ABSTRACT

The objective of this study is to validate method R0034/01 for the analysis of the diastereomeric forms of BAS 311 I (Cis I, Cis II [alpha-cypermethrin], Trans III, and Trans IV), and its three metabolites 3-phenoxybenzoic acid (PBA) and DCVA (*cis*- and *trans*-isomers) in soil by LC-MS/MS.

Principle of the Method. Soil samples (5 g) are extracted for cypermethrin isomers (Cis I, Cis II, Trans III, and Trans IV) by shaking with 50 mL of 0.1% formic acid in acetonitrile. An aliquot (20%) from the extract is evaporated to dryness and then re-dissolved with acetonitrile/water with 0.1% formic acid (50:50, v/v). The residues are determined using LC-MS/MS.

Soil samples (5 g) are extracted for the metabolites of cypermethrin (*cis*-DCVA, *trans*-DCVA, and 3-PBA) by shaking twice with 25 mL acetonitrile/water (70:30, v/v). An aliquot (10%) from the combined extract is evaporated to dryness and then dissolved in methanol/water (20:80, v/v) with 0.1% formic acid. The residues are determined using LC-MS/MS.

Test Conditions. The method was validated at two fortification levels (0.001 and 0.01 mg/kg (ppm)) for sandy and clay loam soil samples. For each fortification level and matrix, five replicates were analyzed. Additionally, a method blank and at least two replicates of unfortified samples were examined.

Primary and confirmatory mass transitions (*m/z*) for the diastereomeric forms of BAS 311 I (Cis I, Cis II, Trans III, and Trans IV) was analyzed using UPLC and HPLC Methods B and C, respectively.

Primary and confirmatory mass transitions for 3-PBA were analyzed using UPLC and HPLC Methods G and E, respectively. The ions monitored for DCVA (Cis and Trans) were analyzed using UPLC and HPLC Methods G and E, respectively. For DCVA (Cis and Trans), Method E was used for primary quantitation in HPLC mode and confirmatory quantitation for Method G. Additionally, Method F was used for confirmatory quantitation both for HPLC mode and for Method E.

Solvent-based and matrix-matched standards were also analyzed within the study to check for possible matrix effects.

Limit of Quantification (LOQ) and Limit of Detection (LOD). The limit of quantification (LOQ) was defined by the lowest fortification level successfully tested. LOQ is 0.001 mg/kg for each analyte, which corresponds to a concentration in the extract of 0.5 ng/mL. The limit of detection in soil is 0.0002 mg/kg (corresponds to 0.1 ng/mL).

Selectivity. The method determines residues of the diastereomeric forms of BAS 311 I (cypermethrin, Reg. No. 127266), and its metabolites, 3-phenoxybenzoic acid (Reg. No. 130213), and DCVA (*cis*- and *trans*-isomers, Reg. No. 180011) in soil. Retention time shifting was observed and is instrument dependent. However, this does not affect quantitation as long as the retention pattern is observed and compared with the reference standards.

Linearity. Good linearity ($r > 0.99$) was observed in the range of 0.05 to 10 ng/mL (nominal) for the two mass transitions of Cis I, Cis II, Trans III, and Trans IV in mixed standard solutions.

Good linearity was observed in the range of 0.1 to 10 ng/mL (nominal) for the two mass transitions of 3-PBA and the one mass transition of *cis*- and *trans*-DCVA in mixed standard solutions.

Extractability. The metabolism extraction solvent was not used to analyze the diastereomeric forms of BAS 311 I. Therefore, an incurred residue was extracted using both the residue and metabolism extraction procedures. The results of the metabolism extraction procedure and the residue extraction procedures were compared. A comparable extractability of the diastereomers of BAS 311 I was demonstrated in this study.

Recovery and Repeatability. It was proven that the method R0034/01 is suitable to determine residues of the diastereomeric forms of BAS 311 I (cypermethrin, Reg. No. 127266), and its metabolites, 3-phenoxybenzoic acid (Reg. No. 130213), and DCVA (*cis*- and *trans*-isomers, Reg. No. 180011) in soil. As shown in the following tables, the mean recovery values were found to be within the acceptable range of 70–120% for all methods tested. The overall relative standard deviations (RSD, %) for all fortification levels were below 20%.

1 INTRODUCTION

1.1 Scope of the Method

BAS 311 I (cypermethrin, Reg. No. 127266) were determined by LC-MS/MS in soil. Cypermethrin is a insecticide used against several diseases in various crops and belongs to the chemistry class of pyrethroid.

A residue analytical method for the detection and quantification of the diastereomers of the active ingredient BAS 311 I (cypermethrin, Reg. No. 127266) and its metabolites, 3-phenoxybenzoic acid (Reg. No. 130213) and DCVA (*cis*- and *trans*-isomers, Reg. No. 180011) in soil was needed for monitoring purposes with a limit of quantification (LOQ) of 0.001 mg/kg.

As described below, the BASF Method No. R0034/01 allows for the determination of the analytes with the required limit of quantification in soil. This method was developed at BASF Crop Protection, located in Research Triangle Park, NC. To demonstrate the validity of the method, recovery trials with spiked soil samples were performed.

The method was validated at two fortification levels (0.001 and 0.01 mg/kg) for sand and clay loam soil types. For each fortification level and matrix, five replicates were analyzed. Additionally, a method blank and two replicates of unfortified samples were examined.

1.2 Principle of the Method

Soil samples (5 g) are extracted for cypermethrin isomers (Cis I, Cis II, Trans III, and Trans IV) by shaking with 50 mL of 0.1% formic acid in acetonitrile. An aliquot (20%) from the extract is evaporated to dryness and then re-dissolved with acetonitrile/water with 0.1% formic acid (50:50, v/v). The residues are determined using LC-MS/MS.

Soil samples (5 g) are extracted for the metabolites of cypermethrin (*cis*-DCVA, *trans*-DCVA, and 3-PBA) by shaking twice with 25 mL acetonitrile/water (70:30, v/v). An aliquot (10%) from the combined extract is evaporated to dryness and then dissolved in methanol/water (20:80, v/v) with 0.1% formic acid. The residues are determined using LC-MS/MS.

