for the remaining solid (soil) in the centrifuge tube (50 mL) from the first extraction. Cap and shake by hand or vortex to mix. If shaking cannot break up the compacted soil, use a suitable implement (e.g., a spatula) to facilitate this process. Swirl the content to facilitate mixing and shake on a mechanical shaker at a speed that visibly agitates the samples for a minimum of 30 minutes. Once again, the tube should be placed in a flat or horizontal orientation.

g) Centrifuge the sample at approximately 6000 rpm with refrigeration at 10°C (or at a speed that visibly separates the solid sample from the supernatant) for about 10 minutes;

h) Decant the supernatant from Section 3.3(g) into the centrifuge tube (Section 3.3 (3)) and combine with the previously collected extract. Carefully adjust the final volume to 50 mL with HPLC grade MeOH.

i) Mix the combined extract well and store in a refrigerator if next step is not conducted immediately.

3.4 Analysis of R182281, R611965, R611968, R417888, SYN546669 and SYN510573

a) Transfer an aliquot (1 mL) from the combined sample extract (Section 3.3(i)) into a clean polypropylene centrifuge tube (15 mL) and add 0.5 mL of MeOH. Dilute the sample to the 10 mL mark with ultrapure water to yield the sample final fraction;

b) Mix well and transfer ~ 1 mL of the aliquot into a HPLC injection vial for LC-MS/MS analysis. See the LC-MS/MS conditions/parameters in Sections 4.1 - 4.3 and use the LC-MS/MS standard solutions as described in Section 2.3.3 for quantitation.

3.5 Optional Dispersive Solid Phase Extraction (*d*-SPE) Procedure

Note: The following *d*-SPE procedure is used only when a direct injection meets sensitivity or interference problems.

a) Weigh approximately 100±10 mg of BONDESIL C18 (40µm; Agilent P/N 12213012) into a 2-mL (or an appropriate size) centrifugal filter device (Millipore; Cat. No. UFC40HV00) in top filter cup.

b) Accurately add 0.5 mL of HPLC grade MeOH to the filter device containing BONDESIL C18 material.

c) Transfer accurately 1.5 mL of the combined extract from Section 3.3 (i) into the BONDESIL C18 containing centrifugal filter device from Section 3.5 (b).

d) Add 20 µL of freshly prepared 1% NH_4OH solution to the sample and cap the vial.

e) Gently mix the mixture with mild swirling action for approximately 30 seconds. Then, allow the vial to sit with occasional swirling for approximately 5 minutes.

f) Centrifuge the sample at approximately 6000 rpm with refrigeration at 10°C (or at a speed that visibly separates the solid sample from the supernatant) for about 45 minutes.

g) Precisely transfer an aliquot (1 mL) of the filtered extract from Section 3.5 (f) into a separate clean polypropylene centrifuge tube (15 mL).

h) Evaporate the sample aliquot to a final volume of approximately 0.4-mL (or less but not dry) under a gentle stream of N_2 at a water bath temperature of approximately 40°C.

i) Remove the polypropylene centrifuge tube from the evaporator and adjust the volume to 1.5 mL with 10/90 (v/v) MeOH/ultrapure water to yield the sample final fraction. Cap and mix well using a vortex mixer. The concentration at this stage is equivalent to 0.2 g/mL (relative to soil).

Note: Higher dilution rate may be required for residues higher than 50 ppb in soil.

j) Transfer ~1 mL of the sample final fraction into a HPLC injection vial for LC-MS/MS analysis. See the LC-MS/MS conditions/parameters in Sections 4.4 - 4.6 and use the LC-MS/MS standard solutions as described in Section 2.3.3 for quantitation.

3.6 Analysis of R611966 and R613636

QuEChERS is used to force distribution/extraction of analytes of interest from aqueous component to volatile organic solvent. Additional solid phase extraction (SPE) procedure is necessary for R611966 and R613636 residue determination on the GC-MSD instrument to minimized matrix interference.

