1. INTRODUCTION

1.1 Scope of the Method

The analytical method presented in draft validation report for Study No. 986.6267 was developed by Smithers Viscient in Wareham, Massachusetts to determine residues of BAS 315 I and its metabolites (Metabolite M12, Metabolite M11, Metabolite M6, Metabolite M1b and Metabolite M1a) in soil using LC-MS/MS (Reference 1). This method was independently validated at ADPEN Laboratories, Inc., Jacksonville, Florida, during this study.

1.2 Principle of the Method

Extraction A: Determination of BAS 315 I and Metabolites M12, M11, M6 and M1b in Soil
An aliquot of soil (5 g dry weight) was weighed into a 50 mL centrifuge tube and fortified with BAS 315 I and Metabolites M12, M11, M6 and M1b, as appropriate. The sample was extracted on a mechanical shaker with 95/5 acetonitrile/purified water (v/v). After centrifugation, the supernatant was transferred into a 50 mL volumetric flask and the extraction steps repeated until the combined supernatants were brought to a final volume of 50 mL with additional 95/5 acetonitrile/purified water (v/v). The sample extract was diluted into the calibration standard range with 50/50 acetonitrile/purified water (v/v) and submitted for LC-MS/MS analysis.

Extraction B: Determination of Metabolite M1a in Soil
An aliquot of soil (5 g dry weight) was weighed into a 50 mL centrifuge tube and fortified with Metabolite M1a, as appropriate. The sample was extracted on a mechanical shaker with acetonitrile. After centrifugation, the supernatant was transferred into a 50 mL volumetric flask and the extraction steps repeated until the combined supernatants were brought to a final volume of 50 mL with additional acetonitrile. The sample extract was diluted into the calibration standard range with 50/50 acetonitrile/purified water (v/v) and submitted for LC-MS/MS analysis.

2. TEST SYSTEM AND TEST/REFERENCE SUBSTANCES

2.1 Test System

The test system used in this study was sandy loam soil.

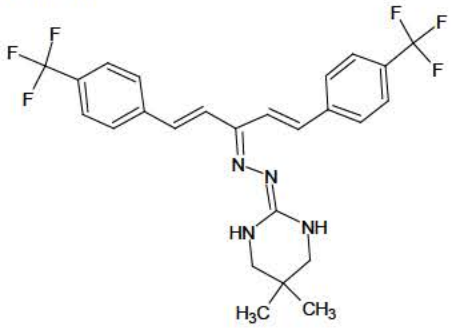
Sample Description	Control Matrix Number
Sandy loam soil	RMN-SL-PF

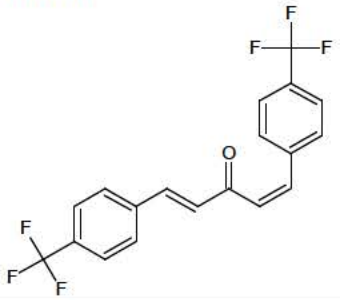
The test system identified in the study protocol (Sandy loam soil, Control Matrix Number: Lot 062618B) was provided by Smithers Viscient and received at ADPEN Laboratories, Inc. on July 11, 2018. Upon arrival at the laboratory, the sample was inspected and assigned a unique sample number through ADPEN's Laboratory Information Management System (LIMS). A second test system (Sandy loam soil, Control Matrix Number RMN-SL-PF) was provided by Smithers Viscient and received at ADPEN Laboratories, Inc. on February 5, 2019. Upon arrival at the laboratory, the sample was inspected and assigned a unique sample number through ADPEN's LIMS. All samples were received ambient and in good condition. The samples were stored in freezer E16 at an average temperature of -20 ºC when not needed for analyses. The characterization report provided for the test system (Control Matrix Number RMN-SL-PF) is presented in Appendix A.

2.2 Test/Reference Substances

The following reference standard substances were provided by the Sponsor and were stored frozen (< -5 °C) upon receipt at the testing facility. Characterization and stability data for the substances is maintained by the Sponsor, and a reserve sample of these standards is retained at BASF, Research Triangle Park, North Carolina. Detailed information regarding the test substances, including the certificates of analysis, is presented in Appendix B.