Primary and confirmatory mass transitions (*m/z*) for the diastereomeric forms of BAS 311 I (Cis I, Cis II, Trans III, and Trans IV) was analyzed using UPLC and HPLC Methods B and C, respectively.

Primary and confirmatory mass transitions for 3-PBA were analyzed using UPLC and HPLC Methods G and E, respectively. The ions monitored for DCVA (Cis and Trans) were analyzed using UPLC and HPLC Methods G and E, respectively. For DCVA (Cis and Trans), Method E was used for primary quantitation in HPLC mode and confirmatory quantitation for Method G. Additionally, Method F was used for confirmatory quantitation both for HPLC mode and for Method E.

Solvent-based and matrix-matched standards were also analyzed within the study to check for possible matrix effects.

1.3 Specificity

The diastereomers of cypermethrin (BAS 311 I, Reg. No. 127266) and its metabolites, 3-phenoxybenzoic acid (Reg. No. 130213) and DCVA (*cis*- and *trans*-isomers, Reg. No. 180011) were identified and quantified as individual compounds.

2 MATERIALS AND METHODS

2.1 Test systems

The following test systems were considered in this study of validation:

Test System 1: **Clay Loam, Louisiana (R1200660030)**

Test System 2: **Sandy Loam, California (R1200690025)**

Test System 3: **Soil, New York (R1200680025) (Extractability)**

Test System 4: **Soil, New York (R1200680400) (Extractability)**

Test System 5: **Solvent, acetonitrile/water (70:30, v/v)**

Test System 6: **Solvent, 0.1% formic acid in acetonitrile**

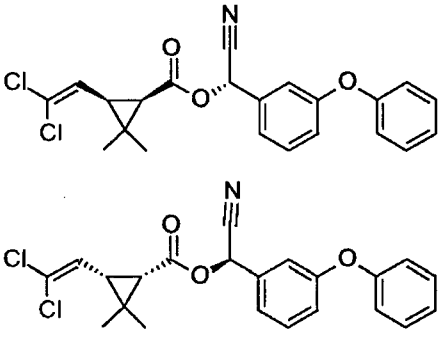
The description and characterization of the soil used is given in the respective attached certificates (Appendix 9.5).

2.2 Test and Reference Items

Alpha-cypermethrin (Cis II)

Common Name	Alpha-cypermethrin (Cis II)
BASF Reg. No.	4078193
CAS-No.	67375-30-8
Molecular Formula	$C_{22}H_{19}NO_3Cl_2$
Molecular Weight	416.3
IUPAC Name	Racemate of (*S*)-cyano-3-phenoxybenzyl (1*R*,3*R*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate and (*R*)-cyano-3-phenoxybenzyl (1*S*,3*S*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarobxylate
Batch No.	AC9575-006
Purity (%)	99.8
Test Substance Type	PAI
Storage Advice	keep in freezer (approx. −18 °C)
GLP	Yes
Expiration Date	01.September.2016

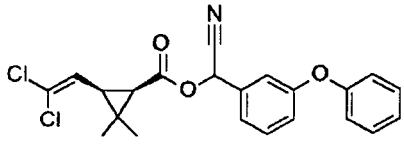
Chemical structure:



Cis I/Cis II Isomers (45:55)

Common Name	Cis I/Cis II Isomers (45:55)
BASF Reg. No.	4111341
CAS-No.	211504-93-7
Molecular Formula	$C_{22}H_{19}NO_3Cl_2$
Molecular Weight	416.3
IUPAC Name	(*RS*)-α-cyano-3-phenoxybenzyl (1*RS*,3*RS*)-3-(2,2-dichlorovinyl)-2,2-dimethylclopropanecarboxylate
Batch No.	AC8949-76
Purity (%)	99.8
Test Substance Type	PAI
Storage Advice	keep in freezer (approx. −18 °C)
GLP	Yes
Expiration Date	01.August.2015

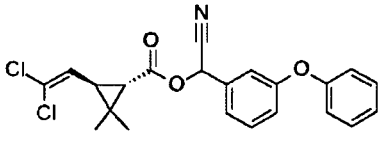
Chemical structure:



Trans III/Trans IV (43.5:56.5)

Common Name	Trans III/Trans IV (43.5:56.5)
BASF Reg. No.	4111342
CAS-No.	211504-94-8
Molecular Formula	$C_{22}H_{19}NO_3Cl_2$
Molecular Weight	416.3
IUPAC Name	(*RS*)-α-cyano-3-phenoxybenzyl (1*RS*,3*SR*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate
Batch No.	AC8949-77
Purity (%)	99.8
Test Substance Type	PAI
Storage Advice	keep in freezer (approx. −18 °C)
GLP	Yes
Expiration Date	01.June.2020

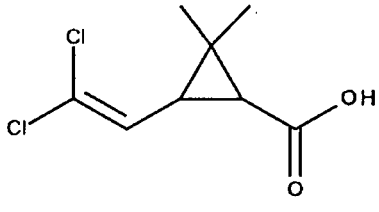
Chemical structure:



DCVA (Mixture of *cis*- and *trans*-isomers, 51.5:48.5)

Common Name	DCVA (Mixture of *cis*- and *trans*-isomers, 51.5:48.5)
BASF Reg. No.	180011
CAS-No.	55701-05-8
Molecular Formula	$C_8H_{10}O_2Cl_2$
Molecular Weight	209.1
IUPAC Name	3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid
Batch No.	AC9966-87
Purity (%)	99.0
Test Substance Type	PAI
Storage Advice	keep in refrigerator (approx.. +4 °C)
GLP	Yes
Expiration Date	01.May.2023

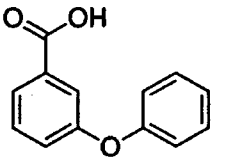
Chemical structure:



3-Phenoxybenzoic acid (3-PBA)

Common Name	3-PBA
BASF Reg. No.	130213
CAS-No.	3739-38-6
Molecular Formula	$C_{13}H_{10}O_3$
Molecular Weight	214.2
IUPAC Name	3-phenoxybenzoic acid
Batch No.	AC12251-34
Purity (%)	100.0
Test Substance Type	PAI
Storage Advice	Keep in refrigerator or freezer
GLP	Yes
Expiration Date	01.December.2020