The sample cleanup and concentration are accomplished by the use of Agilent Bond Elut QuEChERS Dispersive kit (Cat No. 5982-4956; 150 mg C18, 900 mg $MgSO_4$, 15-mL tube) followed by Agilent Bond Elut solid phase extraction (Cat. No. 12102052; 500 mg, 6-mL) cartridge. The QuEChERS procedures are described as below:

a) Condition a QuEChERS tube as follows:
   1. Ethyl acetate; 5 mL, one time.
   2. Mix well, then centrifuge at 3000 rpm for approximately 2 minutes at room temperature.
   3. Decant ethyl acetate to waste leaving solid materials in QuEChERS tube.

b) Add 4 mL of ethyl acetate to a conditioned QuEChERS tube followed by 2.0 mL of the combined extract from Section 3.3(i);

c) Vigorously mix contents of tube both by hand and vortex mixer. Attempt to break up any clumps of solid material as much as possible;

d) Centrifuge mixture at 3000 rpm for approximately 2 minutes at room temperature and decant off liquid into a clean polypropylene centrifuge tube (15 mL);

e) Add an additional 3 mL of ethyl acetate to the used QuEChERS tube and repeat Sections 3.6(c) - 3.6(d) combining respective ethyl acetate decantings.

f) Add 1 mL of ultrapure water to the ethyl acetate decanting and evaporate the sample to aqueous (1 mL or less) under a gentle stream of N_2 at a bath temperature of approximately 40°C.

g) Re-constitute the sample by adding 1 mL of MeOH, then bring up to 5 mL with ultrapure water.

h) Vortex and proceed to Section 3.7 Solid Phase Extraction procedure.

3.7 Solid Phase Extraction (SPE) Clean-up Procedure

A solid phase extraction (SPE) procedure is necessary for R611966 and R613636 residue determination on the GC-MSD instrument to minimize matrix interference.

The sample cleanup and concentration are accomplished by the use of Agilent Bond Elut solid phase extraction (500 mg, 6-mL) cartridge. In general, avoid cartridges from drying during the process unless specified. Allow one solvent to flow through the SPE (no liquid layer on top of bed) before adding the next solvent. The flow rate should be kept at a rate of less than 20 drops per minute (approximately 1 mL/min). Flow efficiency can be improved by controlled vacuum on the SPE extraction box or controlled positive pressure on the SPE cartridge. However, **gravity flow** is highly recommended for the sample loading and final elution. The SPE procedures are described as below:

a) Condition a SPE cartridge as follows:
   1. 3 mL of MeOH;
   2. 3 mL of acetone;
   3. 3 mL of ultrapure water.

b) Load the sample from Section 3.6(h) onto the SPE cartridge, portion-wise and quantitatively. Slight positive pressure or vacuum may be applied if needed; however, the flow rate should be less than 20 drops per minutes (approximately 1 mL/min). Discard the eluents.

c) Wash the cartridge as follows and discard the washes.
   1. Rinse the sample tube with 2 mL of 20/80 (v/v) MeOH/ultrapure water and transfer the rinsate to the SPE cartridge
   2. 2 mL of 20/80 (v/v) MeOHl/ultrapure water, three times

d) Remove residual solvents inside the SPE cartridge by vacuum and allow complete dry of the cartridge (allow air to go through the cartridge) for approximately 10 minutes.

e) Elute the cartridge with 3 mL of 50/50 (v/v) MeOH/acetone and collect into a clean glass tube.

f) Evaporate the eluant to dryness under a gentle stream of N_2 at a bath temperature of approximately 40°C.

g) Re-constitute the sample with 1 mL (adjust to appropriate final volume as needed) toluene using an air-tight syringe to yield the sample final fraction.

h) Vortex the final fraction and transfer ~ 1mL into a GC vial (with an insert as needed) for GC-MSD analysis. See the GC-MSD conditions/parameters in Section 4.7 and use the GC-MSD standard solutions as described in Section 2.3.4 for quantitation.

3.8 Experimental Precautions

a) The *d*-SPE and QuEChERS-SPE procedures have been developed using cartridges and media material from the stated manufacturer. Similar material or cartridges from other manufacturers may be used. In all cases however, it is strongly recommended that the elution profile of the chosen batch of cartridges is checked prior to commencing analysis to assess any variation in manufacturers' products and between batches.

b) Bottled HPLC grade ultrapure water is used to prepare the LC mobile phase, which produces a lower background noise in the MS/MS chromatograms than water taken from a laboratory water purification system.

c) To prevent contamination of the instrument and to minimize possible carry-over issues using LC-MS/MS, it is recommended that high level recoveries (> 0.05 mg/kg) and samples with expected residues greater than 0.1 mg/kg should be diluted so that the final individual analyte concentration does not exceed 0.02 µg/mL. It may also be useful to include blank injections of 10/90 (v/v) MeOH/ultrapure water after high level samples to clear any observed carry-over greater than 10% of the LOQ.