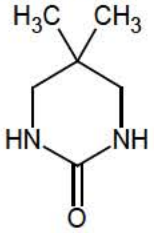
A brief description of each reference substance follows:

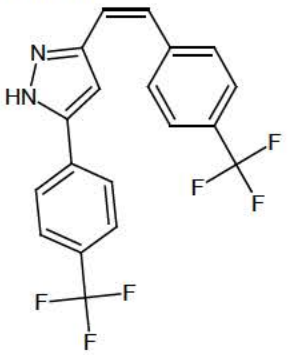
Code No.	BAS 315 I
Chemical Name (IUPAC):	5,5-dimethylperhydropyrimidin-2-one 4-trifluoromethyl-alpha-(4-trifluoromethylstyryl)cinnamylidenehydrazone
CAS Registry No.:	67485-29-4
BASF Reg. No.:	4111109
Molecular Formula:	$C_{25}H_{24}F_6N_4$
Molecular Weight:	494.5 g/mol
Batch No.:	L83-26
Purity:	99.5%
Expiration:	October 1, 2020
Storage:	Frozen
Molecular Structure:	

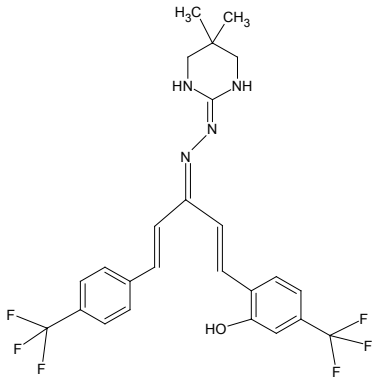
Code No.	Reg. No. 255418 (M12)
Chemical Name (IUPAC):	1,5-bis[4-(trifluoromethyl)phenyl]penta-1,4-dien-3-one
CAS Registry No.:	42160-07-06
BASF Reg. No.:	255418
Molecular Formula:	$C_{19}H_{12}F_6O$
Molecular Weight:	370.3 g/mol
Batch No.:	AC9745-96A
Purity:	99.6%
Expiration:	November 1, 2028
Storage:	Frozen
Molecular Structure:	

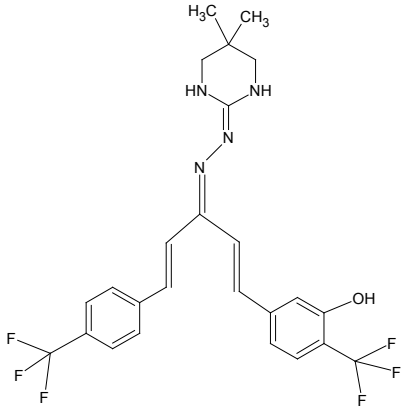
The following reference standard substances were obtained from a commercial source and were stored frozen (< -5 °C) upon receipt at the testing facility. A purity statement accompanied the shipment of these materials to the testing facility. Detailed information regarding the test substances, including the certificates of analysis, is presented in Appendix B.

A brief description of each reference substance follows:

Code No.	Hydramethylnon Metabolite P (M11)
Chemical Name (IUPAC):	N/A
CAS Registry No.:	N/A
Commercial Source:	Concord Biosciences
Molecular Formula:	$C_6H_{12}N_2O$
Molecular Weight:	128.2 g/mol
Batch No.:	55658-25-30
Purity:	94.49%
Expiration:	December 20, 2019
Storage:	Frozen
Molecular Structure:	

Code No.	Hydramethylnon Metabolite M6
Chemical Name (IUPAC):	N/A
CAS Registry No.:	N/A
Commercial Source:	Concord Biosciences
Molecular Formula:	$C_{19}H_{12}F_6N_2$
Molecular Weight:	382.3 g/mol
Batch No.:	55715-06-04
Purity:	95.94%
Expiration:	December 20, 2019
Storage:	Frozen
Molecular Structure:	

Code No.	Hydramethylnon Metabolite M1a
Chemical Name (IUPAC):	N/A
CAS Registry No.:	N/A
Commercial Source:	Concord Biosciences
Molecular Formula:	$C_{25}H_{24}F_6N_4O$
Molecular Weight:	510.5 g/mol
Batch No.:	55878-16-05
Purity:	99.42%
Expiration:	January 10, 2020
Storage:	Frozen
Molecular Structure:	

Code No.	Hydramethylnon Metabolite M1b
Chemical Name (IUPAC):	N/A
CAS Registry No.:	N/A
Commercial Source:	Concord Biosciences
Molecular Formula:	$C_{25}H_{24}F_6N_4O$
Molecular Weight:	510.5 g/mol
Batch No.:	55898-8-34
Purity:	94.5%
Expiration:	December 20, 2019
Storage:	Frozen, dark
Molecular Structure:	

3. ANALYTICAL METHOD

Smithers Viscient Study 986.6267 “Validation of the Analytical Method for the Determination of BAS 315 I and Metabolites in Soil” (draft report, Reference 1) was used for the analysis of the samples. The final report for the validation study is presented in Appendix D.

Final determination of BAS 315 I and its metabolites was conducted using the following transition ions.

Analyte	Polarity	Quantitation (*m/z*)	Confirmation (*m/z*)
BAS 315 I	Positive	495→323	495→368
Metabolite M12	Positive	371→159	371→199
Metabolite M11	Positive	129→69	129→70
Metabolite M6	Positive	383→363	383→151
Metabolite M1b	Positive	511→491	511→364
Metabolite M1a	Positive	511→369	511→142

3.1 Method Selectivity

The method selectivity was demonstrated during method validation with the collection of mass spectra (product ion scans) to justify the selection of ion transitions used for LC-MS/MS determination (Appendix D). The method selectivity was further demonstrated during the conduct of the ILV study by screening the unfortified test system for the quantitation and confirmation ion transitions listed above.