Chemical structure:



2.3 Materials and Methods

Equipment	Size, Description	Manufacturer
Balance, Top Loader	Model PJ3600	Mettler DeltaRange
Balance, Analytical	Model AT100	Mettler
Beakers	Various sizes	PYREX Brand, VWR Scientific Products
Bottle, Amber glass	Qorpak , 2 oz and 4 oz with Teflon®-lined screw cap	VWR Scientific Products Boston Round, Amber
Volumetric, pipettes	0.5 mL, 1 mL, 2.5 mL, 5 mL	Fisher Scientific – Class A
Repeater Pipette	1000 µL 250 µL 25 µL	Gilson Microman Fisher Scientific
Syringe	1 mL	BD, Fisher Scientific
13 mm Syringe Filter	0.45 µm Nylon	Pall,Gelman Acrodisc, VWR
13 mm Syringe Filter	0.45 µm PTFE	Pall,Gelman Acrodisc, VWR
Mechanical shaker	KS501 Digital	IKA Labortechnik
Culture Tubes	Glass, disposable, 16x100mm size	Fisher
Cylinder, Graduated	Various sizes	Various
Evaporator traps	Inner vapor tube on top	VWR
Evaporator traps	Inner vapor tube on bottom	VWR
Flask, Erlenmeyer, 24/40	1000 mL	Various
Flask, Flat bottom, 24/40	125, 250 mL	Various
Centrifuge	Allegra 6	Bechman Coulter
Vortex	Genie 2	VWR Scientific Products
Nitrogen Evaporator	N-EVAP 112	Organomation Associates, Inc.

Equipment	Size, Description	Manufacturer
Glass Centrifuge Tubes	50 mL	VWR
Culture tube caps	16 mm	VWR
HPLC-MS/MS	Agilent 1200 SL / AB Sciex 5500 Mass Spectrometer	Agilent and AB Sciex
HPLC-MS/MS	Agilent 1200 SL / AB Sciex 4000 QTRAP Mass Spectrometer	Agilent and AB Sciex
HPLC Column	Acquity UPLC HSS T3 1.8 µm 2.1x150mm	Waters
HPLC Column	Acquity UPLC HSS T3 1.8 µm 2.1x50mm	Waters
HPLC Column	BEH Phenyl 2.5 µm 2.1x100 mm	Waters
HPLC Column	XSelect HSS T3 2.5 µm 2.1x150 mm	Waters

2.3.1.1 Chemicals

Chemical	Grade	Manufacturer/Supplier
Methanol	HPLC Grade	Fisher Scientific
Acetonitrile	HPLC Grade	Fisher Scientific
Water	HPLC Grade	EMD
Ammonium Formate	>99.0%	Fluka
Formic acid	98% GR ACS	EMD

2.3.1.2 Solutions and Solvent Mixtures

Description	Code	Composition
Extraction solvent [BAS 311 I]	**S1**	0.1% Formic Acid in Acetonitrile Add 1 mL of formic acid to 1000 mL of acetonitrile into a, e.g., 1-L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
Extraction solvent [Metabolites]	**S2**	Acetonitrile/water (70:30,v/v) Add 700 mL of acetonitrile and 300 mL of water into a, e.g., 1-L Erlenmeyer flask and mix well to ensure complete homogeneous solution.

Description	Code	Composition
Final Volume solvent [BAS 311 I]	**S3**	Water/acetonitrile with 0.1% Formic Acid (50:50, v/v) Add 1 ml of formic acid to 500 mL of acetonitrile and 500 mL of water into a, e.g., 1-L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
Final Volume solvent [Metabolites]	**S4**	Methanol/Water with 0.1% Formic Acid (20:80, v/v) Add 1 mL of formic acid to 800 mL of water and add 200 mL of methanol into a, e.g., 1-L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
HPLC mobile phase A [BAS 311 I]	**LC1**	0.1% Formic Acid/4mM Ammonium Formate in Water Add 999 mL of water, 1 mL of concentrated formic acid and 0.252 g of ammonium formate into a 1-L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
HPLC mobile phase B [BAS 311 I]	**LC2**	0.1% Formic Acid/4mM Ammonium Formate in Methanol Add 999 mL of methanol, 0.252 g and 1 mL of concentrated formic acid into a, e.g., 1-L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
HPLC mobile phase A [Metabolites]	**LC3**	Acetonitrile/water (5:95,v/v) Add 50 mL of acetonitrile and 950 mL of water into a, e.g., 1-L Erlenmeyer flask and mix well to ensure complete homogeneous solution
HPLC mobile phase B [Metabolites]	**LC4**	Acetonitrile/water (95:5,v/v) Add 950 mL of acetonitrile and 50 mL of water into a, e.g., 1-L Erlenmeyer flask and mix well to ensure complete homogeneous solution

2.3.1.3 Standard Solutions

2.3.1.3.1 BAS 311 I Diastereomers (Cis I, Cis II, Trans III, and Trans IV)

Stock Solution

Individual 1 mg/mL stock solution of each diastereomeric form was prepared by weighing an appropriate amount of each reference item into a volumetric flask and adding the required volume. The Certificate of Analysis was used to calculate the absolute concentration of each diastereomer in solution.

Fortification Solutions

Standard solutions for fortification were prepared by combining stock solutions of each reference item (0.5 mL) into a 50-mL volumetric flask. Afterwards, dilution series were made up by using 0.1% formic acid in acetonitrile (S1), as exemplified in the table below. The use of sonication or vortexing was also considered for ensuring a complete homogeneous solution.