3.9 Time Required for Analysis

The methodology is normally performed with a batch of 13 samples. One skilled person can complete the extraction procedures in one day (8 hour working period) for LC-MS/MS analysis. An additional working day will be required for sample clean-up for GC-MSD analysis. The analytical sequences were normally carried out with overnight arrangement to maximize the lab output.

3.10 Method Stopping Points

The analytical procedure can be stopped at various points for overnight and weekend breaks unless otherwise specified in the analytical procedure. Acceptable method recoveries will validate any work flow interruptions. Samples should be stored refrigerated in sealed containers where the analysis cannot be completed in a single day.

4.0 FINAL DETERMINATION

The following instrumentation and liquid chromatographic conditions are suitable for analysis of R182281, R611968, R611965, R417888, SYN546669 and SYN510573. The instrumentation and gas chromatographic conditions are suitable for analysis of R611966 and R613636. Other instruments may also be used, however optimization may be required to achieve the desired separation and sensitivity. The operating manuals for the instruments should always be consulted to ensure safe and optimum instrument operation.

4.1 LC-MS/MS Instrument Description

Pump	:	Waters Acquity UPLC® system (I Class) with Sample Manager and Column Manager
Detector	:	Applied Biosystems Sciex API 4000 triple quadrupole mass spectrometer with Analyst TM software version 1.6.2 or equivalent

4.2 LC Chromatographic Conditions for Analysis of R182281, R611965, R611968, R417888, SYN546669 and SYN510573

Column	:	Zorbax SB-CN, 4.6 x 75 mm, 3.5 µm (Agilent Cat. no. 866953-905)
Column Oven Temperature	:	40°C
Injection volume	:	50 µL
Stop Time	:	10.0 minutes
Injection protocol	:	Analyze calibration standard after 4 to 5 sample injections
Sample Tray Temperature	:	10°C
Mobile phase	:	Solvent A: 0.05% formic acid in Optima water Solvent B: 0.05% formic acid in Optima acetonitrile

Mobile Phase Composition

Time (mins)	% solvent 1	% solvent 2	Flow rate (mL/min)
0	90	10	0.6
0.5	90	10	0.6
3	10	90	0.6
8	10	90	0.6
8.1	90	10	0.6
10	90	10	0.6

Column Switching Valve Program

Time (min)	Valve Position
	To waste
1.5	To mass spectrometer

Notes : The column eluate may be diverted to waste for the first 1.5 minutes to prevent ionic material from the sample contaminating the mass spectrometer front plate, if required. A secondary pump providing flow of mobile phase to the mass spectrometer when the column eluate is switched to waste has been found to be unnecessary. It is not necessary to reduce the flow rate into the mass spectrometer when using the API 4000.

4.3 Mass Spectrometer Conditions for Conditions for Analysis of R182281, R611965, R611968, R417888, SYN546669 and SYN510573

Interface	:	TurboIonSpray
Polarity	:	Negative
Curtain gas (CUR)	:	Nitrogen set at 25 (arbitrary units)
Temperature (TEM)	:	550°C
Ionspray voltage	:	-4200
Collision gas setting (CAD)	:	Nitrogen set at 12 (arbitrary units)
Gas 1 (GS1)	:	Air set at 55 (arbitrary units)
Gas 2 (GS2)	:	Air set at 50 (arbitrary units)
Interface heater (ihe)	:	On
Scan type	:	MRM

MRM Operating Parameters:

MS/MS Transitions

Analyte	MS/MS Transition*	Dwell (ms)	DP	EP	CE	CXP	RT (min.)
R182281	ESI Negative						
Quantification	244.9 → 182.0	75	-85	-10	-40	-50*	4.72
Confirmation	244.9 → 174.9	75	-85	-10	-40	-50*	4.72
R611968	ESI Negative						
Quantification	263.0→ 220.0	75	-50	-10	-40*	-10	4.03
Confirmation	265.0 → 222.0	75	-50	-10	-40*	-10	4.03
R611965	ESI Negative						
Quantification	265.8 → 221.8	75	-32	-10	-10	-10	3.37
Confirmation	267.8 → 223.8	75	-32	-10	-10	-10	3.37
R417888	ESI Negative						
Quantification	326.8→ 220.0	75	-70	-10	-32	-10	3.74
Confirmation	328.8 → 222.0	75	-70	-10	-32	-10	3.74
SYN510573	ESI Negative						
Quantification	344.8 → 302.0	75	-70	-10	-29	-14	2.90
Confirmation	346.8 → 304.0	75	-70	-10	-29	-14	2.90
SYN546669	ESI Negative						
Quantification	372.9 → 355.9	75	-48	-10	-16	-14	3.85
Confirmation	374.9 → 357.9	75	-48	-10	-16	-14	3.85

