1. Principle

The residues of cyclanilide and its metabolite 2,4-dichloroaniline in soil are determined by shaking a 20 gram soil sample twice with methanol/water, amending with an internal standard, centrifuging and an aliquot evaporated to reduce volume with analysis by LC/MS/MS.

The residues of cyclanilide in water are determined by amending a sample with an internal standard, with analysis by LC/MS/MS.

Quantification is based on a comparison of peak areas with those of known standards. Two sets of MRM transitions are shown, one for quantitation and the second for confirmatory purposes.

The method limit of quantitation (LOQ) for cyclanilide and its metabolite 2,4-dichloroaniline is 5ng/g in soil and 0.5ng/g for cyclanilide in water.

2. Apparatus

Use as a guide; equivalent apparatus may be substituted.

VWR Pyrex® Brand volumetric pipets, glass class A (Assorted Volumes)
Eppendorf Reference and Repeat pipettes and tips
VWR Pyrex® Brand volumetric flasks, glass class A (Assorted Volumes)
VWR Pyrex® Brand mixing cylinders, glass class A (Assorted Volumes)
VWR Pyrex® Brand disposable Pasteur pipets (Cat. No.: 53283-910 & 53283-914)
BD Falcon 50mL conical centrifuge tubes (Cat. No.: 352070)
National Scientific LC vials, Snap-Its (Cat. No.: C4011-5)
National Scientific LC vial Snap-It Seals, (Cat. No.: C4011-55)
Phenomenex® Luna 2.5µm C18(2)-HST 50x2mm (Cat 00B-4446-B0)
ABSciex 5500 (LC/MS/MS) equipped with an electrospray interface, Shimadzu 20ADXR HPLC pumps (2), CBM 20A communications bus module, CTO-20 A column oven and a CTC autosampler, and data collection/processing software (Analyst 1.6.2)
TurboVap evaporator (Zymark Corporation, Model LV)
Centrifuge
Mechanical Shaker
Various general laboratory glassware and utensils

3. Reagents

Use as a guide; equivalents or different manufactures (brands) may be substituted.

Water (HPLC Grade or Millipore)
Methanol (HPLC Grade)
Acetonitrile (HPLC Grade)
Formic acid (HPLC Grade)
50/50(v/v) Water/Methanol. Combine 500mL water and 500mL Methanol. Mix well.
85/15(v/v) Water/Methanol. Combine 850mL water and 150mL Methanol. Mix well.
0.3% formic acid in water. Add 3mL of formic acid to 1L of water. Mix well.

4. Preparation of Analytical Standards

NOTE: The following procedure is an example description of how standard solutions may be prepared. Alternate or additional standards of appropriate weight and volume may be prepared as needed.

Class "A" volumetric glassware or calibrated pipets should be used in the preparation of all analytical standards. All standard solutions should be stored in a freezer when not in use. Solutions should be allowed to warm to room temperature prior to use.

4.1 Primary Stock Standard Solutions

Prepare individual 100µg/mL stock solutions of cyclanilide and 2,4-dichloroaniline by transferring 0.0100 grams of each analyte to separate 100mL volumetric flasks. Dilute to volume with acetonitrile and mix well. Store frozen when not in use.

NOTE: Corrections for standard purities should be applied when expressing standard concentrations.

4.2 Mixed Standard Solutions

Prepare a mixed 5ug/mL solutions of cyclanilide and 2,4-dichloroaniline by taking a 5.0mL aliquot of the 100µg/mL stock solutions and diluting to 100mL with acetonitrile. Refrigerate when not in use.

Prepare a mixed 500ng/mL solutions of cyclanilide and 2,4-dichloroaniline by taking a 10.0mL aliquot of the 5µg/mL mixed solution and diluting to 100mL with acetonitrile. Refrigerate when not in use.