Preparation of Mixed Fortification solutions

Take solution (µg/mL)	Volume (mL)	Dilute with S1 to a final volume of (mL)	Concentration (µg/mL)
1000	0.50 (of each stock solution)	50	10.0
10.0	5	50	1.0
1	5	50	0.10

Calibration Standard Solutions

Standard calibration solutions for LC-MS/MS analysis were prepared using the solutions described above and by diluting them with water/acetonitrile with 0.1% formic acid (50:50, v/v) (S3) as needed. These solutions were made up as follows:

Preparation of Standard Solutions for Calibration

Take solution (ng/mL)	Volume (mL)	Dilute with S3 to a final volume of (mL)	Concentration (ng/mL)
100	5	50	10
100	2.5	50	5.0
10.0	5	50	1.0
5.0	5	50	0.50
1.0	5	50	0.10
0.5	5	50	0.05

Preparation of Matrix matched Standard Solutions for Calibration

Matrix-matched standards were also needed for matrix effects experimentation by LC-MS/MS analysis by using the solutions that were prepared below. A 10-mL aliquot (volumetrically) of the final extract was transferred to a culture tube and evaporated to dryness using nitrogen at about 50 °C (e.g., in a water bath). The samples were reconstituted using the appropriate standard solution in water/acetonitrile with 0.1% formic acid (50:50, v/v). The matrix-matched standards were made in a way that the matrix load was at least 90% of the matrix load in the unknown samples. In addition, the matrix load was the same for all calibration standard solutions.

Take standard solution (ng/mL)	Reconsititute with volume (mL)	Concentration (ng/mL)
0.250	2	0.250
0.500	2	0.500
2.50	2	2.50

2.3.1.3.2 Metabolites of BAS 311 I (3-PBA, *cis*- and *trans*-DCVA)

Stock Solution

Individual 1 mg/mL stock solution was prepared by weighing an appropriate amount of each reference item into a volumetric flask and adding the required volume. For *cis*- and *trans*-DCVA, the Certificate of Analysis was used to calculate the absolute concentration of each isomer in solution.

Fortification Solutions

Standard solutions for fortification were prepared by combining stock solutions of each reference item (0.25 mL) into a 25-mL volumetric flask. Afterwards, dilution series were made up by using methanol, as exemplified in the table below. The use of sonication or vortexing was also considered for ensuring a complete homogeneous solution.

Preparation of Mixed Fortification Solutions

Take solution (µg/mL)	Volume (mL)	Dilute with MeOH to a final volume of (mL)	Concentration (µg/mL)
1000	0.25 (of each stock solution)	25	10.0
10.0	2.5	25	1.0
1	2.5	25	0.10

Calibration Standard Solutions

Standard calibration solutions for LC-MS/MS analysis were prepared using the solutions, which were prepared in the previous section "Fortification Solutions", and by diluting them with methanol/water (20:80, v/v) with 0.1% formic acid (S4) as needed. These solutions were made up as follows:

Preparation of Standard Solutions for Calibration

Take solution (ng/mL)	Volume (mL)	Dilute with S4 to a final volume of (mL)	Concentration (ng/mL)
1000	0.500	50	10
1000	0.250	50	5.0
100	0.500	50	1.0
100	0.250	50	0.50
10	1.25	50	0.25
10	0.500	50	0.10

Preparation of Matrix matched Standard Solutions for Calibration

Matrix-matched standards were also needed for matrix effects experimentation by LC-MS/MS analysis by using the solutions that were prepared below. A 150-mL aliquot (volumetrically) of the final extract was transferred to 250-mL round-bottom flask and evaporated to dryness using nitrogen at about 50 °C (e.g., in a water bath). The samples were reconstituted using the appropriate standard solution in methanol/water with 0.1% formic acid (20:80, v/v). The matrix-matched standards were made in a way that the matrix load was at least 90% of the matrix load in the unknown samples. In addition, the matrix load was the same for all calibration standard solutions.

Take solution (ng/mL)	Volume (mL)	Add to this vol. of control matrix solution (mL)	Concentration (ng/mL)
2.50	0.1	0.9	0.250
5.00	0.1	0.9	0.500
25.0	0.1	0.9	2.50

2.3.1.4 Stability of Fortification and Calibration Standard Solutions

The stability of the diastereomers of BAS 311 I in the concentrated (approximately 1000 µg/mL, nominal), fortification (1 µg/mL, nominal), and calibration standard (0.01 µg/mL, nominal) solutions were investigated within this study. For this purpose, fortification and concentrated standard solutions were stored refrigerated for 65 days and for the calibration standards for 64 days. At this time point, the concentrations of the diastereomers of BAS 311 I were measured against freshly prepared standards (0 day) within one analytical queue.

The stability of the metabolites of BAS 311 I in the concentrated (500 µg/mL, nominal), fortification (0.1 µg/mL, nominal), and calibration standard (0.01 µg/mL, nominal) solutions were investigated within this study. For this purpose, these standard solutions were stored refrigerated for 38 days (calibration and fortification standard solutions) and 69 days (concentrated stock solution). At this time point, the concentrations of the metabolites of BAS 311 I were measured against freshly prepared standards (0 day) within one analytical queue.

Standard stability data is presented in Appendix 9.6.

2.3.1.5 Stability of Extracts

Since sample extracts at day 0 (starting values) had to be stored prior to final measurement for a short-time period, the stability of the diastereomers of BAS 311 I in soil extracts was also tested (at fortification level 0.001 and 0.01 mg/kg). Data were obtained from the refrigerated stored extracts after zero and 14 days for clay loam.

At each sampling time point, the concentration of BAS 311 I was measured against freshly prepared standards within one analytical queue. Quantification of the analytes was done for both mass transitions.

The stability of the metabolites of BAS 311 I in soil extracts was also tested (at fortification level 0.001 mg/kg). Data were obtained from the refrigerated stored extracts after zero and 25 days for clay loam.

Quantification of 3-phenoxybenzoic acid was done using two separate mass transitions. The ions monitored for *cis*- and *trans*-DCVA were analyzed using UPLC and HPLC Methods G and E, respectively. Recoveries were used to prove the stability of the analytes in the soil extracts. Extract stability data are presented in Appendix 9.6.

Analytical Procedure

2.3.1.6 Weighing and Fortification

For control, fortification, and treated samples extracted for BAS 311 I, 5 g of soil was weighed into a 150-mL centrifuge bottle.