* These parameters are intentionally de-optimized away from the best values: CXP = -9 for R182281 and CE = -25 for R611968

Typical chromatograms are shown in the Figures Section.

4.4 Optional LC-MS/MS Instrument Description

HPLC System	:	Surveyor Plus LC System – A binary solvent system equipped with MS Pump Plus
Autosampler	:	Surveyor MS Plus
Detector	:	Thermo Electron TSQ Quantum Ultra mass spectrometer with Xcalibur™ Software
Collision Gas	:	Purified Argon in compressed cylinder
Gas Supply	:	House Nitrogen supply

4.5 Optional Chromatography Conditions for R182281, R611965, R611968, R417888, SYN546669 and SYN510573

Column	:	Zorbax SB-CN, 4.6 x 75 mm, 3.5 µm (Agilent Cat. no. 866953-905)
Column Oven Temperature	:	30°C
Injection volume	:	50 µL
Stop Time	:	7.0 minutes
Injection protocol	:	Analyze calibration standard after 4 to 5 sample injections
Sample Tray Temperature	:	20°C
Mobile phase	:	Solvent A: 0.05% formic acid in Optima water Solvent B: Optima acetonitrile

Mobile Phase Composition

Time (min)	%A	%B	Flow Rate (µL/min)
0.0	90	10	600
0.5	90	10	600
3.0	10	90	600
6.0	10	90	600
6.1	90	10	600
7.0	90	10	600

The typical retention times for the analytes are listed in Section 4.3 when using this instrumentation and conditions. The retention time may vary depending upon chromatographic conditions and systems. The chromatographic conditions employed in this method are not designed to resolve the stereoisomers in racemic mixtures.

Two injections are required: one for positive detection and one for negative detection.

Note: To help minimizing instrument contamination, a timed event controlled switching valve may be used to divert the LC stream to waste during periods of no data collection.

4.6 Optional Mass Spectrometer Conditions for the Analytes

Ion Source Parameters (HESI-II Probe):

	ESI Negative Mode
Spray Voltage (V)	2400
Vaporization Temperature (°C)	350
Sheath Gas Pressure (psi)	40
Ion Sweep Gas Pressure (psi)	2.0
Aux Gas Pressure (psi)	15
Capillary Temperature (°C)	300
Tube Lens Offset	tuned value(s)
Skimmer Offset (V)	5
Collision Pressure (mTorr)	1.0

Note: The mass spectrometer tuning parameters shown here are for reference only. The analyst should always consult with instrument operation manual to obtain optimum conditions for all the analytes prior to residue analysis.

MRM (SRM) Operating Parameters:

MS/MS Transitions

Analyte	MS/MS Transition*	Scan Width	Dwell (sec.)	CE (Volts)	Q1 PW	Q3 PW	RT (min.)
R182281	ESI Negative						
Quantification	244.9 → 182.0	0.002	0.04	30	0.7	0.7	5.32
Confirmation	244.9 → 175.0	0.002	0.04	30	0.7	0.7	5.32
R611968	ESI Negative						
Quantification	262.9 → 219.9	0.002	0.005	15	0.7	0.7	4.51
Confirmation	264.9 → 221.9	0.002	0.005	15	0.7	0.7	4.51
R611965	ESI Negative						
Quantification	265.9 → 222.0	0.002	0.04	8	0.7	0.7	3.81
Confirmation	267.9 → 224.0	0.002	0.04	8	0.7	0.7	3.81
R417888	ESI Negative						
Quantification	326.9→ 220.0	0.002	0.04	28	0.7	0.7	4.17
Confirmation	328.9 → 222.0	0.002	0.04	28	0.7	0.7	4.17
SYN510573	ESI Negative						
Quantification	344.6 → 301.9	0.002	0.15	22	0.7	0.7	3.13
Confirmation	346.6 → 303.9	0.002	0.15	22	0.7	0.7	3.13
SYN546669	ESI Negative						
Quantification	372.9 → 291.9	0.002	0.15	25	0.7	0.7	4.05
Confirmation	374.9 → 293.9	0.002	0.15	25	0.7	0.7	4.05

ESI negative mode data collection windows:
2.5 – 3.5 minutes for SYN510573
3.5 – 5.5 minutes for R182281, R611968, R611965, R417888 and SYN546669

Typical chromatograms are shown in the Figures Section.