Prepare a mixed 50ng/mL solution of cyclanilide and 2,4-dichloroaniline by taking a 10.0mL aliquot of the 500ng/mL mixed solution and diluting to 100mL with acetonitrile. Store refrigerated when not in use.

4.3 Internal Standard Solution

Prepare an individual 100µg/mL stock solution of 3,5-dichloroaniline-$^{13}C_6$ by transferring 0.005 grams of the analyte into a 50mL volumetric flasks. Dilute to volume with acetonitrile and mix well. Store frozen when not in use.

Prepare an individual 5µg/mL solution of 3,5-dichloroaniline-$^{13}C_6$ by taking a 5.0mL aliquot of the 100ug/mL mixed solution and diluting to 100mL with acetonitrile. Refrigerate when not in use.

Further dilutions of this mixed fortification solution may be made as needed. Note that 3,5-dichloroaniline-$^{13}C_6$ is used as a surrogate internal standard for the cyclanilide analysis.

4.4 Calibration Standard Solutions

Prepare working calibration solutions as described below and dilute to volume with 85/15(v/v) water/methanol.

Conc. of Native Standard Solution (µg/mL)	Aliquot Native Standard Solution Taken (mL)	Conc. of Internal Standard Solution (µg/mL)	Aliquot Internal Standard Solution Taken (mL)	Dilution Volume (mL)	Native Conc in Calibration Solution (ng/mL)	Internal Standard Conc in Calibration Solution (ng/mL)
5.0	0.5	5.0	0.2	100	25	10
5.0	0.2	5.0	0.2	100	10	10
0.5	1.0	5.0	0.2	100	5	10
0.5	0.5	5.0	0.2	100	2.5	10
0.5	0.2	5.0	0.2	100	1.0	10
0.5	0.1	5.0	0.2	100	0.5	10
0.5	0.05	5.0	0.2	100	0.25	10

Additional calibration solutions may be prepared as required. Refrigerate when not in use.

5. Extraction

5.1 Soil Analysis

1. **Transfer 20±0.01 grams of soil into a suitably sized glass jar.**
2. **Fortify the recovery samples at the desired fortification level using the individual standard solutions (see Section 4.2 Mixed Standard Solutions). For fortifications at the method LOQ add, by pipet, 0.2mL of the 500ng/mL mixed standard solution.**
3. **Add ≈40mL of water/methanol 50/50 (v/v) to all samples and shake for ~10 minutes.**
4. **Centrifuge the sample at ~3000rpm for ~5 minutes. Decant the aqueous layer into a stoppered glass container.**
5. **Repeat the extraction by adding an additional ≈40mL of water/methanol 50/50 to the soil extract and shake for ~10 minutes. Add, by pipet, 80uL of the 5ug/mL isotopic internal standard solution. Stopper and shake well.**

6. Centrifuge the sample at ~3000rpm for ~5 minutes. Decant the aqueous layer into a stoppered glass container used in step 4. Dilute the contents to ~80mL with methanol and mix well.

7. Transfer a 10mL aliquot into a suitable container and evaporate the contents to ~5mL using a TurboVap set at 35°C.

8. Transfer ~2mL to a LC vial, cap and analyze the contents by LC/MS/MS.

5.2 Water Analysis

1. Transfer 20±0.1mL of water into a suitably sized glass jar.

2. Fortify the recovery samples at the desired fortification level using the individual standard solutions (see Section 4.2 Mixed Standard Solutions). For fortifications at the method LOQ add, by pipet, 0.2mL of the 50ng/mL mixed standard solution.

3. Add 2mL of methanol, 50uL of formic acid and 50uL of the 5ug/mL isotopic internal standard to each sample. Dilute to 25mL with deionized water.

4. Transfer an aliquot to a LC vial and analyze the contents by LC/MS/MS.

6. Analysis

6.1 Sample Analysis

Step 1. Using the recommended procedures listed below; analyze an aliquot of the 0.25, 0.5, 1.0, 2.5, 5, 10 and 25ng/mL standard solutions. (these are calibration solution analyses).