3.2 Influence of Matrix Effects on Analysis

Matrix effects were evaluated during the conduct of this study. To assess the potential matrix effects of each analyte in the test system, matrix-matched standards, and non-matrix-matched standards were prepared (in triplicate) according to the analytical method, and analyzed on the LC-MS/MS. Matrix effects found to be ≥ 20 % were considered significant, and justified the use of matrix-matched calibration standards for method validation trials. Matrix effects found to be less than 20% were considered insignificant, and did not require the use of matrix-matched standards for method validation trials.

3.3 Validation of Method

For validation of the method/extraction procedure to determine BAS 315 I and Metabolites M12, M11, M6 and M1b, untreated samples of soil were fortified with the necessary analytes then analyzed according to the established method guidelines. For validation of the method/extraction procedure to determine Metabolite M1a, untreated samples of soil were fortified with Metabolite M1a then analyzed according to the established method guidelines. To test the repeatability of the method, the analytical sets for each method/extraction consisted of a reagent blank, two unfortified control samples, five replicates fortified at the method LOQ (50 µg/kg) and five replicates fortified at a higher level, corresponding to 10x the method LOQ (500 µg/kg).

The moisture content of the test system was determined on a halogen moisture analyzer according to ADPEN SOP 5.4.

7. RECOMMENDATIONS/CONCLUSIONS FROM ILV

The independent laboratory validation of the method validated in Smithers Viscient Study 986.6267 was successfully completed for all analytes in sandy loam soil in the first trial. Upon completion of the independent laboratory validation, the following recommendations were noted:

1. *Stability of standards*: The stability of the calibration standards was not evaluated as part of method validation or ILV studies. During the ILV study, the calibration and fortification solutions were prepared on the day of use for each validation set, but future method work should include determination of standard stability to allow the fortification and calibration solutions to be stored and used past the day of preparation.

2. *Stability of extracts*: The stability of the final volume extracts was not evaluated as part of the method validation or ILV studies. During the ILV study, the stability of the final volume solutions was confirmed with acceptable recovery of fortified samples, but future

method work should include determination of the stability of the sample extracts and final volume solutions.

3. *Limit of Detection (LOD):* The analytical method calculated the limit of detection (LOD) for each transition (primary and confirmatory) of each analyte (based on the standard deviation of the average recovery at the LOQ fortification level, see Reference 1) for a total of 12 LOD values. The LOD's provided in the analytical method are instrument-specific, and the analytical method did not state an overall method LOD for future method work. During the ILV, the LOD was defined as 30% of the method LOQ which resulted in a method LOD of 15 µg/kg. In future method revisions, a method LOD should be defined to ensure a consistent LOD is used when the method is implemented at different analytical facilities.

4. *Chromatography*: The initial chromatography observed during the ILV resolved multiple of isomers for metabolites M1b, M12, M6 and M1a. To resolve each isomer as a single peak during the ILV study, the "dwell time" in the mass spectrometer was increased to either 200 or 300 milliseconds (msec, See Table 20 and Table 22). It is recommended that some discussion be added to the method to address the chromatographic discrepancies which may arise during the analysis of compounds containing multiple isomers.

5. *Fortification Volumes*: The fortification volumes listed for M1a range from 5-50% of the sample weight. It is recommended that fortification volumes should consistently be less than or equal to 10% of the sample volume.

6. *Calibration Ranges*: The cited data requirement (SANCO/825/00 rev 8.1) stated that the calibration range should encompass 30% of the method LOQ to at least 20% above the highest level. The calibration standards presented in the analytical method (Reference 1) ranged from 0.10 to 1.0 µg/L, which encompassed 50% of the method LOQ to at least 20% above the highest level. As a result, an additional calibration level (0.06 µg/L, equivalent to 30% of the method LOQ) was added to the calibration range during the ILV study to comply with the data requirements. It is recommended that the calibration range in the final method should be adjusted to agree with the cited data requirement.

8. PROTOCOL, AMENDMENTS, AND DEVIATIONS

A single protocol amendment was issued on March 11, 2019. Protocol amendment #1 updated the study timelines, provided an updated Certificate of Analysis for the reference standard for Metabolite M12, updated the test system to the sandy loam soil received at the testing facility on February 5, 2019, and updated the Experimental Design section of the study protocol. The updated Experimental Design section removed the requirements for product ion scans and stability assessments (extract stability and standard stability), and defined the limit of detection (LOD) as 30% of the limit of quantitation, 15 µg/kg.

No protocol deviations were issued during the analytical phase of the study.