* Minimum of 0.001 amu mass difference for precursor ions in quantification and confirmation detections is required for channel separation of signals on the Thermo Electron TSQ Quantum Ultra mass spectrometer with Xcalibur™ software. The MS/MS transitions listed were the most sensitive and stable transitions for the corresponding analytes based on the optimal tuning parameters obtained prior to method validation with Thermo Electron TSQ Quantum Ultra instrument. Alternative MS/MS transitions may be used if different (or comparable) instrument is applied or if interferences are encountered. Analysts should consult with instrument operation manuals for the specifics and adjustments when using instruments from different manufacturers to obtain optimum results.

4.7 GC-MSD Instrument Description

Instrument Description

The following instrumentation has been found to be satisfactory for the analysis of R611966 and R613636, using an Agilent 6890N gas chromatography fitting with an Agilent 5973 series mass selective detector working under EI mode.

Chromatography Conditions

Column	:	Agilent DB1701 30 m x 0.25 mm x 0.25 µm film thickness (Agilent Cat. no. 122-0732E)
Injector Port	:	Split/splitless injector operating in splitless mode with 4mm double gooseneck inlet liner
Carrier gas and Head pressure	:	Helium at a constant flow of 1 mL min^{-1} with a pressure pulse of 30 psi for 1 minute
Splitless time	:	1.0 minute
Injection volume	:	2.0 µL
Injector temperature	:	250°C
Temperature Program	:	Initial Temperature: 120°C Initial Time: 1.00 min Ramp Rate: 15°C/min Final Temperature: 270°C Final time: 7.0 min Post Temperature: 325°C Post Time: 2.00 min

MSD Conditions

Ionization mode	:	EI
Electron energy	:	70 eV

Analyte	Low Mass Resolution	SIM Mode	
R611966	Yes	Target Ion	231.9 *m/z*
		Qualifier 1	233.9 *m/z*
		Qualifier 2	247.9 *m/z*
		Retention Time	12.73 min.
R613636	Yes	Target Ion	267.9 *m/z*
		Qualifier 1	265.9 *m/z*
		Qualifier 2	269.9 *m/z*
		Retention Time	15.68 min.

Note: Retention Time may vary depending on system and instrument condition.

4.8 Confirmatory Procedures for R182281, R611966, R611965, R611968, R613636, R417888, SYN546669 and SYN510573

Final determination by LC-MS/MS with two transitions is considered to be highly specific; hence no further confirmatory conditions are included. Final determination by GC-MSD with at least two qualifier ions also is considered to be highly specific; hence no further confirmatory conditions are included.

5.0 CALCULATION OF RESULTS

5.1 Multi-point Calibration Procedure

Residues of analytes may be calculated in ppb (µg/kg) for each sample as follows.

a) Prepare standard solutions over a concentration range appropriate to the expected residues in the samples (for example, 50% LOQ to 20x LOQ). An appropriate number of different concentrations within this range should be prepared (at least five).

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to the analytes of interest. Calibration standard solutions should be interspersed throughout the analysis, after a maximum of five injections of sample solutions

c) Generate calibration curve parameters using an appropriate regression package.

d) With linear regression as an example, following equation can be rearranged and used to calculate residues as follows:

$$y = mx + c$$

Where y is the instrument response value, x is the standard concentration, m is the gradient of the line of best fit (or "X-variable 1" in MS Excel) and c is the intercept value. An example of this equation generated using the experimental values of *m* and *c* should be included in the raw data, as should the "R-Squared" (R^2) value for the regression.