Step 2. Analyze an aliquot of each analytical sample from Section 5.1 Step 8 or Section 5.2.

Step 3. Again analyze an aliquot of the 0.25, 0.5, 1.0, 2.5, 5, 10 and 25ng/mL calibration standard solutions.

6.2 LC/MS/MS Standard Calibration and Residue Calculations

Standardize the LC/MS/MS response under the conditions outlined in Appendix 1 by injecting an aliquot of each LC/MS/MS calibration solution interspersed with samples.

The residues of cyclanilide and its metabolite 2,4-dichloroaniline are quantified using internal standard linear regression analysis. A separate calibration curve was produced for each set of samples analyzed on the LC/MS/MS. A calibration curve was generated by linear regression of the standard peak area versus the standard concentrations in ng/mL using ABSciex Analyst Software (Version 1.6.2), a computer-programmed data capturing system. The Analyst Software uses the MS/MS standard responses to

calculate the regression coefficients M and B, respectively called slope and intercept, for each analytical set.

The standards were fit to the linear equation: $Y = MX + B$

where: *X is the concentration of the reference standard in ng/mL*
M is the calibration line slope
B is the calibration line intercept
Y is the native peak area

The calibration points are weighted 1/x to provide better fit near the limit of detection.

After regression coefficients were calculated, the residue in parts per billion was determined. The parts per billion (ppb) of cyclanilide and 2,4-dichloroaniline in soil was calculated using the following equation,

$$\text{residue found (ppb)} = \frac{Y-B}{M} \times D$$

$$\text{Dilution Factor (D)} = \frac{\text{Initial volume (V1)}}{\text{Initial sample wt. (W)}} \times \frac{\text{Final volume (V3)}}{\text{Aliquot (V2)}}$$

For soil: W = 20g
V1 = 80mL
V2 = 10mL
V3 = 5mL

For water: W = 20mL
V1= 25mL

Analyst software was used to calculate the residues of cyclanilide and 2,4-dichloroaniline in ppb for each sample and the percent recovery for the fortified samples.

6.3 Fortification Experiments

Note: Fortification experiments may be performed as needed to monitor method efficiency and reproducibility, but are not required when analysis of samples is performed for tolerance enforcement. Fortification experiments are intended to be used for data collection methods or establishing & validating method efficiency.

With each sample set, analyze an untreated control sample and one or more fortified control samples. Calculate recoveries using the following equation.

$$Recovery\ (\%) = \frac{(R-S)}{T} \times 100$$

Where: R = *ppb of target analyte found in fortified sample*
S = *ppb of target analyte found in control sample*
T = *theoretical ppb in fortified sample*

Recoveries are determined by analyzing fortified control samples alone or in conjunction with a sample set. Samples may be fortified prior to extraction at the LOQ of 5ng/g for soil and 0.5ng/mL for water or other appropriate level with fortification solutions. Calculate the residue for the control (S) and fortified control (R) samples.

Bayer CropScience CC-001-S14-01

Appendix 1 Instrument Conditions for Cyclanilide and Its Metabolite 2,4-dichloroaniline

Equipment with equivalent or better sensitivity and performance may be substituted.

LC/MS/MS Parameters

NOTE: Variations in equipment or sample characteristics may require slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity. Therefore, the given LC/MS/MS parameters listed below are guidelines and may be modified. These parameters should be optimized for the instrument and column actually used. Also, instrument parameters and mobile phase may be adjusted to improve separation from any observed interfering peaks.

The following conditions were used on an ABSciex API 5500 LC/MS/MS system.