For control, fortification, and treated samples extracted for metabolites of BAS 311 I (3-PBA, *cis*- and *trans*-DCVA), 5 g of soil was weighed into a disposable centrifuge tube. For fortified samples, fortification solutions were added to the matrix, as shown in the following table:

Sample Type	Sample Weight	Concentration of Spiking Solution [ng/mL]	Volume of Spiking Solution [mL]	Level of Fortification [µg/g]
Control	5 g	N/A	N/A	0.00
Fortification (LOQ)	5 g	100	0.05	0.001
Fortification (10× LOQ)	5 g	1000	0.05	0.01
Treated	5 g	N/A	N/A	N/A

* Limit of quantification (LOQ)

2.3.1.7 Extraction of Sample Material

2.3.1.7.1 Extraction of BAS 311 I

Exactly 50 mL S1 (0.1% formic acid in acetonitrile, v/v)) was added to the pre-weighed sample and it was shaken using a mechanical shaker for approximately 30 minutes. A 20-mL aliquot (definite volume not necessary) of the extract was centrifuged for about 5 min at 4000 RPM. A 10-mL aliquot (volumetrically) was transferred to a culture tube.

2.3.1.7.2 Extraction of Metabolites (3-PBA, *cis*- and *trans*-DCVA)

Exactly 25 mL S2 was added to the pre-weighed sample and it was shaken using a mechanical shaker for approximately 30 minutes and then centrifuged at about 4000 RPM for 5 minutes at about 0 °C. The sample was decanted into a 50-mL disposable centrifuge tube. Another 25-mL aliquot of S2 (acetonitrile/water (70:30, v/v)) was added to the sample. The sample was vortexed to dislodge the soil marc and shaken on the mechanical shaker for another 30 minutes and centrifuged at about 4000 RPM for 5 minutes at about 0 °C. The sample was decanted into the same 50-mL disposable centrifuge tube containing the first extract. The sample was vortexed thoroughly to ensure a homogenous solution and centrifuged at about 4000 RPM for 5 minutes at about 0 °C.

2.3.1.8 Preparation for Measurement

2.3.1.8.1 Preparation for Measurement of BAS 311 I

The extract was evaporated to dryness using nitrogen at about 50 °C (e.g., in a water bath). The samples were reconstituted using the appropriate volume of S3, water/acetonitrile with 0.1% formic acid (50:50, v/v).

For fortifications at LOQ and the control samples, residues are reconstituted in 2 mL of S3. The sample was sonicated for about 1 minute then vortexed for about 15 seconds. A 1-mL aliquot of the sample was transferred into a HPLC vial using a primed syringe filter (0.45 µm Nylon). The syringe was primed by discarding the first 200 µL of the filtered sample prior to vialing for analysis.

If a residue outside the calibration curve was observed, the samples can be diluted with S3 as needed to fit into the calibration curve.

2.3.1.8.2 Preparation for Metabolites (3-PBA, *cis*- and *trans*-DCVA)

A 5-mL aliquot of the extract was transferred to a culture tube and evaporated to dryness using nitrogen at about 50 °C (e.g., in a water bath). The samples were reconstituted using the appropriate volume of S4.

For fortifications at LOQ and the control samples, residues are reconstituted in 1 mL of S4. The sample was sonicated for about 1–2 minutes and vortexed for about 15 seconds. The sample was transferred into a HPLC vial using a syringe filter (0.45 µm PTFE).

If a residue outside the calibration curve was observed, the samples can be diluted with S4 as needed to fit into the calibration curve.

2.3.1.9 Influence of Matrix effects on Analysis

In order to test the influence of the matrix effects on the analysis, the response of each analyte in the matrix as compared to pure standards was studied during method validation. The standard response was compared at three different levels (e.g., ½ LOQ, LOQ, 5× LOQ) of the diastereomers of BAS 311 I and its metabolites (prepared in S3 and S4 solvent, respectively) against their respective calibration standards prepared in untreated matrix extracts (matrix-matched standards). The evaluation was done by comparing the average area response of three replicate injections of the standards with and without matrix. If significant suppression occurs, matrix-matched standards may be utilized.

The percent matrix interference was determined by the following equation:

$$\text{Matrix Interference (\%)} = \frac{\text{Avg. Area Response of Matrix-Matched Std. - Avg. Area Response of Inj. Std.}}{\text{Avg. Area Response of Inj. Std.}} \times 100$$

2.3.1.10 Extractability

The metabolism extraction solvent was not used to analyze the diastereomeric forms of BAS 311 I [Reference 7]. Therefore, an incurred residue was extracted using both the residue and metabolism extraction procedures. The extractability set consisted of one control, two fortifications at the LOQ (0.001 mg/kg) and one at 10× LOQ for each analyte, and one sample with incurred residues from the terrestrial field dissipation (Study Number 380198) [Reference 8]. The results of the metabolism extraction procedure and the residue extraction procedures were compared. The following procedures were used to test extractability.

A. Residue extraction procedure:

Diastereomeric forms of BAS 311 I: follow the analytical procedure in method R0034/01 for weighing and extracting the soil, and preparation for measurement.

Extraction of diastereomeric forms of BAS 311 I: the samples were extracted with 0.1% formic acid in acetonitrile by shaking in a mechanical shaker for 30 minutes.

B. Metabolism extraction procedure:

1. Extraction of diastereomeric forms of BAS 311 I and its metabolites: the samples were extracted with acetonitrile/water (70:30, v/v; 4 × 6 mL) by shaking in a mechanical shaker for an hour. All four extracts were combined and centrifuged.

2. Determination of the diastereomeric forms of BAS 311 I: an aliquot from the combined extract (from Step 1, listed above) was evaporated to dryness using nitrogen at about 50 °C (e.g., in a water bath). The residue was reconstituted using an appropriate volume of water/acetonitrile with 0.1% formic acid (50:50, v/v) to fit the residues in the calibration curve.

Instrument analysis was achieved using method R0034/01 HPLC-MS/MS method C (Section 3.1) for the diastereomeric forms of BAS 311 I. For the extraction of the metabolites, the original metabolism extraction procedure using acetonitrile/water (70:30, v/v) was used [Reference 7]. The results of the extractability experiments can be found in Section 4.3.

2.3.1.11 Moisture Determination

The recoveries were not corrected for moisture content of the sample. The results of soil analysis were reported on a "dry weight" basis for residue determination. Therefore, soil sample weights were corrected for moisture content when the sample had a residue value above the LOD. The percent moisture was determined using automated moisture determination equipment (Mettler Toledo HR83).