Re-arrangement for *x* gives

$$x = \frac{y - c}{m}$$

e) Alternatively, the analyte found values can be obtained directly from instrument quantification software package when appropriate regression package is applied.

f) Calculate the residue for the analyte of interest in the sample, expressed as ppb (µg/kg), as follows:

$$\text{Residue (ppb)} = \frac{\text{Analyte found } (\mu g/\text{mL})}{\text{Sample conc. (g/mL)}} \times \frac{1{,}000\ g}{1\ kg}$$

Where analyte found (µg/mL) is calculated from the standard calibration curve and sample conc. is the final sample concentration in *g/mL*.

If residues need to be corrected for average percentage recovery; *e.g.* for storage stability studies, then the equation below should be used.

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}} \text{ (ppb)}$$

5.2 Single-Point Calibration Procedure

Residue of analytes may be calculated in µg/Kg for each sample using a mean standard response from each of the injections bracketing the sample as follows. Single point calibrations are generally NOT recommended for this method; particularly when analyte has non-linear detector responses.

a) Make repeated injections of a standard containing analytes of interest at an appropriate concentration into the LC-MS/MS (and GC-MSD) operated under conditions as described in Section 4.0. When a consistent response is obtained, measure the peak areas obtained for the analyte of interest.

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to the analyte of interest.

c) Re-inject the standard solution after a maximum of four injections of sample solutions.

d) Calculate the residues for the analyte of interest in the sample, expressed as µg/Kg using a mean standard response from each of the injections bracketing the sample as follows.

$$\text{Residue (ppb)} = \frac{\text{PK area (SA)}}{\text{PK area (STD)}} \times \frac{\text{Standard Conc.}}{\text{Sample Conc.}} \times \frac{1{,}000\ g}{1\ kg}$$

PK area (SA) = Peak response for sample
PK area (STD) = Average peak response for bracketing standards
Standard Conc. = Concentration of standard (µg/mL)
Sample Conc. = Sample concentration (g/mL)

If residues need to be corrected for average percentage recovery e.g. for storage stability studies, then the equation below should be used.

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}} \text{ (ppb)}$$

Although single point calibration may be used to quantify residues, it is recommended that a calibration curve be generated with each analytical set to demonstrate proper instrument response (Reference 2).

6.0 CONTROL AND RECOVERY SAMPLES

Control samples should be analyzed with each set of samples to verify that the sample used to prepare recovery samples is free from contamination. A minimum of one control should be analyzed with each batch of samples.

At least two recovery samples (control samples accurately fortified with known amounts analytes in methanol) should also be analysed alongside each set of samples. Provided the recovery values are acceptable they may be used to correct any residues found. The fortification levels should be appropriate to the residue levels expected.

Recovery efficiency is generally considered acceptable when the mean values are between 70% and 110% and with a relative standard deviation of $\leq 20\%$.

Where the method is used for tolerance enforcement purposes, control and recovery samples are not required since suitable control samples are not available.

7.0 SPECIFICITY

It is recommended that reagent blank samples be included in a sample set if contamination is suspected.

7.1 Matrix

LC-MS/MS is a highly specific detection technique. If matrix effects arising from the matrices are observed and significant, matrix-matched standards should be used to compensate the matrix effects or by further dilution of the sample final fractions with 10/90 (v/v) MeOH/ultrapure water for LC-MS/MS or toluene for GC-MSD to reduce or eliminate these effects should instrument sensitivity permit.

7.2 Reagent and Solvent Interference

Using high purity solvents and reagents no interference has been found.

7.3 Labware Interference

This method uses mainly disposable labware. All reusable glassware should be detergent washed and then rinsed with HPLC grade methanol, acetone or acetonitrile prior to use.

CHEMICAL STRUCTURES

FIGURE 1 R182281

Compound Code Number	:	R182281
Alternative compound code number	:	SDS3701
CAS Number	:	28343-61-5
IUPAC Name	:	2,4,5-Trichloro-6-hydroxy-isophthalonitrile
Molecular Formula	:	$C_8 H Cl_3 N_2 O$
Molecular Weight	:	247.46
Molecular Mass	:	245.91

FIGURE 2 R611966

Compound Code Number	:	R611966
Alternative compound code number	:	SDS47523
CAS Number	:	not available
IUPAC Name	:	2,4,5-Trichloro-3-cyano-benzamide
Molecular Formula	:	$C_8 H_3 Cl_3 N_2 O$
Molecular Weight	:	249.48
Molecular Mass	:	247.93