HPLC Parameters

Pumps Used:	Two Shimadzu LC-20ADXR pumps with a Shimadzu CBM-20A communications bus module and a Shimadzu CTO20A oven	
Autosampler	CTC PAL	
Column Temperature:	60°C	
Injection Volume:	5uL	
Column:	Manufacturer:	Phenomenex®
	Type:	Luna 2.5µm C18(2)-HST
	Particle Size:	2.5µm
	Diameter:	2.0 mm
	Length:	50 mm
Mobile Phase A:	0.3% formic acid in water	
Mobile Phase B:	Acetonitrile	

HPLC gradient program:

Time (min.)	Module	Flow Rate (mL/min)	A(%)	B(%)
0.0	Pumps	0.40	90	10
5.0	Pumps	0.40	10	90
6.0	Pumps	0.40	10	90
6.1	Pumps	0.40	90	10
9.0	System Controller	Stop		

Diverter valve program :

Time (min.)	Position
0.0	Waste
2.0	Source
5.0	Waste

Bayer CropScience CC-001-S14-01

Mass Spectrometer Instrument Conditions

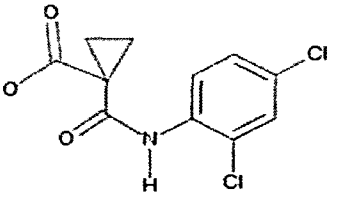
The method is set up with two separate periods, the first with positive ionization for DCA analysis and the second with negative ionization for cyclanilide analysis

Component:	Cyclanilide	Cyclanilide	2,4-DCA	2,4-DCA	3,5-DCA-$^{13}C_6$
Retention Time	4.5 minutes	4.5 minutes	3.9 minutes	3.9 minutes	4.0 minutes
Transition	Quantitation	Confirmation	Quantitation	Confirmation	Internal Standard
Parent Ion	272	272	162	162	168
Product Ion	160	228	126	99	133
Ionization Mode	ESI	ESI	ESI	ESI	ESI
Polarity	-	-	+	+	+
Dwell Time (ms)	50	50	25	25	25
Resolution Q1/Q3	Unit/Unit	Unit/Unit	Unit/Low	Unit/Low	Unit/Low
Declustering Potential (DP)	-35	-35	66	66	70
Entrance Potential (EP)	-10	-10	10	10	6
Collision Energy (CE)	-28	-16	27	27	30
Collision Cell Exit Potential (CXP)	-19	-21	12	12	7
Curtain Gas (CUR)	25	25	25	25	25
Collision Gas (CAD)	10	10	10	10	10
Ion Source Gas 1 (GS1)	40	40	40	40	40
Ion Source Gas 2 (GS2)	65	65	65	65	65
Source Temp (TEM)	750	750	750	750	750
Interface Heater (IHE)	Off	Off	Off	Off	Off
Ion Transfer Voltage (IS)	-4500	-4500	5500	5500	5500

Bayer CropScience CC-001-S14-01

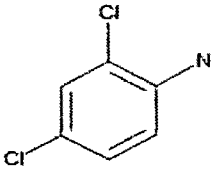
Appendix 2 Structures

Chemical Name: Cyclanilide
(Parent Molecule)



CAS Name: 1-[[(2,4-Dichlorophenyl)amino]carbonyl]cyclopropane carboxylic acid
CAS Number 113136-77-9
Molecular Formula: $C_{11}H_9Cl_2NO_3$
Molecular Weight: 274.1

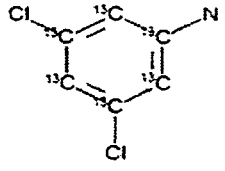
Chemical Name: 2,4-Dichloroaniline
(Metabolite)



CAS Name: 2,4-Dichlorobenzenamine
CAS Number 554-00-7
Molecular Formula: $C_6H_5Cl_2N$
Molecular Weight: 162.2

Bayer CropScience **CC-001-S14-01**

Chemical Name: 3,5-Dichloroaniline-$^{13}C_6$
(Metabolite)



CAS Name: 3,5-Dichlorobenzenamine-$^{13}C_6$
CAS Number 554-00-7
Molecular Formula: $C_6 H_5 Cl_2 N$
Molecular Weight: 168.0