3 Instrumental Analysis

3.1 Instrumentation and Conditions (BAS 311 I)

Method B (UPLC Mode): Primary and Confirmatory Quantitation

	Parameter		
Chromatographic System	Agilent 1200SL HPLC ***		
Analytical-column	Acquity UPLC HSS T3 1.8 µm 2.1x150 mm		
Column Temperature	60 °C		
Injection Volume	30 µL		
Mobile Phase A Mobile Phase B	0.1% formic acid in water, 4 mM ammonium formate 0.1% formic acid in methanol, 4 mM ammonium formate		
Flow Rate	350 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.00	90.0	10.0
	1.00	90.0	10.0
	6.00	22.0	78.0
	18.00	22.0	78.0
	18.10	2.0	98.0
	19.00	2.0	98.0
	19.10	90.0	10.0
	22.10	90.0	10.0
Detection System	AB Sciex 5500 Mass Spectrometer		
Ionisation	Electrospray (ESI)		
Ionisation Temperature	600 °C		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time ****
Alpha-cypermethrin (Cis II isomer)	433 → 191* 435 → 193	positive	~ 13.0 min
CIS I Isomer	433 → 191* 435 → 193	positive	~ 13.1 min
TRANS III Isomer	433 → 191* 435 → 193	positive	~ 12.9 min
TRANS IV Isomer	433 → 191* 435 → 193	positive	~ 12.7 min

* proposed as quantification transition. Any of these transitions could be used for quantitation in case interference is observed at the same retention time.

** Retention time shifting can occur.

***The change in chromatographic systems from Method A using Waters UPLC Acquity to Agilent 1200SL HPLC required a change in gradient parameters to achieve analyte separation. Due to the transfer of the UPLC method to this HPLC method, the peak separation may not be completely baseline separated. However, quantitation can be done successfully by using the peak select tool in the data software or by consistent manual integration to the baseline.

Method C (HPLC Mode): Primary and Confirmatory Quantitation

	Parameter		
Chromatographic System	Agilent 1200SL HPLC		
Analytical-column	XSelect HSS T3 2.5 µm 2.1x150 mm		
Column Temperature	60 °C		
Injection Volume	20 µL		
Mobile Phase A Mobile Phase B	Water 0.1% formic acid, 4 mM ammonium formate Methanol 0.1% formic acid, 4 mM ammonium formate		
Flow Rate	288 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.0	95	5
	1.0	95	5
	6.0	22	78
	18.0	22	78
	27.0	22	78
	27.1	2	98
	28.1	2	98
	28.2	95	5
	31.2	95	5
Detection System	AB Sciex QTRAP 4000 Mass Spectrometer		
Ionisation	Electrospray (ESI)		
Ionisation Temperature	500 °C		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time****
Alpha-cypermethrin (Cis II isomer)	433 → 191* 435 → 193	positive	~ 24.0 min
CIS I Isomer	433 → 191* 435 → 193	positive	~ 25.0 min
TRANS III Isomer	433 → 191* 435 → 193	positive	~ 23.6 min
TRANS IV Isomer	433 → 191* 435 → 193	positive	~ 23.1 min

* proposed as quantification transition. Any of these transitions could be used for quantitation in case interference is observed at the same retention time.

** Retention time shifting can occur.

3.2 Instrumentation and Conditions (3-PBA, *cis*- and *trans*-DCVA)

Method E (HPLC Mode): Primary and Confirmatory Quantitation of 3-PBA; Primary Quantitation in HPLC mode of DCVA (Cis and Trans) and Confirmatory Quantitation for Method G

	Parameter		
Chromatographic System	Agilent 1200SL HPLC		
Analytical-column	BEH Phenyl 2.5 µm 2.1x100 mm		
Column Temperature	60 °C		
Injection Volume	50 µL		
Mobile Phase A Mobile Phase B	Water Acetonitrile		
Flow Rate	350 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.00 0.50 14.00 14.10 17.00	100.0 100.0 25.0 100.0 100.0	0.0 0.0 75.0 0.0 0.0
Detection System	AB Sciex QTRAP 5500 Mass Spectrometer		
Ionisation	Electrospray (ESI)		
Ionisation Temperature	600 °C		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**
3-Phenoxybenzoic Acid	213 → 93* 213 → 169	negative	~ 8.8 min
DCVA Trans	207 → 207* 209 → 209**	negative	~ 9.7 min
DCVA Cis	207 → 207* 209 → 209**	negative	~ 10.4 min

* Proposed as quantification transition. Any of these transitions could be used for quantitation in case interference is observed at the same retention time.
** Listed transitions for *cis*- and *trans*-DCVA are two ions monitored and not transitions. An alternate method is required for confirmation.

Method E could be used for the quantitation for all above analytes specified in the table and should be tested before GLP analysis for recoveries. Method F was used for confirmation of *cis*- and *trans*-DCVA.

Method F (HPLC Mode): DCVA (Cis and Trans) Confirmatory Quantitation both for HPLC Mode and for Method E

	Parameter			
Chromatographic System	Agilent 1200SL HPLC			
Analytical-column	XSelect HSS T3 2.5 µm 2.1x150 mm			
Column Temperature	60 °C			
Injection Volume	50 µL			
Mobile Phase A Mobile Phase B	Water Acetonitrile			
Gradient (including wash and equilibration)	Time (min)	Flow Rate	Phase A	Phase B
	0.00 15.00 15.10 17.00 17.10 20.00	500 500 800 800 500 500	100.0 30.0 5.0 5.0 100.0 100.0	0.0 70.0 95.0 95.0 0.0 0.0
Detection System	AB Sciex QTRAP 5500 Mass Spectrometer			
Ionisation	Electrospray (ESI)			
Ionisation Temperature	600 °C			
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**	
3-Phenoxybenzoic Acid	213 → 93* 213 → 169	negative	~ 10.5 min	
DCVA Trans	207 → 207* 209 → 209**	negative	~ 11.2 min	
DCVA Cis	207 → 207* 209 → 209**	negative	~ 12.2 min	

* Proposed as quantification transition. Any of these transitions could be used for quantitation in case interference is observed at the same retention time.
** Listed transitions for *cis*- and *trans*-DCVA are two ions monitored and not transitions. An alternate method is required for confirmation.