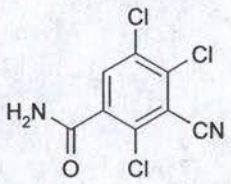


FIGURE 3 R611968

Compound Code Number	:	R611968
Alternative compound code number	:	SDS47525
CAS Number	:	not available
IUPAC Name	:	2,4,5-Trichloro-3-cyano-6-hydroxy-benzamide
Molecular Formula	:	$C_8 H_3 Cl_3 N_2 O_2$
Molecular Weight	:	265.48
Molecular Mass	:	262.92

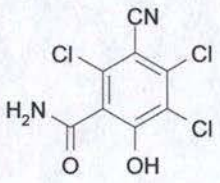


FIGURE 4 R611965

Compound Code Number	:	R611965
Alternative compound code number	:	SDS46851
CAS Number	:	142733-37-7
IUPAC Name	:	2,4,5-Trichloro-isophthalamic acid
Molecular Formula	:	$C_8 H_4 Cl_3 N O_3$
Molecular Weight	:	268.48
Molecular Mass	:	266.93

FIGURE 5 R613636

Compound Code Number	:	R613636
Alternative compound code number	:	SDS19221
CAS Number	:	61073-19-6
IUPAC Name	:	2,3,4,6-Tetrachloro-5-cyano-benzamide
Molecular Formula	:	$C_8 H_2 Cl_4 N_2 O$
Molecular Weight	:	283.92
Molecular Mass	:	281.89

FIGURE 6 R417888

Compound Code Number	:	R417888
Alternative compound code number	:	not available
CAS Number	:	not available
IUPAC Name	:	2-Carbamoyl-3,5,6-trichloro-4-cyano-benzenesulfonic acid
Molecular Formula	:	$C_8 H_3 Cl_3 N_2 O_4 S$
Molecular Weight	:	329.54
Molecular Mass	:	327.89

FIGURE 7 SYN510573

Compound Code Number	:	SYN510573
Alternative compound code number	:	R471811
CAS Number	:	not available
IUPAC Name	:	Potassium; 2,4-dicarbamoyl-3,5,6-trichloro-benzenesulfonate
Molecular Formula	:	$C_8 H_4 Cl_3 K N_2 O_5 S$
Molecular Weight	:	385.64
Molecular Mass	:	344.89 (less K^+)

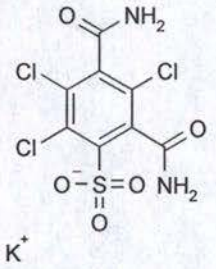
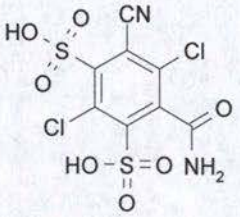


FIGURE 8 SYN546669

Compound Code Number	:	SYN546669
Alternative compound code number	:	R419492
CAS Number	:	not available
IUPAC Name	:	4-Carbamoyl-2,5-dichloro-6-cyano-benzene-1,3-disulfonic acid
Molecular Formula	:	$C_8 H_4 Cl_2 N_2 O_7 S_2$
Molecular Weight	:	375.16
Molecular Mass	:	373.88



APPENDIX 1 Apparatus

Recommended Suppliers [Need update]

Equipment	Description	Supplier
General lab glassware	General lab glassware	www.thermoscientific.com
General lab plastic-ware	General lab plastic-ware	www.thermoscientific.com
Autosampler vials	Snap cap, 2 mL size	www.thermoscientific.com
LC-MS/MS system Includes HPLC and autosampler units	Waters Acquity UPLC (I Class) with AB Sciex 4000	www.waters.com
GC-MSD system Includes GC, MS and autosampler units	6890/5973 series GC-MSD	www.agilent.com
HPLC column	Zorbax SB-CN; 3.5 µm 4.6 mm i.d × 75 mm	www.agilent.com
GC column	J&W DB-1701, 30 m × 0.25 mm, 0.25 µm film thickness	www.agilent.com
Despervive SPE	BONDESIL C18; 40µm	www.agilent.com
SPE Cartidges	Bond Elut C18 500 mg, 6-mL	www.agilent.com
PTFE Syringe Filter	13mm, 0.45 µm	www.thermoscientific.com
QuEChERS Dispersive kit (Cat. #5982-4956)	15-mL Tube; 150 mg C18, 900 mg $MgSO_4$	www.agilent.com
Centrifugal Filter Devices	Ultrafree-CL 2 mL	www.millipore.com

APPENDIX 2 Reagents

Recommended Suppliers

Reagent	Description	Supplier
Ultrapure water	Optima grade	www.thermoscientific.com
Ultrapure water	HPLC grade	www.thermoscientific.com
Acetonitrile	HPLC grade	www.thermoscientific.com
Toluene	HPLC grade	www.thermoscientific.com
Acetone	HPLC grade	www.thermoscientific.com
Ammonium Hydroxide	A.C.S grade	www.thermoscientific.com
Formic Acid	A.C.S. grade	www.thermoscientific.com
Ethyl Acetate	HPLC grade	www.thermoscientific.com
R182281, R611966, R611968, R611965, R613636, R417888, SYN510573 and SYN546669	GLP certified	Syngenta Crop Protection, LLC, P.O. Box 18300, Greensboro, NC 27419-8300.

Preparation of Reagents

a) 0.05% Formic acid in ultrapure water prepared by mixing 0.5 mL of formic acid with 1000 mL of HPLC grade water.

b) 50/50 (v/v) MeOH/ultrapure water; prepared by mixing 250 mL of HPLC grade MeOH with 250 mL of HPLC grade water.

c) 50/50 (v/v) Acetonitrile/ultrapure water; prepared by mixing 250 mL of HPLC grade acetonitrile with 250 mL of HPLC grade water.

d) 10% Ammonium hydroxide in ultrapure water; prepared by mixing 0.5 mL of ammonium hydroxide with 4.5 mL of HPLC grade water.

e) 1.0% Ammonium hydroxide in ultrapure water; prepared by mixing 0.5 mL of 10% ammonium hydroxide in ultrapure water with 4.5 mL of HPLC grade water.

f) 10/90 (v/v) MeOH/ultrapure water; prepared by mixing 50 mL of HPLC grade MeOH with 450 mL of HPLC grade water.

g) 50/50 (v/v) MeOH/acetone, prepared by mixing 50 mL HPLC grade MeOH with 50 mL of HPLC grade acetone.

APPENDIX 3 LC-MS/MS Tuning Procedure

Calibration of Instrument

The instrument must be mass calibrated on a regular basis using polytyrosine-1, 3, 6 solutions according to the manufacturer's instructions. Calibrate both mass resolving quadrupoles (Q1 and Q3).

Tuning Instrument for Analytes

For example, infuse a standard solution of R182281 (0.1 to 1.0 µg/mL) in mobile phase (see section 4) directly into the mass spectrometer interface at a rate at of approximately 5-20 µL/min. Roughly adjust interface parameters (sprayer position and temperature, spray, heater/auxiliary gas flows, as well as voltages of spray, orifice, and focusing ring) for a sufficiently high parent ion signal at *m/z* 244.9 for R182281 in negative ionization mode.

Similarly, infuse individual standard solution of R611968, R611965, R417888, SYN510573 and SYN546669 (0.1 to 1.0 µg/mL) in mobile phase (see section 4) directly into the mass spectrometer interface at a rate at of approximately 5-20 µL/min. Roughly adjust interface parameters (sprayer position and temperature, spray, heater/auxiliary gas flows, as well as voltages of spray, orifice, and focusing ring) for a sufficiently high parent ion signal at *m/z* for individual analyte.

Using the Xcalibur™ Software optimization routine, tune the instrument for R182281, R611968, R611965, R417888, SYN510573 and SYN546669, individually, and ensuring that the correct ion is selected. If desired, manual tuning of the ion optics and collision energy can be carried out to ensure maximum sensitivity.

Finally, connect the LC-pump via the autosampler directly to the MS/MS instrument. Perform repetitive flow injection of standards using mobile phase at the flow rate to be used. Tune the interface parameters (sprayer position and temperature, spray and heater gas flows, spray, orifice, and focusing ring voltages and the collision gas pressure) for maximum sensitivity.

In general, the two most sensitive product ions are selected and used for quantitative and confirmative analysis. The tuned *m/z* values and corresponding MRM transitions for the analytes are listed in Section 4.3.

Final determination by LC-MS/MS with two transitions is considered to be highly specific; hence no further confirmatory conditions are included.

The instrument must be mass calibrated on a regular basis using polytyrosine-1, 3, 6 solutions according to the manufacturer's instructions. Calibrate both mass resolving quadrupoles (Q1 and Q3).

APPENDIX 4 Method Flow Chart