Method F could be used for the quantitation for all above analytes specified in the table and should be tested before GLP analysis for recoveries.

Method G (UPLC Mode): Primary and Confirmatory Quantitation of 3-PBA and Primary Quantitation of DCVA (Cis and Trans)

	Parameter			
Chromatographic System	Agilent 1200SL HPLC			
Analytical-column	Acquity UPLC HSS T3 1.8 µm 2.1x50 mm			
Column Temperature	60 °C			
Injection Volume	50 µL			
Mobile Phase A Mobile Phase B	Water Acetonitrile			
Gradient (including wash and equilibration)	Time (min)	Flow Rate (µL/min)	Phase A	Phase B
	0.00	400	85.0	15.0
	8.00	400	45.0	55.0
	8.10	600	5.0	95.0
	9.00	600	5.0	95.0
	9.10	400	85.0	15.0
	13.00	400	85.0	15.0
Detection System	AB Sciex 5500 Triple Quad Mass Spectrometer			
Ionisation	Electrospray (ESI)			
Ionisation Temperature	600 °C			
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**	
3-Phenoxybenzoic Acid	213 → 93* 213 → 169	negative	~ 5.3 min	
DCVA Trans	207 → 207* 209 → 209**	negative	~ 6.1 min	
DCVA Cis	207 → 207* 209 → 209**	negative	~ 6.7 min	

* Proposed as quantification transition. Any of these transitions could be used for quantitation in case interference is observed at the same retention time.
** Listed transitions for *cis*- and *trans*-DCVA are two ions monitored and not transitions. An alternate method is required for confirmation.

3.3 Calibration Procedures

Calculation of results was based on peak area measurements using a calibration curve. Six calibration levels were injected. The calibration curve was obtained by direct injection of the diastereomers of BAS 311 I standards for LC-MS/MS in the range of 0.05 to 10.0 ng/mL (nominal). The metabolites of BAS 311 I (3-PBA, *cis*- and *trans*-DCVA) standards for LC-MS/MS were injected in the range of 0.1 to 10.0 ng/mL (nominal). In all injection runs for the diastereomers of BAS 311 I, either 20 or 30 µL were used for all samples and standards depending on the method utilized. In all injection runs for the metabolites of BAS 311 I, the same injection volume was used for all samples and standards.

3.4 Calculation of Residues and Recoveries

For the recoveries, the exact sample weight was used in calculating the final calculation of residues (mg/kg or ppm). All calculations were based on unrounded numbers. Percent recoveries and all ppm values have been corrected for the isomeric ratio of Cis I/Cis II/Trans III/Trans IV (45:55:43.5:56.5) and *cis*- and *trans*-DCVA (51.5:48.5).

a) Calibration curve: $y = mx + b$ Solving for x: $x = \frac{y-b}{m}$

Where, m = slope
b = y-intercept
x = Amount found (ng)
y = Peak area

b) $$\text{Amount of sample injected (mg)} = \frac{\text{injection size (µL)}}{\text{final sample vol. (mL)}} \times \text{sample weight (g)} \times \frac{1\text{ mL}}{1000\text{ µL}} \times \frac{1000\text{ mg}}{\text{g}}$$

c) $$\text{Residue found (ppm)} = \frac{\text{ng found}}{\text{Amount of sample injected (mg)}}$$

d) $$\text{Recovery (\%)} = \frac{\text{(Residue found in Sample(ppm))} - \text{(Residue found in Control Sample (ppm))}}{\text{Amount fortified (ppm)}} \times 100$$

Example:* Cis I *(m/z 433.0 → 191.0) in clay loam fortified at 0.001 ppm in WO-13073001.

a) Calibration curve: $y = (7.11e + 005)x + 868$

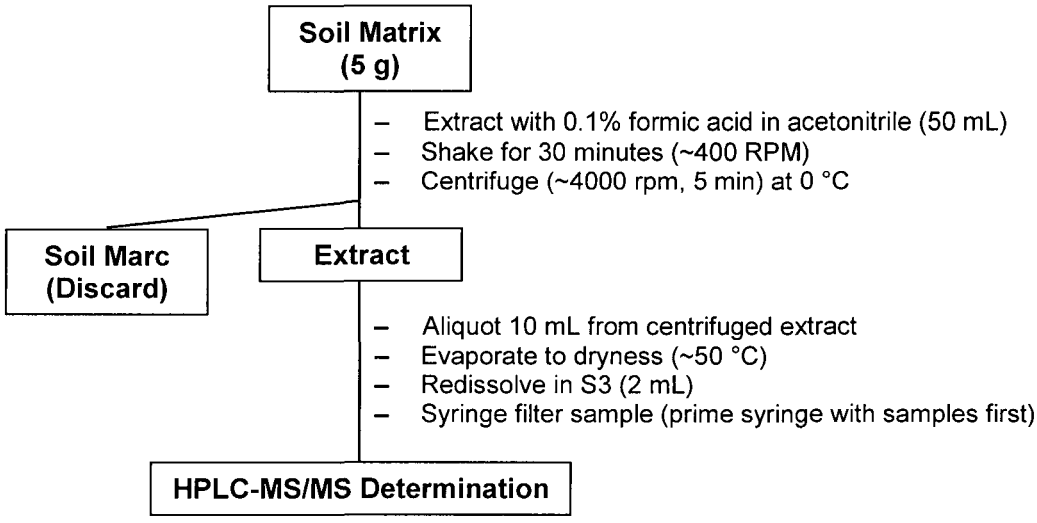
Solving for x: $x = \frac{5723-868}{7.11e+005} = 0.00683$ ng

b) $$\text{Amount of sample injected (mg)} = \frac{20\text{ µL}}{10\text{ mL}} \times 4.99\text{ g} \times \frac{1\text{ mL}}{1000\text{ µL}} \times \frac{1000\text{ mg}}{1\text{ g}} = 9.98\text{ mg}$$

c) $$\text{Residue found (ppm)} = \frac{0.00683\text{ ng}}{9.98\text{ mg}} = 0.000685\text{ ppm}$$

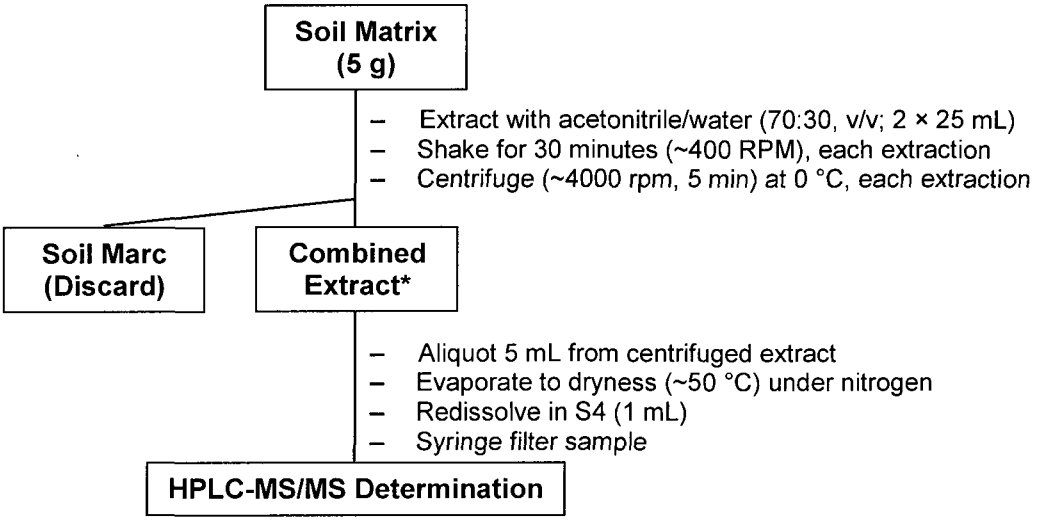
d) $$\text{Recovery (\%)} = \frac{(0.000685\text{ ppm}) - (0.00000\text{ ppm})}{0.000818\text{ ppm}} \times 100 = 84\%$$

Appendix 9.3 Additional Information on the Method
Figure 9.3.1 Method Flowchart – Analysis of BAS 311 on Soil



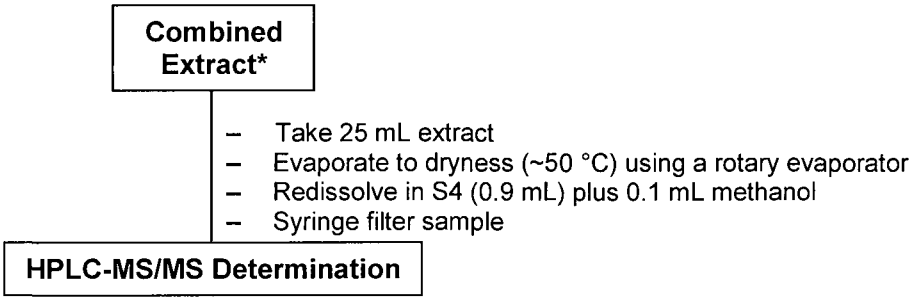
S3 = Water/acetonitrile with 0.1% formic acid (50:50, v/v)

Figure 9.3.2 Method Flowchart – Analysis of Metabolites of BAS 311 (3-PBA, *cis*-DCVA, and *trans*-DCVA) on Soil



S4 = Methanol/water with 0.1% formic acid (20:80, v/v)

* The following procedure is used for matrix-matched standard analysis:



METHOD MANAGEMENT AND TIME REQUIREMENTS

The analysis of one series of samples (one reagent blank, two controls, and 10 fortified samples for recovery experiments) requires 1.5 working day (12 hours) per laboratory assistant. This time includes the calculation of the results, the preparation of the equipment as well as the reporting of all raw data under GLP.

Limit of Quantification (LOQ) and Limit of Detection (LOD)
The limit of quantification is defined as the lowest fortification level successfully tested. The limit of quantification and limit of detection are 0.001 and 0.0002 mg/kg for the diastereomers of BAS 311 I and its metabolites, 3-PBA, *cis*- and *trans*- DCVA, respectively. The lowest standard for each analyte in the calibration curve has good detectability (signal to noise ratio greater than 3:1).

Selectivity
The method R0034/01 determines residues of the diastereomeric forms of BAS 311 I (Cis I, Cis II [alpha-cypermethrin], Trans III, and Trans IV), and its three metabolites 3-phenoxybenzoic acid (PBA) and DCVA (*cis*- and *trans*-isomers), in soil. No interfering peaks were found at the retention times for BAS 311 I or its metabolites. Justification of selection of ions is shown in the Figure 9.4.36.

Confirmatory Techniques
The HPLC-MS/MS final determination for BAS 311 I and its metabolites is a highly selective detection technique. The ions monitored for DCVA (Cis and Trans) were analyzed using UPLC and HPLC methods G and E, respectively. For DCVA (Cis and Trans), Method E was used for primary quantitation in HPLC mode and confirmatory quantitation for Method G. Additionally, Method F was used for confirmatory quantitation both for HPLC mode and for Method E.

Potential Problems
BAS 310 I (alpha-cypermethrin, Cis II isomer) has been shown to be unstable in methanol. In addition, all BAS 311 I standard solutions should be stored to protect them from light (i.e., use amber glassware wherever possible).

The glassware used for the method should be thoroughly rinsed with acetonitrile to prevent contamination.

It has been observed that the diastereomers of BAS 311 I in solution are absorbed to the nylon filters during filtration yielding loss of recoveries. It is recommended that the use of nylon filters should be primed with extracts prior to vialing for analysis.

Interferences may be observed during analysis of the metabolites (PBA and DCVA, Cis and Trans isomers) depending on soil type. It is recommended that the LC-MS/MS gradient to be tested before GLP analysis for interferences. Consequently, LC gradient modifications may be necessary.

Retention time shifting of the diastereomers of BAS 311 I have been observed and is instrument dependent. This will not affect quantitation as long as the analyst observes the retention pattern and quantitates the analytes accordingly comparing with reference standards. The instrument must be sufficiently conditioned with injections of sample matrix prior to analysis.